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A comparative proteomic analysis of *Desulfovibrio vulgaris* Hildenborough in response to the antimicrobial agent free nitrous acid

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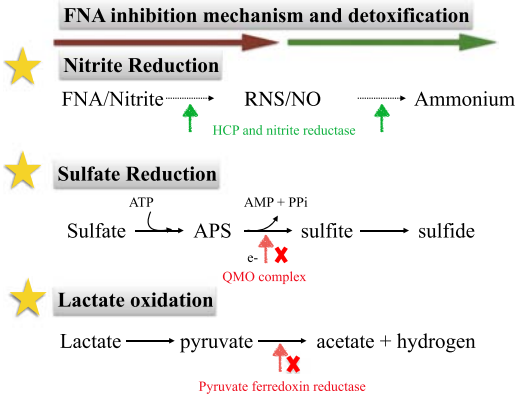
HIGHLIGHTS

- This study investigates the whole genome proteomic dynamics of *D. vulgaris* to FNA.
- The antimicrobial effect of FNA is multi-targeted and concentration dependent.
- FNA exposure stimulated nitrite reduction.
- FNA exposures (4.0 μg N/L) initially inhibited catabolic process then recovered.
- HCP responds to nitrosative stress caused by FNA.

GRAPHICAL ABSTRACT



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ABSTRACT

Sulfate reducing bacteria (SRB) can contribute to facilitating serious concrete corrosion through the production of hydrogen sulfide in sewers. Recently, free nitrous acid (FNA) was discovered as a promising antimicrobial agent to inhibit SRB activities thereby limiting hydrogen sulfide production in sewers. However, knowledge of the bacterial response to increasing levels of the antimicrobial agent is unknown. Here we report the proteomic response of *Desulfovibrio vulgaris* Hildenborough and reveal that the antimicrobial effect of FNA is multi-targeted and dependent on the FNA levels. This was achieved using a sequential window acquisition of all theoretical mass spectrometry analysis to determine protein abundance variations in *D. vulgaris* during exposure to different FNA concentrations. When exposed to 1.0 μg N/L FNA, nitrite reduction (nitrite reductase) related proteins and nitrosative stress related proteins, including the hybrid cluster protein, showed distinct increased abundances. When exposed to 4.0 and 8.0 μg N/L FNA, increased abundance was detected for proteins putatively involved in nitrite reduction. Abundance of proteins involved in the sulfate reduction pathway (from adenylylphophosulfate to sulfite) and lactate oxidation pathway (from pyruvate to acetate) were initially inhibited in response to FNA at 8 h incubation, and then recovered at 12 h incubation. Lowered ribosomal protein abundance in *D. vulgaris* was detected, however, total cellular protein levels were mostly constant in the presence

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or absence of FNA. In addition, this study indicates that proteins coded by genes DVU2543, DVU0772, and DVU3212 potentially participate in resisting oxidative stress with FNA exposure. These findings share new insights for understanding the dynamic responses of *D. vulgaris* to FNA and could be useful to guide and improve the practical applications of FNA-based technologies for control of sewer corrosion.

1. Introduction

The antimicrobial properties of nitrite have been known for a long time, for example it has long been included to be used as a food preservative (Rowe et al., 1979; Cammack et al., 1999). However, it is free nitrous acid (FNA), the protonated form of nitrite, that is thought to be the actual compound responsible for the microbial inhibition (Vadivelu et al., 2006). Recently, FNA has been applied in sewers to control sulfate reducing bacteria (SRB) activity. The study achieved an 80% reduction of hydrogen sulfide production when FNA was intermittently dosed at 0.26 mg N/L for 8–24 h every four weeks (Jiang et al., 2013). Those investigations demonstrated that FNA is highly applicable for controlling SRB activities and limiting sulfide levels that lead to sewer corrosion.

Recently, FNA is becoming a very promising antimicrobial agent in wastewater treatment, for example its potential use for selection of the nitritation pathway for carbon and energy saving purposes (Wang et al., 2014). Consequently, there is great interest to understand how FNA inhibits or kills the bacteria and how SRB may survive in response to FNA. Currently, the antimicrobial effects of FNA are thought to be multi-targeted (Fang, 2004; Gao et al., 2016a, 2016b). FNA can form various reactive nitrogen intermediates in biological conditions. These include NO, N_2O_3, NO_2, and ONOO-, and these reactive nitrogen intermediates can readily diffuse across cell membranes. Iron-sulfur clusters, thiols, heme groups and amines that are biologically important may react with these reactive nitrogen species by nitrosylation to form nitrosyl complexes such as metal nitrosyl complexes, S-nitrosothiols, and N-nitroso compounds (Fang, 2004). These nitrosyl complexes can be cytotoxic to bacteria. There have been some investigations examining the effects of nitrite on *D. vulgaris* Hildenborough (Haveman et al., 2004; He et al., 2006; Yurkiw et al., 2012; Rajeev et al., 2015). During exposure of SRB to nitrite, it is seen that genes coding for dissimilatory sulfite reductase are down regulated, which would potentially disrupt SRB respiration (Haveman et al., 2004; He et al., 2006; Gao et al., 2016a, 2016b). In *Mycobacteirum* it is seen that FNA and perhaps FNA derived reactive nitrogen species (RNS) cause oxidative stress resulting in damage to cell enzymes, cellular membranes and walls, and to nucleic acid (Zahrt and Deretic, 2002). Other hypotheses of the antimicrobial effects suggest that FNA disrupts the proton motive force (Zhou et al., 2011a, 2011b), acts to nitrosylate metal centres or thiol groups in enzymes (O'Leary and Solberg, 1976), and mutates DNA (Fang, 2004). However, most of the conclusions and hypotheses drawn from those investigations are not well corroborated since they are based either on transcriptomics, other physiological/mutant studies, or on low throughput PCR experiments. Moreover, while the transcriptome analysis allows us to examine the changing messenger RNA levels caused by FNA (Wang et al., 2009), not all the differentially expressed transcripts end up as functional proteins, due to post-transcriptional modifications. Quantitative proteomics can explore the metabolic and physiological details of microbes in response to FNA at the functional protein level. A comprehensive and systematic understanding of the antimicrobial mechanisms of FNA via whole genome proteomics on *D. vulgaris* Hildenborough is lacking.

SRB are typically prevalent in anaerobic regions in the sewer. They utilize sulfate as the terminal electron acceptor for respiration to generate energy and produce hydrogen sulfide (Heidelberg et al., 2004). The hydrogen sulfide can then diffuse from the water phase to aerobic parts of the sewers, such as in condensation layers on the sewer wall. In these layers it can be oxidized by sulfur oxidizing bacteria to sulfuric acid and finally induce the corrosion of the concrete surfaces of the sewers. Subsequently, the corrosion leads to serious failure of sewers which requires tremendous repair effort and remediation costs (Jiang et al., 2011; Pikaar et al., 2014). Therefore, there is significant interest to efficiently minimize hydrogen sulfide production by SRB in sewers.

Desulfovibrio species are potentially significant contributors of hydrogen sulfide production in sewers (Sun et al., 2014). The well-studied *Desulfovibrio vulgaris* Hildenborough has a periplasmic cytochrome *c* nitrite reductase converting nitrite to ammonium serving as a detoxifying mechanism (Heidelberg et al., 2004). We have recently studied the response of *D. vulgaris* to FNA exposure and have found the antimicrobial effects are concentration-dependent and multi-targeted (Gao et al., 2016a, 2016b). However, much of the findings in that study are based on a RNA transcriptional response at only one FNA concentration (4.0 μg N/L) and at one time point (1 h after FNA addition). The effect of nitrite on *D. vulgaris* was also examined in other transcriptome based studies using macroarray and microarray analyses (Haveman et al., 2004; He et al., 2006). All these transcriptome-based studies indicate that sulfate reduction was inhibited, that possible oxidative stress was caused, and that iron homeostasis was disrupted in a short time after FNA/nitrite treatment.

To achieve a more comprehensive and systematic picture of the antimicrobial mechanisms of FNA, and of the *D. vulgaris* response, it would be insightful to determine the whole cell proteome dynamics over a range of exposure periods and at different FNA levels. Sequential window acquisition of all theoretical mass spectrometry (SWATH-MS) is a recently developed approach that provides extensive and very convenient label-free quantitation of the measurable peptide ions in a sample (Vowinckel et al., 2013; Schubert et al., 2015). Currently, a proteomic response to bacterial FNA stress exposure has not been conducted. Consequently, in this study we used SWATH-MS to detect whole-cell protein abundance dynamics of *D. vulgaris* in the presence of four FNA concentrations (0, 1.0, 4.0, and 8.0 μg N/L) and at three incubation periods of 2, 8, and 12 h. From this analysis, a more comprehensive understanding of the effects of FNA on *D. vulgaris* was obtained and key responses relating to coping with the FNA exposure were determined.

2. Material and methods

2.1. Culture growth and FNA treatment on D. vulgaris

D. vulgaris Hildenborough (ATCC 29579) was cultured and prepared in triplicate for experiments in a defined lactate-sulfate medium (see the Material and Methods section in the Supplementary Information). Samples from triplicate control cultures (without FNA addition) and from all FNA-treated cultures containing 1.0, 4.0, or 8.0 μg N/L FNA (also performed in triplicate) were taken for total protein extraction after 2, 8, and 12 h FNA exposure. Ten mL of the *D. vulgaris* suspension was taken from the cultures and centrifuged at 12,000 rpm for 5 min, the supernatant was discarded and the cell pellets were immediately frozen in liquid nitrogen and stored at −80 °C prior to total protein extraction. Protein extractions were performed on these cell pellets.

2.2. Whole cell proteome preparation and mass spectrometry analysis

From the above mentioned frozen cell pellets, proteins were extracted using the B-PER method and peptides were prepared by trypsin digestion as previously described (see details in Materials and Methods section in the Supplementary Information) (Grobbler et al., 2015). For each sample the peptides were analysed on a Triple-Tof 5600 instrument (ABSciex) equipped with a Nanospray III interface as previously described (Grobbler et al., 2015). Mass spectrometry (MS) data from the IDA library were combined and then searched using the ProteinPilot software (ABSciex, Forster City CA) against a *D. vulgaris* database obtained from the National Center for Biotechnology Information (NCBI) on the 28th of January 2016. The search settings used were trypsin for enzyme digestion and iodoacetamide for alkylation. The database was searched with the search effort set to "thorough" and a cut off applied >0.05 (10%). The false detection rate was determined using the proteomics system performance evaluation pipeline software, an add-on to ProteinPilot, which uses a decoy database constructed by reversing all the protein sequences in the searched database.

2.3. SWATH-MS data analysis

The IDA library and SWATH-MS data were loaded into PeakView (version 1.2) software for processing using the SWATH micro processing script. To achieve protein identifications the settings of confidence level at 99%, the number of peptides at 5 and the number of transitions at 3, were used. A minimum of 2 peptides and 3 transitions were used for quantitative analysis. The R- based program MSstats (Choi et al., 2014) was used for statistical analysis of the spectral data. A stringency cut-off of log2 fold change (LFC) ≥0.5 or ≤−0.5 (Ting et al., 2009) with q value <0.05 was used to identify the significantly changed protein abundances between the control and FNA treated cultures. All other proteins outside this defined stringency were defined as no change to abundance. The MS proteomics data has been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaíno et al., 2013) with the dataset identifier of PXD004475.

3. Results

3.1. The effect of FNA on protein levels in D. vulgaris

In this study, *D. vulgaris* was cultured using sulfate as the electron acceptor and lactate as the electron donor while producing hydrogen sulfide and acetate. The growth profiles and the proportions of live cells of *D. vulgaris* at different levels of FNA were determined (Figs. S1 and S2 in Supplementary Information). The pH of the medium increased only slightly over the incubation period (Table S1). Similar to previously reported (Gao et al., 2016a, 2016b) the inhibition of growth increased with the increased levels of FNA, to the point when growth was not detected at the FNA level of 8.0 μg N/L. Additionally, 8.0 μg N/L FNA was seen to almost completely kill *D. vulgaris* cultures over the 12 h incubation (Fig. S2), which is lower than that of previous studies showing 4.3 and 5.0 mg N/L FNA could 100% kill *Mycobacterium ulcerans* over a 10 min exposure time (Phillips et al., 2004) and the denitrifier *Pseusomonas aeruginosa* over 20 h exposure (Gao et al., 2015), respectively.

The amount of protein produced by *D. vulgaris* was monitored during the FNA incubations (Fig. 1). Total protein levels (μg/μL) in the absence of FNA addition increased after 8 and 12 h (Fig. 1A). Similar total protein levels were observed for *D. vulgaris* in the control cultures (no FNA addition) and in the cultures exposed to 4.0 and 8.0 μg/L FNA at the 2 h incubation time, while the total protein level of cultures with 1.0 μg/L FNA was higher than that of control cultures (Fig. 1A). However, at the later incubation time points of 8 and 12 h, proteins levels decreased at the higher levels of FNA exposure (Fig. 1A). The decrease of the bacteria numbers would be the main reason for the decrease of the total

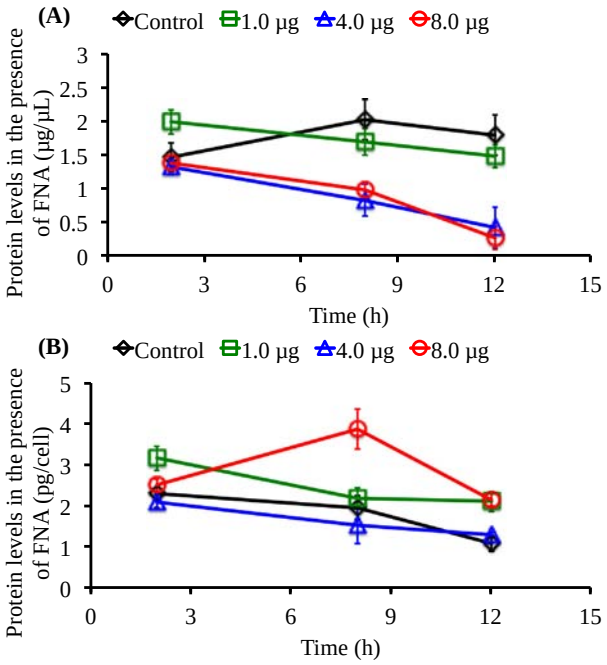

Fig. 1. Total protein levels per microliter of *D. vulgaris* culture in the presence of different FNA levels (control, 1.0, 4.0 and 8.0 μg/L) at different incubation time periods (2, 8 and 12 h) (A), and cellular protein levels per live cells of *D. vulgaris* when exposed to different levels of FNA (control, 1.0, 4.0 and 8.0 μg/L) with different treatment time periods (2, 8 and 12 h) (B). The error bars represent the standard deviation between triplicate samples.

protein levels when exposed to 8.0 μg/L FNA (Fig. 1). Additionally, the activities of *D. vulgaris* were also completely inhibited then the bacteria would not generate new proteins. Protein abundance was also normalised to the number of live cells (Fig. 1B), and protein levels per cell were found to be comparable regardless of the FNA exposure concentration. This suggests that the live cellular components were active and coping with the FNA stress (Fig. 1B). However, it was noticed that a high cellular protein level (4.0 pg/cell) was detected when exposed to FNA at 8.0 μg N/L for 8 h (Fig. 1B). At this FNA exposure, extremely low levels of live cells were detected, and this may account for this outlier cellular protein value. Otherwise, the results indicate that the total protein level per live cell was relatively stable in the viable cells regardless of the FNA level (Fig. 1B).

3.2. Detecting the proteomic response of D. vulgaris at different levels of FNA exposure

To assess the responses of *D. vulgaris* when exposed to different levels of FNA, whole cell quantitative proteome analyses were conducted at the three different treatment periods of 2, 8 and 12 h. These time points were chosen because, on the one hand, it is interesting to examine the immediate and relatively prolonged effect of FNA on protein abundance changes, on the other hand, more protein changes could be detected since the half-life of proteins is variable depending on protein type and many other factors (Nath and Koch, 1971). The high performance liquid chromatography MS/MS spectra for all proteins had appropriate intensity and quality for further analysis (Fig. S3). A total of 863 unique proteins were identified within the IDA library with a false detection rate of 0.01. Proteins with significantly different abundance (adjusted p value <0.05) were determined between pairwise comparisons of the *D. vulgaris* control cultures (no FNA) with cultures at each of the different FNA levels (1.0, 4.0 and 8.0 μg N/L). This was performed for each of the different treatment times of 2, 8, and 12 h (Table 1). For the more extensive treatments of 8 and 12 h at

Table 1
The number of proteins with significantly different abundance levels as determined between the FNA-treated cultures (1.0, 4.0 and 8.0 μg N/L) and the control cultures (no FNA addition) at the different treatment periods.

FNA levels (μg N/L)	FNA treatment time (h)		
	2	8	12
1.0	14	8	11
4.0	10	65	69
8.0	15	51	75

4.0 and 8.0 μg/L FNA, more proteins were detected with significant altered abundance. The potential functions of these proteins include cell metabolism, protein synthesis, oxidative stress response, and other proteins with unknown functions. Details of these proteins are in the following sections.

3.3. Changed protein abundance relevant to metabolism

Changed abundances of proteins relevant to metabolism in the presence of FNA were determined (Fig. 2, Table S2). Cytochrome c nitrite reductase (DVU0625), the hybrid cluster protein (HCP) (DVU2543), and an iron-sulfur cluster-binding protein (DVU2544) are proposed to participate in nitrite reduction in *D. vulgaris* (Zhou et al., 2010; Zhou et al., 2011a, 2011b; Zhou et al., 2013; Zhou et al., 2015). The activity of these proteins is suggested to serve as a global role in regulating the response to nitrosative stress (Cadby et al., 2017). The nitrite reductase component (DVU0625) showed some increase in abundance at the low level FNA exposure (1.0 μg N/L) but not at the higher FNA concentrations (Fig. 2, Table S2). In contrast, the HCP and DVU2544 proteins showed the highest increases in abundance levels detected in this study at all three incubation periods and at all FNA concentrations (excluding the low FNA exposure level of 1.0 μg N/L for 2 h) (Fig. 2, Table S2). These results strongly coincide with a transcriptome investigation of *D. vulgaris* exposed to FNA (Gao et al., 2016a, 2016b) and further implicate the potential importance of HCP in the defence to FNA stress.

In the presence of 1.0 μg N/L FNA at all incubation time points, and at 2 h for all applied FNA concentrations, most of the proteins detected relevant to lactate oxidation and sulfate reduction showed no change in abundance (Fig. 2, Table S2). This likely reflects that the low level of FNA caused no obvious inhibition effect on cell growth, sulfate reduction, and lactate oxidation as previously reported (Gao et al., 2016a, 2016b).

In comparison, some proteins potentially involved in sulfate reduction and lactate oxidation showed different abundance levels at the FNA exposures of 4.0 and 8.0 μg N/L. For instance, adenylyl-sulfate reductase (DVU0846), implicated in the reduction of sulfate to adenylylphophosulfate (APS), was more abundant at 8 h and 12 h incubation when exposed to 4.0 μg N/L FNA. Additionally, sulfate adenylyltransferase (DVU1295) showed increased abundance at 12 h incubations with FNA levels of 4.0 and 8.0 μg N/L (Fig. 2, Table S2). This occurred when sulfate reduction activity was very low (Gao et al., 2016a, 2016b). This apparent anomaly may be explained in that at 8 h incubation with 4.0 μg N/L FNA (starting concentration) the culture had begun to recover respiratory activity as the actual measured FNA level had lowered to around 2.0 μg N/L (Gao et al., 2016a, 2016b). In comparison, the heterodisulfide reductase (DVU0850), part of the quinone-interacting membrane-bound oxidoreductase complex (QMO complex), reportedly involved in the reduction of APS to sulfite (Zhou et al., 2010; Zhou et al., 2011a, 2011b; Zhou et al., 2015), had reduced abundance at 8 and 12 h incubation with starting FNA levels of 4.0 μg N/L (Fig. 2, Table S2). Meanwhile, ATP synthase subunit beta (DVU0775) displayed increased protein levels at 8 h and 12 h incubation with FNA concentrations of 4.0 and 8.0 μg N/L (Fig. 2, Table S2). These results indicate a mix of responses to FNA exposure. The observations indicate that FNA caused increased the abundances of DVU0846 and DVU1295 in *D.vulgaris*, indicating FNA had no inhibitory effect to the reduction of sulfate to APS. However, the lowered abundance of DVU0850 would potentially impede the reduction pathway of APS to sulfite.

In agreement with sulfate reduction, several proteins involved in lactate oxidation in the presence of 4.0 and 8.0 μg N/L FNA showed different abundance levels. These proteins include pyruvate ferredoxin oxidoreductase (DVU1569), alcohol dehydrogenase (DVU2201), formate dehydrogenase (DVU2482), an uncharacterized protein (DVU3032) belonging to luo operon, and an iron-sulfur cluster-binding protein (DVU3033) (Fig. 2, Table S2). DVU1569 and DVU2201 showed decreased abundance at 8 h and 12 h incubation after FNA addition, implying the inhibition of pyruvate to acetate metabolism had occurred. DVU3032 and DVU3033 exhibited increased abundance at 8 h and 12 h after FNA addition. These are elements of the luo (for lactate utilization operon) operon which is reported to function in lactate oxidation in *D. vulgaris* (Vita et al., 2015). Additionally, ATP synthesis protein (DVU0775) and electron transfer protein HMC (DVU0535) (Fig. 2, Table S2) also showed increased abundance at 8 h and/or 12 h with FNA levels of 4.0 and/or 8.0 μg N/L. This could be evidence of possible metabolic activity recovery of *D. vulgaris*. However, the actual measured activities of nitrite reduction, sulfate reduction, and lactate oxidation activities are quite limited at 12 h with FNA concentrations of 4.0 and 8.0 μg N/L (Gao et al., 2016a, 2016b). For the starting FNA concentration of 4.0 μg N/L, at 12 h incubation after FNA addition, the measured FNA level had decreased to approximately 2.0 μg N/L (Fig. S4). Therefore, it seems that by that stage, *D. vulgaris* cultures were attempting to recover metabolic activities. However, for the starting FNA concentration of 8.0 μg N/L at 12 h incubation, higher abundance of proteins involved in sulfate reduction and lactate oxidation still occurred. At the high FNA levels, there was considerable decrease of FNA even when cell numbers were low, which only about 5% of the cells remain viable (Gao et al., 2016a, 2016b). This is possibly due to the occurrence of "persister" cells which have developed resistance and have the potential to detoxify FNA with high efficiency. Additionally, the decrease may be due to potential chemical nitrite reactions being more noticeable at the high nitrite level (Fang, 2004). Moreover, the initial sharp decrease with the addition of 8.0 μg/L FNA could be resulting from potential chemical transformations of nitrite and FNA being more significant at the higher levels (Fang, 2004). In comparison, transcriptomics analysis described previously demonstrated down regulation of all the genes involved in sulfate and lactate reduction. These observed differences may relate to differences reflecting in short term response (only determined 1 h after 4.0 μg N/L addition (Gao et al., 2016a, 2016b), as opposed to the longer term responses determined in the proteomes of this study. Moreover, the higher FNA levels applied coincided with lowered total protein levels (Fig. 1). Likely this is because when exposed to higher FNA levels, there are less live cells of *D. vulgaris* Hildenborough, which resulted in the total protein abundance decrease relevant to metabolism.

3.4. Proteins differentially expressed relevant to protein synthesis

Similar to proteins relevant to metabolism, the levels of most proteins involved in protein synthesis showed no significant changes in the presence of 1.0 μg N/L FNA and at 2 h incubation for 4.0 and 8.0 μg N/L FNA addition. Although, this was in exception to the proteins DVU0874 at 2 h with 4.0 μg N/L FNA, DVU1322 at 8 h with 1.0 μg N/L FNA, and DVU2339 at 2 h with 8.0 μg N/L FNA (Table S3). Of the 55 proteins identified, 18 of them showed increased abundance at 8 h and/or 12 h incubation in the presence of 4.0 and 8.0 μg N/L while 3 of them (DVU1309, DVU1312, and DVU2926) had decreased abundance with FNA exposure (Table S3). Additionally, several proteins (e.g. DVU2981 for 2-isopropylmalate synthase and DVU3168

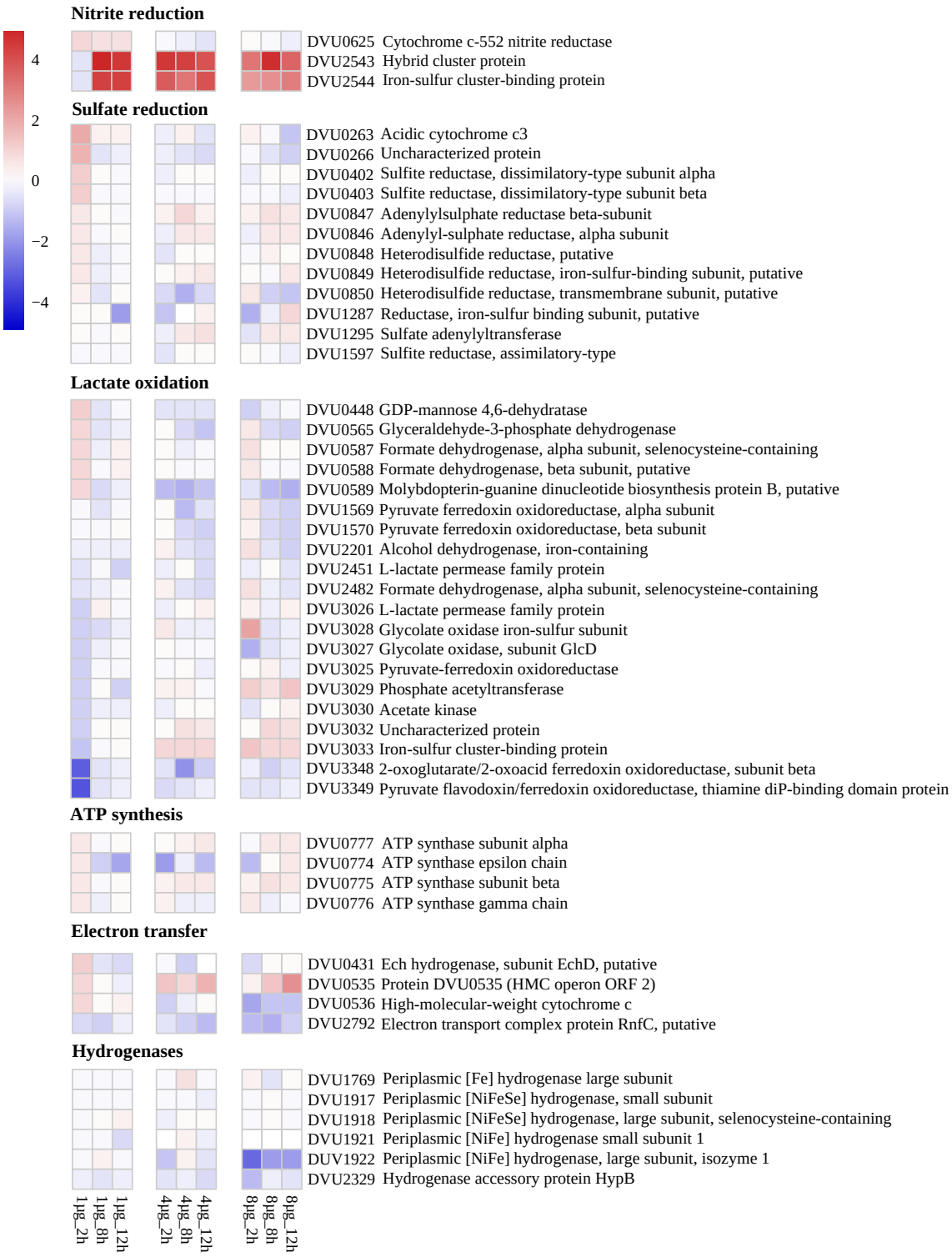

Fig. 2. Proteomic responses of genes involved in metabolism in *D. vulgaris* cultures. Changes in protein abundance over time in response to three different FNA concentrations (1.0, 4.0 and 8.0 μg N/L) are shown. Candidates are grouped by function. The range of changes for the three FNA levels is shown in the key to the left of the heat map. The candidates included are proteins identified by SWATH-MS that are important for central pathways such as lactate oxidation, sulfate reduction, and nitrite detoxification.

for glutamate-1-semialdehyde 2,1-aminomutase) involved in amino acid biosynthesis showed increased protein abundance in the presence of 4.0 and 8.0 μg N/L at 8 h and 12 h incubations. Largely, the components of the protein synthesis pathways seem not much affected by the exposure to low FNA levels, although there was evidence of attempts to increase protein synthesis were detected at the higher FNA levels.

3.5. Evidence of oxidative stress in response to FNA exposure

It is proposed that FNA causes oxidative stress on *D. vulgaris* and this was confirmed by lowered cellular redox detected in the presence of FNA (Gao et al., 2016a, 2016b). Surprisingly, this study revealed that in the presence of FNA, only one protein (DVU0278) at 1.0 μg N/L FNA exposure and two proteins (DVU0772 and DVU3212) at 4.0 and 8.0 μg

N/L FNA exposures, which are proposed to be involved in oxidative stress, had higher abundance compared to that of control cultures (Fig. 3, Table S4). DVU0278 is annotated as a glyoxalase family protein, DVU0772 is annotated as an uncharacterised protein which is part of the PerR regulon, and DVU3212 is annotated for pyridine nucleotide-disulfide oxidoreductase activity. The PerR regulon is predicted to be involved in oxidative stress (Mukhopadhyay et al., 2007). Of the 5 PerR regulon members we detected by SWATH-MS quantitative analysis, DVU0772 for conserved hypothetical protein (Zhou et al., 2010), DVU2247 an alkyl hydroperoxide reductase subunit C-like protein, DVU3094 and DVU3093 proposed rubredoxin oxygen oxidoreductases, and DVU2318 a rubrerythrin, only protein DVU0772 had increased abundance (8 times) in the presence of high levels of FNA at 8 h and 12 h incubation. The other proteins showed either decreased abundance or no change. This indicates that potentially DVU0772 plays a critical role in resisting oxidative stress caused by FNA.

In addition to PerR regulon members, rubredoxin and related proteins including DVU3183 for desulfoferrodoxin, DVU3184 for rubredoxin, DVU3185 a rubredoxin-oxygen oxidoreductase, and DVU3212 a pyridine nucleotide-disulfide oxidoreductase are suggested to have roles in the oxygen defence mechanism of *D. vulgaris* (*Chhabra* et al., 2011). It is demonstrated that DVU3183 and DVU3185 play important roles under aerobic and anaerobic conditions respectively (Wildschut et al., 2006). DVU3212 is suggested to have a flavin monoculeotide cofactor that reduces oxygen to hydrogen peroxide and transfers electrons to APS reductase from NADH (Chen et al., 1994). Although potentially involved in energy metabolism, the primary function of DVU3212 is thought to be for defending against oxidative stress in *D. vulgaris* (Chhabra et al., 2011). The PerR regulon protein DVU3184 was not detected by SWATH-MS analysis in our study. However, we observed increased protein levels for DVU3212 in the presence of 4.0 and 8.0 μg N/L at 8 h and 12 h incubation while decreased protein levels or no change for DVU3183 and DVU3185 in the presence of FNA. These results suggest that DVU3212 rather than DVU3183 and DVU3185 is critical to resist the oxidative stress caused by FNA or RNS.

3.6. Other protein abundance dynamics resulting from the FNA exposure

Apart from the proteins mentioned above in three main aspects of cellular activities (metabolism, protein synthesis, and oxidative stress), many other proteins showed altered abundance when exposed to different FNA levels (Table S5). The increased abundance of proteins DVU0004, DVU0042 and DVU3199, involved in DNA and RNA biosynthesis, indicates that *D. vulgaris* was potentially enhancing DNA and RNA replication at low FNA exposure levels. However, DVU2929 a DNA-directed RNA polymerase subunit displayed decreased abundance at 4.0 and 8.0 μg N/L FNA. This suggests the inhibition of RNA biosynthesis occurs at higher levels of FNA, which is in agreement with previous studies of *P. aeruginosa* and *D. vulgaris* when exposed to FNA (Gao et al., 2016a, 2016b).

In *D. vuglaris*, there are two gene clusters coding for the orange protein complex including DVU2103-DVU2104-DVU2105 (*orp2*) and DVU2107-DVU2108-DVU2109 (*orp1*). This complex is proposed to be involved in cell division of this organism (Neca et al., 2016). DVU2108 and DVU2105 exhibited lowered protein levels in the presence of 8.0 μg N/L at 12 h incubation. Consequently, high FNA levels may cause changes in cell morphology. However, at this stage there is no experimental evidence to directly support this. Protein DVU2569, for peptidyl-prolyl cis-trans isomerase involved in cell wall biosynthesis

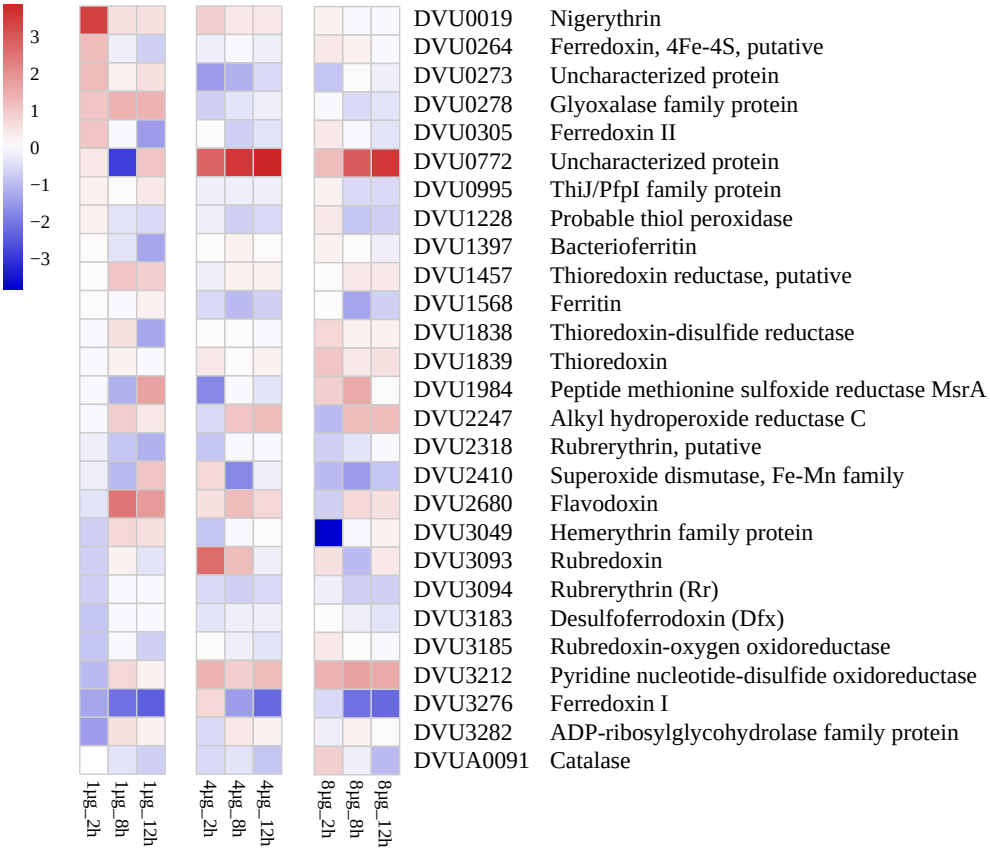

Fig. 3. Proteomics responses of genes involved in oxidative stress in *D. vulgaris* cultures. Changes in terms of protein abundance over time in responding to three different FNA concentrations (1.0, 4.0 and 8.0 μg N/L) are shown. The range of changes for the three FNA levels is shown in the key to the left of the heat map. The candidates included are proteins identified by SWATH-MS that are important for oxidative stress.

(Stolyar et al., 2007), was also down regulated in the presence of 4.0 and 8.0 μg N/L FNA at 12 h incubation. Potentially there is lowered cell wall synthesis in response to the stress, which is also reported in *D. vulgaris* with alkaline stress (Stolyar et al., 2007). Proteins up regulated involved in periplasmic binding, transport, and excretion. This included increased abundance of the proteins DVU1260 an outer membrane protein P1, DVU1343 a cation ABC transporter, and DVU1612 an ACT domain protein, when exposed to 4.0 and 8.0 μg N/L FNA.

Several universal stress proteins (DVU0261, DVU0423, and DVU1030) in *D. vulgaris* had different abundance levels with FNA exposure. DVU0261 showed decreased abundance during increased FNA levels and incubation time, while in contrast DVU1030 exhibited increased abundance and DVU0423 showed almost no change (Table S5). DVU1030 is reported to be a stasis induced protein (Clark et al., 2006), and its down regulation implicating that FNA stress is causing *D. vulgaris* to change from active growth status to stationary phase. The gene DVU2441 codes for a heat shock protein which had increased abundance in the presence of 4.0 and 8.0 μg N/L FNA. The heat shock response, important for cell homeostasis, facilitates the folding of nascent proteins and the repair of damaged proteins (Chhabra et al., 2006). This suggests that protein damage occurred in *D. vulgaris* during exposure to FNA. In comparison, another heat shock protein, DVU0811 a chaperone protein DnaK (Chhabra et al., 2006), showed decreased abundance at 12 h in the presence of 8.0 μg N/L FNA, with no change at other FNA concentrations and incubation times.

4. Discussion

FNA is a promising antimicrobial agent in wastewater treatment and there is increasing successful investigations and high potential for practical application to inhibit or kill SRB in sewers for control of sewer corrosion (Jiang et al., 2011; Jiang et al., 2013). However, the underlining mechanism of how SRB responds to FNA over time is an important factor to be considered for improved FNA applications in wastewater treatment. In this study, protein dynamics in the presence of different levels of FNA were determined in the model SRB *D. vulgaris*. For the first time, a quantitative SWATH-MS whole proteome approach was used to detect and compare protein dynamics in the presence of different FNA levels over time. The proteomic analysis detected significant differences in protein abundances at the different FNA levels. This revealed multiple responses of *D. vulgaris* to FNA exposure that included detoxification mechanisms. These responses were largely in agreement with FNA affected growth profiles and physiological responses previously detected in *D. vulgaris* (Gao et al., 2016a, 2016b). Consequently, our approaches for protein extraction and for quantitative proteomic analysis were appropriate for detection of the bacterial responses to environmental change.

In this study the HCP was one of the most abundant proteins detected in *D. vulgaris* when exposed to FNA. High expression of the gene DVU2543 for the HCP in *D. vulgaris* has been reported previously in response to nitrite/FNA exposure (Haveman et al., 2004; He et al., 2006; Gao et al., 2016a, 2016b). Consequently, the HCP is likely highly relevant for the organism's response to the antimicrobial effects of FNA. The HCP has been shown to respond to nitrosative stress in *Salmonella enterica*, *Escherichia coli*, and *Desulfovibrio species* (Karlinsey et al., 2012; Rowley et al., 2012; Cadby et al., 2016; Wang et al., 2016). In *E. coli* it has been characterised as a high affinity nitric oxide (NO) reductase that reduces NO to nitrous oxide under physiologically relevant conditions. Potentially this is acting to protect against nitrosative stress (Wang et al., 2016). In *Desulfovibrio* species, it is proposed that the HCP is working to relieve RNS/NO stress. Where a lack of HCP results in severely impaired growth under nitrosative stress conditions (Figueiredo et al., 2013; da Silva et al., 2015). In *D. vulgaris*, DVU2543 was one of the most highly abundant proteins in this proteomics study at all treatment times with all FNA concentrations except at 2 h with 1.0 μg N/L. Therefore, this supports the hypothesis that FNA resulted in nitrosative stress to *D. vulgaris*, and this coincides with previous conclusions that the HCP provides protection against nitrosative stress in bacteria (da Silva et al., 2015; Wang et al., 2016; Cadby et al., 2017). This also agrees with transcriptomic studies, where the gene for HCP was one of the most abundantly expressed in response to FNA exposure for 2.5 h (Haveman et al., 2004; He et al., 2006; Gao et al., 2016a, 2016b). Consequently, it is apparent that DVU2543 is critical for responding FNA exposure and important for relieving nitrosative stress in *D. vulgaris*. Proteins responsible for part of the nitrosative stress response in *Desulfovibrio gigas*, such as the rubredoxin oxygen oxidoreductases (DVU3093 and DVU3094), showed either no change or reduced abundance when exposed to FNA. This further emphasizes the importance of the HCP for the *D. vulgaris* response to FNA stress. It is previously reported that *D. vulgaris* experiences oxidative stress during FNA exposure (Gao et al., 2016a, 2016b). It was seen that the potential oxidative stress proteins, DVU0278, DVU0772 (part of PerR regulon) and DVU3212 (Fig. 3, Table S4), did show some increased abundance in response to FNA. The PerR regulon not only responds to oxygen stress, but also is reported to respond to salt (Mukhopadhyay et al., 2006; Zhou et al., 2013), and heat stresses (Chhabra et al., 2006). However, in the conditions of the experiments conducted here with *D. vulgaris*, it is likely these proteins were participating to deal with oxidative stress caused by FNA. It would be useful to understand the metabolism of *D. vulgaris* by validating the functions of proteins DVU2543, DVU0772, and DVU3212, which requires future investigation.

Several proteins responsible for protein synthesis showed no change at 2 h incubation and increased abundance at 8 h and 12 h incubation when exposed to 4.0 and 8.0 μg N/L FNA. In comparison, severe down regulation of the genes involved in protein synthesis was observed in a previous transcriptome study (Gao et al., 2016a, 2016b). Thus, there is a major discrepancy between the transcriptomic and proteomic investigations, although, this can be logically explained. The transcriptome study revealed that during the FNA exposure the cells were very inactive and rather than lose or degrade the ribosomes, these are preserved in an inactive state (Gao et al., 2016a, 2016b). Consequently, the proteins of these preserved ribosomes are still detected in this proteomic response. There is also the possibility that the initial down regulation of the protein synthesis genes was alleviated over incubation time. This could occur through nitrite reduction activity lowering the high FNA levels to around 2.0 μg N/L, especially for the 8 and 12 h exposures (Gao et al., 2016a, 2016b), enabling recovery of some cell activities.

In summary, this proteomic examination revealed that *D. vulgaris* underwent protein abundance variations, and part of that response was to alleviate the nitrite stress by nitrite reduction. The proteomic response dynamics to increasing FNA levels was logical. Firstly, proteins involved in nitrite reduction and nitrosative stress, including the HCP (DVU2543), exhibited highly increased levels at almost all FNA concentrations and at all treatment times (Fig. 4). Secondly, FNA exposures of 4.0 and 8.0 μg N/L initially led to inhibition of sulfate reduction (from APS to sulfite) and lactate oxidation (from pyruvate to acetate) (Fig. 4). Subsequently, these inhibition effects seem to be diminished over time. This can be deduced from the increased protein levels involved in sulfate reduction and lactate oxidation that occurred over the longer exposure times at higher FNA levels. Lastly, the levels of proteins involved in protein synthesis, which do not substantially change, could possibly be explained by the preservation of ribosomes as suggested by the previous transcriptomic study (Gao et al., 2016a, 2016b). This study further emphasizes the importance of the HCP protein in relieving FNA stress, which is likely playing an important role for protecting bacteria from nitrosative stress.

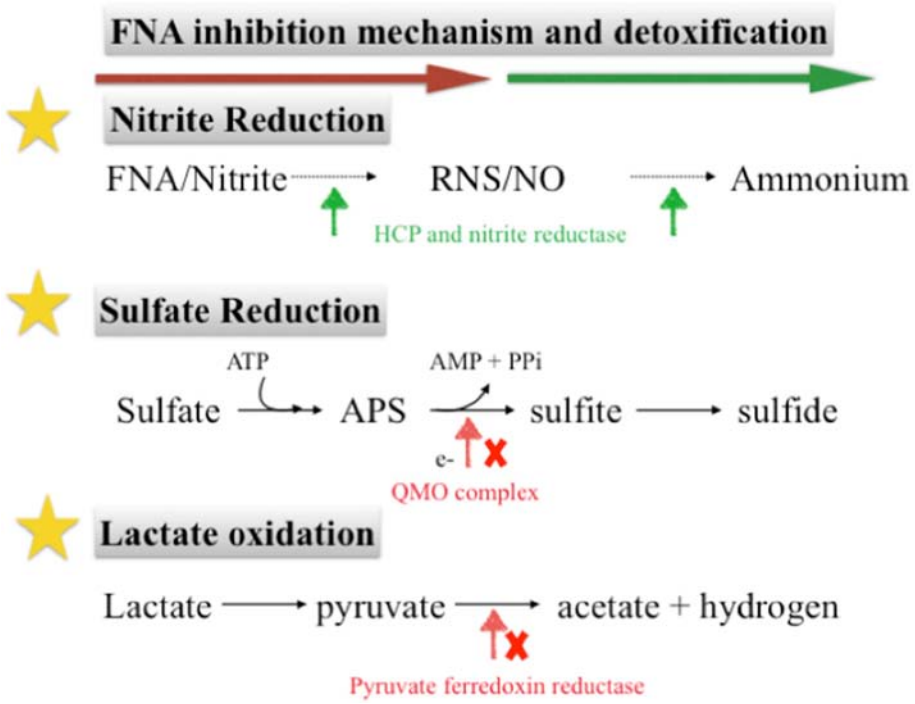

Fig. 4. A representation of the FNA inhibition mechanism on *D. vulgaris* and detoxification strategies based on the interpretations of the proteomic responses. The colored arrows indicate where the protein abundance increased (green) or decreased (red) in response to FNA exposure.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2019.03.442.

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