Summary of results from DOE project DE-FG02-01ER63142

# Metagenomic analysis of uncultured *Cytophaga* and **$**-1,4 glycanases in marine consortia

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July 2005

Most bacteria and archaea in natural environments still cannot be isolated and cultivated as pure cultures in the laboratory, and the microbes that can be cultured appear to be quite different from uncultured ones. Consequently, the phylogenetic composition, physiological capacity and genetic properties of natural microbes have to be deduced from bulk properties of microbial assemblages, fluorescence in situ hybridization (FISH) assays, and from a variety of PCR-based methods applied to DNA isolated directly from natural samples. Another culture-independent approach is to clone microbial DNA directly into appropriate vectors and to screen the resulting metagenomic library, which theoretically consists of all possible genes from the microbial assemblage. We have been using this approach to examine microbial communities in the Delaware Estuary and coastal waters and the western Arctic Ocean.

We have finished the analysis of bacterial community structure in the Delaware River and compared results from the metagenomic approach with other culture-independent approaches (Cottrell et al. 2005a). Results from this part of the project were discussed in detail in our last report. We have also continued work on the aerobic anoxygenic photosynthesizing (AAP) bacteria (Cottrell et al. in press; Waidner and Kirchman 2005). Finally, we examined the role of Cytophaga-like bacteria in degrading high molecular weight organic material and stumbled on a cautionary tale about using sequence data for identifying genes from metagenomic libraries (Cottrell et al. 2005b).

## AAP bacteria in estuaries and the oceans

Current models of carbon cycling in the oceans assume that marine microbes are mainly either photoautotrophic (consume $CO_2$ and produce organic material using light energy) or heterotrophic (consume organic material and produce CO2 independent of light). Recent work has suggested that photoheterotrophic microbes, which use both phototrophy and heterotrophy, may be abundant and biogeochemically important in the oceans. One group of photoheterotrophic bacteria includes the AAP bacteria. (AAP bacteria have bacteriochlorophyll (bchl) rather than chlorophyll a found in other phototrophs.) Photoheterotrophy would fundamentally alter our understanding of the role of prokaryotes in biogeochemical cycles of aquatic ecosystems.

We lack, however, even the most basic data about photoheterotrophs, such as the abundance and growth rates of AAP bacteria in the oceans. Consequently, we examined the abundance of AAP bacteria using IR epifluorescence microscopy in the North Atlantic and Pacific Oceans (Cottrell et al. 2005). We found that AAP bacteria

comprised 5% to 16% of total prokaryotes in the Atlantic but only 5% or less in the Pacific. In the Atlantic, AAP bacterial abundance was as much as 2-fold higher than *Prochlorococcus* and 10-folder higher than *Synechococcus*. In contrast, *Prochlorococcus* outnumbered AAP bacteria 5- to 50-fold in the Pacific. In both oceans, subsurface abundance maxima occurred within the photic zone, and AAP bacteria were least abundant below the 1% light depth.

AAP bacteria appear also to be common in the Delaware Estuary as the abundance of bacteriochlorophyll-containing cells is often high and can exceed 20% of total prokaryotic abundance especially in the turbidity maximum. We also surveyed the Delaware River for pufL and pufM genes, which encode AAP reaction center proteins, in a fosmid library of bacterial genomic DNA. Two fosmid clones containing AAP photosynthetic operons were found, completely sequenced by JGI, and annotated (Waidner and Kirchman 2005).

One of the fosmid clones appears to be from a beta-proteobacterium, based on BLAST analyses of 30+ genes from the fosmid, and is quite likely a new type of AAP bacteria. To determine its distribution in the estuary, PCR primers were designed to specifically detect this AAP beta-proteobacterium. Sequence analysis confirmed that the PCR assay detects only very similar relatives of this bacterium. Qualitative PCR results indicate that the bacterium is found in much of the estuary but not in its most saline waters. A quantitative PCR assay is now being developed to explore this question in greater detail.

**Peptidases in uncultured Cytophaga-like bacteria: a cautionary tale**

One of our general goals is to understand the biogeochemical role of *Cytophaga*-like bacteria and their hydrolyases such as peptidases in the oceans. (Hydrolyases are enzymes that break down high molecular weight organic material; one example is the hydrolysis of peptides by peptidases.) Venter et al. (Science 2004) reported a relatively low number of *Cytophaga*-like contigs, but our analysis suggest that this bacterial group is under-represented by >2-fold in the current annotation of the Sargasso data set. One of the *Cytophaga*-like 16S rRNA genes in the Venter et al. data is similar to a group we initially found in the Delaware Bay, DE Cluster 2 (Kirchman et al. 2003). We detected this bacterial group in the Arctic Ocean, and more recently Abel and Bowman (2004) found DE Cluster 2 to be abundant in Antarctica.

We also detected cellulases apparently from *Cytophaga*-like bacteria in the Venter et al. data. The *Cytophaga*-like contigs in the Venter et al. data include a cellulase similar to one found in the *Cytophaga hutchinsonii* genome which was sequenced by JGI. One of these cellulases (cellulase M) is on a contig also bearing a 16S rRNA of DE Cluster 2. PCR primers were designed to detect cellulase M and were used in a PCR-based screening of available metagenomic fosmid libraries.

One fosmid clone in a library from the western Arctic Ocean was positive for cellulase M. Sequencing of the PCR product confirmed that it was most similar to a

cellulase, although the similarity was low. Additional analyses of the fosmid clones by PCR and denaturing gradient gel electrophoresis revealed that the cellulase-bearing fosmid clone also contains a 16S rRNA gene, which is most similar to *Cytophaga*-like bacterial 16S rRNA. In addition, the complete sequence of this fosmid by JGI revealed four peptidases.

To explore its identity in greater detail, the presumed cellulase M gene was subcloned into an expression vector and its enzymatic properties examined (Cottrell et al. 2005b). SDS PAGE and amino acid sequencing confirmed that the expressed protein was the product expected of the cellulase M gene. However, the enzymatic tests revealed that this enzyme was a peptidase, not a cellulase. In retrospect, this result was not entirely surprising. Although the top BLAST hit was to cellulase M, the cloned gene was also similar to a peptidase from *Lactobacillus*.

These results illustrate the power of the metagenomic approach but also tell a cautionary tale. The metagenomic approach provided another piece of data linking *Cytophaga*-like bacteria to the degradation of high molecular weight organic material. This was possible only because the cloned DNA had both "functional genes" (peptidases) and a phylogenetic marker (16S rRNA). Because the entire gene was cloned and was expressed in *E.coli*, the identity of the gene could be determined experimentally. In contrast, sequence analysis with the current data base lead to the wrong conclusion. Further metagenomic studies such as this one are necessary for not only confirming gene identities, but also for exploring totally unknown genes retrieved from the oceans.

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