

Involvement of a Membrane-Bound Class III Adenylate Cyclase in Regulation of Anaerobic Respiration in *Shewanella oneidensis* MR-1^{∇†}

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Unlike other bacteria that use FNR to regulate anaerobic respiration, *Shewanella oneidensis* MR-1 uses the cyclic AMP receptor protein (CRP) for this purpose. Three putative genes, *cyaA*, *cyaB*, and *cyaC*, predicted to encode class I, class IV, and class III adenylate cyclases, respectively, have been identified in the genome sequence of this bacterium. Functional validation through complementation of an *Escherichia coli* *cya* mutant confirmed that these genes encode proteins with adenylate cyclase activities. Chromosomal deletion of either *cyaA* or *cyaB* did not affect anaerobic respiration with fumarate, dimethyl sulfoxide (DMSO), or Fe(III), whereas deletion of *cyaC* caused deficiencies in respiration with DMSO and Fe(III) and, to a lesser extent, with fumarate. A phenotype similar to that of a *crp* mutant, which lacks the ability to grow anaerobically with DMSO, fumarate, and Fe(III), was obtained when both *cyaA* and *cyaC* were deleted. Microarray analysis of gene expression in the *crp* and *cyaC* mutants revealed the involvement of both genes in the regulation of key respiratory pathways, such as DMSO, fumarate, and Fe(III) reduction. Additionally, several genes associated with plasmid replication, flagellum biosynthesis, and electron transport were differentially expressed in the *cyaC* mutant but not in the *crp* mutant. Our results indicated that CyaC plays a major role in regulating anaerobic respiration and may contribute to additional signaling pathways independent of CRP.

Shewanella oneidensis MR-1 is a metal reducer that uses more than 14 terminal electron acceptors for respiration. These electron acceptors include oxygen, nitrate, fumarate, dimethyl sulfoxide (DMSO), Fe(III) oxides, uranium, and chromium (21, 25, 26). In *Escherichia coli* and other bacteria, the shift from aerobic respiration to anaerobic respiration requires activation of the global transcriptional regulator FNR (11, 34). FNR is an oxygen-sensing protein that is activated under anaerobic conditions by the formation of a [4Fe-4S] cluster (14). The *S. oneidensis* FNR homolog, EtrA, complements an *E. coli* FNR mutant (27) but does not appear to have the same role as the *E. coli* protein in *S. oneidensis* (17). Our previous findings demonstrated that instead of EtrA, the cyclic AMP (cAMP) receptor protein (CRP) controls anaerobic respiration in *S. oneidensis* MR-1 (28). *crp* mutants are deficient in anaerobic respiration of Fe(III), Mn(IV), fumarate, nitrate, and DMSO. Furthermore, fumarate, DMSO, and nitrate reductase activities are either severely decreased or undetectable in the *crp* mutants, suggesting that CRP regulates the expression of these anaerobic reductases (28).

Although genetic and phenotypic data clearly have implicated CRP in the activation of anaerobic reductase systems in *S. oneidensis* MR-1, the mechanisms of this regulation remain unclear. CRP lacks obvious redox-sensing domains and is not expected to respond to changes in oxygen concentrations like FNR. Complementation of the *S. oneidensis* *crp* mutants with

E. coli *crp* indicates that CRP is activated similarly in these two organisms. Furthermore, addition of cAMP to aerobic cultures of *S. oneidensis* MR-1 leads to significant induction of the activity of the anaerobic fumarate reductase (28). Therefore, transcriptional regulation by CRP under anaerobic conditions is likely to be directly linked to adenylate cyclase activity and cAMP production. In this paper, we investigate the role of the *S. oneidensis* adenylate cyclases in the regulation of anaerobic respiration. Genetic, biochemical, and genome-wide transcriptome analyses indicated that although MR-1 has three adenylate cyclases, the membrane-bound class III enzyme, CyaC, appears to play a more significant role in CRP-dependent anaerobic gene expression.

MATERIALS AND METHODS

Bacterial strains and growth conditions. A list of the bacterial strains and plasmids used in this study is given in Table 1. *S. oneidensis* and *E. coli* strains were routinely cultured in Luria-Bertani (LB) medium at 30°C and 37°C, respectively. Antibiotics (20 µg/ml chloramphenicol, 25 µg/ml kanamycin, and 20 µg/ml gentamicin) were added as needed. Anaerobic growth of *S. oneidensis* strains was performed in Hungate tubes filled with minimal medium (28) supplemented with 50 mM lactate and 0.02% Casamino Acids. Disodium fumarate, DMSO, and ferric citrate were used as electron acceptors at a final concentration of 10 mM.

For transcriptome-profiling experiments, O₂-limited cultivation was selected primarily because of the inability of Δ *crp* mutants to grow anaerobically with fumarate or DMSO. Wild-type *S. oneidensis* MR-1 and mutant strains were grown under O₂-limited conditions in chemostats using 6-liter Bioflow 3000 bioreactors (New Brunswick Scientific, Edison, NJ) containing 3 liters of minimal medium (pH 7.0) supplemented with 90 mM DL-lactate and 10 ml of 10× Wolfe's vitamin solution (13). The bioreactors were each inoculated with 1 ml/liter of an overnight LB medium-grown culture and maintained in a batch mode until the late logarithmic stage. Continuous cultures were initiated and maintained at a dilution rate of 0.06/h. The bioreactors were constantly sparged with gas (60% pure N₂, 40% air) at a rate of 4 liters/min. Agitation and temperature were automatically maintained at 150 rpm and 30°C, respectively. The pH was main-

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Description	Reference or source
<i>S. oneidensis</i> strains		
MR-1	Lake Oneida isolate	23
SR660	MR-1 Δ <i>cyaA</i>	This study
SR945	MR-1 Δ <i>cyaB</i>	This study
SR672	MR-1 Δ <i>cyaC</i>	This study
SR665	MR-1 Δ <i>cyaA</i> Δ <i>cyaC</i>	This study
SR946	MR-1 Δ <i>cyaA</i> Δ <i>cyaB</i> Δ <i>cyaC</i>	This study
SR694	MR-1 Δ <i>crp</i>	This study
SR956	SR946 with <i>cyaA</i> in pJB3Cm6, Cm ^r	This study
SR957	SR946 with <i>cyaB</i> in pJB3Cm6, Cm ^r	This study
SR958	SR946 with <i>cyaC</i> in pJB3Cm6, Cm ^r	This study
<i>E. coli</i> strains		
EC100	<i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) <i>recA1</i>	Epicenter Technologies
EC100D ⁺	<i>E. coli</i> EC100 derivative, <i>pir</i> ⁺	Epicenter Technologies
β 2155	<i>pir</i> ::RP4, Km ^r	5
SP850	Δ <i>cyaA1400</i> (::kan)	31
E299	<i>E. coli</i> SP850 with pJB3Cm6, Km ^r Cm ^r	This study
E300	<i>E. coli</i> SP850 with <i>cyaA</i> in pJB3Cm6, Km ^r Cm ^r	This study
E301	<i>E. coli</i> SP850 with <i>cyaC</i> in pJB3Cm6, Km ^r Cm ^r	This study
E347	<i>E. coli</i> SP850 with P _o - <i>cyaB</i> in pJB3Cm6, Km ^r Cm ^r	This study
E456	<i>E. coli</i> SP850 with <i>cyaB</i> in pJB3Cm6, Km ^r Cm ^r	This study
Plasmids		
pJB3Cm6	Cloning and sequencing vector, Cm ^r	2
pDS3.1	R6K <i>ori</i> Gm ^r <i>sacB</i>	38
pPR9TT	Promoter probe vector, <i>lacZ</i> Cm ^r Ap ^r	30
pKB1	<i>cyaC</i> in pJB3Cm6, Cm ^r	This study
pMC2	<i>cyaA</i> in pJB3Cm6, Cm ^r	This study
pMC3	<i>cyaB</i> fused to operon promoter (P _o) in pJB3Cm6	This study
pMC4	<i>cyaB</i> and 300 bp of upstream DNA in pJB3Cm6	This study
pDS10	<i>dms</i> promoter fused to <i>lacZ</i> in pPR9TT	This study
pDS11	<i>fccA</i> promoter fused to <i>lacZ</i> in pPR9TT	This study
pDS12	<i>cyaC</i> promoter fused to <i>lacZ</i> in pPR9TT	This study
pDS13	<i>cyaA</i> promoter fused to <i>lacZ</i> in pPR9TT	This study

tained at 7.0 by automatic addition of 2 M HCl. The dissolved oxygen tension was constantly monitored using an Ingold polarographic oxygen electrode. The onset of O₂ limitation was defined by a decrease in the dissolved O₂ tension to a level below the detection threshold ($\leq 1\%$). To induce the expression of anaerobic respiratory genes, continuous addition of sodium fumarate (final concentration, 10 mM) to the bioreactors was initiated at the onset of steady-state O₂-limited growth. Samples used for RNA isolation and transcriptome analysis were taken after at least five volume changes under steady-state conditions.

Generation of *cya* and *crp* chromosomal deletions. Chromosomal deletions of *crp* and the predicted adenylate cyclase genes were performed using a previously published method (38), with minor modifications. Approximately 1-kb upstream and downstream fragments flanking the target genes were amplified by PCR with Phusion polymerase (New England Biolabs, Ipswich, MA) using the primers listed in Table S1 in the supplemental material. The fragments were fused by overlap extension PCR (12). The fusion fragments generated complete deletions of the *cyaC* and *crp* genes and removed the internal 1,860 bp (bases 435 to 2295) and 430 bp (bases 154 to 585) of *cyaA* and *cyaB*, respectively. The fusion amplicons were ligated into SmaI-digested pDS3.1 (38). Plasmids with the desired inserts were used to transform *E. coli* β 2155 (5) or WM3063 (29) and subsequently transferred to the *S. oneidensis* strains by conjugation. The primary integrants were selected by plating preparations on LB agar supplemented with 25 μ g/ml gentamicin. To isolate mutants with *cya* or *crp* chromosomal deletions, transconjugants were grown on 10% tryptone-5% yeast extract agar plates sup-

plemented with 5% sucrose. Sucrose-resistant colonies were screened for deletion of the genes by PCR.

Complementation of *E. coli* adenylate cyclase mutant. DNA fragments carrying the complete *cyaA* and *cyaC* genes with upstream flanking regions were amplified by PCR using Phusion polymerase and the primers listed in Table S1 in the supplemental material. The fragments were cloned into SmaI-digested broad-host-range vector pJB3Cm6 (2) to generate plasmids pMC2 and pKB1. To clone *cyaB*, which appears to be part of a three-gene operon, we first amplified this gene using primers *cyaBFN1* and *cyaBRE* (see Table S1 in the supplemental material) to introduce an NdeI site at the 5' end of the gene. A second DNA fragment, P_o, which contains the promoter region that lies upstream of *cydDC*, *cyaB*, and an NdeI site at the 3' end, was amplified using primers *cyaBPROF* and *cyaBProR1*. The two fragments (*cyaB* and P_o) were digested with NdeI, ligated, and cloned into SmaI-digested pJB3Cm6 to generate pMC3. We also cloned *cyaB* and 300 bp of DNA directly upstream of this gene to generate pMC4. Plasmids pKB1, pMC2, pMC3, and pMC4 were transferred by electroporation into *E. coli* SP850 (obtained from the *E. coli* Genetic Stock Center, Yale University, New Haven, CT). This strain is deficient in *lacZ* expression because it lacks the adenylate cyclase needed for cAMP production and cannot activate CRP (31). To test for β -galactosidase activity, *E. coli* SP850 cells were grown in LB medium to early mid-log phase in the presence or absence of 3 mM cAMP. Isopropyl- β -D-thiogalactopyranoside (IPTG) was added at a concentration of 1 mM, and the cells were incubated for an additional hour. *E. coli* SP850 cells carrying the *S. oneidensis* *cyaA*, *cyaB*, or *cyaC* gene were grown in a similar manner but without addition of cAMP. β -Galactosidase activity was measured as described previously (19).

Localization of CyaA and CyaC. Antibodies against the CyaA and CyaC peptides (N'-LDEENQLSHYVQSGSDMSELV and N'-ALAGRLDNPDSAYRLTNLGP, respectively) were generated by Protein Tech Group, Inc. (Chicago, IL). Membrane and soluble fractions were obtained from wild-type and mutant cells grown either aerobically or anaerobically as described previously (1). Proteins were separated on 10% or 4 to 20% sodium dodecyl sulfate-polyacrylamide gels (Pierce, Rockford, IL), transferred to a polyvinylidene difluoride membrane, and then incubated with CyaA or CyaC antibodies. Blots were developed using Supersignal West Pico chemiluminescent substrate (Pierce, Rockford, IL).

Enzyme activity and heme staining. Wild-type *S. oneidensis* and mutant strains were grown anaerobically with fumarate or DMSO for 2 h. Cells were pelleted and then lysed using B-PER (Pierce, Rockford, IL). Total protein concentrations were measured using a BCA protein assay kit (Pierce, Rockford, IL). Enzyme activity was detected on native polyacrylamide gels as previously described using methyl viologen as the artificial electron donor and 10 mM DMSO as the electron acceptor (28). Heme staining of proteins in sodium dodecyl sulfate-polyacrylamide gels was performed using 3,3',5,5'-tetramethylbenzidine dihydrochloride as described previously (37).

Generation of promoter-*lacZ* fusions. DNA fragments directly upstream of the DMSO reductase operon (*dmsEFAB*; SO_1427 to SO_1430), the fumarate reductase gene (*fccA*; SO_0970), *cyaA*, and *cyaC* were amplified by PCR using the primers listed in Table S1 in the supplemental material and Phusion polymerase (New England Biolabs). The resulting 597-, 526-, 321-, and 207-bp fragments were digested with HindIII and BamHI and then cloned into pPR9TT (30) that carried a promoterless *lacZ* gene. Following transformation of *E. coli* β 2155 (5), the plasmids were transferred into *S. oneidensis* wild-type and mutant strains by conjugation as described above. Recombinant strains were grown anaerobically for 2 h with either 10 mM fumarate or 10 mM DMSO in minimal medium supplemented with 50 mM lactate and 0.02% Casamino Acids. For aerobic growth, 10-ml cultures were grown in 500-ml flasks with vigorous shaking for 2 h. Cultures were assayed for β -galactosidase activity as described previously (19).

Microarray analysis. Microarray expression profiling of *S. oneidensis* wild-type strain MR-1 and mutants was carried out using whole-genome Affymetrix custom expression arrays (Affymetrix, Inc., Santa Clara, CA) comprised of 103,797 11- μ m synthesis features. The design used contains both antisense oligonucleotides and a complementary set of single-base-mismatched oligonucleotides to facilitate detection of background signals. Total cellular RNA from cultures grown in chemostats, as described above, was isolated using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA samples were treated with RNase-free DNase I (Ambion, Inc., Austin, TX) to digest residual DNA and purified using a mini RNeasy kit (Qiagen, Inc., Valencia, CA). The concentrations and purity of the RNA samples were determined by spectrophotometric measurement at 260 and 280 nm, respectively. Generation of cDNA using reverse transcriptase, cDNA fragmentation, labeling, and hybridization were performed according to the instructions in the manufacturer's GeneChip expression analysis technical manual. Scanning of the hybridized microarrays was performed using an Affymetrix GeneChip 3000 scanner. Probe-level

TABLE 2. β -Galactosidase activity of an *E. coli cyaA* mutant (strain SP850) complemented with *S. oneidensis cya* genes

Strain	β -Galactosidase activity (Miller units) ^a
<i>E. coli</i> SP850	26.0 \pm 10.5
SP850 + pJB3Cm6.....	31.7 \pm 4.2
SP850 + cAMP.....	1,284.6 \pm 143.8
SP850 + <i>S. oneidensis cyaA</i>	1,034.9 \pm 266.7
SP850 + <i>S. oneidensis cyaC</i>	1,057.8 \pm 281.0
SP850 + <i>S. oneidensis</i> P _o - <i>cyaB</i>	747.6 \pm 80.6
SP850 + <i>cyaB</i>	10.7 \pm 1.6

^a The data are the means \pm standard deviations of three independent experiments performed in duplicate.

intensity values from Affymetrix CEL files were processed using the MAS5 and RMA algorithms to compute gene expression values as described previously (6, 7). Using the RMA approach, the mean expression for each gene was compared with a background mean for all other treatments. Using the difference of means, along with a pooled estimate of variance, the significance of up- or downregulation (expressed as a *P* value) was computed using a two-sample *t* test statistic (6). Genes that exhibited a change in expression in $\Delta cyaC$ or Δcnp mutants are listed in Tables S2 to S5 in the supplemental material.

Microarray accession number. The Affymetrix CEL files generated in our experiments have been deposited in the NCBI Gene Expression Omnibus microarray database (<http://ncbi.nlm.nih.gov/geo/>) under accession number GSE15657.

RESULTS

Functional analysis of *S. oneidensis* MR-1 adenylate cyclase genes. The *S. oneidensis* genome (<http://www.tigr.org>) contains three putative adenylate cyclase genes. Two of these genes are designated *cyaA* and *cyaB* (SO_4312 and SO_3778, respectively), while the third is annotated as a putative adenylate cyclase gene (SO_1329) and is designated *cyaC* in this paper. CyaA is predicted to be a cytoplasmic protein consisting of 804 amino acids. It is similar to the bacterial class I adenylate cyclases and shares 30% identity and 49% similarity with *E. coli* CyaA. CyaB is predicted to be a soluble protein consisting of 194 amino acids with 69% identity to the class IV adenylate cyclase of *Aeromonas hydrophila* (33). The third adenylate cyclase, CyaC, consists of 335 amino acids and contains three putative membrane-spanning domains, suggesting that it is anchored to the cytoplasmic membrane. The C terminus of CyaC exhibits significant degrees of identity (33 to 39%) and similarity (54 to 65%) to the catalytic domain of the class III adenylate cyclase from *Rhizobium etli* (gi: 21616131; 35) and to putative class III adenylate cyclases from *Psychroflexus torquis* and *Flavobacterium* species. The predicted membrane-spanning domain of CyaC did not exhibit similarity to other sequences in the databases outside the genus *Shewanella*.

To confirm the predicted function of CyaA, CyaB, and CyaC, plasmids carrying the corresponding *S. oneidensis* MR-1 genes (Table 1) were used to complement *E. coli* SP850, which lacks the adenylate cyclase gene and cannot induce *lacZ* expression. This deficiency can be overcome by addition of cAMP to the growth medium or by introduction of a functional *cya* gene (35). Introduction of pKB1, which carries *cyaC* and 327 bp of upstream DNA, and pMC2, which carries *cyaA* and 200 bp of upstream DNA, into SP850 led to restoration of β -galactosidase expression in this strain. The levels of β -galactosidase activity in SP850 complemented with plasmids carry-

ing *cyaA* and *cyaC* were \sim 1,035 and 1,058 Miller units, respectively (Table 2). Similar levels of β -galactosidase activity were obtained when SP850 was complemented by addition of exogenous cAMP to the growth medium (Table 2), indicating that both genes encode adenylate cyclases, as predicted by sequence analysis.

To determine the role of *cyaB* in cAMP production, two plasmids were generated. pMC4 carries *cyaB* and 300 bp of upstream DNA in *cydC* which is 25 bp upstream of *cyaB*, while pMC3 carries *cyaB* fused to 360 bp of DNA upstream of *cydDC* (designated P_o as described in Materials and Methods). Since *cyaB* is located downstream of *cydDC* and is separated from this operon by 25 bp, generation of the two plasmids served to identify the promoter region of *cyaB* and determine its involvement in cAMP production. Introduction of pMC3 into *E. coli* SP850 restored β -galactosidase activity (Table 2), indicating that *cyaB* encodes an adenylate cyclase, as predicted. Introduction of pMC4, however, failed to complement SP850, suggesting that the DNA directly upstream of *cyaB* lacks a promoter and that *cyaB* is part of the *cydDC-cyaB* operon.

Cellular localization of CyaA and CyaC. Antibodies against CyaA and CyaC peptides were used to determine the locations of these proteins in *S. oneidensis* MR-1 cellular subfractions. A band that reacted with the CyaA antiserum and corresponded to the predicted size of CyaA (\sim 90 kDa) was detected in the soluble fraction of wild-type strain MR-1 but not in the soluble fraction of the *cyaAC* mutant (Fig. 1). When antibodies raised against the CyaC peptide were used, a band at \sim 37 kDa, which corresponded to the predicted size of CyaC, was detected in the membrane fraction of the wild type but not in the *cyaAC* mutant fraction (Fig. 1). These results are in agreement with the initial sequence analysis that predicted cytoplasmic and membrane locations of CyaA and CyaC, respectively.

Effect of adenylate cyclases on anaerobic growth. To further elucidate the role of adenylate cyclases in anaerobic respiration of *S. oneidensis* MR-1, we generated mutants with single, double, and triple chromosomal deletions of *cyaA*, *cyaB*, and *cyaC*. The mutants were tested for anaerobic respiration with fumarate, DMSO, and Fe(III) citrate as electron acceptors. Loss of either *cyaA* or *cyaB* did not affect the ability of the

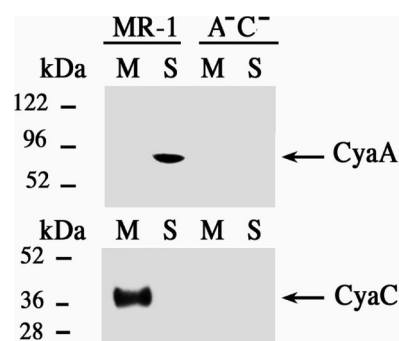


FIG. 1. Localization of CyaA (top blot) and CyaC (bottom blot) by Western blot analysis of soluble (lanes S) and membrane (lanes M) fractions of wild-type strain MR-1 and the *cyaAC* double mutant ($A^{-}C^{-}$). A band that reacted with CyaA antibodies was detected in the soluble fraction of the wild type but not in the mutant fractions. A band that reacted with the CyaC antibodies was detected in the membrane fraction of the wild type but not in the mutant fractions.

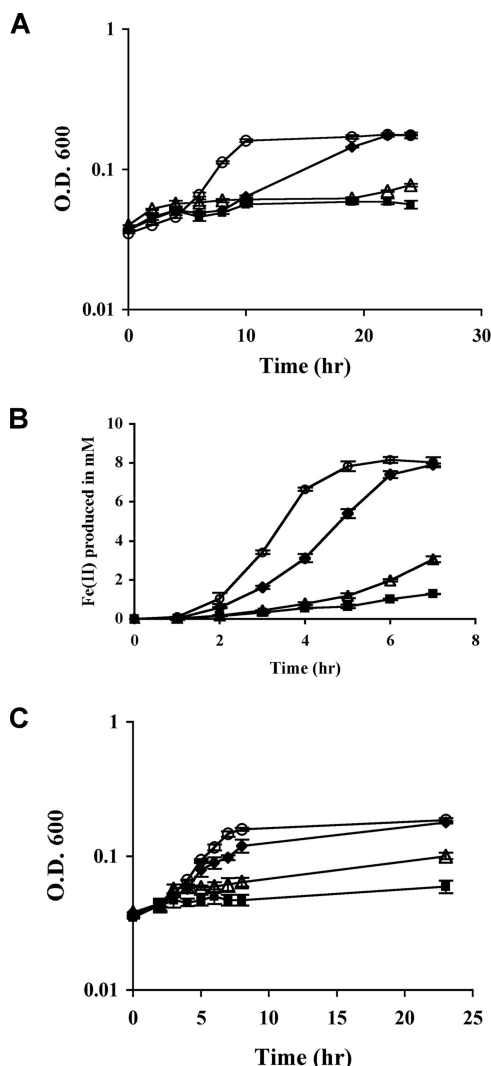


FIG. 2. Anaerobic respiration with DMSO (A), Fe^{+3} (B), and fumarate (C) by wild-type strain MR-1 (○), the $\Delta cyaC$ mutant (◆), the $\Delta cyaA \Delta cyaC$ mutant (△), and the Δcrp mutant (■). The $cyaC$ mutant was deficient in anaerobic respiration with DMSO and $Fe(III)$. The $cyaAC$ double mutant was deficient in anaerobic respiration with the three electron acceptors, and its phenotype mimicked the phenotype of the crp mutant. The data are the means and standard deviations of three independent experiments. O.D. 600, optical density at 600 nm.

mutants to use any of these electron acceptors (data not shown). However, significant deficiencies were observed for the $\Delta cyaC$ mutant that lacked the membrane-bound class III adenylate cyclase. When DMSO was used as the electron acceptor, a lag phase of 10 to 12 h was routinely observed for the $\Delta cyaC$ mutant, compared to a ~ 5 h-lag for the wild type (Fig. 2A). To determine if growth of this mutant with DMSO after the long lag phase was due to suppressor mutations, stationary-phase cells from the cultures were grown aerobically in LB medium and then retested for anaerobic growth in minimal medium with DMSO as described above. Significant changes in the duration of the lag phase were not observed (data not shown), indicating that growth of the $\Delta cyaC$ mutant after a

long lag phase was not due to accumulation of suppressor mutations.

In addition to DMSO growth deficiencies, the $cyaC$ mutant was deficient in $Fe(III)$ reduction (Fig. 2B). At 4 h, ~ 6.7 mM $Fe(II)$ and 3.2 mM $Fe(II)$ were produced by the wild type and the $\Delta cyaC$ mutant, respectively. When fumarate was used as the electron acceptor, the $\Delta cyaC$ mutant exhibited a slight deficiency in growth (Fig. 2C), but this deficiency was not as significant as the deficiency observed when DMSO or $Fe(III)$ was used as the electron acceptor.

More pronounced differences in anaerobic growth with DMSO, fumarate, and $Fe(III)$ were observed for the $cyaAC$ double and $cyaABC$ triple mutants. These two mutants exhibited similar deficiencies in anaerobic respiration with DMSO, fumarate, and $Fe(III)$ (Fig. 2 and Fig. 3). The phenotype of the $cyaAC$ double mutant was essentially identical to the phenotype of the Δcrp mutant (Fig. 2) (28). This suggests that CyaA and CyaC are both involved in CRP activation under anaerobic conditions, while CyaB does not appear to contribute significantly to this process.

Complementation of cya triple mutant with $cyaC$. To determine if CyaC is sufficient for CRP activation under anaerobic conditions or if other adenylate cyclases are essential for this process, we introduced $cyaA$, $cyaB$, and $cyaC$ on plasmids into the cya triple mutant. Introduction of $cyaB$ did not restore anaerobic respiration with any of the electron acceptors tested (Fig. 3), further confirming that this gene does not have a significant role in anaerobic respiration. Interestingly, the expression of $cyaA$ alone restored anaerobic growth with fumarate to levels similar to the wild-type levels, although the growth with DMSO and the reduction of $Fe(III)$ were slow (Fig. 3) and similar to those of the $\Delta cyaC$ mutant (Fig. 2). In contrast, expression of $cyaC$ in the cya triple mutant restored the ability to grow with fumarate and DMSO and the ability to reduce $Fe(III)$ like the wild type (Fig. 3). These results clearly demonstrate that the class III membrane-bound CyaC protein was sufficient for anaerobic reductase gene expression in the absence of other cAMP-producing enzymes.

To begin to understand the reason for the partial redundancy of CyaA and CyaC, we used $cyaA$ and $cyaC$ promoter- $lacZ$ fusions to measure the relative activities of the promoters in wild-type *S. oneidensis* under different growth conditions. The presence of the $cyaA$ and $cyaC$ promoters resulted in ~ 23 to 25 Miller units of β -galactosidase activity when the cells were grown aerobically. Under anaerobic conditions with fumarate, the β -galactosidase activity increased almost threefold (62.4 ± 5 Miller units) in cells carrying the $cyaC$ promoter- $lacZ$ fusion. Surprisingly, the β -galactosidase activity decreased two-fold (9.7 ± 1.1 Miller units) in fumarate-grown cells carrying the $cyaA$ promoter- $lacZ$ fusion plasmid. These results suggest that CyaC may be the dominant adenylate cyclase under anaerobic conditions, which may explain the observation that its presence is sufficient for anaerobic respiration with several electron acceptors.

Microarray analysis of genes regulated by CyaC and CRP. The results described above indicate that the phenotype of the crp mutant is reproduced when both $cyaA$ and $cyaC$ are absent, while loss of $cyaC$ alone results in a partial deficiency in anaerobic respiration. To further explore the roles of CyaC and CRP in the regulation of anaerobic respiration, we compared

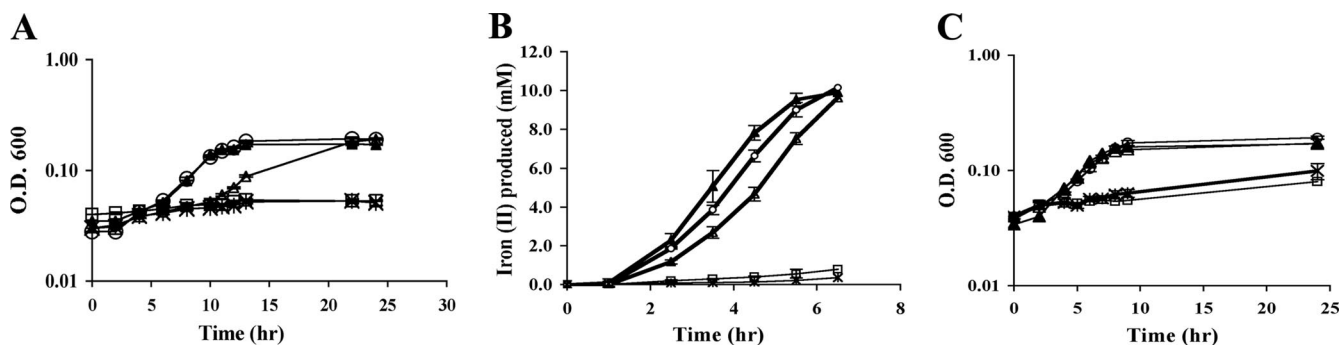


FIG. 3. Complementation of the *cya* triple mutant with *cyaA*, *cyaB*, or *cyaC*. The mutant (\square) was deficient in anaerobic respiration with DMSO (A), Fe(III) citrate (B), and fumarate (C). Introduction of *cyaC* (\circ) restored the ability of the triple mutant to grow with these electron acceptors. Introduction of *cyaA* (\triangle) led to wild-type levels of growth only when fumarate was used as the electron acceptor. Introduction of *cyaB* ($*$) into the mutant did not restore anaerobic respiration with any of the electron acceptors. The data are the means and standard deviations of three independent experiments. O.D. 600, optical density at 600 nm.

the genome-wide transcriptome profiles of $\Delta cyaC$ and Δcrp mutants to those of wild-type strain MR-1. Given the growth deficiencies displayed by the Δcrp and $\Delta cyaC$ mutants under anaerobic conditions, all strains were cultured in chemostats under O_2 -limited conditions. Since both the Δcrp and $\Delta cyaC$ mutants are not impaired in aerobic respiration, oxygen-limited conditions provided the means to maintain equal growth rates (0.06 h^{-1}) for the mutant and wild-type strains, thus eliminating the effect of growth on the overall transcriptome profiles. At the same time, while growing aerobically, the cells decreased the dissolved O_2 concentration in the medium to levels below the detection threshold (dissolved O_2 tension, $\leq 1\%$), allowing expression of anaerobic respiratory genes. The expression of these genes was confirmed by the reduction of fumarate by the wild-type culture, which produced stoichiometric amounts of succinate under O_2 -limited conditions. The optical densities at 600 nm of the wild-type, $\Delta cyaC$, and Δcrp strains in chemostat cultures were 0.50, 0.45, and 0.40, respectively.

Complete lists of genes that exhibited significant changes in expression in the Δcrp or $\Delta cyaC$ mutant are shown in Tables S2 to S5 in the supplemental material. Genes primarily predicted or known to be involved in electron transport and respiration are listed in Tables 3, 4, and 5. Of 5,066 genes represented on the microarray, 280 exhibited an increase or decrease in the expression level in the Δcrp mutant but not in the $\Delta cyaC$ mutant. These genes included genes involved in heme biosynthesis (*hemH-1*, *hemB*, *hemG-3*, and *hemB-2*), whose expression was significantly reduced in the Δcrp mutant (Table 3). The expression data for these genes were consistent with the observed lack of pigmentation of Δcrp colonies compared to the orange wild-type colonies (data not shown). Orange pigmentation is attributed to the large amount of *c* cytochromes produced by *S. oneidensis* wild-type strain MR-1 (3). Heme staining further confirmed the absence of a large number of *c* cytochromes in the Δcrp mutant (Fig. 4A). It should be noted that the genome sequence of *S. oneidensis* contains redundant homologues of several heme biosynthesis genes, and the *crp* mutant does not appear to be completely deficient in heme production. Therefore, it is likely that the significant loss of *c* cytochromes in the Δcrp mutant is due to a combination of

reduced heme synthesis and downregulation of the structural *c*-type cytochrome genes.

Other genes that appeared to be positively regulated by CRP include SO_0970 and SO_0848, which encode the terminal fumarate and nitrate reductases, respectively, as well as SO_4591, which encodes the *c* cytochrome CymA (Table 3). CymA is needed for electron transfer to the fumarate, nitrate, and metal terminal reductases (24). Since loss of CymA affects anaerobic growth with fumarate, we tested the expression levels of the fumarate reductase gene (*fccA*; 28) in the wild-type, Δcya , and Δcrp strains using plasmids that carry the *fccA* promoter fused to a promoterless *lacZ* gene. The levels of β -galactosidase activity were reduced in the Δcrp mutant compared to the wild type (Table 6), in agreement with the microarray data that showed a lower level of *fccA* expression in the mutant. These results confirmed that the deficiency in anaerobic growth with fumarate is due to loss of the terminal reductase and not simply due to a lack of required intermediate electron transport chain components. We have previously shown that *crp* mutants of *S. oneidensis* lack fumarate and nitrate reductase activities, using methyl viologen as an electron donor (28). The results presented here are in agreement with these findings. CRP also appears to positively control the expression of genes that encode regulatory proteins, such as SO0490, SO0940, and SO3538. These proteins were shown to be overexpressed in response to cold shock (9), but their roles are not known.

In addition to positive regulation, microarray data suggested that CRP negatively regulates the expression of some genes, such as *cyaA* and *cyaC* (Table 3; see Table S3 in the supplemental material). The expression levels of *cyaA* and *cyaC* were increased in the Δcrp mutant compared to the wild type. These results were confirmed by β -galactosidase activity measurements for *lacZ*-promoter fusions. The β -galactosidase activity in wild-type cells carrying the *cyaC* promoter-*lacZ* fusion plasmid and incubated anaerobically with fumarate was 62.4 ± 5.0 Miller units. The β -galactosidase activity in the Δcrp mutant was 101 ± 2.1 Miller units. A more significant increase was observed for the Δcrp mutant when the *cyaA* promoter was fused to *lacZ*. The β -galactosidase activity in wild-type strain MR-1 cells carrying the fusion plasmid and incubated anaero-

TABLE 3. Genes with altered expression profiles in the Δcrp mutant compared to the wild type for cells grown under O₂-limited conditions

Locus	Gene	Function or description	Δcrp mutant/wild type ratio ^a
Upregulated genes			
Heme biosynthesis			
SO_0027	<i>hemG-2</i>	Protoporphyrinogen oxidase	0.05
SO_0435	<i>hemE</i>	Uroporphyrinogen decarboxylase	0.22
SO_2109	<i>hemH-1</i>	Ferrochelatase	0.34
SO_2587	<i>hemB-1</i>	Delta-aminolevulinic acid dehydratase	0.20
SO_3720	<i>hemG-3</i>	Protoporphyrinogen oxidase	0.30
SO_4208	<i>hemB-2</i>	Delta-aminolevulinic acid dehydratase	0.25
Respiratory functions			
SO_0396	<i>fdC</i>	Quinol:fumarate reductase	0.13
SO_0848	<i>napA</i>	Periplasmic nitrate reductase	0.30
SO_0902	<i>nqrA</i>	Na-translocating NADH-quinone reductase subunit A	0.21
SO_0903	<i>nqrB</i>	Na-translocating NADH-quinone reductase subunit B	0.14
SO_0904	<i>nqrC</i>	Na-translocating NADH-quinone reductase subunit C	0.11
SO_0970	<i>fccA</i>	Periplasmic fumarate reductase	0.14
SO_2912	<i>pflB</i>	Pyruvate formate lyase	0.17
SO_2913	<i>pflA</i>	Pyruvate formate lyase 1 activating subunit	0.20
SO_3922	<i>fdh</i>	Formate dehydrogenase, cytochrome <i>b</i>	0.32
SO_4591	<i>cymA</i>	Membrane-anchored tetraheme <i>c</i> cytochrome	0.22
Regulatory functions			
SO_0490		Transmembrane transcriptional regulator	0.19
SO_0940		Transmembrane transcriptional regulator	0.07
SO_1415		Transcriptional regulator, TetR family	0.12
SO_3059		Sigma 54 transcriptional regulator, Fis family	0.28
SO_3538	<i>hlyU</i>	Transcriptional regulator	0.06
SO_3627		Transcriptional regulator, TetR family	0.14
Downregulated genes			
SO_0852		Type IV pilin, putative	4.76
SO_1329	<i>cyaC</i>	Adenylate cyclase	2.08
SO_4312	<i>cyaA</i>	Adenylate cyclase	3.97
SO_4609	<i>coxC</i>	<i>aa</i> ₃ -type cytochrome <i>c</i> oxidase, subunit III	2.68

^a Ratio of the mean hybridization signal intensity for the *crp* mutant to the mean hybridization signal intensity for the wild type. Values greater than 1 indicate an increase in the mRNA level in the mutant; values less than 1 indicate a decrease.

bically with fumarate was 9.7 ± 1.1 Miller units, and the activity was 95.9 ± 0.7 Miller units in the Δcrp mutant under the same conditions. These data confirm the role of CRP in the negative regulation of *cyaA* and *cyaC* expression. This situation is similar to the situation in *E. coli*, where CRP negatively regulates the expression of *cyaA* (20).

Analysis of microarray data identified 77 genes that appeared to depend on both CRP and CyaC for proper expression. The transcript levels of the DMSO and metal reductase genes (SO_1427 to SO_1432, which encode DmsEFABGH, and SO_1776 to SO_1779, which encode MtrB, MtrA, MtrC, and OmcA) were reduced in the Δcrp and $\Delta cyaC$ mutants (Table 4). These data are in agreement with the results of a phenotypic analysis of these mutants. The downregulation of *dms* genes was further tested using a promoterless *lacZ* gene fused to the *dms* promoter. The β -galactosidase activity in these strains confirmed that the expression of the DMSO reductase operon was significantly reduced in both mutants (Table 6). We also tested the levels of DMSO reductase activity in the wild-type and mutant strains using methyl viologen as the electron donor. DMSO reductase activity was either severely diminished or absent in $\Delta cyaC$, $\Delta cyaAC$, and Δcrp mutant cell extracts (Fig. 4C), while wild-type activity levels were present in $\Delta cyaA$ mutant cell extracts. These results confirm the major

role of CyaC in addition to CRP in regulation of the expression of the DMSO reductase.

Comparison of gene expression profiles in the *crp* and *cyaC* mutants identified 557 genes that appeared to be regulated by CyaC but not by CRP. Genes that encode *cbh*₃-type cytochrome *c* oxidase subunits and flagellum biosynthesis proteins, as well as genes that encode phage-related proteins (Table 5), were downregulated in the $\Delta cyaC$ mutant. Similarly, genes involved in cytochrome *c* biosynthesis, such as *ccmH-1*, exhibited lower levels of expression in the $\Delta cyaC$ mutant than in wild-type strain MR-1 and the Δcrp mutant (Table 5). Heme staining of $\Delta cyaC$ mutant cell extracts indicated that there was a reduction in the number of proteins that possess peroxidase activity compared to the number in the wild type, suggesting that there were reduced levels of *c* cytochromes in the mutant (Fig. 4A). As observed for the heme biosynthesis genes described above, there are redundant copies of the cytochrome *c* maturation genes in the *S. oneidensis* genome, which may explain the incomplete loss of *c* cytochromes in the $\Delta cyaC$ mutant. Transcriptome analysis and heme staining of cell extracts suggested that CyaC may negatively regulate the expression of some genes (Table 5 and Fig. 4A). A band with increased peroxidase activity was detected in cell extracts of the $\Delta cyaC$ mutant but not in extracts of the wild type or the Δcrp mutant.

TABLE 4. Genes that exhibited decreased or increased expression in Δcrp and $\Delta cyaC$ mutants compared to the wild type for cells grown under O_2 -limited conditions

Locus	Gene	Function or description	Δcrp mutant/wild type ratio ^a	$\Delta cyaC$ mutant/wild type ratio ^a
CRP- and CyaC-upregulated genes				
SO_0827		Lactate transport protein	0.03	0.23
SO_1422	<i>ifcR</i>	Fe(III)-induced transcriptional regulator	0.12	0.29
SO_1427	<i>dmsE</i>	Cytochrome <i>c</i> component of DMSO reductase	0.08	0.38
SO_1428	<i>dmsF</i>	Outer membrane protein	0.07	0.25
SO_1429	<i>dmsA</i>	Outer membrane DMSO reductase	0.09	0.23
SO_1430	<i>dmsB</i>	FeS subunit of DMSO reductase	0.13	0.12
SO_1431	<i>dmsG</i>	DMSO reductase chaperone	0.14	0.16
SO_1432	<i>dmsH</i>	Conserved hypothetical protein	0.24	0.22
SO_1776	<i>mtrB</i>	Outer membrane protein, metal reduction	0.06	0.20
SO_1777	<i>mtrA</i>	Decaheme <i>c</i> cytochrome, metal reduction	0.05	0.31
SO_1778	<i>mtrC</i>	Outer membrane <i>c</i> cytochrome, metal reduction	0.04	0.31
SO_1779	<i>omcA</i>	Outer membrane <i>c</i> cytochrome, metal reduction	0.09	0.24
SO_3285	<i>cydB</i>	Cytochrome <i>d</i> ubiquinol oxidase	0.14	0.20
SO_3286	<i>cydA</i>	Cytochrome <i>d</i> ubiquinol oxidase	0.12	0.19
CRP- and CyaC-downregulated genes				
SO_3503	<i>nagP</i>	<i>N</i> -Acetylglucosamine transporter	6.50	2.58
SO_3504	<i>nagX</i>	<i>N</i> -Acetylglucosamine-related transporter	10.06	8.00
SO_3505	<i>nagA</i>	<i>N</i> -Acetylglucosamine-6-phosphate deacetylase	14.42	8.28
SO_3506	<i>nagB</i>	Glucosamine-6-phosphate deaminase	27.47	17.15
SO_3507	<i>nagK</i>	<i>N</i> -Acetylglucosamine kinase	23.75	14.83
SO_3509	<i>hexB</i>	Beta- <i>N</i> -acetylhexosaminidase	1.00	1.00
SO_3510		Methyl-accepting chemotaxis protein	10.27	6.06
SO_3513		Tryptophan halogenase	9.71	5.06

^a Ratio of the mean hybridization signal intensity for the *crp* or *cyaC* mutant to the mean hybridization signal intensity for the wild type. Values greater than 1 indicate an increase in the mRNA level in the mutant; values less than 1 indicate a decrease.

These results strongly imply that CyaC may have other roles in addition to CRP activation.

DISCUSSION

The role of proteins that belong to the FNR-CRP family of global regulators has been extensively studied in many bacteria. Generally, the oxygen-sensing members of this family, such as *E. coli* FNR, are activated by anaerobiosis and play a major role in regulating anaerobic respiration. Contrary to expectations, *S. oneidensis* uses CRP instead of FNR to regulate anaerobic respiration (28). This finding, however, does not explain how cells sense redox changes in their environment. The *S. oneidensis* CRP lacks redox-sensing domains and appears to be activated solely by cAMP (22, 28). This conclusion is further supported by the observation that addition of cAMP to aerobically growing *S. oneidensis* cells results in anaerobic levels of fumarate reductase activity (28). This suggests that although CRP is a major regulator of anaerobic respiration in *S. oneidensis*, additional oxygen-sensing proteins must be involved. These proteins, such as ArcA (10), may function downstream of CRP and participate in activation of anaerobic gene expression, or they may function upstream of CRP and play a role in its activation. Since cAMP is needed for CRP activation, we investigated the role of three *S. oneidensis* adenylate cyclases in CRP activation and anaerobiosis. Although these three cyclases were capable of activating CRP in *E. coli*, only two enzymes, CyaA and CyaC, were needed for CRP activation under anaerobic conditions in *S. oneidensis*. The membrane-bound

class III cyclase, CyaC, had a prominent role in this process since it was able to fully restore anaerobic respiration to a *cya* triple mutant.

We used gene expression profiling to determine the connection between CyaC and CRP activation. As expected, several genes had similar changes in their expression patterns in the $\Delta cyaC$ and Δcrp mutants, including the DMSO reductase genes, whose expression was reduced in both mutants. The expression of more than 200 genes, however, was altered only in the Δcrp mutant. One example of these genes is *fccA*, which encodes the terminal fumarate reductase (28). Anaerobic growth of the Δcrp mutant with fumarate was severely impaired, while that of the $\Delta cyaC$ and $\Delta cyaA$ mutants was barely affected. Similarly, *fccA* promoter activity was greatly diminished only in the Δcrp mutant. These observations indicate that although CyaC and CyaA contribute to cAMP production and CRP activation, they are not completely redundant. This may be due to the relative abundance and activity of the two enzymes, as well as nature of the cAMP-CRP-regulated promoters. Assays of promoter-*lacZ* fusions in wild-type strain MR-1 indicated that *cyaC* was expressed at higher levels than *cyaA* in anaerobic cells. Based on these results, we expect cAMP production to be higher in the $\Delta cyaA$ mutant than in the $\Delta cyaC$ mutant, which may lead to increased levels of cAMP-CRP. The long lag phase observed for the *cyaC* mutant when it was grown with DMSO may be a reflection of the time required to reach optimum cAMP levels in the absence of CyaC.

One of the most surprising results of this study was the finding that a large number of genes appeared to be regulated

TABLE 5. Genes with altered expression profiles in the Δ *cyaC* mutant compared to the wild type for cells grown under O₂-limited conditions

Locus	Gene	Function or description	Δ <i>cyaC</i> mutant/wild type ratio ^a
Genes upregulated by CyaC			
SO_0026		Transcriptional regulator	0.34
SO_0268	<i>ccmH-1</i>	Cytochrome <i>c</i> biogenesis protein	0.33
SO_0269		Cytochrome <i>c</i> biogenesis thioredoxin	0.28
SO_0282	<i>pilN</i>	Type IV pilus biogenesis protein	0.26
SO_0521		Monooxygenase domain protein	0.28
SO_1215	<i>ompK</i>	Outer membrane protein	0.33
SO_1490	<i>adhB</i>	Alcohol dehydrogenase II	0.35
SO_1824		TonB system biopolymer transport component	0.34
SO_2361	<i>ccoP</i>	<i>cbb</i> ₃ -type cytochrome <i>c</i> oxidase, subunit III, CcoP	0.26
SO_2362	<i>ccoQ</i>	<i>cbb</i> ₃ -type cytochrome <i>c</i> oxidase, subunit IV, CcoQ	0.21
SO_2363	<i>ccoO</i>	<i>cbb</i> ₃ -type cytochrome <i>c</i> oxidase, subunit II, CcoO	0.15
SO_2364	<i>ccoN</i>	<i>cbb</i> ₃ -type cytochrome <i>c</i> oxidase, subunit I, CcoN	0.21
SO_2942		Prophage lambda So transcriptional regulator	0.31
SO_2993		Prophage lambda type II DNA methyltransferase	0.16
SO_3146		DNA-binding protein, H-NS family	0.29
SO_3211	<i>flhG</i>	Flagellar biosynthesis protein	0.21
SO_3212	<i>flhF</i>	Flagellar biosynthesis protein	0.31
SO_3213	<i>flhA</i>	Flagellar biosynthesis protein	0.29
SO_3221	<i>fliM</i>	Flagellar motor switching	0.30
SO_3223	<i>fliK</i>	Flagellar hook length control protein	0.18
SOA_0001	<i>repA</i>	Plasmid replication protein	0.10
Genes downregulated by CyaC			
SO_0312		Outer membrane porin	3.06
SO_0630	<i>nosA</i>	TonB-dependent outer membrane protein	6.87
SO_2318	<i>cheY</i>	Chemotaxis response regulator	5.97
SO_3687	<i>csgE</i>	Curli secretion protein	6.67

^a Ratio of the mean hybridization signal intensity for the *cyaC* mutant to the mean hybridization signal intensity for the wild type. Values greater than 1 indicate an increase in the mRNA level in the mutant; values less than 1 indicate a decrease.

by CyaC but not by CRP. These genes included a large number of prophage and plasmid-related genes, as well as genes involved in cytochrome *c* maturation, regulation, and electron transfer. These results point to additional roles for CyaC in the regulation of gene expression that may be independent of CRP

activation. It is not clear at present what these roles are, but it is possible that additional cAMP-binding proteins, yet to be identified, may rely on CyaC for activation.

Although class III adenylate cyclases have been studied mostly in eukaryotes, they are beginning to receive considerable attention in prokaryotes. Several bacteria use class III adenylate cyclases to respond to osmotic stress, pH, light, and other environmental stimuli (16, 32). In *Pseudomonas aeruginosa*, the membrane-bound CyaB protein is involved in regulation of virulence genes and appears to respond to calcium limitation (39), and in *Myxococcus xanthus* *cyaA* plays a role in signal transduction during osmotic stress (15). Analysis of bacterial genomes has identified several membrane-bound class III adenylate cyclases in *Bradyrhizobium japonicum*, *Sinorhizo-*

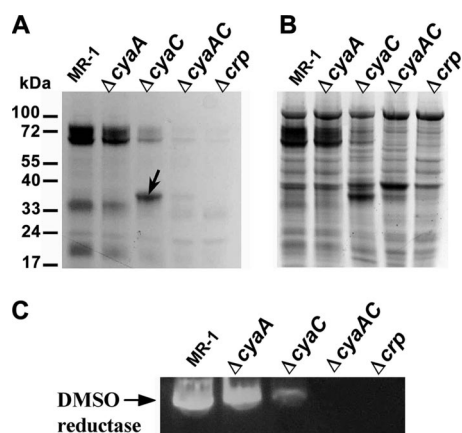


FIG. 4. (A and B) Heme (A) and Coomassie blue staining (B) of polyacrylamide gels with cell extracts from wild-type and mutant strains. The arrow indicates a heme-stained band that was overexpressed in the *cyaC* mutant. Twenty micrograms of protein was loaded in each lane. (C) DMSO reductase activity staining using methyl viologen as the electron donor. Similar levels of DMSO reductase activity (arrow) were detected in the wild type and the *cyaA* mutant. The levels were significantly reduced in the *cyaC*, *cyaAC*, and *crp* mutants. Twenty micrograms of protein was loaded in each lane.

TABLE 6. β -Galactosidase activities for *dms* and *fccA* promoter-*lacZ* fusions under anaerobic conditions in wild-type and mutant strains of *S. oneidensis* MR-1

Strain	β -Galactosidase activity (Miller units) ^a	
	<i>dms</i> promoter ^b	<i>fccA</i> promoter ^c
MR-1	237.1 \pm 23.9	341 \pm 6.2
Δ <i>cyaC</i> mutant	61.9 \pm 1.7	242.8 \pm 5.2
Δ <i>cyaAC</i> mutant	19.9 \pm 0.7	55.9 \pm 1.4
Δ <i>crp</i> mutant	25.0 \pm 5.3	71.4 \pm 1.8

^a The β -galactosidase activity in MR-1 carrying pPR9TT without an insert was 10 Miller units. The data are means \pm standard deviations of three independent experiments.

^b Cultures were incubated anaerobically with DMSO for 2 h.

^c Cultures were incubated anaerobically with fumarate for 2 h.

bium meliloti, and *Mycobacterium tuberculosis* (18, 32). One *M. tuberculosis* adenylate cyclase, Rv1264, is a pH-sensing enzyme (8, 36), while another, Rv1319, is regulated by bicarbonate ions (4). In *S. oneidensis* MR-1 and *Shewanella* sp. strain ANA-3 (22), the class III adenylate cyclases are involved in the regulation of anaerobic respiration, and a shift from aerobic to anaerobic conditions resulted in increased expression of cyclase genes in both organisms. It is interesting that class III adenylate cyclases in bacteria are proving to be versatile enzymes that respond to different stimuli based on the lifestyle of the organism (32). The results presented in this paper and the recently published reports of Murphy et al. (22) suggest that the *Shewanella* class III enzymes may represent a novel group of adenylate cyclases that may, directly or indirectly, sense and respond to anaerobiosis.

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