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## A composite biochemical system for bacterial nitrate and nitrite assimilation as exemplified by *Paracoccus denitrificans*

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1 **A composite biochemical system for bacterial nitrate and nitrite assimilation as**  
 2 **exemplified by *Paracoccus denitrificans***

3  
 4 Andrew J. Gates\*<sup>†1</sup>, Victor M. Luque-Almagro<sup>‡1</sup>, Alan D. Goddard<sup>§</sup>, Stuart J. Ferguson<sup>§</sup>, M. Dolores  
 5 Roldán<sup>‡</sup>, David J. Richardson\*<sup>†2</sup>

6  
 7 \*Centre for Molecular and Structural Biochemistry, University of East Anglia, Norwich Research Park,  
 8 Norwich, NR4 7TJ, UK., <sup>†</sup>School of Biological Sciences, University of East Anglia, Norwich Research  
 9 Park, Norwich, NR4 7TJ, UK., <sup>‡</sup>Departamento de Bioquímica y Biología Molecular, Universidad de  
 10 Córdoba, Edificio Severo Ochoa, 1ª planta, Campus de Rabanales, Córdoba, 14071, Spain.,  
 11 <sup>§</sup>Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, UK.

12  
 13 <sup>1</sup>The authors have contributed equally to this work.

14  
 15 <sup>2</sup>To whom correspondence should be addressed (d.richardson@uea.ac.uk).

16  
 17 **Running title:** Nitrate and nitrite assimilation in *Paracoccus denitrificans*.

18  
 19 **Keywords:** nitrate reductase, nitrite reductase, nitrate transport, *Paracoccus denitrificans*.

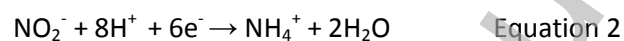
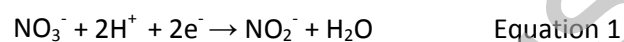
20  
 21 **Abbreviations used:** Nas, assimilatory nitrate reductase; Nar, membrane-bound nitrate reductase;  
 22 Nap, periplasmic nitrate reductase; FAD, flavin-adenine dinucleotide; NAD(P)<sup>+</sup> and NAD(P)H, oxidized  
 23 and reduced nicotinamide-adenine dinucleotide (phosphate); MV, Methyl Viologen;  $K_M$ , Michaelis  
 24 constant; O.D., optical density;  $\mu_{\max(\text{app})}$ , apparent maximum growth rate.

25  
 26 **The denitrifying bacterium *Paracoccus denitrificans* can grow aerobically or anaerobically using**  
 27 **nitrate or nitrite as the sole N-sources. The biochemical pathway responsible is expressed from a**  
 28 **gene cluster comprising a: nitrate/nitrite transporter (NasA), nitrite transporter (NasH), nitrite**  
 29 **reductase (NasB), ferredoxin (NasG) and nitrate reductase (NasC). NasB and NasG are essential for**  
 30 **growth with nitrate or nitrite as N-source. NADH serves as electron-donor for nitrate and nitrite**  
 31 **reduction, but only NasB has a NADH-oxidising domain. Nitrate and nitrite reductase activities**  
 32 **show the same  $K_M$  for NADH and can be separated by anion-exchange chromatography, but only**  
 33 **fractions containing NasB retain the ability to oxidise NADH. This implies that NasG mediates**  
 34 **electron-flux from the NADH-oxidising site in NasB to the sites of nitrate and nitrite reduction in**  
 35 **NasC and NasB, respectively. Delivery of extracellular nitrate to NasBGC is mediated by NasA, but**  
 36 **both NasA and NasH contribute to nitrite uptake. The roles of NasA and NasC can be substituted**  
 37 **during anaerobic growth by the biochemically-distinct membrane-bound respiratory nitrate**  
 38 **reductase (Nar), demonstrating functional overlap. *nasG* is highly conserved in nitrate/nitrite**  
 39 **assimilation gene clusters, consistent with a key role for the NasG ferredoxin, as part of a**  
 40 **phylogenetically widespread composite nitrate and nitrite reductase system.**

41  
 42  
 43 **INTRODUCTION**

44  
 45 The importance of inorganic nitrate as a key nutritional component of the global nitrogen cycle,  
 46 particularly for marine and freshwater autotrophic phytoplankton is long recognised. This highly  
 47 soluble anion can make a significant environmental impact, supporting accelerated biomass  
 48 formation or 'blooms' in nitrate and phosphate polluted water courses that may have an important  
 49 role as CO<sub>2</sub> sinks. Accordingly, the biochemistry of nitrate assimilation has been well studied in  
 50 cyanobacteria where assimilatory nitrate reduction is functionally linked to photosynthetic  
 51 processes, and both nitrate and nitrite reductases use photosynthetically reduced ferredoxin as

52 electron donor. By contrast, the utilisation of nitrate by heterotrophic bacteria has received less  
 53 attention. The ability of heterotrophic bacteria and archaea to metabolise nitrate or nitrite as the  
 54 sole nitrogen source (N-source) for growth is phylogenetically widespread. However, only a few  
 55 physiological, genetic and biochemical studies have been performed, most notably early studies on  
 56 *Enterobacter aerogenes* [1] and more recent studies on the  $\gamma$ -proteobacterium *Klebsiella oxytoca* [2],  
 57 the photoheterotroph *Rhodobacter capsulatus* [3], the Gram positive bacterium *Bacillus subtilis* [4]  
 58 and the diazotroph *Azotobacter vinelandii* [5]. In contrast to cyanobacteria the reductases from most  
 59 heterotrophic bacteria are thought to be dependent on the cytoplasmic reduced pyridine nucleotide  
 60 pool, which enables them to be coupled to organic carbon catabolism [6]. In fact, recent data  
 61 suggest heterotrophic bacterial species that can utilise nitrate for the biosynthesis of essential  
 62 cellular components during growth may also be significant consumers of inorganic nitrogen globally,  
 63 particularly in environments where there are high concentrations of dissolved organic carbon  
 64 relative to dissolved organic nitrogen [7-9]. This is due to the high bioenergetic demand for reducing  
 65 equivalents required for the assimilatory reduction of nitrate to ammonia, which requires eight  
 66 electrons:



69  
 70  
 71  
 72 Consequently assimilatory nitrate reduction is a good route for disposal of the excess reductant  
 73 present in a reduced organic carbon pool [10]. However, the biochemical mechanism by which  
 74 bacterial assimilatory nitrate reductases access this pool of cellular reductant is not well understood,  
 75 particularly because analysis of the primary structure of heterotrophic bacterial assimilatory nitrate  
 76 reductases suggests that they do not have a NAD(P)H binding domain [2, 11].  
 77

78 In addition to being a substrate for nitrogen assimilation in heterotrophs, nitrate can also be  
 79 a substrate for anaerobic respiration, for example in denitrifying bacteria that can reduce nitrate, via  
 80 nitrite, nitric oxide and nitrous oxide, to dinitrogen gas and enterobacteria that can reduce nitrate to  
 81 ammonium [1, 12]. One of the paradigm heterotrophic denitrifiers, *Paracoccus denitrificans*  
 82 synthesises two heterotrimeric respiratory ubiquinol/nitrate oxidoreductases, Nar and Nap (Fig. 1).  
 83 The membrane-bound enzyme (NarGHI) reduces nitrate as the first step of growth-linked anaerobic  
 84 denitrification while the other, a periplasmic system (NapABC), serves to dissipate excess reducing  
 85 equivalents formed during aerobic growth [12, 13]. These enzymes have been studied at the  
 86 biochemical level and derive electrons from the membrane-confined ubiquinol pool [14, 15], which  
 87 can be coupled to NADH generated from oxidative metabolism via the NADH-ubiquinone  
 88 oxidoreductase. In *P. denitrificans* NarGHI, the active site for nitrate reduction is exposed to the  
 89 cytoplasm and therefore it is dependent on a nitrate transport protein NarK, a fusion protein of two  
 90 transmembrane domains NarK1 and NarK2, to deliver nitrate into the cell (Fig. 1). These two  
 91 functional components have putative roles in nitrogen oxyanion trafficking: NarK1 is a proposed  
 92 proton-linked nitrate importer, and NarK2 is a putative nitrate/nitrite antiporter [16, 17].  
 93

94 *Paracoccus* species can also assimilate nitrate via a third cytoplasmic reductase that has not  
 95 yet been characterised, but is known to be distinct from the two respiratory systems [18]. In general,  
 96 a bacterial nitrate assimilation system (Nas) involves a cytoplasmic molybdenum-dependent nitrate  
 97 reductase that reduces nitrate to nitrite (Equation 1), which is further reduced to ammonium by a  
 98 sirohaem-dependent nitrite reductase (Equation 2) [2]. Like the respiratory Nar system, the  
 99 cytoplasmic assimilatory system is also dependent on nitrate transport into the cell. However, a  
 100 major biochemical conundrum is that it is not clear how the assimilatory nitrate reductase is coupled  
 101 to NADH oxidation. This is because primary sequence analysis of bacterial assimilatory nitrate  
 102 reductases suggests that, in contrast to the nitrite reductases, they do not possess an NADH binding

103 domain [2, 11]. The absence of such a site is unimportant for photoautotrophic metabolism in  
104 cyanobacteria, where the electron donor is ferredoxin that is photoreduced by photosystem I [19],  
105 but it is an important issue in organoheterotrophic metabolism where the nitrate reductase needs to  
106 be coupled to NADH released from oxidative metabolism of organic substrates. In this study we  
107 identify a key role for a putative Rieske-type [2Fe-2S] ferredoxin in *P. denitrificans* that is widely  
108 conserved in other bacterial Nas systems and show that this protein is essential for coupling of  
109 NADH oxidation to both nitrate and nitrite reduction. A three-component ferredoxin-nitrate-nitrite  
110 reductase system is proposed, where the ferredoxin mediates electron transfer from a single NADH-  
111 oxidising site within the nitrite reductase to the sites of nitrate and nitrite reduction present in the  
112 nitrite reductase and nitrate reductase components, respectively. Bioinformatic analysis of *nas* gene  
113 clusters suggests that this is a widespread mechanism amongst heterotrophic bacterial species and  
114 so provides the biochemical link between the nitrate reductase and the cytoplasmic NADH pool. In  
115 addition we demonstrate a degree of biochemical overlap between the assimilatory Nas system and  
116 the respiratory Nar system at the level of nitrate transport and reduction.

## 117 118 **EXPERIMENTAL**

### 119 120 **Bacterial strains, media and growth conditions**

121 All bacterial strains and plasmids used in this study are listed in Table 1. *P. denitrificans* was routinely  
122 cultured under aerobic conditions at 30 °C in either Luria-Bertani (LB) medium or a defined mineral  
123 salts medium with 50 mM succinate as the carbon source [20, 21]. Ammonium chloride (10 mM),  
124 potassium nitrate (20 mM), potassium nitrite (10 mM) or sodium L-glutamate (5 mM) were used as  
125 nitrogen sources. For aerobic batch culture, 50 ml volumes were rotated at 250 rpm in 250 ml flasks.  
126 During anaerobic growth, nitrate (30 mM) or nitrite also acted as the respective terminal electron  
127 acceptors for cellular respiration. The *Escherichia coli* strains were cultured aerobically on LB  
128 medium at 37 °C. Cell growth was followed by measuring the optical density (O.D.) of cultures at 600  
129 nm. Antibiotics were used at the following final concentrations ( $\mu\text{g}\cdot\text{ml}^{-1}$ ): ampicillin (Ap), 100;  
130 kanamycin (Km), 25; rifampicin (Rif), 100; spectinomycin (Spec), 25; streptomycin (Sm), 60;  
131 tetracycline (Tc), 10; gentamycin (Gm), 20; chloramphenicol (Cm), 50. Where shown in the  
132 manuscript Figs bacterial growth curves are presented as arithmetic plots to minimise distortion of  
133 small changes in biomass during the early stages of the growth curve. For determination of growth  
134 rates, curves were analysed as semi-log plots from which the apparent maximum growth rate,  
135  $\mu_{\text{max(app)}}$  was determined from the gradient of the exponential growth phase.

### 136 137 **Analytical methods**

138 For preparation of subcellular fractions, *P. denitrificans* strains were cultured in mineral salts  
139 medium with nitrate and glutamate in a BioFlo IV fermenter (New Brunswick Scientific, USA) that  
140 was maintained at 30 °C, pH 7.2 with dissolved  $\text{O}_2 > 95\%$ . Upon reaching an O.D.<sub>600</sub> value of approx.  
141 0.6, a 10 litre culture volume was harvested and cells and fractionated to prepare cytoplasmic  
142 fractions (supplementary information). Assimilatory nitrate or nitrite reductase activity was assayed  
143 spectrophotometrically at pH 7.5 in the presence of the following electron donors: NADH (100  $\mu\text{M}$ ),  
144 NAD(P)H (100  $\mu\text{M}$ ) or dithionite-reduced Methyl Viologen (MV) at 1 mM. Activity assays were  
145 performed in quartz cuvettes of 1 cm path length. The reactions were followed by measuring the  
146 decrease in absorbance observed over time at 340 nm for NADH (or NADPH) dependent oxidation  
147 rates, or at 600 nm when monitoring re-oxidation of the reduced viologen cation radical [22].  
148 Experiments were initiated by addition of  $\text{NaNO}_3$  and  $\text{NaNO}_2$ , as required. NADH dependent assays  
149 performed on cytoplasmic cell fractions showed a constant background oxidation that was  
150 proportional to the amount of extract used. This rate was unaffected when experiments were  
151 performed under strict anaerobic conditions and addition of  $\text{NAD}^+$  up to approx. 1 mM did not affect  
152 the rate of NADH-dependent nitrate or nitrite reduction. MV dependent rates were obtained under  
153 strict anaerobic conditions, using stock solutions of sodium nitrite and dithionite prepared and

154 stored anaerobically at 4 °C prior to use [15]. The following molar extinction coefficients,  $\epsilon_{340\text{ nm}} =$   
155  $6.22 \times 10^3 \text{ M}^{-1}\cdot\text{cm}^{-1}$  (NADH) and  $\epsilon_{600\text{ nm}} = 13.7 \times 10^3 \text{ M}^{-1}\cdot\text{cm}^{-1}$  (MV) were used to calculate specific  
156 activities. Extracellular nitrate concentration was determined using a Dionex ICS-900 HPLC system  
157 fitted with a DS5 conductivity detector and AS40 automated sampler. Samples were diluted into  
158 analytical reagent grade water (Fisher Scientific, total N <0.1 ppm) and passed through a 0.2  $\mu\text{m}$   
159 syringe filter prior to injection onto a 2 x 250 mm IonPac® AS22 analytical carbonate eluent anion-  
160 exchange column. Nitrite concentration present in the extracellular medium was determined  
161 colourmetrically as described by Nicholas and Nason [23]. For separation of nitrate and nitrite  
162 reductase activities by anion-exchange chromatography a DEAE-Sepharose™ (GE Healthcare) column  
163 matrix was equilibrated in 5 mM L-ascorbate, 5mM EDTA, 50 mM Tris-HCl, pH 7.5. The column was  
164 loaded with cytoplasmic extract (obtained from *P. denitrificans* WT cells grown with nitrate as sole  
165 N-source), washed with 2 column volumes and then developed with a linear gradient of 0-0.5 M  
166 NaCl, over 1 column volume, at 1.5 ml·min<sup>-1</sup> flow rate.

167  
168 Samples for two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) were prepared  
169 from *P. denitrificans* WT cells that were washed twice with 50 mM Tris-HCl (pH 8.0) and re-  
170 suspended in 10 mM Tris-HCl (pH 9.0) buffer solution containing DNaseI, RNaseA and protease  
171 inhibitors prior to lysis by sonication. Unbroken cells and cellular debris were removed by  
172 centrifugation (14000 x *g*) for 20 minutes at 4 °C. Supernatants were then subject to  
173 ultracentrifugation (40000 x *g*) for 1 hour at 4 °C to obtain soluble protein extracts. Protein was  
174 quantified using a 2-D Quant kit (GE Healthcare) according to the manufacturer's instructions and  
175 precipitated in 10% (w/v) trichloroacetic acid solution by incubation on ice for 2 hours, followed by  
176 centrifugation (14000 x *g*) for 20 minutes. Protein pellets were washed twice in the following  
177 solutions: 50 mM Tris-HCl (pH 8.0), 50 mM Tris (pH unadjusted) and 80% (v/v) ice-cold acetone. Final  
178 re-suspension was in 800  $\mu\text{l}$  of solubilisation buffer that contained 7 M urea, 2 M thiourea, 4% (w/v)  
179 3-[(3-cholamidopropyl)dimethylammonio]propane-1-sulphonic acid (CHAPS), 1% (w/v) Dithiothreitol  
180 (DTT), 1.5% (v/v) IPG buffer (pH range 4-7, GE Healthcare) and a trace of bromophenol blue. This  
181 mixture was vortexed for 2 hours and centrifuged (14000 x *g*) for 30 minutes after which the  
182 supernatant was recovered for use. Immobiline DryStrips (11 cm in length, pH range 4-7 from GE  
183 Healthcare) were rehydrated with 350  $\mu\text{g}$  of protein for 12 hours into IPTG strip holders (GE  
184 Healthcare). Isoelectric focusing (IEF) of samples was then performed in an IPGphor ceramic  
185 manifold, covered with Plusone DryStrip cover fluid. Sample strips were focused for 20000 Volt  
186 hours in an IPGphor isoelectric focusing system (GE Healthcare). Following IEF, strips were  
187 equilibrated as previously described [24] and applied to 12.5% polyacrylamide gels performed with  
188 30% acrylamide/bis solution, 37.5:1 or 29:1 ratio (Bio-Rad). Second dimension separation was  
189 performed by using the system Hoefer SE600 (GE Healthcare) and protein spots were visualised  
190 using Coomassie staining (2  $\text{g}\cdot\text{l}^{-1}$  Coomassie Brilliant Blue G250 and 0.5  $\text{g}\cdot\text{l}^{-1}$  R250 in 5% methanol,  
191 42.5% ethanol and 10% glacial acetic acid). Triplicate 2D-PAGE separations were generated for each  
192 sample condition and gels were imaged using the GS-800 calibrated densitometer (Bio-Rad).

193  
194 Protein identification was performed in the UCO-SCAI proteomics facility (University of  
195 Córdoba, Córdoba, Spain), a member of ProteoRed network. Protein spots of interest were excised  
196 automatically in a ProPic station (Genomic Solutions) and subjected to automated digestion with  
197 trypsin according to standard protocols in a ProGest station (Genomic Solutions). Peptide fragments  
198 were analyzed in a 4700 Proteomics Analyzer MALDI-TOF/TOF mass spectrometer (Applied  
199 Biosystems) with an accelerating voltage of 20 kV, in the *m/z* range 800 to 4000 (reflectron mode  
200 and delayed extraction were enabled, the elapse time was 120 nanoseconds). Proteins were  
201 identified by peptide mass fingerprinting and confirmed by MS/MS analysis of the 3 most abundant  
202 peptide ions. The MASCOT search engine (Matrixscience) was used for protein identification over  
203 the non-redundant National Center for Biotechnology Information (NCBI) database of proteins. The  
204 confidence in the peptide mass fingerprinting matches ( $p < 0.05$ ) was based on the Molecular weight



205 search method (MOWSE) score (higher than 65) and C.I. > 99.8%, and confirmed by the accurate  
206 overlapping of the matched peptides with major peaks present in the mass spectrum.

207 The detailed methodologies for cell fractionation and the construction and  
208 complementation of the *nas* mutants is described in the Supplementary Material.

209

210

## 211 RESULTS

212

### 213 Nitrate and nitrite as nitrogen sources for assimilation by *P. denitrificans*

214 In the absence of ammonium, *P. denitrificans* 1222 was able to grow aerobically using nitrate (Fig.  
215 2A) or nitrite (Fig. 3A) as the sole N-source with values for  $\mu_{\max(\text{app})}$  of 0.27 and 0.23 ( $\pm 0.01$ )  $\text{h}^{-1}$ ,  
216 respectively at pH 7.2 (Table S2). Approximately 10 mM of nitrate or nitrite was consumed during  
217 batch culture with 50 mM succinate present as carbon substrate (Fig.s 2B and 3B). A maximum O.D.  
218 value of 2.15 ( $\pm 0.05$ ) was reached after approx. 16 h, corresponding to a growth yield of  $\sim 100$  mg  
219 dry wt cells  $\cdot$  mM  $\text{N}^{-1}$  which were comparable to those obtained from cultures assimilating nitrogen  
220 from ammonium under carbon-sufficient growth conditions. With nitrate as sole N-source, a  
221 transient accumulation of  $\sim 1$  mM nitrite in the extracellular medium was observed during growth  
222 (Fig. 2C). To probe the biochemical basis of this growth physiology, spectrophotometric solution  
223 assays were performed on subcellular fractions from *P. denitrificans* using reduced pyridine  
224 nucleotides as electron donor and either nitrate or nitrite as electron acceptor. Cytoplasmic fractions  
225 prepared from cells cultured with nitrate present as the sole N-source showed clear nitrate and  
226 nitrite dependent NADH-oxidation rates (Fig. 4). These activities were not detected in either  
227 periplasmic or membrane fractions prepared from the same cell cultures, or in any cell fraction  
228 when NADPH was used in place of NADH as electron donor. In addition, no activity above a stable  
229 background non-specific oxidation rate, which was proportional to the amount of cell extract used,  
230 was detected in cytoplasmic fractions from *P. denitrificans* cells when ammonium was the sole N-  
231 source (Fig. 4). This pattern of activity is consistent with the regulation of other bacterial *nas* systems  
232 that are subject to ammonium repression and nitrate induction [2, 5, 19, 31].

233

234 Analysis of the *P. denitrificans* genome (<http://genome.jgi-psf.org/parde/parde.home.html>)  
235 reveals the presence of three gene clusters that likely code for different nitrate reductase systems.  
236 The genes for the respiratory systems Nar and Nap are located on chromosome 2 (NC\_008687) and  
237 plasmid 1 (NC\_008688), respectively. These clusters have been characterised previously in the  
238 closely related organism *Paracoccus pantotrophus* [12, 16, 32, 33]. In *P. denitrificans*, a third putative  
239 nitrate reductase gene is present within a cluster on chromosome 2 that is predicted to encode both  
240 the regulatory and structural elements for a cytoplasmic nitrate and nitrite reductase system  
241 (Pden\_4455-4449). This gene cluster comprises seven open reading frames, *nasTSABGHC* (from 5' to  
242 3') and spans some 10 kilobases from base pairs 1,657,840 to 1,667,370. A non-coding region of  
243 approx. 200 bases divides this cluster into two distinct functional units (Fig. 1A). The larger coding  
244 region, i.e., *nasABGHC*, which is the focus of this study, codes for putative redox proteins and  
245 substrate transport proteins and lies downstream of two genes encoding a putative nitrate and  
246 nitrite responsive two-component regulatory system, *nasT* and *nasS* [5]. The role *nasABGHC* in the  
247 assimilation of nitrogen from nitrate was confirmed by insertion of a kanamycin a resistance marker  
248 into the *nasA* gene (*nasA $\Delta$ ::Km\**). Here, the configuration of the resistance cassette was selected  
249 such that transcriptional terminators were present to cause polar effect and prevent expression of  
250 all genes collectively transcribed downstream of *nasA*. Significantly, the *nasA $\Delta$ ::Km\** mutant lost the  
251 capacity of the wild-type (WT) organism for aerobic growth with both nitrate and nitrite and thus  
252 established the importance of the *nasABGHC* region in the assimilation of both N-sources.

253

254

255

**Biochemical properties of cytoplasmic NADH-dependent assimilatory nitrate and nitrite reduction**  
Spectrophotometric assays were performed to define the kinetic properties of the NADH-dependent nitrate reductase and nitrite reductase activities present in cytoplasmic fractions of *P. denitrificans*. Activity was measured as the concentration of NADH was varied at fixed saturating concentrations of nitrate or nitrite (Fig. 5A, outlined symbols), and conversely as the concentration of nitrate or nitrite was varied at fixed saturating NADH (Fig. 5A, solid symbols). In all cases, enzyme activity varied in accordance with the Michaelis-Menten description and Hanes analysis was performed to define kinetic constants for nitrate (Fig. 5B) and nitrite (Fig. 5C) reduction. Values for  $V_{\max}$  of 111 and 302 ( $\pm 12$ ) nmol $\cdot$ min $^{-1}\cdot$ mg protein $^{-1}$  were determined for NADH oxidation with nitrate and nitrite present as electron acceptor, respectively. The  $V_{\max}$  expressed as a function of NADH consumed for the nitrite reductase reaction was  $\sim$ 3-fold higher than that for the nitrate reductase reaction. However, when the electron stoichiometries of each reaction are taken into account, i.e.,  $2e^-/\text{NO}_3^-$  and  $6e^-/\text{NO}_2^-$ , the rates of nitrate and nitrite reduction are evenly matched, which would minimise the accumulation of toxic nitrite in the cytoplasm. The values of the Michaelis constant ( $K_M$ ) for the reduction of nitrate and nitrite were 17 ( $\pm 4$ ) and 5 ( $\pm 2$ )  $\mu\text{M}$ , respectively. By contrast  $K_M$  values determined for NADH-oxidation during the nitrate reductase or nitrite reductase reactions were in good agreement, at 51 ( $\pm 6$ ) and 58 ( $\pm 8$ )  $\mu\text{M}$  respectively.

The elution of nitrate and nitrite reductase activities was monitored when a cytoplasmic fraction was subject to anion-exchange chromatography. Column fractions were assayed for nitrate and nitrite reductase activity using the non-physiological electron donor reduced Methyl Viologen (MV), which has been shown to donate electrons to both nitrate and nitrite reductases (either directly to the active sites or via electron transferring iron sulphur centres [14, 15, 34]). The two activities did not co-elute, with a large peak of MV-dependent nitrate reductase activity eluting at approx. 0.2 M NaCl, and the major peak of MV-dependent nitrite reductase activity eluting at higher approx. 0.3 M NaCl (Fig. 6A). Significantly, the nitrite reductase peak retained NADH-dependent nitrite reductase activity, but the nitrate reductase peak did not (Fig. 6B). A small protein population, eluting at approx. 0.28 M salt, retained both NADH-dependent and MV-dependent nitrate and nitrite reductase activities.

### **Genetic basis for a composite NADH-linked NasBGC nitrate and nitrite reductase system**

Analysis of the *P. denitrificans* NasC primary amino acid sequence suggests that it binds an N-terminal [4Fe-4S] cluster and a molybdenum containing cofactor and so shares a similar general organisation to the assimilatory nitrate reductase of cyanobacteria (NarB) and the catalytic unit of the structurally-defined respiratory periplasmic nitrate reductases (NapA) [35-37]. It is also predicted to contain an additional C-terminal region of  $\sim$ 200 residues that may bind a [2Fe-2S] cluster, as also proposed for the nitrate reductase from *K. oxytoca* (NasA) [2, 31, 38, 39] (Fig. S1). Significantly, none of these nitrate reductases has a canonical NADH binding domain. A *P. denitrificans* strain mutated in *nasC* lost the capacity for aerobic growth with nitrate as N-source ( $\mu_{\max(\text{app})} < 0.01 \text{ h}^{-1}$ , maximum O.D.  $< 0.05$ ), but retained the ability to grow using nitrite and displayed similar growth kinetics to WT ( $\mu_{\max(\text{app})} = 0.23 \pm 0.02 \text{ h}^{-1}$ , maximum O.D. of  $1.21 \pm 0.05$  was reached after approx. 16 h) (Fig. 7A and Table S2). This finding is consistent with NasC being the sole assimilatory nitrate reductase present during aerobic growth, reducing nitrate to nitrite, but playing no further role in the subsequent reduction of nitrite to ammonium. The *nasC* mutant could be grown in the presence of glutamate and nitrate ( $\mu_{\max(\text{app})} = 0.25 \pm 0.02$ , O.D. $_{\max} = 1.3 \pm 0.1$ ). Under these conditions, cytoplasmic fractions from WT cells display both MV- and NADH-dependent nitrate reductase activities, but both activities were absent in the *nasC* mutant (Table 2).

In order for the assimilation of nitrogen from nitrate to proceed, the cytoplasmic nitrite generated by NasC must be further reduced to ammonium. NasB shares significant sequence homology to flavin, Fe-S, sirohaem-containing nitrite reductases [2, 11, 40] (Fig. S2). The *P. denitrificans* NasB polypeptide is predicted to contain N-terminal FAD and NADH binding domains, while highly conserved central and C-terminal sequence regions contain the cysteine residues

307 required for iron-sulphur cluster coordination and the nitrite/sulphite reductase ferredoxin half-  
308 domain associated with sirohaem binding (Fig. S2). A *P. denitrificans nasB* mutant was unable to  
309 grow aerobically with either nitrite or nitrate as sole N-source. In order to confirm that these growth  
310 defects were not caused by a downstream effect of the gene disruption process, the *nasB* strain was  
311 complemented with a pEG276-*nasB* expression construct. The presence of this plasmid allowed the  
312 *nasB* mutant to grow to near WT levels with either nitrate or nitrite as sole N-source, see Table S2  
313 ( $\mu_{\max(\text{app})} = 0.20 \pm 0.02 \text{ h}^{-1}$ , maximum O.D. =  $1.23 \pm 0.05$ ). However, in the absence of the pEG276-  
314 *nasB* expression construct, no growth of the *nasB* mutant was observed, despite prolonged  
315 incubation for several days. This observation excludes the possibility of growth recovery through  
316 spontaneous mutation that may up-regulate a cryptic nitrite reductase. Cytoplasmic fractions  
317 obtained from the *nasB* mutant, grown in the presence of glutamate and nitrate, were assayed for  
318 NADH-dependent nitrate reductase and nitrite reductase activity. By contrast to that observed for  
319 WT cytoplasmic fractions, nitrite reductase activity was not detected in this mutant, consistent with  
320 the loss of the assimilatory nitrate reductase, NasB (Table 2). Significantly, nitrate reductase activity  
321 was also absent with NADH present as electron donor. Analysis of the NasC primary structure  
322 suggests that it lacks the NADH and FAD binding domains present in NasB that would be required for  
323 self-contained coupling of NADH oxidation to nitrate reduction (Figs S1 and S2). Therefore the  
324 absence of NADH-dependent nitrate reduction in the *nasB* mutant suggests a model in which the  
325 NADH-dehydrogenase domain of NasB provides electrons for both nitrite reduction by the sirohaem  
326 domain of NasB and nitrate reduction by NasC (Fig. 1B). Such a model is consistent with the similar  
327  $K_M$  values for NADH observed with nitrate or nitrite in cytoplasmic extracts, and also the loss of  
328 NADH-nitrate reductase activity when nitrate reductase is separated from nitrite reductase by anion-  
329 exchange chromatography. Further evidence in support of this model was forthcoming from  
330 measuring nitrate reductase activity in the NasB mutant using the artificial electron donor MV, which  
331 can donate electrons directly to the nitrate reductase. Significantly, MV-dependent nitrate reductase  
332 activity was detected in the *nasB* mutant, which confirmed the presence of functional NasC that is  
333 unable to couple to NADH oxidation in the absence of NasB.

334  
335 If the NADH-binding domain of NasB also serves NasC, then this raises the question of how  
336 electron transfer between NasB and NasC might occur. NasG is a strong candidate for mediating  
337 such electron transfer since it is predicted to be a Rieske-type iron-sulphur protein in which all  
338 residues (2 cysteine and 2 histidine) essential for coordination of a [2Fe-2S] redox site are conserved  
339 (Fig. S3). A *nasG* mutant was unable to grow with either nitrate or nitrite as sole N-source, under  
340 aerobic conditions. Expression of *nasG* in *trans* from a pEG276-*nasG* expression construct  
341 complemented the growth deficiencies of the *nasG* strain observed with nitrate and nitrite ( $\mu_{\max(\text{app})}$   
342 =  $0.22 \pm 0.07 \text{ h}^{-1}$ , maximum O.D. =  $1.31 \pm 0.05$ ), confirming specific disruption of *nasG* with no  
343 downstream effects, see Table S2. Unlike NasC and NasB there is no direct enzymatic assay with  
344 which to establish the synthesis of NasG. However, two-dimensional polyacrylamide gel  
345 electrophoresis (2D-PAGE) of soluble extracts from *P. denitrificans* WT, grown aerobically in the  
346 presence of glutamate and nitrate, revealed a protein with the mass and charge characteristics (12.1  
347 kDa and pI of 5.6) of NasG that was absent in the *nasG* mutant (Fig. 8). Analysis by mass  
348 spectrometry confirmed that this spot was the NasG polypeptide (MASCOT Protein Score = 314;  
349 Table S3). Cytoplasmic fractions prepared from cells of the *nasG* mutant grown with glutamate and  
350 nitrate were devoid of NADH-dependent nitrate reductase or nitrite reductase activities, but MV-  
351 dependent activities were detected (Table 2). These findings confirm that NasG is essential for both  
352 NADH-dependent nitrate and nitrite reduction, consistent with the protein mediating electron  
353 transfer from NADH to the active sites of both NasB and NasC (Fig. 1B). It was also notable that the  
354 MV-dependent nitrite reductase activity was unstable with a half-life of approx. 50 minutes  
355 following preparation of the cytoplasmic extract. This instability was reflected in 2D-PAGE analysis of  
356 the WT and *nasG* strains. In WT extract a triad of protein spots of ~88 kDa and PI of ~5.6 were each  
357 established by mass spectrometry to be NasB (MASCOT Protein Score = 622; Table S3) (Fig. 8A). As



358 expected this triad was absent in the *nasB* mutant. However, they could also not be identified in  
359 extracts from the NasG mutant despite analysis of a number of samples at different protein loadings.  
360 By contrast the NasG spot could be detected at ~WT levels in 2D-PAGE analysis of the *nasB* strain  
361 (Fig. 8). Thus NasG is stable in the absence of NasB, but may be required for NasB stability. It was  
362 notable that in the *nasB* strain a new protein spot was very prominent in cells grown with nitrate  
363 and glutamate. This spot was identified by mass spectrometry to be a member of the Hsp20 family  
364 of chaperones that protect unfolded proteins from aggregation (MASCOT Protein Score = 632; Table  
365 S3) [41]. This response may reflect the need to protect NasG whilst it is being assembled in the  
366 absence of its NasB partner or a response to protein damage by nitrosative stress arising from a  
367 lesion in cytoplasmic nitrite reduction.

368

### 369 **The contribution of NasA and NasH to nitrate and nitrite transport**

370 Delivery of nitrate and nitrite from the external environment to the cytoplasmic NasBGC system  
371 requires that the two N-oxyanions are transported across the cytoplasmic membrane against a  
372 membrane potential that is negative on the inside of the membrane. NasA and NasH are good  
373 candidate transporters for facilitating assimilatory N-oxyanion import. NasA is predicted to be a 12  
374 trans-membrane helix transporter of the major facilitator super-family (MFS), see Fig.s 1B and S4. *P.*  
375 *denitrificans* synthesizes another well characterised MFS family member in the presence of nitrate  
376 under anoxic conditions, NarK that serves to transport nitrate for the respiratory nitrate reductase  
377 system, NarGHI [16, 17, 33] (Fig. 1B). A non-polar *nasA* strain was constructed and was found to be  
378 strongly attenuated for aerobic growth with nitrate as sole N-source ( $\mu_{\max(\text{app})} = 0.04 \pm 0.01 \text{ h}^{-1}$ ,  
379 maximum O.D. =  $0.38 \pm 0.05$ ), demonstrating that NasA is the major nitrate transporter under these  
380 growth conditions (Fig. 2A and Table S2). Despite disruption of the *nasA* gene this strain retained the  
381 ability to grow aerobically with nitrite as sole N-source albeit with slower growth ( $\mu_{\max(\text{app})} = 0.20 \pm$   
382  $0.01 \text{ h}^{-1}$ , maximum O.D. =  $1.99 \pm 0.05$ ) and nitrite consumption kinetics than that observed for WT  
383 cells, implying that NasA may make a contribution to, but is not essential for, nitrite uptake into the  
384 cell (Fig. 3 and Table S2). Efforts to complement the *nasA* strain using the same complementation  
385 vector system used successfully for *nasB* and *nasG* was unsuccessful, possibly due to a failure to  
386 assemble the integral membrane protein. However, evidence that there were no polar effects of the  
387 *nasA* mutation on downstream gene expression is given by the detection of NADH-dependent  
388 nitrate ( $\sim 10 \text{ nmoles} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ ) and nitrite ( $\sim 30 \text{ nmoles} \cdot \text{min}^{-1} \cdot \text{mg protein}^{-1}$ ) reductase activity  
389 at WT levels in cells grown on glutamate-nitrate medium, which would require transcription of  
390 *nasBGC*, which lies downstream of *nasA*.

391

392 NasH is a putative member of the formate-nitrite transporter super-family that includes NirC  
393 from *E. coli* (Fig. S5) [42-44]. Recent evidence suggests that NirC can move nitrite *bi*-directionally  
394 across the cytoplasmic membrane during anaerobic growth of *E. coli* (29). NasH is thus a prime  
395 candidate for mediating nitrite uptake or export and so may be important for nitrite homeostasis  
396 during nitrate assimilation. Perhaps surprisingly though, the *P. denitrificans nasH* mutant displayed  
397 similar growth kinetics and yields ( $\mu_{\max(\text{app})} = 0.28 \pm 0.01 \text{ h}^{-1}$ , maximum O.D. =  $1.79 \pm 0.09$ ) to that  
398 observed for WT, when cultured aerobically with either nitrate or nitrite as sole N-source at pH 7.2  
399 (Fig.s 2 and 3). The pattern of nitrite consumption from the extracellular medium during growth with  
400 nitrite as sole N-source was also similar in the WT and *nasH* strains (Fig. 3B), as was the transient  
401 accumulation of approx. 1 mM nitrite observed during the mid to late exponential growth phase  
402 when nitrate was present as sole N-source (Fig. 2C). A double *nasA nasH* mutant also retained the  
403 ability to grow with nitrite as sole N-source, at pH 7.2 ( $\mu_{\max(\text{app})} = 0.20 \pm 0.01 \text{ h}^{-1}$ , maximum O.D. =  
404  $1.72 \pm 0.05$ ), displaying similar growth kinetics to the *nasA* strain (Fig. 3A and Table S2). Nitrite is a  
405 protonatable anion that exists in equilibrium with nitrous acid ( $\text{NO}_2^- + \text{H}^+ \leftrightarrow \text{HNO}_2$ ), with a  $\text{pK}_a$  value  
406 of 3.3 [17]. Thus, at pH 7 with an external nitrite concentration of 10 mM, the concentration of  $\text{HNO}_2$   
407 is present in the low micromolar range. This acid could freely diffuse across the phospholipid bilayer,  
408 dissociate in the cytoplasm, and so deliver nitrite to the NasBGC complex without recourse to a

409 specific nitrite uptake system. The extent to which this could occur will be decreased at higher pH. It  
410 was therefore notable that when the *nasH* and *nasA nasH* mutants were grown at pH 9.2 with nitrite  
411 as the sole N-source, significant attenuation in growth and nitrite consumption was observed in both  
412 cases, thus demonstrating a role for NasH in nitrite import (Figs 3C, 3D and Table S2).

413

#### 414 **Functional substitution of Nar components for Nas components in anaerobic nitrate assimilation**

415 *P. denitrificans* could grow anaerobically, as well as aerobically, with nitrate or nitrite as the sole N-  
416 source (Fig. 7 and Table S2). Under these growth conditions energy conservation is via nitrate and  
417 nitrite respiration through the anaerobically synthesised Nar and Nir systems, respectively [12]. The  
418 Nar system includes the respiratory ubiquinol/nitrate oxidoreductase, NarGHI of which the NarG  
419 nitrate-reductase subunit is located at the cytoplasmic face of the cytoplasmic membrane and a  
420 fusion protein of two NarK-type modules facilitates nitrate and nitrite movement across the  
421 membrane (Fig. 1B). To investigate whether the NarG and NarK proteins could functionally  
422 substitute for NasC and NasA, the *nas* mutants were cultured under anaerobic conditions with  
423 nitrate present as sole N-source and electron acceptor anoxic, conditions that result in *nar* gene  
424 expression. Both the *nasA* and *nasC* mutants showed significant growth under these conditions (Fig.  
425 7B), despite being unable to grow aerobically with nitrate as sole N-source (compare Figs 2A and 7B).  
426 The *nasC* mutant was also able to grow anaerobically with nitrite present as both sole N-source and  
427 respiratory electron acceptor (Fig. 7C). Unlike the *nasC* strain, however, neither the *nasB* or *nasG*  
428 mutants retained the ability to grow anaerobically with nitrate or nitrite, demonstrating that the  
429 NADH-dependent NasBG nitrite reductase was indispensable to both oxic and anoxic assimilation of  
430 nitrate and nitrite.

431

#### 432 **DISCUSSION**

433

434 This work has established that *P. denitrificans* NasC and NasB are both part of a cytoplasmic NADH-  
435 dependent assimilatory nitrate and nitrite reduction system. However, analysis of the primary  
436 structures of both proteins revealed that only NasB has a canonical FAD-dependent NADH binding  
437 domain. A bioinformatic analysis of predicted gene products for *nasC* and *nasB* homologues from  
438 the diverse bacterial phyla suggests that this is a common feature (Fig. S6). There is no bacterial  
439 assimilatory nitrate reductase that we can identify that has an NADH binding site. As such, this  
440 makes them quite distinct from plant and fungal assimilatory nitrate reductases in which NAD(P)H  
441 binding domains are ubiquitous [45]. In the present study we have reported genetic and biochemical  
442 data that suggests that the NADH-oxidising FAD domain of NasB provides electrons for both nitrite  
443 reduction by the NasB ferredoxin:sirohaem active site and nitrate reduction by the NasC  
444 molybdenum cofactor active site: (i) NADH-dependent nitrate reductase activity is lost when the  
445 nitrate reductase is separated from the NADH-dependent nitrite reductase; (ii) the  $K_M$  value for  
446 NADH determined with either nitrate or nitrite is the same in cytoplasmic fractions from WT cells;  
447 (iii) non-polar deletion of *nasB* results in loss of aerobic growth with nitrate as sole N-source; (iv)  
448 non-polar deletion of *nasB* results in loss of NADH-dependent nitrate reductase activity, but not MV-  
449 dependent nitrate reductase activity. We also show that the putative Rieske [2Fe-2S] ferredoxin,  
450 NasG is required for these NADH-dependent electron transfer processes because in a *nasG* strain: (i)  
451 no growth is observed with either nitrate or nitrite as sole N-source and (ii) NADH-dependent nitrate  
452 and nitrate reductase activities are absent in cytoplasmic extracts, but MV-dependent activities are  
453 detected. These findings imply that NasG can interact in the cytoplasm with both NasB and NasC,  
454 and lead us to propose a model in which it serves at the interface of a composite NADH-dependent  
455 nitrate and nitrite reductase system, NasBGC (Fig. 1B).

456

457 It is notable that many bacterial *nas* clusters that we have examined encode a NasG-like  
458 protein, in addition to an NADH-oxidising sirohaem-dependent nitrite reductase, suggesting an  
459 important conserved function (Fig. S6). It is absent in the *Synechococcus elongatus* cluster (Fig. 9),

460 but in this case the photoautotroph uses reduced ferredoxin generated from photosystem I to drive  
461 nitrate and nitrite reduction [19]. A *nasG* homologue is also absent from the *nas* cluster of *Klebsiella*  
462 species (Fig. S6) [39]. However, these novel larger NasB proteins show an extended C-terminal  
463 region of approx. 100 residues that shares homology with NasG. In addition, in *Klebsiella oxytoca*  
464 there is a gene in the *nas* operon that codes for a flavoprotein which may substitute for NasG or  
465 support it in enabling electron transfer from NADH to the nitrate reductase subunit. A homologue of  
466 this gene may also be playing such a role in *Bacillus subtilis* [4], whilst in *Klebsiella pneumoniae* this  
467 gene appears to be fused to the gene for the nitrite reductase (Fig. S6). Nevertheless, even taking  
468 these exceptions into account, the widespread distribution of NasG-type modules leads us to  
469 propose that the composite NasBGC system exemplified here by *P. denitrificans* is a very common  
470 feature of Nas systems in phylogenetically diverse bacteria. Though the interaction between NasC  
471 and NasBG does not resist anion-exchange chromatography, there is precedent for a stable  
472 interaction between a molybdenum cofactor dependent enzyme and Rieske-type ferredoxin.  
473 Intriguingly, the catalytic subunit of the arsenite oxidase from *Alcaligenes faecalis* forms a stable  
474 heterodimeric complex and co-crystallises with its redox partner, a Rieske-type [2Fe-2S] protein that  
475 shares sequence similarity with NasG (~40 %) [46, 47]. Structural studies also revealed that the iron-  
476 sulphur centres present in each subunit are ideally situated, at <14 Å (edge-to-edge) from one  
477 another across the heterodimeric interface, to facilitate rapid electron transfer that does not limit  
478 catalysis [47]. Thus, the NasC-NasG interaction may be a weaker manifestation of the protein-  
479 protein interaction observed in the arsenite oxidase complex. The genetic evidence that NasG is a  
480 dedicated ferredoxin for the NasBC system and that the NasB protein is possibly unstable is perhaps  
481 surprising given that *P. denitrificans* genome predicted to encode a number of small cytoplasmic  
482 ferredoxins. However, precedent for this may be found in the *E. coli* cytoplasmic nitrite reductase  
483 system. *E. coli* is unable to grow aerobically with nitrite as sole N-source, but when grown under  
484 nitrate-rich anoxic conditions a respiratory nitrate reductase NarG and a sirohaem:ferredoxin-type  
485 nitrite reductase, NirB operate in the cytoplasm to respire nitrate and detoxify the nitrite product  
486 (Fig. S6) [50]. *E. coli* can also grow anaerobically with nitrite as sole N-source under conditions  
487 where the *nirB* gene encoding this anaerobically-inducible nitrite reductase is expressed [50, 51]. *E.*  
488 *coli nirB* is a homologue of *P. denitrificans nasB* (65% similarity) and both genes are found upstream  
489 of their ferredoxin partners, *E. coli nirD* and *P. denitrificans nasG*, respectively, which share 59%  
490 similarity (Fig. S6). Significantly, like the *P. denitrificans nasG* mutant, NADH-dependent nitrite  
491 reductase activity is also lost in an *E. coli nirD* mutant [52]. In both *E. coli* and *P. denitrificans* the  
492 *nirB/nasB* and *nirD/nasG* loci lie immediately upstream of a gene encoding a transporter *nirC/nasH*  
493 (55% similarity) in their respective genomes (Fig. S6). Here, by growing *P. denitrificans* at high pH to  
494 minimise HNO<sub>2</sub> formation, we have shown that *nasH* contributes to nitrite uptake. These  
495 observations show a clear genetic and biochemical link between two nitrite reductase systems with  
496 distinct primary functions in nitrite assimilation and detoxification.

497  
498 Turning finally to the rescue of *nasA* and *nasC* mutants under anaerobic growth conditions  
499 by the respiratory Nar system. This is consistent with functional overlap between two physiologically  
500 distinct systems whose cellular function commonly requires the import and cytoplasmic based  
501 reduction of nitrate. In this respect, it is notable that an assimilatory nitrate reductase gene is absent  
502 from some assimilatory gene clusters in nitrate-assimilating bacteria where the respiratory nitrate  
503 reductase *narG* gene is present at a different genetic locus in the bacterium. A noteworthy example  
504 is *Mycobacterium* (Fig. S6), in which *narG* mutants cannot grow with nitrate as sole N-source [48,  
505 49]. The interchangeability between NasC and NarGHI in anaerobic assimilatory nitrate reduction is  
506 also consistent with the dissociation of NasC from NasBG observed during anion-exchange  
507 chromatography, suggesting a modular arrangement in which NasBG can exist as a stable functional  
508 entity in the absence of NasC.

509  
510

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## FIGURE LEGENDS

**Fig. 1.** Genetic and proposed functional organisation of the assimilatory nitrate reductase system from *P. denitrificans* PD1222. The *nas* gene cluster includes seven open reading frames, *nasTSABGHC* (A). The gene products have the following putative roles, two regulatory components (*nasS* and *nasT*), two nitrogen oxyanion transporters (*nasA* and *nasH*), nitrate and nitrite reductases (*nasC* and *nasB*, respectively) and a ferredoxin (*nasG*). Three distinct nitrate reductase systems are present in *P. denitrificans* (B). The periplasmic (NapABC) and membrane-bound (NarGH) enzymes are ubiquinol-dependent respiratory nitrate reductases. NarK, a nitrate importer, moves nitrate into the cytoplasm and also exports nitrite, the product of nitrate reduction, to the periplasm to support respiratory denitrification. For assimilatory nitrate reduction, electrons likely derived from NADH within the FAD-containing nitrite reductase (NasB) flow to the nitrate reductase (NasC), possibly via the small ferredoxin (NasG). In the Nas system there are two transporters, NasA and NasH, which are predicted to be involved in nitrate and nitrite transport, respectively. Note that there is no common nomenclature for assimilatory nitrate and nitrite reductase genes in prokaryotes. In the case of nitrite reductase genes the 'nir' prefix is quite widely used, but since *P. denitrificans* has a separate respiratory nitrite reductase 'nir' gene cluster, this term would be inappropriate. Likewise the 'nar' prefix is sometimes used for the assimilatory nitrate reductase genes, but this would also be inappropriate for *P. denitrificans* which also has a *nar* gene cluster encoding the respiratory nitrate reductase system. Hence we have adopted the *nas* prefix for the genes encoding the assimilatory nitrate and nitrite reductase system of *P. denitrificans*. The term 'nH<sup>+</sup>' indicates that the number of protons (n) moved across the membrane is not known.

**Fig. 2.** Aerobic growth of *P. denitrificans* WT (squares), *nasA* (circles), *nasH* (triangles) and *nasA nasH* (inverted triangles) strains with nitrate present as the sole nitrogen source (A). Extracellular nitrate (B) and nitrite (C) concentrations are shown for growth of strains described in (A). Data shown are the average of triplicate determinations.

**Fig. 3.** Aerobic growth of *P. denitrificans* WT (squares), *nasA* (circles), *nasH* (triangles) and *nasA nasH* (inverted triangles) strains with nitrite present as the sole nitrogen source. Cells were cultured at pH 7.2 (A) and pH 9.2 (C), during which extracellular nitrite concentration was determined (shown in B and C, respectively). Data shown are the average of triplicate determinations.

**Fig. 4.** The nitrate and nitrite reductase activity of cytoplasmic fractions of *P. denitrificans*. Fractions prepared from cells grown with either nitrate (solid lines) or ammonium (dashed lines) as sole nitrogen source were assayed for nitrate (A) and nitrate (B) reductase activity. Spectrophotometric assays were performed at pH 7.5 in the presence of NADH (100  $\mu$ M). The reaction was initiated by addition of either nitrate or nitrite and followed by measuring the decrease in absorbance observed over time at 340 nm.

**Fig. 5.** Kinetic properties of NADH-dependent assimilatory nitrate and nitrite reduction. NADH dependent nitrate (squares) and nitrite (circles) reductase activities present in cytoplasmic fractions from *P. denitrificans* grown with nitrate as sole nitrogen source (A). Solid and outlined symbols show (i) NADH oxidation rates observed with substrate concentration varied against fixed NADH (200  $\mu$ M), and (ii) NADH varied against fixed substrate concentration (200  $\mu$ M), respectively. Hanes analysis of nitrate reductase (B) and nitrite reductase activities (C) presented in A. Values for  $K_M$  of 17 and 5 ( $\pm$  2)  $\mu$ M were derived for nitrate and nitrite reduction, respectively. Similar  $K_M$  values for NADH of 58 and 51 ( $\pm$  8)  $\mu$ M were determined with nitrate or nitrite as electron acceptor, respectively. From (i)  $V_{max}$  values for nitrate and nitrite reduction were 95 and 86 ( $\pm$  10) units

respectively, and (ii)  $V_{\max}$  values of 111 and 302 ( $\pm 12$ ) units were determined for NADH oxidation with nitrite and nitrite, respectively (1 unit  $\equiv$  1 nmol $\cdot$ min $^{-1}$  $\cdot$ mg protein $^{-1}$ ).

**Fig. 6.** A representative activity-elution profile observed during anion exchange chromatography. Panels A and B show MV- and NADH-dependent nitrate (solid symbols) or nitrite (outlined symbols) reductase activities present in column fractions, respectively. DEAE-Sephacrose™ column matrix was equilibrated in 5 mM L-ascorbate, 5mM EDTA, 50 mM Tris-HCl, pH 7.5. The column was loaded with a cytoplasmic extract from *P. denitrificans* grown with nitrate as sole nitrogen source, washed with 2 column volumes and then developed with a linear gradient of 0-0.5 M NaCl, over 1 column volume at 1.5 ml $\cdot$ min $^{-1}$  flow rate.

**Fig. 7.** Growth curves for the *nasC* and *nasA* strains under aerobic and anaerobic conditions. Aerobic growth of the *nasC* strain with either nitrate (squares) or nitrite (circles) present as the sole nitrogen source (A). Anaerobic growth of WT (squares), *nasA* (circles) and *nasC* (triangles) strains with either nitrate (B) or nitrite (C) present as the sole nitrogen source. Data shown are the average of triplicate determinations.

**Fig. 8.** 2D-PAGE analysis of soluble extracts from *P. denitrificans*. 350  $\mu$ g of protein was applied to 11 cm strips. Isoelectric focusing was performed in the range 4-7. The 12.5% polyacrylamide gels formed with either with 30% acrylamide/bis solution, 37.5:1 (A) or 29:1 ratio (B).

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**Table 1.** Bacterial strains and plasmids used in this work.

Strain or plasmid	Relevant characteristics	Source or reference
Bacteria		
<i>Paracoccus denitrificans</i>		
wild-type, PD1222	Rif <sup>r</sup> , Spec <sup>r</sup> , enhanced conjugation frequencies	[53]
<i>nasAD::Km*</i>	Km <sup>r</sup> (polar mutation)	This work
<i>nasAD::Km</i>	Km <sup>r</sup>	This work
<i>nasBD::Sm</i>	Sm <sup>r</sup>	This work
<i>nasGD::Sm</i>	Sm <sup>r</sup>	This work
<i>nasHD::Sm</i>	Sm <sup>r</sup>	This work
<i>nasCD::Tc</i>	Tc <sup>r</sup>	This work
<i>nasAD::Km/nasHD::Sm</i>	Km <sup>r</sup> , Sm <sup>r</sup>	This work
<i>Escherichia coli</i>		
DH5 $\alpha$	<i>supE44 lacU169</i> ( $\phi$ 80 <i>lacZ</i> $\Delta$ M15) <i>hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i> plasmid host	[54]
pRK2013	Km <sup>r</sup> , Tra <sup>+</sup> (pRK2013 encoded) helper strain	[29]
Plasmids		
pUC18	Ap <sup>r</sup> , Lac <sup>+</sup> , cloning vector	[26]
pGEM-T Easy	Ap <sup>r</sup> , Lac <sup>+</sup> , cloning vector	Promega
pSUP202	Ap <sup>r</sup> , Tc <sup>r</sup> , Cm <sup>r</sup> , Mob <sup>+</sup> , mobilizable suicide vector	[28]
pSUP202*	pSUP202( $\Delta$ Tc::Km), Ap <sup>r</sup> , Km <sup>r</sup> , Cm <sup>r</sup> , Mob <sup>+</sup> , mobilizable suicide vector	This work
pSUP2021	pSUP202::Tn5, Ap <sup>r</sup> , Km <sup>r</sup> , Cm <sup>r</sup> , source of Km cassette	[28]
pSRA2	Ap <sup>r</sup> , Spec <sup>r</sup> , Sm <sup>r</sup> , source of Sm cassette	[27]
pML5B <sup>+</sup>	Tc <sup>r</sup> , <i>lacZ</i>	[25]
pEG276	Gm <sup>r</sup> , expression vector	[30]
pEG276- <i>nasB</i>	Gm <sup>r</sup> , <i>P. denitrificans nasB</i> expression construct	This work
pEG276- <i>nasG</i>	Gm <sup>r</sup> , <i>P. denitrificans nasG</i> expression construct	This work

**Table 2.** NADH- and reduced MV-dependent nitrate and nitrite reductase activity of cytoplasmic fractions prepared from *P. denitrificans* strains affected in *nasB*, *nasG* and *nasC* grown in the presence of nitrate plus glutamate.

Strain	Electron acceptor	Electron donor	
		NADH	MV
WT	NO <sub>3</sub> <sup>-</sup>	13 ± 4	20 ± 1
	NO <sub>2</sub> <sup>-</sup>	35 ± 2	203 ± 22
<i>nasC</i>	NO <sub>3</sub> <sup>-</sup>	n.d.	n.d.
	NO <sub>2</sub> <sup>-</sup>	39 ± 4	205 ± 20
<i>nasB</i>	NO <sub>3</sub> <sup>-</sup>	n.d.	13 ± 2
	NO <sub>2</sub> <sup>-</sup>	n.d.	n.d.
<i>nasG</i>	NO <sub>3</sub> <sup>-</sup>	n.d.	142 ± 4
	NO <sub>2</sub> <sup>-</sup>	n.d.	103 ± 7*

Activity units = nmoles·min<sup>-1</sup>·mg protein<sup>-1</sup>

n.d. = not detectable

\* Activity decayed with t<sub>1/2</sub> of approx. 50 minutes

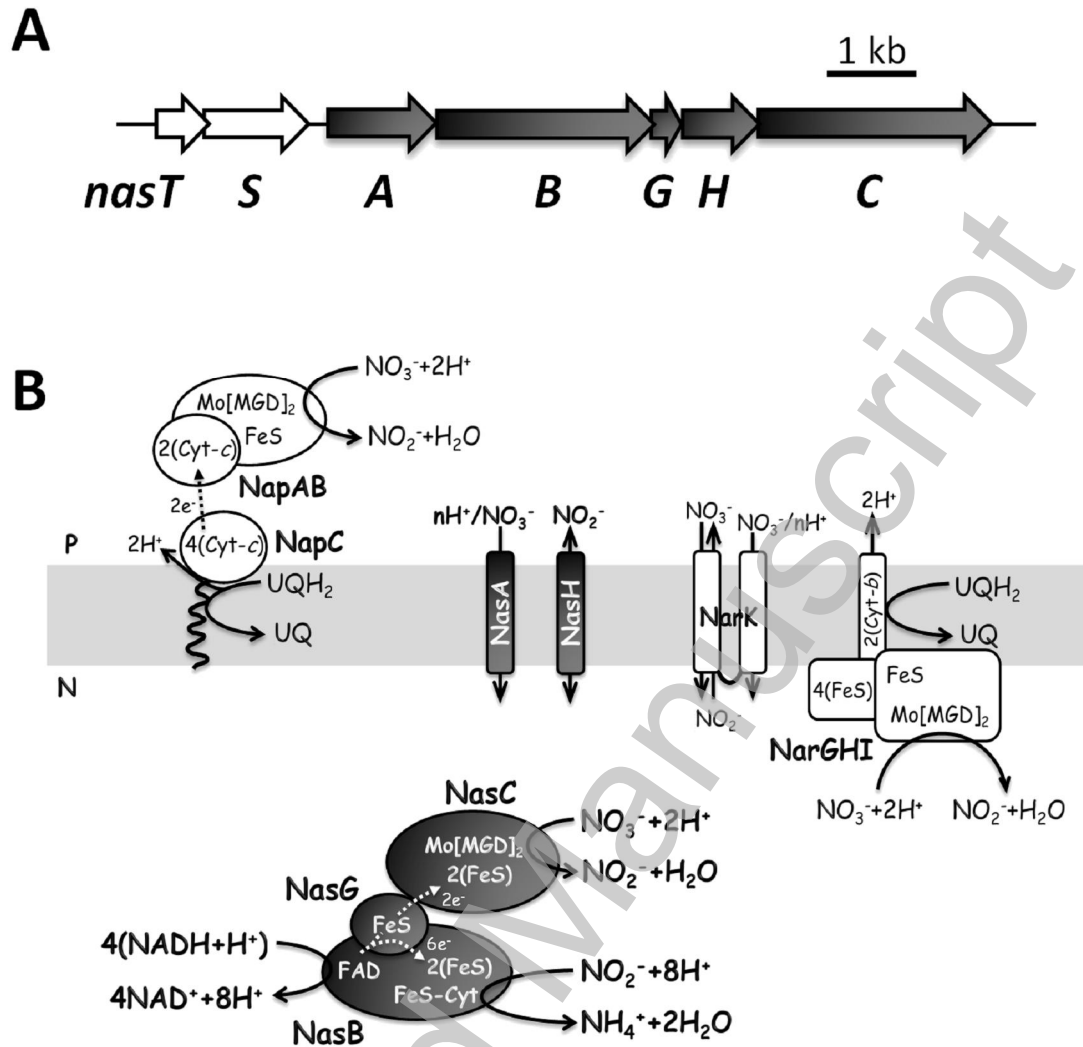


Fig. 1.

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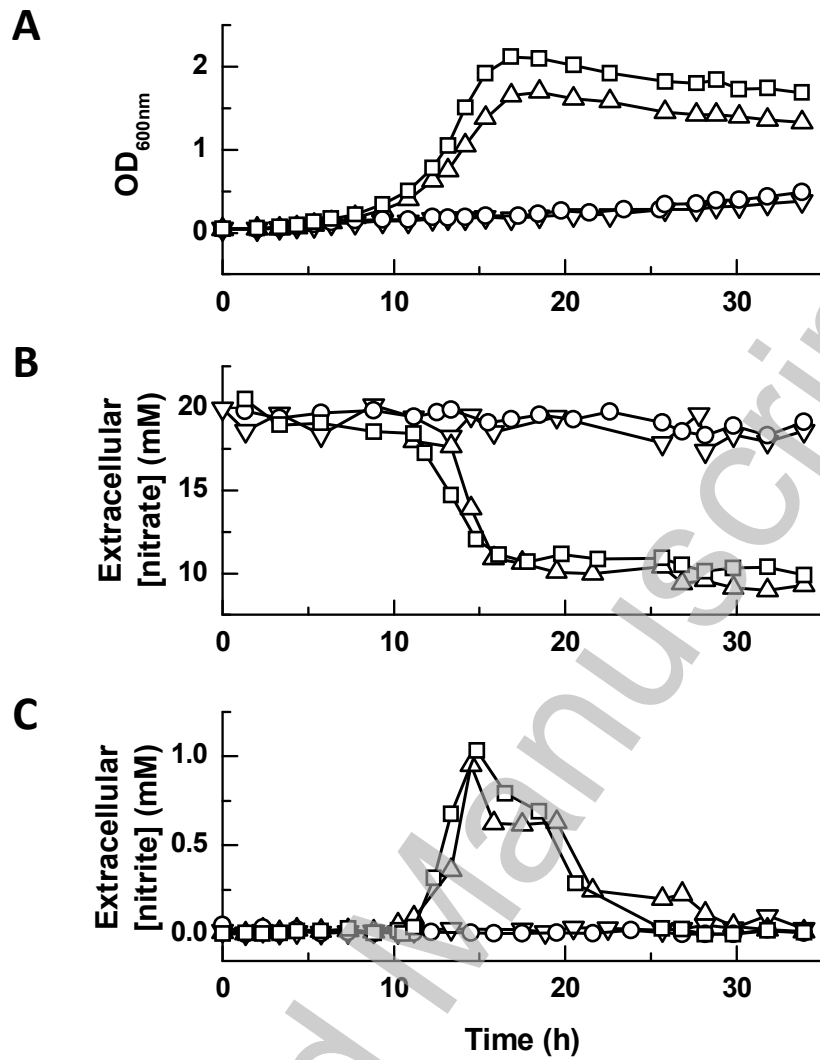


Fig. 2.



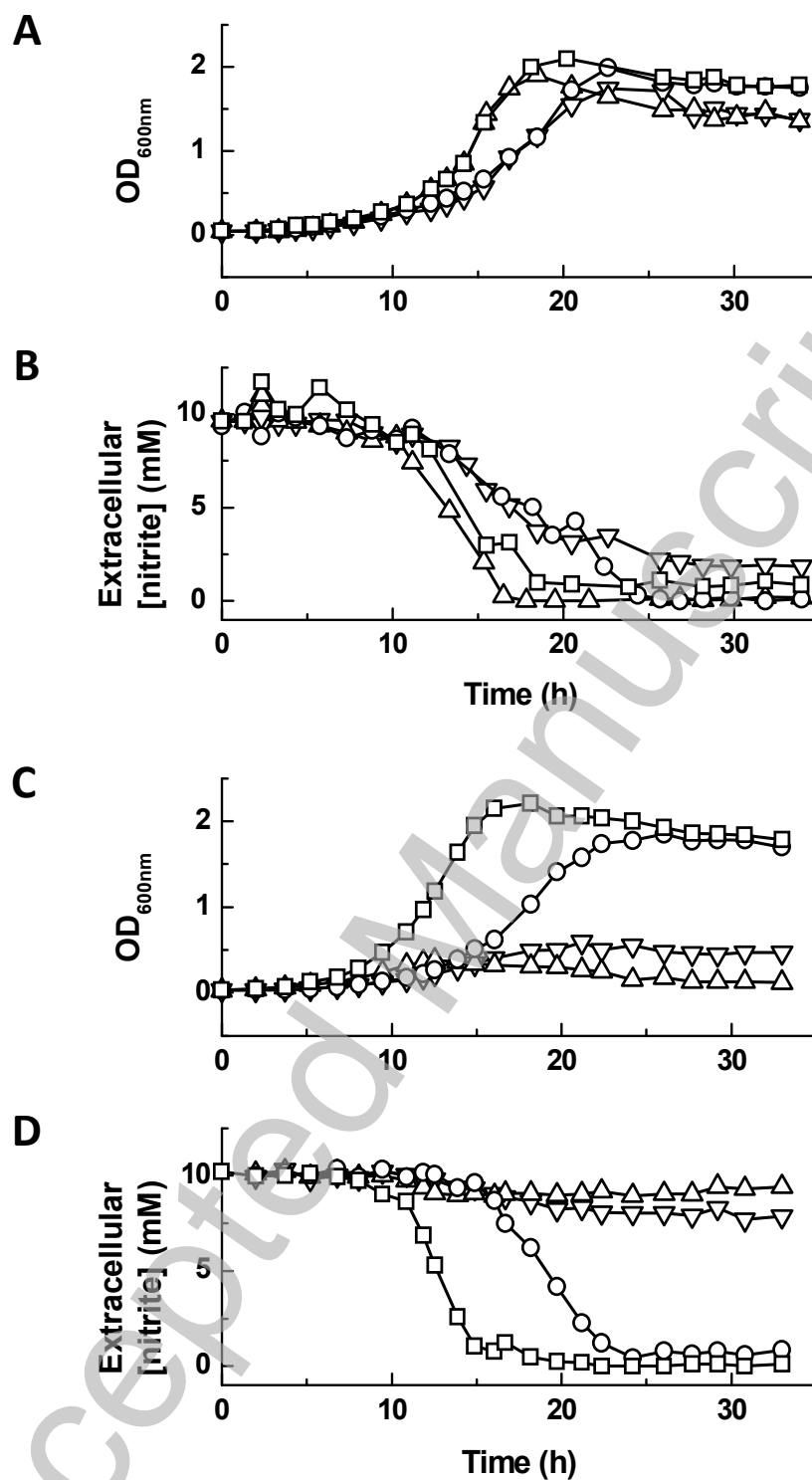


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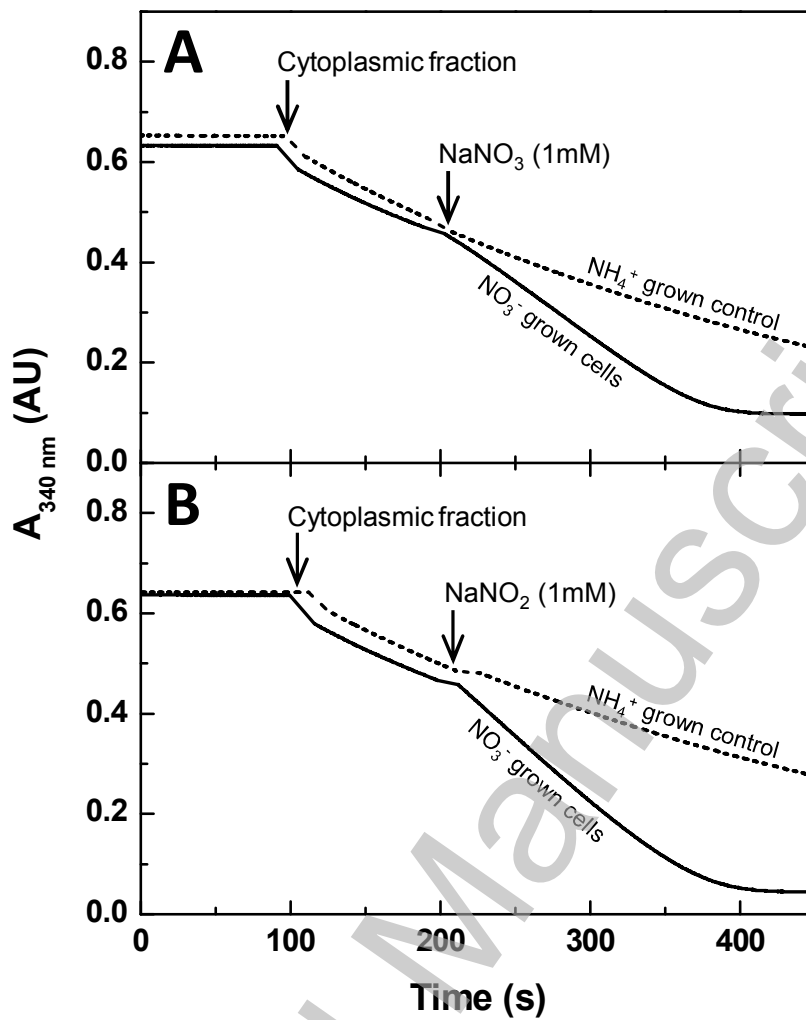


Fig. 4.

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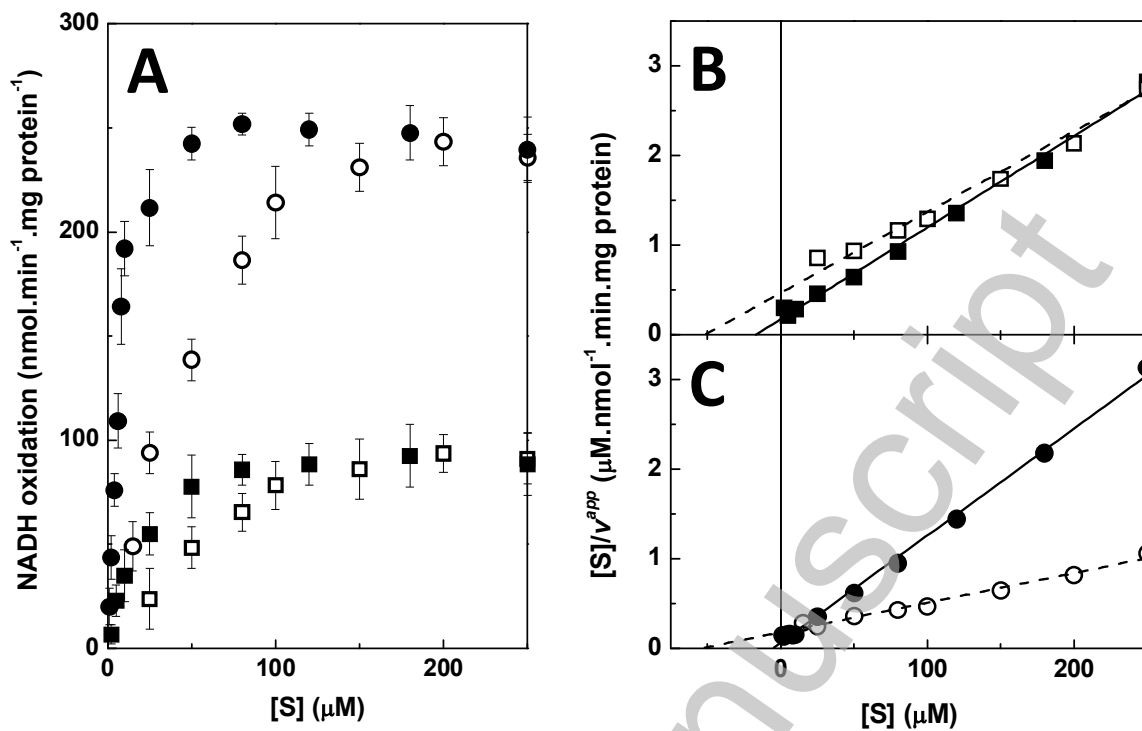


Fig. 5.

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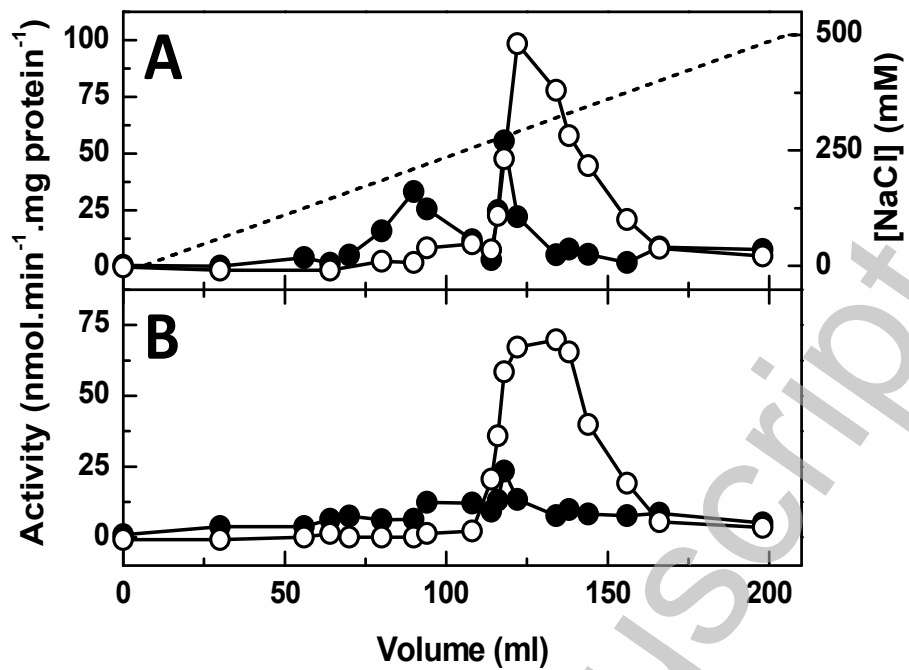


Fig. 6.

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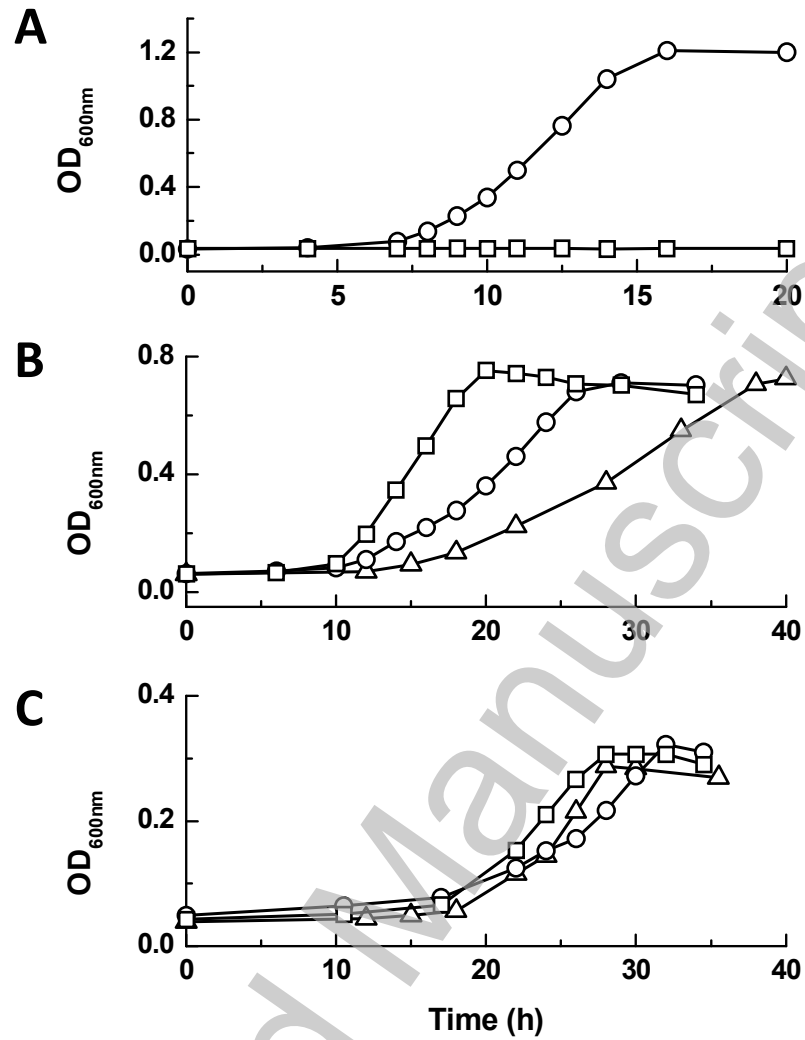


Fig. 7.

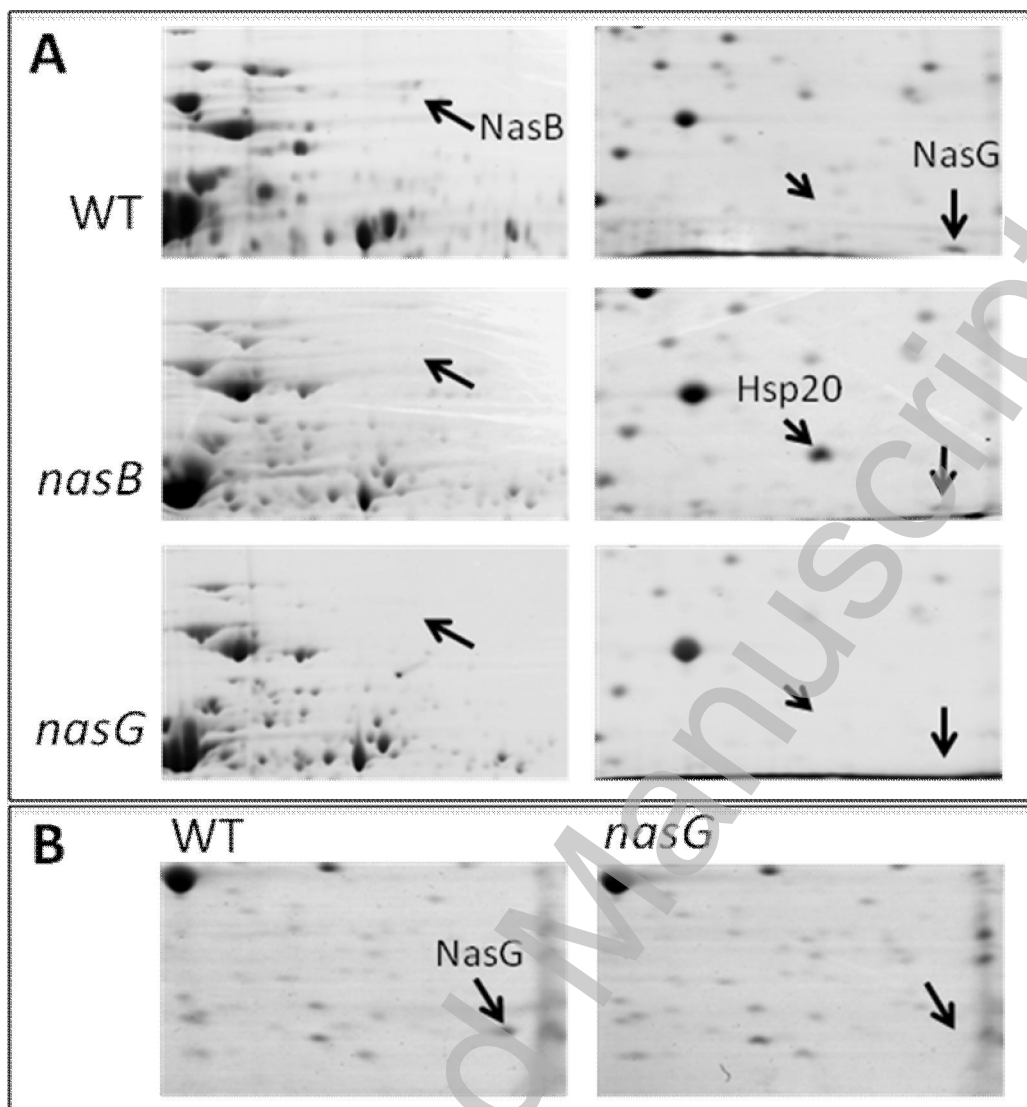


Fig. 8.

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