Genome-enabled insights into the ecophysiology of the comammox bacterium *Candidatus* Nitrospira nitrosa

Pamela Y. Camejo[1], *Jorge Santo Domingo*[2], *Katherine D. McMahon*[1,3], *Daniel R. Noguera*[1,*]

[1]Department of Civil and Environmental Engineering, University of Wisconsin - Madison, Madison, WI, USA

[2]Environmental Protection Agency, Cincinnati, OH, USA

[3]Department of Bacteriology, University of Wisconsin - Madison, Madison, WI, USA

Email addresses:

camejo.pamela@gmail.com

Santodomingo.Jorge@epa.gov

trina.mcmahon@wisc.edu

noguera@engr.wisc.edu

* Corresponding author: Daniel R. Noguera, 1415 Engineering Drive, Madison, WI 53706. Email: noguera@engr.wisc.edu; Tel: 608-263-7783; Fax: 608-262-5199

ABSTRACT

The recently discovered comammox bacteria have the potential to completely oxidize ammonia to nitrate. These microorganisms are part of the *Nitrospira* genus and are present in a variety of environments, including Biological Nutrient Removal (BNR) systems. However, the physiological traits within and between comammox- and nitrite oxidizing bacteria (NOB)-like *Nitrospira* species have not been analyzed in these ecosystems. In this study, we identified *Nitrospira* strains dominating the nitrifying community of a sequencing batch reactor (SBR) performing BNR under micro-aerobic conditions. We recovered metagenomes-derived draft genomes from two *Nitrospira* strains: (1) *Nitrospira* sp. UW-LDO-01, a comammox-like organism classified as *Candidatus* Nitrospira nitrosa, and (2) *Nitrospira* sp. UW-LDO-02, a nitrite oxidizing strain belonging to the *Nitrospira defluvii* species. A comparative genomic analysis of these strains with other *Nitrospira*-like genomes identified genomic differences in *Ca.* Nitrospira nitrosa mainly attributed to each strains' niche adaptation. Traits associated with energy metabolism also differentiate comammox from NOB-like genomes. We also identified several transcriptionally regulated adaptive traits, including stress tolerance, biofilm formation and micro-aerobic metabolism, which might explain survival of *Nitrospira* under multiple environmental conditions. Overall, our analysis expanded our understanding of the genetic functional features of *Ca*. Nitrospira nitrosa, and identified genomic traits that further illuminate the phylogenetic diversity and metabolic plasticity of the *Nitrospira* genus.

44 **INTRODUCTION**

45 Nitrification is a microbiological process that plays an important role in the nitrogen (N)
46 cycle. This process has been conventionally known as a two-step reaction. The first step, oxidation
47 of ammonia to nitrite, is performed by ammonia-oxidizing bacteria (AOB) or archaea (AOA) and
48 the second step, oxidation of nitrite to nitrate, is carried out by nitrite-oxidizing bacteria (NOB).
49 Recently, the discovery of a new player with the potential to completely oxidize ammonia to
50 nitrate, as in the case of complete ammonia oxidizing (comammox) organisms,[1, 2] has dramatically
51 changed our understanding of microbial mediated N transformations in engineered and natural
52 systems.

53 Comammox bacteria have been classified within the genus *Nitrospira*. Members of this
54 genus were conventionally regarded as NOB and were thought to rely only on nitrite for growth.
55 However, the genomes of the four comammox-like *Nitrospira* identified to date (*Ca.* Nitrospira
56 nitrosa, *Ca.* Nitrospira nitrificans, *Ca.* Nitrospira inopinata and *Nitrospira* sp. Ga0074138[1-3]),
57 encode the genes necessary for ammonia and nitrite oxidation, suggesting that *Nitrospira* are much
58 more metabolically versatile organisms. Furthermore, comammox-like *Nitrospira* have been
59 identified in a variety of habitats, including groundwater wells, drinking water biofilters,
60 wastewater treatment plants (WWTPs) and other soil and aquatic environments.[4] These findings
61 have prompted questions regarding the ecological significance and lifestyle of these organisms in
62 each of these ecosystems.

63 Nutrient removal in WWTPs relies on nitrifying organisms to remove N from the
64 wastewater. *Nitrospira*-like bacteria appear to be the dominant nitrite-oxidizers[5-7] in most WWTPs
65 and laboratory scale reactors. The abundance of comammox in WWTPs has been briefly surveyed,

66 and preliminary results show this functional group is present in these systems.[4] However, genetic
67 and functional adaptations of comammox to this environment have not been addressed.

68 In this study, the community performing N removal in a Biological Nutrient Removal (BNR)
69 lab-scale reactor was analyzed to explore the genomic basis for comammox ecophysiology. A
70 sequencing batch reactor (SBR) was operated under cyclic anaerobic and micro-aerobic conditions
71 using two different operational conditions. During the first stage (nitrite addition during a micro-
72 aerobic phase), two *Nitrospira*-like strains were enriched in the reactor. Draft genome sequences
73 of these two strains were assembled from metagenomic data; one of them was identified as a
74 commamox organism, the other as an NOB. Here, we used the draft genomes of these strains, as
75 well as genomes from both NOB- and comammox-related bacteria, to perform a comparative
76 genome analysis of the genus *Nitrospira*.

77 **MATERIAL AND METHODS**

78 **Operation of Lab-Scale Sequencing Batch Reactor**

79 A laboratory-scale SBR was originally inoculated with activated sludge obtained from the
80 Nine Springs WWTP in Madison, WI, which uses a modified University of Cape Town (UCT)
81 process designed to achieve biological P removal[8] and operates with high aeration rates.[9] Synthetic
82 wastewater containing acetate as the sole carbon source was used for the feed, as described
83 elsewhere.[10] The hydraulic retention time (HRT) and solids retention time (SRT) were 24 h and
84 80 days, respectively. The pH in the system was controlled to be between 7.0 and 7.5.

85 The 2-liter reactor was operated under alternating anaerobic and low oxygen cycles. During
86 Stage 1 of operation the cycles consisted of 2 h anaerobic, 5 h micro-aerobic, 50 min settling and
87 10 min decanting. At the beginning of the micro-aerobic phase, sodium nitrite was added to reach

88 an in-reactor concentration of 10 mg N-NO_2^-/L and stimulate the use of nitrite as an electron donor.
89 In addition, an on/off control system was used to limit the amount of oxygen pumped to the reactor
90 (0.02 L/min) and maintain low dissolved oxygen (DO) concentrations in the mixed liquor, as
91 described elsewhere.[10] After 100 days of operation, the nitrite supplement was eliminated and the
92 reactor cycle was changed to: 1.5 h anaerobic, 5.5 h micro-aerobic, 50 min settling and 10 min
93 decanting (Stage 2).

94 **Sample Collection and Analytical Tests**

95 To monitor reactor performance, mixed liquor and effluent samples were collected, filtered
96 through a membrane filter (0.45 μm; Whatman, Maidstone, UK) and analyzed for acetate, PO_4^{3-}–
97 P, NH_4^+-N, NO_3^--N, and NO_2^--N. The concentrations of PO_4^{3-}–P were determined according to
98 Standard Methods.[11] Total ammonia (NH_3 + NH_4^+) concentrations were analyzed using the
99 salicylate method (Method 10031, Hach Company, Loveland, CO). Acetate, nitrite and nitrate
100 were measured using high-pressure liquid chromatography as previously described.[10]

101 Biomass samples from the reactors were collected weekly and stored at -80°C until DNA
102 extraction was performed. DNA was extracted using UltraClean® Soil DNA Isolation Kit (MoBIO
103 Laboratories, Carlsbad, CA). Extracted DNA was quantified using a NanoDrop spectrophotometer
104 (Thermo Fisher Scientific, Waltham, MA) and stored at -80°C.

105 **Metagenome Sequencing, Assembly and Binning**

106 Samples from day 100 (Stage 1) and days 317, 522 and 674 (Stage 2) were selected for
107 metagenomic analysis. Illumina TruSeq DNA PCR free libraries were prepared for DNA extracts
108 according to the manufacturer's protocol and paired-end sequenced on either the Illumina HiSeq
109 2000 platform (v4 chemistry, 2 × 150 bp; 522-day sample), or the Illumina MiSeq platform (v3

110 chemistry, 2 × 250 bp; other samples). Unmerged reads were quality-trimmed and filtered with
111 Sickle (https://github.com/ucdavis-bioinformatics/sickle.git), using a minimum phred score of 20
112 and a minimum length of 50 bp. The metagenomic reads from the 100-day sample (Stage 1) were
113 assembled using IDBA-UD.[12] Individual genome bins were extracted from the metagenome
114 assembly with the R package 'mmgenome'[13] using the differential coverage principle.[14] The bins
115 were initially extracted by plotting the genome coverage of contigs in metagenomes from day 100
116 and 317. During the bin extraction, GC content and taxonomy of contigs were also taken into
117 consideration.

118 After binning, SSPACE was used to filter small scaffolds (length < 1,000 bp), extend
119 scaffolds, and to fill gaps.[15] Genome completeness and contamination was estimated using
120 CHECKM 0.7.1.[16] Table S1 displays quality metrics of the draft genomes after each of the steps
121 previously described. Two putative *Nitrospira*-like bins were identified and annotated using
122 MetaPathways v 2.0.[17] To further reduce contamination in these assembled bins, scaffolds with
123 Open Reading Frames (ORFs) having less than 85% nucleotide identity and 0% protein identity
124 to other *Nitrospira* genomes were removed from the bins.

125 **Average Nucleotide Identity (ANI)**

126 Pair-wise ANI values of *Nitrospira*-like genomes were obtained using the ANIm method[18]
127 and implemented in the Python script 'calculate_ani.py' available at
128 https://github.com/ctSkennerton/scriptShed/blob/master/calculate_ani.py.

129 **Phylogenetic Analyses**

130 The phylogeny of the draft genomes was assessed by constructing a phylogenetic tree using
131 a concatenated alignment of marker genes. First, PhyloSift v 1.0.1[19] was used to extract a set of 38

132 marker genes from each genome. Then, the extracted marker protein sequences were concatenated
133 into a continuous alignment to construct a maximum-likelihood (ML) tree, using RAxML v
134 7.2.8.[20] RAxML generated 100 rapid bootstrap replicates followed by a search for the best-scoring
135 ML tree.

136 For phylogenetic analyses of ammonia monooxygenase subunit A (*amoA*), hydroxylamine
137 reductase (*hao*) and nitrite oxido-reductase subunit A (*nxrA*) genes, full nucleotide datasets were
138 downloaded from the NCBI GenBank database.[21] Alignment was performed on sequences
139 retrieved from the NCBI and the draft genomes using the 'AlignSeqs' command in the DECIPHER
140 "R" package.[22] Phylogenetic trees were calculated using neighbor-joining criterion with 1,000
141 bootstrap tests for every node, using the MEGA6 software package.[23] Trees were visualized with
142 the assistance of TreeGraph.[24]

143 **Population Structure by Metagenomic Analysis**

144 To estimate the abundance of currently known ammonia oxidizers, comammox, and nitrite
145 oxidizers in the reactor over time, paired-end DNA reads from the metagenomic datasets (days
146 100, 317, 522 and 674) were competitively mapped to the published genome sequence of 14 AOB
147 (*Nitrosomonas*, *Nitrosospira* and *Nitrosococcus* genera), 6 AOA (*Nitrososphaera*,
148 *Nitrosoarchaeum* and *Nitrosopumilus*), 5 NOB (*Nitrospira* and *Nitrobacter* lineage), 5 anaerobic
149 ammonia-oxidizing (anammox) bacteria (*Ca.* Brocadia fulgida, *Ca.* Brocadia caroliniensis, *Ca.*
150 Kuenenia stuttgartiensis, *Ca.* Brocadia sinica and *Ca.* Jettenia caeni), 4 comammox bacteria (*Ca.*
151 Nitrospira nitrosa, *Ca.* Nitrospira nitrificans, *Ca.* Nitrospira inopinata and *Nitrospira* sp.
152 Ga0074138) and the two *Nitrospira*-like draft genomes retrieved from the reactor, using the
153 software package BBMap version 35.85 (https://sourceforge.net/projects/bbmap). A list of the
154 genomes included in this analysis and the number of reads mapping to each sequence is found in

155 Table S2. For each organism, the number of unambiguous reads (best hit) mapping to the genomic
156 sequence with a minimum alignment identity of 90% was quantified and normalized by the number
157 of reads in each metagenome, paired-end reads average length and genome size (Table S2).

158 **Orthologous Genes Clusters**

159 To assess the degree of homology in the proteomes of the two *Nitrospira*-like genomes,
160 orthologous genes clusters (OCs) were determined using OrthoMCL.[25] OrthoMCL was run with a
161 BLAST E-value cut-off of 1e-5, and an inflation parameter of 1.5.

162 Protein products of each ortholog set were compared against the Eggnog database[26] using
163 the eggNOG-mapper tool (https://github.com/jhcepas/eggnog-mapper) (*e*-value $<10e^{-2}$) to
164 determine the COG functional and super-functional category to which they belong.

165 **Accession Numbers**

166 Raw reads and draft genome sequences have been submitted to NCBI and are accessible
167 under the BioProject identifier PRJNA322674.

168 **RESULTS AND DISCUSSION**

169 **Nutrient Removal in Lab-Scale Reactor**

170 Results from a typical cycle of the lab-scale SBR at steady-state operation during Stage 1 are
171 shown in Fig. 1. During the anaerobic phase, acetate added at the beginning of the anaerobic phase
172 was completely consumed within an hour (Fig. 1B). P release to the mixed liquor during this
173 condition was not observed (Fig. 1B), indicating the absence of polyphosphate accumulating
174 organisms (PAO) in the reactor. Denitrification was incomplete, with only ~ 60% of the nitrate
175 removed in the anaerobic phase (Fig. 1A), even though the reactor received acetate in this phase.
176 This suggests that efficient acetate uptake was likely performed by glycogen accumulating

177 organisms (GAO), without affecting P concentrations.[27] In addition, nitrite production during this
178 phase (~ 10%) is an indicator of partial denitrification (Fig. 1A).

179 Complete nitrification occurred in the subsequent micro-aerobic stage, where 91% ± 4% of
180 ammonia and 91% ± 8% of the added nitrite were removed (Fig. 1A), with nitrate accumulation
181 accounting for 50% ± 2% of the oxidized nitrogen. During the period of active nitrification, the DO
182 remained below 0.05 mg/L (Fig. 1C), as the oxygen supplied balanced the oxygen uptake rate. The
183 oxygen uptake rate decreased after nitrification ceased, and correspondingly, DO increased. To
184 maintain a low-DO environment, aeration was stopped when DO exceeded the set point (0.2 mg
185 O_2/L) and resumed when DO decreased below the set point. This operation effectively maintained
186 DO below 0.4 mg/L (Fig. 1C).

187 After 100 days of reactor operation under these conditions, the operational parameters were
188 changed to promote simultaneous nitrification, denitrification and P removal (Stage 2). Unlike
189 Stage 1, no nitrite was added to the reactor during micro-aerobic conditions. During this second
190 stage, acetate added at the beginning of the anaerobic phase was used by PAOs for P-cycling, and
191 nitrite and nitrate produced by ammonia oxidization were used as electron acceptors by PAOs
192 during micro-aerobiosis, achieving simultaneous removal of N and P. Results of this stage were
193 described elsewhere.[10]

194 ***Nitrospira*-like Genome Binning**

195 Using a combination of bidimensional coverage and tetranucleotide frequency, two
196 *Nitrospira*-like draft genomes were assembled from a sample collected at the end of Stage 1. The
197 two draft genomes (*Nitrospira* sp. UW-LDO-1 and UW-LDO-2) had 3.9 and 3.5 Mbp in total with
198 average GC content of 54.9% and 59.2%, respectively (Table S1). The reconstructed genomes

199 were assessed to be nearly complete (completeness ≧90%) with low contamination (≦5%),
200 according to the presence of 43 single-copy reference genes (Table S1).

201 Since the composite genomes did not encode complete 16S rRNA genes, the average
202 nucleotide sequence identity (ANI) between the draft genomes assembled herein and formerly
203 published *Nitrospira*-like genomes, was used to determine whether UW-LDO-1 and UW-LDO-2
204 represented distinct species, as this method has been shown to correlate well with previously
205 defined 16S rRNA gene species boundaries.[28] The calculated ANI and fraction of alignment for
206 the *Nitrospira* genomes (Fig. 2) showed that UW-LDO-01 is a representative of the *Ca. Nitrospira*
207 *nitrosa* species (ANI >94%, Fraction Aligned = 74.9%), while UW-LDO-02 had the closest
208 nucleotide identity to *Nitrospira defluvii* (ANI = 92.4%, Fraction Aligned = 72.4%). None of the
209 other ANI values were greater than 88%, indicating that the two genomes were different from each
210 other, and supports their classification as *Nitrospira nitrosa* UW-LDO-01 and *Nitrospira defluvii*
211 UW-LDO-02, respectively.

212 **Phylogenetic Analysis**

213 A genome tree constructed from a concatenated protein alignment of 38 universally
214 distributed single-copy marker genes[29] confirms the affiliation of *Nitrospira* sp. UW-LDO-01 and
215 UW-LDO-02 with *Ca.* Nitrospira nitrosa and *Nitrospira defluvii*, respectively (Fig. 3). Consistent
216 with this phylogeny, UW-LDO-01 encoded the *amoCAB* operon, responsible for ammonia
217 oxidation, while UW-LDO-02 did not.

218 The *amoA* and *hao* genes are functional genes involved in redox nitrogen transformations
219 and are also considered phylogenetic markers to study the diversity of ammonia oxidizing
220 microorganisms (AOM).[30-33] The phylogenetic tree topologies based on these genes (Fig. S1)

221 further confirm the classification of UW-LDO-01 as related to *Ca.* Nitrospira nitrosa, although
222 two paralogs of the *amoA* gene are present in the genome of *Ca. Nitrospira nitrosa*, while only
223 one *amoA* gene was found in UW-LDO-1.

224 In addition, the *Nitrospira* sp. UW-LDO-1 and UW-LDO-2 genomes encoded the key
225 enzyme for nitrite oxidation, *nxr*, which can also be used as a phylogenetic biomarker. UW-LDO-
226 1 encoded two paralogs of the periplasmic NXR enzyme while *Nitrospira* UW-LDO-2 only
227 encoded one copy. The affiliation of UW-LDO-1 with *Ca.* Nitrospira nitrosa and UW-LDO-2 with
228 *N. defluvii* was supported by phylogeny based on the *nxrA* gene sequence (Fig. S2), consistent
229 with the phylogenetic analysis of *amoA* and *hao*.

230 **Nitrifying Prokaryotes in Lab-Scale Reactor**

231 The metagenomic analysis of the Stage 1 sample, which corresponds to the operational
232 conditions when nitrite and ammonia were both present under micro-aerobic conditions, did not
233 result in the assembly of any other genome of nitrifying microorganisms. Thus, in order to assess
234 the relative abundance of other known nitrifying prokaryotes present in the reactor, we mapped
235 metagenomic reads to published genomes of comammox, anammox, AOB, AOA and NOB,
236 including *Nitrospira* sp. UW-LDO-01 and UW-LDO-02 (Fig. 3). After a competitive mapping of
237 short-reads from metagenomic samples to each genome (>90% identity), the number of mapping
238 reads was normalized to both metagenome size and reference genome size, and used as a proxy of
239 genome abundance.

240 The metagenomic data show little evidence of AOA and anammox bacteria during the two
241 stages (0.06% and 0.25% of mapping reads, respectively). AOB were detected in the system, albeit
242 representing a small fraction of the community (0.17% and 0.05% of total number of reads during
243 Stage 1 and 2, respectively). Noticeably, *Nitrospira*-like sequences (including comammox- and

244 NOB-like genomes) recruited the greatest number of metagenomic reads (14.0% of total number
245 of reads) in the Stage 1 sample (Fig. 4A). Within this genus, *Nitrospira sp.* UW-LDO-01 retrieved
246 32.3% of the reads competitively mapping to the *Nitrospira*-like genomes (Fig. 4B). The published
247 genome of *Ca.* Nitrospira nitrosa retrieved 2.3% of the reads, while less than 4% mapped to other
248 comammox genomes. Therefore, with only a small fraction of reads mapping to other ammonia
249 oxidizers, we propose that *Nitrospira* sp. UW-LDO-01 was the main comammox in the reactor,
250 and the main contributor to ammonia oxidation during Stage 1.

251 *Nitrospira* sp. UW-LDO-02 appeared to be the most abundant NOB in the reactor during
252 Stage 1, retrieving 42.4% of the *Nitrospira*-like reads (Fig. 4B), although a large fraction of reads
253 competitively mapping to *N. defluvii* may indicate the presence of other nitrite oxidizing strains in
254 the reactor. Therefore, the nitrite oxidation activity in the reactor was carried out by comammox
255 and NOB.

256 The metagenomic analysis of Stage 2 samples reveals an overall decrease in the relative
257 abundance of nitrifying organisms in the reactor after transitioning to this operational
258 configuration (Fig. 4A). During this stage, metagenomic reads mapping to NOB and comammox
259 genomes (including *Nitrospira* sp. UW-LDO-01 and UW-LDO-02) decreased to less than 1% of
260 the total number of reads. This was in part due to the removal of nitrite addition during Stage 2.
261 However, the decrease in comammox did not correspond to an increase in the number of reads
262 mapping to other known AOM (Fig. 4A), suggesting the presence of still unrecognized AOM in
263 reactors operated with low DO conditions, as previously reported.[34]

264 **Differential Gene Content Among *Ca.* Nitrospira nitrosa Genomes**

265 Since *Nitrospira* sp. UW-LDO-01 is the second comammox genome representative of *Ca.*
266 Nitrospira nitrosa, and the first comammox genome recovered from a nutrient removal bioreactor,

267 a comparative analysis of its genetic content was carried out. First, a comparison of gene content
268 among *Ca.* Nitrospira nitrosa (CZQA00000000) and *Nitrospira* sp. UW-LDO-01 was conducted
269 by BlastP comparison of the translated coding DNA sequence (CDS) set, clustering of ortholog
270 proteins, and annotation of representatives of each ortholog cluster (OC) and genome-unique
271 CDSs.

272 Overall, sequencing and annotation of the UW-LDO-01 genome revealed a genomic
273 inventory highly similar to the genome of *Ca.* Nitrospira nitrosa.[2] The two genomes shared 81.2%
274 of the OCs (3,202 OCs), with UW-LDO-01 and *Ca.* Nitrospira nitrosa having 740 and 857 unique
275 OCs, respectively (Fig. 5A).

276 OCs belonging to the shared and genome-specific fractions of the two genomes were
277 classified according to their predicted functional role (Fig. 5B-C). 2,906 and 3,009 OCs, in the
278 UW-LDO-01 and *Ca*. Nitrospira nitrosa genomes, respectively, were categorized into functional
279 Clusters of Orthologous Groups (COG) categories. The majority of OCs belonged to the group of
280 "Unknown Function (S)" (23% of shared OCs, and 19% and 23% of the genome-specific fraction
281 in UW-LDO-01 and *Ca.* Nitrospira nitrosa, respectively), indicating a large set of metabolic
282 features not yet elucidated (Fig. 5B-C).

283 Besides the genes of unknown function, OCs within this shared fraction were mostly
284 represented by the "Cell wall/membrane/envelope biogenesis (M)" (7%), "Signal transduction
285 mechanisms (T)" (6%) and "Energy production and conversion (C)" (6%) families, indicating
286 general conservation of energy metabolism and regulatory mechanisms.

287 In both genomes, the functional group "Replication, recombination and repair (L)" was over-
288 represented within the genome-specific fraction. This functional category includes transposases,
289 integrases and other mobile genetic elements, and their extensive representation in the genome-

290 specific fraction indicates that horizontal gene transfer has likely played a significant role in the
291 diversification of *N. nitrosa* strains. The greatest difference among the genome-specific fractions
292 of UW-LDO-01 and *Ca*. Nitrospira nitrosa was the proportion of OCs represented by the "Cell
293 wall/membrane/envelope biogenesis (M)" and "Intracellular trafficking, secretion, and vesicular
294 transport (U)" functional families (Fig. 5C). Glycosyltransferases[35-38], Type IV[39-41] secretion
295 system proteins and other enzymes involved in polysaccharide formation (main component of the
296 biofilm matrix) were enriched within these COG categories in the genome-specific fraction of *Ca.*
297 Nitrospira nitrosa. Differences in biofilm formation capabilities between these strains may relate
298 to specific niche adaptation: *Ca.* Nitrospira nitrosa was enriched in a biofilm, whereas UW-LDO-
299 01 was found in a planktonic habitat in wastewater. Analogous findings have been observed in
300 other genera, where differences among biofilm formation capabilities within the same genus were
301 linked to the genome content of different strains.[42-44] Similar to the results presented here, these
302 genetic differences included the presence of type IV secretion systems and enzymes involved in
303 protein glycosylation.

304 The comparative genomic analysis also indicated a higher proportion of gene clusters
305 associated with "Lipid transport and metabolism (I)" in *Nitrospira* sp. UW-LDO-01 (Fig. 5C).
306 Genes related to ß-oxidation of long-chain fatty acids to acetyl-CoA were present in the genome
307 of UW-LDO-01, but absent in *Ca*. Nitrospira nitrosa. These genes include a long-chain fatty acid-
308 CoA ligase, acyl-CoA dehydrogenase, enoyl-CoA hydratase, 3-hydroxyacyl-CoA dehydrogenase
309 and acetyl-CoA acetyltransferase. Presence of these lipid-related metabolic genes in other
310 *Nitrospira* strains was confirmed, although the complete pathway is lacking in *Nitrospira defluvii*,
311 *Ca.* Nitrospira nitrificans and *Nitrospira* sp. Ga0074138. This feature may represent a competitive
312 advantage of some *Nitrospira* strains in habitats rich in long-chain fatty acids, such as WWTPs.[45]

313 **Metabolic Features in *Nitrospira* Genomes**

314 To explore the diverse metabolic capabilities and provide insights into the common and
315 unique metabolic features encoded in the genome of NOB- and comammox-like strains, we
316 compared the gene inventory of 9 complete and draft genomes classified as *Nitrospira*. The
317 analysis was focused on traits associated to energy production, which are summarized in Table S3.

318 In agreement with previous analyses, only comammox-like genomes encoded ammonia
319 monooxygenase (*amoCAB*) and hydroxylamine dehydrogenase (*haoAB-cycAB*) gene clusters,
320 responsible for ammonia oxidation to nitrite (Table S3), reflecting the capability of this novel
321 *Nitrospira* sub-lineage to perform full-nitrification from ammonia to nitrate.

322 Analysis of nitrite reducing genes revealed that all *Nitrospira* strains encoded a copper-
323 containing dissimilatory nitrite reductases (*nirK*), which catalyzes the nitrite reduction to nitric
324 oxide, a key step in the denitrification process. Despite the widespread presence of this enzyme
325 across the *Nitrospira* genus, former studies have documented no activity of this protein in NOB-
326 like[46] or comammox-like strains[1], where N loss caused by formation of gaseous compounds was
327 not observed. Since it has been predicted that the NXR complex of *Nitrospira* can reduce nitrate
328 to nitrite,[46] these microorganisms appear genetically capable of converting nitrate (product of
329 nitrification) to nitric oxide. Additional experiments are still needed to obtain more insights into
330 this *Nitrospira* trait. Other denitrification genes, such as nitrate reductase (*nar*), nitric oxide
331 reductase (*nor*) or nitrous oxide reductase (*nos*), were not found in the *Nitrospira* strains analyzed
332 here.

333 All comammox-like genomes, including UW-LDO-01, encoded the machinery to hydrolyze
334 urea: the *ureABCDFG* urease operon and the *urtABCDE* urea transport system, suggesting that
335 this *Nitrospira* sub-division possesses a high-affinity uptake system for urea and, thus, is adapted

336 to habitats where urea is present at low levels. A gene cluster involved in urea metabolism was
337 also found in *N. moscoviensis* (Table S3), although the urea-binding proteins *urtBCDE* were
338 lacking in the genome. Ureolytic activity of both *N. moscoviensis*, *Ca*. Nitrospira nitrosa and *Ca.*
339 Nitrospira nitrificans was formerly tested by incubation of these strains with urea-containing
340 media, where urea hydrolysis to ammonium was observed in both cases.[2, 46] Former studies have
341 also shown the presence of genes for urea utilization in *Nitrospira lenta,*[47] a novel *Nitrospira*
342 species enriched under low temperatures, suggesting that the ureolytic activity might be associated
343 with this lineage. Since several ammonia oxidizers also possess the capability of hydrolyzing
344 urea,[48-50] the presence of this trait in *Nitrospira* might evidence horizontal gene transfer among
345 these functional groups.

346 A contrasting difference among NOB-like and comammox-like genomes was the capability
347 to convert cyanate into ammonia. Only NOB-like genomes encoded a cyanase hydratase enzyme;
348 former studies have experimentally confirmed cyanate degradation in *N. moscoviensis.*[51] Cyanate
349 is produced intracellularly from urea and carbamoyl phosphate decomposition,[52, 53] and in the
350 environment from the chemical/physicochemical decomposition of urea or cyanide.[54, 55] The
351 presence of a cyanase enzyme benefits nitrite oxidizers because it allows them to detoxify cyanate,
352 and the formed ammonium is then available for assimilation and might also serve as a source of
353 energy for ammonia oxidizers in a process described as "reciprocal feeding".[46, 51] Further
354 experiments analyzing the effect of cyanate in the growth on comammox-like bacteria are needed
355 to understand how cyanate degradation would confer them a biological advantage, besides
356 generation of ammonia.

357 The analysis also revealed the presence of the gene inventory for the uptake and oxidation
358 of formate, an exclusive feature of NOB. Growth on formate as electron donor has been confirmed

359 in *N. moscoviensis* (under both micro-oxic and anoxic incubations),[46] *Nitrospira japonica*[56] and
360 uncultured *Nitrospira* in activated sludge.[57] Although formate oxidation should be advantageous
361 for organisms thriving in hypoxic or anoxic habitats, which also includes comammox-like bacteria,
362 this feature is absent in the genome of these microorganisms.

363 The genome of *N. moscoviensis* encodes a group 2a [Ni-Fe] hydrogenase (*hupS* and *hupL*)
364 and accessory proteins involved in the maturation and transcriptional regulation of hydrogenases
365 (*hypFCDEAB* and *hoxA*). Furthermore, experiments showed that *N. moscoviensis* was capable of
366 growing by aerobic respiration of H_2.[58] Although the comammox-like genomes lack the subunits
367 of the [Ni-Fe] hydrogenase (Hup), the five genomes analyzed here encoded a group 3 [Ni-Fe]
368 sulfur-reducing hydrogenase gene set (*hydBGDA and hybD*) positioned at the same locus where
369 Hup is located in *N. moscoviensis*. This hydrogenase complex is an heterotetramer with both
370 hydrogenase activity and sulfur reductase activity, which might play a role in hydrogen cycling
371 during fermentative growth.[59] Its beta and gamma subunits, that form the sulfur reducing
372 component, catalyze the cytoplasmic production of hydrogen sulfide in the presence of elemental
373 sulfur. The presence of this complex in the genomes indicates the potential of these
374 microorganisms for oxidizing H_2 using sulfur as electron acceptor, a trait that has not been
375 analyzed in comammox before, but that could confer this sub-group an advantage when growing
376 under anaerobic conditions.

377 Furthermore, the presence of a hyf-like operon (*hyfBCEFGI*), which encodes a putative
378 group 4 hydrogenase complex, was detected in every NOB-like genome, as well as *Ca.* Nitrospira
379 nitrosa, *Nitrospira* sp. Ga0074138 and UW-LDO-01. In *Escherichia coli*, this hydrogenase
380 complex forms part of a second formate hydrogenlyase pathway (oxidation of formate to CO_2 and
381 reduction of $2H^+$ to H_2 under fermentative conditions).[60] This is likely the case for the

382 hydrogenase-4 present in the genome of NOB-like strains, which co-occur with genes encoding
383 formate dehydrogenase. In comammox, however, the role of this distinct hydrogenase is not as
384 clear. In *Ca.* Nitrospira nitrosa, this complex is found immediately adjacent to a carbon monoxide
385 dehydrogenase (CODH), an enzyme that catalyzes the interconversion of CO and CO_2[61], a
386 genomic feature that would allow this strain to obtain energy from carbon monoxide.[62] Conversely,
387 the genomes of *Nitrospira* sp. Ga0074138 and UW-LDO-01 lack the CODH at this position, which
388 in the case of UW-LDO-01, was confirmed by alignment of the metagenomic reads to this gene.
389 No other neighboring gene of the hydrogenase-4 complex could be associated with this enzyme in
390 these two strains, therefore, the biological role of these genes is still unclear.

391 Altogether, these results reveal specific traits characterizing the NOB and comammox
392 functional groups: while comammox-like *Nitrospira* has the genomic potential of ammonia and
393 nitrite oxidation and potentially, sulfur reduction, NOB-like strains are distinguished by their
394 cyanate degradation and formate oxidation capabilities; and both urea hydrolysis and H_2
395 respiration are common traits shared by multiple *Nitrospira* strains.

396 **The Role of Transcriptional Regulation in *Nitrospira***

397 Transcriptional regulation of gene expression is the most commonly used strategy to control
398 many of the biological processes in an organism, including progression through the cell cycle,
399 metabolic and physiological balance, and responses to environmental stress. This regulation is
400 generally orchestrated by several transcriptional factors (TFs) that directly coordinate the activity
401 of genes by binding to their promoters. Each *Nitrospira*-like genome codes for at least 100
402 transcriptional regulators, which account for ~3% of the estimated total number of genes, in
403 agreement with TFs in other microorganisms.[63-65] A comparative genomic analysis of full and draft

404 *Nitrospira* genomes was used to investigate the repertoire of TFs potentially involved in the
405 survival of these microorganisms under diverse environmental conditions (Table 1).

406 Among the TFs analyzed, the formate hydrogenlyase transcriptional activator (fhlA)[60, 66] was
407 the only one shared across all the *Nitrospira* genomes, although only NOB-like genomes encode
408 genes of its known regulon, the formate hydrogenase complex. The presence of this transcriptional
409 activator in comammox microorganisms, which appear to be genetically incapable of formate
410 oxidation (Table 1), might represent an ancestral trait shared by *Nitrospira* and lost during
411 diversification. This theory would also support the presence of the group 4 hydrogenase
412 (associated with the formate-hydrogenlyase complex in *E. coli*) in both NOB- and comammox-
413 like groups.

414 A common feature among some NOB and comammox bacteria is the presence of the
415 transcriptional regulators NhaR[67, 68] and OxyR[69] (Table 1). The first one is associated with stress
416 response to alkaline, acidic, saline and osmotic conditions. OxyR regulates hydrogen peroxide-
417 inducible genes, such as alkyl hydroperoxide reductase (*ahpCF*) and glutaredoxin (*grxA*), encoded
418 in all the *Nitrospira* genomes. Presence of these genes would confer *Nitrospira* an improved fitness
419 advantage over other nitrifying bacteria. For instance, NhaR is lacking in *Nitrosomonas* and
420 *Nitrobacter* and OxyR is not present in *Nitrosomonas* and *Nitrosospira* (based on genome search).
421 Furthermore, the role of NhaR during regulation of *pga* expression,[68] allows the biofilm formation
422 process to be considered as a flexible and dynamic developmental process driven by external
423 conditions, representing another means by which NhaR could promote survival of *Nitrospira*.
424 Likewise, the presence of the chemotaxis regulator CheZ in *Nitrospira* suggests chemotaxis as
425 another important mechanism by which these microorganisms efficiently and rapidly respond to
426 changes in the chemical composition of their environment.

427 To date, the role of the Fnr-type regulatory protein in *Nitrospira* has not been determined.
428 In other microorganisms, Fnr is part of the signaling involved in the adaptation to micro-oxic
429 environments,[70-74] where it acts as oxygen sensor and regulator of genes involved in anaerobic and
430 micro-aerobic metabolism. In *Nitrospi*ra, we predict that this TF would regulate similar genes,
431 such as the *frd* operon (fumarate reductase), *sdh* operon (succinate dehydrogenase), *ndh* (NADH
432 dehydrogenase) and *ccb3* complex (cytochrome c oxidase). At least one copy of Fnr in the
433 genomes of UW-LDO-01, *N. moscoviensis*, *N. defluvii* and N. sp. OLB-3 was located upstream of
434 a copper-containing nitrite reductase gene (nirK), suggesting a possible mechanism that controls
435 expression of this denitrification enzyme. The presence of multiple paralog copies of Fnr in several
436 *Nitrospira* genomes may indicate a rigorous regulation of metabolism when these microorganisms
437 are exposed to low levels of oxygen, an important factor affecting *Nitrospira* community
438 compositions in nitrifying systems.[75]

439 Overall, this study sheds light about differences in the physiological role of NOB and
440 comammox-like *Nitrospira*. Specifically, the comparative genomic results evidence traits
441 associated with energy metabolism as characteristic to each of these functional groups.
442 Furthermore, the analysis of TFs in *Nitrospira* reveals the alternative use of organic compounds,
443 response to environmental stress, chemotaxis and anaerobic metabolism as some of the key
444 mechanisms for the adaptive metabolism of the genus to multiple and adverse conditions. Further
445 studies in the field should include experiments that combine omics-analysis (transcriptomics,
446 metabolomics, and proteomics) with chemical data to confirm the ecological role and functionality
447 of each of these functional groups and their interactions with other microorganisms.

448 **ACKNOWLEDGMENTS**

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FIGURES

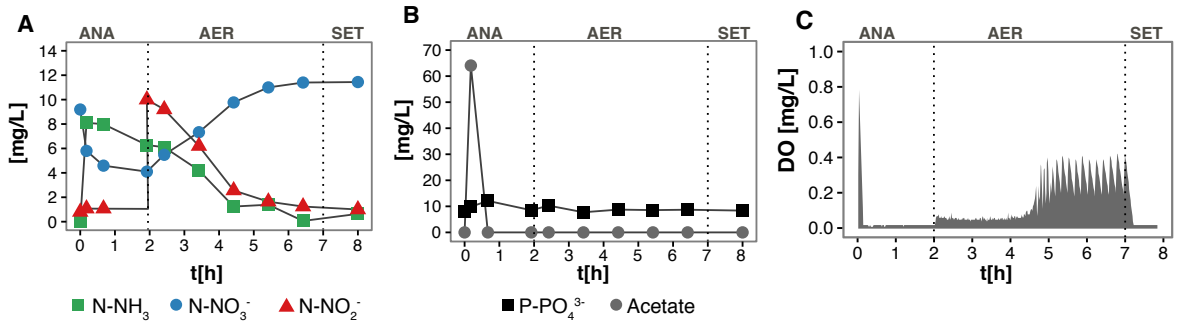

Figure 1. Nutrient profiles of (A) nitrogenous compounds, (B) phosphorous and acetate, and (C) oxygen concentration in a regular cycle of the lab-scale SBR during Stage 1. ANA: Anaerobic, AER: Micro-aerobic, SET: Settling.

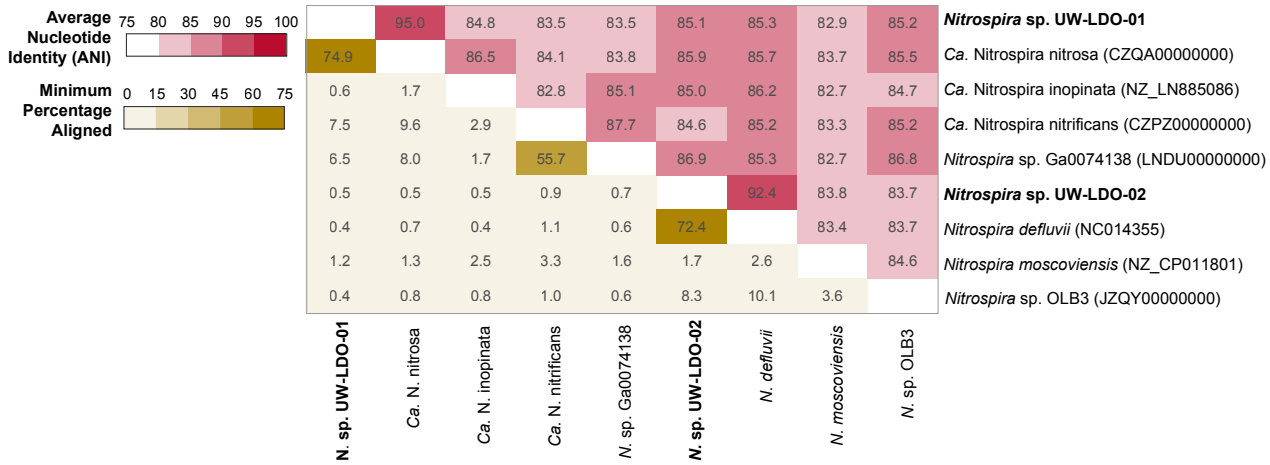

Figure 2. Comparison of the genome-wide average nucleotide identity and alignment percentage, of *Nitrospira*-like genomes. The heatmap shows the average nucleotide identity (red upper section of matrix) and the percentage of the two genomes that aligned (yellow lower section).

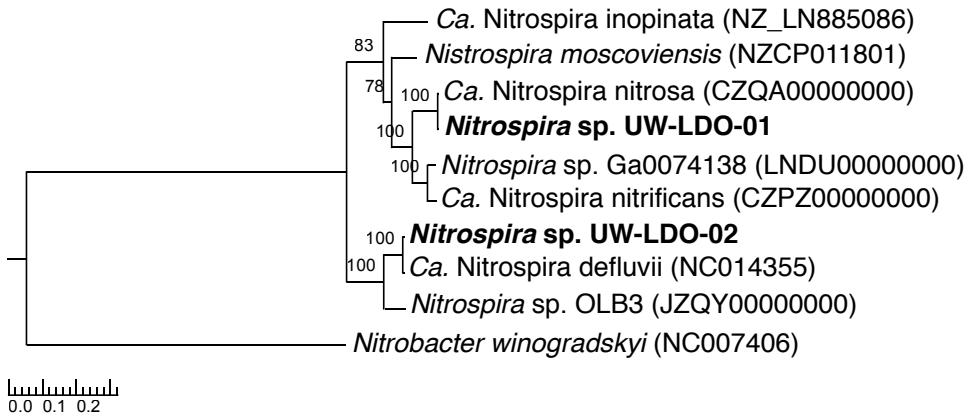

Figure 3. RAxML phylogenetic tree of a concatenated alignment of 37 marker genes (nucleotide sequence) data set with the root placed on the branch leading to *Nitrobacter winogradskyi.* The numbers at the nodes of both trees show support values derived from 100 RAxML bootstrap.

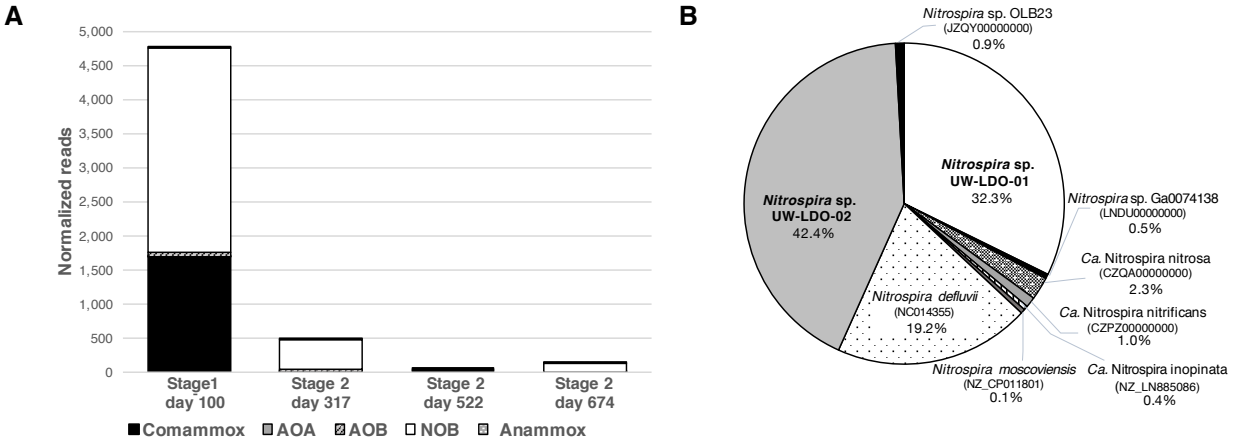

Figure 4. (A) Normalized frequency of metagenomic reads mapping to the genome of Comammox, AOB, AOA, NOB and Anammox-related organisms in samples from Stage 1 and 2 of the lab-scale SBR. (B) Relative abundance of reads mapping to genomes of *Nitrospira*-related bacteria in Stage 1 sample, including the draft genomes retrieved in this study.

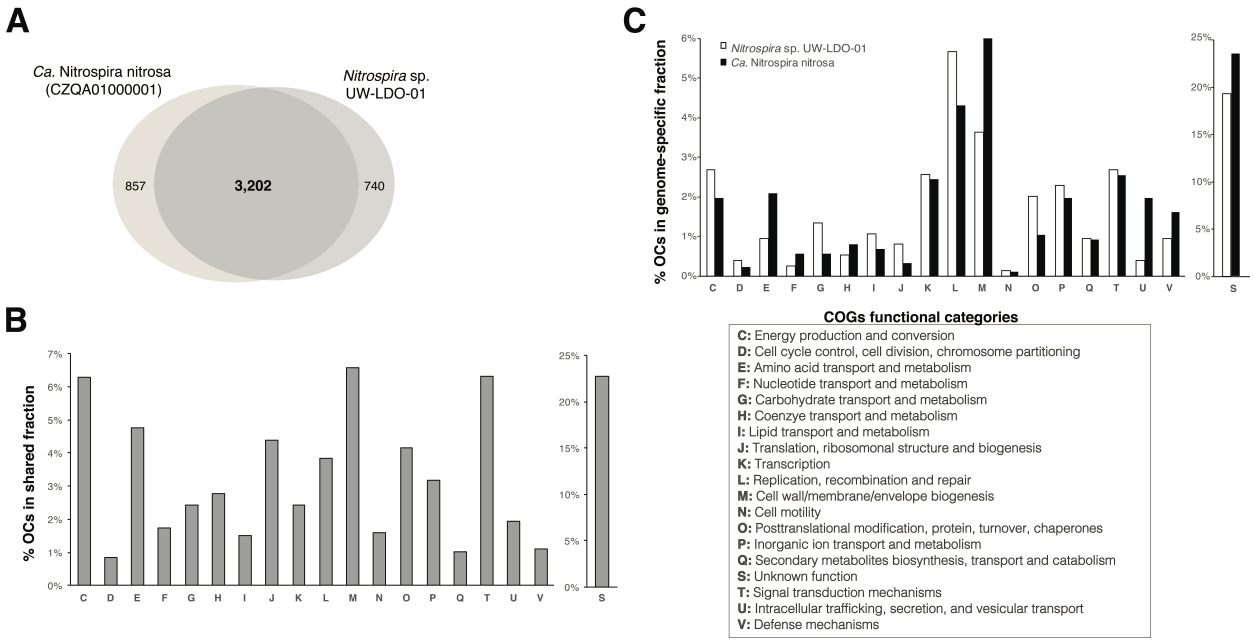

Figure 5. Genomic differences between *Nitrospira sp.* UW-LDO-01 and *Ca*. Nitrospira nitrosa. (A) Venn diagram of ortholog clusters shared between the two draft genomes; (B) distribution of COGs functional classes in the fraction of orthologs shared by the two genomes; (C) distribution of COGs functional classes in ortholog clusters found in only one of the genomes (genome-specific fraction).

487 # TABLES

488 **Table 1.** Inventory of transcriptional regulators with implications on adaptive metabolism,
489 from complete and draft genomes of *Nitrospira*. Grey and white rectangles represent
490 presence and absence of each gene, respectively.

		Comammox					NOB				
Transcriptional Regulator	**Gene**	***Nitrospira* sp. UW-LDO-01**	***Ca.* Nitrospira nitrosa**	***Ca.* Nitrospira nitrificans**	***Ca.* Nitrospira inopinata**	***Nitrospira* sp. Ga0074138**	***Nitrospira* sp. UW-LDO-02**	***Nitrospira moscoviensis***	***Nitrospira defluvii***	***Nitrospira* sp. OL23**	**Function**
Formate hydrogenlyase transcriptional activator	*fhlA*										FhlA binds to formate hydrogenlyase structural genes (formate dehydrogenase and group 4 hydrogenase complex) to activate transcription of their promoters [66].
Transcriptional activator protein NhaR	*nhaR*										NhaR regulates *nhaA*, a pH-dependent sodium-proton antiporter that responds to alkaline and saline conditions [76]. It is also responsible for *osmC* induction [67], required for resistance to organic peroxides and osmotic conditions and for long-term survival in stationary phase [77, 78]. NhaR also stimulates *pga* transcription, a set of genes responsible of poly-β-1,6-N-acetyl-D-glucosamine (PGA) synthesis [68]. PGA is involved in cell-cell adhesion and attachment, which stabilizes biofilm formation [79].
Hydrogen peroxide-inducible genes activator	*oxyR*										OxyR is required for the induction of a hydrogen peroxide-inducible regulon in response to elevated levels of hydrogen peroxide [69].
Chemotaxis regulator CheZ	*cheZ*										CheZ is a component of the chemotaxis signal-transduction pathway [80]. It controls the phosphorylation of CheY, a protein involved in the cell excitation response. Absence of CheZ results in non-chemotactic cells or long stimuli response latencies, demonstrating its critical importance during response to stimuli [81].
Fumarate and nitrate reductase regulatory protein	*fnr*										Fnr is an oxygen-responsive regulator required for the expression of a number of genes involved in anaerobic metabolism [73, 82, 83], including fumarate reductase, nitrate and nitrite reductase, and cytochrome oxidase genes [84]

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499 **ASSOCIATED CONTENT**

500 **Supplementary Material.** Supplementary Figures and Tables supporting the information
501 presented in this manuscript.

502

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