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Brief note

Comparative proteomics of *Acidithiobacillus ferrooxidans* grown in the presence and absence of uranium

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Abstract

Acidithiobacillus ferrooxidans is an acidophile that thrives in metal-contaminated environments and tolerates high levels of uranium. To gain a better understanding of the processes involved in U(VI) resistance, comparative proteomics was used. The proteome of *A. ferrooxidans* was grown in the presence and absence of 0.5 mM U(VI); expression of 17 proteins was upregulated and one was downregulated. Most proteins with increased expression are part of the general stress response or are involved in reactive oxygen species detoxification. Four novel proteins showed increased expression in the presence of U(VI) and may contribute to U(VI) resistance via thiol homeostasis and U(VI) binding.

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1. Introduction

Widespread contamination of the environment with toxic heavy metals and radionuclides is often a result of industrial activities and is of considerable concern for the environment and human health. Microorganisms are capable of rapidly adapting to changes in environmental conditions. Some microbes can tolerate high levels of these contaminants and could be used for bioremediation of the contaminant in question. For such an approach to be undertaken, knowledge of the resistance mechanism(s) employed by the organism is required.

Uranium (U) contamination is of particular concern due to its toxicity and radioactivity, both of which are regarded as ecological and public health hazards. *Acidithiobacillus ferrooxidans* is an acidophilic bacterium that thrives in metal-contaminated environments and is resistant to high

concentrations of U. Some strains of *A. ferrooxidans* can grow in the presence of 9 mM U(VI) [1], whereas *Escherichia coli* growth is inhibited at a concentration of 2 mM U(VI) [2]. *A. ferrooxidans* is capable of U(VI) biosorption [1,3]; U(VI) is mainly found on the cell wall and within the extracellular polysaccharides [3], suggesting U(VI) does not accumulate inside the cell. Comparative proteomic studies on the response of *E. coli*, *Geobacter sulfurreducens* and *Caulobacter crescentus* to U(VI) exposure indicate that there is no specific mechanism for uranium resistance [4–6].

Since genetic studies in *A. ferrooxidans* are difficult, several proteomics studies of *A. ferrooxidans* have been undertaken to gain insight into mechanisms of adaptation in response to environmental changes. Studies have been undertaken to determine how the proteome changes in response to metals such as copper, cadmium, nickel, zinc and potassium [7,8]. To gain insight into how *A. ferrooxidans* is able to resist damage by U(VI), we carried out a comparative proteomics approach where the bacterium was grown in the presence and absence of U(VI) and a comparison was made of proteins that were differentially expressed.

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2. Materials and methods

2.1. Bacterial strains and growth conditions

A. ferrooxidans ATCC 23270 was grown at 28 °C in iron medium, pH 1.6 [9] with and without 0.5 mM U(VI), until cells reached mid-log phase. *A. ferrooxidans* cells were adapted to growth with 0.5 mM U(VI), resulting in approximately 10 subcultures prior to harvesting cells for protein extraction.

2.2. *A. ferrooxidans* cell preparation, lysis and protein precipitation

A. ferrooxidans cells were harvested by centrifugation at $23,700 \times g$ for 30 min; all centrifugation steps were carried out at 4 °C. The pellets from 3×400 ml cultures were pooled together and this was classed as one replicate sample. The cells were washed twice in 1 ml of 0.01 N H₂SO₄ by centrifugation at $10,000 \times g$ for 10 min and stored at –20 °C. Cell pellets (4 from each growth condition) were suspended in 600 µl of protein solubilisation buffer (7 M urea; 2 M thiourea; 4% CHAPS) containing benzonase (250 units; Sigma–Aldrich) and protease inhibitor cocktail (4 µl; GE Healthcare). Approximately 1 g of glass beads (0.1 mm diameter) was added to each tube. The cells were vortexed for 30 s followed by 1 min incubation on ice; this process was repeated 8 times. The suspension was centrifuged for 2 min at $10,000 \times g$, the supernatant was centrifuged for a further 60 min and the supernatant stored overnight at –20 °C. A 2-D Clean-up kit (GE Healthcare) was used according to Procedure A of the manufacturers' instructions. The protein

pellets were suspended in 100 µl of rehydration buffer (30 mM Tris, pH 8.8; 7 M urea; 2 M thiourea; 4% CHAPS). Protein concentrations were determined using the Bradford method [10].

2.3. Differential protein expression analysis

Differential in gel electrophoresis (DIGE) was performed using the Refraction-2D™ labelling kit (NH DyeAG-NOSTICS) according to the manufacturer's specifications. Four replicate experiments were carried out comparing four with U(VI) and four without U(VI) samples. Isoelectric focussing (IEF) was performed using an Ettan™ IPGphor™ IEF system (GE Healthcare) and an 18 cm, pH 4–7, immobiline drystrip. IEF was undertaken at 20 °C using step-and-hold methods as follows: 0 V 5 h; 30 V 5 h; 500 V 2 h; 1000 V 0.3 h; 1500 V 0.3 h; 2500 V 0.3 h; 5000 V 10 h; 8000 V until 69,500 V was achieved. IEF strips were equilibrated as described previously [11]. SDS-PAGE was done using an 11% resolving gel in an Ettan DALT II electrophoresis system (GE Healthcare). Proteins were stained with Brilliant Blue-G-Colloidal (Sigma–Aldrich) and digitised using a Typhoon TRIO variable mode imager (GE Healthcare). Spot detection and differential protein expression analysis were performed using Delta 2D software (Decodon). Differentially expressed spots (Student's *t*-test value of less than $p < 0.05$) were excised from the gel. In gel digestion, analysis by MALDI-TOF/TOF MS and database searching were performed by Plateforme Protéomique Strasbourg Esplanade as described previously [12]. Proteins were identified by searching data against the SwissProt non-redundant protein sequence database. In all results, the probability

Table 1

MALDI-TOF/TOF spectroscopy analysis showing proteins with altered expression following growth of *A. ferrooxidans* in the presence of U(VI).

Locus tag (NCBI)	Gene name	Putative function	MW (Da)	pI	Fold change (U(VI) 0.5 mM/0 mM)
Cellular processes and signalling					
AFE_2086	<i>hsp20</i>	Heat shock protein Hsp20	16,924	6.2	3.7
AFE_1648	<i>hsp20</i>	Heat shock protein, Hsp20 family	16,671	5.43	3.3, 3.6, 3.7
AFE_0200	<i>ftsZ</i>	Cell division protein FtsZ	40,578	4.98	2.2
AFE_2600 or/and AFE_3117		Putative uncharacterised protein	22,830	5.54	2.0
AFE_0375	<i>trxB</i>	Thioredoxin reductase	35,056	5.61	1.7
AFE_0985	<i>ahpC/tsa</i>	Alkyl hydroperoxide reductase/thiol specific antioxidant/Mal allergen	22,534	5.69	1.4
AFE_2666	<i>grpE</i>	Protein GrpE	18,949	5.12	1.4
Cellular processes and signalling, and information storage and processing					
AFE_2017	<i>pspA</i>	Phage shock protein A, PspA	26,454	4.87	1.6, 1.8
Metabolism					
AFE_1545	<i>mop</i>	Molybdopterin binding protein	7,204	5.77	2.1
AFE_0419	<i>tal</i>	Transaldolase	39,609	5.4	1.9
AFE_2155	<i>cbbL2</i>	Ribulose biphosphate carboxylase	50,522	5.69	1.7
AFE_1811		2-Oxo acid dehydrogenase, acyltransferase, putative	44,291	6.26	1.6
AFE_1857	<i>gdh</i>	Glucose 1-dehydrogenase, putative	27,388	5.03	1.4
AFE_2741	<i>omp40</i>	Major outer membrane protein 40	42,225	4.93	0.2, 0.3
Poorly characterised					
AFE_3280		Phospholipid-like binding protein	20,358	5.25	2.1
AFE_2018		Putative uncharacterized protein	30,506	5.04	1.9
AFE_1839		Glyoxalase/bleomycin resistance protein/dioxygenase	15,321	5.06	1.7
AFE_3116 or/and AFE_2599		Putative uncharacterised protein	13,809	5.04	1.5

scores were greater than the score fixed as significant with a *p*-value of 0.05.

3. Results and discussion

Analysis of the *A. ferrooxidans* proteome following growth with or without U(VI) was undertaken to gain a better understanding of the mechanism(s) involved in U(VI) resistance. As has been done previously [3], we adapted the cells to growth with 0.5 mM U(VI); non-adapted cells were severely stressed in the presence of U(VI) and did not reach a cell density that provided enough protein for proteomic studies. A total of 22 differentially expressed protein spots were analysed by MALDI-TOF/TOF MS. In three cases, multiple spots corresponded to the same protein identification (AFE_1648, AFE_2017 and AFE_2741), indicating post-translational modification. Expression of 17 proteins was upregulated and expression of one protein was downregulated in response to growth with U(VI) (Table 1).

Proteins whose expression was up- or downregulated in response to growth of *A. ferrooxidans* in the presence of U(VI) were grouped according to their putative function within the COG (clusters of orthologous groups) functional categories. Most of the proteins were classified into the cellular processes and signalling (39%) group. The remainder of the proteins were classified into cellular processes and signalling and information storage and processing (6%), metabolism (33%) and poorly characterised (22%) groups (Table 1).

3.1. Cellular processes and signalling

The greatest number of changes observed in proteins from *A. ferrooxidans* grown in the presence of U(VI) belonged to the functional group cellular processes and signalling; within this category, 75% belonged to the post-translational modification, protein turnover and chaperones subgroup. Heavy metal ions and metalloids interfere in protein folding affecting cell viability and protein homeostasis [13]. Heat shock proteins (Hsps) are induced in response to stress, and function as protein chaperones, preventing aggregation or aiding in the refolding of partially denatured proteins [14]. AFE_1648 and AFE_2086 are both annotated as Hsps that belong to the Hsp20 family, and their expression was the most upregulated in response to *A. ferrooxidans* growth with U(VI) (Table 1). The expression of the co-chaperone protein GrpE was also upregulated in the presence of U(VI). It had been previously shown that when *A. ferrooxidans* LR was exposed to 40 °C (10 °C above optimum growth temperature) for 20 h, the expression of Hsp20 encoded by AFE_1648 and GrpE was upregulated [15]. Overexpression of Hsps in *A. ferrooxidans* was also observed in response to metals such as copper [16], growth stresses such as pH [17] and changes in energy sources such as iron and sulphur [18], suggesting that Hsps respond to different types of stress in *A. ferrooxidans*.

The expression of PspA was upregulated in the presence of U(VI). PspA is considered part of a cytoplasmic stress response in Gram-negative bacteria and has been shown to

maintain the proton motive force across the membrane, which is required by many divalent metal transporters for function [19]. In *E. coli*, the expression of PspA was upregulated in response to a variety of stresses, indicating that PspA is involved in the general stress response [20] and might be part of the general stress response in *A. ferrooxidans* to U(VI).

Exposure of *A. ferrooxidans* cells to U(VI) triggers a response against oxidative stress to protect cellular functions and maintain thiol homeostasis. The expression of both TrxB and AhpC/Tsa family proteins was upregulated, and they function to combat oxidative stress by decomposition of superoxide radicals or oxidation of sulphur-containing moieties, using electrons donated from redox cofactors. Genes in one operon from *Desulfovibrio desulfuricans* G20 which encode thioredoxin, thioredoxin reductase and an oxidoreductase were heterologously expressed in *E. coli* and shown to increase its resistance to U(VI). These results indicate that thioredoxin may serve as the electron donor for U(VI) reduction and that thioredoxin reductase is the terminal reductase involved in U(VI) reduction [21]. The expression of TrxB is probably upregulated in response to U(VI) in *A. ferrooxidans*, because the reduced redox state of the cytoplasm is compromised due to reactive oxygen species (ROS) oxidising thiols, and therefore TrxB may help to maintain a reduced redox state in the cytoplasm.

3.2. Metabolism

The expression of both transaldolase and ribulose biphosphate carboxylase (RubisCO) was upregulated in the presence of U(VI). Transaldolase is important for the balance of metabolites in the pentose-phosphate pathway. In *Saccharomyces cerevisiae* and *Xanthomonas campestris* pv. *phaseoli* transaldolase mutants, there is a reduction in NADH in the cell, which is required to combat oxidative stress, resulting in higher sensitivity to oxidative stress [22]. The increased expression of transaldolase in response to U(VI) could be a mechanism enabling *A. ferrooxidans* to produce more NADH through the pentose phosphate pathway so as to combat oxidative stress. RubisCO is the enzyme that catalyses CO₂ fixation by the Calvin–Benson–Bassham (CBB) cycle in autotrophs. *cbbL2* (AFE_2155), encodes a Form II RubisCO; it has been suggested that Form II RubisCO may be involved in maintenance of the redox balance of the cell [23]. *A. ferrooxidans* contains two genes encoding a Form I RubisCO (AFE_1690-1 and AFE_3051-2) and a gene encoding a Form IV RubisCO (AFE_0434). Since expression of other RubisCO genes was not upregulated, it is possible that AFE_2155 has an alternative unknown function and could be involved in U(VI) resistance in *A. ferrooxidans*.

Molybdopterin-binding protein (Mop) is involved in Mo transport and ATP binding and its expression was upregulated in the presence of U(VI). Mop from *Haemophilus influenzae* has been shown to bind molybdate at two sites, and can also weakly bind sulphate (bond length = 143 pm) [24]. The oxyanion size is thought to be important in molybdenum binding proteins [25]; therefore due to a similar bond length, they can also bind tungstate (Mo–O bond length = 173 pm,

W–O bond length = 174 pm) [24]. The U–O bond length varies from 167 to 208 pm; therefore, it may be possible that Mop can bind it, but this has never been shown. It could also be possible that Mop can bind phosphate (P–O bond length = 163 pm, P=O bond length = 150 pm) which is bound to U(VI).

The expression of outer membrane protein 40 (Omp40) was downregulated in the presence of U(VI). Omp40 belongs to the Porin_O_P family which are anion-specific porins. Phosphate groups are the main binding sites for U(VI); U(VI) associated with *A. ferrooxidans* is mainly found as uranyl organic phosphate compounds [3]. The expression of Omp40 was downregulated in the presence of copper and it was suggested that a change in the permeability of the outer membrane occurred, decreasing the entrance of copper ions to the cell [16]. Omp40 is predicted to be located in the outer membrane and U(VI) in *A. ferrooxidans* is mostly associated with the cell wall, so it is possible that a change in the permeability of the outer membrane occurs to decrease U(VI) entrance to the cell to prevent toxicity.

3.3. Poorly characterised

The expression of glyoxalase/bleomycin resistance protein/dioxygenase protein (AFE_1839) was upregulated in the presence of U(VI). ArsK in *Bacillus subtilis* is involved in arsenic resistance by maintaining thiol homeostasis, and both *arsK* and AFE_1839 belong to the VOC metallo-enzyme superfamily [26]. An *arsK* deletion in *B. subtilis* was more sensitive to arsenite in the presence of oxygen [26]. Arsenic is known to induce oxidative stress and low-molecular-weight thiols play an important role in the protection against oxidative stress, therefore, the *arsK* mutant may be impaired in its thiol homeostasis, which renders it more sensitive to arsenite-induced oxidative stress [26]. When *Shewanella oneidensis* was exposed to Cr(VI) stress, the expression of a glyoxalase family protein was upregulated; it was hypothesised the gene that codes for the glyoxalase family protein and an adjacent that codes for a hypothetical protein, function in cellular defence against thiol-reactive electrophiles [27]. The protein encoded by AFE_1839 might be involved in thiol homeostasis in *A. ferrooxidans*.

AFE_3280 was upregulated in the presence of U(VI) and is annotated as a phospholipid-like binding protein. In *S. cerevisiae*, a gene knockout of a phospholipid-binding protein resulted in a decrease in U(VI) accumulation compared to the wild-type [28], and AFE_3280 may serve the same role in *A. ferrooxidans*.

The expression of an uncharacterised protein in *A. ferrooxidans* (AFE_2018) was upregulated in the presence of U(VI). The protein encoded by gene AFE_2018 contains two YbjQ-1 domains and the gene is adjacent to gene AFE_2017 coding for PspA. From comparative structural analysis, this family is likely to be involved in heavy-metal binding, and therefore may play a role in U(VI) resistance. AFE_2019 also encodes a region homologous to proteins in the YbjQ-1 superfamily. Although only expression of AFE_2017 and AFE_2018 was upregulated, the the corresponding adjacent gene products could all be involved in responding to metal stress. Given the close proximity of the genes encoding the YbjQ-1 family protein and PspA, it is possible that they form an operon and may be involved in resistance to U(VI) in *A. ferrooxidans*.

Two sets of identical genes (AFE_2599/AFE_3116 and AFE_2600/AFE_3117) encode two identical uncharacterised proteins (Fig. 1), which were upregulated in the presence of U(VI). AFE_2599/AFE_3116 encodes a DsrE domain which is involved in intracellular sulphur reduction [29]. TusD has strong sequence homology to DsrE, and it has been shown that TusA transfers sulphur to TusD [30]. AFE_2600/AFE_3117 encode a TusA domain which functions as a sulphur transferase. Bacterial TusA homologues contain a highly conserved cysteine residue (C19) which is responsible for persulphide formation and sulphur transfer [30]; AFE_2600/AFE_3117 contain this conserved cysteine residue (data not shown). Given that the expression of AFE_2599/AFE_3116 and AFE_2600/AFE_3117 was upregulated in the presence of U(VI), there is a strong likelihood that these proteins interact together in *A. ferrooxidans* with a role in sulphur relay. Low molecular weight thiols maintain the reducing environment of the cytosol and are critical for preventing the oxidation of cysteine residues in proteins.

Transcriptional regulation of genes of interest from the proteomics study was analysed by quantitative-RT-PCR analysis (data not shown). Unfortunately, we did not find any

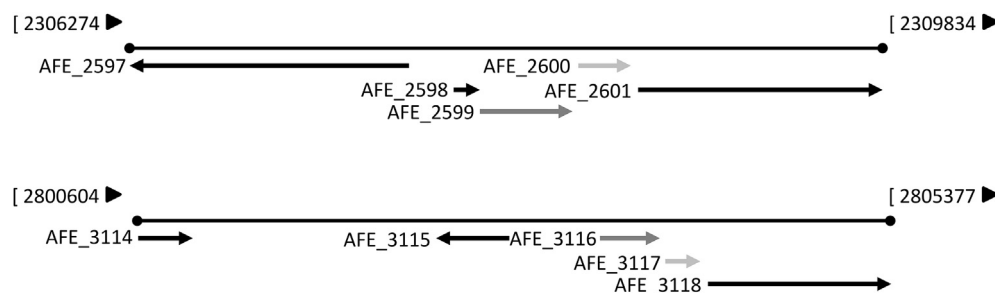


Fig. 1. Two identical copies of two genes encoding proteins with increased expression in the presence of U(VI). AFE_3117/AFE_2600 (light grey arrows) and AFE_3116/AFE_2599 (dark grey arrows) are uncharacterised proteins whose expression was upregulated when grown in the presence of U(VI). AFE_3118/AFE_2601 is annotated as a pyridine nucleotide-disulphide oxidoreductase in *A. ferrooxidans* ATCC 23270. The remainder of the genes encode uncharacterised proteins. The position on the chromosome is shown above each gene arrangement.

correlation between the proteomics and q-RT-PCR results. It is possible that the rate of response to growth with U(VI) differed between the mRNA and protein levels, and hence a non-optimal time point was analysed. This result, however, is not entirely surprising, as previous studies comparing proteomics and q-RT-PCR studies often found no correlation or only a weak one [5,31,32]. Attempts at targeted gene disruptions were also unsuccessful.

3.4. Conclusion

The proteomic analysis presented in this work gives us a better understanding of the adaptive responses of *A. ferrooxidans* when grown in the presence of U(VI). Proteins whose expression were upregulated were mainly involved in either the response to ROS or the general stress response. The data suggest that U(VI) induces oxidative stress in *A. ferrooxidans*, and low molecular weight thiols play an important role in protection against oxidative stress; this was also observed following U(VI) exposure in *G. sulfurreducens* and *E. coli* [4,6], indicating there is also a general response to U(VI) rather than a specific response in *A. ferrooxidans*. Novel proteins, most with unknown functions, are also implicated in the ability of *A. ferrooxidans* to grow in the presence of U(VI).

Conflict of interest statement

The authors have declared no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.resmic.2016.01.007>.

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