Nitrolancea hollandica gen. nov., sp. nov., a chemolithoautotrophic nitrite-oxidizing bacterium isolated from a bioreactor belonging to the phylum Chloroflexi

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A novel nitrite-oxidizing bacterium (NOB), strain LbT, was isolated from a nitrifying bioreactor with a high loading of ammonium bicarbonate in a mineral medium with nitrite as the energy source. The cells were oval (lancet-shaped) rods with pointed edges, non-motile, Gram-positive (by staining and from the cell wall structure) and non-spore-forming. Strain LbT was an obligately aerobic, chemolithoautotrophic NOB, utilizing nitrite or formate as the energy source and CO2 as the carbon source. Ammonium served as the only source of assimilated nitrogen. Growth with nitrite was optimal at pH 6.8–7.5 and at 40 °C (maximum 46 °C). The membrane lipids consisted of C20 alkyl 1,2-diols with the dominant fatty acids being 10MeC18 and C18:1 ν9. The peptidoglycan lacked meso-DAP but contained ornithine and lysine. The dominant lipoquinone was MK-8. Phylogenetic analyses of the 16s rRNA gene sequence placed strain LbT into the class Thermomicrobia of the phylum Chloroflexi with Sphaerobacter thermophilus as the closest relative. On the basis of physiological and phylogenetic data, it is proposed that strain LbT represents a novel species of a new genus, with the suggested name Nitrolancea hollandica gen. nov., sp. nov. The type strain of the type species is LbT (=DSM 23161T=UNIQEM U798T).

Lithoautotrophic nitrite-oxidizing bacteria (NOB) are important players in the global nitrogen cycle, closing the oxidative branch. So far, NOB have been found in three bacterial phyla: Proteobacteria (alpha, beta, and gamma), Nitrospirae (Speick & Bock, 2005) and Nitrosinae (Lücker et al., 2013). Recently we have isolated an NOB strain, which was dominant in a lab-scale nitrifying bioreactor, with an unusual phylogenetic position within the phylum Chloroflexi (Sorokin et al., 2012). This was also the first nitrifying prokaryote with a Gram-positive cell wall and is described here as a new genus and species.

Strain LbT was isolated from a nitrifying bioreactor with a high loading of ammonium bicarbonate as the only substrate, which was oxidized to nitrate via nitrite by a consortium of aggregated Nitrosomonas and an unknown NOB (Vejmelkova et al., 2012). The reactor was inoculated with the sludge of a full-scale nitritation bioreactor operating...
in Rotterdam-Sluisjedijk WWTP. The isolation mineral medium contained 0.2 g l\(^{-1}\) KH\(_2\)PO\(_4\) and 50 mg l\(^{-1}\) CaCl\(_2\) sterilized in closed bottles with 10 % liquid and the gas phase consisting of air/CO\(_2\) (90:10, v/v). After sterilization the medium was supplemented with 1 ml l\(^{-1}\) trace metal solution (Pfenning & Lippert 1966), 0.2 g l\(^{-1}\) MgSO\(_4\), 0.4 g l\(^{-1}\) filter-sterilized NH\(_4\)HCO\(_3\) and 5–50 mM filter-sterilized KNO\(_2\). For mixotrophic growth, 40 mM sodium formate was added. The pH was maintained at 6.9–7.4 by adding CO\(_2\) into the gas phase. The bottles were incubated on a rotary shaker at 100–120 r.p.m. at 37 °C. Solid medium was prepared by 1:1 mixing of double-strength liquid medium and 4 % (v/v) washed Noble agar at 50 °C. The plates were incubated in closed jars containing air/CO\(_2\)/N\(_2\) (50:10:40, v/v) and 4 % (v/v) washed Noble agar at 50 °C. Growth was followed by measurements of OD\(_{600}\) cell protein (Lowry et al., 1951), nitrite consumption (Griess-Romijn van Eck, 1966) and nitrate formation (Bhandari & Simlat, 1986). Formate consumption was analysed by HPLC anionic chromatography (column Aminex HPx-87H, 300 × 7.8 mm, t = 60 °C, flow rate 0.6 ml min\(^{-1}\), eluent 1.5 mM H\(_3\)PO\(_4\); detector – RI).

Phase-contrast microphotographs were obtained using a Zeiss Axiosplan Imaging 2 microscope (Göttingen). For total cell electron microscopy, cells were fixed with glutaraldehyde (final concentration 3 %, v/v) for 2 h at 4 °C, washed with 10 mM potassium-phosphate buffer, pH 7, and positively contrasted with 1 % (w/v) neutralized phosphotungstic acid. For thin sectioning, the cells were fixed in 1 % (w/v) OsO\(_4\) for 24 h at room temperature, washed, stained overnight with 1 % (w/v) uranyl acetate, dehydrated in an increasing ethanol series, and embedded in Epon resin. Thin sections were stained with 1 % (w/v) lead citrate.

Strain Lb\(^T\) was isolated on mineral medium with nitrite as the only energy source after optimization of the growth medium by supplementation with ammonium bicarbonate (5 mM) as an additional N-source. Consistently, later genome analysis (Sorokin et al., 2012) indicated that the organism, in contrast to all known NOB, lacked the potential to utilize N-oxides as an N-source. The isolation approach included several rounds of serial dilutions in liquid medium followed by colony isolation from solid medium. The final purified culture was checked for heterotrophic contaminants using the same medium without nitrite and supplemented with 200 mg l\(^{-1}\) yeast extract.

The isolate had a highly characteristic cell morphology that was different from all known NOB species (Fig. 1a–c). Gram-staining and thin sectioning revealed a Gram-stain-negative type cell wall (Fig. 1d, e), which makes strain Lb\(^T\) unique among the functional group of NOB. The intracytoplasmic membranes (ICM), typical for the genera 

\textit{Nitrooccus}, were clearly absent from strain Lb\(^T\). Carboxysome-like structures were not observed under the growth conditions used. The ultrastructure of cells grown mixotrophically with formate and nitrite was, in general, similar to the cells grown chemolithoautotrophically with nitrite except that many mixotrophic cells contained small mesosome-like membrane vesicles in the polar regions (Fig. S1, available in the online Supplementary Material).

Structural characterization included membrane lipid and cell-wall peptidoglycan analyses. The polar lipids were extracted from cell material of strain Lb\(^T\) by Bligh-Dyer extraction and analysed further by liquid chromatography-mass spectrometry as described previously (Kulichevskaya et al., 2012). The predominant membrane lipids were uncommon and comprised C\(_{20}\) alkyl 1,2-diols with a polar head group (sugar or phosphorus-sugar moiety) attached to the first OH group, whereas the second OH group was predominantly esterified with 12 methyl-octadecanoic acid (12Me-C\(_{17}:0\)). Similar glycolipids have only been identified in thermophilic members of the phylum Chloroflexi including \textit{Thermomicrobium roseum}, a relative of \textit{Nitrolancea} (see below) and \textit{Roseiflexus castenholzii}, and in members of the genus \textit{Thermus} (Pond et al., 1986; Pond & Langworthy, 1987; van der Meer et al., 2002; Wait et al., 1997).

Respiratory lipoquinones were extracted with cold acetone from cells disrupted by grinding in liquid N\(_2\) and further separated by thin-layer chromatography (TLC). The excised bands were analysed by tandem mass spectrometry (LC ADVANTAGE MAX) and the compounds were identified from their ionized masses. The dominant quinone species was identified as MK-8.

For peptidoglycan analysis, the cell-wall fraction was obtained and analysed according to Streshinskaya et al. (1979). A cell suspension in 1 % (w/v) SDS was disrupted by sonication and heated briefly (100 °C, 5 min). Cell walls were separated by fractional centrifugation and purified using trichloroacetic acid and trypsin. Quantitative determination of cell-wall amino acids was performed with a LC 600 E amino acid analyser (Biotronic) after acid hydrolysis (6 M HCl, 100 °C, 18 h). Isomers of diaminopimelic acid (DAP) were determined by TLC on cellulose (modified method of Hasegawa et al., 1983). The results demonstrated the absence of meso-DAP in the murein, a diagnostic di-amino acid characteristic for all Gram-negative bacteria. Instead, the di-amino acids ornithine and lysine were present, along with D-glutamic acid and alanine, which is characteristic of Gram-positive bacteria (Schleifer & Kandler, 1972). This result, taken together with the ultrastructural organization, classical Gram-staining results, and the absence of genes coding for lipopolysaccharide synthesis (as indicated by genomic information; Sorokin et al., 2012) indicate that the novel NOB, in contrast to all nitrifiers known so far, is a Gram-positive bacterium.

Growth was observed under autotrophic conditions with nitrite as the electron donor. Strain Lb\(^T\) tolerated up to 75 mM nitrite, which was stoichiometrically oxidized to nitrate (Fig. 2). None of the organic compounds tested (yeast extract, pyruvate, organic acids, sugars, alcohols) stimulated growth in the presence or absence of nitrite. The
only substrate utilized by strain LbT was formate, but it was only used as a co-substrate after cells were pre-grown with nitrite (Fig. 2), similar to what was described for *Nitrobacter vulgaris* (Bock et al., 1990). The novel NOB also resembled strains of species of the genus *Nitrobacter* with respect to its tolerance of high nitrite concentrations (Van Gool & Laudelout, 1966). However, the growth temperature profile of strain LbT resembled some of the representative species of the genus *Nitrospira* as it was able to grow at up to 46 °C (optimum 40 °C). Furthermore, in contrast to most species of the genus *Nitrobacter* (Spieck & Bock, 2005) strain LbT was not able to grow anaerobically with nitrite or nitrate as the electron acceptor (with formate and pyruvate tested as electron acceptors). The genomic analysis (Sorokin et al., 2012) also indicated CO to be a potential alternative electron donor, since a functional \( \text{cox} \) operon encoding the type II aerobic CODH was present. However, neither growth nor consumption of CO was observed in growing cultures or washed cells of strain LbT. Autotrophic carbon fixation of strain LbT is mediated by the Calvin-Benson cycle. It has a distinct RuBisCO-type related to the ‘red’ form I, which is also found in some Gram-positive facultative autotrophs and members of the only genus of the phylum *Chloroflexi* utilizing the Calvin cycle, *Oscillochloris* (Sorokin et al., 2012).
Fig. 2. Growth dynamics of strain LbT\textsuperscript{T} in autotrophic (open symbols) and mixotrophic (closed symbols) cultures. The autotrophic culture was grown on 50 mM nitrite; the mixotrophic culture received 50 mM nitrite and 40 mM formate. Circles, biomass; diamonds, nitrite; crosses, nitrate in autotrophic culture; triangles, formate.

The isolation of DNA and the subsequent determination of the G+C content was performed according to Marmur (1961) and Marmur & Doty (1962), respectively. The DNA G+C content of strain LbT\textsuperscript{T} was 62.6 mol% consistent with whole genome analysis (Sorokin et al., 2012).

Phylogenetic analyses of 16S rRNA gene sequences were performed within ARB software (Ludwig et al., 2004), as described elsewhere (Daims et al., 2001). Applied treeing methods were tree-puzzle [Version 5.0 (Schmidt et al., 2003)], maximum-parsimony (PHYLIP version 3.66 with 100 bootstrap iterations), maximum-likelihood [RAxML (Stamatakis et al., 2005) with 100 bootstrap iterations], and Bayesian inference using MrBayes [(Ronquist and Huelsenbeck 2003) run for 5 000 000 generations]. Only 16S rRNA gene sequences longer than 1300 nt were used in tree calculations. These analyses placed the novel NOB strain into the class Thermomicrobia of the phylum Chloroflexi and thus into a phylum not known or expected to contain autotrophic NOB (Fig. 3). The closest relative of strain Lb\textsuperscript{T} (with a sequence similarity of 93 %) was Sphaerobacter thermophilus, a moderately thermophilic heterotroph from a high-temperature wastewater treatment plant originally assigned to Actinobacteria (Demharter et al., 1989) and later reclassified as a member of the class Thermomicrobia within the phylum Chloroflexi (Hugenholtz & Stackebrandt, 2004). Among the few 16S rRNA gene sequences available, one sequence obtained from a Sharon bioreactor (converting ammonium to nitrite; GenBank accession number JN087902) in South Korea apparently belongs to the same organism as described here. The natural habitat of this NOB species is unclear to date, as it has not been observed outside

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**Fig. 3.** Phylogenetic affiliation of strain Lb\textsuperscript{T} based on 16S rRNA gene sequence analysis. 16S rRNA gene-based Bayesian inference tree (sd=0.004631) showing the affiliation of strain Lb\textsuperscript{T} (boldface) with the class Thermomicrobia (dark grey box) of the phylum Chloroflexi. Other classes of the phylum are outlined (medium dark and light grey boxes). Pie charts indicate statistical support of nodes based on bootstrap analysis or Bayesian inference. Conflicting topologies among the different methods are also indicated. The arrow indicates the position of the outgroup. Bar, 10 % estimated sequence divergence. MB, Bayesian inference; ML, maximum-likelihood; MP, maximum-parsimony; TP, Treepuzzle.
Table 1. Comparative properties of strain LbT, its closest phylogenetic relative and other known lithotrophic NOB

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
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</thead>
<tbody>
<tr>
<td>Cell morphology and size (µm)</td>
<td>Rods with tapered edges, 1–1.2 × 2–4</td>
<td>Coccoid rods, 1–1.5 × 1.5–3</td>
<td>Budding rods, 0.5–0.9 × 1–2</td>
<td>Cocci, 1.5–1.8</td>
<td>Coccoid rods, 0.4–0.7 × 1.0</td>
<td>Pleomorphic rods to spirilla, 0.2–0.4 × 0.9–2.2</td>
<td>Spindle rods, 0.2–0.4 × 0.9–2.2</td>
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<td>+</td>
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<td>–</td>
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<td>–</td>
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<tr>
<td>Cell wall type</td>
<td>G+</td>
<td>G+</td>
<td>G−</td>
<td>G−</td>
<td>G−</td>
<td>G−</td>
<td>G−</td>
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<tr>
<td>Intracytoplasmic membranes</td>
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<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
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<td>–</td>
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<td>Cytoplasm</td>
<td>Cytoplasm</td>
<td>Cytoplasm</td>
<td>Periplasm</td>
<td>Periplasm</td>
<td>Periplasm</td>
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<td>Major respiratory lipoquinone</td>
<td>MQ−8</td>
<td>MQ−8</td>
<td>UQ</td>
<td>C18:1 cis 11</td>
<td>C18:1 cis 11, C16:0 cis 9</td>
<td>C16:0, C16:1 cis 9 (11)</td>
<td>C16:1 cis 9, C14:0</td>
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<td>10MeC18</td>
<td>ND</td>
<td>C18:1 cis 11</td>
<td>C18:1 cis 11, C16:0 cis 9</td>
<td>C16:0, C16:1 cis 9</td>
<td>C16:1 cis 9, C14:0</td>
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<td>a, c</td>
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<td>a, c</td>
<td>a, c</td>
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<td>b, c</td>
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<td>NOB</td>
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<td>Carbon fixation pathway</td>
<td>Calvin cycle (Cbb ‘red’ type)</td>
<td>Calvin cycle (Cbb ‘green’ type)</td>
<td>Calvin cycle</td>
<td>Calvin cycle</td>
<td>ND</td>
<td>Reversed TCA</td>
<td>Reversed TCA</td>
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<td>Mixotrophy</td>
<td>+ (formate)</td>
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<td>+ (formate)</td>
<td>–</td>
<td>–</td>
<td>–</td>
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<tr>
<td>Anaerobic growth with nitrate</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>μmax with nitrite (h−1)</td>
<td>0.019</td>
<td>ND</td>
<td>0.025–0.058</td>
<td>0.069</td>
<td>ND</td>
<td>0.008–0.058</td>
<td>0.029</td>
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<tr>
<td>Nitrate tolerance (mM)</td>
<td>75</td>
<td>ND</td>
<td>up to 145</td>
<td>60</td>
<td>1.2</td>
<td>6–25</td>
<td>20</td>
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<tr>
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<td>ND</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<tr>
<td>Maximal growth temperature (°C)</td>
<td>46</td>
<td>55</td>
<td>32</td>
<td>&gt;40</td>
<td>22</td>
<td>58</td>
<td>&lt;40</td>
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<tr>
<td>DNA G + C content (mol%)</td>
<td>62.6</td>
<td>66.3</td>
<td>61–62</td>
<td>61.2</td>
<td>ND</td>
<td>50–57</td>
<td>57.7</td>
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<td>Thermomicrobia (Chloroflexi)</td>
<td>Alphaproteobacteria</td>
<td>Betaproteobacteria</td>
<td>Gammaproteobacteria</td>
<td>Nitrospirae</td>
<td>Nitrospinae</td>
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<td>Isolation source</td>
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<td>Bioreactor</td>
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<td>Seawater</td>
<td>Permafrost soil</td>
<td>Thermal springs, seawater, wastewater</td>
<td>Seawater</td>
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</tbody>
</table>
Nitrolancea [Ni.tro.lan’ce.a. L. n. nitrum native soda, natron, nitrate; L. fem. n. lancea a lance; N.L. fem. n. Nitrolancea a nitrate (-forming) lance-shaped bacterium].

A chemolithoautotrophic nitrite-oxidizer that can grow with nitrite and formate. Gram-stain-positive. Thermotolerant, neutrophilic, and with high nitrite tolerance. A member of the class Thermomicrobia within the phylum Chloroflexi of the domain Bacteria. The type species is Nitrolancea hollandica.

**Description of Nitrolancea gen. nov.**

Nitrolancea [Ni.tro.lan’ce.a. L. n. nitrum native soda, natron, nitrate; L. fem. n. lancea a lance; N.L. fem. n. Nitrolancea a nitrate (-forming) lance-shaped bacterium].

Ovoid, sometimes diamond or lancet-shaped rods with pointed edges, 1–1.2 × 2–4 μm. Cells are Gram-stain-positive and the structure of the cell wall is Gram-positive in type. Colonies are circular, smooth, greenish and up to 0.5 mm in diameter after 2 months growth. Grows aerobically with nitrite and nitrite plus formate as the energy source. Nitrate is the sole product of nitrite oxidation. CO₂ is used as a carbon source and ammonium is the sole nitrogen source. The optimal growth temperature is 40 °C, maximum growth temperature is 46 °C and the maximal temperature for activity is 63 °C, with the optimum pH 6.8–7.5. The optimum NO₂⁻ concentration is 5–20 mM, and the maximal tolerated concentration 75 mM. K₈ for nitrate is 1 mM. The membrane lipids consist of C₂₀ alkyl 1,2-diols with the predominant fatty acids 10MeC₁₈ and C₁₈:1. The peptidoglycan structure is of the Gram-positive type (contains ornithine and lysine).

The type strain, LbT (=DSM 23161T=UNIQEM U798T) was isolated from a partial nitritation reactor in Delft (The Netherlands). The DNA G + C content of the type strain is 62.6 mol%.

**References**


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