CHITINOLYTIC BACTERIA AND ENZYMES FROM MONO LAKE, CA, USA

by

GARY R. LECLEIR

(Under the Direction of James T. Hollibaugh)

ABSTRACT

Chitin is an abundant biopolymer whose degradation is mediated primarily by bacterial chitinases. We developed a degenerate PCR primer set to amplify a ~900 bp fragment of family 18, group I chitinase genes and used it to retrieve these gene fragments from environmental samples. Clone libraries of presumptive chitinase genes were created for nine water and six sediment samples from ten aquatic environments including freshwater and saline lakes, estuarine water and sediments and the central Arctic Ocean. Putative chitinase sequences were also retrieved from the Sargasso Sea metagenome sequence database. PCR product using these primers was not obtained from an alkaline, hypersaline lake (Mono Lake, CA). In total, 108 partial chitinase gene sequences were analyzed, with a minimum of 5 and a maximum of 13 chitinase sequences obtained from each library. All chitinase sequences were novel compared to previously identified sequences. Intralibrary sequence diversity was low, while significant differences were found between libraries from different water column samples and between water column and sediment samples. Identical sequences, however, were retrieved from samples collected at widely distributed locations that did not necessarily represent similar environments, suggesting homogeneity of chitinoclastic communities between some environments.

An inability to amplify chitinase genes from Mono Lake, despite high levels of chitinolytic activity, prompted the analysis of the microbial community composition associated with *Artemia monica* exuvia and in chitin enrichments of Mono Lake water. Chitinolytic bacteria from Mono Lake were also isolated. Bacterial assemblages were characterized by cloning and sequencing 16S rDNA amplicons. Isolates were screened for chitinolytic activity using methylumbelliferyl-diacetylchitobioside (MUF-DC) and methylumbelliferyl-triacetylchitotrioside (MUF-TC); for the ability to hydrolyze colloidal chitin; and for growth on medium containing only chitin. Several ribotypes were common to *Artemia* exuvia samples and chitin enrichments. Four Proteobacteria ribotypes were only retrieved from clone libraries of chitin enrichments. The majority of the isolates obtained were Gram-positive bacteria and 70% of the Gram-positive isolates hydrolyzed at least one model substrate.

Chitinolytic genes from two Mono Lake isolates and from an environmental DNA library from Sapelo Island, GA were then obtained by shotgun cloning using fosmid vectors. Fosmid libraries were screened for MUF-DC hydrolysis and six positive clones were analyzed further. Genes of interest were localized by random transposon mutagenesis. One clone from a Mono Lake isolate contains a gene encoding a family 18 glycosyl hydrolase. Two additional clones, one from a Mono Lake isolate and another from the environmental library, contain genes encoding family 20 glycosyl hydrolases. The proteins expressed by these clones were characterized with respect to pH and salt tolerance. An enzyme from Mono Lake clone AI214B1 maintained activity at pH 11 and salinity of 225 ppt. These characteristics have not been previously associated with this enzyme family.

INDEX WORDS: Mono Lake, chitin, chitinolytic enzymes, n-acetylglucosaminidase, Bacteria, Vibrio, diversity, biogeography

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CHAPTER 1

INTRODUCTION

Chitin and chitinolytic enzymes

Chitin is second to cellulose as the most abundant biopolymer on the planet (12). Chitin production has been estimated at 10^{11} metric tons per year (19) in aquatic systems alone, where it is synthesized by a wide variety of organisms, including marine protists, fungi, arthropods, mollusks and annelids. Organisms generally use chitin as a structural support molecule, however, due to its durability, strength and impermeability, it also provides protection against predation and desiccation (12). Pure chitin is a homopolymer of repeating N-acetyl-glucosamine (GlcNAc) units; thus each molecule contains carbon and nitrogen in a ratio of 8:1. In addition, chitin almost always contains structural proteins or minerals interlaced throughout the chitin matrix (25). The high carbon and nitrogen content of chitin, its resiliency to degradation and its large annual production make it a significant component of the organic matter in many environments (19). Thus, the mineralization of chitin, which is performed almost exclusively by microbes, is an essential component of nutrient cycling in most natural systems (12, 27).

Chitin was first discovered in 1811 by Professor Henri Braconnott in the cell walls of mushrooms. Due to its strength, solubility and chelating properties, it was quickly realized that chitin held great biotechnological promise. For instance, the capacity of both chitin and chitosan (a deacetylated form of chitin) to bind heavy metals and other contaminants in water is currently

exploited in a wide range of applications, including water purification and wastewater treatment. Furthermore, consumer products such as dietary supplements, cosmetics, internal sutures, burn dressings and even contact lenses may contain chitin or chitosan (19).

Due to its polymeric nature, chitin must undergo at least partial hydrolysis prior to assimilation by microbial cells (3). This is accomplished by the synergistic action of chitinases (EC 3.2.1.14) (6, 13) and N-acetyl-glucosaminidases (GlcNAcidases) (E.C. 3.2.1.52). Chitinases hydrolyze the bonds between GlcNAc residues in the chitin molecule, typically yielding oligomeric or dimeric products. GlcNAcidases also hydrolyze the glycosidic bonds between GlcNAcs, however, they generally cleave GlcNAc monomers from the oligomeric products produced by chitinases. Some GlcNAcidases are also capable of cleaving GlcNAc monomers directly from the chitin molecule (36), however, this function is somewhat rare. GlcNAc monomers and dimers can be transported across bacterial cell membranes where they undergo further enzymatic processing to acetate, NH_3 and fructose-6-phosphate (1, 19) . These products can be then shuttled to central bacterial metabolism pathways or modified for use in cell wall biosynthesis.

Chitinases are organized into family 18 and 19 of the glycosyl hydrolases based on amino acid sequence similarity (14). These two families are truly distinct; they share no similarity at the amino acid level, have different three-dimensional structures (10) and different mechanisms of hydrolysis (15). The vast majority of bacterial chitinases characterized thus far fall within family 18 and can be further categorized into five different groups (I-V) based on conservation of amino acid residues within the catalytic domain (30). Group I chitinases are widely distributed among members of diverse Proteobacterial lineages (5). Groups II-IV contain chitinases from more narrowly restricted lineages. Group V is a collection of chitinases that do

not fall into one of the other four groups (30). Family 19 chitinases are generally found in higher plants. Bacterial genomes from organisms such as some of the *Burkholderia* and *Streptomyces* have been shown to harbor family 19 chitinase genes. Family 19 chitinases are presumed to have been obtained by bacteria through the process of horizontal gene transfer (7, 18). While some bacterial chitinases are intracellular, many are often associated with the outer membrane or are secreted as extracellular enzymes (19, 20).

GlcNAcidases are classified into family 20 of the glycosyl hydrolases (13, 14). GlcNAcidases are found either extra-, ecto- or intracellularly in bacteria (22, 31, 34). They are widely distributed throughout the domains of life, from archaea and bacteria to higher plants and animals. In addition to their importance in chitin hydrolysis, GlcNAcidases are also crucial in higher organisms; for example, deficiencies in GlcNAcidase functioning causes severe disorders including Tay-Sachs and Sandhoff disease in humans (32).

Chitinases and GlcNAcidases have received a great deal of attention in recent years because of their potential uses in many different industrial and biotechnological fields. They are currently used for shellfish waste processing and for bio-control of insects in agricultural fields (11, 25). Genetically modified plants showed a demonstrable ability to resist infection by various pathogens when expressing these enzymes (16).

The extracellular location of many chitinases and GlcNAcidases suggests that many of these enzymes must be adapted to function under the physicochemical conditions present in the surrounding environment. Thus unique environmental conditions (e.g. high salinity, pH, or extreme temperatures) may select for enzymes with unique amino acid sequences and novel biochemical properties. This hypothesis drove the investigation of chitinases and GlcNAcidases

in microbes from Mono Lake because of the high salinity, high pH and the overall unique environmental conditions there.

Mono Lake: a unique site for the study of chitinases

Mono Lake is an alkaline, hyper-saline lake located east of the Sierra Nevada Mountain range in California, USA (38° 00' N; 119° 02' W http://geopubs.wr.usgs.gov/map-mf/mf2393/). The lake has a pH of 9.8 and a salinity of approximately 85 ppt (17). The extreme environment of Mono Lake results in a relatively simple food web. The primary organisms in this system are archaea, bacteria, phytoplankton, rotifers, brine flies (*Ephedra hians*) and the brine shrimp *Artemia monica*. *Artemia* are the dominant macro-zooplankter in the lake and achieve population densities greater than 80,000 m^{-2} in the summer months (4).

In Mono Lake, brine shrimp nauplii begin to hatch in early spring (8, 9, 23) and adult *Artemia* are observed by mid-May (9). *Artemia* develop and mature through 12 instar stages. At the transition between each stage, they molt and shed their chitin-rich exoskeleton (exuvia) into the water (26). Due to the high population densities attained by *Artemia* in Mono Lake and their frequent molts, chitin is a major biopolymer in the lake. For example, following the fall die-off of the *Artemia* population, exuvia and carcasses sink to the lake bottom to form a flocculent layer that can be more than a centimeter thick (LeCleir and Hollibaugh, pers. obs.). A combination of the unique environment and high quantities of chitin in Mono Lake made it an attractive location for investigations of unique chitinolytic enzymes.

Rationale for the experimental approach

Bacterial chitinase genes have already been retrieved from diverse terrestrial environments, including alkaline soils (33), sandy soils (35) and pastures (21, 24). However, equivalent studies of chitinases in aquatic systems are relatively rare (6, 20, 28). Furthermore,

no studies have compared chitinases across a broad range of distinct environments. Comparison of chitinase genes retrieved from similar, but geographically isolated, environments could yield insight into the biogeography of functional genes. In addition, comparisons of gene sequences retrieved from environments with distinct chemical and physical characteristics (water column versus sediments; estuaries; freshwater and saline lakes; temperate coastal waters, the Sargasso Sea and the Arctic Ocean) may yield insights into how environmental conditions select for enzymes with novel properties.

Chitin degradation in salt lakes has received relatively little attention. In fact, it is not yet known if the phylogeny of the chitinolytic community in salt lakes is similar to those found in other environments. Furthermore, chitin mineralization in these environments is expected to contribute significantly to nutrient cycling because of the high numbers of *Artemia* in the lake and the chitin rich exuvia they produce during the maturation process. Finally, due to the variability associated with these environments (temperature, salinity and O_2 fluctuations), investigations of the microbial community involved in chitin degradation and of their response to environmental changes is relevant to our understanding of these systems as a whole.

Recently, large-insert clones have been used to investigate functional genes from environmental samples (2, 29). These techniques (bacterial artificial chromosome (BAC) and fosmid libraries) avoid the use of PCR and permit the isolation and study of genes and enzymes without the need for a prior knowledge of their sequences. These techniques are especially well suited to environments with organisms or genes that prove difficult to study using PCR. Furthermore, the relatively large size of inserts in BAC and fosmid libraries (30-150 kbp) affords investigators the ability to study complete operons and flanking genes with moderate ease.

The following chapters include studies that help to answer questions about chitin degradation in Mono Lake and other environments. Chapter 2 describes the modification of a degenerate PCR primer set to be more inclusive of chitinases from a wider array of organisms. These primers were then used to examine deduced amino acid sequences of chitinase gene amplicons to try to determine the diversity of microbial chitinases in the environment and if environmental conditions influence microbial chitinases. In Chapter 3, information obtained from enrichment cultures, PCR, DGGE, clone library formation and bacterial isolation helped define a microbial community associated with chitin degradation in Mono Lake. Chapter 4 explains how shotgun fosmid libraries were used to identify, isolate and partially characterize chitinolytic enzymes from two contrasting environments, representing the two enzyme families involved in chitin degradation.

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CHAPTER 2

CHITINASE GENE SEQUENCES FROM DIVERSE AQUATIC HABITATS REVEAL COMPLEX PATTERNS OF DIVERSITY

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ABSTRACT

Chitin is an abundant biopolymer whose degradation is mediated primarily by bacterial chitinases. We developed a degenerate PCR primer set to amplify a ~900 bp fragment of family 18, group I chitinase genes and used it to retrieve these gene fragments from environmental samples. Clone libraries of presumptive chitinase genes were created for nine water and six sediment samples from ten aquatic environments including freshwater and saline lakes, estuarine water and sediments and the central Arctic Ocean. Putative chitinase sequences were also retrieved from the Sargasso Sea metagenome sequence database. We were unable to obtain PCR product with these primers from an alkaline, hypersaline lake (Mono Lake, CA). In total, 108 partial chitinase gene sequences were analyzed, with a minimum of 5 and a maximum of 13 chitinase sequences obtained from each library. All chitinase sequences were novel compared to previously identified sequences. Intralibrary sequence diversity was low, while we found significant differences between libraries from different water column samples and between water column and sediment samples. However, identical sequences were retrieved from samples collected at widely distributed locations that did not necessarily represent similar environments, suggesting homogeneity of chitinoclastic communities between some environments.

INTRODUCTION

Chitin is the second most abundant biopolymer on the planet (15). In aquatic systems alone, chitin production has been estimated at 10^{11} metric tons per year (23). Chitin is composed of repeating units of the monomer N-acetylglucosamine (GlcNAc) and contains carbon and nitrogen in a ratio of 8:1. Chitin degradation is a key step in the cycling of nutrients in the environment (15, 32) and microorganisms are the primary agents of chitin degradation. Due to its polymeric nature, chitin must undergo at least partial hydrolysis prior to assimilation by microbial cells (6); this is accomplished by the enzyme chitinase (EC 3.2.1.14) (8, 18). Chitinases hydrolyze the bonds between GlcNAc residues, typically yielding oligomeric or dimeric products capable of being transported across the cellular membrane where they can be metabolized further (3, 23). Bacterial chitinases are often associated with the outer membrane or are secreted as extracellular enzymes (23, 24). The extracellular location of chitinases suggests that they must be adapted to function under the physicochemical conditions present in the surrounding environment. Thus unique environmental conditions (e.g. high salinity, pH, or extreme temperatures), may select for proteins with unique sequences and thus biochemical properties.

Chitinases are classified as either Family 18 or 19 glycosyl hydrolases based on amino acid sequence similarity (19). These two families are truly distinct; they share no similarity at the amino acid level, have different three-dimensional structures (10) and mechanisms of action (22). The vast majority of bacterial chitinases fall within Family 18 and can be further organized into five different groups (I-V) based on conservation of amino acid residues within the catalytic domain (38). Group I chitinases are widely distributed among members of diverse Proteobacterial lineages (7). Groups II-IV contain chitinases from more narrowly restricted

lineages. Group V is a collection of chitinases that do not fall into one of the other four groups (38).

Bacterial chitinase genes have been retrieved from diverse terrestrial environments, including alkaline soils (40), sandy soils (46) and pastures (25, 27). However, equivalent studies of chitinases in aquatic systems are relatively rare (8, 24, 33). Furthermore, no studies have compared chitinases across a broad range of distinct environments. Comparison of chitinase genes retrieved from similar, but geographically isolated, environments could yield insight into the biogeography of functional genes. In addition, comparisons of gene sequences retrieved from environments with distinct chemical and physical characteristics (water column versus sediments; estuaries; freshwater and saline lakes; temperate coastal waters, the Sargasso Sea and the Arctic Ocean) may yield insights into how environmental conditions select for enzymes with novel properties.

In this study, we used a degenerate primer set to retrieve putative chitinase genes from eight aquatic systems with distinct environmental characteristics. The results suggest that similar environments yield similar chitinase gene sequences. Furthermore, unique signature sequences were retrieved from one set of samples that may translate into fundamental differences in enzyme properties.

MATERIALS AND METHODS

Community DNA. Locations sampled in this study and a summary of the environmental conditions at these locations are given in Table 1. Samples of surficial (0-1 cm) sediments were collected with a plastic spatula or by hand, placed in a glass jar and stored on ice for transport to the laboratory. Water samples were collected with a Niskin sampler (Mono Lake, Soap Lake,

Walker Lake); a clean plastic bottle or bucket (estuarine and coastal water samples); or from a submarine as described in Bano and Hollibaugh (1)(Arctic Ocean samples).

Samples were either frozen or immediately processed in the lab upon return from the field. Microbial biomass was collected from water samples by pressure filtration through Millipore Sterivex® cartridge filters (ca. 50 kPa; 0.22-μm pore size). Excess water was expelled from the capsule. The cartridges were filled (1.8 mL) with a buffer containing 50 mM Tris (pH 8.3), 40 mM EDTA, and 0.75 M sucrose; capped; frozen on dry ice; shipped to the laboratory; and stored at -70°C until processed.

Community DNA was extracted from sediment samples and purified using the Ultraclean® Soil DNA Kit (MoBio Laboratories Inc., Solana Beach, CA) following the manufacturer's instructions. Extraction and purification of DNA from cartridge filters was essentially as described by Ferrari and Hollibaugh (13). Briefly, 40 μL of lysozyme (50 mg mL^{-1}) was added to each cartridge, and the cartridges were incubated for 60 min at 37°C. Fifty microliters of proteinase K (20 mg mL^{-1}) and 100 μL of a 20% (wt/vol) solution of sodium dodecyl sulfate were added to each cartridge, and the cartridges were incubated at 55°C for 2 hours. DNA was purified from 800 μL of the lysate by sequential extraction with 800 μL of phenol:chloroform:isoamyl alcohol (25:24:1), chloroform:isoamyl alcohol (24:1), and finally *n*-butanol. The aqueous phase was removed, placed in a Centricon-100 concentrator (Amicon, Bedford, MA), mixed with 500 μL of TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0), and centrifuged at 1,000 x *g* for 10 min. Next, 500 μL of TE was added to the Centricon-100 concentrator, and the mixture was centrifuged for another 10 min. Successful extraction of high molecular weight DNA was verified for all samples by electrophoresis on 1% agarose gels.

Primer Design. The degenerate primer chiAfor.ext was based on conserved residues identified in chitinases from diverse proteobacteria (Fig. 1). Protein sequences were aligned using the PILEUP tool of the Wisconsin package, version 10.0 (Accelrys, San Diego, CA). chiAfor.ext was used in conjunction with chiA.rev, a primer developed by Cottrell et al (9). This primer set successfully amplified the chitinase gene from *V. harveyii*.

PCR and cloning. PCR primers chiAfor.ext and chiA.rev were used to amplify putative chitinase gene fragments from community DNA. PCR was run with the following conditions on an MJ Research PTC-200 Peltier thermal cycler: Denaturation at 94 °C for 1 min, annealing at 58 ºC for 1 min and an extension step at 72 ºC for 1 min. This sequence was repeated 35 times followed by a 10 min final extension step at 72 ºC.

Products of the appropriate size (~900 bp) were recovered from a 1.5% agarose gel using the QiaQuik Gel Extraction Kit (Qiagen, Valencia, CA) and cloned into the pCR 2.1 vector (Invitrogen Corp., Carlsbad, CA) following manufacturer's protocols. Clone libraries were generated for all samples that yielded a PCR product of the expected size. Colonies were selected randomly then plasmids were isolated from *E. coli* host cells with the Qiaprep Spin Miniprep kit (Qiagen). Insert size was verified by digestion with *Eco*RI, then inserts of the correct size were sequenced using an ABI PRISM 310 Genetic Analyzer and the BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) using primers that recognized the cloning vector (M13 forward and reverse). Reads of approximately 550 bp of nucleotide sequence were obtained in each direction. Sequences were edited and assembled using the AssemblyLign Program (Oxford Molecular, 1998). The forward and reverse reactions resulted in a complete sequence for the amplified region of the chitinase gene with ~200 bp of

overlap. Regions corresponding to the primer binding sites were removed from the sequences prior to analysis.

Phylogenetic analyses. Sequences were analyzed using the Wisconsin Package v 10.2 (Accelrys, San Diego, CA) and homology searches (BLASTX) were carried out at the network server of the National Center for Biotechnology Information. Phylogenetic trees were constructed with the PHYLIP package using evolutionary distances (Jukes-Cantor or Kimura) and the neighbor-joining method (12). A maximum-likelihood tree was also constructed using the phylogenetic analysis program PAUP (39) to verify the results from the Jukes-Cantor algorithm. The sequences have been submitted to GenBank under the following accession numbers: AY674058-AY674165.

Database sequences. Putative chitinase sequences were retrieved from the Sargasso Sea metagenome database (SSMD) (http://www.ncbi.nih.gov/BLAST/Genome/EnvirSamplesBlast.html; 43) by interrogation (BLASTX) using one sequence from each of the five clusters of our tree (refer to Fig. 2; WLS-07 (AY674163), TLS-08 (AY674150), BBW-04 (AY674077), AOW55-10 (AY674066), SLW21-07 (AY674140)). Homology searches were then carried out against the entire GenBank database using each of the SSMD potential chitinase sequences. Criteria for inclusion in our phylogenetic analysis were: (1) the sequenced portion of the gene had to contain the entire region of the gene analyzed in this study and (2) the putative genes had to be capable of being aligned to our existing library of chitinases using the PILEUP tool of the Wisconsin package. Accession numbers of all potential chitinases from the SSMD have been recorded in a spreadsheet that can be accessed at the Mono Lake Microbial Observatory web site

(http://www.monolake.uga.edu/research.htm; "Ancillary Data" section; "Sargasso_Sea_Chitinases.xls").

RESULTS & DISCUSSION

We were unable to amplify chitinase genes from alkaline and estuarine environmental samples using primer sets previously described in the literature (9). Upon inspection and after examining published chitinases sequences, the problem seemed to be related to the sequence of the forward primer. To remedy the problem, we developed a new, degenerate forward primer based on the published forward primer sequence (9) but modified to be consistent with sequences from diverse Proteobacteria (sequence divergence prevented design of a primer set that included chitinases from Gram-positive bacteria; Fig. 1). We used the redesigned forward primer in combination with the published reverse primer (9) to target family 18, group I chitinase genes. Amplification with this primer set yielded a PCR product of the expected size for all samples except those from Mono Lake water and sediment and Soap Lake sediments. Despite repeated attempts to optimize PCR conditions and alter DNA extraction protocols, only non-target amplification products were obtained from Mono Lake samples (both water and sediments) and Soap Lake sediments never yielded products of the correct size.

A total of 160 inserts was sequenced from 15 clone libraries with inserts from at least 10 randomly-selected clones sequenced from each library. Homology searches suggested that the inserts in 52 of the clones were not chitinase genes (Tables 1 and 2). Non-target sequences were retrieved from all environments examined in this study (including 20 from Mono Lake). These typically lacked significant similarity to any database sequence and were not analyzed further. We checked a subset of our remaining sequences (13 total; all of the deeply-branching, unique

sequences in Figure 2, for example SIS-10, SFBW-13 and SFBW-11) for possible chimera formation by BLASTing 200 bp from each end of the sequence against the database to ensure that they returned the same top hits. None of the sequences we examined failed this test; however, some of the 52 discarded sequences may have been chimeras. All 108 putative chitinase genes retrieved were unique when compared to sequences presently in the GenBank database. At the nucleotide level, the sequences were between 57-94% identical to previously identified chitinase genes. At the amino acid level, the sequences were 44-98% identical and 52-98% similar to current (July 2004) GenBank entries.

Phylogenetic analysis (Jukes-Cantor) placed the chitinase nucleotide sequences into five major clusters, designated Clusters A-E (Fig. 2). A maximum-likelihood tree of the nucleotide sequences (not shown) was essentially identical to this tree. A phylogenetic tree (Kimura) was also constructed using deduced amino acid sequences (not shown). The topologies of the nucleotide and amino acid trees were similar, with the composition of the clusters being the same for all trees. Cluster A contained sequences from the sediments collected at Sapelo Island, GA; San Francisco Bay, CA; Tomales Bay, CA; Topaz Lake, NV; and Walker Lake, NV. Cluster B contained sequences retrieved from sediments collected at Sapelo Island, GA; San Francisco Bay, CA; Tomales Bay, CA, and Topaz Lake, NV. In addition, sequences retrieved from the San Francisco Bay and San Joaquin River water samples formed a distinct sub-cluster within cluster B. Cluster C contained sequences retrieved from the Sapelo Island, San Joaquin River and Bodega Bay water column samples. Cluster D consisted of sequences retrieved from Arctic Ocean water samples. These sequences segregated into sub-clusters that typically corresponded to sample depth. Cluster E contained sequences retrieved exclusively from the two Soap Lake, WA water column samples.

We identified 43 potential family 18, group I chitinase sequences (maximum E value of 8e-4) in the Sargasso Sea metagenome database (SSMD). The region possessing the signature motif, [DG]-G-[LIV]-[DG]-[IV]-[DH]-W-[EG], of the family 18, group I chitinase sequences (38), was present in 13 (30%) of these sequences. These putative chitinases appear to be diverse in origin, as the most similar sequences in GenBank were obtained from γ-Proteobacteria (23%), Gram positive (51%) and *Bacterioides* (2%) bacteria, arthropods (9%), mammals (5%), fungi (5%) and *C. elegans* (5%). The majority of the SSMD putative chitinases either did not have any overlap with the region of the gene analyzed in this study (58%) or contained only a portion of the region (30%) . The remaining five (12%) SSMD sequences contained the entire region of the chitinase gene delimited by the primers we used, however, only three of these sequences were similar enough to be included in the tree (Fig. 2). The three SSMD sequences included in the tree fell outside of the clusters (A-E) defined by sequences retrieved from our samples. Two SSMD sequences (EAJ50883 and EAH89100) clustered with a family 18, group I chitinase reference sequence from *Shewanella baltica*, and were most closely related to our Cluster A (Fig. 2). The third SSMD sequence (EAI65414) grouped with one *Enterobacter* and two *Serratia* chitinase sequences. Given the overall dominance of *Shewanella*-like sequences in the Sargasso Sea metagenome library (42), it is not surprising that we retrieved *Shewanella*-like chitinase sequences from it. We were surprised that we did not find sequences similar to those from our Arctic Ocean samples since the 16S rRNA gene libraries from these samples contained sequences similar to those retrieved from Sargasso Sea samples (1).

Some of the chitinase sequences retrieved from different samples were identical (Fig. 2). For example, a sequence from the Sapelo Island library (SIS-01) was identical to three San Francisco Bay sequences (SFBS16-02, SFBS17-05 and SFBS29-01). Both of these samples are

intertidal sediments from salt marshes dominated by *Spartina alterniflora* (Sapelo Island) or *Salicornia virginica* (San Francisco Bay). The estuaries have similar temperature and salinity ranges, which would lead to the expectation that they harbor similar microflora, but they are geographically isolated. We are unaware of other reports of identical functional gene sequences having been retrieved from isolated environments; however, this may simply be due to the smaller database for functional genes, as closely related (16) or identical (1, 2) 16S rRNA genes have been retrieved from distant locations.

Interestingly, some sequences retrieved from sediments collected in freshwater Topaz Lake were identical to sequences retrieved from estuarine sediments of San Francisco Bay and from sediments of alkaline, saline Walker Lake (i.e. TLS-05, SFBS28-06 and WLS-02). Furthermore, another San Francisco Bay sequence (SFBS17-06) was identical to a clone from Topaz Lake (TLS-06) and from Walker Lake (WLS-08). This was a surprising finding as these environments range in salinity from <1 (Topaz Lake) up to ~30 PSU (SF Bay) and in pH from ~7 (Topaz Lake) to 9.8 (Walker Lake) (Table 1).

One factor that these sequences have in common is that they were all retrieved from sediment samples. This suggests that physicochemical properties common to sediments (surfaces, hypoxia/anoxia, elevated DOC and POC concentrations, and likely elevated chitin concentrations since shed arthropod exoskeletons sink), override other environmental factors (temperature, salinity, pH) in determining the distribution of functional gene sequences. Clearly there is a limit to this generalization because Mono Lake chitinases (water column, sediment and isolates) were not amplified by the primer set used in this study, even though enzyme assays demonstrated chitinase activity (LeCleir, unpublished data). DNA extracted from Soap Lake sediment also failed to yield PCR product with our primer set. In contrast to chitinase sequences

retrieved from sediment communities, sequences retrieved from water column samples collected at different locations segregated into separate clades (Fig. 2). Furthermore, within Cluster D, sequences retrieved from mixed-layer (55 m) and halocline (131 m) samples collected at the same station tended to fall into separate sub-clusters. The bacterial assemblages associated with these water masses have been characterized previously and were found to be distinct from one another (1, 2) and from those of temperate coastal water assemblages (1). Because the composition of Soap Lake water differs significantly from either seawater or fresh water, the bacterial assemblages from the lake might also be expected to be phylogenetically distinct. Biodiversity studies of other saline, alkaline lakes have verified that the composition of bacterial assemblages differs from those in other aquatic environments and also that the same suites of organisms are found in lakes from widely separated locations (11, 21, 36).

Alignment of family 18 glycosyl hydrolases shows that a number of residues essential for catalytic activity are conserved (29). The majority of chitinase sequences identified in this study (94%) contain a conserved motif encompassing the catalytic site, [DG]-G-[LIV]-[DG]-[IV]-[DH]-W-[EG], corresponding to positions 308-315 of the *S. marcescens* ChiA protein (29) (Fig. 3). Two additional residues, a tyrosine and an aspartate at positions 390 and 391, respectively, are also conserved in most of our sequences. However, seven of the sequences we obtained contained substitutions at one of these conserved positions. All of these substitutions result from single base-pair changes: six A->G transitions and one G->C transversion. Both SLW23-03 and AOW131-04 contain a glycine instead of an aspartate at position 308. WLS-07 contains histidine rather than aspartate at position 313. Interestingly, this same substitution is found in narbonin, a protein found in plants with high similarity to chitinase but with no known enzymatic function (42). WLS-08, TLS-06 and SFBS17-6 have a glycine instead of glutamate at position

315. This glutamate residue has been shown to be the essential catalytic proton donor in structurally characterized bacterial chitinases(45). Finally, clone SFBS16-01 contains a cysteine rather than the completely conserved tyrosine at position 390. Collectively, these seven sequences may represent pseudogenes. Alternatively, they may correspond to genes that encode for enzymes with unique properties, including different activities, mechanisms of action; or for proteins with no known enzymatic function that share sequence similarity with chitinase (i.e., narbonin). They may also simply be the result of PCR (44); cloning (28, 35); or sequencing errors, although the sequence reads were unambiguous at these positions. In the absence of biochemical data for the expressed protein, it is difficult to evaluate the significance of these substitutions.

All of the sequences retrieved from the two Soap Lake libraries contain aspartate rather than lysine at residue 305 (D305K), as well as a more conserved substitution at position 304 (tyrosine for tryptophan; Fig. 3). The D305K substitution has only been found in a novel chitinase recently identified in the marine bacterium *Microbulbifer degradans* 2-40 (20). The *M. degradans* chitinase has two catalytic domains, each with distinct activities towards polymeric chitin. Despite significant homology between domains at the amino acid level, the D305K substitution is present only in one of the domains, designated GH18C (BK001042). Overall, the Soap Lake sequences share approximately 45% identity and 55% similarity to the GH18C domain. It is speculative to infer the physiological or biochemical implications of these substitutions with only sequences in hand. Nonetheless, this finding raises the possibility that the Soap Lake chitinases may have properties similar to those identified in the *M. degradans* protein.

Chitinases from polar microorganisms appear to have adaptations required to function well in cold environments, as recently demonstrated for two chitinase alleles, ChiA (CAB62382)

and ChiB (CAB62499), from an *Arthrobacter* strain isolated from Antarctic sediment (26). The increased heat lability of these chitinases is believed to be a consequence of structural changes that give the enzymes greater flexibility at lower temperatures, permitting conformational changes necessary for catalysis (14). Similar sequence modifications might be expected in genes from other cold-adapted microbes, regardless of their phylogenetic affiliation, leading to unique sequences for Arctic Ocean genes, as we have found (Fig. 2).

The form and source of chitin found in the environment may also select for specific genes in different environments. There are three major types of chitin, designated α, β, and γ (32). Each has unique physical attributes and chemical properties. Chitin can also vary by the degree of acetylation and the presence of cross-linked structural components (37). The composition of the chitin matrix and its associated molecules is typically organism-dependent (15). Other molecules associated with the chitin matrix often select for specific enzymes and control rates of chitin hydrolysis (32, 37). Therefore, predominance of different structural variants of chitin in the environments we examined may dictate elaboration of what appear to be environmental-specific proteins that are in reality required for efficient hydrolysis of the predominant form of chitin.

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Table 2.1 Location, summary characteristics, and references for further descriptions of the environments where samples used in this study were collected. Entries in the column headed Depth either give the depth from which a water sample was collected or indicates (**SED**) sediment samples.

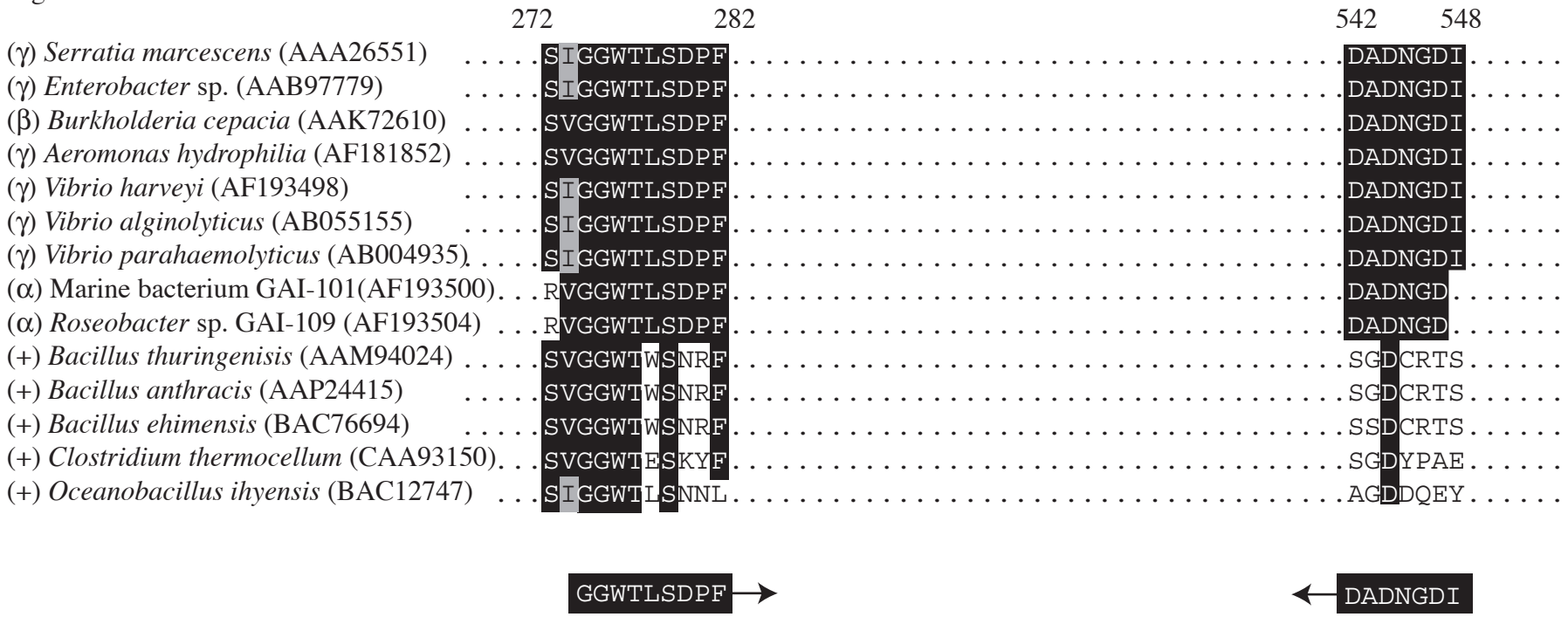
Location	Latitude/Longitude	Depth (m)	Salinity (ppt)	pH	Sample Date	Reference	Clones Sequenced[a]
Arctic Ocean Station 1.33.1	70°53.083' N 141°49.1' W	5	32	8	9/1997	(1)	10 (9)
Arctic Ocean Station 1.33.4	70° 53.033' N 141° 50.033' W	31	33	8	9/1997	(1)	10 (7)
Mono Lake (California, USA)	38° 00.388' N 119° 01.64' W	5	80	9.8	8/2001	(30)	10 (0)
Soap Lake (Washington, USA)	47° 24.3' N 119° 29.85' W	21	18	9.5	9/2003	(31)	10 (7)
Soap Lake (Washington, USA)	47° 24.3' N 119° 29.85' W	23	142	9.9	9/2003	(31)	10 (6)
San Francisco Bay (California, USA)	37° 54.667' N 122° 19.783' W	0	~33	8	6/2003	(17)	10 (8)
San Joaquin River (California, USA)	37° 40.34' N, 121° 15.55' W	0	<1	~7	6/2003	(36)	10 (5)
Sapelo Island (Georgia, USA)	31° 25.05' N 81° 17.75' W	0	~20	8	4/2003	(5)	10 (9)
Bodega Bay (California, USA)	38° 18.3' N 123° 3.95' W	0	33	8	11/2003	(47)	10 (10)
Mono Lake (California, USA)	38° 00.388' N 119° 01.64' W	**SED**	80	9.8	8/2001	(30)	10 (0)
Tomales Bay (California, USA)	38° 13.133' N 123° 56.833'W	**SED**	~33	8	6/2003	(34)	10 (9)

Topaz Lake (Nevada, USA)	38° 41.583' N 119° 31.167' W	**SED**	<1	7	5/2003	(41)	10 (7)
San Francisco Bay (California, USA)	37° 54.667' N 122° 19.783' W	**SED**	~33	8	6/2003	(17)	15 (13)
Sapelo Island (Georgia, USA)	31° 25.05' N 81° 17.75' W	**SED**	~20	8	4/2003	(5)	15 (13)
Walker Lake (Nevada, USA)	38° 43' N 118° 43' W	**SED**	12	9.8	5/2003	(4)	10 (5)
Soap Lake Mixolimnion	47° 24.3' N 119° 29.85' W	**SED**	18	9.5	9/2003	(31)	0
Soap Lake Monimolimnion	47° 24.3' N 119° 29.85' W	**SED**	142	9.9	9/2003	(31)	0

[a]Number of clones showing sequence similarity to previously identified chitinases are given in parentheses.

Figure 2.1. Design of degenerate primers for family 18, group I chitinase genes. Alignments of chitinase amino acid sequences from organisms representing diverse phylogenetic lineages were used to design the degenerate primers. Symbols represent bacterial taxonomic groups: γ = γ-Proteobacteria, β = β-Proteobacteria, α=α-Proteobacteria and + = Gram-positive bacteria. GenBank accession numbers are provided in parentheses. Position designations relative to the *Serratia marcescens* chitinase sequence (P07254) are shown above the alignment. Conserved residues are shown in black, similar residues in grey. I = inosine base; Y = C or T; W = A or T; S = G or C; R = A or G. The degeneracy for both primers in this study is 16-fold. The references for chiAfor.ext and chiA.rev are this study and (9), respectively.

Figure 2.1



Primer Sequence 5' GGI GGI TGG ACI YTI WSI GAY CCI TT 3' 5' ATR TCI CCR TTR TCI GCR TC 3'

Primer Name chiAfor.ext chiA.rev

Figure 2.2. Neighbor-joining tree (partial sequence, ~800 bp) showing phylogenetic relationships between family 18, group I chitinase nucleotide sequences. Clone designations are: AOW55 = Arctic Ocean, 55 m depth, AOW131 = Arctic Ocean 131 m depth, BBW = Bodega Bay water column**,** SIS = Sapelo Island sediments, SIW = Sapelo Island water column, SFBS = San Francisco Bay sediments, SFBW = San Francisco Bay water column, SJRW = San Joaquin River water column**,** SLW21 = Soap Lake, 21 m depth, SLW23 = Soap Lake, 23 m depth, TBS = Tomales Bay sediments, TLS = Topaz Lake sediments, WLS = Walker Lake sediments. Water column samples for which no depth is given were collected at the surface (nominal depth 0.1 m). Each sequence from a given library is also provided with a numerical designation. Branches containing identical sequences are indicated with a filled circle. The scale bar indicates Jukes-Cantor distance. Bootstrap values >50% (for 100 iterations) are shown at branch nodes. The tree is unrooted with the chitinase gene from *Bacillus circulans* (AF154827) as the outgroup. GenBank accession numbers for reference sequences are provided in parentheses.

Figure 2.2

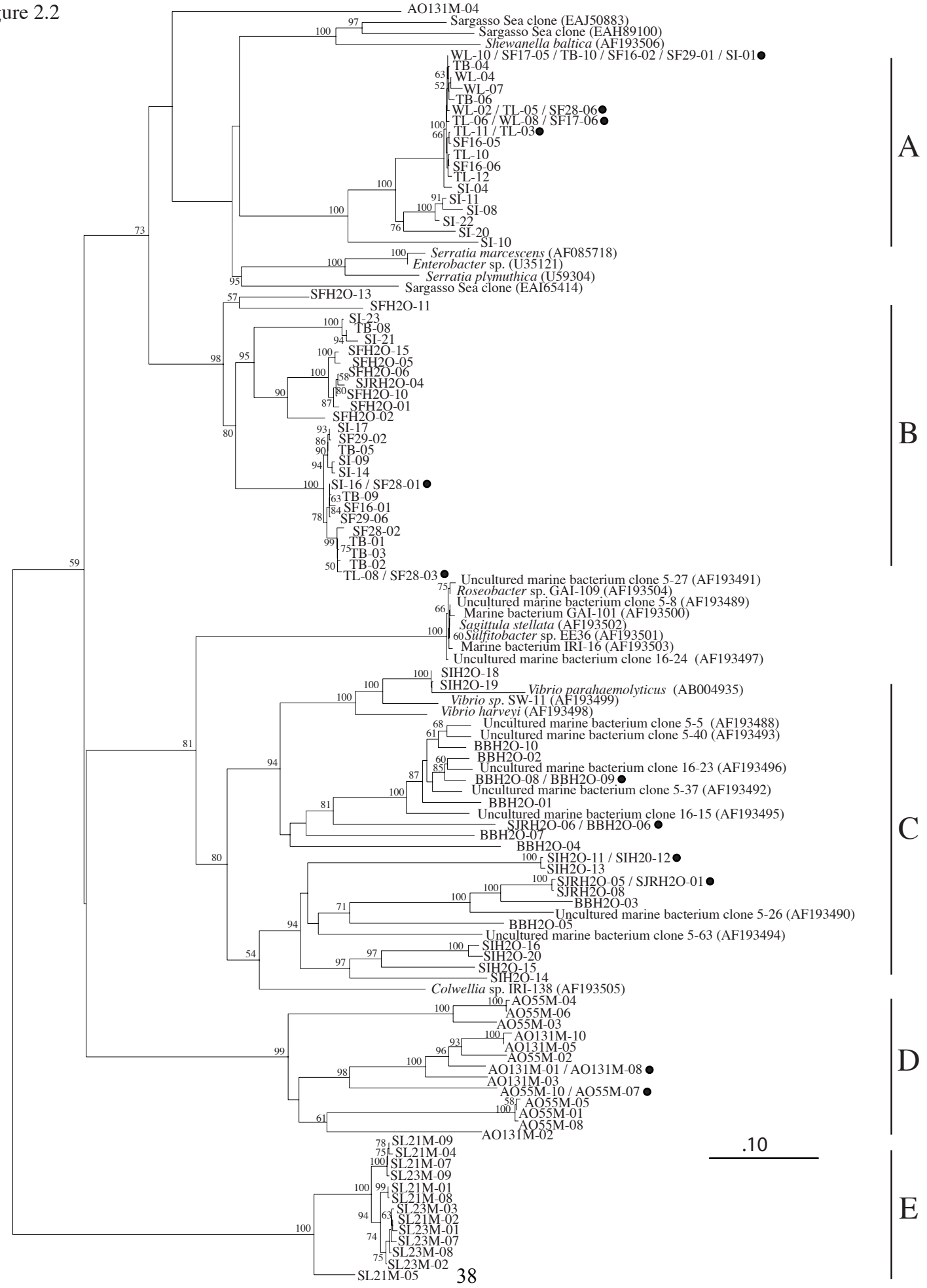
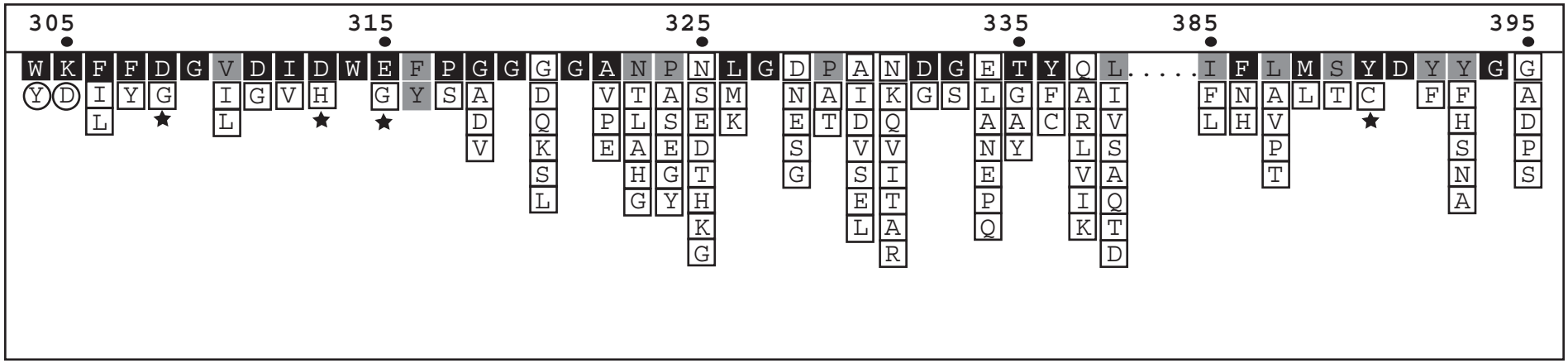

AO131M-04
Sargasso Sea clone (EAJ50883)
Sargasso Sea clone (EAH89100)
Shewanella baltica (AF193506)
WL-10 / SF17-05 / TB-10 / SF16-02 / SF29-01 / SI-01
TB-04
WL-04
WL-07
TB-06
WL-02 / TL-05 / SF28-06
TL-06 / WL-08 / SF17-06
TL-11 / TL-03
SF16-05
TL-10
SF16-06
TL-12
SI-04
SI-11
SI-08
SI-22
SI-20
SI-10
Serratia marcescens (AF085718)
Enterobacter sp. (U35121)
Serratia plymuthica (U59304)
Sargasso Sea clone (EAI65414)
SFH2O-13
SFH2O-11
SI-23
TB-08
SI-21
SFH2O-15
SFH2O-05
SFH2O-06
SJRH2O-04
SFH2O-10
SFH2O-01
SFH2O-02
SI-17
SF29-02
TB-05
SI-09
SI-14
SI-16 / SF28-01
TB-09
SF16-01
SF29-06
SF28-02
TB-01
TB-03
TB-02
TL-08 / SF28-03
Uncultured marine bacterium clone 5-27 (AF193491)
Roseobacter sp. GAI-109 (AF193504)
Uncultured marine bacterium clone 5-8 (AF193489)
Marine bacterium GAI-101 (AF193500)
Sagittula stellata (AF193502)
Sulfitobacter sp. EE36 (AF193501)
Marine bacterium IRI-16 (AF193503)
Uncultured marine bacterium clone 16-24 (AF193497)
SIH2O-18
SIH2O-19
Vibrio parahaemolyticus (AB004935)
Vibrio sp. SW-11 (AF193499)
Vibrio harveyi (AF193498)
Uncultured marine bacterium clone 5-5 (AF193488)
Uncultured marine bacterium clone 5-40 (AF193493)
BBH2O-10
BBH2O-02
Uncultured marine bacterium clone 16-23 (AF193496)
BBH2O-08 / BBH2O-09
Uncultured marine bacterium clone 5-37 (AF193492)
BBH2O-01
Uncultured marine bacterium clone 16-15 (AF193495)
SJRH2O-06 / BBH2O-06
BBH2O-07
BBH2O-04
SIH2O-11 / SIH20-12
SIH2O-13
SJRH2O-05 / SJRH2O-01
SJRH2O-08
BBH2O-03
Uncultured marine bacterium clone 5-26 (AF193490)
BBH2O-05
Uncultured marine bacterium clone 5-63 (AF193494)
SIH2O-16
SIH2O-20
SIH2O-15
SIH2O-14
Colwellia sp. IRI-138 (AF193505)
AO55M-04
AO55M-06
AO55M-03
AO131M-10
AO131M-05
AO55M-02
AO131M-01 / AO131M-08
AO131M-03
AO55M-10 / AO55M-07
AO55M-05
AO55M-01
AO55M-08
AO131M-02
SL21M-09
SL21M-04
SL21M-07
SL23M-09
SL21M-01
SL21M-08
SL23M-03
SL21M-02
SL23M-01
SL23M-07
SL23M-08
SL23M-02
SL21M-05
.10
A
B
C
D
E

Figure 2.3. Conserved residues including and surrounding the catalytic domain of Proteobacterial chitinases. Residues are coded according to degree of conservation as follows: black >75%; gray 50-75%; no color <50%. Positions that are altered in chitinase sequences retrieved in this study are indicated by symbols: starred residues represent those found in a limited number of sequences and are described in the text, circled residues represent those found exclusively in sequences retrieved from Soap Lake samples. Amino acid positions are relative to *Serratia marcescens* ChiA (P07254)

Figure 2.3



CHAPTER 3

CHITINOLYTIC BACTERIA FROM ALKALINE, HYPERSALINE MONO LAKE, CALIFORNIA

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SUBMITTED TO: *Aquatic Microbial Ecology*

ABSTRACT

We have been unable to retrieve chitinase gene fragments from Mono Lake, California, samples by PCR using primers for the family 18, group I chitinase genes commonly found in other aquatic environments, yet measurements made with model substrates clearly indicate chitinase activity. To obtain more information about the chitinolytic bacterial community of this alkaline, hypersaline lake, we analyzed the composition of the bacterial assemblages associated with *Artemia monica* exuvia and in chitinolytic enrichment cultures, and we isolated aerobic and anaerobic chitinolytic bacteria from lake water samples. Bacterial assemblages were characterized by PCR/DGGE using primers specific for 16S rRNA genes, coupled with cloning and sequencing. We screened our isolates for chitinase activity using the fluorogenic chitin analogs methylumbelliferyl-diacetylchitobioside and methylumbelliferyl-triacetylchitobioside; using a clearing assay on chitin plates; and using a growth assay on medium with chitin as the sole carbon and energy source.

Several ribotypes were found to be common to both Mono Lake exuvia samples and chitin enrichments. These ribotypes were most closely related to *Paracoccus* sp. MBIC4036 (AB025192), *Arhodomonas* sp. EL-201 (AJ315984) and *Psychroflexus tropicus* (AF513434). Four Proteobacteria ribotypes were only retrieved from clone libraries of chitin enrichments. Three were most closely affiliated with the γ-Proteobacteria: Strain N10 (AF250323), *Nitrumincola lacisponis*, (AY567473) and *Microbulbifer* strain Th/B/38, (AY224196); while the fourth was most closely related to an unidentified Proteobacterium (strain BD1-5, AB015518). The majority (73%) of the

isolates we obtained were Gram-positive bacteria and 70% of the Gram-positive isolates were capable of hydrolyzing at least one of the model substrates.

INTRODUCTION

Mono Lake is an alkaline, hypersaline lake located east of the Sierra Nevada Mountain range in California, USA (38° 00' N; 119° 02' W, see bathymetric chart at http://geopubs.wr.usgs.gov/map-mf/mf2393/). The lake has a pH of 9.8 and a salinity of approximately 85 ppt (15). Because of the extreme environmental conditions in Mono Lake, the food web is relatively simple, consisting of bacteria, archaea, phytoplankton, rotifers, brine flies (*Ephedra hians*) and the brine shrimp *Artemia monica*. *Artemia* is the dominant macro-zooplankter in the lake and can achieve population densities greater than 80,000 m^{-2} in the summer months (4).

Brine shrimp nauplii begin to hatch in early spring (7, 8, 19) and adult brine shrimp are observed in Mono Lake by mid-May (8). *Artemia* develop and mature through 12 instar stages. At the transition between each stage, they molt and shed their exoskeleton (exuvia) into the water (23). Arthropod exoskeletons are rich in the structural biopolymer chitin. Because of the high population densities attained by *Artemia* in Mono Lake and their frequent molts, chitin is a major biopolymer in the lake. For example, following the fall die-off of the *Artemia* population, exuvia and carcasses sink to the lake bottom to form a flocculent layer that can be centimeters thick (pers. obs.).

Chitin is composed of repeating units of the monomer n-acetyl-D-glucosamine (GlcNAc). Chitin is insoluble in water at circumneutral pH and is resistant to hydrolysis by most enzymes (26). Organisms that degrade chitin do so by producing chitinases (EC 3.2.1.14) (11, 12). Bacteria generally mediate chitin degradation in aquatic systems (10, 26).

Measurements of chitinase activity in Mono Lake made using the fluorogenic chitin analog methylumbelliferyl-diacetylchitobioside (MUF-DC) indicated chitinolytic activity in both the water column and sediment of Mono Lake. However, attempts to clone and sequence chitinase genes from Mono Lake using PCR primers targeting the family 18, group I chitinase genes commonly found in other aquatic environments were unsuccessful (18). Chitin degradation is an extracellular process, thus chitinases have to function in the chemical milieu of the environment in question. Mono Lake presents an unusual chemical environment (elevated pH and salinity with a salt composition that is dramatically different from seawater) to microbes living in its waters. This led us to hypothesize that chitinases (and thus chitinase genes) from Mono Lake would be novel when compared to chitinase genes from terrestrial, freshwater or marine environments and thus would not be detected by PCR primer sets based on chitinase gene sequences from organisms living in these other environments. Evidence in support of this hypothesis is that Mono Lake chitinase activity has a pH optimum of >8, significantly higher than the pH optima of chitinases from other sources (G. R. LeCleir, unpublished data).

An alternative approach to studying chitin degradation that avoids the limitations imposed by primer specificity is to identify the bacteria in chitinolytic assemblages using phylogenetically informative sequences (e.g. 16S rRNA genes) or to isolate and identify chitinolytic microbes. While interpretation of community composition data based on isolate collections is subject to constraints imposed by our inability to isolate and culture many organisms from the environment (1), the genomes of isolates obtained by this approach can be probed to identify novel chitinase genes. Furthermore, these approaches

provide information on the composition of the chitinolytic assemblage that can be compared to other locations.

We analyzed the composition of the mixed microbial assemblage responding to chitin enrichments using PCR amplification of 16S rRNA genes and denaturing gradient gel electrophoresis (DGGE) (22) or by cloning and sequencing PCR-amplified 16S rRNA genes. We compared the bacterial assemblage from enrichment experiments with the assemblage associated with *Artemia* and *Ephedra* exuvia collected from Mono Lake surface water. We used standard culturing techniques and media selective for chitin degraders to obtain isolates that were then screened for their ability to hydrolyze the fluorogenic substrates MUF-DC and methylumbelliferyl-triacetylchitotrioside (MUF-TC) and for the ability to hydrolyze colloidal crab shell chitin. Isolates were probed for family 18, group I chitinase genes using published primer sets (6, 18) and categorized phylogenetically based on their 16S rRNA gene sequences.

METHODS

MUF hydrolysis in Mono Lake samples. We used the fluorogenic chitin analogue methylumbelliferyl-diacetylchitobioside (MUF-DC; Sigma, St. Louis, MO, USA) for routine measurements in Mono Lake samples. Although this substrate may be hydrolyzed by other enzymes (for example lysozyme; (31)), we assume that it represents chitinase activity in our samples. Activity was assayed in water samples from 5 m, 15 m, 20 m, and 35 m; and in oxic and anoxic sediments collected in February, March, April, May and June 2002. Water samples were collected from discrete depths using a Niskin sampler. Gas-tight bottles were filled from the Niskin sampler leaving no head space and

taking care to prevent oxygenation of the sample, then stored in the dark, on ice or at 4°C until assayed (within 4 hours of sample collection). Triplicate MUF-DC hydrolysis assays were performed in 15 mL centrifuge tubes with 4.5 mL of Mono Lake water and 0.5 mL of 0.1 mM MUF-DC (initially dissolved in dimethyl formamide to a concentration of 5 mM and then diluted in autoclaved and filter sterilized Mono Lake water).

Sediment was collected from Station 6 (37° 57.822' N, 119° 01.305' W, 39 m depth) where the overlying water was anoxic, and from a station north of Station 6 near Paoha Island where physical characteristics of the sediment were similar to those at Station 6 (soupy, organic-rich mud), but where the overlying water was oxygenated (O_2 concentration >1 mg/L as determined with a YSI oxygen meter equipped with a Clark type electrode, bottom depth from 10 to 15 m). Sediment was collected using an Eckman grab, then surficial sediment was skimmed from the undisturbed sediment-water interface of the sample with a plastic spoon, placed in a glass jar with an air-tight cap and stored on ice until the assays were performed (within 4 hr). Triplicate sets of tubes containing 2.5 mL of autoclaved and filter-sterilized Mono Lake water and 2.0 mL of sediment were amended with 0.5 mL of 0.1 mM MUF-DC, then incubated at 20°C and 4°C with shaking (100 rpm) for 3 or 96 hrs, respectively.

Fluorescence of 0.2 mL of sample (for mud samples the tubes were centrifuged and only water was used) in 1.8 mL of carbonate buffer (pH 9.7) was measured at 365 nm excitation and 460 nm emission using a Hoefer DynaQuant fluorometer. All environmental measurements of MUF-DC hydrolysis were performed in triplicate. MUF-DC hydrolysis was linear for at least 3 d in pilot experiments with water samples

and comparison of hydrolysis rates in filtered versus whole water samples indicated that the majority of the activity (>90 %) was associated with particles (unpublished data).

Exuvia assemblage. The microbial assemblage associated with brine shrimp and brine fly exuvia was examined using a sample collected in November 2004 immediately following the fall die-off of the *Artemia* population. Exuvia floating on the lake's surface were collected with a plankton net. DNA was extracted and analyzed by PCR/DGGE and cloning of 16S rRNA genes was as described below. Data from this sample were compared to data obtained from chitin enrichment experiments.

Enrichment Cultures. Two replicates of enrichment cultures were prepared in 1 L glass bottles using 500 mL of water for each bottle. Water was collected in February 2002 from 5 m (aerobic) or 35 m (anaerobic) at Station 6. This experiment was performed in February because there are no brine shrimp in the water column at this time of year, thereby eliminating *Artemia* chitin from control samples. Most of the sample (450 mL) was passed through ashed GF/F filters (Whatman, Maidstone, England). The filtrate was then mixed with the remaining 50 mL of raw Mono Lake water. Experimental treatments were amended with 0.5 g L^{-1} autoclaved crab shell chitin (Sigma), while controls received no chitin. Bottles were wrapped in aluminum foil and incubated at in-situ temperatures (4°C) in the dark. The anaerobic samples were manipulated in a Coy anaerobic chamber (Grass Lake, MI, USA).

Following a four-week incubation, water was filtered through a 0.22 μm Sterivex filter cartridge until the filter clogged (~250 mL of sample) to collect bacterial cells. The particulate chitin in experimental treatments was not removed prior to filtration to prevent the exclusion of bacteria attached to chitin particles. Excess water was expelled from the

Sterivex cartridges, 1.8 mL of lysis buffer (50 mM Tris (pH 8.3), 40 mM EDTA, and 0.75 M sucrose) was added, and then the cartridges were stored at –80 °C until processed.

Enumeration. The abundance of bacteria in all treatments of the chitin enrichment experiment was determined at the beginning and end of the incubation. Samples for enumeration were preserved with 2% (final concentration) filtered formalin. Bacteria were enumerated by epifluorescence microscopy using diaminophenyl indole (DAPI) following a protocol modified slightly from (25). Sample (0.75 mL), DAPI solution (0.75 mL of 0.003% w/v) and filter sterilized, 10% acetic acid (0.1 mL, we have found that this enhances staining of these samples) were combined and filtered, after seven minutes incubation, onto black 0.2 μm pore size membrane filters (Osmonics, Livermore, CA) then counted using a Leica DMRXA microscope equipped with epifluorescence optics. At least ten fields and 300 cells were counted per slide.

DNA extraction, purification and PCR/DGGE. DNA was extracted from Sterivex cartridges as described previously (9). Briefly, 40 μL of lysozyme (50 mg mL^{-1}) was added to each cartridge, and the cartridges were incubated for 60 min at 37°C. Fifty microliters of proteinase K (20 mg mL^{-1}) and 100 μL of a 20% (wt/vol) solution of sodium dodecyl sulfate were added to each cartridge, and the cartridges were incubated at 55°C for 2 hours. DNA was purified from 800 μL of the lysate by sequential extraction with 800 μL of phenol: chloroform: isoamyl alcohol (25:24:1), chloroform: isoamyl alcohol (24:1), and finally n-butanol. The aqueous phase was removed, placed in a Centricon-100 concentrator (Amicon, Bedford, MA), mixed with 500 μL of TE buffer (10 mM Tris and 1 mM EDTA, pH 8.0), and centrifuged at 1,000 x g for 10 minutes.

Next, 500 μL of TE was added to the Centricon-100 concentrator, and the mixture was centrifuged for another 10 minutes.

PCR, for DGGE analysis, was performed using the 340-356F and 517-533R primer set (2). PCR conditions were similar to those used by Ferrari and Hollibaugh (9). PCR products were quantified by the Hoechst dye assay (24). DGGE was performed on a 6.5% polyacrylamide gel with a 45%-65% denaturing gradient using a CBS Scientific DGGE apparatus (Del Mar, CA, USA). Gels were loaded with 400 ng of PCR product per lane then electrophoresed at 75 volts (3.75 V cm^{-1}) for 15 hours in 1 X TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA [pH adjusted to 7.4 with acetic acid]) at a constant temperature of 60°C. PCR products in the DGGE gels were visualized using an FMBIO II gel scanner (Hitachi) set to measure fluorescein fluorescence. Selected bands were excised and sequenced on an ABI 310 genetic analyzer with the 340-356F primer.

Gel analysis was performed using the Molecular Analyst-Fingerprint Plus software (BioRad Version 1.12). Phylotypes, defined as bands recognized by the software, were counted for each sample lane. Bands were scored as present or absent at each position. The 20 bands in the 10 samples were ordinated with non-metric multidimensional scaling (MDS), using the ViSta software program (Version 5.6 http://forrest.psych.unc.edu/research/index.html). The MDS was constrained to a 2-dimensional solution and was run from 1,000 randomized starts to avoid local minima. The run with the lowest value of stress among these randomized starts was used for analysis.

Clone libraries. Clone libraries were constructed from PCR amplicons produced using the Bacteria primer set 9F and 1492R. Amplicons of the correct size were separated from non-specific amplification products by agarose gel electrophoresis, then extracted from the agarose using the QiaQuick Gel Extraction Kit (Qiagen) and cloned into *E. coli* using the TOPO TA Cloning Kit for Sequencing (Invitrogen, Carlsbad, CA). Twenty clones were selected randomly from each library. Cloned inserts were sequenced at the University of Georgia's Molecular Genetics Instrumentation Facility (MGIF) on an ABI 3700 genetic analyzer (Applied Biosystems, Foster City, CA) with the 9F primer. Sequences were cut to 800 bp in length and queried against GenBank using BLASTN. Database sequences with highest BLASTN similarity values were imported and compared to cloned sequences using the GAP tool of the Wisconsin package, version 10.2 (Accelrys, San Diego, Calif.).

Chitinolytic Isolates. Chitinolytic bacteria were isolated from water and sediment samples and from chitin enrichment experiments. Samples (0.1 mL) were spread on chitin thin-layer R2A plates (28) or on plates containing colloidal chitin prepared from pulverized, particulate crab shell chitin (Sigma) as the sole carbon and energy source. All plates were made using artificial Mono Lake water (3). Although some colonies showed clearing zones indicating production of soluble chitinase, most did not, so colonies were selected randomly after 2 weeks growth at 20°C. Isolates were re-streaked twice on fresh R2A plates, then on plates containing only chitin as a carbon and energy source and finally (if they grew on the chitin-only plate), on another R2A plate. Anaerobic isolates were cultured from anaerobic regions of the lake on agar plates in a Coy anaerobic chamber.

Phylogenetic characterization of isolates. Cells from single colonies were collected with a loop and lysed in ultra-pure water by boiling. The lysate was centrifuged briefly to collect cellular debris at the bottom of the tube and PCR was performed using the supernatant as template. This method proved efficient and did not require any further sample manipulations for successful PCR using the Bacteria primer set 9F and 1492R (17, 34). PCR products were cleaned using the Qiaquick PCR Cleanup Kit (Qiagen, Valencia, CA). The products were then sequenced on an ABI 310 Genetic Analyzer using a BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) with the 9F primer. Sequences were queried (BLASTN) against the GenBank database. Closest relative sequences were compared to isolate sequences using the GAP tool of the Wisconsin package, version 10.2 (Accelrys, San Diego, Calif.).

Tests for chitinase. Overnight cultures of all isolates were transferred to 96 well plates containing liquid R2A media and autoclaved, particulate chitin and incubated overnight at 20°C to induce chitinase production. The following morning wells were amended with MUF-DC or MUF-TC (10 μM final concentration) and incubated for 24 hours. MUF fluorescence (indicating chitinase activity) was detected visually using a UV trans-illuminator (UVP, Upland, CA).

Chitin plates were routinely inspected to see if clearing zones were formed around individual colonies that would indicate production of soluble chitinase. We also attempted to amplify chitinase genes from Mono Lake isolates and from enrichment cultures using PCR primers targeting family 18, group I chitinases as described in LeCleir et al. (2004). We constructed phylogenetic trees of the isolates using 16S rRNA gene sequences, then selected representative isolates for PCR screening based on the tree

topology and whether or not they hydrolyzed one of the MUF substrates. Representatives of all of the major groups of isolates (all α- and γ-Proteobacteria, 23 Gram-positive bacteria) were screened using these chitinase primers.

RESULTS

Chitinase activity in Mono Lake. Chitinase activity, as measured by the hydrolysis of MUF-DC, was detected at all depths sampled in the water column and in both sediment samples on all sampling expeditions. Chitinase activity in the sediment was at least 5-fold higher than in the water column (Table 3.1). Water column rates of MUF-DC hydrolysis were 0.6-1.6 nmol/hr/ml and 0.5-5.9 nmol/hr/ml for 4°C and 20°C incubations, respectively. Rates of MUF-DC cleavage by sediment slurries were 3.3-81.1 nmol/hr/g and 137-874 nmol/hr/g for 4°C and 20°C incubations, respectively. Reaction Q_{10} values ranged from 1.8 to 4.8 for water column samples and 3.0 to 9.3 for sediment samples. Chitinase activity increased dramatically in May, coincident with the emergence, development and subsequent molting of brine shrimp nauplii (Fig. 3.1).

Exuvia assemblage. We obtained 25 sequences from the Mono Lake exuvia clone library (Table 3.2). These sequences were from a variety of bacterial groups including CFB, α-, δ- and γ-Proteobacteria. Several sequences were obtained from both the exuvia sample and from chitin enrichments. These sequences were most closely related to *Paracoccus* sp. MBIC4036 (AB025192), *Arhodomonas* sp. EL-201 (AJ315984) and *Psychroflexus tropicus* (AF513434). Bacterial assemblages from the exuvia sample, chitin enrichments and controls were compared by PCR/DGGE of 16S rRNA genes. Examination of the gel revealed 5-7 bands in the exuvia sample that had the same

mobility as bands from chitin enrichments (data not shown). Sequences similar to *Vibrio metschnikovii* (X74711, X74712) were found in the exuvia clone library and appeared in our isolate collection.

Enrichment experiments. Bacterial abundance increased significantly in chitin enrichments compared to initial and control treatments (Table 3.3). DGGE banding patterns revealed distinct differences between the microbial assemblages in initial, chitin-amended and control samples (Fig. 3.2). Sequences obtained from dominant bands from the different treatments confirmed these differences (Table 3.4). With the exception of the 5 m chitin enrichment, community profiles of duplicate treatments were similar. The richness of the Bacteria assemblages in all treatments appeared to be relatively low, with no more than 10 distinct bands present in any treatment (Fig. 3.2). MDS analysis was used to analyze the DGGE banding patterns from samples of initial, chitin-amended and control treatments. It is clear from this analysis that incubations containing chitin clustered separately from the control and $T_{initial}$ incubations (Fig. 3.3).

A clone library of PCR amplified 16S rRNA genes was made from one bottle from each treatment. An additional clone library was generated for the replicate 5 m chitin enrichment bottle because of the obvious differences in the DGGE community profiles (Fig. 3.2). Twenty clones were randomly selected for sequencing from each of the libraries. One sequencing reaction failed, resulting in 19 sequences from the 35 m control library. The sequences obtained were most closely affiliated with *Clostridia*, α- and γ-Proteobacteria, *Bacteroidetes*, *Sphingobacter*, *Chlorophyta* and *Planctomycetaceae* (Table 3.2). Similarities to GenBank sequences ranged between 81.3% and 98.9% (Table 3.2). Four sequences were only retrieved from chitin-amended treatments, suggesting

that they are from chitinolytic organisms. These four sequences were most closely affiliated with Proteobacteria sequences: γ-Proteobacterium N10 (AF250323), γ-Proteobacteria strain 4CA (*Nitrumincola lacisponis*, AY567473), an unidentified Proteobacterium (strain BD1-5, AB015518) and *Microbulbifer* (strain Th/B/38, AY224196). Cloned sequences with greatest similarity to strain 4CA and strain N10 were found in chitin-amended samples incubated under both aerobic and under anaerobic conditions.

Isolates. A total of 80 isolates were purified, screened for MUF-DC and MUF-TC activity and sequenced. 46 isolates were capable of hydrolyzing MUF-DC, 49 isolates were capable of hydrolyzing MUF-TC and 43 isolates were capable of hydrolyzing both MUF-DC and MUF-TC under the conditions tested (Table 3.5). With the exception of four *Vibrio* isolates, none of our isolates caused clearings in the colloidal chitin agar that would indicate production of soluble chitinase. With the exception of the same *Vibrio* isolates, we were unable to amplify chitinase genes from any of the isolates using group 1, family 18 chitinase primers.

Sequences of 16S rRNA genes obtained from isolates in this study ranged from 363 bp to 767 bp in length. Phylogenetic affiliation of isolates included α- and γ-Proteobacteria, Bacillus, Clostridia, Lactobacillus, and Fusobacteria. These sequences were between 86.1 and 99.8% similar to 16S rRNA gene sequences in GenBank. The majority (73%) of the isolates were members of the Gram-positive group and 70% of the Gram-positive isolates were capable of hydrolyzing at least one of the model substrates (Table 3.5).

DISCUSSION

In agreement with studies from other environments, chitinase activity is higher in sediments than in the water column of Mono Lake (13, 27). Our data indicate that sediment chitinase activity responds to the emergence and development of the *Artemia monica* population in the water column (Fig. 3.1). The May-June increase in chitinase activity may be due to an increase in the abundance of chitinolytic microbes or to up-regulation of chitinase expression in response to greater substrate availability. Molting arthropods also produce chitinases (20) so chitinase activity, especially of the water column, may increase as the number of molting *Artemia* increase.

The ability of isolates to hydrolyze MUF substrates conveys information about the types of chitinases produced by these bacteria. It is assumed that bacteria capable of hydrolyzing MUF-DC produce exo-chitinases (enzyme cleaves GlcNAc dimers from the non-reducing end of the chitin molecule) and those hydrolyzing MUF-TC produce endo-chitinases (enzyme cleaves glycosidc bonds randomly within the chitin molecule) (5). Microbes hydrolyzing both substrates potentially produce both exo- and endo-chitinases. Although some isolates hydrolyzed only one of the chitin analogues, over half of them could hydrolyze both (Table 3.5). From our results it appears that endo- and exo-chitinolytic activity is evenly distributed in Mono Lake isolates. The production of multiple chitinases by a single organism has been well documented (29) and is not surprising given the complex structure of chitin (10, 29).

The difficulty we encountered in amplifying chitinases from our chitinolytic isolates is not uncommon (6). The negative results are likely due to the high variability found within chitinase gene sequences (30). Another alternative is that the MUF

substrates are hydrolyzed by a completely different enzyme. Lysozyme is also capable of hydrolyzing MUF analogues (31), and may have been responsible for the activity we were measuring with them, hence the lack of chitinase gene products in our PCR. However, since our isolates were capable of growth on chitin as the sole C and energy source, it seems unlikely that they lack chitinase.

Bacterial growth and shifts in community composition in chitin-amended samples indicate that the Mono Lake bacterial community can respond strongly to chitin enrichment. Bacterial abundance increased significantly in chitin enrichments compared to initial and control treatments (Table 3.3). Four sequences retrieved from the 16S rDNA clone libraries were only present in chitin-amended samples and not in control bottles. MDS analysis of DGGE banding patterns also suggests a strong relationship between the presence of chitin and the composition of the bacterial assemblage. Our results suggest that chitin production by *Artemia* may influence the seasonal succession of the microbial community in Mono Lake because of the strongly seasonal population dynamics of *Artemia* (8).

We were able to obtain isolates that grew on chitin plates from a wide variety of phylogenetic groups (Table 3.5). The amount of overlap, however, between the 16S rRNA gene sequences of culturable chitinolytic isolates and those retrieved from our enrichment cultures is small. Only *Rhodobaca bogoriensis* (AF384205) and Gamma-Proteobacterium N10 (AF250323) were found to be common to both the cultured isolate sequences and the enrichment culture sequences. 16S rRNA gene sequences most similar to *Vibrio metschnikovii* (X74712 and X74711) were found among the isolate sequences and also in the Mono Lake exuvia clone library. While there are large discrepancies

between our isolate library and our clone libraries, the taxonomy of bacteria isolated in this study is similar to those found by Cottrell and Kirchman (6). The lack of overlap between isolate and enrichment cultures is likely a result of the difficulties encountered when trying to culture environmentally significant isolates. It is likely that the bacteria we isolated on chitin plates represent only the portion of chitinolytic microbes that are easily cultured on plates. Nevertheless, these isolates are chitinolytic and will be useful for studying chitinases from environments similar to Mono Lake in the future.

Many of the 16S rRNA gene sequences obtained from the enrichment cultures were quite different from any 16S sequences currently in GenBank, despite the fact that similar studies have investigated chitin degradation in other environments (6, 21). The low percent similarity between database sequences and ribotypes retrieved from exuvia or chitin-amended samples makes inference of the functional capabilities of these organisms virtually impossible. However, the unique association of certain ribotypes with chitin-amended treatments suggests that those organisms are important in chitin degradation, or at least that they are associated with organisms responsible for chitin degradation. The fact that these "chitinophilic" sequences are all Proteobacteria is consistent with previous work because Proteobacteria have been identified as playing an important role in chitin hydrolysis in other environments (6, 14, 16, 29). In contrast, Gram-positive ribotypes were not dominant in any of our clone libraries, despite the fact that they comprised 73% of our collection of cultures isolated on media that selected for chitin degrading organisms, and that the majority of the Gram-positive isolates we cultured were capable of cleaving MUF compounds. This discrepancy may be due to PCR or DNA extraction biases, although we have had no trouble extracting or amplifying

DNA from gram-positive cultures. It is more likely that the discrepancy results from strong selection exerted during the isolation procedure.

Our results suggest that α- and γ-Proteobacteria and Gram-positive bacteria are active members of the chitinolytic assemblage in Mono Lake. Members of these lineages were found on *Artemia* exuvia sampled from Mono Lake and in chitin-amended enrichment cultures. Members of these groups have also been isolated on chitin plates and demonstrated MUF substrate (MUF-DC and MUF-TC) hydrolysis. Our results are in agreement with studies conducted in other environments (6, 14, 32, 33).

Using PCR to retrieve family 18, group I chitinase gene sequences from environmental DNA samples collected in Mono Lake has thus far been unsuccessful. Thus, we have turned to other approaches to gain insight into the chitinolytic microbial community of Mono Lake. Using both culture dependent and independent techniques, we have been able to circumvent the difficulties inherent in primer design and PCR analysis of highly variable functional genes to characterize the chitinolytic bacterial assemblage from Mono Lake. These results will help us focus future efforts to study chitinolytic microbes in Mono Lake and similar environments.

ACKNOWLEDGEMENTS

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Table 3.1. Summary of MUF-DC hydrolysis rates in Mono Lake on April 14, 2002. Water samples were collected at St. 6. Oxic sediment is sediment overlain by oxygenated water and anoxic sediment is sediment overlain by anoxic water.

Sample Location	Chitinase activity ($\mu mol\ g^{-1}\ hr^{-1}$)	Chitinase activity ($\mu mol\ ml^{-1}\ hr^{-1}$)
5 m	*	0.00539 ± 0.00085
15 m	*	0.00294 ± 0.00000
25 m	*	0.00392 ± 0.00170
35 m	*	0.00588 ± 0.00147
Oxic Sediment	0.13676 ± 0.0148	*
Anoxic Sediment	0.44767 ± 0.0218	*

Table 3.2. Summary of 16S rDNA sequences obtained from clone libraries. Accession numbers and phylogenetic affiliations are for closest relatives and determined by the BLASTN program of the National Center for Biotechnology Information. Symbols in the column headed "Multi source ?" indicate ribotypes retrieved from different samples across tables 3.2 and 3.5. Symbols are placed next to sequences that appeared in multiple enrichments, clone and isolate libraries (and none of the control libraries). Percent identities were determined using the GAP program of the Wisconsin Package.

Closest Relative	Accession Number	Phylogenetic Affiliation	Multi source ?	Number of Sequences	Percent Similarity
5 m Chitin Enrichment 1 (20 clones)					
Gamma-Proteobacterium N10	AF250323	γ-Proteobacteria	-	9	97.5-97.9
Nitrumincola lacisaponis	AY567473	γ-Proteobacteria	=	1	89
Microbulbifer sp. Th/B/38	AY224196	γ-Proteobacteria	™	4	91.9-92.1
Unidentified Proteobacterium	AB015518	ε-Proteobacteria		1	90
Caulobacter sp.	AJ227811	α-Proteobacteria		1	95
Lake Nakuru isolate 52N3	X92134	γ-Proteobacteria		1	99
Paracoccus sp. MBIC4036	AB025192	α-Proteobacteria	□	1	96
Gamma-Proteobacterium EHK-1	AF228694	γ-Proteobacteria		1	92
Arhodomonas sp. EL-201	AJ315984	γ-Proteobacteria	•	1	92
5 m Chitin Enrichment 2 (20 clones)					
Rhodobaca bogoriensis strain LBB2	AF384205	α-Proteobacteria	Δ	9	96.3-98.9
Microbulbifer sp. Th/B/38	AY224196	γ-Proteobacteria	™	1	91
Bacteroidetes bacterium GMDsbC7	AY162093	Bacteroidetes		2	85.5-86
Unidentified Proteobacterium	AB015518	ε-Proteobacteria		6	88.3-89.3

Psychroflexus tropicus	AF513434	Flavobacteria	]	1	92
Alcanivorax sp. OM-2	AB053128	γ-Proteobacteria		1	97
35 m Chitin Enrichment (20 clones)					
Thiomicrospira sp. JB-A1F	AF013976	γ-Proteobacteria		2	97.7-97.8
Nitrumincola lacisaponis	AY567473	γ-Proteobacteria	=	7	93.1-99.2
Uncultured bacterium SB-83-CS	AJ319865	Bacteroidetes		3	85.2-88.4
Hailaer soda lake bacterium Z4	AF275713	γ-Proteobacteria		1	94
Gamma-Proteobacterium ML-173	AF140006	γ-Proteobacteria		1	94
Gamma-Proteobacterium N10	AF250323	γ-Proteobacteria	-	1	94
Alkalliphilus auruminator	AB037677	Clostridia		1	89
Thialkalimicrobium sibericum	AF126549	γ-Proteobacteria		2	90.6-91.1
Idiomarina fontislapidosi	AY526861	γ-Proteobacteria		1	92
Crater Lake isolate 11C1	X92129	γ-Proteobacteria		1	95
5 m Control (20 clones)					
Lewinella nigricans	AF039294	Sphingobacteria		3	83.0-83.5
Unidentified actinobacterium d13	AJ292034	Actinobacteridae		1	94
chloroplast *Picocystis salinarum*	AF125173	Chlorophyta		7	97.2-97.8
Uncultured bacterium SB-83-CS	AJ319865	Bacteroidetes		3	87.8-88.6
Legionella adelaidensis	Z49716	γ-Proteobacteria		2	87.4-88.4
Sulfur-oxidizing bacterium OAII2	AF170423	γ-Proteobacteria		1	85
Lake Nakuru isolate 19N1	X92149	γ-Proteobacteria		1	98
uncultured bacterium gene	AB062814	Verrucomicrobia		1	86

Pirellula sp.	X81940	Planctomycetacia		1	94
35 m Control (19 clones)					
Thiomicrospira sp. JB-A1F	AF013976	γ-Proteobacteria		4	81.3-97.8
Uncultured bacterium SB-83-CS	AJ319865	Bacteroidetes		7	88.4-88.6
chloroplast *Picocystis salinarum*	AF125173	Chlorophyta		2	98
Bacteroidetes bacterium GMDJE10E6	AY162091	Bacteroidetes		2	89.3-90.8
Unidentified actinobacterium d13	AJ292034	Actinobacteridae		1	95
Brumimicrobium glaciale	AF521195	Flavobacteria		1	90
Firmicutes str.. ikaite c10	AJ431334	Firmicutes		2	93-95.7
Exuvia sample(25 clones)					
Arhodomonas sp. EL-201	AJ315984	γ-Proteobacteria	•	1	96
Uncultured bacterium SBR1071	AF268996	Candidate division TM7		1	
Rhodobaca bogoriensis	AF384205	α-Proteobacteria	Δ	6	96
Paracoccus sp. MBIC4019	AB025190	α-Proteobacteria		1	96
Bacterium str. 77003	AF227847	Gram-positive		1	98
Triticum aestivum (L.) partial chloroplast	AJ239003	Streptophyta		1	96
Vibrio metschnikovii (CIP 69.14T)	X74711	γ-Proteobacteria	©	1	98
Gamma-Proteobacterium M12-26A	AY730246	γ-Proteobacteria		1	99
Gamma-Proteobacterium HTB021	AB010859	γ-Proteobacteria		1	93
Plesiocystis pacifica	AB083432	δ-Proteobacteria		1	90
Chlorella mirabilis	X65100	Chlorophyta		1	93
Halomonas sp. 18bAG	AJ640133	γ-Proteobacteria		1	98
Roseobacter sp. TM1038	AF384205	α-Proteobacteria		1	97
Vibrio sp. M12-2C	AY730244	γ-Proteobacteria		1	99

Natronohydrobacter thiooxidans	AJ132383	α-Proteobacteria		3	97
Psychroflexus tropicus	AF513434	CFB	]	1	92
Bacteroidetes bacterium MO54	AY553122	CFB		1	89
Vibrio metschnikovii (NCTC 11170)	X74712	γ-Proteobacteria	®	1	98

Table 3.3. DAPI bacterial counts of time initial and time final chitin amended and control treatments of the chitin enrichment experiment. Measurements were taken for initial and final samples. At least 10 fields and 300 cells were counted per slide.

Treatment name	No. cells ml^{-1} (10^6)
5 m (initial)	4.30 ± 2.16
5 m chitin-amended (final)	21.62 ± 4.59
5 m control (final)	11.18 ± 2.59
35 m (initial)	3.28 ± 2.27
35 m chitin-amended (final)	16.39 ± 2.27
35 m control (final)	15.46 ± 4.04

Table 3.4. Phylogeny of dominant band sequences excised from figure 3.2.

Band #	Treatment	Closest Relative
1	5 m ($T_{initial}$)	chloroplast *Picocystis salinarum*
3	35 m ($T_{initial}$)	Bacterium Chibacore 1500
8	5 m chitin-amended (T_{final})	**Alkalimonas amylolytica**
9	5 m chitin-amended (T_{final})	*Rhodobaca bogoriensis* strain LBB2
10	5 m chitin-amended (T_{final})	*Synechococcus* sp. MW97C4
11	5 m chitin-amended (T_{final})	*Rhodobaca bogoriensis* strain LBB2
14	5 m control (T_{final})	**Stenotrophomonas maltophilia**
15	5 m control (T_{final})	Bacterium Chibacore 1500
17	35 m chitin amended (T_{final})	**Nitrumincola lacisaponis**
20	35 m chitin-amended (T_{final})	*Hymenobacter* sp. 29F
21	35 m chitin-amended (T_{final})	**Clostridium litorale**
29	35 m control (T_{final})	Bacterium Chibacore 1500
30	35 m control (T_{final})	Proteobacterium Dex60-82
32	35 m control (T_{final})	*Thiomicrospira* sp.

Table 3.5. Phylogenetic affiliation of isolates and their ability to hydrolyze chitin analogs. Accession numbers and phylogenetic affiliations are for closest matches (at least 363 bp of informative sequence information) and determined by the BLASTN program of the National Center for Biotechnology Information. Symbols in the column headed "Multi source ?" indicate ribotypes retrieved from different samples across tables 3.2 and 3.5. "Number of Isolates" indicates the number of isolates that had the same closest relative, the range of similarity values for the 16S rRNA gene sequences for these isolates is given in the column headed "Percent Similarity." The + and - symbols indicate that a substrate was or was not, respectively, hydrolyzed by the isolate and the numbers in parentheses indicate the number of isolates of that ribotype that were active towards the substrate.

Closest Relative	**Accession Number**	**Phylogenetic Affiliation**	**Multi source?**	**Number of Isolates**	**Percent Similarity**	**MUF-DC**	**MUF-TC**
Aerobic Isolates							
Arctic sea ice bacterium ARK10255	AF468429	Flavobacteria		1	92.8	-	-
Halomonas sp. A-07	AY347310	γ-Proteobacteria		4	96.4-99.7	(2) +	(2) +
Rhodobaca bogoriensis strain LBB2	AF384205	α-Proteobacteria	Δ	3	95.3-98.9	(2) +	(1) +
Lake Elmenteita isolate WE1	X92164	Bacillales		1	98.2	+	-
Halomonas sp. EF11	AY332559	γ-Proteobacteria		1	95.4	+	+
Bacillus sp. ZBAW6	AY453415	Bacillales		1	95	+	+
Bacillus sp. GSP75	AY553091	Bacillales		1	96.9	+	+
Gamma-Proteobacterium N10	AF250323	γ-Proteobacteria	-	1	95.0	+	+
Bacillus sp. T41	AB111934	Bacillales		1	97.4	+	+
Azospirillum sp. TTI	AF170353	α-Proteobacteria		1	86.4	-	-
Alpha Proteobacterium ML-168a	AF140003	α-Proteobacteria		4	97.6-98.1	-	(4) +
Vibrio metschnikovii (NCTC 11170)	X74712	γ-Proteobacteria	®	2	98	(2)+	(2)+

Vibrio metschnikovii (CIP 69.14T)	x74711	γ-Proteobacteria	©	1	98	+	+
Anaerobic Isolates							
Paraliobacillus ryukyuensis	AB087828	Bacillales		8	92.4-94.3	(4) +	(4) +
Amphibacillus tropicus	AF418602	Bacillales		26	86.1-94.3	(25) +	(25) +
Natronincola histidinovorans	Y16716	Clostridia		2	90.8-92.7	-	(1)+
Halomonas sp. LBB1	AY334093	γ-Proteobacteria		1	93.8	-	-
Halomonas sp. A-07	AY347310	γ-Proteobacteria		1	99.8	-	+
Hailaer soda lake bacterium F24	AF275702	Lactobacillales		2	98.8-99.2	(2) +	(2) +
Marinobacter flavimaris strain SW-145	AY517632	γ-Proteobacteria		1	90.8	+	+
Bacillus sp. ZBAW6	AY453415	Bacillales		1	95.1	+	-
Hailaer soda lake bacterium F10	AF275698	Bacillales		1	97.4	-	-
Bacillus selenitireducens	AF064704	Bacillales		8	98.3-99.8	-	-
Hailaer soda lake bacterium T2	AF275708	Bacillales		4	97.2-99.5	-	-
Uncultured Fusobacteria bacterium	AJ575990	Fusobacteria		1	89.5	-	-
Vibrio metschnikovii (CIP 69.14T)	X74711	γ-Proteobacteria	©	1	98.4	+	+
Tindallia californiensis	AF373919	Clostridia		1	96.4	-	-

Figure 3.1. Temporal variation of MUF-DC hydrolysis rates in samples of Station 6 sediments during the development of *Artemia monica* population. Dashed line represents *Artemia* population and the solid line represents MUF-DC hydrolytic measurements.

Figure 3.1

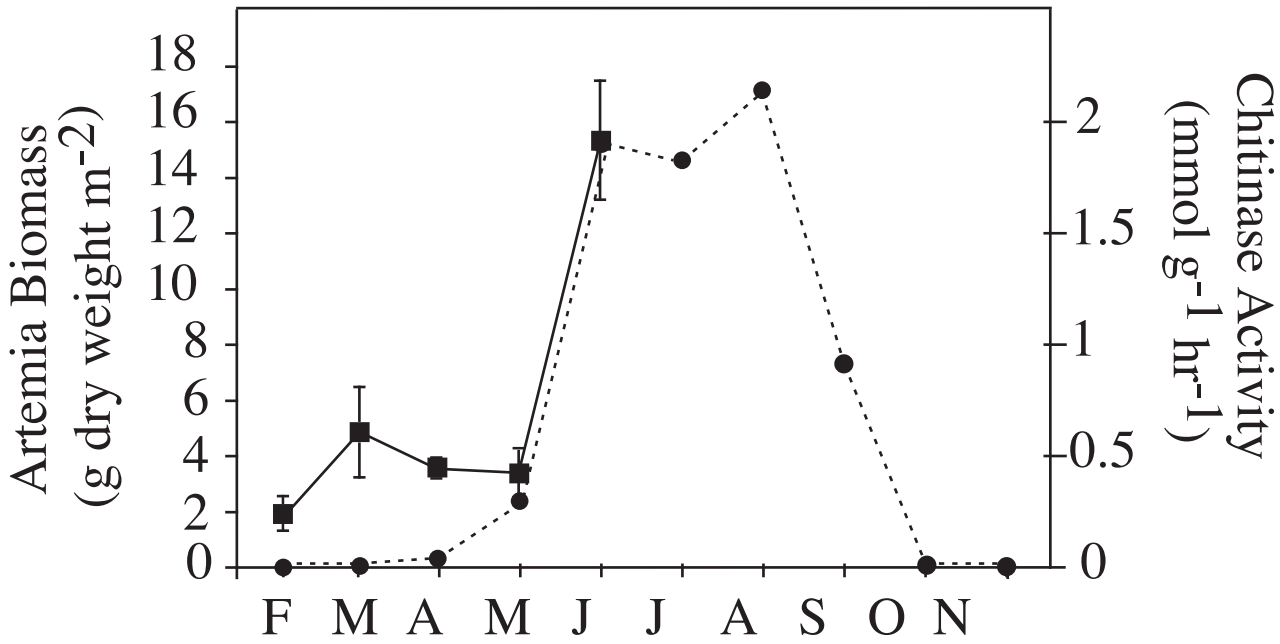

Artemia Biomass
(g dry weight m-2)
18
16
14
12
10
8
6
4
2
0
F M A M J J A S O N
2
1.5
1
0.5
0
Chitinase Activity
(mmol g-1 hr-1)

Figure 3.2. Image of a DGGE gel containing 16S rRNA gene fragments amplified from initial, chitin-amended and control treatments. Phylogeny of numbered bands are found in Table 3.4.

Figure 3.2

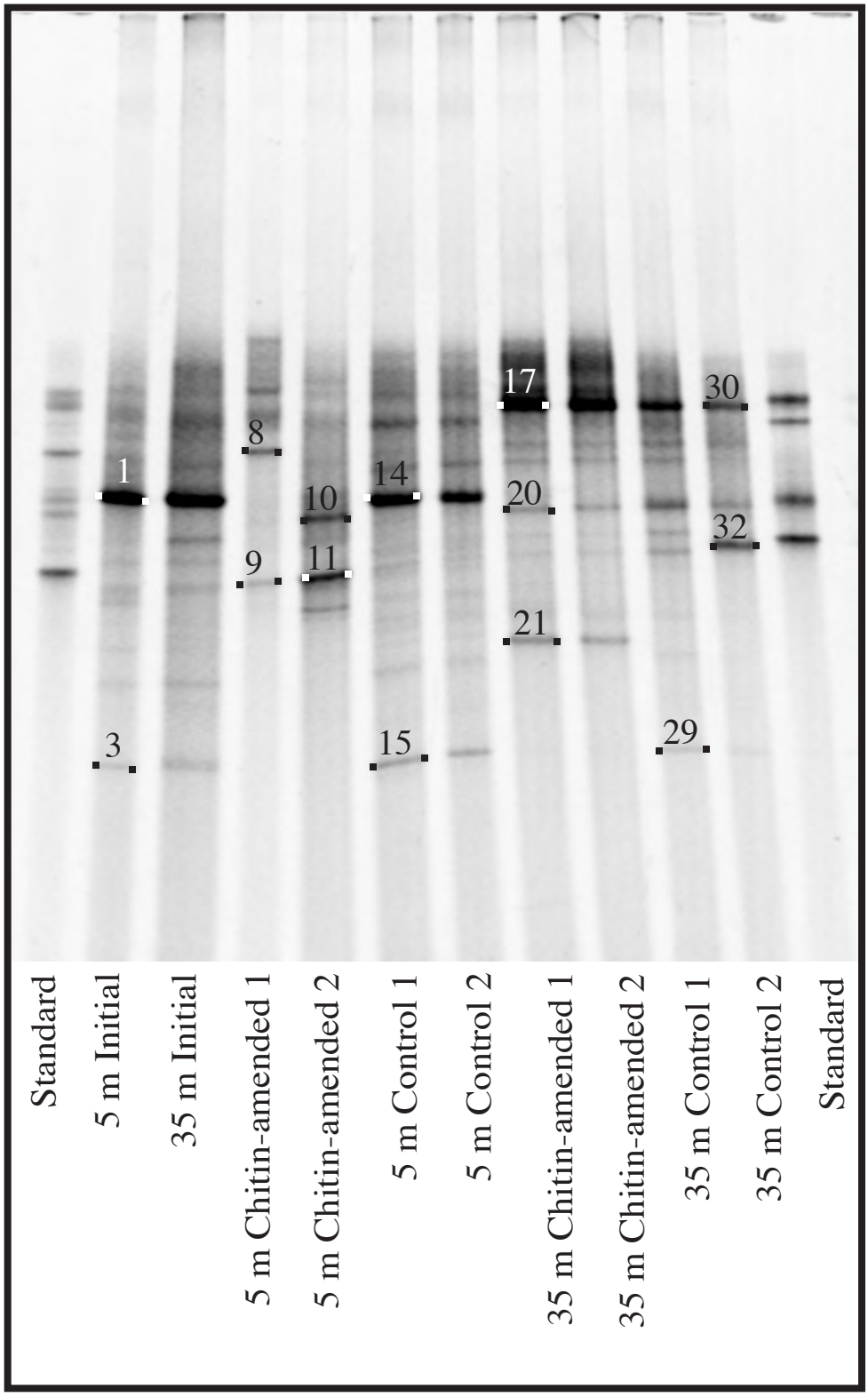
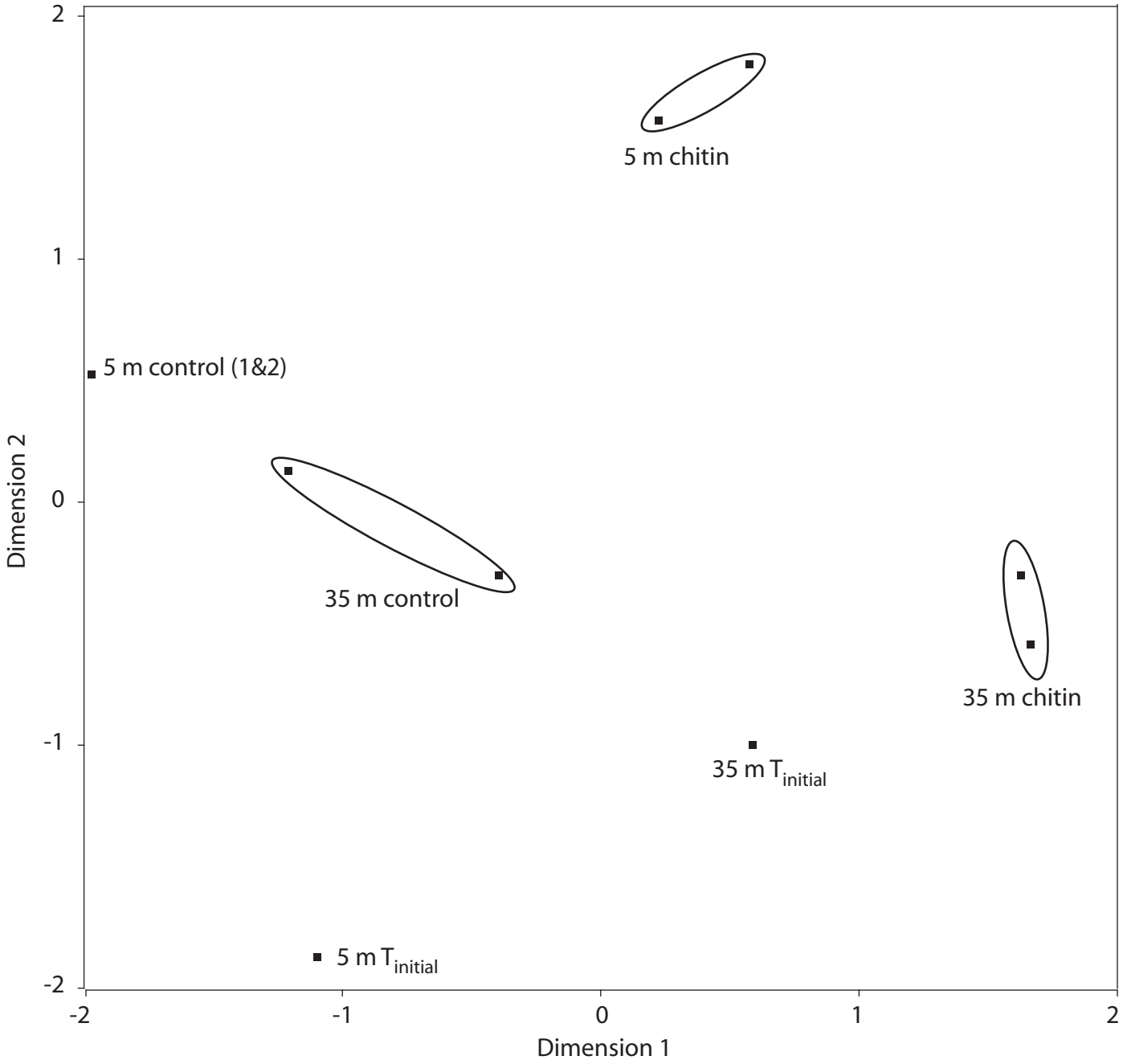

Standard
5 m Initial
35 m Initial
5 m Chitin-amended 1
5 m Chitin-amended 2
5 m Control 1
5 m Control 2
35 m Chitin-amended 1
35 m Chitin-amended 2
35 m Control 1
35 m Control 2
Standard
1
3
8
9
10
11
14
15
17
20
21
29
30
32

Figure 3.3. Plot of sample scores from non-metric multidimensional scaling analysis of DGGE banding patterns from initial, chitin-amended and control treatments. Final stress was 0.1062.

Figure 3.3


2
1
0
-1
-2
-2
-1
0
1
2
Dimension 2
Dimension 1
5 m chitin
5 m control (1&2)
35 m control
35 m chitin
35 m T initial
5 m T initial

CHAPTER 4

AN ANALYSIS OF GENES INVOLVED IN CHITIN DEGRADATION FROM TWO CONTRASTING ENVIRONMENTS: AN ESTUARY AND AN ALKALINE, HYPERSALINE LAKE

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ABSTRACT

We examined the genetic and physiological characteristics of chitin degrading enzymes from alkaline, hypersaline Mono Lake, CA, USA and from an estuarine environment (Dean Creek) on Sapelo Island, GA, USA. Fosmid libraries were created from genomic DNA of two bacterial strains isolated from Mono Lake and for environmental DNA from a natural, estuarine bacterial community (Sapelo Island). The libraries were screened for the ability to hydrolyze fluorogenic analogs of chitin (methylumbelliferyl β-D-N,N'-diacetyl-chitobioside; MUF-DC) and six positive clones were analyzed further. The genes encoding enzymes involved in MUF-DC cleavage were localized on these six clones by random transposon mutagenesis and the regions of interest were sequenced. One of the fosmid clones from Mono Lake isolate 12A contained a gene encoding a family 18 glycosyl hydrolase. Two additional clones, one from Mono Lake isolate AI21 and another from the Sapelo Island environmental DNA library, contained genes encoding family 20 glycosyl hydrolases.

We characterized the chitinolytic proteins expressed by these fosmids. A putative chitin degrading protein from Mono Lake strain AI21 had a pH optimum of 10; several pH units higher than any other enzyme currently assigned to this family, and it retained 80% of its activity at pH 11. The enzyme was also halotolerant, retaining activity in salt solutions of up to 225 g/L. Sequence analysis indicated a molecular weight of 90 kD for this enzyme, and that it contained 2 active sites. Culture supernatant contained two proteins, 45 and 31 kD, that were capable of cleaving MUF compounds, suggesting post-expression modification of the gene product to yield 2 enzymes.

INTRODUCTION

Chitin is the second most abundant biopolymer on the planet with ~10^{10} metric tons produced per year. The polymer provides structural support and protection for the vast array of organisms capable of producing it. The chitin molecule is an N-acetylglucosamine (GlcNAC) homopolymer that is rich in carbon and nitrogen, but is resistant to degradation. Complete hydrolysis of the chitin molecule typically results from the synergistic actions of two enzymes: chitinases (E.C. 3.2.1.14) and N-acetylglucosaminidases (GlcNAcidases; E.C. 3.2.1.52) (4, 22). Chitinases are grouped into families 18 and 19 of the glycosyl hydrolases (10) and cleave GlcNAc oligomers from the chitin molecule. GlcNAcidases are categorized as family 20 glycosyl hydrolases and cleave GlcNAc molecules from chitin oligosaccharides (4) or directly from chitin molecules (31). For simplicity, in this paper we include both chitinases, E.C. 3.2.1.14, and N-acetylglucosaminidases, E.C. 3.2.1.52 in the terms "chitinolytic" or "chitin-degrading." Chitinolytic enzymes are important in the biogeochemical cycling of carbon and nitrogen in the environment and they have potential application in pest control, bioremediation and biotechnology (6, 13, 26).

Our knowledge of environmental chitinases, GlcNAcidases and the genes encoding them is based primarily on work with bacteria from relatively conventional habitats (5, 6, 14, 19, 30). Reports of chitinases from extreme environments are scant. However, microbes from such environments potentially harbor unique enzymes with novel enzymatic properties (i.e. activity at a high pH or salinity, or sustained activity over a broad range of physiological conditions). The database of nucleotide sequences encoding environmental chitinase and GlcNAcidase genes has been obtained primarily

using PCR-based assays. Primers used in these studies are based on sequences obtained from organisms cultured from conventional habitats and thus may not fully reflect the diversity of these genes present in natural assemblages.

In a previous study, we were unable to retrieve chitinase gene sequences from DNA extracted from Mono Lake (an alkaline, hypersaline environment) samples using a PCR-based approach (17), despite measurements indicating high chitinolytic activity in these same samples. Deduced amino acid sequences of chitinase genes retrieved from estuarine and freshwater environments were found to be more similar to database sequences than those retrieved from another "extreme" environment with high alkalinity and salinity (Soap Lake, WA). Unique amino acid substitutions occurred at positions in the Soap Lake sequences that could have conferred novel enzymatic properties to the expressed protein (17). We hypothesized that chitinase genes from Mono Lake were too divergent from database sequences to be amplified by PCR using primers based on current database entries.

We constructed fosmid libraries from environmental DNA and isolates to test our hypothesis and to identify genes encoding putative chitinolytic enzymes. The libraries were screened using an expression-based assay. Transposon mutagenesis was then used to identify and sequence genes encoding chitinolytic enzymes in the fosmids. We found three genes encoding putative chitinolytic enzymes with this approach. Two of these were from Mono Lake isolates while the third was found in a fosmid library constructed from DNA extracted from an estuarine (Sapelo Island, GA) environmental sample. We were able to characterize the chitinolytic proteins expressed by the fosmid containing

hosts and determined that some of the Mono Lake enzymes had high pH optima and salt tolerance.

MATERIALS AND METHODS

Mono Lake isolates. Bacteria were isolated from water collected at station 6 in Mono Lake (38° 00' N; 119° 02' W, see bathymetric chart at http://geopubs.wr.usgs.gov/map-mf/mf2393/) during the summer of 2002. Samples (100 μl) were spread on thin-layer colloidal chitin R2A plates (24, 25). All plates were made using artificial Mono Lake water (AMLW) (3). Although some colonies developed clearing zones in the colloidal chitin indicating production of soluble chitinolytic enzymes, most did not, so colonies were selected randomly after 2 weeks growth at 20°C. Isolates were streaked twice on fresh R2A plates, then on plates containing only particulate chitin as a carbon and energy source and finally (if they grew on the chitin-only plate), on another R2A plate.

Assays for chitinolytic activities. Isolates were grown in liquid R2A in 96 well plates. After a 48 hr incubation, the media was amended with MUF-DC or methylumbelliferyl β-D-N,N',N"-triacetyl-chitotrioside (MUF-TC) (10 μM final concentration) and incubated for another 24 hrs. MUF fluorescence was detected visually using a UV transilluminator (UVP, Upland, CA). For the purposes of this paper, we define chitinolytic activity as the ability to cleave MUF-DC or MUF-TC. We also routinely inspected chitin plates for the formation of a clearing zone around individual colonies indicating production of soluble chitinases. In addition, we attempted to amplify chitinase genes from the Mono Lake isolates using published PCR primers targeting family 18, group I chitinase genes as described previously (LeCleir et al. 2004).

Phylogenetic characterization of bacterial isolates. Cells from single colonies were collected with a loop and lysed by boiling in ultrapure water. The lysate was centrifuged briefly to remove cellular debris, and then PCR was performed using the supernatant as template. This method proved efficient and did not require any further preparation for successful PCR amplification of 16S rRNA genes using the Bacteria primer set 9F and 1492R (16, 29). Products were cloned into the pCR 2.1 vector (Invitrogen Corp., Carlsbad, CA) following manufacturer's protocols. Colonies were selected randomly and plasmids were isolated from *E. coli* host cells using the Qiaprep Spin Miniprep kit (Qiagen). Inserts were sequenced with an ABI PRISM 3700 Genetic Analyzer and the BigDye terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) using primers that recognized the cloning vector (M13F and M13R). Reads of approximately 850 base pairs were obtained in each direction. Sequences were edited and assembled using the AssemblyLign Program (Oxford Molecular, 1998). Regions corresponding to the primer binding sites were removed from the sequences prior to analysis.

Fosmid Library Construction. Fosmid libraries were constructed for two Mono Lake isolates demonstrating chitinolytic activity (AI21 and 12A). Isolates were grown in 50 ml of R2A media made using AMLW to an $O.D._{600}$ of 0.6 - 0.8. Cells were centrifuged and DNA was extracted from cell pellets using the Gnome Genomic DNA extraction kit (Qbiogene, La Jolla, CA). Fosmid libraries were constructed using the Epicentre CopyControl fosmid library production kit (Epicentre, Wisconsin, USA) according to the manufacturer's protocol.

An environmental DNA fosmid library was constructed using DNA from 100L of water collected in Dean Creek, a small tidal creek on Sapelo Island, Georgia, USA in July

2004. Sand and sediment particles in the sample were allowed to settle overnight at 4°C. Bacterial cells were concentrated from the water using a tangential flow filtration apparatus. DNA was extracted using a modified CTAB extraction protocol (2). The environmental DNA was run on a low melting point agarose gel to isolate DNA of approximately 40 kb that was then used to construct a fosmid library using EpiCentre's CopyControl fosmid library production kit according to the manufacturer's protocol.

Screening libraries. Approximately 6,000 clones from the environmental library and 400 clones from each of the isolate libraries were selected randomly and screened for MUF-DC hydrolysis. Clones were grown for 16 hrs at 37°C in 96 well plates containing 500 μl of LB medium with 12.5 μg/mL chloramphenicol. Plates were replicated into 100 μl of the same medium supplemented with 2X Induction Solution (Epicentre). After ~18 h incubation, 50 μl of LB containing MUF-DC was added to each well (final concentrationof 100 μM MUF-DC per well). Following 16 h of incubation at room temperature, plates were viewed on a UVP transilluminator (Upland, CA) to identify clones that hydrolyzed MUF-DC. These clones were grown in 3 mL of LB and induced to high copy number with 2X Induction Solution (EpiCentre). Fosmids were extracted using the FosmidMax extraction kit (Epicentre), fosmid DNA was quantified using a Hoechst Dye assay (23) and stored at –20 °C in TE buffer.

Transposon Mutagenesis. Each of the four environmental fosmid clones expressing MUF-DC hydrolytic activity (Sapelo 2.13B10, Sapelo 77A, Sapelo 33B and Sapelo 99E) and one MUF-DC hydrolytic clone from each isolate library (AI214B1 and 12A2.1B10) were subjected to random transposon mutagenesis using the Hyper-Mu <Kan–1> transposon insertion kit (EpiCentre) according to the manufacturer's protocol.

Representative transposon-containing clones were grown and assayed for MUF hydrolysis as described above, except that all media contained 50 μg/mL kanamycin. At least eight clones per fosmid showing loss of activity were sequenced bi-directionally using a BigDye Terminator cycle sequencing kit (Applied Biosystems, Foster City,CA) with primers targeting the outer regions of the transposon (MUKAN-1 FP-1, MUKAN-1 RP-1). Additional sequence was obtained for fosmid AI214B1 using a primer walking approach. Sequencing was performed at the Molecular Genetics Instrumentation Facility at the University of Georgia.

Sequences were edited using the 4Peaks software package (9) and aligned using the AssemblyLign software program (Oxford Molecular, 1998). The assembled fosmid sequences were then queried against the database at the National Center for Biotechnology Information using the ORF Finder and BLASTP programs. Reference sequences that were most similar to the query sequence were imported into GCG (Wisconsin Package v. 10.2; (1)) and compared using the GAP program.

pH optima of expressed enzymes. Three buffers, encompassing a pH range of 3-11, were used to determine the pH optima of the enzymes expressed from fosmids 12A2.1B10, Sapelo 2.13B10 and AI214B1: McIlvaine's buffer (pH 3-6), Tris-HCl buffer (pH 7-8) and glycine-NaOH buffer (pH 9-11). All assays were performed in triplicate. Clones were grown to an OD_{600} of ~1.0 in LB. One hundred microliters of media and cells was pipetted into micro-centrifuge tubes. Cells were pelleted and the supernatant was removed. The pellet was resuspended in 500 μl of the appropriate buffer then 5 μl of 5 mM MUF-DC was added to each tube. Samples were incubated at room temperature for 4 hr. Sample fluorescence was read on a Hoefer DynaQuant 200

fluorometer. Clones were also screened for the ability to cleave MUF-TC and methylumbelliferyl β-D-N-acetylglucosaminide (MUF-GlcNAc) using the same conditions as described above for MUF-DC hydrolysis. The *E. coli* host strain without a fosmid was used as a negative control for these experiments.

Salt tolerance of expressed enzymes. Supernatant proteins of clone AI214B1 were concentrated from 1 L to 5 mL using an Amicon stirred ultrafiltration cell with a 30,000 NMWL filter. Preliminary experiments demonstrated this to be an effective method of concentrating the enzymes of interest (data not shown). MUF-DC hydrolytic activity was assayed over a salinity range of 0-150 g/L at pH 10.0 using 3 different salt solutions, NaCl, $MgCl_2$ and AMLW. Assays were performed as described above. The *E. coli* host strain without a fosmid was used as a negative control for these experiments.

Purity of supernatant proteins. Preliminary data showed that staining gels with Coomasie Blue destroyed MUF-DC hydrolyzing enzyme activity, so we used an indirect method to detect MUF-DC hydrolysis in mixtures of supernatant proteins resolved by electrophoresis (25 cm, 5% agarose gel run for 5 hr at 200 V). One lane was loaded with a size standard (Biorad, low range standard) and 2 lanes received supernatant proteins concentrated as above. Following electrophoresis, the gel was sliced lengthwise between the two sample lanes. One piece of the gel containing two lanes (the size standard and one sample lane, Piece A) was stained overnight with Coomassie brilliant blue. The other piece of the gel (Piece B) was stored at 4 ºC in a Ziploc bag. The next morning piece A was destained with a solution of 45% methanol, 45% water, 10% glacial acetic acid then protein bands were visualized on a light table. The lane loaded with supernatant contained 2 prominent bands. Piece B was aligned with Piece A and four

samples were excised from Piece B. Two were from areas adjacent to the prominent bands in Piece A, and 2 were from locations adjacent to regions between bands on Piece A. Excised gel samples were incubated with MUF-DC for 4 hours. Sample fluorescence was measured as above. The concentrated supernatant from the host strain without a fosmid was used for comparison to clone AI214B1 supernatant.

Accession numbers. Nucleotide sequences determined for this study have been submitted to GenBank under the following accession numbers: (to be supplied prior to publication.)

RESULTS

Two Mono Lake isolates (AI21 and 12A) capable of hydrolyzing MUF-DC and MUF-TC were chosen for analysis. When screening the isolates with published PCR primers targeting family 18, group I chitinase genes, we only obtained an amplification product from isolate 12A. Furthermore, only isolate 12A formed clearing zones on colloidal chitin thin-layer plates, indicating the presence of a soluble chitinase system in this strain. Phylogenetic characterization by 16S rDNA analysis revealed that both isolates fell within the γ-Proteobacteria. The 16S rRNA genes of isolates AI21 and 12A are 95% identical to γ-Proteobacteria N-10 (AF250323) and 98% identical to *Vibrio* metschnikovii (X74712), respectively.

Twelve clones from the strain 12A fosmid library, five from the strain AI21 fosmid library and 4 from the Dean Creek environmental DNA fosmid library hydrolyzed MUF-DC. After performing random transposon mutagenesis on one clone from each isolate library (AI214B1, 12A2.1B10) and all four environmental DNA clones (Sapelo

2.13B10, Sapelo 77A, Sapelo 99E, Sapelo 33B) we bi-directionally sequenced at least eight fosmids from each transposed fosmid library. We identified sequences in Sapelo 2.13B10, AI214B1 and 12A2.1B10 that encode enzymes identified in chitin degradation pathways (Table 4.1). No sequences obtained from Sapelo 77a, Sapelo 99E or Sapelo 33B were closely related (e-values >10-3) to any genes presently recognized as being involved in chitin degradation. Many of the sixteen sequences obtained for each these fosmids code for proteins related to decarboxylases, isomerases, and heat shock proteins. Several other sequences are most similar to hypothetical proteins with no known function.

The assembled sequence obtained from Sapelo 2.13B10 consists of 2531 bases from a single gene encoding a family 20 glycosyl hydrolase. The deduced amino acid sequence is 55% similar and 46% identical to the most similar reference sequence, a □-N-acetylhexosaminidase (CAC34802) from *Streptomyces olivaceoviridis* (see Fig. 4.1 for conserved domains in all three fosmid sequences). The deduced sequence of Sapelo 2.13B10 contains a region identified in the conserved domain database (CDD) as a glyco_hydro_20 domain (18).

The assembled sequence obtained from fosmid 12A2.1B10 totals 2248 base pairs. The deduced amino acid sequence is 82.9% similar and 78.2% identical to its closest match, a chitodextrinase from *Vibrio cholerae* (AAF96599). This sequence contains two conserved domains, a glyco_18 and a ChiC domain (CDD designations)(18).

The assembled sequence from fosmid AI214B1, obtained by sequencing from the transposons totals 3297 bases and encodes two separate genes, a GlcNAc kinase (297 aa) and a family 20 glycosyl hydrolase (858 aa). Additional sequence obtained by primer

walking increased the total sequence length to >7,000 bases and revealed 3 additional genes, a GlcNAc-6-P deacetylase (263 aa), a hypothetical protein (242 aa) and a tryptophan halogenase (471 aa). The deduced amino acid sequence of the GlcNAc-6-P deacetylase is 60% similar to the closest database match, a GlcNAc-6-P deacetylase from *Shewanella oneidensis* (AAN56496) and contains a putative NagA domain (CDD designation)(18). The deduced amino acid sequence of the gene encoding a GlcNAc kinase is 52% similar to a predicted GlcNAc kinase of *Microbulbifer degradans* (ZP_00317548) and contains domains similar to BcrAD_BadFG and COG2971 (CDD designations)(18). The deduced amino acid sequence of the gene encoding a family 20 glycosyl hydrolase is 59% similar and 52 % identical to the closest database sequence, GlcNAcidase A from *Alteromonas* sp. O-7 (BAB17855). This sequence contains a total of three conserved regions including two different catalytic domains, Glyco_hydro_20 and Glyco_hydro_20b and a chitin binding domain, CHB_HEX, (CDD designations)(18). The deduced amino acid sequence of the gene encoding the hypothetical protein is 43% identical and 64% similar to a hypothetical protein from *Shewanella frigidimarina* NCIMB 400. It contains a conserved domain similar to a SapC domain (CDD designation)(18). The deduced amino acid sequence of the gene encoding a tryptophan halogenase is 50% identical and 65% similar to a putative tryptophan halogenase from *Shewanella oneidensis* MR-1. It has a conserved region similar to a trp_halogenase domain (CDD designation)(18).

Transposed fosmid clones AI214B1, 12A2.1B10, and Sapelo 2.13B10 were also screened for their ability to cleave MUF-TC, the GlcNAc trimer. AI214B1 was the only clone to cleave MUF-TC. When the entire library of transposed-fosmid clones for

AI214B1 was screened with MUF-TC, the clones that did not cleave MUF-TC were the same clones that did not cleave MUF-DC suggesting the same gene(s) encode protein(s) responsible for cleaving both fluorogenic compounds. Neither AI214B1, 12A2.1B10 or Sapelo 2.13B10 hydrolyzed MUF-GlcNAc after 16 hours of incubation.

The pH optima for MUF-DC hydrolytic activity of the enzymes encoded by fosmids Sapelo 2.13B10, AI214B1 and 12A2.1B10 were distinct. Clone Sapelo 2.13B10 exhibited maximal activity towards MUF-DC at pH 4 with a small secondary optimum (<10% of maximum activity) at pH 9 (Fig. 4.2). Clone AI214B1 showed no activity towards the substrate at pH 4; maximal activity was measured at pH 10 (Fig. 4.2). This clone maintained 80% of its maximum activity at pH 11. Clone 12A2.1B10 cleaved MUF-DC optimally at pH 4 with a secondary pH optimum (60% of maximum relative activity) at pH 10 (Fig. 4.2). We also determined the pH response of AI214B1 using MUF-TC as a test substrate. This activity profile was unique in that enzymatic activity appeared to increase monotonically through pH 11 rather than peaking at some lower pH (Fig. 4.3).

The unique pH response profiles of AI214B1 enzymes prompted additional investigations of the expressed proteins. We found activity towards MUF-DC in the culture supernatant, as well as in membrane and intracellular fractions. The greatest activity occurred in the intracellular fraction (42% of total activity), however, a significant amount of activity was found in the supernatant (27% of total activity). The temperature optimum for MUF-DC hydrolysis by the enzyme from AI214B1 was 20 °C, although it remained active at 37°C for more than six hours. MUF-DC hydrolysis by the enzyme from AI214B1 occurred optimally at a salinity of 0 ppt, however, the enzyme

retained 80% of its activity at 225 ppt salinity (using both mono- and divalent salts). A MUF-DC hydrolysis curve was also made using artificial Mono Lake water (AMLW). Again, activity was greatest at 0 ppt. Hydrolytic activity quickly dropped to 20% as salinity increased, but maintained that activity level through 135 ppt (the highest salinity tested) (data not shown).

Two dominant bands of approximately 45 kD and 31 kD were found when concentrated supernatant of AI214B1 was run on a 5% agarose gel. The proteins in both bands were capable of cleaving MUF-DC, while 2 other samples of the gel, from regions lacking protein bands, did not cleave MUF-DC. The molecular weights of these proteins were confirmed on a SDS-PAGE gel (Fig. 4.4). No dominant protein bands were detected in either agarose or SDS-PAGE gels of the concentrated supernatant of *E. coli* host strain lacking the fosmid, nor did any fraction of these controls exhibit MUF-DC hydrolysis under any of the conditions tested.

An alignment of GlcNAcidases, adapted from an alignment in Kubota et al. (2004), shows that the gene sequences retrieved from fosmid AI214B1 and Sapelo 2.13B10 contain many conserved residues common to other GlcNAcidase sequences in the database, including the putative active site glutamic acid (Fig. 4.5). AI214B1 and Sapelo 2.13B10 were also compared to alignments presented in (26) and (8). Many positions along the aligned region are 100% conserved, indicating their potential importance in enzymatic function (Fig.4.5). A single residue in the catalytic regions of Sapelo 2.13B10 and AI214B1 at positions 298 and 317 of NagC, respectively, were unique when aligned with all sequences in (8, 15, 26)(Fig. 4.5).

DISCUSSION

Because we were unable to amplify chitinase genes from Mono Lake samples using published primers, we hypothesized that the physiochemical environment of Mono Lake exerts selective pressure on ectoenzymes such as microbial chitinase, causing their gene sequences to diverge from sequences obtained from organisms living in milieus with circumneutral pH and moderate salinity (LeCleir et al., 2004). Our results support this hypothesis. We found three gene sequences encoding enzymes that hydrolyzed the test substrates, two of the three chitinolytic sequences were most similar to N-acetylglucosaminidases (GlcNAcidases; E.C. 3.2.1.52), and one was similar to chitinase (E.C. 3.2.1.14). All three sequences were novel compared to database sequences with the two GlcNAcidases sharing little more than 50% similarity to current database sequences despite having multiple regions identified as conserved domains.

Microbial chitinases are generally found extracellularly or associated with the cell membrane. Chitinases typically hydrolyze the substrate in two different ways. Exo-chitinases cleave GlcNAc dimers from the non-reducing end of the chitin chain and endo-chitinases randomly cleave glycosidic bonds within the chitin polymer. Chitinases generally have acidic pH optima; however, some have been characterized that are active in alkaline pH ranges (20, 21, 31). Fosmid 12A2.1B10 contained a gene encoding a chitinase with a pH optimum of 4. This enzyme had a second peak in activity at pH 10, the approximate *in-situ* pH of Mono Lake. The secondary peak exhibited ~60% of the maximal activity towards the substrate recorded at pH 4. The deduced amino acid sequence of this enzyme was only 36% identical and 48% similar to another chitinase sequence obtained from this isolate using published PCR primers for family 18 group I

chitinase genes, indicating that this organism contains multiple chitinase genes. The presence of multiple chitinase genes is a common trait in chitinolytic microbes(11, 27, 28). Sequence analysis of the chitinase sequence obtained from the fosmid library shows that it would can not be amplified using family 18 group I primers in (17).

GlcNAcidases can be found intracellularly or in the periplasmic space of microorganisms (13, 15). They cleave GlcNAc monomers directly from chitin molecules (31) or from oligomers produced by chitinases. Optimal pH conditions for GlcNAcidases vary greatly from enzyme to enzyme. The GlcNAcidase expressed in clone Sapelo 2.13B10 has a pH optimum of 4 and demonstrates nominal activity towards the substrate at pH 11. This is a typical pH profile for GlcNAcidases that generally have pH optima between 4 and 6 (12). GlcNAcidase activity between pH 2-10 has been observed in *Paecilomyces persicinus*, however, the level of activity for this enzyme at pH 10 was approximately 5% of its optimal activity (pH 5.8) (7). Keyhani and Roseman (1996) characterized a perplasmic GlcNAcidase from *Vibrio furnissii* and found it to have a pH optimum approaching 7 with substrates $(GlcNAc)_{3\text{-}6}$. For $(GlcNAc)_2$ the authors found the enzyme to have a pH optimum of 5.8 and it demonstrated negligible activity at the *in-situ* pHs of 8-8.3 (12). The authors concluded that the GlcNAcidase they characterized was in some way involved in regulating the chitin catabolic cascade of the organism and did not actually hydrolyze chitin (12). Since the pH optimum of this enzyme is so much lower than *in-situ* pH of the estuary and because it exhibits only negligible activity at *in-situ* pH, it is possible that this enzyme functions similarly to the periplasmic GlcNAcidase described by Keyhani and Roseman (1996).

Mono Lake clone AI214B1 encoded at least one enzyme with hydrolytic activity towards MUF-DC. The enzyme expressed by clone AI214B1 has a pH optimum of 10 and retains 80% of its MUF-DC hydrolytic activity at pH 11. We are not aware of any other GlcNAcidase with such a high pH optimum or relative activity at pH 11. This enzyme was also active at salinity levels 2.5 times the current salinity of Mono Lake. It appears to be genetically novel when compared to other GlcNAcidase sequences currently in the database.

The agarose and SDS-PAGE gel analyses performed on the concentrated supernatant of AI214B1 revealed the presence of two dominant bands. These bands were 45 kD and 31 kD. Proteins in both of these bands were capable of hydrolyzing MUF-DC. The sequence for this GlcNAcidase gene coded a protein with two catalytic domains and was calculated to be approximately 90 kD in size. It is possible that the two bands we found in our gel resulted from proteolysis that yielded two separate and active units. This cleavage may either be part of the physiology of the bacterium or an artifact of the cloning and concentration processes.

Fosmid AI214B1 contained several genes involved in chitin breakdown. It is interesting to note that the GlcNAcidase was coded on the opposite strand, compared to all other genes in this region (Fig. 4.1). Although all of the enzymes encoded by this region of the fosmid were involved in GlcNAc degradation, apparently they are not all regulated by the same operator.

Proteins expressed by three other environmental DNA clones (Sapelo 77A, Sapelo 33B and Sapelo 99E) cleaved MUF substrates, yet the sequences putatively encoding this activity, as indicated by transposon mutagenesis and subsequent

sequencing, were not closely related to known chitinases or GlcNAcidases. This could result from the transposon inserting into a gene coding for protein not yet known to be associated with chitin degradation. Another possibility is that the transposon had a polar effect on expression of the chitinolytic gene, disrupting it from afar. Further analysis of these fosmids will be required to discriminate between these hypotheses.

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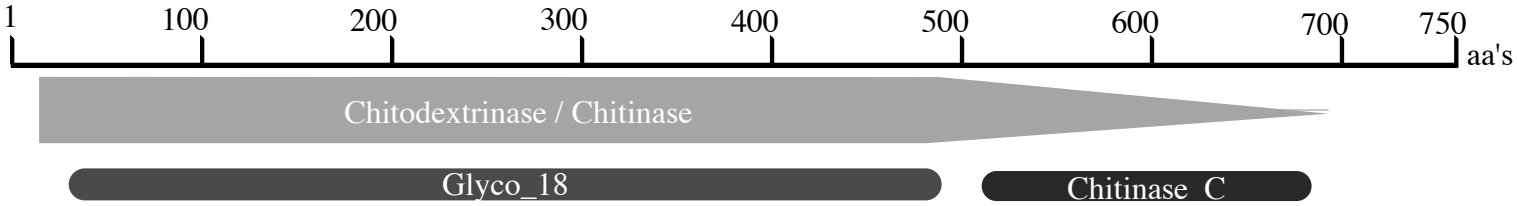
Table 4.1. Fosmid sequence names and similarity of chitinolytic regions to related enzymes. Sequence length refers to the total obtained sequence length in base pairs. Length of chitinolytic region refers to the length of the sequenced region that is similar to a chitinolytic sequence in the database. % similarity and % identity are derived from the closest database matches using the GAP program.

Fosmid Name	Fosmid Accession #	Sequence Length	Length of Chitinolytic Region	# Conserved Domains	Closest Relative	% Similarity	% Identity
12A2.1B10		2,248	2,013	2	AAF96599	82.9	78.2
AI214B1		7,100	2,574	3	BAB17855	59.1	52.7
Sapelo 2.13B10		2,531	1,437	1	CAC34802	55.0	46.0

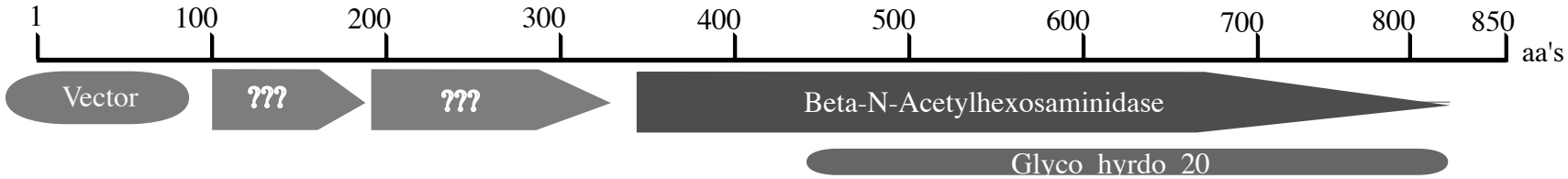
Figure 4.1. Diagram of the genetic organization of three fosmids capable of hydrolyzing MUF-DC. Direction of arrow represents direction of transcription. Each individual arrow represents a conserved domain. Arrows with ??? indicate they are domains with no known function. Domains were identified using the ORF finder and BLASTp programs (http://www.ncbi.nih.gov).

Figure 4.1

Mono Lake Isolate 12A Fosmid 2.1B10 (genome has PCR amplifiable chitinase gene)



Sapelo Fosmid 2.13B10



Mono Lake Isolate AI21 Fosmid 4B1

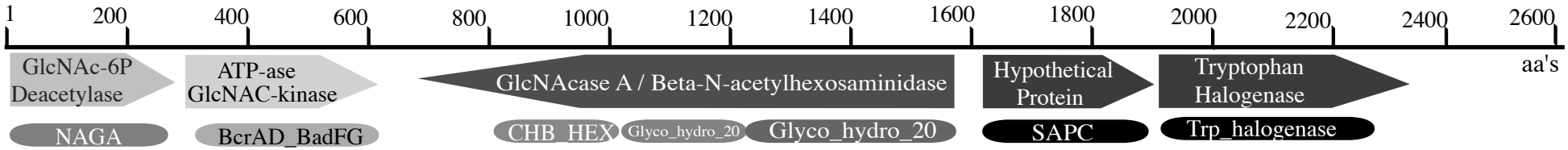


Figure 4.2. pH optima curves for enzymes produced by three fosmids capable of cleaving MUF-DC. ▲= Isolate fosmid AI214B1, △=Isolate fosmid 12A2.1B10, ●=Sapelo 2.13B10.

Figure 4.2

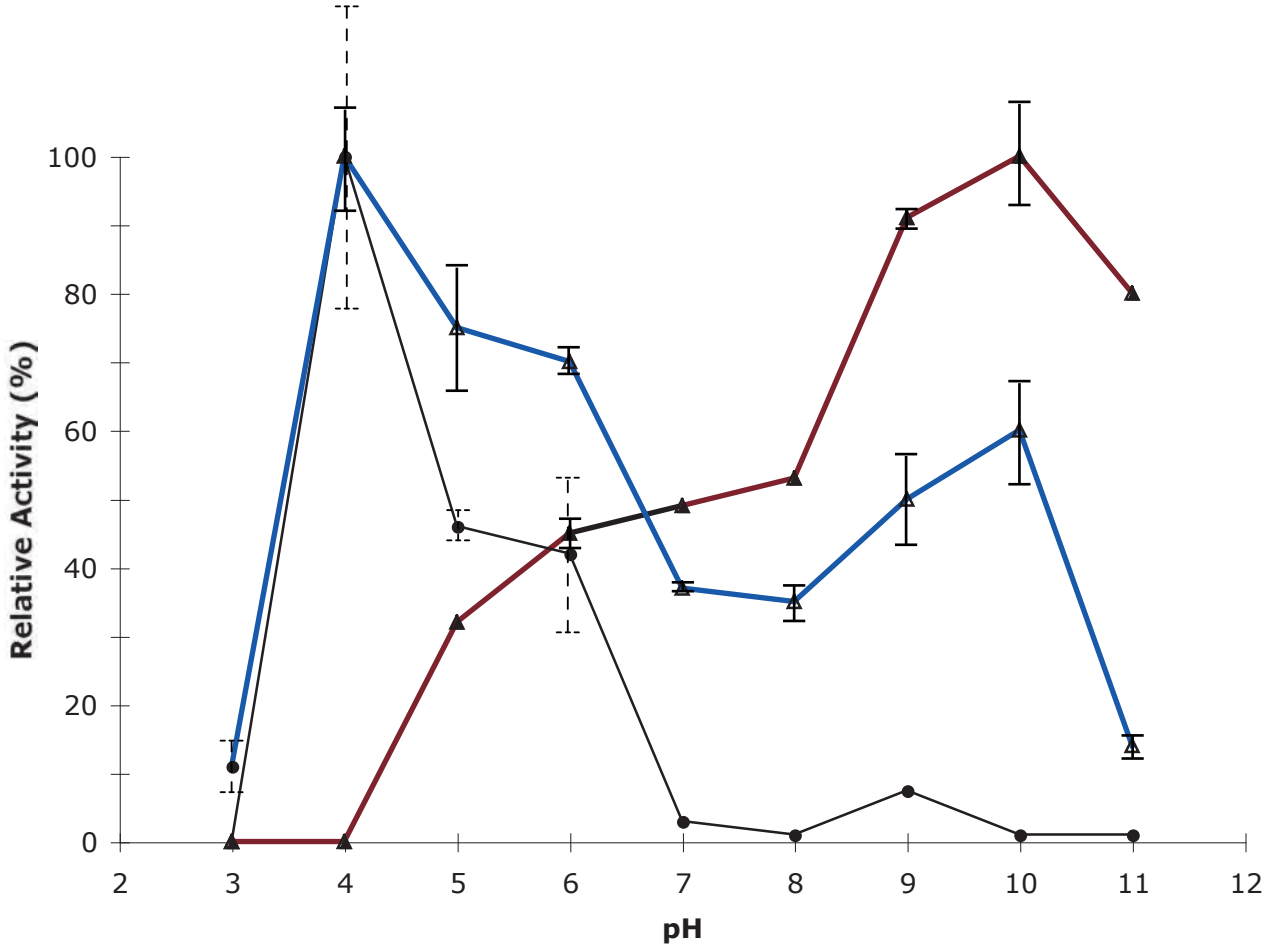


Figure 4.3. pH optimum curve for enzymes produced by AI214B1 towards MUF-TC.

Figure 4.3

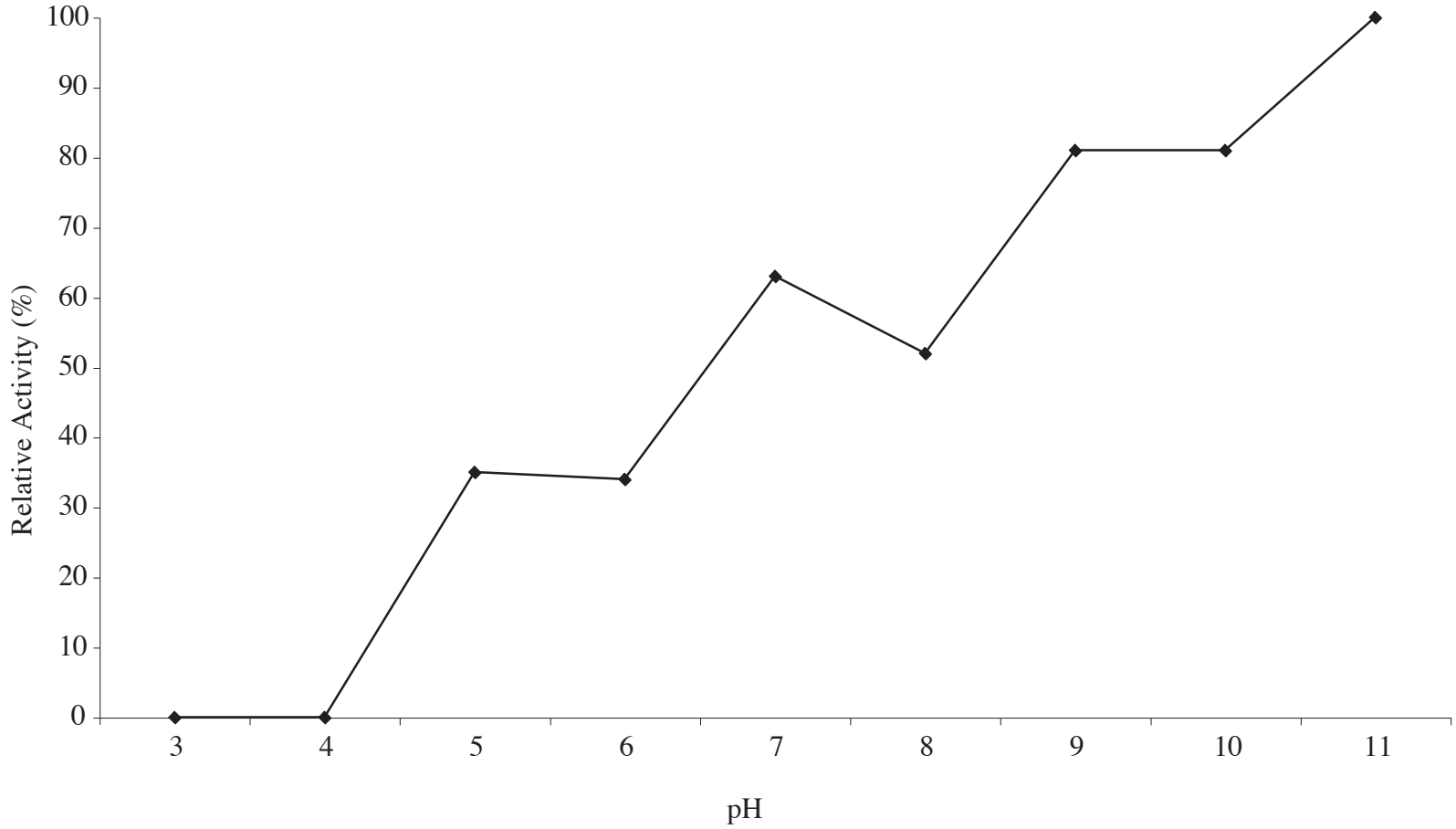

100
90
80
70
60
50
40
30
20
10
0
Relative Activity (%)
3
4
5
6
7
8
9
10
11
pH

Figure 4.4. SDS-PAGE of concentrated supernatant proteins from AI214B1. ★= Sections of gel excised from agarose gel capable of hydrolyzing-DC. ○ = Sections of gel excised from agarose gel incapable of hydrolyzing MUF-DC. Protein band sizes are listed in kD.

Figure 4.4

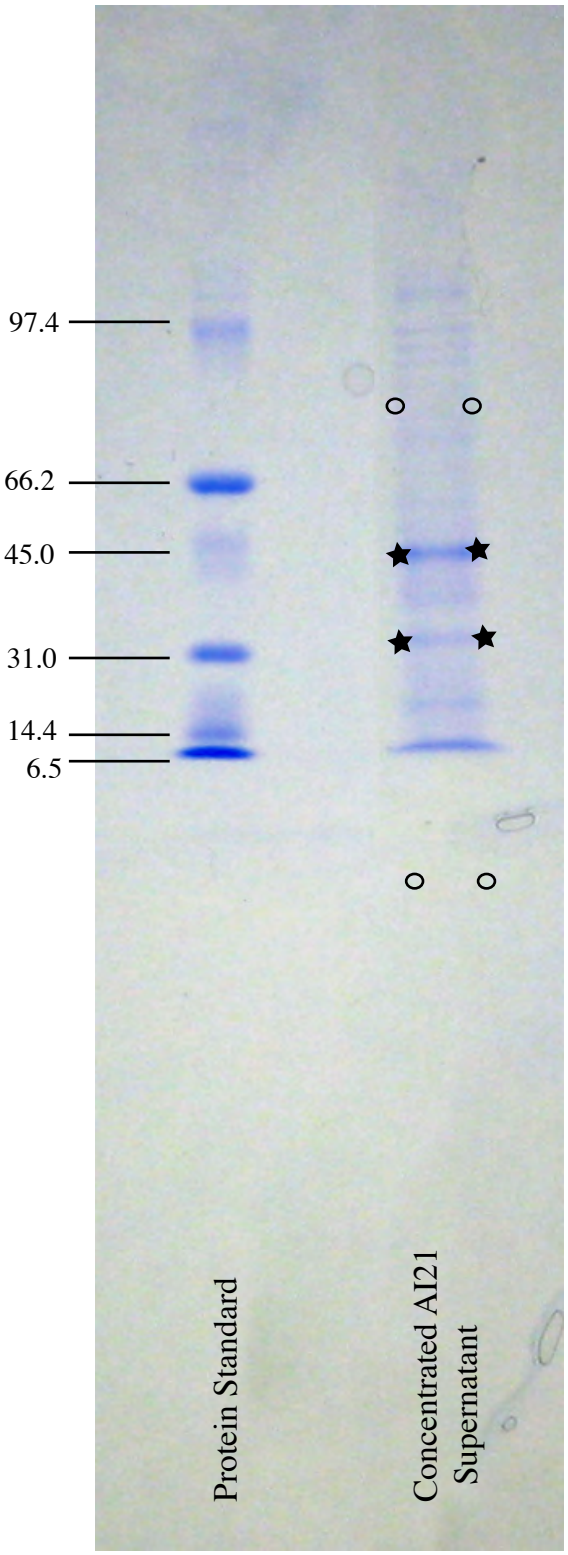
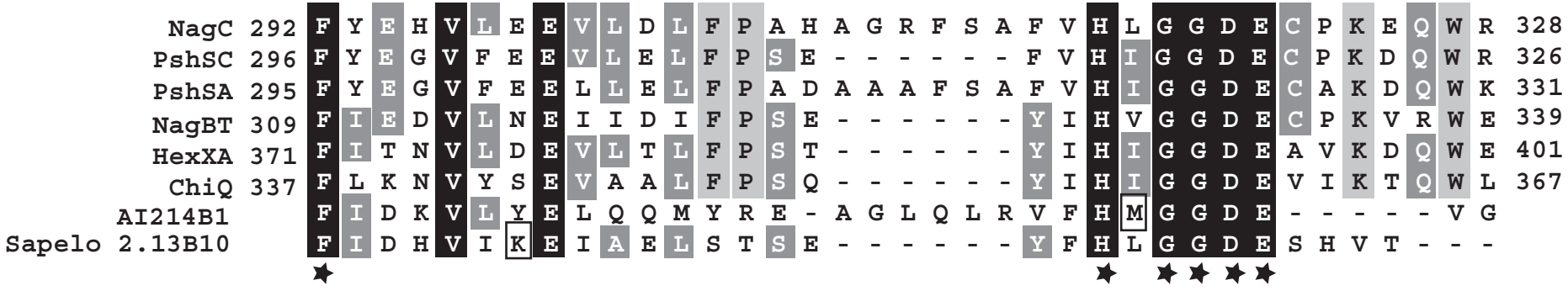

97.4
66.2
45.0
31.0
14.4
6.5
Protein Standard
Concentrated AI21
Supernatant

Figure 4.5. Alignment of deduced GlcNAcidase residues. Black background with white lettering: 100% residue conservation. Dark Gray with white letters: residue is conserved in at least 50% of sequences. Light gray with black letters: 100% conservation of residues from Kubota et al., but different in sequences from this study. ★ = 100% conservation in alignments from (8, 15, 26). Black squared residues: unique residues at this position compared to alignments in (8, 15, 26). Accession numbers for alignment sequences are: NagC (AB110077), PshSC (CAB72189), PshSA (BAC72846), NagBT (AAO76158), HexXA (AAM37919), ChiQ (AAC83237).

Figure 4.5


NagC 292 F Y E H V L E E V L D L F P A H A G R F S A F V H L G G D E C P K E Q W R 328
PshSC 296 F Y E G V F E E V L E L F P S E - - - - - - - F V H I G G D E C P K D Q W R 326
PshSA 295 F Y E G V F E E L L E L F P A D A A A F S A F V H I G G D E C A K D Q W K 331
NagBT 309 F I E D V L N E I I D I F P S E - - - - - - - Y I H V G G D E C P K V R W E 339
HexXA 371 F I T N V L D E V L T L F P S T - - - - - - - Y I H I G G D E A V K D Q W E 401
ChiQ 337 F L K N V Y S E V A A L F P S Q - - - - - - - Y I H I G G D E V I K T Q W L 367
AI214B1 F I D K V L Y E L Q Q M Y R E - A G L Q L R V F H M G G D E - - - - - V G
Sapelo 2.13B10 F I D H V I K E I A E L S T S E - - - - - - - Y F H L G G D E S H V T - - -

CHAPTER 5

CONCLUSIONS

Understanding the roles microbes play in chitin hydrolysis is essential to understanding carbon and nitrogen cycling in natural systems. A first step in understanding these processes is characterization of the chitinolytic microbial community and their associated enzymes. Toward that end, the goals of the studies presented in this dissertation were: [1] compare functional gene sequences retrieved from physiochemically distinct environments, [2] investigate chitinolytic activities in a physiochemically extreme environment (Mono Lake), [3] characterize the phylogeny of the chitin degrading community in Mono Lake, [4] isolate chitinolytic microbes from Mono Lake and [5] implement a cloning and gene expression strategy to obtain chitinase gene sequences without the use of PCR.

Analysis of chitinase gene sequences from physiochemically distinct environments suggests a functional significance to observed variability at the deduced amino acid level. In a phylogenetic context, chitinase sequences obtained from "conventional" environments (e.g. coastal salt marshes) tended to cluster most closely with one another and with database sequences. However, sequences retrieved from relatively "extreme" environments (e.g. the Arctic Ocean and Soap Lake) were unique and phylogenetically distinct from previously characterized sequences (Fig. 2.2). Furthermore, signature residues were identified in Soap Lake sequences (Fig. 2.3) that possibly confer novel properties to the chitinases of Soap Lake. Of the

10 systems analyzed, no chitinase sequences were retrieved using established primers from a system demonstrated to have significant chitinolytic activities, Mono Lake, CA

In a separate analysis of chitin degradation in Mono Lake, seasonal shift-up in MUF-DC cleavage was found to correlate with the emergence of the brine shrimp *Artemia monica* (Fig. 3.1). Clone libraries and DGGE analysis of 16S rRNA genes of chitin-degrading enrichment cultures demonstrated that the presence of chitin could cause shifts in the community profile (Fig. 3.2). MDS analysis of the DGGE banding patterns showed that chitin amendments strongly influenced the microbial assemblage of Mono Lake water incubations (Fig. 3.3). 16S rRNA gene sequences retrieved from chitin enrichments were related to α and γ-Proteobacteria as well as CFB and *Clostridia*. Some 16S sequences appeared in clone libraries from chitin-enriched water samples collected from different depths, as well as from a clone library generated from 16S rRNA amplicons derived from DNA extracted from *Artemia* exuvia and in a collection of Mono Lake isolates. The presence of a sequence in several libraries, but none of the controls, is strong presumptive evidence that the organism containing that sequence plays a role in chitin mineralization.

An inability to retrieve chitinase gene sequences from Mono Lake using degenerate PCR primers based on current database entries forced us to devise a new approach to obtain these gene sequences. An approach that used fosmid libraries coupled with random transposon mutagenesis and expression assays was used on genomic DNA from two chitinolytic isolates from Mono Lake and an estuarine environmental DNA fosmid library. Gene sequences coding for enzymes involved in MUF-DC hydrolysis were related to family 18 and 20 glycosyl hydrolases (Figs. 4.1, 4.5) were found. One family 20 glycosyl hydrolase encoded by fosmid AI214B1 (made from Mono Lake isolate AI21) hydrolyzed MUF-DC over an extremely wide

pH and salinity range (Fig. 4.2). This enzyme remained active at two pH units higher than any other GlcNAcidase previously reported in the literature. The deduced amino acid sequence for this enzyme was novel compared to other family 20 glycosyl hydrolases.

The results obtained in this dissertation can serve as the foundation for a number of future research projects, including: [1] Determining if amino acid substitutions in chitinase genes from "extreme" environments actually confer novel physiological traits to the enzymes. [2] Developing PCR primers targeting enzymes from "extreme" environments, allowing us to track chitinase diversity over an enrichment time course. [3] Re-examining the transposed fosmid libraries of isolates AI21 and 12A for other chitinolytic enzymes with novel properties. [4] Developing quantitative PCR primers targeting 16S rRNA gene sequences specific to chitinolytic microbes allowing us to track the chitinolytic microbial population over a seasonal cycle. [5] Developing RT-PCR primers targeting chitinolytic mRNAs to document the up-regulation of chitinolytic enzymes as the *Artemia* community matures in the spring.

In closing, there are many opportunities for continuing extracellular enzyme research in Mono Lake, specifically with chitinases and GlcNAcidases. It is highly probable that other enzymes can be isolated from Mono Lake with novel physiological capabilities as well. More research is necessary to completely understand chitin degradation in Mono Lake, however, the information presented here provides some valuable insight into the organisms and enzymes responsible for chitin hydrolysis in this environment.