Exploring the upper pH limits of nitrite oxidation: diversity, ecophysiology, and adaptive traits of haloalkalitolerant *Nitrospira*

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Abstract

Nitrite-oxidizing bacteria of the genus *Nitrospira* are key players of the biogeochemical nitrogen cycle. However, little is known about their occurrence and survival strategies in extreme pH environments. Here, we report on the discovery of physiologically versatile, haloalkalitolerant *Nitrospira* that drive nitrite oxidation at exceptionally high pH. *Nitrospira* distribution, diversity, and ecophysiology were studied in hypo- and subsaline, highly alkaline (pH 8.9-10.3) lakes by amplicon sequencing, metagenomics, and cultivation-based approaches. Surprisingly, diverse *Nitrospira* populations from lineages I, II and IV were found. Furthermore, the ability of *Nitrospira* enrichment cultures to oxidize nitrite at neutral to highly alkaline pH of 10.5 was demonstrated. Metagenomic analysis of a newly enriched *Nitrospira* lineage IV species, "*Candidatus* Nitrospira alkalitolerans", revealed numerous adaptive features of this organism to its extreme environment. Among them were a sodium-dependent N-type ATPase and NADH:quinone oxidoreductase next to the proton-driven forms usually found in *Nitrospira*. Other functions aid in pH and cation homeostasis and osmotic stress defense. "*Ca*. Nitrospira alkalitolerans" also possesses group 2a and 3b [NiFe] hydrogenases, suggesting it can use hydrogen as alternative energy source. These results reveal how *Nitrospira* cope with strongly fluctuating pH and salinity conditions and expand our knowledge of nitrogen cycling in extreme habitats.

Introduction

Chemolithoautotrophic nitrite-oxidizing bacteria (NOB) are key players of the nitrogen cycle in virtually all oxic habitats including soil, freshwater and marine ecosystems, engineered environments, and geothermal springs [1–9]. By catalyzing the second step of nitrification, NOB are the main biological source of nitrate, which is an important source of nitrogen and a terminal electron acceptor used by a plethora of other organisms. In most terrestrial and engineered environments, the predominant known NOB are uncultured members of the genus *Nitrospira* [1, 10–13]. Within this highly diverse genus, six phylogenetic lineages (named lineage I to VI) have been described, some of which seem to colonize distinct habitat types [1, 4, 8, 14, 15]. Recent studies revealed an unexpected metabolic versatility of *Nitrospira* beyond nitrite oxidation, such as aerobic growth on hydrogen or formate [16, 17] and, most surprisingly, the capability of complete ammonia oxidation to nitrate by some representatives (the comammox organisms) [18, 19].

Soda lakes and other haloalkaline systems are highly productive environments that harbor diverse, haloalkaliphilic microbial communities capable of rapid biogeochemical cycling [20–27]. Although nitrogen is rapidly cycled in these systems [20, 21, 28], knowledge of the responsible microbes and their ecology, in particular of NOB, is fragmentary [21, 27, 29, 30]. In a pioneering study, the hitherto only known facultatively alkaliphilic nitrite oxidizer, *Nitrobacter alkalicus*, was isolated and analyzed regarding its morphology and tolerance towards elevated pH of around 10 [29].

Shallow, saline-alkaline lakes are a characteristic of the Pannonian steppe in Central Europe – an ecosystem which extends into eastern Austria and is protected in the national park “Neusiedler See - Seewinkel”. The salinity of these lakes varies within the hyposaline range

and the pH is generally above 9 [31, 32]. These lakes exhibit a high turbidity caused by inorganic suspended particles and/or high humic substance content and frequently dry out during summer months [31]. Plant material of the shoreline vegetation and excrements of aquatic birds provide organic carbon and inorganic nitrogen and phosphorous inputs [33, 34]. Taken together, shallowness, intermittent character (periodic desiccation), high turbidity, alkaline pH, polyhumic organic carbon concentration, hypertrophic conditions and during summer high daily water temperature fluctuation create multiple extreme environmental conditions in these lakes [35].

In the present study, we obtained deeper insights into the biology of *Nitrospira* in haloalkaline systems. An investigation of the NOB community structure in sediments of saline-alkaline lakes in the national park “Neusiedler See – Seewinkel”, Burgenland, Austria (Fig. 1), by amplicon sequencing subsequently allowed for the targeted study of the ecophysiology and genomic adaptations in newly discovered alkalitolerant *Nitrospira*.

Materials and Methods

Sediment sampling and enrichment cultures of alkalitolerant Nitrospira

Triplicate sediment samples (approx. 10 g) from nine saline-alkaline lakes in the national park "Neusiedler See - Seewinkel”, Burgenland, Austria (Fig. 1) were sampled from the top 10 cm of the sediments within a radius of 5 m in October 2013. The water temperature and pH of each lake were measured at the time of sampling. Dissolved organic carbon (DOC) concentrations were analyzed from water filtered through precombusted glass fiber filters (GF/F, Whatman) and measured on a Total Carbon Analyzer (Sievers M9 Laboratory Analyzer, GE Analytical Instruments, Boulder, Colorado, U.S.A.). Nutrient concentrations (total nitrogen

[TN], phosphorus-P, ammonium-N, nitrite-N, and nitrate-N) in lake waters were determined using German Standard Methods [36]. Aliquots of both sediment and lake water samples were used as inoculum for nitrite oxidizer enrichment cultures, and the remaining material was stored at -20°C for molecular and chemical analyses. Concentration measurements of calcium carbonate, total nitrogen, and trace metals were performed by the Austrian Agency for Health and Food safety (AGES GmbH, Vienna, Austria) according to their standard protocols.

Enrichment cultures of alkalitolerant NOB were established in mineral nitrite medium with a pH of 9 to 10.2 at 28°C. Physiological tests were performed with selected *Nitrospira* enrichment cultures to determine their pH tolerance (with tested pH values ranging from 7.6 to 11) and nitrite concentration optimum of growth (with tested concentrations ranging from 0.15 to 1 mM NO_2^-). A detailed description of the cultivation procedure and physiological experiments is provided in the supplemental text.

Molecular analyses of Nitrospira *community structures*

DNA extraction, PCR amplification, cloning, Illumina amplicon sequencing, and phylogenetic analyses of 16S rRNA and *nxrB* genes, as well as rRNA-targeted fluorescence *in situ* hybridization (FISH), were performed as described in the supplemental text.

Metagenome sequencing, Nitrospira *genome assembly, and analyses of genes putatively involved in haloalkalitolerance*

Cells of the "*Ca*. Nitrospira alkalitolerans" enrichment culture were harvested by centrifugation at 20.000 x g for 15 min and the cell pellet was used for DNA extraction

according to Angel *et al.* [37]. Metagenome sequencing, assembly, binning, and annotation procedures are described in the supplemental text.

Specific genomic features of "*Ca.* N. alkalitolerans", which are likely important for its adaptation to haloalkaline conditions, were identified by comparison to previously sequenced genomes of *Nitrospira* and *Nitrospina* that did not originate from haloalkaline habitats by using the OrthoFinder software [38] with default settings. Organisms used in these analyses were *Nitrospira moscoviensis [17]* and *N. defluvii* [39] (both canonical NOB), *N. inopinata* [18, 40] (moderately thermophilic comammox organism), "*Ca.* N. nitrosa" and "*Ca.* N. nitrificans" [19] (two mesophilic comammox organisms), and *Nitrospina gracilis* [41] (marine canonical nitrite oxidizer). Phylogenetic trees and the amino acid alignments of ATPase subunit *c* were reconstructed as described in the supplemental text.

Data availability

The raw, demultiplexed amplicon sequencing datasets obtained in this study have been deposited at the European Nucleotide Archive (ENA) database under study accession number PRJEB34917. The raw metagenomic sequence reads obtained from the "*Ca.* N. alkalitolerans" enrichment culture have been deposited at ENA under study accession number PRJEB34830. The metagenome assembled (MAG) sequence and associated annotations of "*Ca.* N. alkalitolerans" are publicly available in MicroScope [42].

Results and Discussion

Community composition of Nitrospira *in the saline-alkaline lakes*

Members of the genus *Nitrospira* are the most diverse and widespread known NOB. However, reports of *Nitrospira* occurrence in alkaline habitats are scarce [23, 30], and a systematic assessment of their presence and activity in such extreme environments is missing. In this study, we discovered and investigated unusually alkalitolerant *Nitrospira* in saline-alkaline lakes of the national park “Neusiedler See – Seewinkel”, Burgenland, Austria using targeted amplicon profiling of the 16S rRNA and *nxrB* genes ,of which the later encodes subunit beta of nitrite oxidoreductase (the key enzyme for nitrite oxidation). In sediment samples from nine lakes, we detected phylogenetically diverse *Nitrospira* phylotypes which predominantly affiliated with *Nitrospira* lineages I, II and IV (Fig. 2) [1].

The genome-sequenced *Nitrospira* possess one to six paralogous copies of *nxrB*, and the *nxrB* copy numbers per genome remain unknown for the majority of uncultured *Nitrospira* [43]. This large variability likely affects relative abundance estimations of *Nitrospira* OTUs based on *nxrB* amplicon data. In contrast, all sequenced *Nitrospira* genomes contain only one ribosomal RNA (*rrn*) operon. Therefore, our further assessment of the *Nitrospira* community structures relies on the 16S rRNA amplicon datasets.

The estimated alpha-diversity of *Nitrospira* 16S rRNA phylotypes was compared across the nine examined lakes (Fig. S2). The inverse Simpson’s index of the *Nitrospira* communities was negatively correlated with pH and the nitrite concentration ($p = 0.00004$, Tau-b = -0.53 for pH and $p = 0.03$, Tau-b = -0.36 for nitrite), indicating that only specific lineages of *Nitrospira* can successfully colonize highly alkaline habitats.

The *Nitrospira* communities clustered into two distinct major groups (Fig. 3). Group 1 mainly comprised the communities from those lakes, which are located closely to the shore of the much larger Lake Neusiedl, whereas group 2 contained the communities from the remaining

lakes that are farther away from Lake Neusiedl (Fig. 1). The average pH and salinity in the water of lakes from the group 1 cluster were 9.97 ± 0.24 .and 6.1 ± 4.1 g/l, respectively. These values were significantly higher (Welch's t-test; p = 0.00001 for pH and p = 0.017 for salinity) than the mean pH of 9.37 ± 0.26 and salinity of 2.74 ± 0.88 g/l in the group 2 lakes (Table 1). None of the other determined lake properties at time of sampling differed significantly between the two groups. The *Nitrospira* phylotypes with the highest relative abundance in the sediments from group 1 were OTU1 and OTU20, both affiliated with *Nitrospira* lineage IV, whereas these OTUs were nearly absent from the sediments of the lakes in group 2 (Fig. 3). In contrast, the predominant phylotypes in the group 2 lake sediments were affiliated with *Nitrospira* lineage II (Fig. 3). Consistent with these results, a principal coordinate analysis showed a clear separation of the *Nitrospira* communities with the same two groups separated on the first axis of the ordination (Fig. S3). These results indicate a strong influence of pH and salinity on the composition of the *Nitrospira* communities. Members of *Nitrospira* lineage IV are adapted to saline conditions and are commonly found in marine ecosystems [15, 44–48]. However, to date no *Nitrospira* species have been described to tolerate elevated pH conditions. Our results show that a substantial diversity of *Nitrospira* is able to colonize alkaline environments. The data also indicate a niche differentiation between lineages IV and II in saline-alkaline lakes, which likely includes a higher tolerance of the detected lineage IV organisms towards an elevated pH and salinity.

Metagenome sequencing and physiology of alkalitolerant Nitrospira *enrichments*

Following the inoculation of mineral nitrite medium flasks with sediment and/or water samples from four saline-alkaline lakes (LL, WW, KS and OEW; abbreviations see Table 1), we

initially obtained 17 enrichment cultures that oxidized nitrite to nitrate. Based on FISH analyses with *Nitrospira*-specific 16S rRNA-targeted probes and Sanger sequencing of cloned *16S rRNA* genes, several of these preliminary enrichment cultures contained co-existing phylotypes from *Nitrospira* lineages I, II, and IV as well as from the genus *Nitrobacter* (data not shown). Members of the genera *Nitrotoga* and *Nitrospina* were screened for by FISH or PCR, but were not detected.

We used three of the enrichments which contained only *Nitrospira* NOB and originated from different lakes (referred to as EN_A from lake OEW, EN_B from lake LL, and EN_C from lake WW comprising approx. 35% *Nitrospira* in relation to the total microbial community based on FISH analysis) to determine the pH range for activity of the enriched *Nitrospira* members. Enrichment cultures EN_A and EN_C contained phylotypes from *Nitrospira* lineages I and II, while EN_B contained phylotypes from lineages I, II, and IV as determined by 16 rRNA gene amplicon cloning and Sanger sequencing (Fig. 2). At first, aliquots of each enrichment culture were incubated with nitrite as the sole added energy source for six weeks at pH 7.6 and 9, respectively. During this period, pH had no significant effect on the amounts of nitrite utilized (Tukey's HSD, $p = 0.82$) and nitrate produced (Tukey's HSD, $p = 0.88$) by any of the three enrichments (Fig. S4). Subsequently, the enrichment culture aliquots that had been incubated at pH 9 were sequentially incubated at pH 10, 10.5, and 11 for eight to nine days at each pH. For all three enrichments, the observed nitrate production was slower at pH 10 and 10.5 than at pH 9 (Fig. S4 and S5). At pH 11, no nitrite consumption was detected (Fig. S5). Based on these results, we conclude that the diverse *Nitrospira* communities in the enrichments consisted of alkalitolerant NOB, whose activity is unaffected by pH at least up to pH 9 and has an upper limit between pH 10.5 and 11. This is remarkable, because previously

enriched or isolated *Nitrospira* strains were not grown above pH 8.0 except for two *Nitrospira* cultures from geothermal springs, which showed activity up to pH 8.8 [4] or pH 9.0 [7]. To our knowledge, this is the first report of nitrite oxidation by *Nitrospira* at pH values above 9 and as high as 10.5.

Further analyses focused on one additional enrichment, which had been inoculated with sediment from lake Krautingsee, belonging to the group 2 of the analyzed lakes (KS, Table 1). In contrast to the other enrichment cultures, this enrichment contained only lineage IV *Nitrospira* based on FISH analysis (Fig. 4a). *Nitrospira*-specific, 16S rRNA gene and *nxrB* gene-targeted PCR and phylogeny detected one phylotype from *Nitrospira* lineage IV that was related to other phylotypes detected from the lakes, specifically OTU 5 and EN_B_1 (16S rRNA gene, 100% and 98% nucleotide sequence identity, respectively; Fig. 2) and OTU 2 (*nxrB* gene, 98.5% nucleotide sequence identity; Fig. S1). The OTU 5 phylotype occurred in most of the analyzed lakes (Fig. 3). Thus, the closely related enrichment from lake KS may represent *Nitrospira* that could adapt to a relatively broad range of conditions, while some of the other OTUs were more abundant NOB in specific lakes (Fig. 3). The enriched *Nitrospira* reached a high relative abundance in the enrichment culture of ~60% of all bacteria based on metagenomic read abundance (see below) and observation by FISH.

High-throughput metagenome sequencing, scaffold assembly, and binning revealed that the enrichment contained three *Nitrospira* strains that could be separated into three genome bins based on sequence coverage data (Table S1, Fig. S7). No other NOB were identified in the metagenome, and the three *Nitrospira* bins represented the most abundant organisms in the enrichment culture (Fig. 4b). Since the genome-wide average nucleotide identity (gANI) values were above the current species threshold of 95% [49] (Table S1), the three bins likely

represented very closely related strains of the same *Nitrospira* lineage IV species with unique genetic components. From the predominant (based on coverage data) *Nitrospira* sequence bin, an almost complete metagenome-assembled genome (MAG) was reconstructed, which met the criteria for a “high-quality draft” genome [50] (Table S1), and used for comparative genomic analysis. Genome-wide, pairwise comparison of the gANI and average amino acid (gAAI) identity between this MAG and *Nitrospira marina* as the only other genome-sequenced and cultured *Nitrospira* lineage IV representative resulted in values of 80.1 and 77.3, respectively. The 16S rRNA gene, which had been retrieved from the MAG, was 97.90% identical to the 16S rRNA gene of *N. marina*, 97.87% identical to “N. strain Ecomares 2.1”, 94.92% to “*Ca*. N. salsa”, and 94.51% to “*Nitrospira* strain Aa01”, which are the other cultured members of *Nitrospira* lineage IV [15, 44, 47, 48]. These values are below the current species threshold of 98.7-99% for 16S rRNA [54]. Based on the low gANI and 16S rRNA sequence identities to described *Nitrospira* species, and additionally considering the distinct haloalkalitolerant phenotype (see also below), we conclude that the enriched *Nitrospira* represent a new species and propose "*Ca*. Nitrospira alkalitolerans” as the tentative name.

The enrichment culture was maintained at a pH of 9 to 10 and a salt concentration of 5 g/l, resembling the natural conditions in the saline-alkaline lakes based on available data from five years. "*Ca*. N. alkalitolerans” grew in dense flocks (Fig. 4a), thereby possibly relieving the pH stress [51]. Its nitrite-oxidizing activity was not affected when the pH in the cultivation medium decreased below 8. However, no nitrite oxidation was observed when the enrichment culture was transferred into medium with 4× to 8× higher salt concentrations, the latter resembling marine conditions. Thus, "*Ca*. N. alkalitolerans” is best described as a

250 facultatively haloalkalitolerant organism that oxidizes nitrite as an energy source over a wide range of pH and under hyposaline conditions. This phenotype is certainly advantageous in the investigated saline-alkaline lakes, as these lakes are prone to evaporation in summer, which causes a temporarily elevated salinity and alkalinity in the remaining water body and the sediment [35].

255 The enrichment culture of "*Ca*. N. alkalitolerans" oxidized nitrite over a broad range of initial nitrite concentrations tested, although an extended lag phase of 10 to 15 days occurred at the higher concentrations of 0.7 and 1 mM nitrite (Fig. S6). Similarly, a lag phase at elevated nitrite concentrations was also observed for the *Nitrospira* lineage II member *Nitrospira lenta* [52]. A preference for low nitrite levels is consistent with the presumed ecological role

260 of nitrite-oxidizing *Nitrospira* as slow-growing K-strategists, which are adapted to low nitrite concentrations [52–54].

Genomic adaptations to the saline-alkaline environment

As described below, comparative genomic analysis of "*Ca*. N. alkalitolerans" revealed several

265 features that distinguish this organism from other known NOB and likely form the basis of its tolerance towards elevated alkalinity and salinity (Fig. 5).

(i) Cytoplasmic pH and ion homeostasis

At high pH, alkaliphilic and alkalitolerant microbes maintain a higher transmembrane

270 electrical potential (ΔΨ) component of the proton motive force (PMF) than usually found in neutrophiles. The high ΔΨ is required to maintain PMF, because the ΔpH component of the PMF is reversed when the extracellular pH is higher than the intracellular pH [55]. Like in

neutrophiles, the ΔΨ of alkaliphiles is negative inside the cell relative to the outside [55]. Furthermore, the intracellular pH must be kept below the (extremely) alkaline extracellular pH. At elevated salinity, resistance against high salt concentrations is an additional, fundamental necessity for survival. All this requires a tightly regulated pH and ion homeostasis, in which cation transmembrane transporters play key roles [55–57]. The "*Ca*. N. alkalitolerans" genome codes for various Na^+-dependent transporters (Fig. 5, Table S2) including secondary Na^+/H^+ antiporters that are involved in pH homeostasis in other organisms: two copies of a group 3 Mrp-type Na^+/H^+ antiporter [58, 59] encoded by the seven genes *mrpA-G*, and monovalent cation-proton antiporters of the types NhaA and NhaB, each of which is encoded by a single gene [60]. The Mrp antiporter is crucial for growth at high pH and elevated salinity in alkaliphilic *Halomonas* spp. and *Bacillus* spp., where it exports Na^+ and imports H^+, thus contributing to the maintenance of a lower intracellular pH compared to the environment (*e.g.*, cytoplasmic pH 8.3 at external pH ~10.5) [50 and references cited therein, 56]. The Mrp proteins may form a large surface at the outside of the cytoplasmic membrane that could support proton capture under alkaline conditions [55, 58]. Nha-type antiporters are widely distributed among non-extremophilic and extremophilic organisms [56]. Being involved in the homeostasis of Na^+ and H^+, they are important for survival under saline and/or alkaline conditions [57]. In *E. coli*, NhaA is regulated by the cytoplasmic pH and it catalyzes the import of $2H^+$ with the concurrent export of one Na^+. This electrogenic activity is driven by ΔΨ and maintains pH homeostasis at elevated external pH [52 and references cited therein]. The simultaneous presence of the two antiporters NhaA and NhaB has been associated with halophilic or haloalkaliphilic phenotypes in other organisms [56, 60]. Although the regulation and cation transport

stoichiometry of the homologs in "*Ca*. N. alkalitolerans" remain unknown, the Mrp- and Nha-family antiporters most likely exhibit important physiological roles in this organism and support its survival under haloalkaline conditions. Possibly, "*Ca*. N. alkalitolerans" can even combine its growth in dense flocks with the extrusion of protons by its numerous proton transporters thereby lowering the pH inside the flock [51].

One of the two *nhaB* genes present in the "*Ca*. N. alkalitolerans" genome is located in an interesting genomic region that also contains all genes encoding the group 3 Mrp-type Na^+/H^+ antiporter (Fig. S8). The two genes downstream from *mrpD* display sequence similarity to the NADH dehydrogenase (complex I) subunits NuoM and NuoL. However, based on the genomic context they are more likely additional *mrpA*- and/or *mrpD*-like genes, as these Na^+/H^+ antiporter subunits are evolutionary related to NuoM and NuoL [62]. Multiple copies of subunits NuoM and NuoL of the NADH dehydrogenase are encoded elsewhere in the genome, partially in larger *nuo* operons (see Table S2). Moreover, the locus contains one gene coding for the low-affinity, high flux Na^+/HCO_3^- uptake symporter BicA [63] and gene *motB* encoding a H^+-translocating flagellar motor component (Fig. S8). In the haloalkalitolerant cyanobacterium *Aphanothece halophytica*, a similar clustering of *bicA* with genes coding for Na^+/H^+ antiporters has been described. The authors proposed a model of cooperation between these transporters, where Na^+ extruded by the Na^+/H^+ antiporters could drive the uptake of HCO_3^- by BicA under alkaline conditions when CO_2 becomes limiting [64]. Sodium-driven import of HCO_3^- could be an essential feature for "*Ca*. N. alkalitolerans", because bicarbonate is the main source of inorganic carbon for autotrophic organisms, but becomes less accessible at high pH >10 [56]. A carbonic anhydrase, which is also present in

the genome (Fig. 5, Table S2), can convert the imported HCO_3^- to CO_2 for carbon fixation *via* the reductive tricarboxylic acid cycle (Fig. 5).

Since cytoplasmic K^+ accumulation may compensate for Na^+ toxicity at elevated intracellular pH [65], many alkaliphiles retain an inward directed K^+ gradient [56]. The potassium uptake transporters of the Trk family contribute to pH and K^+ homeostasis of halo- and/or alkaliphiles [56]. TrkAH catalyzes the NAD^+-regulated uptake of K^+ possibly coupled with H^+ import [66]. Moreover, kinetic experiments revealed that TrkAH of the gammaproteobacterium *Alkalimonas amylolytica* is salt-tolerant and functions optimally at pH >8.5 [67]. "*Ca*. N. alkalitolerans" encodes a TrkAH complex (Fig. 5, Table S2), which may be a specific adaptation to its haloalkaline environment as no homologous K^+ transporter has been identified yet in any other NOB genome. Under more neutral pH conditions, Kef-type K^+ efflux pumps, which are present in two copies in the "*Ca*. N. alkalitolerans" genome, could excrete excess K^+ (Fig. 5, Table S2).

(ii) Adaptations of the energy metabolism

Aside from the different cation transporters (see above), "*Ca*. N. alkalitolerans" also encodes several mechanisms for cation homeostasis that are linked to membrane-bound electron transport and energy conservation. Like in other aerobic alkaliphiles [57], ATP synthesis is likely catalyzed by a canonical, H^+-translocating F_1F_O-ATPase (Fig. 5, Table S2). In addition, the genome contains all genes of a predicted Na^+-translocating N-ATPase [68] (Fig. 5, Fig. S9, Table S2). N-ATPases form a separate subfamily of F-type ATPases and have been suggested to be ATP-driven ion pumps that extrude Na^+ cations [68] or H^+ [69]. The *c* subunit of the N-ATPase in the genome of "*Ca*. N. alkalitolerans" contains the typical amino acid motifs for Na^+

binding and transport [68] (Fig. S10). Subunits *a* and *c* of the N-ATPase, which are involved in ion transport, are most similar to homologs from the halotolerant, sulfate-reducing *Desulfomicrobium baculatum* (81.5% AA identity) and the haloalkalitolerant, sulfur-oxidizing *Sulfuricella denitrificans* (88.2% AA identity), respectively. Hence, in "*Ca*. N. alkalitolerans", the N-ATPase may contribute to the maintenance of ΔΨ, the generation of a sodium motive force (SMF), and salt resistance (Fig. 5).

The genome of "*Ca*. N. alkalitolerans" encodes two different types of NADH:quinone oxidoreductase (complex I of the electron transport chain) (Fig. 5, Table S2). Firstly, the organism possesses all 14 genes of type I NADH dehydrogenase (*nuoA* to *nuoN*). They are present in one to three copies each. The *nuo* genes are mostly clustered at several genomic loci (Table S2) and are most similar to either of the two *nuo* operons present in *Nitrospira defluvii* [39], with AA identities between 41% and 90%. As mentioned above, *nuoL/M*-like genes at loci without other *nuo* genes might represent subunits of cation antiporters.

The genome furthermore contains a locus encoding all six subunits of a Na^+-dependent NADH:quinone oxidoreductase (Nqr or type III NAD dehydrogenase) (Fig. 5, Table S2). The locus is situated on a single contig in the vicinity of transposase genes, indicating that "*Ca*. N. alkalitolerans" might have received this type of complex I by lateral gene transfer. The gene of subunit E, which takes part in Na^+ translocation [70], is most similar to a homolog in the ammonia-oxidizing bacterium *Nitrosomonas nitrosa* (86% AA identity).

The metabolic model for *N. defluvii* [39] assumes that two different versions of the H^+-dependent complex I (Nuo) are used for forward or reverse electron transport, respectively. *Nitrospira* possess a canonical Nuo that is likely used for PMF generation during the forward flow of low-potential electrons from the degradation of intracellular glycogen or from

hydrogen as an alternative substrate (see also below). In addition, reverse electron transport is essential in NOB to generate reducing power for CO_2 fixation. In *Nitrospira*, a second (modified) form of Nuo with duplicated proton-translocating NuoM subunits might use PMF to lift electrons from quinol to ferredoxin [71]. The reduced ferredoxin is required for CO_2 fixation *via* the rTCA cycle. As expected, "*Ca*. N. alkalitolerans" possesses these two Nuo forms that are conserved in other characterized *Nitrospira* members. In addition, the Na^+-dependent Nqr complex might function in two directions in "*Ca*. N. alkalitolerans" as well. During forward electron flow, Nqr would contribute to SMF generation (Fig. 5). Reverse operation of the Nqr could generate NADH while importing Na^+, thus utilizing SMF for the reduction of NAD^+ with electrons derived from quinol (Fig. 5). Hence, the two types of complex I are likely involved in essential electron transport and the fine-tuning of PMF and SMF. They probably cooperate with the Na^+- and the H^+-translocating ATPases and the various cation transporters (see above) to adjust the cytoplasmic ion concentrations and the membrane potential in response to the environmental salinity and pH.

In addition to a novel "*bd*-like" cytochrome c oxidase, which is commonly found in *Nitrospira* genomes [16, 39], the genome of "*Ca*. N. alkalitolerans" contains a locus with fused genes for a cbb_3-type cytochrome c oxidase (Fig. 5, Table S2) similar to the one present in the marine nitrite oxidizer *Nitrospina gracilis* [41]. The cbb_3-type terminal oxidases usually exhibit high affinities for O_2 [72] and may allow "*Ca*. N. alkalitolerans" to sustain respiration at low oxygen levels.

Interestingly, "*Ca*. N. alkalitolerans" encodes two different hydrogenases and the accessory proteins for hydrogenase maturation (Fig. 5, Table S2). First, it possesses a group 2a uptake hydrogenase that is also found in *N. moscoviensis*, which can grow autotrophically on H_2 as

the sole energy source [16]. Second, "*Ca*. N. alkalitolerans" codes for a putative bidirectional group 3b (sulf)hydrogenase that also occurs in other NOB and in comammox *Nitrospira* [18, 41] but has not been functionally characterized in these organisms. The capability to use H_2 as an alternative energy source and electron donor likely confers ecophysiological flexibility, especially if nitrite concentrations fluctuate and H_2 is available at oxic-anoxic boundaries in biofilms or upper sediment layers. While electrons from the group 2a hydrogenase are probably transferred to quinone [16], the group 3b hydrogenase might reduce NAD^+ [41] and fuel forward electron transport through the Nuo and Nqr complexes (see above).

(iii) Osmoadaptation

The intracellular accumulation of compatible solutes is an important mechanism allowing microorganisms to withstand the high osmotic pressure in saline habitats [56]. "*Ca*. N. alkalitolerans" has the genetic capacity to synthesize or import the compatible solutes trehalose, glycine betaine, and glutamate (Fig. 5, Table S2). The genes *opuD* and *opuCB* for glycine betaine import have been identified in the marine *Nitrospina gracilis* [41], but not yet in any *Nitrospira* species. In addition, we identified adaptations of "*Ca*. N. alkalitolerans" to the low availability of iron and the presence of toxic arsenite in saline-alkaline systems (supplemental text).

Conclusions

This study shows that diverse *Nitrospira* phylotypes are able to colonize saline-alkaline lakes, and that members of these lineages carry out chemolithoautotrophic nitrite oxidation under strongly alkaline conditions up to pH 10.5. The genomic analysis of the newly cultured

Nitrospira species "*Ca*. Nitrospira alkalitolerans" has revealed several adaptive features, many of which are also found in other haloalkalitolerant or –philic microorganisms but are missing in other characterized NOB. These results extend our picture of nitrogen cycling in extreme habitats and push the known limits of nitrite oxidation to an unusually high pH. The presence of hydrogenase genes in "*Ca*. N. alkalitolerans" suggests that alkalitolerant NOB can utilize alternative energy metabolisms and thus share at least part of the physiological versatility known from their neutrophilic relatives [13, 16, 17, 73]. As a next step it will be crucial to determine which ammonia oxidizers and/or comammox organisms coexist with alkalitolerant NOB and drive nitrification in saline-alkaline ecosystems.

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Author contributions

AD and HD conceived the study. AD took the samples, performed the physiological experiments and analyzed the data. AD, KK, HK, MS, JS set up the enrichments and collected data. CWH, SMK and MA processed the amplicon and metagenomic raw data. AD performed

phylogenetic and comparative genomic analyses. AD and HD wrote the manuscript with input from all authors.

Conflict of interest statement

The authors declare no conflict of interest.

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Tables and figures

Table 1. Environmental properties determined for sediments and lake water

Lake (group)	AS (1)	MS (1)	OS (1)	OH (1)	KS (2)	WW (2)	OEW (2)	LL (2)	ML (2)
pH*	9.98	9.77	10.3	9.82	9.71	9.18	9.16	9.42	8.9§
Conductivity [mS/ cm]*	16.0§	6.1§	4.4§	4.0§	3.88	2.79	4.66	4.19	1.7§
Salinity [g/l]	12.8	4.88	3.52	3.2	3.1	2.23	3.73	3.35	1.36
DOC [mg/l]*	NA	NA	NA	NA	531.75	139.61	280.22	138.05	NA
TN [mg/l]*	NA	NA	NA	NA	33.54	4.09	15.75	5.69	NA
NH_4^--N [µg/l]*	NA	166	NA	1948	259	179	298	109	578
NO_2^--N [µg/l]*	NA	11	NA	398	30	4	13	7	12
NO_3^--N [µg/l]*	NA	179	NA	328	146	212	129	155	185
PO_4^--P [µg/l]*	NA	3100	NA	7850	133	58	1420	1840	774
$CaCO_3$ [%]	20.6	30.5	17.7	22.2	17.7	46.1	44.5	41.2	5.8
N_{tot} [%]	0.087	0.109	0.092	0.081	0.135	0.293	0.585	0.164	0.032
Fe [mol/gDW]	7.48	10	10.16	10.33	14.97	12.06	NA	12.34	8.1
Mn [mol/gDW]	2.86	2.47	2.31	2.42	2.69	2.03	NA	3.3	7.25
Cu [mol/gDW]	0.1	0.21	0.08	0.12	0.1	0.15	NA	0.19	0.11
Zn [mol/gDW]	0.08	0.06	0.07	0.1	0.1	0.11	NA	0.29	0.05

Table 1. Environmental properties determined for sediments and lake water (indicated by *) sampled in October 2013 from nine saline-alkaline lakes in the national park "Neusiedler See - Seewinkel", Burgenland, Austria. Values marked with § denote data derived from the literature [32]. Missing data marked with NA (not available) was due to insufficient material for determination. Salinity values were estimated using a regionally constant correction factor [31]. Per cent units for $CaCO_3$ and N_{tot} refer to the percentage weight of dried sediment. Abbreviations for lakes are as follows: AS, Albersee; MS, Mittlerer Stinker-See; OS, Oberer Stinker; OH, Obere Höll-Lacke; KS, Krautingsee; WW, Westliche Wörthenlacke; OEW, Östliche Wörthenlacke; LL, Lange Lacke; ML, Südliche Martinhoflacke.

Fig. 1

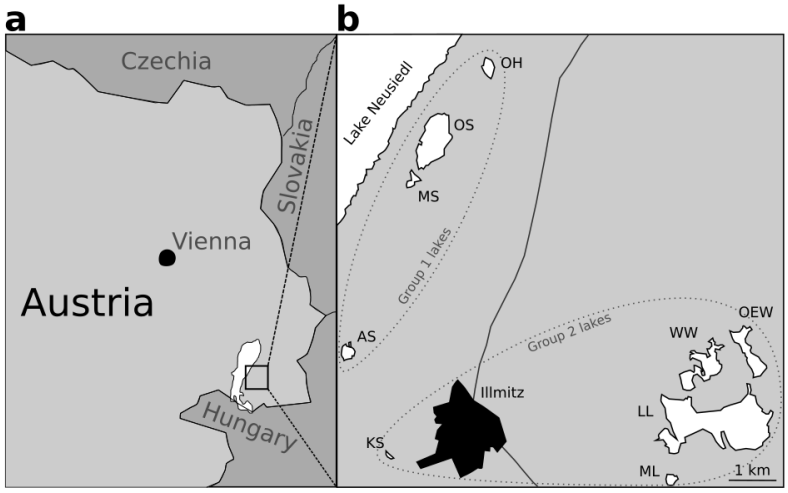


Fig. 1 Map of the sampled aline-alkaline lakes. (a) Location of the sampling region in Austria. (b) Geographic location of the sampled lakes in the national park "Neusiedler See – Seewinkel", Burgenland, Austria. The lakes are shown in white with the corresponding identifier abbreviations (see Table 1). Only lakes sampled for this study are shown. Dashed circles enclose lakes with similar *Nitrospira* communities (see Fig. 3).

Fig. 2

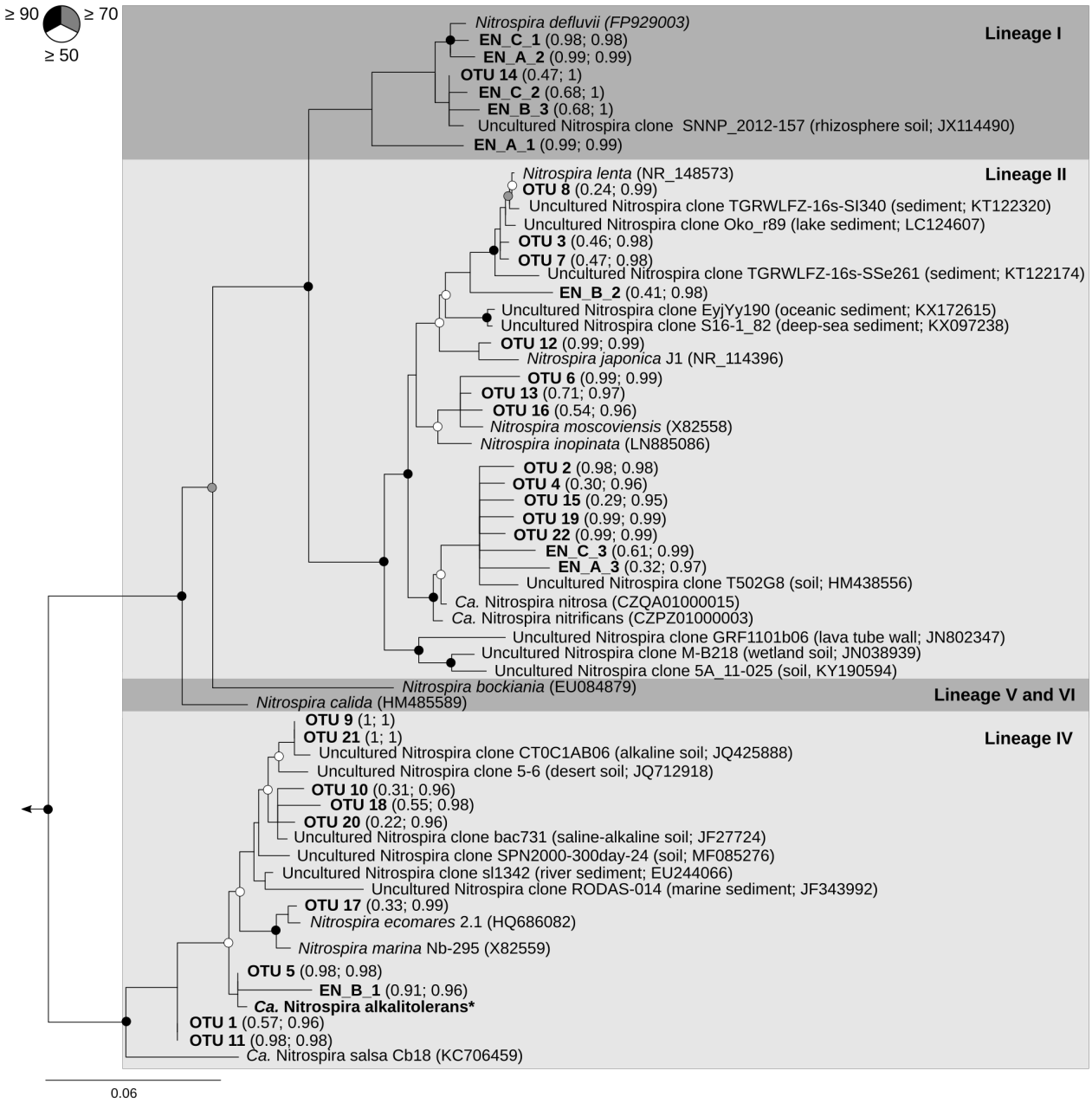


Fig. 2 Phylogenetic maximum likelihood analysis based on the 16S rRNA gene sequences of selected representatives from the genus *Nitrospira* and of the *Nitrospira* members detected in sediments from nine saline-alkaline lakes. Sequences obtained in this study are printed in bold. The organism marked with an asterisk is the *Nitrospira* species cultured and further analyzed in this study. The tree was constructed using full length sequences and a 50% conservation filter resulting in 1310 valid alignment positions. Shorter sequences from this study, generated through

amplicon and Sanger sequencing were added to the tree using the Evolutionary Placement Algorithm (EPA) without changing the overall tree topology. Numbers in brackets behind these sequences firstly denote the likelihood score of the exact placement and secondly the cumulative likelihood score of the placement within the cluster. Filled, grey, and open circles denote branches with ≥ 90%, ≥ 70% and ≥50% bootstrap support, respectively. *Leptospirillum ferrooxidans* (AJ237903), *Ca*. Magnetobacterium bavaricum (FP929063), *Thermodesulfovibrio yellowstonii* DSM 11347 (CP001147), and *Ca*. Methylomirabilis oxyfera (FP565575) were used as outgroup. The scale bar indicates 6% estimated sequence divergence.

Fig. 3

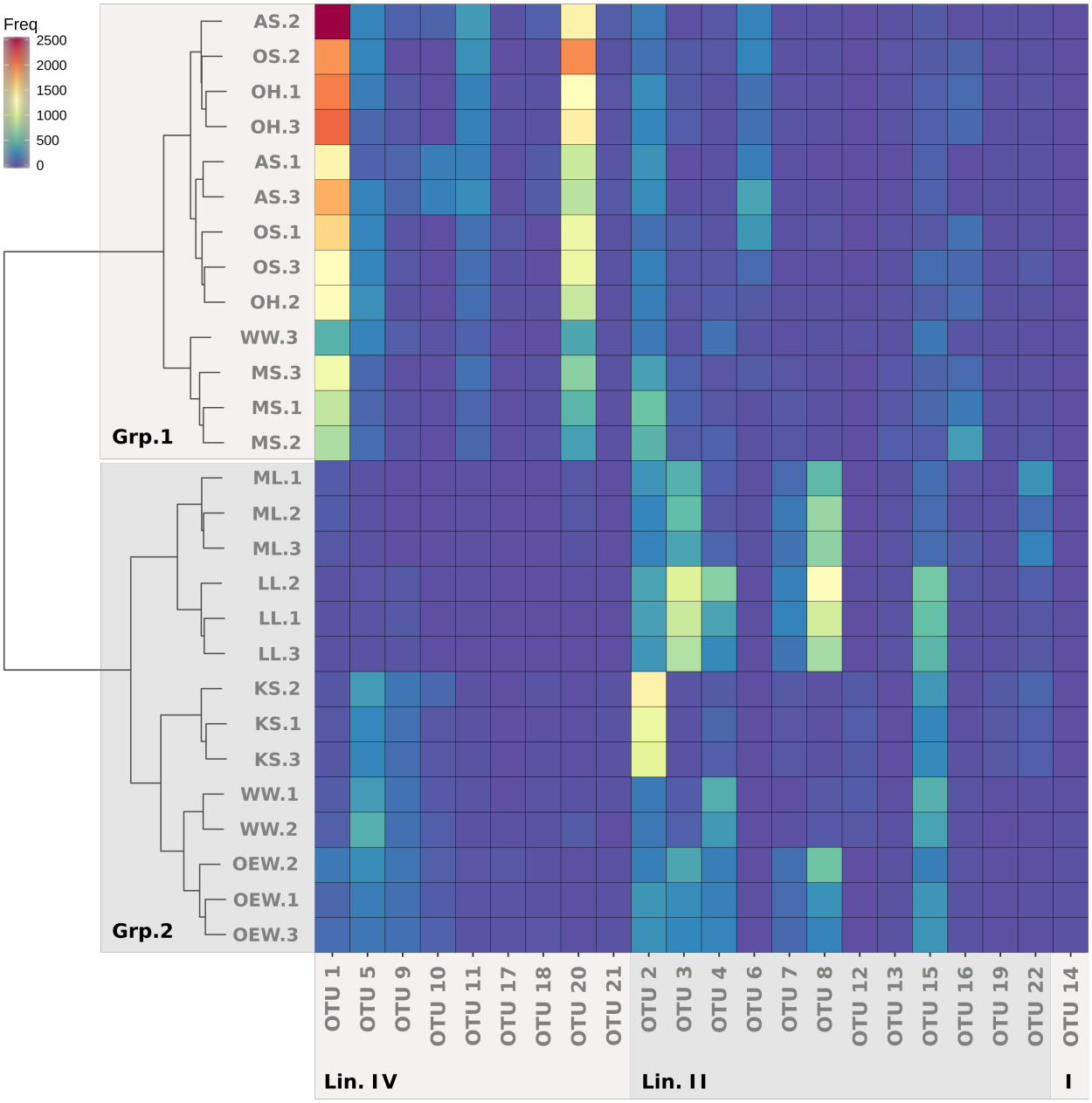


Fig. 3 Normalized abundances of *Nitrospira* 16S rRNA gene phylotypes detected in triplicate sediment samples from nine saline-alkaline lakes. *Nitrospira* communities are grouped by hierarchical clustering on the *y*-axis, and OTUs are grouped by phylogenetic affiliation on the *x*-axis. Lake names are abbreviated as in Table 1. Lin. IV, *Nitrospira* lineage IV; Lin. II, *Nitrospira* lineage II; I, *Nitrospira* lineage I; Freq, normalized frequency counts; Grp.1, group 1 lakes; Grp.2, group 2 lakes (see also Fig. 1).

Fig. 4

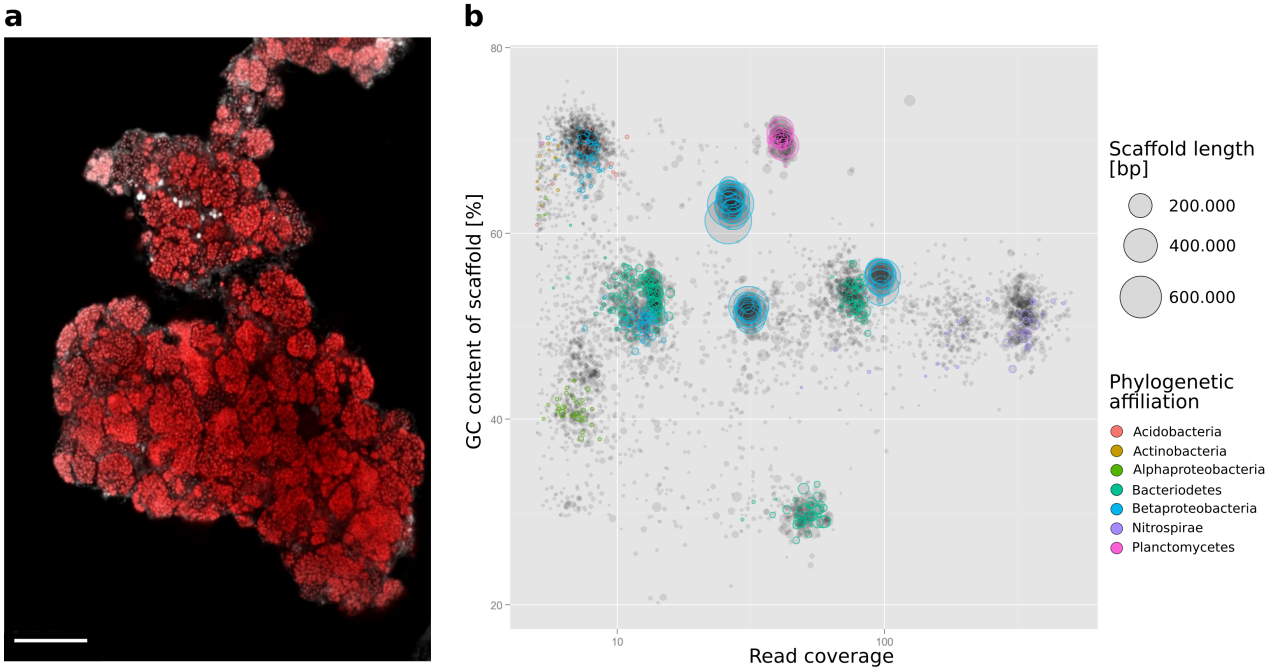
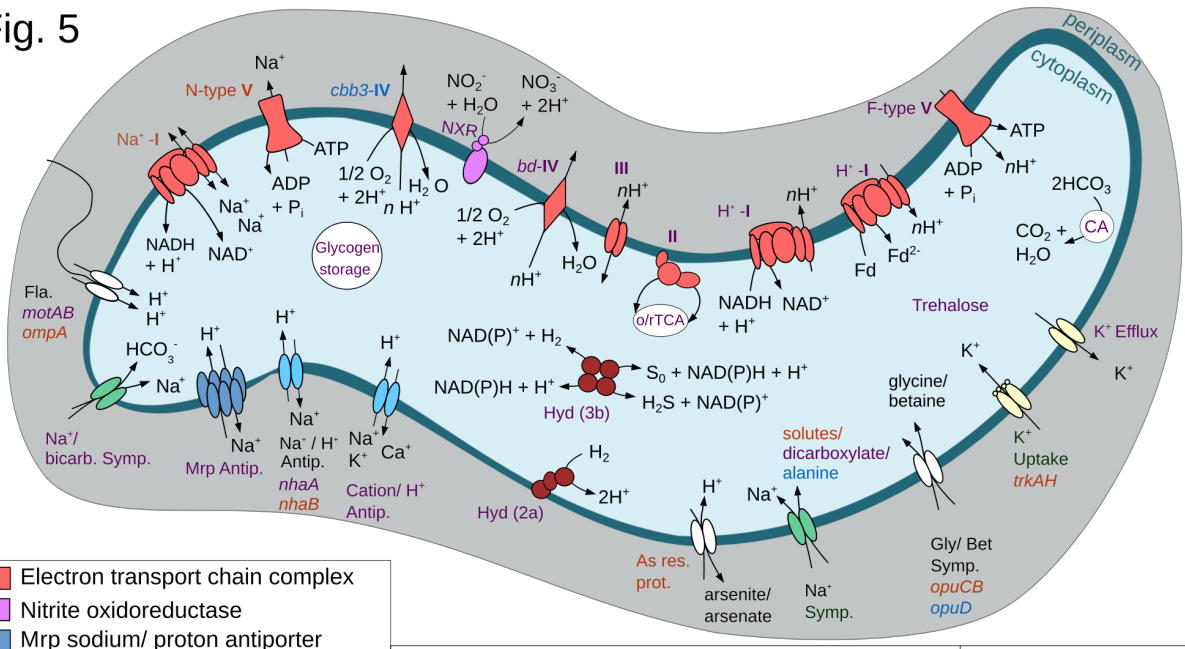


Fig. 4 Visualization and metagenomic analysis of the "*Ca*. N. alkalitolerans" enrichment. (a) FISH image showing dense cell clusters of "*Ca*. N. alkalitolerans" in the enrichment culture. The "*Ca*. N. alkalitolerans" cells appear in red (labelled by probe Ntspa1151 which has 1 mismatch at the 3' end to the 16S rRNA gene sequence of "*Ca*. N. alkalitolerans"; the absence of lineage II *Nitrospira* in the enrichment culture was confirmed by the application of the competitor oligonucleotides c1Ntspa1151 and c2Ntspa1151 as indicated in the supplemental text). Other organisms were stained by DAPI and are shown in light grey. Scale bar, 25 µm. (b) Phylogenetic affiliation of the metagenome scaffolds from the "*Ca*. N. alkalitolerans" enrichment, clustered based on sequence coverage and the GC content of DNA. Closed circles represent scaffolds, scaled by the square root of their length. Clusters of similarly colored circles represent potential genome bins.

Fig. 5



Electron transport chain complex	
Nitrite oxidoreductase	
Mrp sodium/ proton antiporter	
Na^+/ H^+ antiporter	
Na^+ symporter	
K^+ transport	
Other	
Na^+ -I	Complex I (Na^+ -translocating)
H^+ -I	Complex I (H^+ -translocating)
II	Complex II
III	Complex III

cbb3-IV	Complex IV (*cbb*$_3$-type cytochrome oxidase)
bd-IV	Complex IV (*bd*-type cytochrome oxidase)
F-type V	Complex V (F-type)
N-type V	Complex V (N-type)
o/rTCA	Oxidative /reductive TCA cycle
Hyd	Hydrogenase
CA	Carbonic anhydrase
As res. prot	Arsenical resistance protein

Trans.	Transporter
Symp.	Symporter
Antip.	Antiporter
Fla.	Flagellar motor
Present in other NOB	
None	Multiple
N. gracilis	

Fig. 5 Cell metabolic cartoon constructed from the genome annotation of "*Ca*. N. alkalitolerans". Features putatively involved in the adaptation to a high alkalinity and salinity, and selected core metabolic pathways of chemolithoautotrophic nitrite-oxidizing *Nitrospira*, are shown. Note that the transport stoichiometry of the ion transporters in "*Ca*. N. alkalitolerans" remains unknown. Colors of text labels indicate whether adaptive features are present (*i.e.*, have homologs) in the genomes of other NOB (red, feature is not present in any other characterized NOB; blue, feature is present only in the marine *Nitrospina gracilis*; purple, feature is present in several other characterized NOB).