Effect of the anode potential on the physiology and proteome of *Shewanella oneidensis* MR-1

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Running Head: Effect of anodic potential on *Shewanella oneidensis*

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ABSTRACT

Shewanella species respire using iron and manganese oxides as well as electrodes as solid terminal electron acceptors. *Shewanella oneidenis* MR-1 exploits mediated as well as direct extracellular electron transfer (EET) modes to transfer electrons at different formal potentials. These different EET modes at different potentials may utilize alternate electron transfer pathways. Therefore, we investigated how different anode potentials, providing different maximum microbial energy gains impacted *S. oneidensis* microbial physiology. Using quantitative proteomics, comparative analysis of the cellular variations to different anode potentials was performed. A label-free proteomic mass spectrometric analysis method, SWATH-MS, was used to gather quantitative information to determine physiological changes of *Shewanella oneidensis* MR-1 grown at different anodic potentials. *S. oneidensis* was cultured and grown in electrochemical cells at the set anode potentials of +0.71 V, +0.21 V & -0.19 V versus SHE reference electrode, while the current production was monitored. At maximum current, electrodes were removed and whole-cell proteins extracted. Subsequent SWATH-MS analysis revealed information on 740 identified proteins across the three electrode potentials. For the first time, we show the abundance of *S. oneidensis* electron transfer proteins differs with electrode potential.

MANDATORY HIGHLIGHTS:

- *S. oneidenis* EET was characterised at different redox potentials
- Levels of electron transfer proteins differed with applied anode

potentials
- Specific proteins and the outer membrane were determined important for EET

KEYWORDS: *Shewanella oneidenis*, variable electrical potential, extracellular electron transfer, quantitative proteomics

INTRODUCTION

Shewanella oneidensis MR-1 is a commonly studied model organism for extracellular electron transfer (EET) within bioelectrochemical systems (BESs) (1-8). The process of EET involves the transfer of electrons from intracellular metabolites in the reduced form, across the membrane to the extracellular environment where they reduce a terminal, insoluble electron acceptor (1). Additionally, the organism can perform more typical electron transfer processes using a soluble electron acceptor (9).

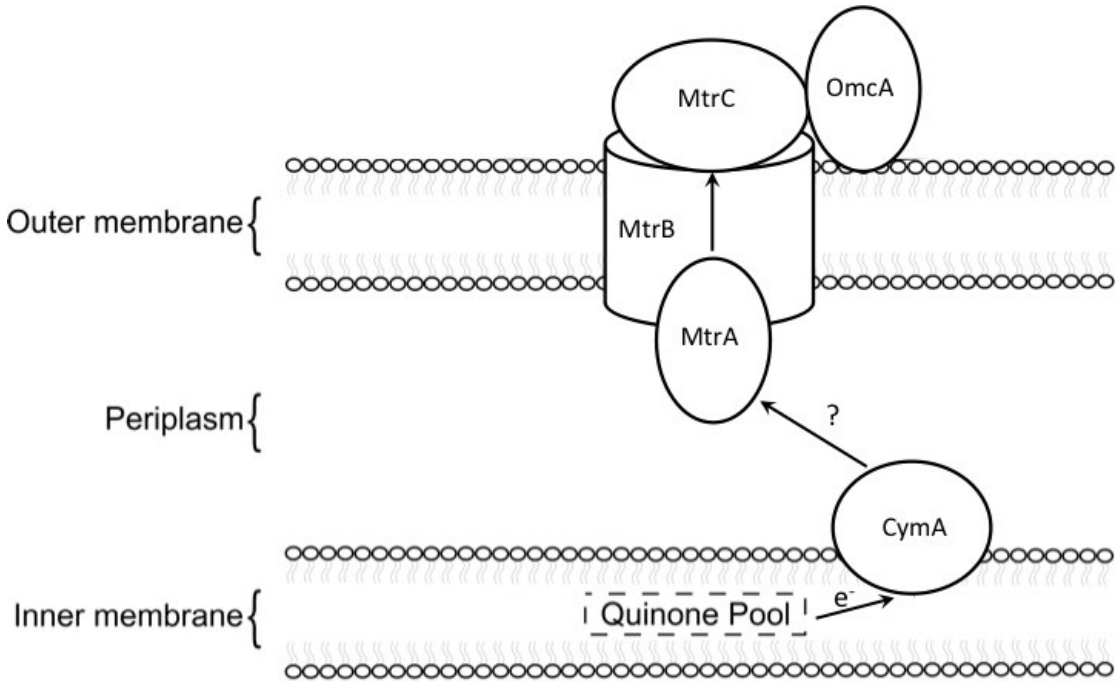
Organisms belonging to the genera *Shewanella* are known to use two mechanisms to achieve EET, namely direct electron transfer (DET) and mediated electron transfer (MET). To achieve the latter process, the organism employs soluble redox mediators (such as flavins) to reversibly transport electrons between the cell and the electrode (1, 7). DET requires the microorganism to establish physical contact with an electron acceptor in the solid state, for example by employing outer membrane cytochromes (10, 11) or electrically conductive appendages produced by the microorganism, referred to as nanowires (1, 12-14). These two mechanisms are not mutually exclusive as *Shewanellaceae* can use these simultaneously to facilitate EET (4, 7, 15). Furthermore, it is speculated that EET can be performed by the combination of both mechanisms, using adsorbed flavins and DET proteins (16).

Shewanella oneidensis MR-1 in particular has attracted interest as it has the ability to reduce a wide array of electron acceptors (17) and it also serves as genetic model for studies of microbial electrosynthesis (18). Its respiratory versatility is reflected in the proteome of the organism, and numerous investigations have focused to determine the proteins involved in *Shewanella* electron transfer pathways (4, 7, 15, 19-26). For example, mutant studies have identified particular cytochromes with certain electron transfer reactions (2, 3). However, with the presence of 42 possible *c*-type cytochromes in the genome (27) it is challenging to determine the exact role that a specific cytochrome may be contributing to in the EET. It is demonstrated that some electron transfer pathways may utilise alternative interchangeable cytochromes, hence displaying modularity of the electron transfer pathway of *S. oneidensis* (3, 25, 28). The current model for extracellular electron transfer by *S. oneidensis* MR-1 is through the use of its metal respiratory system (Mtr) (Schematic 1) (2, 29). In this model EET is facilitated by a set of five key proteins: MtrA; a decahaeme *c*-type cytochrome periplasmic electron carrier (26, 30), MtrC; an outer membrane decahaem c-type cytochrome (31), OmcA; an outer membrane decahaem c-type cytochrome (31), MtrB; which functions

to localize OmcA (a non-haem containing integral outer-membrane protein (23)) and finally CymA; an inner membrane anchored c-type cytochrome (22, 29). In addition, a small periplasmic c-type cytochrome (CctA) has been associated with Mtr reduction in *S. oneidensis* MR-1 and another *Shewanella* species (*S. frigidmarina*) (32, 33).

Several gene and protein expression studies have been conducted to determine how *S. oneidensis* performs EET (5, 8, 9, 34). These studies investigated the molecular mechanisms of *S. oneidensis* electron transfer, using either molecular or electrochemical techniques and were focused mainly on its physiology and response during current production at the single potentials (13, 20, 23, 31). A study by Carmona-Martinez *et al.* investigated the effect of altered electrode potential on the anodic current production and biofilm formation of *Shewanella* (35). They showed that both current density and the electrode biomass increased with increasing electrode potential. Furthermore, through the use of cyclic voltammetry it was shown that, under semi-batch reactor conditions, DET was the most dominant pathway of EET, with a minor contribution from the planktonic culture using MET. Further, a recent study observed the stress and protein turnover responses of *S. oneidensis* biofilms at different applied anodic potentials (8). It was found that EET did not increase the stress response of *S. oneidensis* and that an increase in protein degradation occurred at the higher electrode potentials, which they recognised to be a result of protein damage caused by the oxidating conditions at the electrode surface. This was supported by a decrease in the measured current densities at the higher potential.

As *Shewanella* has modularity in electron transfer proteins and the ability to utilise direct as well as mediated EET modes, there is the likelihood that the electron transfer mechanisms could be fine-tuned to different redox potentials. As the difference in redox potentials between electron donor and acceptor determines the maximum microbial energy gain attainable through respiration, different anode potentials will result in different energy conservation opportunities for electroactive microorganisms. The net share of energy that can be utilized depends on the formal potential of the donor and that of the final electron transfer site accepting electrons (36). Here we test the hypothesis that particular proteins used within the electron transport pathway of *S. oneidensis* will alter to maximise energy conservation when the organism is exposed to different anode potentials. In particular, this study aims to identify the molecular mechanisms employed by biofilms *of Shewanella oneidensis* MR-1 grown at different anode potentials. In addition to electrochemical characterization, SWATH-MS (referring to Sequential Window acquisition of All Theoretical fragment ion spectra) was used to detect changes in protein abundance at the different anode potentials. SWATH-MS is a global, label-free method for relative quantification of the measurable peptide ions across samples (37). The approach rapidly acquires high resolution Q-TOF mass spectrometer data through repeated analysis of sequential isolation windows (swaths) throughout the chromatographic elution range and has been used successfully in a number of studies (37-39) including a quantitative proteomic analysis of an anode grown *S. oneidensis* biofilm (40).



Schematic 1: A model for electron flow through the cytochromes involved in the extracellular electron transfer process in *Shewanella*. It is not presently clear whether an intermediate electron carrier transfers electrons between CymA and MtrA. MtrA is embedded with MtrB and transfers electrons to MtrC. Either MtrC or OmcA may be involved in the electron transfer directly to the metal.

MATERIALS AND METHODS

Unless stated otherwise, all potentials are reported vs. the SHE reference electrode and all chemicals were analytical grade.

Culture preparation

Shewanella oneidensis MR-1 was pre-grown in 20 ml of LB overnight at 30 ˚C in a 50 ml falcon tube while shaking at 200 rpm. The LB culture (3 ml) was used as an inoculum for 300 ml of defined minimal medium (41) that included lactate as the electron donor, a vitamin solution and oxygen as the electron acceptor. The culture was grown at 30˚C shaking at 200 rpm for 24 hours, the cultures were pelleted by centrifuging for 10 minutes at 5000 rpm and resuspended in fresh reactor medium (without added vitamins). The cell density was adjusted to an OD600 of 0.6-0.7 and 5 ml was used as inoculum for each of the BES.

Bioelectrochemical cell set up

A plain, untreated carbon cloth (Fuel Cell Store, USA. Product No. 7302003) working electrode (anode) (2.0 cm x 6.0 cm), a titanium wire counter electrode (cathode) and a Ag/AgCl reference electrode in 3 M KCl (MF-2052, Basi, USA) (+0.210 V vs. SHE) were used in all BES experiments. The BES was a 250 ml Schott glass bottle modified with ports for electrodes and sampling. The BES and electrodes were rinsed thoroughly with deionized water (MilliQ)

and autoclaved separately before being assembled under anaerobic and sterile conditions. Modified minimal medium (41) excluding fumarate was used for all BES experiments, with 18 mM lactate as the carbon and electron source. After autoclaving, the media was placed in an anaerobic chamber, cooled to room temperature, then sterile vitamins were added. Following inoculation of replicate BES*, S. oneidensis* MR-1 biofilms were grown at anode potentials of +0.71 V, +0.21 V & -0.19 V at 30°C and the current was monitored over time for 21 (+0.71 V and +0.21 V) or 29 (-0.19 V) hours. Triplicate BES were operated for both electrochemical measurements and to obtain biomass for protein extractions.

Electrochemical measurements

All experiments were conducted under strictly sterile and anaerobic conditions. The working electrode was poised at the set electrochemical potential using a potentiostat (Potentiostat/Galvanostat VMP3, BioLogic Science Instruments, France). The development of bioelectrocatalytic current production was measured (chronoamperometry) during the batch conditions. Batch experiments were monitored until near maximum current was achieved (usually within 21-29 hours after inoculation). Maximum current densities (j_{max}) were calculated by normalising the measured current (i_{max}) to the projected surface area of the anodes (12 cm^2) (42). For protein extractions, triplicate BES batch experiments were stopped near the point of maximum current, the reactors were placed into an anaerobic chamber and the working electrodes were removed, rinsed with fresh medium and immediately stored at -80 °C. For electrochemical analysis of separate replicate BES, cyclic voltammograms were recorded under turnover conditions (that is, in the presence of metabolic electron donor lactate) at a scan rate of 2 mV s^{-1} and within a potential range between -0.49 V to +0.71 V.

UV-HPLC measurements

Riboflavin and flavin mononucleotide were measured by UV-HPLC using a Shimadzu HPLC system with an LC pump (LC-10ADVP) and an autoinjector (SIL-10ADVP) coupled to a Shimadzu Fluorescence detector (RF-10AXL). The stationary phase was an Altima C8 250 mm x 4 mm, 5 µm HPLC column with a 50: 50 methanol: water mobile phase operated at 35 ˚C. Samples were analysed under a flow rate of 0.7 mL/min with excitation at 450 nm and emission set at 530 nm.

Protein extraction and peptide preparation

The working electrodes were thawed and placed into separate 5 ml aliquots of extraction buffer (containing 77 mg dithiothreitol and 1 tablet of complete Protease Inhibitor Cocktail (Roche) with 10 ml B-PER Bacterial Protein Extraction Reagent (Thermo Scientific)) into 15 ml centrifuge tubes and these were subjected to three freeze/thaw cycles by freezing at -80 ˚C and thawing on ice. The electrode samples were then sonicated on ice three times for 10 second intervals at power level 3 (Branson Sonifier 250, Danbury, USA). The electrode was removed from the solution then rinsed with an additional 5 ml of extraction buffer and discarded. The extraction buffer solutions were combined and cell debris was pelleted by centrifugation at 15,000 g for 15 min. The supernatant was collected and proteins were precipitated from the

solution by adding 10% total volume of 4 mg/ml sodium deoxycholate in 100% trichloroacetic acid and incubated overnight at 4 ˚C. Precipitated proteins were pelleted by centrifugation at 15,000 g for 10 min. The supernatant buffers were removed and the pellets were washed in cold acetone, dried for 5 minutes and then suspended in buffer (2 M thiourea, 7 M urea, 100 mM ammonium bicarbonate). Total protein was quantified by the 2D Quant method (GE Healthcare).

For each replicate extracted sample the suspended proteins were reduced and alkylated by incubation with 5 mM dithiothereitol for 30 min at 56 ˚C, and 25 mM of iodoacetamide at room temperate in the dark for 30 min. Following that the proteins were diluted with 50 mM ammonium bicarbonate buffer to reduce the urea concentration to below 2 M, and then digested overnight with trypsin (Promega) using an enzyme to protein ratio of 1:25 at 37 ˚C.

A portion of the digested proteins (1 µg) of each replicate sample was taken and passed through C18 Zip-tips (Millipore) to remove salts or contaminants. These purified portions were then loaded onto the nanoLC column and subjected to analysis by SWATH-MS as described below (43). In addition, portions of digested protein (2 µg) of each sample were pooled (18 µg total) for duplicate mass spectrometry analysis to create an information dependent acquisition (IDA) ion library.

Mass spectrometry based protein detection and quantification

The purified digested proteins were loaded and separated on a nanoLC system (Shimadzu Prominence) using a modified protocol described by Kappler et al (43). The desalting step was modified with the Agilent C18 trap (0.3 x 5 mm, 5 µm) flow rate running for 3 min at 30 µl/min. Peptides were separated on a Vydac Everest C18 (300 A, 5 µm, 150 mm x 150 µm) column at a 1 µl/min flow rate, using a gradient of 10-60% buffer B with buffer A over 75 min, (buffer A: 1 % acetonitrile (v/v) / 0.1% formic acid (v/v), buffer B: 80% acetonitrile (v/v) / 0.1 % formic acid (v/v)). SWATH mass spectra of the eluted peptides were acquired using a Triple-Tof 5600 instrument (ABSciex, Forster City CA) equipped with a Nanospray III interface. Gas 1 was set to 10 psi, curtain gas to 30 psi, and the ion spray floating voltage was 2700 V. SWATH analyses covering a precursor mass range of 350-1800 m/z for 0.05 sec was followed by high sensitivity IDA mode, using 26 Da (containing 1 Da for the window overlap) isolation windows for 0.1 s, across the m/z range of 400-1250. Collision energy for IDA analyses was set to 45 +/- 15 V, and for SWATH analyses was automatically assigned based on m/z mass windows by Analyst software (ABSciex, Forster City CA).

Mass spectrometry (MS) data from IDA were combined and searched using ProteinPilot software (ABSciex, Forster City CA) with parameters for enzyme digestion set to trypsin, cysteine alkylation set to iodoacetamide, Search Effort was ‘thorough’ and a cut off of > 0.05 (10 %) was applied. The *S. oneidensis* MR1 genome sequence used in the search was obtained from NCBI on the 28th of May 2012. The false discovery rate (FDR) was determined using Proteomics System Performance Evaluation Pipeline software, an add-on to

ProteinPilot, which used a decoy database constructed by reversing all the protein sequences in the searched database.

The IDA ion library and SWATH-MS data for each replicate were loaded into PeakView software for processing using a confidence level of 99, the number of peptides set at 100, the number of transitions used set at 3. Identifications that included modifications or shared peptides were excluded. A minimum of 1 peptide and 3 transitions was used for quantitative analysis. Peak areas for peptide transitions were obtained and analysed using MSstats (44), and used to represent the protein abundance. These values were normalised for each sample, by dividing the sum of the abundance values for all proteins by the abundance of each individual protein. Thus, after loading standard amounts of purified digested protein onto the LC column (1 µg per sample) for the SWATH-MS analysis (see above), and following the normalisation, quantitative comparison of protein abundance between samples was made.

The R- based program MSstats (44) was used for statistical analysis of the spectral data (see Supplementary Information for details). The statistical analysis included calculating the $\log_2$ Fold Change ($\log_2$FC) of each identified protein between the comparisons 0.71 V vs. -0.19 V, +0.21 V vs. -0.19 V and 0.71 V vs. 0.21 V. Pathway Tools software (45) was used to reconstruct metabolic pathways of the identified proteins. The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (46) with the dataset identifier PXD001472.

RESULTS AND DISCUSSION

Electrochemical activity

Catalytic current production of *S. oneidensis* over time was measured at the anodic electrode potentials of +0.71 V, +0.21 V and -0.19 V in three independent biological triplicates, i.e. n=3. (Figure 1). The average maximum current density (j_{max}) produced at these potentials was 24.6 (± 1.9) µA cm^{-2} at t≈18.5 hrs, 16.9 (± 2.8) µA cm^{-2} at t≈23.5 hrs and 0.6 (± 0.2) µA cm^{-2} at t≈29 hrs, respectively (Figure 1a). These current densities are comparable with that reported by others for *S. oneidensis* MR-1 grown at the potentials of +0.71 V (47), 0.251 V (4, 48) and 0.015 V (49, 50) (vs. SHE). Hence, the generated electroactive biofilms performed as expected for *Shewanella oneidensis* MR-1 grown at the respective conditions and therefore allow a representative analysis.

For each BES batch experiment, soon after the catalytic current had reached the maximum value (j_{max}), cyclic voltammetry (CV) in the presence of metabolic electron donor (i.e., under turnover conditions) was performed. The profiles display the characteristic current versus potential response typical of electrocatalytic substrate oxidation (Figure 1b). During CV all systems started producing appreciable current at potentials above approximately -0.19 V, after which the current rises rapidly with the applied potential up until approximately -0.04 V, above which the sharp response in current finishes. At applied potentials between -0.04 V and 0.71 V, the current continues to increase,

albeit at a slower pace. This voltammetric response is typical of *Shewanella* spp. (4) and is explained by assigning the initial fast current response in the mid-potential range to mediated electron transfer (MET) facilitated by self-produced flavins. At higher potentials, however, the slope of the current versus potential trace is less steep, and the current increase is assigned to direct electron transfer (DET) by immediate interaction between cytochromes and the electrode. Indeed, Carmona-Martinez *et al.,* (2011) showed that MET and DET occur at the formal potentials of - 0.330 ± 0.045 mV and - 0.070 ± 0.017 mV (*vs* Ag/AgCl), respectively (3). First derivative traces of the CV profiles (Figure 1c) reveal the presence of two symmetric maxima that correspond to the inflection points of the oxidative and the reductive sweeps in the potential region between -0.29 V and 0.0 V (Figure 1b). This indicates the presence of one apparent major redox centre with formal potential, E_f, of -0.20 ± 0.010 V for anodes grown at +0.71 V, -0.165 ± 0.020 V for the biofilms grown at +0.21 V, and -0.192 ± 0.002 V for the biofilms grown at -0.19 V. The formal potentials for MET of *Shewanella* have been shown previously to range between -0.20 V (4) to -0.12 V (3). Our values are therefore consistent with MET using flavins as shuttling molecules. The small differences observed in the formal potentials amongst the three systems can be ascribed to differences in the local environment at the electrode (e.g., pH), as also suggested by others (3). The similarity in the observed formal potentials indicates similar mechanisms for electron transfer for the three conditions tested, that is, flavin is being used at the different potentials for MET. The difference in peak heights and widths (Figure 1c) may indicate different flavin concentrations at the electrode surface. MET is expected in these batch mode BES experiments as planktonic cells are present and lack physical contact with the electrode and would require the flavin mediator for respiration (51).

Analysis of the first derivative analysis of the CV profiles indicates the presence of a second less distinctive broad peak between the potentials 0.11 V and +0.61 V (Figure 1c), which can be assigned to DET. This pathway, however, seems to be only of minor importance under batch conditions for the overall current production at each of the potentials as it is not associated to an obvious catalytic wave for turnover conditions (Figure 1b). The differences in peak area and height determined at the different potentials were not found to be significant in this study. The DET mechanism likely involves interactions between c-type cytochromes and the electrode (4). The *S. oneidensis* genome codes for up to 42 such cytochromes (27), and the cytochromes that are well established in the EET process of *Shewanella oneidensis* MR-1 include MtrA, MtrB, MtrC, OmcA, CymA and STC (Schematic 1). The MtrCAB complex of cytochromes are found to cover a broad range of formal potentials for DET (52). Absorbed flavins at the electrode (53) in addition to different electron tunnelling distances of the cytochromes as well as their orientation could explain the broadening of the peak covering a large potential range. This is opposed to a distinctive peak at a specific potential (Figure 1c). Further, regarding the electrochemical heterogeneity of redox active species contributing to DET, the residual slope observed in the high potential region of the CVs indicates slow interfacial electron transfer rates at the electrode caused by the different electron transfer rates of each redox active species.

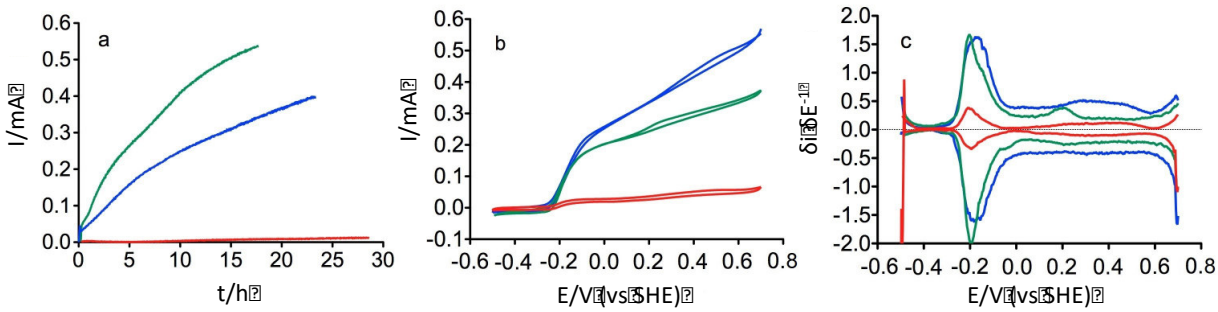


Figure 1: Representative chronoamperometry profiles of *S. oneidensis* in the BES at +0.71 V (—), +0.21 V (—) and -0.19 (—) (a), along with respective turnover CV determined on the differently grown biofilms (b) and the corresponding first derivative analysis of the turnover CV (c).

Proteomic Analysis

Higher amounts of total cellular protein were extracted from biofilms at the higher potentials, with 238 ± 30, 215 ± 20 and 62 ± 13 µg of protein extracted from electrodes poised at +0.71, +0.21 and -0.19 V respectively. As mentioned in the methods section, 1 µg of each of these extracted samples was used for the SWATH-MS analysis.

Differential protein abundance from anodic *S. oneidensis* biofilms grown at different potentials was shown using SWATH-MS (54). Due to the sensitivity of the analysis technique i.e. mass spectrometry, this approach is particularly useful for investigative analysis of low biomass samples, where quantitative proteomics can be challenging. We recently used SWATH-MS successfully for quantitative proteomic analysis of anode grown *S. oneidensis* biofilms (40).

The ion library revealed a total of 740 identified proteins in the anodic biofilm samples, with a false detection rate of 0.01. Initial SWATH-MS analysis detected 697 of these proteins across all biofilm samples. The number of significantly different abundant proteins ($p<0.05$) was determined by pairwise comparisons of the $\log_2$FC of the spectral abundance data. There were 174, 219 and 53 differentially abundant proteins between the comparisons of +0.21 V to - 0.19 V, +0.71 V to -0.19 V and +0.71 V to +0.21 V respectively ($\log_2$FC>1, $p<0.05$) (See SI Tables S2-S4).

Increased substrate turnover at the higher electrode potential

A number of changes in relative protein abundance suggest that an anodic biofilm of *S. oneidensis* at the electrode surface (not taking into account the planktonic fraction) had increased cell activities at the higher electrode potential, i.e. higher substrate turnover at higher levels of current production. Out of the 53 proteins that were more abundant at +0.71 V in comparison to -0.19 V, 24 were ribosomal proteins with $\log_2$FC ranging from 1.5 to 3 (SI Figure S1). Furthermore, elongation factor FusB ($\log_2$FC 1.9) involved in protein synthesis and a ribosome maturation factor RimM ($\log_2$FC 1.8) were also significantly more abundant at the higher potential. As ribosomes are the key component of protein synthesis within the cell, this finding implicates a

higher biosynthetic activity of cells grown at the higher potential. It is seen that protein turnover of *S. oneidensis* differs when respiring different electron acceptors (5, 9), and that ribosomal protein expression is related to the redox potential of the metal electron acceptor (8). However, it was recently discovered that ribosomal protein expression was not positively correlated to electrode potential, but rather to increased rates of EET (i.e. current/substrate turnover) (8). Thus, the increase in biosynthetic activity that we observe may be correlated to the higher rate of EET. However, it should be noted that increased EET occurs because of larger thermodynamic gains resulting from a higher electrode potential, suggesting that potential does in fact play a role. In contrast, there are circumstances where very high oxidative potentials can have a negative effect on current production by *S. oneidensis* biofilm grown on the anode (8). The contrasting results detected may be due to differences in experimental conditions, however, we cannot offer a substantial explanation for the differences detected here (8). Further, we note that the higher amounts of protein extracted from the electrodes poised at higher potentials, would correspond to a higher number of cells and thus thicker biofilms. This is supported by the higher slope for the voltammograms for the electrodes poised at +0.71 V and +0.21 V (Figure 1), as the voltametric curve is a function of the thickness of the bioelectrocatalyst (55). We suggest here that at the higher redox potentials used in this study that *S. oneidensis* had higher production of proteins and higher metabolic activities. This is also supported by the higher current production detected here at +0.71 V, and the increased abundance of proteins involved in the energy generating reactions of the *S. oneidensis* TCA cycle detected at this higher potential (40).

MET is the dominant form of EET for S. oneidensis

The turnover cyclic voltammetry of the biofilms at all anodes suggests the dominance of MET through the use of flavins. This may be due to experiments being performed in batch mode as opposed to a continuous feed mode. In batch mode it is expected that planktonic growth is encouraged as well as riboflavin biosynthesis, as planktonic cells use MET for respiration which does not require physical contact with the electrode (51). SWATH-MS revealed a higher abundance of riboflavin biosynthesis protein, RibBA, at +0.71 V (log_2FC 0.96 compared to -0.19 V). However, in contrast to this, higher concentrations of riboflavin were detected in the reactor medium at the lower potential of -0.19 V in comparison to the higher potentials ($t(4) = 2.77$, $p < 0.05$). These results are confusing. However, while *S. oneidensis* MR-1 uses flavins to mediate remote reactions, there is evidence these could facilitate electron transfer to the electrode directly when bound to the outer membrane cytochromes MtrC and OmcA (53, 56). If this occurred the flavin concentration at the anode could differ significantly from concentrations in the bulk medium. Thus, a possible explanation for the lower mediator concentration in the liquid medium at the higher potential, could be due to an increased concentration of flavin (ad)sorbed at the electrode or 'bound' to OmcA and MtrC, which are found in higher abundance at this potential.

Abundance of specific cytochromes suggests outer membrane involvement in EET

Previous investigations implicate a particular set of proteins important for DET

in *Shewanella* (20, 23, 25, 26) (Schematic 1). Our SWATH-MS revealed a higher abundance of the DET related proteins MtrA, MtrB and MtrC and OmcA at +0.71 V, +0.21 V compared to -0.19 V (Figure 1), suggesting a relationship between the applied potential, current production, and the abundance of these EET related proteins. At the low potential used here (-0.19 V), very little current flows and thus the anode plays a minor role as a terminal electron acceptor. Consequently, fewer EET related proteins were detected at the low potential in comparison to +0.71 V (Figure 2). Details of the regulation of the genes involved in EET (*mtrA*, *mtrB*, *mtrC*, *omcA* & *cymA*) are not well understood. Altered gene expression at the transcription level has been detected (5, 9, 27), however, the mechanisms responsible for the regulation of these genes are yet to be identified.

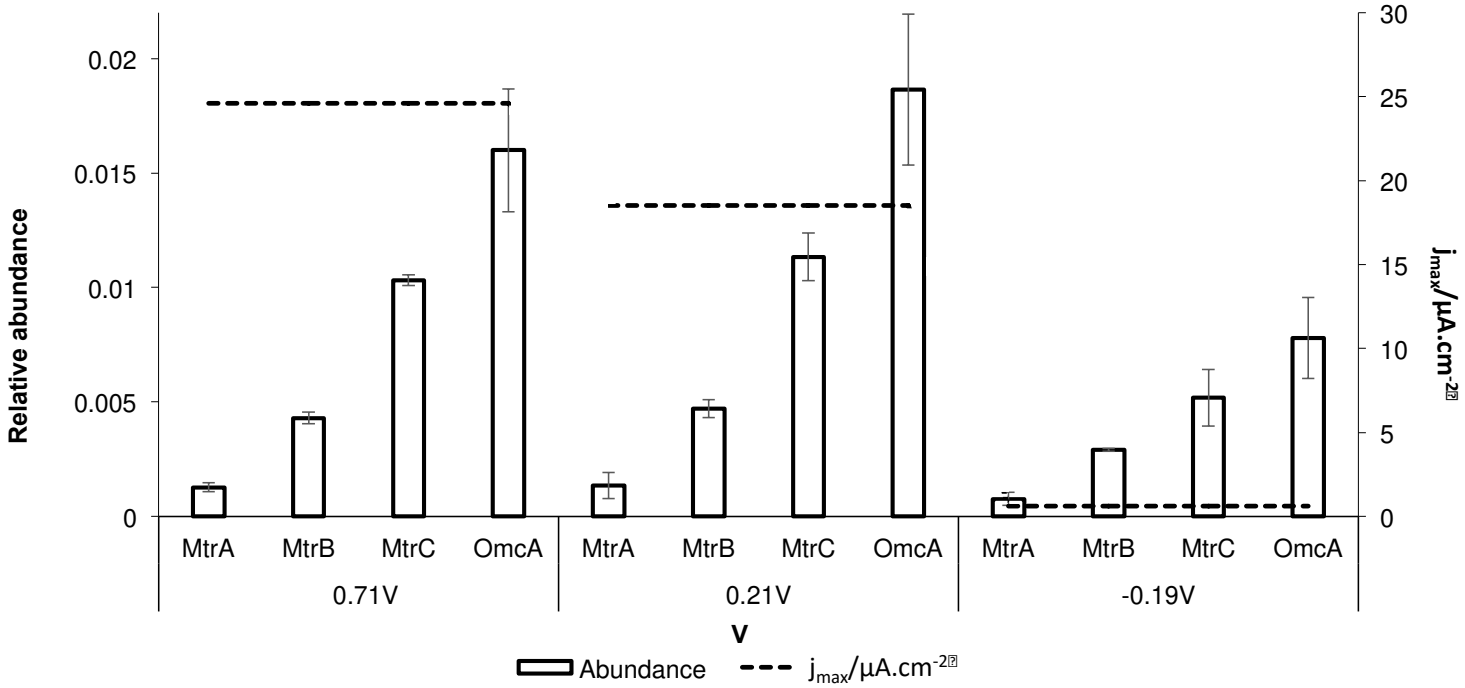


Figure 2: Relative abundance of individual EET proteins at the potentials +0.71 V, +0.21 V and -0.19 V (Ag/AgCl). Error bars indicate the standard deviation between triplicate experiments. The dashed line indicates the average maximum current density, j_{max}, at the different potentials.

Differential gene expression has been compared when *S. oneidensis* was grown using either an electrode (+0.2 V vs Ag/AgCl), oxygen or soluble iron (III) as electron acceptors (5). It was found that the genes *mtrABC* and *omcA* and that coding for riboflavin synthase were all up regulated during oxidation at the anode, in comparison to use of oxygen or iron as the electron acceptor. This coincides with our proteomic findings associating these proteins with EET when using an electrode as an electron acceptor.

CymA, another important EET component (Schematic 1), was detected in our IDA library and thus present in the analysed samples. However, the data obtained from the SWATH-MS analysis for the protein was of insufficient

quality for quantification in this instance. Difficulties in the detection of CymA could be that the protein was simply in low abundance and/or it may be related to the protein extraction method. CymA is an integral membrane protein (22) which may require harsher extraction conditions (57).

In our study the relative abundance of the outer membrane associated proteins MtrCAB and OmcA were higher at +0.71 and 0.21 V compared to -0.19 V (Figure 2). A recent investigation of *S. oneidensis* using an insoluble electron acceptor shows that outer membranes extrude from the cell. It is hypothesised that these extrusions provide structure and the membrane associated proteins for transfer of electrons for the EET (14). Additionally, it has been seen that membrane vesicles produced by *S. putrefaciens* and from *S. oneidensis* are conductive, suggesting their composition includes components for electron transfer (58). This membrane extrusion model does correlate with the increased outer membrane proteins we detected. However, it is important to highlight that further proof is required to support the involvement of these membrane extrusions in EET.

Our results reveal several cytochromes to be present at higher abundance at -0.19 V; these were Cytochrome *c* oxidase, cbb3-type, subunit II (CcoO), *c*-type cytochrome (SO3420) and Periplasmic cytochrome *c* (CytB), with $\log_2$FC of -0.66, -0.43 and -0.96 respectively. CcoO has been found to be associated with the cell membrane (5, 59). Both the genes for CcoO and SO3420 are found to more highly expressed when *S. oneidensis* is grown on an electrode compared to growth on soluble Iron (III) citrate (5). The higher abundance of these cytochromes at -0.19 V may suggest that these proteins are important for survival on an electrode at this potential. Possibly these cytochromes play a role in EET at low potential (i.e. low energy gain) as modularity of these mechanisms is proposed for *Shewanella* (25). However, CcoO is part of an O_2 reduction pathway and plays a critical role in aerobic growth (60) . Although the reactors were prepared and sealed under anaerobic conditions, there is the possibility of some level of oxygen intrusion into the reactors.

Signs of higher motility at lower anode potential.

Our results show a higher abundance of a number of chemotaxis and motility related proteins at -0.19 V (Figure 3). Methyl-accepting chemotaxis proteins (MCPs), which are part of the cells motility mechanism for moving towards attractants, were in higher abundance at -0.19 V compared to +0.21 V and +0.71 V (Figure 3). Additionally, flagellin related proteins were in higher abundance at -0.19 V. This included FliD the flagellar filament capping protein ($\log_2$FC -2.43), and FilC a flagellin filament ($\log_2$FC -1.69). This increased abundance of both chemotaxis and flagellin related proteins at -0.19 V indicate a higher involvement of motility at this potential compared to +0.71 and +0.21 V. This may reflect a higher need for searching for new habitats, when facing low energy yielding conditions. These findings are in contrast to an earlier study where *S. oneidensis* was found to have stronger motility in an anode at high potential in comparison to motility at low potentials (61). However, the cells that exhibited this behaviour were a small proportion of free-swimming cells in the vicinity of the electrode. One explanation for the observed differences is that our proteomic analysis is of cells from an anodic

biofilm. Potentially the free-swimming cells may demonstrate different motility behaviours and may respond differently to electrode potentials compared to cells encased within a biofilm.

Highly abundant energy generating proteins detected at higher potential
Numerous proteins were more abundant at the higher potential (i.e. +0.71 V), that were presumably involved in producing reducing power, passing electrons onto the ubiquinone pool and substrate level phosphorylation. As we have recently reported (40), a number of the TCA cycle enzymes, especially those involved in the production of reducing power, were more abundant at higher potentials. This included the proteins L-lactate dehydrogenase (LldE), NAD-dependant Isocitrate dehydrogenase, L-lactate dehydrogenase iron-sulfur cluster-binding protein (LldF), NAD-dependent malic enzyme (MaeA), Phosphate acetyltransferase (Pta) and Succinyl-CoA ligase [ADP-forming] subunit beta (SucC) with these having Log_2FC of 2.8, 2.8, 2.6, 2.1, 2.1 and 2.0 respectively when compared to low potential. Other associated proteins also more abundant were pyruvate formate lyase (PflB) and formate dehydrogenase (FdhA, FdhB-1and FdhB-2), the activity of these resulting in NADH production. Additionally, increased phosphate acetyl transferase and acetate kinase could result in increased substrate level phosphorylation, and increased levels of NADH-quinone reductase (subunit NqrF), could partake in the reduction of the ubiquinone pool (62). The significant increase in abundance of these proteins at the higher potential indicate increased energy metabolism and this correlates with the higher current production generated by the cells at +0.71 V in comparison to -0.19 V.

Highly abundant proteins detected at lower potential
We identified a putative periplasmic CbiK superfamily protein (SO1190) with a significantly high abundance at low potential, i.e. -0.19 V (log_2FC -2.9). Protein BLAST analysis found a 96% similarity to a nickel transporter of *Shewanella decolorationis* (SI Table S1). Nickel is an essential cofactor for many enzymes and is transported into the cell by specific transport systems. It is a component of Ni-containing hydrogenases that play an important role in energy metabolism through the oxidation and production of hydrogen gas. At the low anode potential *S. oneidensis* would have surplus reducing potential (63). Possibly the increased hydrogenase provides a potential opportunity for alternate mechanisms for electron release, however, this may be futile as hydrogen production is not expected at the anode.

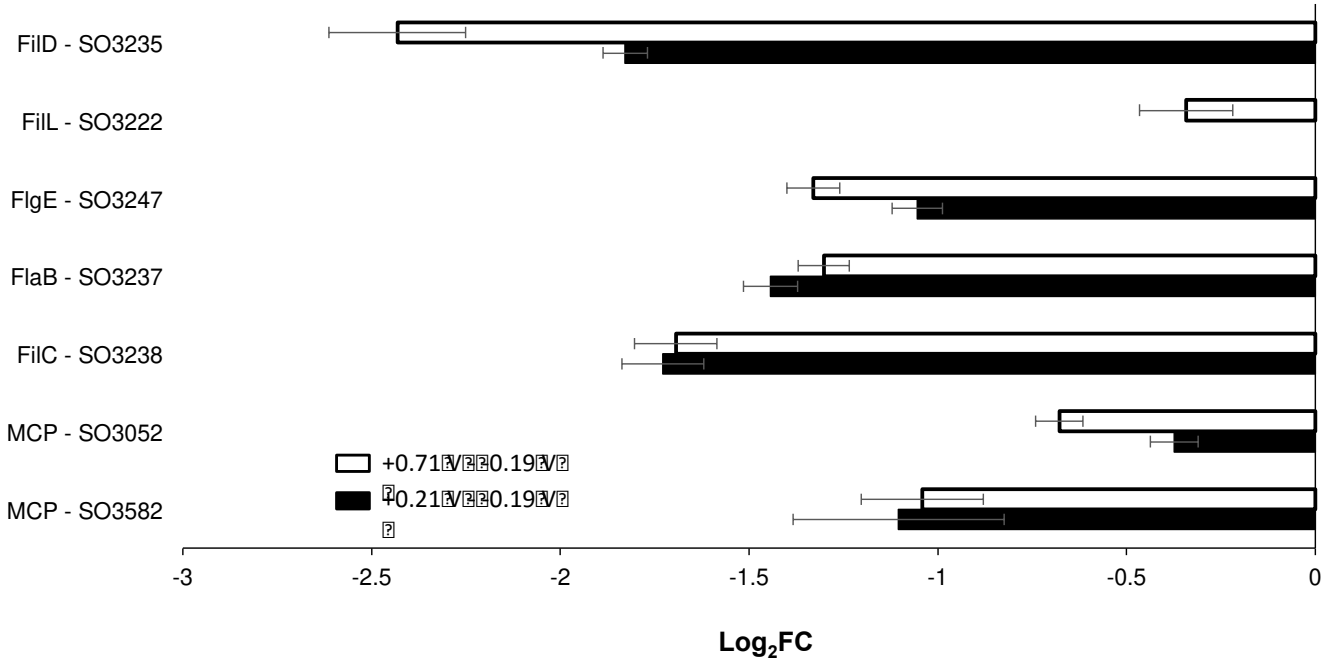


Figure 3: Change in abundance of chemotaxis proteins detected between the potentials +0.71 V vs. -0.19 V and +0.21 V vs. -0.19 V ($p<0.05$). Error bars indicate standard error.

CONCLUSIONS

Using both electrochemical and molecular techniques, this study sheds light on the proteomic profiles of *S. oneidensis* MR-1 grown at different anodic potentials. We found that as current production increases, so does the abundance of EET proteins and proteins for EET mediator synthesis.

This study also confirmed that both MET and DET play a role in EET under batch conditions, with MET acting as the dominant mechanism for all tested potentials for *S. oneidensis*. This agrees with the findings of other studies examining EET of *S. oneidensis* using batch BES electrochemical investigations (4, 7, 64). The CV analysis revealed a broad peak covering a large potential range. Here we hypothesise that this is due to the *Shewanella* EET cytochromes involved in DET possessing a wide range of potentials (52), micro-environmental changes and varying orientations of the EET proteins relative to the electrode surface. Here a detailed analysis using more sensitive electrochemical techniques like square wave voltammetry and Raman microscopy, could shed light on the behaviour of these cytochromes. Our findings support the existing model for the involvement of the *Shewanella* metal-reducing mechanisms in extracellular electron transfer (25, 65).

Acknowledgements
We acknowledge the PRIDE team for the deposition of our data to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org).

Funding information

The authors would like to thank the Australian Research Council for their financial support (through project DP0879245).

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