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Cellulose Fermentation by Nitrogen-Fixing Anaerobic Bacteria

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SUMMARY

In anaerobic natural environments cellulose is degraded to methane, carbon dioxide and other products by the combined activities of many diverse microorganisms. We are simulating processes occurring in natural environments by constructing biologically-defined, stable, heterogeneous bacterial communities (consortia) that we use as *in vitro* systems for quantitative studies of cellulose degradation under conditions of combined nitrogen deprivation. These studies include the investigation of i) metabolic interactions among members of cellulose-degrading microbial populations, and ii) processes that regulate the activity or biosynthesis of cellulolytic enzymes. In addition, we are studying the sensory mechanisms that, in natural environments, may enable motile cellulolytic bacteria to migrate toward cellulose. This part of our work includes biochemical characterization of the cellobiose chemoreceptor of cellulolytic bacteria. Finally, an important aspect of our research is the investigation of the mechanisms by which multienzyme complexes of anaerobic bacteria catalyze the depolymerization of crystalline cellulose and of other plant cell wall polysaccharides. The research will provide fundamental information on the physiology and ecology of cellulose-fermenting, N_2-fixing bacteria, and on the intricate processes involved in C and N cycling in anaerobic environments. Furthermore, the information will be valuable for the development of practical applications, such as the conversion of plant biomass (e.g., agricultural, forestry and municipal wastes) to automotive fuels such as ethanol.

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I. PROGRESS REPORT

The last progress report was written in June 1990, as part of the competitive renewal proposal submitted to the DOE at that time. The present progress report deals with research carried out from that time up to November 1992.

During the period covered by the present report extensive progress has been made in the following three major areas of investigation:

1. The cellulase system of anaerobic bacteria.
2. The cellobiose chemoreceptor of cellulolytic bacteria.
3. Cellulose fermentation by biologically-defined bacterial consortia.

1. The cellulase system of anaerobic bacteria.

Our recent research in this area has yielded a major finding with regard to the composition of the enzyme system utilized by anaerobic bacteria for the hydrolysis of crystalline cellulose. Previous work had shown that an extracellular multi-protein enzyme complex was responsible for cellulose hydrolysis by anaerobic cellulolytic bacteria. Most of the previous work had been carried out by various investigators who characterized cellulase preparations obtained mainly by means of size exclusion or affinity chromatography methods. During the last year, however, we used ion exchange chromatography (Mono Q column) and electron microscopy to study the cellulase system of *Clostridium papyrosolvens* strain C7, and found that the cellulase system of this anaerobic bacterium consists of at least seven distinct extracellular, high-molecular-weight multiprotein complexes, each with different enzymatic and structural properties. We believe that future research performed by using the same experimental approach will reveal that the cellulase system of other species of anaerobic bacteria has a composition similar to that we found in *C. papyrosolvens* C7. A paper describing the details of our research on the cellulase system of the latter organism is in preparation. Within the space limitation of this report the above-mentioned work with *C. papyrosolvens* is summarized as follows.

The molecular weight of the seven complexes, as determined by size exclusion chromatography (Sephacryl S-300 column) ranged from 500,000 to 650,000, and all the complexes showed high affinity for cellulose, except for one which only partially bound to this polysaccharide. These findings may explain the failure, in previous work, to separate the different complexes by size exclusion or affinity chromatography.

The isolelectric points of the complexes were from 4.4 to 4.8. Electron microscopy examination of purified preparations of the complexes revealed distinct ultrastructural features for each complex. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the purified complexes showed that each complex had a distinct polypeptide composition. A 125,000 M_r glycoprotein with no apparent enzyme activity was the only polypeptide present in all fractions. The possibility that this is a scaffolding and/or cellulose-binding protein is under investigation.

Activity zymograms of gels (following electrophoresis using mildly denaturing conditions) revealed different carboxymethylcellulase-active proteins in all complexes, but xylanase-active proteins in only two of the complexes. The specific xylanase activity of each of these complexes was more than 10-fold higher than that of the multicomplex system.

Three of the seven complexes were able individually to hydrolyze crystalline cellulose, but the hydrolysis proceeded at a relatively slow rate. Recombination of fractions resulting from ion exchange chromatography, including four of the complexes, yielded a high cellulase specific activity similar to that found in the original multicomplex system, indicating that at least some of the complexes act synergistically in the hydrolysis of crystalline cellulose.

Synthesis of the multicomplex cellulase system responsible for the hydrolysis of crystalline cellulose was induced in cells growing in the presence of cellulose or of low cellobiose concentrations (as low as 29 μM). Cells growing in media containing other sugars (e.g., D-xylose, D-glucose or others) did not synthesize the multicomplex system, but retained carboxymethylcellulase and xylanase activities. Thus, the latter two activities were produced constitutively, and were found both in culture supernatants and in association with cells.

Fractions from culture supernatants of a mutant lacking the ability to synthesize the multicomplex system, and of cells grown under non-inducing conditions contained polypeptides that, when subjected to PAGE, had migration rates similar to those of polypeptides from the multicomplex system. However, those fractions did not contain the 125,000 M_r glycoprotein present in all complexes and suspected to be a scaffolding protein. Possibly, the synthesis of this protein is inducible, whereas that of the other polypeptides is constitutive. Thus, cells of cellulolytic bacteria that find themselves in the presence of cellulose may release a constitutive endoglucanase which acts on cellulose and generates low levels of cellobiose sufficient to induce the production of the scaffolding protein upon which the complexes can be assembled into a multicomplex. This postulated mechanism is under investigation.

2. <u>The cellobiose chemoreceptor of cellulolytic bacteria</u>.

In the course of our work on bacterial degradation of cellulose and other plant cell wall polysaccharides, we observed that growing cells of motile cellulolytic bacteria accumulated, without attachment, near cellulose fibers present in the cultures. Because it seemed likely that the accumulation was due to chemotactic behavior, we investigated the chemotactic responses of one of the above-mentioned bacteria (*Cellulomonas gelida* ATCC 488). We studied primarily the response toward cellobiose, which is the major product of cellulose hydrolysis by microorganisms, and toward hemicellulose hydrolysis products. We found that cellobiose, cellotriose, D-glucose, xylobiose, and D-xylose, as well as other sugars that are hemicellulose components, served as chemoattractants for *C. gelida* , as determined by a modification of Adler's capillary assay.

Competition and inducibility experiments indicated that *C. gelida* possesses at least two types of separately regulated cellobiose chemoreceptors (Cb1 and Cb2). Cb1 binds cellobiose and xylobiose but does not bind D-glucose, and its synthesis is inducible. Cb2 binds cellobiose, cellotriose, xylobiose and D-glucose, and it is constitutively synthesized. The presence in *C. gelida* of a constitutive response toward cellobiose and of at least two cellobiose chemoreceptors has implications for the survival of this cellulolytic bacterium in nature. In a paper that will appear in number 24 of the two December 1992 issues of the *Journal of Bacteriology* we have proposed a possible mechanism for cellobiose-mediated bacterial chemotaxis toward cellulose. We suggested that, in natural environments, motile cellulolytic bacteria migrate toward plant materials that contain cellulose and hemicellulose by swimming up cellobiose concentration gradients and/or concentration gradients of other sugars (e.g., xylobiose, D-xylose, and D-glucose) formed by enzymatic hydrolysis of plant cell wall polysaccharides. A copy of the galley proofs of the above-mentioned paper, in which this first stage of our chemotaxis research is described in detail, is attached to this progress report (see the Appendix section).

Methyl-accepting chemotaxis proteins (MCPs) are membrane proteins that function as transducers for various amino acids and sugars. During chemotactic responses the MCPs accept methyl groups from S-adenosyl methionine (SAM), an activated form of methionine. We found that ethionine, an inhibitor of SAM synthesis, inhibited *C. gelida* chemotaxis toward cellobiose and other sugars, and that a methionine auxotrophic *C. gelida* mutant exhibited decreased chemotactic responses toward cellobiose and D-glucose in the absence of exogenous methionine, as determined by capillary assays. Because these results suggested that MCPs might be involved in *C. gelida* chemotaxis, we carried out *in vivo* and *in vitro* experiments to investigate this possibility. These experiments showed that a specific 51,000 M_r methyl-accepting chemotaxis protein participates in chemotactic responses toward cellobiose and other sugars in *C. gelida*. This conclusion is based on the following lines of evidence: (1) Under methionine starvation conditions, methionine auxotrophs showed longer smooth swimming periods in tethered cell studies; (2) In the absence of protein synthesis the protein was methylated, and the level of methylation increased upon the addition of cellobiose or other sugar attractants, and decreased after the removal of the attractants; (3) The changes in methylation level were induced by 2-deoxy-D-glucose (a non-metabolizable attractant) but not by glycerol (a non-attractant growth substrate), implying that the methylation changes were not due to energy fluctuation; (4) the attractant L-aspartate or sugars that were not attractants did not elicit the methylation changes; (5) Mutants defective in cellobiose or D-glucose chemotaxis did not show the change in methylation level upon stimulation; (6) The incorporated methyl groups were alkali sensitive; (7) *In vitro* methylation experiments demonstrated that the 51,000 M_r protein was a membrane protein.

On the basis of these findings we concluded that the 51,000 M_r membrane protein is an MCP specifically involved in chemotaxis toward cellobiose and other

sugars. Inasmuch as this protein is constitutively synthesized by *C. gelida* cells, it may correspond to the Cb2 chemoreceptor described above.

3. Cellulose fermentation by biologically-defined bacterial consortia.

We have isolated from natural environments a number of different bacterial strains that we use in constructing anaerobic cellulose-degrading bacterial consortia (communities of different bacteria that carry out processes beyond the capabilities of any individual member of the community). These isolates include various anaerobic cellulolytic bacteria, several facultatively and obligately anaerobic non-cellulolytic bacteria that ferment cellulose-hydrolysis products (e.g., cellobiose), sulfate reducing bacteria that use end products of fermentation (e.g., lactate, acetate, H_2) as electron donors, methanogens that produce methane from H_2+CO_2, and two strains of a previously undescribed species of non-cellulolytic, thermophilic spirochetes that ferment cellobiose and D-glucose. A typical consortium that we have constructed utilizing some of these isolates (all from the same natural environment) consists of the following: a N_2-fixing cellulolytic clostridium, a cellobiose-fermenting anaerobic rod, and a H_2+CO_2-utilizing methanogen. This consortium ferments cellulose under N_2-fixing conditions (or in the presence of NH_4Cl) at a higher rate than the clostridium monoculture and forms methane as a major product. This and other consortia of different composition are being used to study metabolic interactions (see original grant proposal) among members of cellulose-utilizing consortia, especially interactions that affect the rate of cellulose degradation. Of special interest, because of its thermophilic nature, is a coculture consisting of a strain of *Clostridium thermocellum* and a strain of thermophilic, non-cellulolytic, cellobiose-fermenting spirochetes for which we have proposed the name *Spirochaeta caldaria*. This coculture grows luxuriantly at 50° C and ferments cellulose at a much higher rate than the clostridium monoculture.

We have directed much effort in determining growth rates, cellulose degradation rates, end product formation rates, and cellulase levels in monocultures of the cellulolytic *Clostridium* strain AD growing under N_2-fixing conditions and in the presence of combined N (usually NH_4Cl). We used cultures containing growth-limiting amounts of cellulose as the fermentable substrate and continuous cultures in cellobiose-containing medium. Inasmuch as *Clostridium* AD is the cellulose hydrolyzer in most of our consortia, this information was essential to interpret results of future experiments. We found that, even though *Clostridium* AD grew at a higher rate in the presence of combined N, the rates of cellulose degradation and of end product formation by cultures containing combined N were virtually the same as those of N_2-fixing cultures. Assays of cellulase (Avicelase) levels indicated that, when growing under N_2-fixing condition, *Clostridium* AD produced more cellulase per cell than when growing in the presence of combined N. These and other experiments indicated that, during growth under N_2-fixing conditions, *Clostridium* AD ferments cellulose at a higher rate per cell than in the presence of combined N. Cellulose hydrolysis is the limiting

step under both conditions but, when fixing N_2, individual cells have higher rates of i) cellulose hydrolysis and ii) metabolism of the hydrolysis products. Apparently, cells growing in the presence of combined N do not achieve the maximum possible rate of cellulose hydrolysis and metabolism.

To study mechanisms that regulate the synthesis of the enzyme system responsible for the hydrolysis of crystalline cellulose (cellulase) we determined cellulase levels (units per mg of cell protein) in supernatants of *Clostridium* AD cultures in which cellulose, cellobiose, or some other soluble sugar served as the fermentable substrate [Note: a) cellulase is extracellular, b) cellobiose is the major product of cellulose hydrolysis]. Cultures growing in media in which the concentration of cellobiose ranged from 0.1 to 0.2 % (w/v) synthesized very low levels of cellulase (e.g., 5 U/mg cell protein), as compared to levels produced by cultures growing in cellulose-containing media (e.g., 50 U/mg cell protein). However, in cultures growing at cellobiose concentrations ranging from 0.01 to 0.05 % (w/v), the cellulase levels were four-fold higher than those observed at the higher cellobiose concentrations. Similar results were obtained with cells growing with other soluble sugars (e.g., D-glucose or D-fructose) as substrates. These and other experiments indicated that synthesis of cellulose is repressed at the higher soluble sugar concentrations. In contrast, cellulose synthesis is induced at the lower soluble sugar concentrations, in response to an inducer that may be a derivative of the soluble sugar substrate. In addition, we found that, among many soluble sugars tested, only cellobiose (at concentrations higher than 0.05%, w/v) significantly inhibited the activity of cellulase.

II. PUBLICATIONS RESULTING FROM RESEARCH

The following publications appeared in press since the last progress report (June 1990).

-Cavedon, K., Leschine, S.B., and Canale-Parola, E. 1990. Cellulase system of a free-living, mesophilic clostridium (strain C7). J. Bacteriol. 172, 4222-4230. Galley proofs of this paper were previously sent to the DOE. A reprint is attached (see Appendix).

-Cavedon, K., Leschine, S.B., Canale-Parola, E. 1990. Characterization of the extracellular cellulase from a mesophilic clostridium (strain C7). J. Bacteriol. 172, 4231-4237. Galley proofs of this paper were previously sent to the DOE. A reprint is attached (see Appendix).

-Hsing, W., Canale-Parola, E. 1991. Cellobiose chemotaxis in cellulolytic bacteria. Abst. Annu. Mtg. Amer. Soc. Microbiol., I-78, 203.

-Pohlschroeder, M., Leschine, S.B., Canale-Parola, E. 1991. Xylanase activity of a mesophilic cellulolytic clostridium. Abst. Annu. Mtg. Amer. Soc. Microbiol., K-60, 224.

-Cavedon, K., Canale-Parola, E. 1992. Physiological interactions between a mesophilic cellulolytic *Clostridium* and a non-cellulolytic bacterium. FEMS Microbiol. Ecology 86, 237-245. A reprint is attached (see Appendix).

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-Pohlschroeder, M., Seward, R., Leschine, S.B., Canale-Parola, E. 1992. *Spirochaeta caldaria,* sp. nov., a thermophilic spirochete from freshwater hot springs. BioEngineering, 8, 72.

-Hsing, W., Canale-Parola, E. 1992. Cellobiose chemotaxis by the cellulolytic bacterium *Cellulomonas gelida.* J. Bacteriol. Accepted, in print. This paper will appear in number 24 of the two December 1992 issues of J. Bacteriol. A copy of the galley proofs is attached (see Appendix).

The following three papers are in preparation:

- Pohlschroeder, M., Leschine, S.B., Canale-Parola, E. The multicomplex cellulase system of *Clostridium papyrosolvens* strain C7. To be submitted to J. Bacteriol.

-Hsing, W. and Canale-Parola, E. A methyl-accepting chemotaxis protein is involved in cellobiose chemotaxis by *Cellulomonas gelida.* To be submitted to FEMS Microbiol. Lett.

-Pohlschroeder, M., Seward, R., Leschine, S.B., Canale-Parola, E. *Spirochaeta caldaria,* a thermophile from freshwater hot springs that enhances cellulose degradation by *Clostridium thermocellum.*

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