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Hemicellulases from Anaerobic Thermophiles

Progress Report

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Hemicellulases from Anaerobic Thermophiles

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The longterm goal of this research effort is to obtain an anaerobic thermophilic bacterium that efficiently converts various hemicellulose-containing biomass to ethanol over a broad pH range. The strategy is to modify the outfit and regulation of the rate-limiting xylanases, glycosidases and xylan esterases in the ethanologenic, anaerobic thermophile *Thermoanaerobacter ethanolicus*, which grows between pH 4.5 and 9.5. Although it utilizes xylans, the xylanase, acetyl(xylan) esterase and O-methylglucuronidase activities in *T. ethanolicus* are barely measurable and regarded as the rate limiting steps in its xylan utilization. Thus, and also due to the presently limited knowledge of hemicellulases in anaerobic thermophiles, we characterize the hemicellulolytic enzymes from this and other anaerobic thermophiles as enzyme donors. Beside the active xylosidase/arabinosidase from *T. ethanolicus*, exhibiting the two different activities, we characterized 2 xylosidases, two acetyl(xylan) esterases, and an O-methyl glucuronidase from *Thermoanaerobacterium spec.* We will continue with the characterization of xylanases from novel isolated slightly acidophilic, neutrophilic and slightly alkalophilic thermophiles. We have cloned, subcloned and partially sequenced the 165 000 Da (2 x 85 000) xylosidase/arabinosidase from *T. ethanolicus* and started with the cloning of the esterases from *Thermoanaerobacterium spec.* Consequently, we will develop a shuttle vector and continue to apply electroporation of autoplasts as a method for cloning into *T. ethanolicus*.

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The overall goal of this project is to characterize microbiologically and biochemically members of the novel group of organisms, the alkalithermophilic anaerobes.

1) **Characterization of new isolates:** We have completed and published the characterization of the second novel anaerobic alkalithermophile, *Clostridium thermoalcaliphilum* s. nov. (L et al., IJSB 44:111-118, 1994). We further have continued with the identification and characterization of about 200 strains of alkaliphilic and alkalitolerant isolates. Since Sept. 94 we have in cooperation with Fred Rainey (DSM-Braunschweig, Germany; started during a three-week stay in Germany), so far we obtained 12 full 16S rRNA sequences for members of this group. We have consequently identified two novel alkalitolerant genera exhibiting a branched cell morphology: '*Anaerobranca horikoshii*', gen. nov., sp. nov. and '*Thermorhizacus celer*' gen. nov., sp. nov. In contrast to the alkaliphilic strains (T_{max} 58 - 63°C), these alkalitolerant strains have their pH_{opt} below pH 9.0 but some strains can grow up to 73°C. We are in the process of completing their descriptions and preparation of manuscripts for publication. One of the interesting features of the latter organism is that some of the strains exhibit a fast doubling time. Thus, we have analyzed the growth rate in a pH-controlled semi-continuous batch fermentor to obtain pH curves under controlled conditions. The shortest doubling time obtained so far is 12 min over 3-4 doublings at pH 8.0 (alkalitolerant) and 60°C. This is close to the fastest doubling time ever determined for an aerobic organisms (10 min) and it is the fastest doubling time for any anaerobic mesophile or thermophile. Unfortunately, during the 1/2 year of constant transfers in the lab, the originally obtained pH_{max} of 10.5 is reduced to about pH 9.5-10.0, however, the initial marginal growth ($OD < 0.2$) has changed to growth up to nearly 1 OD unit in the fermentor. (There are no indications of contaminations, other properties are the same).

2) **Gene probe for sporulation:** *Clostridium thermoalcaliphilum* has similar pH and temperature characteristics to the previously described *C. paradoxum*. The 16S rRNA sequence analysis places this organism close (2% distant) to *C. paradoxum*. However, the cell wall type of these two organisms differs significantly (m-DAP type versus L-Orn-D-Asp type), justifying their placement into two different species. In contrast to the easily sporulating *C. paradoxum* (up to 95% of the cells), *C. thermoalcaliphilum* does not form spores under any tested conditions. After reaching the stationary growth stage, it starts to lyse and the culture becomes non-viable, i.e., it can no longer be subcultured regardless whether or not heat treated for activating germination of presumptive spores. This problem of inability to demonstrate sporulation by some organisms exhibiting otherwise very similar physiological characteristics to sporulating counterparts is an old one, especially in the taxonomy of thermophilic anaerobes, and has lead to confusion in the past. To resolve this problem, we started to develop a set of DNA-based probes for specific genes involved in sporulation. Identifying the presence of various sporulation genes should help to clarify whether the organisms are truly asporogenic, (i.e., lack sporulation genes), or are just blocked in one or more steps of sporulation and thus may be called cryptic sporeformers, (i.e., are nonsporulating due to lacking an active gene product of any of the many sporulation genes (Wiegel, 1992)). We have identified three different types of suitable genes

which cover quite different aspects of sporulation, as well as specific sequence stretches between 25 and 80 bases suitable for obtaining probes (according to computer analysis of the available sequences). The gene products are the small acid-soluble protein, a spore-specific protein, the sporulation specific sigma E factor (and possibly sigma G factor), and the dipicolinic acid synthase, a unique enzyme synthesizing the major spore coat component dipicolinic acid, which is involved, e.g., in the heat resistance of spores. We are in the process of synthesizing labelled probes; next month we will start with testing the probes for their specificity and reliability in our assay, using known sporulating and nonsporulating test organisms. Consequently, we will test *C. thermoalcaliphilum* and similar organisms (e.g., *T. ethanolicus*) for which no sporulation has been observed, but which otherwise cluster physiologically or in a matrix based on 16S rRNA sequences with the sporulating organism.

3) Testing for useful enzymes: The screening for alkali- and temperature-resistant hemicellulolytic xylanases and EDTA-resistant alkalithermophilic proteases has not yielded, so far, enzymes with optimal activities above pH 9.5 and temperatures above 70°C. Thus we have started enrichments specifically for this purpose.

4) Antibiotics: stability and MIC determination: Furthermore, we are testing the stability and thus usefulness of various antibiotics for developing a genetic system in our alkaliphiles. Using the method previously developed (Peterandel et al., 1990) we determined the stability of the antibiotics under the growth conditions of our alkalithermophiles (pH 9.0 and 11.0 and at 50 and 66°C). As one would expect, some of the antibiotics (e.g., Penicillin G, Streptomycin, Tetracycline) are inactivated rapidly with increasing temperature and pH, and others (e.g., Neomycin, Gentamycin) are fairly stable at all tested conditions, whereas others (e.g., Vancomycin) exhibit an unusual stability, in that they are more quickly inactivated at the lower pH than at the higher pH. We will next determine the MIC values for some of our alkaliphilic strains.

5) Biochemistry: Due to a sudden withdrawal (for personal reasons) of the post-doctoral candidate selected for this project, we have yet to do many of the biochemical tasks. I have now identified another person starting next month. We have noticed during cell wall analysis, that the cell walls of these thermophilic alkalitolerant strains behave differently than other gram-type positive organisms, and thus, do not allow a proper analysis. At this time, we do not know the reason for the unusual behaviour in the analysis process. We are continuing with the analysis using differently grown cells.

Publications and Reports since start of the Project:

INVITED SEMINARS:

- 1) Extremophiles from not so extreme environments (in German) 3. Göttinger Mikrobiologen Forum. University of Göttingen, Germany October 1993 (in honor of Prof. H.G. Schlegel; invited plenum talk).
- 2) Anaerobic degradation of PCB. University of Bergen, Norway, October 1993 (invited seminar).
- 3) Alkalithermophiles, a novel group of extremophiles. University of Bergen, Norway October 1993 (invited seminar).
- 4) Alkalithermophiles. Deutsche Sammlung für Microorganismen (DSM) Braunschweig (Germany), October 1993 (invited seminar).
- 5) Participating in the US-New Zealand Workshop "Extreme thermophiles", December 1993 (invited participant).

ABSTRACTS:

- 1) M. Engle, Y. Li, and J. Wiegel. A novel group of anaerobic (eu)bacteria: alkalitolerant and alkaliphilic thermophiles isolated from various mesobiotic and thermobiotic environments. Thermophiles '93. Intern. Meeting in Hamilton, NZ, December 1993 (invited plenum talk; Ext. Abstr. A10).
- 2) M. Engle, Y. Li, and J. Wiegel. *Clostridium thermoalcaliphilum*, a novel anaerobic alkaliphilic thermophilic isolated from mesobiotic sewage sludge. Thermophiles '93. Intern. Meeting in Hamilton, NZ, December 1993. Ext. Abstr. P43.

ORIGINAL PUBLICATION (Peer-reviewed)

- 1) Y. Li, M. Engle, L. Mandelco and J. Wiegel. 1994. *Clostridium thermoalcaliphilum* sp. nov., an anaerobic and thermotolerant facultative alkaliphile. Int. J. Syst. Bacteriol. 44:111-118.

Experiments planned for the next 12 months:

We will continue

- to prepare the publications for the description of the two new organisms and characterize other new alkaliphilic/alkalitolerant thermophilic isolates;
- to test the synthesized sporulation gene probes for specificity and practical use;
- to determine the MIC for various antibiotics for the 4 different alkaliphilic/alkalitolerant thermophiles and
- screen for extracellular alkalistable thermostable hydrolytic enzymes

With the arrival of the new postdoctoral associate (next month) and a new graduate student in Fall, we will start with the planned biochemical analyses of cell wall components, pH homeostasis and their basic energy metabolism, especially investigating the presence and, if found, the importance of Na^+/H^+ antiport and Na^+ /solute symport of sugars and amino acids as outlined in the Grant application. Furthermore we will start the investigation on the presence of specific phages and plasmids in the alkalithermophiles, especially in *C. paradoxum* and *C. thermoalcaliphilum* strains.

Update on DOE-(DE-FG09-89ER-14059) Research Grant Report

~~The progress report and the grant application for the competitive renewal was sent~~
in May 1994. This is a short summary of additional research accomplishments since May. We think we have made significant progress on our molecular tasks and feel we have now overcome our initial learning period and therefore expect further good progress in the next year.

Ad TASK 1. Purification and characterization of hemicellulolytic enzymes:

XYLANASES: LOCALIZATION in strain JW/SL-YS485: In the May 94 report, we described the isolation and first characterization of an unusually large cell-associated xylanase from *Thermoanaerobacterium* JW/SL-YS485 (*this strain is related to T. saccharolyticum based on 16S rRNA sequence analysis done in the meantime. Two papers on the systematic of Thermoanaerobacterium species and description of new strains and new species are in preparation. Because strain JW/SL-YS485 needed to be classified, this taxonomic study was in part paid from this grant*). At the time the grant application and the manuscript (Appendix 3 of Grant Application) were submitted, we did not know where the xylanase was located (i.e., outside or inside the cells). We have now determined that the cell-associated xylanase activity is located with the surface protein layer (S-layer) fraction on the outside of the cells. The enzyme is released into the medium at late exponential and stationary growth phase. H. Bahl and coworkers in Germany developed the hypothesis that their pullulanase (from *T. thermosulfurigenes* EM1) is specifically anchored in the S-layer. We adopted this possibility for the xylanase in our organism. Besides the above localization data, this theory is also supported by finding conserved binding motifs in the obtained sequence of the gene for our xylanase (see below).

Strain JW/SD-ND190 XYLANASE: We are still in progress with the characterization of the more alkaline xylanase. (We are still unable to stain the enzyme protein and are now obtaining larger quantities of the putative purified enzyme for the full characterization). We also have started to clone this enzyme using the same methods we had employed successfully for the enzyme from strain JW/SL-485.

XYLOSE ISOMERASE from strain JW/SL-YS489: Purification, characterization, and sequence of the gene: (*Only partly supported by funds from this grant during the process of learning molecular techniques in my lab*) In connection with the xylan utilization of anaerobic thermophiles, especially in strains of *Thermoanaerobacter* and *Thermoanaerobacterium* we have purified and characterized an unusual, acidic xylose isomerase from strain JW/SL-YS489 (*Strains JW/SL-YS485 and JW/SL-YS489 exhibit around 97% homology on the 16S-RNA level*). Whereas, all previously reported isolated xylose isomerases have a pH optimum above pH 7.0, this enzyme has a pH optimum around 6.5 (pH 6.4 at 60°C and pH 6.6 at 80°C; temperature optimum 80-85°C). We have cloned and sequenced this gene and have started a detailed comparison with other xylose(glucose) isomerases. We have identified 10 amino acids that could be responsible for the different pH optimum and for activity down to below pH 4.5. We will seek additional funds to do site-directed mutagenesis and crystal structure analysis for this enzyme.

PUBLICATIONS: The three papers (on enzyme characterization of the purified xylanase, α -glucuronidase, and 2 acetyl(xylan)esterases) have been returned, with only minor corrections, to the ASM journal last week and we are confident that they will be published in the beginning of 1995 in Appl. Environ. Microbiol. The MS on enzyme regulation has been modified before it was submitted to Biotechnol. Letters. The paper on the comparison of the purified xylosidases is still in preparation.

Ad TASK 2: Cloning the genes for the purified hemicellulolytic enzymes into *E. coli*:

a) *Thermoanaerobacterium* JW/SL-YS485; XYLANASE: Based on homologous regions in the sequence of the xylanase genes of *Thermoanaerobacterium thermosulfurigenes* EM1, *T. saccharolyticum* B6A-RI, and *C. thermocellum*, we constructed two 18-bp oligonucleotides. The first probe is from a region coding for part of the presumptive xylan binding site and which is located in the N-terminal end of the enzyme. The second probe is from a sequence coding for the C-terminal region. The two probes were used as PCR primers. Using the procedures of Innis and Gelfand (1990) and the chromosomal DNA from *Thermoanaerobacterium* JW/SL-YS485, we obtained a 3800-bp PCR-product. This product was subsequently used as probe to obtain a 4.5-kb *Eco*RI fragment by Southern hybridization; the fragment in subsequent pUC19 clones yielded xylanase activity. The protein exhibited the expected size and thermostability. To our knowledge this is the largest prokaryotic xylanase described so far. We are presently characterizing further the thermostable recombinant enzyme to compare its characteristic properties with those of the purified native enzyme. Subcloning was performed and the gene was sequenced in both directions with M13 forward and reverse, yielding an open reading frame of around 4.0 kb. A putative ribosome binding site (AGGGAGG) and a leader sequence believed to code for a 32-amino acid signal peptide have been identified. Even more exciting, we found in the C-terminal region repeated sequence motifs which are believed to be homologous to conserved sequence motifs in known genes coding for S-layer proteins (Sleytr et al.). These regions code for protein regions in the S-layer subunits which are assumed to be involved in the protein-protein interactions during the self-assembly of the subunits. This observation correlates with our finding (see above) that the xylanase is associated with the S-layer. Thus, **FURTHER EXPERIMENTS are IN PROGRESS AND PLANNED:** Our hypothesis is that the extracellular but cell-associated xylanase from our strain is anchored in the S-layer via the analogous regions (near the C-terminal) to the S-layer subunits. We are now starting to prove this hypothesis further by isolating dissociated S-layer subunits from glucose-grown cells (cells without measurable xylanase activity) and then allow them to reassemble (using a procedure from Sleytr et al. 1988, 1994) in the presence and absence of the isolated native and recombinant xylanase, respectively. We will then analyze the isolated polymers for activity. If the enzyme is incorporated in to the reassociated S-layer matrix to a significant degree, we will do electron microscopy on the self assembled subunit sheets and isolated S-layers (done according to procedures from Sleytr and coworkers) using mouse-derived antibodies to the recombinant enzyme. Finally, we will study the incorporation (or the failure of incorporation) of enzymes truncated at the C-terminal (lacking the binding sites using specific restriction enzymes to cut out the regions with the S-layer analog motifs and/or enzymes modified by site directed mutagenesis in the repeated motives. The group in Germany is presently trying to clone the genes for the S-layer proteins from *T. thermosulfurigenes* EM1. If their and our hypothesis about the anchoring of extracellular hydrolytic enzymes is true, this mechanism represents then a novel principle which is apparently used, at least, by *Thermoanaerobacterium* species to maintain their extracellular enzyme in the vicinity of the cells. This mechanism used by these non-cellulolytic but starch and xylan-hydrolyzing organisms is totally different from the cellulosome principle described for *C. thermocellum* and other cellulolytic anaerobes.

We have submitted an Abstract for a presentation on the sequence and on the hypothesis at the Ann. Meet. of Am. Soc. for Microbiology in Washington DC in May 1995.

β -D-XYLOSIDASE: We have isolated and sequenced a full-length clone (pXYLO-1) from an expression plasmid library made from Hind III digested genomic DNA. The obtained sequence is very similar (>90% identity) to the recently published sequence from *Thermoanaerobacterium saccharolyticum* 6BA-RI (Lee et al. 1993). However, the N-terminal sequence predicted from our DNA sequence does not match the N-terminal sequences obtained for either of the purified β -D-

xylosidases I and II; therefore, we are presently reanalyzing the N-terminal from the isolated enzymes (a reason why the MS on the xylosidases has not been completed yet). The cloned enzyme could theoretically represent a third xylosidase. The presence of three xylosidases, however, is uncommon in anaerobic bacteria. We have further obtained three independent clones which have a high activity on 4-MU-xyloside-containing plates as well as some activity on α -L-arabinopyranoside plates. These clones do not react with probes made from the obtained sequence. Thus, we can assume that these other clones containing the genes for xylosidase I and/or II and we are in the process obtaining the sequences for two these different clones.

ACETYL(XYLAN) ESTERASE: The instability of 4-MU-acetylpyranoside precluded the use of the same method for obtaining expression clones for this enzyme. Using a filter overlay protocol we obtained five putative acetyl (xylan) esterase clones. We are awaiting further sequence results for tryptic peptides made from the native in order enzyme to synthesize corresponding oligonucleotides. These probes will be used to eliminate false putative positives. They also can be used, if necessary, to screen the plasmid library by filter hybridization in the case that all putative clones are false positives.

O-METHYL- α -GLUCURONIDASE: The corresponding indicator substrates pNP- or 4-MU- α -glucuronisides could not be synthesized at a reasonable price. Thus, we have started to obtain internal peptide sequences (awaiting sequence analysis results) to make further oligonucleotides to screen both the plasmid and phage libraries by PCR or oligonucleotide hybridization.

b) *Thermoanaerobacter ethanolicus* JW200; β -D-XYLOSIDASE / α -L-ARABINOSIDASE: We have continued our work on this novel bifunctional enzyme. The full-length gene is located in a 3.5 kb fraction of the genomic DNA partially digested with *Ssp*I. Subclones will be further sequenced. Furthermore, due to the success with the expression library of the xylosidase from *Thermoanaerobacterium* we are screening shotgun libraries constructed with the 3.5 kb *Ssp*I fraction ligated into pUC18 to obtain enzymes which contain only the xylosidase or the arabinosidase activity in order to prove further the bifunctional nature of this enzyme.

AD TASK 3: To clone into *T. ethanolicus* JW 200

We have further optimized the regeneration of autoplasts from this organism: Growth of autoplasts and regeneration on agar plates now requires only 48 h compared to 5-7 days before. Kanamycin at a concentration of 60 mg/ml inhibited *T. ethanolicus* for at least 72 h and thus can be used as a marker for selection over a period of at least 48 h. We have experimented with the broad-host range cosmid pJRD215 (triparental mating with pRK2013) and plasmid pVE6086, into which we inserted the thermostable kanamycin cassette from plasmid pKD102. However, the negative results suggest that the restriction system in *T. ethanolicus* is too active. Therefore, we will use in methylated plasmid DNA the next experiments. In order to elucidate which methylated system can be used, we will test (using agarose gel electrophoresis) different methyltransferases for protecting lambda DNA incubated with crude extract of *T. ethanolicus* JW200. Without transmethylese treatment, λ DNA is highly fragmented by the restriction system of our organism. We continue to screen our collection of *Thermoanaerobacter* and *Thermoanaerobacterium*-like isolates for suitable plasmids to construct a shuttle vector for our thermophiles if necessary.