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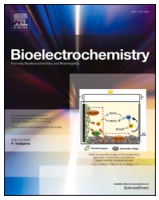

Interfacial extracellular electron uptake is linked to nitrate respiration in the marine heterotroph, *Thalassospira xiamenensis* SN3

Joshua D. Sackett [a,*], Gabriel P. Tonucci [a], Annette R. Rowe [a,b,c]

[a] Department of Biological Sciences, University of Cincinnati, Cincinnati, OH, USA
[b] Department of Earth and Environmental Sciences, Michigan State University, East Lansing, MI, USA
[c] Department of Microbiology, Genetics, and Immunology, Michigan State University, East Lansing, MI, USA

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ABSTRACT

Thalassospira species are ubiquitous marine bacteria with poorly understood ecology, and some have been implicated in iron corrosion. To better elucidate the mechanisms and ecological implications of extracellular electron transfer (EET) in oxidative processes, we conducted genomic and bioelectrochemical characterization of *Thalassospira xiamenensis* strain SN3, an obligate heterotroph isolated from coastal marine sediment cathode-oxidizing enrichments. Physiologic and genomic analyses indicate that SN3 lacks the capacity for lithoautotrophic growth and lacks homologs to genes canonically involved in EET. Bioelectrochemical characterization of SN3 cells shows that inward EET requires a terminal electron acceptor (respiration). Deletion of nitrate reductase catalytic subunit *napA* abolished current consumption and catalytic activity under nitrate-reducing conditions. Media exchange experiments demonstrate that inward EET in SN3 is facilitated by direct contact with the electrode, with a formal midpoint potential of -153 ± 16 mV vs. SHE. Through deletion of the formate dehydrogenase *fdhABCD* and electrochemical characterization of mutant cells, we show that inward EET is not a function of Fdh enzyme sorption to the electrode, as has been demonstrated for other organisms. This provides further evidence of a cell-mediated and contact-dependent EET mechanism. This work provides a foundation for investigating this metabolically versatile organism's yet uncharacterized mechanism of EET.

1. Introduction

Extracellular electron uptake (EEU) is a metabolic process by which certain microorganisms transfer electrons from external surfaces, such as redox-active minerals or electrodes, across their insulating cellular envelope to support their growth, energy conservation, or redox balance. While much of the research on EEU has focused on model organisms, such as *Shewanella oneidensis* MR-1 [59,60], non-model organisms are increasingly recognized for their contribution to biogeochemical cycling and for their potential utility in biotechnological applications and bioremediation [20,35,72]. These organisms may exhibit unique or uncharacterized adaptations to utilize extracellular electron donors, and thus investigating these processes provides valuable insights into the diversity of EEU mechanisms and/or biomarkers for these redox capacities. Further understanding the mechanisms behind EEU in non-model organisms could lead to improved engineering of EEU pathways for energy capture, wastewater treatment, and even renewable energy generation [16,26,57].

Currently the best understood EEU mechanisms stem from those homologous to known direct extracellular electron transfer (EET) mechanisms from model metal oxide-reducing bacteria. In *Geobacter sulfurreducens* and *Shewanella oneidensis* MR-1, EET involves highly specialized networks of multi-heme *c*-type cytochromes and protein complexes that facilitate the transfer of electrons from the cell's cytoplasm to external electron acceptors, such as iron(III) oxyhydroxides or other metal oxides [63,64]. Homologous proteins have been observed in the iron oxidizing microorganisms *Rhodopseudomonas palustris* and *Sideryoxydans lithotrophicus* and are proposed to play a role in electron acquisition from extracellular reduced iron. Genetic evidence in *R. palustris* supports PioAB, a homolog of MtrAB from *S. oneidensis*, playing a significant role in iron oxidation [20,21,30]. In *S. lithotrophicus*, transcriptomic and proteomic data suggest that MtoAB, a homolog of MtrAB from *S. oneidensis*, is used to access and reduce solid Fe(II) in smectite [80]. However, mechanistic work in other lithotrophic iron oxidizing microorganisms has been more challenging [9,10,34,49,80].

* Corresponding author.
E-mail address: Joshua.sackett@uc.edu (J.D. Sackett).

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Several non-model organisms from environmental niches such as sediments, wastewater treatment plants, and bioreactors have shown promising EET mechanisms that lack homology to the well-characterized mechanisms in model organisms [77]. Thus, identification of EEU in environmental organisms is hindered by the number of genetically or biochemically characterized pathways. Recent studies have begun to address these knowledge gaps by utilizing genomics, proteomics, and high-throughput genetics, in combination with electrochemical techniques to explore the EET capabilities of environmentally relevant microorganisms [15,34,50,58,61,68,70,80]. This has included autotrophic, heterotrophic and mixotrophic strains. Larger strides have been made in mixotrophic and heterotrophic organisms due to their compatibility with genetic techniques. With these challenges in mind, focusing characterization efforts on both heterotrophic and autotrophic EEU-capable organisms has merit as a way of more rapidly expanding the number of genetically characterized pathways. This is especially true for widely distributed and metabolically versatile organisms. One such organism, *Thalassosopira* strain SN3, was isolated in our previous electrochemical enrichments from a coastal marine sediment [58].

Thalassospira species are widely distributed in both pelagic and benthic marine environments [13,17,19,27,33,37,41,42,47,62,69,73]. The described species range in metabolic capabilities, from strict aerobic heterotrophs capable of growth under ultra oligotrophic conditions comprised of filtered and autoclaved seawater supplemented with 0.01 g/L of complex organic substrates [47] to facultatively aerobic heterotrophs capable of denitrification, some of which are capable of agar degradation [17] or degradation of polycyclic aromatic hydrocarbons [37,78].

Thalassospira have garnered attention for their potential role in biocorrosion [7,8,46,73], including observations of iron oxidation in a strain of *Thalassospira xiamenensis* [22], and iron reduction in a species of *Thalassospira* [8]. It must be noted that iron oxidation assays utilizing gradient tubes have been shown to have potentially misleading results, especially in organisms that are known to metabolize agar or grow under ultra oligotrophic conditions [28]. Nevertheless, Chen and Zhang [8] have shown that a *Thalassospira* species contributes to pitting corrosion of Q235 carbon steel. Several diverse mechanisms of iron corrosion have been described in marine organisms, including both proposed direct and mediated electron transfer mechanisms (see review [29]). EEU has been shown to both induce and inhibit corrosion processes [10,56], however, it is unclear what role EEU may have in iron corrosion for *Thalassospira* species. In these systems the biological mechanisms of corrosion are largely unknown but may be related to the currently undercharacterized extracellular electron transfer mechanism in *Thalassospira* species. Characterization of an EEU mechanism in *Thalassospira* could serve as a starting point to identifying and characterizing EEU mechanisms more broadly in this group of organisms.

Here, we present an amperometric, voltametric, genetic, and genomic investigation of *Thalassospira xiamenensis* strain SN3, a cathode-oxidizing heterotrophic bacterium isolated from coastal marine sediments [58]. This work describes the capacity of strain SN3 to utilize a poised cathode as a source of electrons in a process that requires direct contact with the electrode surface and is linked with respiration but not growth.

2. Methods

2.1. Bacterial strains and culture conditions

All bacterial strains utilized in this work were stored in 40 % glycerol at −80 °C unless otherwise stated and are listed in Table 1. Prior to each experiment, *Thalassospira xiamenensis* SN3 was grown on LBS + Ions (25 g/L LB [Fisher Bioreagents], 10 g/L NaCl, 3 g/L $MgCl_2$*$6H_2O$, 0.15 g/L $CaCl_2$*$2H_2O$, with 15 g/L agar added for solid media) agar plates incubated at 30 °C [5] and single colonies were used to inoculate LBS + Ions broth or artificial saltwater base (SWB) [58] containing 5 mM sodium acetate (30 °C, 200 RPM). For anaerobic growth, 10 mM KNO_3 was added to SWB media preparations with acetate. Anaerobic growth conditions were maintained by sparging media with N_2 for 45 min following autoclaving, and anaerobically dispensing media into serum vials. Inoculations and culture transfers were performed with sterile anaerobic syringes and needles in an anaerobic chamber (90 % N_2, 5 % CO_2, 5 % H_2 atmosphere). *Escherichia coli* WM3064, a diaminopimelic acid (DAP) auxotroph, was used as a donor strain in conjugation experiments and was grown on lysogeny broth (LB) supplemented with 300 μM DAP. Kanamycin (50 mg/L) was routinely used to maintain plasmids in WM3064 or to select for transconjugants.

Table 1
Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype	Source or refs.
T. xiamenensis SN3 strains		
ARR073	Electrochemically enriched and subsequently isolated from coastal sediments (Catalina Island, CA), wild type.	[58]
ARR294	*ΔfdhABCD*	This study
ARR305	*ΔnapA*	This study
ARR323	ARR305 with p*napA*$^+$, Km^r	This study
E. coli strains		
WM3064	Donor strain for conjugation. *thrB1004 pro thi rpsL hsdS lacZΔM15 RP4–1360 Δ(araBAD)567 ΔdapA1341::[erm pir]*	W. Metcalf, University of Illinois, Urbana
Plasmids/vectors		
pSMV3	9.5 kb mobilizable suicide vector. *oriR6K, sacB,* Km^r	Saltikov and Newman 2003
pSMV3Δ*fdhABCD*	2 kb fusion PCR fragment containing Δ*fdh* cloned into the *Sac*I and *Bam*HI sites of pSMV3; used to make the ARR294 Δ*fdhABCD* strain	This study
pSMV3Δ*napA*	2 kb fusion PCR fragment containing Δ*napA* cloned into the *Sac*I and *Bam*HI sites of pSMV3; used to make the ARR298 Δ*napA* strain	This study
pBBR1MCS-2	5.1 kb broad-host range plasmid: Km^r, *lacZ*	[39]
p*napA*$^+$	*napA* PCR fragment cloned into the *Bam*HI and *Sac*I sites of pBBR1MCS-2	This study

2.2. Genomic DNA isolation, sequencing, and analysis

Genomic DNA for short-read Illumina sequencing was isolated from an overnight culture of SN3 grown in LBS + Ions with the DNeasy PowerSoil Kit (Qiagen) and prepared for sequencing with Illumina's Nextera XT sample prep kit. Illumina 2 × 250 paired-end sequencing was performed at the UC Davis DNA Technologies Core Facility using the Illumina HiSeq platform. Genomic DNA for long-read Nanopore minION sequencing was extracted with the Qiagen Blood and Tissue DNA extraction kit. gDNA was prepped for sequencing using the Oxford Nanopore Ligation Sequencing Kit 1D combined with the Native Barcoding Kit 1D. Sequencing was performed on a SpotON flow cell Mk 1 (FLO-MIN106R9) with a minION device. Base calls were made in real time using Guppy v. 3.0.6.

Long reads were assembled with Flye v. 2.7 [38]. Illumina reads were aligned to the genome using Burrows-Wheeler aligner [43] and used to polish the long-read assembly with Pilon v. 1.23 [71] with option –fix all. Three rounds of polishing were carried out. Assembly quality was assessed with CheckM v. 1.0.18 [55]. The genome was annotated using the NCBI Prokaryotic Genome Annotation Pipeline (PGAP) v. 6.5 [67]. Metabolic pathways were identified using the Kyoto Encyclopedia of Genes and Genomes' BlastKOALA functional characterization tool [31]. SN3's genome was taxonomically classified with the Genome Taxonomy Database Toolkit (GTDB-Tk) v1.7.0 [6]. The genome was queried for

carbohydrate-active enzymes (CAZymes) [45] with dbCAN3 automated CAZyme annotation server [74,76,79]. Protein-encoding genes annotated as CAZymes by a minimum of two independent methods (pHMMER, DIAMOND, or Hotpep) were considered positive hits. Protein localization predictions were made with PSORTb v3.0.3 [75]. Proteins encoding putative *c*-type cytochrome heme-binding motifs ($CX_{2\text{-}4}CH$) were identified with Prosite [25]. FeGenie v1.0 [18] was used to identify potential iron cycling genes. Finally, *cyc2* homologs previously used to construct a comprehensive Cyc2 phylogenetic tree (1593 sequences total, [34]) were queried against the SN3 genome using NCBI's Protein BLAST [2,3].

2.3. Gene deletion and complementation mutant generation

Two mutant strains of SN3 were constructed (ARR294 and ARR305, Table 1), each containing an in-frame deletion of *fdhABCD* or *napA*, respectively. For each deletion vector, 1 kb regions flanking the target gene(s) were PCR amplified using primers listed in Table S1 and NEB Q5 high-fidelity DNA polymerase. PCR amplicons were size selected and gel extracted using the NEB Monarch gel extraction kit. pSMV3 vector was isolated from an overnight culture of *E. coli* WM3064 using the NEB Monarch plasmid miniprep kit, digested with *Sac*I and *Bam*HI, and size selected and gel extracted using the NEB Monarch gel extraction kit. PCR fragments and digested pSMV3 were quantified with a Qubit fluorometer and 1× dsDNA HS assay kit (Invitrogen) and assembled using NEBuilder HiFi DNA Assembly kit. Assembled DNA was processed through the NEB Monarch gel extraction kit directly to remove inhibitors and reaction components. Approximately 10 ng of the clean assembly was then transformed into *E. coli* WM3064 via electroporation (1 mm cuvette gap, 2.1 kV). Transformants were verified for correct assembly by PCR with M13 primers. Verified plasmids were transferred to SN3 by conjugation (1:1 donor to recipient ratio, 16 h mating on an LBS + Ions + DAP plate, 30 °C). SN3 transconjugants containing the integrated mutagenesis vector were selected for on LBS + Ions agar plates supplemented with 50 mg/L kanamycin. Transconjugants were then streaked onto SWB plates containing reduced NaCl (0.5 % NaCl versus 2 % in standard SWB), 5 mM acetate, and 10 % *w/v* sucrose and grown at 22 °C for 24–48 h. Colonies were then replica plated onto LBS + Ions agar and LBS + Ions + kanamycin agar. Kanamycin-sensitive colonies were then screened by PCR for the deletion of *fdhABCD* or *napA*.

Plasmid p*napA*$^{+}$ was constructed to complement the SN3 Δ*napA* mutant. PCR was used to generate *napA* gene fragments flanked with *Bam*HI and *Sac*I sites on the 5′ and 3′ ends, respectively. Broad host complementation vector pBBR1MCS-2 [39] was isolated from an overnight culture of *E. coli* WM3064 with the NEB Monarch plasmid miniprep kit. PCR products and vector were restriction digested with *Bam*HI and *Sac*I and subsequently cleaned and concentrated using the NEB Monarch gel extraction kit. Gene fragments were then ligated into the digested vector and cleaned and transformed into *E. coli* WM3064 as described above. Successful assemblies were verified by PCR using M13 primers. The verified complementation vector was conjugated into the SN3 Δ*napA* strain and transconjugants were selected by plating onto LBS + Ions + kanamycin agar lacking diaminopimelic acid.

2.4. Bioelectrochemical measurements

Bioelectrochemical experiments were conducted in standard three-electrode bioelectrochemical cells as described previously [58]. An indium-tin oxide coated glass electrode (Delta Technologies), with a 10.68 cm^2 surface area was used as the working electrode, platinum wire (Sigma-Aldrich) was used as the counter electrode, and Ag/AgCl (1 M KCl) (CH Instruments) was used as the reference electrode. The reactors were inoculated with 20 mL of SWB cathode media (SWB lacking organic carbon, vitamins, or minerals). For anaerobic experiments, the electrolyte contained either 10 mM KNO_3 or 28 mM Na_2SO_4 as terminal electron acceptors and reactors were constantly bubbled with 0.2 μm-filtered ultrapure N_2 gas. For aerobic experiments, oxygen served as the terminal electron acceptor and reactors were bubbled with 0.2 μm-filtered room air. Chronoamperometry (CA) and cyclic voltammetry (CV) analyses were conducted with either a 16-channel potentiostat (Biologic) or 4-channel Squidstat Prime potentiostat (Admiral Instruments). For CA studies, the working electrode was poised at an electron-donating potential of −278 mV vs. SHE to act as the electron donor for cells. For CV experiments, the working potential was cycled between −378 and 422 mV vs. SHE three times at a scan rate of 1 mV/s for the Biologic potentiostat or 2.4 mV/s for the Squidstat Prime potentiostat. Current consumption was monitored for 24 h following cell inoculation. CVs were recorded following this period of CA. Only the third cycle is plotted for all CVs.

For anaerobic experiments, bioelectrochemical cells were inoculated with SN3 strains grown anaerobically overnight in SWB + acetate + NO_3^-. Cells were pelleted via centrifugation (8000 xg, 5 min) and resuspended in anaerobic SWB cathode medium to a final OD_{600} of 0.2. Cells were inoculated into bioelectrochemical cells once a stable current was observed. For aerobic experiments, cells were grown aerobically overnight in SWB + acetate medium. Regardless of condition, The SN3 Δ*napA* strain was grown aerobically as anaerobic growth with nitrate was not possible. Kanamycin (50 mg/L) was added to the catholyte for experiments utilizing the *napA*$^{+}$ complement strain.

To discern biological electron uptake processes from abiotic processes, we performed a kill experiment. Following the CV and once current had stabilized, sodium cyanide (10 mM final concentration) was added to both abiotic control reactors and biological replicates. Current consumption was monitored until a stable current was observed and a final CV was recorded.

For media exchange experiments to discern the role of electrode-attached cells and cell-containing spent media in electron uptake, the bioelectrochemical cells were transferred to an anaerobic chamber to minimize the influence of oxygen. Spent media was removed from bioelectrochemical cells with a sterile syringe and transferred to a sterile bioelectrochemical cell. Fresh anaerobic SWB cathode media was then added to the electrode-attached biofilms. Bioelectrochemical cells were removed from the anaerobic chamber, connected to the potentiostat, and bubbled with N_2 to maintain anaerobicity. A CV was immediately recorded upon reconnection to the potentiostat.

2.5. Anion analysis by ion chromatography

Fluid samples from wild type and sterile abiotic control bioelectrochemical cells were collected to determine nitrate and/or sulfate concentrations via ion chromatography. Approximately 1 mL of fluid from triplicate reactors was collected at inoculation ($t = 0$ h) and after 24 h of sustained chronoamperometry at −278 mV vs. SHE, filtered through 0.2 μm polyethersulfone filters, and stored at 4 °C until analysis. Samples were diluted 1:5 in MilliQ water and subsequently analyzed with a Dionex Aquion Ion Chromatograph (Thermo Fisher Scientific).

2.6. Microscopy

Electrode-attached biofilms and sterile abiotic electrodes were visualized using fluorescence microscopy. Working electrodes were removed from bioelectrochemical cells and incubated with 5 μg/mL of FM 4-64FX membrane stain (Molecular Probes) for 1 min. The samples were then imaged on a Nikon ECLIPSE TI-E inverted microscope using a TRITC excitation/emission channel (Nikon filter set G-2E/C) under 1000× total magnification with a 500 ms exposure. To ensure images were captured at the appropriate focal plane (electrode surface) of the abiotic electrode, a mark was made to the electrode surface and used as a landmark during focusing. Abiotic and biotic electrode images were false colored using the same red scale lookup table (LUT).

2.7. Data availability

The raw genome sequencing reads (Illumina and Nanopore) have been deposited in the NCBI Sequence Read Archive (SRR23873169 and SRR23873164, respectively). The genome assembly has been deposited in Genbank (CP121124.1).

3. Results and discussion

3.1. General features of the genome and metabolic potential

To gain insight into SN3's genomic potential for extracellular electron uptake, we sequenced and analyzed the genome (see Supplemental Text 1 for complete analysis of the genome). Assembly statistics and genome features can be found in Tables S2 and S3, respectively. Taxonomic assignment was made with GTDB, which identified SN3 as a strain of *Thalassospira xiamenensis* (97.52 % ANI, 0.91 alignment fraction, accession no. GCF_000300235.2), of which type strain M-5 was originally isolated from surface seawater of a waste oil pool from near Xiamen, China [42,44]. SN3 grows heterotrophically in complex media (LBS + Ions) or in defined media (SWB + acetate) *in vitro* with either oxygen or nitrate as the terminal electron acceptor but does not grow with sulfate as the sole electron acceptor. Metabolic pathways reconstructed from the genome sequence corroborate observations that this organism grows heterotrophically under aerobic or nitrate-respiring conditions and is incapable of sulfate reduction or autotrophic growth (Schematic 1). Although the organism was initially isolated on artificial seawater agar plates containing elemental sulfur as the electron donor, growth of SN3 was not observed in liquid media containing elemental sulfur as the sole electron donor, suggesting that residual organic carbon or agar in the plate media served as the electron donor for growth, as has been demonstrated in other species of *Thalassospira* [17]. Genes involved in assimilatory sulfate reduction were annotated, but sulfur oxidation and dissimilatory sulfate reduction genes were absent.

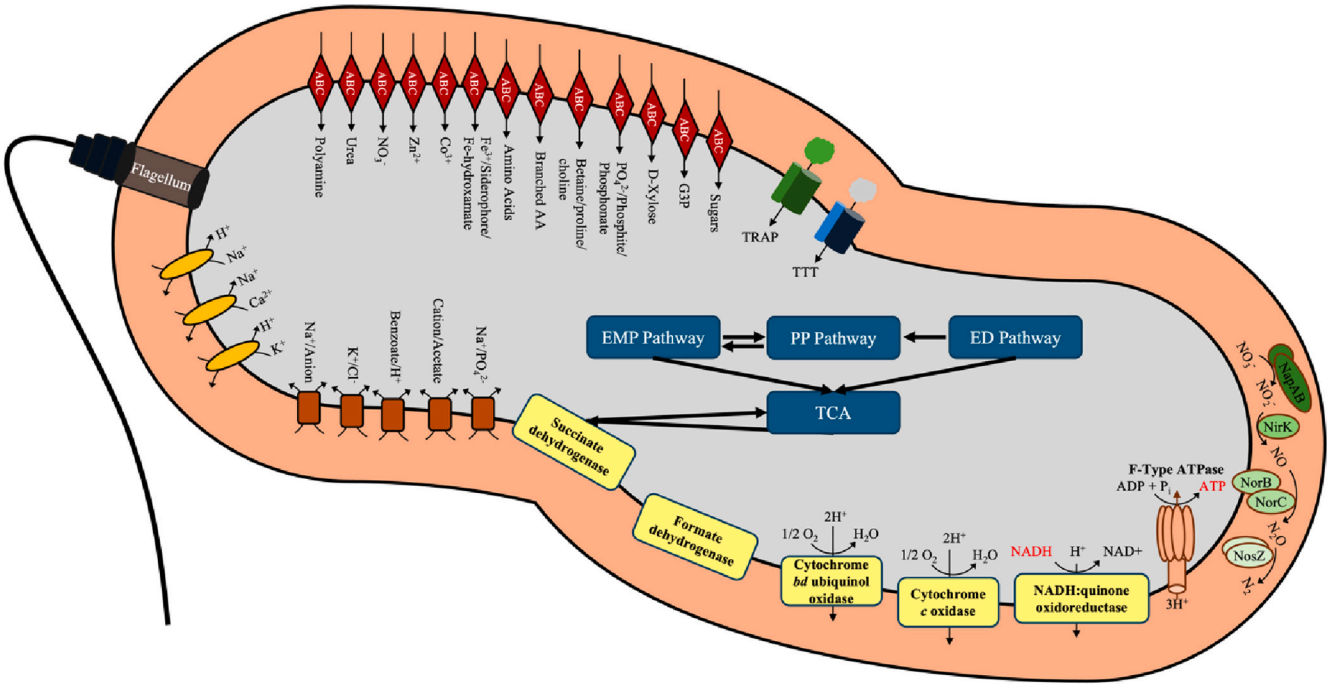
3.2. Genome lacks canonical extracellular electron transfer genes but encodes a formate dehydrogenase

As previous investigations report putative iron oxidation [17] and iron respiration [7,8] in *Thalassospira* species, we used FeGenie to search the genome for genes potentially involved in iron cycling and extracellular electron transfer to/from iron-containing minerals [18]. FeGenie identified 43 genes potentially involved in iron gene regulation and four genes involved in iron storage but failed to identify any genes involved in iron respiration or iron oxidation (Table S4). Based on our KEGG and FeGenie analyses, SN3 lacks the genomic potential for lithotrophic growth, iron respiration, and homologs to canonical EET genes.

In other cathodic systems, extracellular enzymes have been shown to produce background electrochemical signals in bioelectrical systems that are similar to those observed in organisms that engage in direct contact EEU [11]. SN3's genome does not encode any hydrogenases but encodes an NAD^+-dependent formate dehydrogenase *fdhABCD*, which can be tested for an EEU phenotype.

3.3. Putative redox-active proteins in the cell envelope

In model organisms, interfacial EET is facilitated by multi-heme *c*-type cytochromes that physically link the electron transport chain with the extracellular environment [53,54]. As the genome of SN3 lacks homologs to known EET pathways, we sought to identify gene products that are predicted to localize to the cellular envelope that contain putative heme-binding motifs characteristic of *c*-type cytochromes ($CX_{2-4}CH$ motifs, Table S3). Of the 48 heme-binding-motif-containing proteins, ten were predicted to localize to the periplasm, nine to the cell membrane, and 13 had unknown localization predictions. Most of these proteins were predicted to contain a single heme-binding motif. The maximum number of heme-binding motifs found in a single protein was four in a cytochrome *c*3 family protein predicted to localize to the cytoplasmic membrane. Gene product localization predictions were made by PSORTb 3.0.3 [75] and were based solely on amino acid sequence homology and/or the presence/absence of known features indicative of localization (e.g. signal peptides). While some putative *c*-type cytochromes are predicted to localize to the cytoplasm, these are



Schematic 1. Genome-resolved metabolic model for *Thalassospira xiamenensis* SN3. See Supplemental Text S1 and Table S3 for additional details.

predictions, as are the putative *c*-type heme binding motifs identified in these proteins. In Gram-negative bacteria, *c*-type heme maturation occurs in the periplasmic space and *c*-type cytochromes function predominantly in the periplasmic space (reviewed in [40,52]). As such, heme binding and localization would need to be experimentally validated for these putative redox-active proteins.

Although the importance of multi-heme cytochromes in facilitating extracellular electron transfer in model organisms is well established (reviewed in ([4]; Paquete, Morgado, et al. 2022)), attention must also be given to monoheme cytochromes. For example, Strycharz et al. [66] have demonstrated that the monoheme cytochrome PccH is required for electron uptake *by Geobacter sulfurreducens*. Additionally, Keffer et al. [34] have evidence that suggests Cyc2 – a putative monoheme outer membrane cytochrome in *Mariprofundus ferrooxydans* PV-1 – functions as an iron oxidase, facilitating extracellular iron(II) oxidation. While FeGenie failed to identify homologs to Cyc2, given the broad diversity of Cyc2 sequences among the Bacteria, we searched SN3's genome for homologs to all 1593 unaligned sequences used to construct the Cyc2 phylogenetic tree ([34], query sequences available at https://doi.org/10.1128/mbio.01074-21). Protein BLAST failed to identify any putative Cyc2 homologs in the SN3 genome. Based on the gene sequences alone, it is unclear which of these putative heme-binding-motif-containing proteins may facilitate EET. Further investigation into the potential role of these proteins in EEU – both multiheme and monoheme – is warranted and will provide valuable information on the diversity of extracellular electron transfer mechanisms employed by microorganisms.

3.4. Electron uptake is biologically mediated

We then sought to characterize the extracellular electron uptake phenotype previously observed in SN3 [58] using amperometric and voltametric methods. We observed consistent and modest increases in cathodic current upon inoculation of *Thalassospira xiamenensis* SN3 into anaerobic bioelectrochemical cells comprised of indium-tin oxide-coated glass working electrodes poised at −278 mV vs. SHE with nitrate as the sole electron acceptor (Fig. 1A, Fig. S1). Despite the fact that SN3's genome lacks hydrogenases and cannot oxidize hydrogen as an electron source, the applied potential of the working electrode was chosen to be well above that necessary for electrochemical H_2 evolution in seawater [5,58]. To confirm this observed current consumption was due to biological activity, bioelectrochemical cells were treated with 10 mM sodium cyanide, a broad respiration inhibitor. Introduction of cyanide resulted in an immediate and drastic reduction in cathodic current in biological replicates, but no change was observed in abiotic controls (Fig. 1A). The observed reduction in current, down to background current levels observed in abiotic controls, confirms that electron uptake is a function of biological activity.

3.5. Direct electrode contact is required for catalysis

To gain insight into the mechanism(s) of extracellular electron uptake by *Thalassospira xiamenensis* SN3 from a poised electrode, cyclic voltammetry (CV) experiments were performed 24 h following inoculation (Fig. 1B). Under turnover conditions with nitrate as the electron acceptor, a reversible catalytic wave was observed with an onset potential of 88 ± 4 mV vs. SHE and midpoint potential of −153 ± 16 mV vs. SHE (calculated from first derivative analysis of cathodic and anodic scans, Figs. S2 A-C). This catalytic wave is absent from sterile media abiotic controls (Fig. 1B). These electrochemical characteristics were consistent across biological replicates (Figs. S2B, S2C). The catalytic wave reflects sustained electron transfer from the electrode that is quenched with the addition of 10 mM sodium cyanide (Fig. 1B).

To assess whether extracellular electron uptake requires direct contact with the electrode or whether electron uptake is facilitated by soluble mediators, anaerobic turnover CV experiments were performed on

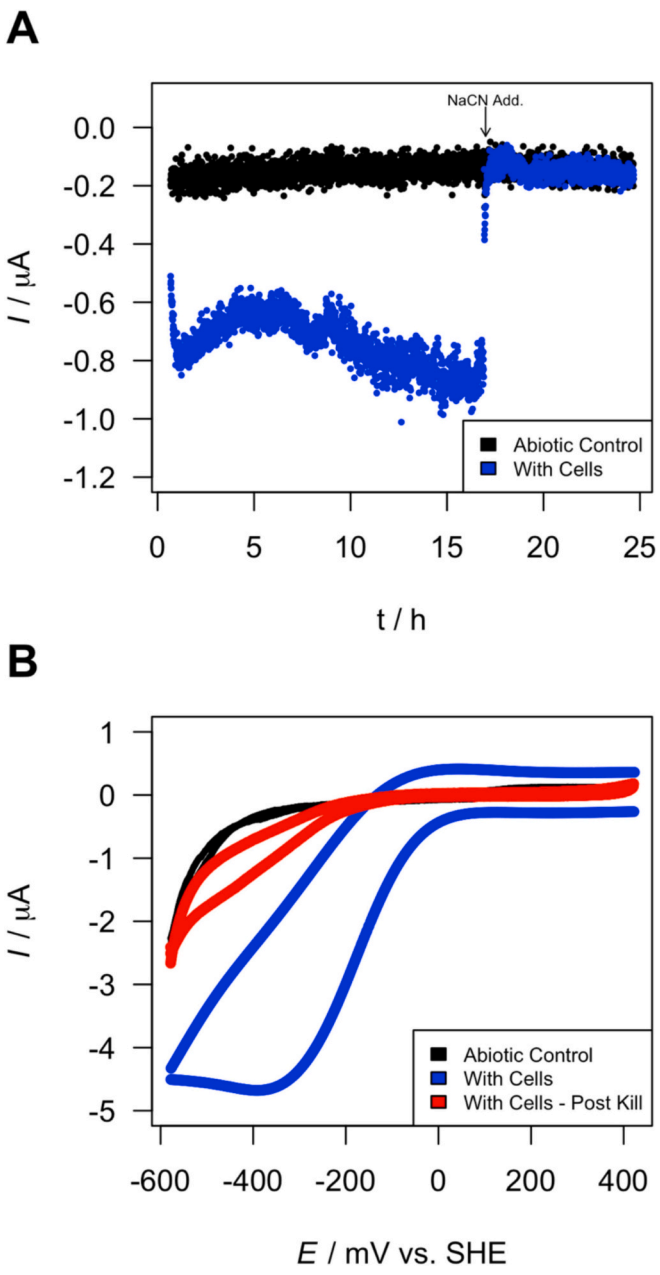


Fig. 1. Cathodic activity of *Thalassospira xiamenensis* SN3 under anaerobic turnover (nitrate reducing) conditions. A) Representative chronoamperometry measurement shows an increase in cathodic current in the condition with wild type cells (blue, $n = 6$) in bioelectrochemical cells containing a working electrode poised at −278 mV vs. SHE. Current consumption was abolished upon addition of 10 mM sodium cyanide (NaCN), indicated by the arrow. No activity was detected in the uninoculated, sterile abiotic control (black). B) Representative turnover cyclic voltammetry reveals a catalytic wave indicative of biologically mediated cathode oxidation (blue, n = 6). Upon treatment with NaCN (red, $n = 3$), catalysis was abolished close to sterile abiotic control levels (black). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

electrode-attached biofilms and on spent media containing planktonic cells following a media exchange in the bioelectrochemical system. Upon media exchange and removal of all planktonic cells and potential soluble mediators, the catalytic waveform associated with the electrode-attached biomass was largely retained although a modest loss in peak cathodic current was observed compared to the CV measured prior to media exchange (Fig. 2A). This reduction in current may be due to cell

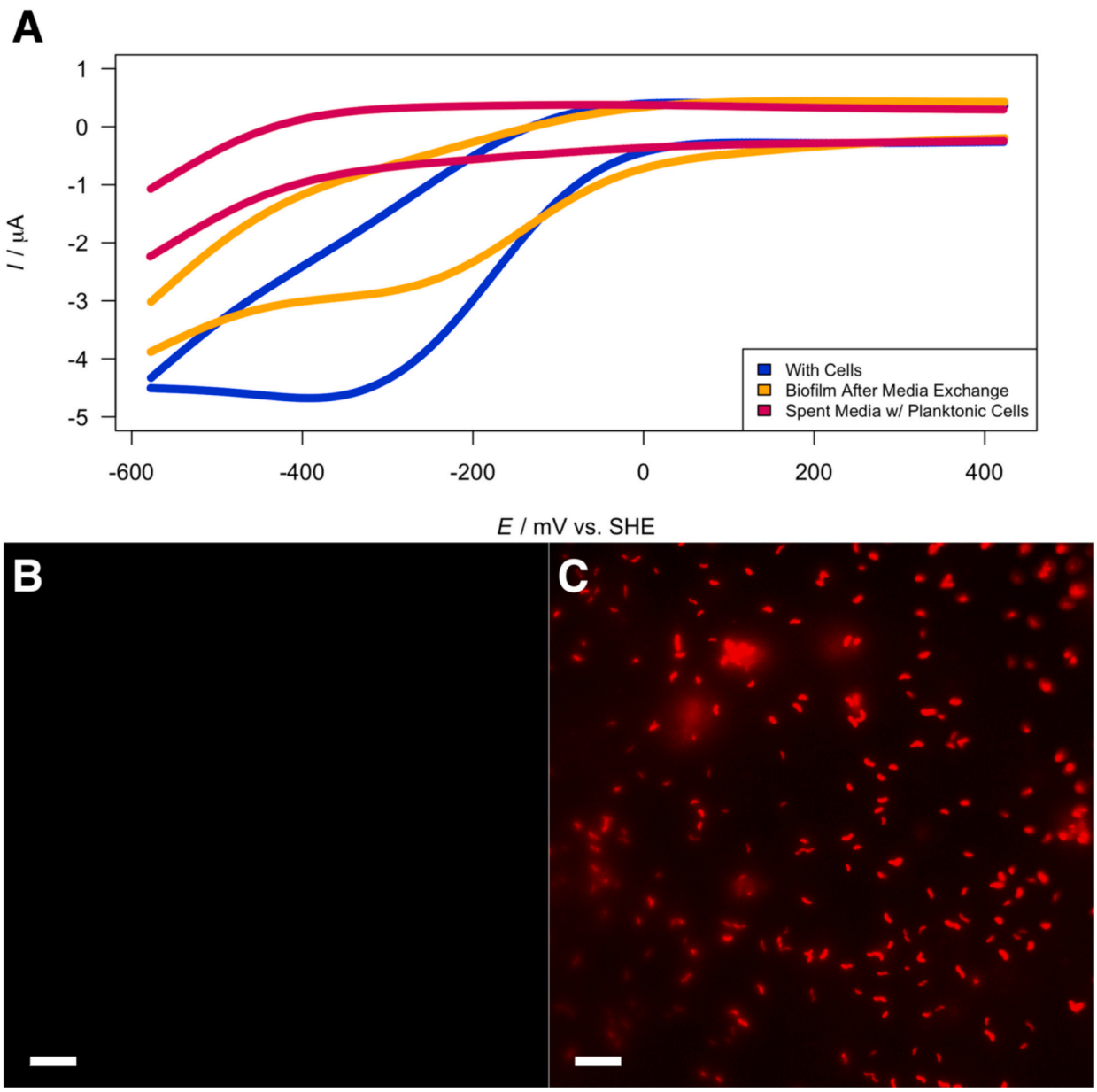


Fig. 2. Catalysis by *Thalassospira xiamenensis* SN3 requires direct cell-electrode contact. A) Representative turnover CVs (nitrate reducing) reveal a catalytic wave indicative of biologically mediated electron uptake (blue, n = 6). This catalytic wave is retained after exchanging spent media with fresh media (orange, n = 3). CVs of spent media containing planktonic cells show no discernable redox features (pink, n = 3). B) Fluorescent microscopy image of FM 4-64FX-stained sterile abiotic ITO-coated glass electrode following media exchange (scale bar = 10 µm). Average pixel fluorescence intensity across the micrograph was 512 ± 17 a.u. C) Fluorescent microscopy image of FM 4-64FX-stained cells attached to an ITO-coated glass electrode following media exchange (scale bar = 10 µm). Average pixel fluorescence intensity in dark regions lacking cells was 729 ± 29 a.u. Average pixel fluorescence intensity of cells was 2566 ± 283 a.u. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

detachment from the electrode surface upon media exchange, as has been postulated for a similar phenomenon observed in other cathode-oxidizing organisms [32].

We then investigated whether planktonic cells and/or soluble mediators were contributing to catalysis. Spent media containing planktonic cells was injected into sterile anaerobic bioelectrochemical cells containing new indium-tin oxide-coated glass working electrodes. Cyclic voltammetry experiments conducted immediately upon injection of spent media to reduce the possibility of cell attachment exhibited a loss in catalytic activity compared to electrode-attached biomass both prior to and after media exchange (Fig. 2A). As these data indicate that extracellular electron uptake requires contact with the electrode surface, we imaged the sterile abiotic control electrode and biofilm-attached working electrodes using fluorescence microscopy and the membrane stain FM 4-64FX following media exchange. Cells were absent in micrographs taken of sterile abiotic electrodes (Fig. 2B). However, micrographs revealed a sparse monolayer of attached cells in biological replicates (Fig. 2C), similar to what has been observed in another *Thalassospira* species on glassy carbon electrodes [73]. The absence of catalytic activity in spent media containing planktonic cells and the retention of catalytic activity in the electrode-attached biofilm upon media exchange indicates that direct electrode-cell or electrode-enzyme contact is required for extracellular electron transfer and catalysis.

3.6. Electron uptake in SN3 requires a terminal electron acceptor (respiration)

To determine whether extracellular electron uptake by SN3 is linked with reduction of a terminal electron acceptor (respiration), we performed chronoamperometry and cyclic voltammetry of wild type SN3 cells under anaerobic conditions with 10 mM nitrate or with 28 mM sulfate (seawater sulfate concentration) as sole electron acceptors (Fig. 3). As SN3 cannot respire sulfate, the sulfate condition is effectively a nonturnover condition. SN3 cells were grown anaerobically in SWB + 5 mM acetate +10 mM nitrate, separated from the growth medium via centrifugation, and inoculated into anaerobic bioelectrochemical cells containing either 10 mM nitrate or 28 mM sulfate. Current consumption was monitored for approximately 24 h at −278 mV vs. SHE (Fig. 3A). Concurrently, samples for anion analysis (specifically nitrate and sulfate) were collected at inoculation ($t = 0$ h) and at 24 h.

Current consumption in the experimental condition containing sulfate with wild type cells was slightly lower than the abiotic control and no loss in sulfate concentration was observed, consistent with prior observations that SN3 cannot respire sulfate (Fig. 3A). In the nitrate reducing condition, current consumption was modest but increased with time and was distinguishable from the abiotic control. After 24 h, nitrate concentrations dropped by 1.9 ± 0.4 mM, indicative of nitrate respiration by SN3. Nitrate concentrations remained unchanged in the abiotic controls throughout the duration of the experiment. Abiotic controls do not serve as a useful baseline measurement for current comparisons with biologic systems, especially in marine fluids that have high non-Faradaic currents due to high salt concentrations. In this work we use abiotic controls exclusively for comparisons of catalytic activity between abiotic and biotic reactors in cyclic voltammetry. The background current in abiotic controls is often larger than in biotic systems, even in highly electrochemically active microorganisms like *Shewanella oneidensis* MR-1, because the effective surface area for background charge transfer interactions (both Faradaic and non-Faradaic) is much larger in abiotic controls (i.e., a blank electrode). Consequently, we use killed controls, which more appropriately estimate the baseline current, as we have shown in our previous work [32,59,70]. However, abiotic controls are indispensable in cyclic voltammetry for establishing baseline catalysis in the absence of added cells or catalytic agents.

CV analysis was performed 24 h post inoculation (Fig. 3B). CVs for the sulfate condition replicates were similar to abiotic and demonstrated a lack of catalysis; however, under nitrate-reducing conditions, the sigmoidal catalytic waveform typical of SN3 cells was observed. We then treated the nitrate reducing condition bioelectrochemical cells and sterile abiotic controls with 10 mM sodium azide, a known inhibitor of some periplasmic nitrate reductases [51,65]. Introduction of azide resulted in an immediate and drastic reduction in cathodic current in nitrate-reducing biological replicates, but sterile abiotic controls were unaffected by azide addition (Fig. 3C). These data indicate that cathode oxidation by SN3 requires a biologically compatible terminal electron acceptor and that extracellular electron uptake is linked with respiration.

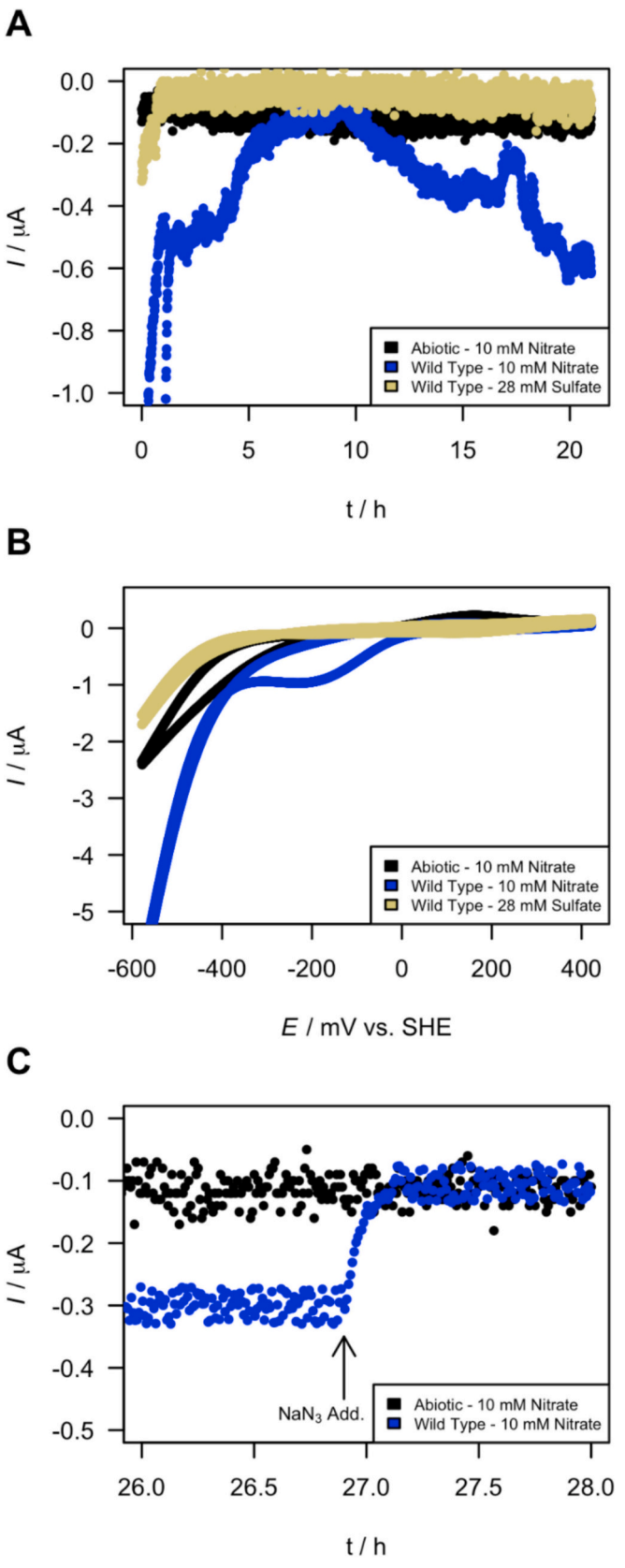
To evaluate the role of SN3's nitrate reductase in cathode oxidation, we constructed a scarless mutation of the *napA* gene. Simultaneously, we complemented the Δ*napA* mutant in *trans* to test for polar effects of deleting *napA*. NapA is the catalytic subunit of the periplasmic nitrate reductase complex NapAB. We tested the Δ*napA* mutant and *napA*$^+$ complement for nitrate reduction capacity under anaerobic heterotrophic conditions with acetate as the sole carbon source. The Δ*napA* mutant was incapable of growth and nitrate reduction (Fig. S3A), but the deletion had no effect on aerobic heterotrophic growth (Fig. S3B). Further, growth of the complementation strain *napA*$^+$ under nitrate reducing conditions was indistinguishable from wild type (Fig. S3A). We then grew the Δ*napA* mutant under aerobic heterotrophic conditions in SWB + 5 mM acetate, separated cells from spent media via centrifugation, and inoculated sterile anaerobic bioelectrochemical cells containing anaerobic cathode medium. Current consumption was monitored for approximately 24 h at −278 mV vs. SHE. Current consumption by the Δ*napA* mutant was indistinguishable from the abiotic control (see Fig. 1A or 3A for abiotic control), but the rate of electron uptake was partially rescued by complementation (Fig. 4A, see Fig. S4 for biological replicates). We then performed cyclic voltammetry to investigate the catalytic activity of the Δ*napA* mutant and *napA*$^+$ complement. The sigmoidal catalytic waveform observed in wild type SN3 was absent in the Δ*napA* mutants but was rescued upon complementation (Fig. 4B), suggesting that respiration of nitrate is required for catalysis under anaerobic conditions. We then performed the same experiment under aerobic conditions with atmospheric oxygen serving as the electron acceptor. CV analysis showed that catalytic activity of the Δ*napA* mutant was indistinguishable from wild type (Fig. S5), suggesting that electrode oxidation requires a terminal electron acceptor and is linked to the organism's respiration. Together, these data indicate that respiration is required for catalysis and that *napA* is not required for electron uptake under aerobic conditions.

These results are in contrast with previous observations in another *Thalassospira* species that cell viability was not required for catalysis and oxygen reduction [73]. The authors treated a *Thalassospira*-colonized glassy carbon electrode with glutaraldehyde – a chemical fixative that induces protein crosslinking – and performed cyclic voltammetry analysis of fixed cells. They observed a trivial decrease in catalytic activity after glutaraldehyde addition and posited that catalysis was due to excretion and sorption of enzymes, porphyrins, or quinones to the electrode and was independent of cell viability. Conversely, in strain SN3, cell viability (Figs. 1A-B), direct cell-electrode contact (Fig. 2A), and respiration (Figs. 3A-C, 4A-B, S5) are required for catalysis.

3.7. Electron uptake is not facilitated by formate dehydrogenases (*fdhABCD*)

As previous investigations into extracellular electron transfer in *Thalassospira* species have posited that EET may be facilitated by excreted enzymes or redox-active metabolites [73], we investigated the potential for extracellular formate dehydrogenases to be involved in the electrochemical phenotype we observed. In the Fe(0)-corroding methanogen *Methanococcus maripaludis*, Deutzmann et al. [11] discovered that free redox-active enzymes, such as hydrogenases or formate dehydrogenases, were adhering to carbon electrode surfaces and converting electrons to either hydrogen or formate, respectively. *M. maripaludis* was rapidly consuming the products of these reactions, which resulted in methane formation that tracked with electrochemical activity (electrochemically, the results were like those observed in direct electron transfer processes) [11]. Our previous work has shown that this enzyme-mediated electrochemical response can be distinguished from direct EEU by perturbing the intermediates and looking for electrochemical responses [32,59].

As described above, the genome of SN3 does not encode any hydrogenases but encodes an NAD^+-dependent formate dehydrogenase (*fdhABCD*). To test whether this NAD^+-dependent formate dehydrogenase was responsible for the observed bioelectrochemical activity, we constructed a deletion mutant of the entire formate dehydrogenase complex, denoted Δ*fdhABCD*. The Δ*fdhABCD* mutant was grown anaerobically and cells were used to inoculate anaerobic bioelectrochemical cells. We performed amperometric and CV analyses on wild type and Δ*fdhABCD* cells (Fig. 5A-B). Current consumption was indistinguishable from wild type cells under anaerobic turnover conditions (Fig. 5A). Catalytic activity was retained in the Δ*fdhABCD* mutant (Fig. 5B), indicating that formate dehydrogenase is not involved in extracellular electron uptake by SN3, in that this deletion mutant should neither be able to support the excretion of extracellular enzymes to generate formate on the electrode surface, nor consume any electrochemically generated formate. This further supports direct electron transfer interactions between SN3 cells and the electrode surface as the



(caption on next column)

Fig. 3. Extracellular electron uptake by wild type *Thalassospira xiamenensis* SN3 requires a terminal electron acceptor. A) Representative turnover chronoamperometry measurement showing current consumption when cells were provided with 10 mM nitrate (blue, n = 6) as a terminal electron acceptor. When cells were provided with sulfate as the sole electron acceptor (tan, n = 3), current consumption was indistinguishable from sterile abiotic controls (black, n = 3). B) Representative CVs (with 10 mM nitrate or 28 mM sulfate) reveal catalysis requires a biologically compatible terminal electron acceptor. C) Current consumption by SN3 under turnover (nitrate reducing) conditions was abolished upon addition of 10 mM sodium azide (NaN_3), a nitrate reductase inhibitor, indicated by the arrow. No activity was detected in the uninoculated, sterile abiotic control (black, n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

predominant mode of EEU.

3.8. Implications of weak electrotrophy

We have demonstrated that *Thalassospira xiamenensis* SN3 is a weak electrotroph that participates in interfacial extracellular electron uptake linked with respiration but not growth, as this strain lacks the capacity for carbon fixation in its genome and was not observed to grow in the absence of an organic carbon source. However, the capacity for an organoheterotroph to engage in EEU, which can serve as an additional electron donor for respiration, is not unprecedented in heterotrophic bacteria. Rowe et al. [58] electrochemically enriched and subsequently isolated a collection of electrode-oxidizing bacteria, including heterotrophic bacteria with weakly electrotrophic phenotypes. Two *Idiomarina* strains isolated as part of that work – obligate organoheterotrophs that grow primarily on amino acids – have been shown to couple interfacial cathode oxidation to aerobic respiration with modest current densities via an unknown mechanism [70]. Even in the model organism *Shewanella oneidensis* MR-1, EEU is not linked with growth [59]. For all these electrotrophic organisms that directly oxidize cathodes but cannot couple this process to a growth phenotype, the physiological and ecological implications of EEU remain speculative. At present, the detection and measurement of EEU in the environment is challenging and is dependent on cultivation and confirmation of these processes *in vitro*. This has limited our ability to investigate the ecologic role of these processes *in vivo* and to interrogate their potential impacts on microbial communities – natural, engineered, or otherwise.

In weakly electrogenic microorganisms – those organisms that utilize solid-phase redox-active substrates located outside the cell as a terminal electron acceptor – it has been postulated that extracellular electron transfer may function as a survival strategy when soluble electron acceptors in the local environment are depleted [1,14,23]. For electrogens, this shift in metabolic strategy would allow for carbon metabolism to continue and for growth to occur, albeit probably with low efficiencies and/or at low rates. These organisms are likely metabolic generalists that occupy a wide variety of ecological niches [14,36]. In EEU-capable organoheterotrophs like SN3, which cannot couple EEU with growth, the benefit of this strategy is less clear. These organisms may oxidize solid-phase extracellular electron donors when soluble electron donors, such as reduced carbon substrates, are exhausted. In energy-limited environments, this would presumably allow for the generation of reducing equivalents and maintenance of proton motive force, potentially allowing the organism to persist during periods of famine [14], as has been postulated for the role of EEU in *Shewanella* [59].

In mixed microbial communities or polymicrobial biofilms where electroactive microorganisms are often found, the benefit of weak electrotrophy is more evident. Mixed species biocathodes are often more stable, perform better than pure cultures in bioelectrochemical systems, and are more adept at responding to environmental perturbations, such as pH changes or other fluctuations in chemistry [12,14,24,48,57,58,72]. This stability and resiliency is likely due to

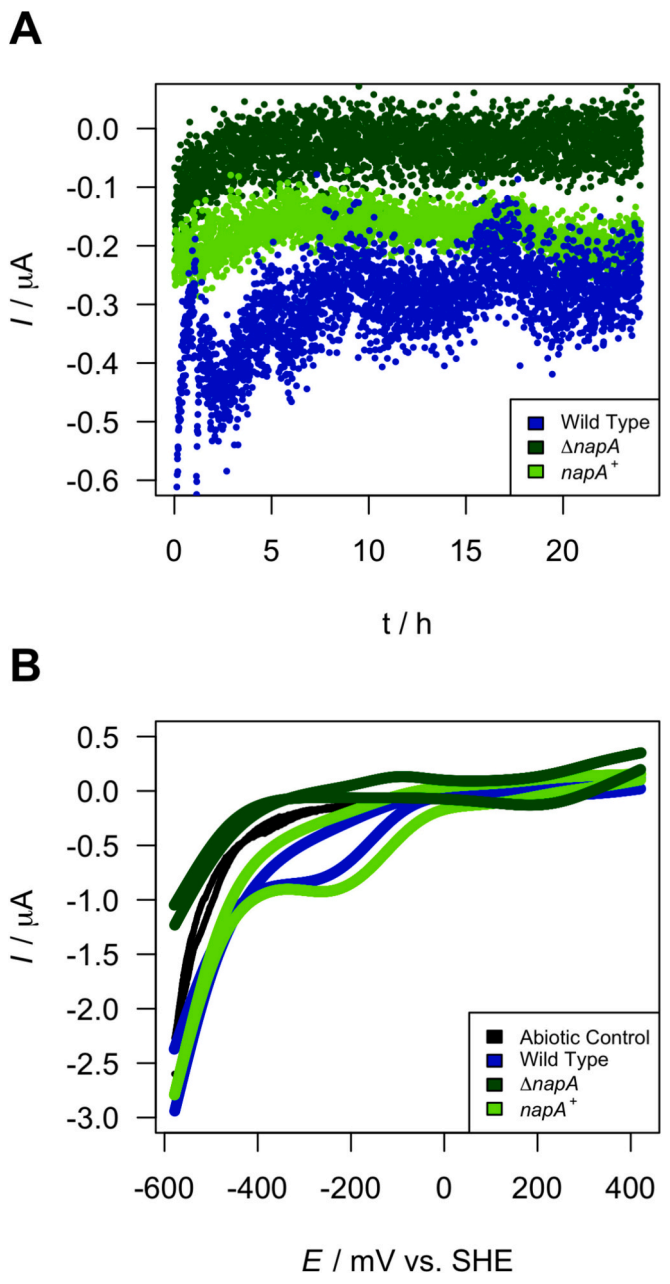


Fig. 4. Deletion of nitrate reductase subunit *napA* abolishes electron uptake and catalysis under anaerobic turnover (nitrate reducing) conditions. A) Representative chronoamperometry measurement shows a lack of cathodic current consumption by Δ*napA* cells (dark green, n = 3) in bioelectrochemical cells containing a working electrode poised at −278 mV vs. SHE. Current consumption was partially rescued upon complementation (light green, n = 3), approaching current consumption rates measured for wild type (blue, n = 6). Abiotic CA data has been omitted from this figure for clarity. B) Representative turnover CVs reveal a catalytic wave indicative of biologically mediated electron uptake in wild type cells (blue, n = 6). Catalytic activity was absent in reactors containing the Δ*napA* deletion strain (dark green, n = 3). Catalysis was rescued upon complementation (light green, n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

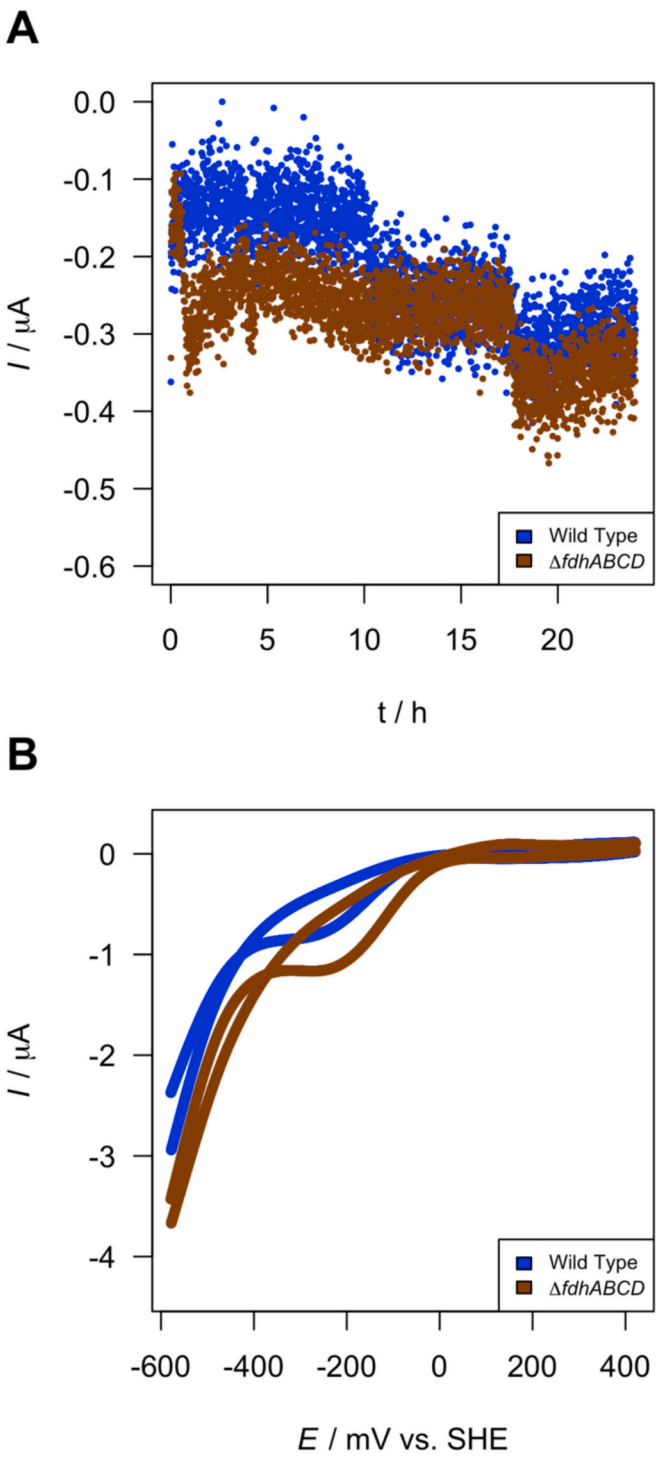


Fig. 5. Deletion of formate dehydrogenase *fdhABCD* has no effect on electron uptake or catalysis under anaerobic turnover (nitrate reducing) conditions. A) Representative chronoamperometry measurement shows similar cathodic current consumption by Δ*fdhABCD* cells (brown, n = 3) and wild type cells (blue, n = 6) in bioelectrochemical cells containing a working electrode poised at −278 mV vs. SHE. B) Representative turnover CVs reveal a catalytic wave indicative of biologically mediated electron uptake in wild type cells (blue, n = 6). Catalytic activity was unaffected in reactors containing the Δ*fdhABCD* deletion strain (brown, n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

sharing of functional tasks (e.g., biofilm formation), metabolites, and/or electrons between organisms, and the presence of diverse metabolic capabilities among community members. In these communities, even weak electrotrophs may contribute directly or indirectly to detoxification of reactive oxygen species, transduction of electrons throughout a

biofilm or via interspecies electron transfer, or potentially act as agents for carbon fixing or bioelectrosynthesis reactions.

A growing body of literature suggests that we are just scratching the surface in terms of the diversity of EEU-capable microorganisms and the EEU mechanisms they employ [5,15,24,50,58,61,70]. Developing a comprehensive understanding of the physiology and mechanisms of EEU, in both strongly and weakly electrotrophic organisms, is a worthy endeavor that will inform the physiologic role of these processes, the ecology of these EEU-capable organisms, and will provide insight into potential bioengineering applications as biocatalysts in energy capture, microbial electrosynthesis, and/or bioremediation. Furthermore, this knowledge will be critical for synthetic biology approaches to improving the efficiency of EEU for industrial applications and engineering synthetic microbial strains and communities for electrosynthesis.

4. Conclusions

We employed amperometric, voltametric, genetic, and genomic investigations to gain insight into the extracellular electron uptake phenotype of *Thalassospira xiamenensis* strain SN3, a Gram-negative bacterium originally isolated from coastal marine sediments. Our results demonstrate that 1) this organism oxidizes a cathode poised at an electron-donating potential, 2) cell viability is required for catalysis, 3) cathode oxidation is interfacial, 4) cathode oxidation requires a biologically compatible terminal electron acceptor (respiration), and 5) EEU is not facilitated by formate dehydrogenase. Although SN3 is a weak electrotroph, this organism consistently performs electron uptake that is uncoupled from growth, which may allow for ATP generation and persistence when environmental conditions become austere: obtaining electrons either from the oxidation of reduced mineral substrates or via conduction of electrons through lithotrophic polymicrobial biofilms. While the ecological and physiological role of oxidative EET in heterotrophs is just beginning to be explored [58,70], this investigation provides new insight into the ecology of a ubiquitous cathode-oxidizing marine heterotroph. As the proteins involved in the extracellular electron uptake conduit remain unknown, future work will focus on disentangling the EET mechanism and improving our understanding of this organism's potential contribution to biocorrosion.

CRediT authorship contribution statement

Joshua D. Sackett: Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization. **Gabriel P. Tonucci:** Writing – review & editing, Investigation. **Annette R. Rowe:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bioelechem.2025.108976.

Data availability

Data will be made available on request.

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