Water Research 113 (2017) 207–214

Contents lists available at ScienceDirect

Water Research

journal homepage: www.elsevier.com/locate/watres

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

K.R. Hartop [a], M.J. Sullivan [a], G. Giannopoulos [a], A.J. Gates [a], P.L. Bond [b], Z. Yuan [b], T.A. Clarke [a], G. Rowley [a], D.J. Richardson [a, *]

[a] School of Biological Sciences, University of East Anglia, Norwich Research Park, Norwich, NR4 7TJ, UK
[b] Advanced Water Management Centre (AWMC), University of Queensland, St Lucia, Brisbane, QLD, 4072, Australia

ARTICLE INFO

Article history:
Received 26 October 2016
Received in revised form
31 January 2017
Accepted 6 February 2017
Available online 7 February 2017

Keywords:
Nitrite
Free nitrous acid
Denitrification
Paracoccus denitrificans
Nitrosative stress
Reactive nitrogen species
Flavohemoglobin
Nitric oxide

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 ($pK_a = 3.25$) 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 Fhp to be involved in endowing nitrite/FNA resistance at high extracellular nitrite concentrations. 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 nitric oxide from the internalisation of FNA.

1. Introduction

The biocidal effect of nitrite in equilibrium with 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, 2006b) and can impact on polyphosphate accumulators (Fux et al., 2003; Zhou et al., 2007, 2008). Nitrite inhibition may be attributable to the protonated conjugate acid of nitrite, free nitrous acid (FNA; $pK_a = 3.25$), 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 nitric oxide (NO) and, if oxygen is present, peroxynitrite. Extensive work by Ye et al. (2010) has suggested that this is 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 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* 0.42 μM FNA completely inhibited phosphate uptake (Zhou et al., 2010). This was corroborated by Jiang et al. (2011) who saw a 75% decrease of biofilm after exposure to 0.42–0.61 μM FNA.

Denitrifying bacteria reduce nitrate sequentially via nitrite, NO 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

* Corresponding author.
E-mail address: d.richardson@uea.ac.uk (D.J. Richardson).

http://dx.doi.org/10.1016/j.watres.2017.02.011

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 extracytoplasmic nitrite and NO that are 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 the oxic-anoxic interface in many environments and the nitrite and nitrate that they use as substrates for denitrification arise 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 *Escherichia coli* were cultured using Lysogeny broth (LB) media containing rifampicin (25 μg mL^{-1}), kanamycin (25 μg mL^{-1}) or gentamicin (25 μg mL^{-1}), where appropriate. For growth experiments, a minimal medium was used as described previously (Felgate et al., 2012) with varying levels of nitrate and nitrite and 30 mM succinate and 10 mM NH_4Cl for carbon and nitrogen sources for growth, respectively. Continuous culture 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 batch culture techniques

Aerobic growth profiles were measured 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 at 400 rpm. Growth was monitored every 0.5 h 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, here termed as apparent value of exponential growth rate μ_{app}, were calculated based on a semi-log plot of OD_{600nm} measurements as a function of time, 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 error.

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 minimal 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 °C and 7.5, respectively, through-out the incubation. A typical continuous culture run consists of an initial batch phase for 22 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 performance liquid chromatography (HPLC). The Dionex® ICS-900 HPLC system was fitted with a 2 mm × 250 mm IonPac® AS22 column and a DS5 conductivity sensor. The system was equilibrated with 4.5 mM sodium carbonate (Na_2CO_3) and 1.4 mM sodium bicarbonate ($NaHCO_3$). The regenerant used was 10 mM sulphuric acid (H_2SO_4). Calculated based on the pK_a (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 *pden*_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 kanamycin 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 broad 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 *P. denitrificans*

RNA was isolated from biological triplicates at similar growth phases for each comparison. Surfaces and equipment used were treated for RNase contamination using RNaseZAP® (Ambion) or autoclaved. For harvesting RNA, cultures of bacteria were stabilised