FINAL TECHNICAL REPORT

MICROBIAL FERMENTATION OF ABUNDANT BIOPOLYMERS: CELLULOSE AND CHITIN

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Susan Leschine, University of Massachusetts

This report covers research performed during the project period, from May 1, 1988 through October 31, 2002. This report covers the entire project period, focusing on research conducted during the final three years. Details of earlier work may be found in publications and in annual and budget-period progress reports. A complete list of publications (journal articles, reviews, and abstracts) resulting from the project is included at the end of this report.

Our research has dealt with seven major areas of investigation: i) characterization of cellulolytic members of microbial consortia, with special attention recently given to *Clostridium phytofermentans*, a bacterium that decomposes cellulose and produces uncommonly large amounts of ethanol, ii) investigations of the chitinase system of *Cellulomonas uda*; including the purification and characterization of ChiA, the major component of this enzyme system, iii) molecular cloning, sequence and structural analysis of the gene that encodes ChiA in *C. uda*, iv) biofilm formation by *C. uda* on nutritive surfaces, v) investigations of the effects of humic substances on cellulose degradation by anaerobic cellulolytic microbes, vi) studies of nitrogen metabolism in cellulolytic anaerobes, and vii) understanding the molecular architecture of the multicomplex cellulase-xylanase system of *Clostridium papyrosolvens*. Also, progress toward completing the research of more recent projects will be briefly summarized.

Major accomplishments include:

1. Characterization of *Clostridium phytofermentans*, a cellulose-fermenting, ethanol-producing bacterium from forest soil. During the past year we completed the characterization of a new cellulolytic species isolated from a cellulose-decomposing microbial consortium from forest soil. This bacterium is remarkable for the high concentrations of ethanol produced during cellulose fermentation, typically more than twice the concentration produced by other species of cellulolytic clostridia. A paper submitted to the *International Journal of Systematic and Evolutionary Microbiology*, which describes this work, is in press ("*Clostridium phytofermentans* sp. nov., a cellulolytic mesophile from forest soil," by T. A. Warnick and S. B. Leschine).

2. Examination of the use of chitin as a source of carbon and nitrogen by cellulolytic microbes. During studies carried out to complete earlier work on nitrogen metabolism and N_2 fixation by cellulose-fermenting bacteria (published in *Science*: Leschine, S. B., K. Holwell, E. Canale-Parola. Nitrogen fixation by anaerobic cellulolytic bacteria. *Science* **242**:1157-1159), we discovered that many cellulolytic anaerobes and facultative aerobes are able to use chitin as a source of both carbon and nitrogen. A paper describing this work has been accepted for publication in *FEMS Microbiology Letters* ("Chitin degradation by cellulolytic anaerobes and facultative aerobes from soils and sediments" by G. Reguera and S. B. Leschine). This major discovery expands our understanding of the biology of cellulose-fermenting bacteria and may lead to new applications for these microbes.

3. Comparative studies of the cellulase and chitinase systems of *Cellulomonas uda*. Other research described in the above-mentioned *FEMS Microbiology Letters* paper focused on the hydrolytic enzyme systems of the facultative aerobe, *C. uda*. Results of these studies indicate that the chitinase and cellulase systems of this bacterium are distinct in terms of the proteins involved and the regulation of their production.

4. Characterization of the chitinase system of *C. uda*. A 70,000-M_r endochitinase, designated ChiA, was purified from *C. uda* culture supernatant fluids. ChiA is the major component of the chitinase system of *C. uda*, accounting for 67% of the total chitinase specific activity present in culture supernatants. We have obtained evidence indicating that the chitinase system of *C. uda* also includes an exochitinase. While most chitinolytic microorganisms secrete a battery of enzymes to hydrolyze the different forms of chitin found in nature, our results indicate that *C. uda* produces a relatively simple chitinase system, which efficiently hydrolyzes this insoluble substrate. Results of this work are presented in the attached manuscript, "Purification and characterization of ChiA, the major component of the chitinase system of *Cellulomonas uda*" by G. Reguera and S. B. Leschine, which has been prepared for the *Journal of Bacteriology*.

5. Analysis of *chiA*, which codes for the major enzymatic component of the chitinase system of *C. uda*. The gene encoding the endochitinase ChiA in *C. uda* was cloned and its complete nucleotide sequence was determined. This gene, *chiA*, specifies a modular protein, 571 amino acids in length, with a signal peptide at its N terminus, a type-2 cellulose binding domain, and a catalytic domain belonging to family 18 of glycoside hydrolases. These studies are described below and details are given in the attached manuscript, "Molecular cloning and sequence analysis of *chiA*, encoding the major component of the chitinase system of *Cellulomonas uda*" by G. Reguera and S. Leschine, which has been prepared for the *Journal of Bacteriology*.

6. Formation of biofilms by *C. uda* on cellulose and chitin. Microscopic observations indicated that, under conditions of nitrogen limitation, *C. uda* cells grew as a biofilm attached tightly to the surface of cellulose or chitin. Cells did not colonize abiotic surfaces such as plastic or the glass surfaces of culture vessels under any growth

condition examined. Results of these studies suggest that biofilm formation may be a general strategy used by bacteria in the degradation of insoluble natural polymers.

7. Development of tools for a genetic approach to studies of cellulose fermentation by cellulolytic clostridia. It is apparent that mutational analysis would be a powerful tool in experiments aimed at advancing understanding of the physiology of cellulolytic clostridia and the production of their cellulase systems. We have explored the potential of various techniques, and obtained evidence indicating that Tn*916* mutagenesis may be particularly effective in this regard. Some of this work is described below in the section, "Investigations of *Clostridium phytofermentans*." Also, as part of research aimed at characterizing cellulolytic microbes from nitrogen-fixing cellulose-decomposing microbial consortia, we identified the presence of a plasmid in one strain. This plasmid was cloned, sequenced, and analyzed, and possibly may prove useful in the development of vectors for genetic studies. A paper describing this work has been published (Chen, T., L. Ouko, T. Warnick, and S. B. Leschine. 2000. Detection, cloning, and sequence analysis of an indigenous plasmid from cellulolytic clostridial strain MCF1, *Plasmid* **43**:153-158).

8. Effects of humic substances on cellulose degradation by anaerobic cellulolytic microbes. An objective of an earlier three-year project was to determine the effects of humic substances on the degradation of cellulose by anaerobic microbial communities, and by the cellulolytic members of these communities. Humic substances are ubiquitous and abundant complex organic materials found in soils and sediments, and they may play an important role in the anaerobic degradation of cellulose in these environments. Microbes from soils and sediments have been shown to use humic substances as electron acceptors for the anaerobic oxidation of organic compounds in a process that yields energy to support growth (Lovley et al., Humic substances as electron acceptors for microbial respiration. *Nature* **382**:445-8, 1996).

As described in Progress Reports for this project, we examined pure cultures of known cellulolytic anaerobes for the ability to transfer electrons to humic substances using the humic model compound, 2,6-anthraquinone disulfonate (AQDS), as a culture medium component. Reduction of this compound results in a change in the color of culture media. In these experiments, we found that most cellulolytic clostridia tested, and also non-cellulolytic clostridia (*Clostridium pasteurianum, Clostridium acetobutylicum*), reduced AQDS. *Escherichia coli* did not reduce AQDS, and served as a negative control. We also investigated the effect of AQDS on growth, cellulose degradation, and product formation by *Clostridium papyrosolvens*. In these experiments, cells were cultured in the absence or presence of 5 mM AQDS in a defined medium supplemented with 0.2% yeast extract. In cultures containing AQDS, cellulose was degraded more rapidly, cell growth rate increased, and cell yields were higher. Also, fermentation products (e.g., ethanol and acetate) accumulated more rapidly in cultures containing AQDS. The results of these experiments indicate that reduction of AQDS dramatically affects growth and cellulose fermentation by *C. papyrosolvens*. Perhaps accessibility of an external electron-accepting molecule

enables this bacterium to more rapidly balance NAD^+/NADH levels and thereby facilitates fermentation.

We have continued these studies in order to confirm the above-mentioned results and to investigate the effects of commercially available preparations of humic acids (e.g., humic acid, sodium salt, Aldrich) on growth and cellulose degradation by *C. papyrosolvens*. Our results showed that the effects of Aldrich humic acids on cellulose fermentation were similar to the effects of AQDS, but were far less pronounced. We plan to complete this study by examining other sources of humic acids and their effects on cellulose fermentation. Results obtained thus far indicate that humic substances may play an important role in the anaerobic cellulose decomposition and in the physiology of cellulose-fermenting soil bacteria.

9. Nitrogenases of cellulolytic clostridia. During an earlier three-year project period, we completed studies aimed at describing the properties of nitrogenases of cellulolytic bacteria. In a manuscript that has been revised for *Applied and Environmental Microbiology* ("Nitrogenase systems of mesophilic cellulolytic clostridia" by T. Chen and S. B. Leschine), we present evidence for the existence of diverse nitrogenase systems in cellulolytic clostridia, including both molybdenum- and alternative-nitrogenases. In a manuscript prepared for *Journal of Bacteriology* ("Cloning and sequence analysis of a *nifH* gene from *Clostridium cellobioparum*, which is followed by a *glnB*-like gene homologous to the methanogen ORF105 family" by T. Chen and S. B. Leschine), we describe a nitrogenase gene from a cellulolytic clostridium and present evidence, based on sequence analyses and conserved gene order, for lateral gene transfer between this bacterium and a methanogenic archaeon.

10. Characterization of *Clostridium hungatei*, a new N_2-fixing cellulolytic species isolated from a methanogenic consortium from soil. This bacterium is described in a paper published in the *International Journal of Systematic and Evolutionary Microbiology* ("*Clostridium hungatei* sp. nov., a mesophilic N_2-fixing cellulolytic bacterium from soil" by E. Monserrate, S. B. Leschine, and E. Canale-Parola).

11. Understanding the molecular architecture of the multicomplex cellulase-xylanase system of *Clostridium papyrosolvens*. As described in several progress reports for the project, *C. papyrosolvens* produces a multiprotein enzyme system that hydrolyzes crystalline cellulose. Enzyme complexes are released into the external environment where they bind to their insoluble substrate and depolymerize it forming, primarily, cellobiose. Various other anaerobic bacteria also have been reported to produce extracellular cellulases that form high-molecular-weight, multienzyme complexes (cellulosomes), which catalyze the hydrolysis of crystalline cellulose. Our studies have shown that the cellulase system of *C. papyrosolvens* consists of at least seven distinct extracellular, high-molecular-weight multiprotein complexes, each with different enzymatic and structural properties, rather than a single complex as had been thought previously. We refer this enzyme system as the "multicomplex cellulase-xylanase system." It is likely that cellulase systems of other anaerobes also consist of multiple protein complexes.

Evidence accumulated to date suggests that cellulosomes assemble by a random association of catalytic subunits with the scaffoldin, and it has been postulated that cellulosomal composition is determined by the relative concentrations of subunits. However, our research has clearly shown that the distribution of catalytic subunits in cellulosomes of *C. papyrosolvens* is not random, and suggests that other mechanisms are involved in cellulosome assembly. A plausible explanation for our observations would be that the diverse cellulosomes of the cellulase system of *C. papyrosolvens* are constructed on more than one scaffoldin. Contrary to this notion, however, our recent results, described below, suggest that a single scaffoldin is used in the assembly of the cellulosomes of the multicomplex cellulase-xylanase system.

In the previous progress report, we described the identification of cellulosomal subunit S4 as the scaffoldin in cellulosomes of *C. papyrosolvens*. Subunit S4 is a 125,000-M_r glycoprotein and the only protein found in all cellulosomes of the multicomplex cellulase-xylanase system of *C. papyrosolvens*. N-terminal sequence analysis indicated that S4 is very similar to scaffoldins from *Clostridium josui* and *Clostridium cellulolyticum*, cellulolytic species that bear a close phylogenetic relationship to *C. papyrosolvens*. We found that the N-terminal sequence of subunit S4 isolated from complex F2, a xylanase-active complex of the *C. papyrosolvens* multicomplex system, was identical to the N-terminal sequence of subunit S4 of the cellulase-active complex F4, the most abundant complex of the multicomplex cellulase-xylanase system. With the exception of subunit S4, these complexes have no major components in common. This result suggests that the very different complexes of the multicomplex system may be built on a single scaffoldin, subunit S4, which we have designated “cellulosome-integrating protein P” or “CipP.” In support of this conclusion, using Southern hybridization analysis, we have obtained evidence suggesting the presence of only one scaffoldin gene (*cip*) in the genome of *C. papyrosolvens*. In these experiments we used, as probe, a cloned DNA fragment encoding the C1 cohesin domain of the scaffoldin from *C. cellulolyticum*. This DNA fragment was obtained from plasmid pETCip1, which was provided by Dr. A. Bélaïch (National de la Recherche Scientifique, Marseilles), and random-primed labeled with digoxigenin-11-dUTP using the Genius System of Boehringer Mannheim. We obtained similar results using, as probe, *Clostridium thermocellum cipA* gene fragments carried by pRG2.0 (provided by Dr. J. H. David Wu, University of Rochester) and a cloned DNA fragment from *C. papyrosolvens*, which we believe contains the *cipP* gene.

Taken together, these results suggest that a single scaffoldin is used in the assembly of the diverse complexes of the multicomplex cellulase-xylanase system of *C. papyrosolvens*. Much of this research has been published in the *Journal of Bacteriology* (see publication list at the end of this report).

Specific results of recent research are summarized below:

INVESTIGATIONS OF *CLOSTRIDIUM PHYTOFERMENTANS*

Characterization of *C. phytofermentans*. During the past year, we completed a basic characterization strain ISDg, a new cellulolytic species that we isolated from a microbial consortium from soil, for which we are proposing the name *Clostridium phytofermentans*. Morphologically, the cells of *C. phytofermentans* are motile long slender rods that form terminal spores, which swell the sporangium. Cells stain gram-negative. An unusually wide range of carbohydrates and polysaccharides, including cellulose, pectin, starch, and xylan, support the growth of this microbe. Ethanol and acetate are major end products of fermentation and formate and lactate are minor products. Also, H_2 is produced. Phylogenetic analyses, based on 16S rRNA sequence comparisons, indicate that *C. phytofermentans* is a member of "cluster XIVa of low G+C gram-positive bacteria" (Collins et al., 1994. *Int. J. System. Bacteriol.* **44**:812-826.), which includes other cellulolytic *Clostridium* species (*C. herbivorans*, *C. polysaccharolyticum*, *C. populeti*). This work is described in detail in the attached preprint of a paper submitted to the *International Journal of Systematic and Evolutionary Microbiology* ("*Clostridium phytofermentans* sp. nov., a cellulolytic mesophile from forest soil," by T. A. Warnick and S. B. Leschine).

Ethanol production by *C. phytofermentans*. As mentioned above, a remarkable characteristic of *C. phytofermentans* is the production of high concentrations of ethanol during cellulose fermentation, typically more than twice the concentration produced by other cellulolytic clostridia. Also, *C. phytofermentans* produces three to eight times more ethanol than acetate (on a molar basis), whereas most other cellulolytic clostridia produce roughly equal amounts of these fermentation products, or less ethanol than acetate. These results suggest that the pathways for fermentation product formation in *C. phytofermentans* may differ from those typically found in cellulolytic clostridia. Some of the research currently in progress in the laboratory is aimed at examining the fermentation pathways of *C. phytofermentans*.

Formation of biofilms by *C. phytofermentans*. The unusual pattern of fermentation products formed by *C. phytofermentans* indicated that this bacterium might be useful in studies of biofilm growth on cellulose as a nutritive surface, primarily because it would be interesting to know the effects of biofilm formation on ethanol production, as well as on cellulose decomposition. Results of our preliminary experiments indicate that *C. phytofermentans* produces biofilms on various forms of cellulose, including filter paper, tissue paper, and cellophane. These initial results suggest that *C. phytofermentans* may serve a good "model bacterium" in experiments that are directed toward examining biofilm formation by an ethanol-producing cellulolytic anaerobe.

Development of tools for a genetic approach to studies of cellulose fermentation by cellulolytic clostridia. As mentioned above, it has become evident that mutational

analysis would be a powerful tool to advance understanding of the physiology of strains of cellulolytic clostridia. For example, conjugative transposons are useful tools for insertional mutagenesis and mutational cloning. Our research has indicated that Tn*916* mutagenesis may be particularly effective in *C. phytofermentans*. We examined several different strains of *Enterococcus faecalis* and *Bacillus subtilis* harboring copies of either Tn*916* or Tn*1545* for the ability to conjugatively transfer their transposons to *C. phytofermentans* using a filter mating procedure. Only one strain (*E. faecalis* CG110, provided by Dr. D. B. Clewell, University of Michigan), containing two copies of Tn*916*, readily transferred Tn*916* to strains of cellulolytic clostridia. To avoid problems encountered in selecting against the donor *Enterococcus* strain, Tn*916* was first transferred into *B. subtilis* W26 and a resulting transconjugant strain was subsequently used as donor. Attempts were made to optimize the filter mating procedure by varying the donor : recipient ratio, the ages of donor and recipient cultures, the type of filter used, and by examining the effect of growth of donor cells with tetracycline and heat shocking recipient cells (55°C for 10 min) before matings. Typically, with *C. phytofermentans*, transconjugation frequencies ranged from 1 to 5 ¥ 10^{-4} transconjugants per recipient, and approximately 5 ¥ 10^{4} transconjugants per mating reaction were obtained. These results suggest that Tn*916* insertional mutagenesis may be a useful technique in future research where we may take a genetic approach to investigations of biofilm formation by *C. phytofermentans.*

CHITIN DEGRADATION BY *CELLULOMONAS UDA*

The chitinase system of *C. uda*. We examined the chitinase system present in supernatant fluids of *C. uda* cultures grown in a colloidal chitin-containing medium, inasmuch as high levels of chitinase activity were produced under these conditions. Basic characteristics of this enzyme system were described in the previous progress report and are detailed in a manuscript accepted for publication by *FEMS Microbiology Letters* ("Chitin degradation by cellulolytic anaerobes and facultative aerobes from soils and sediments" by G. Reguera and S. B. Leschine).

During experiments aimed at purifying components of the chitinase system of *C. uda*, we discovered that gel filtration chromatography on a Sephacryl S-300 column was hindered by aberrant binding of proteins to the column matrix. In contrast, we determined that anion exchange chromatography would be a useful technique for purifying one component of the chitinase system of *C. uda*. Using this technique, we purified the major component of the chitinase system, a glycoprotein with a M_r of 70K and a pI of 8.0, which we designated ChiA. ChiA accounted for 67% of the total chitinase specific activity present in culture supernatants. This enzyme degraded glycol chitin and other chitinous substrates with different degrees of crystallinity, but it did not degrade carboxymethylcellulose. However, it bound to both chitinous and cellulosic substrates. Based on the observation that oligosaccharides larger than chitobiose were formed with colloidal chitin as substrate, and activity with artificial substrates, we concluded that ChiA is an endochitinase. Also, we have obtained evidence indicating that the chitinase system of *C. uda* includes, in addition to ChiA, an exochitinase. While most chitinolytic microorganisms secrete a battery of enzymes

to hydrolyze the different forms of chitin found in nature, our initial results suggest that *C. uda* produces a relatively simple chitinase system, which efficiently hydrolyzes this insoluble substrate. Details of this work are presented in a manuscript, "Purification and characterization of ChiA, the major component of the chitinase system of *Cellulomonas uda*" by G. Reguera and S. B. Leschine, which has been prepared for the *Journal of Bacteriology*.

Cloning and sequence analysis of the gene encoding ChiA. Internal amino acid sequence information indicated that ChiA was closely related to chitinases from *Streptomyces* species, especially ChiC from *Streptomyces lividans*. Drs. A. Saito and K. Miyashita of the National Institute of Agro-Environmental Sciences, Ibaraki, Japan provided us with the cloned *chiC* gene from *S. lividans*, which we used in the construction of nucleotide probes. As detailed in our manuscript entitled "Molecular cloning and sequence analysis of *chiA*, encoding the major component of the chitinase system of *Cellulomonas uda*." we used these probes to clone the gene which specifies ChiA in *C. uda*. Our initial attempts to clone *chiA* and amplify regions of the *chiA* gene were unsuccessful, possibly because of the extraordinarily high G + C content of the DNA of *C. uda* (76.3%). To circumvent this problem, we used a two-step PCR procedure, which resulted in successful amplification of *chiA* gene fragments. The first run was performed in the presence of d-aza-GTP and a hot start was applied to the first five PCR cycles. The second run was performed without either d-aza-GTP or application of a hot start.

The complete nucleotide sequence of *chiA* was determined. This gene specifies a 571-amino acid, 61-kDa modular protein with a signal peptide at its N terminus, a type-2 cellulose binding domain (CBD), and a catalytic domain belonging to family 18 of glycoside hydrolases. Both the CBD and catalytic domain were found to be homologous to modules present in chitinases from a different *Cellulomonas* species and a species of *Streptomyces*. However, in the chitinase from the *Streptomyces* species, the CBD and catalytic domain are connected by a fibronectin type III domain, whereas the linker region in the cellulomonad chitinases is a proline- and threonine-rich module, which is likely to be glycosylated in the mature protein. We also identified sequences upstream of the *chiA* coding region, including a -35 hexamer flanked by two direct repeats, which are similar to those found in the promoters of many *Streptomyces* chitinases and thought to function as binding sequences for regulatory proteins involved in repression by glucose and induction by chitin. These studies are described in detail in the manuscript, "Molecular cloning and sequence analysis of *chiA*, encoding the major component of the chitinase system of *Cellulomonas uda*," which has been prepared for the *Journal of Bacteriology*.

Regulation by nitrogen availability of production of chitinase activity in *C uda*. As described in an earlier progress report for the project and detailed in a paper accepted for publication in *FEMS Microbiology Letters* ("Chitin degradation by cellulolytic anaerobes and facultative aerobes from soils and sediments"), we found that chitin and chitin degradation products, such as *N*-acetyl-D-glucosamine (GlcNAc), chitobiose, and chitotriose, serve as sources of nitrogen for *C. uda*. We also

conducted experiments to examine the effect of nitrogen availability on the production of chitinase activity by *C. uda*. Our results indicated that chitinase activity could not be detected in cultures grown in cellulose- or cellobiose-containing medium when a sufficient source of nitrogen was available. However, chitinase activity was detectable in cultures when cells were grown in a nitrogen-deficient medium containing cellulose or cellobiose as growth substrate. These results suggest that nitrogen depletion may signal *C. uda* cells to produce chitinases in order to use chitin as a nitrogen source.

BIOFILM FORMATION BY *CELLULOMONAS UDA*

Microscopic observations of *C. uda* cells cultured in nitrogen-depleted media revealed that cells were present as large aggregates. Addition of a nitrogen source (e.g., NH_4Cl, urea, GlcNAc) to the growth medium resulted in a rapid dispersal of cells from these aggregates. *C. uda* cells attached tightly to cellulose and chitin, but did not colonize abiotic surfaces such as plastic or the glass surfaces of culture vessels. Cell aggregation observed under conditions of nitrogen depletion coincided with the production of extensive extracellular material. The appearance of *C. uda* cells stained with ruthenium red and examined by transmission electron microscopy suggested that cells grown under conditions of nitrogen depletion are embedded in a thick carbohydrate layer. This layer was absent from cells cultured in media containing sufficient available nitrogen. Under any of a wide range of culture conditions, nitrogen fixation by *C. uda* cells was not detected using acetylene reduction assays. Also, a dependence on N_2 in the atmosphere of growth cultures of *C. uda* could not be demonstrated.

Under conditions of nitrogen limitation, cells appeared to form a biofilm, which was apparent when the carbon source was a crystalline form of chitin, such as squid pen chitin. The squid pen surface was colonized by patches of cell aggregates, which appeared to vary in density and form three-dimensional structures separated by open areas, similar to those observed in other biofilms and described as "pillars" or "mushrooms," separated by "channels." Chitinase activity was detected in supernatant fluids of biofilm-forming cultures, but cell-associated activity was not detected. This result suggests that cells in these biofilms may not degrade the surface to which they are directly attached. *C. uda* cells did not form biofilms when cultured on colloidal chitin. Cells grew as microcolonies on the surface of colloidal chitin particles, but detached as chitin was degraded. These results suggest that, as the readily degradable colloidal chitin was decomposed, sufficient nitrogen became available to signal a switch to planktonic growth.

Biofilm formation by *C. uda* was especially apparent when cellulose served as carbon source. Biofilms formed on cellulose only under conditions of nitrogen limitation, and addition of a nitrogen source to cultures resulted in a rapid dispersal of the cells from cellulose fibers. As shown previously, cells (stained with SYTO® 9 green fluorescent nucleic acid stain from Molecular Probes) were present on the surface of cellulose fibers as patches of cell aggregates that appeared to form

structures similar to those that formed on squid pen, as described above. Cellulose appeared to be degraded by these biofilms, based on visual observations. Also, when *C. uda* was cultured with cellulose bound to Remazol Brilliant Blue 5R ("Cellulose-Azure," Sigma) under conditions of biofilm formation, dye was rapidly released from the substrate as measured by an increase in the optical density of culture supernatants measured at 595 nm.

These results demonstrate the involvement of biofilms in the degradation of natural polymers such as cellulose and chitin and suggest that biofilm formation on biological surfaces may play a nutritive role. Colonization of natural polymers and biofilm formation may confer on *C. uda* cells a selective advantage over other cellulolytic and/or chitinolytic microorganisms, allowing the bacteria to "secure" a nutrient in an environment where competition for food is high. Possibly, biofilm formation is a general strategy used by bacteria in the degradation of insoluble natural polymers. In the renewal application for this project, we are proposing research to identify factors involved in biofilm formation, and examine the effects of biofilm production on cellulose degradation.

PUBLICATIONS RESULTING FROM THE PROJECT

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