1 A novel oxidase from *Alcaligenes* sp. HO-1 oxidizes hydroxylamine 2 to N₂

3 Running Title: A novel oxidase catalyzes hydroxylamine oxidation to N₂

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22

23 Abstract

24 Hydroxylamine is a key intermediate of microbial ammonia oxidation and plays an important
 25 role in the biogeochemical cycling of N-compounds. Hydroxylamine is oxidized to NO or N₂O
 26 by hydroxylamine oxidases or cytochrome P460 from heterotrophic or autotrophic bacteria,
 27 but its enzymatic oxidation to N₂ has not yet been observed. Here, we report on the discovery
 28 of a novel oxidase that converts hydroxylamine to N₂ from the newly isolated heterotrophic
 29 nitrifier *Alcaligenes* strain HO-1. Strain HO-1 accumulated hydroxylamine and produced N₂
 30 from ammonia oxidation. Using transcriptome analysis and heterologous expression via
 31 fosmid library screening, we identified three genes (*dnfABC*) of strain HO-1 that enabled *E.*
 32 *coli* cells not only to produce hydroxylamine from ¹⁵N-labelled ammonium but also to further
 33 convert it to ¹⁵N₂. The three genes were individually cloned and expressed, and their
 34 translational products DnfA, DnfB, and DnfC were purified. *In vitro* DnfA bound to
 35 hydroxylamine and catalyzed the conversion of hydroxylamine to N₂ in the presence of FAD,
 36 NADH and O₂. Thus, DnfA was identified as a novel hydroxylamine oxidase and catalyzed a
 37 previously unknown N-N bond forming reaction with a yet-to-be discovered mechanism. DnfA
 38 homologs were detected in different bacterial groups, suggesting that hydroxylamine
 39 oxidation to nitrogen might occur in additional microbial taxa.

40

41 Introduction

42 Hydroxylamine is formed by diverse microorganisms, including ammonia-oxidizing bacteria
 43 (AOB) and archaea (AOA) (1, 2), complete ammonia oxidizers (3, 4), anaerobic ammonia
 44 oxidizers (5), heterotrophic nitrifiers (6), fungi (7), and methane-oxidizing bacteria (8).
 45 Hydroxylamine is not only an important intermediate/side product in various microbial
 46 physiological pathways, but its production also has significant implications for the health of the
 47 environment. Both biological and non-biological processes are involved in the conversion of
 48 hydroxylamine to N₂O (4, 9-11), which is an important greenhouse gas. Hydroxylamine
 49 production by soil microbes contributes to the formation of HONO, a precursor of the hydroxyl
 50 radical (OH), which represents the primary oxidizing agent in the atmosphere (12).

51 AOBs oxidize hydroxylamine enzymatically to nitric oxide (NO) by the enzyme hydroxylamine
52 oxidoreductase to extract electrons for ammonia oxidation by the ammonia monooxygenase
53 and respiration (13). Similarly, the hydroxylamine oxidase of the anaerobic ammonium
54 oxidizer *Kuenenia stuttgartiensis* detoxifies hydroxylamine to NO and thus generates
55 substrate and electrons for respiration (14). Furthermore, AOB encode a cytochrome P460
56 (also known as CytL) that detoxifies hydroxylamine by oxidizing it to N₂O with an unknown
57 biological oxidant (11). So far to the best of our knowledge, the enzymatic conversion of
58 hydroxylamine to N₂ at physiological conditions has not been reported.

59 Heterotrophic nitrifiers are able to oxidize ammonia (but cannot grow on it as the only source
60 of energy), and hydroxylamine was identified as an immediate metabolite (15-18). Some of
61 those heterotrophic nitrifiers are also able to denitrify at oxic conditions (19-21), a process
62 called heterotrophic nitrification-aerobic denitrification (HNAD). HNAD has been known for
63 many years, and efforts were made to disclose its metabolic pathway and biochemical
64 mechanism. The general understanding of HNAD is that ammonia is sequentially oxidized to
65 hydroxylamine, NO, nitrite and nitrate by not well understood ammonia-oxidizing enzymes,
66 HAOs and other unknown enzymes. Then, nitrite and nitrate are reduced to N₂ by the well-
67 known NarGHI/NapAB, NirK/NirS, NorBC and NosZ enzymes (22) that are frequently
68 encoded in the genomes of HNAD bacteria (23, 24). But the understanding of HNAD is still
69 very fragmentary, and both hydroxylamine and nitrite were postulated as links between
70 nitrification and denitrification (6, 21). Furthermore, very little is known about the enzymatic
71 machinery used by heterotrophic nitrifiers for ammonia oxidation (18, 25). In addition,
72 purification and partial characterization of the HAOs from several heterotrophic nitrifiers
73 including *Thiosphera pantotropha* (26), *Pseudomonas* sp. PB16 (27), *Arthrobacter globiformis*
74 (28), and *Acinetobacter* sp. Y16 (29) showed that HAOs from heterotrophic nitrifiers differ
75 significantly from those found in autotrophic nitrifiers, but the functional consequences of
76 these differences have not yet been fully explored.

77

In this study, we isolated an *Alcaligenes* species strain HO-1, from a wastewater treatment bioreactor. Strain HO-1 was efficiently removing ammonia from wastewater, transiently accumulated hydroxylamine in culture broth, and produced N₂. Here, we report the genetic cloning and biochemical characterization of a novel oxidase that catalyzed a previously unknown N-N bond-forming reaction from hydroxylamine to N₂ at physiological conditions.

Materials and Methods

Bacterial strains, plasmid, media, cultivation, protein purification and sequence alignment

All bacterial strains, plasmids and primers used in this study are listed in Supplementary Tables S1 and S2. Information about media, cultivation, plasmid construction, protein purification and sequence alignment is provided in Supplemental Information (SI).

Isolation, characterization, genome sequencing and phylogenetic analysis of strain HO-1

Strain HO-1 was isolated from a SHARON bioreactor (30) using heterotrophic nitrification medium (HNM) supplemented with 2.0 mM hydroxylamine, which served as a selective pressure. HNM contained 5 mM (NH₄)₂SO₄ and 17.5 mM succinate, and the concentrations of (NH₄)₂SO₄ and succinate were optimized for fosmid library screening with *E. coli* and transcriptome sequencing with HO-1. The 16S rRNA gene of strain HO-1 was PCR-amplified and extracted also from its sequenced genome. Genome sequencing was performed by Single Molecule, Real-Time (SMRT) technology at the Beijing Novogene Bioinformatics Technology Co., Ltd. Additional information is available as SI.

Transcriptome sequencing

For transcriptome sequencing (RNA-seq), strain HO-1 was initially cultured in HNM medium containing 14 mM (NH₄)₂SO₄ and 83 mM succinate at 30 °C with shaking as described in SI. After depletion of ammonium at 58 h, 14 mM (NH₄)₂SO₄ were added and cultivation was

continued. Samples for RNA extraction were taken at the following time intervals after this
(NH₄)₂SO₄ addition, 0 h (C), 3.5 h (T1), 10 h (T2), and 22 h (T3) and then assayed. More
detailed information is provided in SI.

Construction and screening of genomic fosmid library

A genomic fosmid library of strain HO-1 was constructed using the CopyControl™ HTP
Fosmid Library Production Kit (Epicentre) according to the instructions of the manufacturer.
Fosmid clones were picked in duplicate into two 384-well plates and preserved with 20%
glycerol at -80 °C. Clones from one of the duplicate 384-well plates were used as inoculum
for four 96-well plates, which contained in each well 160 µl LB with chloramphenicol (12.5
µg/mL). The inoculated plates were cultivated overnight on a high-speed shaker (INFORS HT
Microtron, Infors) at 37 °C. 18 µl of the cultured clones were transferred to new 96-well plates,
which contained 1.2 mL of HNM with 5 mM (NH₄)₂SO₄, 29.2 mM succinate and 12.5 µg/mL
chloramphenicol. Positive clones that were capable of producing hydroxylamine and nitrite
from ammonium were screened and sequenced using specific pCC2FOS sequencing primers
(Epicentre) at SinoGenoMax (Beijing, China). The genomic locations of inserted DNA
fragments were determined by blasting against the genome of strain HO-1.

Nitrogen-balance experiments

For nitrogen-balance experiments, strain HO-1 or *E. coli* BW25113/pBAD-DnfABC was pre-
cultured in HNM with 5 mM (¹⁵NH₄)₂SO₄ (Spectra Corp., USA) as the sole nitrogen source
and transferred three times in this medium for complete depletion of any ¹⁴N in the cells. Then,
200 µL culture was inoculated into 250 mL sealed bottles containing 20 mL HNM. The
headspace of bottles was filled with 1:1 He/O₂ using a gas displacement system. The bottles
were incubated at 30 °C and 160 rpm on a rotary shaker for 80 h (HO-1) or 120 h (*E. coli*
BW25113/pBAD-DnfABC). Then, bacterial growth (OD₆₀₀), total nitrogen content (TN), NH₄⁺-N
(initial and final amount), gaseous nitrogen products (¹⁵N₂, ¹⁵NO and ¹⁵N₂O), soluble nitrogen
products (NO₂⁻-N, NO₃⁻-N, NH₂OH-N, organic-N) and biomass-N were measured. Results are
presented as mean ± SD from five biological replicates.

131   **Physiological characterization of recombinant *E. coli* clones**

132   *E. coli* BW25113 carrying pBAD-DnfT1RT2ABCD, pBAD-DnfABC or pBAD-DnfAB was
 133   characterized for production of N₂ and N₂O, as well as for hydroxylamine accumulation. *E. coli*
 134   EPI300™-T1^R or *E. coli* BW25113 containing vector pBAD/HisA were used as negative
 135   controls under the same conditions. 50 µL of the respective cell suspension with OD₆₀₀ of 0.5
 136   was inoculated into 20 mL sealed tubes containing 5 mL ¹⁵N-labelled HNM with 0.1% L-
 137   arabinose as inducer. Before inoculation, the top air of the sealed tubes was completely
 138   replaced by 1 : 1 He/O₂ as mentioned above. After incubation at 30 °C and 160 rpm for 5
 139   days, gaseous nitrogen compounds (¹⁵N₂, ¹⁵NO, and ¹⁵N₂O) and NH₂OH were detected.
 140   Results are presented as mean ± SD from three independent experiments.

141   **Analytical methods**

142   Bacterial growth was monitored by measuring OD₆₀₀ (spectrophotometer model UV-7200,
 143   UNICO, Shanghai, China). Concentrations of ammonium (NH₄⁺), nitrite (NO₂⁻) and nitrate
 144   (NO₃⁻) were determined on an Aquakem 600 Analyzer (Thermo Scientific) using the standard
 145   methods recommended by the manufacturer. Hydroxylamine was determined by using 8-
 146   quinolinol to form the stable 5,8-quinolinequinone-5-(8-hydroxy-5-quinolylimide) (31). Total
 147   nitrogen (TN) was determined by using alkaline potassium persulfate digestion with ultraviolet
 148   spectrophotometry (32). To determine the biomass nitrogen, bacterial cells for each of the
 149   samples were harvested by centrifugation (8 000 g, 10 min, 4 °C) and resuspended in
 150   ultrapure water with the same volume of the original sample. The nitrogen content of this
 151   suspension was determined with the same method as for TN determination, and was
 152   calculated as the biomass nitrogen. Quantitative detections of ¹⁵N₂ and ¹⁵NO were performed
 153   by GC/MS (model 7890A/5975C, Agilent) equipped with a CP-Molsieve 5A Plot (25 m×0.32
 154   mm×30 µm, Agilent, USA). ¹⁵N₂ and NO (which might be formed in the source of mass
 155   spectrometers from unlabeled N₂ and O₂ and has a similar mass as ¹⁵N₂) were eluted at
 156   retention times of t=6.897 and t=15.357 min, respectively, and could thus be distinguished
 157   without problem (Figure S2). Quantitative detection of ¹⁵N₂O was performed as previously
 158   described by Liu *et al.* (33).

159 ITC measurements

160 Substrate-enzyme binding experiments were carried out on an ITC200 Isothermal Titration
161 Calorimeter (ITC). All experiments were carried out at 25 °C. The buffer used in this
162 measurement was the same as in the enzymatic assays. 1×0.4 µL followed by 19×2 µL of
163 hydroxylamine were injected into a 200 µL enzyme solution. The data were analyzed using
164 the ORIGIN software (MicroCal Inc.).

165 Hydroxylamine oxidase activity assays

166 *In vitro* enzymatic assays of hydroxylamine oxidase activities were carried out in 300 µL
167 reaction mixtures in buffer (20 mM Tris-HCl, pH 8.5) supplemented with 10 mM ¹⁵NH₂OH, 2
168 mM NADH, 2 mM NADPH and 20 µM FAD. In this experiment, 331 µM DnfA, 3.2 µM DnfB
169 and 311 µM DnfC were added separately or in combination, with mixtures lacking any of
170 these proteins as controls. The reaction was started with the addition of ¹⁵NH₂OH (Cambridge
171 Isotope Laboratories Inc.), and the mixture was directly injected into 10 mL gastight tubes, the
172 air of which had been completely replaced by 1:1 He/O₂. The reactions were incubated at
173 30 °C without agitation in the dark for 100 min. The hydroxylamine consumption in the
174 mixtures and ¹⁵N₂ released in the headspace were assayed. To determinate alternative
175 electron mediators for *in vitro* reconstitution of the hydroxylamine oxidase activity of DnfA, the
176 assay mixture contained 10 µM DnfA, 1 mM ¹⁵NH₂OH, 1 mM NADH and various electron
177 mediators including 100 µM flavin adenine dinucleotide (FAD), 100 µM flavin mononucleotide
178 (FMN), 100 µM phenazine methosulfate (PMS), 100 µM phenazine ethosulfate (PES) and 20
179 µM/10 µM ferredoxin/ferredoxin reductase (Fd/FdR). The reaction mixtures of 300 µL in 10
180 mL gastight tubes were incubated at 30 °C without agitation in the dark for 60 min. Kinetic
181 assays were conducted in 0.6 µM DnfA, 480 µM FAD, 10 mM NADH and 25-400 µM NH₂OH
182 of 100 µL reaction mixture in 1.5 mL vials at 30 °C. Enzymatic assays were performed in
183 triplicate.

184 Involvement of O₂ and H₂¹⁸O measurements

Oxygen (O₂) requirement experiments were carried out using ¹⁸O₂ and detection of the formation of H₂¹⁸O. ¹⁸O₂/He (with different ratios) was purchased from Newradar Special Gas Co., LTD. (Wuhan, China). The components of 300 µL reaction mixture and reaction conditions were: 331 µM DnfA, 120 µM FAD, 10 mM NADH, 10 mM ¹⁵NH₂OH in 20 mM Tris-HCl (pH 8.5), in 10 mL anaerobic tube at different time. The reaction mixtures were diluted 2 times and δ¹⁸O was detected by isotopic water analyzer (L2130-I, PICARRO Inc, USA.). The concentration of H₂¹⁸O was calculated according to the equation: δ (‰) = (R_{sample}/R_{standard}-1) ×1000. The standard was VSMOW2 (International Atomic Energy Agency; IAEA). Milli-Q water (Millipore, USA) was used as blank controls.

194

195 Results

196 *Alcaligenes* sp. strain HO-1 oxidizes ammonia to N₂ and accumulates hydroxylamine

The bacterial strain HO-1 was isolated from a SHARON bioreactor of a combined system termed “UASB + SHARON + ANAMMOX”, which was used for the treatment of ammonia-rich wastewater (30). Strain HO-1 (electron microscopic pictures of its cell morphology are shown in Figure 1A and 1B) was isolated by using HNM supplemented with 2 mM hydroxylamine as a selective pressure. Purity of strain HO-1 was confirmed by microscopic observation, 16S rRNA gene and genome sequencing. Phylogenomic (Figure 1C) and phylogenetic (supplemental Figure S1) analyses indicated that strain HO-1 and several other *Alcaligenes* isolates formed a coherent cluster, and that this cluster is phylogenetically related but clearly separated from the type strain of *Alcaligenes faecalis*. Average nucleic acid identities (ANI, supplemental Table S3) with all published available genomes of *Alcaligenes* suggest that strain HO-1 belongs to a new, not yet formally described species together with a few *Alcaligenes* strains currently classified as *Alcaligenes faecalis*.

Strain HO-1 removed ammonia efficiently under oxic conditions (Table S4) with a tentative accumulation of hydroxylamine in the culture broth (Figure 1D). N-balance experiments of strain HO-1 cultured with 5 mM (¹⁵NH₄)₂SO₄ showed that all nitrogen atoms

were traceable. 48.5% and 2.2% of the consumed ^{15}N -labelled ammonium was recovered as $^{15}\text{N}_2$ and $^{15}\text{N}_2\text{O}$, respectively (Figure 1E), and there was no measurable ^{15}NO production (see Figure S2). Small amounts of nitrite (2.0%) and nitrate (0.7%) were detected. Interestingly, $^{15}\text{N}_2$ production from ammonia by strain HO-1 was not inhibited by 0.1 mM of the copper chelator sodium diethyldithiocarbamate (supplementary Figure S3), which was reported to strongly inhibit at this concentration nitrite reduction to N_2 in other heterotrophic nitrifiers (24). We also observed that neither nitrate nor nitrite could be used as the sole nitrogen source to support growth of strain HO-1 under oxic conditions (supplementary Figure S4), although nitrite but not nitrate supported anaerobic growth of strain HO-1 and was used as electron acceptor in the presence of succinate as electron donor (supplementary Figure S5). Based on those results, we hypothesized that strain HO-1 might possess an oxic pathway for N_2 production different from the previously reported aerobic denitrification pathway in HNAD organisms.

Figure 1. Strain HO-1 converts ammonia mainly into N₂. (A) and (B) Scanning and transmission electronic microscopic pictures of strain HO-1, respectively. (C) Phylogenomic reconstruction of cultivated *Alcaligenes* with published genomes. *Alcaligenes* sp. HO-1 is depicted in bold and type strains are indicated with a (T) and in blue. Genome accession numbers are given in parentheses. (D) Cell growth (OD₆₀₀), consumption of ¹⁵NH₄⁺, transient accumulation of hydroxylamine, and production of ¹⁵N₂, ¹⁵N₂O, and NO₂⁻. Production of ¹⁵NO was not detected. Succinate and (¹⁵NH₄)₂SO₄ were added at initial concentrations of 17.5 and 5 mM, respectively. (E) The N-balance experiment showed that the consumed ammonia is mainly converted into ¹⁵N₂. The data shown in panel (D) and (E) are averages of 3 and 5 replicates, respectively.

Genome sequence, annotation for N-metabolism genes, and transcriptome analysis of strain HO-1 upon ammonia stimulus

A closed genome of strain HO-1 was determined. Sequence quality control did not find contaminating DNA reads from other organisms, thus further confirming the purity of strain HO-1. The genome of strain HO-1 consists of a single chromosome of 3.77 Mb (Figure 2A). Annotation of N-metabolism related genes (Figure 2B) and their predicted involvements in aerobic ammonia oxidation and anaerobic nitrite reduction are depicted in Supplementary Figure S6. The transcription of these genes was significantly regulated upon ammonia stimulus (Figure 2C). The HO-1 genome encodes *nirK* (encoding a nitrite reductase), *norBC* (encoding nitric oxide reductase) and *nosZ* (encoding nitrous-oxide reductase), representing a complete denitrification pathway from nitrite. This nitrite denitrification pathway is likely responsible for the reduction of nitrite to N₂O in the presence of O₂ and to N₂ in the absence of oxygen with succinate as electron donor, as observed in Figure 2D. This observation is consistent with the perception that nitrous-oxide reductase NosZ is more sensitive to oxygen than other enzymes involved in denitrification and thus might not reduce N₂O to N₂ in strain HO-1 under oxic conditions (34). We did not detect any genes coding for an ammonia monooxygenase or hydroxylamine oxidoreductases in the genome of strain HO-1. A pyruvic oxime dioxygenase (POD) is annotated in the genome of strain HO-1, which was previously

254 detected in other *A. faecalis* strains (35). This enzyme catalyzes the oxygenation of pyruvic
 255 oxime [2-(hydroxyimino)propanoic acid] to yield pyruvate and nitrite (35-37). In this context it
 256 is important to note that hydroxylamine reacts non-enzymatically with pyruvate to pyruvic
 257 oxime (38). Consequently, once hydroxylamine is formed from ammonia in this heterotrophic
 258 nitrifier, nitrite can be formed in its cytoplasm by a combination of a non-enzymatic reaction
 259 and the POD activity (Figure 3C). Consistently, small amounts of nitrite were observed in our
 260 ¹⁵N-ammonium incubation experiments. Besides N₂O production from abiotic conversion of
 261 hydroxylamine (4), partial reduction of nitrite represent a possible origin of N₂O in the N-
 262 balance experiments. We did not find any *nrf* and *nap* genes in the genome of strain HO-1,
 263 which are indicative for the absence of dissimilatory or assimilatory nitrate reduction to
 264 ammonia. This is consistent with the observation that strain HO-1 did not use nitrate as
 265 nitrogen source for growth. The small amounts of nitrate detected in the ammonium addition
 266 experiments might be formed by nitrite-oxidizing side-activity of the strain HO-1 catalases
 267 KatA and KatE (39, 40) (Figure 3C). Alternatively, nitrate formation might have been
 268 catalyzed by the NO detoxifying enzyme, flavohemoglobin-NO-dioxidase (Hmp), which
 269 converts NO together with O₂ to NO₃⁻ (41) as strain HO-1 has two genes with significant
 270 homologies to *hmp*.

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274 **Figure 2.** Panel A summarizes general features of the genome of strain HO-1. Panel B
275 shows a circular genome map with predicted N-metabolism-related genes. Circles from the
276 outermost to the center: (1) scale marks of the genome, (2) protein-coding genes on the
277 forward strand, (3) protein-coding genes on the reverse strand, (4) tRNA (purple) and rRNA
278 (dark green) genes on the forward strand, (5) tRNA (purple) and rRNA (dark green) genes on
279 the reverse strand, (6) GC content, (7) GC skew. Panel C shows a schematic representation
280 of the proposed routes for N-metabolism in strain HO-1 based on gene annotation and
281 transcriptomic data (green arrows; for details see Table S5). The routes for aerobic ammonia
282 oxidation are showed in red, and anaerobic denitrification is showed in blue. Panel D shows

283 experimental date of nitrite conversion by strain HO-1 in HNM medium to N₂O at oxic or to N₂
284 at anoxic conditions. The data shown in panel (D) are averages of 3 replicates.

285 In order to identify genes that are involved in ammonia oxidation and N₂ formation and to
286 check whether the above discussed N-metabolism genes were regulated in strain HO-1, we
287 performed stimuli-response transcriptome experiments. Strain HO-1 was cultured with 83 mM
288 succinate and 14 mM of ammonium sulfate until ammonia was completely consumed and
289 then 14 mM of ammonium sulfate was added to the culture. Cells were harvested at 0 h, 3.5
290 h, 10 h and 22 h after the addition of ammonium sulfate, and were subjected to transcriptomic
291 analysis. Supplemental Table S5 show the transcriptional response of N-metabolism-related
292 genes to this ammonia stimulus. The transcription levels of genes involved in denitrification
293 (*nirK*, *norBC*, *nosZ*) and glutamate metabolism (*gltD*, *gltB*, *gltS*, *glnK*) were significantly (fold
294 changes >2) regulated. As discussed above, the POD and KatA activities might catalyze the
295 formation of nitrite and nitrate, respectively, and we found their transcripts upregulated at 22 h
296 upon ammonia stimulus. We also found that the *amtB* (encoding a putative ammonia
297 transporter), *gltD*, *gltB*, *gltS*, and *glnK* were significantly downregulated, which were
298 reasonable responses to the high concentration of ammonia. Interestingly, we observed that a
299 gene without a functional annotation (FE795_13025, named *dnfA*) and its neighboring genes
300 (FE795_13030, FE795_13035, FE795_13040; named *dnfB*, *dnfC*, and *dnfD*, respectively)
301 were all significantly upregulated at 3.5 h and 10 h in response to ammonium stimulation, and
302 their transcriptional levels decreased as ammonium was depleted at 22 h. As shown in Figure
303 2C (inside panel), the transcriptional levels of *dnfA* and *dnfB* were very high after 3.5 h.

304 **The gene cluster *dnfABC* from strain HO-1 enables *E. coli* to accumulate**
305 **hydroxylamine and to produce N₂ from ammonia.**

306 In order to identify the pathway and genes involved in the aerobic ammonia oxidation and
307 N₂ production in strain HO-1, we constructed a fosmid library of the strain HO-1 genome in *E.*
308 *coli* strain EPI300™-T1^R (Figure 3A). We screened 1920 clones of the fosmid library, and six
309 clones that were positive for hydroxylamine accumulation were obtained. Sequencing the 6
310 clones revealed that they shared a cluster of 7 genes (named *dnfT1RT2ABCD*, *dnf* stands for

311 ***d***initrogen formation) (Figure 3C), which were also detected on the chromosome of strain HO-
 312 1 (their position is shown in Figure 2B). As described above, two (*dnfA*, *dnfB*) of the 7 genes
 313 were significantly upregulated and strongly transcribed upon ammonia stimulation. Excitingly,
 314 these 7 genes conferred *E. coli* cells the ability to produce hydroxylamine and nitrite (Figure
 315 3B), N₂ and N₂O from ammonium. We subcloned the *dnfABC* or *dnfAB* genes into *E. coli*
 316 strain BW25113 cells. *E. coli* cells carrying *dnfABC* or *dnfAB* already generated N₂ and N₂O
 317 from added ammonium, although *E. coli*/pBAD-DnfAB showed much decreased N₂ production
 318 when compared to *E. coli*/pBAD-DnfABC (Figure 3D). In N-balance experiments of *E. coli*
 319 harboring *dnfABC* cultured with HNM media, 6.5% of the consumed ¹⁵N-labelling were
 320 recovered as ¹⁵N₂ (Figure 3E).

321 The interpretation of data from physiological experiments with a host carrying
 322 heterologously expressed genes obviously also has to take into account the relevant
 323 pathways naturally encoded by the host cells. Both the *E. coli* strain BW25113 (for
 324 subcloning) and strain BL21(DE3) (for heterologously expressing proteins, see below)
 325 encodes in their genomes genes for nitrate transport (Nrt), dissimilatory nitrate reduction
 326 (NarGHI, NapAB, NirBD, NrfAH), nitrate reduction/nitrite oxidation (NxrAB) and assimilation
 327 (supplementary Figure S6). This *E. coli* strain encodes no enzymes that would catalyze N₂
 328 and NH₂OH production via nitrification and/or denitrification pathways. Thus, the observed
 329 production of N₂ and NH₂OH from ¹⁵NH₄⁺ in the *E. coli* strain carrying *dnfABC* from strain HO-
 330 1 cannot be explained by the metabolism of *E. coli*. We therefore concluded that *dnfABC* is
 331 critical to catalyze the conversion of ammonia to N₂ in the *E. coli* cells.

Figure 3. *dnfABC* genes from strain HO-1 enable recombinant *E. coli* ammonia

conversion into N₂. (A) Flowchart summarizing the construction of the fosmid library and the screen for hydroxylamine accumulation. On the right side, one of the six positive clones (see text) that accumulated hydroxylamine is spotted (framed in red). Ck, a control assay performed using *E. coli* EPI300^{TM}-T1^R that did not harbor any exogenous gene. (B) Demonstration for hydroxylamine and nitrite production in *E. coli* harboring gene cluster *dnfT1RT2ABCD*. (C) Annotation of the *dnf* genes are shown on the right side, and their homolog proteins in UniProtKB/Swiss-Prot database are listed in Table S6. (D) Production of N₂ and N₂O by *E. coli* clones harboring *dnfABC* or *dnfAB* genes. (E) ¹⁵N-balance results from *E. coli* cells carrying *dnfABC*. The numbers indicate the ratio (%) of ¹⁵N₂ or ¹⁵N₂O production to the consumption of ammonia. All experiments were conducted under 50% O₂ + 50% He in HNM medium with 17.5 mM succinate and 5 mM (¹⁵NH₄)₂SO₄ in 10 mL gastight tubes and 250 mL sealed bottles. The data shown in panel (D) and (E) are averages of 3 and 5 replicates, respectively.

349 **DnfA binds and converts hydroxylamine into N₂**

350 Based on our bioinformatic analyses, DnfA, DnfB and DnfC were annotated as diiron
 351 oxygenase, NADH-dependent oxygenase reductase, and glutamine amidotransferase,
 352 respectively. *In silico* analyses of DnfA, DnfB and DnfC did not reveal transmembrane
 353 domains or secretion signals, and thus most likely these proteins have a cytoplasmic
 354 localization. DnfB has binding sites for NAD(P)H, FMN and 2Fe-2S. Since the genetic cluster
 355 *dnfABC* conferred *E. coli* cells the ability to produce hydroxylamine and N₂ from ammonia,
 356 firstly, we individually cloned and expressed *dnfA*, *dnfB*, and *dnfC* in *E. coli*. The DnfA, DnfB
 357 and DnfC were purified to homogeneity (Figure 4A) and their interactions with ammonia and
 358 hydroxylamine were assayed with an isothermal titration calorimeter (ITC). Results showed
 359 that none of them interacted with ammonia, but DnfA bound to hydroxylamine with a
 360 dissociation constant (K_d) of $0.16 \pm 0.02 \mu\text{M}$ (Figure 4B). Next, we tested whether DnfA,
 361 DnfB, or DnfC was able to catalyze the conversion of ammonia and hydroxylamine. An *in vitro*
 362 reconstitution enzymatic activity assay using the chemical electron mediator FAD was
 363 performed following the previously reported method for the DnfA homolog AurF (42). We
 364 found that neither DnfA, nor DnfB, nor DnfC catalyzed the oxidation of ammonia *in vitro*, but
 365 that DnfA, as well as the combinations of DnfA/DnfB and DnfA/DnfB/DnfC, catalyzed the
 366 conversion of hydroxylamine to N₂ in the presence of molecular O₂, FAD and NADH (Figure
 367 4C). We calculated that the consumption of approximate 2 moles of hydroxylamine produced
 368 one mole N₂, suggesting hydroxylamine was almost stoichiometrically converted into N₂.
 369 Considering that hydroxylamine was completely converted to ¹⁵N₂ when DnfA was present but
 370 not so when DnfA was absent, we concluded that DnfA was essential. It was reported that
 371 chemical decomposition of hydroxylamine in soils produced N₂ (43). We observed trace but
 372 detectable amount of N₂ production in the reaction system without DnfA. A possible
 373 explanation might be that O₂ was activated by FAD/NADH and consequently triggered
 374 spontaneous reactions with yet unknown chemistry for N₂ generation. But the amount of N₂
 375 production was much lower than the one observed from the DnfA-catalyzed conversion of
 376 hydroxylamine (Figure 4C), indicating a major role of DnfA catalysis.

377

378

379 **Figure 4. DnfA binds to and catalyzes the conversion of hydroxylamine to N₂.** (A)

380 SDS-PAGE of purified DnfA, DnfB and DnfC. (B) ITC assay of the DnfA-hydroxylamine
381 interaction. 0.2 mM hydroxylamine was titrated into 0.1 mM DnfA. The experiments were
382 repeated three times, and a representative result is shown. (C) Enzymatic assay of DnfA
383 activity (331 μM DnfA, 3.2 μM DnfB and 311 μM DnfC) in presence of 10 mM $^{15}\text{NH}_2\text{OH}$, 20
384 μM FAD, 2 mM NADH or 2 mM NADPH and 50% O₂. (D) Determination of suitable electron
385 mediators for reconstitution of hydroxylamine oxidase activity of DnfA *in vitro*. The assays
386 were performed with 10 μM DnfA, 1 mM hydroxylamine, 1 mM NADH and various electron
387 mediators, including FAD (100 μM), FMN (100 μM), phenazine methosulfate (PMS, 100 μM),
388 phenazine ethosulfate (PES, 100 μM) and ferredoxin/ferredoxin reductase (Fd/FdR, 20 μM
389 and 10 μM). Data shown in panels C and D are averages of 3 replicates.

DnfB contained both ferredoxin (Fd) and ferredoxin reductase (FdR) domains and its gene is located at the immediate vicinity of DnfA. Thus, it seems likely that DnfB is an Fd/Fd reductase and mediates electron transfer to DnfA. This is consistent with our observation that Fd/FdR/NADH supported DnfA activity during *in vitro* assays. In addition, we also found that additional electron donors such as phenazine methosulfate and phenazine ethosulfate also supported DnfA activity in the presence of NADH (Figure 4D).

DnfA is a hydroxylamine oxidase

DnfA contains a diiron-motif and was annotated to be a diiron oxygenase. We determined that iron atoms were associated with DnfA molecules at a ratio of $1.60 \pm 0.01 : 1$, suggesting that one molecule of DnfA bound two iron atoms. DnfA homologues were identified in the genomes of *Burkholderia*, *Delftia*, *Herbaspirillum*, *Microvirgula*, *Pantoea*, and *Pseudomonas* species, in addition to *Alcaligenes* species. Those DnfA homologues formed a coherent clade and they are related to the AurF N-oxygenase (42, 44) and CmlI homologues (Figure 5A), and is distantly related to previously known HAOs (34, 45, 46) (sequence identity < 20% over full length). We demonstrated that DnfA activity depends on molecular oxygen by performing experiments with $^{18}\text{O}_2$ (Figure 4C). By using 10 mM ^{15}N -labelled hydroxylamine and 10% of $^{18}\text{O}_2$ (90% of the gas phase is Helium), we determined the molar ratio (net reaction catalyzed by DnfA) of hydroxylamine consumption to N_2 production to H_2^{18}O production was 2.6 : 1 : 1.9 (Figure 5B). We also calculated this ratio with hydroxylamine consumption rate, $^{15}\text{N}_2$ and H_2^{18}O production rates under the same condition. The consumption rate of hydroxylamine was determined to be $8.71 \pm 1.29 \mu\text{M}/\text{min}$ per mg protein, and the production rates of $^{15}\text{N}_2$ and H_2^{18}O were $3.84 \pm 0.64 \mu\text{M}/\text{min}$ per mg protein and $7.53 \pm 0.64 \mu\text{M}/\text{min}$ per mg protein, respectively (Figure 5C). The molar ratio of hydroxylamine consumption rate to N_2 production rate to H_2^{18}O production rate was calculated to be 2.3:1:1.9, a ratio close to the values in equation (1). Similar assays were carried out under 1% $^{18}\text{O}_2$ and the result also confirmed the equivalent consumption/production rates of NH_2OH , N_2 and H_2O . Further, we determined the kinetic constants of DnfA-catalyzed reactions at atmospheric condition (21% O_2). The Michaelis-Menten constant (K_m) for hydroxylamine and the catalytic efficiency (k_{cat}) were

418 determined to be $48.1 \pm 6.0 \mu\text{M}$ and $18.6 \pm 1.2 \text{ min}^{-1}$, respectively (Figure 5D). The substrate
419 binding affinity and catalytic efficiency of DnfA are comparable with those reported for the
420 DnfA homolog AurF ($K_m 5.24 \pm 0.64 \mu\text{M}$ and $k_{cat} 6.21 \pm 0.52 \text{ min}^{-1}$), supporting the possibility
421 of hydroxylamine as the substrate under physiological conditions. Based on the above results,
422 a model was proposed for the DnfA-catalyzed oxidation of hydroxylamine to N_2 (Figure 5E).

Figure 5. Isotope-labelling experiment with $(^{15}\text{NH}_4)_2\text{SO}_4$ and $^{18}\text{O}_2$ showing the involvement of O_2 during hydroxylamine oxidation and determination of kinetic constants of DnfA. (A) Neighbour-joining phylogenetic tree of DnfA, AurF, CmlI and HAO, and their homologues. Bootstrap percentages based on 1,000 replicates are shown at branch points. AurF, p-aminobenzoate N-oxidase from *Streptomyces thioluteus*. CmlI, N-Oxidase from *Streptomyces venezuelae*. DnfA, AurF and CmlI are indicated by asterisks. The tree showing DnfA, AurF and CmlI fell within the same lineage, and HAOs formed a distinct lineage from them. 24 HAOs leaves were collapsed. (B) The consumption or production of NH_2OH , $^{15}\text{N}_2$, and H_2^{18}O with (left) or without enzyme (right) after 20 min incubation. Experiments were performed in 0.3 ml reaction solution with initial 10 mM $^{15}\text{NH}_2\text{OH}$, 120 μM FAD, 10 mM NADH, 331 μM DnfA, and 10% of $^{18}\text{O}_2$ + 90% of He in the head gas space. The asterisk (*) represents statistically significant ($P < 0.05$). (C) Consumption rate of NH_2OH and production rates of $^{15}\text{N}_2$ and H_2^{18}O . Experiments were performed with the same protocol as in (B). The data shown here are the differences of the amounts of substrates and products from triplicate experiments with and without DnfA, and data are fitted into a linear equation to access the reaction rates. (D) Determination of kinetic constants of DnfA under atmospheric conditions. The assays were performed with 0.6 μM DnfA, 480 μM FAD, 10 mM NADH and 25-400 μM NH_2OH (100 μL reaction mixture in 1.5 mL vials at 30 °C). For kinetic constant determination at another temperature including lower NH_2OH concentrations see Fig. S7 (E) The proposed catalytic model of DnfA. The data shown here are averages of 3 replicates.

Discussion

While a reasonable albeit not complete understanding of the enzymes and pathways involved in aerobic autotrophic ammonia oxidation is available, our knowledge on the physiology and biochemistry of heterotrophic nitrification is surprisingly rudimentary (23, 47). In this study, we discovered in the newly isolated heterotrophic nitrifier *Alcaligenes* strain HO-1 a previously unknown process that leads to the oxidation of ammonia to N_2 gas. Via heterologous expression in *E. coli* and work with purified Dnf proteins/enzymes, we demonstrated that the

gene cluster *dnfT1T2RABCD* is critical for this process. While we did not yet manage to reveal which enzyme(s) is catalyzing the conversion of ammonia to hydroxylamine in the *Alcaligenes* strain HO-1, we demonstrated that the key enzyme DnfA catalyzes a new reaction - the oxidation of hydroxylamine and NADH with oxygen to N₂ and water [reaction 1]. It should be noted that oxygen can be non-enzymatically reduced to water by NADH (Figure 5B), but our data demonstrated that the oxidation of hydroxylamine clearly relied on O₂. Additional research is urgently required to reveal the biochemical mechanism of this novel way of N-N bond formation.

An obvious key question is which physiological role the oxidation of ammonia to N₂ via hydroxylamine plays in *Alcaligenes* HO-1. In the reaction catalyzed by DnfA [reaction 1], two electrons are needed for the formation of one molecule N₂ and four molecules of water from two molecules of hydroxylamine and one molecule of oxygen. In addition, ammonia oxidation to hydroxylamine (if catalyzed by an unknown AMO) costs additional 2 electrons. Thus, it is tempting to speculate that *Alcaligenes* uses this reaction to flare off electrons during rapid growth and that this process serves to maintain redox balance in this organism. The DnfA catalyzed process will also help to reduce the hydroxylamine concentration in the cytoplasm and thus minimize chemical conversion of precious pyruvate to pyruvic oxime (which subsequently needs to be salvaged by the activity of the POD enzyme). However, clearly additional physiological experiments will be needed to investigate this in more detail.

The discovery of hydroxylamine oxidation with an oxidase to N₂ in an *Alcaligenes* strain is particularly noteworthy as several *Alcaligenes* strains were previously reported as heterotrophic nitrifiers producing N₂ gas (6, 16, 20, 21, 48, 49). In this context, it is interesting to note that homologues of *dnfA* are found in other *Alcaligenes* species as well as in other heterotrophic nitrifiers such as *Pseudomonas*, *Microvirgula*, *Pokkaliibacter*, and *Burkholderia*, suggesting that this hydroxylamine oxidase may widely occur in diverse microorganisms. If this reaction indeed is responsible under aerobic conditions for (part of the) N₂-formation from ammonia in many heterotrophic nitrifiers, our discovery will fundamentally change our

482 perception on heterotrophic nitrification and might offer applications in engineered systems for
483 N-removal from contaminated waters.

484

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493

494 **Data and materials availability**

495 The genome sequence of *Alcaligenes* sp. strain HO-1 has been deposited at NCBI GenBank
496 under the BioProject PRJNA543690 with the accession number CP049362.

497

498 **Author Contributions**

499 M-RW, YL, Y-XZ, T-TH isolated the strain HO-1, conducted the N-balance, constructed the
500 fosmid library and finished the genetic cloning and screening; L-LM, M-RW, LM, H-ZZ, X-YG,
501 Y-LQ, T-TH, TW, G-MA finished protein purification, enzymatic assays, biochemical tests and
502 isotope measurements; S-JL, Z-PL, D-FL and X-HS designed and supervised the
503 experiments; M-RW, T-TH, H-ZZ, CWH, S-JL, Z-PL, and D-FL analyzed the data; and MW, Z-
504 PL, D-FL and S-JL wrote the manuscript.

505

506 **Competing Interests statement**

507 The authors declare no competing interests.

508

509 **References**

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