Genomic and kinetic analysis of novel Nitrospinae enriched by cell sorting

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Abstract

Chemolithoautotrophic nitrite-oxidizing bacteria (NOB) are key players in global nitrogen and carbon cycling. Members of the phylum Nitrospinae are the most abundant, known NOB in the oceans. To date, only two closely affiliated Nitrospinae species have been isolated, which are only distantly related to the environmentally abundant uncultured Nitrospinae clades. Here, we applied live cell sorting, activity screening, and subcultivation on marine nitrite-oxidizing enrichments to obtain novel marine Nitrospinae. Two binary cultures were obtained, each containing one Nitrospinae strain and one alphaproteobacterial heterotroph. The Nitrospinae strains represent two new genera, and one strain is more closely related to environmentally abundant Nitrospinae than previously cultured NOB. With an apparent half-saturation constant of 8.7±2.5 μM, this strain has the highest affinity for nitrite among characterized marine NOB, while the other strain (16.2±1.6 μM) and *Nitrospina gracilis* (20.1±2.1 μM) displayed slightly lower nitrite affinities. The new strains and *N. gracilis* share core metabolic pathways for nitrite oxidation and CO_2 fixation but differ remarkably in their genomic repertoires of terminal oxidases, use of organic N sources, alternative energy metabolisms, osmotic stress and phage defense. The new strains, tentatively named "*Candidatus* Nitrohelix vancouverensis" and "*Candidatus* Nitronauta litoralis", shed light on the niche differentiation and potential ecological roles of Nitrospinae.

Introduction

Bioavailable nitrogen is essential to all life on earth and the growth limiting factor in many ecosystems. With that said, in marine habitats, an excess in nitrogen availability due to anthropogenic pollution can cause dramatic effects such as coastal eutrophication and the formation of hypoxic dead zones [1]. The majority of fixed nitrogen in marine systems is composed of nitrate (NO_3^-) [2]. While microbially catalyzed, nitrite oxidation by chemolithoautotrophs is thought to be the most significant biological pathway in terms of nitrate production, surprisingly little is known about the underlying microbiology in the oceans. The known diversity of marine nitrite-oxidizing bacteria (NOB) consists of members of the phylum Nitrospinae and representatives of the genera *Nitrospira*, *Nitrococcus*, *Nitrobacter*, and *Nitrotoga* [3]. Among these, the Nitrospinae (recently proposed to be renamed to Nitrospinota [4]) are the most abundant NOB in the majority of the marine environments studied so far. They have been found to be present in a wide range of habitats including the free water column, oxygen minimum zones (OMZs), sediments, and deep-sea trenches [5-17]. Interestingly, nitrite oxidoreductase (NXR), the key enzyme for nitrite oxidation, was found to be highly abundant in metaproteomic studies of OMZs [11, 12] and high *in situ* nitrite oxidation rates were measured, although nitrite oxidation is considered to be an aerobic process and OMZs are strongly oxygen limited [5, 18, 19]. In addition to their importance in the nitrogen cycle, the Nitrospinae have been suggested to play a major role in dark ocean carbon fixation by contributing up to 15-45% of the fixed inorganic carbon in some environments [20]. The contribution of Nitrospinae to CO_2 fixation is, however, still under debate [21, 22], partially due to a lack of cultured representative organisms.

To date, only two strains from the Nitrospinae have been isolated, both of which are members of the genus *Nitrospina* (*N. gracilis* and *N. watsonii*) and are closely related to each other (97.9% 16S rRNA identity) [23, 24]. Experimental and genomic evidence obtained thus far from these two cultured strains points to *Nitrospina* spp. being chemolithoautotrophic NOB and obligate aerobes [23–25]. However, single-cell genomic analyses of marine bacteria have revealed a much higher diversity of Nitrospinae. Accordingly, the phylum contains at least two additional major phylogenetic lineages, which are only distantly affiliated with the genus *Nitrospina* and are referred to as "Clade 1" ("*Candidatus* (*Ca.*) Nitromaritima") and "Clade 2" [6, 20]. Intriguingly, these uncultured organisms are considerably more abundant in the environment than *Nitrospina* spp. [20]. They were predicted to be NOB based on the presence of *nxr* genes, but direct physiological evidence of this activity has been lacking. Various other NOB, such as *Nitrospira* members, have been shown to be able to utilize alternative energy metabolisms, including the oxidation of hydrogen or formate coupled to oxygen or nitrate reduction [26–28]. There is also some genomic and metagenomic evidence for putative alternative metabolisms in the Nitrospinae, such as hydrogen oxidation, sulfite oxidation, and nitrate reduction, which remain to be tested [6, 7, 20, 25]. Indeed, ecophysiological analyses of Nitrospinae outside the described genus *Nitrospina* are hampered by the lack of any cultivated strains that could systematically be characterized. The major cause of this deficiency is the recalcitrance of most NOB to cultivation and the time needed to isolate these organisms by traditional approaches [24, 29], which can take more than a decade.

We therefore employed an accelerated approach based on live cell sorting and obtained two binary co-cultures, each containing a novel Nitrospinae genus and a heterotrophic bacterium. Excitingly, one of the strains groups with the environmentally abundant but uncultured

Nitrospinae clades 1 and 2 [20]. Here, we describe both strains, including genomic characterization and determination of their nitrite affinities, in order to expand our understanding of the diversity and ecophysiology of the Nitrospinae and provide new model organisms from this globally important phylum.

Materials and Methods

Sample collection, pre-enrichment and cultivation of NOB

Sandy coastal surface sediment (0-3cm) samples were taken at Burrard Inlet, Vancouver, Canada (49°16'22.1"N 123°11'32.5"W) in November 2016 and in Elba, Italy (42°43'48.1"N 10°09'23.2"E) in November 2016. Aliquots of the sediments were inoculated into 50 ml Schott glass bottles filled with 25 ml marine minimal medium at pH 7.4-7.6 containing 4.2 µl l^{-1} supplement solution (0.02g l^{-1} biotin, 0.02g l^{-1} folic acid, 0.10g l^{-1} pyridoxine HCL, 0.05g l^{-1} riboflavin, 0.05g l^{-1} nicotinic acid, 0.05g l^{-1} DL-pantothenic acid, 0.05g l^{-1} P-aminobenzoic acid, 2.00g l^{-1} choline chloride and 0.01g l^{-1} vitamin B_{12}) and 0.5 mM NO_2^-, and incubated at 28°C in the dark without agitation [30] . The marine minimal medium was modified from the medium used for the cultivation of *N. gracilis* and *N. watsonii* by replacing natural seawater with 33.4 g l^{-1} (Vancouver) and 39.9 g l^{-1} (Elba) red sea salts (Red Sea Aquaristic) dissolved in Milli-Q water [23–25]. A higher salinity was chosen for the Elba enrichment to reflect the higher salt concentrations in the Mediterranean Sea. The enrichments were regularly checked for nitrite consumption and nitrate formation by using nitrite/nitrate test stripes (Merckoquant, Merck). Upon consumption of nitrite, the cultures were replenished with 0.5 to 1 mM nitrite (Vancouver Nitrospinae) or up to 5 mM nitrite (Elba Nitrospinae and *N. gracilis*) (final concentrations). Once nitrite oxidation was consistently observed, aliquots of the cultures were transferred at a 1:10

ratio into fresh media. Unless stated otherwise, the Vancouver and Elba Nitrospinae were cultured as described above. *N. gracilis* was grown at the same red sea salt concentration as the Vancouver Nitrospinae. Growth of the Nitrospinae strains on solid media was tested on Marine Broth 2216 (Difco, BD) and the strain specific marine minimal media that were solidified with 15g l^{-1} agar.

Cell sorting and activity screening

Cells were concentrated from 10 ml of the enrichment cultures by centrifugation (4500×g, 20 min, 28°C) using a swing-bucket rotor (Eppendorf) and resuspended in approximately 200 µl of the supernatant. Subsequently, the cells were sorted into sterile 96-well tissue culture plates (VWR, item no. 10062-900) by using the single cell sorting option at a pressure of 60 psi on a MoFlo Astrios Flow Cytometer (Beckman Coulter) equipped with a 70 µm jet in air nozzle. On the instrument, cells were visualized in the forward and side scatter channel and small, non-cell particles were excluded; otherwise no gating was applied. The wells of the microtiter plates contained 200 µl of sterile mineral salt medium amended with supplements (see cultivation details above) and 0.25 mM sodium nitrite. For the cultivation of the Vancouver sourced Nitrospinae strain, the sorting medium additionally contained 0.1 mM sodium pyruvate to alleviate oxidative stress [31]. The 96-well microtiter plates were placed in closed plastic bags to prevent evaporation, incubated at 28°C in the dark without agitation and 10 µl aliquots of each well were regularly checked for nitrite consumption with the Griess assay [32]. Selected wells, which showed nitrite consumption, were gradually scaled up by transferring the cultures into larger volume microtiter plates and doubling the culture volume after each round of nitrite consumption up to 3.2 ml and then to a final volume of 25 or 50 ml in Schott bottles. The

cultures were further cultivated as described for the enrichments (see above). Once sufficient biomass was available, the enriched nitrite-oxidizing organisms were provisionally identified by full-length 16S rRNA gene amplification and Sanger sequencing.

The morphology of the cells was visualized via scanning electron microscopy and catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) with the Nitrospinae specific 16S rRNA-targeted probe Ntspn759 [21] (Supplemental Materials and Methods).

Genomic and phylogenetic analyses

DNA was extracted from the cultures and complete genomes were obtained through Illumina and Nanopore sequence co-assemblies. A 16S rRNA gene phylogenetic tree was calculated and the genomes were annotated on the MicroScope platform (MAGE Workflow version: 1.8 [33] (Supplemental Materials and Methods).

Publicly available Nitrospinae genomes, including metagenome assembled genomes (MAGs) and single-cell amplified genomes (SAGs), were retrieved from NCBI and from the JGI genome portal (see Table S1 for details). Completeness and contamination of the genomes was assessed by CheckM (v. 1.0.18) (Table S1), and phylogenetic analyses were conducted on genomes that were more than 80% complete and less than 10% contaminated [34]. A phylogenetic tree was calculated with an alignment of concatenated conserved bacterial marker proteins, made with the GTDB (Genome Taxonomy Database) toolkit (v. 0.3.2), using IQ-TREE (v. 1.6.11, model: LG+F+R5 as chosen by automatic model selection and 1000 ultrafast bootstrap runs) [4, 35–37]. The genomes of *Nitrospira moscoviensis*, *G. metallireducens*, and *D. multivorans* served as outgroup. Clades within the Nitrospinae were depicted as they have been previously described in the literature [6, 20]. Average amino acid identity (AAI) and whole-genome nucleotide identity

158 (gANI) were calculated as described elsewhere [38–40]and visualized using R (v. 3.6.1) with the
159 R package tidyverse (v. 1.3.0) [41]. The GTDB-TK output (Table S1) was used to delineate
160 phylogenetic affiliations beyond the genus level [4].
161 The raw reads and genomes of the novel Nitrospinae strains were submitted to NCBI under the
162 Bioproject accession PRJNA602816 and the Biosamples SAMN13976148 ("*Ca.* Nitrohelix
163 vancouverensis" VA) and SAMN13976151 ("*Ca.* Nitronauta litoralis" EB). The genomes are
164 further available on the MicroScope platform under the accession NTSPN23.2 ("*Ca.* Nitrohelix
165 vancouverensis" VA) and NTSPN 3.2 ("*Ca.* Nitronauta litoralis" EB).
166

167 **Nitrite oxidation kinetics**

168 Nitrite oxidation kinetics of the Nitrospinae co-cultures and *N. gracilis* were quantified by
169 measuring nitrite-dependent oxygen consumption in a microrespirometry (MR) system
170 (Unisense) as described in detail by Kits *et al.* [42]. To concentrate the biomass for MR, cells
171 were collected from 100 to 300 ml of culture with Amicon Ultra-15 tubes (Millipore) by
172 centrifugation (4500×g, 10 min, 28°C) using a swing-bucket rotor (Eppendorf). Concentrated
173 biomass was washed and resuspended in nitrite free growth medium for the MR experiments.
174 The media did not contain supplements to prevent oxygen respiration due to the degradation of
175 organic compounds by the co-enriched, heterotrophic bacteria during the MR experiment.
176 Culture biomass was incubated in a water bath set to the experimental temperature prior to being
177 transferred to a 2 ml glass MR chamber with a stir bar. All MR experiments were performed with
178 300 rpm stirring at 28°C. Small culture volumes (~10 µl) were taken before and immediately
179 after nitrite injection, and ~5 min after nitrite depletion for nitrite/nitrate measurements to
180 confirm stoichiometric conversion of oxygen, nitrite and nitrate. Protein concentrations were

181 determined with the Pierce BCA protein assay (ThermoScientific) and cell abundances by qPCR
182 (Supplementary Methods). All experiments were replicated three times or more, using at least
183 two different cultures on different days (see Results and Discussion for total biological and
184 technical replicates per strain). For one replicate of each strain, images of the cells stained with
185 DAPI were taken before and after the MR measurements by using an epifluorescence
186 microscope (Zeiss). These images were used to check whether the cells in the MR chamber had
187 formed aggregates, which could cause oxygen diffusion limitations and thus affect the measured
188 respiration rates and the inferred nitrite oxidation kinetics. No aggregates were observed.
189
190 **Results and Discussion**
191 **Cultivation of novel Nitrospinae representatives**
192 For the initial enrichment of marine NOB, nitrite-containing mineral media were inoculated with
193 coastal surface sediment samples taken in Vancouver, Canada, and on Elba, Italy. Within 4
194 weeks of incubation, nitrite oxidation to nitrate was detected in the cultures and this activity
195 continued to be observed after subsequent replenishment of nitrite and transfers of culture
196 aliquots into fresh medium. Usually, the further purification of NOB from accompanying
197 organisms in the enrichment cultures is hindered by the very slow growth and inability of most
198 NOB (including all cultured Nitrospinae) to grow on solid media. To expedite the purification of
199 Nitrospinae strains from our initial enrichments, which was previously a laborious and lengthy
200 process [24], we developed a method for the physical separation, activity-based identification of
201 NOB, and subcultivation in 96-well microtiter plates. This method uses random, non-fluorescent,
202 single-cell sorting using a fluorescence activated single cell sorting (FACS) instrument paired
203 with a nitrite consumption activity screen (Fig. S1a). Thus it differs from a previously reported

FACS isolation approach for *Nitrospira* NOB from activated sludge, where the NOB were targeted based on their known cell cluster size and shape, which had been determined by *Nitrospira*-specific rRNA-targeted FISH analysis before FACS was performed [43]. Our method does not rely on prior knowledge of the identity and morphology of the NOB [43], but is solely based on the detection of nitrite oxidation after sorting. Still, it might allow for a flexible selection of the sorted cell morphologies by adjusting the gating parameters. While the previous method facilitated the isolation of already known NOB, the approach used in our study was designed for the discovery of novel nitrite oxidizers that grow under the given conditions. It may also be suitable for the isolation of other microorganisms that can be efficiently sorted (i.e., grow in suspension or that can be suspended by sonication or other methods) and that perform a specific metabolism of interest, which is detectable by a colorimetric, fluorimetric, or other high-throughput assay. Examples include previously performed, high-throughput enzyme discoveries and the isolation of microalgae [44–46]. After sorting cells from the initial Vancouver and Elba enrichment cultures into one 96-well plate each, several wells showed nitrite-oxidizing activity within 3-4 weeks. Cells from three active wells from the Elba enrichment and 4 for the Vancouver enrichment (>4 were active) were progressively transferred into larger culture volumes. This procedure led to the separate enrichment of two different Nitrospinae, one from each of the initial enrichments, that were identified by 16S rRNA gene sequencing (Fig. S1b): strain VA (Vancouver) and strain EB (Elba). Interestingly, despite attempted single-cell sorting and various dilution to extinction attempts, an axenic culture could not be established for either of the two obtained Nitrospinae strains. Rather, these cultures represent binary co-cultures each containing one Nitrospinae strain, one alphaproteobacterial strain, and no other detectable

226 microorganisms. Overall, using this single-cell sorting and screening method, we were able to
227 obtain the two binary co-cultures from the environmental samples in approximately 10 months.
228
229 **Co-enrichment with Alphaproteobacteria**
230 Pure cultures of the two co-enriched heterotrophic strains were obtained on Marine Broth Agar.
231 Subsequent 16S rRNA gene analysis of these isolates showed that the two Nitrospinae strains
232 had been co-cultured with members of two distinct genera within the Alphaproteobacteria. Strain
233 VA was co-cultured with a bacterium most closely related to *Stappia stellulata* strain
234 (NR_113809.1, 99.58% 16S rRNA gene sequence identity), whereas the EB strain culture
235 contained a *Maritimibacter alkaliphilus* strain (NR_044015.1, 100% 16S rRNA gene sequence
236 identity). Both these species have previously been isolated from marine environments and have
237 been described as alkaliphilic chemoorganoheterotrophs that can use a wide variety of simple
238 and complex organic substrates [47, 48]. In our cultures, which were only provided with nitrite
239 for growth, the alphaproteobacterial strains may have lived off simple organic compounds that
240 were excreted by the autotrophic Nitrospinae. Since the NOB could not be grown separately
241 from the heterotrophs, it is tempting to speculate that the Nitrospinae strains also benefitted, for
242 example, from reactive oxygen species (ROS) protection by the heterotrophs. Superoxide
243 dismutase and catalase genes are present in the genomes of both co-cultured alphaproteobacteria,
244 and other isolates of both species are catalase positive [47, 48]. Similar interactions have already
245 been observed in marine autotroph-heterotroph co-cultures, including other nitrifiers [49–51].
246

Phylogeny of the novel Nitrospinae

Closed genomes were reconstructed for both Nitrospinae strains by co-assembling Illumina and Nanopore sequencing data. Since the genome of *N. gracilis* was nearly completely sequenced but not closed [25] (Table S2), the obtained genomes represent the first closed genomes from Nitrospinae. With a length of >3.9 Mbp, Nitrospinae strain EB has a larger genome than the other cultured Nitrospinae strains sequenced to date (Table S2).

Phylogenetic analysis of the 16S rRNA genes (Fig. S1b) showed that the Nitrospinae strains VA and EB are only distantly related to *N. gracilis* (93% sequence identity with EB and 91% with VA), *N. watsonii* (92% sequence identity with EB and 90% with VA), and to each other (90% sequence identity). Since public databases contained only few 16S rRNA gene sequences closely related to strains VA and EB (Fig. S1b), these strains seem to represent a yet underexplored diversity of Nitrospinae. A phylogenetic tree using conserved concatenated marker proteins from all available Nitrospinae genomes that are >80% complete and <10% contaminated, including MAGs and SAGs, further confirmed that the strains obtained here represent novel lineages (Fig. 1). In particular, strain VA was more closely related with the Clade 1 and 2 Nitrospinae than *N. gracilis*, although it was clearly not a member of either group. In the concatenated marker tree, it belonged to a third well-resolved clade with additional MAGs. The exact branching order between Clade 1, 2, and this third clade remained unresolved (Fig. 1, Fig. S1b).

The novelty of the two cultured Nitrospinae strains was corroborated by genome average nucleotide identity (gANI) (Fig. S2) and average amino acid identity (AAI) (Fig. 2) analyses. Since strain VA, strain EB, and most of the Nitrospinae members in the dataset are quite distantly related (below the 96.5% species level cut-off [39]), gANI (Fig. S2) was not suitable to further resolve their taxonomic grouping. However, AAI analysis revealed a high genus-level

diversity within the Nitrospinae, comprising 15 distinct genera according to a proposed 60% genus level cut-off [52]. Both strains from this study, VA and EB, represent a new genus with 55% and 58% AAI to *N. gracilis*, respectively, and 53% AAI to each other (Fig. 2). The AAI analysis also suggested that each of the environmental clades 1 ("*Ca*. Nitromaritima") and 2 represent separate genera, with the lowest AAI values within each clade being >70%, and that these genera are distinct from the genus containing strain VA (Fig. 2). In order to make taxonomic inferences beyond the genus level, the GTDB-TK tool [38] was applied to the Nitrospinae genome dataset. GTDB-TK confirmed that strains VA and EB belonged to the Nitrospinaceae family but, in agreement with the AAI analysis, did not assign them to a genus (Tab. S2). While we showed that nitrite oxidation is spread among phylogenetically distant members within the Nitrospinaceae, no nitrite oxidation phenotype has been observed yet for the Nitrospinae members that do not belong to the Nitrospinaceae family.

Taken together, the phylogenetic, ANI, AAI, and GTDB-TK analyses revealed a high genus-level diversity within the Nitrospinae, the vast majority remaining uncultured and poorly characterized. Among all cultured Nitrospinae members, strain VA is most closely related with the uncultured but environmentally abundant clades 1 and 2 [6, 7, 20, 21].

Cell morphology

The morphologies of the new Nitrospinae strains were visualized using scanning electron microscopy (SEM), and by 16S rRNA-targeted CARD-FISH using a Nitrospinae-specific oligonucleotide probe (Ntspn759) [21]. Strain VA cells were helically shaped rods (Fig. 3a, d, g), whereas strain EB appeared to be short, slightly curved rods (Fig. 3b, e, h). Interestingly, neither of the two strains displayed the long, slender, rod-shaped morphology that was mostly observed

for the previously isolated *Nitrospina* species, *N. gracilis* (Fig. 3c, f, i) [23]. The shape of strain VA (Fig. 3a, d, g) rather resembled *Nitrospira* species [53]. The short, slightly curved rod morphology of strain EB (Fig. 3b, e, h) resembled the compact cell shape reported for aging cultures of *N. watsonii* [24] and environmental Nitrospinae [5]. While the helical shape of strain VA could be clearly distinguished from the co-cultured *Stappia* sp. using Nitrospinae-specific FISH (Fig. 3g) and SEM of the *Stappia*-like isolate (Fig. S3), assigning a morphology to strain EB was slightly more difficult due a more similar morphotype of the co-cultured *Maritimibacter*-like bacterium (Fig. 3e and Fig. S3b). According to SEM, the isolated *Maritimibacter* had a coccoid morphology (Fig. S3) similar to the slightly smaller coccoid cells that were observed in the active co-culture with strain EB (Fig. 3e). Therefore, we assume that the slightly larger, curved rods in the SEM pictures (Fig. 3b, e) were Nitrospinae strain EB cells. However, the previously described morphological variability suggests that the cell shape of Nitrospinae is influenced by the growth stage and environment [23, 24]. Thus, morphology would be of limited use as the sole criterion to differentiate Nitrospinae strains from each other and from other organisms. We propose the name "*Candidatus* Nitrohelix vancouverensis" VA for strain VA based on its observed morphology and isolation source, and "*Candidatus* Nitronauta litoralis" EB for strain EB based on its isolation source.

Nitrite oxidation: activity and kinetics

Both Nitrospinae strains oxidized nitrite stoichiometrically to nitrate (Fig. S4). Exponential growth of "*Ca.* N. vancouverensis" correlated with the consumption of nitrite (Fig. S4a), whereas "*Ca.* N. litoralis" did not enter the exponential growth phase during the incubation period (Fig. S4b). During the experiment, the relative abundance of "*Ca.* N. vancouverensis" compared to the co-cultured *Stappia* sp. increased pronouncedly from 6 to 75% (Fig. S4a). The

317 relative abundance of "*Ca.* N. litoralis" compared to the *Maritimibacter sp.* could not be reliably
318 determined during the incubation experiment (Fig. S4b), but measurements taken after the MR
319 experiments (see below) showed that the relative abundance of "*Ca.* N. litoralis" ranged from
320 approximately 56 to 99%. Thus, the quantitative composition of each co-culture appeared to
321 fluctuate and likely depended strongly on the availability of nitrite as the substrate for the NOB
322 strains.
323 The kinetics of nitrite oxidation of *N. gracilis* and the two novel Nitrospinae strains were
324 assessed by measuring the nitrite-dependent oxygen consumption in MR experiments. For all
325 three NOB, nitrite oxidation followed Michaelis-Menten kinetics (Fig. S5). The stoichiometry of
326 NO_2^- and O_2 consumption was always close to 1:0.5 (*N. gracilis*: mean=1:0.49; s.d.=0.02; *n*=8;
327 "*Ca.* N. vancouverensis": mean=1:0.51; s.d.=0.01; *n*=4; "*Ca.* N. litoralis": mean=1:0.51;
328 s.d.=0.02; *n*=5). This ratio was expected for NOB [54] and indicates that O_2 consumption by the
329 co-enriched heterotrophs was only minor during the relatively short MR experiments (maximum
330 1 h) and did not affect the kinetic analysis of "*Ca.* N. vancouverensis" and "*Ca.* N. litoralis". The
331 apparent half-saturation constant, $K_{m(app)}$, of *N. gracilis* was determined to be 20.1 µM NO_2^-
332 (s.d.=2.1, *n*=8) and the maximum reaction rate, V_{max}, to be 41.4 µmol NO_2^- mg protein^{-1} hour^{-1}
333 (s.d.=9.4, *n*=6), which is highly similar to the previously reported $K_{m(app)}$ and V_{max} of the closely
334 related *N. watsonii* (18.7±2.1 µM NO_2^- and 36.8 µmol NO_2^- mg protein^{-1} hour^{-1}) [55]. The $K_{m(app)}$
335 measured for "*Ca.* N. litoralis" was 16.2 µM NO_2^- (s.d.=1.6, *n*=7) and thus resembled the values
336 of *N. gracilis* and *N. watsonii*. In contrast, with a $K_{m(app)}$ of 8.7 µM NO_2^- (s.d.=2.5, *n*=3), "*Ca.* N.
337 vancouverensis" showed a higher affinity for nitrite that was comparable with non-marine
338 *Nitrospira* members ($K_{m(app)}$=6 to 9 µM NO_2^-), which have been the cultured NOB with the
339 highest nitrite affinity known so far [54, 56]. Indeed, strain VA turned out to have the lowest

$K_{m(app)}$ of all hitherto analyzed marine NOB in culture (Fig. 4). Interestingly, among all cultured marine NOB, "*Ca.* N. vancouverensis" is also most closely related to the Nitrospinae clades 1 and 2 that are abundant in oligotrophic waters [20] (see above). However, its $K_{m(app)}$ is still 1-2 orders of magnitude higher than the $K_{m(app)}$ values of nitrite oxidation reported for environmental samples from an OMZ (0.254±0.161 μM NO_2^-) and South China Sea waters (0.03 to 0.5 μM) [22, 57]. The very high nitrite affinity observed with these samples might be explained by the presence of uncharacterized nitrite oxidizers, whose nitrite affinity exceeds that of all cultured NOB. However, it remains to be tested whether known NOB can persist under extremely low *in situ* nitrite concentrations. For example, the half-saturation constant of growth for ammonia oxidizing bacteria spans several orders of magnitude under different temperatures [58]. Strongly different substrate affinities have also been observed for *Nitrobacter winogradskyi* and *Escherichia coli* under oligotrophic versus copiotrophic growth conditions [59, 60]. Systematic assessments of the kinetic plasticity of NOB under different conditions are still pending, mainly because the production of sufficient biomass of NOB isolates has been a major obstacle for such studies.

General genomic features of cultured Nitrospinae

A pan-genomic analysis of the two novel cultured Nitrospinae strains and *N. gracilis* showed that these three organisms share a core genome of 1347 proteins, which have at least 50% amino acid sequence identity over 80% of the alignment (Fig. S6). The core genome included universally highly conserved bacterial genes, such as those coding for ribosomal proteins, translational elongation factors and the DNA replication machinery, as well as the genes for the core metabolism of chemolithoautotrophic NOB. Interestingly, among the shared conserved genes we

363 also found highly conserved glutaredoxins, thioredoxin reductases, and peroxidases (>80%
364 amino acid identity among the respective homologs). Like *N. gracilis* [25], "*Ca.* N.
365 vancouverensis" and "*Ca.* N. litoralis" lack the canonical defense systems of aerobic organisms
366 against oxidative stress, catalase and superoxide dismutase. While the aforementioned core genes
367 could thus be essential for the detoxification of peroxides in all three organisms [61, 62], it
368 remains a mystery how Nitrospinae deal with superoxide radicals [25]. Each of the strains
369 encode a number of unique proteins (Fig. S6), many of which are phage related, corroborating a
370 recently proposed hypothesis predicting extensive phage predation on Nitrospinae [21]
371 (Supplemental Results and Discussion, Fig. S8). Yet, the majority of the variable genome
372 content is still functionally uncharacterized. However, a few genes of the variable genome have
373 known functions and might be important for niche adaptations. In the following sections, we
374 address these genes as well as the shared core metabolism of the three analyzed Nitrospinae.
375
376 **Nitrite oxidation and respiration**
377 Among the highly conserved proteins are the three known subunits of a periplasmic nitrite
378 oxidoreductase, NxrABC. Details of the predicted subunit composition and cofactors of the NXR
379 of Nitrospinae, which is closely related to the NXR of *Nitrospira*, have been described elsewhere
380 [3, 25, 63]. Briefly, all three Nitrospinae strains possess two genomic copies of the substrate-
381 binding subunit NxrA, two or three (only "*Ca.* N. vancouverensis") copies of the electron-
382 channeling subunit NxrB (Fig. S7), and several copies of putative NxrC subunits, which may
383 transfer electrons from NXR to a downstream acceptor in the electron transport chain (Table S4).
384 Homologs to all of the different putative NxrC subunits of *N. gracilis* [25] were also found in

385 "*Ca.* N. vancouverensis" and "*Ca.* N. litoralis" (Table S4), but it remains to be determined
386 whether all of these proteins are functionally involved in nitrite oxidation.
387 The respiratory electron transport chain of NOB is short, as electrons derived from nitrite are
388 directly transferred, via *a*- or *c*-type cytochromes, to the terminal oxidase (complex IV) [25, 63,
389 64]. *N. gracilis* carries a *cbb*$_3$-type high affinity heme-copper cyt. *c* oxidase (HCO) [25], whereas
390 both "*Ca.* N. vancouverensis" and "*Ca.* N. litoralis" lack any canonical HCO. However, all three
391 organisms encode highly conserved, putative "*bd*-like oxidases" [25]. These proteins, which also
392 occur in all *Nitrospira* genomes, are phylogenetically related with but clearly distinct from the
393 canonical cyt. *bd*-type quinol oxidases [63]. Interestingly, one variant of the *bd*-like oxidases
394 from *Nitrospira* contains all conserved amino acid residues for heme and Cu binding in HCOs
395 [65], indicating that this enzyme could be a novel cyt. *c*-oxidizing HCO [63]. The *bd*-like
396 oxidases of *N. gracilis*, "*Ca.* N. vancouverensis", and "*Ca.* N. litoralis" have most of these
397 conserved residues; however, one of the three histidine ligands of the Cu_B is replaced with a
398 glutamine, and the histidine ligand of the high-spin heme is replaced with a phenylalanine. Thus,
399 without the *cbb*$_3$-type oxidase found only in *N. gracilis*, it remains unclear how the final electron
400 transport step from cyt. *c* to O_2 occurs in "*Ca.* N. vancouverensis" and "*Ca.* N. litoralis". Future
401 biochemical and protein structural research may reveal whether the cyt. *bd*-like oxidases can
402 catalyze this reaction despite their divergence from *bona fide* HCOs at two of the predicted
403 cofactor-binding residues, and whether these proteins are capable of proton translocation for
404 proton motive force generation. Corroborating evidence for a function of this enzyme in the
405 context of electron transport stems from its highly conserved genetic synteny within Nitrospinae,
406 Nitrospirae and anammox bacterial genomes. A conserved cluster within these organisms
407 contains a cyt. *bd*-like oxidase with the aforementioned glutamine and phenylalanine residues, a

408 diheme- and a triheme- cyt. *c*, and a membrane integral, alternative NxrC subunit (Tab. S4). This
409 putative NxrC might be involved in the electron transfer from NO_2^- to the terminal oxidase [25,
410 63, 66].
411 In addition to the putative cyt. *bd*-like oxidase discussed above, "*Ca.* N. litoralis" possesses a
412 canonical cyt. *bd*-type (quinol) oxidase that is lacking in "*Ca.* N. vancouverensis" and *N.*
413 *gracilis*. Since quinol oxidases cannot accept electrons from the high-potential donor nitrite, we
414 assume that this oxidase receives electrons from quinol during the degradation of intracellular
415 glycogen or during hydrogen oxidation (see below). The cyt. *bd*-type oxidase may also be
416 involved in oxidative stress defense, as homologous oxidases in other organisms can degrade
417 H_2O_2 [67] and protect from dioxygen [68]. Taken together, the diverse repertoire of terminal
418 oxidases may be a key feature of Nitrospinae that contributes to the ecological success of this
419 lineage over a broad range of redox conditions in marine ecosystems.
420
421 **Carbon metabolism and alternative energy metabolisms**
422 Like *N. gracilis* and other Nitrospinae [6, 25], the novel strains encode all key genes of the
423 reductive tricarboxylic acid (rTCA) cycle for CO_2 fixation, including the hallmark enzymes
424 ATP-citrate lyase (ACL), pyruvate:ferredoxin oxidoreductase (POR), and 2-
425 oxogluterate:ferrodoxin oxidoreductase (OGOR). As in *N. gracilis*, all genes required for the
426 oxidative (oTCA) cycle are also present. All three strains can form glycogen as storage
427 compound, which is degraded via glycolysis and the oTCA cycle. Since *N. gracilis* lacks
428 pyruvate kinase, the final step of glycolysis may be catalyzed by pyruvate phosphate dikinase
429 (PPDK) in this organism. In contrast, strains VA and EB possess both pyruvate kinase and
430 PPDK, indicating a strict regulatory separation between glycolysis and gluconeogenesis.

Alternative energy metabolisms such as the oxidation of hydrogen, sulfide or organic carbon compounds have been demonstrated in NOB with representatives from the genera *Nitrospira*, *Nitrococcus*, *Nitrolancea*, and *Nitrobacter* [3, 26–28, 69]. Among the three Nitrospinae strains analyzed here, *N. gracilis* has the largest potential to exploit energy sources other than nitrite: its genome harbors a bidirectional type 3b [NiFe] hydrogenase, which could enable aerobic hydrogen utilization, and a sulfite:cyt. *c* oxidoreductase [25]. In addition, *N. gracilis* contains the genes *prpBCD* for 2-methylisocitrate lyase, 2-methylcitrate synthase, and 2-methylcitrate dehydratase, and might thus be able to catabolically degrade propionate via the 2-methylcitrate pathway (Fig. 5). Of these potential alternative energy metabolisms, "*Ca.* N. litoralis" shares only the type 3b hydrogenase, whereas "*Ca.* N. vancouverensis" seems to be an obligate nitrite oxidizer. No genes for the uptake and utilization of urea and cyanate as organic N sources were found in the genomes of the new strains. These genes show a patchy distribution among Nitrospinae [6, 7, 20, 21, 25], suggesting further niche differentiation of these organisms based on the capacity to use organic compounds as sources of reduced N for assimilation.

Adaptations to saline environments

The intracellular accumulation of ions or the production of organic osmolytes are two main strategies of microorganisms to cope with the osmotic stress in highly saline, marine environments. Interestingly, the three Nitrospinae strains seem to utilize different osmotic stress defense mechanisms. *N. gracilis* has the genetic potential to produce glycine betaine, an organic osmolyte that is ubiquitously found in bacteria (Fig. 5) [70]. It also encodes for OpuD, a betaine/carnitine/choline transporter, whereas the genes for glycine betaine synthesis and import are missing in "*Ca.* N. vancouverensis" and "*Ca.* N. litoralis". These two strains harbor the

454 canonical genes *ectABC* for ectoine biosynthesis (Fig. 5), another widely distributed osmolyte in
455 bacteria [70]. "*Ca.* N. litoralis" additionally encodes the ectoine hydroxylase EctD and is thus
456 able to form hydroxyectoine. Directly downstream of the (hydroxy-)ectoine synthesis cassette,
457 the two strains further encode an ABC transporter that has similarity to ectoine or amino acid
458 transporters from other organisms and may be utilized for (hydroxy-)ectoine import across the
459 cytoplasmic membrane. Since genes for the synthesis and transport of (hydroxy-)ectoine were
460 also found in "*Ca.* Nitromaritima" (Nitrospinae clade 1) [6], we assume that usage of (hydroxy-
461)ectoine is wide-spread among the Nitrospinae. *N. gracilis* and "*Ca.* N. litoralis" may also be
462 able to synthesize sucrose as an additional compatible solute (Fig. 5).
463 Moreover, "*Ca.* N. vancouverensis" and "*Ca.* N. litoralis" genomes harbor the gene *glsA* coding
464 for a glutaminase (Fig. 5), which allows them to deaminate glutamine to glutamate while
465 releasing ammonia. The strains further possess the *gltP* gene coding for a glutamate/H^+
466 symporter. Both *glsA* and *gltP* seem to be lacking in the *N. gracilis* genome. Since glutamate can
467 play a role in osmoregulation [70], the ability to regulate the intracellular glutamate level via
468 transport or the degradation of glutamine may be one of various adaptations by "*Ca.* N.
469 vancouverensis" and "*Ca.* N. litoralis" to rapidly respond to stress caused by fluctuating
470 salinities.
471 All three Nitrospinae strains have the genomic capacity to synthesize the hopanoids hopan-(22)-
472 ol and hop-22(29)-ene (Fig. 5). Hopanoids are pentacyclic, bacterial lipids that integrate into
473 bacterial membranes, much like cholesterol in eukaryotes [71]. They help regulate membrane
474 fluidity and may be important in highly saline environments [70, 72]. Knock-out studies have
475 shown that cells lacking the ability to make hopanoids are more sensitive to various stresses,
476 such as temperature, pH and osmolarity [72]. Interestingly, a metagenomic study of hopanoid-

producing bacteria in the Red Sea revealed Nitrospinae to be among the main organisms harboring squalene hopene cyclase, the key gene for hopanoid production [73]. Other marine NOB (members of *Nitrospira*, *Nitrobacter* and *Nitrococcus mobilis*), and even non-marine *Nitrospira* and *Nitrobacter*, also have the squalene hopene cyclase and therefore the genomic potential to produce hopanoids. Insight into the chemical structure of the hopanoids produced by NOB could help to gain insight into early NOB evolution as hopanoids are important lipid biomarkers and commonly used to deduce ancient microbial activity from sediment fossil records [73].

Vitamin B12 auxotrophy

The three Nitrospinae strains lack multiple genes involved in vitamin B12 (cobalamin) synthesis and seem to be auxotrophic for this vitamin, as previously suggested for *N. gracilis* [74]. To acquire vitamin B12 from the environment, all strains encode the vitamin B12 ABC transporter *btuCDF* and the outer membrane permease *btuB* [75][74]. Alternatively, this transporter may also import cobinamide that may then be converted to vitamin B12 via a salvage pathway that uses the genes *yvqK*, *bluB*, *cobS*, each encoded in all three Nitrospinae genomes [76]. Hence, the availability of externally supplied vitamin B12 or cobinamide is likely of crucial importance for Nitrospinae *in situ* and also in lab cultures. The incomplete cobalamin pathway in the already available *N. gracilis* genome led us to amend the cultivation medium with vitamin B12. Indeed, the addition of vitamin B12, either alone or together with other vitamins, has allowed us to cultivate *N. gracilis* in a more defined medium that is based on Red Sea salt. Previously, the standard medium for this organism had to be prepared from natural seawater [25]. Furthermore, the addition of vitamin B12 was likely an essential prerequisite for our successful enrichment of

novel Nitrospinae after cell sorting. The co-cultured alphaproteobacteria may also provide additional vitamin B12 as they both have the genomic repertoire for its synthesis. In the environment, vitamin B12 could be supplied by different heterotrophic or autotrophic microorganisms including ammonia-oxidizing thaumarchaeota, which have been shown to produce vitamin B12 [77–79] and often co-occur with Nitrospinae [10, 21, 80].

Conclusions

Nitrospinae are important players in marine nitrogen and carbon cycling, but difficulties to cultivate these bacteria have been a major obstacle for their characterization. In this study, the usually very time-consuming enrichment and purification procedure of marine NOB was accelerated by combining cell sorting with genome-informed adjustments to the cultivation medium. By employing this method, we were able to obtain two new, highly enriched Nitrospinae strains, which represent two novel genera in the Nitrospinae and double the number of available cultures from this phylum. A comparison of their completely sequenced genomes and that of *N. gracilis* revealed numerous shared metabolic features, as well as several non-shared, putative adaptations in these distantly related Nitrospinae. With the new cultures at hand, it will now be possible to systematically test such genome-based hypotheses and to elucidate the ecological roles played by members of the Nitrospinae within and beyond the nitrogen cycle.

Taxonomic consideration of "*Candidatus* Nitrohelix vancouverensis" gen. nov. sp. nov.

Ni.tro.he'lix L. n. nitrum: nitrate, L. n. helix: a coil, spiral; N.L. fem. n. *Nitrohelix* nitrate-forming spiral. van.cou.ver.en'sis L. fem. adj. *vancouverensis* of Vancouver.

A nitrate-forming helical bacterium obtained from Vancouver, Canada. Phylogenetically affiliated with the family Nitrospinaceae, phylum Nitrospinae. Cells are gram-negative helically shaped rods with 1-3 turns and a length of approximately 3 µm. The genome consists of a single chromosome of 3,309,797 bp. The DNA G+C content is 51 mol%.

Strain "*Candidatus* Nitrohelix vancouverensis VA" was cultivated from coastal surface sediment from Vancouver, Canada. Marine aerobic chemolithoautotroph that oxidizes nitrite to nitrate. $K_{m(app)}$ is 8.7±2.5 µM NO_2^-. The strain was routinely cultured with 0.5 mM nitrite at 28°C in liquid marine mineral medium. Could not be grown on solid medium. Auxotrophic for vitamin B12 according to genome analysis.

Taxonomic consideration of "*Candidatus* Nitronauta litoralis" gen. nov. sp. nov.

Ni.tro.nau'ta L. n. nitrum: nitrate, L. n. nauta: seaman; N.L. masc. n. *Nitronauta* nitrate-forming seaman. li.to.ra'lis L. masc. adj. *litoralis* coastal.

A nitrate-forming marine bacterium found in a coastal habitat. Phylogenetically affiliated with the family Nitrospinaceae, phylum Nitrospinae. Cells are gram-negative short rods with a length of approximately 1.5 µm. The genome consists of a single chromosome of 3,921,641 bp. The DNA G+C content is 47 mol%.

Strain "*Candidatus* Nitronauta litoralis EB" was cultivated from coastal surface sediment from Elba, Italy. Marine aerobic chemolithoautotroph that oxidizes nitrite to nitrate. $K_{m(app)}$ is 16.2±1.6 µM NO_2^-. The strain was routinely cultured with 1 to 5 mM nitrite at 28°C in liquid marine mineral medium. Could not be grown on solid medium. Auxotrophic for vitamin B12 according to genome analysis.

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Competing Interests

RHK owns part of DNASense ApS. The remaining authors declare no competing interests.

References

1. Diaz RJ, Rosenberg R. Spreading Dead Zones and Consequences for Marine Ecosystems. Science. 2008; 32:926–929.

2. Ward BB. Nitrification in Marine Systems. In: Capone DG, Bronk DA, Mulholland MR,

Carpenter EJ (eds). Nitrogen in the Marine Environment. 2nd edn. (Academic Press, 2008) pp 199-261.

3. Daims H, Lücker S, Wagner M. A New Perspective on Microbes Formerly Known as Nitrite-Oxidizing Bacteria. Trends Microbiol. 2016;24:699–712.

4. Parks DH, Chuvochina M, Waite DW, Rinke C, Skarshewski A, Chaumeil PA, et al. A standardized bacterial taxonomy based on genome phylogeny substantially revises the tree of life. Nat Biotechnol. 2018;36:996.

5. Füssel J, Lam P, Lavik G, Jensen MM, Holtappels M, Günter M, et al. Nitrite oxidation in the Namibian oxygen minimum zone. ISME J. 2012;6:1200–1209.

6. Ngugi DK, Blom J, Stepanauskas R, Stingl U. Diversification and niche adaptations of -*Nitrospina* like bacteria in the polyextreme interfaces of Red Sea brines. ISME J. 2015;10:1–17.

7. Sun X, Kop LFM, Lau MCY, Frank J, Jayakumar A, Lücker S, et al. Uncultured *Nitrospina*-like species are major nitrite oxidizing bacteria in oxygen minimum zones. ISME J. 2019;13:2391–2402.

8. Garcia-Robledo E, Padilla CC, Aldunate M, Stewart FJ, Ulloa O, Paulmier A, et al. Cryptic oxygen cycling in anoxic marine zones. Proc Natl Acad Sci U S A. 2017;114:8319–8324.

9. Levipan HA, Molina V, Fernandez C. *Nitrospina*-like bacteria are the main drivers of nitrite oxidation in the seasonal upwelling area of the Eastern South Pacific (Central Chile ~36°S). Environ Microbiol Rep. 2014;6: 565–573.

10. Mincer TJ, Church MJ, Taylor LT, Preston C, Karl DM, DeLong EF. Quantitative distribution of presumptive archaeal and bacterial nitrifiers in Monterey Bay and the North

Pacific Subtropical Gyre. Environ Microbiol. 2007;9:1162–1175.

11. Hawley AK, Brewer HM, Norbeck AD, Pasă-Tolić L, Hallam SJ. Metaproteomics reveals differential modes of metabolic coupling among ubiquitous oxygen minimum zone microbes. Proc Natl Acad Sci U S A. 2014;111:11395–11400.

12. Saito MA, McIlvin MR, Moran DM, Santoro AE, Dupont CL, Rafter PA, et al. Abundant nitrite-oxidizing metalloenzymes in the mesopelagic zone of the tropical Pacific Ocean. Nat Geosci. 2020;13:355–362.

13. Nunoura T, Takaki Y, Hirai M, Shimamura S, Makabe A, Koide O, et al. Hadal biosphere: Insight into the microbial ecosystem in the deepest ocean on Earth. Proc Natl Acad Sci U S A. 2015;112:E1230–E1236.

14. Rani S, Koh H, Rhee S, Fujitani H. Detection and Diversity of the Nitrite Oxidoreductase Alpha Subunit (*nxrA*) Gene of *Nitrospina* in Marine Sediments. Microb Ecol. 2017;37:111–122.

15. Santoro AE, Casciotti KL, Francis CA. Activity, abundance and diversity of nitrifying archaea and bacteria in the central California Current. Environ Microbiol. 2010;12:1989–2006.

16. Damashek J, Tolar BB, Liu Q, Okotie-Oyekan AO, Wallsgrove NJ, Popp BN, et al. Microbial oxidation of nitrogen supplied as selected organic nitrogen compounds in the South Atlantic Bight. Limnol Oceanogr. 2019;64:982–995.

17. Jorgensen SL, Hannisdal B, Lanzén A, Baumberger T, Flesland K, Fonseca R, et al. Correlating microbial community profiles with geochemical data in highly stratified sediments from the Arctic Mid-Ocean Ridge. Proc Natl Acad Sci U S A. 2012;109:E2846–E2855.

614 18. Bristow LA, Dalsgaard T, Tiano L, Mills DB, Bertagnolli AD, Wright JJ, et al.
615 Ammonium and nitrite oxidation at nanomolar oxygen concentrations in oxygen minimum
616 zone waters. Proc Natl Acad Sci U S A. 2016;113:10601–10606.
617 19. Lipschultz F, Wofsy SC, Ward BB, Codispoti LA, Friedrich G, Elkins JW. Bacterial
618 transformations of inorganic nitrogen in the oxygen-deficient waters of the Eastern
619 Tropical South Pacific Ocean. Deep Sea Res Part A Oceanogr Res Pap 1990;37:1513–
620 1541.
621 20. Pachiadaki MG, Sintes E, Bergauer K, Brown JM, Record NR, Swan BK, et al. Major role
622 of nitrite-oxidizing bacteria in dark ocean carbon fixation. Science 2017; 358: 1046–1051.
623 21. Kitzinger K, Marchant HK, Bristow LA, Herbold CW, Padilla CC, Kidane AT, et al.
624 Single cell analyses reveal contrasting life strategies of the two main nitrifiers in the
625 ocean. Nat Commun. 2020;11.
626 22. Zhang Y, Qin W, Hou L, Zakem EJ, Wan X, Zhao Z, et al. Nitrifier adaptation to low
627 energy flux controls inventory of reduced nitrogen in the dark ocean. Proc Natl Acad Sci
628 U S A. 2020;117:4823–4830.
629 23. Watson SW, Waterbury JB. Characteristics of Two Marine Nitrite Oxidizing Bacteria ,
630 *Nitrospina gracilis nov. ten. nov. sp.* and *Nitrococcus mobilis nov. ten. nov. sp.**. Arch
631 Mikrobiol. 1971;77:203–230.
632 24. Spieck E, Keuter S, Wenzel T, Bock E, Ludwig W. Characterization of a new marine
633 nitrite oxidizing bacterium , *Nitrospina watsonii* sp . nov ., a member of the newly
634 proposed phylum " *Nitrospinae* ". Syst Appl Microbiol. 2014;37:170–176.
635 25. Lücker S, Nowka B, Rattei T, Spieck E, Daims H. The genome of *Nitrospina gracilis*
636 illuminates the metabolism and evolution of the major marine nitrite oxidizer. Front

637 Microbiol Microbiol. 2013;4:1–19.

638 26. Koch H, Galushko A, Albertsen M, Schintlmeister A, Gruber-Doringer C, Lücker S, et al.
639 Growth of nitrite-oxidizing bacteria by aerobic hydrogen oxidation. Science.
640 2014;345:1052–1054.

641 27. Koch H, Lücker S, Albertsen M, Kitzinger K, Herbold C, Spieck E. Expanded metabolic
642 versatility of ubiquitous nitrite-oxidizing bacteria from the genus *Nitrospira*. Proc Natl
643 Acad Sci U S A. 2015;112:11371–11376.

644 28. Sorokin DY, Vejmelkova D, Lücker S, Streshinskaya GM, Rijpstra WIC, Sinninghe
645 Damsté JS, et al. *Nitrolancea hollandica* gen. nov., sp. nov., a chemolithoautotrophic
646 nitrite-oxidizing bacterium isolated from a bioreactor belonging to the phylum
647 *Chloroflexi*. Int J Syst Evol Microbiol. 2014;64:1859–1865.

648 29. Lebedeva E V., Alawi M, Jozsa PG, Daims H, Spieck E. Physiological and phylogenetic
649 characterization of a novel lithoautotrophic nitrite-oxidizing bacterium, '*Candidatus*
650 Nitrospira bockiana'. Int J Syst Evol Microbiol. 2008;58:242–250.

651 30. Lee KS, Palatinszky M, Pereira FC, Nguyen J, Fernandez VI, Mueller AJ, et al. An
652 automated Raman-based platform for the sorting of live cells by functional properties. Nat
653 Microbiol. 2019;4:1035–1048.

654 31. Kim JG, Park SJ, Sinninghe Damsté JS, Schouten S, Rijpstra WIC, Jung MY, et al.
655 Hydrogen peroxide detoxification is a key mechanism for growth of ammonia-oxidizing
656 archaea. Proc Natl Acad Sci U S A. 2016;113:7888–7893.

657 32. Miranda KM, Espey MG, Wink DA. A Rapid, Simple Spectrophotometric Method for
658 Simultaneous Detection of Nitrate and Nitrite. Nitric Oxide - Biol Chem. 2001;5:62–71.

659 33. Vallenet D, Calteau A, Dubois M, Amours P, Bazin A, Beuvin M, et al. MicroScope: an

integrated platform for the annotation and exploration of microbial gene functions through genomic, pangenomic and metabolic comparative analysis. Nucleic Acids Res. 2020;48:D579–D589.

34. Parks DH, Imelfort M, Skennerton CT, Hugenholtz P, Tyson GW. CheckM: Assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. Genome Res. 2015;25:1043–1055.

35. Nguyen LT, Schmidt HA, Von Haeseler A, Minh BQ. IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. Mol Biol Evol. 2015;32:268–274.

36. Kalyaanamoorthy S, Minh BQ, Wong TKF, Von Haeseler A, Jermiin LS. ModelFinder: Fast model selection for accurate phylogenetic estimates. Nat Methods. 2017;14:587–589.

37. Hoang DT, Chernomor O, von Haeseler A, Minh BQ, Vinh LS. UFBoot2: Improving the Ultrafast Bootstrap Approximation. Molecular biology and evolution. Mol Biol Evol. 2018;35:518–522.

38. Konstantinidis KT, Tiedje JM. Towards a genome-based taxonomy for prokaryotes. J Bacteriol. 2005;187:6258–6264.

39. Varghese NJ, Mukherjee S, Ivanova N, Konstantinidis T, Mavrommatis K, Kyrpides NC, et al. Microbial species delineation using whole genome sequences. Nucleic Acids Res. 2015;43:6761–6771.

40. Daebeler A, Herbold CW, Vierheilig J, Sedlacek CJ, Pjevac P, Albertsen M, et al. Cultivation and genomic analysis of '*Candidatus* Nitrosocaldus islandicus,' an obligately thermophilic, ammonia-oxidizing thaumarchaeon from a hot spring biofilm in Graendalur valley, Iceland. Front Microbiol. 2018;9:1–16.

683 41. R Core Team. R: A language and environment for statistical computing. 2019. R
684 Foundation for Statistical Computing, Vienna, Austria.
685 42. Kits KD, Sedlacek CJ, Lebedeva E V., Han P, Bulaev A, Pjevac P, et al. Kinetic analysis
686 of a complete nitrifier reveals an oligotrophic lifestyle. Nature. 2017;549:269–272.
687 43. Fujitani H, Kumagai A, Ushiki N, Momiuchi K. Selective isolation of ammonia-oxidizing
688 bacteria from autotrophic nitrifying granules by applying cell-sorting and sub-culturing of
689 microcolonies. Front Microbiol. 2015;6:1159.
690 44. Uchiyama T, Watanabe K. Substrate-induced gene expression (SIGEX) screening of
691 metagenome libraries. Nat Protoc. 2008;3:1202–1212.
692 45. Ostafe R, Prodanovic R, Commandeur U, Fischer R. Flow cytometry-based ultra-high-
693 throughput screening assay for cellulase activity. Anal Biochem. 2013;435:93–98.
694 46. Pereira H, Barreira L, Mozes A, Florindo C, Polo C, Duarte C V., et al. Microplate-based
695 high throughput screening procedure for the isolation of lipid-rich marine microalgae.
696 Biotechnol Biofuels. 2011;4:61.
697 47. Rüger H-J, Höfle MG. Marine Star-Shaped-Aggregate-Forming Bacteria: *Agrobacterium*
698 *atlanticum* sp. nov.; *Agrobacterium meteori* sp. nov.; *Agrobacterium ferrugineum* sp.
699 nov., nom. rev.; *Agrobacterium gelatinovorum* sp. nov., nom. rev.; and *Agrobacterium*
700 *stellulatum* sp. nov., nom. . Int J Syst Bacteriol. 1992;42:133–143.
701 48. Lee K, Choo Y, Giovannoni SJ, Cho J. *Maritimibacter alkaliphilus* gen. nov., sp. nov., a
702 genome-sequenced marine bacterium of the Roseobacter clade in the order
703 *Rhodobacterales*. Int J Syst Evol Microbiol. 2007;57:1653–1658.
704 49. Morris JJ, Johnson ZI, Szul MJ, Keller M, Zinser ER. Dependence of the cyanobacterium
705 *Prochlorococcus* on hydrogen peroxide scavenging microbes for growth at the ocean's

706 surface. PLoS One. 2011;6.

707 50. Bayer B, Pelikan C, Bittner MJ, Reinthaler T, Herndl GJ, Offre P. Proteomic Response of
708 Three Marine Ammonia-Oxidizing Archaea to Hydrogen Peroxide and Their Metabolic
709 Interactions with a Heterotrophic Alphaproteobacterium. mSystems. 2019;4:1–15.

710 51. Bayer B, Hansman RL, Bittner MJ, Noriega-ortega BE, Niggemann J, Dittmar T, et al.
711 Ammonia-oxidizing archaea release a suite of organic compounds potentially fueling
712 prokaryotic heterotrophy in the ocean. Environ Microbiol. 2019;21:4062–4075.

713 52. Rodriguez-r LM, Konstantinidis KT, Sequence-discrete MF. Bypassing Cultivation To
714 Identify Bacterial Species. Microbe. 2014;9:111–118.

715 53. Watson SW, Bock E, Valois FW, Waterbury JB, Schlosser U. *Nitrospira marina* gen. nov.
716 sp. nov.: a chemolithotrophic nitrite-oxidizing bacterium. Arch Microbiol. 1986;144: 1–7.

717 54. Nowka B, Daims H, Spieck E. Comparison of oxidation kinetics of nitrite-oxidizing
718 bacteria: Nitrite availability as a key factor in niche differentiation. Appl Environ
719 Microbiol. 2015;81:745–753.

720 55. Jacob J, Nowka B, Merten V, Sanders T, Spieck E, Dähnke K. Oxidation kinetics and
721 inverse isotope effect of marine nitrite-oxidizing isolates. Aquat Microb Ecol.
722 2017;80:289–300.

723 56. Ushiki N, Jinno M, Fujitani H, Suenaga T, Terada A, Tsuneda S. Nitrite oxidation kinetics
724 of two *Nitrospira* strains: The quest for competition and ecological niche differentiation. J
725 Biosci Bioeng. 2017;123:581–589.

726 57. Sun X, Ji Q, Jayakumar A, Ward BB. Dependence of nitrite oxidation on nitrite and
727 oxygen in low-oxygen seawater. Geophys Res Lett. 2017;44:7883–7891.

728 58. Jones R, Morita R. Low-temperature growth and whole-cell kinetics of a marine

729 ammonium oxidizer. Mar Ecol Prog Ser. 1985;21:239–243.

730 59. Kovárová-Kovar K, Egli T. Growth kinetics of suspended microbial cells: from single-
731 substrate-controlled growth to mixed-substrate kinetics. Microbiol Mol Biol Rev.
732 1998;62:646–66.

733 60. Martens-Habbena W, Stahl DA. Nitrogen metabolism and kinetics of ammonia-oxidizing
734 archaea. In: Koltz MG, Stein LY (eds). Research on Nitrification and Related Processes,
735 Part B. 1st edn. (Academic Press, 2011) pp. 466-487.

736 61. Serata M, Iino T, Yasuda E, Sako T. Roles of thioredoxin and thioredoxin reductase in the
737 resistance to oxidative stress in *Lactobacillus casei*. Microbiology. 2012;158:953–962.

738 62. Song JJ, Rhee JG, Suntharalingam M, Walsh SA, Spitz DR, Lee YJ. Role of glutaredoxin
739 in metabolic oxidative stress: Glutaredoxin as a sensor of oxidative stress mediated by
740 H2O2. J Biol Chem. 2002;277:46566–46575.

741 63. Lücker S, Wagner M, Maixner F, Pelletier E, Koch H, Vacherie B, et al. A *Nitrospira*
742 metagenome illuminates the physiology and evolution of globally important nitrite-
743 oxidizing bacteria. Proc Natl Acad Sci U S A. 2010;107:13479–13484.

744 64. Bock E, Wagner M. Oxidation of Inorganic Nitrogen Compounds as an Energy Source.
745 In: Dworkin M, Falkow S, Rosenberg E, Schleifer K-H, Stackebrandt E (eds). The
746 Prokaryotes: Volume 2: Ecophysiology and Biochemistry. 3rd edn. (Springer New York,
747 New York, 2006) pp 457–495.

748 65. Pereira MM, Santana M, Teixeira M. A novel scenario for the evolution of haem-copper
749 oxygen reductases. Biochim Biophys Acta. Bioenerg. 2001;1505:185–208.

750 66. Mundinger AB, Lawson CE, Jetten MSM, Koch H, Lücker S. Cultivation and
751 Transcriptional Analysis of a Canonical *Nitrospira* Under Stable Growth Conditions.

Front Microbiol. 2019;10:1325.

67. Borisov VB, Forte E, Davletshin A, Mastronicola D, Sarti P, Giuffrè A. Cytochrome bd oxidase from *Escherichia coli* displays high catalase activity: An additional defense against oxidative stress. FEBS Lett. 2013;587:2214–2218.

68. Das A, Silaghi-Dumitrescu R, Ljungdahl LG, Kurtz DM. Oxidase, Oxidative Stress, and Dioxygen Tolerance of the Strictly Anaerobic Bacterium. J Bacteriol. 2005;187:2020–2029.

69. Steinmüller W, Bock E. Growth of Nitrobacter in the Presence of Organic Matter. Arch Microbiol. 1976;108:299–304.

70. Banciu H, Sorokin DY. Adaptation in Haloalkaliphiles and Natronophilic Bacteria. In: Seckbach J, Oren A, Stan-Lotter H (eds). Polyextremophiles. (Springer, Dordrecht, 2013) pp 249-267.

71. Sáenz JP, Grosser D, Bradley AS, Lagny TJ, Lavrynenko O, Broda M, et al. Hopanoids as functional analogues of cholesterol in bacterial membranes. Proc Natl Acad Sci U S A. 2015;112:11971–11976.

72. Belin BJ, Busset N, Giraud E, Molinaro A, Silipo A, Newman DiK. Hopanoid lipids: From membranes to plant-bacteria interactions. Nat Rev Microbiol. 2018;16:304–315.

73. Kharbush JJ, Thompson LR, Haroon MF, Knight R, Aluwihare LI. Hopanoid-producing bacteria in the Red Sea include the major marine nitrite oxidizers. FEMS Microbiol Ecol. 2018;94:1–9.

74. Tian R-M, Sun J, Cai L, Zhang W-P, Zhou G-W, Qiu J-W, et al. The deep-sea glass sponge *Lophophysema eversa* harbours potential symbionts responsible for the nutrient conversions of carbon , nitrogen and sulfur. Environ Microbiol. 2016;18:2481–2494.

775 75. Klein JS, Lewinson O. Bacterial ATP-driven transporters of transition metals:
776 Physiological roles, mechanisms of action, and roles in bacterial virulence. Metallomics.
777 2011;3:1098–1108.
778 76. Fang H, Kang J, Zhang D. Microbial production of vitamin B12: A review and future
779 perspectives. Microb Cell Fact. 2017;16:1–14.
780 77. Doxey AC, Kurtz DA, Lynch MDJ, Sauder LA, Neufeld JD. Aquatic metagenomes
781 implicate *Thaumarchaeota* in global cobalamin production. ISME J. 2015; 9: 461–471.
782 78. Heal KR, Qin W, Ribalet F, Bertagnolli AD, Coyote-maestas W, Hmelo LR. Two distinct
783 pools of B12 analogs reveal community interdependencies in the ocean. Proc Natl Acad
784 Sci USA. 2016;114:364–369.
785 79. Heal KR, Qin W, Amin SA, Devol AH, Moffett JW, Armbrust EV, et al. Accumulation of
786 NO_2-cobalamin in nutrient-stressed ammonia-oxidizing archaea and in the oxygen de fi
787 cient zone of the eastern tropical North Pacific. Environ Microbiol Rep. 2018;10:453–457.
788 80. Park BJ, Park SJ, Yoon DN, Schouten S, Damsté JSS, Rhee SK. Cultivation of
789 autotrophic ammonia-oxidizing archaea from marine sediments in coculture with sulfur-
790 oxidizing bacteria. Appl Environ Microbiol. 2010;76: 7575–7587.
791 81. Kitzinger K, Koch H, Lücker S, Sedlacek CJ, Herbold C, Schwarz J, et al.
792 Characterization of the first "*Candidatus* Nitrotoga" isolate reveals metabolic versatility
793 and separate evolution of widespread nitrite-oxidizing bacteria. MBio. 2018;9:1–16.
794
795

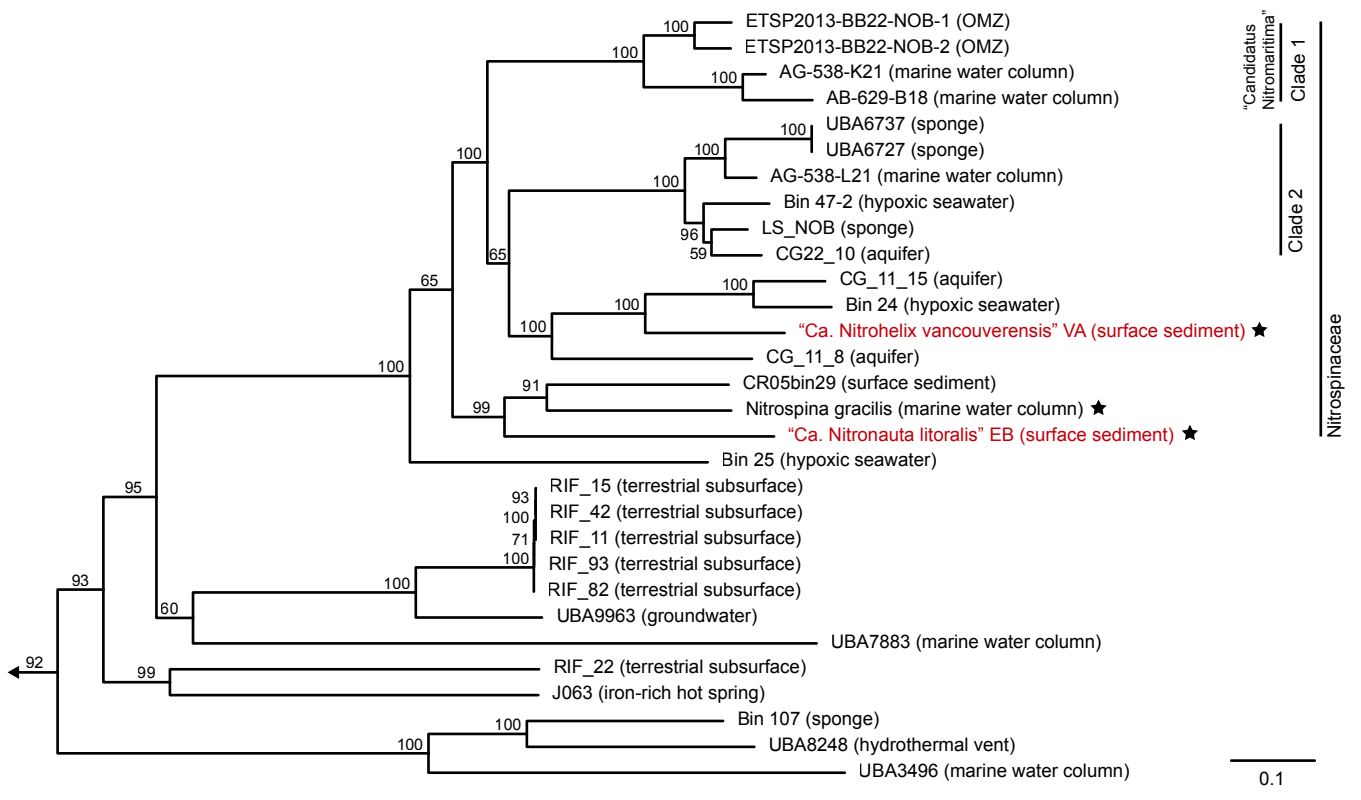
Figure legends

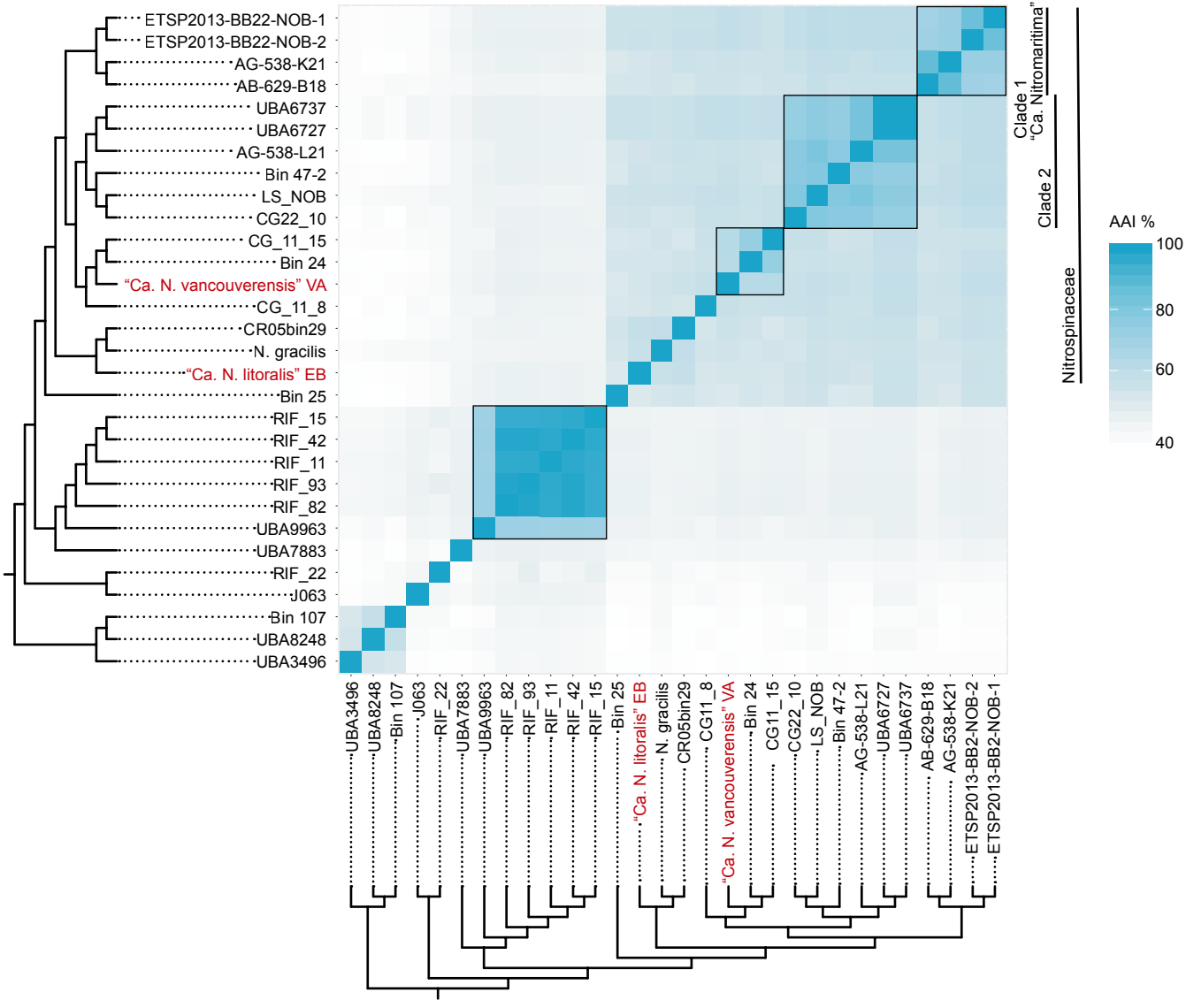
Figure 1. Concatenated marker gene tree of the Nitrospinae. The maximum likelihood tree shows the phylogenetic positions of the two newly cultured strains (highlighted in red) within the phylum Nitrospinae. The tree was calculated from a concatenated alignment of 120 conserved bacterial marker proteins. Details of the Nitrospinae genomes, which were used to calculate this tree, are listed in Table S1. Numbers at branches indicate ultrafast bootstrap (n=1,000) support. *Nitrospira moscoviensis*, *Desulfococcus multivorans*, and *Geobacter metallireducens* were used as an outgroup. Cultured organisms are marked with an asterisk. Clades of the Nitrospinae are indicated as proposed elsewhere [6] and the family Nitrospinaceae is indicated as determined by GTDB-TK. The scale bar indicates 0.1 estimated substitutions per residue. The sample source is indicated in parentheses.

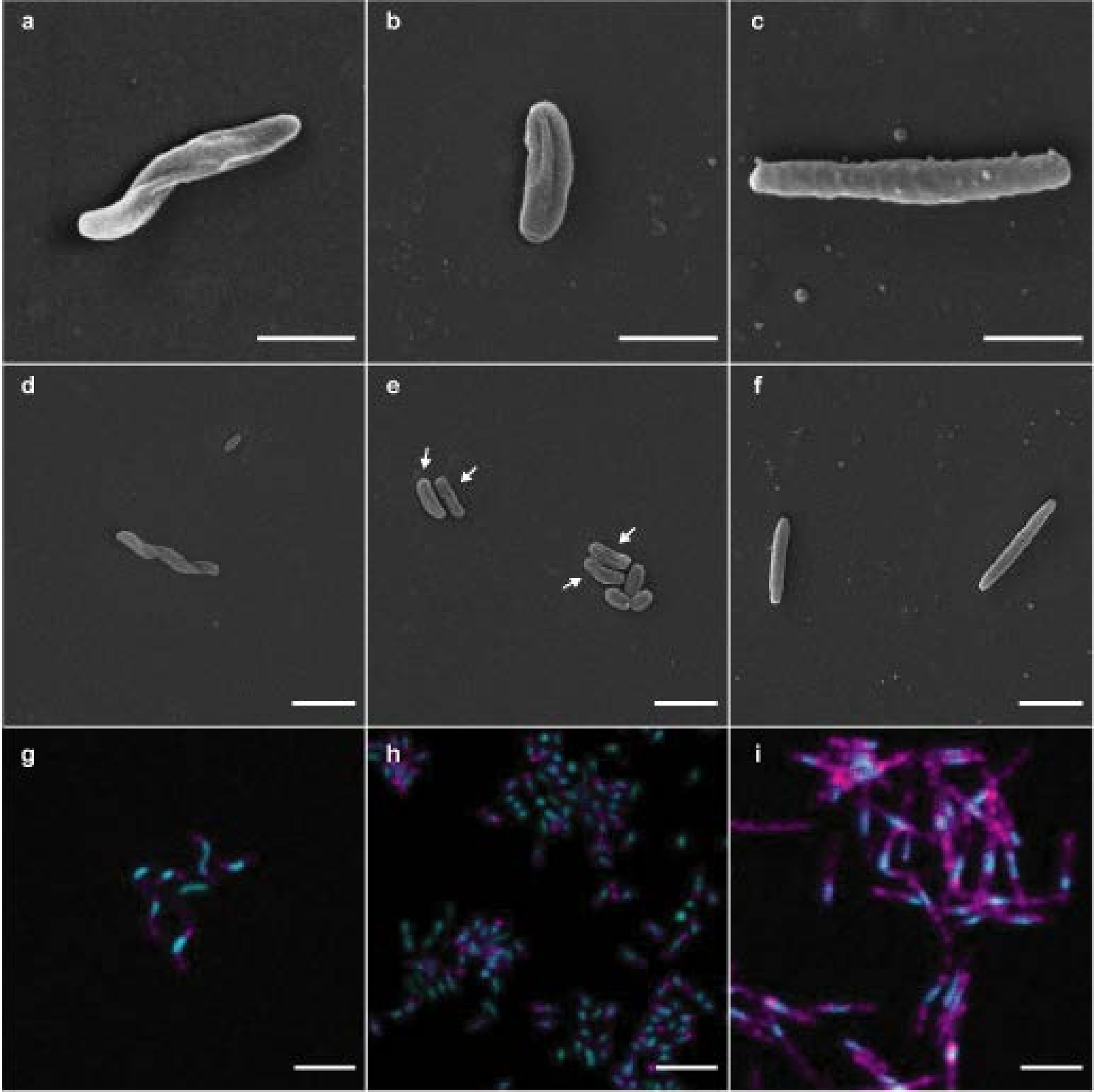
Figure 2. Average amino acid identity (AAI) analysis of the Nitrospinae. Pairwise AAI values were calculated for the same set of Nitrospinae genomes that was used to reconstruct the phylogenetic tree in Fig. 1. The same tree is used here to annotate the heatmap. The two newly cultured strains are highlighted in red. Clades of the Nitrospinae are indicated as proposed elsewhere [6] . The boxes indicate the genus boundary at 60% identity.

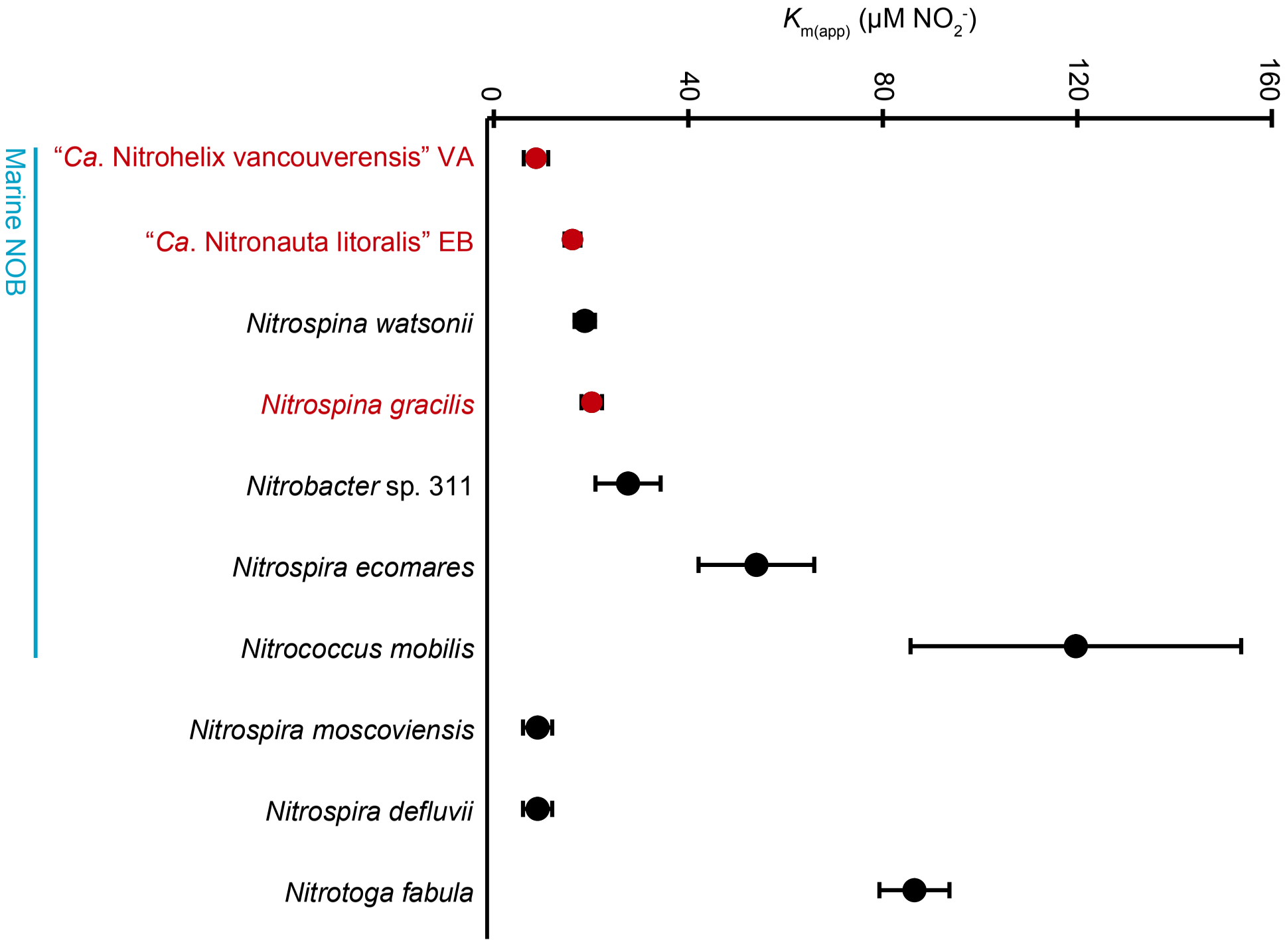
Figure 3. Scanning electron microscopy (SEM) and CARD-FISH images of the two newly cultured Nitrospinae strains and *N. gracilis*. **a, d, g** "*Ca*. Nitrohelix vancouverensis" strain VA. **b, e, h** "*Ca*. Nitronauta litoralis" strain EB. **c, f, i** *N. gracilis*. The short rod in **d** is a cell of the accompanying heterotroph *Stappia* sp. in the enrichment. **e** Shows slightly longer and slightly curved rods (strain EB, arrows) and shorter rods, which are cells of the accompanying

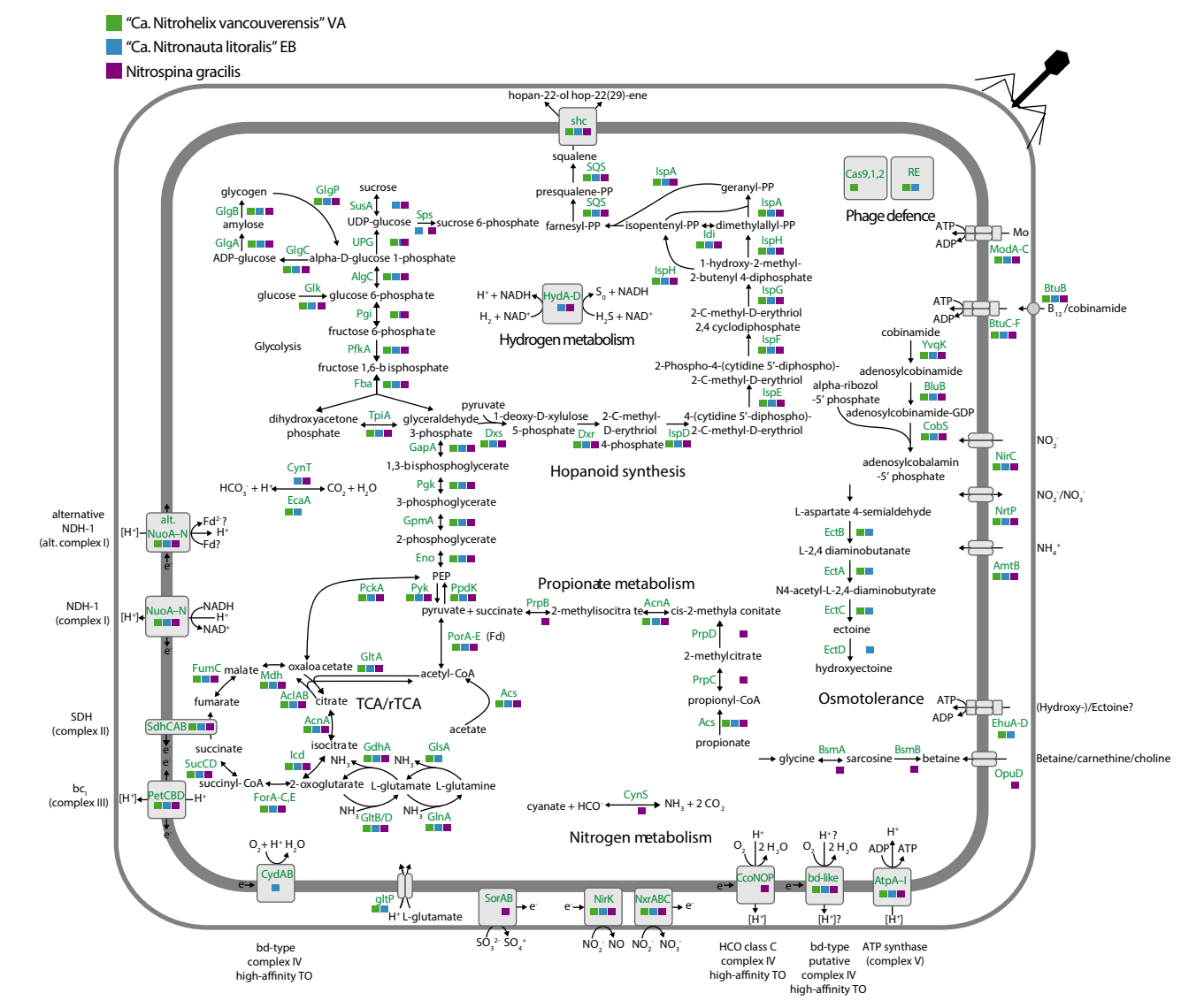
819 heterotroph *M. alkaliphilus* in the enrichment. The scale bars in **a-c** depict 1 µm; all other scale
820 bars depict 2 µm. **g-i** The 16S rRNA-targeted probe Ntspn759 was used to detect Nitrospinae
821 cells (magenta), and total nucleic acids were stained with SYBR Green (cyan).
822
823 **Figure 4.** Comparison of the whole-cell apparent half-saturation constants ($K_{m(app)}$) for nitrite
824 between marine and non-marine NOB. The $K_{m(app)}$ values measured in this study (highlighted in
825 red) are the mean from all biological replicates (n=3 for "*Ca.* Nitrohelix vancouverensis" VA;
826 n=5 for "*Ca.* Nitronauta litoralis" EB; n=8 for *N. gracilis*). The other $K_{m(app)}$ values were
827 retrieved from previous studies [54, 55, 81].
828
829 **Figure 5.** Cell cartoon based on the annotation of "*Ca.* Nitrohelix vancouverensis" VA, "*Ca.*
830 Nitronauta litoralis" EB and *N. gracilis 3/211*. The colored squares indicate the presence or
831 absence of the respective genes, "*Ca.* Nitrohelix vancouverensis" VA is shown in green, "*Ca.*
832 Nitronauta litoralis" EB in blue, and *N. gracilis 3/211* in purple. The gene annotations are
833 detailed in Table S3. Abbreviations: Fd, ferredoxin; RE, restriction enzymes; TO, terminal
834 oxidase.
835


ETSP2013-BB22-NOB-1 (OMZ)
ETSP2013-BB22-NOB-2 (OMZ)
AG-538-K21 (marine water column)
AB-629-B18 (marine water column)
UBA6737 (sponge)
UBA6727 (sponge)
AG-538-L21 (marine water column)
Bin 47-2 (hypoxic seawater)
LS_NOB (sponge)
CG22_10 (aquifer)
CG_11_15 (aquifer)
Bin 24 (hypoxic seawater)
"Ca. Nitrohelix vancouverensis" VA (surface sediment) ★
CG_11_8 (aquifer)
CR05bin29 (surface sediment)
Nitrospina gracilis (marine water column) ★
"Ca. Nitronauta litoralis" EB (surface sediment) ★
Bin 25 (hypoxic seawater)
RIF_15 (terrestrial subsurface)
RIF_42 (terrestrial subsurface)
RIF_11 (terrestrial subsurface)
RIF_93 (terrestrial subsurface)
RIF_82 (terrestrial subsurface)
UBA9963 (groundwater)
UBA7883 (marine water column)
RIF_22 (terrestrial subsurface)
J063 (iron-rich hot spring)
Bin 107 (sponge)
UBA8248 (hydrothermal vent)
UBA3496 (marine water column)
"Candidatus Nitromaritima"
Clade 1
Clade 2
Nitrospinaceae
0.1


ETSP2013-BB22-NOB-1
ETSP2013-BB22-NOB-2
AG-538-K21
AB-629-B18
UBA6737
UBA6727
AG-538-L21
Bin 47-2
LS_NOB
CG22_10
CG_11_15
Bin 24
"Ca. N. vancouverensis" VA
CG_11_8
CR05bin29
N. gracilis
"Ca. N. litoralis" EB
Bin 25
RIF_15
RIF_42
RIF_11
RIF_93
RIF_82
UBA9963
UBA7883
RIF_22
J063
Bin 107
UBA8248
UBA3496
Clade 1
"Ca. Nitromaritima"
Clade 2
Nitrospinaceae
AAI %
100
80
60
40


a
b
c
d
e
f
g
h
i


$K_{m(app)}$ (μM NO_2^-)
0
40
80
120
160
Marine NOB
"Ca. Nitrohelix vancouverensis" VA
"Ca. Nitronauta litoralis" EB
Nitrospina watsonii
Nitrospina gracilis
Nitrobacter sp. 311
Nitrospira ecomares
Nitrococcus mobilis
Nitrospira moscoviensis
Nitrospira defluvii
Nitrotoga fabula


"Ca. Nitrohelix vancouverensis" VA
"Ca. Nitronauta litoralis" EB
Nitrospina gracilis
hopan-22-ol hop-22(29)-ene
shc
squalene
SQS
presqualene-PP
farnesyl-PP
IspA
geranyl-PP
isopentenyl-PP
dimethylallyl-PP
Idi
IspH
1-hydroxy-2-methyl-2-butenyl 4-diphosphate
IspG
2-C-methyl-D-erythriol 2,4 cyclodiphosphate
IspF
2-Phospho-4-(cytidine 5'-diphospho)-2-C-methyl-D-erythriol
IspE
4-(cytidine 5'-diphospho)-2-C-methyl-D-erythriol
IspD
Dxr
1-deoxy-D-xylulose 5-phosphate
Dxs
pyruvate
glyceraldehyde 3-phosphate
TpiA
dihydroxyacetone phosphate
Fba
fructose 1,6-b isphosphate
PfkA
fructose 6-phosphate
Pgi
glucose 6-phosphate
Glk
glucose
Glycolysis
GlgP
GlgB
amylose
GlgA
ADP-glucose
GlgC
alpha-D-glucose 1-phosphate
UPG
UDP-glucose
SusA
sucrose
Sps
sucrose 6-phosphate
AlgC
HydA-D
H+ + NADH
H2 + NAD+
S0 + NADH
H2S + NAD+
Hydrogen metabolism
Hopanoid synthesis
Cas9,1,2
RE
Phage defence
ATP
ADP
Mo
ModA-C
BtuB
B12 /cobinamide
BtuC-F
cobinamide
YvqK
adenosylcobinamide
alpha-ribozol -5' phosphate
BluB
adenosylcobinamide-GDP
CobS
adenosylcobalamin -5' phosphate
NO2-
NirC
NO2-/NO3-
NrtP
NH4+
AmtB
L-aspartate 4-semialdehyde
EctB
L-2,4 diaminobutanate
EctA
N4-acetyl-L-2,4-diaminobutyrate
EctC
ectoine
EctD
hydroxyectoine
Osmotolerance
(Hydroxy-)/Ectoine?
EhuA-D
glycine
BsmA
sarcosine
BsmB
betaine
Betaine/carnethine/choline
OpuD
CynT
HCO3- + H+
CO2 + H2O
EcaA
CynS
cyanate + HCO-
NH3 + 2 CO2
Nitrogen metabolism
CydAB
O2 + H+ H2O
bd-type complex IV high-affinity TO
gltP
H+ L-glutamate
SorAB
SO3 2- SO4+
NirK
NO2- NO
NxrABC
NO2- NO3-
CcoNOP
HCO class C complex IV high-affinity TO
bd-like
bd-type putative complex IV high-affinity TO
AtpA-I
ATP synthase (complex V)
alternative NDH-1 (alt. complex I)
alt. NuoA-N
[H+]
Fd2+?
Fd?
H+
e-
NDH-1 (complex I)
NuoA-N
NADH
NAD+
SDH (complex II)
SdhCAB
succinate
fumarate
bc1 (complex III)
PetCBD
malate
FumC
oxaloacetate
Mdh
citrate
AclAB
GltA
acetyl-CoA
TCA/rTCA
AcnA
isocitrate
Icd
2-oxoglutarate
succinyl-CoA
SucCD
ForA-C,E
GdhA
NH3
GlsA
L-glutamate
L-glutamine
GltB/D
GlnA
Acs
acetate
PorA-E (Fd)
PEP
PckA
Pyk
PpdK
pyruvate + succinate
Propionate metabolism
PrpB
2-methylisocitrate
AcnA
cis-2-methyla conitate
PrpD
2-methylcitrate
PrpC
propionyl-CoA
Acs
propionate
Pgk
3-phosphoglycerate
GpmA
2-phosphoglycerate
Eno
1,3-bisphosphoglycerate
GapA
4-phosphate