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

Dimitry Y. Sorokin,[1,2] Dana Vejmelkova,[3] Sebastian Lücker,[4] Galina M. Streshinskaya,[5] W. Irene C. Rijpstra,[6] Jaap S. Sinninghe Damsté,[6] Robbert Kleerbezem,[2] Mark van Loosdrecht,[2] Gerard Muyzer[7] and Holger Daims[4]

Correspondence
Dimitry Y. Sorokin
soroc@inmi.ru

[1]Winogradsky Institute of Microbiology RAS, Moscow, Russia

[2]Department of Biotechnology, TU Delft, The Netherlands

[3]Department of Water Technology, ICT Prague, Czech Republic

[4]Department of Microbial Ecology, Ecology Centre, University of Vienna, Vienna, Austria

[5]Microbiology Department, Faculty of Biology, Lomonosov Moscow State University, Moscow, Russia

[6]Department of Marine Organic Biogeochemistry, NIOZ Royal Netherlands Institute for Sea Research, Den Burg, The Netherlands

[7]Department of Aquatic Microbiology, Institute for Biodiversity and Ecosystem Dynamics, University of Amsterdam, Amsterdam, The Netherlands

A novel nitrite-oxidizing bacterium (NOB), strain Lb[T], 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 Lb[T] was an obligately aerobic, chemolitoautotrophic NOB, utilizing nitrite or formate as the energy source and CO_2 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 C_{20} alkyl 1,2-diols with the dominant fatty acids being $10MeC_{18}$ and $C_{18:1\omega9}$. 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 Lb[T] 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 Lb[T] 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 Lb[T] (=DSM 23161[T]=UNIQEM U798[T]).

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* (Spieck & Bock, 2005) and *Nitrospinae* (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 Lb[T] 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 nitration bioreactor operating

in Rotterdam-Sluisjesdijk 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 (Pfennig & 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, by vol.) in the gas phase. 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 Axioplan 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 NOBs, 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-positive 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 Nitrobacter and Nitrococcus, 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 Thermomicrobium roseum, a relative of Nitrolancea (see below) and Roseiflexus castenholzii, and in members of the genus Thermus (Pond et al., 1986; Pond & Langworthy, 1987; van ver 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 (LCQ 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
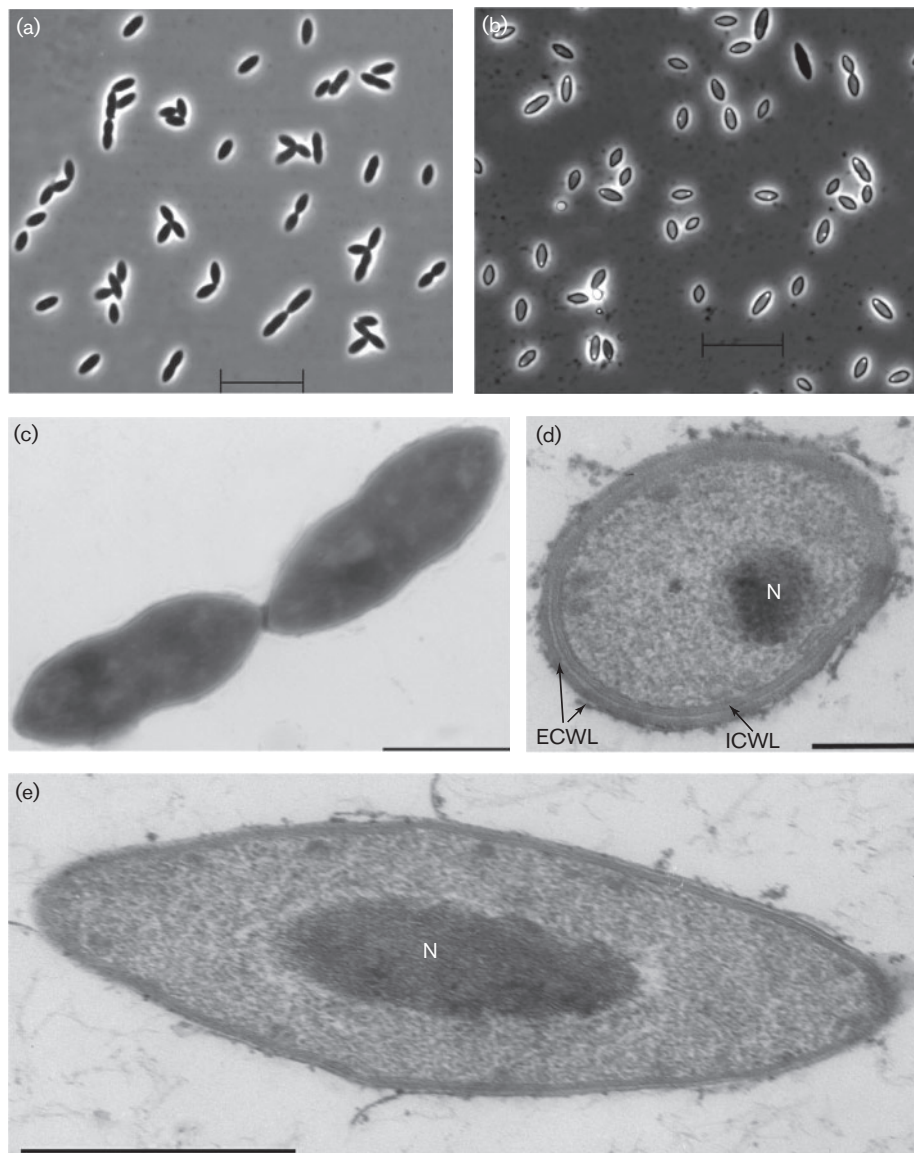
Fig. 1. Cell morphology of strain Lb^T. Phase-contrast microphotographs of cells grown autotrophically (a) and mixotrophically (b); electron micrograph of total cells (c); perpendicular (d) and longtitudal (e) thin sections of the nitrite-grown cells showing the ultrastructural organization (multilayered cell envelope and unusually condensed nucleoid). N, Nucleoid; ECWL external cell wall layers; ICWL, internal cell wall layers. Bars, 10 μm (a, b), 1 μm (c), 0.2 μm (d) and 0.5 μm (e).

only substrate utilized by strain Lb^T 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 Lb^T 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 Lb^T 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 *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 Lb^T. Autotrophic carbon fixation of strain Lb^T 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).
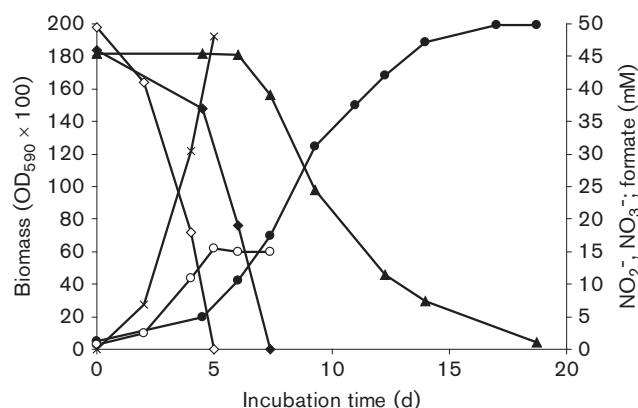
Fig. 2. Growth dynamics of strain Lb^T in autotrophic (open symbols) and mixotrophic (closed symbols) cultures. The autotrophic culture was grown on 50 mM nitrite; the mixotrophic culture received 50 mM nitrite and 40 mM formate. Circles, biomass; diamonds, nitrite; crosses, nitrate in autotrophic culture; 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 Lb^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^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
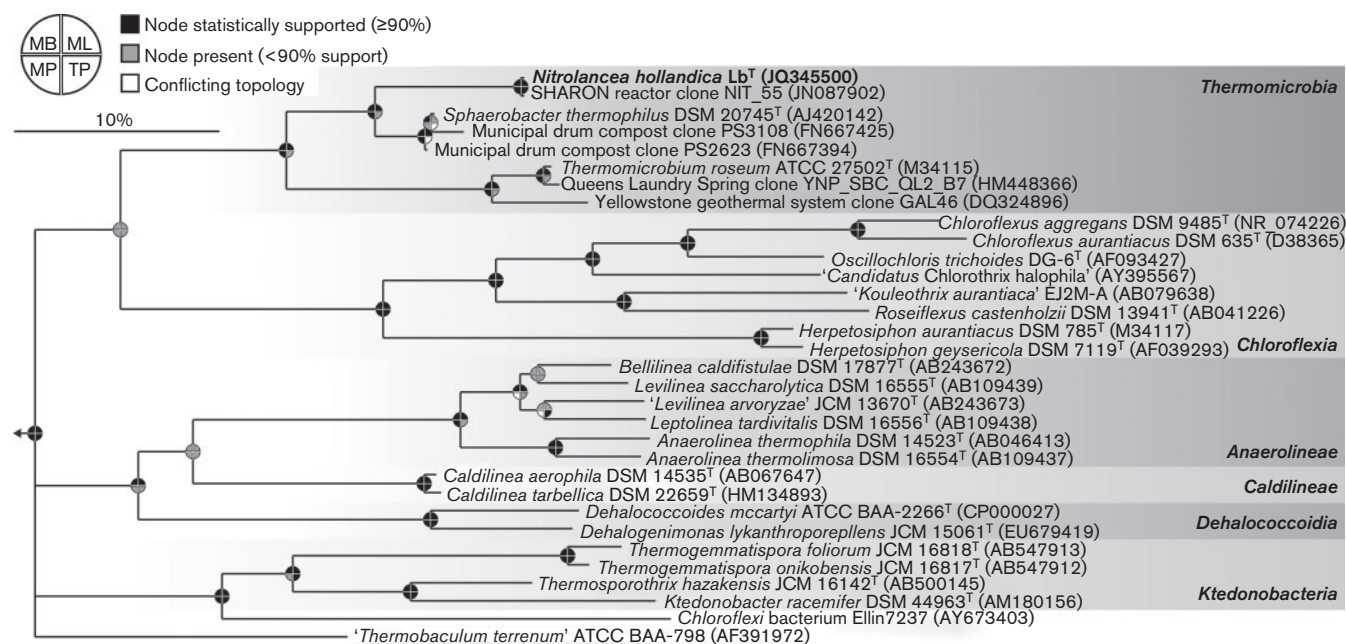

Fig. 3. Phylogenetic affiliation of strain Lb^T based on 16S rRNA gene sequence analysis. 16S rRNA gene-based Bayesian inference tree (SD=0.004631) showing the affiliation of strain Lb^T (boldface) with the class *Thermomicrobia* (dark grey box) of the phylum *Chloroflexi*. Other classes of the phylum 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 Lb[T], its closest phylogenetic relative and other known lithotrophic NOB

Taxa: 1, strain Lb[T] (data from this study); 2, *Sphaerobacter thermophilus* strain DSM20745[T] (Demharter *et al.*, 1989); 3, *Nitrobacter* (*n*=4) (Spieck & Bock, 2005; Spieck & Lipski, 2011); 4, *Nitrococcus mobilis* strain ATCC25380[T] (Spieck & Bock, 2005; Watson & Waterbury, 1971); 5, 'Nitrotoga arctica' (Alawi *et al.*, 2007); 6, *Nitrospira* (*n*=4) (Spieck & Bock, 2005; Spieck & Lipski, 2011); 7, *Nitrospina gracilis* strain ATCC25379[T] (Spieck & Bock, 2005; Watson & Waterbury, 1971; Lücker *et al.*, 2013). +, Positive; –, negative; ND, not detected; G[+], Gram-stain-positive; G[−], Gram-stain-negative; NXR, nitrite oxido-reductase; PLFA, polar lipid fatty acids.

Characteristic	1	2	3	4	5	6	7
Cell morphology and size (µm)	Rods with tapered edges, 1–1.2 × 2–4	Coccoid rods, 1–1.5 × 1.5–3	Budding rods, 0.5–0.9 × 1–2	Cocci, 1.5–1.8	Coccoid rods, 0.4–0.7 × 1.0	Pleomorphic rods to spirilla, 0.2–0.4 × 0.9–2.2	Spindle rods, 0.2–0.4 × 0.9–2.2
Motility	–	–	+	+	–	–	–
Cell wall type	G[+]	G[+]	G[−]	G[−]	G[−]	G[−]	G[−]
Intracytoplasmic membranes	–	–	+	+	–	–	–
Location of NXR	Cytoplasm		Cytoplasm	Cytoplasm	Periplasm	Periplasm	Periplasm
Major respiratory lipoquinone	MQ–8	MQ-8	UQ	ND	ND		ND
Dominant PLFA	$10MeC_{18}$	ND	$C_{18:1}$ *cis* 11	$C_{18:1}$ *cis* 11, $C_{16:0}$, $C_{16:1}$ *cis* 9	$C_{16:0}$, $C_{16:1}$ *cis* 9	$C_{16:0}$, $C_{16:1}$ *cis* 7 (11)	$C_{16:1}$ *cis* 9, $C_{14:0}$
Dominant cytochromes	a, c	ND	a, c	a, c	ND	b, c	c
Catabolic type	NOB	Organotroph	NOB	NOB	NOB	NOB	NOB
Carbon fixation pathway	Calvin cycle (Cbb 'red' type)	Heterotroph	Calvin cycle (Cbb 'green' type)	Calvin cycle (Cbb 'green' type)	ND	Reversed TCA	Reversed TCA
Mixotrophy	+ (formate)	ND	+ (formate)	–	–	–	–
Anaerobic growth with nitrate	–	–	+	–	–	–	–
μ_{max} with nitrite (h^{-1})	0.019	ND	0.025–0.058	0.069	ND	0.008–0.058	0.029
Nitrite tolerance (mM)	75	ND	up to 145	60	1.2	6–25	20
Nitrite as N-source	–	ND	+	+	+	+	+
Maximal growth temperature (°C)	46	55	32	>40	22	58	<40
DNA G+C content (mol%)	62.6	66.3	61–62	61.2	ND	50–57	57.7
Affiliation	*Thermomicrobia* (*Chloroflexi*)	*Thermomicrobia* (*Chloroflexi*)	*Alphaproteobacteria*	*Betaproteobacteria*	*Gammaproteobacteria*	*Nitrospirae*	*Nitrospinae*
Isolation source	Bioreactor	Bioreactor	Natural soils and waters, wastewater	Seawater	Permafrost soil	Thermal springs, seawater, wastewater	Seawater

bioreactors, not even in large-scale molecular studies utilizing 16S rRNA tag sequencing or metagenomics.

The novel NOB isolate differs significantly from all known NOBs (Table 1) due to its unusual phylogenetic position, cell architecture, and polar lipid composition. Therefore, we propose strain Lb[T] represents a novel species of a new genus, *Nitrolancea hollandica* gen. nov., sp.nov., within the phylum *Chloroflexi*.

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].

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 hollandica* sp. nov.

Nitrolancea hollandica (hol.lan′di.ca. N.L. fem. n. *hollandica* from Holland, pertaining to the country of origin).

Ovoid, sometimes diamond or lancet-shaped rods with pointed edges, $1–1.2 \times 2–4$ 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. CO_2 is used as a carbon source and ammonium (but not nitrite or nitrate) as a 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 NO_2^- concentration is 5–20 mM, and the maximal tolerated concentration 75 mM. K_S for nitrite is 1 mM. The membrane lipids consist of C_{20} alkyl 1,2-diols with the predominant fatty acids $10MeC_{18}$ and $C_{18:1}\omega 9$. The dominant respiratory lipoquinone is MK-8. The peptidoglycan structure is of the Gram-positive type (contains ornithine and lysine).

The type strain, Lb[T] (=DSM 23161[T]=UNIQEM U798[T]) was isolated from a partial nitritation reactor in Delft (The Netherlands). The DNA G+C content of the type strain is 62.6 mol%.

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