Amphibacillus iburiensis sp. nov., an alkaliphile that reduces an indigo dye

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An indigo-reducing alkaliphilic strain, designated strain N314 7, was isolated from a fermented Polygonum tinctorium (L.) liquor sample, aged for 10 months, that was obtained from Date City, Iburi Branch, Hokkaido, Japan. The 16S rRNA gene sequence phylogeny suggested that strain N314 7 is a member of the genus Amphibacillus, with the closest relatives being Amphibacillus indicireducens (98.9% similarity to the type strain) and Amphibacillus xylanus (98.0% similarity to the type strain), the only species with 16S rRNA gene sequence similarities higher than 97% to strain N314 7. The cells of the isolate stained Gram-positive and were facultatively anaerobic, straight rods that were motile by means of peritrichous flagella. The strain grew at 26–39 °C with optimum growth at 36 °C. It grew at pH 8.0–9.1, with optimum growth at pH 8.9–9.1. No isoprenoid quinone was detected, and the DNA G+C content was 38.4 mol%. The whole-cell fatty acid profile consisted mainly of iso-C15 : 0 and anteiso-C15 : 0. Analysis of DNA–DNA hybridization with the type strains of Amphibacillus indicireducens and A. xylanus revealed 29±2% and 10±2% relatedness, respectively. Owing to differences in phenotypic characteristics from reported species of the genus A. and results of phylogenetic analyses based on 16S rRNA gene sequences and DNA–DNA relatedness data, the isolate merits classification within a novel species, for which the name Amphibacillus iburiensis sp. nov. is proposed. The type strain is N314 7 (=JCM 18529 7=NCIMB 14823 7).

The traditional method of indigo dying relies on the indigo-reducing ability of bacteria. Before 2000, indigo-reducing bacteria were isolated by Takahara & Tanabe (1960) and Padden et al. (1999). They were identified as ‘Bacillus alkaliphilus’ and Clostridium isatidis, respectively. In addition to these isolates, we have also isolated the indigo-reducing bacteria Alkalibacterium psychrotolerans and Alkalibacterium ibuiense from indigo fermentation liquor obtained from Date City, Iburi, Hokkaido, Japan (Yumoto et al., 2004; Nakajima et al., 2005). In addition, Alkalibacterium indicireducens was isolated from indigo fermentation liquor obtained from Tokushima Prefecture, Shikoku, Japan (Yumoto et al., 2008).

Aino et al. (2010) demonstrated that several isolates other than the above-mentioned species of micro-organisms from indigo fermentation liquor can reduce indigo. On the basis of these findings, it is clear that indigo-reducing ability is present not only in the genus Alkalibacterium but also in other genera belonging to the phylum Firmicutes, including the genera Amphibacillus, Bacillus and Oceanobacillus. Among these isolates, the novel indigo-reducing species Amphibacillus indicireducens (Hirota et al., 2013a) and Oceanobacillus indicireducens (Hirota et al., 2013b) have been reported.

The genus Amphibacillus was proposed by Niimura et al. (1990). A strain from an alkaline compost of manure with grass and rice straw was identified as Amphibacillus xylanus. Since the first report on the genus Amphibacillus, additional species have been described as follows: Amphibacillus fermentum and Amphibacillus tropicus from sediment of a soda lake, Lake Magadi, Kenya (Zhilina et al., 2001), Amphibacillus sediminis from the sediment of Lake Hamana, Shizuoka Prefecture, Japan (An et al., 2007), Amphibacillus jilinensis from sediment of a soda lake in Jilin province, China (Wu et al., 2010), Amphibacillus cookii from a sediment sample taken from the southern arm of the Great Salt Lake (Pugin et al., 2012), Amphibacillus indicireducens from indigo fermentation liquor (Hirota et al., 2013a) and Amphibacillus marinus from the South China Sea (Ren et al.,

Abbreviation: DAP, diaminopimelic acid.

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain N314 7 is AB736274.

Three supplementary figures and a supplementary table are available with the online version of this paper.

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Members of the genus *Amphibacillus* are moderately alkaliphilic and lack isoprenoid quinones and oxidase activity (Arai et al., 2009).

During the screening of indigo-reducing micro-organisms from a fermented polygonum indigo (*Polygonum tinctorium* Lour.) liquor sample obtained from Date City, Iburu Branch, Hokkaido, Japan, fermented indigo liquor aged for 10 months (pH 10.5) was used as a sample for the screening of indigo-reducing bacteria (Aino et al., 2010). An aliquot of the sample was inoculated onto a reinforced clostridial agar (RCA; Sigma) plate containing 100 mM NaHCO₃/Na₂CO₃ buffer (pH 10.0) (alkali RCA) and incubated aerobically at 30 °C for 10 days. NaHCO₃/Na₂CO₃ buffer for media used in this study was sterilized separately by autoclaving. The colonies that appeared were inoculated onto a PYA (peptone/extract/alkaline)—indigo carmine agar plate containing 8 g peptone (Kyokuto), 3 g yeast extract (Merck), 1 g K₂HPO₄, 2 g indigo carmine and 15 g agar in 1 l NaHCO₃/Na₂CO₃ buffer (100 mM in deionized water, pH 10.0), followed by incubation at 30 °C for 2 days. After determining the microbial reduction of indigo carmine, the culture was transferred to another 100 ml fresh 0.01 % indigo-containing PYA broth to examine the indigo-reducing ability of the isolates further. The colonies obtained were transferred to an alkali RCA plate and incubated at 30 °C. One of the strains obtained, N314T, appeared to be different from the other strains in terms of 16S rRNA gene sequence and was used for taxonomic characterization in this study because there was a possibility that the strain represented a novel species, as determined in preliminary experiments.

The proposed minimal standards for description of endospore-forming bacteria reported by Logan et al. (2009) were applied for phenotypic characterization of strain N314T. *A. xylanus* JCM 7361T, *A. indicireducens* JCM 17250T, *A. sediminis* IAM 15428T, *A. jilinensis* JCM 16149T and *A. cookii* DSM 23721T were used as reference strains. Cells for chemotaxonomic analysis (fatty acid composition, DNA G+C content, isoprenoid quinone, polar lipid and cell wall analyses) were harvested in the late exponential phase after cultivation with rotational shaking (50 strokes min⁻¹) at 30 °C in clostridial nutrient medium (CNM; Fluka BioChemika) containing 100 mM NaHCO₃/Na₂CO₃ buffer (pH 10.0) (alkali CNM).

For morphological and phenotypic characterization, alkali RCA or alkali CNM was used as the basal medium. Gram-staining was performed as described previously (Bartholomew & Mittwer, 1952). The culture was incubated at 35 °C for 2 weeks and experiments were performed three times to confirm the reproducibility of the results. API ZYM (bioMérieux) was used according to the manufacturer’s instructions. Growth experiments at pH 7–12 were performed at 30 °C using PYMSG medium containing 1 g peptone (Kyokuto), 5 g yeast extract (Kyokuto), 1 g meat extract (Kyokuto), 1 g starch, 5 g D-glucose and 1 ml metal mixture (Yumoto et al., 1998) dissolved in 1000 ml buffer solution: 100 mM NaH₂PO₄/Na₂HPO₄ (pH 7.0 and 8.0), 100 mM NaHCO₃/Na₂CO₃ (pH 9.0 and 10.0), NaHPO₄/NaOH (pH 11.0) or KCl/NaOH (pH 12.0). The broth at pH 10.0, 11.0 and 12.0 were sterilized by passing through a 0.22 μm pore filter. The initial pH of the broth adjusted to pH 8.0, 9.0, 10.0, 11.0 and 12.0 after inoculation of the seed culture was 8.0, 9.1, 9.8, 10.6 and 11.7, respectively. The pH of the spent medium dropped from 8.0, 9.1, 9.8, 10.6 and 11.7 to 7.6, 7.8, 7.9, 9.1 and 9.2, respectively, at the corresponding growth period. Growth under anaerobic conditions was determined by examining growth on an alkali RCA plate in an argon gas exchange jar. Other physiological and biochemical characteristics were examined according to the methods described by Barrow & Feltham (1993) and Yumoto et al. (1998). Acid production from carbohydrates was examined using alkali-OF medium (pH 10.0) containing 2 g peptone (Kyokuto), 2 g yeast extract (Kyokuto), 0.3 g K₂HPO₄, 0.03 g thymol blue, 3.0 g agar, 0.29 g NaHCO₃, 0.7 g NaCO₃ and 1 % carbohydrate. Anaerobicism in the inoculated test tubes was obtained by overlaying with sterile paraffin oil. Hydrolysis of xylan and cellulose was determined using alkali-RCA containing 1 % of each substrate and determined by the method of Teather & Wood (1982). The requirement for and tolerance of NaCl were determined using medium containing 1 g peptone (Kyokuto), 0.2 g yeast extract (Kyokuto) and 0–200 g NaCl in 1 l KHCO₃/K₂CO₃ (50 mM; pH 10.0) in distilled water. Antibiotic sensitivity was investigated by placing a paper disc, impregnated with a small amount of antibiotic, on an alkali RCA plate. For the observation of negatively stained cells by transmission electron microscopy (TEM), the cells were grown on alkali RCA. TEM preparation and observation were performed as described previously (Yumoto et al., 2001). Peritrichously flagellated straight rods were observed by TEM (Fig. S1, available in IJSEM Online). Spherical endospores were found in the terminal position (Fig. S1). Growth of the isolate at pH 10 under aerobic conditions in a temperature gradient incubator (Bio-Photorecorder, type TN-2612; Advantec) in the temperature range 10–40 °C was determined by monitoring the OD₆₅₀. The isolate can be differentiated from previously reported phylogenetically neighbouring species belonging to the genus *Amphibacillus* on the basis of the 16S rRNA gene sequence, and their characteristics are listed in Table 1.

Bacterial DNA was prepared according to the method of Marmur (1961) and the DNA base composition was determined by the method of Tamaoka & Komagata (1984). The DNA G+C content of strain N314T was 38.4 mol%. Whole-cell fatty acids and isoprenoid quinones were analysed as described previously (Yumoto et al., 2002). The presence of meso-diaminopimelic acid (meso-DAP) in the cell wall was analysed in strain N314T by TLC (Staneck & Roberts, 1974). GC analysis was used to determine the cellular fatty acid composition of strain N314T, as shown in Table S1 (means of two datasets obtained from independent cultures). The fatty acid composition of the isolate was similar to those of other species of the genus *Amphibacillus* in the sense that...
anteiso-C15:0 and iso-C15:0 were the predominant fatty acids. The proportions of anteiso-C13:0, C14:0 and C16:0 in strain N314T were higher than those in other species of the genus Amphibacillus. Polar lipids were analysed by the methods of Minnikin et al. (1979) and Collins & Jones (1980). Phosphatidylethanolamine, diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol and phosphatidylserine were used as standards for TLC. Fifteen polar lipids, which include unidentified lipids including one glycolipid, two aminolipids and three phospholipids, were detected by TLC (Fig. S2). Although the polar lipid composition of strain N314T was different from that of A. xylanus JCM 7361T, neither strain possessed phosphatidylglycerol or diphosphatidylglycerol (Fig. S2). Unidentified glycolipids in similar positions to those detected in strain N314T have been detected in TLC analysis of A. xylanus JCM 7361T and A. cookii DSM 23721T (Fig. S2; Pugin et al., 2012). Isoprenoid quinones were not detected by TLC or HPLC in fractions of concentrated extracts from strain N314T. The cell wall of strain N314T contained meso-DAP.

The 16S rRNA gene was amplified by PCR using primers 9F (5'-GAGTTTGATCCTGGCTCAG) and 1541R (5'-AAGGAGGTGATCCAGGC). The resulting PCR product was purified with a QIAquick PCR purification kit (Qiagen) and sequenced directly by the dideoxynucleotide chain-termination method using a DNA sequencer (ABI PRISM 3100; Applied Biosystems) with BigDye Termination RR mix version 3.1 (Applied Biosystems) according to the manufacturer’s instructions. Sequences were aligned and the consensus sequence was determined using the program

### Table 1. Differential characteristics of strain N314T and related type strains

<table>
<thead>
<tr>
<th>Characteristic</th>
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<td>Growth temperature (°C)</td>
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<td>Optimum</td>
<td>36</td>
<td>35</td>
<td>40</td>
<td>27</td>
<td>32</td>
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<td>NaCl concentration for growth (%)</td>
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<td>Range</td>
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<td>0–7</td>
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<td>0–1.6</td>
<td>0–2.8</td>
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<td>Neomycin (30 µg)</td>
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<td>+</td>
<td>–</td>
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<td>Novobiocin (30 µg)</td>
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<td>Nystatin (100 µg)</td>
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<td>DNA G+C content (mol%)</td>
<td>38.4</td>
<td>37.5</td>
<td>36.0</td>
<td>42.3</td>
<td>37.7</td>
</tr>
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</table>
13869T.

Gascuel, 2003) methods in MEGA 5 (Tamura et al., 2003) and maximum-likelihood (Guindon & Gascuel, 2003) methods in MEGA 5 (Tamura et al., 2011). For neighbour-joining analysis, the distance between sequences (K_{\text{max}} value) was calculated using Kimura’s two-parameter model (Kimura, 1980). The similarity between sequences was calculated using the GENETYX program. The 16S rRNA gene sequence of strain N314T (1526 bp) was obtained and compared with those of previously reported strains to determine sequence similarity. The maximum-likelihood phylogenetic tree is shown in Fig. 1. The maximum-likelihood and maximum-parsimony (Fig. S3c) phylogenetic trees show that the node involving strain N314T and A. xylanus JCM 7361T is most closely related to the independent node of A. indicireducens JCM 17250T. On the other hand, the node involving strain N314T and A. indicireducens JCM 17250T is most closely related to the independent node of A. xylanus JCM 7361T in the neighbour-joining and minimum-evolution phylogenetic trees. The neighbour-joining and minimum-evolution trees were very similar (Fig. S3a, b). Strain N314T showed the highest similarity to A. indicireducens JCM 17250T (similarity 98.9 %). It showed the following similarities to the type strains of other species of the genus Amphibacillus: 98.0 % to A. xylanus JCM 7361T, 94.3 % to A. sediminis IAM 15428T, 94.4 % to A. cookii ATCC BAA-2118T, 94.4 % to A. jilinensis JCM 16149T, 94.1 % to A. tropicus DSM 13870T and 93.9 % to A. fermentum DSM 13869T.

On the basis of the results of 16S rRNA gene sequence analysis, A. indicireducens JCM 17250T and A. xylanus JCM 7361T were used as reference strains for DNA–DNA hybridization. The reference strains and isolate N314T were cultivated by the same method. The hybridization temperature was 36.2 °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. DNA–DNA hybridization results indicated that strain N314T is different from A. indicireducens JCM 17250T (29 ± 2 % relatedness, n = 3) and A. xylanus JCM 7361T (10 ± 2 %, n = 3). It is concluded that the isolate is distinct from other reported species of the genus Amphibacillus.

In addition to the phylogenetic position based on the 16S rRNA gene sequence, the characteristic of a lack of a requirement of NaCl for optimum growth was specific to strain N314T in comparison with all species of the genus Amphibacillus except A. indicireducens. The phenotypic characteristics of strain N314T were compared under the same experimental conditions using the phylogenetic nearest neighbours based on the 16S rRNA gene sequence: A. indicireducens JCM 17250T, A. xylanus JCM 7361T, A. sediminis IAM 15428T and A. jilinensis JCM 16149T (Table 1). Strain N314T can be differentiated from A. indicireducens JCM 17250T, A. xylanus JCM 7361T, A. sediminis IAM 15428T and A. jilinensis JCM 16149T on the basis of several phenotypic and chemotaxonomic characteristics. For example, strain N314T can be differentiated from A. indicireducens JCM 17250T on the basis of negative reactions for sensitivity to amikacin, gentamicin and polymyxin B, esterase (C4) and z-galactosidase and its growth characteristics (optimum growth temperature and NaCl concentration for growth).

**Fig. 1.** Maximum-likelihood phylogenetic tree reconstructed from 16S rRNA gene sequences of strain N314T and related micro-organisms. Bootstrap values (>50 %) based on 1000 replicates are shown as percentages at branch nodes. Bar, 0.01 changes per nucleotide position.
It can also be differentiated from *A. xylanus* JCM 7361<sup>T</sup>, *A. sediminis* IAM 15428<sup>T</sup> and *A. jilinensis* JCM 16149<sup>T</sup> on the basis of its negative reactions for hydrolysis of Tween 20, valine arylamidase and α-galactosidase and positive reactions for β-glucuronidase and β-glucosidase (API ZYM), its lack of sensitivity to several antibiotics and growth characteristics (temperature and pH). Strain N314<sup>T</sup> can also be differentiated from other phylogenetically neighbouring strains of the genus *Amphibacillus* on the basis of fatty acid composition, namely, the ratio of the amount of anteiso-C<sub>15</sub>:0 to that of iso-C<sub>15</sub>:0 and the high percentages of branched C<sub>13</sub> and straight C<sub>16</sub>:0 fatty acids (Table S1).

Although strain N314<sup>T</sup> can be differentiated from *A. indicireducens* JCM 17250<sup>T</sup>, which was isolated from the same source, they exhibited similar characteristics, especially in terms of substrate utilization. This may mean that the environment that induces the fermentation of the indigo plant at high pH causes the accumulation of microorganisms that exhibit similar characteristics. At the same time, the existence of similar micro-organisms may have a role in the buffering effect for the maintenance of the microbiota in the environment. The indigo fermentation fluid maintains its indigo-reducing state even though there is a high possibility of contamination by foreign microorganisms during the dyeing process.

On the basis of the above results, strain N314<sup>T</sup> was assigned to a novel species of the genus *Amphibacillus*, for which the name *Amphibacillus iburiensis* sp. nov. is proposed.

**Description of *Amphibacillus iburiensis* sp. nov.**

*Amphibacillus iburiensis* (i.bu.ri.en’xis. N.L. masc. adj. *iburiensis* from Iburi, the place where the type strain was isolated).

Cells are Gram-staining-positive, peritrichously flagellated, straight rods (0.3–0.4 × 1.7–3.0 µm) and facultatively anaerobic. Spherical endospores are formed in the terminal position. Colonies are circular, convex and white. Catalase and oxidase reactions are negative. Growth temperature is 26–39 °C, with optimum growth at 36 °C (pH 10.0). NaCl is not required for its growth; the NaCl concentration for growth is 0–7 %, with optimum growth at 0–3 % (pH 10.0, 35 °C). Although growth is apparent from acid production by the inoculated cells in broth adjusted to above pH 10, actual growth is initiated at pH 8.0–9.1, with the optimum at pH 8.9–9.1 (30 °C). Hydrolyses starch, xylan and cellulose but not casein, gelatin, DNA or Tweens 20, 40, 60 or 80. API ZYM test results are positive for esterase lipase (C8), leucine arylamidase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, β-glucuronidase and β-glucosidase, and negative for esterase (C4), alkaline phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, acid phosphatase, α-galactosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase. Ferments (no gas) L- and D-arabinose, D-xyllose, D-glucose, D-fructose, D-mannose, D-rhamnose, D-galactose, sucrose, trehalose, cellobiose, raffinose, lactose and melibiose. Does not ferment xylitol, sorbitol, mannitol or myo-inositol. Contains the following cellular fatty acids: iso-C<sub>13</sub>:0 anteiso-C<sub>13</sub>:0, iso-C<sub>14</sub>:0, anteiso-C<sub>14</sub>:0, iso-C<sub>15</sub>:0 anteiso-C<sub>15</sub>:0, isC<sub>16</sub>:0, iso-C<sub>16</sub>:0, anteiso-C<sub>17</sub>:0, iso-C<sub>18</sub>:0, ε9c and C<sub>20</sub>:0. Fifteen polar lipids, including one unidentified glycolipid, two unidentified aminolipids and three unidentified phospholipids, are detected on TLC. The cell-wall peptidoglycan is based on meso-DAP. No isoprenoid quinones are detected.

The type strain is N314<sup>T</sup> (=JCM 18529<sup>T</sup> =NCIMB 14823<sup>T</sup>), isolated from a *Polygonum tinctorium* (Lour.) fermentation liquor. The DNA G+C content of the type strain is 38.4 mol%.

**Acknowledgements**

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**References**


