nosX is essential for whole-cell N₂O reduction in Paracoccus denitrificans but not for assembly of copper centres of nitrous oxide reductase

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Abstract
Nitrous oxide (N₉O) is a potent greenhouse gas that is produced naturally as an intermediate during the process of denitrification carried out by some soil bacteria. It is consumed by nitrous oxide reductase (N₂OR), the terminal enzyme of the denitrification pathway, which catalyses a reduction reaction to generate dinitrogen. N₂OR contains two important copper cofactors (Cu₅ and Cu₆ centres) that are essential for activity, and in copper-limited environments, N₂OR fails to function, contributing to rising levels of atmospheric N₂O and a major environmental challenge. Here we report studies of nosX, one of eight genes in the nos cluster of the soil dwelling α-proteobacterium Paracoccus denitrificans. A P. denitrificans ΔnosX deletion mutant failed to reduce N₂O under both copper-sufficient and copper-limited conditions, demonstrating that NosX plays an essential role in N₂OR activity. N₂OR isolated from nosX-deficient cells was found to be unaffected in terms of the assembly of its copper cofactors, and to be active in in vitro assays, indicating that NosX is not required for the maturation of the enzyme; in particular, it plays no part in the assembly of either of the Cu₅ and Cu₆ centres. Furthermore, quantitative Reverse Transcription PCR (qRT-PCR) studies showed that NosX does not significantly affect the expression of the N₂OR-encoding nosZ gene. NosX is a homologue of the FAD-binding protein ApbE from Pseudomonas stutzeri, which functions in the flavinylation of another N₂OR accessory protein, NosR. Thus, it is likely that NosX is a system-specific maturation factor of NosR, and so is indirectly involved in maintaining the reaction cycle of N₂OR and cellular N₂O reduction.

INTRODUCTION
Nitrous oxide is a potent greenhouse gas which has rapidly increased in the atmosphere over the past century [1]. The rise in N₂O coincides with the introduction and application of anthropogenic nitrogen species in agriculture, to improve crop yield and ultimately feed the growing global population [2, 3]. Of the total N₂O released, 40% is produced by soil bacteria [4]. Soil dwelling denitrifying micro-organisms such as Paracoccus denitrificans consume nitrate as an alternative electron acceptor during anaerobic growth conditions. N₂O is an intermediate substrate in the denitrification pathway; it is reduced to N₂ by the copper enzyme nitrous oxide reductase (N₂OR). N₂OR-containing bacteria can be separated into two clades, and a feature that distinguishes the clades is the ability of the micro-organism to produce and consume, or only consume, N₂O [5, 6]. Clade-I members are complete denitrifiers with the nitrite reductase genes nirS or nirK present in their genome. In contrast, about half of the clade-II members are non-denitrifying N₂O reducers, and are therefore N₂O sinks [5]. Ammonia-oxidizing bacteria (AOB) are another microbial source of N₂O in coastal ecosystems, through a process named ‘nitrifier denitrification’. However, they do not harbour genes encoding N₂O reduction activity [7]. Environmental factors such as soil pH, Cu content, and moisture impact on N₂O emissions from soil [8–10]. In order...
to identify N₂O mitigation strategies, we are trying to understand the optimal genetic components needed to biologically remove N₂O.

Nitrous oxide reductase (N₂OR) is a homo-dimeric, ~120 kDa, multi-Cu protein. Each monomer contains two Cu cofactors, the Cu₄ and Cu₆ centres, responsible for electron transfer and the catalytic reduction of N₂O, respectively. The Cu₆ centre is a bis-thiolate-bridged di-nuclear Cu centre, accommodated within a cupredoxin fold domain, similar to that of subunit II of cytochrome c oxidase. The Cu₄ centre is a unique [Cu-S] cluster ligated by seven conserved histidine residues within a β-barrel domain. It comprises four Cu atoms and one or two sulphur atoms, depending on the purification method [11–13]. Notably, the subunits of the active homodimer are orientated in a head to tail configuration, with one Cu₆ centre in close proximity to the Cu₄ centre of the other monomer. N₂O is encoded by the nosZ gene, which, in denitrifying organisms such as Paracoccus denitrificans and Pseudomonas stutzeri, is translated and exported through the twin-arginine transport [14] pathway to the periplasm, as a folded apo-protein, before acquiring its Cu cofactors. Consistent with this, a TAT signal leader sequence mutant accumulated unprocessed, dimeric, apo-protein in the cytoplasm of the cell [15]. In contrast, the N₂OR of clade-II members is transported through the Sec pathway [16]. The functional significance of this is currently unknown.

The nosZ gene is found among the nos gene cluster (NGC), which comprises eight genes in P. denitrificans: nosCRZD-FYXL. The nosC and nosR genes are copper responsive in P. denitrificans and function in the regulation of nosZ transcription. During Cu limitation, nosCR transcription is increased, whilst nosZ transcription is reduced [17]. In Pseudomonas stutzeri, NosR is a cytoplasmic membrane protein with two soluble domains located at either side of the membrane: the N-terminal periplasmic domain covalently binds a flavin mononucleotide, while the C-terminal cytoplasmic domain binds two [4Fe-4S] clusters [18]. The P. denitrificans homologue (44.3% identical) is predicted to have similar features. The function of NosR is not well understood; in addition to the regulatory role mentioned above, it is important for whole-cell N₂O reduction [17, 18], with evidence indicating that it is not involved in the assembly of the Cu centres of N₂OR, but may be the physiological electron donor to NosZ [18].

nosDFY encode a cytoplasmic membrane spanning ABC-type transporter that functions in the maturation of the Cu₆ centre of N₂OR, as illustrated by an insertional mutation in P. stutzeri nosD, which produced an N₂OR without the key spectroscopic signal of the Cu₆ centre [19]. Similarity to mitochondrial ABC transporters that export a sulphur species to the cytoplasm for iron-sulphur cluster biogenesis suggests a role for NosDFY in providing the essential sulphur atoms of the catalytic Cu₄ centre [20]. The nosL gene is well conserved across NGCs and is essential for whole-cell N₂O reduction in P. denitrificans. NosL is a Cu-binding lipoprotein, putatively anchored to the outer membrane of the cell. The properties of N₂OR purified from a PdΔnosL strain revealed that Cu-binding NosL is a component of the Cu₆ maturation apparatus under Cu replete conditions and, more importantly, is an essential maturation factor for both Cu centres during Cu limitation [21].

The nosX gene is predominantly found in α- and β-proteobacterial NGCs in clade I but does not feature among γ-proteobacteria nor clade-II NGCs (Fig. 1). NosX is a soluble protein of ~30 kDa, which is exported to the periplasm by the Tat pathway. Previously, it was reported that insertional mutagenesis of P. denitrificans nosX resulted in wild-type-like growth [22]. Interruption of both nosX and the homologue nirX did, however, present a Nos-negative (Nos⁻) phenotype, leading to the conclusion that NosX and NirX are functional homologues [22]. Furthermore, this study demonstrated that the nosX nirX double mutant strain contained N₂OR that was deficient in its Cu₄ centre, implicating these proteins in copper cofactor assembly [22].

The γ-proteobacterium P. stutzeri, which does not feature nosX in its NGC, instead contains a NosX homologue encoded elsewhere on the genome. The protein, called ApbE, was shown to be a FAD-binding flavin transferase that serves as a flavin donor to NosR, which in turn activates N₂OR [23]. PaApbE and PdNosX share 32% amino acid homology, in particular the conservation of key amino acid residues associated with flavin binding suggest that their roles are similar while their genetic context implies they may differ in system specificity. Here, we present a re-examination of the role of NosX in P. denitrificans, through the analysis of full nosX deletion in P. denitrificans, in terms of cell growth and the properties of N₂OR purified from an unmarked mutant background. The data show that NosX is essential for N₂OR activity and cannot be substituted by NirX. Furthermore, NosX plays no role in assembly of the NosZ Cu cofactors, nor does it have a major function in the regulation of nosZ expression. Instead, the role of NosX is consistent with a system-specific maturation factor for NosR to support the activity of NosZ in vivo.

**METHODS**

**Construction and complementation of a nosX-deficient strain of P. denitrificans**

A double allelic exchange method was employed to generate a whole nosX gene deletion strain (Table S1, available in the online version of this article), as described previously [17, 21]. Briefly, the suicide plasmid pK18mobsacB containing DNA regions that flank the nosX gene (pSPBN4) was conjugated into PD1222 using the E. coli helper plasmid pRK2013. Single cross-over recombination events resulted in Spec⁸/Km⁸ transconjugants, from which a double cross over mutant (Spec⁸), named PD2502, was generated. The mutated region was PCR amplified and confirmed by sequencing.

PdΔnosX (PD2502) was complemented in trans using pSPBN5, which contains the coding sequence of Pden_4214. The gene was synthesized by Genscript with flanking 5′ NdeI and 3′ EcoRI restriction sites and subcloned into a taurine inducible modified pLMB509 derivative with gentamycin
resistance (20 µg ml⁻¹) to generate pSPBN5. The complementation plasmid was conjugated into the mutant strain using the helper *E. coli* pRK2013 strain, with successful conjugants identified as SpecR/GmR. Expression of *nosX* from the plasmid was induced by adding 1 mM taurine to the medium at the start of growth.

**Growth and phenotypic analysis of cultures**

Anaerobic minimal media batch cultures (400 ml) were grown in sealed Duran flasks fitted with a septum seal to allow for gas-tight sample extraction. Minimal media consisted of: 30 mM succinate, 20 mM nitrate, 11 mM dihydrogen orthophosphate, 29 mM di-sodium orthophosphate, 0.4 mM magnesium sulphate, 1 mM ammonium chloride, pH 7.5. The minimal media was supplemented with a 2 ml l⁻¹ Vishniac and Santer trace element solution [24] where copper sulphate was present (Cu-sufficient, 12.8 µM) or excluded (Cu-limited, <0.5 µM) from the original recipe. Media were inoculated using a 1% inoculum from a starter culture to give a starting OD₆₀₀ nm of ~0.02 and incubated at 30 °C. Samples of the liquid culture were taken in 1 ml aliquots and OD₆₀₀ nm measured. The 3 ml gas samples were removed from the headspace of the cultures and stored in pre-evacuated 3 ml Exetainer vials. A 50 µl gas sample was injected into a Clarus 500 gas chromatograph (PerkinElmer) equipped with an Elite-PLOT Q (30 m×0.53 mm internal diameter) and an electron capture detector. Carrier gas was N₂, make-up gas was 95% (v/v) argon, 5% (v/v) methane. Standards containing N₂O at 0.4, 5, 100, 1000, 5000 and 10000 p.p.m. (Scientific and Technical Gases) were measured and total N₂O was determined as previously described [17].
Purification and characterization of affinity-tagged N$_2$OR from *P. denitrificans* strains

Plasmid pMSL002, which encodes NosZ (N$_2$OR) with a C-terminal Strep-tag II, was conjugated into wild-type (PD1222), *PdΔnosZ* (PD2303) and *PdΔnosX* (PD2502) strains using the *E. coli* pRK2013 helper strain. Strep-tagged N$_2$OR was overproduced and purified as previously described [21]. Briefly, this involved applying the soluble portion of cell lysates to a Hi-Trap HP Strep II affinity column (5 ml, GE Healthcare) and eluting with 20 mM HEPES, 150 mM NaCl and 2.5 mM desthiobiotin, pH 7.2, before exchanging into 20 mM HEPES, 150 mM NaCl, pH 7.2. Sample purity was confirmed using SDS-PAGE analysis and LC-MS. Protein concentrations were determined using the Bradford assay (BioRad) and bovine serum albumin as a protein standard.

UV-visible absorbance spectra of N$_2$OR-Strep-tag II from different backgrounds were recorded on a Jasco V-550 spectrophotometer. Samples were made anaerobic by sparging with nitrogen gas for 5 min and oxidized or reduced with 5 mg ml$^{-1}$ stocks of potassium ferricyanide and sodium dithionite, respectively, in 20 mM HEPES, 150 mM NaCl, pH 7.2. Sample purity was confirmed using SDS-PAGE analysis and LC-MS. Protein concentrations were determined using the Bradford assay (BioRad) and bovine serum albumin as a protein standard.

RNA isolation, cDNA synthesis and qRT-PCR experiments

Expression of the *nosZ* gene was determined by qRT-PCR, using an AriaMx Real-Time PCR System G9930A (Agilent Technologies). The *nosX* mutant and PD1222 wild-type strains were cultivated under anoxic conditions as mentioned above for 12 h, reaching final OD$_{600nm}$ of 0.6. Total RNA extraction, RNA quality and integrity assays, and RNA quantification were performed using the methodology previously described [17]. Briefly, 2 µg of total RNA were used for cDNA synthesis using RevertAid First Strand cDNA synthesis kit (Thermo Scientific) and random hexamers following the supplier’s instructions. qRT-PCR reactions were run in triplicate in a total volume of 20 µl containing 10 µl of SensiFAST SYBR No-ROX Mix (Bioline), 0.7, 7 or 70 ng of cDNA and 2 µM of each primer. Melting curves were generated to verify the specificity of each amplification reaction. Expression of *nosZ* gene was determined using the oligonucleotide pair nosZ2F/nosZ2R [17] and normalized against the housekeeping gene *gapA* (glyceraldehyde-3-phosphate dehydrogenase; GAPDH1F/GAPDH1R [17]). The changes in gene expression were analysed accordingly to Plaffl methodology [28]. The data presented correspond to the average of three independent biological replicates.

RESULTS

**NosX is essential for whole-cell N$_2$O reduction in *P. denitrificans***

Wild-type *P. denitrificans* (PD1222), *ΔnosZ* (PD2303, missing the gene encoding N$_2$OR) and *ΔnosX* (PD2502, missing the gene *Pden_4214*) were grown in batch culture, in minimal medium, under Cu-sufficient and limited conditions. The wild-type culture produced a small amount of N$_2$O (~1 mM) in Cu-deficient conditions, but this was no longer detected as the culture moved into the stationary phase of growth. For 2. A N$_2$O-negative phenotype (*Nos*), in terms of growth and N$_2$O production, was observed in the *ΔnosZ* strain under both Cu regimes. For the *ΔnosX* strain, growth was affected both under Cu-sufficient and limited conditions, and N$_2$O levels were similar to those of the *ΔnosZ* strain, demonstrating the absence of a functioning enzyme.

The Nos$^-$ phenotype of the *ΔnosX* strain was almost fully complemented under both Cu regimes by a plasmid-borne...
nosX gene copy (pSPBN5) expressed in trans from a taurine inducible promoter (Fig. 3), demonstrating that the Nos- phenotype is associated with the absence of nosX and not a downstream effect of the deletion. The data demonstrate that the nosX deletion mutant strain of P. denitrificans is unable to catalyse N₂O reduction. This is in contrast to a previous study by Saunders and co-workers [22] involving a marked nosX deletion, where it was concluded that that NosX and NirX are functionally redundant, such that only one is required for N₂O reduction.

NosX is not involved in maturation of either Cu cofactor in N₂OR

Three possible explanations for the Nos- phenotype in the ∆nosX mutant are apparent: the incomplete maturation/assembly of copper centres of N₂OR; the failure to activate N₂O catalytic activity, for example through disruption of supply of electrons; or, the severe down-regulation of nosZ transcription. To investigate this further, a C-terminal strep II-tagged N₂OR was purified from the ∆nosX mutant strain and the properties of the N₂OR analysed with respect to the status of the Cu₁ and Cu₂ centres.

Aerobically purified N₂OR, also known as the pink form of N₂OR, has been spectroscopically well characterized and all oxidized spectra were normalised to ε₅₈₀ nm = 5000 M⁻¹ cm⁻¹ per monomer, as described by Rasmussen et al. [13]. Absorbance spectra of N₂OR enzymes isolated from cultures grown under Cu-sufficient conditions are shown in Fig. 4a. Spectra of N₂OR from wild-type cells and ∆nosX and ∆nosZ mutants have features at 480, 540 and 640 nm, in agreement with the previous literature on N₂OR from P. denitrificans [21], P. pantotrophus (PpN₂OR) [13], Pseudomonas stutzeri (PsN₂OR) [29], Pseudomonas nautica (PnN₂OR) [30], Achromobacter cycloclastes (AcN₂OR) [31] and Marinobacter hydrocarbonoclasticus (MhN₂OR) [32]. Features in the absorption spectrum at these wavelengths arise from S²⁻ to Cu(II) charge-transfer bands and additional optical bands due to interactions between the Cu(I) and Cu(II) ions of the centres [13]. Spectra of N₂OR isolated from wild-type cells have lower extinction coefficients than those from the mutant strains, suggesting that it contains lower levels of Cu cofactors. N₂OR activity was measured using a methyl viologen assay in which the reduced MV extinction coefficient, ε₆₀₀ nm = 13600 M⁻¹ cm⁻¹ [27], was used to quantify activity, and N₂OR was pre-incubated with a 500-fold excess reduced methyl viologen (MV) before initiating the reaction with N₂O (Table 1). Each N₂OR sample was active, with values for the enzyme from the wild-type and ∆nosZ

![Fig. 3](image_url)  
**Fig. 3.** Complementation of the nosX mutant. (a) Growth characteristics (optical density, OD₆₀₀ nm), left, and N₂O production (N₂N₂O, mM N in the form of N₂O), right for the mutant ∆nosX PD2502 complemented under (a) Cu-limited, and (b) Cu-sufficient conditions in anaerobic batch culture. The pSPBN5 plasmid was conjugated into the ∆nosX PD2502 strain and cultured in the absence of taurine and in the presence of 1 mM taurine. For reference, the ∆nosZ PD2303 strain and wild-type PD1222 are shown. Experiments were repeated in triplicate and bars represent se.

![Fig. 4](image_url)  
**Fig. 4.** UV-visible absorbance characterisation of strep-tagged N₂OR purified from different P. denitrificans backgrounds. Data are shown for N₂OR from wild-type PD1222, ∆nosX PD2502 and ∆nosZ PD2303 in 20mM HEPES, 150mM NaCl, pH 7.2. Spectra of ferricyanide-oxidized (a), sodium dithionite-reduced (b) and the oxidized minus reduced difference (c) are shown for enzymes isolated from cultures grown under Cu-sufficient conditions. Equivalent spectra (d–f), respectively, were measured for enzymes isolated from cultures grown under Cu-limited conditions.
strains consistent with those previously reported [21, 26, 30]. Activity for N\(_2\)OR from the ΔnosX mutant was similar to that from wild-type, even though it contained significantly more Cu, suggesting that the enzyme from the ΔnosX mutant has a slightly lower activity.

Reduction of N\(_2\)OR samples with dithionite leads to reduction of the Cu\(_{A}\) centre to a [Cu\(^{2+}\):Cu\(^{2+}\)] diamagnetic species, which is colourless and thus does not contribute in the visible region of the absorbance spectrum. Thus, in Fig. 4b, bands at 480, 540 and 900 nm are lost to leave a Cu\(_{Z}\) signature, consisting of a peak at 640 nm, in agreement with the literature for pink N\(_2\)OR [13]. The oxidized minus reduced difference spectrum, Fig. 4c, revealed the spectrum due to the Cu\(_{A}\) centre. The close similarity of spectral form and absorption extinction coefficients for N\(_2\)OR from ΔnosZ and ΔnosX mutants demonstrate that the assembly of the Cu cofactors of N\(_2\)OR is not affected by the nosX deletion when grown under Cu sufficiency [13].

An equivalent spectroscopic analysis of N\(_2\)OR enzymes isolated from cultures grown under Cu limitation (Fig. 4d–f) revealed spectra similar to those of Fig. 4a–c for enzymes from ΔnosZ and ΔnosX mutants, but with lower extinction coefficients, suggesting lower incorporation of Cu. Spectra for enzyme isolated from wild-type cultures, however, indicate very low levels of Cu incorporation. Determination of Cu content (Table 1) revealed that N\(_2\)OR from ΔnosZ and ΔnosX mutants contain ~4 Cu per N\(_2\)OR monomer, while that recovered from wild-type cells contains <1 Cu per monomer, consistent with absorbance data (Fig. 4d–f). As above, the close similarity between N\(_2\)OR enzymes isolated from ΔnosZ and ΔnosX mutants demonstrate that NosX does not play a role in assembly of the Cu cofactors of N\(_2\)OR under Cu-limited conditions.

**Table 1.** Summary of some characteristics of strep-tagged N\(_2\)OR purified from *P. denitrificans* strains PD1222, PD2502 and PD2303

|                  | Cu ions/monomer\(^a\) | Specific activity\(^b\) (µmol N\(_2\)O min\(^{-1}\) mg\(^{-1}\) enzyme) |
|------------------|------------------------|---------------------------------------------------------------|
|                  | Cu-sufficient | Cu-limited | Cu-sufficient | Cu-limited |
| Wild-type PD1222/ pMSL002 (StrepII tagged-NosZ) | 5.6±0.1 | 0.4±0.27 | 171±13 |
| ΔnosX/pMSL002    | 6.4±0.2 | 4.2±0.2 | 172±12 |
| ΔnosZ/pMSL002    | 5.9±0.6 | 4.8±0.4 | 196±9 |

\(^a\)Total copper per monomer was determined using the BC5 Cu assay (see Methods).

\(^b\)N\(_2\)O reductase activity was determined for enzymes isolated from cultures grown under Cu-sufficient conditions using a reduced methyl viologen assay (µmol N\(_2\)O min\(^{-1}\) mg\(^{-1}\) enzyme). Proteins were pre-incubated with a 500-fold excess reduced methyl viologen for 150 min prior to activity assay. All reactions were carried out in triplicate and sd is shown. nd. The data show that even though the ΔnosX strain has a Nos- phenotype, N\(_2\)OR isolated from it is fully or close to fully active in an in vitro assay.

**DISCUSSION**

The nosX gene is conserved across the NGC of \(\alpha\)- and \(\beta\)-proteobacteria, but not among \(\gamma\)- or clade-II members of N\(_2\)O-reducing bacteria. Here, we have demonstrated a Nos\(^{-}\) phenotype for a nosX deletion mutant in *P. denitrificans* (PD2502), which was complemented in trans using a functional nosX plasmid-borne gene copy under taurine.
inducible control. NosX is a member of the AbpE protein family, which bind flavin adenine dinucleotide [33, 34]. Some AbpE proteins are flavinyl transferases, functioning in the post-translational maturation of another flavin-requiring protein. For example, Vibrio cholera ApbE transfers a flavin mononucleotide (FMN) to a threonine residue in NqrC [35]. In P. denitrificans there are three apbE homologues: nosX, encoded by pden_4214, nirX (pdn_2485) and pden_3291. NosX and NirX are exported to the periplasm via the Tat pathway while Pden_3291 is predicted to be cytoplasmic.

An earlier study of an antibiotic cassette insertion mutation in the P. denitrificans nosX gene reported no effect on N_2 OR activity [22]. This led to the proposal that the nirX gene in P. denitrificans is a functional homologue of nosX, such that mutation of both genes are required in order to observe a Nos phenotype. This previous conclusion is clearly at odds with the data presented here. One possibly important observation is that the previous mutagenesis study did not involve full nosX deletion. Conserved residues within the putative FAD binding pocket in NosX are now known, including Ser68, Tyr70, Thr174 and Gly256, based on sequence similarities with the SeApbE (Fig. S1) [34]. If these residues are important for NosX function, then the previous mutation strategy for P. denitrificans nosX, in which a kanamycin insertion was made 469bp into the gene, would not have disrupted the conserved Ser68 and Tyr70 residues. The resulting truncated NosX may have retained some function, which would account for why a clear phenotype was not observed in the single nosX insertional mutant. We note that the requirement for nosX in N_2 O reduction has also been demonstrated in Sinorhizobium meliloti. In that case, a Tn5-mediated mutation 31 nucleotides into the total 966 nucleotide sequence downstream of nosDFYL, a region now recognized as nosX, abolished N_2 OR activity [36].

In the earlier report of a double nosXnirX mutant of P. denitrificans, it was reported that the N_2 OR present in unfraccionated periplasm from this mutant was deficient in the Cu_4 centre, leading to the conclusion that NosX and NirX play a role in the assembly of this cofactor [22]. However, subsequent studies of anaerobically purified N_2 OR from the double nirXnosX mutant and a single nirX mutant indicated that the absence of NosX resulted in N_2 OR with both Cu cofactors assembled, but with Cu_4 exhibiting a spectroscopically distinct from, termed pink Cu_4*, that is normally only observed upon reaction with O_2 [37]. This Cu_4* form is not catalytically active, but is proposed to represent a catalytically relevant intermediate oxidation state of the Cu_4 centre ([4CuS]^{3+}), which binds N_2 O and proceeds through a state denoted as Cu_4 → [38].

Here, to determine the effect of the absence of nosX/NosX alone on N_2 OR, we utilized a previously reported plasmid-encoded Strep-tagged N_2 OR that can be readily isolated from different background strains and characterized in terms of its Cu cofactor content and spectroscopic properties. These experiments demonstrated unequivocally that the assembly of the Cu_4 and Cu_4 centres was unaffected in the absence of nosX. Thus, the phenotype exhibited by the mutant does not arise because of a deficiency in the insertion of Cu into N_2 OR. We note that the spectroscopic properties of N_2 OR from the ΔnosX mutant strain are the same as those of the Cu_4 centre from purified from the nosXnirX mutant. This may suggest the Cu_4 centre was purified in a catalytically inactive redox state. However, the pink form reported in this work was generated by aerobic purification, with nirX remaining in the genome and under conditions where we expect to observe the Cu_4 centre is this pink Cu_4* form, as demonstrated by the control experiments with N_2 OR isolated from the wild-type strain.

ApbE from the N_2 O-reducing bacterium P. stutzeri is a monomeric FAD-binding protein [23]. In the absence of nosX in the NGC of P. stutzeri, AbpE functions as a flavin donor, catalysing the covalent flavinylation of a threonine residue of NosR [23]. Importantly, the post-translationally modified, FMN-bound NosR is proposed to be the electron donor to N_2 OR, such that in the absence of NosX, NosR is not functional. Our data indicate that N_2 OR Cu cofactor maturation is unaffected by the loss of NosX, and we conclude that in P. denitrificans it most likely functions as the main system-specific maturation factor for NosR and thus as an indirect activator of N_2 OR. If this is the case, then a Nos phenotype would be expected for a ΔnosX strain. This was recently demonstrated: a P. denitrificans ΔnosR strain exhibited a vastly decreased capacity to reduce N_2 O, irrespective of the levels of Cu in the cell [17]. However, we note that the ΔnosR strain did retain some ability to reduce N_2 O, whereas the nosX mutant investigated here did not, and so the nos phenotype is actually more dramatic than the nosR phenotype. Why this is the case is not clear. One possibility is that NosX does not only mature NosR, such that the absence of NosX, there is a further effect on NosZ activity. Alternatively, having a non-flavinyalted NosR present might somehow inhibit NosZ more severely than having no NosR present at all. We also note that the previously reported transcription data revealed the loss of Cu-responsive transcription of nosZ in the nosR deletion strain [17], suggesting that NosR itself may be multifunctional, or that its absence leads to pleiotropic effects, some of which may be indirect. Clearly, further studies are needed to investigate directly the role of NosX in NosR maturation, and more generally other possible roles of NosX and the function(s) of NosR.

An intriguing observation reported here is the lower levels of Cu cofactor incorporation observed under Cu-limited conditions for the Strep-tagged N_2 OR from wild-type cells compared to that from the nosZ and nosX mutants. One possibility that we examined was that nosX/NosX is involved in the regulation of nosZ, such that in the absence of nosX/ NosX, lower amounts of chromosomally encoded N_2 OR were present, perhaps leading to less competition for copper and higher incorporation of Cu into the plasmid-encoded Strep-tagged form. While the absence of nosX did result in a twofold reduction of nosZ expression under Cu-sufficient conditions, no significant difference between the wild-type and nosX mutant strains was detected under Cu-limited conditions where the incorporation of Cu was most pronounced. The very low expression of the chromosomal nosZ gene under Cu-limited conditions suggests that a simple competition
between chromosomal- and plasmid-encoded N\textsubscript{2}OR enzymes for Cu is unlikely. A further possibility is that the presence of the Strep-tag required for rapid recovery and biochemical analysis of NosZ results in modest perturbation of Cu cofactor assembly factor interactions such that the wild-type enzyme is a preferred substrate, an effect that only becomes apparent under very low Cu conditions. Clearly, further studies are needed to explore this possibility.

In summary, the data presented here show that nosX is essential for whole-cell N\textsubscript{2}O reduction in the \textit{\alpha}-proteobacterium \textit{P. denitrificans}, and that the nosX and nirX gene products are not functionally redundant under our experimental conditions. The function of NosX is not associated with the assembly of the Cu cofactors of N\textsubscript{2}OR. Instead, based on homology between NosX and ApbE proteins, and the recent demonstration of an essential role for an ApbE family flavin transference in the maturation of NosR in \textit{P. stutzeri}, it is likely that NosX is involved in indirectly maintaining the reaction cycle of N\textsubscript{2}OR through the flavinylation of another accessory protein, NosR.

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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

**References**

1. Ravishankara AR, Daniel JS, Portmann RW. Nitrous oxide N\textsubscript{2}O: the dominant ozone-depleting substance emitted in the 21st century. \textit{Science} 2009;326:123–125.

2. Fowler D, Coyle M, Skiba U, Sutton MA, Cape J et al. The global nitrogen cycle in the twenty-first century. \textit{Philos Trans R Soc Lond B Biol Sci} 2013;368:20130164.

3. Thomson AJ, Giannopoulos G, Pretty J, Baggs EM, Richardson DJ. Biological sources and sinks of nitrous oxide and strategies to mitigate emissions. \textit{Philos Trans R Soc Lond B Biol Sci} 2012;367:1157–1168.

4. Hu H-W, Chen D, He J-Z, HW H, JZ H. Microbial regulation of terrestrial nitrous oxide formation: understanding the biological pathways for prediction of emission rates. \textit{FEMS Microbiol Rev} 2015;39:729–749.

5. Hallin S, Philippot L, Löffler FE, Sanford RA, Jones CM. Genomics and ecology of Novel N\textsubscript{2}O-reducing microorganisms. \textit{Trends Microbiol} 2018;26:43–55.

6. Jones CM, Graf DRH, Bru D, Philippot L, Hallin S. The unaccounted yet abundant nitrous oxide-reducing microbial community: a potential nitrous oxide sink. \textit{ISME J} 2013;7:417–426.

7. Stein LY. Insights into the physiology of ammonia-oxidizing microorganisms. \textit{Curr Opin Chem Biol} 2019;49:9–15.

8. Domeingo-Horta LA, Philippot L, Peyrard C, Bru D, Breuil M-C et al. Peaks of in situ N2O emissions are influenced by N2O-producing and reducing microbial communities across arable soils. \textit{Glob Chang Biol} 2018;24:360–370.

9. Griffis TJ, Chen Z, Baker JM, Wood JD, Millet DB et al. Nitrous oxide emissions are enhanced in a warmer and wetter world. \textit{Proc Natl Acad Sci U S A} 2017;114:12081–12085.

10. Liu B, Frostegård Åsa, Bakken LR. Impaired reduction of N\textsubscript{2}O to N\textsubscript{2} in acid soils is due to a posttranscriptional interference with the expression of nosZ. \textit{mBio} 2014;5:e01383–01314.

11. Brown K, Djinnovic-Carugo K, Haltia T, Cabrito I, Saraste M et al. Revisiting the catalytic Cu\textsubscript{1} cluster of nitrous oxide (N\textsubscript{2}O) reductase. Evidence of a bridging inorganic sulfur. \textit{J Biol Chem} 2000;275:41133–41136.

12. Pomowski A, Zumft WG, Kroneck PMH, Einsle O. N\textsubscript{2}O binding at a [4Cu:2S] copper-sulphur cluster in nitrous oxide reductase. \textit{Nature} 2011;477:234–237.

13. Rasmussen T, Berks BC, Butt JN, Thomson AJ. Multiple forms of the catalytic centre, Cu\textsubscript{c}, in the enzyme nitrous oxide reductase from \textit{Paracoccus pantotrophus}. \textit{Biochem J} 2002;364:807–815.

14. Mattatall NR, Jazairi J, Hill BC. Characterization of HpmQ, an accessory protein required for the expression of cytochrome c oxidase in \textit{Bacillus subtilis}. \textit{J Biol Chem} 2000;275:28802–28809.

15. Heikilä MP, Honisch U, Wunsch P, Zumft WG. Role of the Tat transport system in nitrous oxide reductase translocation and cytochrome cd\textsubscript{b} biosynthesis in \textit{Pseudomonas stutzeri}. \textit{J Bacteriol} 2001;183:1663–1671.

16. Simon J, Einsle O, Kroneck PM, Zumft WG. The unprecedented nos gene cluster of \textit{Wolinella succinogenes} encodes a novel respiratory electron transfer pathway to cytochrome c nitrous oxide reductase. \textit{FEBS Lett} 2004;569:7–12.

17. Sullivan MJ, Gates AJ, Appia-Ayme C, Rowley G, Richardson DJ. Copper control of bacterial nitrous oxide emission and its impact on vitamin B\textsubscript{12}-dependent metabolism. \textit{Proc Natl Acad Sci U S A} 2013;110:19926–19931.

18. Wunsch P, Zumft WG. Functional domains of NosR, a novel transmembrane iron-sulfur flavoprotein necessary for nitrous oxide respiration. \textit{J Bacteriol} 2005;187:1992–2001.

19. Riester J, Zumft WG, Kroneck PM. Nitrous oxide reductase from \textit{Pseudomonas stutzeri}. Redox properties and spectroscopic characterization of different forms of the multicopper enzyme. \textit{Eur J Biochem} 1989;178:751–762.

20. Zumft WG. Biogenesis of the bacterial respiratory Cu\textsubscript{c}, Cu-S enzyme nitrous oxide reductase. \textit{J Mol Microbiol Biotechn} 2005;10:154–166.

21. Bennett SP, Soriano-Laguna MJ, Bradley JM, Svistunenko DA, Richardson DJ et al. NosL is a dedicated copper chaperone for assembly of the Cu\textsubscript{c} center of nitrous oxide reductase. \textit{Chem Sci} 2010;1:4985–4993.

22. Saunders NF, Hornberg JJ, Reijnders WN, Westerhoff HV, de Vries S et al. The NosX and NirX proteins of \textit{Paracoccus denitrificans} are functional homologues: their role in maturation of nitrous oxide reductase. \textit{J Bacteriol} 2000;182:5211–5217.

23. Zhang L, Trncik C, Andrade SLA, Einsle O. The flavinyl transference ApbE of \textit{Pseudomonas stutzeri} matures the NosR protein required for nitrous oxide reduction. \textit{Biochim Biophys Acta Bioenerg} 2017;1858:95–102.

24. Vishniac W, Santer M. The thiobacilli. \textit{Bacteriol Rev} 1957;21:195–213.

25. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of proteindye binding. \textit{Anal Biochem} 1976;72:248–254.

26. Ghosh S, Gorelsky SI, Chen P, Cabrito I, Moura JG et al. Activation of N\textsubscript{2}O reduction by the fully reduced μ\textsubscript{4}-sulfide bridged tetranuclear Cu\textsubscript{b} cluster in nitrous oxide reductase. \textit{J Am Chem Soc} 2003;125:15708–15709.

27. Kristjansson JK, Hollocher TC. First practical assay for soluble nitrous oxide reductase of denitrifying bacteria and a partial kinetic characterization. \textit{J Biol Chem} 1980;255:704–707.

28. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. \textit{Nucleic Acids Res} 2001;29:e45–.

29. Coyle CL, Zumft WG, Kroneck PM, Körner H, Jakob W. Nitrous oxide reductase from denitrifying \textit{Pseudomonas putida}. Purification and properties of a novel multicopper enzyme. \textit{Eur J Biochem} 1985;153:459–467.

30. Prudêncio M, Pereira AS, Tavares P, Besson S, Cabrito I et al. Purification, characterization, and preliminary crystallographic study
of copper-containing nitrous oxide reductase from *Pseudomonas nautica* 617. *Biochemistry* 2000;39:3899–3907.

31. Paraskevopoulos K, Antonyuk SV, Sawers RG, Eady RR, Hasnain SS. Insight into catalysis of nitrous oxide reductase from high-resolution structures of resting and inhibitor-bound enzyme from *Achromobacter cycloclastes*. *J Mol Biol* 2006;362:55–65.

32. Dell’Acqua S, Pauleta SR, Moura JJG, Moura I. Biochemical characterization of the purple form of Marinobacter hydrocarbonoclasticus nitrous oxide reductase. *Philos Trans R Soc Lond B Biol Sci* 2012;367:1204–1212.

33. Bertsova YV, Fadeeva MS, Kostyrko VA, Serebryakova MV, Baykov AA et al. Alternative pyrimidine biosynthesis protein ApbE is a flavin transferase catalyzing covalent attachment of FMN to a threonine residue in bacterial flavoproteins. *J Biol Chem* 2013;288:14276–14286.

34. Boyd JM, Endrizzi JA, Hamilton TL, Christopherson MR, Mulder DW et al. FAD binding by ApbE protein from *Salmonella enterica*: a new class of FAD-binding proteins. *J Bacteriol* 2011;193:887–895.

35. Hayashi M, Nakayama Y, Yasui M, Maeda M, Furuishi K et al. FMN is covalently attached to a threonine residue in the NqrB and NqrC subunits of Na (+)-translocating NADH-quinone reductase from *Vibrio alginolyticus*. *FEBS Lett* 2001;488:5–8.

36. Chan YK, McCormick WA, Watson RJ. A new nos gene downstream of nosDFY is essential for dissimilatory reduction of nitrous oxide by *Rhizobium* (*Sinorhizobium*) *meliloti*. *Microbiology* 1997;143:2817–2824.

37. Wunsch P, Körner H, Neese F, van Spanning RJM, Kroneck PMH et al. NosX function connects to nitrous oxide (N₂O) reduction by affecting the Cu⁺ center of NosZ and its activity in vivo. *FEBS Lett* 2005;579:4605–4609.

38. Carreira C, Pauleta SR, Moura I. The catalytic cycle of nitrous oxide reductase - The enzyme that catalyzes the last step of denitrification. *J Inorg Biochem* 2017;177:423–434.

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