

Analysis of multiple haloarchaeal genomes suggests that the quinone-dependent respiratory nitric oxide reductase is an important source of nitrous oxide in hypersaline environments.

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Summary

Microorganisms, including Bacteria and Archaea, play a key role in denitrification, which is the major mechanism by which fixed nitrogen returns to the atmosphere from soil and water. Whilst the enzymology of denitrification is well understood in Bacteria, the details of the last two reactions in this pathway, which catalyse the reduction of nitric oxide (NO) via nitrous oxide (N₂O) to nitrogen (N₂), are little studied in Archaea, and hardly at all in haloarchaea. This work describes an extensive interspecies analysis of both complete and draft haloarchaeal genomes aimed at identifying the genes that encode respiratory nitric oxide reductases (Nors). The study revealed that the only *nor* gene found in haloarchaea is one that encodes a single subunit quinone dependent Nor homologous to the qNor found in bacteria. This surprising discovery is considered in terms of our emerging understanding of haloarchaeal bioenergetics and NO management.

1. INTRODUCTION

1.1. Denitrification and nitric oxide reductases.

Denitrification is a form of anaerobic respiration found in microorganisms and some fungi that plays an important role in the biogeochemical nitrogen cycle because of its potential to regenerate atmospheric nitrogen (N₂) from oxidized inorganic nitrogen compounds such as nitrate (NO₃⁻) and nitrite (NO₂⁻) (Berks, 1995; Zumft, 1997; Zumft and Körner, 1997; Zumft and Kroneck, 2006). During the denitrification process, two nitrate ions are sequentially reduced to N₂ in a series of four reactions, each catalysed by a metalloenzyme, that consume a total of ten electrons (Fig. 1). Under anaerobic conditions these four respiratory metalloenzymes, nitrate-(Nar), nitrite-(Nir), nitric oxide-(Nor) and nitrous oxide-(Nos) reductases, replace the terminal oxidases of the aerobic electron transport chain (Richardson and Watmough, 1999; Lledó *et al.*, 2004).

Nitric oxide (NO), is the product of the reaction catalysed by the respiratory nitrite reductases (Nir) and a free intermediate in the bacterial denitrification pathway (Carr and Ferguson, 1990). NO is also a potent cytotoxin and consequently bacteria must control the levels and activity of the NO-generating (Nir) and consuming (Nor) reactions to maintain steady-state [NO] at <50 nM (Poole, 2005; Bergaust *et al.*, 2012). The absence of a functional Nor leads to accumulation of NO and has been reported as lethal (Poole, 2005; Falk *et al.*, 2010). However, this detoxification leads directly to the production of the potent greenhouse gas nitrous oxide (N₂O).

The bacterial respiratory Nors can be classified according to the number of subunits in the respiratory complex, the nature of the immediate electron acceptor and the length of the polypeptide harbouring the bimetallic heme/non-heme iron active site (Zumft, 2005). There are three main groups

i) short-chain respiratory NORs (scNORs) These are characterised by a catalytic subunit, NorB, that is an integral membrane protein of *ca* 450 amino acids which forms a complex with a membrane anchored *c*-type cytochrome (NorC) that is the electron receiving domain. These NorBC complexes are also known as cNORs to reflect the fact that they receive their electrons from soluble *c*-type cytochromes and/or cuprodoxins (Hendriks *et al.*, 2000; Thorndycroft *et al.*, 2007).

ii) long-chain respiratory NORs (lcNors) These have a single subunit, NorZ, containing approximately 780 amino acids. Also referred as qNors, these enzymes accept electrons directly from the reduced quinol pool rather than from cytochrome *c*, and therefore lack the NorC subunit (Hendriks *et al.*, 2000). The single subunit has a catalytic C-terminal domain that is substantially homologous to NorB, which is fused to a N-terminal extension that is proposed to mediate electron transfer from quinol (Cramm *et al.*, 1999).

iii) Cu₄Nor This is a distinctive two-subunit enzyme recently described in *Bacillus azotoformans* that is characterised by a catalytic subunit whose structure is closer to that of

cytochrome ba_3 terminal oxidase than to NorB. Instead of a c-type heme subunit II contains a dinuclear Cu_A site which receives electrons from the physiological donor, cytochrome c_{551} (Suharti *et al.*, 2001, Al-Attar and de Vries 2015).

1.2. Denitrification in Archaea.

The regulation and enzymology of denitrification have been extensively studied in Bacteria (Berks *et al.*, 1995, Zumft, 1997, Richardson and Watmough, 1999, Spiro, 2012). In comparison, Archaeal denitrification remains poorly understood because of those species of Archaea grown in laboratory cultures only a few are capable of respiring NO_3^- (Offre *et al.*, 2013), or NO_2^- (Völkl *et al.*, 1993; Martínez-Espinosa *et al.*, 2006; Nájera-Fernández *et al.*, 2012). These NO_3^-/NO_2^- respiring Archaea include several species of an evolutionarily distinct class known as haloarchaea whose members express proteins that are adapted to enable them to thrive in hypersaline environments (Fukuchi *et al.*, 2003; Oren, 2008; 2013a, 2013b; Longo *et al.*, 2013; Reed *et al.*, 2013; Ortega *et al.*, 2015).

With the exception of the nitrate (Nar) and nitrite reductases (Nir) from some members of the genera *Haloferax* and *Haloarcula* (Hochstein and Lang, 1991; Inatomi and Hochstein, 1996; Ichiki *et al.*, 2001; Yoshimatsu *et al.*, 2002; Lledó *et al.*, 2004; Martínez-Espinosa *et al.*, 2006; Martínez-Espinosa *et al.*, 2007; Bonete *et al.*, 2008; Esclapez *et al.*, 2013; Torregrosa-Crespo *et al.*, 2016), the enzymes of denitrification in the haloarchaea, and by extension Archaea, have not been characterised to the same extent as their bacterial counterparts. For example, just one Archaeal NOR, a menaquinone dependent NO reductase from *Pyrobaculum aerophilum* that contains o-type rather than b-type hemes, is described in the literature (de Vries *et al.*, 2003).

To date neither NO- (Nor) or N_2O -reductase (Nos) has been isolated from any species of haloarchaea. However, both *Haloferax denitrificans* and *Haloferax mediterranei* grown anaerobically on NO_3^- can generate N_2O and/or N_2 (Tindall *et al.*, 1989, Mancinelli & Hochstein 1986, Bonete *et al.*, 2008). Gas formation is not only consistent with the ability of these two

species to reduce NO but also the presence of a gene encoding qNOR in the type species for the genus *Haloferax volcanii* (Zumft and Kroneck, 2006). Otherwise, the type(s) of respiratory Nor found in Haloarchaea and their relationship to the well-characterised bacterial enzymes has been uncertain.

Over the past 15 years, an increasing number of completed or partially sequenced genomes have provided an alternative basis on which to understand the respiratory networks of haloarchaea. Direct interrogation of these genomes avoids the problems associated with erroneous and inconsistent automated annotations and reveals that the *nor* gene found in 96 species of haloarchaea may have been recently acquired and encodes a protein closely related to the bacterial qNORs. This discovery is considered in the context of both the bioenergetics of haloarcheal denitrification and the potential contribution of this class of micro-organism to N₂O emission from hypersaline environments.

2. RESULTS AND DISCUSSION

2.1. The respiratory NOR of *Hfx. mediterranei* is a qNor related to bacterial qNors.

Inspection of the annotated *Hfx. mediterranei* genome revealed a chromosomal gene encoding for a putative nitric oxide reductase that is annotated as *norB* (Han *et al.*, 2012; Becker *et al.*, 2014). This notation is usually reserved for genes that encode the catalytic subunit (NorB) of a bacterial scNOR. Although, *norB* genes are invariably found in the same transcriptional unit as *norC*, (a gene that encodes NorC), there was no evidence of *norC*, or any homologue, in the *Hfx. mediterranei* genome. Moreover, the “*norB*” gene encodes a protein of 761 amino acids, which has a molecular mass that is more typical of a long-chain bacterial qNor than NorB.

To determine the relationship between the *Hfx. mediterranei* Nor and the bacterial lcnors the derived amino acid sequence of the *Hfx. mediterranei* respiratory NOR was used as a query in a BlastP search of the bacterial databases and a subsequent ClustalW alignment.

Several authentic bacterial qNORs exhibit significant homology (>72% identity) with the *Hfx. mediterranei* respiratory NOR (Fig. S1, supporting information). These include two biochemically well characterised enzymes from *Cupriavidus necator* (Cramm *et al.* 1999) and *Geobacillus stearothermophilus* (Salomonson *et al.*, 2012; Shiro, 2012; Matsumoto *et al.*, 2012; Terasaka *et al.*, 2014).

The high degree of sequence conservation allowed the published coordinates of the *G. stearothermophilus* qNor (PDB 3AYF or 3 AYG (in complex with N-oxo-2-heptyl-4-Hydroxyquinoline (HQNO)) to be used as the basis of a model of the *Hfx. mediterranei* enzyme. The model reveals that three key features observed in the *G. stearothermophilus* qNor structure are well conserved in the *Hfx. mediterranei* enzyme; (i) the residues contacting the bound inhibitor HQNO; (ii) a water channel lined by several conserved hydrophilic residues leading from the cytoplasm to the active site (Fig. 2A); (iii) a hydrophilic domain in the N-terminus that lacks a consensus sequence for binding either a c-type heme or Cu_A (Fig. 2B).

HQNO is an inhibitor of several quinone-dependent oxido-reductases and suggests the presence of a quinone binding site which along with the lack of a consensus sequence for a metal centre in the N-terminal domain suggest that the *Hfx. mediterranei* enzyme is a qNOR. Presumably the substrate protons are derived from the quinol substrate and the water channel is there to move the co-product water (Fig. 1) away from the active site. These structural similarities along with the apparent absence of any proton conducting channels support the proposition that the *Hfx. mediterranei* respiratory NO-reductase is a non-electrogenic single subunit qNOR closely related to the bacterial NorZs that derive their electrons directly from the quinone pool.

2.2. How widespread are qNors in haloarchaea?

Interrogation of 141 complete and draft haloarchaeal genomes (Table S1, supporting information) using the *Hfx. mediterranei* qNOR protein sequence as a query revealed the presence of single *norZ* orthologue in 96 species (Fig. 3). The outputs from these analyses

(Table S2, supporting information) showed that *norZ* of *Hfx. mediterranei* has >90% identity with the predicted sequences of the respiratory NOR found in other species of *Haloferax* (Fig. 3; Fig. S2, supporting information), >85% identity to the enzymes found in the genus *Haloarcula* and between 60% and 83% identity to the derived amino acid sequences of the *nor* genes found in other haloarchaeal genera including *Natrinema*, *Halobacterium*, *Halococcus*, *Halobiforma* and *Haloterrigena*. Genes encoding NorZ like proteins were not identified in the following genera: *Haloquadratum* (6 strains analysed), *Natronorrubrumi* (6 strains analysed) and *Halorrrhabdus* (3 species analysed).

The gene encoding qNOR was correctly annotated in just 10 of the 96 genomes containing *norZ*. For example, in the available genomes from the genus *Haloarcula* the annotations are essentially accurate (product="nitric oxide reductase, NorZ apoprotein"; function="nitric oxide reductase, NorZ apoprotein"). Examination of the annotated GeneBank files allowed the identification of three common errors that lead to the incorrect annotation or apparent absence of *norZ*: i) the ORF is correctly identified as a respiratory Nor but incorrectly annotated; ii) the ORF is well described, but not annotated as a respiratory Nor; iii) the identification of other potential ORFs in a region of the genome where there is clear evidence of a *norZ* gene due to errors in identifying initiation or termination codons (Table S1, supporting information). It is suggested that the annotation "*norZ*" is used to identify a gene encoding qNor, the single subunit lcNor (NorZ) in both bacteria and haloarchaea, rather than the alternative *qnorB* (Cramm *et al.*, 1997; Casciotti and Ward, 2005). This avoids confusion with *norB*, an annotation that should be reserved for genes encoding the catalytic subunit (NorB) of the two-subunit bacterial scNORs.

2.3. Organization of haloarchaeal genomes in the region of *norZ*.

The organization of ORFs around the *norZ* gene was examined in both fully sequenced and draft haloarchaeal genomes from ten species that are representative of the genera *Haloarcula*, *Halobiforma*, *Halococcus*, *Haloferax*, *Halogeometricum*, *Halopiger*, *Halorubrum*,

Haloterrigena, *Natrialba* and *Natrinema*. Although there is little evidence of conservation in this region of the genome, it appears that a gene encoding the respiratory nitrite reductase (NirK) is frequently found close to *norZ* (Fig. 4) along with a gene coding for a peripheral membrane protein known as halocyanin which is a cupredoxin (Mr ~ 15.5 kDa) that is believed to serve as a mobile electron carrier on outer face of the cytoplasmic membrane (Scharf and Engelhard, 1993; Mattar *et al.*, 1994). In principle, halocyanin could serve as the physiological electron donor to both NirK and other Cu-containing proteins involved in denitrification such as nitrous oxide reductase (Nos). This arrangement is not entirely unexpected as co-expression of *nirK* (its cognate electron donor) and *norZ*, as found in Bacteria, is desirable to avoid cytotoxic steady state levels of nitric oxide during active denitrification (Gómez-Hernández *et al.*, 2011).

The considerable diversity in the organization of genes around *norZ* in haloarchaea could be accounted for by the recent acquisition of *norZ* through horizontal gene transfer and subsequent recombination events. Evidence for two potential examples of duplication through or homologous recombination were identified in the genera *Halopiger*, *Haloterrigena* and *Natrialba* (Tables S1 and S2, supporting information). Each of the species belonging to the genera *Halopiger* and *Haloterrigena* that were examined have a copy of *norZ* flanked by the same cluster of four genes involved in heme biosynthesis. One of these genes is *hemL* which encodes glutamate-1-semialdehyde aminotransferase and is incorrectly annotated as "product=nitric oxide reductase subunit B" (Tables S1 and S2, supporting information). Three species, *Halopiger aswanensis* DSM-13151 (scaffold Ga0074794_103), *Halopiger* sp. IIH3 (scaffold Ga0036367_109) and *Haloterrigena salina* JCM-13891 (scaffold AOIS01000063) contain a second copy of *norZ* that is not surrounded by the heme biosynthesis genes. Given that duplication of *norZ* appears to be an infrequent event in haloarchaeal genomes, it is suggested that where an extra copy exists it has arisen as the result of a recent incorporation.

In the case of the *Natrialba aegyptia* DSM-13077 (scaffold AOIP01000040) genome the genes located both upstream and downstream of each of the two copies of *norZ* are unlike those found in any other haloarchaeal species that we examined. One copy of *norZ* is close to a nitrate reductase (*nar*) like gene, whilst the second copy is close to a gene encoding XerD which is a serine recombinase-like protein involved in intraspecific homologous recombination processes (Mau *et al.*, 2006). Typically, XerD is found flanking a recombination target site or as part of the sequences to be recombined allowing it both to promote exchanges between the core genome and accessory regions and incorporating new genetic elements to synthetic regions. XerD-like proteins are also abundant in those regions of high genomic interchange, like those belonging to genomic islands (Fernández Gómez *et al.*, 2012; Bellanger *et al.*, 2014-3).

2.4. Haloarchaea represents a significant class of NO-reducers/N₂O producers.

The increase in the number of available haloarchaeal genomes makes it possible to analyse their genetic organization and conduct *in silico* studies to identify functional proteins. Unfortunately, this is not straightforward for three reasons: i) most genome sequences are incomplete and/or not fully annotated; ii) the gene annotations are not always correct; iii) where *nor* genes are found the conventions of nomenclature are not consistently applied. Consequently, identifying genes based solely on inspection of the annotated genomes is not possible and it was necessary to adopt a rigorous two step *ab initio* bioinformatic approach to identify *nor* genes in haloarchaea and classify the proteins they express.

Many existing gene annotations imply that haloarchaeal nitric oxide reductase genes are related to bacterial *norB* genes that encode the large subunit of the NorBC complex. The results presented here clearly show that this is not the case and that the respiratory nitric oxide reductases found in haloarchaea are closely related to the single subunit long-chain quinol-dependent bacterial enzymes (qNor) encoded by *norZ*. Although *norZ* genes are widely distributed amongst the haloarchaea, the organisation of the genome around the *norZ* gene is

not conserved. These differences in genetic organization suggest that the ability to reduce NO to N₂O is a trait that has been acquired recently and the genomic variation is due to subsequent recombination and duplication events. It is suggested that the annotation of these haloarchaeal genomes is reviewed to clearly identify these nitric oxide reductase genes as *norZ*.

It is not clear why haloarchaea express a qNOR rather than a NorBC complex, but there may be two important bioenergetic considerations. Firstly, nitrate reduction in haloarchaea is catalysed by an energy conserving nitrate reductase (pNar) that has not been reported in bacteria (Martínez-Espinosa *et al.*, 2007). The product of this reaction, nitrite, is reduced either enzymatically by *nirK*, or abiotically to form NO which, in the absence of any NO-consuming enzyme, would rapidly accumulate to cytotoxic levels. Co-expression of qNor and NirK counteracts this by catalysing the reduction of NO₂⁻ via NO to form N₂O which is relatively benign. The advantage for haloarchaea of *nirK* and *norZ* is that they are both discrete genetic units that express catalytically active enzymes that allow the organism to denitrify without any need for any complex maturation processes such as those associated with cytochrome c maturation and heme *d*₁ biosynthesis in mesophilic bacteria (Watmough *et al.*, 2009). This feature may explain the abundance of qNOR in non-denitrifying strains of isolates from freshwater and marine sediments (Bracker and Tiedje, 2003).

The second consideration is that although both qNOR and NorBC terminate electron transfer chains there is no evidence for either enzyme being electrogenic (Bell *et al.*, 1992; Salomonsson *et al.*, 2012). However, bacteria that express NorBC derive electrons for NO reduction from the cytochrome *bc*₁ complex which catalyses a protonmotive Q-cycle that couples NO detoxification to energy conservation (Watmough *et al.*, 2009). A qNor deriving electrons directly from the quinone pool would bypass the *bc*₁ complex limiting an organism's ability to conserve energy when respiring NO. This limitation may be overcome by

haloarchaea through expression of the energy conserving pNar (Martínez-Espinosa *et al.*, 2007).

Haloarchaea inhabit large areas across the planet including salt marshes, salty ponds and saline lagoons and are the dominant group of microorganisms in these ecosystems. Saline environments are increasingly polluted by nitrate and nitrite because of anthropogenic activities (Martínez-Espinosa *et al.*, 2011). The ability of haloarchaea to respire nitrate and nitrite together with their potential to express a qNOR capable of reducing nitric oxide suggest they may contribute to the production of the potent greenhouse gas N₂O. The ubiquity of nitric oxide reductases in a significant majority of haloarchaea genera along with the crucial role that this class plays in maintaining their ecosystems suggests that the contribution of haloarchaea to global N₂O emissions may be greater than previously thought.

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Legends to figures:

Fig. 1: Summary of the four key reactions involved in denitrification. The pathway occurs under microaerobic/anaerobic conditions. The four respiratory metalloenzymes, nitrate-(Nar), nitrite-(Nir), nitric oxide-(Nor) and nitrous oxide-(Nos) reductases, replace the terminal oxidases of the aerobic electron transport chain (Richardson and Watmough, 1999; Lledó *et al.*, 2004).

Fig. 2: Structural model of the qNOR encoded by *Haloferax mediterranei*. Panel A: Structure of the quinol binding site and the water channel. Panel B: Structure of the N-terminal hydrophilic domain. The published coordinates of the *G. stearothermophilus* qNOR as isolated (PDB 3AYF) or complexed with HQNO (PDB 3AYG) were used as the basis for the homology model of the *Hfx. mediterranei* enzyme (Matsumoto *et al.*, 2012).

Fig. 3: Cladogram of genes encoding Nor-like proteins in Haloarchaea. The outer circles represent *norZ* and the inner circles represent *norB*. The presence of a gene encoding a Nor like protein is indicated by a filled symbol, whereas an open white circle indicates the absence of

such a gene. Note that the inner circles are open in most species indicating the absence of *norB* genes in most Haloarchaea. *Sulfolobus islandicus* has been included as the root of the cladogram.

Fig. 4: Organization of the genome around the genes encoding copper containing nitrite reductase (*nirK*) and qNor (*norZ*) in selected Haloarchaeal species. Arrows show the direction of transcription, but the genes are not drawn to scale. The genes shown are assigned as follows: *arsR*: Putative transcriptional regulator, ArsR family; *cbr*: Carotene biosynthesis associated membrane protein; *cofG*: FO synthase subunit 1; *cox*: Cytochrome c oxidase subunit I; *csd*: Cysteine desulfurase; *fhu*: ferrichrome-binding protein; *gdhA1*: Glutamate dehydrogenase (NAD(P)+); *gluTR*: Glutamil-tRNAGlu reductase; *hcy*: Halocyanin precursor-like protein; *hth*: HTH DNA binding domain family protein; HYP: Hypothetical protein; *lclR*: lclR-like transcriptional regulator; *lip*: Lipoprotein; *mcoA*: Multicopper oxidase; *mscS*: Mechanosensitive ion channel; *nirK*: Copper containing nitrite reductase ; *norZ*: quinol dependent nitric oxide reductase, NorZ apoprotein; *oxr*: Predicted Fe-S oxidoreductase; *pbp*: Pterin cluster protein; *phd*: Phytoene desaturase; *pqqE*: Coenzyme PQQ synthesis protein; *sce*: Sodium/calcium exchanger membrane region; *sdr*: Short-chain dehydrogenase/reductase; *ubiA*: UbiA prenyltransferase. The authors have retained the existing annotations found in the databases for the following genes: *gdhA1* and *arsR* in *Hfx. mediterranei*; *mcoA* and *nirK* in *Hfx. volcanii*, and *cofG* in *Halorubrum lacusprofundi*. The remaining of the genes have been annotated by the authors in this work based on their predicted biological function to avoid confusion.

Supporting information

Additional supporting information may be found in the online version of the article at the publisher's website:

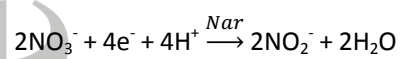
Table S1. Genomes database. It summarises the main details about the haloarchaeal genomes included in this study (27 completely sequenced genomes and 114 draft or permanent drafts status)

Table S2. BlastN and BlastP qNor results.

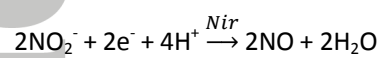
Fig. S1: Comparison of nitric oxide reductase amino acid sequences A) *Haloferax mediterranei* nitric oxide reductase (Nor) sequence is aligned with five bacterial and one archaeal qNor. The enzymes from *Geobacillus stearothermophilus* (Matsumoto et al., 2012), *Synechocystis* sp. (Büsch et al., 2002) and the hyperthermophilic denitrifying archaeon *Pyrobaculum aerophilum* (de Vries et al., 2003) have been isolated and characterized. The enzymes from *Staphylococcus aureus*, *Cupriavidus necator*, *Neisseria sicca* have been assigned as qNor by sequence similarity (Hendriks et al., 2000; Braker and Tiedje, 2003; Heylen et al., 2007); B) qNOR enzymes are also aligned with NorB and NorC from *Pseudomonas aeruginosa* and *Paracoccus denitrificans* (Hino et al., 2010). The residues involved in binding the *c*-type heme in cNor are highlighted in blue. The residues in red are in the same position as the bulky residues filling the potential *c*-type heme binding site in qNor from *Geobacillus stearothermophilus* (Matsumoto et al., 2012), and would hinder heme binding; C) The quinol binding site. The residues in the same position of thus involved in the interaction with the quinol analogue, 2-heptyl hydroxyquinoline N-oxide, in *Geobacillus* are highlighted in red *stearothermophilus* (Matsumoto et al., 2012).

Fig S2. qNor Heatmap. Summary of the percentage of strains per specie showing significant hits with regard to the 8 references considered (see experimental procedures. Supporting information).

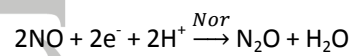
Experimental procedures section



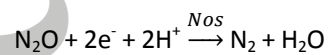
(Reaction 1)



(Reaction 2)



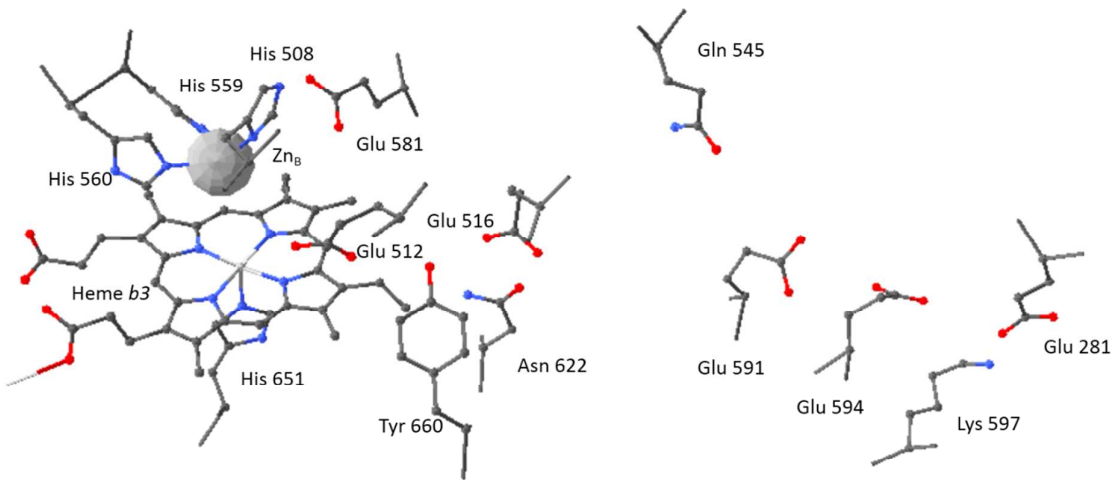
(Reaction 3)



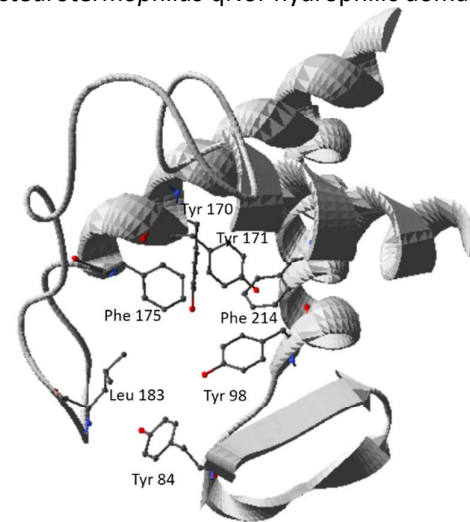
(Reaction 4)



Active site and water channel: *G. stearotermophilus* qNor



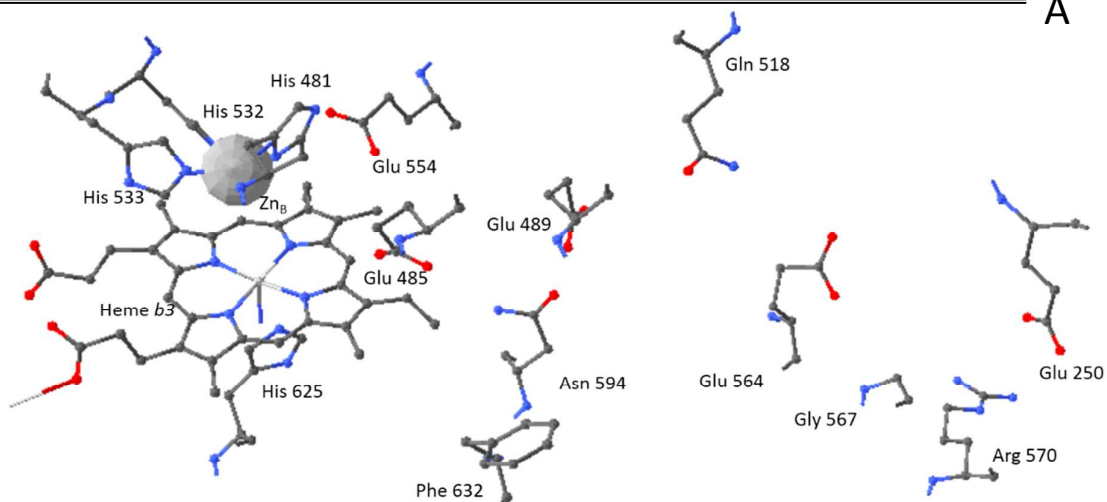
G. stearotermophilus qNor hydrophilic domain



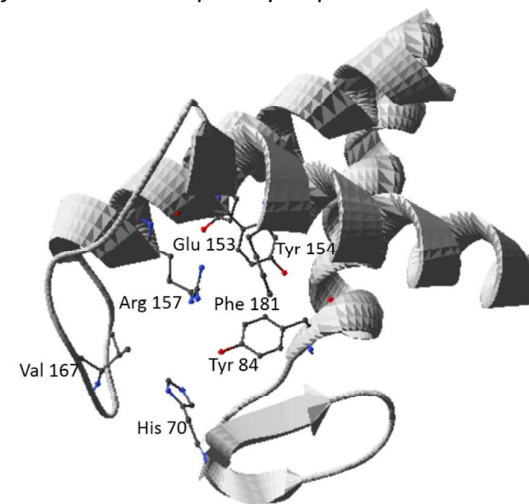
B

A

Hfx. mediterranei qNor hydrophilic domain



Active site and water channel: *Hfx. mediterranei* qNor



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Fig. 3.

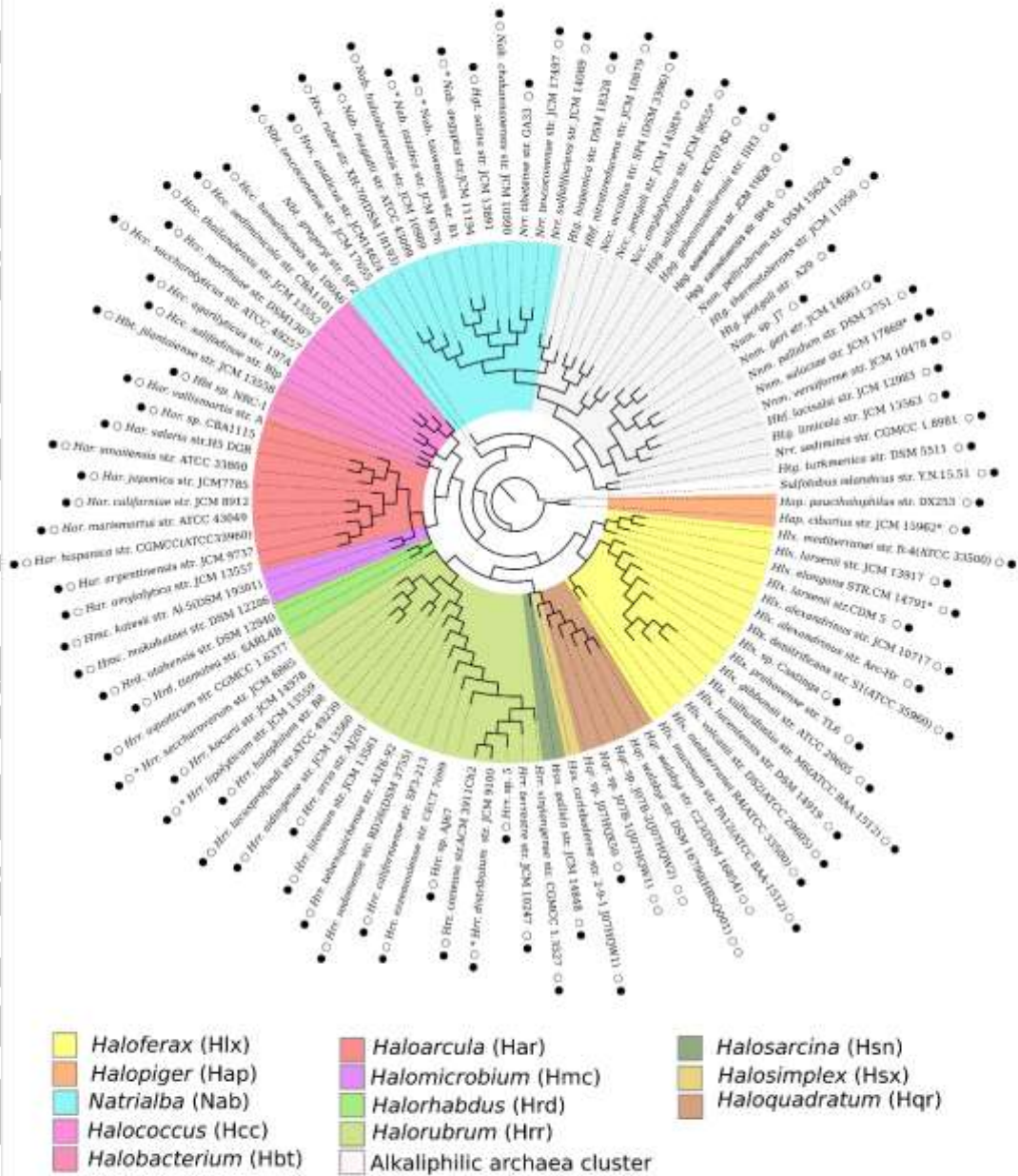
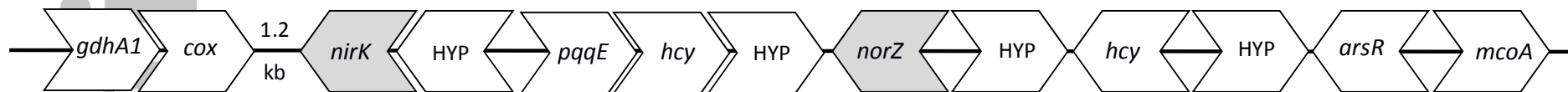
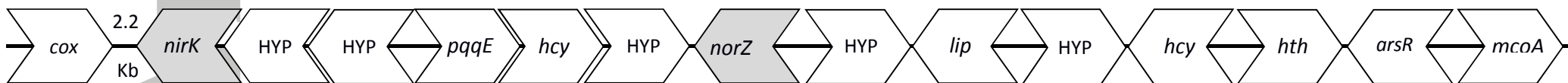


Fig. 4

Haloferax mediterranei R-4, ATCC 33500. Chromosome.



Haloferax volcanii DS2, ATCC 29605. Chromosome.



Halogeometricum borinquense PR3, DSM 11551



Halorubrum lacusprofundi ATCC 49239

