Regulation of anaerobic respiration by cAMP receptor protein and role of adenylate cyclases.

Previous work in my laboratory identified the cAMP receptor protein, CRP, as the major regulatory protein that controls anaerobic respiration in *S. oneidensis* MR-1. We have additionally identified three adenylate cyclases that are involved in cAMP production, and subsequently in CRP activation. One of these enzymes, CyaC, is a membrane-bound enzyme that is a member of the class III adenylate cyclases. Deletion of the gene that encodes this enzyme results in deficiencies in anaerobic respiration with fumarate, DMSO and Fe(III). We determined the expression profiles of genes in the *crp*, *cyaC*, and *S. oneidensis* MR-1 wild type using microarray analysis. The results indicated that CRP regulates the expression of more than 200 genes, a large number of which is involved in electron transport and anaerobic respiration such as the terminal fumarate reductase, CymA which is involved in reduction of several electron acceptors, and heme biosynthesis. CyaC, the membrane-bound adenylate cyclase, was found to regulate the expression of more than 500 genes, none of which is regulated by CRP. These included genes that are involved in flagella biosynthesis and motility, and phage and plasmid replication functions, among others. Based on microarray data, 77 genes were regulated by both CRP and CyaC and included the terminal DMSO reductase and metal reductase genes. Our results indicated that CyaC is required for activation of CRP through cAMP production. They also indicate that CyaC is involved in gene regulation independent of CRP. The work described here represents the first report of a class III adenylate cyclase that is involved in anaerobic respiration. This work has been submitted for publication to the Journal of Bacteriology.

The microarray data, described above, identified several genes involved in anaerobic respiration that may be regulated by CyaC alone. These included genes that are predicted to be involved in the reduction of various sulfur compounds such as sulfite. Examples include SO_0714-SO_0717 that are predicted to encode a sulfite dehydrogenase, SO_1413-SO_1414 that are predicted to encode an ABC sulfur transporter, and SO_0482 that is predicted to encode a flavocytochrome of unknown function. We have generated a chromosomal deletion mutant that lacks SO_0714-0717. This mutant was able to reduce sulfite, and we plan to test it for tetrathionate and sulfur reduction to determine if the deleted genes play a role in these processes. We will generate mutants of the other genes that are predicted to play a role in sulfur or sulfite reduction and determine their phenotypes.

To further investigate the effect of CRP and CyaC on gene expression, we generated a promoter probe vector, pMC10, which contains a promoterless *lacZ* gene. The utility of plasmid has been verified by testing the expression levels of the fumarate and DMSO reductases in wild type and *crp* mutant backgrounds. We used this plasmid to test the expression of cytochromes and reductase genes that are predicted to be involved in reduction of sulfur compounds, and other electron acceptors, and that appear to be regulated by CyaC and CRP as determined by microarray analysis (see below).

Identification of an octaheme *c* cytochrome as the terminal sulfite reductase in *S. oneidensis* MR-1.

In an effort to identify additional proteins involved in the reduction of S compounds, we generated mutants in *c* cytochrome genes and in genes that are predicted to encode oxidoreductases. One of the mutants, with a chromosomal deletion of SO_0479 was completely deficient in sulfite reduction. This gene encodes an octaheme *c* cytochrome that we designated SirA. The amino acid sequence of this protein includes an atypical heme binding site that is predicted to require a specialized heme ligation system. The genes that encode this atypical system are located within the *sirA* locus (*sirFEG*; SO_0477, SO_478, SO_0482, respectively). Preliminary results suggest that copper may be required for sulfite reduction in *S. oneidensis* MR-1. Genes that encode an ATP-binding cassette (ABC) copper transport system (SO_485-SO_0488) are located in what appears to be an operon that includes the terminal sulfite reductase gene (*sirA*) and the atypical heme lyase system described above. The role of copper and the role of the copper transport genes in sulfite reduction is currently under investigation.

One of the surprising results of our work is the finding that the terminal sulfite reductase consists of a *c* cytochrome but does not appear to bind siroheme. All terminal sulfite reductases identified to date from Bacteria and Archaea are siroheme proteins. This suggests that SirA is a novel type of sulfite reductases that uses different mechanisms for reduction than other known enzymes. Our results further suggest that reduction of sulfite by SirA proceeds directly by a 6-electron reduction mechanism and does not generate intermediates such as thiosulfate or trithionate. Interestingly, all the genes described above that appear to be involved in sulfite reduction are conserved in *Shewanella* species that are able to reduce sulfite. The work described here has been published in Environmental Microbiology.

Identification and analysis of components of the electron transport chains that lead to reduction of thiosulfate, tetrathionate, and elemental sulfur in MR-1.

Shewanella oneidensis MR-1 is able to use S-compounds as electron acceptors for anaerobic respiration. These include tetrathionate, thiosulfate, sulfite, and elemental sulfur. The cAMP receptor protein (CRP) positively regulates the reduction of these compounds in *S. oneidensis*. In the previous progress report, we described our results that indicated *S. oneidensis* MR-1 uses one enzyme (PhsABC; Locus tag SO_4061-SO_4063) to reduce thiosulfate, tetrathionate, and polysulfide. In addition, we described the roles of the two-component system TtsSR and CRP in the regulation of thiosulfate and tetrathionate reduction. We have recently developed methods to determine the concentrations of thiosulfate, tetrathionate and sulfite. The development of these methods allowed us to monitor more accurately the process of thiosulfate and tetrathionate reduction and determine the concentrations and identity of the reduction products. As a result, we found that although PhsABC is involved in tetrathionate reduction, it only accounts for 50% of this reduction. This suggests that another enzyme, yet to be identified is involved in tetrathionate reduction. To identify this enzyme, we used the mini-transposon that we developed previously (mini-*himar* *RBI*) to generate mutations in the *phsABC* mutant described above. Since the *phsABC* mutant is partially deficient in tetrathionate reduction, mutagenesis of this strain would lead to the generation of mutants that are completely deficient in tetrathionate reduction. We have identified several

mutants that are completely deficient in tetrathionate reduction. These mutants are currently being analyzed to identify the genes that were disrupted by the transposon insertions.

We have tested the *phsABC* mutant for reduction of polysulfide (which is soluble) and elemental sulfur (which is insoluble). Our results indicated that the mutant was able to reduce elemental sulfur but not polysulfide. This was surprising since we expected the same enzyme to be responsible for the reduction of the two forms of this electron acceptor. Our preliminary results suggested that a second enzyme, in addition to PhsABC, may be involved in reduction of elemental sulfur. We further tested mutants that are deficient in type II secretion system. In *S. oneidensis* this secretion system is involved in the proper localization of *c* cytochromes such as MtrC and OmcA that are involved in metal reduction. Type II secretion mutants were deficient in reduction elemental sulfur, but not in polysulfide reduction. These preliminary results suggest that outer membrane proteins may be involved in the reduction of elemental sulfur, similar to Fe(III) and Mn(IV) reduction. We are currently analyzing mutants deficient in outer membrane proteins to identify additional components that are involved in insoluble sulfur reduction.

In addition to the results described above, we have tested mutants deficient in cytochrome *c* maturation and menaquinone biosynthesis. Our results indicated that *c* cytochromes are not required for thiosulfate, tetrathionate, and polysulfide, while menaquinones were essential. This is in contrast to sulfite reduction that requires a *c* cytochrome for the reduction of sulfite. Surprisingly, reduction of all sulfur compounds tested to date does not appear to require the intermediate *c* cytochrome CymA. The work described here is being prepared for publication.

Involvement of pili and flagella in metal reduction by *S. oneidensis* MR-1

One of the aims of the proposal was to study metal reduction in MR-1, and determine the mechanisms of reduction. Metal reduction has been hypothesized to require pili or nanowires to allow extracellular transfer to metal oxides or cathodes of microbial fuel cells. To test this hypothesis, we generated mutants that are deficient in type IV pili, mannose sensitive hemagglutinin (MSH) pili, and flagella. Our results indicated that cells lacking both types of pili are able to reduce iron oxides and generate electricity in microbial fuel cells. Mutants that lack flagella and are unable to swim were also able to reduce Fe(III) similar to the wild type, and to generate electricity in fuel cells. Cells that lack the outer membrane *c* cytochromes, MtrC and OmcA, were completely deficient in both processes. Furthermore, wild type and mutants deficient in pili or flagella produced similar amounts of riboflavin in the medium. Riboflavins are thought to act as electron shuttles during metal reduction or current production. Our results indicated that pili and flagella are not required for extracellular electron transfer, and that the essential components are outer membrane *c* cytochromes. These results further indicate that riboflavins may act as electron shuttles if they come in contact with, and are reduced by, outer membrane *c* cytochromes. These results have been published in *Electroanalysis*.

HemN1 is the major enzyme that is involved in heme biosynthesis under anaerobic conditions.

We have identified a transposon mutant that is deficient in cytochrome *c* biosynthesis under anaerobic conditions. The mutant had an insertion in SO_4720 that is predicted to encode an oxygen-independent coproporphyrinogen III oxidase HemN1. The genome sequence of *S. oneidensis* MR-1 contains three genes that are predicted to encode HemN-like proteins (SO_4720, SO_3359 and SO_4520, designated, *hemN1*, *hemN2*, and *hemN3*, respectively). The encoded HemN proteins do not share a high degree of similarity with each other, but they are highly similar to HemN proteins from other bacteria. HemN1 (SO4730) is 65% identical to HemN from *E. coli*, while SO3359 (HemN2) and SO4520 (HemN3) are 61% identical to the predicted HemN proteins from *Vibrio cholerae*.

Analysis of the transposon mutant indicated that disruption of *hemN1* (SO4720) causes severe deficiencies in mature *c*-type cytochromes synthesis in *S. oneidensis* under anaerobic condition. This is due to the inability of the mutant to synthesize heme under oxygen limiting conditions, as indicated by complementation of the mutant with exogenous heme. The *hemN1* mutant was able to synthesize *c*-type cytochromes similar to the wild type when cells were grown aerobically. As mentioned above, the genome sequence of *S. oneidensis* contains three *hemN* genes, but the severe loss of mature *c* cytochromes in the *hemN1* mutant suggests that HemN1 plays a major role in heme biosynthesis under anaerobic conditions. The detection of some heme *c* staining bands in the *hemN1* mutant cell extracts, however, suggests that HemN2, HemN3, or both are functional proteins that may play a minor role in anaerobic heme biosynthesis. Future work will determine which of these proteins is involved in this process.

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