Supporting information

Growth of nitrite-oxidizing *Nitrospira* and ammonia-oxidizing *Nitrosomonas* in marine recirculating trickling biofilter reactors

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This manuscript contains 2, 8, and 2 supplementary figures, table and text.

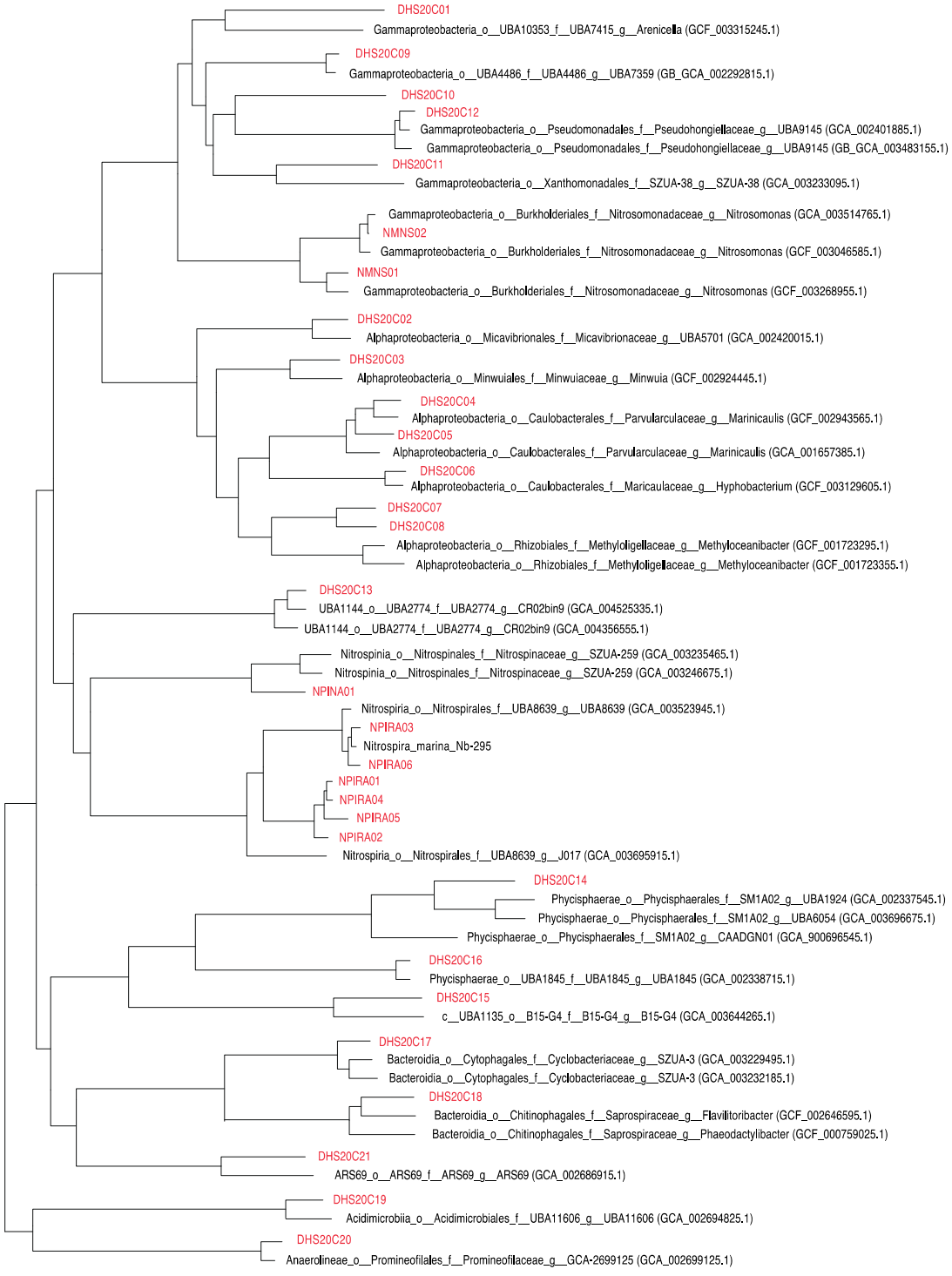

Figure S1. Phylogeny of the 30 metagenomic bins obtained in the present study. The phylogenetic position of the obtained metagenomic bins were shown with red color. The scale bar represents 20% sequence divergence.

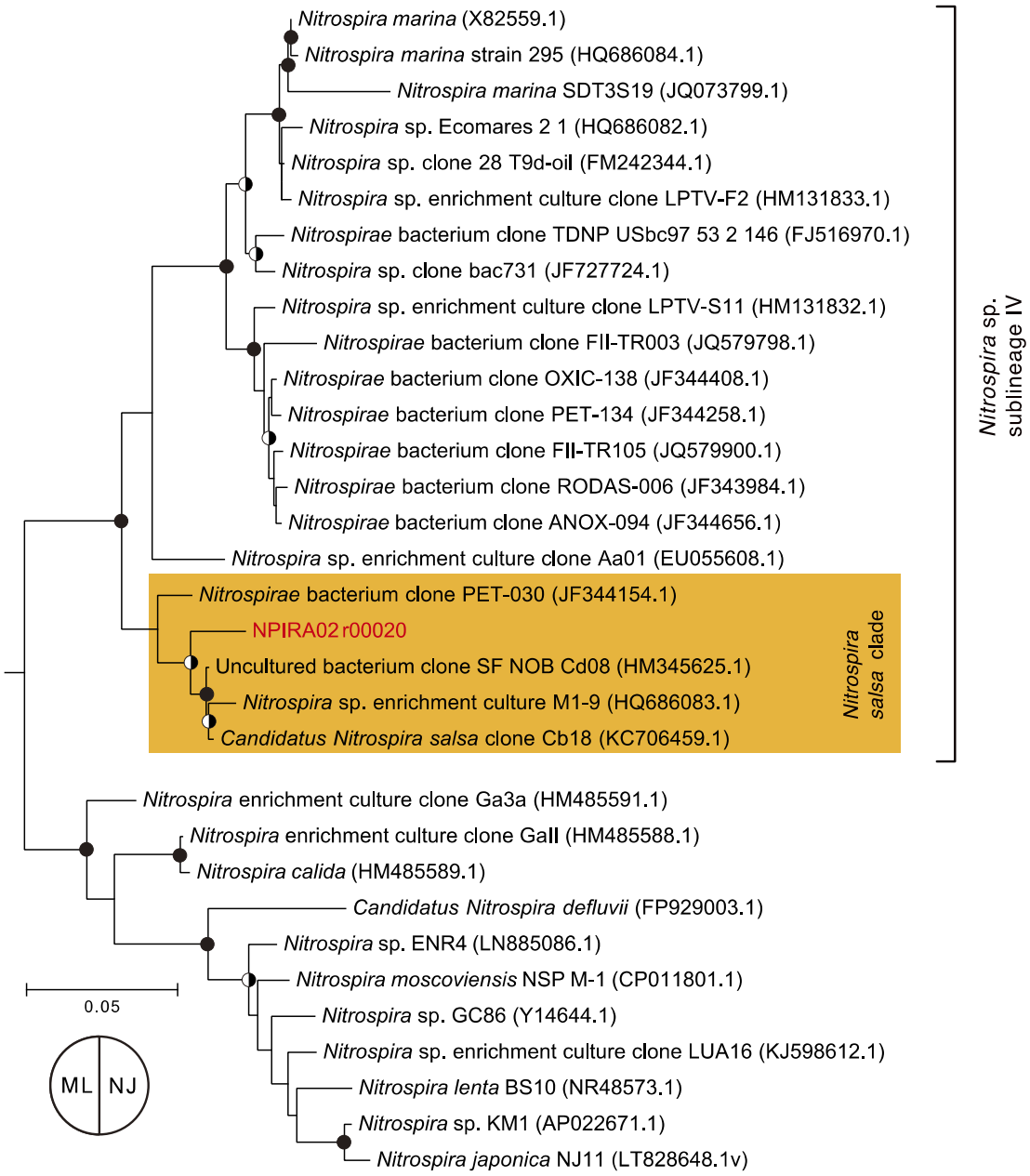

Figure S2. 16S-rRNA gene based maximum likelihood (ML) tree showing phylogenetic position of the NPIRA02 bin. Branching points that support probability >80% in the bootstrap analysis (based on 500 replicates), estimated using the ML method and the neighbor joining (NJ) method are shown as filled symbol. The 16S rRNA gene sequence located on the

17 NPIRA02 bin was affiliated into the clade containing *Nitrospira salsa* 16S rRNA gene

18 sequence (KC706459.1). The scale bar represents 5% sequence divergence.

Table S1 Average nucleotide identity (ANI) values of the obtained *Nitrospira* bins. 1; NPIRA01, 2; *Nitrospirae* bacterium SPGG5 (GCA_001643555.1), 3; *Nitrospirae* bacterium (GCA_003228495.1), 4; MAG-cas150m-170 (PRJNA548657), 5; NPIRA02, 6; *Nitrospiraceae* bacterium (GCA_003523945.1), 7; NPIRA03, 8; *Nitrospirae* bacterium (GCA_003233615.1), 9; NPIRA06, 10; NPIRA04, 11; NPIRA05, 12; *Nitrospirae* bacterium (GCA_003696975.1), 13; *Nitrospira* sp. bin75 (GCA_002238765.1), 14; *Nitrospirales*_bacterium_isolate_MH-Pat-all_autometa_1-10 (WLXC01000001.1), 15; *Nitrospirae* bacterium (GCA_003235785.1), 16; *Nitrospira marina* Nb-295 (PRJNA262287), and 17; MAG-cas50m-175 (PRJNA548657).

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	100%	69.9%	71.0%	70.6%	78.8%	71.0%	71.6%	70.7%	70.8%	89.3%	85.4%	69.7%	69.9%	70.9%	70.7%	70.7%	70.6%
2		100%	71.7%	77.4%	70.2%	70.1%	70.3%	71.4%	70.2%	70.1%	70.2%	70.1%	72.0%	70.5%	70.4%	70.2%	77.4%
3			100%	70.9%	71.4%	73.4%	73.1%	79.9%	73.1%	71.7%	70.6%	69.9%	70.7%	71.2%	78.2%	73.1%	71.1%
4				100%	70.6%	70.3%	70.3%	70.9%	70.1%	70.6%	70.3%	69.9%	71.5%	71.1%	70.4%	70.7%	99.9%
5					100%	71.4%	71.5%	70.8%	70.9%	78.3%	77.6%	69.8%	70.3%	71.6%	70.9%	71.0%	70.7%
6						100%	83.3%	72.7%	84.2%	72.5%	75.3%	70.0%	70.5%	70.6%	74.0%	84.1%	70.7%
7							100%	72.5%	85.1%	71.8%	74.7%	69.5%	70.1%	70.6%	73.5%	88.2%	70.7%
8								100%	72.6%	71.4%	70.7%	69.9%	70.1%	71.1%	76.8%	72.7%	71.0%
9									100%	71.4%	77.3%	69.7%	70.3%	70.6%	73.7%	86.1%	70.6%
10										100%	84.6%	69.9%	70.4%	72.1%	71.1%	71.6%	71.1%
11											100%	69.5%	70.0%	70.6%	71.1%	74.9%	70.5%
12												100%	70.7%	70.2%	69.9%	69.9%	70.1%
13													100%	70.4%	69.9%	70.4%	71.6%
14														100%	70.6%	70.9%	71.2%
15															100%	73.8%	70.8%
16																100%	71.0%
17																	100%

25 **Table S2 Gene sets involved in central metabolism found in *Nitrospira* (i.e., NPIRA)**

26 **bins.**

27 (**Table S2** is available as the separated excel file.)

28 **Table S3 The blastP analysis of Hao protein found in the obtained *Nitrospira* bin.** The

29 blastP analysis was performed using the NPIRA02_01710 protein as the query sequence and

30 *nr* database. *: canonical nitrite-oxidizing *Nitrospira*, †: complete ammonia oxidation

31 (commamox) *Nitrospira*.

Organisms	Product	Accession No.	Identity	*e*-value
Nitrospirales bacterium	cytochrome C_{552}	NKB80548.1	82.6	0
Nitrospiraceae bacterium	TPA: cytochrome C_{552} partial	HBP89707.1	85.7	0
*Nitrospira moscoviensis**	putative octaheme cytochrome *c*	ALA57709.1	45.7	2.1E-139
*Nitrospira japonica**	conserved protein of unknown function	SLM48584.1	42.0	9.0E-137
Nitrospira nitrosa†	Hydroxylamine dehydrogenase Candidatus	CUS31385.1	25.6	1.4E-17
Nitrospira nitrificans†	Hydroxylamine dehydrogenase Candidatus	CUS32689.1	24.9	1.6E-17
Nitrospira inopinata†	Hydroxylamine dehydrogenase Candidatus	CUQ65042.1	23.8	2.0E-17

32

33 **Table S4 Gene sets involved in central metabolism found in *Nitrosomonas* (i.e., NMNS)**

34 **bins**

35 (**Table S4** is available as the separated excel file.)

36 **Table S5 Operational conditions of the marine recirculating bioreactors where**

37 ***Nitrospira* sublineage IV population outnumbered AOB/AOA population.**

Reactor	Salinity	Temp.	pH	DO mg/L	NH_4^{+*} µM	NO_2^- µM	NO_3^- µM	Reference
down-hanging sponge (DHS) reactor	33‰	20ºC	7.3	8	7.9	9.3	4,900	This study
Moving-bed biofilter	24–30‰	15–20ºC	7	9–10	5–60	10–40	6,000	[1]
Bio ball filter	26.3 psu	*n.a.*	8.8	6.5	18	*n.d.*	1,800	[2]
Trickling filter	20‰	19–30ºC	7.7	>6	35	15.7	2,000	[3], [4]

38 *n.a.*; not available, *n.d.*; not detected

39 * total ammonia concentration, TAN

40 **References**

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45 oxidizing nitrospiras in the biofilter of a shrimp recirculating aquaculture system. *FEMS*

46 *Microbiol Ecol* 2013; **83**: 17–25.

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54 **Table S6. Oligonucleotide primers used for quantitative PCR (qPCR) assays.** Annealing

55 temperature and extension time are 60ºC and 20 s, respectively.

Target	Primers	Sequence (5'-3')	Reference
Prokaryotic 16S rRNA gene	515F 806r	GTGCCAGCMGCCGCGGTAA GGACTACHVGGGTWTCTAAT	Caporaso *et al.* (2011)
AOB *amoA*	amoA1F amoA2Rv1	GGGGTTTCTACTGGTGGT CCCCTSKGSRAAKCCTTCTTC	Rotthauwe *et al.,* 1997 This study
Nitrospira nxrB	nxrB169f nxrB638r	TACATGTGGTGGAACA CGGTTCTGGTCRATCA	Pester *et al.,* 2014
Nitrospira 16S rRNA gene	Nspra675F Nspra746R	GCGGTGAAATGCGTAGAKATCG TCAGCGTCAGRWAYGTTCCAGAG	Graham *et al.,* 2007

56 **References**

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58 P.J., *et al*. (2011) Global patterns of 16S rRNA diversity at a depth of millions of

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60 Graham, D.W., Knapp C.W., Van Vleck, E.S., Bloor, K., Lane, T.B., and Graham, C.E.

61 (2007) Experimental demonstration of chaotic instability in biological nitrification.

62 *ISME J* **1**: 385–393.

63 Pester, M., Maixner, F., Berry, D., Rattei, T., Koch, H., Lücker, S., *et al.* (2014) NxrB
64 encoding the beta subunit of nitrite oxidoreductase as functional and phylogenetic
65 marker for nitrite-oxidizing *Nitrospira*. *Environ Microbiol* **16**: 3055–3071.
66 Rotthauwe, J-H., Witzel, K-P., and Liesack, A.W. (1997) The ammonia monooxygenase
67 structural gene *amoA* as a functional marker: molecular fine-scale analysis of natural
68 ammonia-oxidizing populations. *Appl Environ Microbiol* **63**: 4704–4712.

69 **Table S7 Accession numbers of the obtained bins.**

Bin	Accession No.
NPIRA01	BQIU01000001-BQIU01000203
NPIRA02	BQIV01000001-BQIV01000092
NPIRA03	BQIW01000001-BQIW01000218
NPIRA04	BQIX01000001-BQIX01000633
NPIRA05	BQIY01000001-BQIY01000595
NPIRA06	BQIZ01000001-BQIZ01000303
NMNS01	BQJA01000001-BQJA01000199
NMNS02	BQJB01000001-BQJB01000117
NPINA01	BQJC01000001-BQJC01000014
DHS20C01	BQJD01000001-BQJD01000119
DHS20C02	BQJE01000001-BQJE01000012
DHS20C03	BQJF01000001-BQJF01000234
DHS20C04	BQJG01000001-BQJG01000133
DHS20C05	BQJH01000001-BQJH01000386
DHS20C06	BQJI01000001-BQJI01000271
DHS20C07	BQJJ01000001-BQJJ01000027
DHS20C08	BQJK01000001-BQJK01000006
DHS20C09	BQJL01000001-BQJL01000420
DHS20C10	BQJM01000001-BQJM01000065
DHS20C11	BQJN01000001-BQJN01000210
DHS20C12	BQJO01000001-BQJO01000282
DHS20C13	BQJP01000001-BQJP01000173
DHS20C14	BQJQ01000001-BQJQ01000273
DHS20C15	BQJR01000001-BQJR01000011
DHS20C16	BQJS01000001-BQJS01000478
DHS20C17	BQJT01000001-BQJT01000462
DHS20C18	BQJU01000001-BQJU01000161
DHS20C19	BQJV01000001-BQJV01000589
DHS20C20	BQJW01000001-BQJW01001044

70

71 **Table S8 Calculation of ΔG_r.** The calculations were performed under 1) the standard

72 condition at pH7, 2) the condition found in NH_4^+-feeding DHS reactor, 3) the initial condition

73 of batch incubation, and 4) the condition where the concentrations of reactant and products

74 are one millimolar at pH 7. Additional explanations are available as **Supplementary text 1.**

75 (**Table S8** is available as the separated Excel file.)

76

Supplementary text 1

Gibbs free-energy change (ΔG_r) of aerobic ammonia and nitrite oxidation in the operated DHS reactors

The DHS reactors fed with NH_4^+ or NO_2^- have been operated at 20ºC for > 1 y continuously without the change of operational conditions and disturbance (See the section Experimental procedures for details of operational conditions). Typical concentrations of NH_4^+, NO_2^-, NO_3^- and dissolved oxygen (DO) and pH values were as following [2]; 7.9 μM of NH_4^+, 9.3 μM of NO_2^-, 4.9 mM of NO_3^-, 250 μM of DO, and pH 7.3.

The ΔG_r of aerobic ammonia (Eq. 1) and nitrite oxidation reaction (Eq. 2) were calculated by using the standard equation ($\Delta G_r = \Delta G_r^{\circ} + RT\ lnk$), where, ΔG_r^{o}; Standard free-energy changes, R; gas constant (0.008314 kJ mol^{-1} K^{-1}), T; temperature (K), k; equilibrium constant calculated from Eq.1 and Eq.2. The ΔG_f^{o} were as following; –26.57 kJ mol^{-1} for NH_3, 16.4 kJ mol^{-1} for dissolved oxygen (*i.e.*, $O_{2\ aq}$), 0 kJ mol^{-1} for H^+, –37.2 kJ mol^{-1} for NO_2^-, –111.34 kJ mol^{-1} for NO_3^-, –23.4 kJ mol^{-1} for NH_2OH [3], and –237.17 kJ mol^{-1} for H_2O. NH_3 concentration was calculated from the NH_4^+ concentration by considering pH equilibrium (pKa = 9.25).

$$1\ NH_3 + 1.5\ O_2 = 1\ NO_2^- + 1\ H_2O + 1\ H^+ \quad (1)$$

$$2\ NO_2^- + O_2 = 2\ NO_3^- \quad (2)$$

95 The ΔG_r were calculated under 1) the standard condition at pH7, 2) the condition

96 found in NH_4^+-feeding DHS reactor (described above), 3) the initial condition of the batch

97 incubations with the addition of 0.5 mM NH_4^+ or 0.5 mM NO_2^- and $^{14}CO_2$, and 4) the

98 condition where the concentrations of reactant and products are one millimolar. As for the

99 condition 3), initial concentration of oxidized products (NO_2^- or NO_3^-) was assumed to be

100 100-folds lower than the initial NH_4^+ or NO_2^- concentration.

101 The ΔG_r of aerobic ammonia oxidation in the operated DHS reactor and at the batch

102 incubation were –271.7 and –283.3 kJ mol-NH_3^{-1}, respectively (**Table S8**). The ΔG_r of

103 aerobic nitrite oxidation in the operated DHS reactor and at the batch incubation were –57.0

104 and –83.5 kJ mol-NO_2^{-1}, respectively. Therefore, aerobic ammonia oxidation yields 4.8- and

105 3.4-folds higher free energies than aerobic nitrite oxidation in the DHS reactor and at the

106 batch incubation, respectively.

107 The oxidation reaction of ammonia to NH_2OH is described as following [1], and the

108 $\Delta G_r'^o$ of the reaction is –170.5 kJ mol-NH_3^{-1};

109 $$1\ NH_3 + 1\ O_2 + 2\ H^+ + 2\ e^- = 1\ NH_2OH + 1\ H_2O \qquad (3)$$

110 Calculations are available as **Table S8**.

111 **Reference**

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120 **Supplementary text 2**

121 ***2-1) Metabolic potentials of the NPIRA bins***

122 Metabolic potentials of the obtained NPIRA bins were examined based on

123 presence/absence of functional genes required. In the following description, the NPIRA05 bin

124 was not included because the completeness of the NPIRA05 bin (59%) was lower than others

125 (81 to 97%) (**Table 1**). Commamox *Nitrospira* oxidizes ammonia to NH_2OH by ammonia

126 monooxygenase (Amo), and the formed NH_2OH is subsequently oxidized to NO by

127 hydroxylamine dehydrogenase (Hao) (Daims *et al.*, 2015; van Kessel *et al.*, 2015). The

128 formed NO is finally oxidized to NO_2^- by the as-yet unidentified enzymes and/or abiotic

129 reaction (Carantoa and Lancaster, 2017). Orthologues of commamox *Nitrospira amoCAB*

130 (threshold *e*-value of blastp search 10^{-15}) were not found in the NPIRA bins and also in the

131 other known *Nitrospira* sublineage IV genomes (**Fig. 3**). As for the *hao*, the NPIRA2 and

132 NPIRA3 bins had the orthologues of *Nitrospira* octaheme cytochrome *c* (OCC) instead of

133 commamox *Nitrospira hao*, which showed high similarities with the OCC found in the

134 canonical NO_2^--oxidizing *Nitrospira* genomes (*Nitrospira moscoviensis* and *Nitrospira*

135 *japonica* genomes) (**Table S3**). In the *Nitrospira moscoviensis* genome, the OCC consisted of

136 a gene cluster containing a Rieske/cytochrome *b* complex and other genes involved in

137 nitrogen acquisition, whereas such a gene cluster was not located in the NPIRA bins. The

138 *Nitrospira* OCC is currently believed to contribute to assimilatory nitrite reduction and/or the
139 detoxification of hydroxylamine (Koch *et al.*, 2015; Ushiki *et al.*, 2018).
140 As for aerobic nitrite oxidation, orthologue of *nxrABC* was found in the NPIRA4 bin,
141 whereas one or two genes among the *nxrABC* were often missing in other NPIRA bins (the
142 NPIRA01, NPIRA02, NPIRA03, and NPIRA06 bins). This is likely due to the incomplete
143 nature of the NPIRA bins (**Table 1**). As shown in **Table 1**, the obtained NPIRA bins showed
144 high strain heterogeneity, which became a bottleneck at the assembly and binning of contig
145 sequences, and the genes highly conserved among *Nitrospira* genomes (*i.e.*, *nxr*) were often
146 not assigned into specific NPIRA bins. Indeed, the *nxrA* of the NPIRA03 bin, the *nxrB* of the
147 NPIRA01 and NPIRA04 bins, and the *nxrC* of the NPIRA03 and NPIRA04 bins were
148 annotated as the fragmented genes located at the end of the contigs.
149 As a component of respiratory chain, *nuo*-operons (*nuoNMLKJIHGFECDBA* and
150 *nuoNMMLKJIGDCBA*) encoding NADH:ubiquinone oxidoreductase (complex I) were found
151 from the NPIRA bins except for NPIRA06 bin in which the *nuoNMLKJIHGFECDBA* operon
152 was missing. The NuoNMMLKJIGDCBA is a 2M-type complex I (Chadwick *et al.*, 2018)
153 and likely involved in the reverse electron flow from quinol to ferredoxin which is required
154 for CO_2 fixation via rTCA cycle (Lawson *et al.*, 2021). Two copies of *qcr*-operon encoding
155 quinol-cytochrome *c* reductase (complex III) were found from the NPIRA01, NPIRA02,
156 NPIRA03, and NPIRA04 bins, and alternative complex III module (ACIII) previously found

157 in the *Nitrospira marina* genome was located in the NPIRA01, NPIRA03, and NPIRA06

158 bins. *Nitrospira* use a novel cytochrome *bd*-like heme-copper oxidase as a terminal oxidase

159 (Lücker *et al.*, 2010; Simon and Klotz, 2013). Multiple copies of the genes encoding putative

160 cytochrome *bd*-like oxidase were found in the NPIRA bins, and the heme *b* and copper-

161 binding sites conserved in canonical cytochrome *bd* oxidase were found in the

162 NPIRA01_31930, NPIRA02_18030, NPIRA03_03640, and NPIRA04_28020 proteins. The

163 recently determined *Nitrospira marina* genome showed that this *Nitrospira* has an additional

164 putative terminal oxidase with high O_2 affinities, cbb_3-type terminal oxidase (Bayer *et al.*,

165 2021). The gene encoding cbb_3-type terminal oxidase was found from the NPIRA01,

166 NPIRA03, and NPIRA04 bins. In *Nitrobacter*, soluble and membrane bound monoheme

167 cytochrome c_{550} transfer the electrons released from Nxr to terminal oxidase (Nomoto *et al.*,

168 1993). The orthologue of the genes encoding *Nitrobacter winogradskyi* soluble and

169 membrane-bound c_{550} (the Nwi_2582 and Nwi_0712 genes) (Starkenburg *et al.*, 2006) was

170 not found in the NPIRA bins. The NPIRA bins had the 57 to 72 genes encoding monoheme

171 cytochrome proteins as well as the *Nitrospira defluvii* genome (Lücker *et al.*, 2010), and those

172 cytochrome proteins supposedly participate the electron transferring from *Nitrospira* Nxr to

173 the above terminal oxidase(s). The H^+-translocating F_1F_0-ATPase was found in the NPIRA

174 bins, and putative Na^+-translocating ATPase previously found in the *Nitrospira marina*

175 genome was located in the NPIRA03, and NPIRA06 bins.

176 The NPIRA bins had the genes encoding 2-oxoglutarate:ferredoxin oxidoreductase
177 (OGOR), five- and/or four-subunit pyruvate:ferredoxin oxidoreductase (POR), and ATP-
178 citrate lyase (ACL) that are key enzymes in the rTCA cycle (Lücker *et al.*, 2010; Bayer *et al.*,
179 2021) (**Table S2**). Four-subunit POR is thought to be more oxygen sensitive but with higher
180 specific activity (Bayer *et al.*, 2021). The gene encoding four-subunit POR was only found
181 from the NPIRA03, NPIRA04 and NPIRA06 bins, whereas the genes encoding the five-
182 subunit POR were well conserved in the NPIRA bins.
183 *Nitrospira marina* (Bayer *et al*., 2021) and *Nitrospira moscoviensis* (Koch *et al*.,
184 2015) are capable of the growth utilizing formate, and the gene encoding formate
185 dehydrogenase (Fdh) was located in the NPIRA03 and NPIRA06 bins affiliated into the
186 *Nitrospira marina* clade (**Fig. 2**). On the other hand, the NPIRA bins affiliated into the
187 *Nitrospira salsa* clade were commonly lacking the gene encoding formate dehydrogenase
188 (**Table S2**), suggesting those *Nitrospira* are incapable of utilizing formate or just reflecting
189 incomplete nature of the NPIRA bins. It would be of interest to examine formate utilization of
190 *Nitrospira salsa* because the formate utilization would be a niche determinant for *Nitrospira*
191 *salsa* and *Nitrospira marina* who are capable of formate utilization.
192 As an oxidative stress defense system, the NPIRA bins carried the genes encoding
193 superoxide dismutase (SOD), catalase, glutathione peroxidase, and cytochrome *c* peroxidase.
194 Sulfite oxidation coupled with the reduction of cytochrome *c* by *Nitrospira* remains

195 controversial due to lack of experimental demonstration, whereas the *sorAB* encoding

196 sulfite:cytochrome *c* oxidoreductase was conserved among the NPIRA bins.

197 ***2-2) Metabolic potentials of the NMNS bins***

198 The *amoCAB* gene cluster was found in the NMNS01 bin (**Table S4**). The *amoCAB* gene

199 cluster (NMNS01_40000 to NMNS01_40020 genes) was located in the short contig (2.6 kb),

200 and no neighbor gene was located in the contig. In the NMNS02 bin, *amoCAB* gene cluster

201 was not found, and only *amoC* was found (NMNS02_32030 gene). Hydroxylamine oxidation

202 to NO and electron transferring to ubiquinone molecules are carried out by a hydroxylamine

203 ubiquinone redox module (Simon and Klotz, 2013) composed of hydroxylamine

204 dehydrogenase (Hao), cytochrome c_{554}, and cytochrome c_{552}. The *haoAB*, c_{554}, and c_{552} genes

205 are composed of a gene cluster in *Nitrosomonas* sp. Nm143 and *Nitrosomonas aestuarii*

206 genomes, and the *haoAB*, c_{554}, and c_{552} gene cluster was found in the NMNS02 bin but not in

207 the NMNS01 bin. Hydroxylamine oxidation reaction of *Nitrosomonas europaea* Hao

208 produces nitric oxide (NO), which is subsequently oxidized to NO_2^-. Nitrosocyanin (Arciero

209 *et al.*, 2002) is a candidate of NO oxidase (Lancaster *et al.*, 2018), and the gene encoding

210 nitrosocyanin was found from both the NMNS01 and NMNS02 bins. Apart from the above

211 genes, the NMNS01 and NMNS02 bins had the genes encoding copper-containing NO-

212 forming nitrite reductase (NirK), cytochrome-containing nitric oxide reductase (cNor),

213 cytochrome *c'* beta (Cyt b'), and cytochrome P460 (Cyt_{P460}), and those enzymes may

214 contribute to nitrogen oxide transformation other than aerobic ammonia oxidation.

215 The electrons released from hydroxylamine (and NO) oxidation enter into a

216 respiratory chain in the form of ubiquinol for the generation of proton motive force across a

217 membrane and also of reducing power (*i.e.*, NAD(P)H) required for CO_2 fixation. The *pet*-

218 operon (*petABC*) encoding cytochrome bc_1 complex (complex III) was found in both the

219 NMNS01 and NMNS02 bins. The electrons released from ubiquinol oxidation are transferred

220 from the complex III to terminal oxidase using a soluble cytochrome *c* (c_{552}) (Simon and

221 Klotz, 2013), and the c_{552} gene was located in both the NMNS01 and NMNS02 bins. The

222 NMNS01 and NMNS02 bins had the genes encoding an aa_3-type terminal oxidase with low

223 O_2 affinity but not a cbb_3-type terminal oxidase with high O_2 affinity and putative high-O_2-

224 affinity terminal oxidase sNor (Sedlacek *et al.*, 2020). The NMNS01 and NMNS02 bins had

225 the two copies of *nuo*-operon (*nuoABCDEFGHIJKLMN* and *nuoABB/C/DEFGHIJKLMN*)

226 encoding NADH:quinone oxidoreductase (complex I) as well as the *Nitrosomonas aestuarii*

227 genome.

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