Bioelectrical redox cycling of anthraquinone-2,6-disulfonate coupled to perchlorate reduction†

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Electrochemical reactors can be used to deliver electrons to bacteria with soluble, redox-active shuttles, and this has been proposed for both biosynthetic and bioremediation purposes. In the case of perchlorate reduction in bioelectrical reactors, very little is known about the mechanism of shuttle oxidation, although this understanding is important for treatment system design and scaleup. In this work, electrochemical, physiological, and proteomic experiments were performed to investigate anthrahydroquinone-2,6-disulfonate (AH2DS) oxidation by Azospira suillum PS under perchlorate reducing conditions. In inoculated bioelectrical reactors with AH2DS as the sole electron donor, only small amounts of cathodic current were observed. However, acetate addition resulted in a rapid increase in cathodic current, concomitant with acetate oxidation. This effect was not seen with nitrate as the terminal electron acceptor or when cells were lysed. We hypothesized that co-utilization of electron donors was a result of normal organotrophic perchlorate reduction, in addition to non-enzymatic reactions between AH2DS and intermediates (chlorite, oxygen) of the pathway. These reactions are expected to result in the production of reactive oxygen species (ROS) and toxicity, which was confirmed in batch growth experiments. Shotgun proteomics revealed significantly increased tandem spectral peptide counts of a diheme c-type cytochrome peroxidase; again, only when perchlorate, acetate, and AH2DS were present. Homologs of this protein relieve oxidative stress by reducing hydrogen peroxide to water. This work highlights the fact that oxidation of inorganic electron donors can occur non-enzymatically, as well as the challenge of targeting a specific metabolism with chemical shuttles that are highly reactive.

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Broader context

Electrochemical reactors are a promising technology for connecting microbial metabolisms to a power supply. Potential applications include water treatment and electrosynthesis. Electron shuttles bypass the requirement for direct microbe-electrode contact, increasing the diversity of metabolisms that can be stimulated. However, soluble electron shuttles are generally non-specific in their ability to donate electrons, and shuttle oxidation alone should not be interpreted as signifying a robust microbial metabolism. A mechanistic understanding of shuttle oxidation is therefore important to applying such technologies. In this paper we develop a model for anthrahydroquinone-2,6-disulfonate (AH2DS) oxidation coupled to perchlorate respiration that includes both enzymatic and abiotic reactions. Abiotic reactions between cathodically reduced AH2DS and transient metabolites of microbial respiratory pathways, or oxygen contamination, can result in the production of reactive oxygen species and be detrimental to the target metabolism. This understanding has important implications for the scaling of shuttle-driven bioreactors that are susceptible to minor oxygen leaks. The results of this study also extend to the development of perchlorate biosensors, and provide insight into Fe(II) and H2S bio-oxidation under perchlorate reducing conditions.
Introduction

The U.S. EPA has recently announced its intention to regulate perchlorate under the Safe Drinking Water Act and to set a national primary drinking water standard. This was based on detection of perchlorate in over 35 states, including in public water systems, surface waters and groundwater. Previously, states had independently regulated perchlorate with state advisory levels that ranged from 1–50 μg L⁻¹. A number of technologies have been developed to remove perchlorate from water, including ion exchange, electro-dialysis reverse filtration, high pressure membrane filtration, carbon adsorption, and biological reduction, but only biological reduction results in complete conversion of perchlorate into innocuous chloride and water. Existing biological treatment systems stimulate microbial perchlorate reduction using an organic electron donor or hydrogen gas. It is difficult to control biomass growth using an organic electron donor because the same molecule is both a carbon and energy source. This results in significant biomass production and large disposal costs, and unused electron donor has the potential to form disinfection byproducts during subsequent water treatment. Hydrogen circumvents these issues, but is costly, difficult to deliver, and has safety concerns.

A new technology for treating perchlorate in groundwater involves electrochemically stimulating bacterial reduction in a bioelectrical reactor (BER). One mechanism of connecting bacterial respiration to a charged cathode is by using soluble electron shuttles. Shuttles, including humic acids, have been shown to serve as electron donors for nitrate and fumarate reduction by a variety of bacteria. In a recent study, the redox shuttle anthraquinone-2,6-disulfonate (AQDS) was reduced electrochemically to anthrahydroquinone-2,6-disulfonate (AH2DS) to provide electrons for biological perchlorate reduction. The mechanism of AH2DS oxidation was not elucidated, and perchlorate reduction may have been a result of electrolytically produced H₂ in some of the experiments. However, AH2DS oxidation by *Azospira suillum* PS, which does not use hydrogen, was also observed.

In order to assess the feasibility of shuttle driven BERs for perchlorate removal, it is necessary to understand the mechanism of microbial AH2DS oxidation coupled to perchlorate reduction. In this study, we use electrochemical current as a measure of AH2DS oxidation, allowing for near real time monitoring of electron consumption. This approach, combined with growth experiments, proteomics, and physiological studies has led us to propose a mechanistic model for AH2DS oxidation that includes both enzymatic and abiotic reactions. This study extends our understanding of dissimilatory perchlorate respiration and informs work aimed at connecting any bacterial metabolism to an electrochemical system. It also has direct relevance to the future development of a specific biosensor for the measurement of perchlorate.

Results

**Cathodic current is proportional to starting cell density**

In order to assess the ability of *A. suillum* PS to reduce perchlorate with AH2DS as the sole electron donor, cell suspension experiments were conducted in BERs at different cell densities. Current (proportional to AH2DS oxidation), and perchlorate concentration were measured over time. A linear relationship between the amount of cells and the average current was observed over the range of cell densities tested (Fig. 1A). However, very high starting cell densities (OD₆₀₀ = 0.35, approximately 2 × 10⁷ cells mL⁻¹) were required to obtain substantial rates of perchlorate removal (~0.25 mM in 24 hours). In this time, the same culture could reduce 10 mM perchlorate with acetate as the electron donor, suggesting that AH2DS was not as efficiently coupled to perchlorate reduction as acetate. No growth was observed in any of the reactors with AH2DS as the sole electron donor.

Interestingly, a single reactor drew more current than its duplicate (Fig. 1 inset, Fig. S1A†). At 200 hours, a sample from this reactor was streaked onto aerobic agar plates containing 10 mM acetate, a medium on which *A. suillum* readily grows. In addition, one millilitre of sample was transferred into a 9 mL anaerobic culture tube with 10 mM acetate and 10 mM perchlorate (normal growth conditions). Unexpectedly, and in contrast to cells removed from other reactors, no colonies were formed on the plates and no growth was observed in the anaerobic tube, indicating that few viable cells remained in the reactor, despite its ability to reduce perchlorate.

**Living cells are not required for perchlorate reduction in a BER**

The high cathodic current and lack of viability seen in the single reactor could be explained if cells had lysed, and the enzymes released were catalyzing AH2DS oxidation coupled to perchlorate reduction. Whole cell lysate from *D. agitata* CKB has previously been shown to catalyze the reduction of perchlorate coupled to a variety of reduced shuttles, including AH2DS, and this concept is the basis of a perchlorate bioassay. Purified perchlorate reductase is also capable of directly accepting electrons from AH2DS and reducing perchlorate. When tested, cell
lysate mediated perchlorate reduction in a BER using AH2DS as the electron donor and the perchlorate removal predicted from current (assuming 8 e− transfer) correlated well with concentrations empirically determined using ion chromatography (Fig. 2). Lysate addition to a BER with 500 mM pre-reduced AH2DS immediately resulted in cathodic current, while intact cells initially drew less current. This apparent difference in current may be a result of the outer membrane (OM) of intact cells serving as a barrier to AH2DS entry into the periplasm, or the capacity of intact cells to export anthraquinones. This latter possibility is supported by evidence of AQDS export by a TolC transporter in *Shewanella oneidensis* MR-1.9

Acetate stimulates AH2DS oxidation in bioelectrical reactors

*A. suillum* PS is a heterotroph, requiring organic carbon for biosynthesis. In order to create conditions for growth and cell maintenance, acetate was added to a BER-cell suspension experiment. Interestingly, with 500 μM AH2DS as the electron shuttle the addition of acetate stimulated current draw (Fig. S2†). The increase in current could not solely be a result of higher cell numbers due to growth on acetate, because the response was immediate, and upon complete utilization of acetate, current returned to initial levels while perchlorate reduction ceased.

Acetate dependent current draw was an enigmatic phenomenon that required explanation. To determine if the current spike corresponded to more perchlorate reduction, reactors with power were compared to reactors with an open circuit. All reactors contained 500 μM of fully reduced AH2DS at the start of the experiment. Utilization of the acetate occurred rapidly, and only slightly more perchlorate was removed in reactors with power (Fig. 3). No increase in current was observed upon addition of acetate without perchlorate in the media or when cells were omitted (Fig. 3, inset). Biological perchlorate reduction was necessary for the observed current spike, as this phenomenon was never observed with pre-lysed cells (Fig. 4). In addition, the current spike occurred within 15 minutes of the addition of the acetate (Fig. 3, inset) suggesting that AH2DS oxidation was not dependent on changes in protein content or increased cell number.

Identical experiments performed with nitrate in place of perchlorate revealed that the acetate-stimulated current draw was specific to perchlorate (Fig. 5) and could potentially be used as the basis of a perchlorate-selective biosensor.

The reaction of AH2DS with intermediates of the perchlorate reduction pathway

A possible explanation for the acetate dependence on current (Fig. 3 and 5) is that AH2DS is chemically oxidized by a metabolite of the perchlorate reduction pathway. The rate of
Perchlorate reduction, and therefore chlorate, chlorite and oxygen production is significantly greater when electrons from acetate serve as the electron donor. This rate difference is clearly observed in Fig. 5, which shows faster perchlorate reduction upon the addition of acetate, and much slower perchlorate reduction when only AH2DS is present.

The reactivity of electron shuttles has been widely reported. Quinones, flavins, and phenazines are excellent sources of single electrons and reduce oxygen rapidly to produce superoxide and hydrogen peroxide.

Chlorite also has the potential to react with AH2DS, forming hypochlorite, which is a strong oxidant and reacts with many biomolecules including proteins and lipids. Potential reactions are shown in reactions (1)–(3), all of which produce reactive oxygen species (ROS) that are toxic to bacteria.

\[
O_2 + AH_2DS^{2-} \rightarrow O_2^- + AHDS^{2-} + H^+ \quad (1)
\]

\[
O_2^- + H^+ + AH_2DS^{2-} \rightarrow H_2O_2 + AHDS^{2-} \quad (2)
\]

\[
ClO_2^- + AH_2DS^{2-} \rightarrow HCl + OH^- + AQDS^{2-} \quad (3)
\]

Of the perchlorate reduction intermediates, chlorite and oxygen react more quickly with AH2DS than chlorate (data not shown). The reaction of oxygen with shuttles is a universal concern for any biocathode system using electron shuttles, especially in continuous reactors that contain dissolved oxygen in the feedwater.

**AH2DS results in a growth defect under perchlorate reducing conditions**

ROS can be detrimental to bacteria in a number of ways. Both superoxide and hydrogen peroxide can oxidize and inactivate iron–sulfur proteins, and this is an important mode of toxicity. Further damage can result as the released iron reacts with hydrogen peroxide to form hydroxyl radicals via Fenton chemistry. For this reason, bacterial response to ROS in *E. coli* includes control of iron import. The possibility of ROS toxicity was explored with batch growth experiments. Under perchlorate reducing conditions, the addition of the AH2DS caused a large growth defect (Fig. 6A). In contrast, no substantial difference was observed with AQDS or under nitrate reducing conditions (Fig. 6B), indicating that the inhibitory effect was specific to perchlorate reduction.

To test if abiotically produced hydrogen peroxide (reactions 1 and 2) contributed to the lag observed on AH2DS, growth experiments were conducted in the presence of catalase and yeast extract. Hydrogen peroxide toxicity in *E. coli* can be partially overcome by the addition of catalase to the media or by the addition of branched chain fatty acids, which can bypass damage to biosynthetic enzymes caused by ROS. Both catalase and yeast extract improved growth under all conditions (perchlorate, nitrate; with and without AH2DS and AQDS), but had the greatest effect on growth under perchlorate reducing cultures in the presence of AH2DS (Fig. S3 and S4†). However, attempts to
directly measure hydrogen peroxide using a horseradish peroxidase (HRP) coupled dye absorbance assay were unsuccessful. Presumably low steady state concentrations and decomposition during sample preparation made it difficult to directly measure hydrogen peroxide in the presence of AH$_2$DS using this method. As a point of reference, when 500 μM of AH$_2$DS was oxidized by exposure to air and immediately assayed, only 3 μM H$_2$O$_2$ was detected with the HRP assay.

**Upregulation of enzymes involved in ROS defense**

To better understand the physiological response to AH$_2$DS, the proteomes of *A. suillum* PS cells grown on perchlorate and acetate, with and without AH$_2$DS, were compared. *t*-tests using a α = 0.005 cutoff were used to screen for differential expression of proteins between these two conditions. Boxplots of the four differentially expressed proteins (Fig. 7) reveal increased expression of a di-heme cytochrome-c peroxidase (Dsui_1166); a tripartite ATP-independent periplasmic (TRAP) transporter (Dsui_3155); an aminotransferase, putatively involved in biosynthesis (Dsui_2433); and peroxiredoxin family protein (Dsui_1901).

Increased tandem spectral peptide counts from the di-heme cytochrome-c peroxidase (Dsui_1166) were seen in perchlorate reducing conditions in the presence of AH$_2$DS. Dsui_1166 was among the top ten most highly expressed proteins under these conditions: in trypsin-shaved samples, an average of 7.5 ± 1.87 (s.d.) spectral counts were observed, compared to the highest counts of 23 ± 7.5 for PcrA. This level of expression, as well as the fact that peptides from this protein were never observed in other conditions, suggests that Dsui_1166 has a physiologically role during growth on acetate/perchlorate/AH$_2$DS. Characterized homologs of Dsui_1166 are found in *Nitrosomonas europaea* (68.9% amino acid similarity) and *Shewanella oneidensis* (63.4% amino acid similarity), both of which have in vitro peroxidase activity. Dsui_1166 has a predicted N-terminal signal peptide (SignalP 3.0 HMM), and is likely to be localized to the periplasm. Consistent with this, trypsin shaving of whole cells, which enriches for cell surface and periplasmic proteins, had increased peptide counts compared to the lysed samples (Fig. 7). The relationship between electron shuttles and ROS has been highlighted recently with the finding that redox active compounds can directly induce ROS defense in *E. coli*. However, in *A. suillum* PS, Dsui_1166 was never observed in controls with nitrate/acetate/AH$_2$DS, indicating that AH$_2$DS alone was not sufficient to activate its expression.

A predicted periplasmic solute binding protein DctP (Dsui_3155), part of a tripartite ATP-independent periplasmic (TRAP) transporter, was also highly expressed during perchlorate reduction in the presence of AH$_2$DS (Fig. 7). Inspection of the genome showed the archetypical dctPQM operon organization observed in *R. capsulatus*. The membrane components of this system, DctQ and DctM, are predicted to have four and thirteen transmembrane helices (TMHMM Server v. 2.0), respectively, but peptides from these proteins were not observed in proteomics, likely because proteins buried in membranes are less accessible to trypsin. Most characterized TRAP transporters studied to date mediate solute uptake, including the transport of a number of small organic acids, sugars and amino acids. Given the broad range of solute specificities, it is difficult to predict the function of this transport system under AH$_2$DS and perchlorate conditions. However, it is tempting to hypothesize its involvement in ROS defense.

Dsui_1901 and Dsui_2433 also showed increased spectral counts on perchlorate in the presence of AH$_2$DS. Dsui_1901 is in the alkyl hydroperoxide reductase subunit C – thiol specific antioxidant family and possibly involved in protection against reactive oxygen or reactive nitrogen species. Dsui_2433 is an aromatic amino acid transferase putatively involved in biosynthesis. H$_2$O$_2$ damage to aromatic biosynthesis pathways has been observed previously.

A less stringent screening process using α = 0.05 identified 25 additional proteins that may be differentially expressed. Box plots (Fig. S5†) and *t*-test summaries (Table S1†) for these proteins, along with the full proteomic dataset (Table S2†) can be found in the ESI†. Of interest is Dsui_1541, a cytochrome c553, which had slightly increased expression on AH$_2$DS under both perchlorate and nitrate reducing conditions (Fig. S5 and Table S1†). Next to Dsui_1541 on the chromosome is an annotated sulfide dehydrogenase flavoprotein subunit (Dsui_1540). In addition, two citric acid cycle enzymes (Dsui_2214, Dsui_3296) had increased spectral counts. In contrast, several proteins involved in iron trafficking (Dsui_1571, Dsui_1658, Dsui_1666) had decreased spectral counts. Controlling iron transport is consistent with iron being an important catalyst of hydroxyl radical formation in the presence of hydrogen peroxide.
Taken together, the proteomics data provides strong evidence that AH$_2$DS generates increased levels of ROS under perchlorate reducing conditions.

A model of AH$_2$DS oxidation under perchlorate reducing conditions

The data presented supports a model, presented in Fig. 8, in which AH$_2$DS is oxidized by multiple mechanisms under perchlorate reducing conditions. The first mechanism is direct coupling to the perchlorate reductase (PcrAB). In this work, we show that AH$_2$DS can donate electrons to cell lysate and catalyze perchlorate reduction in a BER. The perchlorate reduction rate was faster when cells were lysed, implying that an intact outer membrane slows the reaction, presumably by preventing AH$_2$DS access to periplasmic enzymes. Recent *in vitro* work demonstrating that AH$_2$DS can directly donate electrons to the perchlorate reductase and the fact that PcrA (Dsui_0149) and PcrB (Dsui_0148) consistently had among the highest number of spectral counts in proteomics experiments, suggests that the AH$_2$DS oxidation in lysate reactors is catalyzed, at least in part, by this enzyme. However, AH$_2$DS oxidation in the absence of acetate in both whole cell and lysate experiments was slow, and its physiological significance is questionable under the experimental conditions tested.

In the presence of acetate, the mechanism includes oxidation of AH$_2$DS by intermediates of the perchlorate reduction pathway. Addition of acetate to cell suspensions resulted in an immediate and rapid increase in AH$_2$DS oxidation, seen by measuring current with a potentiostat. This response was dependent on whole cells, perchlorate, and acetate, and was not observed if cells were pre-lysed by sonication. When acetate is added to an anaerobic cell suspension, the flux of oxygen and chloride increases. These oxidants can react with AH$_2$DS *in vivo*, consuming current in a bioelectrical reactor, and generating toxic ROS. In support of this, growth inhibition and expression of a cytochrome-$_c$ peroxidase were only observed under perchlorate reducing conditions in the presence of reduced AH$_2$DS.

**Experimental**

**Media composition**

Experiments were conducted using basal media with 80 : 20 N$_2$ : CO$_2$ headspace and 30 mM bicarbonate buffer or with 30 mM HEPES and a N$_2$ headspace. The basal media, per litre, consisted of 0.25 g ammonium chloride, 0.6 g sodium phosphate (monobasic), 0.1 g potassium chloride, with vitamins and minerals as previously described. The pH was 7.2. Electron donors and acceptors were prepared anaerobically from their respective sodium salts, autoclaved and added to cultures using anoxic, sterile technique.

**Cell suspensions**

Pure cultures of *A. suillum* PS were used in all experiments. Cultures were revived from freezer stocks every two weeks, plated, and single colonies picked into liquid culture. For cell suspensions, cells were harvested in late log phase, centrifuged for 10 minutes, washed thrice in basal media, and re-suspended in an anaerobic serum bottle. Cells were injected into reactors at the start of an experiment using a de-gassed syringe. For the lysate experiments, washed cells were sonicated (550 Sonic Dismembrator, Fisher Scientific, Waltham, Massachusetts) on ice for 30 seconds every minute for 4 minutes under an N$_2$ gas headspace, and the same volume of lysate was injected as had been injected into reactors with whole cells.

**Growth experiments**

A 10% inoculum (v/v) of approximately 5 $\times$ 10$^8$ cell mL$^{-1}$ was used, and growth experiments were performed in an anaerobic chamber (Coy Labs, Grass Lake, Michigan) at 37 °C using a UV-Vis spectrophotometer equipped with a 96 well plate reader (Agilent Cary 50 Bio & MPR, Santa Clara, California). Absorbance at 660 nm was used to decrease interference of AH$_2$DS (500 $\mu$M). No lid was used on the 96 well plate, but 80 mL of mineral oil was placed on the 300 $\mu$L sample to prevent evaporation. Growth under nitrate reducing conditions produced N$_2$ gas, which had the potential to distort the optical density measurements by producing gas bubbles. For this reason, a larger number of replicates ($n$ = 6) were generally used, and data points that were clearly a result of gas bubble formation were discarded. Growth curves with active catalase (32 mg L$^{-1}$, 1500 U mL$^{-1}$, Sigma-Aldrich), heat-inactivated catalase (32 mg L$^{-1}$, inactivation for 3 minutes at 80 °C followed by sonication for 30 seconds) or bovine serum albumin (32 mg L$^{-1}$, Sigma-Aldrich) were completed using the same 96-well plate reader.

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**Fig. 8** Proposed model of periplasmic perchlorate reduction coupled to AH$_2$DS oxidation. Thick arrows denote flux under normal perchlorate reducing conditions. AH$_2$DS oxidation is postulated to occur in *A. suillum* PS via perchlorate reductase as well as oxidation by perchlorate derived oxygen or chloride. CI = Complex I, NADH dehydrogenase, QDH = quinol dehydrogenase, CCO = cytochrome-$_c$ oxidase, PcrA = perchlorate reductase subunit A, PcrB = perchlorate reductase subunit B, PcrC = perchlorate reductase subunit C.
method. Background absorbance of catalase and AH₂DS was subtracted from growth curves. For batch growth experiments, 4 mM AH₂DS stock was prepared electrochemically (as described later) and added to a final concentration of 500 μM.

Anion analysis

Perchlorate, nitrate and acetate were measured with an ion chromatograph (ICS-2100, Dionex, Sunnyvale, California) using a method developed to quantify all three analytes simultaneously. The guard and analytical columns were IonPac AG16 (4 x 50 mm) and IonPac AS16 (4 x 250 mm), respectively, with an ASRS-300 4 mm suppressor system and a DS6 heated conductivity cell. A KOH gradient was generated using a EGC III KOH generator at an isocratic flow rate of 1.5 mL min⁻¹. The KOH concentration was 1.5 mM from 0–7 min, ramped to 10 mM from 7–13 min, was held at 10 mM from 13–16 min, ramped to 35 mM from 16–17 min, was held at 35 mM from 17–27 min and ramped back down to 1.5 mM from 27–30 min.

Electrochemistry

Acid washed glass H-cell electrochemical reactors were assembled from pairs of custom-built glass chambers, separated by a Nafion 117 membrane (Sigma-Aldrich) using o-rings and clamps as previously described. Graphite TTK-50 electrodes (Tri-gemini, Hillside, Illinois) were soaked in 10% hydrochloric acid for two days, polished with P400 grit alumina oxide–silicon carbide sandpaper (3M, Minneapolis, Minnesota), sonicated in deionized water for 30 minutes (FS20 sonic cleaner, Fisher Scientific, Waltham, Massachusetts), soaked in acetone overnight, and stored in deionized water until use. Electrodes were connected to the potentiostat using watertight connectors (RMA-FS & XSA-BC, Impulse, San Diego, California). Chromatography experiments (and stock AH₂DS preparation) were conducted at −500 mV vs. saturated standard calomel electrode (SCE) (Fisher Scientific), using a multichannel potentiostat (Biologic VMP3, Claix, France). All reported potentials are referenced to SCE. The reference electrode was placed into a Luggin capillary style apparatus filled with 0.5 M potassium chloride in 1.5% agar. This was fitted through the top of the cathodic chamber.

AH₂DS media for growth experiments (500 μM) and proteomics (4 mM) was prepared using the media recipe described previously. AQDS was added to the media, reduced electrochemically in H-cell reactors, transferred anaerobically to degassed serum bottles, and autoclaved.

Bioelectrochemical reactor studies were conducted with a working volume of 150 mL media. N₂ was continuously sparged through the liquid to maintain anaerobicity and provide mixing. In these experiments, 500 μM AQDS was reduced to AH₂DS prior to the addition of cells. Any AH₂DS that was oxidized by bacteria was re-reduced in situ during chromatography experiments and measured as current. Baseline current was usually 50–100 μA, and was subtracted from the data presented. A delay occurred between bacterial oxidation and re-reduction due to transport of AH₂DS to the electrode surface or electrode kinetics. By convention, cathodic current is represented with negative values. AQDS cyclic voltammetry, redox potentials as a function of pH, and self-exchange kinetics have been reported elsewhere.

Hydrogen peroxide assay

An Amplex Red Hydrogen Peroxide Assay kit (Invitrogen) was used to measure H₂O₂ according to the manufacturers instructions. Absorbance at 571 nm was recorded using a UV-Vis spectrophotometer equipped with a 96 well plate reader (Agilent Cary 50 Bio & MPR, Santa Clara, California) in an anaerobic chamber. The detection limit was 0.5 μM H₂O₂, but this increased by an order of magnitude when 100 μM AH₂DS was present.

Proteomics

Cells grown with the following electron donors and acceptors were analysed with proteomics: acetate/perchlorate/AH₂DS, acetate/perchlorate, acetate/nitrate/AH₂DS and acetate/nitrate. Fifty millilitres of mid-log phase cells (OD₆₆₀ = 0.1–0.15) were harvested in an anaerobic chamber, centrifuged at 6 k rpm for five minutes, and washed with anaerobic 30 mM HEPES buffer in the anaerobic chamber. This was repeated, and cells were re-suspended into 1 mL of 100 mM NH₄HCO₃. Samples were not aerobic until the final re-suspension. Each sample was divided into two sub-samples, which are termed “lysed” or “trypsin shaved.” Lysed sub-samples were subjected to three rounds of 30 second sonication at a power of 1.5 (550 Sonic Dismembrator, Fisher Scientific, Waltham, Massachusetts) at 4 °C. Both sub-samples were proteolyzed with 50 ng trypsin and incubated for 1.5 hours at 37 °C. Trypsin shaved samples were used to profile the bacterial surface proteome, because these samples contain an enriched fraction of periplasmic and surface exposed proteins. Although some cell lysis and release of cytoplasmic proteins is expected during trypsin shaving, it is a valuable technique for observing non-cytoplasmic components of Gram-negative bacteria. Samples were centrifuged for 10 minutes and supernatant was transferred into fresh tubes. Each sample was incubated with 1 mM dithiothreitol and 1 mM iodoacetamide (Sigma-Aldrich, St. Louis, Missouri), in two sequential 30 minute incubations at room temperature. A second round of trypsin digestion was completed for 12 hours at 37 °C. C₁₈ Zip Tips (Millipore, Billerica, Massachusetts) were used to concentrate peptides and remove salts. Peptides were eluted with 85% acetonitrile and 0.1% trifluoroacetic acid and vacuum centrifuged to 30 μL. Trypsin-digested proteins were analyzed by liquid chromatography tandem mass spectrometry as described below.

Data were normalized by the total tandem mass spectral peptide counts per sample, and multiplied by one hundred to give a percentage of the total spectral counts associated with a single protein. Spectral peptide counting has been validated as a semi-quantitative measure of relative protein abundance. Proteins were removed from the dataset if no peptides were observed across all treatments. Expression differences between treatments were determined with multiple t-tests using the MultiTest class in R. Statistical tests used pooled data from the lysed and trypsin shaved sample preparations but un-pooled data is presented to visualize possible differences in protein localization. To
distinguish expression changes that were specific to perchlorate/acetate/AH2DS, controls with nitrate/acetate/AH2DS were also run. t-Tests were used to screen the data for differential expression using a stringent cutoff of \( \alpha = 0.005 \), and \( p \)-values were interpreted with caution because the normality of the data could not be rigorously established with small (\( n \approx 4 \)) data sets, and the fact that falsely detecting differential expression is more likely with multiple t-tests.

**Liquid chromatography-tandem mass spectrometry (LC-MS/MS)**

Acetonitrile (Fisher Optima grade, 99.9%), formic acid (Pierce, 1 mL ampules, 99%+), and water purified to a resistivity of 18.2 M\( \Omega \) cm (at 25 \( ^\circ \)C) using a Milli-Q Gradient ultrapure water purification system (Millipore, Billerica, MA) were used to prepare mobile phase solvents for LC-MS/MS. Samples of trypsin-digested proteins were analyzed using an ultraperformance liquid chromatograph (UPLC) that was connected in-line with an orthogonal acceleration quadrupole time-of-flight (Q-tof) mass spectrometer. Peptides were separated using a nanoAcquity UPLC (Waters, Milford, MA) equipped with C18 trapping (180 \( \mu \)m x 20 mm, 5 \( \mu \)m particles) and analytical (100 \( \mu \)m x 100 mm, 1.7 \( \mu \)m particles) columns and a 10 \( \mu \)L sample loop. Solvent A was 99.9% water/0.1% formic acid and solvent B was 99.9% acetonitrile/0.1% formic acid (v/v). Sample solutions contained in 0.3 mL propylene glycol snap-top vials sealed with septa caps (Wheaton Science, Millville, NJ) were loaded into the nanoAcquity autosampler compartment prior to analysis. Following sample injection (10 \( \mu \)L), trapping was performed for 5 min with 100% A at a flow rate of 15 \( \mu \)L min\(^{-1} \). The injection needle was washed with 500 \( \mu \)L of solvent A and 200 \( \mu \)L of solvent B after each injection to avoid cross-contamination between samples. The elution program consisted of a linear gradient from 10% to 35% B over 80 min, a linear gradient to 95% B over 0.33 min, isocratic conditions at 95% B for 3.67 min, a linear gradient to 1% B over 0.33 min, and isocratic conditions at 1% B for 12.67 min, at a flow rate of 500 \( \mu \)L min\(^{-1} \). The analytical column and sample compartment were maintained at 35 \( ^\circ \)C and 8 \( ^\circ \)C, respectively.

The UPLC column exit was connected to a Universal Nano-Flow Sprayer nanoelectrospray ionization (nanoESI) emitter that was mounted in the nanoflow ion source of the mass spectrometer (Waters Q-tof Premier). The nanoESI emitter tip was positioned approximately 3 mm from the sampling cone aperture. The nanoESI source parameters were as follows: nanoESI voltage 2.4 kV, nebulizing gas (nitrogen) pressure 0.15 mbar, sample cone voltage 35 V, extraction cone and ion guide voltages 4 V, and source block temperature 80 \( ^\circ \)C. The Tof analyzer was operated in “V” mode. Under these conditions, a mass resolving power of 1.0 \( \times \) 10\(^4 \) (measured at \( mlz = 498 \)) was routinely achieved, which was sufficient to resolve the isotopic distributions of singly and multiply charged precursor and fragment ions measured in this study. Thus, an ion’s mass and charge were determined independently, i.e., the ion charge was determined from the reciprocal of the spacing between adjacent isotope peaks in the \( mlz \) spectrum. External mass calibration was performed prior to analysis using a sodium formate solution. Survey scans were acquired in the positive ion mode over the range \( mlz = 400–1800 \) using a 0.45 s scan integration and a 0.05 s interscan delay. In the data-dependent mode, up to five precursor ions exceeding an intensity threshold of 25 counts per second (cps) were selected from each survey scan for tandem mass spectrometry (MS/MS) analysis. Real-time deisotoping and charge state recognition were used to select 2\(^+\), 3\(^+\), 4\(^+\), and 5\(^+\) charge state precursor ions for MS/MS. Collision energies for collisionally activated dissociation (CAD) were automatically selected based on the mass and charge state of a given precursor ion. MS/MS spectra were acquired over the range \( mlz = 100–2000 \) using a 0.20 s scan integration and a 0.05 s interscan delay. Ions were fragmented to achieve a minimum total ion current (TIC) of 50 000 cps in the cumulative MS/MS spectrum for a maximum of 2 s. To avoid the occurrence of redundant MS/MS measurements, real-time dynamic exclusion was used to preclude re-selection of previously analyzed precursor ions over an exclusion width of \( \pm 0.2 \) \( mlz \) unit for a period of 15 min.

Raw LC-MS/MS data were processed using ProteinLynx Global Server software (version 2.3, Waters), which performed background subtraction (35% threshold and fifth order polynomial), smoothing (Savitzky–Golay algorithm, performed 10 times, over three channels), and centroiding (top 80% of each peak, minimum peak width at half height \( = \) four channels) of mass spectra and MS/MS spectra. Processed data were searched against the \textit{A. suillum} protein database. The following criteria were used for the database search: precursor ion mass tolerance 100 ppm, fragment ion mass tolerance 0.15 Da, digest reagent trypsin, allowing for up to three missed cleavages, and methionine sulfoxide as a variable post-translational modification. The identification of at least three consecutive b- or y-type fragment ions was required for assignment of a peptide to an MS/MS spectrum. Protein identifications obtained from MS/MS measurements of single tryptic peptides were validated by manual inspection of the MS/MS spectra.

**Conclusions**

The goal of this project was to understand the molecular mechanisms of AH2DS oxidation coupled to perchlorate reduction by \textit{A. suillum}. We provide evidence that the mechanism includes both enzymatic oxidation (slow) and abiotic oxidation by intermediates of the perchlorate reduction pathway (fast). Given the reactivity of many inorganic electron donors with oxygen and chloride, abiotic reactions must be considered. These abiotic reactions may have integrated with enzymatic steps as part of a metabolic strategy, or they may be solely an inadvertent consequence of generating extremely oxidized intermediates in an anaerobic environment. The implications of this work may also extend to Fe(II) and H\textsubscript{2}S oxidation under perchlorate reducing conditions.

AH2DS oxidation by \textit{A. suillum} PS under nitrate reducing conditions has been reported\textsuperscript{49} and a growth advantage on AH2DS was observed in \textit{D. aromatica} RCB.\textsuperscript{48} We saw very little AH2DS oxidation by \textit{A. suillum} PS coupled to nitrate reduction during exponential growth in both batch and reactor studies; it may be that AH2DS oxidation coupled to nitrate reduction is a stationary phase phenomenon or only induced at the lower concentrations of acetate used in previous studies. It would be interesting to investigate AH2DS oxidation and the proteomes of cells in stationary phase or with low concentrations of organic co-substrate.
Recently, there has been interest in using shuttles to deliver electrons for bioelectro-synthesis.\textsuperscript{31,32} Anaerobicity in these systems is paramount. Contact with air, possibly at the membrane junction of electrochemical cells, will result in superoxide and hydrogen peroxide production. The scale up of any shuttle driven BER technology must consider this possibility.

As bioelectrosynthesis research progresses, shuttle oxidation alone should not be interpreted as signifying a robust microbial metabolism. As our work has shown, shuttles are highly reactive and non-specific. Somehow the desired enzymatic steps must be targeted and side reactions minimized, possibly by matching redox potentials, preventing shuttles from entering the periplasm (i.e., extracellular oxidation), or by isolating bacteria specialized in accepting electrons from these inorganic donors.

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Notes and references