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14. ABSTRACT
We exploited a novel spectrophotometer where the cuvette is a reflecting cavity completely filled with an absorbing suspension of live, intact bacteria to monitor the in situ absorbance changes in bacteria as they respired aerobically on soluble ferrous ions. Our prior observations suggested the following hypothesis: acidophilic bacteria that belong to different phyla express different types of electron transfer proteins to respire on extracellular iron. We tested this hypothesis using six different organisms that represented each of the six phyla of microorganisms that respire aerobically on iron. Each of these six organisms expressed spectrally different biomolecules that were

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a. REPORT	b. ABSTRACT	c. THIS PAGE			Robert Blake
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Report Title

Final Report: Optimizing the Electron Transfer Reactions at the Cathode of Microbial Fuel Cells

ABSTRACT

We exploited a novel spectrophotometer where the cuvette is a reflecting cavity completely filled with an absorbing suspension of live, intact bacteria to monitor the in situ absorbance changes in bacteria as they respired aerobically on soluble ferrous ions. Our prior observations suggested the following hypothesis: acidophilic bacteria that belong to different phyla express different types of electron transfer proteins to respire on extracellular iron. We tested this hypothesis using six different organisms that represented each of the six phyla of microorganisms that respire aerobically on iron. Each of these six organisms expressed spectrally different biomolecules that were redox-active during aerobic respiration on iron. In all six cases, compelling kinetic evidence was collected to indicate that the biomolecules in question were obligatory intermediates in their respective respiratory chains. Additional experiments with intact Acidithiobacillus ferrooxidans revealed that the crowded electron transport proteins in this organism's periplasm constituted a semi-conducting medium where the network of protein interactions functioned in a concerted fashion as a single ensemble. Thus the molecular oxygen-dependent oxidation of the multi-center respiratory chain occurred with a single macroscopic rate constant, regardless of the proteins' individual redox potentials or their putative positions in the aerobic iron respiratory chain.

Enter List of papers submitted or published that acknowledge ARO support from the start of the project to the date of this printing. List the papers, including journal references, in the following categories:

(a) Papers published in peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in peer-reviewed journals:

(b) Papers published in non-peer-reviewed journals (N/A for none)

Received Paper

TOTAL:

Number of Papers published in non peer-reviewed journals:

(c) Presentations

Robert Blake II and Megan Griff (2013) "Respiratory enzymes of Leptospirillum ferriphilum: Cytochrome 579 behaves differently in situ than it does in vitro", poster presented at the DOE's 2013 Annual Genomic Science Meeting, February 25 to 28, Bethesda, MD

Blake, R.C., II, Li, T.F., Painter, R.G., and Ban, B. (2014) “The multicenter aerobic iron respiratory chain of Acidithiobacillus ferrooxidans functions as an ensemble with a single macroscopic rate constant”, poster presented at the DOE's 2014 Annual Genomic Science Meeting, February 10 to 12, Crystal City, Washington, D.C.

Blake, R.C., II, Li, T.F., Painter, R.G., and Ban, B. (2015) “A spectroscopic device to monitor respiratory electron transfer in suspensions of live organisms”, platform talk presented at the DOE's 2015 Annual Genomic Science Meeting, February 23 to 25, Washington, D.C.

Number of Presentations: 3.00

Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Non Peer-Reviewed Conference Proceeding publications (other than abstracts):

Peer-Reviewed Conference Proceeding publications (other than abstracts):

Received	Paper

TOTAL:

Number of Peer-Reviewed Conference Proceeding publications (other than abstracts):

(d) Manuscripts

Received		Paper
08/29/2014	1.00	Robert C. Blake II, Megan N. Griff. Respiratory enzymes of Leptospirillum ferriphilum: cytochrome 579 behaves differently in situ than it does in vitro, Journal of Biological Chemistry (04 2014)
08/29/2014	2.00	Robert C. Blake II, Ting-Feng Li, Bhupal Ban, Richard G. Painter. The multicenter aerobic iron respiratory chain of Acidithiobacillus ferrooxidans functions as an ensemble with a single macroscopic rate constant, Journal of Biological Chemistry (06 2014)
TOTAL:		**2**

Number of Manuscripts:

Books

Received	Book
TOTAL:	

Received	Book Chapter
TOTAL:	

Patents Submitted

Patents Awarded

Awards

Graduate Students

NAME	PERCENT_SUPPORTED
FTE Equivalent:	
Total Number:	

Names of Post Doctorates

NAME	PERCENT_SUPPORTED
Tingfeng Li	0.33
Richard Painter	0.33
Bhupal Ban	0.25
FTE Equivalent:	**0.91**
Total Number:	**3**

Names of Faculty Supported

NAME	PERCENT_SUPPORTED	National Academy Member
Robert Blake II	0.10	
FTE Equivalent:	**0.10**	
Total Number:	**1**	

Names of Under Graduate students supported

NAME	PERCENT_SUPPORTED	Discipline
Michael Tran	0.00	Biosciences
FTE Equivalent:	**0.00**	
Total Number:	**1**	

Student Metrics

This section only applies to graduating undergraduates supported by this agreement in this reporting period

The number of undergraduates funded by this agreement who graduated during this period: 0.00

The number of undergraduates funded by this agreement who graduated during this period with a degree in science, mathematics, engineering, or technology fields:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and will continue to pursue a graduate or Ph.D. degree in science, mathematics, engineering, or technology fields:..... 0.00

Number of graduating undergraduates who achieved a 3.5 GPA to 4.0 (4.0 max scale):..... 0.00

Number of graduating undergraduates funded by a DoD funded Center of Excellence grant for Education, Research and Engineering:..... 0.00

The number of undergraduates funded by your agreement who graduated during this period and intend to work for the Department of Defense 0.00

The number of undergraduates funded by your agreement who graduated during this period and will receive scholarships or fellowships for further studies in science, mathematics, engineering or technology fields: 0.00

Names of Personnel receiving masters degrees

NAME

Total Number:

Names of personnel receiving PHDs

NAME

Total Number:

Names of other research staff

NAME PERCENT_SUPPORTED

FTE Equivalent:
Total Number:

Sub Contractors (DD882)

Inventions (DD882)

Scientific Progress

See Attachment

Technology Transfer

Final Progress Report for Grant Number W911NF-12-1-0042

We developed and demonstrated the means to directly observe and document respiratory electron transfer reactions in live bacteria as they exchanged electrons with soluble iron under physiological conditions. We exploited a new type of spectrophotometer known as an integrating cavity absorption meter (ICAM). The premise was that accurate UV-visible spectroscopy of electron transfer reactions among colored cytochromes could be conducted in highly turbid suspensions if the live bacteria were irradiated in an isotropic homogeneous field of incident measuring light. Under these conditions, the absorbed radiant power was expected to be independent of scattering effects. We cooperated with On Line Instrument Systems (Bogart, GA) to use an experimental beta unit of a commercial integrating cavity absorption meter where the cuvette was a reflecting cavity completely filled with the absorbing suspension.

Our initial hypothesis was that acidophilic bacteria with different types of cell walls would express different types of electron transfer proteins to respire on extracellular iron. The rationale for this hypothesis was that the electron transfer chains of single-celled organisms that respire aerobically on iron must conduct electrons from the extracellular milieu to the interior of the cell because there is no evidence that soluble iron ever enters the cell in bulk quantities. The cell wall architectural features of the Gram-negative, Gram-positive, and archaea bacteria are so structurally different that it is difficult to imagine how all three types of organisms could express the identical biomolecules to conduct electrons from the exterior of the cell to their interior to accomplish oxidative phosphorylation. Thus the premise is that bacteria with different types of cell walls must express different electron transfer proteins to respire aerobically on extracellular ferrous ions.

We collected spectrophotometric data over the 3-year course of this project that were consistent with the hypothesis stated above. Our ongoing spectroscopic observations led to the following new hypothesis that extended the initial hypothesis: acidophilic microorganisms that belong in different genetic phyla also express different types of electron transfer proteins to respire on extracellular iron. An evolutionary tree that shows the approximate locations of acidophilic microorganisms that are capable of aerobic respiration on extracellular iron is shown in Figure 1. The organisms identified in Fig. 1 are distributed among 6 different phyla in the bacterial and archaea kingdoms, as indicated in the figure.

We conducted functional studies using the ICAM with representative members of each of the six phyla identified in Fig. 1. The results of these studies are summarized in Figure 2. The difference spectra shown in Fig. 2 were obtained when soluble ferrous ions were rapidly mixed with live, intact strains of iron-oxidizing Gram-negative eubacteria (Nitrospiraceae and Proteobacteria), Gram-positive eubacteria (Firmicutes and Actinobacteria) and archaea (Euryarchaeota and Crenarachaeota) under acidophilic physiological solution conditions and

Figure 1. Evolutionary tree showing the approximate locations of acidophilic microorganisms capable of aerobic respiration on extracellular iron. Each of the six relevant phyla is further represented by a dendrogram that contains the genus or species of interest.

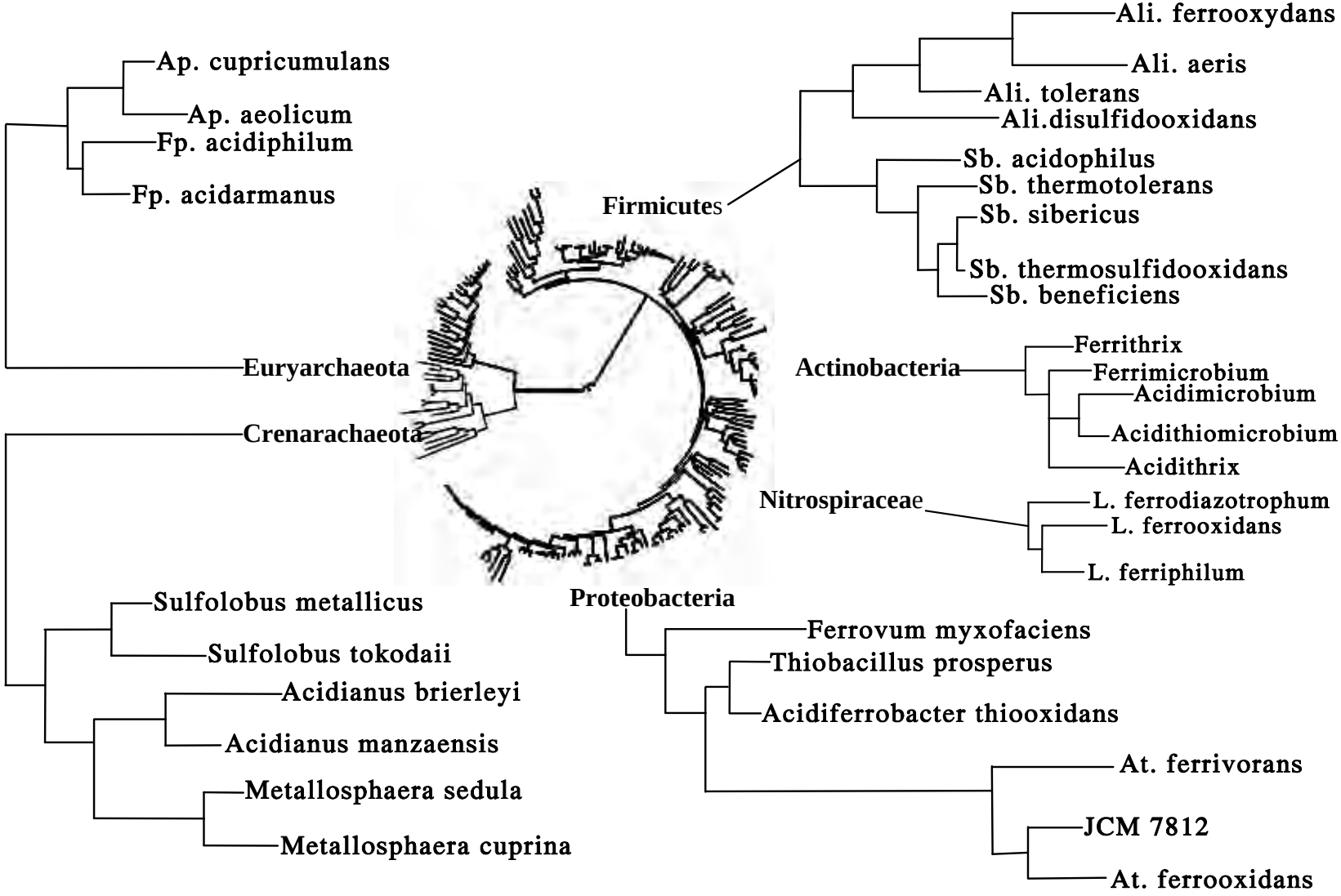
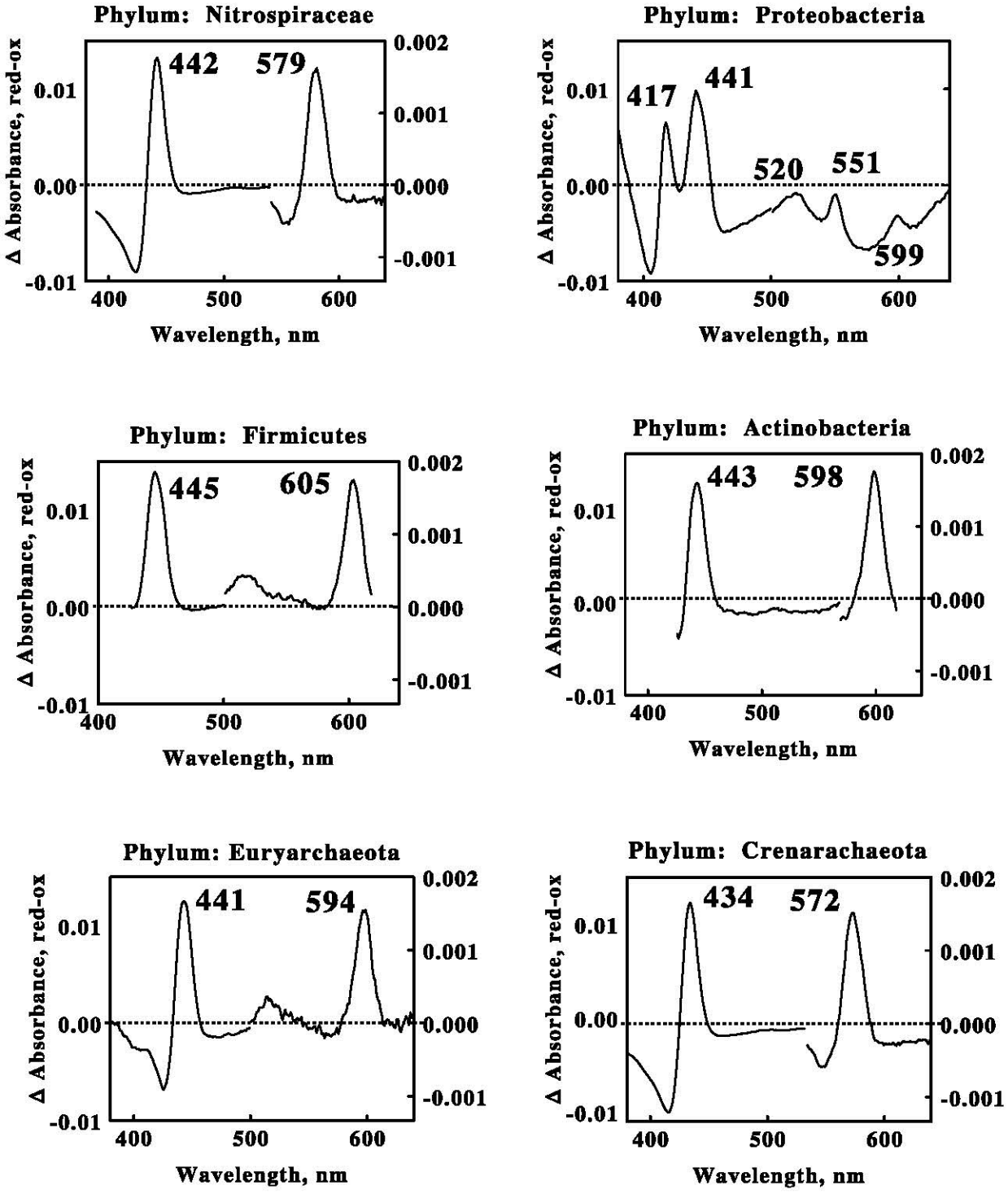


Figure 2. Iron-reduced minus oxidized difference spectra of representative intact micro-organisms in six different phyla. Spectra were obtained using an integrating cavity absorption meter. Organisms: Nitrospiraceae, *Leptospirillum ferriphilum*; Proteobacteria, *Acidithiobacillus ferrooxidans*; Firmicutes, *Sulfobacillus thermosulfidooxidans*; Actinobacteria, *Ferrimicrobium acidiphilum*; Euryarchaeota, *Acidiplasma aeolicum*; Crenarachaeota, *Acidianus brierleyi*.



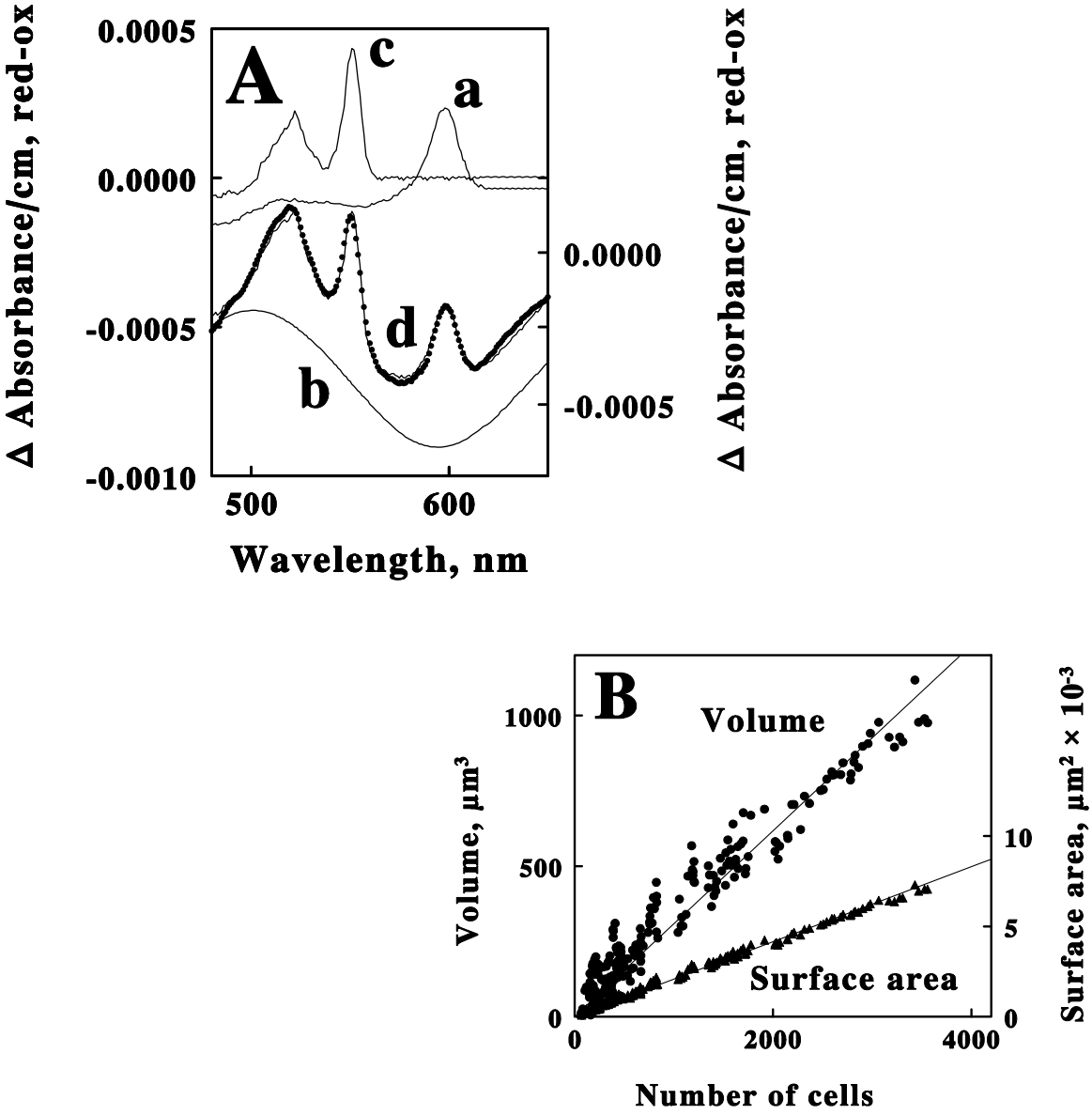
temperatures appropriate for each organism. In each case, a spectrally unique, prominent redox-active electron transfer protein was immediately reduced when the intact cells were mixed with soluble ferrous ions. In each case, subsequent steady state turnover experiments were conducted where the initial concentrations of ferrous iron were less than or equal to that of the oxygen concentration. Under these conditions, the resting absorbance spectrum of the bacterium observed under air-oxidized conditions was always regenerated from that of the Fe(II)-reduced bacterium initially observed in the presence of Fe(II). These observations confirmed that each different type of electron transfer protein participated in the respective iron respiratory chain of each organism during aerobic respiration.

Manuscripts that describe the studies obtained with two of these organisms were prepared and submitted to the *Journal of Biological Chemistry*; the current status of these two manuscripts is summarized below. It is anticipated that the remainder of these data will form the basis for four additional manuscripts for a peer-reviewed journal that describes the functional respiratory enzymes of *Sulfobacillus thermosulfidooxidans, Ferrimicrobium acidiphilum, Acidiplasma aeolicum,* and *Acidianus brierleyi*. We intend to continue to work on these manuscripts and submit them for publication in the coming year even though funds from the ARO for this project have terminated. We will keep the ARO informed if and when additional manuscripts are accepted for publication.

The functional ICAM data obtained with intact *At. ferrooxidans* was of particular interest due to the insights that we obtained regarding the high concentrations of electron transfer proteins in the periplasm of that organism. Fig. 3A shows the difference spectrum obtained when *At. ferrooxidans* was rapidly mixed with 400 µM Fe(II) in sulfuric acid, pH 2.0, at 30^{o} C. The electron transfer pathway for the flow of electrons from iron to molecular oxygen in *At. ferrooxidans* is hypothesized from earlier studies by many laboratories to consist of an initial electron transfer from extracellular ferrous ions to a cytochrome *c* located in the outer membrane of this Gram-negative organism. The periplasmic blue copper protein, rusticyanin, then transfers the electron from the cytochrome *c* in the outer membrane to a different periplasmic cytochrome *c*. The final electron transfer is from the periplasmic cytochrome *c* to the terminal oxidase, an *aa3*-type cytochrome that is located in the cytoplasmic membrane and reduces molecular oxygen. All three types of electron transport proteins were identified and readily visible in the difference spectrum shown in Fig. 3A. The participation of *c*-type cytochromes was indicated by the peaks at 417, 520, and 551 nm, while the participation of *a*-type cytochromes was indicated by the peaks at 441 and 598 nm. Finally, the broad trough in the difference spectrum from 500 to 650 nm was consistent with the hypothesis that large quantities of rusticyanin were also reduced by soluble iron.

The data shown in Fig. 3A illustrate the approach taken to quantify the visible electron transport proteins in intact *At. ferrooxidans* that were transiently reduced by soluble iron. *Curve a* is a reduced minus oxidized difference spectrum for 13.4 nM cytochrome *a* that was calculated

Figure 3. Determination of the redox-active protein concentrations in the periplasm of *Acidithiobacillus ferrooxidans*. **A**, deconvolution into individual components of the difference spectrum obtained when intact *At. ferrooxidans* was reduced soluble iron. Curves *a*, *b*, and *c* are the reduced minus oxidized difference spectra for 13.5 nM cytochrome *a*, 311 nM rusticyanin, and 20.6 nM cytochrome *c*, respectively; curve *d* is the sum of curves *a*, *b* and *c*. The *data points* are those taken 0.5 seconds after 5.8×10^8 cells of *At. ferrooxidans* were mixed in the ICAM with 300 µM ferrous sulfate, pH 1.5, at 30^o C. **B**, dependencies of the volume and surface area of *At. ferrooxidans* as a function of the number of cells as measured by electrical impedance using a Multisizer IV.



from spectra published using the electrophoretically homogeneous cytochrome oxidase purified from cell-free extracts of *At. ferrooxidans*. Similarly, *curve c* is a difference spectrum for 20.6 nM cytochrome *c* that was calculated from published spectra of cytochrome *c* also purified from the same organism. Curve *b* is a difference spectrum for 311 nM rusticyanin that was calculated from the spectra of rusticyanin purified by my laboratory and others. *Curve d* is the sum of *curves a*, *b*, and *c*. The data points in Fig. 3A represent the actual difference spectrum that was observed experimentally. The close correspondence between the calculated and the observed spectra indicated that (i) these three components were the principal electron transport proteins that were reduced by extracellular iron and (ii) we had the means to quantify the concentration of each type of reduced protein. When the soluble ferrous concentrations were 2 to 4 mM and far exceeded the limited electron-accepting capacity of the soluble molecular oxygen, the maximum concentrations of reduced proteins in the organism were observed to be 510, 22, and 36 nM for rusticyanin and cytochromes *a* and *c*, respectively.

Fig. 3B shows the dependencies of the volume and the surface area of intact *At. ferrooxidans* on the number of cells derived from over 220 assays using electrical impedance measurements. Each value of the volume represents the sum of the individual volumes for all of the cells measured in that particular assay. Each value of the surface area is the sum of individual surface areas calculated from the corresponding volumetric data assuming that each bacterial particle is a perfect sphere. An average volume and surface area for intact *At. ferrooxidans* of 0.31 ± 0.01 μm^3 and 2.1 ± 0.05 μm^2, respectively, were obtained from the slopes of the two lines in Fig. 3B.

The data in Figs. 3A and B were subsequently used to estimate the concentrations of the respiratory components in the periplasm of the bacterium. The maximum rusticyanin concentration was determined spectrophotometrically as 510 nM, which corresponds to 4.08 nmoles in the 8 mL volume of the observation cuvette. At 0.31 μm^3 per cell, the total cellular volume of the 4.6×10^9 cells in the cuvette was 1.43×10^9 μm^3. If 10 to 20% of that total cellular volume were periplasmic space, then the concentration of rusticyanin in the periplasm was between 14.3 and 28.5 mM, or 240 and 470 mg/mL. Taking 15% as a compromise value, our data yield a periplasmic concentration for rusticyanin of 21.4 mM, or 350 mg/mL. Similarly, the concentration of cytochrome *c* in the periplasmic space was 25 mg/mL. The volume of a single rusticyanin molecule is approximately 20 nm^3, as determined previously by my laboratory using the actual dimensions of the purified rusticyanin obtained from structural studies by both X-ray crystallographic and multidimensional NMR means. Consequently, the rusticyanin protein at 350 mg/mL occupies 4.6×10^{16} nm^3 or 21% of the total volume in the periplasmic space! What emerges from these calculations is the realization that the concentrations of respiratory proteins in the periplasm are orders of magnitude higher than any encountered in published *in vitro* functional studies. We hypothesize that these respiratory proteins have evolved to function as an ensemble in this crowded environment and that the crowded periplasmic space has a structure and order that we haven't yet appreciated or characterized.

These observations were summarized in a manuscript entitled "The multicenter aerobic iron respiratory chain of *Acidithiobacillus ferrooxidans* functions as an ensemble with a single macroscopic rate constant" that was submitted to the *Journal of Biological Chemistry*. The manuscript received a favorable review, but final formal acceptance awaits (relatively minor) revisions that are currently in progress.

The functional ICAM data obtained with intact *L. ferriphilum* was also of particular interest due to the insights that we obtained by comparing the properties of the redox-active cytochrome expressed in the periplasm of the organism with the functional properties of the same protein as observed in the intact bacterium under physiological conditions. The purified cytochrome had subunit and native masses of 16 and 125 kilodaltons, respectively. The purified cytochrome *in vitro* reacted relatively sluggishly with soluble iron at pH 1.7. In contrast, the red cytochrome was electrochemically reduced relatively rapidly *in situ* when a suspension of live bacteria was mixed with soluble iron at pH 1.7 in the ICAM. Steady state turnover experiments were conducted where the initial concentrations of ferrous iron were less than that of the oxygen concentration. Under these conditions, the initial absorbance spectrum of the *in situ* cytochrome observed under air-oxidized conditions was always regenerated from that of the electro-chemically reduced cytochrome observed in the presence of Fe(II). The kinetics of aerobic respiration on soluble iron by the intact bacterium conformed to the Michaelis-Menten formalism. The reduced intracellular red cytochrome represented the Michaelis complex whose subsequent oxidation appeared to be the rate-limiting step in the overall aerobic respiratory process. These data were consistent with the hypothesis that the reduced red cytochrome is an obligatory steady state intermediate in the iron respiratory chain of this bacterium. The most interesting feature of our observations was that the kinetic behavior of the cytochrome 579 observed in live *L. ferriphilum* was far different from that obtained with the purified protein. The same reduction reaction that was complete within 0.5 s when intact *L. ferriphilum* was mixed with 100 µM Fe(II) was predicted from our *in vitro* functional data to occur with an extrapolated pseudo-first order rate constant of 7×10^{-10} s^{-1} using the purified protein, a difference in reactivity of over a billion-fold!

These observations were summarized in a manuscript entitled "Respiratory enzymes of *Leptospirillum ferriphilum*: cytochrome 579 behaves differently *in situ* than it does *in vitro*" that was also submitted to the *Journal of Biological Chemistry*. The manuscript received an unfavorable review. The reviewers felt that the description of the structural and functional characterization of the purified cytochrome 579 could be worthy of publication in this journal, but that the description of the experiments with intact cells was not. Consequently, we are dividing the data into two manuscripts: one manuscript focuses on the properties of the purified cytochrome and will be resubmitted to the *Journal of Biological Chemistry*; the other manuscript focuses on the spectroscopic measurements conducted using the ICAM and will be submitted to *Applied and Environmental Microbiology*.