Microbiology and Physiology of Anaerobic Fermentation of Cellulose

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A. PROGRESS REPORT - Lars G. Ljungdahl

Microbiology and Physiology of Anaerobic Fermentation of Cellulose

In our grant submitted 1989 involving an integrated study of anaerobic bacteria important for the conversion of plant polymers (cellulose mainly) to methane, work was proposed as follows:

- a) *Clostridium thermocellum* - the structure and enzymatic activity of the cellulosome and polycellulosome.
- b) *Anaerobic fungi* - extracellular enzymes involved in degradation of plant material (leaves, grasses, husk) and physiology of fermentation.
- c) *Acetogenic clostridia* - enzymes dealing with CO, CO₂, H₂, CH₃OH, and electron transport and energy generation coupled to the acetyl-CoA autotrophic pathway.

a) *Clostridium thermocellum*. The cellulosome of *C. thermocellum* first described by Lamed et al (1) is a large enzyme complex, consisting of tightly associated polypeptides. A number of *C. thermocellum* strains have been described. Much progress has been achieved by several groups in elucidating the structure and function of the cellulosome. We have recently reviewed this progress (Felix and Ljungdahl). (3)

The cellulosome from the different strains may differ in masses (from 2×10^6 to 6.5×10^6 Da) and in numbers of different polypeptides (14-26) constituting the complex (12). Our work has been exclusively with *C. thermocellum* JW20, isolated and described by Wiegel (2,4), who found the bacterium tightly adhering to cellulosic fibers. This adherence is clearly mediated by polycellulosomes, which have masses between 50×10^6 to 100×10^6 Da (5,6). It has been suggested that the polycellulosomes which apparently are formed outside on the cell surface of the bacterial cell are inside a skin-like cover perhaps consisting of peptidoglycan (6,7). This material may form contact corridors between the cellulosic fiber and the bacterial cell allowing the cellulosomes to move from the cell surface to the cellulosic fiber. The cellulosome is attached to the fiber by a large (210kDa) noncatalytic polypeptide previously called C1/S_L, but now designated CipA (cellulose integrating protein) and coded by gene *CipA* (8), which has been sequenced. It encodes a protein with a mass of 196,700 Da. CipA has a cellulose binding domain and contains also 9 internal repeated sequences which are postulated to bind 8 catalytic subunits and a special peptide (ORF3p) anchoring CipA to the cell surface (9). Thus, the attachment of the bacterium to the cellulose may be by the skin-like cover and by the CipA and ORF3p polypeptides.

Extensive genetic work has been performed with *C. thermocellum*, especially by groups led by Beguin and Aubert (14) in France, Hazelwood (15) in England and Demain (8) at MIT. Thus more than 30 genes have been identified coding for proteins associated with cellulolytic activity of *C. thermocellum* (3). The genetic work confirms the multiple polypeptide structure of the cellulosome. It also confirms a multiple-cut event mechanism proposed by us based on electron microscopic studies (Fig. 1A) (6). According to this mechanism from 6 to 8 catalytic subunits are attached to a scaffold. The scaffold has now

been identified with the CipA polypeptide (9) (Fig 1B), that as mentioned may bind 8 catalytic subunits. They line up with the cellulose fiber in an arrangement that allows the multi-cutting of the glucan chain.

Fig. 1. Models of the action of cellulosomes on cellulose. The hydrolysis is postulated to involve a multiple cutting event. A. This diagram is based on electron microscopic observations (6). B. Model based on sequencing of polypeptides by cloning (9). In a C₄, is the cellulose chain; C₄, C₂, are cello dextrins and C₁, cellobiose. CS and HS are catalytic subunits and hydrolytic sites respectively. LF is a long fiber serving as scaffold and now postulated to be identical with CipA. In B D.S. are duplicated segments binding catalytic subunits and ORF3p (8,16) CipA contains in addition a cellulose binding site (17,18).

In addition to the many polypeptides the cellulosome contains carbohydrates (10,11), and requires Ca²⁺ and thiols for activity (12). A recent report also suggests that a lipid component, cardiolipin, is a part of the cellulosome (13). This lipid could be similar or identical to the yellow activity substance (YAS) previously described by us (10). The roles of the nonproteinaceous components are unknown.

The cellulosome is a remarkably stable polypeptide complex. It withstands organic solvents and 8M urea. In 2% SDS at room temperature it slowly loses activity against crystalline cellulose. It is stable in 50% ethanol for months at 25°C. This has made it difficult to purify individual enzyme; they cling together. It is remarkable but only a few of the endoglucanases and other hydrolytic enzymes found by gene-cloning of *C. thermocellum* have actually been purified from the bacterium itself and identified with the cellulosome.

We have now been able to dissociate the cellulosome by treatment with calcium binding reagents like EDTA and EGTA. First to be noted is that these reagents are strong inhibitors of activity toward crystalline cellulose (avicelase) whereas no inhibition is observed of endoglucanase (carboxymethyl cellulase, CMCase) activity. Secondly, the SDS-PAGE profile of the complete cellulosome preparation changed much upon EDTA-treatment. This

change is time dependent and was noticed after 2 hours of exposure of the cellulosome to EDTA. The reaction seems to be complete after 36 hours. The change involved a decrease in the M_r of most of the catalytic polypeptides. It did not change the 210 kDa scaffolding polypeptide CipA. Most notable changes included the disappearance of bands indicating polypeptides of M_r -s of 160,000; 98,000; 76,000; 65,000; and 54,000, and the appearance of new bands of polypeptides with M_r -s of 150,000; 132,000; 91,000; 71,000; 57,000; and 45,000 (Fig. 2). We assume that the large peptides were converted to those of the smaller.

Fig. 2. Changes in SDS-PAGE pattern of the cellulosome from *C. thermoaceticum* upon treatment with EDTA. The 98 and the 76 bands (before treatment) are converted to the 91 and 71 bands (after treatment) respectively.

This has been confirmed so far in two cases by N-terminal amino acid analyses, which are identical for the 98 and 91 kDa bands and the 76 and 71 kDa bands. The N-terminal sequence of the 98 kDa polypeptides does not match up to any known sequence of cellulolytic peptides identified from *C. thermocellum* and may constitute another yet unknown endoglucanase. The sequence of the 71 kDa polypeptide perfectly matches that of the S_1 endoglucanase isolated and sequenced in by Wang et al (19) and postulated as the major endoglucanase of *C. thermocellum*. The production of the new smaller polypeptides apparently does not involve proteolysis. The presence of proteinase inhibitors did not change the results, neither could we detect any proteolytic activity in the cellulosome preparation.

The EDTA treated cellulosome dissociated into subfractions, which separated by gel filtration and on mono-Q-columns. On the latter four major fractions were obtained each constituting one of the major components of the cellulosome: the CipA-protein, the 91 kDa, 71 kDa, and 57 kDa polypeptides. We are now able to investigate these peptides, their physical and catalytic properties.

Noticing the apparent role of Ca^{2+} in the integrating of the cellulosome, we grow *C. thermocellum* in medium containing ^{45}Ca . The cellulosome contains 47 nMol of Ca per mg of protein. Repeated dialysis and gel filtrations do not change this content indicating that Ca is tightly bound to the cellulosome. SDS-PAGE gels of the labeled cellulosomes demonstrated that major Ca-binding components are the polypeptides that upon EDTA-treatment changes M_r -s. Also two smaller Ca-binding components were observed traveling with M_r -s around 4 to 5 kDa. They are not proteinaceous. We feel Ca plays an important role in the integrity and catalytic activity of the cellulosome and speculate that it is involved in the binding of the catalytic subunits to the scaffold protein CipA. One or two additional components may be involved in this binding as indicated by the discovery of two small Ca^{2+} binding compounds with masses around 5 kDa. The nature of these is unknown. The work on Ca^{2+} and EDTA will be presented at the ASM meeting 1993. A manuscript for submission to Applied and Environmental Microbiology is ready for final internal review.

Work on *C. thermocellum* is carried out in close cooperation with Juergen Wiegel, who is studying the xylanolytic activity, which he demonstrated is associated with the cellulosome (2).

b) *Anaerobic fungi*. Until 1975 the rumen microbiota was considered composed of bacteria and protozoa. However, Orpin (20) isolated *Neocallimastix frontalis* and demonstrated that it is an anaerobic fungus. Now 11 different anaerobic fungi have been described belonging to seven genera. They differ by being mono- or polycentric and producing either mono- or polyflagellated spores. All major polysaccharides of plants are fermented by rumen fungi and they are even able to solubilize, but perhaps not degrade, lignin to some extent (21). Degradation of lignin is supposed to occur strictly by an aerobic process.

In 1989 we reported the isolation of 5 different fungi from a bovine rumen (22). Two of these were monocentric; one of these was monoflagellated, *Piromyces* MC-1, the other polyflagellated, *Neocallimastix*, MC-2. The other three were polycentric and have been characterized as *Orpinomyces* species, strains PC-1 to PC-3. The fermentation balances with glucose and Coastal Bermuda grass as substrates were determined; formate acetate, ethanol, CO_2 and H_2 were the main products. Lesser amounts of lactate, and succinate were also produced. We are now investigating two aspects of the fungi using *Neocallimastic*, MC-2, and *Orpinomyces*, PC-2. The first aspect deals with secreted enzymes involved in the degradation of polymers; the second is the production of formate, CO_2 and H_2 which are energy yielding substrates for acetogenic and other CO_2/H_2 utilizing microorganism like methanogenes and sulfur reducers. The latter project just started will be in cooperation with

Harry Peck, Alan Przybyla and Barny Whitman. It is interesting to note that rumen fungi interact with rumen bacteria and methanogens to effectively ferment cellulose to methane (23,24).

The metabolism of formate, CO_2 and H_2 in anaerobic fungi has not been investigated much. Instead of mitochondria they contain a microbody-like organelle termed hydrogenosome that possesses respiratory function and produces acetate, H_2 and CO_2 (25).

The work on secreted enzymes was at the beginning aimed at cellulolytic enzymes and to include a comparison with those enzymes from bacteria, e.g., *C. thermocellum* and aerobic fungi. However, in surveying the anaerobic fungi, we found them to produce high xylanolytic and esterolytic activities (26). Subsequently we developed assays for *p*-coumaroyl and feruloyl esterases (27) and purified from *Neocallimastic*, MC-2, one *p*-coumaroyl esterase (28) and two specific feruloyl esterases (29). The substrates for the esters were isolated from Coastal Bermuda grass, and have been designated PAXX = *O*-{5-*O*[(*E*)-*p*-coumaroyl]- α -L-arabinofuranosyl}-(1 $\rightarrow$ 3)-*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylopyranose and FAXX = *O*-{5-*O*[(*E*)-feruloyl]- α -L-arabinofuranosyl}-(1 $\rightarrow$ 3)-*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylopyranose. The structures are shown in Fig. 3 together with some PAXX and FAXX derivatives.

Fig. 3. Abbreviations used: PAXX, *O*-{5-*O*[(*E*)-*p*-coumaroyl]- α -L-arabinofuranosyl}-(1 $\rightarrow$ 3)-*O*- β -D-xylopyranosyl-(1 $\rightarrow$ 4)-D-xylopyranose; PAXX-1, PAXX containing an additional xylopyranose residue on C-2 of the arabinosyl residue (more correctly referred to as XPAXX); PAXX-II, PAXX containing an additional β -1,4-linked xylosyl residue; FAXX, FAXX-1 and FAXX-II are the corresponding feruloyl analogues.

The importance of the esterases in the degradation of plant tissue was demonstrated with Coastal Bermuda grass as substrate (Table 1). The esterases alone released only

TABLE 1. Release of phenolics and reducing sugars from Coastal Bermuda grass cell walls by FAE-1, FAE-II, CAE and other wall-degrading enzymes.

Enzyme Treatments	<i>p</i> -Coumaroyl groups released (ng)*	Feruloyl groups released (ng)*	Reducing sugars released (μ g)*
Buffer control (no enzyme)	21	4	5
1. CAE	320	17	6
2. FAE-I	91	287	8
3. FAE-II	18	463	11
4. Xylanase †	20	13	38
5. Driselase ‡	34	12	87
6. CAE + Xylanase	956	49	44
7. FAE-I + Xylanase	383	1002	80
8. FAE-II + Xylanase	31	2073	69
9. CAE + Driselase	1087	40	115
10. FAE-I + Driselase	515	1287	228
11. FAE-II + Driselase	62	3079	207
12. FAE-I + FAE-II + Driselase	645	3445	312
13. CAE + FAE-I + FAE-II + Driselase	1190	3507	333

Enzymes and amounts used were as follows: CAE, FAE-I and FAE-II, 2.5 mU; xylanase, 1 unit; driselase (amount containing 1 unit xylanase). Incubations were for 5 h at 40°C in 2 ml of 1- mM BTP buffer, pH 7.0, containing 10 mg or CBG (ground to 80 μ m). *Values are the mean of a triplicate analysis and varied < 7%.

†Xylanase from *Trichoderma viride* (Sigma X 3876).

‡Driselase from *Basidiomycetes* (Sigma D 9515).

moderate amounts of ferulic acid (300-400 ng/10 mg of CBG) and *p*-coumaric acid (320 ng/10 mg of CBG). Together with a purified xylanase these figures increased from 3-to-5-fold and with mixture of several hydrolytic enzymes even higher amounts of the esters were released. In addition the esterases effected the release of reducing sugars and thus increased the overall degradation of CBG. We have interpreted these results as follows: Xylanase and other glycosidases produce phenolic oligosaccharides, which are rather resistant to further degradation unless esterases remove the phenolics. The free oligosaccharides are then

hydrolyzed to simple sugars. A case can perhaps be made that the phenolics are bridges between hemicellulosics and lignin. Thus the breaking of these bridges by the esterases may even aid in solubilizing lignin. We have recently summarized our work on the esterase, which has been in cooperation with Danny E. Akin's group at the Richard B. Russell Agricultural Research Center (30).

In addition to the esterases we have from *Orpinomyces*, PC-2, isolated β -glucosidase (cellobiose), endoxylanase, and a protein with cyclophilin type structure. The β -glucosidase is monomeric with a M_r of 85,400, as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis. It has a pI of 3.95. It contains about 8.5% (wt vol⁻¹) carbohydrates. The N-terminus appears to be blocked. The enzyme catalyzes the hydrolysis of cellobiose and *p*-nitrophenyl- β -D-glucoside (pNPG). The K_m and V_{max} values with cellobiose as substrate at pH 6.0 and 40°C are 0.25 mM and 27.1 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$, respectively; with pNPG as substrate corresponding values are 0.35 mM and 27.7 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$. Glucose ($K_i=8.75\text{mM}$ with pNPG as substrate) and gluconolactone ($K_i=1.68 \times 10^{-2}$ mM and 2,57 mM, with pNPG and cellobiose as substrates, respectively) are competitive inhibitors. Optimal activity with pNPG and cellobiose as substrates is at pH 6.2 and 50°C. The enzyme has high activity against sophorose (β -1,3), but has no activity against gentiobiose (β -1,6). The activity of the β -glucosidase is stimulated by Mg^{++} , Mn^{++} , Co^{++} and Ni^{++} , and inhibited by Ag^+ , Fe^{++} , Cu^{++} , Hg^{++} , SDS and *p*-chloromercuribenzoate.

The endoxylanase (one of several) is a monomeric protein having a mass of 29 kDa, as estimated by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. It has a pI above 8.0. The K_m and V_{max} values with water-soluble oat spelts xylan as substrate at pH 5.5 and 40°C were 2.15 mg ml⁻¹ and 1,770 $\mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$, respectively. The enzyme has pH optimum of 5.4 and is stable at 45°C and pH 5.5 for 30 min. It is specificity for xylan and has no detectable activity against crystalline cellulose, carboxymethyl cellulose, *p*-nitrophenyl- β -glucoside, *p*-nitrophenyl- α -L-arabinoside, *p*-nitrophenyl- β -xyloside, and *p*-nitrophenylacetate. The activity of the xylanase was inhibited by N-bromosuccinimide, *p*-hydroxymercuribenzoate, SDS, Ag^+ , Cu^{2+} , Hg^{2+} , and Fe^{2+} . Activities of both the endoxylanase and the β -glucosidase were higher in cultures grown on CBG compared to those grown on glucose.

When purifying the endoxylanase an unknown protein was obtained. Of interest is that this protein has high homology (60% of the first 30 N-terminal amino acids) with human, mouse and yeast cyclophilins. Cyclophilins are peptidyl-prolyl *cis-trans*-isomerases and believed to be involved in folding of newly synthesized proteins (37, 38). They have not been previously described to occur in anaerobic microorganisms.

One of our long term goals is to understand the structure and active sites of the enzymes we isolated. For that reason we have adapted the polymerase chain reaction (PCR) to clone and sequence the genes of the respective enzymes. To facilitate the application of PCR we started with an endoxylanase from the aerobic fungus *Aureobasidium pullulans* Y-2311-1. This enzymes (APX-II) studied by K.-E.L. Eriksson in our department, has now been characterized (31). It has the highest endoxylanase activity reported ($V_{max}=2,650 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$). We have cloned and sequenced the enzyme. It has a mass of 25 kDa and it is

different from another xylanase (APX-I) also from *A. pullulans* studied by Leathers (32). APX-II has high homology with bacterial xylanases. These results will be reported of ASM meeting 1993 (Abstr. 12 in publication list).

c) **Acetogenic clostridia.** To understand the pathway of acetate synthesis from CO, CO₂/H₂ has been the major goal of this laboratory. Over 30 years of work was recently summarized and reviewed (33). The pathway now well established is the autotrophic acetyl-CoA or Wood-pathway. The latter is in honor of H.G. Wood. *Clostridium thermoaceticum*, and *Clostridium thermoautotrophicum* the organism in which the Wood-pathway was discovered have growth yields fermenting sugars higher than can be accounted for by substrate phosphorylation (34), contains cytochromes and menaquinone (35), and growth autotrophically on CO, and a mixture of CO₂/H₂ (36). Formation of acetate by the Wood-pathway does not generate any ATP. Therefore this pathway must involve a chemosmotic mechanism for ATP generation. We have now isolated membrane vesicles from the two acetogenic clostridia in which we identified components of a anaerobic electron transport chain. They are summarized in Fig. 4 (39).

Fig. 4. Scheme of electron transport and Δp generation in the membrane of *C. thermoautotrophicum*. 1. Ferredoxin NADP: oxidoreductase. 2. ferredoxin NAD: oxidoreductase. 3. NADH dehydrogenase. 4. methanol dehydrogenase. 5. carbon monoxide dehydrogenase. 6. methylenetetrahydrofolate reductase. 7. F₁F₀-H⁺ ATPase. 8. hydrogenase. FAD, flavin adenine dinucleotide; FP, flavoprotein; MQ, menaquinone; PQQ, pyrroloquinoline quinone; Fd, ferredoxin. (From Hugenholtz and Ljungdahl, [39]).

The flavoprotein (FP), and the two b-type cytochromes, a hydrogenase, and ATP have been isolated (manuscripts are being prepared). The menaquinone (MQ) have been characterized and found to mediate the electron transfer between the two cytochromes (40). Using EPR (in cooperation with Vance Morgan and D. DerVartanian) we have obtained evidence for the presence of rubredoxin, ferredoxin, and a Cu-protein in the membranes. Neither of these have been isolated from the membranes and it will be part of the future project together with D. DerVartanian and J. Wampler. An ATPase has been purified, which has a somewhat different composition than that of *Escherichia coli*. Thus its F_1 -component has only four different types of polypeptides designated α , β , γ , and δ instead of 5 in *E. coli* (49) and the F_0 -compound consists of only two different polypeptides instead of 3 (manuscript in preparation). We have now shown that the F_0 and F_1 parts of ATPases of *C. thermoautotrophicum* and *E. coli* are not functionally compatible (43). Previously we demonstrated that CO dehydrogenase/ acetyl-CoA synthase and methylene-THF reductase are membrane associated (42). Now we shown that the membranes actively transport amino acids with CO as the only source of energy (44). We feel that the coupling of the acetyl-CoA pathway with electron transport and ATP synthesis is now on rather firm footing. The CO dehydrogenase/acetyl-CoA synthase has been sequenced. A work carried out in cooperation with S. Ragsdale and H.G. Wood (45).

With the discovery of electron transport by membranes of *C. thermoaceticum* and *C. thermoautotrophicum* we tested several electrons acceptors using CO as the source of electron. This still preliminary work has shown that thiosulfate, DMSO and DMSO_2 serve as terminal electron acceptors. Furthermore, whole cells grow with these compounds as electron acceptor using methanol and higher alcohols as electron donors. It was possible to demonstrate a membrane bound methanol dehydrogenase (perhaps PQQ-dependent) as well as a FAD-dependent formaldehyde dehydrogenase.

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A. PROGRESS REPORT - Juergen Wiegel

Two separate reports A.1. and A.2. are given for the two presently funded grants A.1. Hemicellulases from ethanologenic thermophile *T. ethanolicus* and similar thermophiles. (6 months after renewal of DOE-grant DE-FG09-89ER-14059: (\$ 56,280 direct, \$ 25,720 indirect costs for current year; total for 3 years \$ 246,000))

We studied the regulation of the synthesis of the xylanase (activity), xylosidases, acetylases by pH, T, growth phase and carbon source; e.g., xylanases (mainly extracellular): Increased activities were observed when growing on salicin, lactose, cellobiose and oat spelt xylan. Except for xylobiose (0.5%), the highest activities were reached when the inducer was added to the cell suspension at concentrations between 0.05 and 0.1 % inducer. The temperature had a strong influence: strain JW/SD-YS485 although growing up to 75°C, produced the highest activity around 50°C with less than 10% of this activity at 60°C and above, EPP100 and NDF190 produced maximal activities around 54-58 and 65°C, respectively. Strain JW/SL-YS485 exhibits in the culture supernatant the maximum activity around pH 4.5-5.0 in accordance with its pH_{opt} for growth. The other two strains had the maximal activity around 6.0 and 7.0. The more alkaline activity in strain JW/SD-EPP100 got lost during additional picking of single colonies. We have regained a culture with the high activity, but it has not been studied further at this time. In strain JW/SD-NDF190, under all induction conditions tested, the same activity bands (4% PAGE) were induced. Xylosidases were studied in *T. ethanolicus* (see publication), *C. thermohydrosulfuricum* JW102, *Thermoanaerobacterium* spec. JW/SL-YS485 (containing 2 different xylosidases), and strain JW/SD-NDF190. The O-methyl glucuronidase in strain JW/SL-YS485 is produced in mid-exponential growth phase, whereas the two different acetyl(xylan) esterases are formed at a later stage. Both enzymes are found at similar levels intracellular and extracellular.

Xylanases: From the three above mentioned strains, several enzymes have been highly purified (although we have problems with the staining of the enzyme protein) and are partly characterized. The pI values vary from 4.5 to 9.0. Strain JW/SD-NDF190 has 5 different enzymes with pI values of 5.5(purified), 7.7, 8.0, 8.6, and 9.0. Strain JW/SL-YS485 has one with a pI of 4.5 and 8.4. The MW are around 30,000-40,000.

Xylosidases: Whereas *T. ethanolicus* and *C. thermohydrosulfuricum* have mixed function xylosidase (containing also a xylan-arabinosidase activity exhibiting different pH and T-optima than the xylosidase activity) the other strains contain two separate enzymes for these activities. The enzymes have been purified and characterized including N-terminal sequences.

O-methyl glucuronidase: So far, only strain JW/SL-YS485 exhibited a high enough activity to warrant purification. The enzyme has been highly purified (gel contains three additional faint bands) and we are in the process of characterization.

Acetyl(xylan) esterase: Two enzymes have been purified to gel electrophoretic purity from strain JW/SL-YS485. Isoelectric points Acetyl(xylan) esterase II exhibits a remarkable stability with a $t_{1/2}$ of 17 min at 100°C although the $T_{opt; 2min}$ is only around 85°C and is stable for at least 1 h in the pH range 5.5 and 8.0 (higher pH not tested). Whereas using the artificial substrate 4 methylumbelliferon, both enzymes exhibit similar activities (75 and 80 μ mol/min and mg protein), Arrhenius energies (27 and 29 kJ/mol), isoelectric points (pH 4.3), pH-optima (pH_{opt} 7.0 and 7.5), acetylase II (MW 106,000; tetramer of 25,000) has much a higher specific activities than acetylase I (MW = 194,000, hexamer(?) of 32,000) using acetylated xylan. N-terminal sequences have been determined.

Publications supported from this grant A.1. for the period August 1993 - May 1994:

PRESENTATIONS WITH ABSTRACTS:

S.-Y. Liu, S. DeBlois and J. Wiegel. Isolation and characterization of xylose utilizing anaerobic thermophiles. Thermophiles: Science and Technology. Reykavik, Iceland, August 1992. Extended Abstr. IV-P-06 (p. 123).

W. Shao, S. DeBlois and J. Wiegel. Hemicellulases from thermophilic anaerobic thermophiles. Thermophiles: Science and Technology. Reykavik, Iceland, August 1992. Extended Abstr. IV-P-09 (p. 125).

Shao, W., S. DeBlois, and J. Wiegel. Acetyl(xylan)esterases from an anaerobic thermophile. Ann. Meet. Am. Soc. Microbiol. Atlanta. May 1993.

PRESENTATIONS (INVITED)

J. Wiegel. Thermophilic Clostridia. Ann. Meet. Am. Soc. Microbiol. Seminar presentation and Seminar Chair.

JOURNAL PUBLICATIONS:

In preparation:

Shao, W. and J. Wiegel. Characterization of acetyl(xylan) esterases from *Thermoanaerobacterium* spec. strain JW/SL-YS485.

Submitted:

Peteranderl, R. F. Canganella, A. Holzenburg and J. Wiegel. (1993) Induction and regeneration of autoplasts from *Clostridium thermohydrosulfuricum* JW102 and *Thermoanaerobacter ethanolicus* JW 200. Appl. Environ. Microbiol.

A.2. Microbiology and Physiology of Anaerobic Fermentations of Cellulose

(DE-FG09-86ER-13614; Section: Wiegel)

(For this period: DIRECT COST \$ 20,000, INDIRECT \$ 9,140)

The multi-P.I. grant on cellulose degradation deals with physiologically diverse organisms involved in the overall conversion of cellulose to feed stock chemicals. Part of this grant section is to provide microbiological cooperation for other sections of this multi-P.I. grant. During the last period we have worked mainly on two different projects i) the fermentation capabilities of *C. thermobutyricum* in batch and continuous culture (work is presently prepared for publication) and ii) on the description and physiology of several newly discovered alkali-thermophilic anaerobes (in cooperation with a industrial grant).

***C. thermobutyricum*:** The biological production of butyric acid by thermophiles is of interest since butyric acid is a feed stock chemical for the food industry e.g., for producing the banana aroma a butyric acid ester. We showed that the organism can tolerate up to about 350 mM butyrate. Using the rotary fermentor and thus a continuous culture approach, a maximal butyrate concentration of 18.4 g/l and a productivity of about 2.4 g/l and h at a concentration of 27 mM butyrate in the culture. However, during the continuous culture, a shift in the fermentation products from predominantly butyrate to acetate was observed. Interestingly, a disposed oscillatory mode of butyrate and acetate formation was observed. Consequently, the addition of ^{14}C -acetate lead to an increased butyrate formation from glucose as well as through ^{14}C -acetate conversion into ^{14}C -butyrate. (Two publications in preparation). Also during growth in the rotary fermentor a shift in the morphology to long (100 μm) twisted ("spaghetti-like") cells were observed.

Novel alkali-thermophilic anaerobes: Within an industrial grant searching for some alkaline organisms with very specific enzyme properties, we isolated some very interesting anaerobic, alkaliphilic thermophiles able to grow at temperatures as high as 63°C and at pH values as high as 11.3. Such organisms were unknown so far and extended the temperature range for alkaliphiles and the pH range for thermophiles markedly (Kristjansson and Stetter, 1992). However, since they did not contain the industrial enzymes of interest, the organisms were further studied under the DOE grant since a coupling of these organisms to cellulolytic organisms could be of high interest. We also will continue to look for anaerobic alkaline cellulolytic thermophiles, organisms unknown so far. However, some of the strains contain xylanases. We have in the mean time identified four different new species, of which two are presently classified as clostridia (*C. 'paradoxum'* {Li et al., 1993} and *C. 'thermoalkaliphilum'* {Li et al., in prep}) and the other two are tentatively grouped with propionibacteria. The latter ones are branched cells and represent a new morphogroup among the thermophiles. *C. paradoxum*, has a t_d of 16 min at its pH_{opt} of 10.5 and T_{opt} of 55°C. It forms terminally located spores. Totally unusually, the sporulated cells exhibited a more straight swimming motion instead of the mainly tumbling motion of exponentially growing cells. This is a very atypical behavior, since sporulated cells normally cease to produce energy which is required for motility.

Publications supported from this grant A.2: (1992/1993)

PRESENTATIONS WITH ABSTRACTS:

F. Canganella and J. Wiegel. Cultivation of *C. thermobutyricum* in a rotary fermentor system. Thermophiles: Science and Technology. Reykjavik, Iceland, 1992. Extended Abstract p. 119.

JOURNAL PUBLICATIONS:

In preparation:

Canganella, F. S-U. Kuk, H. Morgan, and J. Wiegel. *Clostridium thermobutyricum*: Growth studies and stimulation of butyrate formation by acetate supplementation. Submitted to Appl. Environ. Biotechnol.

Canganella, F. and J. Wiegel. Cultivation of *Clostridium thermobutyricum* in a rotary fermentor system (to be submitted to J. Ind. Microbiol.)

Submitted:

Li, Y. L. Mandelco, and J. Wiegel. 1993. *Clostridium thermoalkaliphilum*, sp. nov. Int. J. Syst. Bacteriol.

Zhang, X., L. Mandelco, and J. Wiegel. *Clostridium hydroxybenzoicum* sp. nov. Int. J. Syst. Bacteriol.

In press:

Li, Y. L. Mandelco, and J. Wiegel. 1993. Isolation and Characterization of a moderately thermophilic anaerobic alkaliphile, *Clostridium paradoxum* sp. nov. Int. J. Syst. Bacteriol. 43: (3).

BOOKCHAPTER:

Canganella, F. and J. Wiegel The potential of thermophilic clostridia in Biotechnology. 1993. IN The Clostridia and Biotechnology (D.R. Woods, ed). Butherworths Publ. Stoneham, MA (in press).

Wiegel, J. Applications of homoacetogenic bacteria. In: Acetogenesis (H. Drake, ed.). Chapman and Hall, N.Y. (in preparation).

B. SUMMARY OF 5 PERTINENT PUBLICATIONS

J. Wiegel (1990) Temperature spans for growth: A hypothesis and discussion. FEMS-Microbiol. Rev. 75:155-170.

This paper, based on a presentation at a workshop, presents two main theories from the author on thermophilic bacteria: the cryptic thermophiles and the temperature tolerant organisms growing over a wide range and exhibiting a bi-phasic curve. It also presents otherwise unpublished data on the temperature span of *Methanobacterium thermoautotrophicum*, exhibiting the widest temperature range for growth of about 50°C. ABSTRACT: In recent years the upper and lower temperature limits for responsible growth of pure cultures of microorganisms have been extended to 115°C and -20°C, respectively. There are no organisms which grow at both 0°C and 100°C

and, therefore, organisms are grouped according to their ranges of growth temperatures. Thus, the questions of importance are: 1) What is the widest temperature range (temperature span) over which a single organism can grow? and 2) How much can one alter the temperature spans of an organism? The concept of 'cryptic thermophiles' is used to explain some of the published data on the latter question. A wider temperature range can be very important for organisms in various ways, since it makes an organisms more versatile with regard to changes in the environment. Also, it enables the organism to utilize a wider range of ecological niches. Some aerobic and anaerobic extreme thermophiles will grow within a span of more than 40°C. Furthermore, such organisms are regarded suitable for biotechnological applications as well. A hypothesis is presented and discussed, that these organisms have two sets of key enzymes, and that their synthesis is regulated by temperature. These organisms are capable of growing in two different ranges, such as the mesophilic and thermophilic ranges. The hypothesis is based on the fact that these bacteria exhibit broken Arrhenius plots (growth temperature versus doubling time as parameters), and is presented using 'temperature tolerant extreme thermophiles' as the major example. However, the hypothesis is not restricted to this group, but is also applicable to the 'temperature tolerant thermophiles and mesophiles'.

J. Wiegel (1992) The anaerobic thermophilic bacteria. Chapter 6 In: Thermophilic Bacteria. J. K. Kristjansson (ed.), CRC-Press, Boca Raton, FL, pp. 105-184.

This extensive review describes all (as of Jan. 1992) thermophilic anaerobic (eu)bacteria. Besides comparing properties such as habitats, morphology, phylogenetic data (e.g., making the case for the unimportance of observed spore formation), temperature ranges, etc., the organisms are described according their major physiology, i.e., fermentation substrates and product. This review contains ample comparative data in tables to facilitate an overview on the diversity of thermophilic anaerobic (eu)bacteria.

For the first time, an identification key for this group of organism has been prepared and is presented at the end of the chapter.

W. Shao and J. Wiegel (1992) Purification and characterization of a thermostable β -xylosidase from *Thermoanaerobacter ethanolicus*. J. Bacteriol. 174:5848-5853.

A highly thermostable β -xylosidase, exhibiting similar high activities with arylxylose and arylarabinose, was purified to gel electrophoretic homogeneity from the ethanologenic thermophilic anaerobe *Thermoanaerobacter ethanolicus*. The isoelectric point is pH 4.4; the molecular weight is around 165,000 for the native enzyme (gel filtration, gradient polyacrylamide gel electrophoresis) and 85,000 for the two subunits (SDS-PAGE). The enzyme exhibited the highest affinity towards p-NO₂-phenyl xyloside (pNPX) ($K_M = 0.018$ mM at 82°C and pH 5.0), but the highest V_{max} with p-NO₂-phenylarabinopyranoside. $T_{opt, 5min}$, the temperature for the maximal initial activity in a 5 min assay of the purified enzyme was observed around pH 5.9 and 93°C; however at 65 and 82°C, the pH optimum was 5.0-5.2, and at this pH the maximal initial activity was observed at 82°C. The pH curves and temperature curves for arylxylosides as substrates differed significantly from those for arylarabinosides as substrates. An incubation for 3 h at 82°C in the absence of substrate reduced the activity to around 75%. At 86°C the half life was around 15 min. An Arrhenius energy of 69 kJ/mol (Substrate = pNPX) was determined. The NH₂-terminal sequence (34 residues) did not reveal a high similarity to those from other published enzyme sequences.

LI, Y., L. MANDELCO AND J. WIEGEL (1993) Isolation and Characterization of a Moderately Thermophilic Anaerobic Alkaliphile, *Clostridium paradoxum* sp. nov. Int. J. Syst. Bacteriol. 43:(3) (in press)

Alkaliphilic, moderately thermophilic anaerobic bacteria able to grow above pH 10.5 and 55°C, were isolated from various sewage plants in the United States. The strains were motile with 2-6 peritrichous flagella and formed round to slightly oval terminal spores in terminal distended and slightly enlarged cells. Sporulated cells remained motile. The pH range for growth was between 7.0 and 11.1 with the optimum of around 10.1. At pH 10.1 the temperature range for growth was between 30 and 63°C, with an optimum of 56°C. The shortest observed doubling time (glucose) was around 16 min at 56°C and pH 10.1. No dissimilatory sulfate reduction was detected. The organism utilized glucose, fructose, sucrose, maltose, and pyruvate but required yeast extract or tryptone for growth. Optimal NaCl concentrations for growth were between 50 and 200mM. The guanine-plus-cytosine content was 30.0 ± 0.10 mol%. Based on the unique properties and 16S rRNA analysis, the strains are placed in a new species, *Clostridium paradoxum*, referring to its unusual retainment of motility by sporulated cells. Strain JW-YL-7 is designated as type strain.

Peteranderl, R. F. Canganella, A. Holzenburg and J. Wiegel (1993) Induction and regeneration of autoplasts from *Clostridium thermohydrosulfuricum* JW102 and *Thermoanaerobacter ethanolicus* JW 200. Appl. Environ. Microbiol. (submitted)

Autolysis was induced to form stable, cell wall-free cells of *Clostridium thermohydrosulfuricum* strain JW 102 and *Thermoanaerobacter ethanolicus* strain JW 200. For this, the bacteria were grown in a complex medium containing glycine (0.4% w/v), sucrose (10% w/v), or a combination of both. The optimum temperature to induce autolysis of C.

thermohydrosulfuricum was 64°C. Autoplasts of both bacteria were grown as L-phase colonies on solid medium; more than 50% of these colonies regenerated spontaneously to the walled form. For the autoplasts of *C. thermohydrosulfuricum* the removal of the cell wall was confirmed by electron microscopic examination using ruthenium red as a specific stain for the bacterial cell walls. (This was an important first step for us in the development of a genetic system for these organisms. Apparently due to the outer protein layer of these organisms, the uptake of DNA was hindered).

C. PROJECT DESCRIPTION.

This contains 2 dissimilar projects and thus, they are described separately under C.1. and C.2.

C.1. TITLE: The hemicellulases from *Thermoanaerobacter ethanolicus* and similar anaerobic thermophiles

SPECIFIC OBJECTIVES: The ULTIMATE GOAL is to obtain a thermophilic anaerobic bacterium that efficiently converts hemicellulose-containing biomass to ethanol. The strategy to reach this goal is to genetically modify the formation and regulation of the rate-limiting hemicellulases in the ethanologenic, thermophilic, anaerobe *Thermoanaerobacter ethanolicus* JW200. This requires knowledge of the involved hemicellulases. Thus, for this application the SHORT TERM GOALS are :

- 1) to complete the purification and biochemical characterization of enzymes involved in the efficient hydrolysis of hemicellulosic material to monomeric sugars. The enzymes and their main sources are:
 - * xylanases working in the pH-range from 4.5 to 9.0 from the slightly alkaliphilic strain FPP100; Strain NDF 190; and the slightly acidophilic *Thermoanaerobacterium* spec. JW/SL-YS485.
 - * xylosidases and arabinosidases as single and as bifunctional enzymes (Shao and Wiegel, 1992) {*Thermoanaerobacter ethanolicus* JW200, *Thermoanaerobacterium* spec. JW/SL-YS485}
 - * O-methyl glucuronidase {*Thermoanaerobacterium* spec. JW/SL-YS485, the only isolate with high activities for this reaction}
 - * acetyl(xylan) esterases {*Thermoanaerobacterium* spec. JW/SL-YS485, which has the highest deacetylating activity}
- 2) to identify and isolate specific genes encoding hemicellulolytic enzymes in *T. ethanolicus*, and the above identified anaerobic thermophiles. Sequence homology analysis with known DNA and protein sequence data obtained from similar genes of bacterial and fungal origin will allow determination of any existing evolutionary relationship and, possibly, identification of motifs necessary for the function of these enzymes. This is of particular interest for the bifunctional xylosidase/arabino(xylan)sidase purified from *T. ethanolicus* JW200.

A. PROGRESS REPORT - Harry D. Peck Jr.

Microbiology and Physiology of Anaerobic Fermentations of Cellulose (DE-FG09-86ER 13614)

Harry D. Peck, Jr., Neeraj Datta and Paul King

In the period from 1989 to the present, our knowledge of the molecular biology, biochemistry and physiology of hydrogenases has been greatly expanded. Until 1991, our group and others directed efforts at the characterization and comparison of over 25 cloned hydrogenase genes. The extensive differences between these hydrogenases was initially stressed, but it has become apparent that even though total amino acid homologies are often as low as 30% between hydrogenases, there are two basic gene families, the [Fe]-only and [NiFe] hydrogenases (First cloned by our group, Menon et. al., 1990) of which the later is by far the most common in nature. The [NiFe] enzymes can be dimeric or multimeric, can reduce F420 or NAD⁺, and in apparently rare cases can contain selenocysteine encoded by TGA (first shown by our group with G. Voordouw, 1989).

Comparison of numerous [NiFe] hydrogenases (our group has cloned at least 10) revealed that several domains are highly conserved in all enzymes of this group. In particular, the sequences R-X-C-X-X-C beginning at about amino acid 75 and D-P-C-X-X-C at about amino acid 575 of the large subunit are ubiquitous (Li et. al., 1987, Menon et. al., 1990). In the latter manuscript, we proposed that as these domains contained cysteines and the sequences were not present in [Fe]-only hydrogenases, that they represented putative ligands for the active site nickel. We have recently demonstrated (Przybyla et. al., 1992) that Cys₇₅, Cys₇₉, Asp₅₇₄, Cys₅₇₆ and Cys₅₇₉, components of the two conserved domains mentioned above, indeed serve as nickel ligands in *E. coli* HYD1. These assignments are consistent with EPR and EXAFS spectroscopy results.

All [NiFe] hydrogenases also contain at least one subunit in addition to the large subunit with a number of cysteinyl residues capable of serving as iron-sulfur ligands. In related organisms such as *E. coli*, *B. japonicum* and *R. capsulatus*, up to 11 cysteines are spatially conserved throughout the small subunit also suggesting they are cluster ligands. Interestingly, the total number of cysteines in this subunit is not sufficient to ligand all xFeS clusters detected by physical techniques. Thus at least one of these clusters must be associated with the large subunit or "straddle" the two subunits. Our group (Menon et. al., 1993) and others have shown that these cysteines are essential for activity, but they have yet to be assigned specific clusters, nor has the function of individual clusters been elucidated.

Our group has also cloned and sequenced the *E. coli* operon (hyb) for HYD2, a [NiFe] enzyme required for growth on fumarate and H₂. The 35kD (small) subunit of this protein contains cysteines to ligand four 4Fe4S clusters and although their structure is not at all homologous to the cysteines in HYD1, at least three are similar to 4Fe4S ligands found in ferredoxins. This general pattern holds true for F420 and NAD⁺ reducing enzymes and suggests that the small subunits, and thus the non-heme iron clusters, do not participate directly in the activation of H₂, but rather channel electron to or away from the active site. The substrate specificity of hydrogenases is thus most likely determined by the small, iron-sulfur cluster liganding subunit. Additionally, at least one of the clusters in *E. coli* HYD1 may serve a structural

function by using ligands from both the large and small subunits. We are currently finishing a manuscript describing these findings.

Since it was first realized that hydrogenases were encoded in most prokaryotes in operons (Menon et. al., 1990) that are now known to consist of between 6 and 20 genes, it has become clear that the biosynthesis of the enzyme is a very complex process. This process involves synthesis of native subunits, insertion of nickel into the active site, carboxy-terminal cleavage of the large subunit resulting in the tight and relatively stable liganding of nickel in the active site and subcellular localization of the nascent enzyme complex which contains Ni and x(XFexS) clusters in and/or around the cell membrane or periplasmic space. Mutagenesis has shown that the additional "accessory" genes found in hydrogenase operons are essential to these processes and that they are very specific for their "own" nascent hydrogenases precluding expression of active hydrogenase in heterologous cells. It has not been possible to decipher the sequence of events in the processing procedure. It is accepted for example, that carboxy-terminal cleavage of the large subunit does not occur unless nickel is associated with the nascent subunit. Thus mutants deficient in the uptake or mobilization of nickel are unable to process the large precursor. Yet it is interesting that all hydrogenase found in the cytoplasm is unprocessed and does not contain bound nickel. It thus appears that the binding of nickel initiates cleavage and simultaneously "traps" the nickel in the active site as a result of the cleavage. But hydrogenase with nickel in the active site is only found tightly associated with the cell membrane. These data make it impossible so far to dissect the sequence of events which take place during processing and subcellular localization. One can only suggest that the processes occur simultaneously.

The apparent species-specificity of active site maturation and cellular localization have resulted in one of the most disappointing failures of the last five years. It was originally hoped that the role of hydrogenases and other electron carriers in the physiology of anaerobic bacteria belonging to the consortia of cellulose fermenting organisms, would be better understood by analyzing their biosynthesis in *E. coli*, a more manageable genetic system. Our findings and those of others have demonstrated that the use of *E. coli* as a host for these studies is very limited and further progress on the physiology of the hydrogenases of the SRB and other anaerobes awaits the refinement of homologous genetic systems.

We have begun to develop a genetic system for the SRB as have others in the field. We and others have shown that plasmids with IncQ replicons are replicated in the SRBs. Unfortunately, direct transformation of these prokaryotes has not been obtained and conjugation with *E. coli* is still necessary. We have successfully used *E. coli* S17-1 which contains chromosomally integrated mobilization functions to transfer plasmids to several SRBs, predominantly *D. vulgaris* at this point as it has a very high plating efficiency. The plasmid pSUP104Ap¹ (Berg et. al., 1989) was used for our original studies but in our hands transformants lost their resistance to ampicillin within two generations suggesting that the vectors were not maintained in the cells. We also experienced considerable contamination even though all transformations were done under anaerobic conditions. As *D. vulgaris* was sensitive to only high levels of ampicillin, we modified the vector by replacing this resistance with a tetracyclin resistance gene (pSUP104Tet¹) since *D. vulgaris* is sensitive to extremely low (1 µg/ml) levels of the drug. To overcome the contamination problems, we have isolated nalidixic acid resistant (nal^r) mutants of *D. vulgaris* which grow at wild type rates on plates or liquid media containing

naladixic acids at concentrations nonpermissive to the conjugation donor, *E. coli*. Using standardized anaerobic conjugation procedures and pSUP104Tet^r, we have transformed *D. vulgaris* and other SRBs to tetracycline resistance which is stably maintained in liquid or on plates.

We have inserted the *D. vulgaris* [Fe]-only hydrogenase gene, the *D. gigas* ferredoxin gene and the *D. africanus* ferredoxinIII gene into pSUP104Tet^r both with and without the homologous promoters. Tests are currently underway to determine if these genes are expressed in *D. vulgaris* after conjugation and to what extent they are expressed.

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Li, C., H.D. Peck, Jr., J. LeGall and A.E. Przybyla. 1987. Cloning, characterization and sequencing of the genes encoding the large and small subunits of the periplasmic (NiFe) hydrogenase of *Desulfovibrio gigas*. DNA 6:344-352.

Menon, N.K., J. Robbins, H.D. Peck, Jr., C.Y. Chatelus, E.S. Choi and A.E. Przybyla. 1990. Cloning and sequencing of six genes in the (NiFe) hydrogenase-1 operon of *Escherichia coli*. J. Bacteriol. 172:1969-1977.

Van den Berg, W.A.M., P.W.G. Stokkermans and W.M.W.M. van Dongen. 1989. Development of a plasmid transfer system for the anaerobic sulphate reducer *Desulfovibrio vulgaris*. J. of Biotech. 12:173-184.

Voordouw, G., N.K. Menon, J. LeGall, E.S. Choi, H.D. Peck, Jr., and A.E. Przybyla. 1989. Analysis and Comparison of the nucleotide sequences encoding (NiFe) and (NiFeSe) hydrogenases from *Desulfovibrio gigas* and *Desulfovibrio baculatus*. J. Bacteriol. 171:2894-2899.

B. SUMMARY OF 5 PERTINENT PUBLICATIONS - Harry D. Peck, Jr.

Voordouw, G.,H.D. Peck, Jr. and A.E. Przybyla. 1989. Analysis and comparison of the nucleotide sequences encoding (NiFe) and (NiFeSe) hydrogenases from *D. gigas* and *D. baculatus*. J. Bacteriol. 171:2894-2899.

This manuscript was the first comparison of the (NiFe) and (NiFeSe) hydrogenases. It demonstrated that both were in the same family structurally and that the difference in redox potential was a result of the selenocysteine ligand in the (NiFeSe) type.

Fauque, G., H.D. Peck, Jr.,J. LeGall. 1989. The three classes of hydrogenases from sulfate-reducing bacteria of the genus *Desulfovibrio*. FEMS Microbiol. Revs. 54:299-344.

For the first time the three types of hydrogenases from the sulfate reducing bacteria were compared with respect to physical properties and molecular biology. The findings reported here concerning the structural subunits of the enzymes have not been added to significantly since that time.

Rhode, M.Peck, H.D., Jr. and N.K. Menon. 1989. Localization of membrane-associated (NiFe) and (NiFeSe) hydrogenases of *Desulfovibrio vulgaris* using immunoelectron microscopic procedures. Eur. J. Biochem. 191:389-396.

This manuscript is important to the hydrogen cycling model as it directly demonstrates that hydrogenase activity can be associated with the cytoplasmic aspect of the cellular membrane. The presence of a cytoplasmic hydrogenase is essential to the model.

Przybyla, A.E.and H.D. Peck, Jr. 1992. Structure/function relationships among the nickel-containing hydrogenases. FEMS Microbiology Revs. 88:109-136.

The data accumulated over several years with physical techniques, especially EXAFS, was used in conjunction with molecular data to identify the amino acid ligands to the active site nickel of *E. coli* hydrogenase-1. This is a prime example of how physical studies and molecular biology can complement each other in approaching difficult problems. These studies will be repeated and verified by many groups over the next several years.

Peck, H.D., Jr. 1993. Bioenergetic Strategies of the Sulfate Reducing Bacteria in R. Singleton, Jr. and J.M. Odom (eds.) The Sulfate-Reducing Bacteria - Contemporary Perspectives. Brock/Springer Series in Contemporary Bioscience, pp. 41-76.

This manuscript summarizes what is currently known about sulfate metabolism in *Desulfovibrio* and includes an extensive and comprehensive reference section.

C. PROJECT DESCRIPTION - Harry D. Peck, Jr.

Physiology of sulfate reducing bacteria

H.D. Peck, Jr., N. Datta and P. King

SPECIFIC AIMS

- 1) Construction and characterization of a plasmid vector for site-specific mutation of genes from sulfate reducing bacteria (SRB) by site-specific integration of *in vitro* mutagenized genes. This project is in progress and will continue for the first year or two of this proposal.
- 2) Refinement of plasmid vectors to be used for the over expression of SRB genes in homologous cells or other closely related SRB. This project is in progress and will continue throughout this proposal.
- 3) Determination of the physiological role of currently cloned SRB genes through site directed mutagenesis. This will begin as soon as the vector described in 1) is completed.
- 4) Analysis of the biochemistry of electron transport proteins by expression of proteins altered by site-directed mutagenesis in homologous or related SRBs.

A. Progress Report

L.E. Mortenson

Hydrogenases

Clostridium pasteurianum

In the previous grant periods we were concentrating on obtaining more information on the two hydrogenases of *Clostridium pasteurianum*. There now appear to be five iron sulfur centers in the bidirectional hydrogenase. The spectroscopic data is consistent with the presence of 4 $[4Fe_4S]^+$ Ferredoxin-like clusters with one of the clusters exhibiting an $s = 1/2$ ground state in addition to $s = 3/2$ and another center, called the H center, which all data indicates is the site of hydrogenase that reacts with molecular hydrogen. The uptake hydrogenase in addition to an H center with some properties like that of the bidirectional H center, has two Ferredoxin-like centers, one with a midpoint potential similar to low potential ferredoxins and the other with an unusual potential around -180mV. The current mechanistic models for the hydrogenases are seen in the accompanying figures (1 and 2).

In summary, the H center of each hydrogenase reacts with and activates molecular hydrogen. In the case of the bidirectional hydrogenase the electrons from molecular hydrogen are transferred to the F centers and it is only from the F centers that electrons can be transferred to external electron carriers such as Ferredoxin. Since the mid-point of the F centers is close to that of the H center, the reaction, as predicted, is freely reversible. In the uptake hydrogenase however, the F centers have potentials much more positive than the H center and can only reduce external carriers of comparable potential (-180mV). Electrons from more reduced carriers (-400mV), although capable of reducing F centers, are not easily transferred from the F centers to the H center because of the large potential difference (-180mV vs. -420mV). It is proposed that the structural constraints preventing the H center from contact with the external environment is what makes this an uptake hydrogenase. This model is consistent with the apparent K_m (H_2) of 30 micromolar for the uptake and 300 micromolar for the bidirectional hydrogenase.

The sequence of the bidirectional hydrogenase of *C.p.* has been obtained and is consistent with the centers predicted from the biophysical analyses. Unfortunately, a genetic system to allow us to modify the *C.p.* hydrogenases and determine the effects of the modifications on their properties is not available. The lack of this powerful tool to analyze the clostridial system convinced us to change emphasis from studying *C.p.* hydrogenases to studying the hydrogenase of *Azotobacter vinelandii*, an organism where a facile genetic system does exist.

Azotobacter vinelandii

Over the past 3 years, we have made substantial progress with the *Azotobacter* system and the results are summarized in a diagram showing the genes of *A.v.* that are required for an active cellular hydrogenase (Fig. 3). and a table showing the relationship among the hydrogenase genes of the *A.v.* genes and the hydrogenase genes of three other organisms (Table 1). One can easily see that there is high sequence homology among the genes of the four hydrogenases, *A.v.*, *E. coli*, *A. eutrophus*, and *R. capulatus*. Putative functions of these genes based on the phenotypes of mutations in the genes and on comparisons with sequences of other genes and their transcribed/translated protein products are also given in Table 1. The structural genes for the alpha and beta subunits (ORF1 and 2) are established. ORF3 appears to be a b type cytochrome thought to be an electron carrier associated with hydrogenase function. There is considerable evidence suggesting that the other genes (ORF4 - ORF16) are involved in a) processing hydrogenase, b) in the electron transport pathway coupled to hydrogenase and c) in synthesizing the Ni and iron sulfur centers required for hydrogenase to function.

We have already inserted two of the genes, ORFs 12 and 13, onto *E. coli* behind a strong promoter and such cells produce both proteins at concentrations estimated from disc gels to be 10 - 25% of the total protein. These two proteins, containing unusually high concentrations of histidine (see accompanying publications), will be isolated and their ability to bind Ni will be ascertained under a variety of conditions. The ultimate goal is to determine the specific role of each of the hydrogenase genes in the synthesis and function of hydrogenase in *A.v.* Within these goals is the goal of obtaining an *in vitro* system to assemble active *A.v.* hydrogenase from apoprotein, Ni, Fe and sulfide using other supporting substrates (probably MgATP), the Ni processing proteins, and the components concerned with FeS insertion.

Three publications from this work are included with this report. Others are in preparation.

Crystallography

We have obtained good diffracting crystals of the *C.p.* bidirectional hydrogenase and Jeff Bolin and his group at Purdue University have already obtained both native and heavy metal derivative data on the crystals. We are optimistic that a preliminary 3D structure will come from the data we are now processing.

- B. SUMMARY OF 5 PERTINENT PUBLICATIONS - (Not Applicable)
- C. PROJECT DESCRIPTION - (Not Applicable)

Fig. 1

Fig. 2

Jack C. Chen and Leonard E. Mortenson (1992). Two open reading frames (ORFs identified near the hydrogenase structural genes in *Azotobacter vinelandii*, the first ORF may encode for a polypeptide similar to rubredoxins. *Biochimica et Biophysica Acta*. 1131:122-124.

Jack C. Chen and Leonard E. Mortenson (1992). Identification of six open reading frames from a region of the *Azotobacter vinelandii* genome likely involved in dihydrogen metabolism. *Biochimica et Biophysica Acta*. 1131:199-202.

Angeli L. Menon, Leonard E. Mortenson, and Robert L. Robson (1992). Nucleotide Sequences and Genetic Analysis of Hydrogen Oxidation (*hox*) Genes in *Azotobacter vinelandii*. *J. Bacteriol.* 174:p.4549-4557.

THESE PUBLICATIONS WERE SUPPORTED BY DOE GRANT #DE-FG09-86ER13514.

Fig. 3

Table 1

SEQUENCE ANALYSIS OF PREDICTED *Av* hox GENE/ORF PRODUCTS

ORF	gene	aa	mol. wt. (Da)	pI	Identity of hydrogenase genes from			function/similarity
					<i>E. coli</i>	<i>A. eutrophus</i>	<i>R. capsulatus</i>	
ORF 1	hox K	358	39,259	8.05	hya A (68%)	hox K (83%)	hup S (79%)	β subunit
ORF 2	hox G	602	66,736	6.62	hya B (60%)	hox G (74%)	hup L (72%)	α subunit
ORF 3	hox Z	204	27,738	9.12	hya C (50%)	hox Z (61%)	orf X (59%)	membrane protein?
ORF 4	hox M	207	22,654	4.58	hya D (43%)	hox M (56%)		} processing?
ORF 5	hox L	106	11,335	4.70	hyb G (31%) hya C (31%)	hox L (38%)		
ORF 6	hox Q	148	16,165	4.36	hya E (24%)	hox Q (37%)		} processing?
ORF 7	hox Q	283	30,866	4.88	hya E (32%)	hox Q (42%)		
ORF 8	hox R	72	8,054	4.36		hox R (74%)		- rubredoxin?
ORF 9	hox I	183	16,342	6.12		hox I (41%)	(hup ORF 1) (30%)	
ORF 10	----	348	36,929	7.38			hup ORF 2 (33%)	} Ni binding?
ORF 11	----	113	12,610	4.75	hya A (35%)		hup ORF 3 (52%)	
ORF 12	----	303	33,186	6.73	hya B (64%)		hup ORF 4 (59%)	} processing?
ORF 13	----	776	82,714	7.81				
ORF 14	----	84	8,983	4.28	hyb G (33%) hya C (31%)			} processing?
(ORF 15)	----	>296	>32,246	----	hya D (48%)			