Noviherbaspirillum denitrificans sp. nov., a denitrifying bacterium isolated from rice paddy soil and Noviherbaspirillum autotrophicum sp. nov., a denitrifying, facultatively autotrophic bacterium isolated from rice paddy soil and proposal to reclassify Herbaspirillum massiliense as Noviherbaspirillum massiliense comb. nov.

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Abstract

Thirty-nine denitrifying bacterial strains closely related to one another, represented by strains TSA40T and TSA66T, were isolated from rice paddy soils. Strains TSA40T and TSA66T were Gram-stain-negative, slightly curved rod-shaped, and motile by means of polar flagella. They were able to reduce nitrate, nitrite and nitrous oxide, but unable to fix atmospheric N2. While strain TSA66T was able to grow autotrophically by H2-dependent denitrification, strain TSA40T could not. Phylogenetic analysis suggested that they belong to the family Oxalobacteraceae, the order Burkholderiales in the class Betaproteobacteria. Major components in the fatty acids (C16:0, C17:0 cyclo, C18:1ω7c and summed feature 3) and quinone (Q-8) also supported the affiliation of strains TSA40T and TSA66T to the family Oxalobacteraceae. Based on 16S rRNA gene sequence comparisons, strains TSA40T and TSA66T showed the greatest degree of similarity to Herbaspirillum massiliense JC206T, Noviherbaspirillum malthae CC-AFH3T, Noviherbaspirillum humi U15T, Herbaspirillum seropedicae Z67T and Paucimonas lemoignei LMG 2207T, and lower similarities to the members of other genera. Average nucleotide identity values between the genomes of strain TSA40T, TSA66T and H. massiliense JC206T were 75–77%, which was lower than the threshold value for species discrimination (95–96%). Based on the 16S rRNA gene sequence analysis in combination with physiological, chemotaxonomic and genomic properties, strains TSA40T (=JCM 17722T=ATCC TSD-69T) and TSA66T (=JCM 17723T=DSM 25787T) are the type strains of two novel species within the genus Noviherbaspirillum, for which the names Noviherbaspirillum denitrificans sp. nov. and Noviherbaspirillum autotrophicum sp. nov. are proposed, respectively. We also propose the reclassification of Herbaspirillum massiliense as Noviherbaspirillum massiliense comb. nov.

We previously obtained 39 denitrifying strains by using a functional single cell isolation method [1] from rice paddy soils [2, 3]. Their 16S rRNA gene sequences were 98–100% similar to those obtained by culture-independent analyses [3–5], but were <98% similar to those of the type strains within the genus Herbaspirillum. Based on 16S rRNA gene sequence analysis using the MOTHUR program [6], the 39 strains were divided into two groups, each represented by strain TSA40T (=JCM 17722T=ATCC TSD-69T) and strain TSA66T (=JCM 17723T=DSM 25787T). The phylogenetic relatedness of the 39 strains based on the nearly full-length 16S rRNA gene sequences is shown in Fig.

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Abbreviations: ANI, average nucleotide identity; POCF, percentage of conserved proteins; RubisCO, ribulose-1,5-bisphosphate carboxylase/oxygenase.

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The GenBank/EMBL/DBJ accession numbers for the 16S rRNA gene sequence and the genome sequence of strain TSA40T are AB542397 and LST000000000, respectively, and those for strain TSA66T are AB542418 and JWJG00000000, respectively. Five supplementary figures and five supplementary tables are available with the online Supplementary Material.
S1 (available in the online Supplementary Material). The 16S rRNA gene sequences of strains TSA40\(^T\) and TSA66\(^T\) were 97.4 % similar to each other. The two strains were characterized in more detail by using a polyphasic study. A third strain, TSO23-1 (=JCM 17720), was also included in the analysis because this strain has a distinct rep-PCR DNA fingerprinting pattern from strain TSA66\(^T\) (Fig. S2), although the 16S rRNA gene sequence of this strain is highly similar to that of strain TSA66\(^T\) (98.9 %). The results showed that strains TSA40\(^T\) and TSA66\(^T\) were phylogenetically and physiologically unique to each other and to other species within the genus Novibasirilla.

Strains TSA40\(^T\) and TSA66\(^T\) were isolated from a rice paddy soil under denitrification-inducing conditions [2], while TSO23-1 was isolated from the same rice paddy soil under N\(_2\)O-reducing conditions [3]. In both cases, succinate was used as an electron donor. The culture was maintained on 100-fold diluted nutrient broth (DNB; Difeo) supplemented with 3 mM nitrate and 4.4 mM succinate (DNBNS medium) or 1.5 % agar plates of the DNBNS medium (DNBNS agar medium) under anaerobic incubation at 30 °C as described previously [2].

Flagellation and morphological characteristics were determined by transmission electron microscopy after negative staining of the cells, grown on DNBNS agar medium at 30 °C for 1 week, with 1 % (w/v) phosphotungstic acid. Filamentous motilility was assayed on semi-solid DNBNS medium that contained 0.075 % agar. The DNBNS medium was used as a basal medium to test growth at various temperatures (from 5 to 42 °C), pH (from 4.5 to 9.0) and NaCl concentrations (from 0 to 3 %, w/v). Utilization of carbon substrates was tested using minimal medium (1.5 g NH\(_4\)Cl, 0.6 g KH\(_2\)PO\(_4\), 0.1 g KCl, 10 ml vitamin solution, 10 ml trace element solution per litre; pH adjusted to 7.0 with 5 M NaOH) supplemented with 10 mM (final concentration) of the following carbon sources: D-fructose, D-galactose, D-glucose, lactose, maltose, D-mannose, L-rhamnose, D-sorbitol, D-xylene, glucuronate, glycerol, ethanol, butanediol, acetate, succinate, pyruvate, butyrate, valerate or lactate. High-molecular-weight carbon sources were tested at the following concentrations: 0.5 % (w/v) starch, cellolose, Tween 40 and Tween 80 or 0.3 % (w/v) poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid). Contents of the vitamin solutions and trace element solutions were described elsewhere [7]. Herbasirilla seropedicae JCM 21448\(^T\), Herbasirillum massiliense DSM 25712\(^T\) (=JCM 17265\(^T\)), Novibasirillum malthae JCM 18414\(^T\) (=CC-AFH3\(^T\)) and Psychomonas lemoignei LMG 2207\(^T\) were used as reference strains.

Potential nitrogen fixation ability was examined by measuring acetylene reduction activity [8]. In brief, cells were grown on 5 ml semisolid modified Rennie medium [8] without out rice extract in a 10 ml serum vial. After 4 days of aerobic incubation at 30 °C, acetylene gas was injected to the vial at a final concentration of 10 %. The vial was further incubated for 2 days and acetylene and ethylene contents were analysed using a gas chromatograph equipped with flame ionization detector (GC9A; Shimadzu) and SHINCARBON-ST column (Shinwa Chemical Industries). Furthermore, growth on nitrogen-free JNFB agar medium [9] was examined. H. seropedicae JCM 21448\(^T\) was used as a positive control for these tests.

Denitrification abilities were analysed by the acetylene block method using DNBNS medium as described previously [10]. N\(_2\)O-reducing ability was examined using \(^{15}\text{N}\)-labelled N\(_2\)O (\(^{15}\text{N}, 99 \text{ at.} \%; \text{Cambridge Isotope Laboratories}) and GC/MS as described previously [3]. In addition, anaerobic growth under denitrification conditions with acetate, succinate or H\(_2\) as an electron acceptor was tested using bicarbonate-buffered basal medium (2.5 g NaHCO\(_3\), 1.5 g NH\(_4\)Cl, 0.6 g KH\(_2\)PO\(_4\), 0.1 g KCl, 10 ml vitamin solutions and 10 ml trace element solutions per litre) [11]. The medium was dispensed into pressure tubes, sealed with a butyl rubber stopper and the air-phase was replaced with N\(_2\)/CO\(_2\) (80:20) gas or N\(_2\)/H\(_2)/CO\(_2\) (80:10:10) gas when grown under autotrophic denitrification conditions.

Potential for metal respiration was tested using bicarbonate-buffered basal medium supplemented with 5 mM succinate and 20 mM Fe(III) citrate or Fe(III) pyrophosphate under N\(_2)/CO\(_2\) (80:20) atmosphere. Anaeromyxobacter dehalogenes 2CP-C was used as a positive control for this test.

For fatty acid and quinone analysis, cells were aerobically grown on R2A agar (Difeo) and in R2A liquid medium, respectively, at 30 °C and harvested at exponential growth phase (after 24 h of incubation). Cellular fatty acids were saponified, methylated, extracted and analysed by GC (Hewlett Packard 6890) according to the standard protocol of the MIDI/Hewlett Packard Microbial Identification System. Polar lipids were extracted from the cells, separated by two-dimensional TLC and identified by spraying with the appropriate detection reagents as described elsewhere [12, 13]. Quinones were extracted from cells and analysed as described elsewhere [13], using reversed-phase HPLC. H. seropedicae JCM 21448\(^T\) was used as a reference strain.

For the analysis of polyamines, strains were grown in liquid medium containing 1 % peptone, 0.2 % yeast extract and 0.1 % MgCl\(_2\),H\(_2\)O (pH 7.0) on a reciprocal shaker at 30 °C. Cells harvested in the late exponential growth phase were washed with saline and freeze-dried. Extraction, analysis and quantification of polyamines were carried out as described by Scherer and Kneifel [14] and Busse and Auling [15]. The detection of polyamines was carried out on a Triant C18 Plus column (250 by 4.6 mm; YMCA Co.) using a Shimadzu LC20A gradient liquid chromatograph equipped with a Hitachi model L-7485 fluorescence detector. The excitation wavelength was 280 nm and the emission wavelength was 451 nm. A linear gradient of 40–80 % acetoni trile/water was applied for 30 min (plus 30 min at the final concentration) with a flow rate of 1 ml min\(^{-1}\) at 40 °C to elute the dansylated polyamines. Polyamine concentrations were calculated from peak areas using the method of internal standardization. The identity of the separated polyamines was confirmed by comparison with authentic reference standards.
samples: putrescine, cadaverine, spermidine and 1,8-diamo-octane purchased from Sigma-Aldrich, and 2-hydroxy-putrescine obtained from Herbaspirillum frisingense NBRC 102522\textsuperscript{T}, which was purchased from the National Biorepository Center (NBRC), NITE, Japan.

Genomic DNA was extracted and PCR was performed using a Viriti 96-well thermal cycler (Applied Biosystems) to detect nitrite reductase genes (\textit{nirK} and \textit{nirS}), nitrous oxide reductase gene (\textit{nosZ}), and nitrogenase gene (\textit{nifH}) as described previously [1, 16]. The nearly full-length sequences of the 16S rRNA gene previously obtained [2, 3] were used to analyse the phylogenetic positions of the strains relative to other \textit{Oxalobacteraceae} strains. Sequences were aligned with the reference sequences retrieved from the DDBJ/EMBL/GenBank databases by using \textsc{clustal w} [17]. Phylogenetic trees were reconstructed based on the neighbour-joining, maximum-likelihood and maximum-parsimony methods by using \textsc{megax} v.6.06 [18]. Bootstrap analysis (\(n=1000\)) was performed to evaluate the topology of the phylogenetic trees.

For genome analysis, DNA from strains TSA40\textsuperscript{T} and TSA66\textsuperscript{T} was extracted by using a PowerSoil DNA Isolation Kit (MoBio Laboratories). PCR-free libraries were prepared using a KAPA HyperPlus Kit (Kapa Biosystems) and TruSeq DNA Sample Prep Kit (Illumina), for strains TSA40\textsuperscript{T} and TSA66\textsuperscript{T}, respectively, according to the manufacturers' instruction. The genome of strain TSA40\textsuperscript{T} was analysed using the Illumina MiSeq with a 250 bp paired-end library, while the genome of strain TSA66\textsuperscript{T} was analysed using the Illumina HiSeq 2000 with a 101 bp paired-end library. Resulting high-quality sequences were assembled using \textsc{velvet} v.1.2.08 [19] to total lengths of 5,306,140 and 5,384,605 bp, respectively, for the genome of strains TSA40\textsuperscript{T} and TSA66\textsuperscript{T}. Gene prediction and annotation were performed by the NCBI Prokaryotic Genomes Annotation Pipeline. Genome sequencing and annotation results are summarized in Table S1. The genome sequences of strains TSA40\textsuperscript{T} and TSA66\textsuperscript{T} have been deposited at DDBJ/EMBL/GenBank under accession numbers LSTM0000000 and JWJG0000000, respectively.

Average nucleotide identity (ANI) values between the genomes of strains TSA40\textsuperscript{T}, TSA66\textsuperscript{T} and \textit{H. massiliense} JC206\textsuperscript{T} (GenBank accession no. CAHF00000000; [20]) were calculated using \textsc{jspecies} [21]. Percentages of conserved proteins (POCP) were calculated [22] to assess the genus-level relationships between strains TSA40\textsuperscript{T}, TSA66\textsuperscript{T}, \textit{H. massiliense} JC206\textsuperscript{T} and \textit{H. seropedicae} SmR1 (GenBank accession no. CP002039; [23]).

The morphological, cultural, physiological and biochemical characteristics of strains TSA40\textsuperscript{T} and TSA66\textsuperscript{T} are summarized in Table 1. Identical results were obtained from strains TSA66\textsuperscript{T} and TSO23-1. Cells of strains TSA40\textsuperscript{T}, TSA66\textsuperscript{T} and TSO23-1 were Gram-stain-negative, slightly-curved rods, 0.4–0.6 µm wide and 1.4–2.2 µm long, with one or two polar flagella (Fig. S3). Colonies appeared white, circular and smooth when grown on DNBNS agar medium. Cells were able to grow aerobically on R2A agar and weakly on nutrient agar, but unable to grow in trypticase soy broth unlike other \textit{Herbaspirillum} species. In DNBNS broth, strains TSA40\textsuperscript{T}, TSA66\textsuperscript{T} and TSO23-1 grew at 10–42 °C, with optimum growth temperature of 30 °C, and at pH 5.5–9.0. Cells could not grow in DNBNS medium supplemented with >1% (w/v) NaCl. Cells were positive for catalase and oxidase activity, but negative for urease activity.

Strains TSA40\textsuperscript{T}, TSA66\textsuperscript{T} and TSO23-1 were able to grow with low-molecular-weight organic acids (e.g. acetate, butyrate, lactate, pyruvate, succinate, valerate) and their polymers [e.g. Tween 40, Tween 80, poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid)], but not with many sugars (e.g. fructose, galactose, glucose, lactose, maltose, mannose, sorbitol, rhamnose, sorbitol, xylose), sugar acids (e.g. glucurate), alcohols (e.g. butanediol, ethanol), or polysaccharides (e.g. starch, cellulose). This is in contrast to other \textit{Herbaspirillum} species [9], but similar to \textit{H. massiliense} DSM 25712\textsuperscript{T}, \textit{N. malthae} JCM 18414\textsuperscript{T} and \textit{P. lenoignei} LMG 2207\textsuperscript{T} [24]. Unlike \textit{H. massiliense} DSM 25712\textsuperscript{T} and \textit{P. lenoignei} LMG 2207\textsuperscript{T}, strains TSA40\textsuperscript{T}, TSA66\textsuperscript{T} and TSO23-1 utilized glycerol for growth.

Strains TSA40\textsuperscript{T}, TSA66\textsuperscript{T} and TSO23-1 were able to grow anaerobically using nitrate, nitrite and N\textsubscript{2}O as an electron acceptor, whereas they did not reduce the Fe(III) substrates tested. Genes responsible for nitrate and N\textsubscript{2}O reduction were previously identified [2, 3]. In addition, gene clusters for membrane-bound nitrate reductase (Nar), periplasmic nitrate reductase (Nap), cytochrome \textit{cd}_{1}-containing nitrite reductase (Nir), nitric oxide reductase (Nor) and nitrous oxide reductase (Nos) were found in the genome of strains TSA40\textsuperscript{T} and TSA66\textsuperscript{T}. In contrast, \textit{H. massiliense} JC206\textsuperscript{T} did not show nitrate-reducing activity [20]. In addition, genes associated with denitrification were not detected in the genome of \textit{H. massiliense} JC206\textsuperscript{T}. Similarly, \textit{N. malthae} JCM 18414\textsuperscript{T} did not show denitrification activity nor grew under denitrification conditions in this study, although previous API 20E tests showed a positive result for nitrate reduction to N\textsubscript{2} gas [25]. In addition, denitrification functional genes (\textit{nirK}, \textit{nirS} and \textit{nosZ}) were not detected by PCR in \textit{N. malthae} JCM 18414\textsuperscript{T}.

Strains TSA40\textsuperscript{T}, TSA66\textsuperscript{T} and TSO23-1 were able to use acetate, glycerol, succinate, pyruvate, butyrate, valerate and lactate as an electron donor for denitrification. Strains TSA66\textsuperscript{T} and TSO23-1 grew autotrophically using H\textsubscript{2} as an electron donor, nitrate as an electron acceptor and bicarbonate as a carbon source, whereas strain TSA40\textsuperscript{T} could not. The presence of genes encoding ribulose-1,5-biphosphate carboxylase/oxygenase (RubisCO), phosphoribulokinase and fructose-1,6-bisphosphatase/sedoheptulose-1,7-bisphosphatase in the genome of TSA66\textsuperscript{T} also supports the autotrophic lifestyle of this strain. The RubisCO gene is present in the genomes of several \textit{Herbaspirillum} species (but not in the genome of \textit{H. massiliense} JC206\textsuperscript{T}), a strain closest to strain TSA66\textsuperscript{T} based on 16S rRNA gene sequence.
not determined. All strains are motile by means of flagella.

Strains: 1, TSA40<sup>T</sup>; 2, TSA66<sup>T</sup>; 3, *Noviherbaspirillum malthae* JCM 18414<sup>T</sup> [25]; 4, *Noviherbaspirillum humi* [30]; 5, *Noviherbaspirillum massiliense* DSM 25712<sup>T</sup> [20]; 6, *Herbaspirillum seropedicae* JCM 21448<sup>T</sup> [9]; 7, *Pacimonas lemoignei* LMG 2207<sup>T</sup> [24]. +, Positive; −, negative; w, weakly positive; nd, not determined. All strains are motile by means of flagella.

**Table 1.** Phenotypic and chemotaxonomic characteristics that separate strains TSA40<sup>T</sup> and TSA66<sup>T</sup> from closely related phylogenetic neighbours.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
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<th>5</th>
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<th>7</th>
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</thead>
<tbody>
<tr>
<td>Cell shape</td>
<td>Slightly curved rods</td>
<td>Slightly curved rods</td>
<td>Short rods</td>
<td>Short rods</td>
<td>Rods</td>
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<td>Rods</td>
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<tr>
<td>Cell size (µm)</td>
<td>0.4–0.6×1.4–2.2</td>
<td>0.4–0.6×1.4–2.2</td>
<td>1.0–1.2×1.4–1.6</td>
<td>0.9–1.1×1.0–1.2</td>
<td>0.44</td>
<td>0.6–0.7×1.5–5.0</td>
<td>0.6–0.8×1.5–3.0</td>
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<td>Pigment</td>
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<td>–</td>
<td>Red-orange</td>
<td>Light brown</td>
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<td>Ranges for growth:</td>
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<td>pH</td>
<td>5.5–9.0</td>
<td>5.5–9.0</td>
<td>5.0–10.0</td>
<td>6.0–9.0</td>
<td>ND</td>
<td>5.3–8.0</td>
<td>5.5–9.0</td>
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<td>NaCl concentration (%)</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
<td>0–1.0</td>
<td>&lt;1.0</td>
<td>ND</td>
<td>&lt;5.0</td>
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<td>Nitrate reduction</td>
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<td>–</td>
<td>+</td>
<td>ND</td>
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<td>Denitrification</td>
<td>+</td>
<td>+</td>
<td>–</td>
<td>ND</td>
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<td>Nitrogen fixation</td>
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<td>ND</td>
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<td>Growth on:</td>
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<td>Acetate</td>
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<td>+*</td>
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<td>Succinate</td>
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<td>+*</td>
<td>ND</td>
<td>4*</td>
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<td>+</td>
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<td>Pyruvate</td>
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<td>+</td>
<td>+*</td>
<td>ND</td>
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<td>Butyrate</td>
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<td>Valerate</td>
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<td>+*</td>
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<td>d-Mannose</td>
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<td>Sorbitol</td>
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<td>Glycerol</td>
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<td>+*</td>
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<td>Ethanol</td>
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<td>+*</td>
<td>ND</td>
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<td>Starch</td>
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<td>ND</td>
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<td>R2A agar</td>
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<td>Nutrient agar</td>
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<td>Trypticase soy agar</td>
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<tr>
<td>DNA G+C content (mol%)</td>
<td>61.8</td>
<td>59.9</td>
<td>63.4</td>
<td>61.2</td>
<td>59.7</td>
<td>63.4</td>
<td>59±2</td>
</tr>
</tbody>
</table>

* Determined in this study.
16S rRNA gene sequence similarity between strains TSA40T and TSA66T, and the type species of the genera Herbaspirillum, Noviherbaspirillum, Herminimonas, Oxalicybacterium, Collimonas, Paucimonas and Glacimonas were 95.6–97.5, 96.4–96.9, 94.8–95.2, 94.4–96.5, 95.0–95.3, 95.6 and 95.5 %, respectively. Based on the phylogenetic analysis, strains TSA40T and TSA66T were closely related to H. massiliense JC206T, N. malthae JCM 18414T, Noviherbaspirillum humi U15T and P. lemoignei LMG 2207T, by the neighbour-joining, maximum-likelihood and maximum-parsimony methods (Fig. 1). 16S rRNA gene sequence similarities were <97 % between strains TSA40T, TSA66T, and N. malthae JCM 18414T, N. humi U15T and Paucimonas lemoignei LMG 2207T, however, those between between strains TSA40T, TSA66T and H. massiliense JC206T were 96.9–97.7 %. The ANI values between strains TSA40T, TSA66T and H. massiliense JC206T were 75–77 % (Table S2), which are below the cutoff value for species discrimination (95–96 %; [21, 28]). The POCP values between strains TSA40T, TSA66T and H. massiliense JC206T were 48–54 % (Table S3), which are around the proposed cutoff value (50 %) for genus discrimination [22]. H. seropedicae SmR1 showed much lower POCP values (32–36 %) to strains TSA40T, TSA66T and H. massiliense JC206T, suggesting that strains TSA40T, TSA66T and H. massiliense JC206T may belong to another genus different from Herbaspirillum based on the comparative genome analysis.

Fig. 1. Phylogenetic relationships between strains TSA40T, TSA66T and TS023-1 and some members of the family Oxalobacteraceae based on nearly full-length 16S rRNA gene sequences. The phylogenetic tree reconstructed by the neighbour-joining method is shown here, but similar trees were obtained by using the maximum-likelihood and maximum-parsimony methods. The nodes supported by bootstrap values (>70 %) from 1000 replicates in the phylogenetic trees reconstructed by the neighbour-joining (NJ), maximum-likelihood (ML) and maximum-parsimony (MP) methods are indicated as grey in the pie charts next to the branches. The accession numbers of the reference strains in the DDBJ/EMBL/GenBank databases are indicated in parentheses.
The DNA G+C contents of strains TSA40<sup>T</sup> and TSA66<sup>T</sup> were 61.8 and 59.9 mol% based on the draft genome sequencing. Strains TSA40<sup>T</sup> and TSA66<sup>T</sup> had Q-8 as the only ubiquinone, similar to other Noviherbaspirillum and Herbaspirillum species [25, 29]. The major components in the fatty acid profiles of strains TSA40<sup>T</sup> and TSA66<sup>T</sup> were C<sub>16:0</sub>, C<sub>17:0</sub> cyclo, C<sub>18:1ω7c</sub> and summed feature 3 (Table S4), similar to Noviherbaspirillum species [25, 30]. Major polyamines were putrescine and 2-hydroxyputrescine (Fig. S4 and Table S5), similar to other species within the genus Noviherbaspirillum [25, 29, 30]. Phosphatidylethanolamine and phosphatidylglycerol were commonly detected major polar lipids in strains TSA40<sup>T</sup>, TSA66<sup>T</sup> and <i>H. massiliense</i> DSM 25712<sup>T</sup> (Fig. S5). Similar to the previous report [29], the presence of phosphatidyldicholine varied between strains: it was only detected in strains TSA66<sup>T</sup> and TSO23-1. While unknown amino lipids and unknown lipids were commonly present in strains TSA66<sup>T</sup> and TSO23-1, diphosphatidylglycerol was only detected in strain TSO23-1. In addition, small amounts of phosphatidylinositol and an unknown aminophospholipid were detected only in <i>H. massiliense</i> DSM 25712<sup>T</sup>. Unknown glycolipids were detected only in strain TSA40<sup>T</sup>, whereas an unknown phospholipid was detected in strain TSA66<sup>T</sup> and <i>H. massiliense</i> DSM 25712<sup>T</sup>. Unknown amino phospholipids were detected in strain TSA40<sup>T</sup> and <i>H. massiliense</i> DSM 25712<sup>T</sup>.

Based on the 16S rRNA gene sequence analysis in combination with physiological, chemotaxonomic and genomic properties, strains TSA40<sup>T</sup> (=JCM 17722<sup>T</sup>=ATCC TSD-69<sup>T</sup>) and TSA66<sup>T</sup> (=JCM 17723<sup>T</sup>=DSM 25877<sup>T</sup>) are the type strains of two novel species within the genus Noviherbaspirillum, for which we propose the names <i>Noviherbaspirillum denitrificans</i> sp. nov. and <i>Noviherbaspirillum autotrophicum</i> sp. nov., respectively. In addition, based on the 16S rRNA gene sequence analysis as well as physiological, chemotaxonomic and comparative genome analysis, we propose to reclassify <i>Herbaspirillum massiliense</i> as <i>Noviherbaspirillum massiliense</i> comb. nov.

**DESCRIPTION OF NOVIHERBASPIRILLUM AUTOTROPHICUM SP. NOV.**

<i>Noviherbaspirillum autotrophicum</i> (au.to.tro’phi.cum. Gr. pronoun autos self; Gr. adj. trophikos nursing, tending, or feeding; N.L. adj. autotrophicus self-nursing or self-feeding, i.e. able to grow with carbon dioxide as a sole carbon source).

Cells are Gram-stain-negative, facultatively anaerobic, motile, slightly curved rods. Cells have one or two polar flagella. Colonies formed on R2A agar medium are smooth, circular, white and convex. Cells are 0.4–0.6 μm in diameter and 1.4–2.2 μm in length. Cells are positive for catalase and oxidase activity, but negative for urease activity. Cells can grow at 10–42 °C (optimum 30 °C) and at pH 5.5–9.0. No growth occurs in the presence of 1% (w/v) NaCl on 100-fold diluted nutrient broth supplemented with 3 mM nitrate and 4.4 mM succinate. Cells can grow both aerobically and anaerobically with nitrate as an electron acceptor. Preferred organic sources are low-molecular-weight organic acids such as acetate, butyrate, lactate, pyruvate, succinate and valerate and their polymers such as Tween 40, Tween 80 and poly(3-hydroxybutyric acid-co-3-hydroxyvaleric acid). Sugars such as fructose, galactose, glucose, lactose, maltose, mannose, sorbitol, rhamnose, sorbitol and xylose, sugar acids such as gluconate, alcohols such as butanediol and ethanol, and polysaccharides such as cellulose and starch do not support growth. The major cellular fatty acids (>10% of total fatty acids) are C<sub>16:0</sub>, cyclo C<sub>17:0</sub>, C<sub>18:1ω7c</sub> and summed feature 3 (C<sub>16:1ω7c/iso-C15 2-OH</sub>). The predominant ubiquinone is Q-8. Major polar lipids are phosphatidylethanolamine and phosphatidylglycerol. Small amounts of an unknown aminophospholipid, an unknown phospholipid and unknown glycolipids are detected.

The type strain, TSA40<sup>T</sup> (=JCM 17722<sup>T</sup>=ATCC TSD-69<sup>T</sup>), was isolated from rice paddy soil originally collected from Tokyo, Japan. The DNA G+C content of the type strain is 61.8% (as determined by draft genome sequencing).
The type strain, TSA66<sup>T</sup> (=JCM 17723<sup>T</sup>=DSM 25787<sup>T</sup>), was isolated from rice paddy soil originally collected from Tokyo, Japan. The DNA G+C content of the type strain is 59.9 % (as determined by draft genome sequencing).

**DESCRIPTION OF NOVIHERBASPIRILLUM MASSILIENSE COMB. NOV.**

Noviherbaspirillum massiliense (mas.si.li.en’se. N.L. neut. adj. massiliense of Massilia, the Latin name of Marseille where the type strain was cultivated).

Basonym: Herbaspirillum massiliense (Lagier et al. 2014).

Cells are rod-shaped with a mean diameter of 0.44 μm. Motile with tufts of polar flagella. Optimal growth occurs under aerobic conditions. Weak growth is observed under microaerophilic conditions and with 5 % CO<sub>2</sub>. No growth is observed under anaerobic conditions. Growth occurs at 30–37 °C, with optimal growth at 37 °C. Catalase, oxidase and arginine dihydrolase activities, as well as aesculin hydrolysis are present. Nitrate reduction and indole production are absent. Preferred organic sources are low-molecular-weight organic acids such as acetate, butyrate and succinate, but not pyruvate. Sugars such as fructose, galactose, glucose, lactose, maltose, mannose, sorbitol, rhamnose, sorbitol and xylose, sugar acids such as gluconate, and alcohols such as butanediol, ethanol and glycerol do not support growth. xylose, sugar acids such as gluconate, and alcohols such as butanediol, ethanol and glycerol do not support growth. Sugars such as fructose, galactose, glucose, lactose, maltose, mannose, sorbitol, rhamnose, sorbitol and xylose, sugar acids such as gluconate, and alcohols such as butanediol, ethanol and glycerol do not support growth. Sugars such as fructose, galactose, glucose, lactose, maltose, mannose, sorbitol, rhamnose, sorbitol and xylose, sugar acids such as gluconate, and alcohols such as butanediol, ethanol and glycerol do not support growth.

The type strain, JC206<sup>T</sup> (=CSUR P159<sup>T</sup>=DSM 25712<sup>T</sup>), was isolated from the faecal flora of a healthy patient in Senegal. The DNA G+C content of the type strain is 59.73 % (as determined by draft genome sequencing).

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**Ethical statement**

No experiment was conducted with human or animal subjects.

**References**


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