Comparative biochemistry and physiology of iron-respiring bacteria from acidic and neutral-pH environments.

Final Technical Report

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Principle Investigator: Timothy S. Magnuson, Idaho State University

Collaborator: David E. Cummings, Point Loma Nazarene University (formerly of Idaho National Laboratory)

Summary: Below are a list of project goals addressed, and a brief summary of findings in those areas:

Acidophilic dissimilatory iron-reducing bacteria (DIRB) are now being detected in a variety of 'extreme' low-pH, radionuclide- and heavy-metal contaminated habitats where Fe(III) reduction is taking place, and may represent a significant proportion of metal-transforming organisms in these environments. *Acidiphilium cryptum* is our model organism, a facultative iron-respiring Alphaproteobacterium. Major findings of this project have been 1) Discovery of novel outer-membrane and periplasmic cytochromes c in acidophiles that are reactive with Fe and Cr, 2) Discovery of Cr(VI) reduction mechanisms in acidophiles, mediated by c-type cytochromes and other reductase activity, 3) Development of enzyme detection methods specific for Cr-reducing enzymes, 4) Characterization of biofilm formation in *A. cryptum*, and 5) Annotation of the *Acidiphilium cryptum* genome (Magnuson, Kusel, and Cummings, DOE-JGI 2005-2006). Two manuscripts and one book chapter have been published, and several more mauscripts are prepared for submission.

Research questions posed. We have listed some research questions and the findings made in these areas:

Do acidophilic iron reducers possess the genes and physiology to form biofilms?

Comparative studies conducted thus far suggest that both acidophilic and neutrophilic DIRB form biofilms on mineral surfaces. Batch cultures of *A. crptum* and *G. sulfurreducens* were grown using anaerobic conditions with either schwertmannite or ferrihydrite as electron acceptor/mineral surface respectively. In both cases, cell attachment was observed, and staining with fluorescent lectins reveals exopolysacharride matrix surrounding the cells and mineral particles. More detailed analysis revealed that *A. cryptum* biofilms show characteristic structure and development (Figure 1).

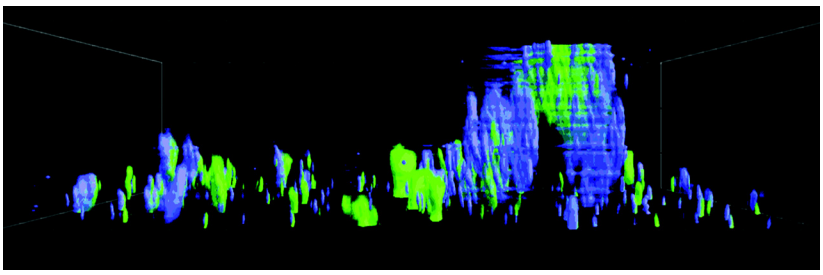

Figure 1. Confocal image of *A. cryptum* biofilm on silica surface. Cells were visualized by a fluorescent esterase substrate that appears green in metabolically active cells, while cells in blue (DAPI counter-stained) are blue. Typical biofilm architecture

Analysis of the *A. cryptum* genome for biofilm-related genes. Brandon Briggs, a Masters Degree student in our group (MS 2007), identified a number of potential biofilm-relate genes in the genome of A. cryptum. Among these genes are those encoding polysaccharide biosynthesis and attachment functions (Table 1).

Table 1. *A. cryptum* EPS genes related to biofilm formation. Relatedness to other EPS genes is shown.

Accession Number	Function	Name	Species	%ID %Sim
ZP_01143962	Biofilm Architecture maintenance	MbaA	*E. coli*	40/57
ZP_01143865	GDP-mannose dehydrogenase	Vps21		35/57
ZP_01146336	glycosyl transferase	Vps32	*Vibrio cholerae*	53/68
ZP_01144541	Polysaccharide synthesis/export protein	Vps69		35/62
ZP_01144540	exopolysacchjaride exporter	Cps19fD		42/63
ZP_01144603	UDP-N-acetyl-D-mannosamine transferase	Cps19fF		41/65
ZP_01147027	glucose-1-P thymidyltransferase	Cps19fL		59/76
ZP_01147028	dTDP-4-keto-6-deoxyglucose-3,5-epimerase	Cps19fM	*Streptococcus pneumoniae*	37/62
ZP_01147026	dTDP-glucose-4,6-dehydratase	Cps19fN		44/64
ZP_01147044	dTDP-4-dehydrorhamnose reductase	Cps19fO		48/50
ZP_01144985	Chain length regulator	EpsA		22/48
ZP_01144992	glycosyltransferase	EpsD	*Lactococcus lactis*	59/76
ZP_01145886	galactosyltransferase	EpsG		32/56
ZP_01147029	galactosyltransferase	EpsK		30/64
ZP_01146503	Type I capsule synthesis	CapD		55/73
ZP_01145277	Type I capsule synthesis	CapG	*Staphylococcus aureus*	40/60
ZP_01147150	Type I capsule synthesis	CapL		38/55
ZP_01145433	galactosyltransferase	EpsE	*Staphylococcus thermophilus*	70/83
ZP_01143818	glycosyltransferase	EpsF		28/52
ZP_01146869	glycosyltransferase	EpsI		51/74

Do acidophilic iron reducers produce electron transport proteins and enzymatic activities that are significantly different than their neutrophilic counterparts?

Studies with the cell surface fractions of *A. cryptum* have revealed two distinct c-type cytochromes, of 42 and 48 kDa molecular mass. Periplasmic fractions contain two major c-type cytochromes of 10.1 and 11.7 kDa molecular mass. These proteins have been purified and preliminary characterization has revealed some interesting features and distinct differences between these proteins and their neutrophilic counterparts (Figures 2 and 3). All of the *A. cryptum* cytochromes purified are monohemic, whereas *Geobacter* cytochromes contain at least three, and up to 12, heme c groups per molecule of cytochrome. The periplasmic cytochromes have the ability to reduce both Fe(III)-NTA and Cr(VI), as does the 42 kDa OM cytochrome c. This suggests a role in thermodynamically favorable electron transfer from these proteins to the Fe(III) or Cr(VI) acceptors.

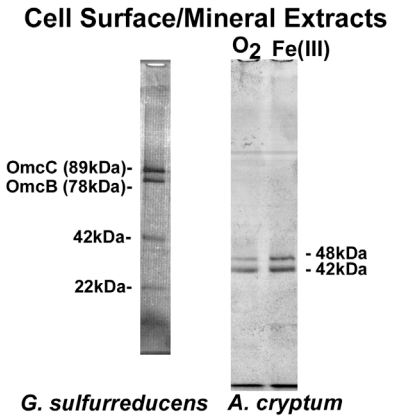

Figure 2. Comparison of cytochrome profiles obtained from cell surface fractions of model organisms. Whereas *G. sulfurreducens* produces at least 4 cell surface cytochromes c, *A. cryptum* has only two. Additional evidence suggests that the 48 kDa is differentially expressed, with more produced under Fe(II) grown conditions.

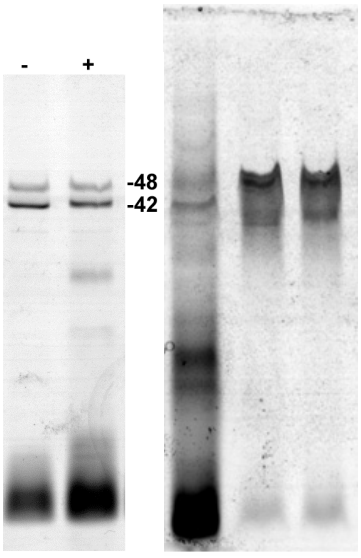

Figure 3. Localization experiments showing outer membrane location of the 42 and 48 kDa ctochromes c of *A. cryptum*. Left gel shows protease digestion results, which suggest that the protein have limited exposure to the outside of the cell, but are still susceptible to digestion, implying a cell surface location. Right gel shows total soluble (first lane) and membrane (second and third lanes) fractions. Both gels were stained for heme c.

Ferric iron seems to be required during growth to stimulate *A. cryptum* into producing or at least up-regulate a 48 kDa cytochrome containing protein. Evidence of this exists from an observation that during extended periods of growth on only oxygen this 48 kDa protein became

less prevalent -in fact it disappeared- in SDS-PAGE protein profiles. The same long-term oxygen culture was then supplemented with ferric sulfate and allowed to clear (about 3 days). Side by side comparisons, of the original long-term oxygen culture with the ferric supplemented culture, on SDS-Page gels reveled that the 48 kDa protein was virtually absent from the long-term oxygen culture but was prevalent in iron supplemented culture.

What ability does *A. cryptum* have to metabolize or reduce chromium under acidic conditions?

We established that, contrary to previous reports, *A. cryptum* indeed reduces Cr(VI) to Cr(III). Reduction was biological in nature – heat-killed cells displayed no capacity for Cr(VI) reduction; however, reduction proceeded regardless of the availability of a suitable electron donor. The reduction product was found to be largely soluble or associated with the cells, but no evidence was found to indicate that a Cr(III) precipitate, such as the $Cr(OH)_3$ commonly produced during reduction at circumneutral pH, was produced. Similar to previous reports of Fe(III) reduction by this organism, Cr(VI) was reduced across a broad range of solution pH, from pH 1.7 to as high as pH 4.7. At the growth optimum pH of 3.2, the reduction rate was estimated to be 0.58 ± 0.02 mg L^{-1} h^{-1} mg-protein^{-1}, within an order of magnitude of the maximal rates reported for many neutrophiles. We now believe that our experiments were Cr-limited, and thus underestimate the true potential for Cr(VI) reduction by *A. cryptum*. Undoubtedly due to the high redox potential of soluble Fe(III) at low pH, Kusel et al. reported the *A. cryptum* reduces Fe(III) and O_2 concurrently, an unusual phenomenon with alternative electron acceptors. Similarly, the presence of oxygen had very little effect on Cr(VI) reduction, while 1 mM Fe(III) (as ferric sulfate) dramatically accelerated the reaction rate, presumably by shuttling electrons through the highly favorable Fe(III).

We also focused our efforts on possible abiotic pathways of Cr(VI) reduction, and the toxicity of the soluble reduction product. The first such pathway considered was that of a hypothetical heat-labile reductant that might have been destroyed during the heat-killing process (used as our negative control). The requirement for live cells was readily substantiated with a general biocide; in the presence of 5 mM Hg^{2+} no Cr(VI) reduction was observed. Another possible abiotic pathway for Cr(VI) reduction was that of electron shuttling via Fe(III)/Fe(II). Although no Fe(III) was intentionally added to the reaction buffer, we speculated that contaminating Fe from the water supply or medium components might be sufficient to catalyze the reduction observed. In contrast to the $HgCl_2$ experiments, this possibility proved to be more problematic to examine. We first performed experiments in the presence of the Fe chelator deferroxamine to bind all traces of contaminating Fe. While the treatment halted all Fe(III) reduction in control experiments amended with ferric sulfate, it also bound Cr(VI), making the assessment of Cr(VI) reduction in its presence impossible. As an alternative to deferroxamine, the cation exchange resin Chelex 100 (Bio-Rad) is frequently used by investigators working with metalloproteins to remove all contaminating metals from working solutions. We found that Cr(VI) reduction in Chelex 100-treated buffer was indistinguishable from reduction in our standard (untreated) buffer. This observation supports our contention that Cr(VI) reduction in *A. cryptum* is enzyme-catalyzed rather than Fe-mediated. Thus, it would appear that *A. cryptum* reduces Cr(VI) by both a direct enzymatic mechanism and a coupled biotic-abiotic mechanism when Fe(III) is present.

Techniques for discovery of Cr(VI) reductase activities. Traditionally, chromate reduction has been monitored by quantifying Cr(VI) disappearance using UV-VIS spectroscopy and the Cr(VI) capture reagent 1,5-diphenylcarbazide (DPC). A chemiluminescence based method for Cr(III) detection in abiotic systems and immunological assays has been previously described but has not been adapted for use in direct monitoring Cr(III) in bacterial cultures or bacterial protein activity assays. We are currently adapting this assay for use in detecting chromate-reducing proteins extracted from cultures of *A. cryptum* JF-5. The assay has been applied to native polyacrylamide gel electrophoresis gels (PAGE) loaded with crude protein extracts from chromate-reducing cultures of *A. cryptum*. Chemiluminescence is detected in the PAGE gel where the chemiluminescent substrate reacts with Cr(III). This preliminary method for detecting Cr(III) has promise for developing activity-based gel assay to identify chromate-reducing proteins (Figure 4).

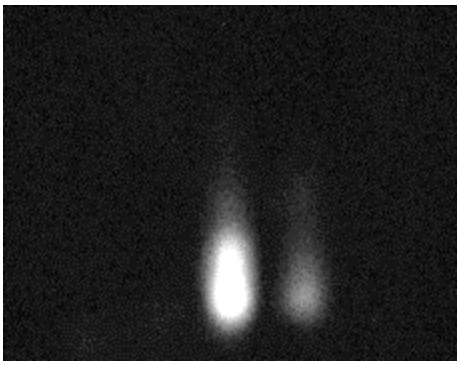

Figure 4. Native PAGE gel image showing chemiluminescent signal from *A. cryptum* JF-5 protein extracts. Chemiluminescence is produced where proteins are reducing Cr(VI) to Cr(III).

Biochemistry and physiology of Cr reduction.

In laboratory studies using pure cultures of JF-5, an NADPH-dependent chromate reductase activity has been detected primarily in soluble protein fractions, and a purified periplasmic and extracellular *c*-type cytochrome (ApcA) also show the ability to reduce Cr(VI). The NADPH-dependent activity was inducible by Cr(VI), and is not produced when chromium is absent. ApcA is reducable by menadiol, and in turn can reduce Cr(VI). Genomic data has revealed the presence of putative chromate reductase and chromate transport genes in the JF-5 genome, as well as of the genes that encode ApcA (Tables 2,3). Proteomic data did not show detectable amounts of the chromate reductase, however, ApcA was expressed in cells grown with Cr(VI) (Table 4). From these studies, it can be concluded that *A. cryptum* has the physiologic and genomic capability to reduce Cr(VI) to the less toxic Cr(III), however, the expected "Chromate reductase" mechanism may not be the primary means of Cr reduction in this organism (Figure 5). Our studies do confirm previous published work with whole cells that this acidophile, and perhaps others, has the potential for use in remediation of toxic metals at low pH.

Table 2. *A. cryptum* predicted proteins hypothetically involved in Cr(VI) metabolism.

Locus ID	Function	Closest Match	Source of Match	BLAST E-value	Comments
Acry_1913[a]	Chromate Transport	ChrA	*Burkholderia* ZP_02377597	7e-37	Paired with Acry_1914
Acry_2828[b]	Reduction	ChrR	*Comamonas* ZP_01521939	4e-29	
Acry_1073[c]	Reduction	ArsH	*Phenylobacterium* YP_002130282	4e-95	Next to Phosphate Transporter

Table 3. *A. cryptum* proteins encoding Cr(VI) reducing c-type cytochromes.

Locus ID	Name	Length(aa)	Signal	Functional Description
Acry_2099	ApcA	123	Yes	10.1 kDa monoheme cytochrome c Class I
Acry_3471	ApcB	114	Yes	11.7 kDa monoheme cytochrome c Class I
Acry_2798	OacA	410	Yes	42.0 kDa monoheme cytochrome c Class I

Table 4. Proteins expressed under Cr-grown conditions. These proteins were not detected in non-Cr(VI) cultures.

Growth Condition	Accession Number	Protein Name	Peptides
6 ppm Cr(VI)	YP_001235217.1	cytochrome c class I (ApcA)	5
	YP_001234854.1	citrate synthase I	4
	YP_001234897.1	NifU domain containing protein	4
	YP_001235148.1	isocitrate dehydrogenase	4
	YP_001235863.1	acetoacetyl CoA reductase	4
	YP_001236132.1	hypothetical protein Acry 3023	4
20 ppm Cr(VI)	YP_001234897.1	NifU domain containing protein	6
	YP_001233708.1	isocitrate dehydrogenase	5
	YP_001235217.1	cytochrome c class I (ApcA)	5
	YP_001234854.1	citrate synthase I	4

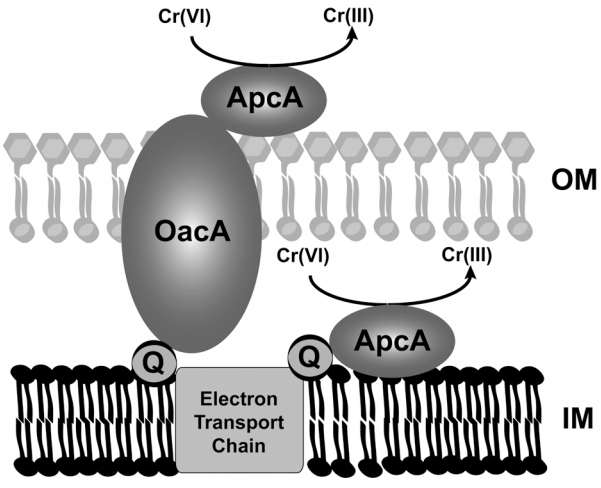

Figure 5. Electron transport model for *A. cryptum* JF-5 chromate reduction. ApcA can be reduced by the quinol pool, and then react with Cr(VI). Alternatively, extracellular ApcA can be reduced by other cell-associated cytochromes (e.g. OacA), and then in turn reduce Cr(VI).

Characterization of purified redox proteins.

ApcA: This protein has one heme group, complexed by His and Met axial ligands in the 5th and 6th coordinate positions. The redox behavior of this cytochrome towards different metals and metal complexes was assessed using spectrophotometry. The protein was dissolved in citrate buffer pH 3 (the same pH as found in the periplasm of the organism) and titrated with reductant until reduced. The electron acceptor under study was then added until the protein was re-oxidized. This protein appears to be a high-potential monoheme cytochromes which, in its reduced state, can in turn reduce Cr(VI) and Fe(III)-citrate. There appears to be no pH-dependent redox behavior, since the same results were obtained at pH 7.0 as well. Figure 6 shows a summary of redox spectra obtained. This protein was also the first to be extensively characterized, and the interaction of this protein with metal oxide surfaces was investigated by differential pulse voltammetry. This work was performed by collaborator Carrick Eggleston and his research group at the University of Wyoming. Our working hypothesis, that cytochrome c proteins undergo conformational change as a result of interaction with mineral surfaces, was confirmed. This work was subsequently published (see Publications section below).

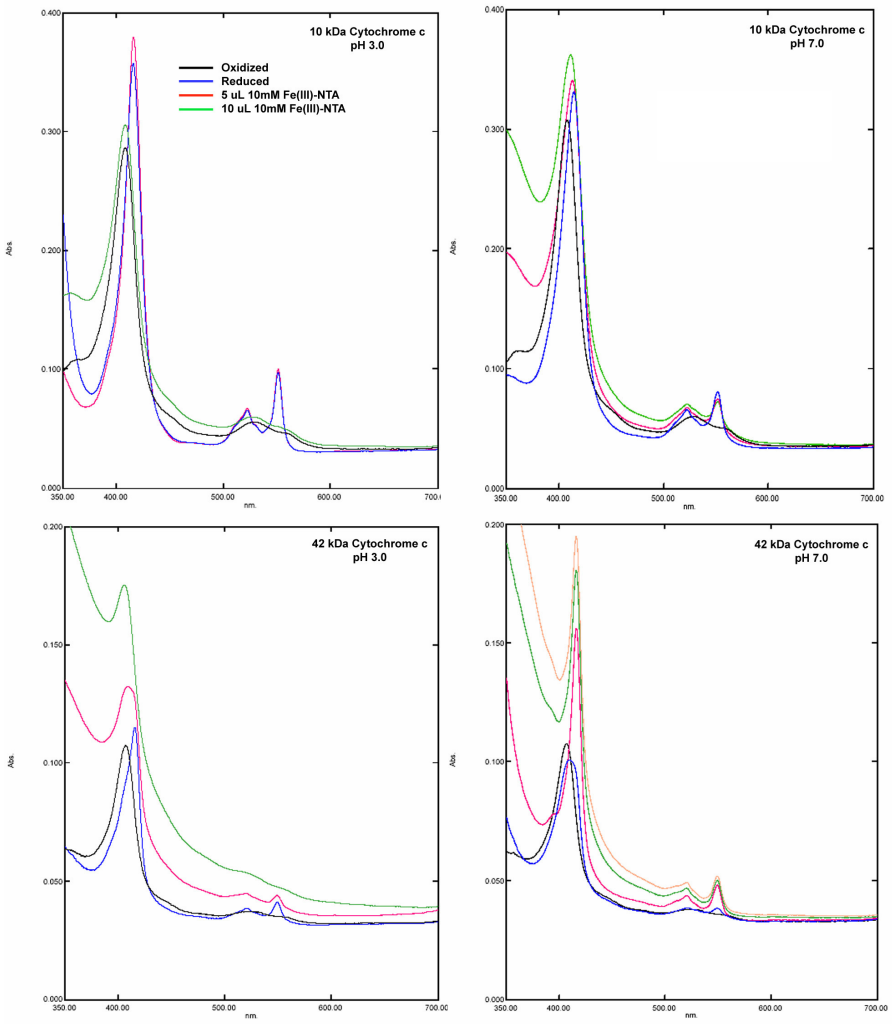

Figure 6. Redox spectra of purified cytochromes c from the acidophile *A. cryptum*. Upper panels-10 kDa cytochrome c; Lower Panels-42 kDa cytochrome c. Both proteins show pH dependent reoxidation when reacted with an Fe(III) chelate, with low pH being favored. This is consistent with these proteins being functional in acidic environments.

OacA: This protein was shown to reside in the outer membrane, as judged by differential extraction and sucrose gradient ultracentrifugation experiments. The redox properties were measured as described above for PacA. This cytochrome was reactive towards Fe(III)-citrate and Cr(VI), and showed optimal redox activity at pH 3.0. This is understandable, since this protein is exposed to the outer face of the cell surface, where pH is low (Figure 5).

Publications:

B.R. Briggs, T. Mitton, R. Smith, T.S. Magnuson. 2009. Teaching cellular respiration & alternate energy sources with a laboratory exercise developed by a scientist-teacher partnership. Amer. Biology Teacher 71(3): 164-167.

Eggleston, C.M, T. S. Magnuson, N. Khare, P.J.S. Colberg, M.W. Swenson, and L. Sycheva. 2009. Properties of adsorbed cytochromes from dissimilatory metal reducing bacteria: Density and rigidity of periplasmic vs. outer-membrane cytochromes. In Preparation.

Magnuson, T.S., M.W. Swenson, T.T. Gresham, L.A. Deobald, A.J. Paszczynski, and D.E. Cummings. 2009. Physiology and proteogenomics of Cr(VI) reduction in *Acidiphilium cryptum* JF-5. In Preparation.

Ray, A.E., P.P. Sheridan, M.S. Shields, J. Gilbreath, S.A. Connon, D.T. Newby, Y. Fujita, and T.S. Magnuson. 2009. 16S rRNA pseudogene detection in an environmental isolate. In Preparation.

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Cummings, D.E., and T.S. Magnuson. 2007. Microbial Fe(III) reduction: ecological and physiological considerations. *In* Manual of Environmental Microbiology, 3rd Edition. American Society for Microbiology Press. Ch. 101.

Cummings, D. E., S. Fendorf, N. Singh, R. K. Sani, B. M. Peyton, and T. S. Magnuson. 2007. Reduction of Cr(VI) under acidic conditions by the facultative Fe(III)-reducing bacterium *Acidiphilium cryptum*. Env. Sci. Technol. 41:146-152.

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Meeting Presentations:

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Swenson, M.W., and T.S. Magnuson. 2009. Identification of Excreted and Surface Associated Proteins in *Acidiphilium cryptum* JF-5. 109th General Meeting, American Society for Microbiology, Philidelphia PA.

Cummings, D.E., D. Kerk, D. Sims, P. Richardson, B. Briggs, M. Swenson, and T.S. Magnuson. 2008. The *Acidiphilium cryptum* genome reveals capacities for metal transformation and mineral colonization. UCLA-Lake Arrowhead Conference on Microbial Genomics, Lake Arrowhead, CA.

M.W. Swenson, T.S. Magnuson, P.J.S. Colberg, and C.M. Eggleston. 2008. Functional Characterization of c-type Cytochromes from Iron-Respiring Bacteria. 18th V.M. Goldschmidt Conference, Vancouver, BC.

Swenson, M.W., P.J.S. Colberg, C.M. Eggleston, and T.S. Magnuson. 2008. Purification and Functional Characterization of Two Periplasmic C-Type Cytochromes from *Acidiphilium cryptum.* American Society for Microbiology General Meeting, Boston, MA.

Gresham, T.G., B. Briggs, M. Swenson, M. Day, L. Yang, M. A. Thomas, P. P. Sheridan, D. Sims, P. Richardson, D. Kerk, D. E. Cummings, and T. S. Magnuson. 2007. Genome Sequencing and Annotation of the Acidophilic Metal-reducing Bacterium *Acidiphilium cryptum* JF-5. Department of Energy-Joint Genome Institute Users Meeting, Walnut Creek, CA.

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Magnuson, T.S., M.W. Swenson, R.N. Ledbetter. 2007. Purification of redox active biomolecules from pure cultures and complex samples. Inland Northwest Research Alliance Environmental and Subsurface Science Symposium, Logan, UT.

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Magnuson, T.S. et al. 2006. Biochemical and genomic inquiries into the metal-reducing acidophile *Acidiphilium cryptum* JF-5. INRA Environmental Biofilms Symposium, Bozeman MT.

Tyler, T.L., D.E. Cummings, and T.S. Magnuson. 2006. Chemiluminescence-based detection of a novel chromate reductase on polyacrylamide gels. 106th General Meeting, American Society for Microbiology, Orlando, FL.

Cummings, D.E., T.L. Tyler, M.E. Swenson, and T.S. Magnuson. 2005. Comparative biochemistry of cytochromes c from acidophilic and neutrophilic iron-reducing bacteria. 15th Annual Goldschmidt Conference, Moscow, ID.

Magnuson, T.S., D.E. Cummings, T.L. Tyler, M.E. Swenson. 2005. Emerging mechanisms of electron transport in iron reducing bacteria. INRA Environmental and Subsurface Science Symposium, Big Sky, MT.

Tyler, T.L., M.W. Swenson, D.E. Cummings, and T.S. Magnuson. 2005. Chromate reduction by the acidophilic metal-reducing bacterium *Acidiphilium cryptum* JF-5**.** The Joint International Symposia for Subsurface Microbiology (ISSM 2005) and Environmental Biogeochemistry (ISEB XVII), Jackson, WY.

Swenson, M.W, D.E. Cummings, and T.S. Magnuson. 2005. Purification and biochemical characterization of four *c*-type cytochromes from the Fe(III)-respiring bacterium *Acidiphilium cryptum*. The Joint International Symposia for Subsurface Microbiology (ISSM 2005) and Environmental Biogeochemistry (ISEB XVII), Jackson, WY.

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Cummings, D.E., M.E. Swenson, T.L. Tyler, and T.S. Magnuson. 2004. Comparative physiology and the discovery of 'metal reductase' proteins in an acidophilic metal-reducing bacterium. West Coast Bacterial Physiologists Annual Meeting, Pacific Grove, CA.

Magnuson, T.S., A. Rasmussen, R.K. Sani, B.E. Peyton, C.R. Breckenridge, and D.E. Cummings. 2004. Acidophilic iron-reducing bacteria: shared traits and unique characteristics as compared with their neutrophilic counterparts. 104th General Meeting, American Society for Microbiology, New Orleans, LA.