Manuscript received for “The Book”
“Biochemistry and Physiology of Anaerobic Bacteria”

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Harold Drake harold.drake@bioek.uni-bayreuth.de and Kirsten Küsel	University of Bayreuth Germany	“How The Diverse Physiological Potentials of Acetogens Determine Their <i>In situ</i> Realities”
Jim Ferry jfg3@psu.edu	The Pennsylvania State University, University Park, PA	“One-carbon Metabolism in Methanogenic Anaerobes”
Howard Gest hgest@sunflower.bio.indiana.edu	Indiana University Bloomington, IN	“Anaerobes In The Recycling of Elements In The Biosphere”
Boi Hanh”Vincent”Huynh vhuyh@emory.edu	Emory University Atlanta, GA	“Reductive Activation of Aerobically Purified <i>Desulfovibrio vulgaris</i> Hydrogenase: Mössbauer Characterization of the Catalytic H Cluster”
Michael Johnson, johnson@sunchem.chem.uga.edu Jeffrey N. Agar, and Dennis R. Dean	The University of Georgia Athens, GA	“Iron-Sulfur Cluster Biosynthesis”
Donovan P. Kelly	University of Warwick Germany	“Inorganic Sulfur Oxidation: An Anaerobe-Aerobe Interface”
Don M Kurtz, Jr. kurtz@sunchem.chem.uga.edu	The University of Georgia Athens, GA	“Oxygen and Anaerobes”
Lars Ljungdahl larsljd@arches.uga.edu Amaresh Das adas@arches.uga.edu	The University of Georgia	“Electron Transport Systems in Anaerobes”

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Rob Maier, mmaier@calc.vet.uga.edu J. Olson, and N. Mehta	The University of Georgia	"Genes and Proteins Involved in Nickel Dependent Hydrogenase Expression"
Ed Laishley elaishle@ucalgary.ca and R.D. Bryant	University of Calgary Alberta, T2N 1N4, Canada	"Electron Flow in Ferrous Biocorrosion"
Hans Günter Schlegel hschlegi@gwdg.de	Institut für Mikrobiologie der George-August-Universität Germany	"The Diversity of Energy Sources of Microorganisms"
Theresa Stadtman tcstadtman@NIH.gov and William T. Self	National Institutes of Health	"Selenium-Dependent Enzymes from <i>Clostridia</i> "
Gerrit Voordouw Voordouw@acs.ucalgary.ca	University of Calgary T2N 1N4 Canada	"Function and Assembly of Electron Transport Complexes in <i>Desulfovibrio vulgaris</i> Hildenborough"
Judy Wall, wallj@missouri.edu Christopher L. Hemme, Barbara Rapp-Giles, Joseph A. Ringbauer, Jr., Laurence Casalot, and Tara Giblin	University of Missouri- Columbia Columbia, MO	"Genes and Genetic Manipulations of <i>Desulfovibrio</i> "
Juergen Wiegel jwiegel@uga.edu and Justin Hanel	The University of Georgia	Chemolithoautotrophic Thermophilic Iron (III)- reducer

The Power of Anaerobes

May 18 - 19, 2000

The University of Georgia
Athens, Georgia

THE POWER OF ANAEROBES

WELCOME RECEPTION:

May 17, 2000, from 7:00 - 9:00 P.M. in Hill Atrium 1 at
The Georgia Center for Continuing Education.

BANQUET: May 18, 2000, in Room 103, Banquet
area at The Georgia Center for Continuing Education

◆ **SOCIAL HOUR - 6:00 - 7:00 p.m.**

◆ **DINNER - 7:00 p.m.**

NOTE: AAA SHUTTLE
TELEPHONE # 1-800/354-7874

POWER OF ANAEROBES SYMPOSIUM

GERTI SCHUT ATHENS-ATLANTA ON FRIDAY, MAY 19TH

	<u>Depart Athens</u>	<u>Arrive Atlanta Airport</u>	<u>Depart Atlanta</u>
Barnoy	4:45 PM	6:30 PM	
Gest (2)	5:15 PM	6:30 PM	Staying over w/friends
Thauer	5:15 PM	6:30 PM	8:05 PM
Self	5:15 PM		10:05 PM
Wall (3)	(Car rental)		6:50 PM

AAA SHUTTLE ATHENS-ATLANTA ON SATURDAY, MAY 20TH

	<u>Depart Athens</u>	<u>Arrive Atlanta Airport</u>	<u>Depart Atlanta</u>
Barton (2)	6:45 AM	8:30 AM	10:00 AM
Wolfe	6:45 AM	8:30 AM	10:02 AM
Statman	6:45 AM	8:30 AM	10:40 AM
Akagi (2)	9:15 AM	11:00 AM	12:30 PM
Bock	9:15 AM	11:00 AM	1:45 PM
Albracht	11:45 AM	1:30 PM	4:30 PM
Ferry	11:45 AM	1:30 PM	2:25 PM
Voordouw	2:15 PM	4:00 PM	6:20 PM
Schlegel (2) (Will be picked up)			
Ragsdale (Car rental, own expense)			

AAA SHUTTLE ATHENS-ATLANTA ON SUNDAY, MAY 21ST

	<u>Depart Athens</u>	<u>Arrive Atlanta Airport</u>	<u>Depart Atlanta</u>
Cammack	11:45 AM or 2:15 PM	1:30 PM or 4:00 PM	6:20 PM
Truper (Car rental, own expense)			4:05 PM
Frey	11:45 AM	1:30 PM	4:30 PM
Drake (Will travel w/Whitman)			
Andreesen (Will travel w/Whitman)			

Welcome to *THE POWER OF ANAEROBES*

We welcome you to *The Power of Anaerobes*. This conference serves two purposes. One is to celebrate the life of Harry D. Peck, Jr., who was born May 18, 1927 and would have celebrated his 73rd birthday at this conference. He died November 20, 1998. The second is to gather investigators to exchange views within the realm of anaerobic microbiology, an area in which tremendous progress has been seen during recent years. It is sufficient to mention discoveries of a new form of life (the archaea), hyper or extreme thermophiles, thermophilic alkaliphiles and anaerobic fungi. With these discoveries has come a new realization about physiological and metabolic properties of microorganisms, and this in turn has demonstrated their importance for the development, maintenance and sustenance of life on Earth.

Harry was a philosopher of science and he had wide perspectives. He clearly predicted the recent developments in microbiology. He adapted modern biochemical, physiological, and molecular biological methods to studies of metabolism of hydrogen and respiratory sulfate reduction. He laid a foundation for the study of energy generation in anaerobes by chemiosmosis. The Power of Anaerobes conference reflects on Harry's scientific contributions.

Harry also had a great impact on The University of Georgia. He built the Department of Biochemistry, now the Department of Biochemistry and Molecular Biology, which he served as chairman and head for 26 years (1965-1991). He made major contributions to the quality and growth of the Biological Sciences Division of Franklin College. He was a key player in attracting outstanding faculty members and in the formation of research centers including the Complex Carbohydrate Research Center and the Center for Metalloenzyme Studies. Many faculty members are grateful for Harry's contributions both to science and to the University. We can not thank him personally, but we can celebrate his life, his many contributions and that we had the opportunity to know and work with him. Because of him we are all better off. What better way is there to celebrate Harry than *The Power of Anaerobes* conference?

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"A Way to Say Yes"

Harry Dowd Peck, Jr. came to the emerald canopied red hills of Georgia
via oak covered rocky ridges.

Inspired in youth by the storied accounts of Roy Chapman Andrews
uncovering 200 million year old dinosaur bones.

Yes, he knew life with microbes was older.

Little did he know about stromatolites or cherts off Darwin or oceanic plumes

But much he knew about sulfur and
the love of microbes for such odorous homes.

With mechanisms for gathering energy from the products of prehistoric volcanos,
migrating mantle plates, and their plumes, ridges, and trenches.

He imagined the sulfur life of microbes independent of sunlight.
Eubacteria living on Earth's geochemistry.

Pushed along by the Maddox-led creation of 450 new faculty posts,
Biochemistry was formed.

Harry moved into constantly stirring administration in an amorphous mixture and
into new research beginnings on the red Athenian hills.

He began to craft a bold inquisitive search
into life processes with a new faculty and new labs.

Graduate Research Studies was built and
Harry was in log phase growth, fermenting the UGA administration.

Microbial-like, Harry was absolutely opportunistic.

Aerobically or anaerobically Harry continuously thought, it can be done!

On every occasion with the faculty he searched for "A Way to Say Yes."

Never was Harry heard to say no—when a chance existed,
an idea was expressed, an opportunity arose,
a bending was possible, or a position could be twisted, moved or circumvented.
— Harry never said no.

Like a microbe in a hot marine sulfur soup off Darwin
he searched for a way and made new life on the red rocky hill of Life Sciences.

What a refreshing administrator
Harry always searched for "A Way to Say Yes!"

C. C. Black (December, 1994)

The Power of Anaerobes

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Georgia Center for Continuing Education

Athens, Georgia

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Atlanta, GA

Thursday, May 18

Diversity of Metabolism in Anaerobes I

Co-Chairs: Ralph Wolfe and Barney Whitman

8:25 a.m.	Welcome	J. David Puett
8:30 a.m.	Welcome	Karen Holbrook
8:40 a.m.	Diversity of Energy Sources for Microorganisms	Hans Schlegel
9:00 a.m.	Anaerobes in the Cycles of Nature	Howard Gest
9:30 a.m.	One-Carbon Metabolism in Anaerobes	Greg Ferry
10:00 a.m.	Break	Poster viewing
10:45 a.m.	Anaerobes and Selenium Containing Enzymes	Terry Stadtman
11:15 a.m.	Anaerobic Eukaryotes	Dick Cammack
11:45 a.m.	Dissimilatory Oxidation of Reduced Sulfur Compounds in Chromatiaceae	Hans Trüper
12:15 p.m.	Lunch	

Diversity of Metabolism in Anaerobes II

Co-Chairs: Harold Drake and Jim Akagi

1:15 p.m.	Reduction of Heavy Metals and Non-essential Elements by Anaerobes	Larry Barton
1:45 p.m.	Iron III as Electron Acceptor in Thermophilic Anaerobes	Juergen Wiegel
2:15 p.m.	Denitrification: A Key Step for Ecological Equilibrium—New and Old Enzymes	José Moura
2:45 p.m.	Hyperthermophilic Anaerobes	Mike Adams
3:15 p.m.	Break	Poster viewing

Molecular Biology of Anaerobes

Chair: Martin Odom

4:00 p.m.	Genes and Genetic Manipulations of <i>Desulfovibrio</i>	Judy Wall
4:30 p.m.	Function and Assembly of Electron Transport Complexes in <i>DusulfovibrioVulgaris</i> , Hildenborough	Gerrit Voordouw

Friday, May 19

Energetics of Anaerobes

Co-Chairs: Jack Payne and Jan Andreessen

8:30 a.m.	Energetics of Methanogens	Gerhard Gottschalk
9:00 a.m.	Methyl Coenzyme M Reduction: The Irreversible Step in Methanogenesis	Rolf Thauer
9:30 a.m.	Electron Transport Systems in Anaerobes	Lars Ljungdahl
10:00 a.m.	Break	Poster viewing
10:45 a.m.	Acetogens	Steve Ragsdale
11:15 a.m.	Electron Flow in Ferrous Biocorrosion	Ed Laishley
11:45 a.m.	Oxygen and Anaerobes	Don Kurtz
12:15 p.m.	Lunch	

Hydrogenases and Redox Proteins

Co-Chairs: Dan DerVartanian and Bob Scott

1:15 p.m.	Structural Analysis of Hydrogenases	Michel Frey
1:45 p.m.	Mössbauer Investigation of the Iron Cluster in <i>Desulfovibrio vulgaris</i> Hydrogenase	Vincent Huynh
2:15 p.m.	Mechanism of Hydrogen Activation	Siem Albracht
2:45 p.m.	Break	Poster viewing
3:30 p.m.	Synthesis of Hydrogenase in <i>E. coli</i>	August Böck
4:00 p.m.	Genes and Proteins in Ni-Dependent Hydrogenase Expression	Rob Maier
4:30 p.m.	Iron-Sulfur Cluster Biosynthesis	Mike Johnson
5:00 p.m.	Closing Comments	Lars G. Ljungdahl

With special thanks to Mrs. Le Bradham, Mrs. Geri McGuire, and Mrs. Angie Stockton, for their many contributions to the success of this event.

DIVERSITY OF ENERGY SOURCES FOR MICROORGANISMS

Hans Günter Schlegel

Institut für Mikrobiologie, Georg-August-Universität, Grisebachstraße 8, 37077 Göttingen, Germany

The talk aims at reminding the audience of the milestones of biological chemistry (the description of metals and gases and the elementary analysis of organic substances) as well as the recognition of the modes of energy conversion among living organisms (photosynthesis, respiration, fermentation and putrefaction, anoxygenic photosynthesis, chemolithoautotrophy, anaerobic intramolecular respiration) in order to outline the highlights of research on bacterial metabolism in the nineteenhundredfifties when Harry Peck started his scientific career.

Lengeler, J.W., Drews, G., Schlegel, H.D. (edt) Biology of the prokaryotes. Thieme, Blackwell, Stuttgart, 1999. (which is a handbook on biochemistry and regulation)

Schlegel, H.G. Geschichte der Mikrobiologie. Acta historica Leopoldina Nr. 28 Deutsche Akademie der Naturforscher Leopoldina, Halle (Saale) 1999.

ANAEROBES IN THE CYCLES OF NATURE

Howard Gest

Photosynthetic Bacteria Group, Department of Biology, Indiana University, Bloomington, IN 47405, USA

Anaerobes play important roles in the cycle transformations of C,N,S,O and H in the biosphere. The assortment of electron donors [e.g., organic compounds, Fe^{2+} , H_2 , S^{2-}] and electron acceptors [CO_2 , N_2 , H^+ , Fe^{3+} , S^0 sulfate, nitrate, fumarate] used by anaerobes attests to their virtuosity as “redox specialists.” Anaerobes are found in environments where dioxygen has been displaced by gaseous products of anaerobic metabolism such as CH_4 , CO_2 , H_2 , and H_2S . Despite sensitivity to O_2 , anaerobic bacteria also persist in circumstances thought to be aerobic in character. Thus, they commonly occur in micro-environments where O_2 is constantly removed by the respiration of aerobes (as in soil particles). In addition, some species are able to detoxify O_2 and its reactive reduction products by special mechanisms. A wealth of biogeochemical and fossil evidence supports the conclusion that for ca. 1.5 billion years, anaerobes were the only inhabitants of the early Earth. About 2 billion years before the present, O_2 produced by oxygenic photosynthesis began to accumulate in the atmosphere and this paved the way for evolution of aerobes. The presence of genes for proteins functional in anaerobic bioenergetics (e.g., fumarate and nitrate reductase genes) in extant obligate aerobes is one indicator of the great complexity of bacterial evolutionary phylogeny. Anaerobes prominent in several major natural cycles will be discussed.

Madigan, M.T., Martinko, J.M., and Parker, J.: *Brock Biology of Microorganisms*, Prentice Hall, Upper Saddle River, NJ. [Ninth ed., 2000]

ONE-CARBON METABOLISM IN ANAEROBES

James G. Ferry

Department of Biochemistry & Molecular Biology, Center for Microbial Structural Biology, The Pennsylvania State University, 205 Frear Laboratory, University Park, PA 16802

The conversion of acetate to methane accounts for approximately two-thirds of the nearly six million metric tons of biological methane produced each year. The pathway involves several one- and two-carbon reactions, many of which are also important in energy-yielding pathways of metabolically diverse anaerobes from both the Archaea and Bacteria domains. The first step in the pathway of methanogenesis from acetate by *Methanosarcina thermophila* involves activation to acetyl-CoA by acetate kinase and phosphotransacetylase, two enzymes that are of central importance for ATP synthesis in almost all anaerobes from both domains. An understanding of the catalytic mechanism for acetate kinase has been advanced by the recent crystal structure and development of site-directed mutagenesis approaches for the enzyme from *M. thermophila*. The second enzyme in the pathway is the acetyl-CoA synthase which catalyzes the cleavage of acetyl-CoA into a methyl group for reduction to methane and a carbonyl group for oxidation to carbon dioxide. The synthase has properties similar to the enzyme from homoacetogenic anaerobes. The carbon dioxide is hydrated to bicarbonate outside the cell membrane by a novel class of carbonic anhydrase found in a variety of prokaryotes. Electrons derived from the oxidation of carbon dioxide enter a membrane bound electron transfer chain coupled to the generation of a proton gradient that drives ATP synthesis. Transfer of electrons to the membrane is dependent on a ferredoxin and a novel iron-sulfur flavoprotein, both of which are common in anaerobes from both domains. The remaining methyl transfer and electron transfer reactions leading to methane formation involve enzymes and cofactors that are largely restricted to the methane-producing Archaea.

ANAEROBES AND SELENIUM CONTAINING ENZYMES

Thressa C. Stadtman, Laboratory of Biochemistry, National Institutes of Health, Building 3, Room 108, Bethesda, MD 20892-0320, phone: 301-496-3002, FAX: 301-496-0599, tcstadtman@nih.gov

Many mammalian and bacterial proteins contain selenium in the form of selenocysteine residues that are specifically incorporated, cotranslationally, as directed by UGA codons in the message. Additionally, three bacterial enzymes contain selenium in a bound labile cofactor form that is essential for catalytic activity. To date the occurrence of this type of selenoenzyme in eukaryotes is unknown. Selenium in the labile cofactor is coordinated to Mo of a bound molybdopterin cofactor in nicotinic acid hydroxylase from *Clostridium barkeri*. Likewise, selenium is coordinated to Mo of a molybdopterin cofactor in *Escherichia coli* formate dehydrogenase but in this enzyme the selenium is present in selenocysteine in the polypeptide. Selenocysteine derivatives that are specifically incorporated into polypeptides are synthesized from a special seryl-tRNA and selenophosphate. This oxygen-labile highly reactive biological selenium donor, monoselenophosphate, is formed from ATP and selenide by selenophosphate synthetase. The reaction mechanism leading to the formation of selenophosphate, orthophosphate and AMP (1:1:1) by the *E. coli* enzyme involves the initial phosphorylation of a group on the enzyme. Subsequently, this phosphoryl group is transferred to selenide and hydrolysis of bound ADP occurs. Various isoenzyme forms of selenophosphate synthetase have been identified in eukaryotes, prokaryotes, and archae. Some of these fail to utilize free selenide as substrate and require a selenium delivery enzyme system that utilizes selenocysteine as source of [Se⁰] (analog of sulfane sulfur). Some selenoenzymes that occur in *Methanococcus vannielii* and *Clostridium sticklandii* will be discussed.

IRON-SULFUR PROTEINS IN ANAEROBIC EUKARYOTES

Richard Cammack¹, David Horner², Mark van der Giezen², Jaroslav Kulda³

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²Molecular Biology Unit, Department of Zoology, The Natural History Museum, Cromwell Road, London SW7 5BD, U.K.

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Certain species of eukaryotes live in anaerobic environments. Some, such as the protozoa *Trichomonas vaginalis*, *Tritrichomonas foetus* and *Giardia lamblia* are pathogenic in Man or animals. Others, such as the rumen fungus *Neocallimastix frontalis*, are found in the digestive tracts of animals, or insects. These anaerobic eukaryotes contain intracellular organelles known as hydrogenosomes instead of mitochondria. Like the mitochondria, hydrogenosomes oxidize substrates such as pyruvate, but release H₂ instead of consuming O₂.

Genome sequencing indicates that they the hydrogenosomes share a common origin with the mitochondria; for example proteins targetted at the organelles have similar signal sequences. The course of evolution of these organisms is controversial. There is an increasing amount of information about gene sequences in these organisms, but less about the proteins involved. The hydrogen-producing metabolism involves the iron-sulfur proteins pyruvate: ferredoxin oxidoreductase (PFOR), ferredoxin and hydrogenase. The PFOR and [Fe]-hydrogenase are similar to those seen in anaerobic bacteria, but the [2Fe-2S] ferredoxins of the trichomonads have more in common with those from vertebrates. The iron-sulfur proteins of the trichomonads and *N. frontalis* have been surveyed by EPR spectroscopy. They appear to contain similar membrane-bound proteins of the [2Fe-2S] type. Other signals have been associated with hydrogenase and PFOR.

The low redox potentials generated by PFOR are the basis for the selective toxicity of the broad-spectrum nitroimidazole drug metronidazole toward the anaerobic pathogens. Reduction of metronidazole leads to the formation of free radicals. Strains of *T. vaginalis* which are resistant to metronidazole are now emerging, and more resistant strains have been developed in the laboratory by growth in the presence of increasing concentrations of the drug. Examination of the highly resistant strains shows that ferredoxin and other iron-sulfur proteins are at lower levels or, in extreme cases, absent.

Supported by grants from BBSRC and the European Union

DISSIMILATORY OXIDATION OF REDUCED SULFUR COMPOUNDS IN CHROMATIACEAE

Hans G. Trüper

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Purple sulfur bacteria (Chromatiaceae) generate electrons for photosynthesis by oxidizing reduced sulfur compounds. As the best known representative of this group we have chosen *Allochromatium* (formerly: *Chromatium*) *vinosum* as subject of our research.

One can differentiate 3 main steps in the oxidative pathway between sulfide and sulfate:

(1) Formation of "elemental sulfur" (S^0) from sulfide (by membrane bound sulfide:quinone oxidoreductase or by periplasmic flavocytochrome *c*) or from the sulfane group of thiosulfate (TS). The so-called S^0 is stored within protein envelopes that are topologically located outside the cytoplasm, i.e. in the periplasm. TS is split to S^0 and sulfite, the former stored, the latter oxidized and excreted as sulfate. (2) Further oxidation of the stored S^0 is probably initiated by reductive activation. The activated sulfur is oxidized to sulfite by the siroheme sulfite reductase and the other proteins encoded in the *dsr* operon. (3) The oxidation of sulfite occurs by two mechanism that are located in different compartments: (a) Direct oxidation to sulfate is catalyzed via sulfite:acceptor oxidoreductase located on the periplasmic side of the cytoplasmic membrane; (b) cytoplasmic oxidation to APS via APS reductase and subsequent release of sulfate by ATP sulfurylase. - As a consequence of the topological localization of the different metabolic steps several trans-membrane transport systems are required.

Brüser, T., Lens, P.N.L., Trüper, H.G.: The Biological Sulfur Cycle. In: P.N.L. Lens and L. Hulshoff Pol: Environmental Technologies to Treat Sulfur Pollution; p. 47-85, London 2000.

REDUCTION OF METALS AND METALLOIDS BY SULFATE-REDUCING BACTERIA

Larry L. Barton¹, Richard M. Plunkett¹, and Bruce M. Thomson².

¹Department of Biology and ²Department of Civil Engineering, University of New Mexico, Albuquerque, New Mexico. 87131.

As a result of natural or artificial processes, oxidized metals or metalloids accumulate in anaerobic sediments at toxic levels. While certain of these oxidized elements may be required by microorganisms at trace level, others have no known role in biology. Anaerobic bacteria have been demonstrated to transform toxic elements through various processes including volatilization, precipitation, or reduction. While there certainly may be numerous examples of anaerobic organisms with this capability of detoxifying metals and metalloids, bacteria that have received greatest attention at this time include members of the following genera: *Desulfovibrio*, *Shewanella*, *Geobacter*, *Desulfotomaculum*, and *Clostridium*. Most of these bacteria have the capability of using molecular hydrogen, lactate, or pyruvate as the electron donor and several are able to grow by dissimilatory metal reduction. Organisms such as *Desulfovibrio desulfuricans* produce high levels of sulfide when using sulfate as the terminal electron acceptor and this final product of anaerobic respiration may chemically react with toxic metals and metalloids. Precipitates from reaction vessels containing suspended or immobilized cells were examined under a JEOL transmission electron microscope with metals determined by analyzing electrons scattered by the specimen, and crystals identified by micro X-ray analysis. Cell-free fractions of *D. desulfuricans* couple the oxidation of molecular hydrogen to reduction of Fe(III) to Fe(II), Cr(VI) to Cr(III), and U(VI) to U(IV). Molybdate at sub-lethal levels is reduced by *D. desulfuricans* and results in the formation of amorphous crystals of MoS₂. It was noted that Te(VI) as tellurate is not reduced to Te⁰ but is reduced outside of the cell to Te(IV) as crystalline TeO₂. Uraninite, U(IV), was formed outside of the cells of *D. desulfuricans* by the reduction of uranyl acetate. Based on the size of and distribution of crystalline deposits of uraninite, it can be determined if the cells are living or dead. While crystals of iron arsenate are formed with the addition of Fe(III) to As(V), cell respiration resulting in the transformation of Fe(III) to Fe(II) and As(V) to As(III) results in the disappearance of insoluble iron arsenate. Metal reduction occurs with physiologically young cells as well as stationary-phase cells. Acknowledgment: This research was supported, in part, by grants from the Department of Energy (WERC Consortium and NABIR Program).

Reference: Tucker, M.D., L.L. Barton, and B.M. Thomson. (1998) Reduction of Cr, Mo, Se, and U by *Desulfovibrio desulfuricans* immobilized in polyacrylamide gels. J. Industrial Microbiol. Biotechnol. 20:13-19.

IRON III AS ELECTRON ACCEPTOR IN THERMOPHILIC ANAEROBES

Juergen Wiegel and Justin Hanel, Dept. Microbiology, University of Georgia, Athens, GA 30602

The recent studies of dissimilatory bacterial iron reduction forming magnetite have yielded ample evidence that this process plays an important role in carbon cycling and in the biogeochemistry of Earth. During the last decades many mesophilic iron (III) reducers have been described, however very few thermophilic chemolithoautotrophs have been found. The obligately anaerobic *Bacillus infernus*, *Thermoterrabacterium ferrireducens*, *Thermoanaerobacter siderophilus*, and facultative microaerophilic *Deferribacter thermophilus* are the validly published thermophilic iron (III) reducers, but only *T. ferrireducens* was known so far as being able to grow with hydrogen and carbon dioxide. Since we believed similar thermophilic iron reducers probably were important in early biogeochemical process, when most of the earth environment was thermobiotic, we looked for novel thermophilic chemolithoautotrophic iron-reducers. Such bacteria could have been involved in the formation of the banded iron formations. Here we describe several novel isolates including "*Ferribacter yellowstonensis*" from the Calcite springs at Yellowstone National Park, which grows preferably chemolithoautotrophically although it also can assimilate carbon from yeast extract and casamino acids. Species of *Desulfotomaculum* are the closest neighbors to this novel isolate, however they do not reduce iron (III) in our hands. Interestingly, some of our chemolithoautotrophic isolates are phylogenetically (based on 16s rRNA sequence analysis) very closely related (above 99%) to species which, so far, were only found to grow heterotrophically. This includes strains related to *Clostridium thermobutyricum* and alkalithermophilic *Thermobrachium celere*. Resting cells of "*F. yellowstonensis*" form magnetite up to pH values close to pH^{60°C} 11. This strain also can use fumarate and thiosulfate as electron acceptor. However, in contrast to the mesophilic iron (III)-reducers, all thermophilic iron (III)-reducing bacteria belong to the Gram-type positive branch, i.e., the firmicutes, and, as recently shown by the group of Lovley, to the *Thermotogales*. Thus, the phylogenetic biodiversity among the described thermophilic iron reducers is still significantly smaller than among the mesophilic ones.

In addition we have shown that several well known heterotrophic thermophiles can reduce iron(III) hydroxide to iron (II). Fermentation product analysis and growth yield studies indicated, however, that this process is probably only assimilatory. Examples include *Thermoanaerobacter brockii* and *Thermoanaerobacterium* species.

In conclusion, these results together with the published data from other laboratories, indicate that iron (III) is an electron acceptor used by many anaerobic mesophilic and thermophilic bacteria, as well as thermophilic archaea. We speculate that probably many more thermophilic anaerobes can utilize this electron acceptor and thus, that iron reduction could have been an important biogeochemical process in the early earth.

DENITRIFICATION – A KEY STEP TO ECOLOGIC EQUILIBRIA : OLD AND NEW ENZYMES

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Denitrification plays a central role on the global nitrogen biocycle, with an enormous relevance for agriculture and environmental related problems. Denitrifiers contribute to depletion from N-containing fertilizers and a way of preventing this process is to till the soils, keeping aerobiosis. Nitrate is a major pollutant of underground water and denitrification may also be used as a bioremediation step. Also N_2O is the third major gaseous contributor to the green house effect and is also responsible for the destruction of the ozone layer.

During denitrification, the nitrate molecule is reduced to molecular dinitrogen in a series of reactions:

Four enzymes have been identified in this inducible pathway: nitrate reductase, nitrite reductase, nitric oxide reductase and nitrous oxide reductase, named after the substrate they transform. The expression of these enzymes is triggered by growth on nitrate or nitrite, and low oxygen pressure.

A brief description of the first three enzymes will be done. 3D structures are available for nitrate reductase [$Mo(MGD)_2$ and $[4Fe-4S]$ center] and for different types of nitrite reductases (cytochrome cd_1 and copper enzymes – *multiheme proteins may also be involved in nitrite reduction*). The reduction of NO is not so well detailed studied and the last reaction is catalysed by nitrous oxide reductase (N2OR) in a two electron step, whose crystal structure was recently solved at 0.24 nm.

N2OR was isolated from different organisms and described as a dimeric protein of ~130 kDa. Based on the studies performed in the last decade the enzyme was thought to contain two binuclear copper centers. One, denominated Cu_A , is responsible for electron transfer to the catalytic site that was designated Cu_Z . The structure of Cu_A is similar to the one observed in cytochrome c oxidase, with two copper ions with two bridging cysteines in a distorted square planar geometry. A histidine and a methionine are also ligands of Cu_1 and a histidine and a tryptophan in the case of Cu_2 .

The studies performed by us on the N₂OR from *Ps.nautica* revealed the unusual structure of Cu_Z . The Cu_Z center belongs to a new type of cluster, never described in biology and chemistry, in which four copper ions are ligated by seven histidine residues of the polypeptide chain and three hydroxyl groups or water molecules. Based on the spectroscopic studies we propose that three of the four copper ions of Cu_Z are in the reduced state (+1) whereas the fourth copper atom is in the +2 oxidation state. A mechanistic proposal will be presented and the binding of N_2O discussed.

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DIVERSITY OF METABOLISM IN HYPERTHERMOPHILIC ANAEROBES

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Hyperthermophilic microorganisms grow at temperatures above 90°C with a current upper limit of 113°C. By 16S rRNA analyses all but two of the more than twenty known genera are classified as *Archaea* (formerly archaebacteria). Almost all hyperthermophiles are strict anaerobes although some are microaerophilic. Of the hyperthermophilic bacteria, *Thermotoga* grows by carbohydrate fermentation and produces hydrogen, while *Aquifex* is an autotrophic, microaerophilic nitrate-reducer that uses hydrogen as an electron donor. The hyperthermophilic archaea include methanogens, which grow only on hydrogen and carbon dioxide; sulfate-reducers, which use both organic substrates and hydrogen as electron donors; and iron-oxidizers, which utilize hydrogen and sulfide as electron donors in addition to ferrous iron. The majority of the known species of hyperthermophilic archaea, however, are sulfur-dependent organisms that reduce elemental sulfur (S^0) to hydrogen sulfide. The autotrophic S^0 -reducers use hydrogen as the electron donor and appear to have a respiratory chain with S^0 as the terminal electron acceptor. The heterotrophic S^0 -reducers utilize proteinaceous materials and in some cases polysaccharides as carbon and energy sources. Sugar fermentation is achieved by a novel glycolytic pathway involving unusual ADP-dependent kinases and ATP synthetases, and novel oxidoreductases that are ferredoxin- rather than NAD(P)-linked. Similarly, peptide fermentation appears to involve several unusual ferredoxin-linked oxidoreductases not found in mesophilic organisms. Most of the heterotrophs are obligately dependent upon S^0 reduction for growth, although a few, such as *Pyrococcus furiosus*, are able to grow well in the absence of S^0 and produce hydrogen as a means of disposing of the excess reductant that is generated during catabolism. The mechanisms by which such organisms reduce S^0 and evolve hydrogen, and whether these processes lead to energy conservation, are not clear. As a model system we are using *P. furiosus*, which grows optimally at 100°C using sugars and peptides as carbon sources. This organism contains three distinct NiFe-hydrogenases, the functions of which are not understood (1). Two are cytoplasmic and are thought to evolve hydrogen during growth utilizing NADPH as the electron donor. NADP is reduced by ferredoxin:NADP oxidoreductase. The third hydrogenase is membrane-bound and appears to be part of a respiratory complex. This enzyme does not utilize NAD(P)H or ferredoxin and its physiological electron donor is not known. The two cytoplasmic hydrogenases also catalyze the NADPH- and hydrogen-dependent reduction of S^0 to H_2S in vitro, but the membrane-bound enzyme does not catalyze these reactions. Insight into the role of these hydrogenases and of associated oxidoreductases involved in S^0 reduction and hydrogen production is being obtained by detailed growth studies coupled with genomic-based analyses using proteomics and DNA microarrays. Preliminary results indicate that a variety of enzymes are strongly regulated by the presence (or absence) of S^0 , peptides and sugar (maltose), although a clear picture of how *P. furiosus* metabolizes S^0 and hydrogen has yet to emerge.

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GENES AND GENETIC MANIPULATION OF *DESULFOVIBRIO*

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The first genetic manipulations of the sulfate-reducing bacteria were reported only a decade ago. Members of the genus *Desulfovibrio* remain the only sulfate reducers to have been the subject of significant genetic and molecular biological analyses. Advances have occurred in the development of expression vectors, cloning tools, transposon mutagenesis, and gene transfer and interruption. While the application of these tools is becoming routine, success is not yet trivially obtained. However, a revolution in approaches looms on the horizon with the determination of the complete sequence of *Desulfovibrio vulgaris* Hildenborough. Whole genome approaches are becoming available to explore coordinated gene expression that would have been prohibitively time consuming and expensive.

FUNCTION AND ASSEMBLY OF ELECTRON TRANSPORT COMPLEXES IN *DESULFOVIBRIO VULGARIS* HILDENBOROUGH

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The *hmc* operon of *Desulfovibrio vulgaris* subsp. *vulgaris* Hildenborough encodes a transmembrane redox protein complex (the Hmc complex) that consists of redox proteins HmcA to HmcF. HmcA is the periplasmic high molecular weight cytochrome containing 16 *c*-type hemes. HmcB to HmcE are integral membrane proteins, containing iron-sulfur clusters or *b*-type heme. HmcF is a cytoplasmic protein containing iron-sulfur clusters. All protein components of the Hmc complex are either membrane-bound or membrane-associated. Its proposed physiological function is to catalyze electron transport from periplasmic hydrogen oxidation to cytoplasmic sulfate reduction reactions. We have constructed a mutant strain *D. vulgaris* H801, in which a 5-kb DNA fragment containing most of the *hmc* operon is replaced by the *cat* gene. Growth of *D. vulgaris* H801 and of the wild-type strain with lactate or pyruvate as electron donors for sulfate reduction was similar. However, growth of strain H801 with hydrogen as electron donor for sulfate reduction (acetate and CO₂ as the carbon source) was significantly slower than of the wild-type strain. The growth yields per mol of sulfate were similar for strains H801 and wild-type. These results prove the importance of the Hmc complex in electron transport from hydrogen to sulfate. Electron transport through the Hmc complex is not linked to energy conservation, leading to additional ATP synthesis and *Desulfovibrio* must have additional transmembrane complexes catalyzing this same function.

Mutant H801 was also found to be deficient in establishing a low-redox-potential niche. On minimal medium plates in which hydrogen serves as the sole electron donor colonies of the wild-type and H801 strains formed after 14 and 30 days, respectively. These results suggest that, in addition to transmembrane electron transport from hydrogen to sulfate, the redox reactions catalyzed by the Hmc complex are important for establishment of the low-redox-potential environment that allows single cells to grow into colonies.

Assembly of the Hmc complex requires transport of polypeptides across or insertion of polypeptides into the cytoplasmic membrane. For periplasmic hydrogenases from *Desulfovibrio* a mechanism was proposed more than 10 years ago in which a single signal peptide at the N-terminus of the small subunit allowed transport of a folded and assembled complex of the large and small subunits. This has recently been referred to as the "hitchhiker mechanism". Strong evidence for a unique mechanism was provided by the fact that all small subunit hydrogenase signal sequences share a consensus sequence (RRxFxK). Database searching and recent genetic studies with *Escherichia coli* have shown that membrane transport of all proteins binding redox cofactors (except *c*-type cytochromes) is catalyzed by the *tat* system (for twin arginine translocation). Mutations in the *E. coli* *tat* operon are pleiotropic and are defective in the export and assembly of many enzymes involved in anaerobic respiration. Assembly of the Hmc complex requires protein export through both the standard *sec* (HmcA) and *tat* (HmcB) pathways.

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NOVEL REACTIONS INVOLVED IN ENERGY CONSERVATION BY METHANOGENIC ARCHAEA

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Methanogenic archaea of the order Methanosarcinales which utilize C₁ compounds such as methanol, methylamines or H₂+CO₂, employ two novel membrane-bound electron transport systems generating an electrochemical proton gradient: the H₂:heterodisulfide oxidoreductase and the F₄₂₀H₂:heterodisulfide oxidoreductase. The systems are composed of the heterodisulfide reductase and either a membrane-bound hydrogenase or a F₄₂₀H₂ dehydrogenase which is functionally homologous to the proton-translocating NADH dehydrogenase. Cytochromes and the novel electron carrier methanophenazine are also involved. In addition, the methyl-H₄MPT:HS-CoM methyltransferase is bioenergetically relevant. The enzyme couples methyl group transfer with the translocation of sodium ions and seems to be present in all methanogens. The proton-translocating systems with the participation of cytochromes and methanophenazine have been found so far only in the Methanosarcinales.

Raw sequencing of the genome of *Methanosarcina mazei* strain Göl in which the energy conserving reactions have been extensively studied is finished. More than 4000 open reading frames have been identified; those encoding components of the F₄₂₀H₂ dehydrogenase and the various methyltransferases will be highlighted.

METHYL-COENZYME M REDUCTION: THE IRREVERSIBLE STEP IN METHANOGENESIS

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Methane is formed in all methanoarchaea by the same reaction, namely the reduction of methyl-coenzyme M ($\text{CH}_3\text{-S-CoM}$) with coenzyme B (HS-CoB) to CH_4 and the heterodisulfide CoM-S-S-CoB . This essentially reversible reaction, which is associated with a free energy change ΔG° of -45kJ/mol , is catalyzed by methyl-coenzyme M reductase. The enzyme harbors a nickel porphyrinoid prosthetic group F_{430} which has to be in the Ni(I) oxidation state for the enzyme to be active. The crystal structure of the nickel enzyme has been elucidated and a catalytic mechanism involving a coenzyme M thiyl radical and a heterodisulfide anion radical as intermediates has been proposed (1). In the active site region of the methyl-coenzyme M reductase, close to where the coenzyme B sulfur in the enzyme product complex is located, a thiopeptide bound thioglycine was found to be conserved (2). Thiopeptides can be reduced by one electron generating a thioketyl radical at a redox potential predicted to be more positive than that of a disulfide anion radical ($E^\circ = -1.6\text{ V}$) but still negative enough for the reduction of Ni(II) F_{430} to Ni(I) F_{430} ($E^\circ = -650\text{ mV}$) considered to be the final step in the catalytic cycle. It is therefore proposed that the reduction of Ni(II) F_{430} to Ni(I) F_{430} involves a thioketyl radical as intermediate. Results supporting the hypothesis will be discussed.

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ELECTRON TRANSPORT SYSTEMS IN ANAEROBES.

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The generation of energy in anaerobic bacteria follows the same basic principle that applies to aerobic bacteria. A trans membrane proton gradient is generated by activities of a membrane located electron transport system and the proton gradient is driving the synthesis of ATP as catalyzed by a membrane bound F_1F_0 ATP synthase. The most investigated bacterial ATP synthase, which is used as a model, is that from *E. coli*. Its cytoplasmic component F_1 has 5 subunits, α , β , γ , δ , ϵ and the membrane part, F_0 , has 3 subunits, a , b , and c . The *E. coli*, *atp* operon contains nine genes arranged in the order *atpI* (i), *atpB* (a), *atpE* (c), *atpF* (b), *atpH* (δ), *atpA* (α), *atpG* (γ), *atpD* (β), and *atpC* (ϵ). The product of the first gene, the i subunit, has never been found in an ATP synthase purified from any source and its function is not known. Functional ATP synthases purified from the gram positive anaerobic bacteria *Clostridium pasteurianum*, *C. thermoaceticum*, *C. thermoautotrophicum*, and *Acetobacterium woodii*, have fewer than eight subunits. They are similar in that the F_0 moiety is functional with only the c subunit and lacks the a and b subunits which are essential for the function ATP synthases in aerobic bacteria. This led to the investigation of the *atp* operon of the above bacteria. As with *E. coli* the *atp* operons of *C. thermoaceticum* and *C. pasteurianum* have 9 genes whereas the operon of *A. woodii* has eleven (1,2). The two extra genes in the *atp* operon of the latter bacterium are additional copies of *atpE* encoding the c subunit. (2) Why the a and b subunits are not found in the active ATPases of the anaerobic bacteria and are not needed for activity is puzzling. In *C. thermoaceticum* the genes of *atpI* and *atpB* overlap and their transcripts are unstable. Furthermore, rare codons are found in the reading frame of *atpB*. The primary structures of genes for the i , a , and ϵ subunits of *C. pasteurianum* also have unique features. These properties may explain poor expressions of the genes and ultimately effect the functions of their products. *C. thermoaceticum* and other acetogens grow autotrophically on CO or CO_2/H_2 . The formation of acetate from these substrates via the acetyl-CoA pathway does not provide any net gain of ATP at substrate level. Energy for growth is generated via electron transport (ET) coupled phosphorylation. The proposed ET chain consists of ferredoxin, flavoprotein, cyt b_{559} , menaquinone, cyt b_{554} and rubredoxin. The physiological electron acceptor of the ET chain in acetogenic clostridia has not been clarified. Rubredoxin has been considered an universal electron acceptor but methylene- H_4 - folate, an intermediate in the acetyl-CoA pathway may be a terminal electron acceptor. The ET chain in acetogenic clostridia may contain other components in addition to those mentioned. Sequencing of the gene of one of the rubredoxin (Rub) found in *C. thermoaceticum* and around *rub* reveals the presence of several redox proteins including rubredoxin oxidoreductase (Rbo), rubrerythrin (Rbr), type A flavoprotein (fprA) and a high molecular rubredoxin (Hrb). The Rbo and Rbr are ubiquitous in anaerobic species and their functions are attributed to defend oxygen toxicity. We assume similar functions of these proteins in acetogenic clostridia. Based on these and other results it is assumed that the electron transport system in acetogenic clostridia is more complex than what was earlier proposed.

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ACETOGENESIS.

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Acetogenic bacteria are strict anaerobes that produce 10^{13} kg of acetic acid annually by metabolizing sugars, aromatic compounds, and inorganic gases like CO, H_2 , and CO_2 . They are important in the biology of the soil and of organisms that house them in their digestive system, like humans, termites, and ruminants. Acetic acid synthesis is accomplished by the Wood/Ljungdahl pathway, which contains an *Eastern* and a *Western* branch. This pathway generates energy for growth of acetogens and for generation of acetyl-CoA, which is used for biosynthesis by autotrophic anaerobes, like acetogens and methanogens. Acetoclastic methanogens catalyze the reversal of the pathway to convert acetyl-CoA into CO_2 and methane. Studies of this pathway have uncovered novel enzymology, including organometallic intermediates, substrate-derived organic radical intermediates, substrate channeling, new heterometallic clusters, and metal ions acting as nucleophiles.

We have been studying several enzymes at the heart of the Wood-Ljungdahl pathway. Pyruvate ferredoxin oxidoreductase (PFOR) provides a source of reductant and, during heterotrophic growth, provides the CO_2 that is converted to acetate. We have been elucidating the mechanism of PFOR and the electronic properties of the radical intermediate(s). There are several key methyl transfer reactions in the pathway. The first is catalyzed by a CH_3 - H_4 folate:corrinoid iron-sulfur protein (CFeSP): methyltransferase (MeTr). We have determined the TIM barrel structure of this protein, which is a prototype of the MT1-type B_{12} -dependent methyltransferase family. We also have elucidated the reaction mechanism, which involves an S_N2 attack of Co(I) on the methyl group of CH_3 - H_4 folate to generate a methyl-Co(III) species on the CFeSP. A proton transfer step is shown to be key in the electrophilic activation of the methyl group. The methyl group is then transferred to acetyl-CoA synthase, which is one of the subunits of the dimeric CO dehydrogenase/acetyl-CoA synthase (CODH/ACS). Our results indicate that the methyl group is transferred to a NiFeS cluster (Cluster A) in the ACS subunit by an S_N2 mechanism. Cluster A was the first example of a metal cluster containing both Ni and Fe. This general motif is repeated in hydrogenases. We propose that the ACS mechanism involves nucleophilic attack of Ni(I) on the methyl-Co(III)-CFeSP to generate Co(I) and methyl-Ni(III), which then accepts an electron to generate a methyl-Ni(II) state. Apparently this is an intramolecular electron transfer that could help coordinate CO_2 reduction with acetyl-CoA synthesis. The methylation of ACS is coupled to carbonylation of the NiFeS center by CO, which is generated from CO_2 at the CODH active site. Thus, carbon monoxide is generated as a key intermediate that is subsequently utilized to form the carbonyl group of acetyl-CoA. Lindahl's and my laboratories have recently gained evidence that CO is channeled from the CODH to the ACS active site. ACS then catalyzes condensation of the bound methyl and carbonyl groups with Coenzyme A to form acetyl-CoA.

Work on the enzymology of acetogenesis and methanogenesis has been supported by the Department of Energy and the National Institutes of Health since 1987.

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ELECTRON FLOW IN FERROUS BIOCORROSION

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It has been well established that sulfate reducing bacteria (SRB) are major contributors to the anaerobic corrosion of mild steel. Evidence has accumulated from whole cell experiments which suggest that the hydrogenase enzyme system plays an important role in the biocorrosion process.

We have reported Fe^{2+} availability regulated *Desulfovibrio vulgaris* (Hildenborough) periplasmic [Fe] hydrogenase, by a derepression/repression control mechanism. The synthesis of this enzyme was regulated by ferrous ion concentration, low Fe^{2+} [<5.0 PPM] caused a significant derepression of the periplasmic [Fe] hydrogenase, whereas the presence of Fe^{2+} greater than 5.0 PPM repressed the enzyme's expression. This finding may explain the lack of correlation between SRB cell counts and oil pipeline corrosion found during our field studies. In our laboratory biocorrosion studies, we noted the mild steel process was stimulated by the presence of phosphate buffer (pH 7.0) which chemically reacted with the metal to produce H_2 and vivianite ($\text{Fe}_3(\text{PO}_4)_2$). In this neutral environment, the phosphate buffer also acted as a source of protons in the cathodic depolarization reaction resulting in H_2 evolution as outlined in the following equation: $3\text{Fe} + 4\text{H}_2\text{PO}_4^- \rightarrow \text{Fe}_3(\text{PO}_4)_2 + 2\text{HPO}_4^{2-} + 3\text{H}_2$. This H_2 generating process led us to develop a unique *in vivo* Warburg assay system for evaluating the corrosive effect of cell free hydrogenases on mild steel rods by stoichiometry analysis of Fe^{2+} : H_2 ratio. We were able to clearly demonstrate that *Clostridium pasteurianum* hydrogenase 1 and *Desulfovibrio vulgaris* periplasmic [Fe] hydrogenase were corrosive enzymes, accelerating the dissolution of iron from the mild steel surfaces.

Outer and cytoplasmic membranes were isolated from *D. vulgaris* cells grown under low (5 PPM) and high (100 PPM) Fe^{2+} conditions. The outer membrane (OM) isolated from low iron cultured cells was found to contain two derepressed proteins, 77.5 and 62.5 kDa by SDS-PAGE analysis. These proteins reacted with a heme-specific stain and were referred to as high molecular weight cytochromes. This isolated OM containing the cytochromes and a small amount of contaminating [Fe] hydrogenase accelerated iron corrosion from the mild steel rods as measure by the *in vivo* assay system.

From our combined findings we proposed a novel biocorrosion model whereby the inducible OM cytochrome(s) removed electrons from the cathodic site on the mild steel surface and interacted with the inducible periplasmic [Fe] hydrogenase to produce H_2 gas. This gas diffuses through the periplasmic space to the cytoplasmic membrane where it is oxidized by the constitutive [NiFe] hydrogenase, the electrons are passed through an electron transport system to the terminal electron acceptor SO_4^{2-} , which is reduced to H_2S and evolved by the cell.

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OXYGEN AND ANAEROBES

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The seemingly oxymoronic title stems from the fact that many strictly anaerobic bacteria and archaea exhibit some degree of aerotolerance. The "classical" oxidative stress enzymes, superoxide dismutase and catalase, which catalyze disproportionation of superoxide and hydrogen peroxide, respectively, do not have recognizable counterparts in the genomes of several anaerobes. We have undertaken both in vitro and in vivo investigations of some candidates for alternative oxidative stress enzymes in anaerobes. These include hemerythrin-like proteins, desulfoferrodoxin, and rubrerythrin. These three enzymes may sense and/or reduce dioxygen, superoxide, and hydrogen peroxide, respectively, without regenerating dioxygen. All of these candidate enzymes are non-heme iron proteins and their genes are found in several anaerobic bacteria and archaea, sometimes in multiple copies. This talk will focus on examples of each of these proteins from the anaerobic sulfate-reducing bacterium, *Desulfovibrio (D.) vulgaris* (Hildenborough).

Hemerythrin is an O₂-carrying diiron protein that had heretofore been found only in a few marine invertebrate species. We have found a hemerythrin-like domain on a *D. vulgaris* gene originally reported by Voordouw and co-workers to encode an inner-membrane-spanning methyl-accepting chemotaxis protein. We have established that the C-terminal domain of this protein closely resembles hemerythrin in both structure and function. This C-terminal domain contains a diiron site and forms an O₂ adduct very similar to but less stable than that of Hr. Given the air-sensitive nature of *D. vulgaris* and the putative chemotactic function, one possible role for the hemerythrin-like domain is O₂ sensing, which may trigger the anaerotaxis exhibited by *D. vulgaris*.

Desulfoferrodoxin, which occurs in several *Desulfovibrio* species, contains an FeS₄ center and a second novel iron center with a 4His, 1Cys coordination sphere. Touati et al. showed that desulfoferrodoxin can complement a superoxide-sensitive mutant of *E. coli* and also presented evidence that desulfoferrodoxin is a superoxide reductase rather than a superoxide dismutase. In contrast we have shown that desulfoferrodoxin actually *reduces* the viability of an *E. coli* catalase-deficient strain. This reduced viability is attributed to a desulfoferrodoxin-catalyzed increase in the level of intracellular peroxide via reduction of superoxide.

NADH peroxidase activity has recently been reported in crude extracts from a few *Desulfovibrio* species. We have found that rubrerythrins can serve as the terminal component of an NADH peroxidase in vitro. This activity requires both the rubredoxin-type center and the diiron-oxo center characteristic of all rubrerythrins. Expression of *D. vulgaris* rubrerythrin complemented the catalase-deficient strain of *E. coli* against exposure to hydrogen peroxide, but did not complement the superoxide-sensitive strain. We propose that desulfoferrodoxin and rubrerythrin function together as a novel oxidative stress defense in anaerobes and microaerophiles.

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MOLECULAR ARCHITECTURE AND FUNCTION OF HYDROGENASES

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Hydrogenases are H₂-activating metalloenzymes which play a central role in hydrogen metabolism in many microorganisms [1]. These proteins show the unique ability to produce or to consume molecular hydrogen by catalyzing the simplest of chemical reaction: $H_2 \rightarrow 2H^+ + 2e^-$. Biophysical and biochemical studies of the structural and catalytic properties of the two major families of this enzyme, known as NiFe and Fe-only hydrogenases, have been pursued for many years with in mind the design of new strategies to produce cheap hydrogen, using the proteins as templates.

The determination of the first three-dimensional crystal structure of a hydrogenase, the NiFe hydrogenase from the sulfate-reducing bacterium *Desulfovibrio gigas* [2] has given a new impetus to studies on this enzyme. For the recent years, joint crystallographic and infrared spectroscopic studies of several NiFe and Fe-only [3] hydrogenases have led to the complete elucidation of the complex structure of the respective active sites of the two families of enzymes. These sites are characterized by a binuclear, NiFe or FeFe, center with CN and CO ligands to the iron. The latter feature is unprecedented in biology. For both NiFe and Fe hydrogenases it has been also established that specific routes exist to transfer the "partners" of the catalytic reaction, i.e. molecular hydrogen, electrons and protons, between the molecular surface and the active sites which are deeply buried inside the respective proteins. These wealth of structural results, together with synthesis and studies of model compounds and theoretical calculations have given new insights on the catalytic mechanism.

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MÖSSBAUER INVESTIGATION OF THE IRON CLUSTERS IN DESULFOVIBRIO VULGARIS HYDROGENASE

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The periplasmic hydrogenase of *Desulfovibrio vulgaris* (Hildenborough) belongs to the category of Fe hydrogenase. Metal content, amino-acid sequence analysis, and spectroscopic evidence indicate that *D. vulgaris* hydrogenase contains two ferredoxin-type [4Fe-4S] clusters and a catalytic Fe-S cluster, designated as the H cluster. Aerobically purified *D. vulgaris* hydrogenase is inactive and requires reductive activation. This activation process is irreversible and has previously been monitored by EPR spectroscopy.¹ The inactive H cluster exhibits a rhombic $g = 2.06$ EPR signal that is different from the rhombic $g = 2.10$ signal observed for the active H cluster. These observations suggest that the reductive activation process involves an irreversible conformational change. Here, we employ Mössbauer spectroscopy to characterize the Fe-S clusters in *D. vulgaris* hydrogenase at the various redox states generated during a reductive titration. Detailed analysis of the Mössbauer data was made possible by a recent crystallographic investigation on the periplasmic Fe hydrogenase from *D. desulfuricans*, which reveals an unusual structure for the H cluster, a ferredoxin-type [4Fe-4S] cluster and a binuclear Fe center bridged by a cysteine residue.² The results of the Mössbauer analysis indicate that the activation process requires the transfer of an electron from the [4Fe-4S] cluster of the H cluster to the binuclear Fe center. Possible conformational changes that promote the relocation of the electron are proposed.

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2. Y. Nicolet, C. Piras, P. Legrand, C.E. Hatchikian, and J.C. Fontecilla-Camps (1999) *Structure* 7, 13-23.

MECHANISM OF HYDROGEN ACTIVATION

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From the combination of FTIR studies and the X-ray structure of [NiFe]- and [Fe]-hydrogenases, it has become clear that Fe, Ni, CN⁻ and CO are essential components of the H₂-activating centres. The splitting of hydrogen is heterolytic and so the basic redox reaction is a two-electron process. The [NiFe]- and the [Fe]-hydrogenases both have a [4Fe-4S] cluster close to the bimetallic site (proximal cluster). We presently assume that the bimetallic site activates H₂. Subsequently, this site plus the proximal cubane cluster act as a two-electron acceptor for a hydride.

For [NiFe]-hydrogenases we hold the opinion that only two of the seven possible states of the active site are involved in turnover, namely the Ni_a-C* state and the Ni_a-SR (completely reduced) state. Both states contain a hydride close to the Fe-site; the hydride binding is light sensitive in the Ni_a-C* state. We believe that this bound hydride is not involved in turnover. The activation of H₂ during turnover takes place at Ni only.

Recent studies on the soluble, NAD-reducing hydrogenase from *Ralstonia eutropha* [1] indicate that in this enzyme the Ni-Fe site contains two extra CN⁻ ligands, one of which is bound to nickel. We propose that the extra CN⁻ groups make the active site inaccessible for CO and O₂. As the Fe atom is six-coordinate and the Ni ion has five ligands, we propose that H₂ activation solely takes place at Ni. FTIR and EPR studies suggest that the Ni-Fe site is not redox active. * Hence, a suitable two-electron acceptor may be required for activity. Recent results concerning this question will be shortly discussed.

R. eutropha also contains a H₂-sensing protein, which regulates the biosynthesis of hydrogenases. The amino-acid sequence suggests the presence of a normal Ni-Fe site, and this was confirmed by EPR and FTIR studies [2]. The active site can only have two states (both active). Binding of H₂ to the Ni_a-S state causes the oxidation of the Ni site and formation of the Ni_a-C* state. The produced electron is assumed to be located in the Fe-S clusters. The protein is a sensor rather than an enzyme: its activity is two orders of magnitude lower than that of regular [NiFe]-hydrogenases. This is in line with the idea, that the Ni_a-S/Ni_a-C* conversion is not involved in turnover in standard [NiFe]-hydrogenases.

The FTIR spectra of [Fe]-hydrogenases indicate that the bridging CO molecule in the resting (inactive) enzyme is no longer bridging in the reduced (active) enzyme. Some current ideas about the possible mechanism of action will be discussed.

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BIOSYNTHESIS OF HYDROGENASES IN *E. COLI*

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The biosynthesis of active [NiFe] hydrogenases in *E. coli* involves a cascade of reactions in which at least seven auxiliary proteins are involved in addition to the structural proteins. These are the products of the *hyp* genes (HypA to HypF) plus HycI. The biochemical function of three of them has been elucidated: HypB is a GTPase, HypC is a chaperone-like protein and HycI an endopeptidase. With the paradigmatic example of hydrogenase 3 from *E. coli* the following scenario for the maturation has been established. HycE, the large subunit polypeptide is synthesized as a precursor protein with a 32 amino acid C-terminal extension. One of the first reactions consists of the formation of a complex between the large subunit and HypC in which cys-241 of the large subunit (which is one of the side chains anchoring the metal center) and the N-terminal cysteine of HypC are involved. Iron (ligated to cys-244) and the CN/CO ligands are incorporated before and independently of the insertion of nickel (ligated to cys-531) which most probably is donated by the HypB protein. After nickel has been inserted HypC has to leave the complex with the large subunit before the endopeptidase - which recognises its substrate by binding to the nickel - can remove the C-terminal extension. Cleavage triggers a conformational switch in which the most C-terminally located cysteine (cys-534) which carries a free thiol during maturation closes the bridge between iron and nickel resulting in the complete binuclear center. The whole maturation pathway takes place in the absence of the small subunit. Docking to the membrane, however, requires the heterodimeric enzyme.

Reference:

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GENES AND PROTEINS INVOLVED IN NICKEL-DEPENDENT HYDROGENASE EXPRESSION

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Many genes are involved in the synthesis of NiFe hydrogenases, and the best characterized of these are from aerobic H₂-oxidizing bacteria. HoxN (from *Alcaligenes eutrophus*) and HupN (from *Bradyrhizobium japonicum*) are involved in nickel transport and both contain the nickel-binding motif HAVDADH within the second transmembrane helix. In addition to HupN, *B. japonicum* contains HupO and HupP that play roles in nickel sequestering in low nickel conditions; *hupO* and *hupP* have not been found in other organisms. A "second hydrogenase" with similarities to NiFe hydrogenases has been found in some aerobic H₂ oxidizers (HupUV for *B. japonicum* and *R. capsulatus*, and HoxBC for *A. eutrophus*), and it is proposed to be a hydrogen sensor, as hydrogen is one of the environmental signals required for hydrogenase expression. An interesting nickel-metabolism related protein, HypB, is encoded as part of a large "hydrogenase accessory gene" operon in aerobic H₂ oxidizers. *Bradyrhizobium japonicum* HypB binds 18 nickel ions (per HypB dimer) and it has been termed nickelin due to its role in nickel storage. The dual roles of nickelin in nickel storage and in GTP-dependent nickel mobilization can be separated functionally and structurally, and the separate functions assigned to different domains of the protein. This was done by physiological analysis of mutants and by biochemical studies of purified mutant versions of nickelin that lacked the histidine-rich area or the GTP binding domain.

Another H₂-oxidizing organism of interest to us is *Helicobacter pylori*, the cause of peptic ulcer disease and gastric cancer. Some, but not all, of the nickel metabolism hydrogenase accessory proteins (encoded by *hyp* genes) of *H. pylori* are involved in urease activity. Mutation of the hydrogenase structural genes of *H. pylori* did not affect urease activity, and the deleterious effects of mutation of the *hypA* or *hypB* genes on both urease and hydrogenase was overcome by adding nickel to the culture medium. As urease is a virulence factor for colonization of the human stomach and *H. pylori* appears to have the *ure* genes needed for urease synthesis, the roles of the *hyp* genes in synthesis of two dissimilar nickel metalloenzymes needs to be critically addressed.

Olson, J.W., and R.J. Maier. 2000. Dual roles of *Bradyrhizobium japonicum* nickelin protein in nickel storage and GTP-dependent Ni mobilization. *J. Bacteriol.* 182:1702-1705.

IRON-SULFUR CLUSTER BIOSYNTHESIS

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Iron-sulfur clusters are ubiquitous prosthetic groups in biology, occurring in more than 120 different types of proteins, but relatively little is known about their biosynthesis. *Azotobacter vinelandii* has been used as a model system to investigate Fe-S cluster biosynthesis, since it has *nif* genes that specifically target nitrogenase cluster biosynthesis and *isc* genes that are used for general cluster biosynthesis and widely conserved in both prokaryotic and eukaryotic organisms. *A. vinelandii* NifS and IscS have been shown to be pyridoxal 5'-phosphate containing cysteine desulfurases, which form complexes with NifU and IscU, respectively, and supply the sulfur for transient cluster assembly on the NifU or IscU scaffold. *A. vinelandii* NifU has been shown to be a modular protein, with an N-terminal iron binding domain (NifU-1) corresponding to IscU, and a C-terminal [2Fe-2S]²⁺ domain (NifU-2). To eliminate the spectroscopic background of the permanent [2Fe-2S]²⁺ cluster, the N-terminal NifU-1 domain was overexpressed and purified from *E. coli*. The addition of ferric citrate to NifU-1 resulted in a UV-visible absorption spectrum indicative of a rubredoxin-like iron site and resonance Raman spectra were consistent with a tetrahedrally ligated ferric site with at least three cysteine ligands. *In vitro* cluster assembly in *A. vinelandii* NifU-1 and IscU, using NifS or IscS, ferric citrate, cysteine, and mercaptoethanol is initially marked by the appearance of a UV-visible absorption spectrum indicative of a [2Fe-2S]²⁺ cluster and this was confirmed by resonance Raman and Mössbauer studies. More detailed IscS-mediated cluster assembly studies with IscU revealed sequential cluster assembly with the initial product containing one [2Fe-2S]²⁺ cluster per dimer, converting first to a form containing two [2Fe-2S]²⁺ clusters per dimer, and finally to a form that contains one [4Fe-4S]²⁺ cluster per dimer. Both the [2Fe-2S]²⁺ and [4Fe-4S]²⁺ clusters in IscU are reductively labile and are degraded within minutes on exposure to air. On the basis of sequence considerations and spectroscopic studies, the [2Fe-2S]²⁺ clusters in IscU are shown to have incomplete cysteinyl ligation. The ability to assemble both [2Fe-2S]²⁺ and [4Fe-4S]²⁺ clusters in IscU supports the proposal that this ubiquitous protein provides a scaffold for IscS-mediated assembly of clusters that are subsequently used for maturation of apo Fe-S proteins. These results also indicate a common mechanism for [4Fe-4S]²⁺ cluster biosynthesis involving reductive coupling of two preformed [2Fe-2S]²⁺ clusters.

Yuvanityama, P., Agar, J. N., Cash., V. L., Johnson, M. K. and Dean, D. R. (2000) NifS-directed assembly of a transient [2Fe-2S] cluster within the NifU protein. *Proc. Natl. Acad. Sci. USA* **97**, 599-604.

POSTERS

Regulation of Cytochrome c_3 IN
Desulfovibrio desulfuricans G20.

Laurence Casalot, Barbara J. Rapp-Giles and
Judy D. Wall

Stimulation of Chloroethene-Respiring
Population by Acetogenesis

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Characterization of Selenium-Dependent
Purine Hydroxylase and Axanthine
Dehydrogenase from *Clostridium Purinolyticum*

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Anaerobic Fermentation of *Salmonella*
Typhimurium LT2 with and without Pyruvate
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Metabolic engineering of *Escherichia Coli* with
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Characterization of the 20S proteasome and
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Heme Utilization and Aerotolerance in
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Characterization of Hydrolytic Enzyme
Systems of Anaerobic Fungi

Xin-Liang Li, Huizhong Chen, David L.
Blum, Yi He, Eduardo A. Ximenes, E.
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Ljungdahl

Clostridium Thermocellum Cellulosome
Reveals specific Interactions and Internal
and Internal Synergism Between Domains
Favoring Decomposition of Plant Cell Wall

Irina Kataeva, Huizhong Chen, David Blum,
Ashit Shah, Jason Cloward, Xin Liang Li,
and Lars G. Ljungdahl

The Primary Structure and Analysis of the
ATP Operon Encoding Subunits of F_1F_0
ATP Synthase from *Clostridium Pasteurianum*

Amaresh Das and Lars G. Ljungdahl

1.4Å Crystal Structure of Hyperthermophilic
Glycerol Dehydrogenase from *Thermotoga*
Maritima

Vasundara Srinivasan, Kesen Ma, M.W.W.
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Purification, Characterization and Analysis
Of the Membrane Bound Hydrogenase from
the Hyperthermophilic Achaeon *Pyrococcus*
Furiosus

Rajat Sapra, Gertie Schut and M.W.W.
Adams

REGULATION OF CYTOCHROME c_3 IN *Desulfovibrio desulfuricans* G20

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Several *c*-type cytochromes have been identified in *Desulfovibrio desulfuricans* G20 and a genetic approach has been chosen to explore the role and the regulation of those cytochromes. Among them, the most abundant is the tetraheme cytochrome c_3 and its gene has been cloned and a mutant generated (Rapp-Giles *et al*, 2000).

To evaluate regulation of the gene, sequences necessary for expression are being sought. Analyses indicate that the gene is monocistronic. A putative promoter has been identified that is a perfect match to the consensus derived for *Desulfovibrio* by Voordouw and coworkers. Evidence for transcripts initiated upstream of that putative promoter has been obtained, suggesting multiple promoters may exist. Further analysis, such as S1 mapping, is in progress in order to characterize the promoter(s) of *cycA*.

Expression of cytochrome c_3 has been followed by Western analyses of periplasmic extracts of *D. desulfuricans* G20. On lactate/sulfate medium, the amount of cytochrome c_3 increases five times in stationary phase. In contrast, growth on pyruvate/sulfate medium shows a constitutive production of cytochrome c_3 . Other studies indicated a reduction in the amount of cytochrome c_3 in absence of iron.

A 1.2-kb fragment upstream of the *cycA* gene, that has the *cycA* start codon at the 3'-end, has been cloned in-frame with *lacZ* on a sulfate-reducing bacteria (SRB) non-replicative plasmid. The recombinant plasmid has been named pc3prom111. The β -galactosidase activity in *E. coli* is twice that of another strain containing *lacZ* under the control of promoter of the β -lactamase gene, *bla*. Attempts to introduce pc3prom111 into *Desulfovibrio desulfuricans* G20 have not been successful to date. It appears that the strength of the promoter makes these analyses difficult.

Rapp-Giles, B.J., Casalot, L., English, R.S., Ringbauer, J.A. Jr., Dolla, A. and Wall, J.D. (2000) Cytochrome c_3 Mutants of *Desulfovibrio desulfuricans*. *Applied and Environmental Microbiology*. 66: 671-677.

STIMULATION OF CHLOROETHENE-RESPIRING POPULATIONS BY ACETOGENESIS

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Chlorinated ethenes are widespread groundwater pollutants. Under anaerobic conditions and in the presence of a suitable electron donor complete reductive dechlorination has been observed. The majority of chloroethene-respiring populations available as pure cultures, including *Dehalospirillum multivorans*, *Dehalobacter restrictus*, and *Dehalococcoides ethenogenes* use hydrogen, but not acetate, as the electron donor for reductive dechlorination. Hydrogen is generally considered to be the ultimate electron donor for reductive dechlorination of chloroethenes. In contrast, "*Desulfuromonas ottawaensis*" strains BB1 and BRS1, as well as *Desulfuromonas chloroethenica*, couple the reductive dechlorination of tetrachloroethene (PCE) to *cis*-1,2-dichloroethene (*cis*-DCE) to the oxidation of acetate, and cannot use hydrogen as an electron donor. PCE-dechlorinating *Desulfuromonas* species are widespread in nature (1), suggesting that acetate, rather than hydrogen, is the relevant electron donor to drive the reductive dechlorination process in many chloroethene-contaminated anaerobic environments.

In anaerobic microcosms established with aquifer solids collected from two PCE-contaminated sites (Cape Canaveral, FL, and the Bachman Road site, MI), PCE was rapidly dechlorinated in acetate-amended treatments. In hydrogen-amended microcosms dechlorination activity commenced only after acetate was accumulated by H_2/CO_2 acetogenesis. Acetate, rather than hydrogen, was the preferred electron donor to stimulate PCE to *cis*-DCE dechlorination at the chloroethene-contaminated sites investigated. Similarly, sustained dechlorination of PCE to *cis*-DCE with "*Desulfuromonas ottawaensis*" strain BB1 and hydrogen as the electron donor only occurred in co-cultures with an acetogen, such as *Sporomusa ovata*. These findings indicate that acetogenesis is an important process to stimulate the reductive dechlorination of chloroethenes by aceticlastic PCE-respirers.

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CHARACTERIZATION OF SELENIUM-DEPENDENT PURINE HYDROXYLASE AND XANTHINE DEHYDROGENASE FROM *CLOSTRIDIUM PURINOLYTICUM*.

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Clostridium purinolyticum is a selenium-requiring purine fermentor. This requirement for selenium has been at least in part attributed to selenium-dependent xanthine dehydrogenase (XDH) and formate dehydrogenase (FDH) enzymes which have been previously studied in cell extracts (Dürre et al., 1981). In the current work, XDH, purified from *C. purinolyticum*, required selenium for xanthine-dependent reduction of 2,6-dichloroindophenol (DCIP) as an electron acceptor. Kinetic analysis of XDH revealed that xanthine is the preferred substrate. An enzyme that also contained selenium and displayed similar properties to XDH was uncovered during a subsequent purification of XDH. This enzyme was found to reduce DCIP with either purine or hypoxanthine as substrate and therefore was termed purine hydroxylase (PH). Selenium was found to be required for this activity. Kinetic analysis suggests purine and hypoxanthine act as substrates for PH *in vivo*. The product of the hydroxylation of either purine or hypoxanthine by PH was determined to be xanthine. The isolation of a novel, purine-specific hydroxylase other than XDH in *C. purinolyticum* suggests the pathway previously described for the interconversion of purines in purinolytic *Clostridia* should be expanded to include PH.

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ANAEROBIC FERMENTATION OF *SALMONELLA TYPHIMURIUM* LT2 WITH AND WITHOUT PYRUVATE CARBOXYLASE

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S. typhimurium LT2-pTrc99A-*pyc* was constructed carrying the *pyc* gene expressing for pyruvate carboxylase from *Rhizobium etli*. Anaerobic fermentation patterns of *S. typhimurium* with and without *pyc* were compared using glucose as a carbon source. The succinate yield of LT2-pTrc99A-*pyc* was 0.231 g/g glucose, about five times the succinate yield of wild type strain (0.046 g/g glucose). Compared to wild type LT2, fermentations with the strain containing *pyc* resulted in lactate, formate and acetate yields reduced by 55.9%, 43.8% and 17.9%, respectively. Metabolic flux calculations indicate that LT2-pTrc99A-*pyc* and LT2 had indistinguishable specific growth rates and ATP yields. However, the glucose uptake rates and specific ATP generation rates were significantly lower in the *pyc*-containing strain than in the wild type LT2.

R.R. Gokarn, M.A. Eiteman, E. Altman, "Expression of pyruvate carboxylase enhances succinate production in *Escherichia coli* without affecting glucose uptake," Biotechnology Letters, 20, 8, 795-798 (1998).

METABOLIC ENGINEERING OF *ESCHERICHIA COLI* WITH PYRUVATE CARBOXYLASE TO PRODUCE SUCCINIC ACID

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Escherichia coli produces acetic, lactic and formic acids and ethanol as major products of anaerobic fermentation and a small quantity of succinic acid. *E. coli* AFP111 is a genetically altered strain in which the genes expressing pyruvate-formate lyase (*pfl*) and lactate dehydrogenase (*ldh*) are deleted, resulting in the absence of lactic and formic acids during anaerobic fermentation but the accumulation of some pyruvic acid. AFP111 was transformed with *Rhizobium etli* *pyc* gene expressing pyruvate carboxylase, an enzyme which converts pyruvate into oxaloacetate and absent in wild-type *E. coli*. We compared the growth and succinic acid production of AFP111 and AFP111-*pyc* on several carbon sources and fermentation conditions. On rich media, AFP111 produced succinic acid with a mass yield of 84% from glucose, while AFP111-*pyc* produced succinic acid with a mass yield of 96%. On glucose minimal media, AFP111 produced succinic acid with a yield of 63%, while AFP111-*pyc* resulted in a yield of 88%. Fructose and xylose as substrates resulted in lower yields and productivities than glucose. The opportunities for industrial succinic acid production with these strains are also discussed.

R.R. Gokarn, M.A. Eiteman, E. Altman, "Expression of pyruvate carboxylase enhances succinate production in *Escherichia coli* without affecting glucose uptake," Biotechnology Letters, 20, 8, 795-798 (1998).

CHARACTERIZATION OF THE 20S PROTEASOME AND PAN PROTEINS FROM THE METHANOARCHAEONS *M. JANNASCHII* AND *M. MARIPALUDIS*.

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The 26S proteasome, which is composed of a 20S proteolytic core and a 19S regulatory cap, is responsible for the majority of the degradation of ubiquitinated proteins in the eucaryotic cell. Genomic sequence analysis reveals that the 20S core subunits as well as two members of the six regulatory particle triphosphatase (Rpt) protein subunits of the 19S cap are conserved in the Archaea. In this study, both the 20S proteolytic core and an Rpt homolog or proteasome-activating nucleotidase (PAN) from the archaeon *Methanococcus jannaschii* were expressed and purified from recombinant *Escherichia coli*. Comparison of the full-length PAN protein to PAN(Δ 1-73) reveals that the N-terminus modulates the rate of NEM-inhibitable ATP and CTP hydrolysis, provides nucleotide specificity to PAN, and contributes to the extreme thermal stability of the protein which hydrolyzes ATP at an optimum of 80° C. Both PAN and PAN(Δ 1-73) purified as ~550- and ~500-kDa complexes, respectively, and were stable in the absence of ATP. Protein degradation assays using β -casein as a substrate indicate that the PAN protein interacts with archaeal 20S proteasomes to form a functional energy-dependent proteolytic complex. In addition, transmission electron microscopy of mixtures of PAN and the 20S proteasome suggest that these proteins interact to form complexes of 20S cores singly and doubly capped with the PAN complex.

Even though the entire genome has been sequenced, little is known about the genetics of *M. jannaschii*. In contrast, *Methanococcus maripaludis*, which has a doubling time of ~2.3 hours with an optimal growth temperature of 37° C, has become a popular model in which to perform genetic analyses in methanogens. Recently, both integrative and expression shuttle vectors, as well as an efficient PEG-based transformation method, have been developed for use in *M. maripaludis*. The expression vector could be especially useful, as the reducing conditions found in the methanogenic cytosol may be necessary in order for the methanococcal 20S proteasome to form a stable complex with the regulatory ATPase component(s). In the present study, a 20S proteasome-like particle has been purified from *M. maripaludis*, which hydrolyzes the peptide substrate LLVY-Amc at an optimum of ~65° C. In addition, two complexes of ~200kDa with significant NEM-inhibitable ATP and CTPase activity have been partially purified, and studies are currently underway to determine if these complexes are analogous to the *M. jannaschii* PAN complex. It is hoped that the preliminary work done with recombinant proteins from *M. jannaschii* will aid in our studies with the mesophilic and genetically-amenable microorganism *M. maripaludis*.

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HEME UTILIZATION AND AEROTOLERANCE IN *BACTEROIDES FRAGILIS*

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The gram-negative bacterium *Bacteroides fragilis* is an obligately anaerobic, opportunistic pathogen. Though this bacterium is a minor constituent of the normal human colonic flora, it is the bacterial species most frequently isolated from intra-abdominal abscesses involving anaerobes. *B. fragilis* requires exogenous heme for optimal growth *in vitro*. Recent experiments have shown that this bacterium displays a dramatic change in its protein expression profile under heme limitation. In addition to the role of heme in the growth of *B. fragilis*, this nutrient is also required for survival of the bacterium during stationary phase, and in the presence of oxygen and hydrogen peroxide. A bi-cistronic operon bearing homology to eubacterial ABC siderophore, vitamin B₁₂, and heme transport systems has been identified. The first open reading frame, *hemO*, bears weak homology to the *fatB* homologue of *Bacillus subtilis*. In *Vibrio anguillarum*, the product of the *fatB* gene is a membrane-associated periplasmic-protein required for transfer of ferric-anguibactin across the cytoplasmic membrane. The second open reading frame, *hemP* (hemin permease), bears homology to the ATP-dependent integral membrane permease component of the ABC siderophore, B₁₂, and heme transport systems. In *B. fragilis*, deletion of *hemOP* results in a two-fold increase in doubling time during growth in medium containing excess hemin (5 µg/ml), and a six-fold increase in doubling time during growth in medium containing limiting hemin (5 ng/ml). Promoter-reporter fusions are being used to study *hemOP* expression during heme limitation. A role for *hemOP* in bacterial survival during exposure to O₂ and H₂O₂ is under investigation. The data presented suggest that the products of *hemOP* are likely to play a role in the virulence of *B. fragilis*.

CHARACTERIZATION OF HYDROLYTIC ENZYME SYSTEMS OF ANAEROBIC FUNGI

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Anaerobic fungi were discovered in 1975. So far 17 species of anaerobic fungi have been isolated from the alimentary tracts of various herbivorous animals. Like other anaerobic eukaryotes such as trichomonads and ciliates, anaerobic fungi lack mitochondria but have hydrogenosomes, special organelles involved in energy metabolism. The anaerobic fungi are very effective degraders of plant cell wall structures. This can be attributed to the production of a number of highly active hydrolytic enzymes by the organisms. The enzymes are either secreted into the culture medium separately or attached to the mycelium surface as high molecular mass complexes, which are analogs of the cellulosomes produced by various species of cellulolytic clostridia. *Orpinomyces* strain PC-2, an anaerobic fungus obtained from the rumen of a cow, produces a cellulosomal enzyme complex with a mass of about 2,000 kDa and a polycellulosomal complex of about 50,000 kDa. The complexes are visualized with a scanning electron microscope and they were shown to bind tightly to cellulose microfibrils. The complexes have identical patterns on SDS-PAGE gels and consist of at least 20 different polypeptides. Most of the polypeptides are enzymatically active with activities such as cellulase, xylanase, mannanase, β -glucosidase and phenolic esterase. Evidence has been obtained for scaffolding polypeptides that bind the catalytic subunits in the complexes. Fifteen genes encoding cellulases and hemicellulases have been cloned, sequenced and expressed. They all contain domains, which may include in addition to the catalytic domain dockerins to bind to the scaffolding subunits, cellulose binding domains and domains of unknown functions. Comparison of sequences with similar enzymes from other sources revealed that the genes encoding hydrolytic enzymes from *Orpinomyces* may have aerobic fungal, or bacterial origins and that horizontal gene transfer between anaerobic fungi appears to be common.

***CLOSTRIDIUM THERMOCELLUM* CELLULOSOME REVEALS SPECIFIC INTERACTIONS AND INTERNAL SYNERGISM BETWEEN DOMAINS FAVORING DECOMPOSITION OF PLANT CELL WALLS**

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C. thermocellum secretes a high molecular mass cellulase/hemicellulase complex termed cellulosome. The cellulosome comprises more than twenty polypeptides with various catalytic and non-catalytic domains. In the present study a new gene, *celK*, coding for CelK, a major component of the cellulosome, has been sequenced. It is composed of an N-terminal signal peptide, a family IV cellulose-binding domain (CBD_{CelK}), a family 9 glycosyl hydrolase domain (Gh9_{CelK}), and a dockerin domain. The *celK* is highly identical to *cbhA* encoding the cellulosomal cellobiohydrolase CbhA. The main difference between CelK and CbhA is an internal region in CbhA encoding a family III CBD which is absent in CelK. The data indicate that *celK* and *cbhA* have evolved through duplications and recombinations of different domain-coding sequences. CelK is a true cellobiohydrolase releasing cellobiose from non-reducing end of cellulosic substrates. CelK has no homology to cellobiohydrolase CelS suggesting different mode of action of the latter. CBD_{CelK} which contains calcium binds cellulose and soluble polysaccharides, and enhances activity of Gh9_{CelK} towards cellulose. Site-directed mutagenesis of CBD_{CelK} showed that calcium is not involved in the binding, but that four aromatic residues are important for the biological function of the domain. Other cellulosomal components, XynY and XynZ, contain domains previously designated as domains of unknown function (DUF). We have demonstrated that the DUFs act as feruloyl esterases (FAE_{XynY} and FAE_{XynZ}). FAE_{XynZ} and xylanase A from *Orpinomyces PC-2*, an anaerobic fungus, significantly enhance release of both ferulic acid and xylose from hemicellulosic material suggesting the presence of internal synergism between the FAE and the xylanase domains in XynY and XynZ. Comparison of stability and binding properties of CelK, CBD_{CelK}, Gh9_{CelK}, FAE_{XynZ} and chimeric proteins composed of FAE_{XynZ} and CBD_{CelK} revealed presence of specific domain interactions affecting final properties of the domain-structured protein.

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The primary structure and analysis of the *atp* operon encoding subunits of F₁F₀ ATP synthase from *Clostridium pasteurianum*

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The proton translocating functionally active F₁F₀ ATP synthase from the gram positive anaerobic bacterium *Clostridium pasteurianum* has the simplest composition yet reported for an ATP synthase (Clarke et al. 1979. Eur. J. Biochem. 98:597-612). It contains only four subunits instead of eight commonly found in ATP synthases from most aerobic bacteria. To understand how this composition of ATP synthase correlates at gene level, the *atp* operon of *C. pasteurianum* (*Cpatp*) was cloned and sequenced. It contains nine genes arranged in the order *atpI* (*i*), *atpB* (*a*), *atpE* (*c*), *atpF* (*b*), *atpH* (*δ*), *atpA* (*α*), *atpG* (*γ*), *atpD* (*β*), and *atpC* (*ε*). This organization of *atp* genes is identical to those found in most bacteria. Northern blot analysis of total RNA from *C. pasteurianum* revealed the presence of a full length transcript (approximately 7.0 kb) of the *Cpatp* operon, indicating that all *atp* genes are transcribed. Apparently, several *atp* genes are either not expressed or their products are not associated with the purified enzyme complex. Similar findings have been previously reported for other gram positive anaerobic bacteria including *Clostridium thermoaceticum* and *Acetobacterium woodii*. Search for homologous proteins in the database reveals strong similarity of the *α* (55-65% identical residues), *β* (61-73% identical residues) and *c* (35-50% identical residues) subunits whereas differences in the *i*, *a*, and *ε* subunits of the *C. pasteurianum* ATP synthase were found in comparison with those of the subunits of ATP synthases from other origins. The N-terminal amino acid sequence of the *i* subunit has similarity with a signal peptide of bacterial lipoproteins. The signature of the *a* subunit does not match with that of the proposed signature of (STAGN)_x(STAG)(LIVMF)RL_x(SAGV)N (LIVMT) which has been found in every *a* subunit sequenced so far. The corresponding region of this signature of the *a* subunit in *C. pasteurianum* is ¹⁵³SLCLRLFCNM¹⁷⁷ which has two cysteine at positions 3 and 8. The *ε* subunit of the *C. pasteurianum* ATP synthase has the smallest size of all *ε* subunits known. It lacks 30-40 residues at the carboxyl end commonly found in this subunit. Antibodies raised against the purified F₁-ATPase of *C. thermoaceticum* react with *α* and *β* subunits of F₁-ATPase from *C. pasteurianum* and with the *β* subunit of F₁-ATPase from *E. coli*. Peptide specific antibodies raised against the *δ* subunit of *C. thermoaceticum* F₁-ATPase react with the *δ* subunit of F₁-ATPase from *C. pasteurianum* but not with the *δ* subunit of F₁-ATPase from *E. coli*. These results indicate that the F₁-ATPase of *C. pasteurianum* is antigenically more related to the F₁-ATPase from *C. thermoaceticum* than that from *E. coli*. Furthermore, the results also provide the first biochemical evidence for the presence of the *δ* subunit in *C. pasteurianum* F₁-ATPase. We have no data to explain the apparent lack of correlation between the composition of ATP synthase at the gene level and that at the protein level in gram positive anaerobic bacteria. Since no significant differences have been found between the primary structure of *atp* genes from aerobic bacteria and those from anaerobic bacteria it is assumed that the regulation of expression of *atp* genes in anaerobic bacteria may be different from those in aerobic bacteria.

1.4Å CRYSTAL STRUCTURE OF HYPERTHERMOPHILIC GLYCEROL DEHYDROGENASE FROM *THERMOTOGA MARITIMA*

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Crystal structures of glycerol dehydrogenase(GLDH) and its complex with NAD have been determined to 1.4Å and 2.5Å respectively. *Thermotoga maritima*, a rod-shaped anaerobic bacterium, isolated from geothermal heated marine sediment, can thrive up to a temperature of 80°C. The bacterium is, evolutionary of great importance as it forms one of deepest and slowly evolving lineages in the bacterial domain.

Glycerol dehydrogenase from *T.maritima* is a NAD⁺ dependent enzyme and catalyzes the reaction:

The enzyme is a soluble, cytoplasmic protein and is a homotetramer. Each monomer consists of 364 amino acid residues with a molecular weight of approximately 42 kDa. Crystals were grown by the process of vapor diffusion using 2-methyl 2,4 pentane diol as the precipitant. There is no representative crystal structure of a glycerol dehydrogenase in the Protein Data Bank at present. The NAD and 'Zn' binding characteristics and structure-based evolutionary significance of GLDH will be discussed.

PURIFICATION, CHARACTERIZATION AND ANALYSIS OF THE MEMBRANE BOUND HYDROGENASE FROM THE HYPERTHERMOPHILIC ARCHAEON PYROCOCOCCUS FURIOSUS

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Highly-washed membrane preparations from cells of the hyperthermophilic archaeon, *Pyrococcus furiosus*, contain high hydrogenase activity (9.4 $\mu\text{mol H}_2$ evolved/mg at 80°C). The enzyme was solubilized with n-dodecyl-b-D-maltoside and purified by multistep chromatography in the presence of Triton X-100. The purified preparation contained two major proteins (ab) in an approximate 1:1 ratio with a minimum molecular mass near 65 kDa and contained ~ 1 Ni and 4 Fe atoms/mol. The reduced enzyme gave rise to an electron paramagnetic resonance signal typical of the so-called Ni-C center of mesophilic NiFe-hydrogenases. Using N-terminal amino acid sequence information, the genes proposed to encode the a- and b-subunits were located in the genome database within a putative fourteen gene operon (termed mbh). The deduced sequences of the two subunits (Mbh 11 and 12) were distinctly different from those of the four subunits that comprise each of the two cytoplasmic NiFe-hydrogenases of *P. furiosus* and show that the a-subunit contains the NiFe-catalytic site. Six of the ORFs in the operon, including those encoding the a- and b-subunits, show high sequence similarity (>30% identity) with proteins associated with the membrane-bound NiFe-hydrogenase complexes from *Methanosarcina barkeri*, *Escherichia coli* and *Rhodospirillum rubrum*. The remaining eight ORFs encode small (< 19 kDa) hypothetical proteins. These data suggest that *P. furiosus*, which was thought to be solely a fermentative organism, may contain a previously unrecognized respiratory system in which H₂ metabolism is coupled to energy conservation. Targeted transcriptional analysis of RNA isolated from *P. furiosus* cells grown under different conditions shows that all three of *P. furiosus* hydrogenases are induced when sulfur is not present in the growth medium.

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