Gracilibacillus alcaliphilus sp. nov., a facultative alkaliphile isolated from indigo fermentation liquor for dyeing

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A facultatively alkaliphilic, lactic-acid-producing and halophilic strain, designated SG103T, was isolated from a fermented Polygonum indigo (Polygonum tinctorium Lour.) liquor sample for dyeing prepared in a laboratory. 16S rRNA gene sequence phylogeny suggested that SG103T is a member of the genus Gracilibacillus with the closest relatives being ‘Gracilibacillus xinjiangensis’ J2 (similarity: 97.06 %), Gracilibacillus thailandensis TP2-8T (97.06 %) and Gracilibacillus halotolerans NN7 (96.87 %). Cells of the isolate stained Gram-positive and were facultatively anaerobic straight rods that were motile by peritrichous flagella. The strain grew at temperatures between 13 and 48 °C with the optimum at 39 °C. It grew in the range pH 7–10 with the optimum at pH 9. The isoprenoid quinone detected was menaquinone-7 (MK-7) and the DNA G+C content was 41.3 mol%. The whole-cell fatty acid profile mainly consisted of iso-C15 : 0, anteiso-C15 : 0 and anteiso-C17 : 0. Unlike other reported species of the genus Gracilibacillus, the strain lacked diphosphatidylglycerol as a major polar lipid. DNA–DNA hybridization experiments with strains exhibiting greater than 96.87 % 16S rRNA gene sequence similarity, ‘G. xinjiangensis’ J2, G. thailandensis TP2-8T and G. halotolerans NN7, revealed 2 ± 4 %, 4 ± 9 % and 3 ± 2 % relatedness, respectively. On the basis of the differences in phenotypic and chemotaxonomic characteristics, and the results of phylogenetic analyses based on 16S rRNA gene sequences and DNA–DNA relatedness data from reported species of the genus Gracilibacillus, strain SG103T merits classification as a members of a novel species, for which the name Gracilibacillus alcaliphilus sp. nov. is proposed. The type strain is SG103T (=JCM 17253T=NCIMB 14683T).

During indigo fermentation, which proceeds under alkaline conditions (higher than pH 10), the pH gradually decreases owing to the action of micro-organisms (Aino et al., 2010). We have isolated indigo-reducing and lactic-acid-producing bacteria, namely Alkalibacterium psychrotolerans, Alkalibacterium iburiense and Alkalibacterium indicireducens, from indigo fermentation liquor obtained from Date City, Iburi, Hokkaido and Tokushima City, Shikoku in Japan (Nakajima et al., 2005; Yumoto et al., 2004, 2008). A lactic-acid-producing bacterium, Oceanobacillus polygoni, has also been isolated from a fermented indigo liquor sample prepared in a laboratory (Hirota et al., 2013). In the present study, we further screened for new lactic-acid-producing micro-organisms from indigo fermentation liquor and found that one strain belonging to the genus Gracilibacillus produced lactic acid.

The genus Gracilibacillus is one of the moderate halophilic genera belonging to the family Bacillaceae. Since the first description of the genus Gracilibacillus with Gracilibacillus halotolerans, which was isolated from surface mud of the Great Salt Lake in the USA (Wainø et al., 1999), and Bacillus dipsosauri (Lawson et al., 1996) from the salt...
glands of a desert iguana, which was transferred to the genus *Gracilibacillus*, additional species have been described, namely *Gracilibacillus orientalis* from a salt lake in Inner Mongolia (Carrasco et al., 2006), *Gracilibacillus boraciitoleras* from soil (Ahmed et al., 2007), *Gracilibacillus halophilus* from saline soil (Chen et al., 2008a), *Gracilibacillus quinghaiensis* from salt-lake sediment (Chen et al., 2008b), *Gracilibacillus lacisalsi* from a salt lake in China (Jeon et al., 2008), *Gracilibacillus saliphilus* from a salt lake in China (Tang et al., 2009), *Gracilibacillus thailandensis* from fermented fish (Chamroensaksri et al., 2010), *Gracilibacillus urelyticus* from saline-alkaline soil (Huo et al., 2010), *Gracilibacillus kekensis* from a salt lake in China (Gao et al., 2012), *Gracilibacillus bigeumensis* from solar saltern soil (Kim et al., 2012), *Gracilibacillus xinjiangensis* from soil (Yang et al., 2013), and *Gracilibacillus marinus* from the northern South China Sea (Huang et al., 2013).

A fermented *Polygonum* indigo (*Polygonum tinctorium* Lour.) liquor sample was prepared in our laboratory as reported previously (Aino et al., 2010). Screening for lactic-acid-producing bacteria was attempted using the fermented indigo liquor. An aliquot of the sample was inoculated onto a peptone/yeast extract/glucose (PYG) medium containing 3 g peptone (Kyokuto), 8 g yeast extract (Kyokuto), 10 g D-glucose, 10 g Na2CO3 and 15 g agar in 1 litre distilled water (pH 10). A 10-fold concentrated alkali solution (10% Na2CO3) was separately sterilized and then mixed with the autoclaved medium at 10% ratio. The inoculated medium was incubated at 27°C for 2 days under N2 gas-exchanged anaerobic conditions in an anaerobic jar. Colonies that appeared on the medium were transferred to 10 ml fresh peptone/yeast extract/glucose (PYG-2) broth (pH 10) containing 5 g peptone (Kyokuto), 10 g yeast extract (Kyokuto), 20 g D-glucose, 1.5 g sodium acetate, 1.5 g K2HPO4, 0.2 g MgSO4.7H2O and 0.05 g MnSO4.4H2O in 900 ml distilled water and mixed with 100 ml 10% Na2CO3 solution (dissolved in distilled water). Cultures were incubated for 3 days at 27°C under static aerobic conditions. The lactic acid concentration was determined by HPLC with a TSKgel OApak column (7.8 mm × 30 cm × 2; Tosoh); column temperature, 50°C; solvent for elution, 0.75 mM H2SO4; flow rate, 1 ml min⁻¹. After checking for lactic acid production in more than 60 strains, 10 strains were identified that exhibited 0.5% lactic acid production. Among them, strain SG103T was selected, which was transferred to PYG agar plates mixed with 10% Na2CO3 solution (final concentration: 1%) and then incubated at 27°C. Strain SG103T and the reference strains ‘*G. xinjiangensis*’ JCM 18859 (=J2), ‘*G. thailandensis*’ JCM 15569T (=TP2-8T) and ‘*G. halotolerans*’ DSM 11805T (=NNT) were used for taxonomic characterization in this study. Cells for DNA G+C content analysis, DNA–DNA relatedness, fatty acid composition, and isoprenoid quinone and polar lipid analyses of strain SG103T were harvested in the late exponential phase of growth after cultivation with aerobic rotary shaking (105 r.p.m.) at 35°C in PYG-2 broth containing 3 g peptone (Kyokuto), 8 g yeast extract (Kyokuto), 10 g D-glucose, 10 g NaCl and 1 ml metal mixture (Yumoto et al., 1998) in 1 liter distilled water containing 100 mM NaHCO3/Na2CO3 buffer (pH 10.0). *G. halotolerans* DSM 11805T and *G. thailandensis* JCM 15569T were also grown in PYG-2 broth. ‘*G. xinjiangensis*’ JCM 18859 was grown in PYG-3 broth containing 3 g peptone (Kyokuto), 8 g yeast extract (Kyokuto), 5 g D-glucose, 1 g sodium glutamate, 3 g 3-sodium citrate and 1 ml metal mixture (pH 7). It was difficult to harvest enough cells of ‘*G. xinjiangensis*’ JCM 18859 in PYG-2 broth for fatty acid analyses owing to their low growth intensity. Therefore, for comparison, strain SG103T was grown in PYG-2 and PYG-3 broths.

For phenotypic characterization, PYG-2 medium containing 100 mM NaHCO3/Na2CO3 buffer (pH 10.0) 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 using API 50CH (bioMérieux). Growth experiments at pH 6–12 were performed using PYG medium containing 100 mM NaH2PO4/Na2HPO4 buffer (pH 6–8), 100 mM NaHCO3/Na2CO3 buffer (pH 9–10), Na2HPO4/NaOH buffer (pH 11) or KCl/NaOH buffer (pH 12). Media with pH 11 and 12 were sterilized by passing through a 0.22 μm pore filter membrane owing to the decrease in pH after autoclaving. The pH of the spent medium decreased from pH 6, 7, 8, 9, 10, 11 and 12 to pH 5.9, 6.5, 6.8, 7.4, 8.4, 8.6 and 10.4, respectively, at the corresponding growth period. Growth was observed within the range pH 7–11. Although immediate growth was observed within the range pH 7–10, growth at pH 11 was observed after the initial medium pH was decreased. Therefore, it was considered that real pH range for growth of the isolate was pH 7–10. Other physiological and biochemical characteristics were examined in accordance with the methods described in Cowan and Steel’s Manual (Barrow & Feltham, 1993) and in a report by Yumoto et al. (1998). Hydrolyses of xylan (Serva) and cellulose (Wako) were examined by the method of Teather & Wood (1982) using 1% each of the substrates with PY medium containing 10 g peptone (Kyokuto) and 3 g yeast extract (Kyokuto) in 1 litre 100 mM NaHCO3/Na2CO3 buffer (pH 10). For the observation of negatively stained cells by transmission electron microscopy (TEM), cells were grown on PYG-2 agar (containing 1.5% agar; pH 10) for 2 days. TEM preparation and observation were performed as described previously (Yumoto et al., 2001). Growth intensities of the isolates at pH 10 under aerobic conditions in the temperature range 10–40°C were determined using a temperature gradient incubator (Bio-Photorecorder, type TN-2612; Advantec) by monitoring at OD650.

Cells were Gram-stain-positive, peripherically flagellated, straight rods (0.5–0.7 × 1.5–3.6 μm) (Fig. S1, available in the online Supplementary Material) and produced ellipsoidal spores in the terminal position within swollen sporangia. Phenotypic characteristics are described in the species description.
Bacterial DNA was prepared in accordance with the method of Marmur (1961). DNA base ratios were determined by the method of Tamaoka & Komagata (1984). The DNA G+C content of strain SG103T was 41.3 mol%. Whole-cell fatty acids and isoprenoid quinones were analysed as described previously (Yumoto et al., 2002). *meso*-Diaminopimelic acid (*meso*-DAP) in the cell wall was identified by TLC (Staneck & Roberts, 1974). Polar lipids were analysed by the methods of Mininnik et al. (1979) and Collins & Jones (1980). Polar lipids consisted of phosphatidylglycerol, one unidentified aminophospholipid, one unidentified phospholipid and five unidentified lipids (Fig. S2). There is no report of a bacteria belonging to the genus *Gracilibacillus* which does not possess diphosphatidylglycerol. The novel isolate is different from reported species of the genus *Gracilibacillus* in that optimal growth occurs at pH 9 and diphosphatidylglycerol is absent. In addition, the isolate is not able to grow at pH 6, while most species of the genus *Gracilibacillus* are able to grow pH 6. Although the presence of diphosphatidylglycerol in the membrane is generally favourable for alkaline adaptation in alkaliphilic members of the genus *Bacillus* (Clejan et al., 1986; Enomoto & Koyama, 1999), lower diphosphatidylglycerol content has been reported following growth at pH 7.5 (Clejan et al., 1986). Therefore, the absence of diphosphatidylglycerol may be related to the growth pH characteristics of the isolate. GC revealed that the cellular fatty acid composition (percentages refer to fraction of total fatty acid) of strain SG103T grown in PYG-2 broth was as follows: C14:0 (2.3%), iso-C15:0 (10.4%), anteiso-C15:0 (34.0%), iso-C16:0 (6.1%), C16:0 (8.6%), isoC17:0 (8.6%), anteiso-C17:0 (18.1%), C17:0 (2.0%), C18:0 (4.9%), C18:1ω9c (2.3%), C18:1ω9t (4.1%) and C20:0 (2.1%). The fatty acid composition of the isolate was similar to that of other species of the genus *Gracilibacillus* in the sense that iso-C15:0, anteiso-C15:0 and anteiso-C17:0 are the predominant fatty acids (Table S1). However, the fatty acid composition of strain SG103T was different from those of other phylogenetically related species of the genus *Gracilibacillus*. Menaquinone-7 was detected by TLC and HPLC of the fraction extracted from the cells. The cell wall of strain SA103T contained *meso*-DAP.

The 16S rRNA gene sequence of strain SA103T was amplified by PCR using primers 9F (GAGTTTGATCCTGCTCAAG) and 1541R (AAGAGGTTGATCCAGCC). The approximately 1.5 kb PCR product was sequenced directly by the dideoxyxynucleotide chain-termination method using a DNA sequencer (ABI PRISM 3100; Applied Biosystems) with a BigDye Termination RR mix version 3.1 (Applied Biosystem) according to the manufacturer’s instructions. Primers 9F, 686F (TACGGGTGAAATGCGTGA), 1099F (GCAACGACGGACGAC) and 357R (CTGCTGCTCCCGTAG) were used in the gene sequencing reaction. The sequences were aligned and the consensus sequence was determined using the program CLUSTAL W (Thompson et al., 1994). The similarity between sequences was calculated using the GENETYX computer program (Genetyx). The 16S rRNA gene sequence (1534 bp) of strain SG103T was obtained and compared with those of previously reported strains to determine sequence similarities. Strain SG103T showed sequence similarity with *G. halotolerans* NN7T (similarity: 96.87%), which is located at the same node with strain SG103T. It also showed similarities with other species of the genus *Gracilibacillus*, *G. thailandensis* TP2-8T (97.06%), ‘*G. xinjiangensis*’ J2 (97.06%), *G. urelyticus* MF38T (96.77%) and ‘*G. quinghaiensis*’ YIM-C229 (96.77%), whereas the similarity of the isolate with the other species of the genus *Gracilibacillus* was less than 96.77%. Phylogenetic trees were reconstructed by the neighbour-joining (Saitou & Nei, 1987), maximum-parsimony (Felsenstein, 1981), minimum-evolution (Fitch, 1971) and maximum-likelihood (Guindon & Gascuel, 2003) methods in MEGA 5 (Tamura et al., 2011). For the neighbour-joining method, the distance between sequences (Ks value) was calculated using Kimura’s two-parameter model (Kimura, 1980). A phylogenetic tree of the strain together with its closely related neighbours was reconstructed. A maximum-likelihood phylogenetic tree is shown in Fig. 1. The minimum-evolution tree was very similar to the neighbour-joining tree (Fig. S3). Although the phylogenetic relationship between the isolate and the group of *G. bigeumensis* BH097T and *G. halophilus* YIM-C55.5T and the group of *G. kekensis* K170T and ‘*G. marinus*’ HB09003 in the maximum-likelihood and maximum-parsimony phylogenetic trees was different from that in the neighbour-joining tree, the phylogenetic position of strain SG103T in the genus *Gracilibacillus* was similar in all three trees (Fig. S3). Phylogenetic analysis results and sequence similarity values determined on the basis of the 16S rRNA gene sequence showed that the isolate can be discriminated from previously reported members of the genus *Gracilibacillus* except *G. thailandensis* TP2-8T and ‘*G. xinjiangensis*’ J2 according to the criterion of gene sequence similarity below 97%.

On the basis of the results of 16S rRNA gene sequence analysis, ‘*G. xinjiangensis*’ JCM 18859, *G. thailandensis* JCM 15569T and *G. halotolerans* DSM 11805T (type species of the genus *Gracilibacillus*) were used as reference strains for DNA–DNA hybridization. The hybridization temperature was 37.4 °C. 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 in triplicate. DNA–DNA hybridization results indicated that strain SG103T is different from ‘*G. xinjiangensis*’ JCM 18859 and *G. thailandensis* JCM 15569T, and revealed 2 ± 4 % and 4 ± 9 % relatedness, respectively. Although strain SG103T showed similarity not higher than 97.0 % with *G. halotolerans* DSM 11805T, the two strains are located at the same node in phylogenetic trees reconstructed on the basis of 16S rRNA gene sequence. The novel strain also showed 3 ± 2 % relatedness to *G. halotolerans* DSM 11805T. It is concluded that the isolate is different from other reported species of the genus *Gracilibacillus*. 
Phenotypic characteristics and fatty acid composition were compared under the same experimental conditions using the phylogenetically nearest neighbours based on 16S rRNA gene sequence. *G. xinjiangensis* JCM 18859, *G. thailandensis* JCM 15569T and *G. halotolerans* DSM 11805T were compared with strain SG103T (Table 1). Strain SG103T could be differentiated from *G. xinjiangensis* JCM 18859, *G. thailandensis* JCM 15569T and *G. halotolerans* DSM 11805T on the basis of several phenotypic characteristics: for example, positive results for the Voges–Proskauer test, urease, citrate utilization and leucine arylamidase, and acid production from rhamnose, mannitol, arbutin, lactose, glycogen, gentiobiose and D-arabitol, and negative results for esterase/lipase (C8). Strain SG103T could be differentiated from *G. xinjiangensis* JCM 18859 and *G. thailandensis* JCM 15569T on the basis of positive results for lysine decarboxylase, 2-naphthyl β-D-galactosidase and acid production from d-arabinose. In addition, strain SG103T could be differentiated from *G. thailandensis* JCM 15569T and *G. halotolerans* DSM 11805T on the basis of positive results for acid production from D-glucose, methyl α-D-glucopyranoside, salicin, cellobiose and maltose. Furthermore, there are several additional phenotypic differences other than those described above between strain SG103T and each of the three reference type strains (Table 1). Strain SG103T could also be differentiated from *G. halotolerans* DSM 11805T on the basis of fatty acid content, namely percentages of iso-C15 and anteiso-C17:0 fatty acids (Table S1).

On the basis of the above results, the isolate was designated a novel species of the genus *Gracilibacillus*, for which the name *Gracilibacillus alcaliphilus* sp. nov. is proposed; the type strain is SG103T. A description of the novel species is given below.

**Emended description of the genus *Gracilibacillus* (Wainø, Tindall, Schuman and Ingvorsen 1999)**

*Gracilibacillus* (Gra.ci.li.bacillus. L. adj. gracilis slender; Gr. n. baktron rod; M.L. masc. Gracilibacillus the slender bacillus/rod).

The description is based on that given by Wainø *et al.* (1999) with the following addition. The DNA G+C content is 34.1–41.3 mol%.
Table 1. Differential characteristics of strain SG103T, ‘G. xinjiangensis’ JCM 18859, G. thailandensis JCM 15569T and G. halotolerans DSM 11805T

| Strains: 1, Gracilibacillus alcaliphilus sp. nov. SG103T; 2, ‘G. xinjiangensis’ JCM 18859; 3, G. thailandensis JCM 15569T; 4, G. halotolerans DSM 11805T. All data except DNA G+C content are from this study using API 20E, API 20NE, API ZYM and API 50CH (aerobic conditions; bioMérieux). Data for DNA G+C content of ‘G. xinjiangensis’ JCM 18859, G. thailandensis JCM 15569T and G. halotolerans DSM 11805T are from Yang et al. (2013), Chamroensaksri et al. (2010) and Wain et al. (1999), respectively. All strains were negative for arginine dihydrolase, ornithine decarboxylase, H2S production, ONPG, indole production, alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, α-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase, α-mannosidase and β-fucosidase. All strains were positive for hydrolysis of gelatin and ascus, p-nitrophenyl β-D-galactopyranoside and naphthyl-AS-Bl-phosphohydrolase. None of the strains produced acid from glycerol, erythritol, L-xylene, D-adonitol, methyl β-D-xylopyranoside, D-mannose, L-sorbose, dulcitol, inositol, sorbitol, methyl α-D-mannopyranoside, melibiose, inulin, melezitose, xyitol, D-lyxose, D-fucose, L-arabitol, gluconate or 2-ketogluconate. All strains produced acid from L-arabinose, ascorbic acid, ferric citrate and 5-ketogluconate. +, Positive; −, negative. |

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Description of Gracilibacillus alcaliphilus sp. nov.

Gracilibacillus alcaliphilus [al.ca.li’phi.lus. N.L. n. alcali (from Arabic article al; Arabic n. qaliy ashes of saltwort) alkali; N.L. adj. phils -a -um (from Gr. adj. philos -é –on) friend, loving; M.L. masc. adj. alcaliphilus liking alkaline media].

Cells are Gram-stain-positive, straight rods (0.5–0.7×1.5–3.6 μm) and motile by peritrichous flagella. Spherical endospores are produced at the terminal position in swollen sporangia. Colonies are circular, slightly convex, creamy white and 2–3 mm in diameter after 2 days of growth on PYG-2 agar medium. Facultatively anaerobic growth is observed. Lactic acid is produced from D-glucose. Catalase reaction is positive but not oxidase reaction. The temperature range for growth is 13–48 °C with the optimum at 39 °C (pH 10). The NaCl concentration range for growth is 0–15 % with the optimum at 3 % (pH 10). Grows at pH 7–10 with the optimum at pH 9 (35 °C). Hydrolyses starch, gelatin and Tweens 20, 40, 60 and 80 but not casein, xylan or cellulose. Acid is produced from D-arabinose, L-arabinose, D-ribose, D-xylene, D-glucose, D-fructose, rhamnose, mannitol, methyl α-D-glucopyranoside, N-acetylgalactosamine, lactose, amygdalin, arbutin, ascorbic acid, ferric citrate, salicin, cellobiose, maltose, sucrose, trehalose, raffinose, starch, glycogen, gentiobiose, D-arabitol and 5-ketogluconate, but not from the other carbohydrates of the API 50CH system under aerobic conditions. Other characteristics are listed in Table 1. The major cellular fatty acids are iso-C15:0, anteiso-C15:0, C16:0, iso-C17:0 and anteiso-C17:0. Polar lipids consist of phosphatidylglycerol, one unidentified phospholipid and five unidentified lipids. The cell-wall peptidoglycan is based on meso-DAP. Menaquinone-7 is detected as isoprenoid quinone.

The type strain is SG103T (=JCM 17253T=NCIMB 14683T) isolated from a Polygonum indigo (Polygonum tinctorium Lour.) fermentation liquor for dyeing prepared in a laboratory. The DNA G+C content of the type strain is 41.3 mol%.

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References


