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Diversity and evolution of nitric oxide reduction

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25   **Nitrogen is an essential element for life, with the availability of fixed nitrogen**

26   **limiting productivity in many ecosystems. The return of oxidized nitrogen species to**

27   **the atmospheric N_2 pool is predominately catalyzed by microbial denitrification**

28   **($NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$)[1]. Incomplete denitrification can produce N_2O as**

29   **a terminal product, leading to an increase in atmospheric N_2O, a potent greenhouse**

30   **and ozone-depleting gas[2]. The production of N_2O is catalyzed by nitric oxide**

31   **reductase (NOR) members of the heme-copper oxidoreductase (HCO) superfamily[3].**

32   **Here we use phylogenomics to identify a number of previously uncharacterized**

33   **HCO families and propose that many of them (eNOR, sNOR, gNOR, and nNOR)**

34   **perform nitric oxide reduction. These families have novel active-site structures and**

35   **several have conserved proton channels, suggesting that they might be able to**

36   **couple nitric oxide reduction to energy conservation. We isolated and biochemically**

37   **characterized a member of the eNOR family from *Rhodothermus marinus*, verifying**

38   **that it performs nitric oxide reduction both *in vitro* and *in vivo*. These newly**

39   **identified NORs exhibit broad phylogenetic and environmental distributions,**

40   **expanding the diversity of microbes that can perform denitrification. Phylogenetic**

41   **analyses of the HCO superfamily demonstrate that nitric oxide reductases evolved**

42   **multiple times independently from oxygen reductases, suggesting that complete**

43   **denitrification evolved after aerobic respiration.**

44   The HCO superfamily is extremely diverse, with members playing crucial roles in both

45   aerobic (oxygen reductases) and anaerobic (nitric oxide reductases) respiration. The

46   superfamily currently consists of at least three oxygen reductase families (A, B and C)

47   and three NOR families (cNOR, qNOR, and qCu$_A$NOR)[4]. The oxygen reductases

48   catalyze the reduction of O_2 to water ($O_2 + 4e_{out}^- + 4H_{in}^+ + nH_{in}^+ \rightarrow 2H_2O + nH_{out}^+$) and

49   share a conserved reaction mechanism[5,6], where three of the electrons required to reduce

50   O_2 are provided by the active-site metals, heme-Fe and Cu_B, while the fourth electron is

51   derived from a redox-active cross-linked tyrosine cofactor[7] (**Figure 1**). The free energy

52   of the reaction is converted to a transmembrane proton electrochemical gradient by two

53   different mechanisms, charge separation across the membrane and proton pumping[8–10].

54   Both the chemical and pumped protons are taken up from the electrochemically negative

55   side of the membrane (bacterial cytoplasm) via conserved proton-conducting channels

56   that are comprised of conserved polar residues and internal water molecules. The

57   different oxygen reductase families exhibit differential proton pumping stochiometries

58   (n=4 for the A-family, and n=2 for the B and C-families)[8–10], depending on the details of

59   their conserved proton channels. The oxygen reductases also vary in their secondary

60   subunits that function as redox relays from electron donors to the active-site, with the A

61   and B-families utilizing a Cu_A-containing subunit[11–13] and the C-family containing one or

62   more cytochrome c subunits[14] (**Figure 1**).

63

64   Nitric oxide reductases (NORs) catalyze the reduction of nitric oxide to nitrous oxide

65   ($2NO + 2H_{out}^+ + 2e_{out}^- + nH_{in}^+ \rightarrow N_2O + H_2O + nH_{in}^+$). Nitric oxide reduction is only a 2-

66   electron reaction, so it does not require the cross-linked tyrosine cofactor for catalysis.

67   There are currently three known HCO NORs, the cNOR, qNOR, and qCu_ANOR. The

68   cNOR and qNOR families have a four amino acid coordinated Fe_B ion in their active-

69   sites, in contrast to the three amino acid coordinated Cu_B found in oxygen reductases[15,16].

70   The cNOR and qNOR families are closely related to the C-family oxygen reductases and

71   likely evolved from this oxygen reductase family. In accordance with this relationship,

72   the cNORs have a secondary cytochrome c-containing subunit, while in the qNORs the

73   two subunits corresponding to those in the cNORs have been fused to a single subunit

74   that lacks the heme c binding. Although, the qNOR from *N. meningitidis*[16] is proposed to

75   take up protons from the cytoplasm for NO reduction, neither the cNOR nor qNOR have

76   conserved residues that could form a proton channel from the cytoplasm, therefore it is

77   unlikely that they conserve energy via either charge separation or proton pumping (n=0

78   for the cNOR and qNOR families). Phylogenomic analysis shows that the qCu$_A$NOR

79   from *Bacillus azotoformans*[17,18] is unrelated to the qNOR family, and has been

80   reclassified here as the bNOR family. The bNOR active-site structure is fundamentally

81   different than those from the cNOR and qNOR families **(Figure 1)**. It also contains a

82   conserved proton channel that is very similar to that found in the B-family oxygen

83   reductases **(Figure 1)**, and has been shown to be electrogenic[18]. This has important

84   consequences for the efficiency of energy conservation associated with denitrification[19].

85

86   **Novel heme-copper oxygen reductase homologs**

87   Phylogenomic analyses of genomic and metagenomic data have identified at least six

88   new families belonging to the HCO superfamily (**Figure 2**). All of these families are

89   missing the active-site tyrosine, suggesting that they do not catalyze O_2 reduction.

90   Furthermore, their active-sites exhibit structural features never seen before within the

91   superfamily (**Figure 1**). One of these families is closely related to qNOR and has been

92   proposed to be a nitric oxide dismutase contributing to O_2 production in 'Candidatus

93   Methylomirabilis oxyfera'[20]. Another family is closely related to cNOR and might be a

94   sulfide and acetylene insensitive nitrous oxide reductase[21,22]. The remaining four families

95   (eNOR, sNOR, nNOR and gNOR) are closely related to the B-family of oxygen

96   reductases (**Figure 2**) and encode for homologs of the Cu_A-containing secondary

97   subunits, consistent with this evolutionary relationship (**Figure 1, Table S1**). Based on

98   modeled active-site structures and genomic context we propose that these four families

99   perform nitric oxide reduction (**Figure 1**).

100

101   **Biochemical characterization of eNOR**

102   To validate these predictions we isolated and biochemically characterized a member of

103   the eNOR family from *Rhodothermus marinus* DSM 4252, a thermophilic member of the

104   Bacteroidetes phylum. *Rhodothermus marinus* was originally classified as a strict

105   aerobe[23], but its genome encodes a periplasmic nitrate reductase (NapA), two nitrite

106   reductases (NirS and NirK), and a nitrous oxide reductase (NosZ), suggesting that it may

107   also be capable of denitrification (**Extended Data Figure 1**). Denitrification was not

108   observed under strictly anaerobic conditions, however under microoxic conditions

109   isotopically labeled $^{15}NO_3^-$ was converted to $^{30}N_2$ (**Extended Data Figure 2**)

110   demonstrating that *R. marinus* DSM 4252 is capable of complete aerobic denitrification

111    $(NO_3^- \rightarrow N_2)$. Blockage of the nitrous oxide reductase with acetylene led to the

112   accumulation of N_2O (**Figure 3**), suggesting that a nitric oxide reductase was also present

113   in *R. marinus* DSM 4252. No known NORs (cNOR, qNOR, qCuANOR) were found in

114   the genome. However, *R. marinus* DSM 4252 does encode for a member of the eNOR

115   family (**Extended Data Figure 1**).

116

117   Isolation and biochemical characterization of the *R. marinus* DSM 4252 eNOR protein

118   verified that it catalyzes NO reduction (at 25°C, k_{cat} = 0.68 $\pm$ 0.21 NO.s^{-1} (n = 4))

119   (**Figure 3**). eNOR was unable to catalyze O_2 reduction using various electron donors

120   (**Extended Data Figure 3**), clearly showing that it functions solely as a nitric oxide

121   reductase. UV-Vis spectroscopy and heme characterization via mass spectrometry

122   demonstrated that the *R. marinus* DSM 4252 eNOR contains a novel modified heme *a*

123   that is used in both heme sites (**Figure 3 and Extended Data Figures 3 and 4**). A

124   member of the eNOR family was previously isolated from the aerobic denitrifier

125   *Magnetospirillum magnetotacticum* MS-1[16,17], however its function was never

126   determined. The UV-Vis spectra of the *M. magnetotacticum* eNOR[17] is identical to the *R.*

127   *marinus* eNOR, suggesting that the modified heme *a* is a general feature of the family.

128   Mass spectrum analysis of the hemes extracted from eNOR suggest that this heme is A$_s$, a

129   previously isolated heme *a* with a hydroxyethylgeranylgeranyl side chain first identified

130   in the B-family oxygen reductase from *Sulfolobus acidocaldarius*[24]. Many eNOR operons

131   contain a CtaA homolog, an O_2-dependent enzyme that converts heme *o* to heme *a*[25].

132   This is consistent with the observation that eNOR requires microoxic conditions for

133   expression and suggests that aerobic denitrification might be common in nature.

134

135   **Active-site features of novel NORs**

136   In addition to the experimental evidence that both eNOR and bNOR enzymes are NO

137   reductases, there is good evidence that the other newly identified families also perform

138   nitric oxide reduction. The sNOR family has the same active-site structure as the bNOR

139   family, strongly suggesting that it also performs nitric oxide reduction. However, the

140   sNOR and bNOR families are not closely related, providing an example of convergent

141   evolution of active-site structures within the HCO superfamily (**Figures 1 and 2**).

142   Another example of convergent evolution is the nNOR family. It has the same conserved

143   active-site residues as the cNOR and qNOR families (**Figure 1**), but is very distantly

144   related to them (nNOR is related to the B-family, while cNOR and qNOR are related to

145   the C-family). Interestingly, the low-spin heme in nNOR is ligated by a histidine and

146   methionine, which likely raises its redox potential by ~150 mV[26]. This is similar to a

147   modification found in some eNORs, where the low spin heme is ligated by histidine and

148   lysine. The gNOR is the first example of a HCO family that has replaced one of the

149   active-site histidines, residues completely conserved in all other families. A bioinorganic

150   mimic of the gNOR active-site exhibited nitric oxide reduction capability[27], suggesting

151   that it is likely a functional NOR *in vivo*. The gNOR has a secondary subunit with a

152   cupredoxin fold that is missing the residues required to bind Cu_A, similar to the quinol

153   oxygen reductase from *E. coli*. Conserved residues that could bind quinol have been

154   identified in gNOR, suggesting that it is a quinol nitric oxide reductase similar to qNOR.

155

156   The biochemically characterized (eNOR and bNOR) and proposed (sNOR and gNOR)

157   HCO NORs have active-sites that differ significantly from those utilized by cNOR and

158   qNOR (**Figure 1**). This demonstrates that while oxygen reduction chemistry is

159   constrained to require a redox active tyrosine cofactor, multiple HCO active-site

160   structures are compatible with nitric oxide reduction chemistry. Interestingly, in the

161   currently characterized HCOs Cu_B is utilized for O_2 reduction chemistry and Fe_B for NO

162   reduction chemistry. If this pattern is verified for the other predicted NOR families it

163   would suggest that the chemistry performed by HCOs is partially determined by the

164   electronic properties of the active-site metal.

165

166   **Bioenergetics of novel denitrification pathways**

167   Although both denitrification and aerobic respiration are highly exergonic processes,

168   most of the enzymes in the denitrification pathway are not coupled to energy

169   conservation, making denitrification significantly less efficient than aerobic respiration[28].

170   In the HCO oxygen reductases conserved proton channels deliver protons from the

171   cytoplasm to the active-site for chemistry. These same channels are used to pump protons

172   to the periplasmic side. The cNORs and qNORs do not have conserved proton channels

173   from the cytoplasm[15,29], making them incapable of conserving energy. The eNOR has

174   conserved residues that closely resemble those found in the proton-conducting K-channel

175   within the B-family of oxygen reductases[30] (**Supplementary Table 1, Extended Data**

176   **Figure 5**). The sNOR family also has conserved residues in the K-channel region,

177   however they are different than those found in the eNOR and bNOR families.

178   Interestingly, the nNOR family, which has the same active site as cNOR and qNOR, also

179   has a conserved proton channel (**Supplementary Table 1, Extended Data Figure 5**),

180   suggesting that the lack of a proton channel in the cNOR and qNOR may not be due to

181   energy constraints[31]. The conserved proton channels in the eNOR, bNOR, sNOR, and

182   nNOR families would allow them to conserve energy via charge separation, and

183   potentially by proton pumping. Characterization of these new NOR families will be

184   critical for a full understanding of the mechanism of proton pumping in the HCO

185   superfamily, one of the most important unanswered question in bioenergetics.

Distribution and environmental relevance of NORs

The new HCO NOR families have broad phylogenetic and environmental distributions (**Table 1, Supplementary Tables 2, 3**). The eNOR, sNOR, gNOR, and nNOR families are found in both Bacteria and Archaea. To date, the bNOR family has been found only in the Bacillales order of Firmicutes (**Supplementary Table 2**). Phylogenetic analysis of metagenomic data shows that the majority of eNOR, sNOR, gNOR, and nNOR enzymes are from uncharacterized organisms, suggesting that many more organisms are capable of nitric oxide reduction than previously suspected. Furthermore, the new HCO NOR families are found in a wide variety of environments (**Table 1, Supplementary Table 2**). The sNOR are found in the majority of ammonia oxidizing bacteria sequenced to date and likely play a role in this process. The gNORs are predominantly found in sulfidic environments and may be an adaptation that allows for denitrification in the presence of sulfide, which inhibits other NOR families. The eNOR family is very common in nature, having a broad distribution similar to the cNOR and qNOR families. The eNOR family appears to play key roles in a number of important microbiological processes. They are found in many strains of *Candidatus Accumulibacter phosphatis*, a microbe utilized for phosphate accumulation in wastewater treatment plants during enhanced biological phosphorus removal. The eNOR is highly expressed in transcriptomic datasets from these facilities, demonstrating that *Accumulibacter phosphatis* is capable of complete denitrification *in situ*[32]. eNOR has also been found in microbes capable of performing autotrophic nitrate reduction coupled to Fe(II) oxidation (NRFO). *Gallionellaceae* KS and related strains express an eNOR under denitrifying conditions, suggesting that an individual organism is capable of complete NRFO[33]. eNOR is also commonly found in

209   hypersaline environments (**Supplementary Table 2**) where is might play a role in the

210   adaptation of denitrification to high salt conditions.

211

212   Many organisms encode NORs from multiple families (e.g., *Ca.* Methylomirabilis

213   oxyfera has a qNOR, sNOR and gNOR, and *Bacillus azotoformans* has a qNOR, sNOR,

214   and bNOR). This suggests that selection for different enzymatic properties (NO affinity,

215   enzyme kinetics, energy conservation, or sensitivity to inhibitors) or the concentration of

216   O_2 may be important factors in determining their distribution, similar to what is observed

217   for the HCO oxygen reductase families[8]. Analysis of the presence of denitrification genes

218   (nitrite reductases, nitric oxide reductases, and nitrous oxide reductases) within

219   sequenced genomes indicates that many more organisms are capable of complete

220   denitrification than previously realized. Our current understanding of the diversity of

221   organisms capable of performing denitrification is far from complete.

222

223   Our evolutionary analysis shows that nitric oxide reductases have evolved many times

224   independently from oxygen reductases (**Figure 2**). The current data show that NORs

225   have originated from both the B and C-families of oxygen reductases, enzymes that are

226   adapted to low O_2 environments. These oxygen reductases can reduce NO at high

227   concentrations *in vitro*[34] so it is not surprising that small evolutionary modifications

228   would lead to enzymes capable of NO reduction at the lower NO concentrations

229   produced during denitrification. The fact that NO reductases are derived from oxygen

230   reductases strongly suggests that complete denitrification evolved after aerobic

231 respiration. This places important constraints on the nitrogen cycle before the rise of

232 oxygen.

233

234 **Competing Interests**

235 The authors declare no competing interests.

236 **Acknowledgment**

250
251 **References**
252
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349

List of Figures

350

351 **Figure 1. Comparison of HCO active sites.** a) Active-site and proton channel properties
352 of the five characterized HCO families (A-family, B-family, C-family, cNOR, and
353 qNOR). The oxygen reductases all have an active-site composed of high-spin heme, a
354 redox-active cross-linked tyrosine cofactor, and a copper (Cu_B) ligated by three
355 histidines. The A-family has two conserved proton channels, whereas the B and C-
356 families only have one. The active-sites of the nitric oxide reductases are composed of a
357 high-spin heme and an iron (Fe_B) that is ligated by three histidines and a glutamate.
358 Notably they are missing the tyrosine cofactor. The cNOR and qNOR are also missing
359 conserved proton channels, making them non-electrogenic. b) Sequence alignment of the
360 active-sites of the newly discovered HCO families that are related to the B-family. c)
361 Predicted active-sites and proton channels for the new HCO families. The eNOR, bNOR,
362 sNOR, and nNOR families contain completely conserved proton channels shown here as
363 arrows. The putative proton channel in the bNOR and eNOR families are highly similar
364 to the K-channel from the B-family oxygen reductase and are colored in red. The K-
365 channel in the B-family is also similar to the K-channel in the A-family oxygen reductase
366 which is colored in dark red. The proton channel in the C-family is different from these
367 channels and is marked in yellow. The putative proton channels in sNOR and nNOR are
368 marked in cyan and differentiated from the other channels with a dashed black outline.

369

370 **Figure 2. Evolution of nitric oxide reductases.** Phylogenetic tree of HCO families. All
371 of the new NOR families are derived from oxygen reductase ancestors. Oxygen
372 reductases are in shades of blue, whereas nitric oxide reductases are in shades of yellow,
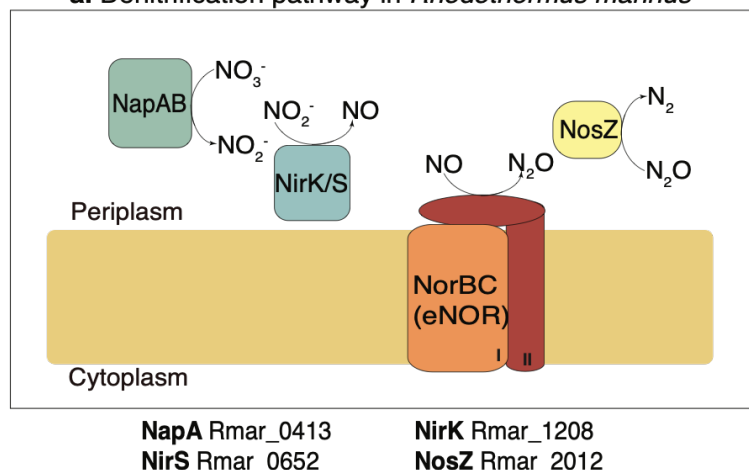373 green and red.

374 **Figure 3. Biochemical Characterization of the eNOR from *Rhodothermus marinus*.**
375 a) UV-Vis spectrum of isolated eNOR indicates the presence of an unusual heme *a*
376 signature at 589 nm. b) NO reductase activity was measured with the use of a Clark
377 electrode in the presence of TMPD and ascorbate as electron donor. c) N_2O accumulation
378 observed in a culture of *Rhodothermus marinus*, in the presence of acetylene d) N_2O
379 production by eNOR from *R. marinus* and qNOR from *Persephonella marina*.

380 **Extended Data Figure 1: Genome of *R. marinus* encodes for the complete**
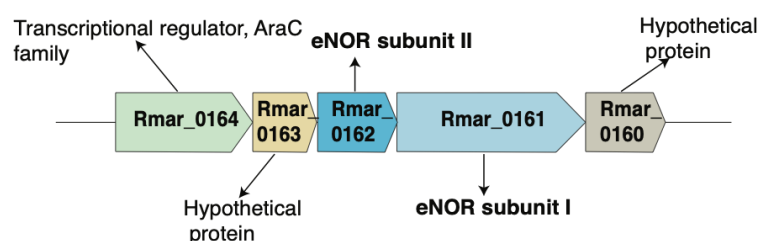381 **denitrification pathway.** a. The genes for NapAB, the periplasmic nitrate reductase
382 (Rmar_0413), the nitrite reductases nirK (Rmar_1208) and nirS (Rmar_0652) and nitrous
383 oxide reductase, nosZ (Rmar_2012) are encoded in the *R. marinus* genome. b. The gene
384 neighborhood of eNOR in *R. marinus*. c. The gene neighborhood of eNOR in *Halovivax*
385 *ruber* includes ctaB, an enzyme involved in the biosynthesis of heme *a*.

386

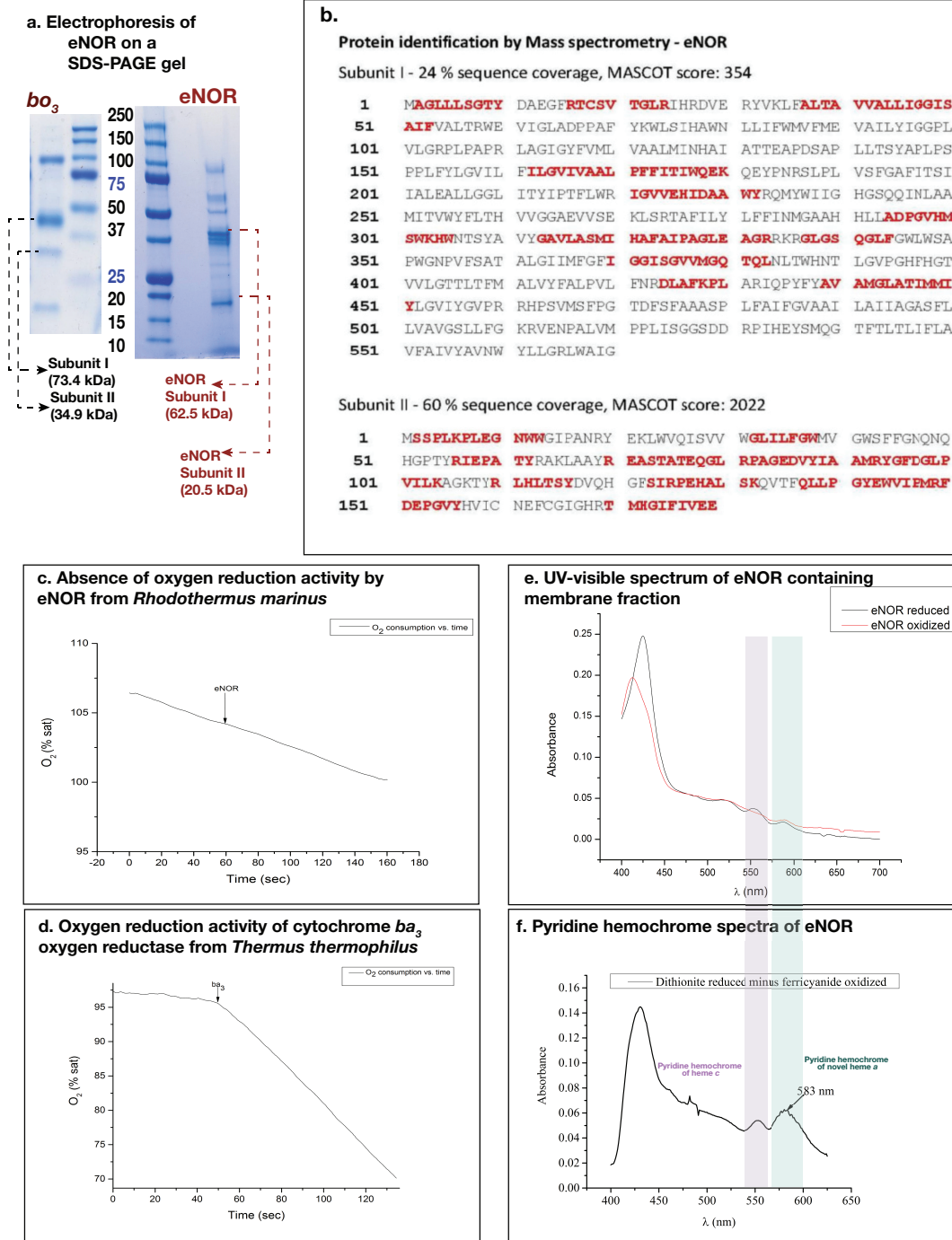**Extended Data Figure 2: *Rhodothermus marinus* does perform complete denitrification. a.** *R. marinus* converts $^{15}NO_3^-$ to $^{30}N_2$. Ratio of $^{30}N_2$ to $^{28}N_2$ for each sample. Air is ambient atmosphere as a standard. C0 is a nitrate-free control. 14 0-17 are cultures grown with unlabeled nitrate, transferred to sealed vials after 0-17 hours, respectively. 15 0-17 are the equivalent samples grown with ^{15}N-labeled nitrate. Error bars represent two standard deviations from three replicate GC/MS measurements. $^{30}N_2$ enrichments from the ^{15}N-labeled samples are over 30-60x higher than background atmospheric ratios, while unlabeled samples have no significant enrichment over background. b. Growth of *R. marinus*, measured using OD_{600nm} over 39 hours. NO_3^- utilization was established by measuring the concentrations of nitrate in the media using a calorimetric assay. c. *R. marinus* growth in rich media was compared under denitrifying and non-denitrifying conditions using OD_{600nm}. d. Phenotypic differences of *R. marinus* cultures, under denitrifying and non-denitrifying conditions.
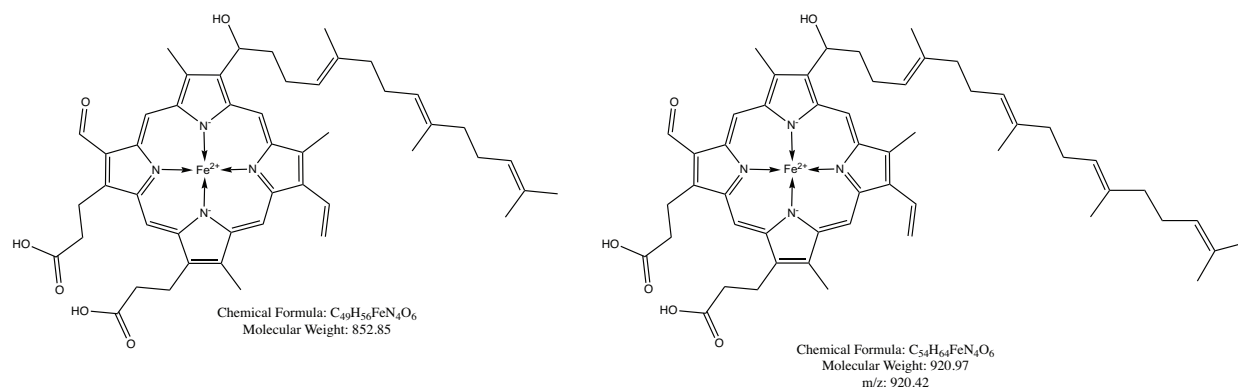
Extended Data Figure 3: Characteristics of eNOR from *Rhodothermus marinus* a. SDS-PAGE gel electrophoresis of eNOR shows two bright bands which are estimated to subunits of I and II of the complex. Both subunits appear to run faster than their estimated molecular weight. This is typical for membrane proteins. For comparison, an SDS-PAGE gel of cytochrome *bo₃* oxidase from *E. coli* is included. b. Mass spectrometric identification of eNOR is confirmed by LC/MS/MS analysis. c,d. Absence of O_2 reduction by *R. marinus* eNOR, in comparison to robust O_2 reduction by *T. thermophilus ba₃*-type oxygen reductase. e. UV-visible spectrum of eNOR f. Pyridine hemochrome-spectra of extracted hemes from eNOR showing a peak which is atypical of hemes *a, b* or *c*.
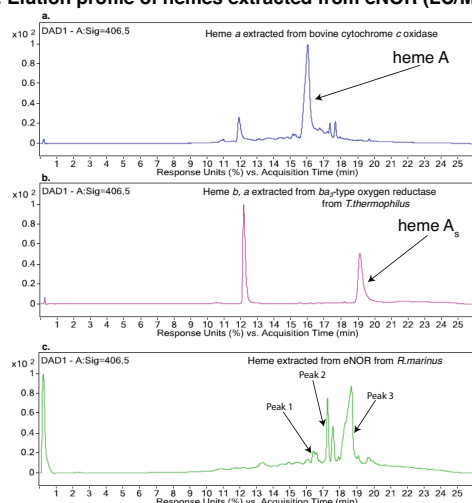
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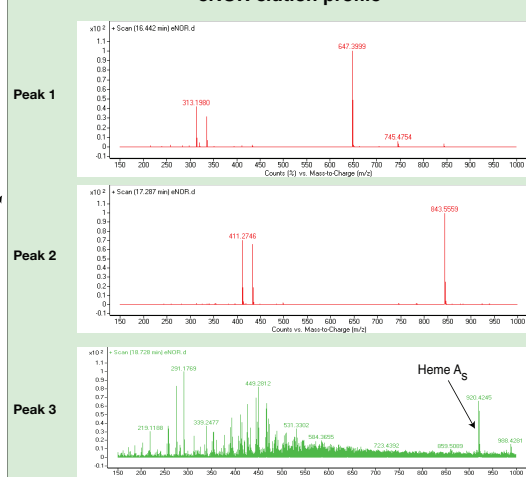
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Extended Data Figure 6: Conserved amino acids in the eNOR family of enzymes. Multiple sequence alignment of 23 eNOR sequences from various taxonomically divergent organisms reveals conserved residues that correspond to the active site ligands, proton channel residues and other sequence features that are unique to eNOR. The active

432　site residues are highlighted in maroon while the proton channel residues are highlighted
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440　**Table 1. Environmental distribution of the HCO NOR families.** Distribution of NOR
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465　for each of the proteins.

Table 1. Environmental distribution of the HCO NOR families. Distribution of NOR families in sequenced genomes versus environmental datasets. The newly discovered NOR families account for approximately 2/3 of currently known diversity and 1/2 of the abundance of NORs in nature.

	NCBI-Genomes	IMG-metagenomes	GTDB-genomes
A-family	20290	102368	45135
B-family	1238	4683	2021
C-family	13976	23015	14981
qNOR	4388	7680	3458
cNOR	2801	4824	2594
eNOR	68	2709	547
sNOR	95	872	344
bNOR	51	12	200
nNOR	6	289	32
gNOR	10	913	156
NOD	8	539	108
N2O	25	597	n.a.

Figure 1. Comparison of HCO active sites. a) Active-site and proton channel properties of the five characterized HCO families (A-family, B-family, C-family, cNOR, and qNOR). The oxygen reductases all have an active-site composed of high-spin heme, a redox-active cross-linked tyrosine cofactor, and a copper (Cu_B) ligated by three histidines. The A-family has two conserved proton channels, whereas the B and C-families only have one. The active-sites of the nitric oxide reductases are composed of a high-spin heme and an iron (Fe_B) that is ligated by three histidines and a glutamate. Notably they are missing the tyrosine cofactor. The cNOR and qNOR are also missing conserved proton channels, making them non-electrogenic. b) Sequence alignment of the active-sites of the newly discovered HCO families that are related to the B-family. c) Predicted active-sites and proton channels for the new HCO families. The eNOR, bNOR, sNOR, and nNOR families contain completely conserved proton channels shown here as arrows. The putative proton channel in the bNOR and eNOR families are highly similar to the K-channel from the B-family oxygen reductase and are colored in red. The K-channel in the B-family is also similar to the K-channel in the A-family oxygen reductase which is colored in dark red. The proton channel in the C-family is different from these channels and is marked in yellow. The putative proton channels in sNOR and nNOR are marked in cyan and differentiated from the other channels with a dashed black outline.

A

A-family   B-family   C-family   cNOR   qNOR

B

```
A-family_1M56   PVLYQHILWFFGHPEVYIIVLPAFGIVSHVIATFAK--GYLPMVYAMVAIGVLGFVVWAHHMYT
B-family_3S8F   PLVARTLFWWTGHPIVYFWLLPAYAIIYTILPKQAG--SDPMARLAFLLFLLLSTPVGFHHQFA
eNOR            AAWYRQMYWIIGHGSQQINLAAMITVWYFLTHVVGG--SEKLSRTAFILYLFFINMGAAHHLLA
bNOR            VMVARTLFWAFGHTAVNIWYLTAVSAWYVIVPKIIG--SDMLTRVVIIALVIMNITGGFHHQII
sNOR            PLLSKNLIYAFGHIFANSIIYMGVIAVYEIFPKYTG--VYGNFLIAWNASTLFTMIIYPHHLLM
gNOR            ALLYKNVYWWGLDLIADGLVLIYVAGSWYLLAMLLT--MQHIARAALFVELVVSWFVWSHHLLS
nNOR            PWPFNVAFWLFAHNLMEAMGIMALAAVYALVPLYTR--SPGMGVLAVGLYTLSAIPAFGHHLYT
C-family_3MK7   GATDAMVQWWYGHNAVGFFLTAGFLGIMYYFVPKQA--SYRLSIVHFWALITVYIWAGPHHLHY
cNOR_300R       LTRDKFYWWWVVHLWVEGVWELIMGAILAFVLVKIT--IEKWLYVIIAMALISGIIGTGHHYFW
qNOR_3AYF       FTMADFWRWWIIHLWVEGIFEVFAVVVIGFLLVQLR--TVRALYFQFTILLGSGVIGIGHHYYY
                --------------Helix VI--------------  ---------Helix VII--------
```

C

eNOR   bNOR   sNOR   nNOR   gNOR

Figure 2. Evolution of nitric oxide reductases. Phylogenetic tree of HCO families. All of the new NOR families are derived from oxygen reductase ancestors. Oxygen reductases are in shades of blue, whereas nitric oxide reductases are in shades of yellow, green and red. The biochemically characterized eNOR from *Rhodothermus marinus* is marked with a black star.
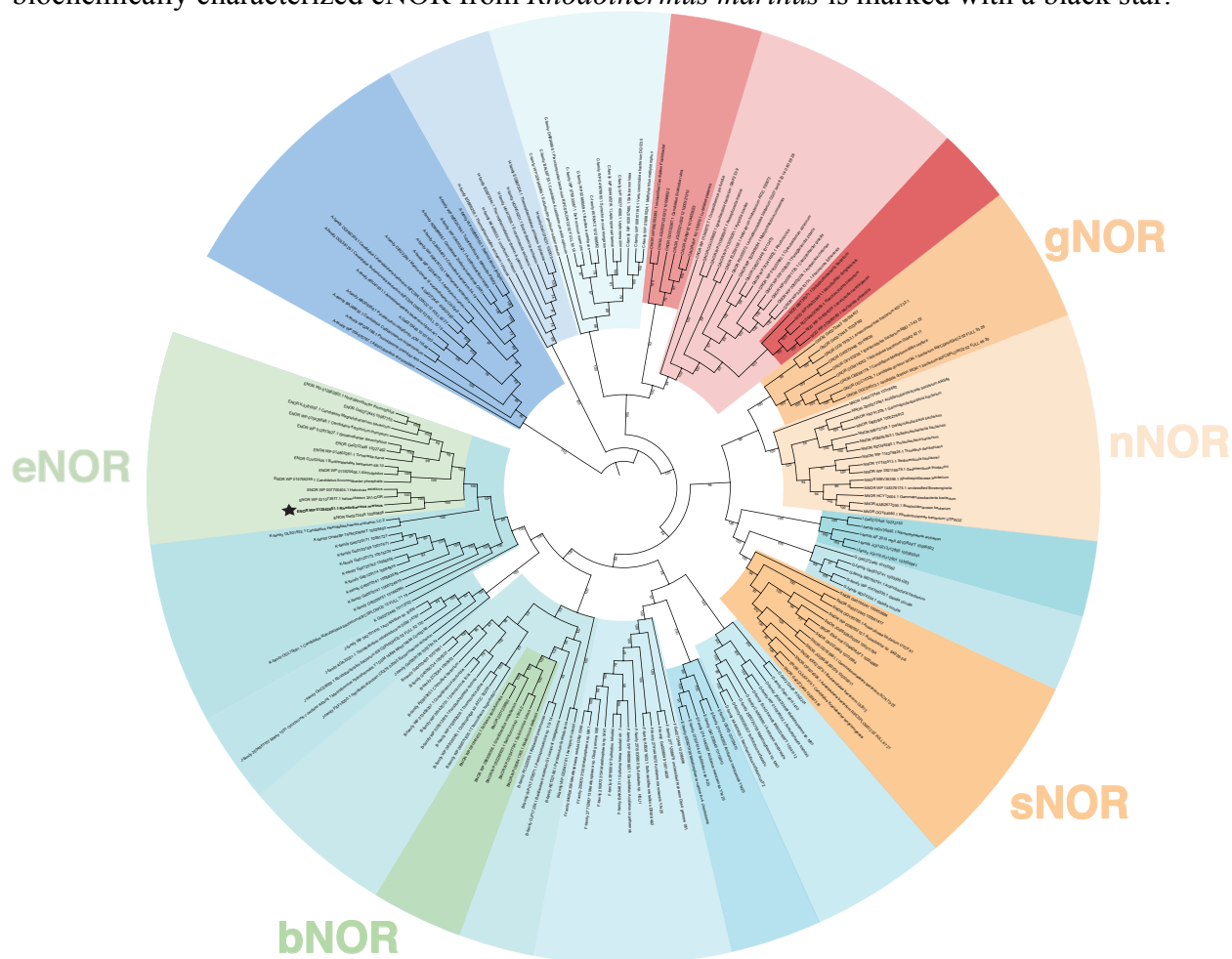
Figure 3. Biochemical Characterization of the eNOR from *Rhodothermus marinus*. a) UV-Vis spectrum of isolated eNOR indicates the presence of an unusual heme *a* signature at 589 nm. b) NO reductase activity was measured with the use of a Clark electrode in the presence of TMPD and ascorbate as electron donor. c) N_2O accumulation observed in a culture of *Rhodothermus marinus*, in the presence of acetylene d) N_2O production by eNOR from *R. marinus* and qNOR from *Persephonella marina*.
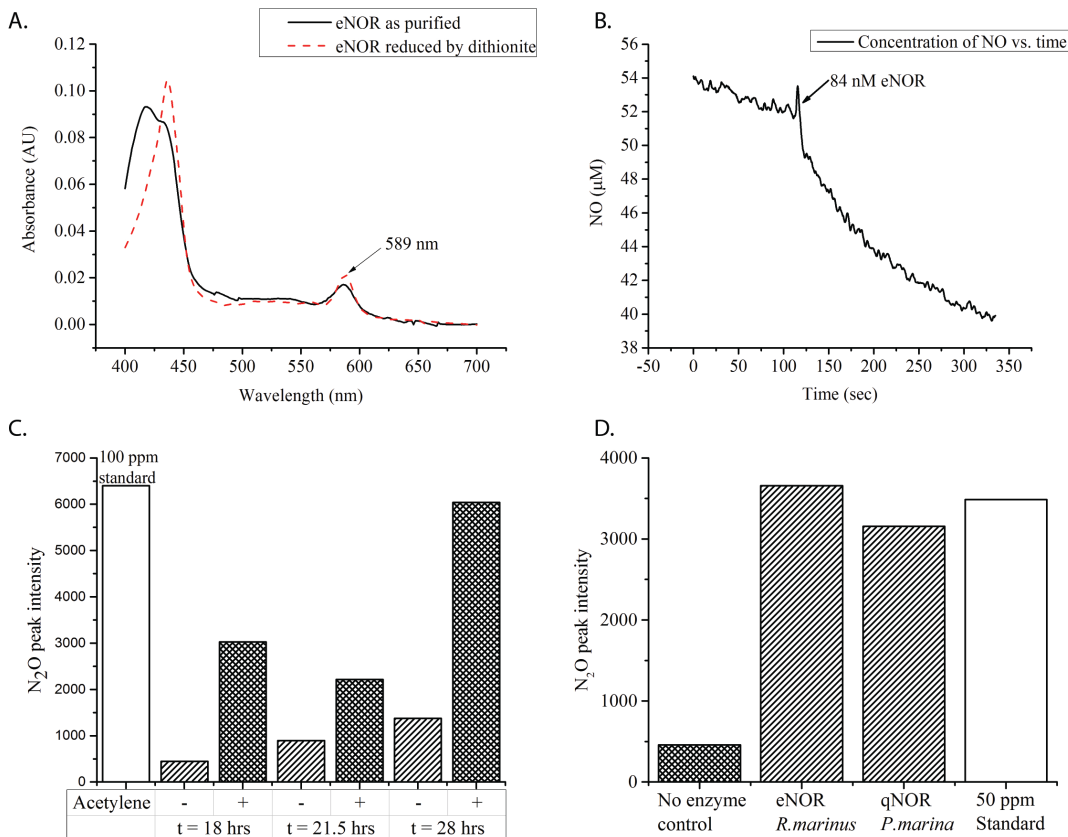
Extended Data Figure 1: Genome of *R. marinus* encodes for the complete denitrification pathway. a. The genes for NapAB, the periplasmic nitrate reductase (Rmar_0413), nitrite reductases nirK (Rmar_1208) and nirS (Rmar_0652), nitric oxide reductase eNOR(Rmar_0161) and nitrous oxide reductase, nosZ (Rmar_2012) are encoded in the *R. marinus* genome. b. The gene neighborhood of eNOR in *R. marinus*. c. The gene neighborhood of eNOR in *Magnetospirillum magneticum AMB-1* includes ctaA and ctaB, enzymes involved in the biosynthesis of heme *a*.

a. Denitrification pathway in *Rhodothermus marinus*

NapA Rmar_0413   **NirK** Rmar_1208
NirS Rmar_0652   **NosZ** Rmar_2012

b. Gene neighborhood of eNOR in *Rhodothermus marinus*

c. Gene neighborhood of eNOR in other organisms include genes for heme *a* synthesis

Extended Data Figure 2: *Rhodothermus marinus* **does perform complete denitrification.** a. *R. marinus* converts $^{15}NO_3^-$ to $^{30}N_2$. Ratio of $^{30}N_2$ to $^{28}N_2$ for each sample. Air is ambient atmosphere as a standard. C0 is a nitrate-free control. 14 0-17 are cultures grown with unlabeled nitrate, transferred to sealed vials after 0-17 hours, respectively. 15 0-17 are the equivalent samples grown with ^{15}N-labeled nitrate. Error bars represent two standard deviations from three replicate GC/MS measurements. $^{30}N_2$ enrichments from the ^{15}N-labeled samples are over 30-60x higher than background atmospheric ratios, while unlabeled samples have no significant enrichment over background. b. Growth of *R. marinus*, measured using OD_{600nm} over 39 hours. NO_3^- utilization was established by measuring the concentrations of nitrate in the media using a calorimetric assay. c. *R. marinus* growth in rich media was compared under denitrifying and non-denitrifying conditions using OD_{600nm}. d. Phenotypic differences of *R. marinus* cultures, under denitrifying and non-denitrifying conditions.

a. Conversion of $^{15}NO_3^-$ to $^{15}N_2$ in pure cultures of *Rhodothermus marinus*

b. Nitrate consumption evaluated by a colorimetric assay

c. Optical density (OD_{600nm}) of Rhodothermus marinus grown with and without nitrate

d. Phenotypic differences between cultures of *Rhodothermus marinus* grown with and without nitrate

Extended Data Figure 3: Characteristics of eNOR from *Rhodothermus marinus* a. SDS-PAGE gel electrophoresis of eNOR shows two bright bands which are estimated to subunits of I and II of the complex. Both subunits appear to run faster than their estimated molecular weight. This is typical for membrane proteins. For comparison, an SDS-PAGE gel of cytochrome *bo₃* oxidase from *E. coli* is included. **b.** Mass spectrometric identification of eNOR is confirmed by LC/MS/MS analysis. **c,d.** Absence of O₂ reduction by *R. marinus* eNOR, in comparison to robust O₂ reduction by *T. thermophilus ba₃*-type oxygen reductase. **e.** UV-visible spectrum of eNOR **f.** Pyridine hemochrome-spectra of extracted hemes from eNOR showing a peak which is atypical of hemes *a, b* or *c*.

a. Electrophoresis of eNOR on a SDS-PAGE gel

Extended Data Figure 4: Identification of hemes extracted from eNOR. Comparing the elution profile of extracted hemes from partially purified *R. marinus* eNOR to bovine cytochrome *c* oxidase (A-type, t=16 min) , *T. thermophilus ba₃*-type oxygen reductase (*b*- and A_s-type hemes, t=12 min and t=19 min) reveals that the heme is most likely an A_s-type heme. Mass spectra of the peak at ~19 min from the eNOR hemes elution profile confirms that the heme is an A_s-type heme with a molecular weight of 920 Da.

a. Chemical structures of hemes A and heme As.

Chemical Formula: $C_{49}H_{56}FeN_4O_6$
Molecular Weight: 852.85

Chemical Formula: $C_{54}H_{64}FeN_4O_6$
Molecular Weight: 920.97
m/z: 920.42

b. Elution profile of hemes extracted from eNOR (LC/MS)

c. Mass spectra corresponding to prominent peaks in the eNOR elution profile

Extended Data Figure 5: Proton channel in eNOR of *Rhodothermus marinus* and in the NOR families bNOR, sNOR and nNOR. a. eNOR contains conserved residues in Helix VII, similar to the location of K-proton channel residues in *T. thermophilus ba₃*-type oxygen reductase. b. A multiple sequence alignment of the NOR families eNOR, bNOR, sNOR and nNOR show conserved amino acids in analogous location to the K-channel in the B-type oxygen reductase. Some conserved residues are also identified in gNOR and may indicate the presence of a conserved proton channel but they do not map to corresponding residues in the B-type oxygen reductase.

a. Comparison of known proton channel from ***ba₃*** oxygen reductase and putative proton channel in eNOR
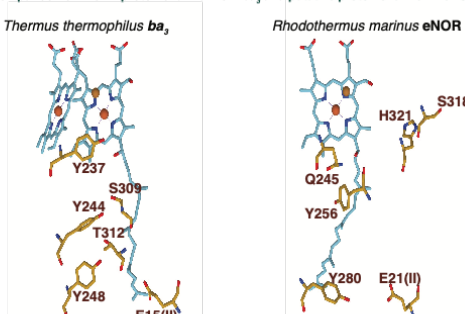
b. Conserved residues forming putative proton channels in eNOR, sNOR, bNOR and nNOR. Conserved proton channel residues are marked in black, while the active site residues are marked in red. Each group is numbered according to the protein whose accession number is in blue.

Extended Data Figure 6: Conserved amino acids in the eNOR family of enzymes. Multiple sequence alignment of 23 eNOR sequences from various taxonomically divergent organisms reveals conserved residues that correspond to the active site ligands, proton channel residues and other sequence features that are unique to eNOR. The active site residues are highlighted in maroon while the proton channel residues are highlighted in blue.

a. Sequence alignment of eNOR - Subunit I showing conserved amino acid residues according to *R.marinus* eNOR numbering

Materials and Methods

Growth and Expression Conditions

Rhodothermus marinus DSM 4252 was inoculated from frozen stock and grown in 5 ml of DSM Medium 630 with 10 g/L NaCl at 60 °C for 36 hrs. It was then inoculated into a larger secondary culture and grown overnight. 25 ml of the culture was inoculated into 1 L of medium with 30 mM nitrate added. The cells were shaken at 75 rpm and grown at 60 °C. The cells were pelleted by centrifugation at 8000 rpm. The cell pellet was either directly used for protein purification or frozen at -80 °C until the time of use.

Labeled ^{15}NO experiments

We used labeled nitrate ($^{15}NO_3^{2-}$) to verify that *Rhodothermus marinus* DSM 4252 was capable of complete denitrification (NO_3^- to N_2) using eNOR as the sole nitric oxide reductase. Cultures were inoculated into flasks containing media with either $^{14}NO_3^{2-}$, $^{15}NO_3^{2-}$, or no nitrate. The cultures were then allowed to grow microaerobically for a period of time before being subsampled for transfer to sealed vials in order to allow accumulation of gaseous end products. Samples were taken from each media composition after 0, 3, 6, 10, and 17 hours. The headspace was sampled after 20 hours of growth in sealed vials via gastight GC syringe and immediately injected into a Hewlett Packard 5972 gas chromatograph/mass spectrometer. Chromatogram peaks corresponding to isotopologues of NO, N_2O, and N_2 were identified by their mass spectra and peak areas were quantified relative to ambient air. As ^{15}N cultures were grown in isotopically pure $^{15}NO_3^{2-}$, complete denitrification should result in accumulation of $^{30}N_2$ at a 1:2 ratio relative to nitrate consumption. $^{30}N_2$ should only accumulate if eNOR is functioning as part of a complete denitrification pathway. If eNOR does not function effectively as a nitric oxide reductase, then ^{15}NO should be seen to accumulate. Instead, only the $^{30}N_2$ peak was observed, indicating the eNOR

functioned effectively as a nitric oxide reductase for denitrification. Over the course of incubations, $^{30}N_2$ was seen to accumulate to more than 50x background. ^{14}N samples showed no significant accumulation of $^{30}N_2$ above background, confirming that the $^{30}N_2$ in ^{15}N samples was due to denitrification of labeled nitrate. NO was not seen to accumulate in any of the cultures. These results demonstrate that eNOR is a functional nitric oxide reductase and can be used as part of a complete denitrification pathway.

Purification of eNOR

The culture of *Rhodothermus marinus*, once harvested, was re-suspended in 100 mM Tris-HCl, pH 8 with 10 mM $MgCl_2$ and 50 µg/ml DNase, using a Bamix homogenizer. The resulting solution was spun down at 42000 rpm in a Beckman Ultracentrifuge. The membrane pellet was collected and re-suspended in 20 mM Tris-HCl, pH 7.5, 1 % CHAPS (Affymetrix) to a final concentration of 40-50 mg/ml. The solution was stirred at 4 °C for 1 hr. In this step a lot of peripheral membrane proteins appear to be solubilized and the remaining protein is pelleted by spinning down at 42000 rpm for 1 hr. The remaining pellet is then solubilized in 20 mM Tris-HCl, pH 7.5, 1 % DDM (Affymetrix) at a final protein concentration of around 5-10 mg/ml. The DDM solubilized fraction was once again centrifuged at 42000 rpm to pellet down protein that was not solubilized.

The solubilized protein was then loaded on a DEAE CL-6B (Sigma) column, pre-equilibrated in 20 mM Tris-HCl, pH 7.5, 0.05 % DDM, and subjected to a linear gradient spanning from 0 to 500 mM NaCl. The fraction containing the eNOR, identified using a peak at 591nm, corresponding to the peak of cytochrome 'a1' in *Magnetospirillum magnetotacticum*[1], eluted at around 200 mM salt. This fraction was then loaded on a Q Sepharose High Performance (GE Healthcare) column, pre-equilibrated with 20 mM Tris-HCl, pH 7,5, 0.05 % DDM and then eluted in a gradient from 0 to 1 M NaCl. The eNOR containing fraction was eluted at around 250 mM salt and the eluted

fraction was then loaded on a Chelating Sepharose (GE Healthcare) column, loaded with Cu^{2+} and equilibriated with 20 mM Tris-HCl, 500 mM NaCl, as previously described for cytochrome caa_3 from *Rhodothermus marinus*[2]. The eNOR fraction was once again loaded on a Q Sepharose High performance column, and a gradient was run between 0 and 300 mM NaCl at low flow rates (0.5 ml/min) and the first peak was found to be the eNOR.

Gel Electrophoresis

The purified eNOR was run on a Tris-Hepes 4-20 % acrylamide gel (NuSep) in the recommended Tris-Hepes-SDS running buffer at 120 V for ~1 hr. The protein was visualized and compared to the Precision Plus Protein™ Dual Color Standards (BIO-RAD).

UV-Visible Spectroscopy

All spectra were recorded on a HP Agilent 8453 UV-Vis spectrophotometer using a quartz cuvette from Starna Cells (No. 16.4-Q-10/Z15). Potassium Ferricyanide was used to obtain the oxidized spectrum, and dithionite was used to obtain the reduced spectrum.

Pyridine Hemochrome Assay

The hemes in eNOR were analyzed using a pyridine hemochrome assay[3]. A stock solution of 200 mM NaOH with 40 % pyridine was prepared. The stock solution was mixed 1:1 with the protein and an oxidized spectrum was obtained by adding 3 µl of 100 mM $K_3Fe(CN)_6$. A reduced spectrum was similarly prepared by adding a few crystals of sodium dithionite. The reduced minus oxidized spectrum was used to identify the heme co-factors

Heme extraction and HPLC Analysis

The hemes from eNOR were extracted and analyzed using an HPLC elution profile according to established protocols[4,5]. 50 µl of eNOR was mixed with 0.45 ml of acetone / HCl (19:1) and incubated for 20 minutes at room temperature after shaking. The mixture was centrifuged at 14,000 rpm for 2 minutes, followed by addition of 1 ml of ice cold water, and 0.3 ml of 100% ethyl acetate to the supernatant. The water/ethyl acetate mixture was vortexed and centrifuged again for 2 minutes. The ethyl acetate phase was recovered and concentrated using a speed vac.

The extracted hemes were analyzed using an Agilent 1290 Infinity LC attached to an Agilent 6230 TOF LC/MS equipment by separation using an Agilent Eclipse Plus C18 column (2.1x300 mm, 1.8 µm, 600 bar) and an acetonitrile (0.05 %TFA) / water (0.05 % TFA) gradient from 20 to 95 %.

NO reductase activity verification using GC

Anaerobic reaction conditions were set up in a 5 mL clear serum vial (Voigt Global Distribution, Inc) sealed with a 20 mm rubber stopper, by passing N_2 through 2 ml of 20 mM KPi, 0.05 % DDM, pH 7.5 with 1 mM TMPD, 5 mM Ascorbate. A control was performed by adding only 50 µM NO. Sample reactions were begun by adding eNOR to a final concentration of 100 nM. The reaction was incubated at 42 °C for half an hour before the headspace was injected into an HP Agilent 5890 Series GC, fitted with a TCD and ECD (SRI Instruments) for verification of N_2O production.

Turnover measurement using a Clark electrode

A sealed chamber fitted with an ISO-NO (World Precision Instruments) electrode was used for NO reductase activity measurements. 1 mM TMPD or 100 µM PMS and 4 mM Ascorbate were was added to 2 ml 50 mM Citrate, pH 6, 0.05 % DDM in the reaction chamber and all traces of oxygen were removed by passing water-saturated Argon for 20 minutes through the solution. This

is similar to the protocol described for cNOR from *Thermus thermophilus*[6]. The buffer system also contained an oxygen scavenging system constituting 100 nM catalase, 35 nM Glucose oxidase and 90 nM Glucose. The NO reduction traces were recorded using a Duo-18 (World Precision Instruments), and activities calculated from the slope of the traces.

LC/MS/MS analysis

Mass spectrometric analysis was conducted at the Protein Sciences Facility, Roy J Carver Biotechnology Center, University of Illinois, Urbana, IL 61801 using a Thermo LTQ Velos ETD pro mass spectrometer. For liquid samples, the samples were cleaned up using G-Biosciences Perfect Focus (St. Louis MO) prior to digestion with trypsin. Digestion was done using proteomics grade trypsin 1:20 (G-Biosciences, St. Louis, MO) and a CEM Discover Microwave Reactor (Mathews, NC) for 15 minutes at 55° C at 50 Watts. Digested peptides were extracted 3X using 50% acetonitrile containing 5% formic acid, pooled and dried using a Speedvac (Thermo Scientific). The dried peptides were suspended in 5% acetonitrile containing 0.1% formic acid and applied to LC/MS.

HPLC for the trypsin digested peptides was performed with a Thermo Fisher Dionex 3000 RSLCnano using Thermo Acclaim PepMap RSLC column (75 μm x 15 cm C-18, 2 μm, 100Å) and a Thermo Acclaim PepMap 100 Guard column (100 μm x 2 cm, C-18, 5 μm, 100Å), solvents were water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B) at a flow rate of 300 nanoliters per minute at 40° C.   Gradient was from 100% A to 60% B in 60 minutes. The effluent from the UHPLC was infused directly into a Thermo LTQ Velos ETD Pro mass spectrometer.

Control and data acquisition of the mass spectrometer was done using Xcalibur 2.2 under data dependent acquisition mode, after an initial full scan, the top five most intense ions were subjected to MS/MS fragmentation by collision induced dissociation. The raw data were processed by Mascot Distiller (Matrix Sciences, London, UK) and then by Mascot version 2.4. The result was searched against NCBI NR Protein database.

Analysis of heme-copper oxygen reductase phylogeny and distribution in environmental datasets

We performed a large-scale analysis of heme-copper oxygen reductase (HCO) protein sequences in the NCBI and IMG databases with BLASTP using an e-value of 1e^{-3} to generate a database of HCO sequences that had at least some of the conserved amino acids previously identified in subunit I[7]. We then used the database of HCOs, filtered it with a sequence cut-off of 50% to generate the multiple sequence alignment, **MSA1**. A phylogenetic tree (**Figure 2**) was inferred using IQ-TREE 2[8] with the Dayhoff substitution matrix, Gamma model of rate heterogeneity and 1000 ultrafast bootstraps. Using the curated HMMs for each of the HCO family oxygen reductases[9], we probed release 202 of the Genome Taxonomy Database[10] for distribution of the NOR families – eNOR, bNOR, sNOR, nNOR, gNOR, cNOR and qNOR – in bacteria and archaea. Curated HMMs for the nitrate reductases (NapAB, NarGH), nitrite reductases (NirK, NirS) and nitrous oxide reductases (NosD and NosZ) were sourced from the HMMs database of MagicLamp[11].

Analysis of HCO distribution in various ecosystems were performed using the metagenomes in the IMG database. Approximately 2300 metagenomes were identified which were sourced from 44 environments identified by IMG. The number of different HCOs in each of these environments were extracted using BLASTP and query sequences that belong to each of the different HCO families.

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