MICROBIAL ECOLOGY OF SOUTH SLOUGH SEDIMENTS: COMMUNITY COMPOSITION OF BACTERIA AND PATTERNS OF OCCURRENCE

by

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Title: MICROBIAL ECOLOGY OF SOUTH SLOUGH SEDIMENTS: COMMUNITY COMPOSITION OF BACTERIA AND PATTERNS OF OCCURRENCE

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The description of any community depends on the spatial, temporal, and organizational parameters chosen. It is essential to understand how patterns and dynamics vary at multiple scales in order to fully understand the community. In this dissertation, I exercised a spatially explicit approach to ask the following fundamental questions about microbial community structure in estuarine soft sediment habitats. (1) What are the patterns of variability within a site and among estuarine sites? (2) Are the same patterns found in all intertidal soft sediment habitats or only in the estuary? (3) Do restored and estuarine sediments have similar microbial assemblages? (4) Is there a relationship between the growth dynamics of algal mats and the organization of bacteria communities? The nuclear gene encoding the small subunit of the ribosome, 16S rDNA, was used to identify characteristics of the community and to identify unknown bacteria. The difference between estuarine and outer coast soft sediments was comparable to genetic diversity differences observed between restored and mature estuarine sites.

Bacteria communities among mature estuarine sites differed, though to a lesser degree. Each mature site had a distinct community fingerprint, which suggested that the environment selects for a suite of species. Similarly, there was not an estuary wide community fingerprint associated with algal mats. However, when each site was analyzed separately, the bacteria associated with algal mats were distinct from bacteria in sediments with no mat. Observed differences in genetic diversity between a restored site and a mature estuarine suggested several testable hypotheses about succession of bacteria communities after habitat restoration. Restored estuarine sediments provide a greater supply of available energy to the community, and observed diversity differences may have resulted from reduced competition for substrate. A number of newly discovered sequences demonstrated that the majority of bacteria from intertidal soft sediment habitats have not been cultured. The phylogenetic position of the newly contributed sequences may help optimize culture strategies, thus contribute to an understanding of the relationship between genetic diversity and ecosystem function.

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CHAPTER I

GENERAL INTRODUCTION

Bacteria are extremely diverse and they occur in a range of habitat types. For example, bacteria have been discovered deep in the lithosphere (Ghiorse 1997), in hot springs (Pace 1997), and in close association with hydrothermal vents (Reysenbach and Cady 2001). Bacteria are a ubiquitous component of the biosphere, but their organization is not haphazard and random. These observations pose a series of fundamental questions: (1) how are communities of bacteria organized, (2) what are the limits of distribution of a bacterium, (3) how do natural and anthropogenic gradients affect the spatial distributions of bacteria, and (4) how can variability in spatial distribution improve our understanding of estuarine ecosystems?

Estuaries are ideal places to understand the organization and variability of microbial communities. Salinity, temperature, and redox potential are some of the gradients that affect distributions of organisms. Bacteria have an important role as prey for other estuarine dependant species and as the mediators of decomposition. Microbes, particularly bacteria, are central to the process of decomposition in marine and estuarine ecosystems. A description of the community composition across abiotic and anthropogenic gradients will improve our understanding of estuaries. The anoxic intertidal sediments of estuaries are also an ideal place to look for taxa that

biodiversity and improve our understanding of bacterial phylogeny. Estuaries are often developed for their capacities to deliver goods or provide rich soils for agriculture. Therefore, they have been impacted by human activities which have included dredged channels and drained salt marshes. The loss of coastal habitat in estuaries has prompted restoration activities, which provide an opportunity for improving degraded habitats. The pattern of recovery of bacteria in restored sediments may provide insights about restoration.

This dissertation details the application of DNA-based markers to describe phylotype distribution patterns of bacteria that occur in marine and estuarine habitats on the coast of southern Oregon, USA. The distribution of bacteria phylotypes across natural and anthropogenic gradients was determined with denaturing gradient gel electrophoresis of the 16S rDNA molecule. 16S rDNA is a gene common to all prokaryotic life forms, plus mitochondria and chloroplasts. It is a well known marker that has an extensive database of published sequences that can be used for detecting the relatives of previously uncultured bacteria. It does not have information about the metabolic capacity or the physiological state, but can be amplified directly from the environment.

The next chapter, Chapter II, uses a spatially explicit approach to understand the patterns of occurrence of microbial communities in a small estuarine system. The striking conclusion from this chapter is that the scale of an investigator's perspective reveals distinct patterns among estuarine sites. For example, there was no pattern across the redox gradient but bacterial communities were consistently different between raked plots (homogenized) and control plots. These within site patterns were insignificant when considering the variability among multiple estuarine, which showed a strong site specificity. On larger scales, bacteria communities in estuaries showed distinctive differences from communities in marine habitats. Chapter III examines genetic diversity following the restoration of tidal circulation to a degraded wetland

examines genetic diversity following the restoration of tidal circulation to a degraded wetland habitat. The genetic diversity was greater in the restoration site than a mature site, which may reflect the pre-restoration impoundment. Chapter IV investigates the percent cover of an algal mat species, whose peak cover during the summer months follows the peak in biomass shown for other estuarine primary producers. Finally, Chapter V reconstructs a phylogenetic hypothesis of the phylotypes that were isolated from the intertidal mudflats. These results are the first known description of the uncultivated bacterial component from this habitat and represent a significant contribution to estuarine microbiology. Chapters II and III are co-authored with my major professor, Dr. Lynda Shapiro. Chapter III has been submitted for publication in Applied and Environmental Microbiology.

CHAPTER II

LANDSCAPE-LEVEL PATTERNS OF OCCURRENCE OF BACTERIA FROM COASTAL SOFT-SEDIMENT HABITATS

Abstract

The description of any community depends on the spatial, temporal, and organizational perspective chosen. It is essential to understand how patterns and dynamics vary at multiple scales in order to develop a predictive capability. In this study, I exercised a spatially explicit approach to ask fundamental questions about the structure of microbial communities in estuarine soft-sediments. The co-Author of Chapter II was my major professor, Lynda Shapiro, who has guided me through the thought process and assisted in editing drafts. The ideas and arguments that accompany these data were collected and analyzed by my hand. A bacterial community fingerprint was generated from amplified 16S rDNA genes that were separated with Denaturing Gradient Gel Electrophoresis (DGGE). Molecular sequence data for bacteria isolated from any given habitat circumvents the need for cultivation and provides a molecular-phylogenetic framework to describe microbial diversity based on 16S rRNA sequence. Results from this study demonstrate that patterns of variation at small scales were not universal and important only in select locales. The redox potential discontinuity layer (RPD) was expected to be a physiological boundary separating the aerobic community from the anaerobic community. Surprisingly, only Hidden Creek, showed evidence that the RPD layer was an important feature separating

microbial communities. The amount of variability at larger spatial scales showed a striking pattern of site-specific identity. With few exceptions, samples collected within a site were more similar than samples from different sites. Bacterial communities collected from estuarine sites were distinct from the bacteria collected from intertidal marine mud. To clarify the differences in bacterial communities that inhabit marine and estuarine soft-sediments, DGGE bands were reamplified and sequenced. The higher salinity marine site yielded sequences that were related to the α-proteobacteria. We included an estuarine restoration site in the analysis and found that the restoration site is becoming more similar to other mature estuarine sites through time. This observation shows that communities of bacteria may be a promising metric for gauging success of future restoration projects. Transplant experiments and other field manipulation studies are needed to follow up on the growing list of hypotheses that have been developed to explain why specific lineages are restricted to precise environments.

Introduction

Communities of heterotrophic bacteria are associated with oxidation of deposited organic matter, regeneration of inorganic nutrients, and food web support (Fenchel *et al.* 1998). Cultivated isolates and biogeochemical field measurements have provided limited information about the role of microbes in marine and freshwater sediments (Capone and Kiene 1988). Estuarine and marine sediments were shown to have strong redox gradients in which steepness varies depending on location and sediment size (Fenchel and Riedl 1970). Theoretical calculations predict the occurrence of thermodynamic zones of respiration (Chester 1990) Associated with these zones, is a progression of bacterial functional groups. It is unknown whether the thermodynamic zones of respiration are also associated with a change in species composition.

Historically, natural abundance and diversity of microbial populations were estimated by routine culture techniques. Anaerobic bacteria, common in estuarine and marine sediments, were difficult to identify due to the absence of information about enrichment conditions. Studies from several types of habitats estimate that more than 99% of microscopic organisms cannot be cultivated by routine techniques (Amann *et al.* 1995, Pace 1997). Molecular sequence data isolated from any given habitat circumvents the requirement of cultivation and provides a molecular-phylogenetic framework (Pace 1997) to describe microbial diversity based on molecular sequence. The conservative properties of the 16S rDNA gene have allowed alignment of sequences spanning over 1 billion years of microbial evolution. It is an ideal molecule to use as a marker in the environment because it is shared by all bacteria.

Recent research suggests that environmental conditions at distinct geographic locations select for a distinct assemblage of prokaryotic organisms. Crump *et al.* (1999) used this postulate to explain why distinct bacteria assemblages were observed along a gradient in the Columbia River estuary, Oregon, USA. The hydrodynamic regime of the estuary mixed a distinct riverine and a marine bacterioplankton community. The free-living fraction never formed a distinct estuarine assemblage and was rapidly flushed out of the estuary. In contrast, the particle-associated fraction was retained long enough to develop a third, uniquely estuarine assemblage. Similarly, the spatial distribution of free-living heterotrophic bacterioplankton assemblage was observed in two sub-estuaries of the Chesapeake Bay (Bouvier and del Giorgio 2002). Members of the Class β-proteobacteria dominated the upper estuary, the class α-proteobacteria dominated the lower estuary and the Phylum *Bacteroidetes* prevailed in the middle estuary. This observation suggested that some lineages were restricted to specific environments because of physiological tolerances, growth optima, Dissolved Organic Carbon (DOC) concentrations, and associations with the estuarine turbidity maximum (ETS). Populations of small-sized organisms are not

ubiquitous; they appear to be subject to selective forces at kilometer scales that influence diversity and abundance. Patterns at smaller scales, however, are less apparent due to technological restraints on a scientist's ability to sample at extremely fine scales.

Spatial variability in distributions of soil organisms is thought to be accompanied by a predictable spatial structure (Ettema and Wardle 2002). Land use (Fromm *et al.* 1993), topography (Robertson *et al.* 1997), tree species patch size (Saetre 1999) and corn plant spacing (Cavigelli *et al.* 1995) have been suggested as major factors influencing microbial spatial patterns. In creek bank sediments (Franklin *et al.* 2002), elevation above sea level and the degree of tidal flooding were postulated to be important determinants of community composition and structure.

Field studies that described the distribution and genetic diversity of bacteria are particularly important in coastal and estuarine ecosystems where efforts are underway to restore drained and diked marshes (Zedler 2000). Sediments undergo visible and measurable changes following reintroduction of tidal flooding to previously diked marshes (Portnoy and Giblin 1997). The sediment and its biological components set an ecological foundation for later colonization by plants and invertebrates (Callaway 2001). Restoration success and the timing of re-colonization rely on the return of proper soil conditions, including the presence of a bacterial community. Short and long term monitoring goals may be set more accurately with a description of the number of species, genetic diversity in the bacterial community, and the variability of bacterial communities in nature.

The description of any community depends on the spatial, temporal, and organizational perspective chosen. It is essential to understand how patterns and dynamics vary at multiple scales in order to develop a predictive capability. In this study, the bacteria assemblage was observed and compared on several scales. A gene common to all bacteria, 16S rDNA, was

chosen to track the distributions of taxa. I used a spatially explicit approach to ask the following fundamental questions about microbial community structure in estuarine soft sediment habitats. (1) Does the microbial community show variability above and below the redox potential discontinuity? (2) Do meter scale patches of benthic algae limit the distributions of microbial populations? (3) Are the same patterns found in all intertidal soft-sediment habitats or only in the estuary? I predicted that the redox potential discontinuity (RPD) layer would limit microbial populations so that the assemblage above the RPD would be different than below, and that similar taxa would be found above and below the RPD at a number of estuarine sites. I also postulated that tidal pools and algal mats at m-scales could explain the variation within each site. Finally, I predicted that a distinct estuarine community could be identified that was different from the intertidal sediment community outside the estuary.

Materials and Methods

Study Areas

The South Slough National Estuarine Research Reserve (SSNERR) is a small drowned river mouth estuary that is heavily influenced by marine conditions (Figure 1). Summer months bring limited rainfall which produces a salinity range of 27-32 PSU in the upper estuary and 30-33 PSU in the mid estuary (NOAA, Central Data Management Office, 09/01). Nutrients (unpublished data), phytoplankton (Hughes 1999, Cowlishaw 2002), and chlorophyll a (Roegner and Shanks 2001) are advected into the estuary from the nearshore ocean during flood tides.

The estuarine sites in SSNERR are characterized by broad mudflats, channelization, patches of algal mats and fringing salt marsh vegetation (Rumrill 2002).

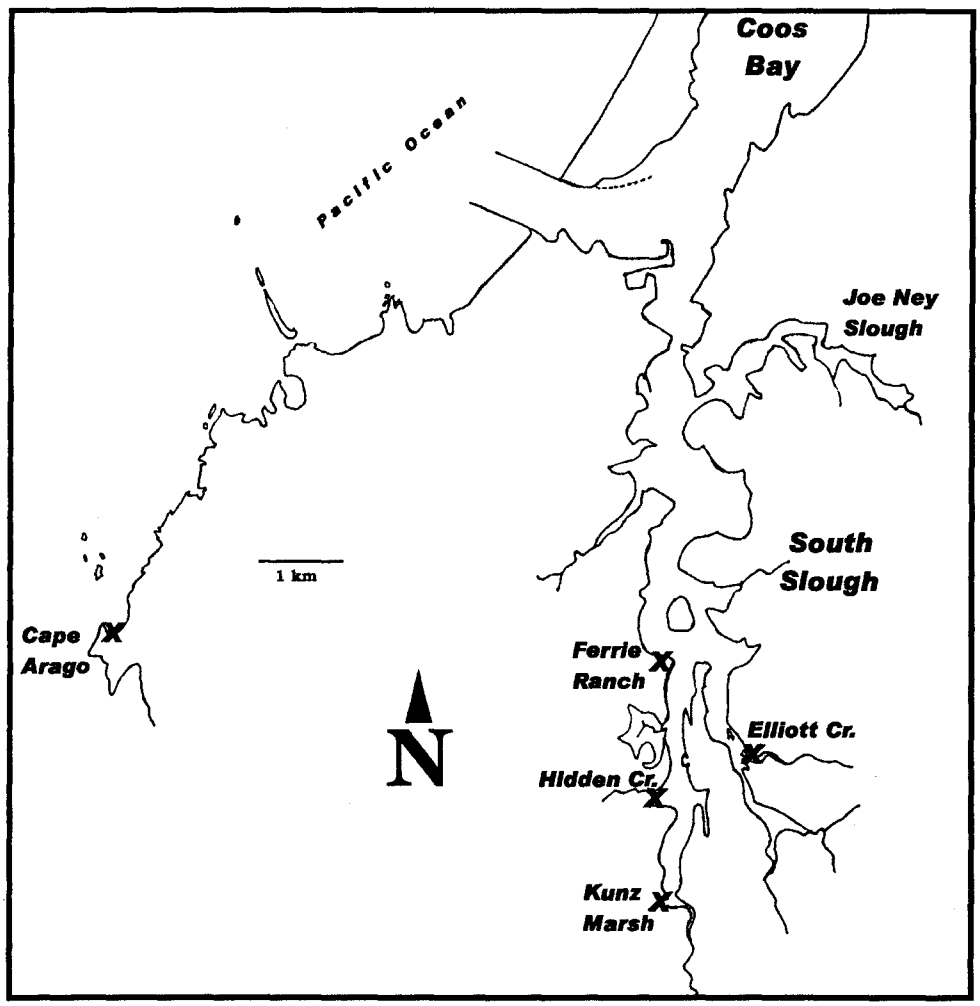

Figure 1. Map of the South Slough Arm of Coos Bay, Oregon, USA. X indicates the location of 5 sample sites that span a gradient from fully marine sediments of Cape Arago to the estuarine sediments of Ferrie Ranch, Elliott Creek, Hidden Creek, and Kunz Marsh.

Unvegetated tideflats free of emergent salt marsh plants were chosen to measure communities of bacteria in the absence of any habitat complexity introduced by plant communities. These mudflats were devoid of vascular plants, but had a mosaic of mat forming algae (*Oscillatoria* spp., *Vaucheria longicaulis, Rhizoclonium* spp., *Chaetomorpha* spp.).

The North Cove of Cape Arago (CA) is a marine site protected from the open ocean by an expansive rock reef that extends around the cape (Figure 1). Sediment has accumulated in the protected waters of North Cove resulting in a soft-sediment macrofaunal community typical of an embayment or estuary.

Sampling Design

A. I focused on centimeter (cm) and meter (m) scale variability within South Slough National Estuarine Research Reserve (SSNERR) in August 2001. Core samples were collected in a cut off 140 cc syringe, with a diameter of 3.2 cm (Walters and Moriarty 1993). I collected samples in four estuarine sites in two replicate groups separated by a distance of 3 m (Figure 2). In each group, two depth fractionated core samples (140 cc modified syringe) were collected at a separation distance of 3 cm.

Genomic DNA was extracted from the sediments and used to amplify the 16S rDNA gene. Amplified sequences from PCR were separated with Denaturing Gradient Gel Electrophoresis (DGGE). I tested two *a priori* hypotheses to describe within site patterns of genetic composition: (1) All replicates within a depth fraction are more similar than between depth fractions. (2) Replicates collected within a patch are more similar than between patches within a site.

B. A second component of the 2001 sampling was to determine if sampling within a m-scale disturbance plot would yield less variability than sampling haphazardly in undisturbed plots.

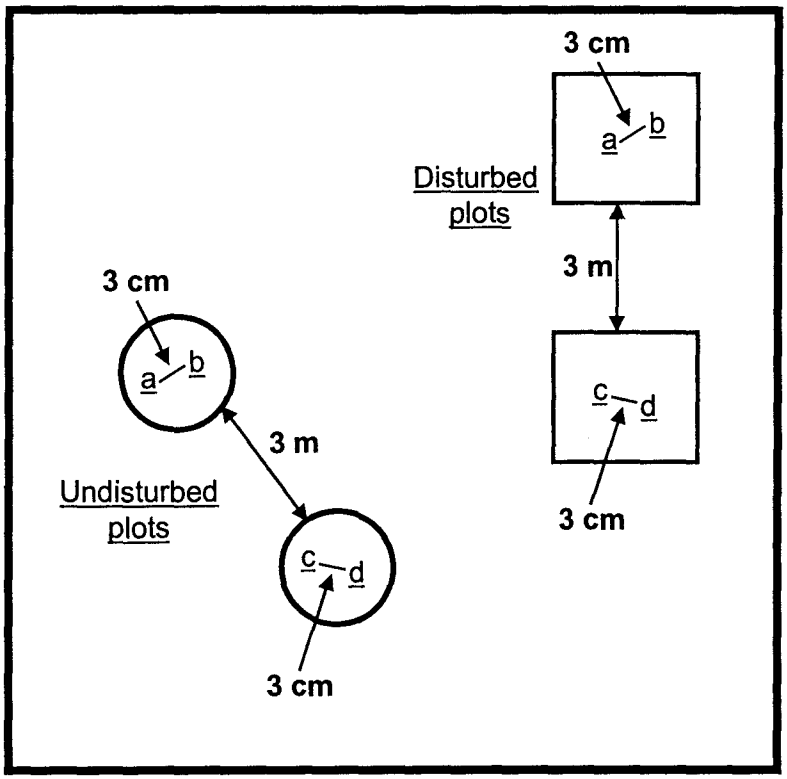

Figure 2. Sampling Design for Assessment of Within-site Variability. Core samples were collected in a spatially arranged pattern drawn in the above figure. Four replicates from undisturbed and four replicates from disturbed plots were collected from all four estuarine study sites in August 2001.

Mudflats in SSNERR are a patchwork of algae, water, and sediment. An artificial mosaic in all four estuarine sites was created by homogenizing the upper 2-3 cm of sediment in 2, 1 X 1-m plots. Disturbance plots were separated by a distance of 3 m. A garden rake was used to homogenize the upper 2-3 cm of the sediment's surface and samples were collected 24-hours later. I hypothesized that similarity within the disturbance plot would be greater than between disturbance plots.

C. The 2002 sampling regime focused on placement of the estuarine sites in context with a marine site. In order to do this, fewer replicates from Hidden Creek and Kunz Marsh and one from a marine 3 m. A garden rake was used to homogenize the upper 2-3 cm of the sediment's surface and samples were collected 24 hours later. It was hypothesized that replicates within the disturbance plots would show less variation than in surrounding undisturbed sediment. The success of 16S rDNA amplication in disturbed plots was greatly reduced, which resulted in missing data in Hidden Creek and Elliott Creek.

Extraction of DNA from Estuarine Sediments

DNA extraction was conducted by a direct lysis method modified from Zhou *et al.* (1996). Upon returning to the lab, DNA was extracted immediately from the sediments or stored at -80°C. We added 100 µl of 5M NaCl, 1 g of sediments, and then added 750 µl of CTAB Lysis buffer (pH 8.0, 1% CTAB, 0.05M Tris, 0.05M EDTA, 0.05M NaH_2PO_4, 1.5M NaCl). The sediment slurry was mixed and incubated at 37°C for 30 min with Proteinase K (5 µl, 20 mg/ml). We added 100 µl of 20% SDS to the mixture followed by an incubation at 65°C for 1 hour, at -80°C for 1 hour, and at 65°C for 1 hour. The boil/freeze/boil sequence was followed by a 25:24:1 extraction in phenol/chloroform/isoamyl. The aqueous extract was removed, precipitated with

0.6 volumes isopropanol, and incubated overnight at –20°C. Precipitated nucleic acids were pelleted for 30 minutes at 15,000g, washed with 70% ethanol, and air dried. Pellets were resuspended in 100 µl Sigma molecular-grade water and incubated overnight at 4°C. Crude extracts were not amendable to enzymatic reactions or restriction digestion, so they were agarose gel purified (Li and Ownby 1993). Genomic DNA extracts may not be uniform with regards to taxonomic affiliation. It is thought that some taxa are extracted and amplified more efficiently (Hugenholtz *et al.* 1998). It has been shown that heteroduplexes on DGGE gels can contain bands with multiple sequence identities (Sekiguchi *et al.* 2001). We attempted to minimize bias in the genomic DNA extraction by following the methods of Zhou *et al.* (1996). We also minimized the amount of PCR inhibitors, common to sediment samples, by gel purifying all crude extractions prior to PCR analysis.

PCR Amplification of 16S rDNA Gene

Ten µl (~40 ng) of gel purified template was used in a 50 µl PCR reaction to amplify small subunit (SSU) 16S rDNA. Primers gc338F and 519R amplified a hypervariable region of the 16S rRNA gene (Muyzer *et al.* 1993, Ferrari and Hollibaugh 1999, Jackson *et al.* 2001). These PCR products were used in DGGE to observe community profiles of bacteria. Each reaction contained 5 µl 10X buffer, 5 µl 25 mM $MgCl_2$ (Applied Biosystems) 1.2 µl 2.5 mM dNTP, 10 U Amplitaq LD, 1.5 µl 10 pmol forward primer and 1.5 ul 10 pmol reverse primer. The reaction was brought to 95° C for 3 minutes then cycled 35 times at 95°C for 30 seconds, annealed at 48°C for 30 seconds, and extended at 72°C for 30 seconds. After cycling, the reactions were held at 72°C for 10 minutes. Given the universal nature of the Domain level primers (gc338F, 519R) and the ubiquity of bacteria in the environment, contamination of

samples was of great concern. We frequently checked for contamination by routinely running negative controls (PCR reactions with no template DNA added).

Separation of 16S rDNA by Denaturing Gradient Gel Electrophoresis (DGGE)

A Biorad D-Code gel rig was used to separate similarly sized, amplified rDNAs by melting domain (Biorad, Hercules, CA). All reagents were prepared as described in the D-Code Instruction manual and Applications Guide (Biorad, Hercules, CA). The denaturing gradient was 20% to 60% by weight urea and formamide (Piceno *et al.* 2000). Gels were loaded with 15 µl of the PCR reaction plus 5 µl of loading dye (0.01% Bromphenol Blue, 40% glycerol), and run for 3 hours at 200 V. Gels were stained with SYBR Gold nucleic acid gel stain (Molecular Probes, Eugene, OR), and photographed on Polaroid film. Polaroid gel images were scanned into digital format and inverted for analysis.

DGGE Gel Analysis and Statistics

A series of DGGE gels were run in order to compare replicates from environmental samples. Gels were analyzed following van Hannen *et al.* (1998, 1999), and Schäfer and Muyzer (2000). Individual bands that composed the bacterial community fingerprint were identified using Gel Pro 4.0, 1-D gel routine. All bands, including heteroduplexes, were included in the analyses since their appearance was characteristic of the sample and informative. A binary matrix was derived by identifying presence (1) or absence (0) of a band using 1.15% minimum peak height in GelPro Analyzer 4.0 (Media Cybernetics). A total of 28 bands in 2001 and 27 bands in 2002 were used for analyses of similarity. Binary matrices were used to calculate Bray-Curtis similarity coefficients (PRIMER v.5.2, Clarke and Gorley 2002). A hierarchical cluster analysis and a non-metric MDS plot (Clarke and Warwick 2001) were used to visualize spatial

patterns of variability. To test the similarity within *a priori*-defined groups, a one-way ANOSIM (PRIMER-E) was applied. ANOSIM applies a non-parametric permutation procedure to test the null hypothesis that there are no differences in similarity between or among test groups (Clarke and Green 1998). ANOSIM was applied because the multivariate dataset did not meet the normality or homoscedasticity assumptions required by parametric statistics.

Sequences of Prominent DGGE Bands

DGGE bands of interest were selected for reamplification. Direct sequencing of reamplified bands was not reliable. Therefore, selected bands were reamplified with 338F and 519R and cloned with a Invitrogen TA cloning kit (Invitrogen CA). Clones were screened by DGGE and run alongside an environmental sample. Clones that contained the desired insert were prepared for sequencing with a mini-prep kit following manufacturer's instructions (CPG inc.). The insert was sequenced with M13 primer at the Center for Gene Research and Biotechnology, Oregon State University, Corvallis, OR.

Missing Data

There were cases where the PCR amplification did not produce a product that could be visualized on the DGGE gel. In 2001, the following lanes contained no data: K0b, F0c, F0d, Fd0c, Fd0d, Fd1a, Fd1b, E1d. The disturbance treatment caused higher than usual failure of the PCR amplification and therefore no disturbance plot data was collected in either Hidden Creek or Elliott Creek.

Results

Variability in Bacterial Communities within Sites

Six DGGE gels were run in 2001 and used to examine within site (m, cm) spatial heterogeneity of bacterial communities (Figure 3). After within site comparisons were made, the gels were compared with internal markers and a universal ladder to make between site comparisons (Figure 4). Three lanes containing a marker plus a set of internal standards were used to determine the position of DGGE bands (Gel Pro 4.0). The marker lane contained four bands whose migration served as reference for the migration distance of unknowns. Bands that were used as internal standards were darkly staining among all estuarine sites.

Bacterial communities collected from the Hidden Creek site showed high similarity among fingerprints from a single depth fraction (Figure 4). Hidden Creek 0-1 cm fraction and 1-2 cm fraction were significantly different ($p<0.03$, Global $R = 0.81$). The null hypothesis that the genetic composition of the bacterial community was identical in 0-1 cm and 1-2 cm depth fractions was rejected for the Hidden Creek site. The bacterial assemblage from depth fractions 0-1 cm and 1-2 cm in Kunz Marsh ($p = 0.54$, Global $R = 0.03$), Kunz Marsh disturbance plot ($p<0.09$, Global $R = 0.354$), Elliott Creek ($p = 0.68$, Global R 0.056), and Ferrie Ranch ($p = 0.27$, Global R 0.143), Ferrie Ranch disturbance plot ($p = 0.33$, Global R 0.0) were not significantly different. The samples collected 3 centimeters apart (a,b or c,d) were not more similar than those collected 3 meters apart (Figure 4). Replicates a,b,c,d were randomly grouped in control plots and therefore there was no visible pattern of organization at the meter scale. No differences were observed between patches and therefore statistical tests were not applied.

Samples collected in disturbed plots showed variability between patches, but not within a patch. Samples collected 3 cm apart were more similar then those collected 3 m apart. Disturbed plots in Kunz Marsh (Kd) showed that replicates a, b had high similarity (80) regardless of the depth fraction (Figure 4). There was variability between patches but not within patch.

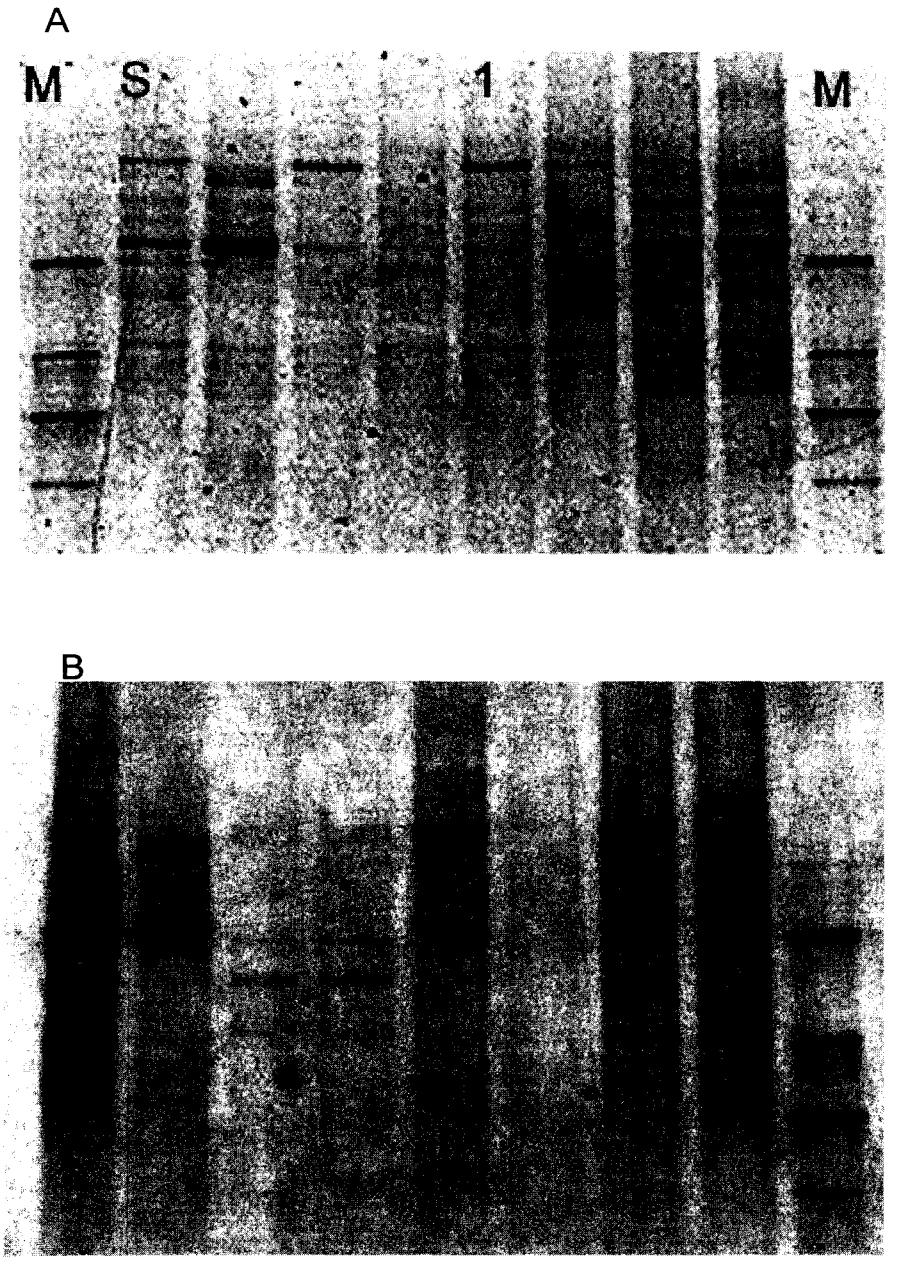

Figure 3. DGGE Gel Images from 2001. The first four lanes are replicates a, b, c, and d from the 0-1 cm fraction. The second four lanes are replicates from the 1-2 cm fraction. Lanes marked M were lanes with known sequences and served as a marker. (A) Ferrie Ranch, (B) Ferrie Ranch disturbance plots, (C) Kunz Marsh, (D) Kunz Marsh disturbance, (E) Hidden Creek, (F) Elliott Creek.

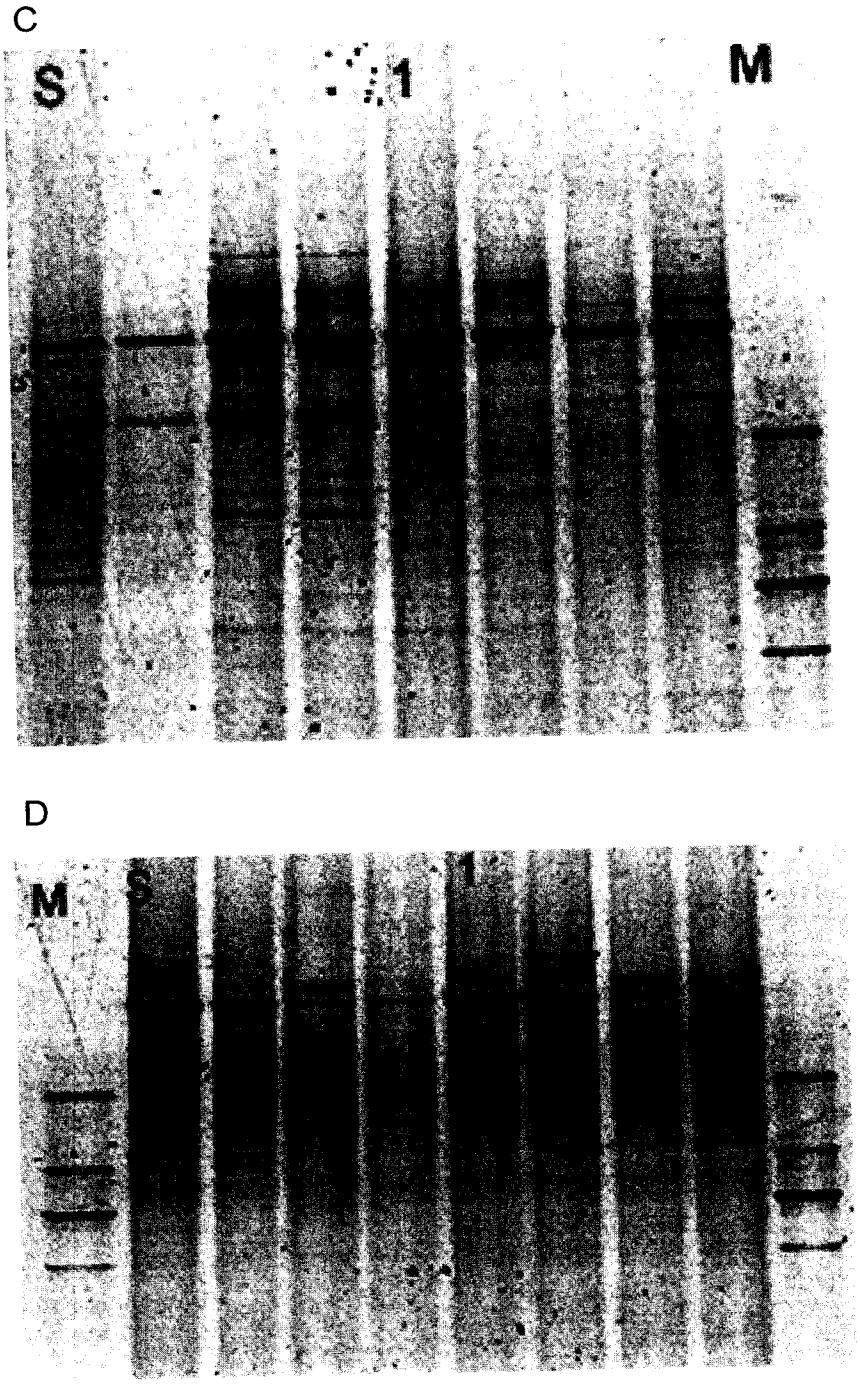

Figure 3 (cont.). DGGE Gel Images from 2001.

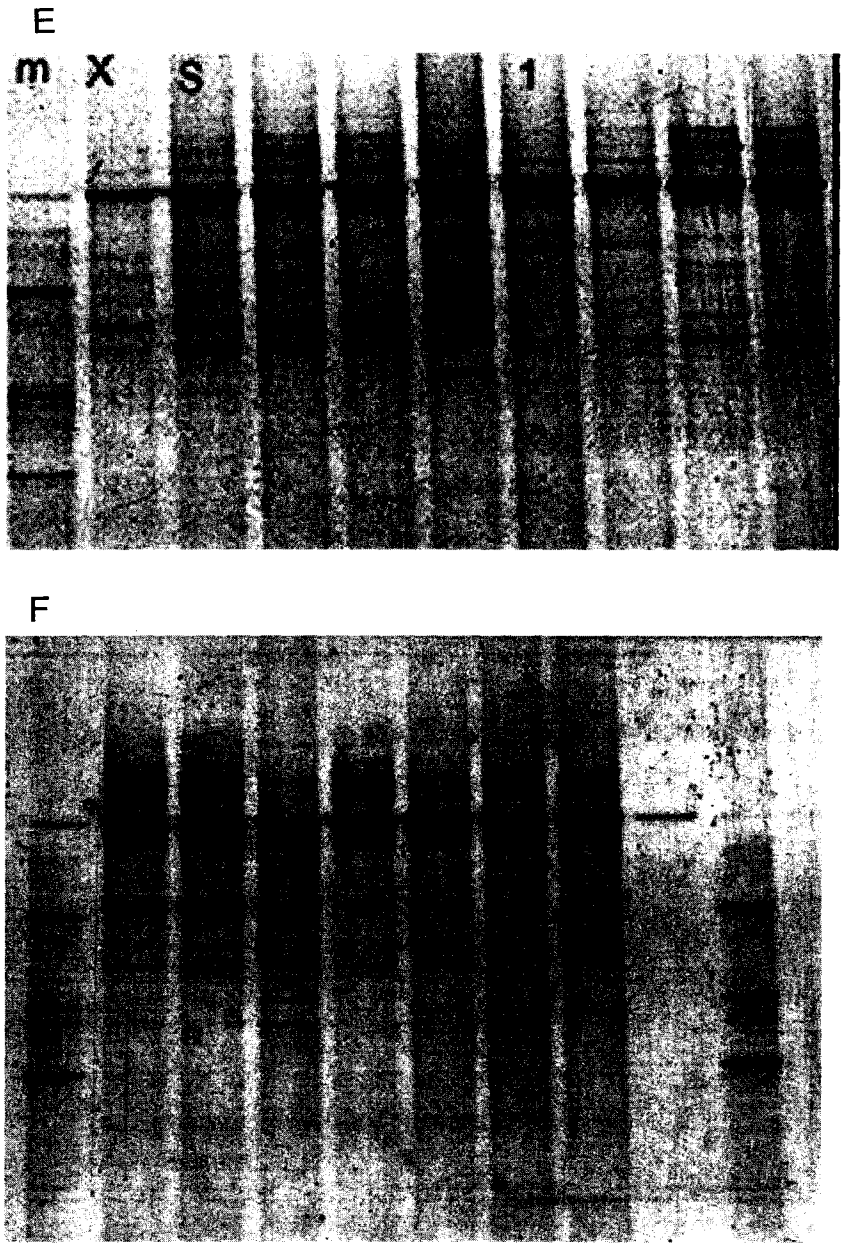

Figure 3 (cont.). DGGE Gel Images from 2001.

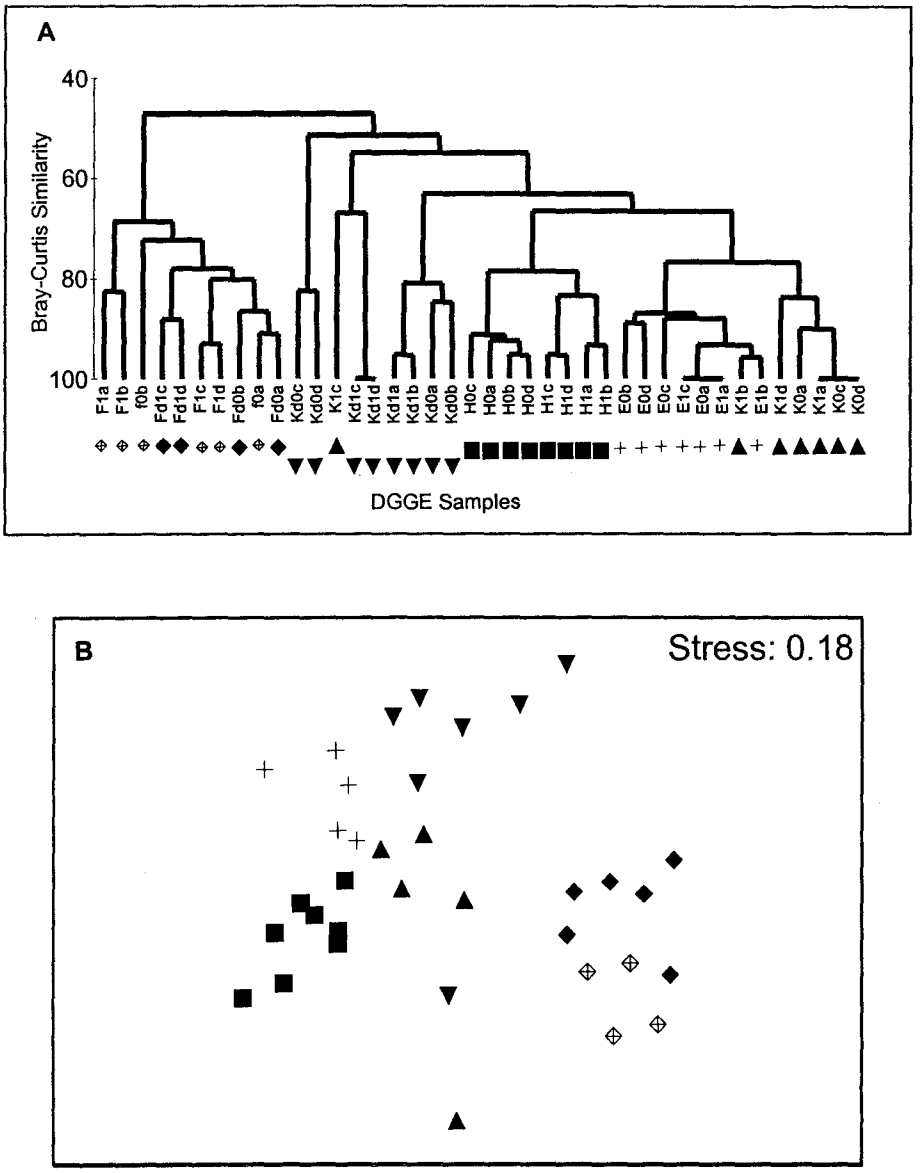

Figure 4. Cluster Analysis and MDS from 2001 DGGE Gels. A similarity table was calculated and used to plot dendrogram (A) and non-dimensional MDS plots (B). Site designations are: F, Ferrie Ranch, K, Kunz Marsh, H, Hidden Creek, E, Elliott Creek. Disturbed plot samples are marked with (d), depth fractions are marked: 0 (0-1 cm), or 1 (1-2 cm) and replicate is marked (a,b,c,d). Symbols in the MDS plot correspond to the designations in the cluster dendrogram.

Replicates c,d from the 0-1 cm fraction (80) and c,d from 1-2 cm fraction grouped together (100). The same pattern was found in Ferrie Ranch, site of a second set of disturbance plots. Disturbance (fd) replicates c,d grouped together (88) and a,b grouped together (85). The number of bands that could be counted from DGGE gels was always higher in disturbance treated samples than in undisturbed samples.

Variability in Bacterial Communities within South Slough Estuary

Generally, sites exhibited a high degree of site-specificity supported by site groupings in cluster analyses (Figure 4). Similarity within site was highest within Elliott Creek (88), followed by Hidden Creek (78), Ferrie Ranch (68) and Kunz Marsh (52). Similarity among all estuarine sites in 2001 was only 47. Similarity values for comparing estuarine sites were higher when the samples from the disturbance treatment were excluded. A one-way ANOSIM was used to test the null hypothesis that communities of bacteria were identical at the four estuarine sites. The null hypothesis was rejected ($p<0.01$, global $R = 0.77$). Pairwise comparisons showed significant differences among all 8 possible combinations ($p<0.01$, $R>0.64$).

Variability in Bacterial Communities between Estuarine and Marine Sediments

Replicates from two estuarine sites and a marine site were collected and run on a single DGGE gel (Figure 5). Branch tips are labeled according to site (CA, H, K), treatment (D), depth fraction (0,1), and replicate (a,b,c,d). Two marker lanes (M) were also included on the DGGE gel. DGGE bacterial community fingerprints collected in the South Slough estuary were more similar to each other than to marine sediment samples from Cape Arago. Similarity among estuarine sites was 50 while similarity between marine and estuarine sites was 30.

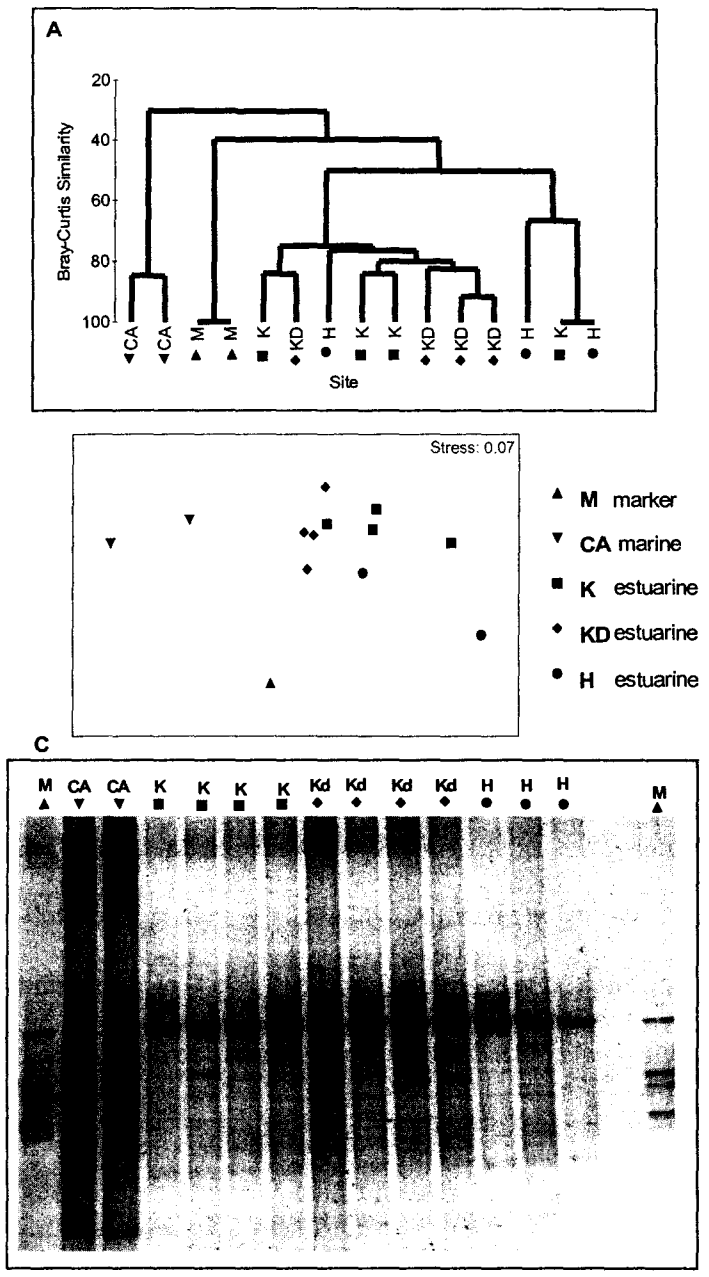

Figure 5. Cluster Analysis, MDS Plot, and DGGE Gel Image of 2002 Samples. (A) Cluster analysis and (B) MDS plot were derived from a Bray-Curtis Similarity Matrix. (C) DGGE gel image from 2002. (M) Marker (CA) Cape Arago, (K) Kunz Marsh (Kd) Kunz Marsh disturbance and (H) Hidden Creek samples were run on a single DGGE gel.

Identity of DGGE Bands

Several prominent bands were picked from the 2002 DGGE gel, reamplified, and sequenced (Figure 6). Most of the sequences from marine soft sediments were related to members of the α-proteobacteria group. Estuarine soft sediments yielded sequences that were related to organisms in the γ-proteobacteria and δ-proteobacteria. The best matched sequence from the NCBI database is listed in Table 1 along with the % similarity and NCBI accession number.

Discussion

The description of any community depends on the spatial, temporal, and organizational perspective chosen. It is essential to understand how patterns and dynamics vary at multiple scales in order to develop a predictive capability. A pattern at one scale may be a manifestation of a pattern at a different scale (Levin 1992), or patterns may be apparent only at certain scales. The detection and description of occurrence patterns in bacteria have often been restricted to either a large scale (km) or a fine scale (cm). The challenge lies in not oversimplifying the community at large scales while maintaining a realistic level of complexity at small scales. I found that patterns of variation at small scales were not universal and were important only in select locales. The question of why lineages are restricted to specific habitats remains largely unknown, with notable exceptions (Rappe *et al.* 2002). The RPD layer was expected to be an important physiological boundary separating the aerobic community and anaerobic community. Surprisingly, only Hidden Creek showed convincing evidence that the RPD layer was an important feature separating microbial communities. Indistinguishable assemblages above and

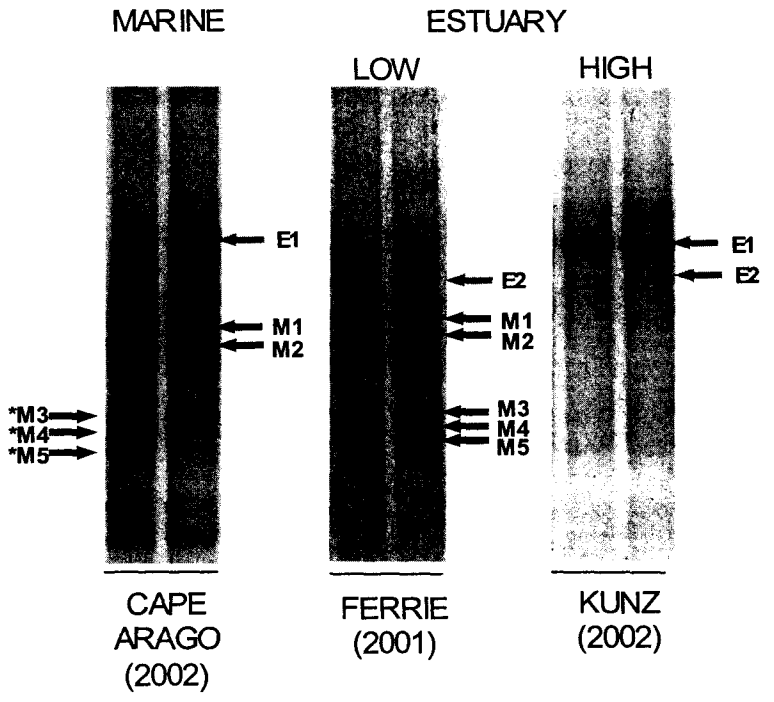

Figure 6. DGGE Community Fingerprints and Position of Selected Bands. These images were taken from the same gel and re-positioned to highlight the differences between marine and estuarine microbial fingerprints from intertidal mudflats.

below the redox boundary may also be due to a lack of metabolic information at the 16S level, since the distributions of different metabolic capacities and functional genes rarely conform to the evolutionary pattern of 16S rDNA (Woese 1987, Devereux *et al.* 1989, Garcia-Pichel *et al.* 1998, Nadeau *et al.* 2001). Braker *et al.* (2001) also observed indistinguishable assemblages above and below the redox boundary. They hypothesized that burrowing marine invertebrates could cause sediments to be homogenized, which would cause few apparent vertical differences in the microbial community structure despite strong redox gradients. Deposit feeding polychaetes and amphipods were abundant in the South Slough sediments. Their burrows may have created microscale heterogeneity that was missed by fractionating core samples at 1 cm depth increments.

At intermediate (m) scales, algae, burrowing invertebrates, and shallow tidal channels create subtle natural boundaries. No pattern was detected within undisturbed estuarine plots when distance between paired samples was set at 3 m. However, when an artificial mosaic was created on the mudflat by raking the sediment, communities separated by 3 m were measurably different. I postulate that the manipulation of the mud's surface homogenized some of the natural variability at the m scale. Future sampling designs should be organized around visible features rather than at a set distance. This design, however, would be difficult to repeat at multiple sites because visible features are often site specific. I am unaware of any manipulative field experiments testing the effects of disturbance on the genetic diversity of microbes. There were a greater number of bands in disturbed plots than in control plots. This was an unexpected observation. It is unlikely that these were new colonizers, given the ambient temperature and the slow doubling times in natural populations. One scenario to explain the greater number of bands in disturbed plots is to consider the penetrating depth of the garden rake. It is likely that the disturbance treatment brought populations of bacteria that normally live in deeper sediments

Table 1. Identity of DGGE bands from Estuarine and Marine Intertidal Mudflat Habitats.

DGGE band	ID%	Best matched organism	Accession no.	Group
M1	93	uncultured CFB	AY133094	CFB
M2	91	*Phaeosirillum fulvum*	AF508113	α-proteobacteria
M3	98	*Antarctobacter heliothermus*	AF513928	α-proteobacteria
M4	95	*Rhodobacter sphaeroides*	AF468821	α-proteobacteria
M5	95	*Rhodovulum* sp. CP-10	AB079682	α-proteobacteria
E1	100	*Pseudomonas fluorescens*	AF094731	γ-proteobacteria
E2	97	uncultured sva0999	UDE241013	δ-proteobacteria

to the surface and mixed them with populations found at the surface. This would create the illusion of greater species richness, but is an artifact of the treatment. A second scenario is that the disturbance physically disrupted consortia, which increased the probability of detection by molecular methods.

The amount of variability at larger spatial scales showed a striking pattern of site specific identity. With few exceptions, samples collected within a site were more similar than samples from different sites. Bray-Curtis similarity values between sites were greater than 47, indicating that half of the bands were found in both sites. These findings are in agreement with other descriptions of communities of bacteria from continental shelf (Scala and Kerkoff 2000) and in Puget Sound (Braker *et al.* 2001) sediments. Although these studies used a different molecular technique, T-RFLP, they came to the same conclusion. I concur that environmental conditions at distinct geographic locations select for distinct assemblages of bacteria.

The site specific pattern observed in 2001 led to a better sampling design for perceiving large scale patterns. By adding a coastal soft sediment habitat outside of the estuary, we placed site-specific differences in a context of a larger spatial scale. We also ran fewer estuarine sites and replicates together with marine samples on a single DGGE gel to minimize error stemming from improper band and gel alignment. The results were comparable to those found when multiple DGGE gels were compared. Bray-Curtis values similarity values between estuarine sites were greater than 50, a value that was in perfect agreement with that observed in 2001. The intertidal mudflat habitat outside the estuary was clearly distinct from the estuarine sites.

In order to elucidate differences between marine and estuarine soft sediments, DGGE bands were reamplified and sequenced. In congruence with the findings of Bouvier and del Giorgio (2002), higher salinity habitats yielded sequences that were related to the Class α-

proteobacteria. This finding may have important applications in determining the timing and quantity of freshwater releases from Lake Okeechobee on estuarine communities (Doering and Chamberlain 2003). There was one sequence found in both marine and estuarine samples that was related to the CFB group. Estuarine sites yielded a sequence that matched (>99% similarity) 16S rDNA from *Pseudomonas fluorescens*. The only other sequence obtained from the estuarine samples was related to a member of the δ-proteobacteria.

A restoration site, Kunz Marsh, was included in 2001 and 2002 analyses. Kunz Marsh was restored in 1996 after being diked for agricultural uses (Rumrill and Cornu 1995). In Chapter III, the communities of bacteria in Kunz Marsh were different than in the mature site, Hidden Creek. These differences were manifest in both species richness and genetic composition. Bray-Curtis similarity values were 20 when sampled in 2000, strikingly less similar than when the same sites were compared in 2001 and 2002. This suggests that the restoration site is on a trajectory toward recovery. The observation that the restoration site is becoming more similar to other mature estuarine sites through time represents a promising short term performance metric for gauging success of future restoration projects.

Any PCR based method for describing natural genetic composition of communities is biased toward the most abundant populations in the sample. Genomic DNA extractions can preferentially extract DNA from some taxa and not others. PCR amplification can fail if the quality of the template or any of the reagents is compromised. Negative PCR controls (no template) were frequently run in order to check for contamination. Interpretation of multiple DGGE gels also presents some difficulties. The darkly staining bands in our Marker lane plus recognizable bands in each environmental sample assisted the identification of bands common in multiple DGGE gels. These biases were universally applied and one assumes that their strength among sites was equal.

Despite these limitations, our descriptions of the bacterial assemblage in shallow coastal and estuarine soft sediments have helped to clarify the the organization of bacterial communities on a number of scales. Patterns at small scales were obscure when using a landscape level approach. A different assemblage above and below the RPD layer was found at only one estuarine site. We suggest that the lack of a difference in bacteria assemblage around the RPD layer may be due to the lack of metabolic information in the 16S rDNA gene or to burrowing activity. The 16S rDNA gene is useful for identifying unknown sequences from the environment, but does not have information about the physiological state or the metabolic capacity of the bacterium where it originated. Therefore, the strong gradient in available electrons observed in these estuarine sediments may influence the distributions of bacteria but these patterns were undetectable using the 16S gene. I proposed that natural variability was caused by algal mats, channels and puddles at the meter scale. Disturbance homogenizes the variability at the meter scale which allows differences at the m scale to be observed. Additional manipulative experiments are needed to confirm this hypothesis.

I presented preliminary evidence in favor of a relatively rapid change in the bacteria community after restoration project in a trajectory that pointed toward a more mature condition. Transplant mesocosm experiments could be used to replicate restoration activities and detail the succession of bacteria after reintroduction of seawater. This critical step is necessary before bacteria community composition can be used as a quantified performance metric.

Finally, environmental conditions at distinct geographic locations select for communities of bacteria. The enormous diversity observed at the 16S rDNA level (Giovannoni *et al.* 1990) is not surprising when there appears to be a great deal of site-specific selection. While the evidence

is not yet overwhelming, data from the water column and soft sediments suggest that a distinct community develops under specific environmental and biological conditions which operate at the kilometer scale.

CHAPTER III

BACTERIA DIVERSITY AND ANTHROPOGENIC HISTORY: A CASE STUDY OF RESTORED AND MATURE ESTUARINE WETLAND SEDIMENTS.

Abstract

Fringing and pocket salt marshes in Pacific Northwest estuaries were drained and diked for agricultural uses at the turn of the 20th century; many were subsequently abandoned. Habitat restoration recovers coastal wetlands from these abandoned sites. In this manuscript, I have described communities of bacteria from a coastal wetland restoration site and an adjacent mature wetland. The co-Author of Chapter II was my major professor, Lynda Shapiro, who has guided me through the thought process and assisted in editing drafts. The ideas and arguments that accompany these data were collected and analyzed by my hand. Restored and estuarine sediments were expected to have a similar microbial assemblage. Genetic diversity was first evaluated by separating 16S rDNA products with Denaturing Gradient Gel Electrophoresis (DGGE). Site variability was evaluated from DGGE gels using the Bray-Curtis similarity index and hierarchical clustering. Genetic diversity was greater in the restored site than the mature site. This observation was confirmed through construction and screening of two 16S rDNA clone libraries. The restoration site had a greater number of Restriction Fragment Length Polymorphism (RFLP) profiles and a greater number of unique sequences than the mature site. Greater genetic diversity in the restoration site may reflect the greater habitat diversity of its recent freshwater past. This comparative study of genetic diversity in two adjacent wetland

habitats with unique histories provides insight into local processes influencing microbial diversity and succession of microbial communities after restoration.

Introduction

Salt marshes throughout Pacific Northwest estuaries were historically diked and drained at the beginning of the 20th century for agricultural uses such as livestock grazing and crop production (Boule and Bierly 1987). Earthen levees constructed from dredge spoils severed the hydrologic connection between estuarine tidal channels and the adjacent marsh. Experimental work conducted in temperate salt marshes throughout the United States has demonstrated that tidal exclusion results in the loss of critical habitat and dampened nutrient exchange (Portnoy and Giblin 1997a). In many coastal landscapes, impounded freshwater wetlands (Montague 1987) have come to replace salt marshes. The historic free exchange of nutrients and production critical to estuarine function has been severely restricted. Statewide losses of tidal wetlands are staggering in the Pacific Northwest region: 90% loss for California, 50% loss for Oregon and 55% loss for Washington (Cortright *et al.* 1987). In some estuaries, over 90% of the historic tidal marshes have been altered, filled or lost to agriculture, municipal or industrial development.

Restoration of tidal wetlands has emerged as a viable mechanism to recover lost habitat functions and ecological values (Frenkel and Morlan 1991, Costanza 1997). Restoration activities can potentially return pre-disturbance ecosystem function to a severely degraded habitat (National Research Council, 1992). The success or failure of these efforts, however, is difficult to measure because the goals set can differ between locations and the nature of the restoration project (Zedler 2000).

Salt marsh, invertebrate, and fish communities have been used to evaluate post-restoration habitat function by comparing a restored community to a reference community (Cornu

and Sadro 2002). Salt marsh plants were targeted because they provide primary production to the estuarine food web and they provide physical structure for a variety of fish, invertebrates and birds. A mature salt marsh occupies an elevation approximating the mean higher high water (MHHW), and thus provides a biological measure of hydrological conditions exposed to a full tidal prism and a target for habitat recovery. Biotic and abiotic components of the restored habitat have developed in a dynamic equilibrium with salt marsh plants.

Bacteria that inhabit interstitial spaces and attached to sediment grains are a poorly understood biotic component of estuarine habitats. Bacteria use potential energy from oxygen, sulfate and ferrous iron to decompose and recycle large pools of proteins, amino acids and lipids in shallow coastal ecosystems (Fenchel *et al.* 1998). By utilizing an abundant supply of reductants, communities of bacteria metabolize carbon molecules and grow. Their biomass provides food for bactivorous invertebrates and products of their metabolism provide important nutrients for photosynthetic and chemosynthetic primary producers (Fenchel and Reidl 1970). Description of the genetic diversity of microbes that inhabit estuarine tideflats may lead to the development of a novel metric for assessing habitat disturbance and recovery.

In this study, I describe communities of bacteria from a coastal wetland restoration site and in a nearby mature salt marsh. Genetic diversity was measured using Denaturing Gradient Gel Electrophoresis (DGGE) and a clone library was constructed for each site, providing the means to describe multi-species assemblages and patterns of their organization.

Materials and Methods

Study Areas

South Slough National Estuarine Research Reserve (SSNERR) is a sub-basin of the Coos Estuary system on the Oregon coast, USA (Figure 1). South Slough is a relatively undisturbed representative of the Lower Columbian coastal ecosystem (Rumrill 2002). Kunz Marsh was restored (Rumrill and Cornu 1995) with the intent of reestablishing estuarine channels, mudflats, and salt marsh. In 2000, the site was largely devoid of vascular plants and soil was watery and black. Hidden Creek is a nearby site that is broadly typical of a mature marsh. Soils were stratified, with a black band that extended 2 cm below the sediment surface. An elevation of 1.2 m above MLLW (mean lower low water) was targeted for sampling because it was devoid of emergent marsh vegetation. Four replicate core samples were collected from each $100m^2$ site and depth-fractionated into 1 cm increments. Clone libraries were constructed from the 1-2 cm depth fraction, whose blackened sediment is characteristic of permanently anoxic conditions. Core samples were collected with a 140 cc modified syringe with a diameter of 4 cm (Walters and Moriarty 1993). Upon returning to the lab, DNA was extracted from the sediments immediately.

Redox Microelectrode Measurement

Replicate profiles of relative redox potential were collected with an ORP Redox Combination Electrode (Lazar Laboratories Inc., Los Angeles, CA). Redox profiles were measured within the Kunz Marsh and Hidden Creek marsh at the same time core samples were collected. Duplicate profiles were run at two randomly chosen locations within the site. A platinum band metallic sensor was used in combination with a double junction reference electrode connected to a digital mV meter. Depth of the tip of the electrode was measured using a ruler on a micromanipulator. Relative redox potential (Δ mV) quantifies the change in electron flow at a specified depth relative to surface sediments (0 mm). Absolute redox potentials were

not reported because electron flow of surface sediment against a standard hydrogen electrode was not measured (Skoog and Leary 1992).

DNA Extraction

DNA extraction was conducted by a direct lysis method modified from Zhou *et al.* (1996). Details of the extraction procedure can be found in Chapter II.

PCR Amplification of the 16S rDNA Gene

Ten µl (~40 ng) of gel purified template was used in a 50 µl PCR reaction to amplify small subunit (SSU) 16S rDNA. Primers gc338F and 519R amplify a hypervariable region of the 16S rRNA gene (Muyzer *et al.* 1993, Ferrari and Hollibaugh 1999, Jackson *et al* .2001). These PCR products were used in DGGE to observe community profiles of bacteria. After differences in DGGE profiles were observed between sites, two clone libraries were constructed from the same template used for DGGE. A PCR reaction with primers 8F and 1392R amplified the 16S rDNA gene. Each reaction contained 5 µl 10X buffer, 5 µl 25 mM $MgCl_2$ (Applied Biosystems) 1.2 µl 2.5 mM dNTP, 10 U Amplitaq LD, 1.5 µl 10 pmol forward primer and 1.5 ul 10 pmol reverse primer (Muyzer *et al.*1993). The reaction was brought to 95° C for 3 minutes then cycled 35 times 95°C for 30 s, 48°C for 30 s, 72°C for 30 s and held at 72°C for 10 minutes. Another potential source of PCR bias is the presence of contaminating DNA. Given the universal nature of the Domain level primers (8F, gc338F, 519R, 1392R) and the ubiquity of bacteria in the environment, contamination of samples was of great concern. I frequently checked for contamination by routinely running negative controls, PCR reactions with no template DNA added.

Estimating Genetic Diversity using Denaturing Gradient Gel Electrophoresis

A Biorad D-Code gel rig was used to separate similarly size, amplified rDNAs by melting domain (Biorad, Hercules, CA). All reagents were prepared as described in the D Code Instruction manual and Applications Guide. The denaturing gradient was 20% to 60% by weight urea and formamide (Piceno *et al.* 2000), 1 mm, 7 % gels were loaded with 15 µl of the PCR reaction plus 5 µl of loading dye (0.01% Bromphenol Blue, 40% glycerol), and run for 3 hours at 200 V. Gels were stained with SYBR Gold nucleic acid gel stain (Molecular Probes, Eugene, OR), and photographed on Polaroid film. Polaroid gel images were scanned into digital format and inverted for analysis.

DGGE provided a profile of a mixed community amplified by PCR to visually compare replicates from environmental samples (Muyzer *et al.* 1993, Van Hannen 1999). Gels were analyzed following van Hannen *et al.* (1998, 1999), and Schäfer and Muyzer (2000). Individual bands that composed the community profile were identified using Gel Pro 4.0, 1-D gel routine. All bands, including heteroduplexes, were included in the analysis since their appearance was characteristic of the samples. A binary matrix was derived by identifying presence (1) or absence (0) of a band. Binary matrices from Kunz Marsh and Hidden Creek were used to calculate a Bray-Curtis similarity coefficient PRIMER v.5.2 (Clarke and Gorley 2002). A hierarchical cluster analysis was performed on the similarity matrix using the group-averaged setting (Clarke and Warwick 2001).

Clone Library Construction and Screening

The 1-2 cm depth fraction was selected for construction of both libraries to eliminate any depth effect. Amplified SSU rDNA (8F, 1392R) was cloned with an Invitrogen TOPO TA

cloning kit. Inserts were amplified with M13F, M13R primers and screened using double digest restriction fragment length polymorphism (RFLP). Abnormally sized inserts were removed from analysis and 10 μl of PCR product was digested. One μl 10X Buffer, 1 μl BSA, 0.2 μl Alu I and 0.4 μl Msp I, 7.4 μl water were incubated with amplified inserts for 1 hr at 37°C. Ten ul of the digest were run on a 2% agarose gel at 100V for 1.5 hrs. Unique RFLP patterns were identified with Gel Pro and unique RFLP were grown up in 1.5 mL LB amp. Plasmid DNA was extracted using CPG mini-prep kit and sequenced. Oregon State University's Center for Genetics Biotechnology Lab sequenced the insert with sp6 and t7 primers. Sequences were formatted to 5' to 3' and edited with the assistance of the sequence aligner in RDP (www.rdp.msu.edu). Full length sequences were analyzed individually using BLAST-n program at the Genbank website.

Results

Redox potential from all sites (Δ mV) showed a characteristic pattern of stratified estuarine and marine sediments (Fenchel and Reidle 1970). Redox potential became increasingly negative as the electrode was positioned deeper into reducing sediments (Figure 7). Kunz marsh redox potential showed a sharp negative gradient in the upper 1 cm of sediments. Kunz Marsh also showed more variability in reduction strength below 5 mm depth. Hidden Creek redox potential and redox potential from two additional mature sites, Ferrie Ranch and Elliott Creek, showed a gradual negative gradient and less variability below 5 mm depth. Average Δ mV at 5 mm was -160 in the restoration site (Kunz Marsh) and -75, -40 and -60 in the mature sites (Hidden Creek, Elliott Creek, Ferrie Ranch), respectively.

DGGE gels from Kunz Marsh showed greater genetic diversity than Hidden Creek (Figure 8). There were a greater number of bands at Kunz Marsh than Hidden Creek. Kunz Marsh had 8 or more prominent bands per lane. Hidden Creek had two prominent bands and 5

faint bands. The average number of bands per lane, averaged across depths, was higher in Kunz Marsh (mean=8.9, n=12, sd=2.7) than in Hidden Creek samples (mean=3.75, n=12, sd= 2.6).

Genetic composition at Kunz Marsh and Hidden Creek had low Bray-Curtis similarity (19). Genetic composition between estuarine sites in 2001 showed higher similarities, ranging between 48 and 65 (Milbrandt, in prep). A one-way ANOSIM (Clarke and Warwick 2001) performed on the similarity matrix was used to test the null hypothesis that Kunz Marsh and Hidden Creek clusters were indistinguishable. The null hypothesis was rejected at $R=0.91$, $p<0.01$.

Higher genetic richness in the restoration site was confirmed with a second PCR-based molecular approach. Clone libraries were constructed from a single replicate 1-2 cm fraction collected in Kunz Marsh and Hidden Creek. A greater number of unique RFLP profiles were observed in the restoration site. To ensure that sample size (# clones screened) did not bias the apparent differences in diversity between the reference and restoration site, a rarefaction curve was plotted (Figure 13). Eighty clones containing the ~1400 b.p. insert were screened from Kunz Marsh, yielding 27 unique clones. Only six unique RFLP profiles were found in Hidden Creek of the 50 clones that were screened.

Most of the sequences generated in this study were different from any of the cultivated taxa chosen for this analysis. Kunz Marsh sediments had sequences that were related to cultivated bacteria in many of the major lineages in Bacteria. There were 19 sequences unique to Kunz Marsh, 4 sequences shared by both sites, and 1 sequence unique to Hidden Creek. Hidden Creek sequences and those sequences found in both sites were related to members of one major clade, the proteobacteria. All of these sequences were best matched to sequences of bacteria isolated from soil enrichments or from environmental DNA collected from soils or anoxic marine sediment. No sequences were best matched to bacteria found in the water column.

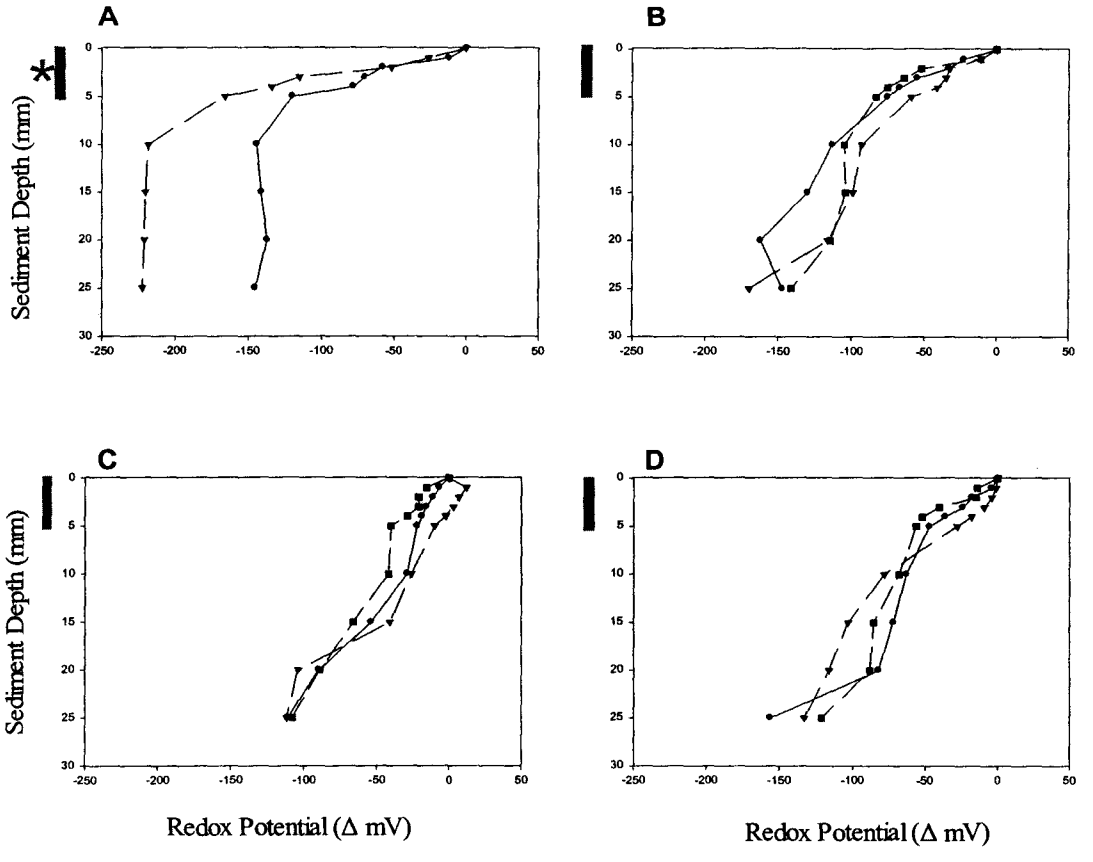

Figure 7. Vertical Microelectrode Redox Potential Profiles from Restored and Mature Intertidal Mudflats. Δ mV is the change in redox potential from the sediment water interface to the depth of the probe tip, A: Kunz Marsh, B: Hidden Creek. Gray bars denote the depth range where restored sediments showed steeper gradients than any of the three mature sediments. Gray star denotes the restored site.

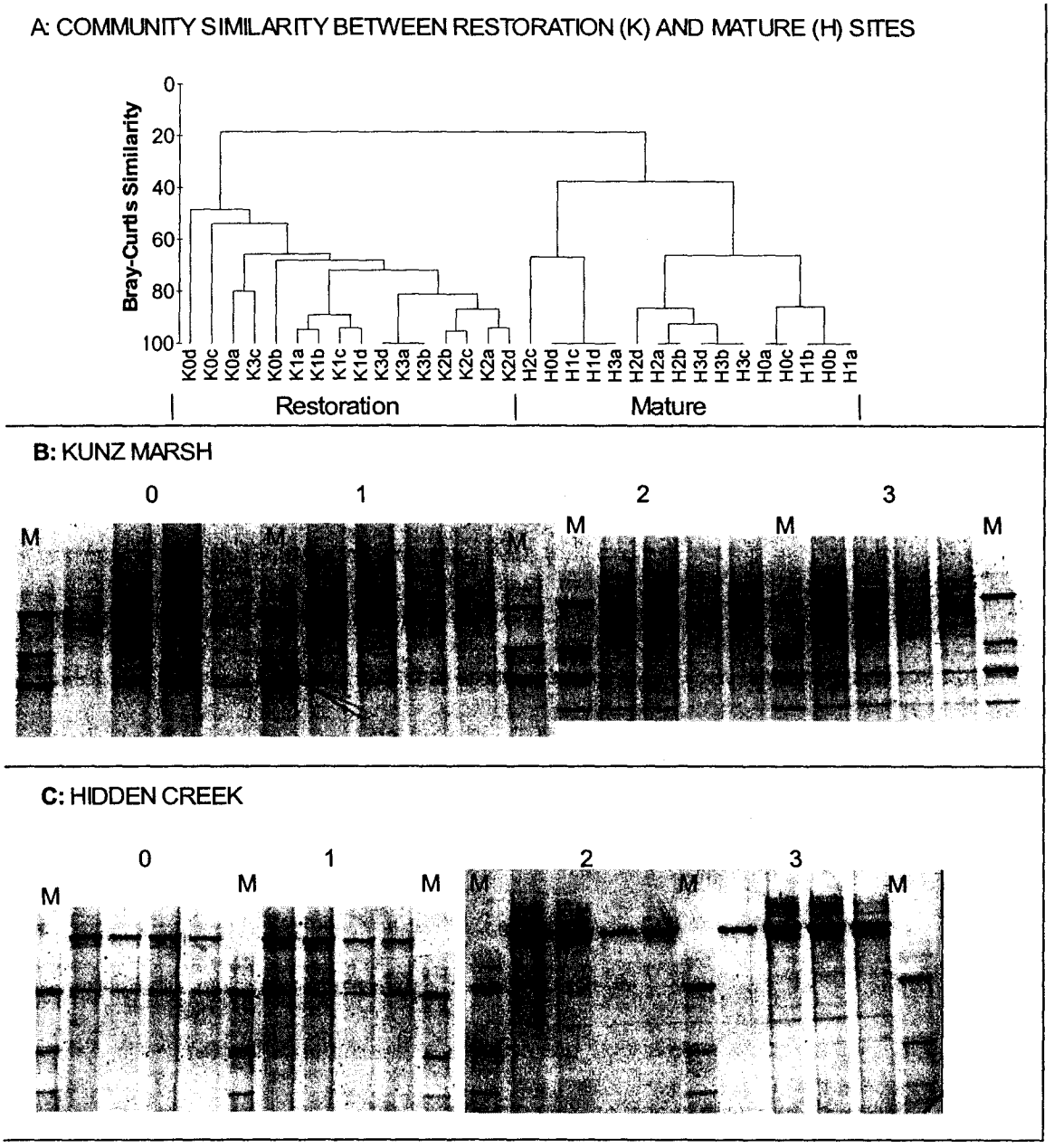

Figure 8. Cluster Analysis and DGGE Gel Images from Restored and Mature Intertidal Mudflats. A. Hierarchical cluster analysis (group averaged) drawn from a Bray-Curtis similarity matrix. DGGE gels shown in B and C were used to compare restored and mature sites. Bands on these gels were scored for presence (1) or absence (0). a,b,c,d: replicate core samples, M: DNA from known taxa, depth fraction 0: 0-1 cm, 1: 1-2 cm, 2: 2-3 cm, 3: 3-4 cm.

Discussion

Estuarine wetlands in Pacific Northwest estuaries, including the Coos Estuary system, characteristically have fringing or pocket salt marshes with extensive mudflats (Rumrill 2002). Hidden Creek is broadly typical of a mature wetland based on two attributes, wetland age and marsh surface elevation relative to the tide (Callaway 2001). Hidden Creek wetland formed in a protected embayment where a lower order perennial freshwater stream flowed into a higher order tidal channel. The delta at the confluence of Hidden Creek consisted of a relatively large and persistent mudflat and sinuous tidal creeks. Each tidal cycle delivered and deposited sediment and organic material. A combination of several dynamic feedback processes affecting the relative elevation of the marsh surface resulted in an equilibrium state when the marsh surface roughly equals mean higher high water (MHHW) (Redfield 1972).

Kunz Marsh was most likely a mature tidal wetland that was restricted from tidal flooding at the turn of the century. Linearized drainage channels and tidegates were installed by early settlers to drain the estuarine wetland for crop production and livestock grazing. The effects of these modifications decoupled marsh primary marsh production from the estuarine food web and eliminated critical habitat for waterfowl, fish and invertebrates. Neglected linear drainage channels and marsh subsidence pooled freshwater on the landward side of the levee. Waterlogged soils provided ideal conditions for colonization by the cattail *Typha latifolia* and other freshwater marsh plants. An impounded freshwater marsh (Montague 1997) replaced the continuum of habitats found in an estuarine wetland. Restoration of tidal flooding to Kunz Marsh and to recovery of estuarine wetlands was initiated in 1996. The restoration project removed an

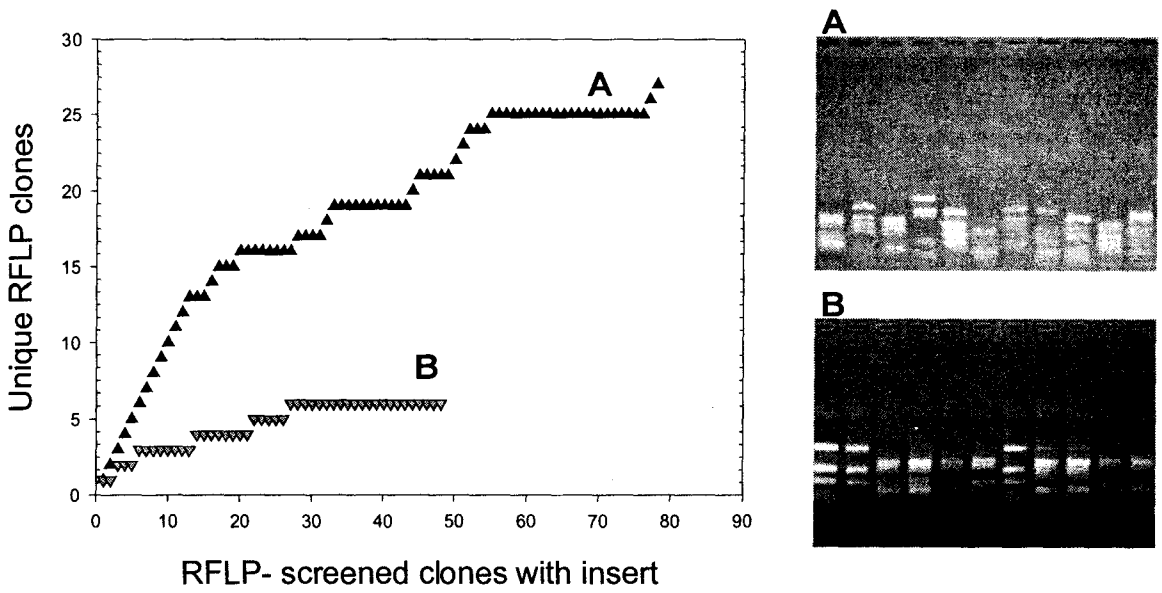

Figure 9. Rarefaction Curve and Restriction Fragment Length Polymorphism Gel Images. A greater number of unique sequences were found in the restored site than the mature site. This is reflected in the number of recombinant clones plotted against the cumulative number of unique RFLP fingerprints. A: restored Kunz Marsh, B: mature Hidden Creek.

earthen levee and filled linear drainage channels. Natural drainage channels were allowed to develop after reintroduction of tidal flooding.

Direct experimental evidence offers the best demonstration of processes that are important in species coexistence or exclusion. This comparative study of genetic diversity in two adjacent wetland habitats with similar geomorphologies and unique histories provides insight into processes that influence microbial diversity. Genetic diversity was measured four years after restoration in Kunz Marsh and Hidden Creek. Observed differences in genetic diversity between Kunz Marsh and Hidden Creek suggested several testable hypotheses about succession of bacteria species and diversity of bacteria after habitat restoration.

Variation in local biodiversity has been attributed to three broad explanations; spatial heterogeneity and organism size, productivity gradients, and temporal heterogeneity (disturbance). Spatial heterogeneity provides a greater variety of microenvironments. These provide the basis for niche partitioning so that more species can avoid competitive exclusion (May 1986). The scale of sampling and species size are important in determining spatial heterogeneity (Tilman and Pacala 1993). Since the average size of bacteria species can vary greatly depending on whether it is a multi-celled species like *Oscillatoria* spp. filament (100-200 μm) or *Pseudomonas* spp. (<1 μm), species richness may be affected by the size of species. However, it is unlikely that observed differences in species richness at Kunz Marsh and Hidden Creek was due to the scale sampling because of the consistency of sample size (1 g) among sites.

Core samples collected from Kunz Marsh had peat that contained roots, branches, leaves, and other large pieces of organic material. This visible material was a remnant of the recent history of the Kunz Marsh as an impounded freshwater wetland, and may have provided greater microhabitat complexity than Hidden Creek. The peat in Kunz Marsh likely influenced the amount and type of carbon source delivered to the sediment-water interface. Carbon sources in a

mature estuarine wetland include complex structural polysaccharides from wetland plants and algae as well as more labile phytoplankton exudates (Haddad and Martens 1987). Stratigraphic analysis of mature and impounded wetlands showed 30 cm of freshwater wetland peat overlying dead *Spartina* rhizomes (Portnoy and Giblin 1997b). A similar stratigraphy was observed in Kunz Marsh which likely provides a greater variety of carbon sources. The additive effect of estuarine carbon sources plus carbon from the remnant freshwater marsh peat may have led to higher species richness in Kunz Marsh. This pattern is supported by evidence from redox potential profiles in the restored and mature wetlands. Greater available carbon resources would increase the electrons needed for oxidation. There were a greater number of available electrons in the restored site, as reflected by strongly negative redox potential data in Kunz Marsh. The greater number of available electrons could provide a larger and more varied pool of resources to support a greater number of taxa (Wright 1983).

Models that explain long-term persistence of a large number of species assume that there are one or more resources that constrain individual fitness (MacArthur 1968) and that species have unavoidable trade-offs in their ability to respond to those constraints (Tilman 1982). Since Hidden Creek had reached a dynamic equilibrium, it may have had sufficient time to allow a few competitively dominant species to exclude inferior competitors. Temporal heterogeneity or disturbance can change availability of resources and thereby reduce the probability of competitive exclusion (Lubchenco 1978, Sousa 1979). In Kunz Marsh, the reintroduction of tidal flooding was a disruptive event. Freshwater wetland plants could not survive in the salty brine and died. Bacteria in Kunz Marsh may have shared a similar fate. In the days and weeks following restoration, a few species may have survived the transition. When we sampled the restoration site four years of development and succession in the bacterial community had occurred. The community had reached an intermediate state with high genetic diversity. It follows that low

genetic diversity found in Hidden Creek is the eventual equilibrium state which will be reached by Kunz Marsh in time. Any combination of these hypotheses may be at work in South Slough sediments.

Kunz Marsh and Hidden Creek are separated by 1 km along the estuarine gradient in the South Slough. These data were collected in September, in the middle of a six month dry season that lasts from June through November. The strength of the estuarine gradient during the summer is considerably lower than during the rainy season (Rumrill 2002). The rainy season brings periodic discharges of freshwater. Given these properties, the Kunz Marsh site likely experiences a different salinity regime than Hidden Creek during the rainy months and a similar salinity regime during summer months. Differences in diversity between the two sites may therefore be less a reflection of history and more a reflection of the differences in the salinity regime during periods of high freshwater dishcharge. Monthly collections of bacterial community data over the course of one year may clarify the influence of freshwater discharge and the response of the community.

Restoration ecology suffers from pseudoreplication because restoration projects are typically not designed for scientific experiments. Site specific differences were partially addressed in this study by selecting a nearby mature estuarine wetland for comparison (Zedler *et al.* 2000). However, there is rarely a perfectly suited reference site (Zedler and Callaway 1999). Multiple paired restoration and mature sites are needed to confirm or refute the patterns that we found. An alternative sampling design would be to transplant several large cores (5 gallon bucket) from an impounded site to a mature site and measure changes in species richness and community composition. In a replicated sampling design, the connection between species richness and carbon availability could be tested in an experimental matter by manipulating amount and variety of carbon or reduction/oxidation ions. Local patterns of biodiversity could

also be attributed to salinity (Crump *et al.* 1999) temperature (Ferris and Ward 1997), and the presence of eelgrass or saltmarsh plants (Cifuentes *et al.* 2000, Hines *et al.* 1999).

Despite the limitations imposed by a molecular approach (Hugenholtz *et al.* 1998), theories governing local species richness patterns can be applied to communities of bacteria. Communities of bacteria have great potential to test hypotheses of biodiversity and may serve as an ideal community for understanding succession following habitat restoration.

CHAPTER IV

SPATIAL AND TEMPORAL DISTRIBUTION OF MAT-FORMING ALGAE AND THE GENETIC DIVERSITY OF THEIR ASSOCIATED MICROBIAL COMMUNITIES

Abstract

Mats of bacteria and algae grow on the sheltered, unvegetated tideflats of Pacific Northwest Estuaries. Algal mats are thought to retain water and trap suspended sediments, thus modifying intertidal mudflat habitats. Intertidal mudflats support a rich assemblage of burrowing deposit feeders that feed on a functionally diverse community of bacteria. Development and variability of algal mats was determined in South Slough National Estuarine Research Reserve. These data were coupled with the patterns of genetic diversity in mat-associated microbial communities and non-mat communities. Spatial cover was determined with monthly photoquadrats and genetic fingerprints of the bacterial community were determined using Denaturing Gradient Gel Electrophoresis of PCR amplified 16S rDNA. Algal mats showed significant seasonal variability and significant differences in the pattern of seasonality in each of the four sites sampled. A distinct mat-associated bacterial community was observed in individual sites. However, the communities were not different when DGGE data from all 4 sites were combined. An annual peak and valley in percent cover that was observed to coincide with the

summer and winter solstice may be coincidental, but it offers evidence that the algal mats are limited by the availability of light. A second peak in mat cover was postulated to be a relaxation of grazing pressure, though data on grazer density was not collected. The patterns that were observed in algal mat cover and genetic diversity of mat associated communities of bacteria suggest that the interactions between a producer and decomposers are complex. Algal mats exhibited an assemblage of bacteria that was distinct from the non mat assemblage in all four sites, but the distinctiveness disappeared when multiple sites were compared.

Introduction

Mats of bacteria and algae (Cyanobacteria: *Oscillatoria* spp., *Lyngbya* sp., *Microcoleus* sp., and yellow green algae (Xanthophyceae: *Vaucheria longicaulis*) exhibit patchy, but prominant distributions in sheltered mudflats of South Slough National Estuarine Research Reserve (NERR). The size and density of the mats in a year are largely unknown. It is thought that algal mats may be an important source of primary production and habitat to bacteria and the deposit feeding invertebrates that feed on them.

Mats can modify the light quality (Pierson *et al.* 1990), salinity, temperature, and the intensity of the redox gradient (Minz *et al.* 1999). At larger scales, marine microbial mats make large contributions to primary production, biomass (Pregnall and Rudy 1985, Zedler 1980), nitrogen fixation, and denitrification (Joye and Paerl 1996). Green macrophyte biomass (Chlorophyceae: *Enteromorpha spp.*) was measured to be from 10% to 70% of the total macrophyte biomass in Coos Bay (Gonor *et al.* 1979). Mats grow, retain water, and trap sediments, characteristics that may be important to sustain secondary production.

Mats may impose selective conditions favoring an assemblage of bacteria that differs from the assemblage found in adjacent, non-mat covered mudflats. Producers depend on

remineralization properties of the decomposers, while decomposers depend on production from plants. Recently, it was shown that manipulating either producer or heterotrophic bacteria diversity in freshwater mesocosms causes changes in algal biomass production (Naeem *et al.* 2000). This finding suggests that there is considerable feedback between groups such as primary producers and heterotrophic bacteria. The suggested co-dependency between producers and decomposers would lead to a unique mat-associated bacteria assemblage that differs from the assemblage found in nearby adjacent intertidal mudflats without algal mats.

I followed the development and variability of algal mats monthly from May 2000 to April of 2001 in the South Slough NERR. Four sites that were distributed throughout the mid and upper estuary were chosen for study. This is the first study to measure percent cover of the alga, *Vaucheria longicaulis* that grows on intertidal mudflats. Anecdotal evidence was provided by Trowbridge (1993), who marveled at the large and frequent patches of this species. Bacteria community fingerprints were collected in September 2000 in mat and non-mat intertidal mudflats. Genetic diversity and bacteria community fingerprints were determined by PCR amplification of 16S rDNA genes and Denaturing Gradient Gel Electrophoresis. Investigation of the growth dynamics of the producer and the spatial distribution of the decomposers was intended to provide new information about the strength and direction of feedback between these groups.

Materials and Methods

Algal Mat Cover Sampling Design and Statistics

Percent cover of intertidal mats was measured monthly at four sites in the South Slough NERR (Figure 1). Data were collected once per month from May 2000 through April 2001,except Febuary 2001. Photoplots were shot using the phytomegatron (Figure 10), a 0.25 m^2

quadrat with a mounted Pentax waterproof camera zoomed to a 400 cm^2 area. A 30 m transect was used to guide the 8 randomly selected quadrats that were approximately 3 m apart. A random number table was used to designate the first plot, followed by the addition of 3 m to determine the next plot. A total of three transects were used to generate 24 photoquadrats per site per month. Developed photographs were analyzed using a point intercept method (Elzinga *et al.* 1998). Percent cover was estimated by the number of points intersecting an algal mats to the total number of points (49). Algal mats were identified in photographs and during the first three months were confirmed with microscopic examinations of collected material.

Percent cover data were sorted by mat type and analyzed with Systat 9.0 (SPSS 2000). Probability plots of the percent cover data were used to test for normality. A two-way ANOVA was performed on *V. longicaulis* percent cover data with the following treatments; site, and month. *A priori* null hypotheses were (1) percent cover of *Vaucheria longicaulis* mats are not different among sites, (2) percent cover of *V. longicaulis* mats are not different among months and (3) there are no differences in *V. longicaulis* mat percent cover by site and month. A post-hoc Bonferroni test was performed to determine the source of differences in percent cover.

Microbial Community Analysis

Genetic diversity of the bacterial community was determined from core samples collected in September 2000. Four replicate core samples were haphazardly chosen from both mat covered and non-mat covered sediments for a total of 8 replicates per site. Community fingerprints were generated by using culture independent molecular techniques (Head *et al.* 1998, Hugenholz *et al.* 1998).

DNA extraction was conducted by a direct lysis method modified from Zhou *et al.* (1996). The steps of this procedure are detailed in Chapter II. DNA extracts were gel purified

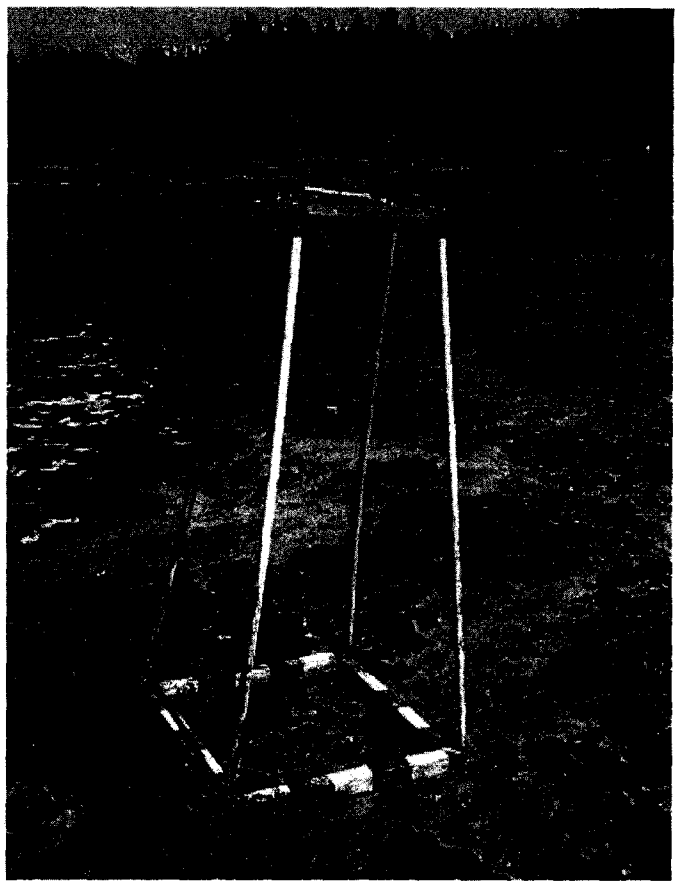

Figure 10. The "Phytomegatron." Yellow-green mats can be seen in the photographs as dark green patches, while the lighter colored areas was an ephemeral bacterial biofilm composed of purple photosynthetic bacteria and sulfur oxidizing bacteria.

following (Li and Ownsby 1994). The template was used in a PCR reaction to amplify a 338 – 519 (*E.coli* numbering) part of the 16S rDNA gene. The details of the PCR amplification can be found in Chapter II. Routine negative controls were run to check for contamination of the PCR reagents. Once the 16S rDNA gene fragment was amplified, the sample was separated with Denaturing Gradient Gel Electrophoresis. A denaturant concentration of 20% to 60% was used to create a community fingerprint. The reagents and procedures that accompany DGGE can be found in Chapter II.

DGGE gels were used as a community fingerprint to compare replicates from environmental samples. Gels were analyzed following van Hannen *et al.* (1998, 1999), and Schäfer and Muyzer 2000). Individual bands that composed the community profile were identified using Gel Pro 4.0, 1-D gel routine. All bands, including heteroduplexes, were included in the analysis since their appearance was characteristic of the samples and informative. A binary matrix was derived by identifying presence (1) or absence (0) of a band using 1.15% minimum peak height in GelPro Analyzer 4.0 (Media Cybernetics). A total of 28 bands in 2001 and 27 bands in 2002 were used for analyses of similarity. Binary matrices were used to calculate Bray-Curtis similarity coefficient PRIMER v.5.2 (Clarke and Gorley 2002). A hierarchical cluster analysis and a non-metric MDS plot (Clarke and Warwick 2001) were used to visualize any spatial patterns of variability. To test the similarity within *a priori*-defined groups (mat and non mat), a one-way ANOSIM (PRIMER-E) was applied. ANOSIM applies a non-parametric permutation procedure to test the null hypothesis that there are no differences in similarity between or among test groups (Clarke and Green 1998).

Results

Seasonal Dynamics of *Vaucheria longicaulis* Mats

Algal mats showed significant seasonal variability and significant differences in the pattern of seasonality in each of the four sites sampled. A parametric two way ANOVA was used to test the statistical significance of the observed patterns. There are four key assumptions of the parametric two-way ANOVA; independent samples, random collection, normal distribution, and homoscedasticity. Samples were independent from each other and collected at random. The assumption of normal distribution was tested by graphing a probability plot of the data. Results from the probability plot of *V. longicaulis* mat percent cover showed a non-normal distribution of data (Figure 11). These data had a higher number of 0.0 and 1.0 than expected from a normally distributed population (y=1x+B). To correct this anomaly, data were rank ordered and subjected to a non-parametric two-way ANOVA. The results of a parametric test with untransformed data were identical to the non-parametric test with transformed data. Therefore, since the parametric statistics were robust to violation of normal distribution, a parametric analysis was performed with no apparent consequences to the integrity of the test.

A strong seasonal pattern in percent cover was observed (Figure 12). Peak mean percent cover was found in June of 2000, while lowest mean percent cover was found during winter and early spring. The two-way ANOVA was used, in part, to test whether mean percent cover of *V. longicaulis* was significantly different among the eleven months. The two-way ANOVA (Table 2) returned highly significant differences in percent cover among months. A post-hoc Bonferroni

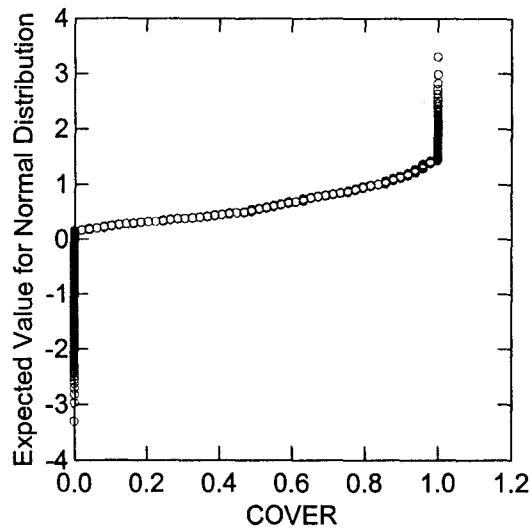

Figure 11. Probability Plot of *Vaucheria longicaulis* Mat Percent Cover. Normally distributed data fall along the y=1x+0 equation. Percent cover data from *V. longicaulis* was not normally distributed, with a higher number of zeros and 1.0 than would be expected from a normally distributed population.

test was applied to find the source of significance. June 2000 was significantly different from all other months ($p<0.005$).

The four sites studied showed significant variability in the percent cover of *Vaucheria longicaulis* mats (Figure 13). Kunz Marsh had the highest mean percent cover over the 1-year sampling period (0.40 ± SE 0.02), followed by Ferrie Ranch (0.39 ± 0.02), Elliott Creek (0.21 ± 0.02), and Hidden Creek (0.09 ± 0.02). Results from the two-way ANOVA showed highly significant differences among sites (Table 2) during the sampling period. Post-hoc Bonferroni test showed the source of significance among sites, Kunz Marsh and Ferrie Ranch were not significantly different, but were significantly different from both Elliott Creek and Hidden Creek ($p <0.000$). Elliott Creek and Hidden Creek were significantly different than all other sites.

Seasonally, each of the four estuarine sites showed significantly different patterns of cover. Results from the two way ANOVA showed significant differences in the month*site cross term (Table 2). Peak cover in three sites was observed in June 2000 (Figure 13). Peak cover in Elliott Creek was in May 2000, a pattern that was different than other sites. A second peak in cover was observed in September 2000 at Ferrie Ranch, and in October 2000 at Kunz Marsh and Elliott Creek. Hidden Creek lacked a minor peak during September or October 2000. At the onset of December, the lowest percent cover in all sites was observed and remained low through the remaining winter and spring months.

Bacterial Community Associated with Algal Mats

A distinct mat-associated bacterial community was observed in individual sites. However, the communities were not different when DGGE data from all 4 sites were combined. A statistical test was employed to determine if the community fingerprints associated with mats significantly differed from non mat sediments. The results of the statistical analyses are listed in

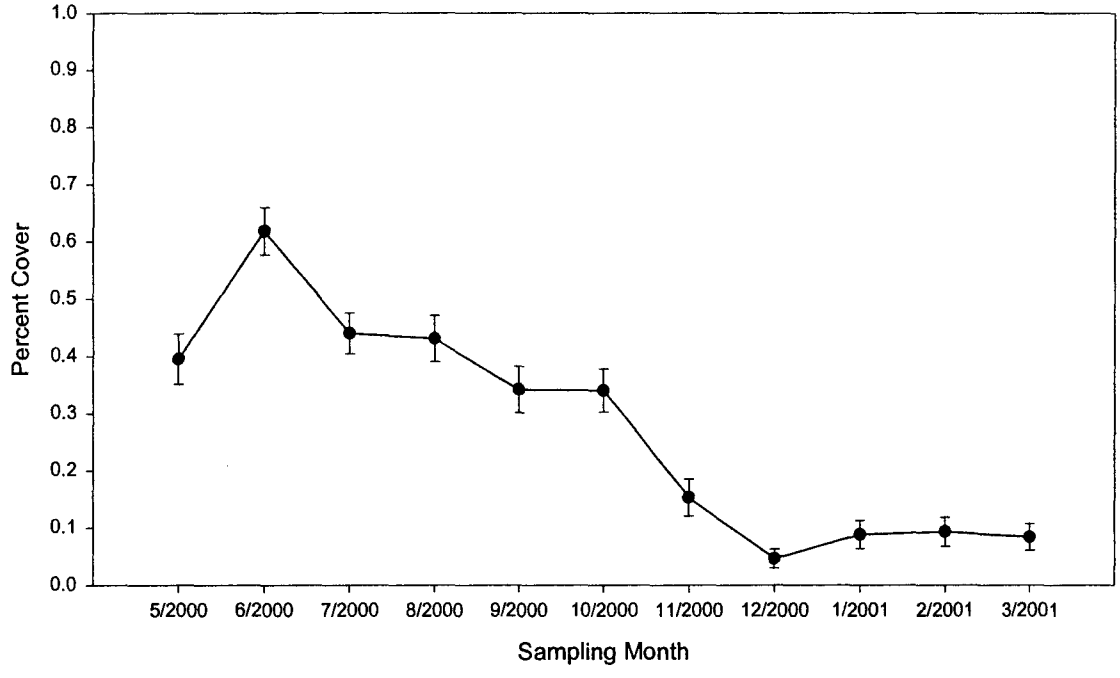

Figure 12. Seasonal Variability of *Vaucheria longicaulis* Mats in South Slough National Estuarine Research Reserve. Peak mean percent cover of the mats was observed in June 2000. The mats nearly disappear during the winter and early spring months. Data represents the mean percent cover measured at four sites; Kunz Marsh, Elliott Creek, Hidden Creek, and Ferrie Ranch. Error bars are the standard error for the monthly means.

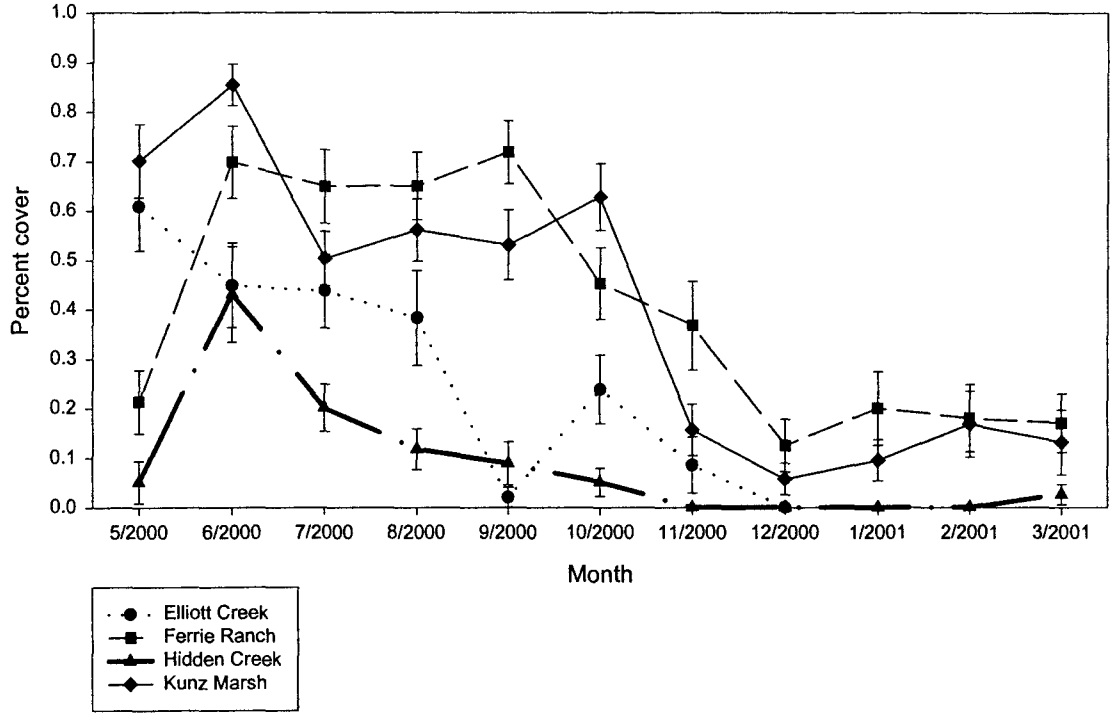

Figure 13. Spatial and Temporal Variability of *Vaucheria longicaulis* Mats in South Slough NERR. The highest mean percent cover of *V.longicaulis* mats was found in Kunz marsh in June 2000. Ferrie Ranch and Hidden Creek also show peak mean percent cover in June 2000, while Elliott Creek's peak mean percent cover was in May 2000. A second peak occurs sometime between September and October at all sites except Hidden Creek. Error bars are the Standard Error calculated from monthly means at each site.

Table 3. A one way ANOSIM tested the null hypothesis that mat associated and non mat communities were identical in all sites, Kunz Marsh, Elliott Creek, Ferrie Ranch and Hidden Creek. The patterns observed in the MDS plots were robust to statistical analyses. The null hypothesis was accepted when all sites were considered together, while it was rejected ($p<0.05$) for the individual sites. The value of the Global R-statistic best indicates the degree of difference between treatments (mat, non-mat), since it is a value based on the rank similarities of between samples in the underlying similarity matrix (Clarke and Warwick 2001). The degree of difference between mat and non-mat associated communities of bacteria was greatest in Ferrie Ranch and Elliott Creek and lower in Hidden Creek and Kunz Marsh. Kunz Marsh had the lowest Global R-statistic at 0.176.

Discussion

Algal mats were a spatially dominant feature of intertidal mudflats during the summer of 2000. The intertidal mudflat at Kunz Marsh was 90% covered by *Vaucheria longicaulis*. The summer peak in *Vaucheria longicaulis* percent cover was consistent with the long days of the year. Peaks were observed in June, while the lowest spatial cover was observed in December. The peak and valley in percent cover coincided with the summer and winter solstice. This may be coincidental, but it offers some evidence that the algal mats are limited by the availability of available light. In the absence of empirical data from productivity measurements, nutrient spike experiments, and shading experiments, the environmental drivers of mat cover is left to speculation. Light limitation was concluded to be the best explanation for the seasonal patterns in mats of *Enteromorpha spp.* in South Slough based on cover observations and a predictive model (Pregnall and Rudy 1983).

Table 2. Two-way ANOVA of Yellow-green Algal Mat *Vaucheria longicaulis* Percent Cover.

Source	Sum-of-Squares	df	Mean-Square	F-ratio	p-value
Month	34.11	10	3.41	42.05	0.000
Site	17.87	3	5.96	73.44	0.000
Month*Site	14.05	30	0.47	5.77	0.000

Table 3. One-way ANOSIM of Mat-associated and Non-mat Bacteria Community. A detailed explanation of this multivariate test of differences can be found in the text and (Clarke and Warwick 2002).

	Sample statistic (Global R)	p-value	Permutations
ALL SITES	0.011	0.150	999
Kunz Marsh	0.176	0.006	999
Elliott Creek	0.380	0.001	999
Ferrie Ranch	0.454	0.003	999
Hidden Creek	0.204	0.012	999

A second peak in mat cover was observed in Kunz and Hidden Creek. Relaxation of light limitation could not account for the timing of the second peak during the fall months. An alternative explanation for the second peak is relaxation of grazing pressure (Lalli and Parsons 1997). The adult phase in the life history of the sea slug, *Alderia modesta*, depends on *Vaucheria longicaulis* for nutrition (Krug 1999). It is the primary grazer of the mats and their abundance peaks in mid-summer. Density data were not available for *A. modesta* populations in Coos Bay, therefore a direct correlation of grazer abundance or pressure with the 2nd peak in spatial mat cover was not possible.

There were differences in total cover and in the seasonal dynamics of algal mats among study sites. The South Slough arm of Coos Bay (19,600 ac) and other small estuaries offer considerable challenges to ecological replication. South Slough drains at the mouth of Coos estuary near the confluence with the Pacific Ocean. As a result, the South Slough estuary covers the entire range of salinity, temperature and dissolved oxygen associated with the estuarine gradient. Replication is difficult, in part, because of the need to sample similar habitats without pseudoreplication. The sites chosen in this study were comparable intertidal mudflat habitats with similar elevations, topography and hydrology. The position of the sites along the estuarine gradient, however, was not comparable. The environmental features that determined the percent cover of algal mats are not known, but may be related to the km scale variation in salinity, nutrients, and temperature associated with the estuarine gradient. Clearly, more work is needed to determine the factors that influence the growth and dynamics of intertidal algal mats. The difficulties lie in the problems associated with morphological differences of laboratory culture. Laboratory cultures of *Vaucheria longicaulis* do not have the same morphological characteristics as mats that grow in the field. The laboratory cultures are collections of thalli that do not form the same dense aggregations attained by this species in the field. There is considerable interest in

understanding the relationship between genetic and phenotypic variability (Mokady *et al.* 1999, Balaguer *et al.* 2001, Perez and Garcia 2002) and algae are good models because many exhibit extreme fluctuations in morphology.

There are many examples in the literature of distinct microbial communities associated with primary producers in marine systems. Plant rhizomes (Hines *et al.* 1999, Bergholz *et al.* 2001, Bagwell *et al.* 2001), dead aboveground biomass (Lovell *et al.* 2001) and eelgrass beds (Cifuentes *et al.* 2000) have been investigated. The most common hypothesis to explain the existence of a distinct community associated with a primary producer is that the primary producer imposes strong selective conditions on the populations of bacteria. This hypothesis was invoked to explain the high numbers of culturable sulfate reducing bacteria and the high relative abundance of sulfate reducing bacteria rRNA compared to other bacteria rRNAs (Hines 1999). Plant root exudates were thought to release substrates used exclusively by sulfate reducing bacteria which created a microbial food web that circumvented fermentation. In this framework, algal mats are postulated to increase the availability and types of carbon available to bacteria. Yellow-green algal mats have been shown to exude photosynthate and proteins which cue settlement of a sea slug planktotrophic larvae (Ascoglosson: *Alderia modesta*). The adult stage of the life history depends on the mat for nutrition (Krug 1999). The exudates that cue settlement likely leak into surrounding sediments and provide nutrition for bacteria. The algal mat may alter the sediment habitat in other ways. Algal mats may influence the steepness of the redox gradient and the availability of terminal electron acceptors. It is not unreasonable to suspect that a population of bacteria will be found in association that has characteristics optimized through selection to grow under these conditions. The thallus of the yellow green alga that forms intertidal mats may also provide a medium for attachment for heterotrophic bacteria as an alternative to organic matter and sediment particles.

There was weak evidence in favor of a distinct microbial community associated with algal mats. An overall estuarine pattern was not detected; however, in each estuarine site a distinct mat associated community was observed. The patterns that were observed in algal mat cover and genetic diversity of mat associated communities of bacteria suggest that the interactions between a producer and decomposers are complex. Algal mats had a distinct community of bacteria from non-mat sediments in all four sites, but the distinctiveness disappeared when multiple sites were compared. Selection in a particular habitat may be different in mat covered sediments than non-mat sediments. This subtle selectional discrepancy between mat and non-mat is overwhelmed by a stronger selection by environmental conditions in each locale. Therefore the existence of a uniquely mat associated community is likely very probable within each site, but the selection regime is not common to all estuarine sites.

CHAPTER V

PHYLOGENY OF UNCULTIVATED BACTERIA FROM ESTUARINE AND MARINE INTERTIDAL MUDFLATS

Abstract

In order to examine the number of species in a community and their patterns of distribution in nature, one must first identify the species. Microbial ecologists identify uncultivated species and unknown molecular DNA sequences from the environment by aligning several sequences from known, cultivated species. In this chapter, I describe the unknown bacteria sequences from intertidal mudflats. Phylogenetic trees of 16S rDNA were used to determine the relationship of unknown 16S rDNA gene sequences to molecular sequences found in the National Center for Biotechnology Information (NCBI) and the Ribosomal Database Project (RDP) database. I found uncultivated members of the Phylum *Chloroflexi*, Phylum *Proteobacteria*, Phylum *Firmicutes*, Phylum *Bacteroidetes*, and Phylum *Verrucomicrobia*. The newly contributed sequences from this research represent the first characterization of 16S rDNA gene sequences from intertidal mudflats. There were several molecular sequences from the sulfate reducing δ-proteobacteria that were also found in the deeper sediments of Puget Sound.

This contributions will assist in understanding a poorly understood community in estuarine ecosystems.

Introduction

Systematics is an organizational tool used by biologists to classify the enormous diversity of organisms in the natural world. In the history of science, numerous classification schemes have been proposed, but a classification system based on heredity and phylogenetic relationships prevailed. Phylogenetic classification uses shared and derived characters to understand phylogenetic and genetic relationships among species. These characters can also be used to identify a species in nature. Ecological studies use systematics to identify organisms and their patterns of distribution and abundance in their natural habitats.

A biological species was defined by Mayr (1942) as groups of interbreeding natural populations which are reproductively isolated from other such groups. Mayr (1963) outlined several characteristics of a biological species; a biological species (a) is defined by distinctness (b) consists of populations (c) is defined by the reproductive isolation of populations (d) is an ecological unit, and (e) is a genetic unit, whereas the individual is a temporary vessel holding a portion of the gene pool for a short time. This definition is problematic when applied to bacteria for several reasons. First, while plants and animals are rich in morphological features, bacteria have relatively simple life forms which make defining a species using morphologically distinct characters difficult. At a molecular scale, related bacteria share phenotypic features such as enzymatic pathways (Holt *et al.* 1994). Until recently, bacteria were classified under a paradigm based on cultivated species, which was devoid of evolutionary concepts (Woese 1987). Second, bacteria do not undergo meiosis or sexual reproduction. The extent of variability among microbial populations is unknown and therefore the amount of variability that defines a species is

also unknown.

A more useful definition of species applied to bacteria is the evolutionary species concept (Wiley 1978). In this definition, a species is a single lineage which maintains its identity and has its own evolutionary tendencies and historical fate. Nucleic acid sequences have shifted bacterial taxonomy from a convenient classification to a phylogenic one. Sequences from several species can be aligned and related using a model of sequence evolution (Hillis *et al.* 1996). The molecule 16S rRNA has been used extensively to identify and classify organisms in nature (Giovannoni *et al.* 1990, Devereux 1994, Risatti *et al.* 1994, Gray and Herwig 1996, Hines *et al.*1999). Other functionally related molecules have also been used for this purpose (Wawer *et al.* 1997, Lovell *et al.* 2001).

Ribosomal RNAs are useful because they are functionally constrained and found universally in all bacteria. While it is functionally constrained, there are domains of rapid and slow rates of positional change within the molecule. This characteristic makes it possible to analyze seemingly distantly related groups together, while maintaining resolution among related groups (Woese *et al.* 1983).

Few cultivation-independent studies of microbial diversity in marine sediments have been published (Gray *et al.* 1996, Kato *et al.* 1997, Rochelle *et al.* 1994). This study is the first from intertidal, coastal marine soft sediments. I included a detailed description of the community composition and four phylogenetic reconstructions of unknown sequences from intertidal mudflats.

In order to provide a systematic framework for identifying unknown sequences, I reconstructed a phylogeny of the major bacterial groups that included 32 newly contributed sequences. In addition, sequences of both cultured and uncultured rDNAs from the Genbank database were used to represent the major groups within the bacteria, as defined by 16S rDNA

sequences. I am not aware of any similar work with the exception of studies directed toward the intertidal salt marsh sediments on the eastern seaboard (Lovell *et al.* 2001). Intertidal mudflats were also the focus of a comprehensive approach to understand microbial communities in the European Wadden Sea (Bottcher *et al.* 2000), although the aforementioned research did not determine the identity and composition of the community. Consequently, this research makes a significant contribution to an improved understanding of the diversity of bacteria in estuarine ecosystems.

Materials and Methods

Extraction of Genomic DNA

Sediment core samples were collected and DNA extraction was conducted by a direct lysis method modified from Zhou *et al.* (47). 100 µl of 5M NaCl was added to 1 g of sediments, then 750 µl of CTAB Lysis buffer was added (pH 8.0, 1% CTAB, 0.05M Tris, 0.05M EDTA, 0.05M NaH_2PO_4, 1.5M NaCl). The sediment slurry was incubated at 37°C for 30 min after Proteinase K (5 µl, 20 mg/ml) was added. After 100 µl of 20% SDS was added, the mixture was incubated at 65°C for 1 hour, -80°C for 1 hour, and 65°C for 1 hour. The boil/freeze/boil step was followed by a 25:24:1 phenol/chloroform/isoamyl extraction. The aqueous extract was removed and precipitated with 0.6 volumes isopropanol, and incubated overnight at –20°C. Precipitated nucleic acids were pelleted for 30 minutes at 15,000g, washed with 70% ethanol and air dried. Pellets were resuspended in 100 µl Sigma molecular-grade water and incubated overnight at 4°C. Crude extracts were not amendable to enzymatic reactions or restriction digestion, so they were agarose gel purified (Li and Ownby 1993). Biases associated with DNA extraction and subsequent amplification of 16S rDNA have been discussed (Sekiguchi *et al.*

2001). I attempted to minimize PCR inhibitors by gel purifying all crude extractions prior to PCR analysis.

Amplification of the 16S rDNA Gene

Ten µl (~40 ng) of gel purified template was used in a 50 µl PCR reaction to amplify small subunit (SSU) 16S rDNA. Primers gc338F and 519R amplify a hypervariable region of the 16S rRNA gene (Muyzer 1993, Ferrari and Hollinbaugh 1999). These PCR products were used in DGGE to observe community profiles of bacteria. After differences were observed between sites, two clone libraries were constructed from the same template used for DGGE. A PCR reaction with primers 8F and 1392R amplified the 16S rDNA gene. Each reaction contained 5 µl 10X buffer, 5 µl 25 mM $MgCl_2$ (Applied Biosystems) 1.2 µl 2.5 mM dNTP, 10 U Amplitaq LD, 1.5 µl 10 pmol forward primer and 1.5 ul 10 pmol reverse primer. The reaction was brought to 95° C for 3 minutes then cycled 35 times 95°C, 30 s, 48°C, 30 s, 72°C 30 s and held at 72°C for 10 minutes. Another potential source of PCR bias is the presence of contaminating DNA. Given the universal nature of the Domain level primers (8F, gc338F, 519R, 1392R) and the ubiquity of bacteria in the environment, contamination of samples was of great concern. I frequently checked for contamination by routinely running negative controls, PCR reactions with no template DNA added.

Clone Library Construction and Screening

The 1-2 cm depth fraction was selected for construction of a clone library estuarine intertidal muflats at two locations. Previous data (Milbrandt 2003b) indicated that within-site variability was negligible compared to the variability observed between estuarine sites. It was concluded that any of the within site replicates would adequately represent the community. The

two sites chosen for clone library construction represented a mature intertidal mudflat (Hidden creek, see Figure 1), while the second site was a recently restored intertidal mudflat (Kunz marsh, see Figure 1). Blackened sediment was found at 1 cm depth in both estuarine sites and was used to construct the clone library. Amplified SSU rDNA (8F, 1392R) was cloned with an Invitrogen TOPO TA cloning kit. Inserts were amplified with M13F, M13R primers and screened (Vergin and Rappe 2002) using a double digest restriction fragment length polymorphism (RFLP). One ul 10X Buffer, 1 ul BSA, 0.2 ul Alu I and 0.4 ul Msp I, 7.4 ul water were incubated with amplified inserts for 1 hr at 37°C. Ten ul of the digest were run on a 2% agarose gel at 100V for 1.5 hrs. RFLP patterns were identified with Gel Pro and colonies containing unique RFLP patterns were grown up in 1.5 mL LB amp. Plasmid DNA was extracted using CPG mini-prep kit and sequenced. Oregon State University's Center for Genetics Biotechnology Lab sequenced the insert with sp6 and t7 primers. Sequences were formatted to 5' to 3' and edited with the assistance of the Ribosomal Database Project (RDP) sequence aligner (www.rdp.msu.edu). Full length sequences were analyzed individually using BLAST-n program at the Genbank website (http://www.ncbi.nlm.nih.gov). The sequences generated from the clone libraries have been registered in Genbank, accession numbers AY216437-AY216460.

Phylogenetic Reconstruction

All sequences were checked for chimera formation with CHECK_CHIMERA software of the Ribosomal Database Project. Sequences of cultivated and uncultivated taxa were downloaded from the RDP website. Taxa were chosen to represent major Bacteria groups as defined by sequence divergence and the Ribosomal Database Project backbone tree. Downloaded sequences were aligned with sequences generated in this study using Clustal X ver. 1.81 (Thompson). All gaps were removed and a multiple sequence alignment was performed. Sequences were truncated

at the 3' end at position1392 (*E. coli* numbering) to ensure that the length of the sequence would not bias the analysis. Phylogenetic trees were constructed with Phylip 3.6 alpha-release software for Windows. One thousand bootstrap replicates of the alignment file were generated and subjected to parsimony and maximum likelihood analysis. Parsimony analysis searched for the best tree using the more-thorough search option and used an archaeon *Methanocaldococcus vulcanius* as the outgroup. An Archaeon was used as an outgroup to show the strength of tree support for the Domain Bacteria. Maximum likelihood searched for the best tree and used a transition/ transversion ratio of 2.000. It assumed a constant rate of evolution and global rearrangements were not made for a speedier analysis. The consensus tree was chosen with majority rule (extended) option with *Methanocaldococcus vulcanius* as the outgroup.

Identity of DGGE Bands

DGGE bands of interest were selected for reamplification. Direct sequencing of reamplified bands was not reliable. Therefore, selected bands were reamplified with 338F and 519R and cloned using Invitrogen TA cloning kit (Invitrogen CA). Clones were screened using DGGE with clones and an environmental sample run on the same gel. Clones containing the desired insert were prepared for sequencing with a CPG mini-prep kit following manufacturer's instructions. The insert was sequenced with M13 primer at the Center for Gene Research and Biotechnology, Oregon State University, Corvallis, OR.

Results

Molecular sequences of bacteria collected from Kunz Marsh and Hidden Creek sediments were placed in a phylogenetic context with sequences from major Bacteria phyla (Cole *et al.* 2000). Cultivated taxa that have assigned characters based on morphological, physiological, and

16S rDNA sequence data are identifiable by scientific epithet (Figure 14). The previously uncultivated bacteria have names taken from their positions in the original clone library. Taxa were chosen according to two criteria: (a) to include a representative with a high similarity to unknown sequences isolated from South Slough and (b) to assure that one representative from the major phyla was included plus representatives from the classes in the Phylum Proteobacteria (Cole *et al.* 2000, Dojka *et al.* 2000).

Our phylogenetic hypothesis (Figure 14) was robust with respect to the choice of either maximum parsimony or maximum likelihood. High bootstrap values were consistently found at similar branch points under both models. The tree shown is the consensus tree from 1000 bootstrap replicates analyzed by maximum likelihood. Sequences that are highlighted in gray were found in 2 of the 2 clone libraries constructed. The sequence highlighted in black was found only in the mature mudflat. The majority of sequences and genetic diversity were found only in the restored site that was once an impounded marsh. The Bacteria formed a well-supported group that was distinct from the Archaeon, demarcated by node (a). The classes δ-proteobacteria (b), γ-proteobacteria (c), and α-proteobacteria (d) form well supported clades within the Phylum Proteobacteria. (g) The class ϵ-proteobacteria was not positioned in with the rest of the Phylum Proteobacteria, and grouped with Phylum *Bacteroidetes*. The bootstrap support at the node (e) was moderately supported by maximum likelihood and weakly supported by parsimony. Five sequences isolated from the intertidal mud were found in the Phylum *Bacteroidetes*. All five were isolated in a restored freshwater impoundment. The Phylum Firmicutes and Phylum Cyanobacteria formed a single clade with high bootstrap support, shown at node (h). There were two contributed taxa that were related to *Clostridium tetani* and *Entercococcus faecium*. These taxa formed the moderately supported clade of the Phylum Firmicutes. The Phylum Firmicutes, the Phylum Cyanobacteria and The basal lineages within the Bacteria were collapsed into one

weakly supported clade (i). There was strong support for the Phylum Chloroflexi (j), a deeply branching phylum which contained one contributed sequences.

A table of the sequences contributed by this manuscript (Table 4) lists the clone abbreviation, the ID percentage and the best matched organism or sequence in the NCBI database. In general, there was agreement in both the NCBI BLAST algorithm and our phylogenetic hypothesis. Two classes within the Phylum Proteobacteria held 10 of the newly contributed sequences. Additional phylogenies were reconstructed to show the within group diversity which included a greater number of reference taxa. For the three phylogenetic reconstructions in the Phylum Proteobacteria, *Aquifex pyrophilus,* was designated as the out group. *Aquifex pyrophilus* was chosen because of its basal position in the Domain Bacteria trees (Cole *et al.* 2003).

The Class δ-proteobacteria tree showed a quarter of the total diversity observed was associated with this group of anaerobic bacteria (Figure 15). It also showed that clones KM15, KM88, and KM95 were found in both clone libraries which suggested a broader range of distribution than the clones found at only one site.

In the Class γ-proteobacteria tree (Figure 16), a darkly staining DGGE band that was found in many estuarine locations was found to be identical to clone KM94. This sequence was 100% similar to a cultivated soil bacterium, *Pseudomonas fluorescens* and 97% similar to *Pseudomonas tolassii*. Their relationship was confirmed when the clade was supported in all 100 replicate trees.

The Class α-proteobacteria phylogeny was constructed to show the genetic diversity of the DGGE bands picked outside the estuary (Figure 17). Four sequences reamplified from intertidal sediments outside the estuary were related to the α-proteobacteria. Low bootstrap values were common for relationships among DGGE fragments and reference taxa. The only

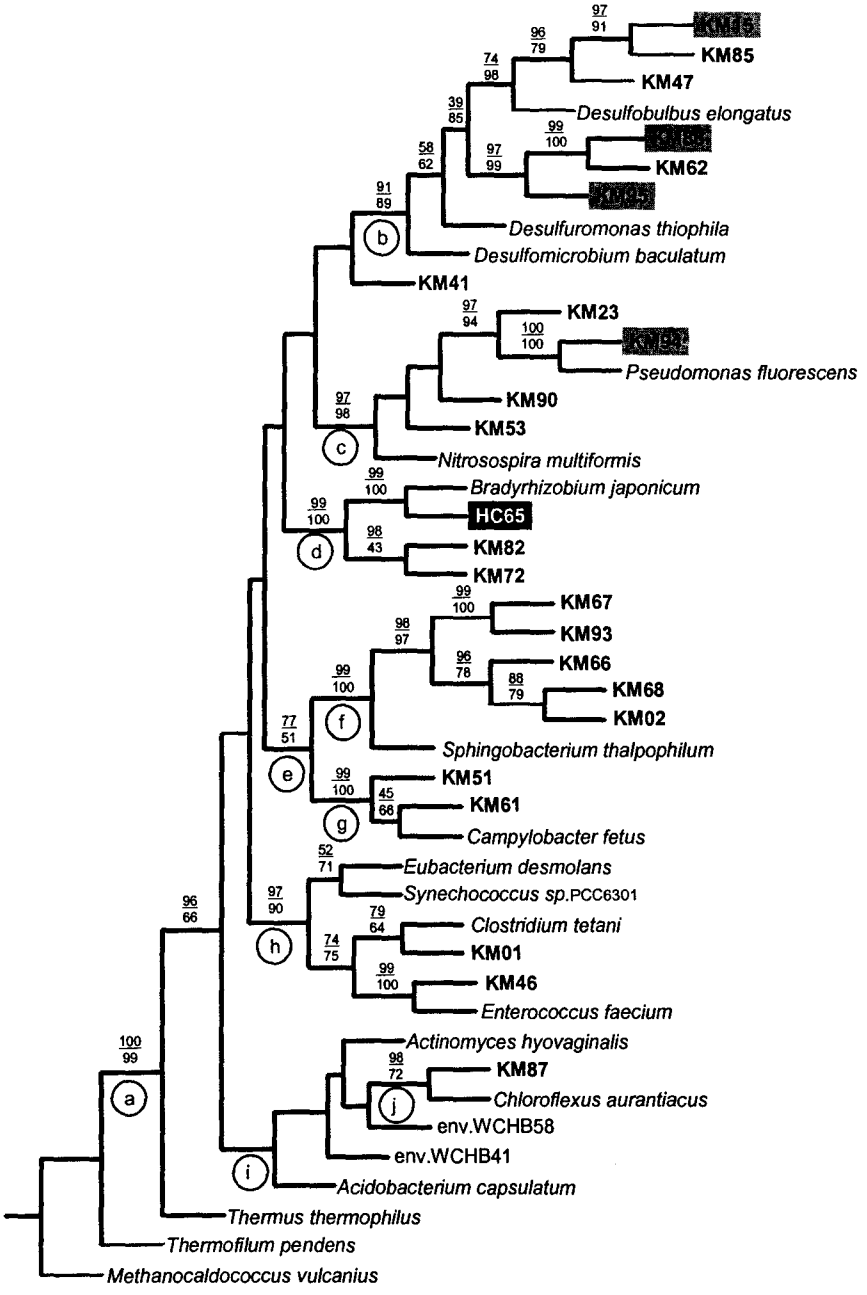

Figure 14. Phylogenetic Reconstruction of Unknown Estuarine Bacteria with Selected Known Taxa. Bootstrapping was performed on 1000 replicate data sets whose values are reported above for maximum likelihood and below for parsimony. Contributed sequences were found in the clone library constructed for a restored site (bold), mature site (black highlight), and both sites (gray highlight).

Table 4. Best Matched 16S rDNA Sequences to the Unknown Sequences Found in South Slough National Estuarine Research Reserve[a]. Contributed sequences were found in the clone library constructed for a restored site (bold), mature site (black highlight), and both sites (gray highlight). Except where designated, all sequences were 1400 b.p. in length.

Clone	ID%	Best matched organism	Accession no.	Group
KM23	93	uncultured OM241	U70702	γ-proteobacteria
KM53	97	*Thiomicrospira* sp. JB-A2	AF013974	γ-proteobacteria
KM90	96	uncultured Sva0091	AJ240987	γ-proteobacteria
KM94	97	*Pseudomonas tolassii*	AF320989	γ-proteobacteria
E1dgge	100	*Pseudomonas fluorescens*	AF094731	γ-proteobacteria
KM62	92	*Desulfobacterium Indolicum*	AJ237607	δ-proteobacteria
KM85	96	*Desulfobacterium catecholicum*	AJ237602	δ-proteobacteria
KM47	99[c]	uncultured CRE-PA66	AF141537	δ-proteobacteria
KM15	96	uncultured sva0999	AJ241013	δ-proteobacteria
E2dgge	97	uncultured sva0999	UDE241013	δ-proteobacteria
KM88	77	*Desulfonema magnum*	U45989	δ-proteobacteria
KM95	87	srb, strain mXyS1	AJ006853	δ-proteobacteria
KM70	97	uncultured *Desulforhopalus* sp.	AY177796	δ-proteobacteria
KM72	96	*Rhodobacter azotoformans*	D70847	α-proteobacteria
HC65	94	*Bradyrhizobium* sp.mc6	AF041446	α-proteobacteria
KM82	96	*Hyphomonas* sp. MHS3	M83812	α-proteobacteria
M2dgge	91	*Phaeosirillum fulvum*	AF508113	α-proteobacteria
M3dgge	98	*Antarctobacter heliothermus*	AF513928	α-proteobacteria
M4dgge	95	*Rhodobacter sphaeroides*	AF468821	α-proteobacteria
M5dgge	95	*Rhodovulum* sp. CP-10	AB079682	α-proteobacteria

Table 4 (continued).

Clone	ID%	Best matched organism	Accession no.	Division
KM61	91	*Campylobacter helveticus*	UO3022	ϵ-proteobacteria
KM51	73	*Thiomicrospira* sp. NKB11	AB013263	ϵ-proteobacteria
KM02	89	Uncultured SB-5	AF029041	CFB
KM93	64	envPAD30	D26217	CFB
KM67	64	envPAD30	D26217	CFB
M1dgge	93	uncultured CFB	AY133094	CFB
KM01	89	Clostridiaceae str.80Wc	AV078860	Eubacterium
KM46	97	Streptococcus infantis	AB008315	Firmicutes
KM19	93[c]	uncultured *Actinobacteria*	AJ229241	High G+C
KM31	94[c]	uncultured *Verrucomicrobia*	AF449258	Prosthecobacter
KM41	96	uncultured NKB17	AB013269	Nitrospina
KM87	90	*Chloroflexus* sp. STL-6-O1	AB06747	Green non-sulfur

[a] Listed are the percent identities (ID%) to previously identified sequences, the number of similar clones from restored and pristine, respectively, the accession numbers and divisions of the best matched organism in Genbank.
[b] 500 bp sequence

1400 b.p. contributed sequence was included. HC65 was the only sequence unique to the clone library constructed from the mature site.

Discussion

The newly contributed sequences from this research represent the first characterization of 16S rDNA sequences from intertidal mudflats. I also included sequences from a DGGE analysis of the distribution patterns of bacteria both within and outside of the estuary (Milbrandt and Shapiro 2003b). The newly contributed sequences were most closely related to cultivars and environmental sequences isolated from soils or sediments, based on BLAST searches in the Genbank database (http://www.ncbi.nlm. nih.gov).

Research on the ecology of sediment dwelling microbes has focused on measuring fluxes and decomposition rates with little attention on community structure. There are considerably more data about the physical and chemical properties of the Sargasso Sea, which has fueled progress in recognizing the ecological niche and culturing a previously uncultured bacterium (Rappe *et al.* 2002). Cultures of SAR11 clade have characteristics that are well suited for an oligotrophic marine environment. There were no sequences related to the SAR11 bacteria found in estuarine mudflats. There does not appear to be a microhabitat for water column associated bacteria in the sediments. This existence of a distinct community that lives in intertidal mudflats leads to a more general conclusion about communities of bacteria; there is a lineage-specific response to selection by environmental conditions. Our data suggests that there is selective pressure against bacteria that are related to the SAR11 lineage and other lineages associated with the water column.

The sediments of Puget Sound provided the best comparison for these data (Gray and Herwig 1996). Sediment samples collected at a depth of 13 m had 22 rDNA sequences that were

placed into the Classes δ–proteobacteria, γ–proteobacteria, and α–proteobacteria, the Phylum *Firmicutes*, Phylum *Actinobacteria*, and the Phylum *Planctomycetes*. I found a similar number of sequences (23) spread among these phyla. I found a number of taxa that were related to published sequences in three classes of the Phylum *Proteobacteria* and to sequences in the Phylum *Bacteriodetes*. I did not find any sequences related to marine snow clone AGGB related to the Phylum *Planctomycetes*. In this study, I found greater genetic diversity in the intertidal mudflats than reported for Puget Sound sediments. I contributed sequences of 16S rDNA related to the Phylum *Chloroflexi*, Phylum *Proteobacteria*, Phylum *Firmicutes*, Phylum *Bacteroidetes*, and Phylum *Verrucomicrobia*. In addition, I collected 4 replicates in two sites to check that the similarity was high between the cloned replicate and the three other replicates collected in each site (Milbrandt and Shapiro 2003b). Many published phylogenies did not consider variability before cloning the amplified 16S rDNA from a habitat. The unreplicated approach (Gray and Herwig 1996, Crump et al. 2000) to describe bacterial communities does not adequately address aspects of variability. I screened 130 clones containing the 1400 b.p. insert, which was more than the number screened by Gray and Herwig (1996). A few rare taxa were recovered by screening a greater number of clones.

Ravenshlag *et al.* (1999) reported high genetic diversity in Arctic Ocean sediments. Their findings of high genetic diversity can be attributed to a more sensitive method of screening. Dot blot hybridization was used with probes designed to distinguish among all Bacteria, the Class α-proteobacteria, a number of the Class δ-proteobacteria genera, Gram positive bacteria, Phylum *Bacteriodetes*, and endosymbionts in the Class γ-proteobacteria. The more sensitive hybridization screening method is evident in their two phylogenies, where a number of newly described sequences have very short branch lengths. The percent similarity for these short branched relatives were not reported but are likely above the 98% similarity sometimes used to

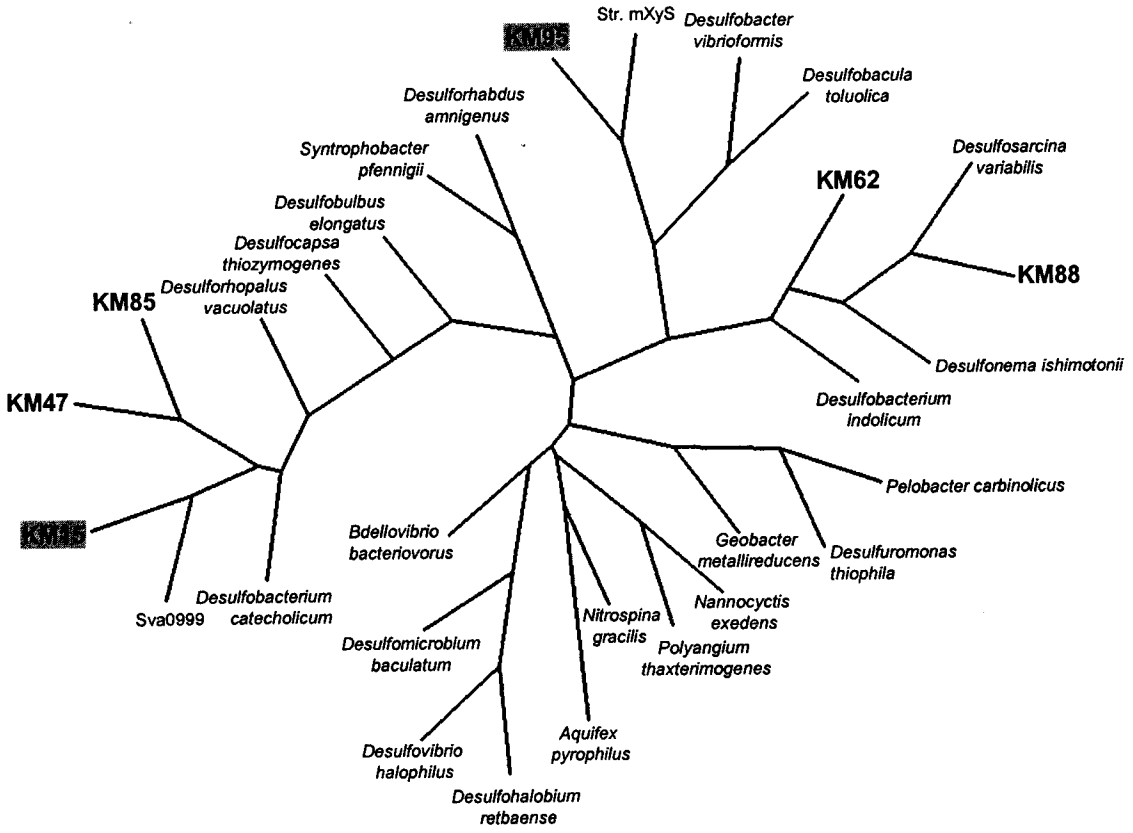

Figure 15. Phylogenetic Reconstruction of δ-proteobacteria.

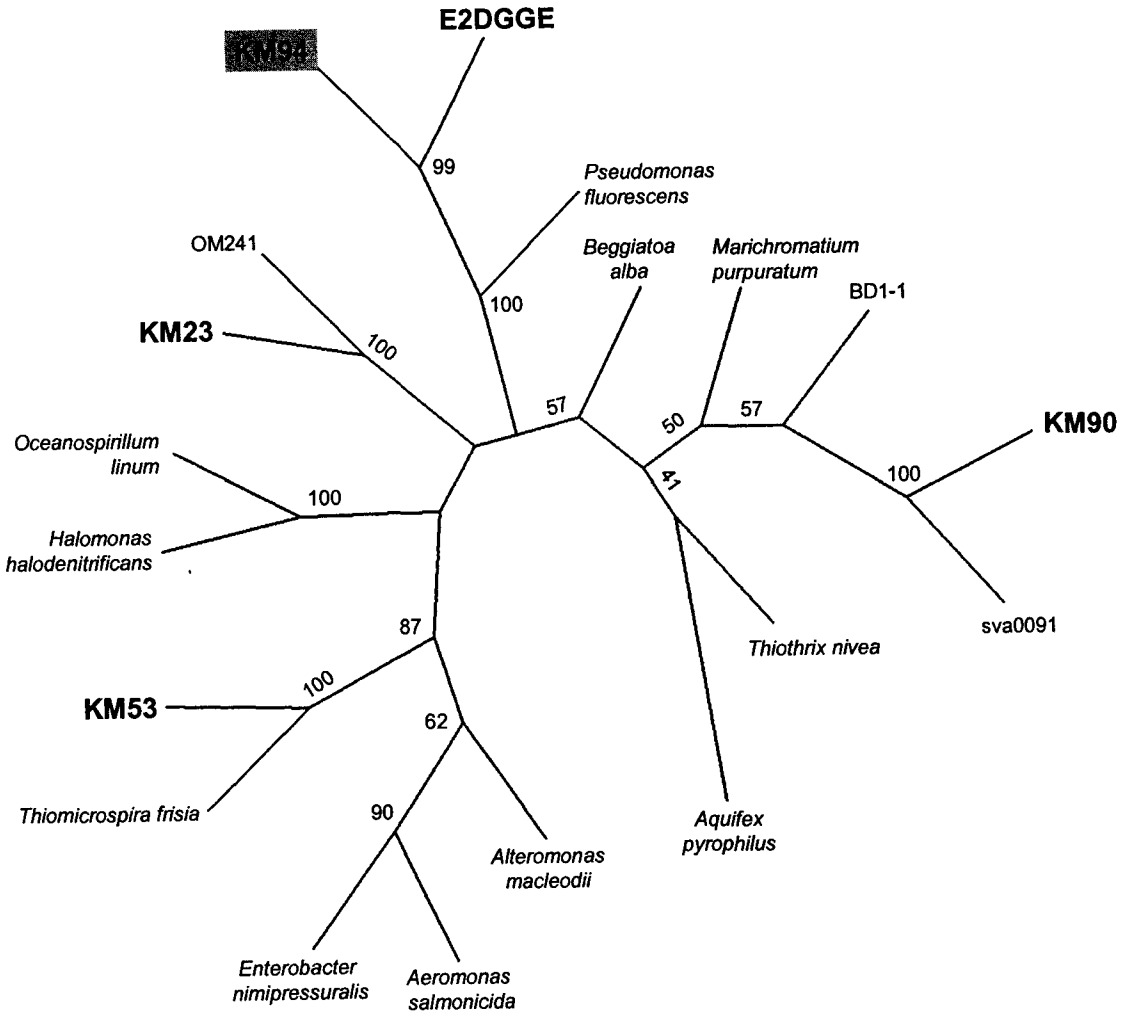

Figure 16. Phylogenetic Reconstruction of γ-proteobacteria.

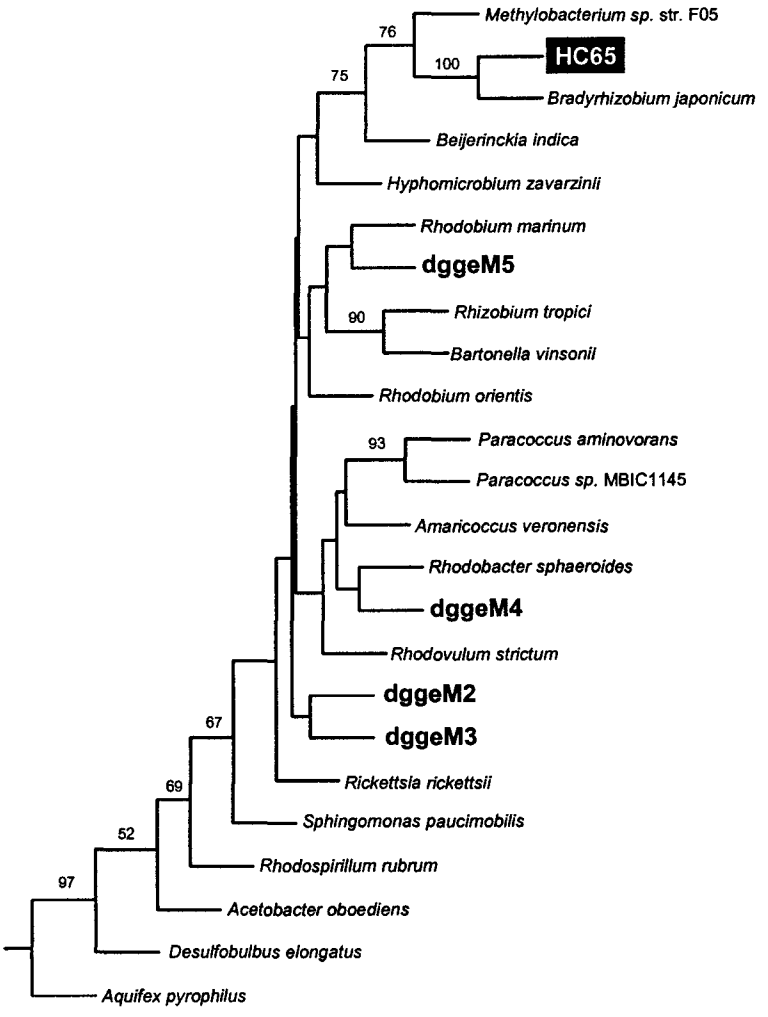

Figure 17. Phylogenetic Reconstruction of α-proteobacteria.

define a species (Pace 2000). The hybridization method detects a greater number of base pair mismatches, which does not equate to greater genetic diversity. I propose that genetic diversity of uncultivated bacteria be defined as the spread of sequences in the Domain Bacteria. Under this definition, I feel that a less-sensitive screening method is more appropriate. Our phylogenetic reconstruction shows that the intertidal mudflat has sequences related to a greater number of bacterial groups than reported for Arctic Ocean sediments.

Estuaries have important parallels in the genetic composition of bacterial communities. Gradients in salinity, temperature, redox potential, dissolved oxygen and light availability are stronger in estuaries than deep-sea sediments. Information about the occurrence and diversity of bacteria in estuaries could streamline the scientific approach to understanding deep-sea sediments, which are more difficult to sample. The scale of resolution and precision of sampling deep-sea communities is limited by ship time and collecting samples under hundreds of meters of water. Estuarine tideflats can provide a source of data for modeling, which can be used to frame better questions and improve the understanding of deep-sea sediments.

Unlike deep ocean sediments (Gray *et al.* 1996), intertidal mudflats are exposed to rapidly attenuated light (Pierson *et al.* 1996). Intertidal mudflats can be frequently exposed to air, and solar heating which has an unknown effect on the survival of microbes. More physical and chemical data about pore water at high and low tides plus culture independent surveys are needed to understand the effect of tidal variation on microbial communities. This course of research may also improve our understanding of the relationship between bacteria and microhabitats. A sequence found in the mature clone library, but not in the restored library was related to the Genus *Bradyrhizobium.* The presence of this taxon suggests that there may be an ecologically important function that is lacking in restored site sediments (del Giorgio and Newell 2000).

Bacterial communities were remarkably different on the outer coast than within the estuary when measured with DGGE (Milbrandt and Shapiro 2003b). The source of difference in the DGGE fingerprint was traced to four DGGE bands (M2-M5). Sequences of bands from marine influenced habitats were related to the α-proteobacteria lineage. The α-proteobacteria lineage has been observed to be associated with marine dominated water column habitats along an estuarine gradient (Bouvier and del Giorgio 2002). The phylogenetic relationships among DGGE fragments and reference taxa were not well supported within the α-proteobacteria tree. A longer sequence (HC65) had high bootstrap support which leads to the conclusion that the robustness of the phylogenetic reconstruction using maximum likelihood is compromised by small sized fragments. This artifact was again illustrated when a DGGE band (E1) from estuarine samples was included in the γ-proteobacteria tree. E1 was found in all DGGE gels that I ran with estuarine samples and it was often the darkest staining band. Clearly, this band and sequence represents a taxon that has a wide distribution and may also be numerically dominant (Shafer and Muyzer 2000). Although a greater amount of DNA on a DGGE gel cannot be directly correlated with numerical abundance, several have argued that DGGE band intensity is an indirect measure of abundance (Nübel *et al.* 1999, Jackson *et al.* 2001, Braker *et al.* 2001).

The soft-sediment habitat is considered a critical component to the estuarine and coastal marine ecosystems. Despite the limitations of molecular methods (Head *et al.* 1998, Hugenholz *et al.* 1996, Kemp 2003), it is the best way to detect taxa that have never been brought into culture. Phylogenetic analysis of one conservative gene sheds no light on the phenotypic diversity in bacterial communities or metabolic function. The presence of a taxa with 100% similarity does not mean that it is the bacterium identical to the strain in the culture collection. However, it is useful to know the relationships of newly contributed sequences to those in culture

to better target growth media. It also provides insight about the extraordinary genetic diversity in the Domain Bacteria in the environment.

CHAPTER VI

GENERAL CONCLUSIONS

The nature and strength of interactions within a multi-species assemblage are likely to be complex. I examined the species distribution patterns and phylogenetic relationships of bacteria across natural and anthropogenic gradients. I used spatial patterns of organization across gradients to provide information about the community. These results tell little about the nature of the interactions, but were useful in determining phylogenetic relationships and genetic diversity patterns. This approach has led to several novel contributions to the field of microbial ecology; (1) intertidal mudflats in the estuary have distinct assemblages from those found outside the estuary, (2) the Class α-proteobacteria, was found in euryhaline sites and may be a useful biological indicator of salinity, (3) centimeter scale and meter scale variability within a site were insignificant to the variability observed among estuarine sites, (4) a salinity dependant pattern in community similarity was demonstrated by several taxa belonging the Class α-proteobacteria, (5) the genetic diversity of the restored site was higher than the mature site, (5) community similarity was higher in three successive year between the restored and mature sites (6) seasonal patterns of cover for the mat forming alga, *Vaucheria longicaulis*, were documented, (7) the newly contributed sequences isolated from intertidal mudflats were placed in a phylogenetic context for the first time.

Estuaries are ideal places to understand the organization and variability of microbial communities. Salinity, temperature, and redox potential are some of the gradients that affect distributions of organisms. Bacteria have an important role as prey for other estuarine dependant species and as the mediators of decomposition. It was surprising to find that kilometer scale variability was more important than the variability at smaller scales. There was variability at small scales, however, it was relatively low within site compared to among sites. Bacteria communities showed patterns of variability shown in macroscopic organisms. The estuarine gradient affects the distribution of bacterial taxa as it does for other estuarine dependant species regardless of the size of an individual.

Estuaries are often developed for their capacities to deliver goods or provide rich soils for agriculture. Therefore, they have been impacted by human activities which have included dredged channels and drained salt marshes. These activities and the loss of coastal habitat in estuaries have prompted restoration activities, which provide an opportunity for improvement of degraded habitats. The science of restoration has overtaken the ability to evaluate success or failure, which has led to the need for biological metrics. The recovery of bacteria in restored sediments may preclude colonization by salt marsh plants or invertebrates, and may serve as a metric for gauging successful restoration.

The anoxic intertidal sediments of estuaries are an ideal place to look for taxa that thrived in the early earth atmosphere. I found one sequence related to an early branching group, the Phylum *Chloroflexi*. The extent of the genetic diversity of bacteria was unknown from intertidal mudflats. DAPI-stained counts of bacterial abundance from sediment habitats are exceedingly high, but their phylogenetic position was unknown. This is the first report of the extent of genetic diversity among such bacteria. Bacteria from five Phyla were reported with a number of newly contributed sequences related to the Phylum *Proteobacteria* and the Phylum *Bacteriodetes*.

Hurst (2002) postulated that communities of bacteria function as interacting, co-evolved assemblages. The nature of interactions between microbial species, families, and phyla has yet to be discovered. It is an exciting time to practice science with this large question looming over the scientific community. Microbes are more abundant and hold more genetic diversity than any known life form. It is difficult to estimate whether humankind will fully comprehend these microscopic organisms, but there will be many discoveries that may help clarify our role as the conscious observer.

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