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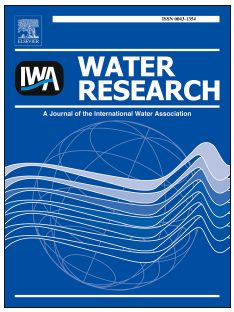

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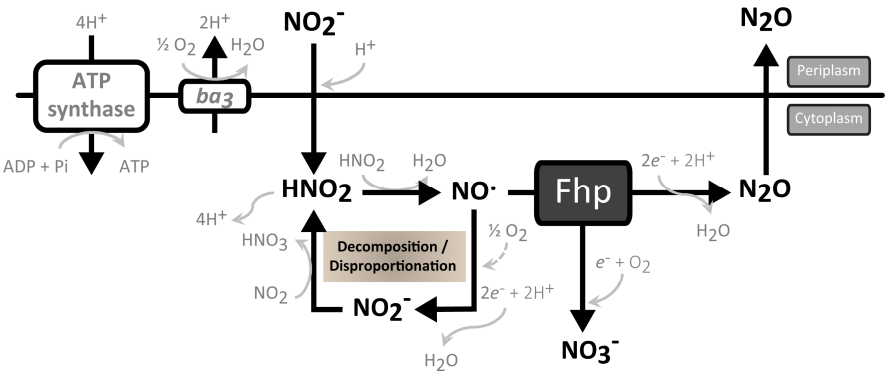

The metabolic impact of extracellular nitrite on aerobic metabolism of *Paracoccus denitrificans*

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Abstract

Nitrite, in equilibrium with free nitrous acid (FNA), can inhibit both aerobic and anaerobic growth of microbial communities through bactericidal activities that have considerable potential for control of microbial growth in a range of water systems. There has been much focus on the effect of nitrite / FNA on anaerobic metabolism and so, to enhance understanding of the metabolic impact of nitrite / FNA on aerobic metabolism, a study was undertaken with a model denitrifying bacterium *Paracoccus denitrificans* PD1222. Extracellular nitrite inhibits aerobic growth of *P. denitrificans* in a pH-dependent manner that is likely to be a result of both nitrite and free nitrous acid (FNA) (pKa = 3.25) and subsequent reactive nitrogen oxides generated from the intracellular passage of FNA into *P.*

denitrificans. Increased expression of a gene encoding a flavohemoglobin protein (Fhp) (Pden_1689) was observed in response to extracellular nitrite. Construction and analysis of a deletion mutant established the Fhp to be involved in endowing nitrite / FNA resistance at high extracellular nitrite concentrations. Micro-array global transcriptional analysis confirmed nitrite-dependent expression of *fhp* and indicated that *P. denitrificans* expressed a number of stress response systems associated with protein, DNA and lipid repair. It is therefore suggested that nitrite causes a pH-dependent stress response that is due to the production of associated reactive nitrogen species, such as NO from the internalisation of FNA.

1. Introduction

The biocidal effect nitrite in equilibrium for free nitrous acid (nitrite / FNA) has recently been harnessed in wastewater treatment to control unwanted growth of microorganism communities (Vadivelu *et al*. 2007, Wang *et al*. 2016). However, accumulation of nitrite can inhibit the metabolism of several groups of bacteria involved in nitrogen removal in wastewater treatment plants, including ammonia oxidisers ($NH_3 \rightarrow NO_2^-$) and denitrifiers ($NO_2^- \rightarrow N_2$) that together can remove harmful levels of reactive nitrogen species from wastewater effluents (Almeida *et al*. 1995a, Anthonisen *et al*. 1976, Vadivelu *et al*. 2006a, Vadivelu *et al*. 2006b) and can impact on polyphosphate accumulators (Fux *et al*. 2003, Zhou *et al*. 2007, Zhou *et al*. 2008). Nitrite inhibition may be attributable to the protonated conjugate acid of nitrite, free nitrous acid (FNA; pKa = 3.25), which can cross the cytoplasmic membrane as the freely diffusing uncharged lipophilic species. Once it is in the cytoplasm FNA can disproportionate to form cytotoxic reactive nitrogen species such as NO^- and, if oxygen is present, peroxynitrite. Extensive work by Ye *et al* (2010) has suggested that this is a likely to be the case for many observations of nitrite linked growth inhibition, with both catabolic and anabolic processes being affected. For example, in a mixed culture of enriched polyphosphate accumulating (PAO) and glycogen accumulating bacteria, comprising largely of *Competibacter*,

consumption of polyhydroxyalkanoate and glycogen production were both impacted, with complete inhibition of growth occurring at an FNA concentration of 0.14 μM (Ye *et al*. 2010). In a study with *Accumulibacter,* a PAO, 0.42 μM FNA completely inhibited phosphate uptake (Zhou *et al*. 2010). This was corroborated by Jiang (2011) who saw a 75% decrease of biofilm after exposure to 0.42 – 0.61 μM FNA.

Denitrifying bacteria reduce nitrate sequentially via nitrite, nitric oxide and nitrous oxide to nitrogen. These reductive reactions are an alternative to oxygen respiration and are coupled to the generation of a proton motive force and so to cell maintenance and growth under anoxic conditions (Berks *et al*. 1995, Richardson 2000). The generation of reactive nitrogen species is an 'occupational hazard' for denitrifying bacteria since both nitrite and nitric oxide are cytotoxins. Under anoxic conditions denitrifying bacteria express respiratory enzymes that can serve to reductively destroy extra-cytoplasmic nitrite and nitric oxide that is generated in the periplasm from nitrate reduction or produced in a microbial community by other organisms. These are the nitrite reductase (Nir) and the nitric oxide reductase (Nor). Indeed, in *P. denitrificans nir* and *nor* gene expression is co-regulated by the same transcriptional regulator, NnrR, an NO sensor (Van Spanning *et al*. 1995). This ensures that the production and consumption of reactive nitrogen species is tightly coupled. However, expression of the *nir* and *nos* systems is repressed by oxygen and activity of the enzymes themselves is inhibited by oxygen. Denitrifying bacteria live at oxic-anoxic interface in many environments and the nitrite and nitrate that they use as substrates for denitrification arises from the aerobic nitrification process. Thus denitrifying bacteria will frequently be exposed to nitrite / FNA in oxic environments leading to the generation of additional reactive nitrogen species as a consequence. With this in mind we have sought to explore the effect of nitrite / FNA on aerobic *P. denitrificans* metabolism and we report here the identification of a cytoplasmic system that contributes to survival at high nitrite / FNA concentrations similar to those reached in some wastewater treatment processes. The research provides molecular information on the response of a denitrifying organism to nitrite / FNA that can

inform those in the water industry assessing the biological impact of nitrite / FNA in various applications.

2. Materials and Methods

2.1. Bacterial strains, media and plasmids.

P. denitrificans PD1222, derivative strains and *E. coli* were cultured using Lysogeny broth (LB) media containing rifampicin (25 $\mu g.mL^{-1}$), kanamycin (25 $\mu g.mL^{-1}$) or gentamicin (25 $\mu g.mL^{-1}$), where appropriate. For growth experiments, a minimal medium was used as described previously (Felgate *et al*. 2012) with varying level of nitrate and nitrite and 30 mM succinate and 10 mM NH_4Cl for carbon and nitrogen sources for growth, respectively. Continuous cultures experiments were performed as described by Felgate *et al*. (2012) Aeration was maintained throughout to maintain a concentration of 0.236 mM (% air saturation).

2.2. Aerobic plate reader and shaking flask batch culture techniques

Aerobic growth profiles were acquired in a 96 well plates format (FLUOstar Omega, UK) containing 100 µL minimal medium and 1% inoculum. Plates were incubated at 30°C with orbital shaking 400 rpm. Growth was monitored every 0.5 hours as optical density (OD) at 600 nm and adjusted to a pathlength of 1 cm. Additional aerobic growth profiles were performed in shaking flasks to facilitate liquid, gas and RNA sampling based on using 50 mL of minimal medium added into a 250 mL conical flask and incubated at 30°C with orbital shaking (200 rpm). Each flask was sealed using a gas permeable foam bung and aluminium foil lightly pressed around the edge to enable gas exchange. Bacterial growth was monitored spectrophotometrically using an Eppendorf® Biophotometer at 600 nm. Growth rates and profiles were calculated based on a semi log plot of OD_{600nm} measurements, here termed as apparent value of exponential growth rate μ_{app}, using the OriginPro 9.0 (OriginLab). The Y_{max} is defined as the maximum OD_{600nm} reached on the growth curve. All growth curves

presented are derived from an average of 6 independent experiments and error bars are +/- the standard deviation.

2.3. Aerobic continuous culture technique

Continuous cultures were established in 2.5 L bio-reactors (BioFlo 310, New Brunswick Scientific) similarly to the study of Felgate *et al.* (2012). Bacteria were incubated in 1.5 L contained media saturated with air. Vigorous agitation (400 rpm) and continuous air flow maintained the dissolved oxygen levels at 100% (air saturation). Temperature and pH were maintained at 30^{o}C and 7.5 respectively through-out the incubation. A typical continuous culture run consists of an initial batch phase for 20 h followed by continuous culture with a dilution rate set at 0.05 h^{-1}.

2.4. Analytical methods

Concentrations of extracellular nitrate and nitrite were determined with high pressure ion chromatography. The Dionex® ICS-900 HPLC system was fitted with a 2 mm x 250 mm IonPac® AS22 column and a DS5 conductivity sensor. The system was eluted with 4.5 mM sodium carbonate (Na_2CO_3) and 1.4 mM sodium bicarbonate ($NaHCO_3$) and regeneration with 10 mM sulphuric acid (H_2SO_4). Calculated based on the pKa (3.25) of the equilibrium for NO_2^-/ FNA using a formula derived from the Henderson-Hasselbalch equation. Nitrous oxide detection was carried out using a Perkin Elmer Clarus® 500 gas chromatographer equipped with an electron capture detector (ECD) and Elite-PLOT Q using nitrogen as the carrier gas: nitrogen and a mixture of 95% argon/5% methane as the make-up gas as in Sullivan *et al* (2013). Calibration gases were acquired from Scientific and Technical Gases Ltd, UK.

2.5. Construction of fhp$^-$ *deficient P. denitrificans*.

An in-frame deletion of Pd_1689 (*fhp)* was generated using the mobilisable suicide plasmid pK18*mobsacB* by allelic exchange via homologous recombination, essentially as described in Sullivan *et al* (2013). Briefly, regions directly upstream and downstream of the DNA to be deleted were amplified by PCR using oligonucleotides incorporating restriction enzyme sites (Supplementary Table 2). These were cloned into pK18*mobsacB* and the resultant plasmid pKH001 was conjugated into *P. denitrificans* PD1222 via triparental mating with *E. coli* harbouring the plasmid pRK2013 Transconjugants were selected first by Kan resistance, and subsequently, by selection on LB media containing sucrose. Double-cross over events were screened by PCR and isogenic *fhp*$^-$ *P. denitrificans* was verified by PCR and sequencing.

2.6. Complementation of fhp *in trans*

To complement the *fhp* mutant *P. denitrificans* strain *in trans*, oligonucleotides were used to amplify the entire *fhp* locus plus 290 bp of DNA upstream of the ATG start codon to include any native *cis*-acting elements required for expression. The PCR product was cloned into the MCS of the wide host-range plasmid *p*OT2 (Allaway *et al*. 2001) and conjugated into *fhp*$^-$ *P. denitrificans* as described above. Prior to growth experiments, persistence of the *fhp*::pOT2 construct was maintained by selecting on gentamicin (20 µg.mL^{-1}).

2.7. RNA extraction from whole cells

RNA extraction was carried out on comparable conditions and in biologically differing triplicates. Surfaces and equipment were treated using RNaseZAP® from Ambion or autoclaved. Whole cell samples were immediately put on ice using addition of 2:5 ratio phenol 5%-ethanol 96% : bacteria culture (v/v) and 1 h incubation on ice for the stabilisation of RNA. RNA extraction and purification was carried out using the SV Total RNA Isolation System from Promega® Z3100 as per manufacturer's instructions for both qRT-PCR and microarray analysis. Quantification of RNA yield was obtained spectrophotometrically in a Thermo Scientific NanoDrop 2000™ Spectrophotometer at

260 nm. DNA contamination was removed using Ambion™ TURBO™ DNase. RNA degradation was checked by RNA electrophoresis using the Bio-Rad® Experion™ RNA StdSens Analysis Kit and DNA contamination checked by PCR using genomic *P. denitrificans* DNA and Bioline® MyTaq™ polymerase. The Bio-Rad® Experion™ RNA StdSens Analysis Kit determined RNA integrity. RNA samples were examined using the Experion Automated Electrophoresis System (Bio-Rad®) by manufacturer's instruction. Each RNA sample was examined using the Experion RNA StdSens Analysis kit (Bio-Rad®) reagents and 1 µL of sample loaded on to an Experion RNA StdSens chip.

2.8. Quantitative real-time reverse transcription PCR

Quantitative real-time reverse transcription PCR (qRT-PCR) was used to quantify mRNA for gene expression profiling and validation of type II microarray analysis. Total RNA was reversed transcribed using Superscript III (Invitrogen, UK) according to the manufacturer's protocol. Oligonucleotides (primers) for the target genes (Supplementary Table 1) were selected based on the genome of *P. denitrificans* PD1222 using Primer3 and ordered through Eurofins MWG® Operon (DE). Gene expression was assessed with a Bio-Rad®C1000 Thermal Cycler and CFX96 Real-time PCR detection system using SensiFAST™ SYBR® Green Master Mix (Bioline, UK) according to the manufacturer's instructions The PCR assays were subjected to melt-curve analysis to ensure that non-specific products and primer-dimer formation was minimal. Gene expression was normalised to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene using primers previously described (Sullivan *et al.* 2013) and relative expression ratios were calculated using primer efficiencies derived from standard curves as described previously (Pfaffl, 2001). The melt-curve analyses for GAPDH and *fhp* amplicons are presented in the supplementary data (Supplementary Fig. 1). All qRT-PCR experiments conformed to the Minimum Information of Quantitative Real-time PCR Experiment (MIQE) guidelines.

2.9. Microarray analysis

RNA was reverse transcribed (RT) using Agilent Technologies AffinityScript™ and then bonded to Cy5-dCTP Blue fluorescent dye. Genomic DNA was extracted using the QIAamp® DNA Mini Kit from QIAGEN® following manufacturer's instructions. RNA Cy3-dCTP Red labelled chromosomal DNA of 2 mg was combined with labelled cDNA 1/5 (v/v) with a Gibco Bioprime DNA labelling system and Klenow enzyme (Bioprime Invitrogen Kit). The cDNA (reverse transcribed RNA) samples were hydrolysed using 15 µL of 0.1 M NaOH and incubation at 70°C for 10 min. This was neutralised with 15 µL of 0.1 M HCl and pipette mixed, making a total volume of 50 µL. The QIAGEN® QIAquick® PCR Purification Kit to clean-purify DNA and RNA (now cDNA) samples from excessive dye and enzymes. A QIAquick® spin column is used for each sample as per manufacturer's instructions. Hybridisation of complementary DNA and genomic DNA to slides. gDNA and cDNA samples were dried to 30 µL by vacuum drying while covered, at 35°C. The volume of gDNA was measured by pipette and 1/5 of gDNA volume added to each cDNA sample. Samples were hybridised on an Agilent custom made *P. denitrificans* oligonucleotide array and hybridised at 55°C for 60 h in a darkened hybridisation oven at 8 rpm. The array slide was washed before being dried in an ArrayIt® Microarray High-Speed Centrifuge for 30 seconds remove the slide from the gasket and carefully open (separate) the DNA array slide before placing it into a glass container and washed as per manufacturer's instructions.

Microarray slides were scanned with a GenePix 4000A scanner (Axon, USA). The fluorescence intensity was imaged with Genepix Pro 7.0 Software. Saturation tolerance was typically set at 0.05 or 5%. Wavelengths are set at 635 and 532 for green / red laser beam. Fluorescence intensity of each dot was quantified by subtraction of background fluorescence and by red/green (Cy5/Cy3) ratio. Intensity values were normalised with the Batch Anti-Banana Algorithm in R (BABAR) algorithm and software package. Statistical analysis of the microarray datasets was done with Genespring 7.3 (Agilent, UK). Genes were filtered with a ≥2 fold expression filter ($p \leq 0.1$) and exported into Microsoft Excel.

3. Results and Discussion

3.1. The effect of extracellular nitrite on aerobic growth of P. denitrificans in batch cultures.

P. denitrificans was grown in aerated batch cultures, in either shake flask or agitated micro-titre wells, over the initial pH range of 6 – 9 (Fig. 1). Growth was very poor at pHs 6 and 6.5 and moderate at pH 9. Good growth to high cell yield was though observed at pHs 7, 7.5, 8 and 8.5. These pHs were therefore selected as the culture conditions for exploring the effect of extracellular nitrite on aerobic growth. Over the range of 2 – 10 mM initial extracellular nitrite an apparent stimulation in the growth of the cultures was observed (Fig. 2). This is a counter intuitive observation suggesting the possibility that between 2 – 10 mM nitrite, there is an energetic gain to the cells. This gain reaches a point at which tolerance is no longer possible and the cells begin to suffer the biocidal effect to an extent that growth is inhibited with respect to nitrite concentration addition. Over the range of 10 – 145 mM extracellular nitrite a degree of growth inhibition was observed that was notably pH dependent, with cultures initiated at pH 8.5 being much less sensitive to nitrite than cultures initiated at pH 7. For example, no growth was observed at 30 mM nitrite at pH 7, whilst at pH 8.5 addition of 145 mM nitrite was required to completely inhibit growth (Figs. 3 & 4). Growth of *P. denitrificans* was not recovered above these pH-specific, growth-inhibiting concentrations of nitrite, therefore these data are excluded from figures for clarity. The pH dependence of sensitivity points towards FNA being a key biocidal factor.

3.2. Physiological analysis of the fhpA locus.

Bioinformatic analysis of the *P. denitrificans* genome enabled identification of a gene (*pden_1689*) coding for a putative NO oxygenase / reductase member of the flavohemoglobin protein family epitomised by well characterised *Escherichia coli* and *Salmonella enterica* serovar Typhimurium

222 flavohemoglobin proteins (*pd1689* will therefore henceforth be referred to as *fhpA*).
223 Flavohemoglobins are able to convert NO to either nitrate or nitrous oxide, depending on the
224 presence or absence of oxygen (Poole and Hughes 2000, Stevanin *et al*. 2002). The *P. denitrificans*
225 *fhpA* gene clustered with a gene (divergently transcribed) that encoded for a putative homologue of
226 the transcriptional repressor NsrR from *E. coli* which responds to nitrosative stress. Expression of *P.*
227 *denitrificans fhpA* was examined at pH 7.5 at a range of sub-lethal extracellular nitrite
228 concentrations. Analysis by qRT PCR confirmed the nitrite-dependent expression of *fhpA*, with
229 expression increasing to a maximum at around 10 mM extracellular nitrite (~2 µM FNA) (Fig. 5).

230 To establish the physiological importance of *fhpA* during aerobic metabolism in the presence of
231 nitrite a deletion mutant was constructed. This mutant was able to grow at up to 45 mM nitrite, but
232 at a consistently lower apparent growth than the WT (wild type) parent strain (Fig. 6 A & B).
233 However, unlike the parent strain the *fhpA* strain was unable to grow in the presence of 50 mM
234 nitrite (~10 µM FNA). This capacity was recovered in full when the strain was complemented with
235 the *fhpA* gene *in trans*. (Fig. 6C).

236

237 *3.3. Aerated continuous culture studies of P. denitrificans in the presence of extracellular nitrate or*
238 *nitrite.*

239 During shake flask or microtitre batch cultures of *P. denitrificans* it is not possible to continuously
240 monitor or control the combination of pH, oxygen, nitrite or biomass levels. Hence in the description
241 of the batch culture studies the external environment and the biomass levels will be changing as a
242 function of time. To explore the effect of nitrite on steady-state *P. denitrificans* cultures, where the
243 external pH, nitrite and biomass level can be clearly defined, we performed some additional
244 experiments using continuous cultures (Fig. 7). The cultures were operated broadly as described by
245 Felgate *et al*. (2012). Aeration was maintained throughout the culture and monitored continuously

246 to ensure 100% air saturation and pH was maintained at 7.5. The cultures were operated as batch
247 cultures for around 24 hours during which time biomass increased. The cultures were then switched
248 to continuous mode with medium flowing through the system to give a dilution rate of 0.05 h^{-1}.
249 Cultures were judged to have reached a biomass steady state at around 80 h (~ 4 vessel volumes).
250 Two comparisons were made. One culture was run with nitrate, which is not considered a reactive
251 N-oxyanion and does not directly yield FNA, and the other with nitrite, which does generate FNA. A
252 flow concentration of nitrite of 35 mM was chosen as this was just below the threshold of tolerance
253 determined in batch cultures (Fig. 2), but would be expected to yield a nitrite / FNA stress response,
254 as indicated by the qRT-PCR data on *fhp* expression in batch cultures (Fig. 5).

255 Both the aerated nitrate and nitrite cultures achieved similar biomass steady states (~0.35 OD units)
256 and in both cases the steady state levels of nitrate or nitrite were similar to the levels in the
257 reservoir medium suggesting there is little, if any, aerobic denitrification occurring (Fig. 7). Thus both
258 cultures were behaving similarly in the continuous culture systems and this was also observed with
259 respect to expression of the genes encoding the denitrification enzymes *nirS*, *norB* and *nosZ*
260 assessed by qRT-PCR which showed similar low levels in both cultures when measured at 120 h in
261 the biomass steady-state. Again, this was consistent with the absence of 'aerobic denitrification'.
262 The similar behaviour of the two cultures deviated though when expression of *fhpA* was assessed
263 and which was 35-fold higher in the steady-state 'nitrite' compared to the 'nitrate' continuous
264 cultures (Fig. 5). This confirmed that *fhpA* was being expressed in order to detoxify nitrite or, a nitrite
265 product, in the system. Since the concentration of extracellular nitrite remained constant at around
266 30 mM throughout the ~100 h steady-state of the experiment it is clear that tolerance through
267 extensive consumption of extracellular nitrite is not taking place. However, if the reactive species is
268 actually FNA then 35 mM extracellular nitrite will equate to only around 3 μM FNA, which could
269 diffuse into the cell and be oxidatively detoxified to nitrate by the FhpA. The 3 μM nitrate generated
270 by this oxidation would not be detectable against the background level of nitrate in the chemostat
271 system (Fig. 7). It was notable though that nitrous oxide, which is a potential reductive product of

FNA detoxification, did accumulate to up to 0.5 µM in the steady state. Thus the up-regulation of *fhpA* and the absence of significant levels of nitrite consumption suggest the intracellular FNA, rather than extracellular nitrite, is the reactive species being detoxified in the system. However, because it is not possible to observe the direct effects of nitrite and FNA in isolation due to their conjugate acid-base relationship, a contributing nitrite-specific, biocidal effect cannot be disregarded. To test the relationship of *fhpA* expression and extracellular nitrite further we set up a number of continuous culture systems at pH 7.5 with the aim of achieving steady states at a range of nitrite concentrations between in 10 – 35 mM. Expression of *fhpA* was proportional to the steady-state level of extracellular nitrite over this range (Fig. 5).

3.4. The transcriptional response of oxic batch cultures of P. denitrificans to sub-lethal extracellular nitrite.

The phenotype of the *fhpA* mutant suggests a role for the flavohemoglobin in tolerance to extracellular nitrite and the resultant intracellular FNA. However, since the mutant was still able to grow at similar growth rates to WT at initial extracellular nitrite concentrations of up to 40mM (Fig. 6A&B), there may be other systems expressed that also contribute to nitrite / FNA tolerance. To explore this further, *P. denitrificans* was cultured aerobically in the presence of sub-inhibitory concentrations of nitrite / FNA and the transcriptome established. A sub-inhibitory concentration of 12.5 mM nitrite was selected because this concentration did not affect the Y_{max} or μ_{app} of WT or *fhpA* mutant cultures, but did induce *fhpA* as indicated by qRT-PCR (Fig. 5). It was therefore anticipated that metabolism would have adjusted to confer resistance to the nitrite, but that the transcriptomic analysis would not be excessively complicated by cellular responses to low growth rates or cell damage that could occur at much higher, more inhibitory, nitrite concentrations. Consistent with this view, only a small number of genes (~1.5% of the genome) were up-regulated (22 genes out of ~5000) or down-regulated (62 genes) more than 2-fold (≥95% significance) in the cultures growing in

297 the presence of nitrite compared to its absence. Verification of the microarray was carried out by
298 qRT-PCR on a number of selected target genes (Table 1 & Supplementary Table 2). Consistent with
299 the qRT-PCR data reported in the previous section, the *fhpA* gene was significantly up-regulated in
300 the micro-array experiments. In addition, the adjacent gene *pd1690,* divergently transcribed from
301 *fhpA,* was also up-regulated ~2-fold in the presence of nitrite. This gene encodes for a putative
302 transcription factor homologous to members of the NsrR family of transcriptional repressors that
303 regulate gene expression in response to nitrosative stress. In *P. denitrificans* it seems likely that the
304 NsrR homolog is regulating transcription of the *fhpA* flavohemoglobin gene. In *E. coli* and *Salmonella*
305 NsrR binds to the DNA upstream of the genes it regulates and prevents its transcription. It features a
306 nitric oxide-sensitive iron-sulphur cluster, the destruction of which, by reactive nitrogen species,
307 leads to release of NsrR protein from the DNA binding site allowing transcription to occur
308 (Bodenmiller and Spiro 2006).

309 Although widely considered as a respiratory process associated with anaerobic metabolism, *P.*
310 *denitrificans* is also reported to catalyse aerobic denitrification. Since nitrite is a substrate for
311 denitrification the expression of the genes associated with denitrification was also assessed during
312 aerobic growth in the absence and presence of nitrite. No significant change was observed in
313 expression of either of the two respiratory nitrate reductases (*napA* and *narG)*, the respiratory
314 nitrite reductase (*nirS*), the nitric oxide reductase (*norB*) or the nitrous oxide reductase (*nosZ*) when
315 examined by both micro-arrays and qRT-PCR. This suggests that respiratory-based detoxification of
316 nitrite during aerobic metabolism is not occurring. In addition to NirS, *P. denitrificans* also has a
317 second nitrite induced nitrite reductase (NasB) in the cytoplasm associated with nitrite assimilation
318 when ammonium is absent. This system was also not up-regulated by nitrite in the experiments
319 reported here, consistent with the presence of ammonium in the growth medium and so again is not
320 likely to be a major contributor to nitrite tolerance.

A gene encoding a respiratory protein that was affected by the presence of nitrite in the growth medium is that encoding the cytochrome ba_3 oxidase (*pd5108*), which was up-regulated by ~2.5 fold, a change that was verified by qRT-PCR (Tables 1 & Supplementary Table 2). The cytochrome ba_3 oxidase mediates electron transfer between ubiquinol and oxygen. The enzyme is a membrane bound proton pump that moves protons from inside to outside the cytoplasmic membrane. One reason for up-regulation of cytochrome ba_3 may therefore be a response to the uncoupling effect of FNA diffusion into the cell that effectively serves to move a proton from the periplasm into the cytoplasm and which could be an important means of tolerating lower levels of intracellular FNA. We note that the aa_3-type cytochrome c oxidase pathway was not observed to be up-regulated in the microarray dataset, though it could also drive such proton translocation. Of the other genes that are up or down regulated it is not clear that any would be specifically involved in tolerance, with many being annotated as hypothetical proteins and a number being involved in various apparently un-related metabolic pathways. Perhaps of some note is the 2.3-fold up-regulation of *pd4586* that is predicted to encode the chaperonin cpn10 (groES) and the 3-fold up-regulation of *pd3605* which is predicted to code for a postulated 93 aa protein shows weak similarity (~50%) with members of YvrJ protein family of characteristically short proteins that are part of the acid stress response in *Bacillus* species through regulation of oxalate decarboxylase acts to rebalance excess protons by catalysing the conversion of oxalate into formate and carbon dioxide with the consumption of a proton (MacLellan *et al*. 2009).

4. Conclusions

The tolerance of a denitrifying bacterium, *P. denitrificans,* to extracellular nitrite during aerobic metabolism has been assessed. Nitrite exists in equilibrium with FNA and the mechanisms by which nitrite and FNA have been reported to act as cytotoxins include the following: (i) FNA can lead to the formation of reactive nitrogen and oxygen species in the cytoplasm including nitric oxide (NO), nitrogen dioxide (NO_2), peroxynitrite ($ONOO^-$), hydroxide ion (OH^-) and hydrogen peroxide (H_2O_2), all

of which exhibit toxicity towards bacterial cells, damaging cellular function and metal centres in protein active sites, disrupting biofilm attachment and causing cell death; (ii) FNA has been suggested to act as an uncoupler whereby it acts to circumvent the ATP synthesis as a short-circuit is formed by FNA transporting protons across the inner membrane and back into the cell and increasing the conductance of the cytoplasmic membrane; and (iii) FNA may be able to directly inhibit electron carriers (Almeida *et al*. 1995a, Almeida *et al*. 1995b, Barraud *et al*. 2006). These possible cytotoxic processes are shown in the schematic (Fig. 8). In the present work the increased sensitivity of aerobic metabolism of *P. denitrificans* to nitrite at pH 7 compared to pH 8.5 is consistent with FNA being the active component and sensitivity was in the range of 1 – 6 μM FNA depending on the growth condition. In principle *P. denitrificans* has enzymes in the periplasm that are able to contribute to detoxification of nitrite and nitric oxide, namely the nitrite reductase (NirS) and NO reductase (NorB). The genes encoding these systems were not induced under oxic conditions in the presence of nitrite and nitrite levels remained high throughout growth of cultures indicating that it itself was not the toxic species and was tolerated at levels up to 140 mM at high pH. Rather, the key response to extracellular nitrite was induction of a gene predicted to encode a flavohemoglobin, which could potentially oxidise nitric oxide to nitrate or reduce nitric oxide to nitrous oxide. This gene was co-located with a putative NO-responsive regulator NsrR that is known to regulate a number of NO-detoxification systems in *E. coli* and *Salmonella* (Spiro 2007). The active range of nitrite / FNA observed in some complex community environments is similar to that identified here in the pure cultures of *P. denitrificans* and it will be of interest to explore gene expression in complex communities in wastewater-treatment systems exposed to sub-lethal FNA concentrations to examine if there is evidence for tolerance to FNA associated with increased expression of flavohemoglobins to further correlate the studies.

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455

456 **Figure legends**

457 **Figure 1. The effect of pH on the aerobic growth of *P. denitrificans* PD1222.** Minimal salts media,
458 supplemented with ammonium chloride, 10 mM, sodium succinate, 30 mM and Vishniac trace
459 element solution. Measured by optical density at 600 nm (OD_{600nm}) adjusted to a pathlength of 1 cm
460 against time (h). Cells were grown aerobically in 100 µl total volume with shaking at 400 rpm.
461 OD600nm was measured every 0.5 h but only the 1 h values shown. Error bars denote standard
462 error n = ≥3.

463 **Figure 2. Stimulation of aerobic growth of *P. denitrificans* PD1222 at low concentrations of nitrite**.
464 Growth experiments were carried out in minimal medium supplemented with ammonium chloride,

10 mM, sodium succinate, 30 mM and Vishniac trace element solution. Measured by optical density at 600 nm (OD600nm) adjusted to a pathlength of 1 cm against time (h). OD_{600nm} was measured every 0.5 h, 1 h values shown. Cells were grown in 100 µl total volume with shaking at 400 rpm. Sodium nitrite concentration addition shown in legend. Error bars denote standard error n = ≥3.

Figure 3. Aerobic of *P. denitrificans* PD1222 at pH 7.0, 7.5, 8.0 and 8.5, with sodium nitrite concentrations in the range of 10 mm to 145 mM. Growth was in minimal salts media, supplemented with ammonium chloride, 10 mM, sodium succinate, 30 mM and Vishniac trace element solution, Measured by optical density at 600 nm (OD600nm) adjusted to a pathlength of 1 cm against time (h). Cells were grown in 100 µl total volume with shaking at 400 rpm. OD600nm was measured every 0.5 h, 1 h values shown. Error bars denote standard error n = ≥3.

Figure 4. Maximum optical density produced (Y_{max} AU) at various sodium nitrite concentrations for the growth of *P. denitrificans* PD1222 at pH 7.0, 7.5, 8.0 and 8.5. Growth was in minimal medium supplemented with ammonium chloride, 10 mM, sodium succinate, 30 mM and Vishniac trace element solution. Measured by optical density at 600 nm (OD_{600nm}) adjusted to a pathlength of 1 cm against time (h). Error bars denote standard error n = ≥3.

Figure 5. Normalised expression of the flavohemoglobin (*fhp*) (gene Pd1689) in *Paracoccus denitrificans* in the presence of nitrite in Batch (diamond and cross points) and continuous (triangle and circle points) cultures. Expression shows *fhp* expression in conditions of nitrite and nitrite and nitrate exposure. Expression was normalised to the glyceraldehyde 3-phosphate dehydrogenase (GAPDH) housekeeping gene. Error bars for cross points only denote standard error n=3. 'Assayed' denotes nitrite (and nitrate for circle points) levels were quantified by HPLC; 'added' nitrite denotes the nitrite concentration added at the start of the batch culture without quantification by HPLC during the culture experiment.

Figure 6: Summary of apparent maximum growth rate (A) and biomass (B) produced at various nitrite concentrations for the growth of *P. denitrificans* PD1222 and the *fhp* mutant. Growth in minimal salts media, pH 7.5, supplemented with ammonium chloride, 10 mM, sodium succinate, 30 mM and Vishniac trace element solution. Measured by optical density at 600 nm (OD_{600nm}) adjusted to a pathlength of 1 cm against time (h). Error bars denote standard error n=3<. C. Growth of *Paracoccus denitrificans* PD1222 wild type (WT), FHP deletion mutant and FHP complement strain in minimal salts media, pH 7.5, supplemented with 50 mM sodium nitrite. Cells were grown in 100 µl total volume with shaking at 400 RPM. OD_{600nm} was measured every 0.5 h, 1 h values shown. Error bars denote standard error n=3.

Figure 7. Chemostat cultures of *P. denitrificans* cultured aerobically in the presence of nitrate. Continuous culture of *Paracoccus denitrificans* PD1222 under nitrosative stress with nitrite addition to minimal salts media at pH 7.5 and 10 mM ammonium chloride (n = 3). At 22 hours feed was applied at a dilution rate of 0.05 h^{-1} (black line at 22 h). Feed was replenished at 72 hours (black line at 72 h). Top panel: average dissolved oxygen (DO), optical density (OD_{600nm}) and biomass. Lower panel: concentration of nitrate, nitrite and nitrous oxide throughout the incubation period. (Error bars denote standard deviation; n = 3.)

Figure 8. A scheme for nitrite and FNA tolerance in aerobic cultures of *P. denitrificans*

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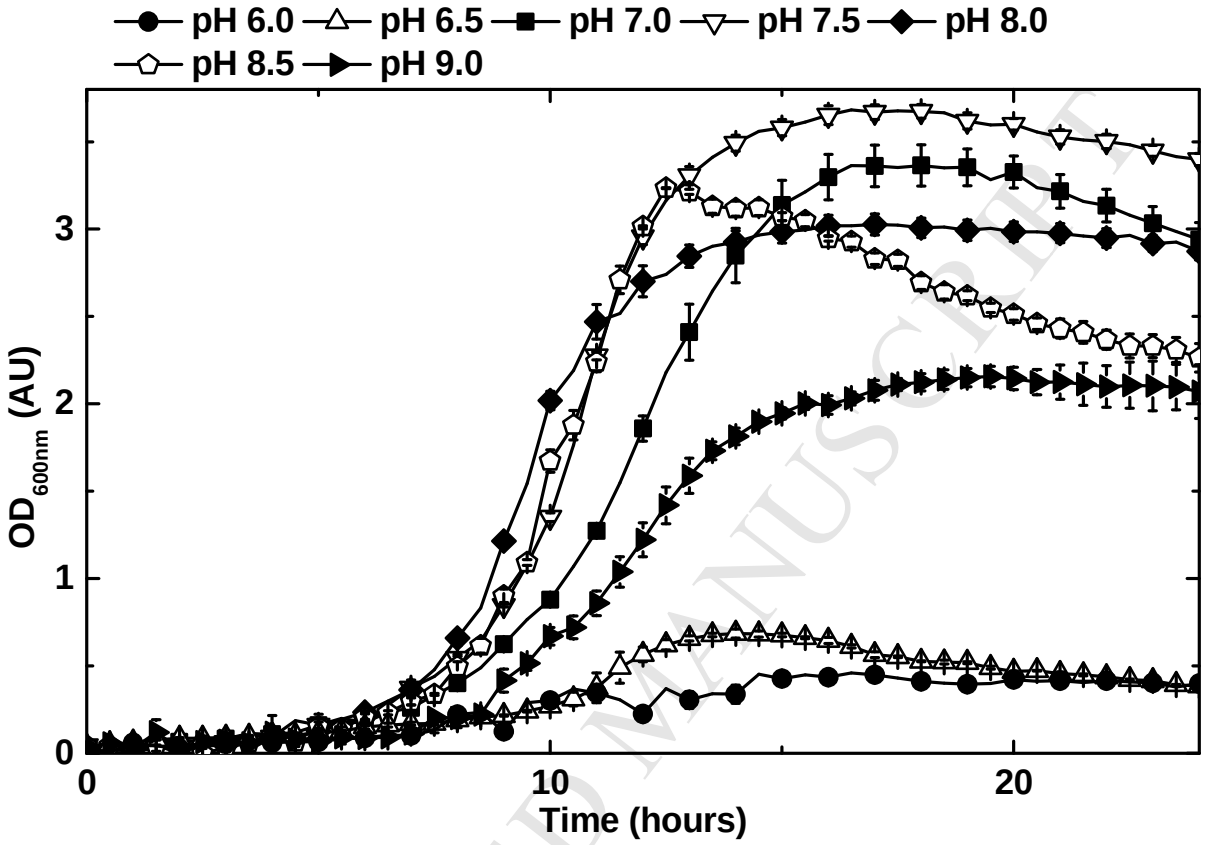
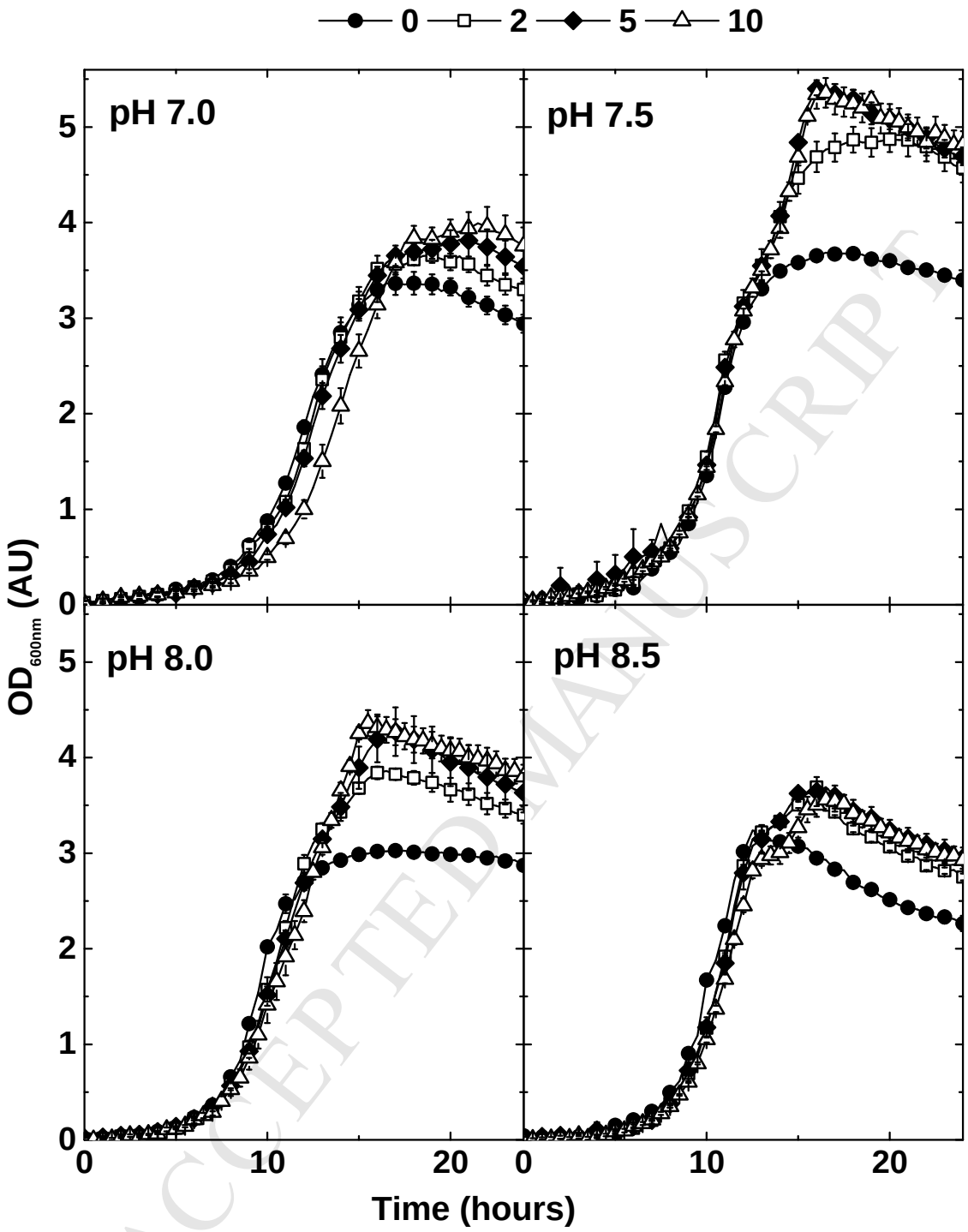
Table 1. Verification of the microarray analysis by qRT-PCR transcriptional analysis of selected genes of *P. denitrificans* grown aerobically at pH 7.5 in the presence of 12.5 mM nitrite.

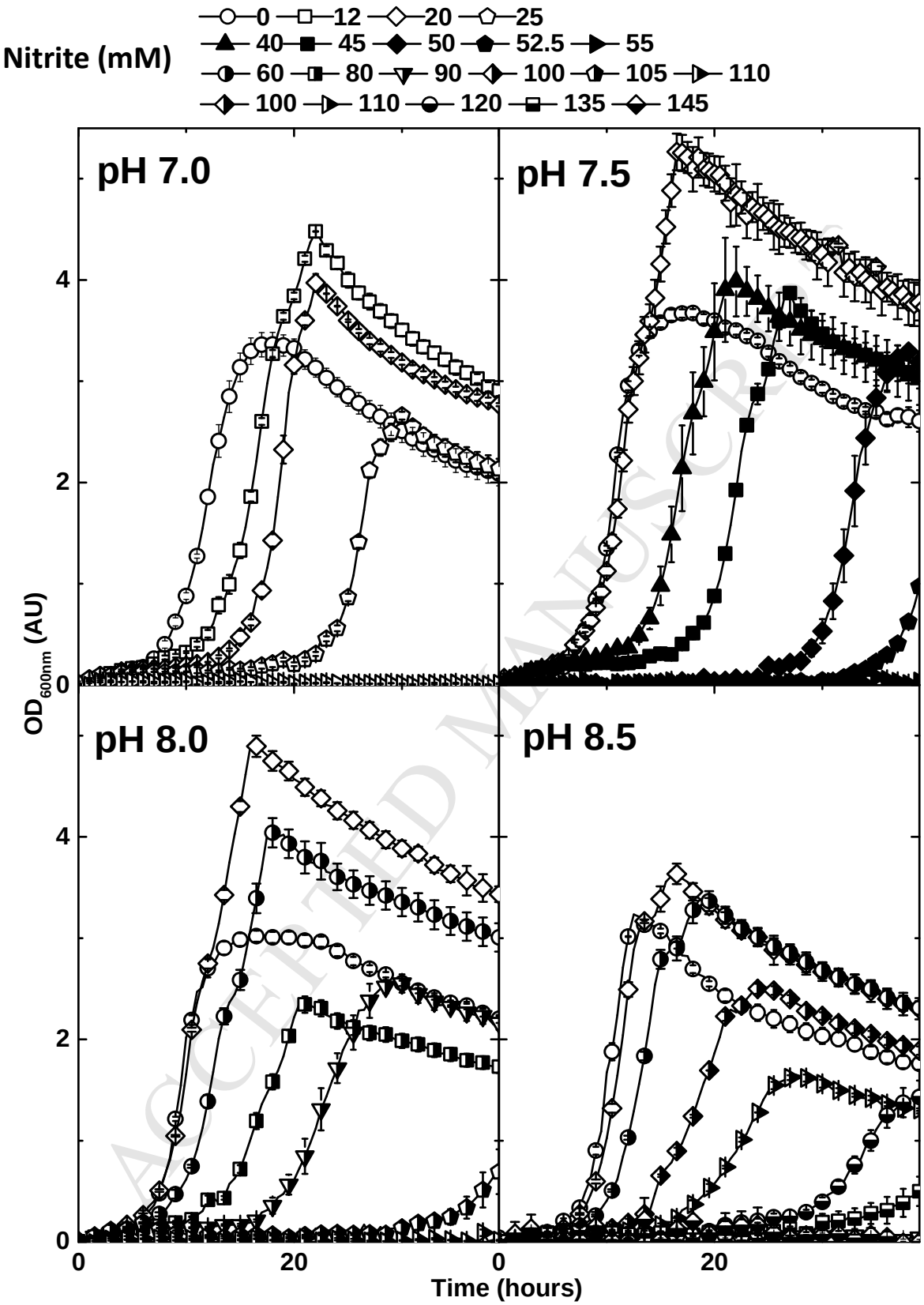
Gene identifier (Pden_)	Gene name	Microarray transcription	qRT-PCR transcription*	±SD
3605	Acid stress1	3.00	2.06	1.27
1690	fhp BadM reg.	2.16	3.61	1.88
1129	Von Willebrand	0.21	0.43	0.25
5108	ba3	2.45	2.68	0.89
1629	sigma-24	0.45	0.39	0.13
3429	DctM subunit	0.44	0.47	0.10
1689	fhp	2.17	3.43	0.37
4721	napA	0.89	0.38	0.10
4236	narG	1.72	0.84	0.15
2487	nirS	0.59	1.12	0.13
2483	norB	0.54	0.65	0.05
4452	nasB	0.93	1.18	0.14
4465	gapdh*	1.18	0.98	0.04
0342	polB	1.12	1.03	0.04

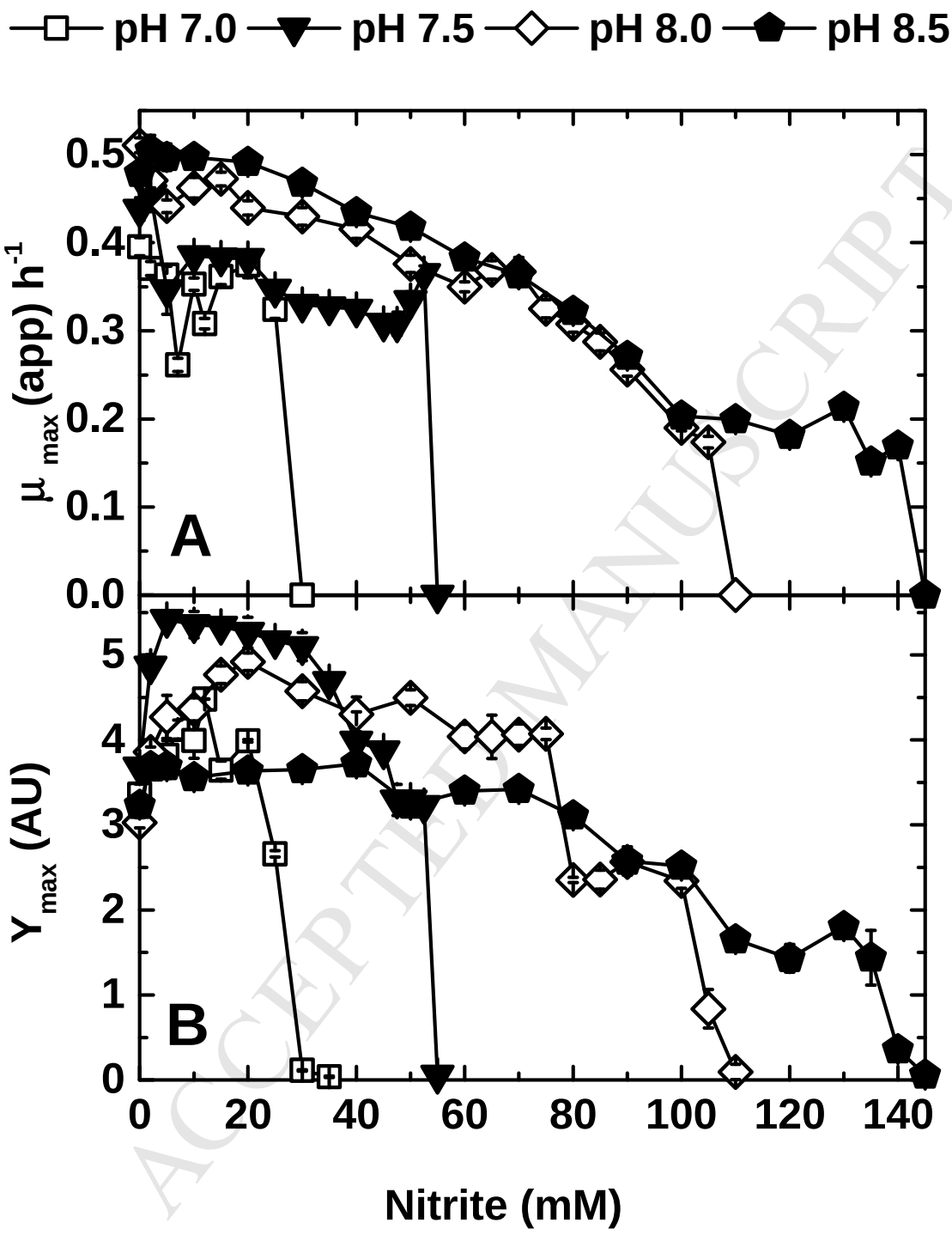
*all qRT-PCR results use gapdh as housekeeping gene for normalisation, with the exception of gapdh itself whose transcription was verified with polB as housekeeping gene

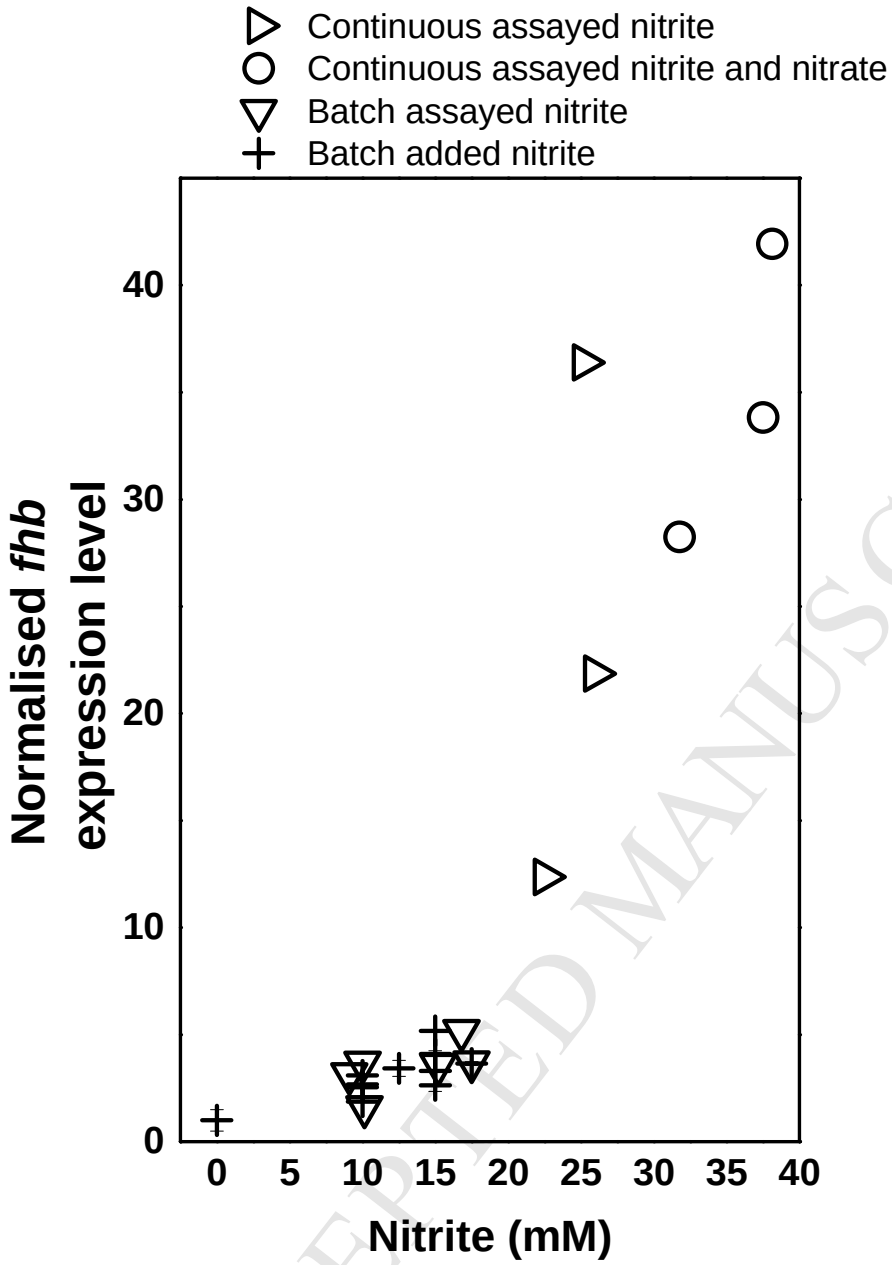
±SD standard deviation n = 3 biologically independent replicates (additional 3 technical replicates)

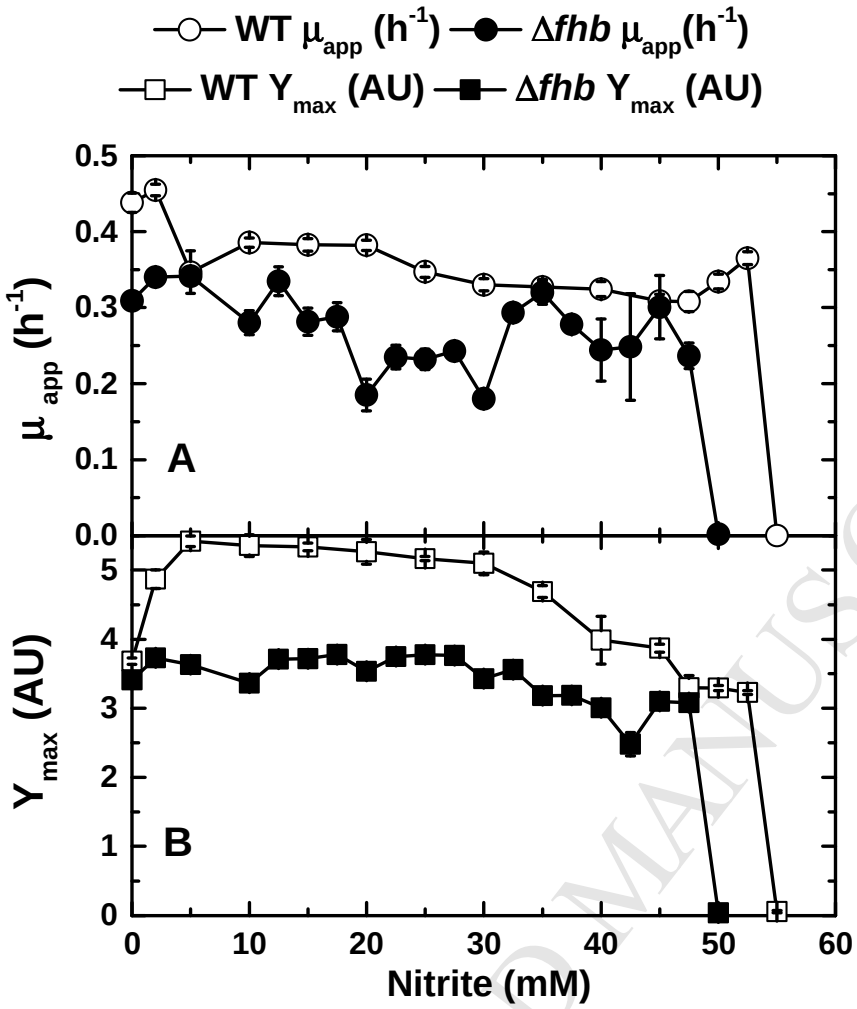


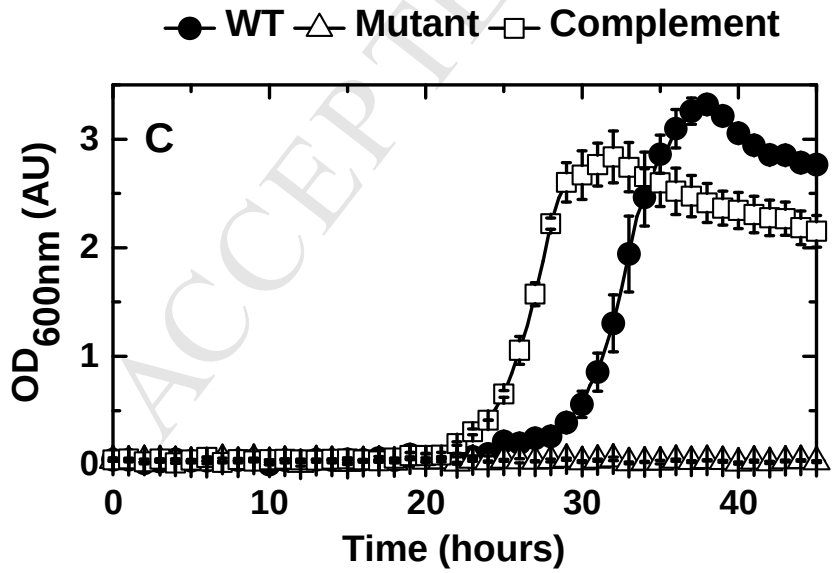


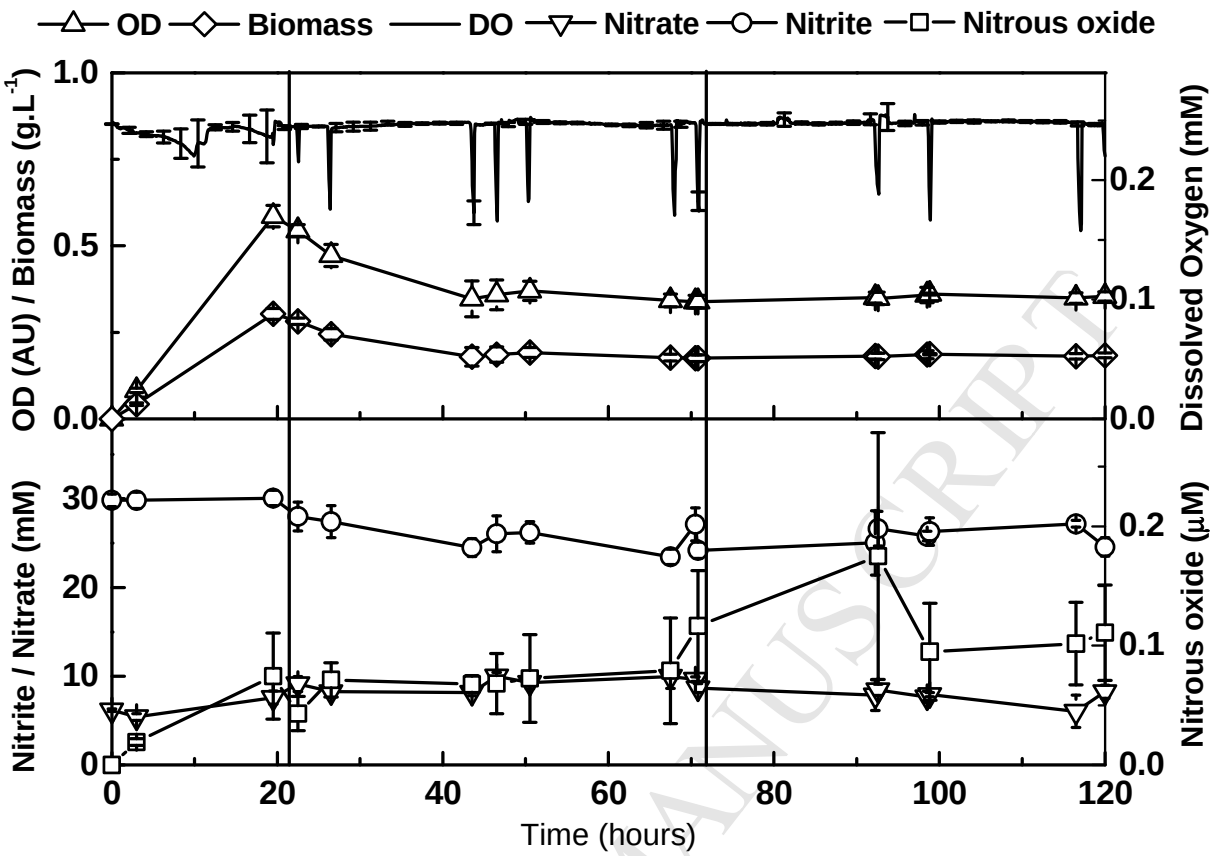


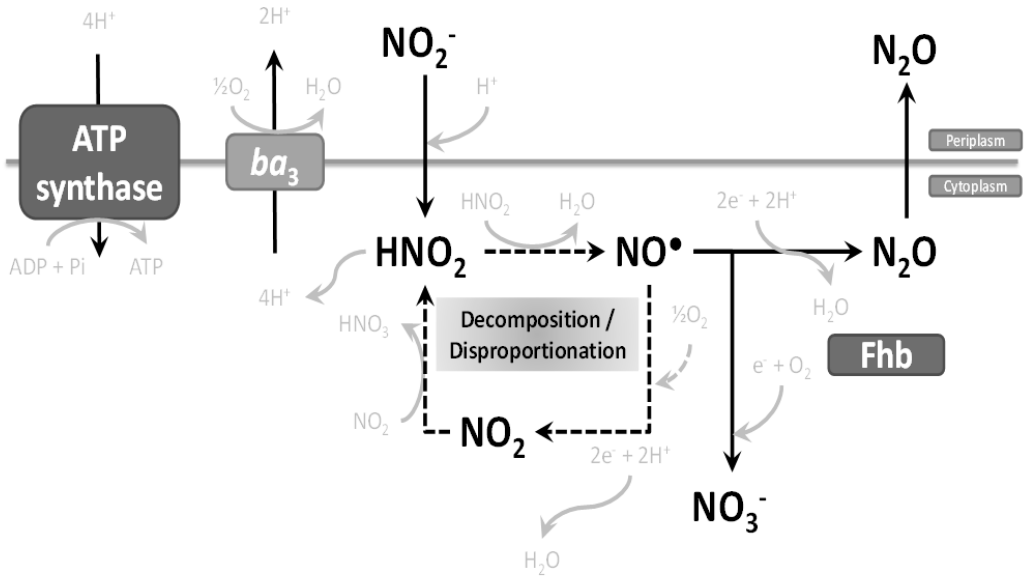












Hartop et al. Highlights

The metabolic impact of extracellular nitrite on aerobic metabolism of *Paracoccus denitrificans*

KR Hartop[a], MJ Sullivan[a], G Giannopoulos[a], AJ Gates[a], P Bond[b], Z Yuan[b], TA Clarke[a], G Rowley[a] and DJ Richardson[a]*

1. *Paracoccus denitrificans* was studied to assess the impact of nitrite on aerobic metabolism
2. Extracellular nitrite inhibits aerobic growth of *P. denitrificans* in a pH-dependent manner
3. Increased expression of a flavohemoglobin was observed in response to extracellular nitrite
4. Analysis of a mutant established flavohemoglobin to be involved in nitrite tolerance
5. Only ~ 1.5% of the genome was differentially regulated in response to nitrite 'stress'