From West Point to the Battery:
Bacterial Diversity Along the Lower Hudson Estuary

A Final Report of the Tibor T. Polgar Fellowship Program

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ABSTRACT

The goal of this study is to categorize bacterial diversity along the salinity gradient of the lower Hudson River Estuary. Previous research has shown that lower salinities support higher diversity, while more saline environments host lower, but more specialized diversity. Mid-river samples were taken in the middle of July from the Battery, Piermont, and West Point, New York. Both subsurface and deepwater samples were taken as the salinity changes by both distance from the mouth of the river and depth. Samples were vacuum filtered to collect the bacteria on 0.2um pore size filters and bacterial DNA was extracted using mechanical and chemical techniques. The 16S region of the bacterial genomes were then extracted and replicated via a PCR reaction, and the sequences were separated by inserting them in vectors in *E.coli* and allowing the *E. coli* cells to form colonies. Colonies with the insert were then selected and the 16S regions amplified by colony PCR. Sequencing of this region was accomplished through cycle sequencing and sequences were read using an ABI automatic sequencer machine. Sequences were analyzed by comparing them to a national database (NCBI BLAST analysis) and also by completing a phylogenetic analysis of the aligned sequences. BLAST analysis of the Battery and West Point samples revealed a higher diversity in the West Point samples (fresh water), with the presence of gamma proteobacteria in the West Point subsurface sample. Phylogenetic analysis provides evidence of eight major bacterial groups among all four samples, but does not provide evidence for location-specific bacteria clusters. Further analysis will include sequencing clones from the Piermont samples as well as analyzing more clones from the Battery and West Point locations. Useful future research includes analyzing seasonal components to bacterial diversity as well as examining the morphological characteristics of Hudson River bacteria.

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INTRODUCTION

Bacterial Diversity in the Lower Hudson Estuary

Understanding the diversity and ecological processes of the Hudson River Estuary is essential to the continued improvement and protection of this unique ecosystem. The high diversity in the Hudson River is partly due to significant influxes of salt that wash upriver from the Atlantic Ocean (Stanne et al. 1996). Incoming salt water usually influences approximately 60 miles of the river; however, in drier years, the salt front has reached points as far north as Poughkeepsie, approximately 85 miles upriver (Stanne et al. 1996). Although much is known about the diversity and distribution of higher-level taxa along the river's salinity gradient, very few studies have attempted to categorize bacterial communities along it.

Bacteria are essential components of ecosystems, providing essential services and stability to natural systems. Most of the essential biogeochemical nutrient cycles rely on bacteria, including the carbon and nitrogen cycles. In the Hudson River, for example, bacteria serve as the largest carbon producers (Findlay et al. 1991). Bacteria also help stabilize ecosystems through their high diversity levels. Diversity has been linked to ecosystem stability, productivity, and resistance to stress; therefore, the high diversity of microbes serves as a stabilizing force in environmental systems (Torsvik and Ovreas 2002).

The study presented here aims at providing a baseline index of bacterial diversity along the salinity gradient in the lower Hudson Estuary. Previous studies of aquatic bodies encompassing differing salinities have revealed significant changes in bacterial species composition (Benlloch et al. 2002). In the study by Benlloch et al. (2002), lower

salinity levels supported multiple assemblages of bacteria, while higher salinities supported one dominant yet diverse bacterial group. Additionally, comparisons of freshwater and oceanic bacterial communities revealed an overall absence of one major type of bacteria, the *ß-Proteobacteria*, in salt water (Methe et al. 1998). *ß-Proteobacteria* have been uncovered in most freshwater systems; however, their presence or absence in aquatic systems with both freshwater and saltwater components has yet to be determined. Based on the aforementioned studies by Benlloch et al., it is hypothesized that the more saline environments house lower, but more specialized bacterial diversity, while the lower saline environment (West Point) supports higher, but less specialized diversity.

Study Approach

Presently, two main approaches are used to study bacterial diversity – culturing bacterial cells and obtaining genetic sequences from bacterial cells. Although culture techniques better correlate biological diversity with functional diversity, less than one percent of bacteria can be cultured with current techniques, making it difficult to obtain information on total diversity (Torsvik and Ovreas 2002). Sequencing the highly conserved 16S region of bacterial DNA allows a significant portion of bacterial genomes to be amplified and eventually sequenced (Amann and Kuhl 1998). Colony cloning techniques by PCR reactions involving 16S rRNA are better at detecting overall diversity and also have the capability of detecting bacteria in low quantities. Thus, this study utilized colony cloning and sequencing techniques for categorizing bacterial samples.

METHODS

Sample Collection

Sampling took place on July 12, 2003. Mid-river samples were taken along the Hudson River in New York State at West Point (N 41° 23.809, W 073° 56.938), Piermont (N 41° 02.263, W 073° 52.865), and the Battery (N 40° 41.77, W 74° 01.76). At each location, subsurface (15 cm below surface), and deepwater samples were taken. The deepwater sample depths were dependent on the depth where salinity was at its maximum. Replicate samples were taken at each site. Samples were collected using a sterilized mechanical release collection bottle, which was rinsed with distilled water between samples to reduce cross contamination of samples. Samples were stored on ice in sterilized Nalgene 500 ml bottles. At each location, temperature, water quality (appearance), and salinity (measured with a conductivity meter) were recorded.

Filtration and DNA Extraction

Bacteria were collected via vacuum filtration using sterilized Nalgene disposable filter units. Water samples were first pre-filtered to remove large particles, then poured through filters with 0.2 um pore sizes to trap bacteria. Samples were then stored at –20° C until further processing.

DNA was extracted from the bacterial cells using both mechanical and chemical extraction techniques. Filters were combined with extraction buffer (100mM Tris-HCL, 100mM sodium EDTA, 100mM sodium phosphate, 1.5 M NaCl), proteinase K, and lysozyme (final concentrations 10 mg/ml). The samples were then shaken at 225 rpm for 30 minutes at 37° C. Next, 1.5 ml of 20% SDS solution was added to the samples, after

which the samples were incubated for 2 hours at 65° C. Samples were then centrifuged at 6400 rpm for 10 minutes, and the supernatant was collected. The pellet was resuspended in 4.5 ml extraction buffer and 0.5 ml SDS, incubated for 10 minutes and centrifuged for 10 minutes. This process was repeated once, following which the supernatant was pooled and combined with an equal amount of chloroform: isoamyl alcohol mix (24:1). The mixture was centrifuged at 6400 rpm for 10 minutes, and 0.6 vol of isopropanol was added to the supernatant. DNA precipitated out overnight, and the pellet was collected after centrifuging for 30 minutes at 10,000 rpm. The pellet was washed with 70% ethanol, collected again after centrifugation at high speed, and the remaining ethanol was evaporated by letting the pellet air dry in a sterile environment.

Cloning Techniques

The 16S rRNA region of the bacterial cells was isolated and replicated via a standard PCR reaction (33 cycles of 94° C for 1 minute, 55° C for 1 minute, and 72° C for 1 minute). Bacteria-specific 16S primers were used for the reaction. The targeted genome region of the bacterial samples were then implanted in *E. coli* cells using a TOPO TA cloning kit (Invitrogen), following product protocols. Cells were incubated overnight at 37°C, white colonies were selected (white colonies indicate the presence of the16S bacterial insert), and reference plates were made of the white colonies.

Colony PCR and Cycle Sequencing

The 16S inserts were isolated and replicated via colony PCR using M13 forward and reverse primers (30 cycles, see above settings). The PCR products were purified by

combining 1/10 vol ammonium acetate and 2 vol 100% isopropanol, incubating at –20° C, and centrifuging at 3500 rpm for 30 minutes. The pellet was then combined with 2 vol 70% isopropanol and centrifuged at 3500 rpm, and then dried with a speed vacuum.

Products were sequenced via standard cycle sequencing protocols (30 cycles of 95° C for 15 seconds, 50° C for 15 seconds, and 60° C for 4 minutes) and cleaned with ethanol and isopropanol. Sequences were read on an ABI automatic sequencer (Sessitsch et al. 2001).

Data Analysis

Sequences were compared to known sequences in the NCBI database (see www.ncbi.nlm.nih.gov, http://rdp.cme.msu.edu/html/index.html) using a standard BLAST search (default settings). Sequence alignment was completed using Clustal-X (Thompson et al. 1994), and phylogenetic analysis was performed using PAUP software.

RESULTS

Sampling Data

Temperature, salinity, and water quality (appearance of water) were recorded for both subsurface and deepwater samples at all three locations (Table 1). With the exception of the Battery deepwater sample, water temperatures across the remaining samples were equivalent. Salinity ranged from 0.53 mS (millisiemens) to 33.90 mS. West Point, being just north of the salt front, had a constant, extremely low salinity, placing it in a freshwater category. Both the deepwater sample at Piermont and the Battery were more saline than their corresponding subsurface samples, due to the angle of

the salt wedge. Battery water samples were clear with no obvious sediment in the samples, whereas the water from the West Point and Piermont samples were more turbid with a yellowish tint.

Table 1: Water Sample Data – Salinity and Temperature

Sample	Salinity (mS)	Temperature (°C)	Water Quality
Battery Subsurface	29.00	23	Clear, no sediment
Battery Deepwater	33.90	20	Clear, no sediment
Piermont Subsurface	10.19	24	Yellow tint, some sediment
Piermont Deepwater	16.22	24	Yellow tint, some sediment
West Point Subsurface	0.53	23	Light yellow tint, some sediment
West Point Deepwater	0.53	23	Yellow tint, sediment

BLAST Results

Further sequencing and analysis is still being completed; therefore, the results presented here deal specifically with the Battery and West Point samples. As Table 2 indicates, every sample location had overlapping bacterial groups, such as *Citrobacter freundii*. Additionally, many of the closest matches in the NCBI database were uncultured or unidentified bacteria – that is, the only information known about them is their sequence information. All the samples had gram-negative bacteria, but only the West Point subsurface sample had Delta proteobacteria.

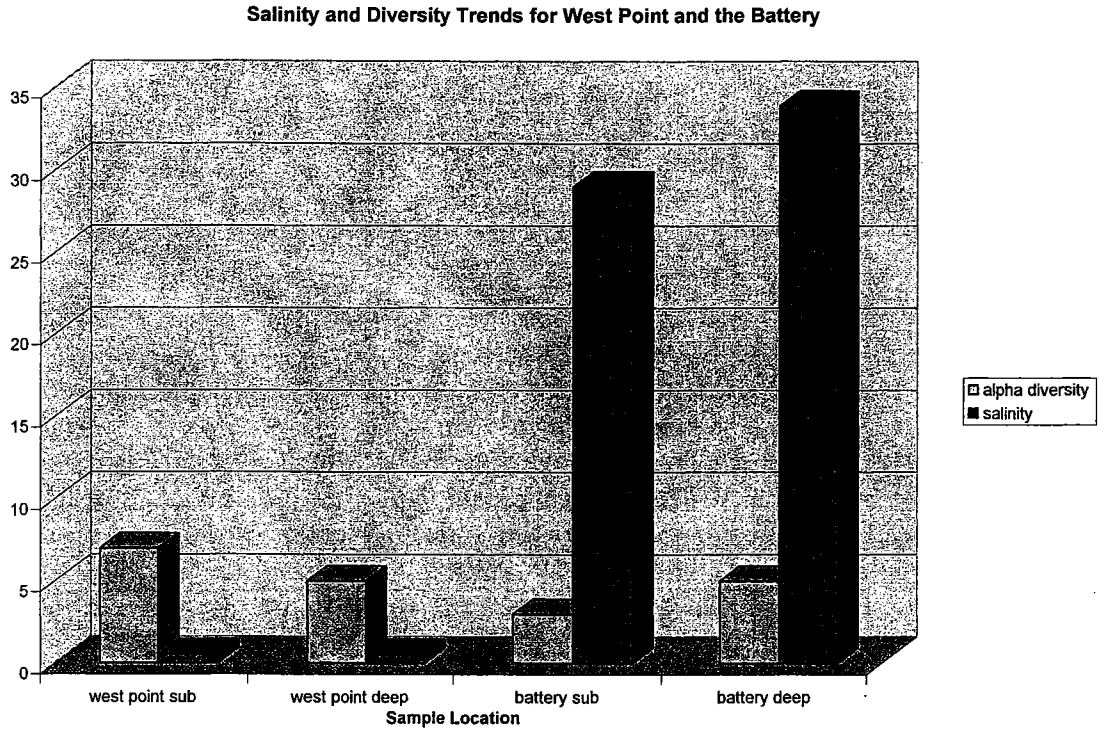
Table 2: BLAST results showing closest matches in the NCBI database to Hudson River sequences and the major bacterial groups represented based on these identifications.

Sample	BLAST Matches	Major Bacterial Groups
Battery Subsurface	*Citrobacter freundii* Uncultured bacterium clone Phs139 Uncultured bacterium clone Phs112	Gram negative Gamma Proteobacteria
Battery Deepwater	*Bacillus cereus*_SH 01 *Citrobacter freundii* Uncultured candidate division OD1 bacterium clone Bol79 Uncultured bacterium clone Phs139 Uncultured bacterium clone Phs131	Gram negative Firmicutes Gamma Proteobacteria
West Point Subsurface	*Bacillus cereus*_SH 01 *Citrobacter freundii* *Stigmatella aurantiaca* Uncultured bacterium clone Phs112 Uncultured bacterium clone Phs139 Uncultured bacterium clone Phs131 Uncultured candidate division OD1 bacterium clone Bol79	Gram Negative Firmicutes Delta Proteobacteria Gamma Proteobacteria
West Point Deepwater	*Bacillus cereus*_SH 01 *Citrobacter freundii* Uncultured bacterium clone BM89PA4BbC5 Uncultured bacterium clone Phs112 Uncultured bacterium clone Phs139	Gram Negative Firmicutes Gamma Proteobacteria

Diversity and Salinity

The West Point subsurface sample displayed the highest diversity, while the Battery subsurface sample displayed the lowest diversity (Figure 1). There is evidence of a trend between salinity and diversity, with higher salinity having lower bacterial diversity, while lower salinity hosts more bacterial types.

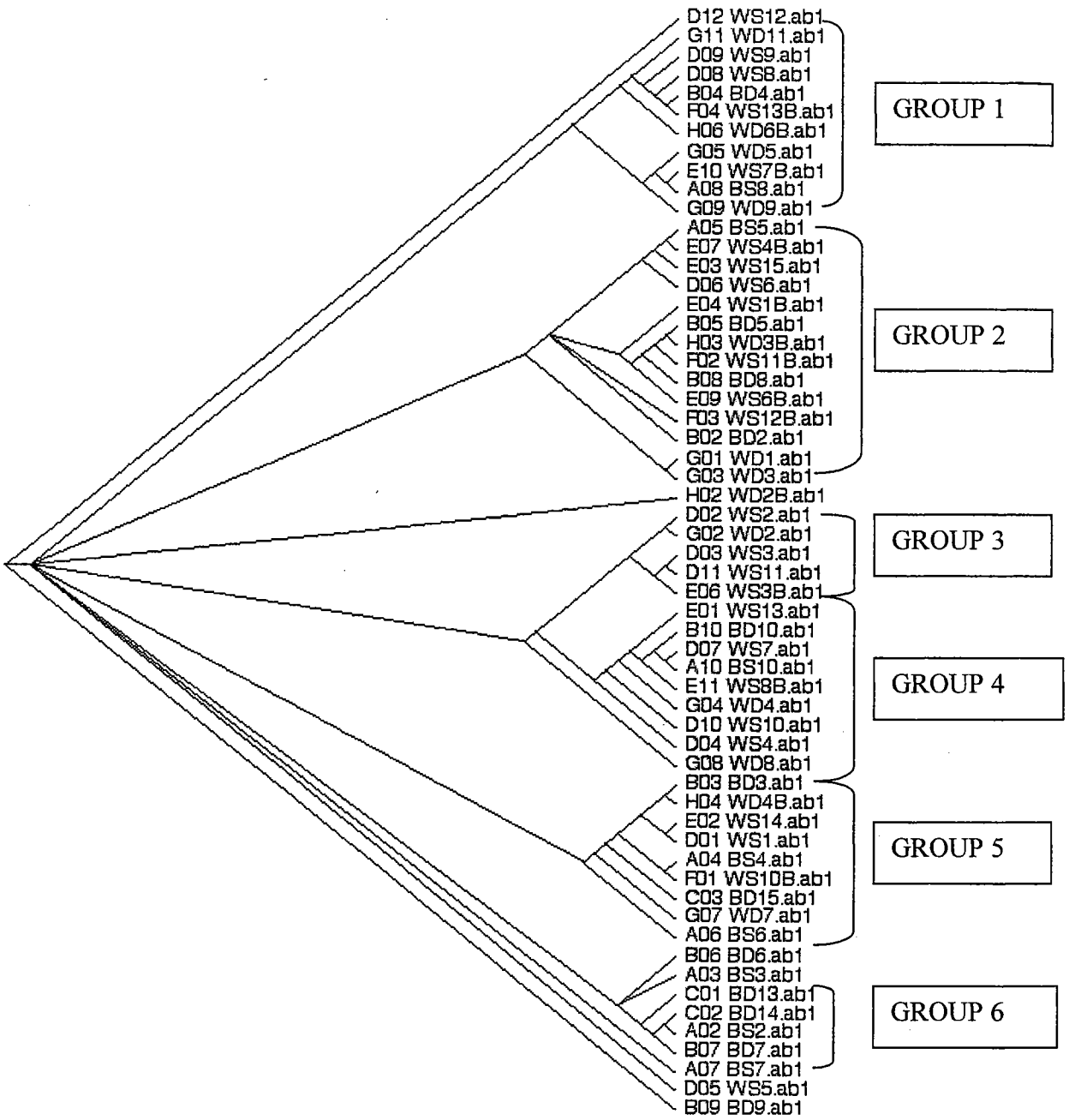
Figure 1: Salinity and Diversity Trends for West Point and the Battery. This figure is based on BLAST matches from the NCBI Database.



Phylogenetic Analysis

Figure 2 shows the majority rule consensus tree of the four sample locations. Using sequence alignment data, PAUP analyzes each base pair as a separate character, compares each character across every sequence, and outputs phylogenetic trees based on the interpretation of these data. These trees show phylogenetic, or evolutionary relationships among the different bacteria. The majority rule consensus tree is the composite of the optimal trees found through the PAUP program, in which at least 50% of the trees had the structure shown in Figure 2. Although the bacterial groups did not separate based on sample location, the tree offers support for six major groups of bacteria.

Figure 2: Majority Rule Consensus Tree Produced by PAUP. This tree is based on the aligned sequence data from every sample thus far processed. Six major bacterial groups are recognized in this tree. WS = West Point Subsurface; WD = West Point Deepwater; BS = Battery Subsurface; BD=Battery Deepwater.



DISCUSSION

Bacterial diversity was assessed by comparing the unknown sequences to known sequences in a national database and by performing a phylogenetic analysis on the aligned sequences. The NCBI database provided a source with which to help identify bacterial species in the water samples, while the phylogenetic analysis showed the overall number of bacterial groups represented across the four samples.

BLAST results supported the hypothesis of higher diversity in more freshwater environments and a lower diversity in more saline conditions. However, it should be noted that there were not equal numbers of sequence clones, and that the sample with highest diversity (West Point subsurface), also had the highest number of clones sequenced. Common species of bacteria were evident in most, if not all of the clones (e.g., *C. freundii*). The West Point Subsurface sample had one bacterial species (*Stigmatella aurantiaca*) that was absent in the other samples. This species is a member of the gamma proteobacteria, and is the only member of this group that was found within the analyzed samples. Further sequencing will reveal the presence or absence of this group from the other samples. Many of the closest matches were with uncultured bacteria, meaning that only the sequence data are known. Phylogenetic analysis of these sequences in reference to known bacterial species' sequences can be a powerful tool in elucidating what major taxonomic groups these samples belong to.

The phylogenetic analysis presented in this paper served to examine the relationships among all the bacterial samples. Parsimony analysis showed evidence for six major bacterial groups among all four sample locations. However, the tree was not resolved (i.e., polytomic), thereby preventing any conclusions to be drawn on the

phylogenetic relationships among the bacterial groups. It is interesting to note, however, that the bacterial sequences did not group according to location, suggesting either the homogeneity of the species or that species at different locations have interactive opportunities in which gene sharing can occur.

In order to resolve these issues, more data are being collected, including sequencing the bacterial clones from the mid-saline location (Piermont) and sequencing more clones from the locations discussed in this paper. Additionally, different 16S primers will be used that will create longer sequences, and thus higher data resolution.

For future research beyond the scope of this project, it would be useful to analyze the morphological characteristics of the bacterial species at these locations by microscopy or culturing in order to gain more information about the functional diversity of the bacterial species at these locations. Additional studies of the same locations throughout the year would help elucidate any seasonal variation or patterns in bacterial diversity. The study presented here and any future studies on bacterial diversity in the Hudson River will help serve as a baseline against which future environmental monitoring studies can be compared with and will also serve to increase our understanding of the complex roles that bacteria serve in the Hudson River.

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