NapB in excess inhibits growth of *Shewanella oneidensis* by dissipating electrons of the quinol pool

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*Shewanella*, a group of ubiquitous bacteria renowned for respiratory versatility, thrive in environments where various electron acceptors (EAs) of different chemical and physiological characteristics coexist. Despite being extensively studied, we still know surprisingly little about strategies by which multiple EAs and their interaction define ecophysiology of these bacteria. Previously, we showed that nitrite inhibits growth of the genus representative *Shewanella oneidensis* on fumarate and presumably some other CymA (quinol dehydrogenase)-dependent EAs by reducing cAMP production, which in turn leads to lower expression of nitrite and fumarate reductases. In this study, we demonstrated that inhibition of fumarate growth by nitrite is also attributable to overproduction of NapB, the cytochrome c subunit of nitrate reductase. Further investigations revealed that excessive NapB per se inhibits growth on all EAs tested, including oxygen. When overproduced, NapB acts as an electron shuttle to dissipate electrons of the quinol pool, likely to extracellular EAs, because the Mtr system, the major electron transport pathway for extracellular electron transport, is implicated. The study not only sheds light on mechanisms by which certain EAs, especially toxic ones, impact the bacterial ecophysiology, but also provides new insights into how electron shuttle c-type cytochromes regulate multi-branched respiratory networks.

*Shewanella*, a group of ubiquitous γ-proteobacteria, exhibit a remarkable versatility in respiration, allowing the use of a diverse array of electron acceptors (EAs), including fumarate, nitrate, nitrite, thiosulfate, trimethylamine N-oxide (TMAO), dimethylsulfoxide (DMSO), Fe(III), Mn(III) and (IV), Cr(VI), U(VI), and so forth. This feature confers the bacteria great potential in bio remediation of heavy metals and energy generation via microbial fuel cells. Moreover, because many physiological traits are either not found in or distinct from well-characterized model microorganisms such as *Escherichia coli*, the genus representative, *Shewanella oneidensis* is now emerging as a research model for general bacterial physiology. As the signature of the genus, respiratory versatility has been extensively studied and a number of respiratory pathways have been elucidated over the last two decades. However, in environments where *Shewanella* thrive it is common that multiple EAs are present, but up to date, how such a situation, especially with toxic EAs, defines ecophysiology of these bacteria remains largely elusive.

Nitrite, which is ubiquitous and highly toxic, is such an EA. To respire nitrite, *S. oneidensis* uses atypical periplasmic nitrite reduction (NRF) system, featured by the lack of the NrfH or NrfBCD complex, which in many bacteria are essential and dedicated quinol dehydrogenases to reductase NrfA. CymA, a cytoplasmic–membrane-bound cytochrome c, is recruited to complete the system in *S. oneidensis*. CymA in fact functions as an electron transport hub, connecting the quinol pool to a number of reductases, such as those for nitrate, fumarate, DMSO, and metals, either directly or via electron shuttles such as CctA and ScyA. Conceivably, there would be preferential electron acceptors for CymA. Indeed, a case has been reported. CymA favors NapB, the small subunit of periplasmic nitrate reductase (NAP) which transports electrons to NapA (the large subunit of NAP), preventing nitrate and nitrite respiration from occurring simultaneously. In the absence of NapB, CymA delivers electrons to both NapA and NrfA, enabling nitrate and nitrite to be respired at the same time.
Nitrite has been traditionally used as a preservative in meat products to inhibit growth of bacterial pathogens for centuries. The antimicrobial action of the nitrite moiety is through either interfering with protein cofactors, such as Fe-S clusters, heme, and lipoamide, or promoting the formation of reactive nitrogen species. Although nitrite has been reported to be able to directly react with the ferric heme, forming an intermediate species with ferrous-nitrogen dioxide character, it is widely accepted that the molecule exerts antimicrobial activity largely via nitric oxide (NO), which can be generated from nitrite spontaneously in highly acidic or reducing environments or enzymatically.

However, this is not the case in S. oneidensis given that nitrite is similarly effective in a NO-free environment. During aerobic growth of S. oneidensis, the primary target of nitrite is the cytochrome cbb oxidase, the enzyme complex predominately responsible for oxygen respiration. When non-oxygen EAs are used to support growth, nitrite inhibits respiration of CymA-dependent but not -independent EAs. This is achieved via repression of cyclic adenosine monophosphate (cAMP) production, a second messenger required for activation of cAMP-receptor protein (Crp). While as a global regulator Crp-cAMP mediates transcription of a large number of genes, it plays a particularly primary role in regulation of CymA-dependent respiration. In the case of fumarate, if nitrite is not promptly removed, intracellular CAMP levels drop; this impairs Crp activity, leading to substantially reduced production of both nitrite reductase and fumarate reductase FccA. In contrast, nitrite can be simultaneously respired with CymA-independent EAs, such as TMAO, resulting in enhanced biomass.

In this study, we continued our effort to unravel mechanisms underlying nitrite inhibition of non-oxygen EA respiration. By using a transposon-based random mutagenesis, we identified that the napB gene is associated with growth inhibition by nitrite. Growth inhibition is in fact due to excessive NapB induced by nitrite. Further investigations revealed that NapB in excess inhibits respiration of all EAs tested, including oxygen. This is seemingly due to that NapB dissipates electrons from the quinol pool, presumably to some extracellular EAs, resulting in an electron shortage for growth-supporting EAs. Additionally, we showed that NrfA production is linked to NapB levels, which relies on both Crp-cAMP and NarQP regulatory systems for nitrate/nitrite sensing and respiration.

Methods

Bacterial strains, plasmids and culture conditions. All bacterial strains and plasmids used in this study were listed in Table 1. Information for primers used in this study was available upon request. For genetic manipulation, E. coli and S. oneidensis were grown in Lysogeny broth (LB, Difco, Detroit, MI) under aerobic conditions at 37 and 30 °C, respectively. When appropriate, the growth medium was supplemented with chemicals at the following final concentrations: 2.6-diaminopimelic acid (DAP), 0.3 mM; ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; gentamycin, 15 μg/ml; and streptomycin, 100 μg/ml.

Growth of S. oneidensis strains under aerobic or anaerobic conditions was determined by recording the optical density of cultures at 600 nm (OD600). M1 defined medium containing 0.02% (w/v) of vitamin free Casamino Acids and 30 mM lactate as electron donor was used as previously described. For aerobic growth, mid-log cultures were inoculated into fresh medium to an OD600 of ~0.05 and shaken at 200 rpm at 30 °C. For anaerobic growth, cultures were purged with nitrogen and inoculated into fresh media prepared anaerobically to an OD600 of ~0.05. EAs used in this study included nitrite (2 mM), fumarate (20 mM), TMAO (20 mM), and goethite (10 mM).

In-frame mutant construction and complementation. In-frame deletion strains for S. oneidensis were constructed using the att-based fusion PCR method as described previously. In brief, two fragments flanking the gene of interest were amplified by PCR, which were linked by the second round of PCR. The fusion fragments were introduced into plasmid pHG01 by using Gateway BP clonase II enzyme mix (Invitrogen) according to the manufacturer’s instruction. Verified mutagenesis vectors were maintained in E. coli WM3064, which was used as the donor for subsequent conjugation, resulting in vector transfer into S. oneidensis. Integration of the mutagenesis construct into the chromosome was selected by resistance to gentamycin and confirmed by PCR. Verified transconjugants were grown in LB broth in the absence of NaCl and plated on LB supplemented with 10% sucrose. Gentamycin-sensitive and sucrose-resistant colonies were screened by PCR for deletion of the target gene. Mutants were verified by sequencing the site for intended mutation.

The majority of mutants used in this study are previously constructed and verified by genetic complementation (Table 1). For newly constructed mutants, plasmid pHG02 was used in genetic complementation of mutants. The coding sequence of the target genes was amplified and inserted into multiple cloning site of pHG02 under the control of the S. oneidensis arcA promoter, which is constitutively active. For inducible gene expression, gene of interest generated by PCR was introduced into pHEG-P tac under the control IPTG-inducible promoter. After sequencing verification, resulting vectors were transferred into the relevant strains via conjugation for complementation and/or expression.

Transposon mutagenesis. A random mutation library for the S. oneidensis wild-type was constructed with pHG01, which is a transposon vector with a strong promoter embedded in the transposable region. Nitrite resistance suppressor strains were selected with gentamycin and 2 mM nitrite. According to the number of colonies on the control plates (Gm ‘Nitrite’), the transposon library was estimated to contain more than ~20,000 individual insertion mutants. Colonies, obtained from Gm ‘Nitrite’ plates, were subjected to the mapping of the transposon insertion sites by using the arbitrary PCR.

Chemical assays. Mid-log phase cultures were used to inoculate Bis-tris propane (BTP) buffer (pH 5.8) with goethite as sole EA to ~0.1 of OD600 and incubated under anaerobic condition. Concentrations of Fe (II) and nitrite of cultures were quantified using ferrozine reagent and a modified Griess assay spectrophotometrically at
562 nm and 540 nm, respectively. Standard curves were made using ferrous sulphate dissolved in 0.5 mol/L hydrochloric acid.

**Nitrite sensitivity assay.** Cells of *S. oneidensis* strains grown to the late-log phase were adjusted to approximately 10^7 CFUs/ml, followed by 10-fold serial dilutions. Ten microliter of each dilution was spotted onto plates containing nitrite of varying concentrations. Nitrite-free plates were included as control. The plates were incubated at 30 °C before being read. The assays were repeated at least three times with similar results.

**Cytochrome oxidase activity assay.** Visual analysis of the cbb_3-HCO activity was done by staining colonies with the agents for the Nadi Assay. Nadi reactions were carried out by the addition of α-naphthol and N′,N′-dimethyl-p-phenylenediamine (DMPD) on LB agar plates. Colonies were timed for formation of the indophenol blue.

**Promoter activity assay.** The activity of promoters of interest was assessed using a single-copy integrative lacZ reporter system as described previously. A fragment covering the sequence upstream of each operon tested from −300 to +1 was then amplified and cloned into the reporter vector pHGE101, verified by sequencing, and the correct plasmid was then transferred into relevant *S. oneidensis* strains by conjugation. Once transferred into *S. oneidensis* strains, pHGE101 containing promoter of interest integrates into the chromosome and the antibiotic marker is then removed by an established approach. Cells grown to the mid-exponential phase under experimental settings were collected and β-galactosidase activity was performed with an assay kit as described previously.

**SDS-PAGE, heme-staining, and Immunoblotting assays.** Unless otherwise noted, cells of the late exponential phase were harvested, washed with phosphate buffered saline (PBS), resuspended in the same buffer,

| Strain or plasmid | Description | Reference or source |
|-------------------|-------------|---------------------|
| **Strain**        |             |                     |
| E. coli           |             |                     |
| DH5α              | Host for cloning | Lab stock |
| WM3064            | Donor strain for conjugation, ΔdapA | W. Metcalf, UIUC |
| S. oneidensis     |             |                     |
| MR-1              | Wild type   | Lab stock |
| HG0608-10         | Δpet derived from MR-1 | 14 |
| HG0624            | Δcrp derived from MR-1 | 37 |
| HG0845            | ΔnapB derived from MR-1 | 10 |
| HG0848            | ΔnapA derived from MR-1 | 10 |
| HG0849-6          | ΔnapDAGH derived from MR-1 | This study |
| HG09970           | ΔficaA derived from MR-1 | 61 |
| HG1778            | ΔmtrC derived from MR-1 | 61 |
| HG2364-1          | Δcco derived from MR-1 | 28 |
| HG3990            | ΔnarA derived from MR-1 | 10 |
| HG3992            | ΔnarF derived from MR-1 | 44 |
| HG4951            | ΔcymA derived from MR-1 | 10 |
| HG0845-3980       | ΔnapBΔnarA derived from MR-1 | This study |
| **Plasmid**       |             |                     |
| pHG01             | Ap^r, Gm^r, Cm^r, att-based suicide vector | 35 |
| pHG102            | Km^r, promoterless broad-host vector | 36 |
| pHGC01            | Integrative vector for complementation | 25 |
| pHGEI01           | Integrative E. coli lacZ reporter vector | 14 |
| pHBR-Cre          | Helper vector for antibiotic marker removal | 25 |
| pHGE-Petac        | Km^r, IPTG-inducible P_α promoter expression vector | 38 |
| pHGT01            | Promoter-embedded transposon vector | 28 |
| pHGE-Petac-napB   | Inducible expression of napB | This study |
| pHGEI-Petac-lacZ  | *E. coli lacZ* under control of pet promoter | 14 |
| pHGEI-PccO-lacZ   | *E. coli lacZ* under control of cco promoter | 28 |
| pHGEI-PnapB-lacZ  | *E. coli lacZ* under control of nap promoter | This study |
| pHGEI-PnapB-lacZ  | *E. coli lacZ* under control of napB promoter | This study |
| pHGEI-ParfA-lacZ  | *E. coli lacZ* under control of arcA promoter | This study |
| pHGEI-PnarA-lacZ  | *E. coli lacZ* under control of narA promoter | This study |
| pHGEI-Pfica-lacZ  | *E. coli lacZ* under control of ficaA promoter | This study |

**Table 1. Strains and plasmids used in this study.**
and sonicated. Protein concentrations of the cell lysates was determined by the bicinchoninic acid assay (Pierce Chemical). For heme-staining, the cell lysates were separated on SDS-PAGE using 12% polyacrylamide gels and stained with 3,3′,5,5′-tetramethylbenzidine (TMBZ) as described elsewhere43. Immunoblotting analysis was performed essentially the same as previously described 44. Proteins separated on SDS-PAGE were electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane according to the manufacturer’s instructions (Bio-Rad). The gels were blotted for 2 h at 60 V using a Criterion blotter (Bio-Rad). The blotting membrane was probed with rabbit polyclonal antibodies against NrfA. The goat anti-rabbit IgG-HRP (horseradish peroxidase) (Roche Diagnostics) was used as the secondary antibody (1:5,000) and the signal was detected using a chemiluminescence Western blotting kit (Roche Diagnostics) in accordance with the manufacturer’s instructions. Images were visualized with a UVP imaging system.

Identification of transcriptional start sites.  S. oneidensis cells were grown in medium with required additives (either 5 mM nitrate or 2 mM nitrite for induction) to the mid-log phase, collected by centrifugation, and applied to RNA extraction using the RNeasy minikit (Qiagen, Shanghai) as described before 27. RNA was quantified by using a NanoVue spectrophotometer (GE healthcare). The transcriptional start sites of acpP and fabF1 were determined using Rapid Amplification of cDNA Ends (RACE) according to the manufacturer’s instruction (Invitrogen, Shanghai) as recently used 30. In brief, reverse transcription was conducted on preprocessed RNA without 5′-phosphates followed by nested PCR using two rounds of PCR reactions. PCR products were applied to agarose gel separation, purification of the 5′-RACE products, and inserted into the pMD19-T vector (Takara, Dalian) for direct DNA sequencing. The first DNA base adjacent to the 5′-RACE adaptor was regarded as the transcription start site.

Figure 1. Transposon screens and terminal phenotypes. (A) Suppressors for nitrite inhibition of growth on fumarate. Shown were three representatives of 19 stable suppressor strains derived from the wild-type (WT): WT5-21, WT5-35, and WT5-57 had insertions within the cyaC promoter region, the cpdA gene, and the napB gene, respectively (refer to B). Densities of mid-log phase cultures were subject to 10-fold serial dilution, and 5 μl of each dilution was spotted onto plates containing 2 mM nitrite with 20 mM fumarate as EA for growth. Experiments were repeated at least three times and similar results were obtained. (B) Schematics indicating the approximate locations of the transposon insertions. Arrows represent transposon insertion points.

Figure 2. Impacts of loss of NapB. (A) Growth of WT, ΔfccA, ΔnrfA, ΔnapB and ΔnapBΔnrfA on 20 mM fumarate in the presence of 2 mM nitrite. The nitrite-associated defect in growth on fumarate was rescued by the loss of NapB (ΔnapB). Inability to reduce nitrite had no significant impact on growth (ΔnrfA vs. WT). (B) Nitrite reduction of WT, ΔfccA, ΔnrfA, ΔnapB and ΔnapBΔnrfA. All experiments were performed at least three times with standard deviations presented as error bars.
Figure 3. **Effect of nitrite on expression of the nap operon.** (A) Promoter activity measurement of $P_{nap}$ by an integrated lacZ reporter in cells grown on fumarate, fumarate + $\text{NO}_2^-$, TMAO (20 mM), and TMAO + $\text{NO}_2^-$.

Other analyses. To estimate relative abundance of proteins in gels, the intensities of bands were quantified using ImageJ software\(^{45}\). Student’s $t$ test was performed for pairwise comparisons. In figures, values are presented as $+/−$ standard deviation (SD).

**Results**

**Screening for suppressors of nitrite inhibition in *S. oneidensis***. Nitrite inhibition of growth with fumarate is due to lowered intracellular cAMP levels in *S. oneidensis*\(^{24}\). As multiple factors are involved in cAMP homeostasis\(^{31,33}\), we reasoned that at least some in their altered abundance or absence may suppress nitrite inhibition. In attempts to identify such factors, a random mutation library was constructed from the wild-type strain using PHGT01, a mariner-based transposon vector derived from pFAC\(^{28,46}\). This vector has been not only used for construction of transposon insertion libraries but also applicable for cryptic operon screening because of a robust promoter embedded in the transposable sequence\(^{28,47}\).

We screened a total of \(\sim 20,000\) colonies (estimated on nitrite-free control plates) on plates containing fumarate and nitrite under anaerobic conditions, and obtained 72 colonies whose nitrite inhibition is suppressed. Among them, 53 were found to be unstable with respect to resistance to nitrite (data not shown), which varied in experiments from one round to another. In addition, the insertion sites in some (16 tested) of these suppressors were either not identified successfully or within genes encoding a variety of proteins not implicated in respiration. The remaining 19 displayed much elevated resistance to nitrite consistently (Fig. 1A). Three were found to have transposon insertions in the *cyaC* promoter region (Fig. 1B). Given that the *cyaC* mutant is hypersensitive to nitrite\(^{24,25,31}\), this observation suggests that the *cyaC* gene is overexpressed because of the transposon-borne promoter. In parallel, 6 suppressors had transposons that mapped within the promoter and coding sequence of the *cpdA* gene (Fig. 1B), which encodes an enzyme dictating cAMP degradation; the *cpdA* mutant had substantially elevated CAMP levels\(^{31}\). These results are expected because nitrite inhibition can be relieved by increased cAMP intracellular levels, either by altering production/degradation or by adding the molecules exogenously\(^{24}\).

Strikingly, the remaining 10 suppressors had transposons that mapped within the *napDAGHB* region, which encodes periplasmic nitrite reductase and accessory proteins (Fig. 1B). For nitrate respiration, out of the NapDAGHB proteins only NapA is completely required as CymA is in place of otherwise essential NapC, which transports electrons from the quinol pool to NapA, via NapB if it is present\(^{10,13}\). The majority of Insertions were within either the *nap* promoter region or the *napB* gene and its 5′ proximity, implying a possibility that the *napB* gene is the target. Given that insertions within the coding sequence most likely destroy the function, we proposed that the NapB loss is likely to suppress nitrite inhibition of growth on fumarate.

**Nitrite inhibition is closely associated with NapB.** To confirm the involvement of NapB in growth inhibition of nitrite on fumarate, we monitored growth of a Δ*napB* strain on fumarate with or without nitrite. There was no difference in growth between the wild-type and the mutant in the absence of nitrite (Fig. 1A). But when nitrite was present the Δ*napB* strain grew much better than the wild-type (Figs 1A and 2A). In addition, the capacity of reducing nitrite in the Δ*napB* strain was substantially stronger than the wild-type (Fig. 2B), implying that the improved growth is probably due to the depletion of nitrite. We then constructed a double mutant lacking both napB and *nrfA* genes to test this notion. Although the Δ*napBΔnrfA* strain could not consume nitrite (Fig. 2B), it still exhibited growth better than the wild-type did when both fumarate and nitrite were served (Fig. 2A). In comparison, growth of an *nrfA* mutant\(^{10}\), which is deficient in nitrite respiration, was similar to that of the wild-type in the presence nitrite. Importantly, the wild-type phenotypes were restored by expression of respective *napB* and *nrfA* in trans (Fig. S1A). These results indicate that nitrite respiration has an important but not essential role in supporting growth on fumarate, implying that NapB functions as an inhibitor of nitrite reduction.
In most characterized bacterial NAP reductases, NapA and NapB, the large and small subunits respectively, are essential to nitrate respiration. Although *S. oneidensis* NapB is not fully required for the process, we assessed the role of NapA in nitrite inhibition. The result revealed that a *napA* null mutant was as sensitive as the wild-type to nitrite (Fig. S1). To test whether other proteins encoded by the *napDAGHB* operon are involved in nitrite inhibition, we knocked out *napDAGH* and assessed the impact of the loss. Similar to the *napA* mutant, this deletion mutant was indistinguishable from the wild-type when cultivated on fumarate in the presence or absence of nitrite (Fig. S1B). Thus, we conclude that NapB is the only one encoded by the *napDAGHB* operon being involved in nitrite inhibition.

Nitrite induces production of NapB. In *S. oneidensis*, the *napB* gene is the last gene in the nitrate-inducible *napDAGHB* operon. We have previously shown that both *napDAGHB* and *nrfA* are regulated by the NarQ-NarP two-component system, which responds to nitrate and nitrite. Apparently, the *napB* gene is expressed in cells grown on fumarate and nitrite given the significant impact of its loss; but there is no nitrate in cultures to induce the *nap* operon. In an attempt to address this dilemma, we used 5′-RACE to determine whether there is a nitrite-responsive promoter for the *napB* gene. However, no promoter in the sequence immediately upstream of the *napB* coding region was identified (data not shown). The lack of an internal promoter for *napB* was also supported by using the lacZ reporter (Fig. S2).

The operon promoter (P_{nap}) activity was then analyzed. In the wild-type grown on fumarate, nitrite elicited P_{nap} induction of at least 5-fold (Fig. 3A). When CymA-independent TMAO was used as EA to support growth (not subjected to nitrite inhibition), a similar result was obtained. To confirm this, we compared cytochrome c profiles in cells grown on fumarate and TMAO with or without nitrite (Fig. 3B). In agreement with our previous data, a band corresponding to NrfA was present in cells grown on TMAO with nitrite but barely visible in those grown on fumarate with nitrite. Evidently, a cytochrome c of ~16 kD in size was present in a large amount in the presence of nitrite but regardless of EAs used to support growth. By compared to the Δ*napB* strain, we predicted that the protein is NapB. This was then confirmed by mass spectrometry (MS) analysis of the ~16 kD band. It is worth noting that CymA was produced at relatively constant levels under all test conditions as previously reported. All these data, collectively, manifest that NapB is subjected to induction of nitrite.
the standard error or representative results were presented. integrative the NarQP system (control. Shown was the cropped blot and the full-length gel is given in the Supplementary Information file oxygen were used to illustrate nitrite induction of (growth defect but could not block growth (refer to Fig. 4A). Promoter activity was assayed the same as in Fig. 3. fumarate (IPTG concentration: 0.05 mM), TMAO (IPTG concentration: 0.2 mM), and oxygen (IPTG expression of the cytochrome lacZ 

tuations as a quinol dehydrogenase, mediating electron transfer from the quinol pool to the was apparently more tolerant to NapB overdose (Fig. 4A). We supposed that the defect is due to an electron shortage for the pool. Indeed, 0.5 mM IPTG caused a severe growth defect under aerobic conditions although oxygen respiration would be affected by excessive NapB as the process also relies on electrons from the quinol TorA for growth on TMAO in above described cases, leading to impaired growth. If this holds, we reasoned that respective terminal reductases, such as fumarate reductase FccA for growth on fumarate and TMAO reductase of the quinol pool, presumably via quinol dehydrogenases. As a result, there could be an electron shortage for this, NapB in essence is an electron shuttle protein; thus this small cytochrome NapB in excess inhibits growth. Our results thus far illustrate that NapB is highly produced in the presence of nitrite. To investigate whether nitrite inhibition of growth on fumarate, at least in part, is due to NapB in overabundance, we assessed effect of NapB in varying amounts on respiration of fumarate in the absence of nitrite. The napB gene was expressed in the wild-type strain under the control of IPTG-inducible promoter P_tac, which is slightly leaky. Overexpression of the napB gene led to severe growth defect on fumarate (Fig. 4A). The inhibitory effect was evident with IPTG of 0.05 mM and growth was hardly visible with 0.5 mM. For comparison, we repeated the experiment with TMAO was sole EA. Surprisingly, NapB in excess also inhibited growth, albeit less severely (Fig. 4A). Cells grew normally with IPTG up to 0.1 mM but displayed substantially impaired growth with IPTG of 0.5 mM. To assess NapB production levels in cells induced by IPTG of various levels, total proteins were extracted, separated on SDS-PAGE, and applied to heme staining. Compared to the NapB level in the wild-type grown with nitrite, IPTG at 0.05 mM and 0.2 mM had expression of more than 5 and 20-fold (estimated by ImageJ), respectively (Fig. S3).

In bacteria having a typical NAP system (NapC as quinol dehydrogenase), NapB is essential to nitrate respiration by complexion with NapA and NapA is the preferred, if not exclusive, electron acceptor for NapB. Despite this, NapB in essence is an electron shuttle protein; thus this small cytochrome c in excess may dissipate electrons of the quinol pool, presumably via quinol dehydrogenases. As a result, there could be an electron shortage for respective terminal reductases, such as fumarate reductase FccA for growth on fumarate and TMAO reductase TorA for growth on TMAO in above described cases, leading to impaired growth. If this holds, we reasoned that oxygen respiration would be affected by excessive NapB as the process also relies on electrons from the quinol pool. Indeed, 0.5 mM IPTG caused a severe growth defect under aerobic conditions although oxygen respiration was apparently more tolerant to NapB overdose (Fig. 4A). We supposed that the defect is due to an electron shortage to the cytochrome cbb oxidase. To test, we conducted Nadi assay, which specifically detects the activity of cytochrome c oxidase. We have previously demonstrated that an electron shortage for the cbb oxidase leads to a hyperactive phenotype, as illustrated in a strain deficient of the cytochrome bc1 complex (Fig. 4B), which functions as a quinol dehydrogenase, mediating electron transfer from the quinol pool to the cbb oxidase. Because the cbb oxidase works with electrons either from the cytochrome bc1 complex or from the Nadi reagents, when electrons are no longer delivered to the cbb oxidase in the absence of the cytochrome bc1 complex the enzyme exclusively reacts with the Nadi reagents, exhibiting enhanced activity. Although the NapB loss did not affect activity of the cbb oxidase, overproduced NapB also resulted in a hyperactive phenotype of the cbb oxidase, albeit not so strong as that in the bc1-deficient mutant, supporting that NapB in excess reduces the amounts of electrons to the cbb oxidase (Fig. 4B). To further confirm this notion, we examined influences of NapB in varying amounts on expression of the petABC operon (encoding the cytochrome bc1 complex) by using the integrative lacZ-reporter. The differences in expression levels were insignificant (Fig. S4). All of these results, collectively,
Figure 6. NapB in overproduction facilitates extracellular electron transfer. (A) Effect of overproduced NapB partially relies on the Mtr pathway. Growth of indicated strains on fumarate was measured, with NapB production induced by 0.2 mM IPTG when necessary. (B) Overproduced NapB promotes iron reduction. Concentrations of Fe(II) were monitored instead of growth. Production of NapB was induced by 0.2 mM IPTG. All experiments were performed at least three times and error bars indicate the standard error.

indicate that NapB in excess inhibits respiration of many EAs, at least including oxygen, fumarate, and TMAO, possibly by dissipating electrons of the quinol pool.

NapB impacts NrfA production significantly in a Crp- and NarQP-dependent manner. Given that NapB in excess inhibits growth on fumarate, TMAO, and oxygen, we tested whether such inhibition is due to impaired expression of the nrfA, fccA, tor, and cco genes/operons. We overexpressed the napB gene in cells grown on nitrite, fumarate, TMAO, and oxygen as sole EA, respectively, and monitored expression of the genes of interest by using the integrative lacZ reporter system14. Because susceptibilities of S. oneidensis cells to NapB amounts with respect to growth differ significantly (Fig. 4A), we adjusted IPTG to concentrations that allow growth but at the same time elicit significant inhibition. Mid-log phase cells were collected and assayed. Clearly, in cells grown on nitrite nrfA expression diminished substantially, to approximately 68% relative to the control sample (the wild-type with empty vector) (Fig. 5A). In contrast, results revealed that all other tested genes were expressed in a manner independent of excessive NapB (Fig. 5A). Moreover, we repeated the experiment in the ΔnapB strain. Consistently, in the ΔnapB strain nrfA was expressed at enhanced levels but all other genes/operons, fccA, tor, and cco, were unaffected (Fig. 5A). The influence of NapB on production of NrfA in cells grown on nitrite was further confirmed by Western blot with antibodies against NrfA (Fig. 5B). All of these results manifest that NapB abundance is a critical factor governing NrfA production.

NapB resides in the periplasm and is not a DNA-binding protein, thus the protein must exert its regulatory role in an indirect manner. In S. oneidensis, both nap and nrf operons are controlled by the NarQ-NarP two-component system (TCS) and the Crp-cAMP complex. While NarQP TCS is an immediate regulator, Crp-cAMP functions at the higher level as a global regulatory system44. Based on the understanding, we predicted that NapB may rely on these systems to influence nrfA expression. To test this, we examined effects of NapB produced at varying levels on nrfA expression in relevant mutants as described above. In strains lacking either the crp or narP gene (encoding DNA-binding regulator of the TCS), the nrfA promoter was no longer responsive to changes in NapB quantity (Fig. 5C). Therefore, the physiological impact of NapB on nrfA expression depends on both Crp-cAMP and NarQP TCS.

NapB dissipates electrons at least in part through the Mtr pathway. The presented data strongly suggest that NapB dissipates electrons of the quinol pool via CymA, TorC, and the cytochrome bc1 complex, in addition to indirectly repressing nrfA expression. A key issue remains unaddressed is where the electrons shuttled by NapB end given that there is no exogenous nitrate in cultures for dumping via NapA. This was further validated by the observation that nitrate was not produced endogenously to physiological relevant levels and a napA mutant behaved like the wild-type in the presence of overexpressed NapB (Fig. 5A). As EAs whose respiration is inhibited by NapB in overabundance are inclusively located in the periplasm, we predicted that those eventually accept electrons from NapB are likely extracellularly present.

To test this, we examined the influence of the Mtr respiratory pathway on the proposed role of NapB given that Mtr dictates electron transport to extracellular EAs in S. oneidensis52–54. When fumarate was used as EA, a strain lacking the mtrC gene, encoding the major component of the Mtr pathway55, displayed significantly elevated growth compared to the wild-type upon excessive NapB (Fig. 6A). This result implicates a role of the Mtr pathway in electron dissipation mediated by NapB. To provide additional evidence, we assayed effect of NapB in varying amounts on extracellular EA goethite (FeO(OH)). We monitored Fe(III) reduction of relevant strains as growth was too poor to be measured reliably. We found that NapB in overproduction significantly enhanced Fe(III) reduction compared to the wild-type without induction (Fig. 6B). However, the loss of NapB did not elicit any notable difference. Nevertheless, these results support that NapB diverts electron flux from CymA to goethite, presumably some other extracellular EAs. All together, we conclude that NapB dissipates electrons through the Mtr pathway, at least in part, in S. oneidensis.
Discussion

It is well established that toxicity of nitrite to cells is largely attributed to either interfering with protein cofactors, such as Fe-S clusters, heme, and lipoamide, or promoting the formation of reactive nitrogen species\(^{18}\). As a molecule with negative charges, nitrite could not cross the inner membrane of Gram-negative bacteria freely; specific transporters, such as _E. coli_ NarK and alike, are needed to mediate uptake of nitrogen oxyanion (nitrate and nitrite)\(^{36,37}\). However, a large number of bacteria that are equipped with the respiratory nitrate/nitrite reductases only, including _S. oneidensis_, could not import nitrate/nitrite for the lack of such transporters. Thus, it is conceivable that some of nitrite targets may reside in the periplasm, where it is at the highest concentrations. Clearly, it is true during aerobicism in _S. oneidensis_; the primary target of nitrite is the cytochrome _cbb_\(_3\) oxidase, the predominant enzyme for oxygen respiration\(^{25,26}\).

During anaerobiosis, however, scenarios are drastically different. In our previous study, we illustrate a mechanism for nitrite inhibition in which the molecule functions as a signal molecule rather than a toxic agent\(^{24}\). Although the exact receptor for nitrite remains elusive, the result is clear: lowered production of CAMP. In this presented study, we uncovered a parallel mechanism underlying nitrite inhibition during anaerobiosis. Although nitrite also acts as a signal molecule as in to the former, it exerts inhibition through up-regulating NapB production.

In most bacteria possessing a NAP system, NapB is a non-catalytic but essential subunit that shuttles electrons from NapA-specific quinol dehydrogenase NapC to NapA\(^{51}\). In _S. oneidensis_, NapB is not essential to nitrate reduction but improve the efficiency of NapA, conferring cells a fitness gain in nitrate-containing environments\(^{40}\). To date, there has not been any report linking NapB to other physiological processes. Nevertheless, due to its low midpoint reduction potentials, it is proposed that NapB has great thermodynamic advantage for drawing electrons from the quinol pool\(^{38}\). The data presented here support the proposal. As a periplasmic cytochrome _c_, to obtain electrons from the quinol pool NapB has to interact with cytoplasmic membrane-bound quinone dehydrogenases, such as established _c_-type cytochromes NapC and CymA\(^{15,51,58}\). By overproducing NapB, we showed that this protein likely at least interacts with, in addition to CymA, the cytochrome _bc_\(_2\), complex and cytochrome _c_ TorC, electron mediators specific for respiration of oxygen and TMAO, respectively\(^{42,59}\). Given that NapB also promotes Mtr-mediated respiration of extracellular EAs, it is reasonable to predict that NapB may interact with the Mtr pathway. Interestingly, although more than 40 _c_-type cytochromes are encoded in the _S. oneidensis_ genome\(^{60–62}\), the bacterium routinely uses this strategy to increase the complexity of its respiratory pathways. For instance, periplasmic _c_-type cytochromes FccA and CctA can work with the Mtr pathway and ScyA partners with cytochrome _c_ peroxidase and the _cbb_\(_3\) oxidase\(^{17,28,62}\).

Although the new role that NapB plays is significantly beyond being the small subunit of NAP, its coding gene is not independently transcribed but as a member of the _napDAGHB_ operon. Nitrite induces expression of the _napDAGHB_ operon, whose transcription is subjected to regulation of NarQ-NarP two-component system and global regulator Crp\(^{44}\). In line with this, it was revealed in this study that up-regulation of the _napDAGHB_ operon by nitrite is dependent on both NarQ-P and Crp. This is somewhat unexpected because nitrite also impairs Crp activity by diminishing CAMP levels, resulting in a reduced level of overall cytochrome _c_ production\(^{24,37}\). This is particularly true for CymA-dependent reductases, such as NrfA and FccA, whose down-regulation largely accounts for nitrite inhibition of growth on fumarate\(^{24}\). However, NapA seems exceptional. How the _napDAGHB_ operon is immuned from Crp deactivation with lowered cAMP levels is unknown and worthy of further investigation. Another interesting question needed to be addressed is how NapB in excess represses expression of the _nrfA_ gene. Given that NapB diverts electrons from CymA to terminal reductases rather than NrfA as presented here and before\(^{10}\), it seems that cells can sense the electron flux and regulate expression of respective reductases accordingly.

The previously undescribed mechanism by which nitrite can regulate multi-branched respiratory networks has important implications in ecophysiology. To unravel the functional mechanism of NapB, we manipulated NapB overproduction to levels sufficiently high to display inhibitory effects on growth on CymA-independent EAs, such as oxygen and TMAO. However, this would not occur in the natural environment. As a matter of fact, NapB induced by 2 mM nitrite had no inhibition on respiration of these EAs and more importantly, improved growth on TMAO, presumably some other CymA-independent EAs too, such as sulfur species\(^{24}\). By this way, nitrite conceivably provides a fitness gain when cells live in environments containing both nitrite and CymA-independent EAs. It is worth mentioning that most of _Shewanella_ thrive in marine environments where TMAO and sulfur species are relatively rich. In parallel, nitrite facilitates respiration of extracellular EAs, implying that nitrite also likely confers cells an advantage in surviving and proliferating in environments that this group of EAs dominate. In _S. oneidensis_, extracellular electron transport mainly relies on the Mtr pathway, despite the existence of a variety of respiratory strategies, including direct enzymatic reduction, bacterial nanowire, or flavin shuttles\(^{54,63}\). We have previously shown that _S. oneidensis_ prefers insoluble to soluble EAs for respiration, such as Fe(III) oxide versus oxygen\(^{46,48}\). By inducing NapB expression, nitrite likely contributes to this living style in the environment.

References

1. Fredrickson, J. K. et al. Towards environmental systems biology of _Shewanella_. Nat Rev Micro 6, 592–603 (2008).
2. Li, W.-W., Yu, H.-Q. & He, Z. Towards sustainable wastewater treatment by using microbial fuel cells-centered technologies. Energy Environ Sci 7, 911–924 (2014).
3. Kato, S. Microbial extracellular electron transfer and its relevance to iron corrosion. Microb Biotechnol 9, 141–148 (2016).
4. Fu, H., Yuan, J. & Gao, H. Microbial oxidative stress response: Novel insights from environmental facultative anaerobic bacteria. Arch Biochem Biophys 584, 28–35 (2015).
5. Janda, J. M. & Abbott, S. L. The genus _Shewanella_ from the briny depths below to human pathogen. Crit Rev Microbiol 40, 293–312 (2014).
6. Gon, S., Patte, J.-C., Dos Santos, J.-P. & Méjean, V. Reconstitution of the trimethylamine oxide reductase regulatory elements of _Shewanella oneidensis_ in _Escherichia coli_. J Bacteriol 184, 1262–1269 (2002).
26. Zhou, G.
24. Jin, M., Fu, H., Yin, J., Yuan, J. & Gao, H. Molecular underpinnings of nitrite effect on CymA-dependent respiration in *Shewanella oneidensis* MR-1. *Environ Microbiol* 13, 108–115 (2011).

25. Burns, J. L. & DiChristina, T. J. Anaerobic respiration of elemental sulfur and thiosulfate by *Shewanella oneidensis* MR-1 requires *cya*, a homolog of the *phdA* gene of *Salmonella enterica* Serovar Typhimurium LT2. *Appl Environ Microbiol* 75, 5209–5217 (2009).

23. Simpson, P. J. L., Richardson, D. J. & Codd, R. The periplasmic nitrite reductase in *Shewanella*; the resolution, distribution and functional implications of two NAP isoforms, NapEDAB and NapDAGHR. *Microbiology* 156, 302–312 (2010).

21. Reddy, D., Lancaster, J. & Cornforth, D. Nitrite inhibition of *cya* expression and growth rate of *Shewanella oneidensis* MR-1. *Microbiology* 150, 3181–3195 (2014).

20. Ford, P. C. Reactions of NO and nitrite with heme models and proteins. *Acc Chem Res* 44, 626–629 (2010).

19. Gladwin, M. T., Grubina, R. & Doyle, M. P. The new chemical biology of nitrite reactions with hemoglobin: R-state catalysis, oxomet, and nitrite reductase/anhdyrase. *Acc Chem Res* 42, 157–167 (2009).

18. Reddy, D., Lancaster, J. & Cornforth, D. Nitrite inhibition of *Cya* expression and growth rate of *Shewanella oneidensis* MR-1. *Microbiology* 150, 3181–3195 (2014).

17. Fonseca, B. M., Mason, M. G. & Elzeneiny, A. S. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite in aqueous solutions. *Anal Biochem* 429, 67–71 (2016).

16. Marritt, S. J. Combined effect of loss of the *arcA* operon and *cya* on nitrite reduction by *Shewanella oneidensis* MR-1. *Environ Microbiol* 17, 1154–1164 (2015).

15. Schwalb, C., Chapman, S. K. & Reid, G. A. The tetraheme cytochrome *Cya* is required for anaerobic respiration with dimethyl nitrite and nitrite in *Shewanella oneidensis*. *Biochemistry* 42, 9491–9497 (2003).

14. Fu, H., Jin, M., Ju, L., Mao, Y. & Gao, H. Evidence for function overlapping of CymA and the cytochrome oxidases by cytochrome *Cya* in *Shewanella oneidensis*. *Environ Microbiol* 15, 465–474 (2012).

13. Simpson, P. J. L., Richardson, D. J. & Codd, R. The periplasmic nitrate reductase in *Shewanella;* the resolution, distribution and functional implications of two NAP isoforms, NapEDAB and NapDAGHR. *Microbiology* 156, 302–312 (2010).

12. Burns, J. L. & DiChristina, T. J. Anaerobic respiration of elemental sulfur and thiosulfate by *Shewanella oneidensis* MR-1 requires *cya*, a homolog of the *phdA* gene of *Salmonella enterica* Serovar Typhimurium LT2. *Appl Environ Microbiol* 75, 5209–5217 (2009).

11. Shirodkar, S., Reed, S., Romine, M. & Saffarini, D. The octaahem SirA catalyses dissipative sulfite reduction in *Shewanella oneidensis* MR-1. *Environ Microbiol* 13, 108–115 (2011).

10. Miranda, K. M., Espey, M. G. & Wink, D. A. A rapid, simple spectrophotometric method for simultaneous detection of nitrate and nitrite in aqueous solutions. *Anal Biochem* 429, 67–71 (2016).

9. Cruz-Garcia, C., Murray, A. E., Klappenbach, J. A., Stewart, V. & Tiedje, J. M. Respiratory nitrate ammonification by *Shewanella oneidensis* MR-1. *Proc Natl Acad Sci USA* 103, 4669–4674 (2006).

8. Granick, J. A., Vai, H., Lies, D. P. & Newman, D. K. Extracellular respiration of dimethyl sulfoxide by *Shewanella oneidensis* strain MR-1. *Proc Natl Acad Sci USA* 103, 4669–4674 (2006).

7. Maier, T. M., Myers, J. M. & Myers, C. R. Identification of the gene encoding the sole physiologically important nitrate reductase in *Shewanella oneidensis* MR-1. *Journal of Bacterial* 189, 656–662 (2007).

6. Gao, H. et al. Reduction of nitrate in *Shewanella oneidensis* depends on atypical NAP and NRF systems with NapF as a preferred electron transport protein from CymA to NapA. *ISME J* 3, 966–976 (2009).

5. Ford, P. C. Reactions of NO and nitrite with heme models and proteins. *Acc Chem Res* 42, 157–167 (2009).

4. Reddy, D., Lancaster, J. & Cornforth, D. Nitrite inhibition of *Cya* expression and growth rate of *Shewanella oneidensis* MR-1. *Microbiology* 150, 3181–3195 (2014).

3. Saffarini, D. A., Schultz, R. & Beliaev, A. Involvement of cyclic AMP (cAMP) and cAMP receptor protein in anaerobic respiration of *Shewanella oneidensis*. *Environ Microbiol* 15, 2198–2212 (2013).

2. Zhou, G. et al. Combined effect of loss of the *cya* operon and *cya* regulation drives *Shewanella* to thrive in redox-stratified environments. *ISME J* 7, 1752–1763 (2013).

1. Chen, H., Luo, Q., Jin, Y., Gao, T. & H. Evidence for the requirement of CydX in function but not assembly of the cytochrome *bd* oxidase in *Shewanella oneidensis*. *Biochim Biophys Acta* 1850, 318–328 (2015).

63. Schütz, B., Seidel, J., Sturm, G., Einsle, O. & Gescher, J. Investigation of the electron transport chain to and the catalytic activity of the diheme peroxidase CcpA of Shewanella oneidensis. Biochem J 356, 851–858 (2001).
64. Marsili, E. et al. Investigation of roles of divalent cations in electron transport to extracellular electron acceptors. J Biol Inorg Chem 12, 1083–1094 (2007).
65. Yuan, J., Chen, Y., Zhou, G., Chen, H. & Gao, H. Investigation of roles of divalent cations in electron transport to extracellular electron acceptors. J Biol Inorg Chem 12, 1083–1094 (2007).

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Author Contributions
H.G. conceived the idea and designed the project. J.M., Q.Z. and Y.S. carried out the experiments. J.M., Q.Z. and H.G. wrote the paper. All authors reviewed the manuscript.

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