Description of *Nitrospira inopinata* sp. nov.

Nitrospira inopinata (in.o.pi.na'ta. L. fem. adj. *inopinata* unexpected, surprising). Cells are tightly coiled spirals with a diameter of 0.2 to 0.3 µm and a length of 0.7 to 1.7 µm, which grow both planktonically and on surfaces. Like other *Nitrospira* species, *N. inopinata* often grows in aggregates or flocs. Surface growth is characterized by a transparent biofilm. *N. inopinata* is motile and exhibits a single polar flagellum[1]. It is a chemolithoautroph, which oxidizes ammonia via nitrite to nitrate and fixes CO_2 through the reductive tricarboxylic acid cycle for cellular energy and carbon, respectively[1]. Ammonium is required for cellular growth, as *N. inopinata* does not appear to be able to assimilate nitrogen from nitrite[1]. Growth of *N. inopinata* is totally inhibited in medium containing >16 mM NH_4^+. Utilization of organic substrates has not been observed. The tested temperature range for growth is 28 to 50 °C with the optimum at 37 °C. Phylogenetically (based on 16S rRNA, NxrA protein, and *nxr*B gene sequences), *N. inopinata* belongs to the widely distributed lineage II of the genus *Nitrospira*, which were previously believed to solely contain strictly nitrite-oxidizing bacteria[1].

Supplementary Discussion

Genome-based hypotheses on the niche specialization of *Nitrospira inopinata*

The annotation of the completely sequenced genome of *N. inopinata* has generated interesting insights into the ecological niche specialization and possible alternative lifestyles of this comammox organism. These hypotheses are based on *in silico* comparisons of predicted *N. inopinata* gene products to their homologs in other organisms and will need experimental verification to enable final conclusions on the ecophysiology of *N. inopinata*. Putative coding sequences (CDS) were detected and functions of the encoded proteins were predicted using the MicroScope genome annotation platform[2]. The annotated contig is available at the ENA under project accession PRJEB10818.

Aside from dissolved ammonium, urea is an additional source of ammonia for nitrification and a source of nitrogen for growth by *N. inopinata*[1]. A gene cluster for the utilization of urea

contains the genes of an ATP binding cassette (ABC) transporter for urea, the *ureABC* genes of the three subunits of a nickel-containing urease, and the *ureFGD* genes of accessory proteins for urease maturation[3]. Genes of urea transporters and urease occur also in canonical nitrite-oxidizing *Nitrospira* members, where they enable a 'reciprocal feeding' interaction with canonical ammonia oxidizers and function in the acquisition of nitrogen for assimilation[4]. The nitrite-oxidizing *Nitrospira* possess cyanase[5], which is lacking in *N. inopinata* as in most other characterized ammonia oxidizers. Thus, *N. inopinata* is not able to use cyanate as a source of energy or nitrogen.

N. inopinata shares key metabolic pathways with genome-sequenced, canonical nitrite-oxidizing *Nitrospira*. These core functions include chemolithotrophic nitrite oxidation by NXR that is associated with the cytoplasmic membrane and whose catalytic site is located in the periplasmic space, the electron transport chain from NXR to oxygen, and autotrophic carbon fixation *via* the reductive tricarboxylic acid (rTCA) cycle[4,6]. The rTCA cyle is more common in anaerobic than in aerobic autotrophs, because its key enzymes 2-oxoglutarate:ferredoxin oxidoreductase and pyruvate:ferredoxin oxidoreductase are oxygen sensitive[7]. Utilization of this pathway implies that *Nitrospira* likely are inhibited by high oxygen tensions, although they possess distinct five-subunit forms of the aforementioned enzymes[6], which were shown to be relatively oxygen resistant and enable the rTCA cycle under microoxic conditions in other bacteria[8,9]. In addition, the *N. inopinata* genome lacks any CDS coding for a known form of superoxide dismutase (SOD), whereas it contains genes of catalase, thioredoxin-dependent peroxiredoxins, and cytochrome (cyt.) *c* peroxidases that likely enable H_2O_2 detoxification. The absence of SOD is highly unusual in aerobic organisms but not uncommon in the genus *Nitrospira*, as the canonical nitrite oxidizer *Nitrospira defluvii* also lacks SOD and also catalase[6]. Inhibition of the rTCA cycle by high oxygen levels and a limited enzymatic repertoire for defense against reactive oxygen species might explain why *Nitrospira* members, including *N. inopinata*, do not grow on the surface of solid media exposed to air and tend to form flocs and biofilms in liquid cultures, which may offer protection from high dissolved oxygen concentrations in the bulk medium[6]. A lifestyle of *N. inopinata* in microcolonies and sessile biofilms could be supported by a number of CDS coding for proteins involved in the assembly of type IV pili, whose functions include bacterial adhesion to surfaces, cell aggregation, and twitching motility[10]. Interestingly, the genome of *N. inopinata* encodes a putative N-acyl-L-homoserine lactone (AHL) synthase (LuxI) and a

transcriptional regulator from the LuxR family, whose genes are adjacent albeit on opposite DNA strands. The LuxR homolog contains both a predicted N-terminal AHL-binding domain and a C-terminal DNA-binding, helix-turn-helix domain, whose presence is indicative for a functional transcriptional regulator in AHL-based quorum sensing (QS)[11]. The presence of both genes in *N. inopinata* is consistent with the recent detection of *luxI* and *luxR* homologous genes on a metagenomic DNA fragment, which probably originated from an uncultured *Nitrospira*-like bacterium, and the confirmed synthesis of AHL by this LuxI following heterologous gene expression in *E. coli*[12]. AHL-based QS is mostly found in Proteobacteria and its occurrence in the genus *Nitrospira*, which belongs to the distinct phylum Nitrospirae[13], is remarkable. This cell-cell communication mechanism is known to regulate bacterial activities and growth modes, such as biofilm formation[14], in response to cell density. The roles of QS in *N. inopinata* remain unknown, but one could speculate that QS may be involved in cell aggregation and biofilm formation by this organism. Adaptations of *N. inopinata* to growth in cell clusters and biofilms are fully consistent with the theoretically predicted ecological niche of comammox organisms within cell aggregates in biofilms, where conditions are characterized by a slow influx of substrates and slow-growing organisms benefit from a high growth yield per consumed substrate[15]. This niche is also consistent with the physiological data from this study (see main text).

At least some canonical nitrite-oxidizing *Nitrospira* are metabolically flexible organisms and can utilize alternative electron donors and energy sources. Aerobic growth of *Nitrospira moscoviensis* on molecular hydrogen (H_2) and on formate was demonstrated[4,16], and use of formate by uncultured *Nitrospira* in activated sludge was shown by FISH-microautoradiography[17]. Interestingly, *N. moscoviensis* can use H_2 or formate and nitrite simultaneously, transferring electrons from these donors to O_2, nitrate, or both at the same time[4,16]. The genome analysis of *N. inopinata* suggests a considerable ecophysiological versatility of this comammox organism, too. The genome encodes a group 3b [NiFe]-hydrogenase, homologs of which are found in diverse bacteria and archaea including *Nitrospina gracilis*, a marine nitrite oxidizer[18]. In *Mycobacterium smegmatis* from soil, a group 3b hydrogenase catalyzes the NAD(P)H-dependent evolution of H_2 during fermentation under oxygen-depleted conditions[19]. The group 3b hydrogenase of the hyperthermophilic archaeon *Pyrococcus furiosus* also acts as sulfhydrogenase, which dissipates reductant from fermentation by reducing elemental sulfur (S^0) or polysulfide to hydrogen sulfide[20]. No

nitrifier has been shown yet to adopt a fermentative metabolism, but we cannot exclude this possibility for *N. inopinata*. Its genome encodes several putative alcohol dehydrogenases, the Embden–Meyerhof–Parnas pathway for glycolysis, and a putative ABC transporter for sugars that might be substrates for fermentation. *N. inopinata* also possesses the gluconeogenesis pathway and the capability to form and degrade glycogen, a carbohydrate storage compound that may be respired under oxic conditions or be fermented in hypoxia and anoxia. Dissipation of excess reductant as H_2 by the group 3b hydrogenase could then support fermentation as a strategy of *N. inopinata* to survive oxygen depletion, as was shown previously for *M. smegmatis*[19]. The group 3b hydrogenase of *P. furiosus* can also oxidize hydrogen[21] and is to some extent oxygen-tolerant[22]. We cannot exclude the possibility that the homologous enzyme of *N. inopinata* also catalyzes the $NAD(P)^+$ dependent oxidation of H_2 and might enable *N. inopinata* to use H_2 as a source of electrons and energy. In *N. moscoviensis*, aerobic hydrogenotrophic growth is enabled by an oxygen-tolerant group 2a [NiFe]-hydrogenase[16]. Another potential electron donor for *N. inopinata* is hydrogen sulfide. The genome codes for a putative sulfide:quinone oxidoreductase (Sqr), a membrane-associated enzyme that would oxidize H_2S or S^{2-} to polysulfide chains or elemental sulfur and transfer the electrons into the quinone pool[23]. For respiration, *N. inopinata* is able to use O_2, nitrate, and nitrite as terminal electron acceptors. Since the genome encodes a complete respiratory chain with complexes I to V[1], electrons derived from low-potential donors like H_2 and from organic compounds like glycogen could be transferred to O_2 for proton motive force generation and oxidative phosphorylation. As in other *Nitrospira* strains[4,6], the genome of *N. inopinata* does not encode any known heme-copper oxidase (cyt. *c* oxidase) and complex IV most likely is a putative novel, cyt. *bd*-like heme-copper oxidase[6]. Two gene clusters coding for complex I were identified, one probably functioning in respiration and the second copy likely being involved in the reverse transport of electrons from ammonia *via* quinol to NAD^+. Nitrate may be reduced to nitrite by NXR, which can act in the reverse direction in *Nitrospira*[4], or by a putative periplasmic nitrate reductase (Nap). *N. inopinata* encodes the molypdopterin cofactor-containing, nitrate-reducing subunit NapA. The gene directly upstream of *napA* codes for a membrane-associated, tetraheme cyt. *c* that exhibits low similarity to NapC from other bacteria. The tetraheme cyt. *c* membrane protein NapC usually extracts two electrons from quinol and transfers them onto the periplasmic NapB, which forms a complex with NapA and channels the electrons to NapA for nitrate reduction[24]. A

homolog of NapB was not found in *N. inopinata*, but a functional Nap system lacking NapB has been described for *Desulfovibrio desulfuricans*, where NapC may transfer the electrons from quinol directly to NapA[25]. The composition of known Nap systems is diverse and includes additional components in some microorganisms[24], but none of these have been identified with sufficient confidence in the genome of *N. inopinata*. If the NapA-NapC system is functional, *N. inopinata* may utilize it for anaerobic respiration, regeneration of oxidized quinone, or as electron sink to maintain redox homeostasis[24]. *N. inopinata* should be able to reduce nitrite to nitric oxide by copper-dependent dissimilatory nitrite reductase (NirK), but other known denitrification enzymes are missing. Interestingly, the *N. inopinata* genome encodes a periplasmic, pentaheme cyt. *c* nitrite reductase (NrfA) and a tetraheme cyt. c (NrfH) that likely form a NrfHA complex for the respiratory reduction of nitrite to ammonium (respiratory ammonification). According to previous genome analyses[4,6] of *N. defluvii* and *N. moscoviensis*, these canonical nitrite oxidizers possess neither the Nap nor the NrfHA systems.

Past studies showed that at least some *Nitrospira* strains, when oxidizing nitrite, can utilize simple organic compounds as additional sources of carbon and possibly of energy[26,27]. As mentioned above, *N. inopinata* might be able to ferment carbohydrates. In addition to the rTCA cycle, the genome encodes the complete oxidative tricarboxylic acid cycle, suggesting that organic compounds (including intracellular glycogen deposits) could also be respired with O_2 or nitrate as terminal electron acceptor. However, chemoorganoheterotrophic growth has not been observed yet for any *Nitrospira* member.

In summary, *N. inopinata* appears to be a metabolically flexible comammox organism and may not be an obligate nitrifier. Its predicted ecological niche is within oligotrophic biofilms, where it is a highly competitive ammonia oxidizer (see main text). We assume that it can survive periods of ammonia and/or oxygen depletion by using H_2 or sulfide as alternative sources of energy and reductant, and by using O_2 or nitrate as terminal electron acceptors. Intracellular glycogen deposits formed during substrate-replete periods, and external organic compounds, may also be aerobically or anaerobically metabolized to overcome ammonia starvation. Habitats of *N. inopinata* might be characterized by fluctuating oxygen concentrations, because nitrification depends on oxygen, whereas H_2 and sulfide would be produced by other organisms especially under oxygen-depleted conditions. Other comammox

Nitrospira that share the metabolic versatility of *N. inopinata* would be well adapted to life in biofilms, sediments, soils, and other spatially complex habitats where they may encounter changing concentrations of ammonia, oxygen, and alternative substrates.

Supplementary References

1 Daims, H. *et al.* Complete nitrification by *Nitrospira* bacteria. *Nature* **528**, 504-509 (2015).

2 Vallenet, D. *et al.* MicroScope-an integrated microbial resource for the curation and comparative analysis of genomic and metabolic data. *Nucleic Acids Res.* **41**, E636-E647, doi:10.1093/nar/gks1194 (2013).

3 Mobley, H. L. T., Island, M. D. & Hausinger, R. P. Molecular biology of microbial ureases. *Microbiol. Rev.* **59**, 451-480 (1995).

4 Koch, H. *et al.* Expanded metabolic versatility of ubiquitous nitrite-oxidizing bacteria from the genus *Nitrospira*. *Proc. Natl. Acad. Sci. U. S. A.* **112**, 11371-11376 (2015).

5 Palatinszky, M. *et al.* Cyanate as an energy source for nitrifiers. *Nature* **524**, 105-108 (2015).

6 Lücker, S. *et al.* A *Nitrospira* metagenome illuminates the physiology and evolution of globally important nitrite-oxidizing bacteria. *Proc. Natl. Acad. Sci. U. S. A.* **107**, 13479-13484 (2010).

7 Campbell, B. J., Engel, A. S., Porter, M. L. & Takai, K. The versatile epsilon-proteobacteria: key players in sulphidic habitats. *Nat. Rev. Microbiol.* **4**, 458-468 (2006).

8 Yamamoto, M., Arai, H., Ishii, M. & Igarashi, Y. Role of two 2-oxoglutarate:ferredoxin oxidoreductases in *Hydrogenobacter thermophilus* under aerobic and anaerobic conditions. *FEMS Microbiol. Lett.* **263**, 189-193 (2006).

9 Ikeda, T. *et al.* Anabolic five subunit-type pyruvate:ferredoxin oxidoreductase from Hydrogenobacter thermophilus TK-6. *Biochem. Biophys. Res. Commun.* **340**, 76-82 (2006).

10 Craig, L., Pique, M. E. & Tainer, J. A. Type IV pilus structure and bacterial pathogenicity. *Nat. Rev. Microbiol.* **2**, 363-378 (2004).

11 Miller, M. B. & Bassler, B. L. Quorum sensing in bacteria. *Annu. Rev. Microbiol.* **55**, 165-199 (2001).

12 Nasuno, E. *et al.* Phylogenetically novel LuxI/LuxR-type quorum sensing systems isolated using a metagenomic approach. *Appl. Environ. Microbiol.* **78**, 8067-8074 (2012).

13 Ehrich, S., Behrens, D., Lebedeva, E., Ludwig, W. & Bock, E. A new obligately chemolithoautotrophic, nitrite-oxidizing bacterium, *Nitrospira moscoviensis* sp. nov. and its phylogenetic relationship. *Arch. Microbiol.* **164**, 16-23 (1995).

14 Labbate, M. *et al.* Quorum sensing-controlled biofilm development in *Serratia liquefaciens* MG1. *J. Bacteriol.* **186**, 692-698 (2004).

15 Costa, E., Perez, J. & Kreft, J. U. Why is metabolic labour divided in nitrification? *Trends. Microbiol.* **14**, 213-219 (2006).

16 Koch, H. *et al.* Growth of nitrite-oxidizing bacteria by aerobic hydrogen oxidation. *Science* **345**, 1052-1054 (2014).

17 Gruber-Dorninger, C. *et al.* Functionally relevant diversity of closely related *Nitrospira* in activated sludge. *ISME J.* **9**, 643-655 (2015).

18 Lücker, S., Nowka, B., Rattei, T., Spieck, E. & Daims, H. The genome of *Nitrospina gracilis* illuminates the metabolism and evolution of the major marine nitrite oxidizer. *Frontiers Microbiol.* **4**, 27, doi:10.3389/fmicb.2013.00027 (2013).

19 Berney, M., Greening, C., Conrad, R., Jacobs, W. R. & Cook, G. M. An obligately aerobic soil bacterium activates fermentative hydrogen production to survive reductive stress during hypoxia. *Proc. Natl. Acad. Sci. U. S. A.* **111**, 11479-11484 (2014).

20 Ma, K., Schicho, R. N., Kelly, R. M. & Adams, M. W. W. Hydrogenase of the hyperthermophile *Pyrococcus furiosus* is an elemental sulfur reductase or sulfhydrogenase: Evidence for a sulfur-reducing hydrogenase ancestor. *Proc. Natl. Acad. Sci. U. S. A.* **90**, 5341-5344 (1993).

21 Bryant, F. O. & Adams, M. W. W. Characterization of hydrogenase from the hyperthermophilic archaebacterium, *Pyrococcus furiosus*. *J. Biol. Chem.* **264**, 5070-5079 (1989).

22 Kwan, P. *et al.* The NiFe -hydrogenase of *Pyrococcus furiosus* exhibits a new type of oxygen tolerance. *J. Am. Chem. Soc.* **137**, 13556-13565 (2015).

23 Cherney, M. M., Zhang, Y. F., Solomonson, M., Weiner, J. H. & James, M. N. G. Crystal structure of sulfide:quinone oxidoreductase from *Acidithiobacillus ferrooxidans*: Insights into sulfidotrophic respiration and detoxification. *J. Mol. Biol.* **398**, 292-305 (2010).

24 Simon, J. & Klotz, M. G. Diversity and evolution of bioenergetic systems involved in microbial nitrogen compound transformations. *Biochim. Biophys. Acta-Bioenerg.* **1827**, 114-135 (2013).

25 Marietou, A., Richardson, D., Cole, J. & Mohan, S. Nitrate reduction by *Desulfovibrio desulfuricans*: A periplasmic nitrate reductase system that lacks NapB, but includes a unique tetraheme *c*-type cytochrome, NapM. *FEMS Microbiol. Lett.* **248**, 217-225 (2005).

26 Daims, H., Nielsen, J. L., Nielsen, P. H., Schleifer, K. H. & Wagner, M. In situ characterization of *Nitrospira*-like nitrite-oxidizing bacteria active in wastewater treatment plants. *Appl. Environ. Microbiol.* **67**, 5273-5284 (2001).

27 Watson, S. W., Bock, E., Valois, F. W., Waterbury, J. B. & Schlosser, U. *Nitrospira marina* gen. nov. sp. nov.: a chemolithotrophic nitrite-oxidizing bacterium. *Arch. Microbiol.* **144**, 1-7 (1986).