Reduced expression of cytochrome oxidases largely explains cAMP inhibition of aerobic growth in *Shewanella oneidensis*

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Inhibition of bacterial growth under aerobic conditions by elevated levels of cyclic adenosine 3′,5′-monophosphate (cAMP), first revealed more than 50 years ago, was attributed to accumulation of toxic methylglyoxal (MG). Here, we report a Crp-dependent mechanism rather than MG accumulation that accounts for the phenotype in *Shewanella oneidensis*, an emerging research model for the bacterial physiology. We show that a similar phenotype can be obtained by removing CpdA, a cAMP phosphodiesterase that appears more effective than its *Escherichia coli* counterpart. Although production of heme c and cytochromes c is correlated well with cAMP levels, neither is sufficient for the retarded growth. Quantities of overall cytochromes c increased substantially in the presence of elevated cAMP, a phenomenon resembling cells respiring on non-oxygen electron acceptors. In contrast, transcription of Crp-dependent genes encoding both cytochromes bd and cbb₃ oxidases is substantially repressed under the same condition. Overall, our results suggest that cAMP of elevated levels drives cells into a low-energetic status, under which aerobic respiration is inhibited.

Among living organisms, prokaryotes thrive in every potential habitat on the Earth suitable for life because of their unparallel metabolic diversity. In many bacteria, central to regulation of metabolism is the cAMP (cyclic adenosine 3′,5′-monophosphate)-Crp (cAMP receptor protein) regulatory system, as clearly illustrated in many bacteria, *Escherichia coli* in particular.¹² The primary role of the canonical cAMP-Crp system, revealed mostly by early studies on *Escherichia coli*, is to regulate uptake of preferred carbon sources and repression of genes required for utilization of less preferred ones, a process called carbon catabolite repression (CCR)³. However, this turns to be only the tip of the iceberg as more and more biological processes are reported to be regulated by the system in diverse bacteria.¹ By using a robust top-down physiological approach, You et al. recently demonstrated that the physiological function of the cAMP-Crp system is to coordinate the allocation of proteomic resources with different metabolic demands in different nutrient environments.⁴ Although environmental cues that modulate cAMP signals vary depending on species or even strains (for example, several α-ketoacids in *E. coli*), there is a possibility that most, if not all, of relevant bacteria use the cAMP-Crp system the same way that *E. coli* does. That is, the transcription of Crp-dependent genes could be differently regulated by altered cAMP levels in response to environmental changes.

*Shewanella*, a genus of Gram-negative γ-proteobacteria thriving in diverse environments, possess highly adaptable metabolism, a quality that could be exploited for potential applications in bioremediation of heavy metals and energy generation via fuel cells.⁵⁶ While this subject has been a focus for more than two decades, the genus is now emerging as an important research model for general bacterial physiology. Many physiological traits displayed by shewanellae, mostly based on studies of the genus representative *Shewanella oneidensis*, are distinct, not found in *Escherichia coli* and other well-characterized model microorganisms. In addition, shewanellae are regarded as a reservoir for antibiotic resistance and the number of *Shewanella* species identified as pathogenic to animals including human being has been increasing with time.⁷⁸

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S. oneidensis is a strictly respiratory organism because the gene encoding 6-phosphofructokinase (PFK), an essential enzyme of glycolysis, is missing. Moreover, the ability of S. oneidensis to utilize five- and six-carbon carbohydrates is rather poor because of the scarcity of enzymes for such sugars and their transport. Despite this, the bacterium, probably all of shewanellae, is regarded respiratory versatile because it derives energy by coupling organic matter oxidation to the respiration of an array of terminal electron acceptors (EAs), such as oxygen, fumarate, nitrate, and metal oxides. To date, how this bacterium adopts different metabolic modes in response to the availability of different EAs has been intensively studied, and some progresses have been made. First, Fnr (fumarate/nitrate regulator), whose E. coli counterpart is the major player in respiration, has no significant role in bacterial physiology. Second, S. oneidensis uses the Arc (aerobic respiration control) system for regulating aerobic respiration without affecting genes in the tricarboxylic acid (TCA) cycle. Third, it is evident that Crp is crucial in respiration because crp mutants are defective in utilizing several EAs, including oxygen, Fe³⁺, Mn⁴⁺, nitrate, nitrite, fumarate, and dimethyl sulfoxide (DMSO).

During aerobic respiration, the primary targets of the cAMP-Crp regulatory system are genes encoding terminal reductases, including those reducing oxygen but traditionally called as cytochrome oxidases. Cytochrome oxidases generate energy by coupling the oxidation of a respiratory substrate such as a c-type cytochrome or quinol to the reduction of oxygen to water. Like in most bacteria, there are multiple cytochrome oxidases in S. oneidensis, two cytochrome c oxidases (a aa₃-type and a cbb₃-type) and a bd-type quinol oxidase. For respiration of oxygen, cytochrome cbb₃ is the predominant system whereas cytochrome aa₃ is not of significance. Cytochrome bd, on the other hand, appears to mainly facilitate adaptation to a variety of stress conditions, especially nitrite, although it is able to support growth when cytochrome cbb₃ is absent.

Initially observed in E. coli and later in other bacteria, aerobic growth is impeded when cAMP is present at concentrations of 0.5 mM or higher with certain sugars as carbon sources. This effect of cAMP is attributed to accumulation of methylglyoxal (MG), which is a toxic intermediate produced from dihydroxyacetone phosphate (DHAP) by MG synthase (MGS), a product of crp.

We have found by chance, in the course of studies on the CAMP-CRP regulation of genes for nitrate and nitrite reductases, that cAMP at 2 mM also retarded aerobic growth in S. oneidensis. However, a gene encoding an E. coli MGS homologue is missing in the S. oneidensis genome. Thus, possibilities for the growth defect associated with cAMP include i) another protein functioning as MGS if MG is responsible, and ii) a different mechanism. In this report, we describe the investigation of the subject. Our results demonstrate that cAMP at elevated levels retards growth mainly by compromising transcription of Crp-dependent genes for both the cytochrome cbb₃-type and bd oxygen oxidases.

Methods

Bacterial strains, plasmids and culture conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Sequences of the primers used in this study are available upon request. All chemicals are from Sigma-Aldrich Co. unless otherwise noted. E. coli and S. oneidensis were grown aerobically in Lysogeny broth (LB, Difco, Detroit, MI) at 37 and 30 °C for genetic manipulation. When appropriate, the growth medium was supplemented with the following: 2,6-diaminopimelic acid (DAP), 0.3 mM; ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; gentamycin, 15 μg/ml; and/or kanamycin, 50 μg/ml; gentamycin, 15 μg/ml.

Growth of S. oneidensis strains under aerobic conditions was measured at 600 nm (OD₆₀₀) in either LB or MS defined medium, which contains 30 mM lactate as electron donor used as previously described. For aerobic growth, mid-log phase cultures were inoculated into fresh media to an OD₆₀₀ of ~0.02 and shaken at 200 rpm at 30 °C.

In-frame mutant construction and complementation. In-frame deletion strains were constructed using the att-based fusion PCR method as described previously. In brief, two fragments flanking the genes of interest were amplified by PCR, and then linked by a second round of PCR. The fused fragments were introduced into plasmid pHGM01 using the Gateway BP clonase II enzyme mix (Invitrogen) according to the manufacturer’s instruction. Vectors carrying mutational constructs in E. coli WM3064, were subsequently transferred into S. oneidensis via conjugation. Integration of the mutagenized constructs into the chromosome was selected by resistance to gentamycin and confirmed by PCR. These 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 deletions of the target genes. Mutants were verified by sequencing the region containing the intended mutations.

Plasmids pHG101 were used for genetic complementation of the mutants. Wild-type genes and their adjacent promoters, were generated by PCR and cloned into pHG101. For inducible gene expression, genes of interest generated by PCR were introduced into pHGE-Ptac under the control IPTG-inducible promoter. After verification by sequencing, the vectors were transferred into the relevant strains via conjugation for complementation and/or expression.

Chemical assays. Cultures of 3 ml grown to an OD₆₀₀ of ~0.2 were subjected to filtering through a 0.22 μm nylon membrane for separation of cells and cell-free filtrate. The filtrate was immediately for cAMP assay, which was performed by using a commercially available kit (cAMP direct immunoassay kit, BioVision, http://www.biovision.com/camp-direct-immunoassay-kit-colorimetric-2862.html) according to the manufacturer’s instructions. The external cAMP levels were used to estimate the cAMP excretion rate by multiplying the specific growth rate and normalizing to OD₆₀₀ values as described elsewhere. The relative cAMP excretion rate for each mutant strain was given by comparing to that of the wild-type, representing the relative internal cAMP level because it is proportional to the cAMP excretion rate. Amounts of MG and heme c from cells were measured following the procedures described elsewhere. Standard curves were made with commercial agents each time.
Viability assay. S. oneidensis strains grown to an OD$_{600}$ of ∼0.2 were incubated with 0.4 mM MG or 4 mM cAMP for half an hour, then adjusted to approximately 10$^7$ CFUs/ml, and followed by 10-fold serial dilutions. Ten microliters of each dilution was spotted onto LB plates. For nitrite susceptibility assay, ten microliters of each dilution of the untreated was spotted onto LB plates containing 5 mM nitrite. The plates were incubated at 30 °C before being read.

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

SDS-PAGE and heme-staining. Unless otherwise noted, mid-log phase cells were harvested, washed with phosphate buffered saline (PBS), resuspended in the same buffer, and sonicated. Protein concentrations of the cell lysates were determined by the bicinchoninic acid assay (Pierce Chemical). The cell lysates were resolved by SDS-PAGE using 12% polyacrylamide gels and stained with 3,3$′$,5,5$′$-tetramethylbenzidine (TMBZ) as described elsewhere.

Promoter activity assay. The activity of various promoters was assessed using a single-copy integrative lacZ reporter system as described previously. A fragment containing the sequence upstream of each operon from −300 to +1 (relative to the translation start codon) was amplified and cloned into the reporter vector pHGEI01 and verified by sequencing. These plasmids were then transferred by conjugation into relevant S. oneidensis strains. Plasmid pHGEI01 containing promoters of interest integrates into the chromosome and the antibiotic marker is then removed by an established approach. Cells grown to the mid-log phase were collected and β-galactosidase activity assays were performed with an assay kit as described previously.

| Strain or plasmid | Description | Source or reference |
|-------------------|-------------|---------------------|
| **E. coli strain** |             |                     |
| DH5α              | Host strain for plasmids | Lab stock          |
| WM3064            | Donor strain for conjugation; ΔdapA | W. Metcalf, UIUC   |
| **S. oneidensis strain** |             |                     |
| MR-1              | Wild type | Lab stock          |
| HG0266            | ΔccmF derived from MR-1 | 26                 |
| HG0624            | Δerp derived from MR-1 | 11                 |
| HG2364            | ΔccoN derived from MR-1 | 17                 |
| HG3901            | ΔcpdA derived from MR-1 | This study         |
| HG3901            | Δcya (ΔcyaAΔcyaBΔcyaC) derived from MR-1 | This study |
| HGGOO              | Δcso (ΔcsoNOPQ) derived from MR-1 | 8                 |
| HG3901            | Δcysl (ΔcysABX) derived from MR-1 | 21                 |
| HG3901-0266       | ΔcpdAΔccmF derived from MR-1 | This study         |
| HG3901-0624       | ΔcpdAΔerp derived from MR-1 | This study         |
| HG3901-CYA        | ΔcpdAΔcya derived from MR-1 | This study         |
| HG3901-CCCO       | ΔcpdAΔcco derived from MR-1 | This study         |
| HG3901-CYD        | ΔcpdAΔcysl derived from MR-1 | This study         |
| **Plasmid**       |             |                     |
| pHGM01            | Ap$^r$ Gm$^r$ Cm$^r$ suicide vector | 26                 |
| pHGE-Ptac         | IPTG-inducible P_tac expression vector | 28                 |
| pHGEI01           | Integrative lacZ reporter vector | 33                 |
| pHGE-Ptac-cpdA    | Vector for expressing cpdA | This study         |
| pHGE-Ptac-EcpdA   | Vector for expressing E. coli cpdA | This study       |
| pHGE-Ptac-hemA    | Vector for expressing hemA | This study         |
| pHGE-Ptac-ccmF    | Vector for expressing ccmF | This study         |
| pHGE-Ptac-cyd     | Vector for expressing cydARX | 8                  |
| pHGEI01-cpdA      | Vector for measuring cpdA expression | This study |
| pHGEI01-hemA      | Vector for measuring hemA expression | This study |
| pHGEI01-ccmF      | Vector for measuring ccmF expression | This study |
| pHGEI01-cco       | Vector for measuring cco expression | This study |
| pHGEI01-cyd       | Vector for measuring cyd expression | This study |
| pHGEI01-hemG2     | Vector for measuring hemG2 expression | This study |
| pHGEI01-hemC      | Vector for measuring hemC expression | This study |
| pHGEI01-ccmA      | Vector for measuring ccmA expression | This study |

Table 1. Strains and plasmids used in this study.
MG (0–0.4 mM) was inoculated with mid-log phase *S. oneidensis* cultures (∼0.2 of OD<sub>600</sub>), incubated (200 rpm) under aerobic conditions. (B) Viability assessment. Mid-log phase *S. oneidensis* cultures were incubated with 4 mM cAMP or 0.4 mM MG, serially diluted, and plated on LB plates. Photos were taken after 24 h. All experiments were performed at least three times with standard deviations presented as error bars in (A) and representative results presented in (B).

**Figure 1.** Effect of cAMP and MG on *S. oneidensis* physiology. (A) LB broth containing cAMP (0–4 mM) or MG (0–0.4 mM) was inoculated with mid-log phase *S. oneidensis* cultures (∼0.2 of OD<sub>600</sub>), incubated (200 rpm) under aerobic conditions. (B) Viability assessment. Mid-log phase *S. oneidensis* cultures were incubated with 4 mM cAMP or 0.4 mM MG, serially diluted, and plated on LB plates. Photos were taken after 24 h. All experiments were performed at least three times with standard deviations presented as error bars in (A) and representative results presented in (B).

**Other analyses.** Student’s *t* test was performed for pairwise comparisons. Values are presented as means +/- standard deviation (SD) in the relevant figures.

**Results**

**Growth inhibition by cAMP is not due to accumulation of methylglyoxal in *S. oneidensis*.** This investigation began with the chance observation that cAMP at 2 mM significantly retards aerobic growth of *S. oneidensis* in LB broth. To further assess the effect of cAMP on growth, we added cAMP of varying concentrations into liquid cultures (∼0.05 of OD<sub>600</sub>) prepared from the mid-log phase cells and monitored the consequences (Fig. 1A). While the addition of 1 mM cAMP hardly affected growth, the molecule at higher concentrations (2 and 4 mM) inhibited growth significantly and inhibition increased with cAMP levels. A similar trend was observed from the MS defined medium, but inhibition appeared more severe, with no visible growth in concentrations (2 and 4 mM) inhibited growth significantly and inhibition increased with cAMP levels. A similar trend was observed from the MS defined medium, but inhibition appeared more severe, with no visible growth in the presence of 4 mM cAMP (Fig. S1). Nevertheless, in both cases cell densities increased constantly when growth trend was observed from the MS defined medium, but inhibition appeared more severe, with no visible growth in.

To rule out the possibility that MG underlies the growth defect in the presence of cAMP, we assessed impact of MG on growth. As shown in Fig. 1A, influences of CAMP and MG on growth were clearly different. We then examined MG on viability with cells prepared the same as above. Cells incubated with 0.4 mM MG for half an hour exhibited significantly reduced viability (Fig. 1B). Furthermore, despite of the lack of an *E. coli* MGS homologue, we examined levels of MG produced endogenously. In either rich or defined medium containing cAMP at concentrations that displays the strongest inhibition, MG was below the detection limit (data not shown). Thus we concluded that cell viability is not affected significantly by cAMP, distinct from the fact that *E. coli* cells die rapidly because of the MG accumulation<sup>22</sup>. These contrasting phenotypes suggest that the growth defect of *S. oneidensis* resulting from exogenous cAMP may not be due to a toxic metabolite.

To further provide evidence for the role of *S. oneidensis* CpdA as a cAMP phosphodiesterase, we assayed cAMP levels in relevant strains. Consistent with a previous report about an *E. coli* Δ*cpdA* strain<sup>16</sup>, intracellular levels of cAMP in an *S. oneidensis* Δ*cpdA* strain increased by over 2.5-fold (Fig. 2B). When either the *S. oneidensis* or *E. coli* cpdA gene was expressed, cAMP levels reduced greatly. Notably, with IPTG at 0.2 mM, cAMP levels between cells expressing the *S. oneidensis* cpdA and *E. coli* cpdA gene differed markedly, implying a difference in the efficacy of these two enzymes. Together with functional prediction based on the sequence, these data manifest that *S. oneidensis* CpdA functions to decompose cAMP.
Figure 2. *S. oneidensis* CpdA is a cAMP phosphodiesterase. (A) Complementation of growth defect of the *cpdA* mutant in LB broth with the wild-type as control (WT). *S. oneidensis cpdA* (So*cpdA*) and *E. coli* cpdA (*EccpdA*) were placed behind the IPTG-inducible P_{lac} promoter as described in the experimental procedures. The *cpdA* mutants carrying empty vector (vec), *S. oneidensis cpdA* (So*cpdA*) and *E. coli* cpdA (*EccpdA*) were examined without IPTG or with 0.2 mM IPTG. (B) cAMP levels in cultures in (A). The averaged cAMP level in WT was set to 100%, to which cAMP levels in other strains were normalized. Experiments were performed at least three times with error bars representing the standard deviation.

**Growth inhibition by cAMP is dependent on CRP in *S. oneidensis***. To unravel the mechanism responsible for the cAMP inhibition in *S. oneidensis*, we first examined whether such effect of cAMP requires Crp. A *crp* deletion strain (Δ*crp*), whose aerobic growth is only slightly impaired, was subject to the analysis of cAMP effect. In contrast to the wild-type, the Δ*crp* strain was resistant to exogenous cAMP with respect to growth (Fig. 3A). As this observation was confirmed by genetic complementation with an integrative system described in our previous study, it supports that Crp is essential for cAMP-induced growth deficiency.

In bacteria, cAMP is synthesized by adenylate cyclases (ACs). The *S. oneidensis* genome encodes three functional ACs, GyaA (SO_4312), GyaB (SO_3778) and Gyac (SO_1329), which have been characterized with respect to cAMP synthesis. Among them, Gyac is the major AC for cAMP production; the loss of all three ACs results in a phenotype similar to that of a *crp* mutant, in line with that both cAMP and Crp are essential to the physiological role of the cAMP-Crp complex. To confirm that the growth defect requires the cAMP-Crp complex, we removed *crp* and *cyt* (all three genes for ACs) from the Δ*cpdA* strain. In contrast to the Δ*cpdA* strain, the newly constructed Δ*cpdAΔcrp* and Δ*cpdAΔcyt* strains displayed normal growth, comparable to that of the Δ*crp* strain (Fig. 3B). Based on these results, we conclude that the growth defect, resulting from either addition of exogenous cAMP or the *cpdA* mutation, is dependent on the cAMP-Crp complex.

**Intracellular CAMP influences quantities of cytochromes c**. *S. oneidensis* colonies are brown-red on plates, largely because of more than 40 c-type cytochromes. Previously, we reported that the loss of Crp decreases the levels of c-type cytochromes approximately by 60%. During this investigation, we noticed that the color of Δ*cyt* colonies (or cell pellets) was similar (Fig. 4A). In contrast, the color of Δ*cpdA* colonies was much deeper, so was the wild-type with 4 mM cAMP. We therefore hypothesized that the levels of c-type cytochromes increase with intracellular cAMP. To test this, heme *c* levels in relevant strains were determined with a Δ*ccmF* mutant used as negative control (Fig. 4A). The *ccmF* gene encodes a cytochrome c heme lyase, which is essential to c-type cytochrome maturation in *S. oneidensis*. Compared to the wild-type, deletion of *cyt* significantly lowered levels of heme *c*, which was comparable to that of the Δ*crp* strain and could be recovered by exogenous cAMP. In contrast, inactivation of *cpdA* elevated levels of heme *c*. Moreover, levels of heme *c* in the Δ*cpdAΔcrp* and Δ*cpdAΔcyt* strains were similar to those of the Δ*crp* and Δ*cyt* strains. This observation was further confirmed by the profile of c-type cytochromes revealed by heme-staining (Fig. 4B). In a word, these data clearly show that the levels of c-type cytochromes in *S. oneidensis* increase with cAMP.

**cAMP-CRP regulates heme biosynthesis and cytochrome c maturation**. To elucidate the mechanism underlying growth defect and/or increased production of c-type cytochromes caused by the *cpdA* mutation, we focused on the heme synthetic pathway and the cytochrome c maturation system. *S. oneidensis* possesses the most common pathway for heme synthesis (Fig. 5A), as illustrated in *E. coli*, which entails nine reactions that convert glutamyl-tRNA to protoporphyrin IX. Interestingly, there are multiple candidates for HemB, HemG, and HemH. To determine which of heme synthetic genes are affected by cAMP, we monitored abundance of the transcripts of these heme genes by qRT-PCR provided that they are not organized into operons except hemC and hemD. In the Δ*cyt* and Δ*cpdA* strains, the *hemA* gene was repressed and induced approximately 2-fold respectively, whereas the other heme genes were affected insignificantly (Fig. 5A). To confirm this observation, we used a lacZ-reporter to assay β-galactosidase activities driven by *hemA*, *hemG2*, and *hemC* promoters. Although robustness of these promoters differed substantially, they showed the same trend as observed from qRT-PCR (Fig. 5B).

HemA (glutamyl-tRNA reductase) catalyzes the first dedicated, rate-limiting step in heme synthesis. To test whether HemA accounts for the phenotype of the Δ*cyt* and Δ*cpdA* strains, we placed the *hemA* gene under the control of IPTG-inducible promoter P_{lac} to examine effects of HemA of varying quantities on heme *c* levels.
Surprisingly, HemA influenced the heme $c$ levels in a dose-dependent way (Fig. 5C). With IPTG at no more than 0.05 mM, the heme $c$ levels increased with HemA but further enhanced production of HemA by IPTG at 0.1 mM and above played an inhibitory role, resulting in significant reduction in heme $c$ levels.

Altered production of HemA also had an apparent impact on growth (Fig. 5D). When IPTG was added to levels more than 0.1 mM, growth was significantly retarded. In contrast, HemA induced by IPTG at 0.05 mM or lower did not exert any negative effect on growth.

We then examined whether the cytochrome $c$ maturation system may be the cause for growth defect and increased heme $c$ levels of the $cpdA$ mutant. In contrast to the $hem$ genes, the $ccm$ genes are organized into three operons, $ccmABCDE$, $ccmI$, and $ccmFGH$. qRT-PCR analysis of the transcript of the $ccmA$, $ccmI$, and $ccmF$ genes revealed that the $ccmF$ operon but not others was affected by the both $cya$ and $cpdA$ mutations (Fig. 5A). This observation was then confirmed by using the $lacZ$-reporter (Fig. 5B). Interestingly, forced production of CcmF by IPTG displayed an effect on heme $c$ levels similar to that observed from HemA, although it appeared milder in the overproduction end (Fig. 5C). Consistently, growth was also similarly impacted (Fig. 5D). All together, these data suggest that HemA and CcmF, when present in certain range, can modestly affect quantities of $c$-type cytochromes, but in large excess exert a significant negative impact on $c$-type cytochrome production. Despite this, it is clear that neither of these two proteins appears to be critical for the growth defect of the $cpdA$ mutant because their overproduction compromises quantities of $c$-type cytochromes.

**cAMP in excess inhibits activity of both cytochrome bd and cbb$_3$ oxidases.** To look further for answers addressing the growth defect of the $cpdA$ mutant, we turned to cytochrome oxidases because these enzymes provide proton motive force for energy under aerobic conditions. Moreover, prior studies showed that the functional oxidases, $bd$-type (encoded by $cydABX$) and $cbb$_3-type (encoded by $ccoNOPQ$), are under the direct control of the cAMP-Crp complex. To test activities of the $bd$-type and $cbb$_3-type oxidases in the $ΔcpdA$ strain, we performed nitrite susceptibility assay and Nadi plate assay, respectively. Consistently with the notion that cytochrome $bd$ confers resistance to nitrite in $S. oneidensis$, a $cyd$ null mutant ($Δcyd$) was hypersensitive to nitrite (Fig. 6A). Like the $Δcrp$ and $Δcya$ strains, the $ΔcpdA$ strain displayed substantially increased susceptibility...
to nitrite, and loss of both crp and cpdA did not further elevate susceptibility. Importantly, this increased susceptibility due to the CpdA loss was restored to the level of wild-type by its expression in trans, only in the presence of cytochrome bd. Moreover, the phenotype was also complemented by forced production of cytochrome bd.

In the case of the cytochrome cbb3, similar results were obtained. With Nadi assay, which specifically detects cytochrome c oxidase-dependent respiration, we visualized activities of the cytochrome cbb3 in relevant strains (Fig. 6B). As shown before, loss of Crp compromised the cytochrome cbb3 activity. Surprisingly, the cytochrome cbb3 activity was most drastically reduced in the ΔcpdA strain, with the indophenol blue ring barely visible in one minute. This severe defect was dependent on Crp as the ΔcpdAΔcrp strains were indistinguishable.

Under standard conditions, the cytochrome bd is dispensable for aerobic growth of the S. oneidensis wild-type. However, this was not observed with the cpdA mutation as the ΔcpdAΔcyd strain had growth defect more severe than the ΔcpdA strain (Fig. 6C), suggesting that the cytochrome bd is crucial for supporting growth when the cpdA gene is absent. Similar results were obtained from the ΔcpdAΔccoN strain (Fig. 6D). Notably, the ΔcpdAΔcco strain had the slowest growth rate when compared to the wild type, ΔcpdA and ΔcpdAΔcyd strains, suggesting that in the ΔcpdA strain the cytochrome cbb3 still plays a predominant role in supporting growth as in the wild-type. These data, collectively, indicate that activities of both cytochrome bd and cbb3 oxygen reductases are impaired in the ΔcpdA strain, leading to growth deficiency.

To unravel the mechanism for reduced activities of both oxidases, we examined their expression levels in the ΔcpdA strain. As shown in Fig. 6E, there was no difference in activities of the cyd promoter in strains lacking any of tested genes, crp, cya, cpdA, or even two of them combined, suggesting that CAMP in absent and in excess has a similar regulatory effect on cyd expression. On the contrary, loss of crp or cya resulted in a modest reduction in cco expression but CAMP in overabundance was more detrimental. Altogether, these results suggest that the growth defect of the cpdA mutant is due to reduced production of both cytochrome bd and cbb3.

**Discussion**

Cyclic nucleotides act as second messengers in diverse signaling cascades throughout all kingdoms of life, among which CAMP is first discovered and most extensively studied in bacteria. The actions of CAMP are mediated by downstream CAMP-binding proteins, which are involved in diverse processes. In bacteria, the central to CAMP-mediated regulation is formation of the CAMP-Crp complex, a transcriptional regulator of a number of metabolic operons, including those involved in the transport of substrates, glycolysis, the tricarboxylic acid cycle,
and aerobic respiration. It has been proposed that a key physiological role of the cAMP-Crp complex is to ensure the proteomic resources to be spent on distinct metabolic sectors as needed in different nutrient environments. As a consequence, some carbon sources are transported and utilized when cAMP levels are manipulated. One of such examples is xylose, which can be converted to be DHAP, leading to production of toxic MG and thereby growth defect. However, this is not the case in S. oneidensis. As our data presented here eliminate the possibility that MG is accountable for the growth phenotype in S. oneidensis, a different mechanism must exist.

The purpose of this study was to unravel the mechanism. The study was facilitated by an S. oneidensis cpdA mutant that stably maintains intracellular cAMP at levels sufficiently high to elicit a similar growth defect as the wild-type with 2 ~ 4 mM cAMP.

S. oneidensis CpdA, a homolog of the E. coli counterpart, is verified to be a cAMP phosphodiesterase by cross-complementation. It should be noted that the E. coli CpdA could not fully complement the phenotype of the S. oneidensis cpdA mutant. With IPTG at 2 mM, forced production of S. oneidensis CpdA reduces the cAMP concentration below the wild-type level whereas cells producing E. coli CpdA exhibit only a 2-fold decrease in cAMP concentration (Fig. 2). This may not be surprising as the cAMP phosphodiesterase activity of E. coli CpdA is poor, with a rather high Km for cAMP (~500 μM) relative to intracellular cAMP concentration. Thus, at least in the context of in vivo data presented here, S. oneidensis CpdA functions more effectively than its E. coli counterpart.

As the growth defect of S. oneidensis caused by cAMP at elevated concentrations is dependent on Crp, we adopt the non-hypothesis-driven approach of testing whether some members of the cAMP-Crp regulon might be accountable for the defect when expressed differently. In addition to growth defect, the cpdA mutant, as well as strains lacking either ACs or Crp, differs from the wild-type in color of colony/pellet, which largely reflects the

Figure 6. cAMP in excess inhibits cytochromes cbb3 and bd. (A) Effect of cAMP on susceptibility of indicated strains to 5 mM nitrite on LB plates. Cytochrome bd confers S. oneidensis resistance to nitrite. ∆cpdA/cpdA, ∆cpdA∆crp/cpdA, and ∆cpdA∆crp/cyd represent indicated mutants expressing cpdA or cyd by IPTG at levels sufficiently high for successful complementation as described in the text or previous publications. (B) Effect of cAMP on cytochrome cbb3 activity of indicated strains on LB plates by the Nadi assay. The method is based on the rapid formation of indophenol blue from colorless a-naphthol catalyzed by cytochrome c oxidase, using N,N’-dimethyl-p-phenylenediamine monohydrochloride as an exogenous electron donor. Photos were taken at indicated times after the reaction started. The wild-type and ∆ccoN strains serve as positive and negative controls. (C) Growth of ∆cpdA∆cyd in LB. (D) Growth of ∆cpdA∆cco in LB. (E) Impacts of cAMP on expression of cyd and cco operons. Activities of the cyd and cco operon promoters from mid-log phase samples were assayed by a lacZ-reporter system used previously. ∆cpdA/cpdA, and ∆cpdA∆crp/cpdA represent indicated mutants expressing cpdA by IPTG at levels sufficiently high for successful complementation as described in the text or previous publications. All experiments were performed at least three times with standard deviations presented as error bars or similar results were obtained.
cellular amount of c-type cytochromes. Apparently, cAMP levels correlate well with overall production of c-type cytochromes. Given that reduced quantities of c-type cytochromes, as due to loss of either AcP or Crp, do not significantly impede aerobic growth, we tested whether c-type cytochromes in increased production could lead to retarded growth under aerobic conditions.

Amounts of c-type cytochromes are determined by two systems, responsible for the heme synthesis and cytochrome c maturation respectively. The heme synthesis is carried out by 10 enzymes (HemN and HemF for the same reaction under different conditions), of which only a few are found to be conditionally inducible. In the present study, we found that hemA, whose product catalyzes the rating-limiting step, is induced about 2-fold in the cpdA mutant. Previous studies have shown that HemA in Salmonella typhimurium responds to heme availability at the level of protein lifetime, when heme is abundant, it binds to HemA to promote degradation of the latter. 

While the stabilization of S. oneidensis HemA is also an issue remains unknown, its induction by increased concentrations of cAMP, to our knowledge, is unprecedented. Seemingly, this regulation by cAMP-Crp is indirect because by prediction there is no Crp-binding site located upstream of the hemA gene. However, given the negative effect of overproduced HemA on overall amounts of c-type cytochromes, the possibility that HemA plays an important role in the growth defect appears small. Intriguingly, this is also true of CcmF, the cytochrome c lyase. We therefore conclude that neither heme synthesis nor cytochrome c maturation is accountable for growth defect or increased level of c-type cytochromes observed from the cpdA mutant.

Rather, increased levels of heme c may be a result of concerted upregulation of many c-type c genes because more than two thirds of them are predicted to be under the direct control of the cAMP-Crp complex. This surely gains support from heme-staining analysis (Fig. 4). We have previously shown that anaerobic respiration of various EAAs favors overall cytochrome c production, a scenario resembling the cpdA mutant to some extent. It has been suggested that cAMP concentrations increase in response to the low internal energetic status in E. coli, promoting catabolism and inhibiting anabolisms. In S. oneidensis, a similar notion has been proposed. Thus, it seems logic that elevated cAMP drives cells into a low-energy mode, favoring respiration of non-oxygen EAAs. As a consequence, genes encoding proteins important for respiration of oxygen are repressed, such as those for cytochrome cbb3 and bd. We propose that this explains the growth defect.

According to previous reports, S. oneidensis cAMP-Crp binds to DNA motifs similar to its E. coli counterpart whereas Crp alone fails in binding. Data presented here reveal that cAMP at varying levels impacts expression of Crp-regulon members differently. The cyd operon (bd) behaves the same in cAMP-deficient and -overproduction strains whereas the cco operon (cbb3) is further repressed by increased concentrations of cAMP. This is consistent with the finding that S. oneidensis Crp functions in a dose-dependent manner. A study of E. coli demonstrates that cAMP-binding has a biphasic effect on site-specific DNA-binding by Crp.

In recent years, cAMP-Crp complexes with distinct features have been found. In Mycobacterium, Crp can not only operate at extremely high levels of CAMP, based on the finding that the intracellular CAMP levels are as high as 3–4 mM, but also bind to DNA in a specific manner and regulate transcription without cAMP. In Pseudomonas putida, a bacterium that also has an incomplete glycolysis pathway (lacking PFK), Crp exhibits an affinity binding of cAMP approximately 1000 times higher than that of E. coli Crp. A consequence of these differences is that the Crp regions of these bacteria, including S. oneidensis, differ drastically from that of E. coli, as suggested in P. putida.

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Author Contributions
H.G. conceived the idea and designed the project. J.Y., M.Q. and H.F. carried out the experiments. J.Y., M.Q. and H.G. analyzed data. J.Y., M.Q. and H.G. wrote the paper.

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