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ORIGINAL ARTICLE

Combined effect of loss of the caa_3 oxidase and Crp regulation drives *Shewanella* to thrive in redox-stratified environments

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Shewanella **species are a group of facultative Gram-negative microorganisms with remarkable respiration abilities that allow the use of a diverse array of terminal electron acceptors (EA). Like most bacteria, *S. oneidensis* possesses multiple terminal oxidases, including two heme-copper oxidases (*caa_3*- and *cbb_3*-type) and a *bd*-type quinol oxidase. As aerobic respiration is energetically favored, mechanisms underlying the fact that these microorganisms thrive in redox-stratified environments remain vastly unexplored. In this work, we discovered that the *cbb_3*-type oxidase is the predominant system for respiration of oxygen (O_2), especially when O_2 is abundant. Under microaerobic conditions, the *bd*-type quinol oxidase has a significant role in addition to the *cbb_3*-type oxidase. In contrast, multiple lines of evidence suggest that under test conditions the *caa_3*-type oxidase, an analog to the mitochondrial enzyme, has no physiological significance, likely because of its extremely low expression. In addition, expression of both *cbb_3*- and *bd*-type oxidases is under direct control of Crp (cAMP receptor protein) but not the well-established redox regulator Fnr (fumarate nitrate regulator) of canonical systems typified in *Escherichia coli*. These data, collectively, suggest that adaptation of *S. oneidensis* to redox-stratified environments is likely due to functional loss of the *caa_3*-type oxidase and switch of the regulatory system for respiration.**
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Introduction

To respire on oxygen (O_2), all aerobic organisms utilize terminal oxidases to catalyze the oxidation of a respiratory substrate such as *c*-type cytochrome and quinol, and the reduction of O_2 to water (Borisov *et al.*, 2011). In prokaryotes, there are two major groups of terminal oxidases: the universal heme-copper oxidases (HCO) and the *bd*-type quinol oxidases (Pereira *et al.*, 2001; Borisov *et al.*, 2011). The HCO is further divided into three families: A, B and C (Wikström and Verkhovsky, 2007; Borisov *et al.*, 2011; Lee *et al.*, 2012). The A-family includes the aa_3-type cytochrome *c* oxidase such as that in *Paracoccus denitrificans* (caa_3-type in some cases, the aa_3-type enzymes with a *c* heme-containing domain, as observed in *Bacillus stearothermophilus*) and the bo_3-type quinol oxidase as in *Escherichia coli* (Puustinen *et al.*, 1991; Giuffrè *et al.*, 1996; Baker *et al.*, 1998). The B-family includes a number of oxidases from extremophilic prokaryotes, such as the ba_3-type enzyme of *Thermus thermophilus* (Chang *et al.*, 2009). The enzymes of the C-family are all cbb_3-type cytochrome *c* oxidases (Ekici *et al.*, 2012).

Unlike eukaryotes carrying the single cytochrome *c* oxidase, most bacteria characterized so far host multiple respiratory oxidases. It has been suggested that differences in O_2 affinity, proton-pumping efficiency and availability of electron donors are critical in determining expression of individual terminal oxidases in widely varying environmental conditions (Han *et al.*, 2011). Given that the proton-pumping stoichiometry in bo_3- and cbb_3-HCOs ($0.5H^+/e^-$) is half of that in aa_3-HCOs ($1H^+/e^-$), aa_3-HCO is energetically advantageous when O_2 is abundant. As a consequence, in organisms carrying an aa_3-HCO it is the predominant enzyme under O_2-rich growth conditions whereas cbb_3-HCO is expressed only under low O_2 or microaerobic conditions (Baker *et al.*, 1998). In bacteria lacking aa_3-HCO, either bo_3- or cbb_3-HCO is able to become the major terminal oxidase supporting aerobic growth. For instance, *E. coli* and *Rhodobacter capsulatus* utilize bo_3- and cbb_3-HCOs as the dominating driving force for aerobic respiration, respectively (Puustinen *et al.*, 1991; Ekici *et al.*, 2012).

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Facultative anaerobes such as *E. coli* adopt different metabolic modes in response to the availability of electron donors and acceptors: aerobic respiration, anaerobic respiration and fermentation (Perrenoud and Sauer, 2005; Vemuri and Aristidou, 2005). Owing to the amount of energy released by each process, aerobic respiration is preferred over anaerobic respiration, which in turn is preferred over fermentation (Green and Paget, 2004). This hierarchy is maintained by monitoring environmental O_2 and cellular redox state, predominantly by Fnr (fumarate nitrate regulator) as well as the Arc (aerobic respiration control) two-component system. Under microaerobic and/or anaerobic conditions, these regulators activate the expression of genes encoding components of alternative electron transport chains, and simultaneously repress the expression of some aerobic functions.

Shewanella are Gram-negative facultative anaerobes predominantly residing in redox-stratified environments, which compel this group of microorganisms to accommodate different O_2 concentrations and use a variety of electron acceptors (EA) such as trimethylamine-*N*-oxide, dimethyl sulfoxide, NO_3^-, Fe^{3+}, Mn^{4+}, and so on, when O_2 is depleted (Fredrikson et al., 2008). To facilitate the adaptation, *Shewanella* have evolved a large number of the *c*-type cytochromes as well as some *b*- and *d*-type cytochromes to respire these EAs, as exemplified in the model species *S. oneidensis* (Heidelberg *et al.*, 2002; Meyer *et al.*, 2004; Bretschger *et al.*, 2007; Gao *et al.*, 2010a). The genome of *S. oneidensis* encodes two cytochrome *c* terminal oxidases: SO4606-4609 (caa_3-HCO) and SO2364-2361 (CcoN-O-Q-P, cbb_3-HCO), and a quinol oxidase SO3286-3285 (CydA-B, *bd*-type) (Heidelberg *et al.*, 2002). It is natural to assume that the caa_3-HCO is largely responsible for respiration when O_2 is abundant whereas the cbb_3-HCO is of importance under O_2 limitation (Marritt *et al.*, 2012). However, our previous study on *c*-type cytochromes revealed that mutants missing either *ccoP* or *ccoO* displayed a defect in growth under O_2-rich conditions much more severe than that missing *SO4606* (an essential subunit II of caa_3-HCO), suggesting that cbb_3-HCO rather than caa_3-HCO dominates in aerobiosis of *S. oneidensis* (Gao *et al.*, 2010a).

In an attempt to decipher why *Shewanella* have resided in redox-stratified niches throughout evolution, we take on to assess the function of oxidases in *S. oneidensis*. We present evidence suggesting that cbb_3-HCO, rather than caa_3-HCO, is indeed the major oxidase functioning under both aerobic and microaerobic conditions. The *bd*-type enzyme, although dispensable under aerobic conditions, confers a significant contribution to respiration of O_2 under microaerobic conditions. Further exploration has revealed that Crp (cAMP receptor protein), but not Fnr or Arc, is the global regulator directly controlling expression of these oxidases. Our report therefore demonstrates that adaptation of *S. oneidensis* to redox-stratified environments is likely due to a combined effect of both Crp regulation and loss of the caa_3-HCO terminal oxidase.

Materials and methods

Bacterial strains, plasmids and culture conditions
A list of all bacterial strains and plasmids used in this study is given in Table 1 and a list of primers used in this study is provided in Supplementary Table S1. For genetic manipulations, *E. coli* and *S. oneidensis* strains were grown under aerobic conditions in Luria-Bertani (LB, Difco, Detroit, MI, USA) medium at 37 °C and room temperature, respectively. When needed, the growth medium was supplemented with chemicals at the following concentrations: 2, 6-diaminopimelic acid, 0.3 mM; ampicillin, 50 μg ml^{-1}; gentamycin, 15 μg ml^{-1}; kanamycin, 50 μg ml^{-1}; and tetracycline, 15 μg ml^{-1}.

In-frame deletion mutagenesis, complementation and physiological characterization
In-frame deletion mutagenesis, complementation and physiological characterization were carried out in essentially the same manner as described previously (Gao *et al.*, 2008a; Wu *et al.*, 2011). M1-defined medium containing 0.02% (w/v) of vitamin-free casamino acids was used in all physiological experiments (Gao *et al.*, 2008a). Aerobic cultures were grown with rigorous shaking (250 r.p.m.) in 500 ml Erlenmeyer flasks containing 20 ml of medium. Microaerobic cultures of 20 ml were grown in 500 ml rubber-stoppered serum bottles, with a gas atmosphere of 1% O_2 and 99% N_2. Anaerobic media and cultures were prepared as reported earlier (Gao *et al.*, 2009). For the viable assays, cells of *S. oneidensis* grown in LB at 30 °C to an OD_{600} of ~0.6 were adjusted to ~10^7 CFU ml^{-1} with fresh LB, followed by three 10-fold serial dilutions. Ten microlitres of each diluted sample (from 10^4 to 10^7 CFU ml^{-1}) was spotted onto LB plates. All plates were incubated at 30 °C before being read. The assay was repeated at least for three times with similar results.

Promoter activity assay
To locate the promoters of the *cco*, *cox* and *cyd* operons, upstream sequences of these operons were analyzed using the promoter prediction program Neutral Network (Reese, 2001). To construct the P_{cco}-*lacZ*, P_{cox}-*lacZ* and P_{cyd}-*lacZ* reporters, ~400 bp DNA fragments upstream of the *cco*, *cox* and *cyd* operons were amplified by PCR with primers listed in Supplementary Table S1 and cloned into pTP327 (Gao *et al.*, 2010b). After verification by DNA sequencing, the reporter plasmids were transferred into each *S. oneidensis* strain by conjugation. Cells grown to an OD_{600} of ~0.1 (early exponential phase), ~0.3 (mid-exponential phase) and ~0.8 of OD_{600} (early stationary phase) under aerobic

Table 1 Strains and plasmids used in this study[a]

Strain or plasmid	*Description*	*Reference or source*
E. coli		
DH5α	Host for regular cloning	Lab stock
WM3064	Host for *pir*-dependent plasmids and donor strain for conjugation; Δ*dapA*	W Metcalf, UIUC
BL21(DE3)	Expression host for pTP247	Lab stock
S. oneidensis		
MR-1	Wild type	Lab stock
HG0610	*petC* deletion mutant derived from MR-1; Δ*petC*	Gao *et al.*, 2010a
HG0624	*crp* deletion mutant derived from MR-1; Δ*crp*	Gao *et al.*, 2010b
HG0624-2356	*crp* and *fnr* double deletion mutant derived from MR-1; Δ*crp*Δ*fnr*	Gao *et al.*, 2010b
HG2356	*fnr* deletion mutant derived from MR-1; Δ*fnr*	Gao *et al.*, 2010b
HG2361	*ccoP* deletion mutant derived from MR-1; Δ*ccoP*	Gao *et al.*, 2010a
HG2363	*ccoO* deletion mutant derived from MR-1; Δ*ccoO*	Gao *et al.*, 2010a
HG2364	*ccoN* deletion mutant derived from MR-1; Δ*ccoN*	This study
HG2364-3285	Δ*ccoN*Δ*cydB*	This study
HG2364-4606	Δ*ccoN*Δ*coxB*	This study
HG3285	*cydB* deletion mutant derived from MR-1; Δ*cydB*	This study
HG3285-4606	Δ*cydB*Δ*coxB*	This study
HG3988-0624	Δ*arcA*Δ*crp*	Gao *et al.*, 2008a
HG3988-2356	Δ*arcA*Δ*fnr*	Gao *et al.*, 2008a
HG3988-0624-2356	Δ*arcA*Δ*crp*Δ*fnr*	Gao *et al.*, 2008a
HG4606	*coxB* deletion mutant derived from MR-1; Δ*coxB*	Gao *et al.*, 2010a
HG4607	*coxA* deletion mutant derived from MR-1; Δ*coxA*	This study
HGTRIOX	Δ*ccoN*Δ*cydB*Δ*coxB*	This study
Plasmids		
pDS3.0	Ap^r, Gm^r, derivative from suicide vector pCVD442	Lab stock
pHG101	Promoterless broad-host Km^r vector	Wu *et al.*, 2011
pHG102	pHG101 containing the *S. oneidensis arcA* promoter	Wu *et al.*, 2011
pTP247	Gateway destination His-tag expression vector	Gao *et al.*, 2008b
pTP247-Crp	pTP247 containing *crp*	This study
pTP327	Ap^r, Tet^r, *lacZ* reporter vector	Gao *et al.*, 2010b
pTP327-P_{cco}-*lacZ*	pTP327 containing ~400 bp upstream sequence of *cco*	This study
pTP327-P_{cyd}-*lacZ*	pTP327 containing ~400 bp upstream sequence of *cyd*	This study
pTP327-P_{cox}-*lacZ*	pTP327 containing ~400 bp upstream sequence of *cox*	This study

[a]plasmids containing mutational structures were constructed, as described in the text and not included in the table.

conditions and grown to ~0.1 of OD_{600} under microaerobic conditions were harvested by centrifugation. Cell pellets were washed once with phosphate-buffered saline, and resuspended in phosphate-buffered saline to an optical density of 1.0 (OD_{600}) for sonication. The total protein concentration of the cell lysates was determined by the bicinchoninic acid assay (Pierce Chemical, Dallas, TX, USA). β-galactosidase activity assay was performed using an assay kit (Beyotime, Dalian, China), as described previously (Wu *et al.*, 2011).

Some samples were subjected to quantitative real-time reverse transcription-PCR (qRT-PCR) analysis for verification, which was performed on an ABI7300 96-well qRT-PCR system (Applied Biosystems, Foster City, CA, USA), as described previously (Yuan *et al.*, 2011; Dong *et al.*, 2012).

Nadi assay

The Nadi test was used for visual assessment of cytochrome *c* oxidase-dependent respiration (Marrs and Gest, 1973). A solution of 1% ∂-naphthol in 95% ethanol and 1% *N*,*N*-dimethyl-*p*-phenylenediamine monohydrochloride was applied to cover colonies grown on LB agar plates. Formation of indophenol blue was timed as an indicator of cytochrome *c* oxidase activity.

Expression and purification of S. oneidensis Crp protein and EMSA

The entire clone set of *S. oneidensis* open reading frames has been constructed, as reported previously (Gao *et al.*, 2008b). The *crp* gene within pDONR221 (the entry vector) was transferred to pTP247 (the destination His-tag expression vector). Protein expression and purification was performed, as previously described (Gao *et al.*, 2008b). The probes used for electrophoretic motility shift assay (EMSA) were prepared by PCR with ^{33}P end-labeled primers. The binding reaction was performed with ~25–50 fmol (~2–5 nM) of labeled probes and various amounts of protein with or without 10 μM cAMP in 12 μl binding buffer containing 100 mM Tris/HCl (pH 7.4), 20 mM KCl, 10 mM $MgCl_2$, 2 mM dithiothreitol, 0.2 μg μl^{-1} poly (dI·dC), and 10% glycerol at 15 °C for 60 min and resolved on pre-run 4.8%

polyacrylamide native gels (Gao *et al.*, 2008a). The band shifts were visualized by autoradiography.

Results

c-type cytochromes that are important for growth with abundant O_2

Impacts of *c*-type cytochromes on aerobic growth of *S. oneidensis* have been investigated previously with 36 single knockout strains (Gao *et al.*, 2010a). Although removal of most of these *c*-type cytochromes resulted in growth that was comparable to that of the parental wild-type strain, 10 mutants displayed distinguishable growth defect, of which Δ*petC*, Δ*ccoP* and Δ*ccoO* were most significant. PetC is an essential component of the cytochrome bc_1 complex, which transfers electrons to all *c*-type cytochrome oxidases (Londer *et al.*, 2008; Gao *et al.*, 2010b). Thus, the impaired growth observed in the *petC* mutants is not unexpected. However, the similar observation from the *ccoP* and *ccoO* mutants was surprising, given that the genome encodes a caa_3-HCO (*SO4606-4609*), which is supposed to operate under O_2-replete conditions. To further confirm that Δ*ccoP* and Δ*ccoO,* rather than Δ*SO4606* (Δ*coxB*), are defective in aerobic growth, we created mutants lacking one of the other essential subunits in these oxidases, Δ*ccoN* (encoding the reductase subunit) and Δ*coxA* (*SO4607*, encoding subunit I), and assayed their growth. Results in Figure 1 show that Δ*ccoN* and Δ*coxA* are indistinguishable from Δ*ccoO* or Δ*ccoP* and Δ*coxB,* respectively.

To rule out polarity issues introduced by the mutations, genetic complementation for mutants with growth defects was carried out using pHG101 or pHG102, as described previously (Wu *et al.*, 2011). In all cases, phenotypic differences were insignificant between the mutant strains containing the corresponding plasmid-borne gene and the wild type containing the empty vector, indicating that the observed phenotype of the mutants was due to the introduced mutation (Table 2). Collectively, these data suggest that the complexes within the electron transfer pathway from cytochrome bc_1 to cbb_3-HCO are required for optimal growth under aerobic conditions.

The cytochrome cbb_3 oxidase is crucial for growth with abundant O_2

To determine whether caa_3-HCO retains some capacity of aerobic respiration, we performed the Nadi assay, which can specifically detect cytochrome *c* oxidase-dependent respiration. Using *N*, *N*-dimethyl-*p*-phenylenediamine monohydrochloride as an exogenous electron donor, cytochrome *c* oxidase catalyzes the rapid formation of indophenol blue from colorless α-naphthol. Although formation of indophenol can occur spontaneously, the process is extremely slow, resulting in a significant delay in strains devoid of a functional cytochrome *c* oxidase. In *S. oneidensis*, both caa_3- and cbb_3-HCOs are supposed to be able to carry out the Nadi reaction. Compared with the wild-type strain, Δ*coxB* exhibited a similar reaction rate, forming indophenol blue visibly in <1 min and developing maximum coloration within 5 min (Figure 2a). In contrast, formation of indophenol blue in Δ*ccoN* did not occur before colonies became completely blue through spontaneous indophenol formation (results at 30 min are shown). Given that the Nadi reaction only requires a terminal oxidase and a *c*-type cytochrome, it is possible that removal of either CcoP or CcoO may not annul the reaction. To test this hypothesis, we performed the experiment with Δ*ccoP* and Δ*ccoO,* and found that both of these two mutants behaved exactly like Δ*ccoN*, indicating that the integrity of the Cco complex is essential for the reaction. In addition, the defective phenotype in the Nadi assay that resulted from the *ccoN* deletion was corrected by its expression *in trans*. All together, these data

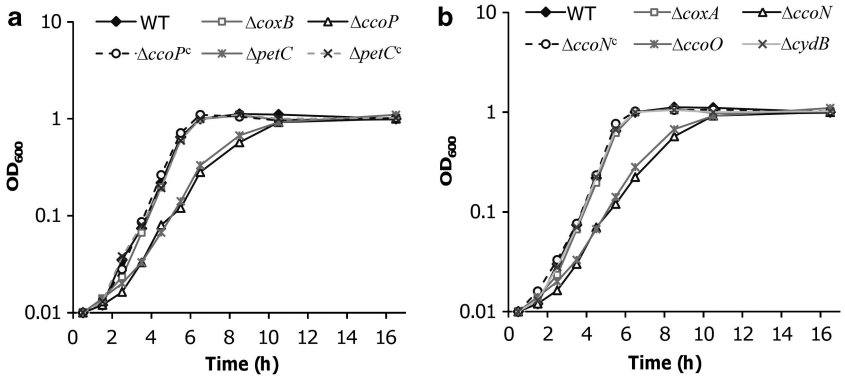


Figure 1 Growth of *S. oneidensis c*-type cytochrome mutants compared with their parental wild-type strain. Superscript 'c' represents the mutant strain containing a copy of the corresponding gene on the complementation vector. All strains were cultured under vigorously agitated conditions. The data are averages from at least three independent cultures. For clarity, error bars (s.d. $<5\%$ of presented data) are omitted.

Table 2 *S. oneidensis* strains subjected to genetic complementation

Strain	*Plasmid*[a]	*Gene(s) on plasmid*[b]	*Generation time (M/C)*[c]
WT			1
WT	pHG101		1.03 ± 0.06
WT	pHG102		0.98 ± 0.04
Δ*ccoO*	pHG101	*ccoNO*	0.79 ± 0.04/0.99 ± 0.05
Δ*ccoP*	pHG102	*ccoP*	0.82 ± 0.06/1.04 ± 0.04
Δ*ccoN*	pHG101	*ccoNO*	0.77 ± 0.05/1.00 ± 0.04
Δ*cydB*	pHG101	*cydAB*	0.47 ± 0.06/1.06 ± 0.08[d]
Δ*coxB*	—	—	0.97 ± 0.03
Δ*coxA*	—	—	1.02 ± 0.05
Δ*cydB*Δ*coxB*	pHG101	*cydAB*	0.44 ± 0.05/1.01 ± 0.06[d]
Δ*ccoN*Δ*coxB*	pHG101	*ccoNO*	0.79 ± 0.04/1.04 ± 0.07
Δ*ccoN*Δ*cydB*	pHG101	*coxB*	No growth/no growth
Δ*ccoN*Δ*cydB*	pHG101	*cydAB*	No growth/0.74 ± 0.07
Δ*ccoN*Δ*cydB*	pHG101	*ccoNO*	No growth/0.47 ± 0.06[d]

Abbreviations: M/C, generation time of the mutant/generation time of the mutant complemented; WT, wild type.
[a]The designated vectors for complementation.
[b]Genes on the designated vector for complementation.
[c]Generation time of each strain grown under aerobic conditions is normalized to that of the wild-type strain.
[d]The maximum cell densities were compared as the generation times differed insignificantly.

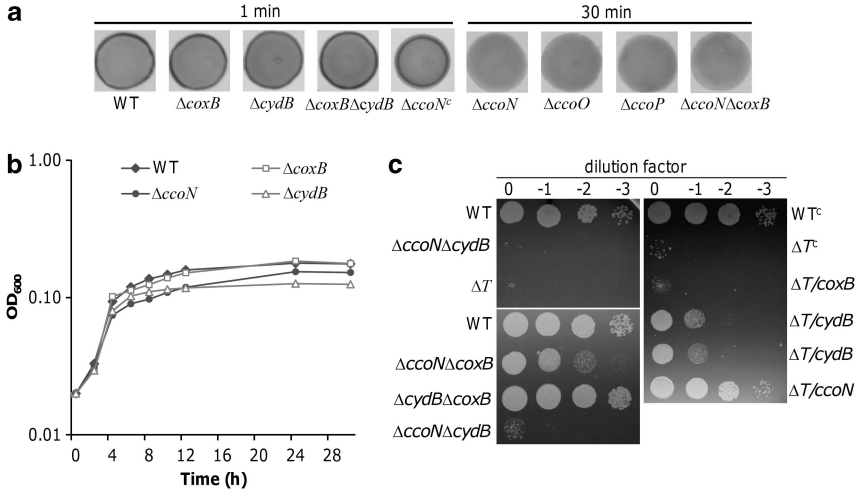


Figure 2 Physiological characteristics of *S. oneidensis* oxidase-deficient mutants. (**a**) Nadi assay. The method is based on the rapid formation of indophenol blue from colorless α-naphtol catalyzed by cytochrome *c* oxidase, using *N*,*N*-dimethyl-*p*-phenylenediamine monohydrochloride as an exogenous electron donor. Nadi-positive and -negative strains were photographed 1 and 30 min after the reaction, respectively. Δ*ccoN*[c] represents Δ*ccoN* containing a copy of *ccoN* on the complementation vector. (**b**) Growth under microaerobic conditions. All strains were grown in the defined medium with 1% O_2 in the gas atmosphere and growth was monitored at OD_{600}. For clarity, error bars (s.d. < 5% of presented data, $n \geqslant 3$) are omitted. (**c**) Drop-plate assay. Cultures at the mid-log phase were adjusted to $\sim 10^7$ CFU ml^{-1}, 10 μl of which were dropped on the LB agar plates in the absence (left panel) or presence of kanamycin (right panel). Cells were incubated at 30 °C for 18 h except those on the lower left panel, which were incubated for additional 6 h for further confirmation of growth of Δ*ccoN*Δ*cydB*. Δ*T* represents the triple mutant, Δ*ccoN*Δ*cydB*Δ*coxB*. WT[c] and Δ*T*[c] represent these strains containing the empty vector. Experiments were performed at least three times and consistent results were obtained.

suggest that cbb_3-HCO dominates aerobic respiration under test conditions.

Both cytochrome cbb_3 and bd oxidase are important under microaerobic conditions

The observation that *S. oneidensis* is able to carry out aerobic respiration without cbb_3-HCO indicates that at least one of other oxidases is functional. In addition to caa_3- and cbb_3-HCO, *S. oneidensis* also carries a *bd*-type quinol oxidase encoded by *cydAB*. To explore the role of the *bd*-type quinol oxidase in O_2 respiration, we constructed Δ*cydB*, in which the essential subunit II was removed, and characterized the mutant under aerobic conditions. Consistent with the lack of a cytochrome *c* component, cells without the *bd*-type quinol oxidase were positive in the Nadi assay (Figure 2a). Moreover, compared with its parental strain, Δ*cydB* did not elicit any noticeable difference in either growth rate or

maximum cell density, indicating that the enzyme has a negligible impact on growth when O_2 is abundant (Figure 1).

We then examined growth of mutants devoid of one of the oxidases with O_2 of microaerobic levels, as both cbb_3-HCO and the *bd*-type oxidase are proposed to function preferentially under these conditions (Borisov *et al*., 2011). The mutation in *coxB* did not elicit any noticeable difference compared with the wild-type strain, reinforcing the idea that caa_3-HCO is dispensable under test conditions (Figure 2b). On the contrary, loss of either cbb_3- or *bd*-type oxidase caused significant reduction in maximum cell density, indicating that both enzymes contribute to O_2 respiration under microaerobic conditions (data at a linear scale are shown in Supplementary Figure S2A). Notably, the biomass of Δ*cydB* cells was much lower than that of Δ*ccoN*, which is likely due to the low efficiency of the *bd*-type oxidase.

To further test whether the caa_3-type enzyme is completely dispensable for aerobic respiration, we made attempts to construct double mutants devoid of two oxidases under aerobic conditions, including Δ*ccoN*Δ*coxB*, Δ*ccoN*Δ*cydB* and Δ*coxB*Δ*cydB*. Although construction of both Δ*ccoN*Δ*coxB* and Δ*coxB*Δ*cydB* went smoothly, with numerous tries no Δ*ccoN*Δ*cydB* colonies were obtained after the resolution (the last step of the mutagenesis procedure), which was supposed to theoretically produce a population of a 50:50 mixture of the mutant and wild-type cells. When the resolution was performed under anaerobic conditions as reported earlier (Kouzuma *et al*., 2012), Δ*ccoN*Δ*cydB* and Δ*ccoN*Δ*cydB*Δ*coxB* were obtained, suggesting that cbb_3- and *bd*-type oxidases are synthetic lethal under aerobic conditions. To confirm this, we performed the drop-plate assay of the triple mutant Δ*ccoN*Δ*cydB*Δ*coxB* strains complemented with each of the deleted genes. The cells were prepared from cultures grown on fumarate under anaerobic conditions. As shown in Figure 2c, under aerobic conditions both Δ*ccoN*Δ*cydB* and Δ*ccoN*Δ*cydB*Δ*coxB* were deficient in growth, whereas the double mutants lacking *coxB* were able to grow. In addition, expression of *coxB in trans* was unable to restore its growth. In contrast, the synthetic lethal phenotypes resulting from the *ccoN* or *cydB* deletions were corrected by their expression *in trans*. These data, collectively, indicate that aerobic growth of *S. oneidensis* requires either cbb_3- or *bd*-type oxidase.

Expression levels of cco, cox and cyd operons likely account for their roles in respiration

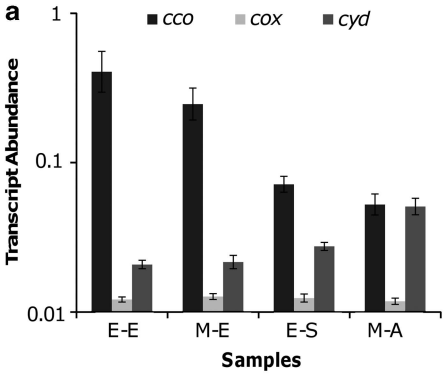
Data presented thus far establish that in *S. oneidensis* the cbb_3- and *bd*-type oxidases are involved in aerobic respiration and the caa_3-type is negligible. Given that transcription is the primary level of regulation, we reasoned that the operons encoding these oxidases may be transcribed differently. The messenger RNA abundance of the *cco*, *cox* and *cyd* operons in samples of various growth stages was therefore measured using qRT-PCR (Figure 3a). Transcription of the *cox* operon was extremely low regardless of growth conditions. On the contrary, both *cco* and *cyd* operons responded to growth conditions at the transcriptional level. The abundance of the *cco* messenger RNA, lowest under microaerobic conditions, was inversely proportional to cell densities under aerobic conditions, suggesting that expression of *cco* is favored in O_2-rich environments. Expression of the *cyd* operon, at a limited level under aerobic conditions, was enhanced substantially under microaerobic conditions.

To confirm these results, we then employed a *lacZ*-based reporter system to assess the promoter activity of *cco*, *cox* and *cyd* operons *in vivo*, represented as P_{cco}, P_{cox} and P_{cyd}, respectively. Analysis of upstream sequences of these operons by the promoter prediction program Neutral Network (Reese, 2001) revealed that the most confident transcription initiation sites of *cco* and *cyd* are located much closer to the translation starting sites (-42 and -87, respectively) than that of *cox* (-259) (Figure 3b). Accordingly, the $\sim$400 bp upstream sequences of *cco*, *cox* and *cyd* operons were amplified and placed in front of the full-length *E. coli lacZ* gene on plasmid pTP327. The resulting vectors, verified by sequencing, were introduced into *S. oneidensis* strains cultured under aerobic or microaerobic conditions. Results obtained from these samples using the *lacZ*-based reporter system were comparable to those from qRT-PCR. These data, consistent with their significance in aerobic respiration, indicate that cbb_3-HCO is the predominant driving force for aerobic respiration. whereas the *bd*-type oxidase facilitates the process when O_2 becomes limited.

Compensatory expression of the cyd operon

In combination, the cbb_3- and *bd*-type oxidases appear to be synthetic lethal, conferring a possible regulatory interplay between these two systems. To test this hypothesis, we examined the activity of P_{cco} and P_{cyd} in cyd^- and cco^- backgrounds, respectively. Results showed that the absence of Cyd had no effect on *cco* transcription (Figure 4a). However, when *cco* was removed transcription of *cyd* increased substantially when O_2 was abundant, reaching a level close to that observed under microaerobic conditions, under which the activity of P_{cyd} was hardly affected. This result implies that expression of *cyd* is possibly maximized under microaerobic conditions.

The compensatory expression of the *cyd* operon under aerobic conditions suggests that cbb_3-HCO, when abundant, represses production of the *bd*-type oxidase. To confirm this, expression of *cyd* in Δ*petC* was examined. We expected that expression of *cyd*



b

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>cco
AGCCAAAATT ACGATTTAGC ACAAAAATTA ACCTTTTGAT GCAAGATGTT AACATTCTAA -220
GGAGCAGATT GATCTAGATC AACTTTCCAA CCCCCTTCTT TGGTATCTTT TTTGACATGG -160
CTCAAGTATC ATTGCGAAAC CTCACGTTTG CGCACAAATG TGAAGGATAA CGCATTGACA -100
TAGCTCAACA ACCCTTATAG GgagaTTTGA GTTTTGTGTC ATAtataacA GGTAAAGCTT -40
>cyd
TGTGTGCCCC GTAGCAAAAC CCATCTAAAC CCCTGAGTTT GATTGATTTG AATCAAAGGC -320
GGGTGGTATA AAATACTCAC AAATGAAAGC GATTAAATAT CTTAACTATC TGATATTTAT -260
ATCGATGGGA TCATTTGTAG GATTGGGTGG TGAGGTGGAA GTCTCAAAGC TGAACGATAG -200
TGTGACTAGG GTCTCAGTAC GTGCGGAAAA AGGGGATTTT TAGGCCTTGC TGCCTCGATG -140
TAATGAAAAA GATgataTTT AACGGTTTAT ACTGGCCTCA GCtataaaCA CAAAATGAAA -80
>cox
TTGTACTCCG TATTTAGCCT TGAAAGCCGC CTCACCAATG AATGTGAGGC TTTTTTTTGG -340
CAATTTTTCA GACAATTAGA AAAAGAAGTG ATCTTGATCA ATTTTTAaaac aGCAAGTATG -280
GTTGattaag GCGTGAATAA TATATCGCCA GTTTAGGCAT AGATATCGAG TTTTTATCGA -220
```

Figure 3 Expression of the *cco, cox* and *cyd* operons. (**a**) Expression of the *cco*, *cox* and *cyd* operons by qRT-PCR. Samples were collected at the early exponential phase (E-E), the mid-exponential phase (M-E) and the early stationary phase (E-S) of aerobic cultures and the M-E phase of microaerobic cultures (M-A). Experiments were performed independently at least three times and error bars represent s.d. (**b**) Upstream sequences of the *cco*, *cox* and *cyd* operons (numbered relative to translation start sites). Transcription starting sites are pointed by dots. Predicted −35 and −10 boxes are in italics and bold lower case, respectively. Predicted Crp- and Fnr-binding sites are in italics and underlined, respectively.

would increase as in Δ*ccoN*, as cbb_3-HCO is unable to function in the *petC*$^-$ background. Strain Δ*petC* was cultured under the same conditions as described above and β-galactosidase activity was measured (Figure 4b). In the absence of PetC, expression of *cco* was significantly reduced, with the largest difference (~25% remaining) observed in the early exponential phase samples. In contrast, expression of *cyd* in Δ*petC* was increased to a level comparable to that observed in Δ*ccoN*, thus confirming that *cyd* is indeed subjected to compensatory induction once *cco* is missing. As expected, expression of *cox* remained at the extremely low level, regardless of PetC.

Impacts of global regulators ArcA, Crp and Fnr on expression of cco, cox and cyd operon

In *S. oneidensis*, global regulators mediating adaptation of metabolic modes in response to the availability of O_2 include the Arc system, Crp and Fnr (Gao *et al.*, 2010b). On one hand, *S. oneidensis* Fnr, unlike its *E. coli* analog, which is the primary factor controlling the switch between aerobic and anaerobic metabolism, has no significant role in the process (Maier and Myers, 2001; Cruz-Garcia *et al.*, 2011). On the other hand, both the Arc system and Crp have roles in respiration, with the former primarily functioning under aerobic conditions and the latter being predominant under anaerobiosis (Saffarini *et al.*, 2003; Gao *et al.*, 2010b).

Regions upstream of all *cco*, *cox* and *cyd* operons are predicted to contain Crp- and Fnr- but not ArcA-binding motifs, implicating that these terminal oxidases may be subjected to direct regulation by Crp and Fnr (Gao *et al.*, 2010b) (Figure 3a). To gain insight into effects of such control *in vivo*, we measured the activity of P_{cco}, P_{cox} and P_{cyd} in the *arcA*$^-$, *crp*$^-$ or *fnr*$^-$ background, respectively. As shown in Figure 5a, the promoter activity of the *cox* operon was too low to be meaningfully compared between the wild type and any mutant strains. Consistent with the lack of ArcA-binding motifs, the expression levels of *cco* and *cyd* operons were hardly altered in the *arcA*$^-$ background compared with the wild-type strain. Interestingly, removal of Crp and Fnr elicited different impacts on the activity of P_{cco} and P_{cyd}, respectively, despite the coexistence

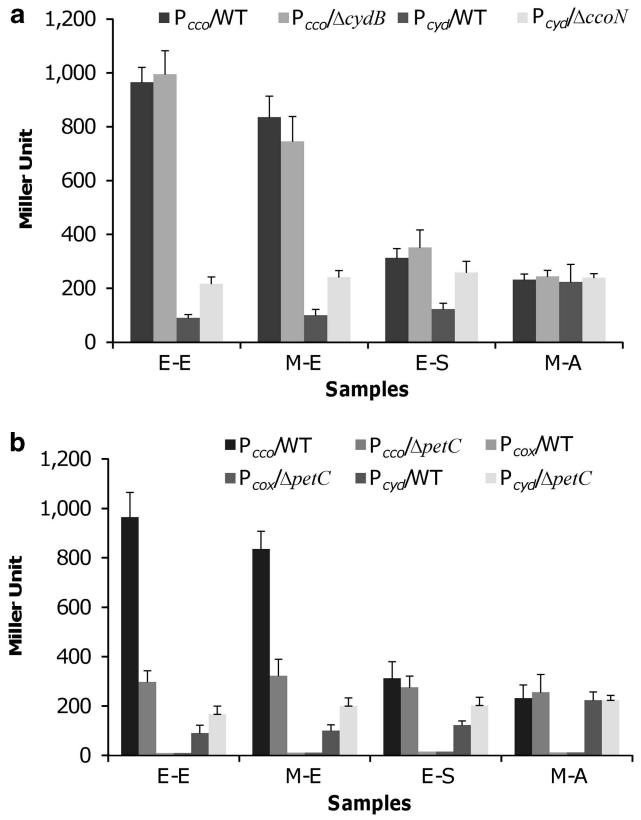

Figure 4 Promoter activities of the *cco*, *cox* and *cyd* operons. Approximate, 400 bp upstream sequences were transcriptionally fused to full-length *lacZ* for β-galactosidase activity assay. Data are given in Miller units. (**a**) Activities of the *cco* promoter in Δ*cydB* and the *cyd* promoter in Δ*ccoN,* grown to phases the same as in Figure 3a. (**b**) Activities of the *cco*, *cox* and *cyd* promoters in Δ*petC* grown to phases the same as in Figure 3a.

of well-conserved Crp- and Fnr-binding sites. Under all tested conditions, response of neither P_{cco} nor P_{cyd} to the loss of Fnr was statistically significant, reinforcing the idea that Fnr has an extremely limited role in regulation. By contrast, Crp was essential for expression of the *cyd* operon but exerted a relatively moderate impact on P_{cco} under all tested conditions.

We have previously shown that both Δ*arcA* and Δ*crp* grow significantly slower, whereas Δ*fnr* is not distinguishable relative to the wild-type strain under vigorously agitated conditions (Gao *et al.*, 2010b). Given that the promoter activities of *cco*, *cox* and *cyd* are affected by Crp only, we reasoned that Δ*crp* would be defective more severely in growth under microaerobic conditions where both the cytochrome cbb_3- and *bd*-type oxidases are involved. To test this, growth of Δ*arcA*, Δ*crp*, Δ*fnr*, Δ*arcA*Δ*crp*, Δ*crp*Δ*fnr*, Δ*arcA*Δ*fnr* and Δ*arcA*Δ*crp*Δ*fnr* under microaerobic conditions was assayed (Figure 5b). As expected, the *crp* mutant displayed a significant defect in growth. On the contrary, both Δ*arcA* and Δ*fnr* grew similarly in comparison with the wild-type strain, and so did Δ*arcA*Δ*fnr* (data at a linear scale shown in Supplementary Figure S2B). Moreover, other strains carrying multiple mutations were not distinct from Δ*crp*, implicating that loss of Crp in these strains was accountable for their growth defect. Collectively, these data converge on the idea that Crp is the regulator controlling respiration of not only a number of EAs anaerobically, but also O_2.

DNA-binding characteristics of Crp

Although predicted Crp-binding motifs are identified in upstream regions of both *cco* and *cyd* operons, different effects of Crp on P_{cco} and P_{cyd} warrants an EMSA assay to determine whether Crp binds directly to the *cco* and *cyd* promoter regions. The His-tagged Crp protein was produced in *E. coli* and purified from inclusion bodies (Gao *et al.*, 2008b). It has been previously shown that expression of the *dms* operon (encoding dimethyl sulfoxide reductase) is dependent on Crp. We therefore chose its upstream sequence for calibration of the Crp binding in a preliminary experiment (Saffarini *et al.*, 2003; Gralnick *et al.*, 2005). A DNA fragment of ~200 bp covering the predicted Crp-binding site was amplified with ^{33}P end-labeled primers, and assayed with the purified His-tagged Crp with or without cAMP in EMSA. Significant binding to the DNA probe occurred at a protein concentration of 0.25 μM for Crp in the presence of 10 μM cAMP (Figure 6a). In contrast, Crp did not bind in the absence of cAMP, even when the protein concentration was increased to 4 μM. The binding of Crp-cAMP to the target promoter was not reduced by addition of the nonspecific competitor poly(dI·dC) DNA, but was outcompeted by adding 100-fold excess unlabeled probe. These results demonstrate that Crp binds the *dms* promoter in a sequence-specific manner and such a capacity is dependent on cAMP.

We then applied EMSA to upstream fragments of the *cco*, *cox* and *cyd* operons covering predicted Crp-binding sites with Crp and 10 μM cAMP. A similar length upstream fragment of *gyrB* (encoding DNA gyrase subunit B) was included in the assay as a negative control, according to the method established previously (Gao *et al.*, 2008a). A gel shift band was observed with all three of targeted upstream sequences when 0.5 μM of Crp was added to the reaction mixture and the intensity of the shifted band became stronger with 2 μM of Crp. In contrast, the *gyrB* fragment was unable to cause a visible motility shift (Figure 6b). These results provide evidence for the direct binding of Crp to the *cco*, *cox* and *cyd* promoter regions, although regulatory effects of these interactions differ.

Discussion

Once regarded to be present only in proteobacteria (Pereira *et al.*, 2001), cbb_3-HCOs have been

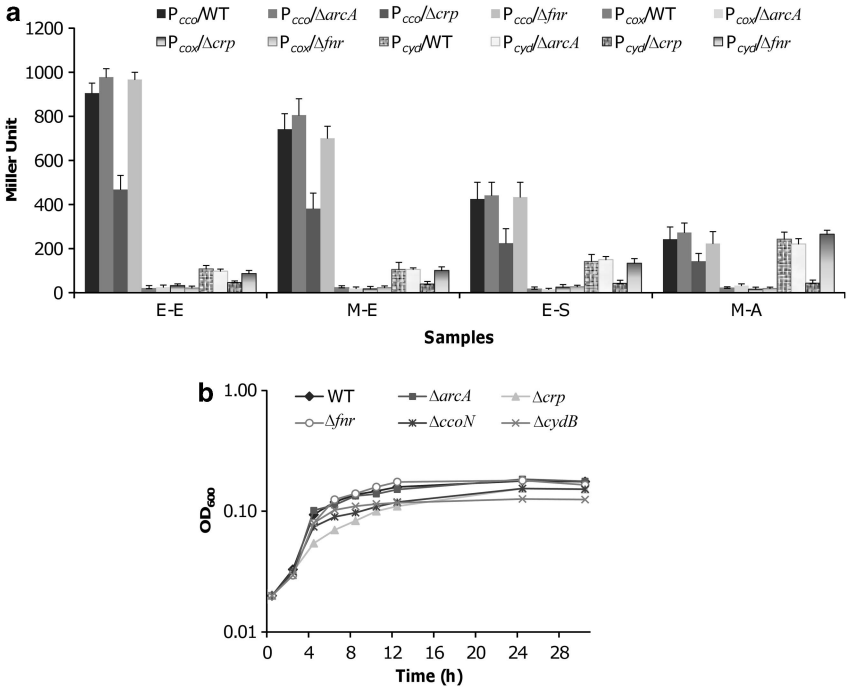

Figure 5 Impacts of ArcA, Fnr and Crp on oxidases in *S. oneidensis*. (**a**) Promoter activities of the *cco*, *cox* and *cyd* operons in strains lacking ArcA, Fnr or Crp, respectively. Cells were prepared the same as in Figures 3b and 4. (**b**) Growth of the *S. oneidensis* wild-type and mutant strains under microaerobic conditions. All strains were grown in the defined medium with 1% O_2 in the gas atmosphere and growth was monitored at OD_{600}. Strains with indistinguishable phenotypes: WT = Δ*arcA* = Δ*fnr* = Δ*arcA*Δ*fnr*; Δ*crp* = Δ*arcA*Δ*crp* = Δ*crp*Δ*fnr* = Δ*arcA*Δ*crp*Δ*fnr*. For clarity, only wild-type and single mutants Δ*arcA*, Δ*crp* and Δ*fnr* are presented in the figure and error bars (s.d. < 5% of presented data, $n \geqslant 3$) are omitted.

suggested to exist in all bacteria with exception of *Thermotogales*, *Deinococcales* and *Firmicutes* on the basis of the occurrence of CcoN and CcoO (Ducluzeau *et al.*, 2008). In a number of species, cbb_3-HCO is the sole HCO, and more rarely, as in *Helicobacter pylori*, serves as the only terminal oxidase (Pitcher and Watmough, 2004; Ekici *et al.*, 2012). In these cases, it is not surprising that cbb_3-HCO have been found to be highly expressed under O_2-saturating conditions (Swem and Bauer, 2002). However, in most bacteria carrying cbb_3-HCO, it coexists with other HCO(s), which are preferentially expressed over cbb_3-HCO under O_2-rich conditions (Pereira *et al.*, 2001; Han *et al.*, 2011). Here, we report on a new twist on the utilization of terminal oxidases during aerobic growth. *S. oneidensis* predominantly applies cbb_3-HCO for respiration of O_2, whereas caa_3-HCO is dispensable, likely due to the substantial difference in their expression.

We present evidence suggesting that transcription of cbb_3-HCO is directly proportional to the O_2 level and the *bd*-type terminal oxidase is preferentially expressed under microaerobic conditions. This is not surprising because cbb_3-HCO is thought to have a lower affinity for O_2 than the *bd*-type terminal oxidase, as evidenced in *R. capsulatus*, which contains these two enzymatic complexes only (Swem and Bauer, 2002). In *S. oneidensis*, the *bd*-type terminal oxidase alone, although expressed at an elevated level in the absence of cbb_3-HCO, supports impaired growth under O_2-saturating conditions. Along with findings that the *bd*-type oxidases are found to account for nitric oxide resistance, we believe that the oxidase primarily has alternative functions relevant to physiology, such as adaptation to a wide variety of stress conditions (Giuffrè *et al.*, 2012; Fu *et al.*, 2013).

Why does caa_3-HCO lose its primary position in aerobic respiration of *S. oneidensis*? By using O_2 as an electron acceptor, facultative anaerobic bacteria like *S. oneidensis* conserve larger amount of energy in comparison with other EAs, thereby supporting much better growth. As a result, respiration of EAs other than O_2 requires low O_2 environments, as evidenced by findings that the expression of some terminal reductases is not allowed or limited under O_2-rich conditions (Baraquet *et al.*, 2009; Dong *et al.*, 2012). To survive and proliferate at submicromolar O_2 levels, *S. oneidensis* utilizes the C-family heme-copper oxidase that can likely either tolerate or adapt to low O_2 environments. In this regard, we propose two evolutionary mechanisms underlying the loss of caa_3-HCO from *S. oneidensis*. The enzyme may not be advantageous in its O_2-limited natural habitat, given its low affinity for O_2, thereby relieving any selective pressure to retain it. Alternatively, the loss of caa_3-HCO may have decreased the competitiveness of *S. oneidensis* in O_2-rich environments, forcing it to occupy redox-stratified niches.

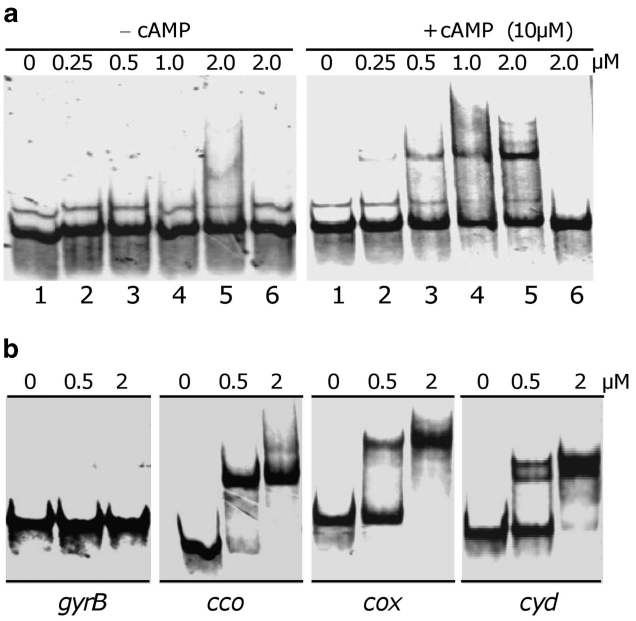

Figure 6 Crp binding to selected promoters by EMSA. (**a**) Interaction of the *dms* promoter DNA with *S. oneidensis* His-tagged Crp. The probe was prepared by PCR with ^{33}P end-labeled primers. The EMSA assay was performed with 2 nM ^{33}P end-labeled probes and various amounts of Crp (left panel) or Crp and cAMP (right panel). The protein concentrations for lanes 1–5 are 0, 0.25, 0.5, 1.0, 2.0 μM, respectively. Non-specific competitor DNA (0.2 μg poly dI·dC) was added to all lanes and specific competitor (10 μM unlabeled *dms* probe) was added (lane 6). (**b**) The binding assay was performed in the presence of 0, 0.5 or 2 μM Crp, 10 μM cAMP, and 2–5 nM radiolabeled promoter DNA. 0.2 μg μl^{-1} poly(dI·dC) was used in all these binding reactions to block nonspecific interactions. Promoter region of *gyrB* was used as negative control.

In *S. oneidensis*, multiple lines of evidence suggest that reduced O_2 concentration, and the lowered internal energetic status that results, are being sensed such that expression of different terminal oxidases can be regulated. Intriguingly, neither Fnr nor ArcA has a significant role in regulation of these oxidases. In the case of Fnr, although this can be readily explained by the inactivation of the protein in the presence of O_2, previous studies suggest that the regulator is not of significance in physiology in general (Maier and Myers, 2001; Cruz-Garcia *et al*., 2011). Unlike its *E. coli* counterpart, ArcA of *S. oneidensis* shows profound impacts on aerobic growth without directly mediating expression of any of the terminal oxidases (Gao *et al*., 2008a, 2010b). The growth defect of an *arcA*-null mutant has been suggested to result from a reduced rate of protein synthesis (Yuan *et al*., 2012). By contrast, Crp has a predominant role in mediating the expression of different terminal oxidases. Unlike its *E. coli* counterpart, which is mainly responsible for the activation of genes involved in the catabolism of organic carbon substrates, *S. oneidensis* Crp appears to control genes that are functionally more diverse (Saffarini *et al*., 2003; Görke and Stülke, 2008; Murphy *et al*., 2009; Murphy and Saltikov, 2009). Nevertheless, the EMSA results presented here are consistent with previously reported *in vivo* data (Charania *et al*., 2009), and argue for an identical mechanism for the activation of Crp. It appears that the low internal energetic status favors the production of cAMP, as evidenced by a twofold increase in Crp under O_2-limited conditions, and Crp primarily functions under anaerobic conditions (Gao *et al*., 2010b). Regulation by cAMP-Crp may be particularly critical in adaptation of *S. oneidensis* to redox-stratified environments, as the expression of different electron transport chains can fluctuate with the levels of intracellular cAMP. We speculate that metabolic fine-tuning offered by this differential regulatory mechanism is advantageous over on-off switching by Fnr and/or Arc, especially for microorganisms that are competitively inferior to those with an aa_3-type oxidase.

Conflict of Interest

The authors declare no conflict of interest.

Acknowledgements

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