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Comparative proteomics implicates a role for multiple secretion systems in electrode-respiring *Geobacter sulfurreducens* biofilms.

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Abstract.

Geobacter sulfurreducens is a dissimilatory metal-reducing bacterium capable of forming thick electron-conducting biofilms on solid electrodes. Here, we employ for the first time comparative proteomics to identify key physiological changes involved in *G. sulfurreducens* adaptation from fumarate-respiring planktonic cells to electron-conducting biofilms. Increased levels of proteins involved in outer membrane biogenesis, cell motility and secretion are expressed in biofilms. Of particular importance to the electron-conducting biofilms are proteins associated with secretion systems of Type I, II, V and Type IV pili. Furthermore, enzymes involved in lipopolysaccharide and peptidoglycan biosynthesis show increased levels of expression in electron-conducting biofilms compared to planktonic cells. These observations point to similarities in long-range electron transfer mechanisms between *G. sulfurreducens* and *Shewanella oneidensis*, while highlighting the wider significance of secretion systems beyond that of Type IV pili identified to date in the adaptation of *G. sulfurreducens* to electrode respiration.

Keywords: *Geobacter sulfurreducens*, electrode biofilm, proteomics, electron transfer, secretions systems

Introduction.

Geobacter sulfurreducens is an anaerobic, gram negative, bacterium capable of utilising a wide range of soluble (e.g. fumarate) and insoluble (e.g. Fe(III) oxide) electron acceptors for respiration¹. In the absence of an alternative electron acceptor *G. sulfurreducens* has been shown to form thick electron-conducting biofilms (>50 µm) on an adequately polarised solid electrode surface (anode) which can act as an artificial, inexhaustible, electron sink^{2,3}. Our previous work examining the charge transfer properties of *G. sulfurreducens* biofilms, grown under controlled applied potentials, has shown that biocatalytic current, generated from oxidation of acetate, scales with applied potential³. The acetate oxidation currents do not scale directly to biofilm thickness⁴, however, suggesting that either mass or charge transport limits the overall current or that the biofilm formed over time displays differences in bacterial cell densities, viability and/or redox response. Besides this uncertainty, the mechanism by which electrons are transferred over long distances (>50 µm) through *G. sulfurreducens* biofilm to the solid electrode still remains unclear. Two mechanisms are proposed; i) electron hopping through redox proteins (c-type cytochromes) present in the outer membrane^{5,6} and, ii) metallic-like conductivity through Type IV pili (nanowires)⁷. Evidence has been presented to support and oppose both conjectures but, as yet, no published consensus has been reached⁸. The identification of key proteins, particularly outer membrane cell components, implicated in electron transfer (ET) to solid electron acceptors is an important step to unravelling the long-range ET mechanism(s) through *G. sulfurreducens* biofilms. In addition to the Type IV pili structural protein, PilA, shown to be essential for long-range ET through thick *G. sulfurreducens* biofilms², a number of cytochromes have been identified which are thought to be either directly or indirectly involved in this ET process². Gene deletion studies have revealed that outer membrane cytochrome OmcZ is essential for long-range ET through biofilms⁹ while others, namely OmcB, OmcE, and OmcS, are required for optimal current

generation^{2, 9}, although biofilms can produce varying degrees of current in their absence. In addition, OmcF has been implicated in the export of outer membrane proteins necessary for ET from biofilms to a solid electrode¹⁰. Gene deletion studies provide useful information on the roles of specific proteins in the ET process but gaps remain in knowledge of the overall physiology of *G. sulfurreducens* electron-conducting biofilms. More holistic approaches such as transcriptomics or proteomics can provide insights into the bacterium respiratory system, by encompassing a comprehensive representation of cell components *via* the characterisation of gene or protein expression, respectively. A comparative transcriptomic study for *G. sulfurreducens* biofilms using graphite electrode versus fumarate as terminal electron acceptors revealed increased levels of 13 gene transcripts including PilA-N, PilA-C, OmcB and OmcZ, at the electrode, although the transcription of OmcS and OmcT was found to be decreased⁹. Information on gene expression is valuable but the lack of systematic correlation between gene transcription and protein expression renders transcript detection only indicative of the role played by the proteins they encode for. As such, proteomics, and particularly comparative proteomics, represents a powerful tool to identify microbial adaptations characteristic of electrode biofilms. Here we employ iTRAQ (isobaric tag for relative and absolute quantification) to provide a ‘snapshot’ of differential protein expression by a mature *G. sulfurreducens* electrode-respiring biofilm compared to fumarate-respiring planktonic cells. Advantages of iTRAQ include the ability to simultaneously access protein identification and protein relative abundance across eight samples. This, in turn, allows investigations of technical and biological replicates, leading to robust experimental designs and the identification of significant differentially expressed proteins. To our knowledge the present study represents the first proteomic investigation examining protein expression in *G. sulfurreducens* biofilms grown on electrodes compared to (fumarate respiring) planktonic cells. In this study, protein location prediction and functional category classification (Cluster

of Orthologous Groups: COG) is carried out for all the proteins detected. Prediction of functional partners for proteins showing differential expression is then performed and finally a metabolic model of *G. sulfurreducens* is inferred from the proteomic data, with a focus on proteins involved in ET processes.

Experimental Section.

Bacterial strain and growth conditions. *G. sulfurreducens* PCA (DSMZ 12127) was used as a source of electroactive bacteria. The strain was grown anaerobically using the growth medium recommended by the culture centre (DSMZ; medium 826) with acetate as electron donor and fumarate as electron acceptor. The cultures were incubated with shaking at 30°C and grown to stationary phase prior to inoculation in five H-type fuel cells. The anode compartment comprised a carbon cloth (50 cm² area E-TEK) electrode immersed in 125 mL of growth medium (without fumarate). The cathode compartment consisted of a graphite rod (3 mm diameter, Graphite Store product NC001300) electrode (area of 65 cm²) immersed in 50 mM ferricyanide in 100 mM phosphate buffer (Sigma), pH 7.0. Compartments were separated by a Nafion 117 proton-exchange membrane (Sigma). Biofilms were grown under a constant applied potential (0 V vs Ag/AgCl) using a multichannel potentiostat (PG580RM Uniscan Instruments) at 30°C under anaerobic conditions. Fresh growth medium was supplied to each fuel cell periodically (8 batches; 24 h per batch).

Protein extraction, iTRAQ labeling and LC-MS/MS analysis. Protein extracts were prepared from two separate experiments (Fig. S1). For each experiment 4 samples were analysed as follows: planktonic cells from 2 anaerobic bottles and biofilms recovered from five H-type fuel cells (by pooling the recovered biofilms into 2 distinct samples). At the time of sampling, 10 µg ml⁻¹ chloramphenicol (Fisher Scientific) were added to both planktonic

cells (as inoculated into H-type fuel cells) and biofilms to block further protein expression. Biofilms were gently detached from the anode surfaces using a sonication bath (Ultrawave Ltd, Cardiff, UK). Biofilms and planktonic cells were harvested at 7000 *g* for 7 min at 4 °C. The resulting pellets were washed with 2 ml of triethylammonium bicarbonate (TEAB; Sigma) buffer. Protein extraction and quantification were conducted as previously described¹¹. Briefly, the cells were lysed by sonication (MSE Soniprep 150) at 40% amplitude for 30 s on ice. A total of 15 pulses were applied with 30 s intervals. The resulting lysates were centrifuged at 10,000 *g* for 30 min at 4°C, and proteins were precipitated from the supernatants with ice-cold acetone (3:1, vol/vol; Sigma) at 20°C for 1 h. The samples were then centrifuged at 10,000 *g* for 15 min and the resulting pellets were resuspended in 200 µl of TEAB buffer. Protein quantification was carried out using a Calbiochem Non-Interfering Protein Assay kit (Merck KGaA, Darmstadt, Germany) following the manufacturer's instructions. After protein concentration normalization (100 µg in 20 µl), iTRAQ labeling was carried out following the manufacturer's recommendation (Sciex, Foster City, CA). Reagents 113, 114, 115 and 116 were employed to label the planktonic cells (113 and 114 for the first experiment and 115 and 116 for the second experiment), while reagents 117, 118, 119 and 121 were used to label the biofilm samples (117 and 118 for the first experiment and 119 and 121 for the second experiment). Following iTRAQ labeling, the eight samples were combined and resuspended in 1 mL cation exchange load buffer (10 mM KH₂PO₄ pH 3.0 in 25 % acetonitrile) with sonication. The pH was assessed and if necessary adjusted to 3.0 with 0.5 M H₃PO₄. The peptides were then separated by cation exchange chromatography on a PolySulfoethyl A column (200 mm x 2.1 mm, 5 µm, 200 Å pore size, PolyLC, Columbia, MD). The column was washed with 100% Buffer A_{scx} (10 mM KH₂PO₄, 20% acetonitrile (MeCN), pH 3.0) at 1 mL min⁻¹ for 22 min to allow the OD on the UV chromatogram to return to baseline. A gradient of 0-50% B_{scx} (10 mM KH₂PO₄, 20% MeCN,

500 mM KCl, pH 3.0) was applied for 20 min, 50-100% B_{scx} for 3 min, followed by 100% B_{scx} for a further 3 min to wash the column, before reequilibration in 100% A_{scx} for another 11 min. 0.5 mL fractions were collected every 30 sec. Fractions were pooled to give 7 fractions of approximately equal peptide concentration, as judged by inspection of the chromatogram, evaporated to dryness, resuspended in 0.1% trifluoroacetic acid and desalted (PepClean C18 spin columns, ThermoFisher Scientific) using the manufacturer's instructions, eluting in 20 μ L 70% MeCN. The eluate solvent was removed (SpeedVac) and the fractions resuspended in 20 μ L 0.1% FA prior to mass spectrometric analysis. Half of each fraction was injected on to a nanoLC Ultra 2D plus loading pump and nanoLC AS-2 autosampler (Eksigent, Redwood City, CA). The peptides were then separated on an Acclaim PepMap 100 C18 trap and an Acclaim PepMap RSLC C18 column (ThermoFisher Scientific). After washing the trap column for 20 min at 5 μ L min⁻¹ with 98% H₂O, 2% MeCN, 0.5% FA, the trap was switched in line with the column and the peptides eluted with a gradient of increasing acetonitrile from 85% Buffer A (98% H₂O, 2% MeCN, 0.1% FA), 15% Buffer B (2% H₂O, 98% MeCN, 0.1% FA) to 49% Buffer A, 51% Buffer B over 48 min, then to 5% Buffer A, 95% Buffer B over a further 10 min. The column was then washed with 95% Buffer B for 9.5 min before re-equilibration to 95% Buffer A. A flow rate of 300 nL min⁻¹ was employed. The eluate was sprayed into a TripleTOF 5600 tandem mass spectrometer (Sciex, Foster City, CA), using a NANOSpray III source, and analysed in Information Dependent Acquisition (IDA) mode, performing 250 ms of MS followed by 100 ms MSMS analyses on the 20 most intense peaks with a charge state of 2+ to 5+. Parent (MS) ions were accepted with a mass tolerance of 50 mDa and MSMS was conducted with a rolling collision energy (CE) inclusive of preset iTRAQ CE adjustments, to give sufficient peptide fragmentation and generation of the iTRAQ reporter groups. Analysed parent ions were then excluded from analysis for 13 seconds after 3 occurrences. The mass spectrometry

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2
3 proteomics data have been deposited to the ProteomeXchange Consortium¹² via the PRIDE
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5 partner repository with the dataset identifier PXD002667.
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10 ***Protein identification and relative expression.*** The MS/MS data were processed with
11 ProteinPilot v4.0 software using the ParagonTM and ProGroupTM algorithms (Sciex). The data
12 were searched against the UniProt-Trembl database, October 2012, containing 58,533,874
13 protein sequences, with no species restriction. The following settings were used: peptide
14 labelling with iTRAQ 8-plex reagents, methyl methanethiosulfonate as the cysteine
15 alkylation reagent and trypsin as the digestion enzyme. The ID focus was on 'Biological
16 modifications' and the Search Effort 'Thorough'. A threshold of unused Protscore (from
17 ProteinPilot) of 2 (corresponding to protein detection with $\geq 99\%$ confidence) was employed
18 for protein identification. For relative protein quantification purposes, the Pro Group
19 Algorithm assigns each detected peptide an expression ratio based on the iTRAQ reagent
20 (113 to 121) peak intensities in the MS/MS spectra. The algorithm excludes peptides with a
21 peptide ID confidence of $< 15\%$, or a combined feature probability of $< 30\%$ (e.g. semi-tryptic
22 peptides, peptides missing an iTRAQ reagent label, or with low probability modifications or
23 large delta masses). In addition, if the MS/MS spectrum matches to two (or more) different
24 proteins and the alternative protein is identified as being present with a higher confidence
25 (Unused ProtScore), the peptide is excluded from the quantification calculation of the lowest
26 scoring protein(s). Protein ratios are then calculated by ProteinPilot from the ratios of the
27 individual unique peptides that matched these criteria (P-values, Error Factors and
28 Confidence Intervals for protein quantification are presented in Table S1). For simplicity, the
29 two independent experiments analysed in the present study were considered as separate.
30 Consequently, each protein was associated with eight ratios: 117/113-117/114-118/113-
31 118/114 reflecting the relative protein expression in the biofilm versus planktonic cells in the
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first experiment and 119/115-119/116-121/115-121/116 reflecting the relative protein expression in the samples in the second experiment. All the corresponding output protein and peptide summary files generated by ProteinPilot were exported to the ProteinPilot Descriptive Statistics Template (PDST; Sciex, Foster City, CA) for data analysis. In order to determine the statistical significance of the differentially expressed proteins between the two conditions investigated (planktonic cells and biofilms) ratio channels (113-114-115-116 and 117-118-119-121) were selected alternatively as decoy or target within PDST. A total of eight PDST analyses were therefore carried out (Table S2). As indicated in Table S2, using each iTRAQ channel as denominator led to different sets of differentially expressed proteins, highlighting the need to apply such approach to select the truly reproducibly differentially expressed proteins. Only proteins identified at the 1% global false discovery rate (FDR) level (Table S3) and with unused Protscore ≥ 2 were retained. ProteinPilot performs FDR analysis at the spectral, peptide and protein level using a non-linear fitting method to determine both global and local FDR from the decoy database search.¹³ After extracting the differentially expressed proteins common to the eight output files (resulting from the eight PDST analyses), a threshold of ≥ 1.3 fold difference in expression between samples was applied for biological significance¹⁴⁻¹⁷.

Data analysis. Clusters of Orthologous Groups (COG) analysis was carried out by searching the identified proteins using Blastp from the National Center for Biotechnology Information (NCBI) against the COG protein database. Rate ratios were calculated using $(nc/n)/(Nc/N)$, where nc is the number of hits to a given COG category in the proteome (the differentially expressed proteins; the proteins expressed at a higher or reduced level in the biofilms); n is the total number of hits in all categories in the same system (proteome, differentially expressed proteins and proteins expressed at a higher or reduced level in biofilms), Nc is the

number of hits to that category in the genome and in the proteome and N is the number of hits to all categories in the same system (genome and proteome). Further functional analysis was conducted using the Kyoto Encyclopedia of Genes and Genomes (KEGG) Mapper tool and the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING version 9.1)¹⁸ for the identification of active metabolic pathways and the prediction of functional protein partners, respectively. Finally, in order to predict their cellular location, the identified proteins were analysed using PSORTb version 3.0¹⁹.

Results and Discussion

Membrane protein levels increase in electron-conducting biofilms. Overall, a total of 1316 proteins were identified with a minimum of 99% confidence in both biofilm and planktonic *G. sulfurreducens* samples (Table S4), amongst which 77 were significantly differentially expressed (Table S5). These included 40 and 37 proteins that were detected at higher and reduced levels in biofilms, respectively (Table S5). The prediction of protein location indicated that a change in the nature of the electron acceptor (soluble fumarate versus solid electrode) in the growth medium of *G. sulfurreducens* resulted in alteration of the expression pattern of proteins located mainly in the membrane (inner and outer) and periplasm (Fig. 1). Indeed, while proteins from these cellular locations accounted for a combined 14% of the whole proteome, 28% of proteins differentially expressed were predicted to belong to the inner membrane (12%), the outer membrane (8%) and the periplasm (8%; Fig. 1A and B). More specifically: 20% of proteins whose level of expression is higher in the electrode biofilms compared to planktonic cells are associated with the inner membrane; an additional 12% are associated with the outer membrane; while 13% of the proteins with a reduced expression in biofilms are located in the periplasm (Fig. 1C and D). This indicates that *G. sulfurreducens* adaptation from planktonic to electron-conducting biofilms, induced by a shift

from soluble to insoluble electron acceptor, involves the preferential increased expression of membrane-associated proteins.

The nature of electron acceptor impacts preferentially on the levels of proteins belonging to certain functional categories. The identification of 1316 *G. sulfurreducens* proteins corresponds to 38% genome coverage. This relatively low coverage can be attributed to the stringent conditions employed and the previously reported reduced performance of iTRAQ 8-plex set-up intrinsically linked to replication discrepancies²⁰. When comparing 4-plex to 8-plex iTRAQ, Mahoney et al.²¹ reported a reduction in protein identification rate of 40%. This was attributed to a reduced ionisation efficiency as well as a higher average charge for 8-plex compared to 4-plex labeled peptides²²⁻²³. Interestingly, when using the KEGG Mapper tool to investigate the functional roles of the proteins identified in the present study, 70% of *G. sulfurreducens* protein-encoding genes involved in metabolic pathways are expressed under the conditions of the present study (Fig. S2). In addition, a proteome coverage of 21% and 42% are reported for membrane proteins and proteins involved in secretion systems. When comparing the COG distribution from *G. sulfurreducens* genome to its proteome (expressed under the conditions investigated and using rate ratios), several COG categories are under-represented or over-represented (Fig. 2A). Of particular note is the under-representation in the proteome of proteins involved in cell motility (N), defense mechanisms (V) and proteins assigned to no COG category. Conversely, proteins involved in amino acid and nucleotide transport and metabolism (E and F, respectively), as well as proteins involved in translation (J) are over-represented in the proteome (Fig. 2A). Even though in the overall proteome proteins can be classified into 21 COG categories (including no COG category), proteins that are differentially expressed can only be assigned to 16 COG categories (Fig. 2). Amongst these, proteins expressed at higher and reduced levels in electrode biofilms belong to 14 and

eight COG categories, respectively. Strikingly, an over-representation of proteins involved in cell envelope biogenesis and outer membrane (M), cell motility (N) and intracellular trafficking and secretion (U) prevails specifically amongst proteins expressed at higher levels in biofilms, with no proteins belonging to these COG categories showing a reduced level of expression in the same cohort of samples (Fig. 2B and C). This observation suggests important roles for proteins involved in functional categories M, N and U in electron-conducting biofilms, particularly in the light of their under-representation in the overall proteome (Fig. 2A). Similarly, proteins associated with energy production and conversion (C), amino acid transport and metabolism (E), carbohydrate transport and metabolism (G) and DNA replication, recombination and repair (L) are over-represented amongst the proteins with reduced levels of expression in biofilms, likely reflecting decreased metabolic activities in these samples compared to planktonic cells (Fig. 2C). Taken together, these observations highlight that the change in the nature of the electron acceptor for *G. sulfurreducens* results in targeted alterations in the proteome, specifically involving proteins belonging to relevant functional groups.

Metabolic reconstruction of G. sulfurreducens inferred from proteomic data. Under both planktonic and biofilm conditions, *G. sulfurreducens* expresses proteins involved in gluconeogenesis/glycolysis, likely entering this pathway by converting acetate (present in the growth medium) to acetyl-CoA using acetate kinase and phosphate acetyltransferase (proteins 20 and 21; Fig. 3; Table S6). In addition to expressing proteins of the pentose phosphate pathway, *G. sulfurreducens* also has an active tricarboxylic acid (TCA) cycle, under both conditions investigated, leading to the generation of reducing power and energy. The resulting NADH and NADPH could be further used as electron donors in oxidative phosphorylation, with complexes I, II and V detected across all samples (represented by

proteins 34, 53-56; Fig. 3). Complex I and II proteins are expressed at higher levels in biofilms (proteins 34 and 56; Fig. 3), while the F₀F₁ ATP synthase of complex V shows a reduced level of expression in biofilms (protein 56; Fig. 3). Proteins from several two-component systems were found to be expressed in both planktonic and biofilm states (Fig. 3) including proteins from the NtrC family, possibly indicating that *G. sulfurreducens* experienced nitrogen limitation at the time of sampling (Fig. 3: proteins 41, 57-61). Proteins involved in glutamate and nitrogen metabolism are also detected, several of which are differentially expressed (Fig. 3). A total of 38 ABC transporters are identified across all samples (represented by proteins 155, 166-187; Fig. 3). Amongst these, five are differentially expressed, including four that are detected with decreased abundances in the biofilms (Fig. 3). Numerous proteins involved in porphyrin metabolism, important for cellular respiration, are also identified across all samples (proteins 98-115; Fig. 3).

It has been proposed that electrons harvested from the cytoplasmic metabolic processes of surface-attached *G. sulfurreducens* are transported to the solid electrode *via* a complex assembly of cytochrome conduits spanning the inner membrane through to the extracellular milieu^{8,24}. Specifically, electrons may be transferred from the menaquinone pool to periplasmic c-type cytochromes, such as GSU1996²⁴, *via* an inner membrane-associated redox protein, such as cytochrome c ImcH²⁵. Transfer across the outer membrane to the solid electrode is thought to involve either specific cytochromes, which can connect directly to the electrode (e.g. OmcZ)²⁶, or through a combination of cytochrome c/pilus assemblies (e.g. OmcS/Type IV pili)²⁷. Studies have highlighted OmcS, OmcB, OmcC, OmcE and OmcZ as being directly or indirectly involved in ET to solid electron acceptors^{2,9,28-29}. In the present study, a total of 20 c-type cytochromes are detected with similar levels of expression for both planktonic and biofilm samples. Amongst these, two proteins are located in the cytoplasm

(proteins 88 and 89; Fig. 3), four in the inner membrane (proteins 79-82; Fig. 3), five in the periplasm (proteins 83-87; Fig. 3) and seven in the outer membrane/extracellular milieu (proteins 91-97; Fig. 3). In addition to the outer membrane cytochromes (Omc's) previously identified to contribute to *G. sulfurreducens* ET processes (detailed above), three additional Omc's are detected: OmcN and two monoheme cytochrome c's (protein 94, 91 and 97; Fig. 3). Periplasmic cytochrome c, GSU1996 (protein 84; Fig. 3), previously implicated in ET to soluble and insoluble Fe(III)³⁰, is detected, although the triheme cytochromes, PpcA to PpcE, proposed as periplasmic electron carriers²⁴, are not detected. Furthermore, cytochrome c ImcH (protein 80; Fig. 3), expressed under both conditions investigated and located in the inner membrane, was previously demonstrated to be essential for *G. sulfurreducens* respiration on high redox potential (> 0 V vs. SHE) insoluble electron acceptors³¹. Direct differential cytochrome expression between planktonic and biofilm samples is not observed. However, analysis of putative functional partners for proteins differentially expressed (using STRING v9.1¹⁸) identified three proteins with increased expression in biofilms (proteins 196, 197 (Fig. 3) and GSU2496; Table S5) predicted to interact with a range of cytochromes, including OmcB (predicted partner of protein 196) and OmcS (predicted partner of protein GSU2496; Table S7). Likewise, two proteins with reduced level of expression in biofilms, GSU0617 and an outer membrane porin (protein 164; Table S6; Fig. 3) are predicted to partner with several cytochromes (Table S7). Differential expression of such proteins may indirectly represent a variation in cytochrome c content for electrode-respiring versus fumarate-respiring *G. sulfurreducens*, although such a variance may simply not be represented at this specific stage of cell sampling. Other cytochromes detected include inner membrane-associated cytochrome peroxidase, MacA (protein 90; Fig. 3), which, although not previously implicated specifically for electrode respiration, was shown to be required for reduction of both soluble and insoluble Fe(III)³², and may be involved in OmcB gene

transcription³³. Also identified are cytochrome c nitrite reductase (protein 76; Fig. 3), and two inner membrane proteins ResA and ResB (proteins 77, and 78; Fig. 3), involved in cytochrome c maturation³⁴.

ET associated flavoproteins are detected at similar levels in planktonic and biofilm samples (proteins 73 and 74; Fig. 3). These could be involved in ferredoxin reduction, as proposed previously³⁵. Indeed, several ferredoxin-dependent proteins are detected including the NfnAB complex (proteins 38 and 75; Fig. 3), which couples the reduction of NADP⁺ and NAD⁺ to ferredoxin oxidation³⁵. In addition, both an indolepyruvate ferredoxin oxidoreductase and a nitrite reductase containing a conserved ferredoxin nitrite reductase domain (as indicated by Blastp), are expressed at lower levels in biofilms (proteins 199 and 222; Fig. 3). Recent evidence suggests that, analogous to *Shewanella oneidensis*³⁶, monolayer thick *G. sulfurreducens* biofilms may secrete flavins which bind to the outer membrane and act as terminal electron carriers for ET to solid electron acceptors. Here, several proteins associated with riboflavin biosynthesis (proteins 116-119; Fig. 3) are detected with similar abundances across all samples with no evidence of expression at the time of sampling of the corresponding flavin export protein, GSU2653³⁶.

Bacterial secretion systems permit cells to transfer small molecules, proteins and DNA across the membrane into the extracellular milieu or into nearby target cells³⁷. To date, six secretion systems have been identified in gram-negative bacteria, classified as Type I secretion system (T1SS) through to Type VI (T6SS)³⁷. All but T5SS comprise protein assemblies which span both inner and outer membranes. In the present study, proteins associated with five of these systems (apart from T3SS; predominately found in bacterial pathogens) are detected, with significant increased levels of expression for T1SS, T2SS, T5SS and Type IV pili secretion

reported in biofilm samples. T1SS, T2SS, T4SS and T6SS all contain inner membrane transporters allowing secretion of substrates directly from the cytoplasm to the extracellular milieu. T1SS, composed of three subunits; an inner membrane ATP binding cassette (ABC) transporter, a periplasmic membrane fusion protein and an outer membrane pore-forming protein, permits substrate secretion in a single step³⁸. Substrates include diverse, largely unfolded, proteins of various size (19 kDa to 900 kDa) and non-proteinaceous toxins³⁸. Sixteen T1SS associated proteins common to both samples are detected (Fig. 3; Table S6), including two membrane fusion proteins detected with increased level of expression in biofilms (represented by protein 131; Fig. 3). The large number of T1SS associated proteins detected suggests an important role for this secretion system under the two conditions investigated. T2SS, T3SS, T4SS and T6SS incorporate pilus or needle-like structures which extend from the inner membrane, through the outer membrane and beyond (except Type II pseudopili which are localised in the periplasm) and facilitate direct or indirect translocation of substrates from the cytoplasm into the extracellular milieu (T2SS) or into neighbouring cells (T3SS, T4SS and T6SS)³⁷. T2SS and T5SS both lack an inner membrane transporter and therefore depend on the general secretion system (Sec pathway) for translocation of unfolded proteins across the inner membrane and into the periplasmic space, many of which are eventually utilised in outer membrane assembly (*vide infra*). In addition, the Sec pathway is also responsible for the insertion of the majority of proteins into the inner membrane³⁹. Six Sec pathway related proteins (represented by proteins 146 to 148; Fig. 3) common to both samples are detected, with an increased level of expression for SecD (protein 148), a subunit of the main Sec channel, in biofilms. Differential expression was also found for two Sec related chaperones, SurA (protein 153; increased expression in biofilms) and DegP (protein 152; decreased expression in biofilms), whose function is to stabilise and translocate Sec secreted proteins through the periplasm to their specific destinations. SurA is considered the

primary chaperone for protein translocation to T5SS Bam machinery which facilitates β -barrel porin assembly in the outer membrane³⁷. *G. sulfurreducens* also encodes several genes (GSU0022, GSU0781, GSU0787, GSU1486 and GSU3190) related to the Twin-arginine translocation (Tat) pathway (which can translocate folded proteins across the inner membrane) although no proteins associated with this system were detected in this study. T2SS is a *ca.* 12-15 component secretion system consisting of four subunits; a cytoplasmic APTase, an inner membrane platform, a periplasmic pseudopilus and an outer membrane secretin which can secrete a wide range of folded proteins⁴⁰. For example, it is estimated that >25 proteins are secreted via the T2SS pathway in the gram negative bacterium *Legionella pneumophila*, with 20 identified in a single study⁴¹. Substrates are delivered to the T2SS *via* the Sec (or Tat) pathway and secreted by the pseudopilus in a rotary piston-like motion which pushes the substrate through outer membrane pores into the extracellular milieu³⁷, although some are known to remain surface-associated upon secretion⁴². Interestingly, T2SS represents the only viable route for secretion of cofactor-containing proteins to outer surfaces implying that it plays a significant role in surface-associated redox protein secretion for *Geobacter* and *Shewanella* species⁴². MrtC and OmcA cytochromes are known substrates of T2SS in *S. oneidensis*⁴³ which remain surface bound upon secretion⁴² and participate in ET to solid electron acceptors⁴³. Both are involved into the MtrABC conduit architecture which facilitates extracellular respiration in *S. oneidensis*⁴⁴. OmpB (detected here; Table S4), a loosely attached outer membrane multicopper oxidase, is the sole protein identified to date that is secreted via T2SS in *G. sulfurreducens*⁴⁵, though it is probable that other, so far unidentified, outer membrane surface-bound redox proteins may also use this route. Here, a total eight T2SS associated proteins common to both samples are detected (represented by proteins 133-136; Fig. 3) including three with increased level of expression in biofilms (represented by proteins 133 and 136; Fig. 3), suggesting an escalation of T2SS secretion in

the biofilm samples compared to planktonic cells. T4SS is associated with bacterial cell adhesion and motility, and can facilitate the transport of a diverse array of substrates from DNA to effector proteins⁴⁶. Analogous to T2SS, it comprises *ca.* 15 proteins to form a four subunit structure spanning both membranes. However, unlike T2SS pseudopili, T4SS pili extend all the way through to the extracellular milieu. Although *G. sulfurreducens* encodes genes associated with T4SS, it is yet unknown if the bacterium utilises this secretion system for extracellular export of DNA and/or proteins. Gene deletion studies, though, have established that protruding Type IV pili are essential for facilitating long range ET through *G. sulfurreducens* biofilms to solid electron acceptors^{2,9}. A total of 17 Type IV associated proteins (represented by proteins 62, 64 and 137-145; Fig. 3) are detected in both samples, including PilQ (protein 144; Fig. 3) with increased level of expression in biofilms. PilQ forms the outer membrane pore which facilitates the extension of the Type IV pili from the periplasm, through the outer membrane and into the extracellular milieu (Fig. 3). Four cytoplasmic ATPases are also detected (represented by proteins 137-139; Fig. 3), including PilT (protein 139; Fig. 3) which powers pilus retraction, aiding cell motility^{47,48}. PilR, also identified here, (protein 62; Fig. 3) is a cytoplasmic transcriptional response protein responsible for the regulation of PilA⁴⁹. Inner membrane proteins PilC, PilO, and PilL, which are also detected (represented by protein 143; Fig. 3), form the platform for pilus assembly⁴⁸. Although differential expression of PilA (protein 64; Fig. 3), seen in other transcriptomic studies⁹, is not observed under the conditions tested, the increased expression of PilQ in biofilm samples (protein 144; Fig. 3) supports the hypothesis of the integral involvement of Type IV pili in long-range ET through electrode-respiring *G. sulfurreducens* biofilms. Spanning only the outer membrane, T5SS (also known as the autotransporter system) is the simplest secretion system, facilitating the secretion of proteins⁵⁰ and is implicated in cell-to-cell adhesion, biofilm formation and outer membrane biogenesis⁵¹. Like T2SS, T5SS depends

on the Sec pathway for delivery of substrates into the periplasm³⁷. BamA (showing increased expression in biofilms; protein 154; Fig. 3) is the main subunit of the T5SS machinery which assists with insertion and assembly of β -barrel proteins into the outer membrane⁵². A total of three outer membrane β -barrel proteins, namely OmpA, OmpJ and a putative Omp (proteins 158, 161 and 162; Fig. 3), are detected in both samples, with increased level of expression for OmpA and OmpJ in biofilms. Previous studies on *G. sulfurreducens* demonstrate the necessity of OmpJ for extracellular ET to Fe(III) and Mn(IV) metal oxides, but report that OmpJ is not required for fumarate reduction⁵³. The increased expression of the Bam complex and several outer membrane β -barrel protein channels (and differential expression of Sec-related periplasmic chaperones known to deliver T5SS substrates, described earlier; Fig. 3; Table S6) in biofilm samples suggests an important role for T5SS in electron conducting *G. sulfurreducens* biofilms. T6SS facilitates the translocation of proteins with roles in pathogenicity and bacterial competition and is composed of two main complexes involving over 13 components; a membrane complex comprising inner membrane proteins with high levels of similarity to T4SS and a tail complex made of proteins evolutionary related to bacteriophage contractile components³⁷. TssD, a needle tube protein belonging to the T6SS tail complex is detected at a similar level of expression in planktonic and biofilm samples (protein 157; Fig. 3), indicating a role for this secretion system under both conditions investigated. Taken together, these results highlight the importance of multiple secretion systems in *G. sulfurreducens* electron conduction biofilms.

Conclusions.

Comparative proteomics has been utilized to aid the elucidation of key physiological changes involved in *G. sulfurreducens* adaptation from fumarate-respiring planktonic cells to electron-conducting biofilms. Analysis of differential protein expression indicates that

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3 microbial adaptation involves targeted alterations of protein levels from relevant functional
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5 groups and from specific cellular location. Specifically, proteins associated with energy
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7 production and conservation, amino acid and carbohydrate metabolism and DNA replication
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9 are expressed at lower levels in biofilms, possibly reflecting reduced metabolic activities
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11 compared to planktonic cells. Conversely, increased levels of expression of proteins involved
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13 in outer membrane biogenesis, cell motility and secretion are reported in biofilm samples.
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15 Overall, similar metabolic pathways are employed under both conditions investigated and
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17 include gluconeogenesis/glycolysis, the pentose phosphate pathway and the TCA cycle. The
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19 increased expression of proteins involved in outer membrane biogenesis and secretion in
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21 biofilm samples suggest a pivotal role for these processes to enable long range ET through
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23 biofilms. However, it should be noted that such observations may not be exclusive to
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25 electrode respiring *G. sulfurreducens* biofilms and may represent a required adaptation for
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27 biofilm formation. Specifically, cytoplasmic proteins involved in lipopolysaccharide and
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29 peptidoglycan biosynthesis (Fig. 3: proteins 124 and 190, respectively; Table S6) show
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31 increased levels of expression in biofilms compared to planktonic cells. A number of recent
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33 studies have emphasised the importance of extracellular polysaccharide networks for
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35 electrical conduction through *G. sulfurreducens* biofilms^{54,55}. Rollefson *et al.*⁵⁴ showed that
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37 such networks aid attachment and colonisation of *G. sulfurreducens* to electrodes and provide
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39 an extracellular scaffold for spatial cytochrome assembly. The importance of outer membrane
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41 assembly and composition in biofilms is further underlined by the increased expression of
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43 multiple secretion systems involved in outer membrane biogenesis, particularly T2SS and
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45 T5SS. T2SS facilitates the secretion of co-factor containing proteins to the extracellular
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47 milieu⁴². Consequently, T2SS likely plays a central role in the assembly of outer membrane-
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49 associated redox co-factor containing protein conduits required for extracellular ET²⁴.
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51 Although the relevance of T2SS in *G. sulfurreducens* extracellular ET processes has received
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little attention to date, the importance of T2SS in *S. oneidensis* is well documented^{42,43,56-59} with studies showing that T2SS is responsible for the translocation of multiple cytochromes, implicated in extracellular ET, across the outer membrane to the cell exterior^{43,59}. The increased expression of T5SS associated proteins in biofilm samples, particularly BamA and a number of β -barrel proteins (OmpA, OmpJ and a putative Omp), might support the recent proposal that a *G. sulfurreducens* ‘porin-cytochrome’ protein complex embedded in the outer membrane may provide a route for trans-membrane ET to solid electron acceptors⁶⁰. Moreover, a similar role is proposed for the previously described *S. oneidensis* ‘porin-cytochrome’ (MtrABC) module⁶¹, further emphasising potential similarities between *G. sulfurreducens* and *S. oneidensis* extracellular ET mechanisms.

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Author Contributions.

P.K., F.A. and D.L. designed the research; F.A. and P.J. prepared, extracted and labelled planktonic and biofilm iTRAQ samples; C.B. performed iTRAQ analysis; F.A. and P.K. analysed the data; P.K. and F.A. wrote the paper with input from D.L. and C.B.

Conflict of Interest Disclosure.

The authors declare no competing financial interest.

Supporting Information.

Figure S1: Schematic diagram of the experimental set-up (.pdf).

Figure S2: Metabolic pathways generated using the KEGG Mapper tool and inferred from proteomic data (.pdf). Red lines indicate reactions catalysed by *G. sulfurreducens* enzymes detected in the present study. Grey lines represent reactions belonging to metabolic pathways involving enzymes for which *G. sulfurreducens* genome does not encode the corresponding proteins. Lines of other colors than red or grey indicate reactions that can be carried out by *G. sulfurreducens* involving enzymes not detected in the present study. Green and black stars represent proteins expressed at higher or reduced levels in the biofilm samples, respectively.

Table S1: Protein Summary file from ProteinPilot (.xlsx). N represents the protein rank. The Unused ProtScore (Unused) is calculated using only peptides from spectra that have not been associated to higher scoring proteins, while the Total ProtScore (Total) uses all the peptides associated to a protein. % Cov represents the protein sequence coverage of the identified peptides. Accession numbers and protein names are provided. The quantification ratios are presented from 114/113 to 121/113. For each ratio, P-values, error factors and confidence intervals are provided.

Table S2: Summary of PDST analyses carried out to access statistically significant differentially expressed proteins (.pdf).

Table S3: Summary of false discovery rate analysis from PDST (.xlsx).

Table S4: Proteins identified in *G. sulfurreducens* grown in the absence (electrode) and in the presence of a soluble electron acceptor (fumarate; .xlsx). Unused Protscore is provided by ProteinPilot and is calculated using only peptides from spectra that have not been associated to higher scoring proteins. A Unused Protscore of 2 corresponds to 99% confidence with Unused Protscore = $-\log(1-(\text{Percent Confidence})/100)$. The percentage sequence coverage is also displayed along with the number of distinct peptides with 95% confidence.

Table S5: Differentially expressed proteins for *G. sulfurreducens* grown on electrodes and in planktonic state (.pdf).

Table S6: *G. sulfurreducens* proteins with functions represented in Figure 3 (.xlsx). Protein numbers correspond to the numbers displayed in Figure 3. Enzyme Commission number (EC) are provided. Proteins highlighted in red and in green were found to be expressed at lower and higher level in electrode-respiring biofilms compared to planktonic cells, respectively.

Table S7: Summary of relevant predicted functional partners of differentially expressed proteins (.pdf).

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Figure legends

Figure 1: Prediction of cellular location for **A.** the whole proteome (1318 proteins); **B.** the differentially expressed proteins (77 proteins); **C.** the proteins expressed at a higher level in the electrode biofilms (40 proteins) and; **D.** the proteins expressed at a reduced level in the electrode biofilms (37 proteins).

Figure 2: Rate ratios of COG categories in **A.** the overall proteome (1318 proteins); **B.** the proteins expressed at a higher level in the electrode biofilms (40 proteins) and; **C.** the proteins expressed at a reduced level in the electrode biofilms (37 proteins). Rate ratios were calculated using $(n_c/n)/(N_c/N)$, where n_c is the number of hits to a given COG category in the proteome (**A**), the proteins expressed at a higher or reduced level in the biofilms (**B** and **C**

respectively), n is the total number of hits in all categories in the same system (proteome, differentially expressed proteins and proteins expressed at a higher or reduced level in biofilms), N_c is the number of hits to that category in the genome (**A**) and in the proteome (**C** and **D**) and N is the number of hits to all categories in the same system (genome and proteome).

Figure 3: Reconstructed metabolism of *G. sulfurreducens* inferred from proteomic data. For full protein information and box numbers see Table S6. Dash dot arrows indicate an effect of one protein on the expression of another protein. Dash arrows indicate an indirect effect from one protein on another. Red circles represent cytochrome c. Protein numbers in red and in green were found to be expressed at lower and higher level in electrode-respiring biofilms compared to planktonic cells, respectively. G3P stands for glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; CHM, 3-carboxy-3-hydroxy-4-methylpentanoate; PPRP, 5-phosphoribosyl-1-pyrophosphate; D-ribu 5P, D-ribulose 5-phosphate; Oxaloa, oxaloacetate; Form, formate; Cit, citrate; Cis, cis-aconitate; Iso, isocitrate; Oxalos, oxalosuccinate; 2-oxoglu, 2-oxoglutarate; Suc-coA; succinyl-CoA; Suc, succinate; GABA, γ -aminobutyric acid; D-Gluco-6P, D-glucosamine-6-phosphate; 5P-Ribo, 5-phosphate-ribosylamine; L-Gln-tRNA(Gln), L-Glutaminyl-tRNA(Gln); D-ala-D-Ala, D-alanyl-D-Alanine; L-Sacc, L-Saccharopine.

Figure 1.

Figure 2.

Figure 3

For TOC only