ISSN 0026-2617, Microbiology, 2010, Vol. 79, No. 4, pp. 509–519. © Pleiades Publishing, Ltd., 2010.

EXPERIMENTAL ARTICLES

Metagenomic Characterization of Oyster Shell Dump Reveals Predominance of *Firmicutes* Bacteria[1]

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Received November 9, 2009

Abstract—Metagenomic analyses were conducted to evaluate the biodiversity of oyster shell bacteria, under storage conditions, on the basis of 16s rDNA sequences. Temperature was recorded during a one year storage period, and the highest temperature (about 60°C) was observed after five months of storage. Bacterial diversity was greatest in the initial stage sample, with 33 different phylotypes classified under seven phyla (*Proteobacteria, Bacteroidetes, Firmicutes, Actinobacteria, Planctomycetes, Verrucomicrobia* and unclassified bacteria), with 42.22% of phylotypes belonging to *Proteobacteria*. The lowest diversity was found in the high temperature (fermentation) stage sample, with 10 different phylotypes belonging to *Firmicutes* (78.57%) and *Bacteroidetes*. In the final stage sample, bacteria were found belonging to *Proteobacteria, Bacteroidetes*, and *Firmicutes,* and some were unclassified bacteria. Of the bacteria constituting the final stage metagenome, 69.70% belonged to *Firmicutes*. Our results show that bacteria belonging to phylum Firmicutes were predominant during fermentation, and during the final stages of oyster shell storage, which suggests that these bacteria supposed to be the key players for oyster shell biodegradation.

Key words: biodiversity, degradation, *Firmicutes*, metagenome, oyster shell.

DOI: 10.1134/S0026261710040132

The southeastern coastal sea of Korea is one of the most productive oyster-farming waters in the world, and shellfish-farming constitutes a large portion of the regional economy. Shellfish farms encompass 4100 hectares (ha) of coastal ocean and produce approximately 300 000 tons of oyster shell every year [1]. The oyster is a dominant product of shellfish farms and oyster industries are associated directly with considerable technological and economic development [2]. Shellfish farms are faced with the problem of oyster shell waste disposal. Enormous amounts of oyster-shell waste have been illegally disposed of at oyster farm sites along the southern coast of Korea [3]. In order to solve this problem, programs under government control were carried out, and many calcium and fertilizer factories were built to increase the amount of reused oyster shell [4]. Therefore, recycling of waste oyster shell has arisen as an imminent issue in mariculture areas. Most oyster shell waste (60%) is now recycled into materials for shell meal fertilizer and for oyster seeding, but remaining 40% has been dumped in the coastal region, causing environmental problems [5]. Waste that is left untreated becomes an environmental hazard due to the decay and microbial decomposition of flesh remnants attached to the oyster shells.

Oyster shell meal can be used as fertilizer, where it increases soil organic matter, available phosphate, and exchangeable cation concentration. As fertilizer it also improved soil pH and nutrient status significantly, increased the microbial biomass carbon and nitrogen concentrations, stimulated soil enzyme activities, and resulted in increased crop productivity [6]. Moreover, Lee et al. [6] demonstrated a high yield of Chinese cabbage following the application of 8 ton ha^{-1} of oyster-shell meal. However, more research is needed to develop efficient methods for processing oyster shells.

The oyster shell is a hard tissue consisting of calcium carbonate and organic matrices. Organic matrices are believed to play an important role in shell formation [7]. Various biominerals, such as the exoskeletons of crustaceans and the nacreous layer of seashells, are thought to contain chitin [8]. Suzuki et al. [9] used IR and NMR spectral analyses to identify the nacreous organic matrix of the central and prismatic layers

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of the Japanese pearl oyster shell (*Pinctada fucata*) as beta-chitin. Many bacteria belonging to the genera *Bacillus*, *Clostridium*, *Serratia*, *Streptomyces*, and *Xanthomonas* have the capability to degrade chitin [10], and chitinase activity plays an important role in the ecology of many marine bacteria [11]. Chitinase-producing organisms can be effectively used in the bioconversion process to treat shellfish waste and obtain value-added products [12]. Microbial degradation of oyster-shell has the potential to be the major pathway of oyster-shell waste management.

It is hypothesized that traditional culturing methods fail to reconstitute the scope of microbial diversity found in nature, because only a small proportion of viable microorganisms in a native sample are recovered by culturing techniques [13]. In addition, only a small fraction (1%) of the bacteria in seawater can be cultured [14] and those bacteria in culture are thought to be unrepresentative of uncultured, native bacteria [15]. However, Cottrell et al. [16] found that culture-dependent methods do not greatly underestimate the proportion of marine bacterial communities capable of chitin degradation, and suggested that a closer examination of chitin degradation by α-proteobacteria will lead to a better understanding of chitin degradation in the ocean. Moreover, 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 [17]. Lecleir et al. [17] also examined the genetic and physiological characteristics of chitin degrading enzymes expressed from fosmids cloned from two strains of chitinolytic gammaproteobacteria phylotyped from alkaline, hypersaline Mono Lake, California, and from a metagenomic library derived from an estuarine bacterial community (Dean Creek, Sapelo Island, GA, USA) and obtained a novel family 20 glycosyl hydrolase sequences from Mono Lake strain AI21. In addition, [18] measured genetic diversity by PCR-DGGE of total DNA extracted from garden/park waste compost, and a source separated organic household waste compost. They were then able to establish a relationship between genetic diversity and chitinase activity.

Many researchers [13, 14, 16] observed that culturable bacterial diversity analysis do not reflect the total microbial community. Moreover, uncultivated marine bacteria also produce chitinase [16], so it is important to determine the major groups of metagenomic bacteria in oyster shells, which are supposed to play key role for oyster shell biodegradation. We have presented a metagenomic assessment of bacterial diversity in oyster shells in a waste dump setting.

MATERIALS AND METHODS

Sample collection. The samples were collected from Haesung shell meal fertilizer factory (Tongyeong, Korea). The sample was collected 50 cm down from the top and classified into three stages of decomposition such as (i) initial stage sample (collected after one month of open-air storage), (ii) high temperature stage sample (collected after six months of open-air storage), (iii) final stage sample (collected after one year open-air storage). For each stage, there were five replications and 10 kg sample was collected for each replication. The base of oyster shell dump was approximately 20 m (width) × 40 m (length) × 15 m (height). The temperature of the oyster shell dump was recorded every month; monthly average ambient temperature during sample collection period was also recorded.

Analysis of microstructure of oyster shell by field emission-scanning electron microscope (FE-SEM). The microstructure of oyster shell was analyzed by FE-SEM at the initial stage, the high temperature stage, and the final stage sample. Incised oyster shells were sputter-coated with gold (JFC-1100E ion sputtering device, EG and G, USA) and were analyzed by a Philips XL30S FEG (Eindhoven, Netherland) FE-SEM which was operated at 10 kV.

Analysis of atomic force microscope. The used atomic force microscope (AFM) was XE-100 (PSIA Corp., Korea). The spatial and vertical resolutions are less than 1 nm and the field is between 5 and 5 μm. The images were taken at high resolution 256 × 256 pixels) by using an intermittent-contact mode (called Tapping Mode™) coupled with phase detection imaging (PDI). The probe is in silicon (Si) chip of the NSC36 series (PSIA, Suwon, Korea) with a round tip of 0.4 mm. It has features such as 30°C of full tip cone angle, a range of 20 to 25 μm of tip height, typical tip curvature radius of uncoated probe of <10 nm, and the reflective side coated with Al. The resonant frequency, the stiffness and the amplitude of cantilever was 155 kHz,1.75 Nm^{-1} and 35 μm, respectively. The scanning rate was 1 Hz. Data was collected in air and at room temperature after polishing parallel to the nacre surface.

Bacterial strains, growth conditions and isolation of bacteria. *Escherichia coli* DH5α was cultured in Luria-Bertani broth (LB, Difco, NJ, USA) at 37°C. For the culture of recombinant *E. coli* DH5α, ampicillin (50 μg ml^{-1}) was added to LB broth. TSA (TSA, Difco, NJ, USA) medium was used for the isolation of oyster shell bacteria at 30 and 37°C [19].

Isolation of genomic DNA. The metagenomic bacterial DNA was isolated according to the method described by the Binary Jungle and PowerSoil™ DNA isolation kit (MO BIO Laboratories, Inc., Carlsbad, CA 92008, United States).

Cloning of 16S rDNA genes. PCR amplification of 16S rDNA fragments of bacterial DNA was conducted according to the previous work [20–22]. The universal primers (877F, 5'-CGGAGAGTTTGATCCTGG-3'; 878R, 5'-TACGGCTACCTTGTTAGCGAc-3') were used with Super-Therm DNA polymerase (JMR, Side

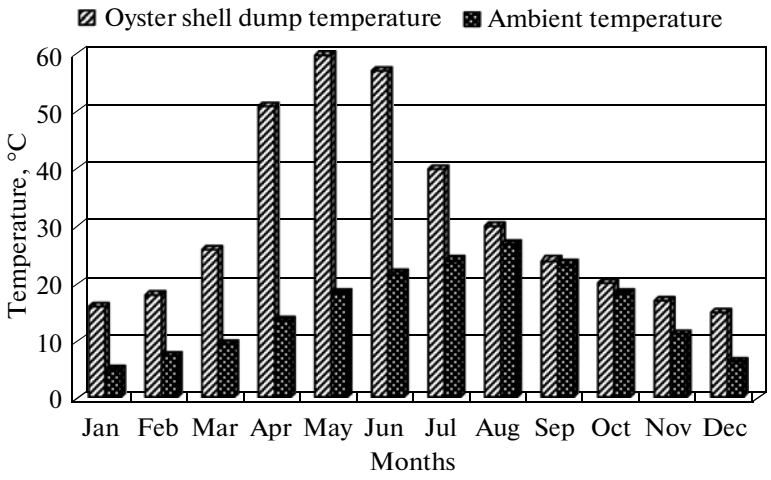

Fig. 1. Temperature changes of the open-air oyster shell dump with monthly average ambient temperature during the one year sample collection period.

Cup, Kent, UK), 1.5 mM $MgCl_2$, 2 mM dNTP in a final volume of 50 μl for thirty cycles (denaturation at 94°C for 30 s, annealing at 50°C for 30 s and extension at 72°C for 90 s followed by final incubation at 72°C for 10 min) [19]. The anticipated product of approximately 1,500 bp was isolated after agarose gel electrophoresis of PCR product using a gel extraction kit (NucleoGen, Seoul, Korea). Amplified 16S rDNA fragments were cloned into the pGEM-T Easy vector (Promega, WI, USA) and transformed into *E. coli* DH5α. *E. coli* cells harboring the recombinant plasmid were grown and selected on LAXI agar plate [(LB medium containing 50 mg ampicillin/ml, 6 g X-gal ml^{-1}, 5 g IPTG ml^{-1}, and 1.5% agar (w/v)]. Recombinant *E. coli* DH5α colonies were randomly picked. Plasmid DNAs from recombinant colonies of *E. coli* DH5α were isolated by the NucleoGen Plasmid Mini Kit (NucleoGen, Seoul, Korea). Standard procedures for restriction endonuclease digestion, agarose gel electrophoresis, purification of DNA from agarose gels, DNA ligation and other cloning related techniques were carried out as described by Sambrook and Russel [23]. Restriction enzymes and DNA modifying enzymes were purchased from Gibco-BRL (Gaithersburg, MD, USA), Promega (Madson, WT, USA) and Bohringer Mannheim (Indianapolis, IN, USA). Other chemicals were purchased from Sigma Chemical Co. (Louis, MO, USA).

16S rDNA sequencing and analysis. Nucleotide sequencing of 16s rDNA was conducted by dideoxy-chain termination method using the PRISM Ready Reaction Dye terminator/primer cycle sequencing kit (Perkin-Elmer Corp., Norwalk, CN, USA). The samples were analyzed with an automated DNA sequencer (Applied Biosystems, Foster City, CA, USA). Assembly of the nucleotide sequences was performed with the DNAMAN analysis system (Lynnon Biosoft, Quebec, Canada). All reference sequences were obtained from the National Center for Biotechnology Information (NCBI) and Ribosomal Database Project (RDP) databases. The 16S rDNA sequences similarity was observed using the BLASTN in the NCBI website [24]. Sequences were aligned using the multiple sequence alignment program, CLUSTAL W. Gaps and positions with ambiguities were excluded from the phylogenetic analysis. Phylogenetic analysis was performed using neighbor-joining methods. Bootstrap analysis was carried out using dataresampled 1000 times using the DNAMAN analysis system.

Nucleotide sequence accession numbers and nomenclature. Nucleotide sequences of oyster shell metagenomic bacteria have been deposited in the GenBank database. For initial stage metagenomic bacteria, clones name begin with the letters "MBIOS" (metagenomic bacteria of initial oyster shell)-01 to MBIOS-33. For the H library (high temperature stage), the prefixes areMBHOS-01 to MBH10. For the F library (final stage), the prefixes are MBFOS-01 to MBFOS-14 for metagenomic bacteria.

RESULTS

Temperature and structural changes in the oyster shell. Temperature (taken at a depth of 50 cm from the top of the pile) was recorded monthly after oyster shell dumping for a period of one year (Fig. 1). The temperature of the initial stage sample (17°C) gradually increased up to 60°C at the high temperature (fermentation) stage, which was about six months of dumping, and then decreased at the final stage (after one year of dumping) to 16°C. Oyster shell dump temperature did not appear to be influenced by the ambient temperature; the high temperature stage occurred in spring when the average ambient temperature was 18.4°C. The microstructure of oyster shell from the three stages was analyzed by FE-SEM. In the initial stage,

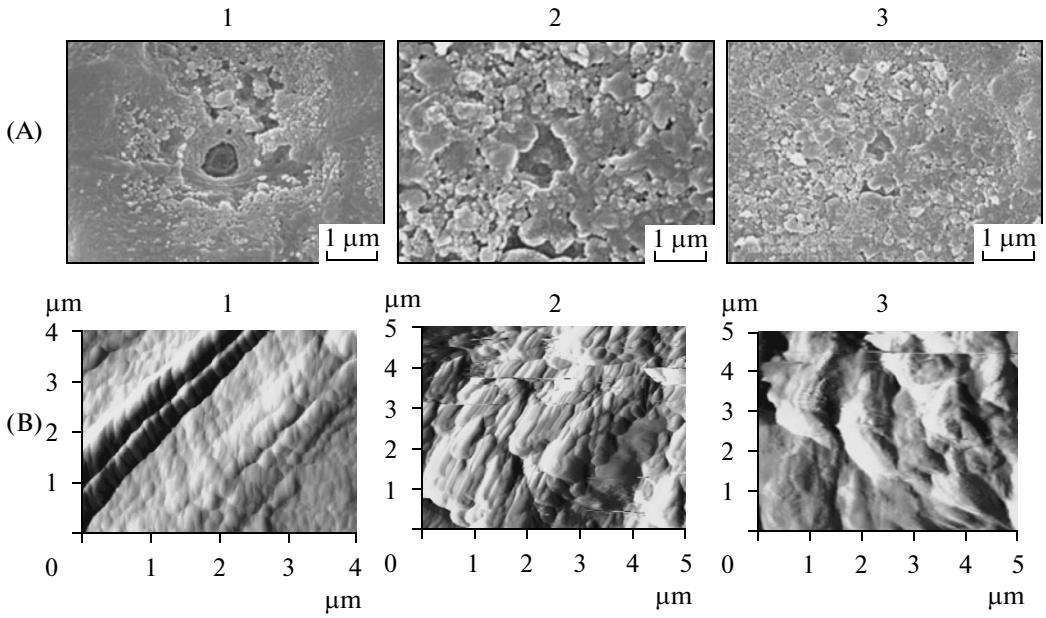

Fig. 2. (A) Field emission-scanning electron micrographs showing the nanograin structure of oyster shells (back region) at three stages: (1) initial stage, (2) high temperature stage, (3) final stage. Magnification is ×2000; scale bar is 10 μm. (B) AFM images of three oyster shell samples: (1) initial stage sample, (2) high temperature stage sample, and (3) final stage sample, AFM analyzed 5 μm.

the crystal structure and nanograin of the oyster shell surface were sharp and large, respectively, while those characteristics were blunt and fine-grained in the high temperature and final stage sample (Fig. 2A). Phase contrast maps from AFM images of initial stage, high temperature stage and final stage samples ere analyzed (Fig. 2B). The intracrystalline organic matrix has a foam-like structure. The surface of the oyster shell in the initial stage sample is more or less planar. The surface is rough at the high temperature stage, but then is smoother in the final stage sample.

Representation of Bacteria. Clones (44) from the initial stage sample were analyzed by a metagenomic approach. The clones were distributed into six phyla i.e., *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Verrucomicrobia*, *Planctomycetes*, and *Actinobacteria* (table). Sixteen clones (36.4%) possessed sequences that exhibited similarity to those from culturable phylotypes: MBIOS-17 (4 clones) with *Piscibacillus salipiscarius*, MBIOS-03 (2 clones) with *Flexibacter aggregans*, MBIOS-08 (1 clone) with *Sphingopyxis mactogoltabida*, MBIOS-10 (1 clone) with *Legionella*-like amoebal pathogen, MBIOS-12 (1 clone) with *Aequorivita antarctica*, MBIOS-13 (1 clone) with *Mesorhizobium* sp., MBIOS-16 (1 clone) with *Mesonia motionis*, MBIOS-22 (1 clone) with *Arenibacter latericius*, MBIOS-23 (1 clone) with Gamma proteobacterium, MBIOS-24 (1 clone) with "*Rubritalea tangerina*," MBIOS-26 (1 clone) with "*Algoriphagus antarcticus*, and MBIOS-28 (1 clone) with *Formosa agariphila*. Twenty-eight clones (63.6%) had sequences that exhibited similarity with those from uncultured phylotypes: MBIOS-01, 11, 20, 25, and 31 (18 clones) with uncultured bacterium, MBIOS-21 (4 clones) with uncultured compost bacterium, MBIOS-32 (2 clones) with uncultured alpha proteobacterium, MBIOS-04 (one clone) with uncultured soil bacterium, MBIOS-06 (one clone) with uncultured gamma proteobacterium, MBIOS-07 (one clone) with uncultured delta proteobacterium, MBIOS-09 (1 clone) uncultured organism and MBIOS-33 (1 clone) with uncultured *Cytophaga* sp. Approximately 97.7% of the sequences in this library exhibited a similarity level with database sequences in the range of 91 to 100%. The remaining sequences (2.3%) were less than 90% similar.

Clones (54) were analyzed from the high temperature stage sample, and were distributed into two phyla: *Bacteroidetes* and *Firmicutes* (table). Twenty-five clones (46.3%) had sequences that exhibited similarity with those from cultured phylotypes: MBHOS-02 (5 clones) with *Bacillus smithii*, MBHOS-04 (5 clones) with *Ureibacillus thermosphaericus*, MBHOS-06 (5 clones) with *Clostridium hastiforme*, MBHOS-08 (5 clones) with *Bacillus* sp., MBHOS-09 (5 clones) with *Virgibacillus necropolis*. Twenty-nine clones (53.7%) had sequences that exhibited similarity to those from uncultured bacteria: MBHOS-01, 03, 05 and 07 (24 clones) with uncultured bacterium, MBHOS-10 (5 clones) with uncultured compost bacterium. Among all clones, uncultured bacterium(MBHOS-07), which was the nearest relative of uncultured bacterium AKIW500 (DQ129377), was found to occur most frequently (9 clones). Approximately 79.6% of the sequences in this library exhibited

Similarity values of 16S rDNA sequences retrieved from the metagenomic bacteria collected from oyster shells at initial stage, high temperature stage and final stage sample

Group	Clone (Accession No.)	Number of colonies	Phylum	Nearest relative* (Accession No.)	Similarity (%)
Initial stage sample					
MBIOS-01	EU369117	1	*Proteobacteria*	Uncultured bacterium SC-144 (AB255109)	99
MBIOS-02	EU369118	1	*Planctomycetes*	Uncultured bacterium 101-74 (EF157236)	89
MBIOS-03	EU369119	2	*Bacteroidetes*	*Flexibacter aggregans* IFO15974 (AB078038)	99
MBIOS-04	EU369120	1	*Proteobacteria*	Uncultured soil bacterium 1_BMK_MJK (EF540368)	96
MBIOS-05	EU369121	1	*Proteobacteria*	Uncultured bacterium 101-99 (FE)	97
MBIOS-06	EU369122	1	*Proteobacteria*	Uncultured gamma proteobacterium SM1D02 (AF445671)	99
MBIOS-07	EU369123	1	*Proteobacteria*	Uncultured delta proteobacterium AKYG967 (AY922176)	100
MBIOS-08	EU369124	1	*Proteobacteria*	*Sphingopyxis macrogoltabida* (AB255383)	100
MBIOS-09	EU369125	1	*Proteobacteria*	Uncultured organism ctg_CGOAB08 (DQ395494)	99
MBIOS-10	EU369126	1	*Proteobacteria*	*Legionella*-like amoebal pathogen CC99 (EF492067)	100
MBIOS-11	EU369127	1	*Actinobacteria*	Uncultured bacterium AKIW874 (DQ129383)	99
MBIOS-12	EU369128	1	*Bacteroidetes*	*Aequorivita antarctica* S4-8 (AY771732)	99
MBIOS-13	EU369129	1	*Proteobacteria*	*Mesorhizobium* sp. R8-Ret-T53-13d (AM183167)	99
MBIOS-14	EU369130	1	*Proteobacteria*	Uncultured bacterium 271c2 (EF460033)	93
MBIOS-15	EU369131	4	*Bacteroidetes*	Uncultured bacterium EV818CFSSAHH221 (DQ337021)	95
MBIOS-16	EU369132	1	*Bacteroidetes*	*Mesoniamotionis* KMM6059 (DQ367409)	93
MBIOS-17	EU369133	4	*Firmicutes*	*Piscibacillus salipiscarius* (AB194046)	95
MBIOS-18	EU369134	1	*Bacteroidetes*	Uncultured soil bacterium (EF688374)	94
MBIOS-19	EU369135	1	*Proteobacteria*	Uncultured bacterium Asc-w-60 (EF632708)	89
MBIOS-20	EU369136	1	Unclassified bacteria	Uncultured bacterium MSB-1C5 (EF125397)	91
MBIOS-21	EU369137	4	*Bacteroidetes*	Uncultured compost bacterium 1B24 (DQ346456)	99
MBIOS-22	EU369138	1	*Bacteriodetes*	*Arenibacter latericius* KMM426T (AF052742)	99
MBIOS-23	EU369139	1	*Proteobacteria*	Gamma proteobacterium 12IX/A01/168 (AY576751)	97
MBIOS-24	EU369140	1	*Verrucomicrobia*	*Rubritalea tangerina* YM27-005 (AB297806)	93
MBIOS-25	EU369141	1	*Bacteroidetes*	Uncultured bacterium CS399 (AY382614)	94
MBIOS-26	EU369142	1	*Bacteroidetes*	*Algoriphagus antarcticus* LMG21983 (AJ577142)	95
MBIOS-27	EU369143	1	*Proteobacteria*	Uncultured alpha proteobacterium Biri24 (AJ893518)	91
MBIOS-28	EU369144	1	*Bacteroidetes*	*Formosa agariphila* KMM3962 (AJ893518)	95
MBIOS-29	EU369145	1	*Bacteroidetes*	Uncultured bacterium ELB25-087 (DQ015772)	91
MBIOS-30	EU369146	1	*Proteobacteria*	Uncultured bacterium B-45 (AY676483)	92
MBIOS-31	EU369147	1	*Planctomycetes*	Uncultured bacterium SS1_B-03_21 (EU050874)	99
MBIOS-32	EU369148	2	*Proteobacteria*	Uncultured alpha proteobacterium LC1-34 (DQ289904)	99
MBIOS-33	EU369149	1	*Bacteriodetes*	Uncultured *Cytophaga* sp. JTB248 (AB015263)	88

Table. (Contd.)

Group	Clone (Accession No.)	Number of colonies	Phylum	Nearest relative* (Accession No.)	Similarity (%)
High temperature stage sample					
MBHOS-01	EU369150	6	*Firmicutes*	Uncultured bacterium DA114 (AJ000980)	95
MBHOS-02	EU369151	5	*Firmicutes*	*Bacillus smithii* R-7170 (AY373319)	90
MBHOS-03	EU369152	4	*Bacteroidetes*	Uncultured bacterium EV818BHEB5102702SAS60 (DQ256356)	95
MBHOS-04	EU369153	5	*Firmicutes*	*Ureibacillus thermosphaericus* (AB101594)	91
MBHOS-05	EU369154	5	Unclassified bacteria	Uncultured bacterium F37-814 (EF174264)	90
MBHOS-06	EU369155	5	*Firmicutes*	*Clostridium hastiforme* DSM5675 (X80841)	95
MBHOS-07	EU369156	9	*Firmicutes*	Uncultured bacterium AKIW500 (DQ129377)	93
MBHOS-08	EU369157	5	*Firmicutes*	*Bacillus* sp. CHNTR52 (DQ337594)	96
MBHOS-09	EU369158	5	*Firmicutes*	*Virgibacillus necropolis* LMG19488T (AJ315056)	95
MBHOS-10	EU369159	5	*Firmicutes*	Uncultured compost bacterium 4-11 (AB034705)	97
Final stage sample					
MBFOS-01	EU369160	2	*Bacteroidetes*	*Brumimicrobium mesophilum* YH207 (DQ660382)	92
MBFOS-02	EU369161	6	*Firmicutes*	Uncultured bacterium G55_D5_H_B_B11 (DQ887925)	99
MBFOS-03	EU369162	3	*Proteobacteria*	Iodide-oxidizing bacterium Mie-8 (AB159210)	99
MBFOS-04	EU369163	2	*Firmicutes*	*Peptostreptococcaceae* bacterium 19gly3 (AF550609)	99
MBFOS-05	EU369164	2	*Firmicutes*	*Bacillus smithii* R-7170 (AY373319)	90
MBFOS-06	EU369165	4	*Bacteroidetes*	*Salegentibacter* sp. 18III/A01/068 (AY576719)	97
MBFOS-07	EU369166	2	*Firmicutes*	Uncultultured compost bacterium 2B16 (DQ346500)	89
MBFOS-08	EU369167	2	*Firmicutes*	*Bacillus polygonumi* (AB292819)	93
MBFOS-09	EU369168	5	*Proteobacteria*	Uncultured bacterium nsmpVI13 (AB212893)	91
MBFOS-10	EU369169	8	*Firmicutes*	Uncultured bacterium AKIW500 (DQ129377)	93
MBFOS-11	EU369170	2	*Firmicutes*	*Bacillus* sp. PeC11 (AM177061)	99
MBFOS-12	EU369171	3	*Bacteroidetes*	*Flavobacteriaceae* bacterium YIMC338 (EU135614)	94
MBFOS-13	EU369172	4	*Bacteroidetes*	Uncultured bacterium MSB-1A4 (EF125382)	99
MBFOS-14	EU369173	2	*Firmicutes*	*Planomicrobium* sp. EP20 (AM403522)	97

* Accession number of the nearest relative. When more than one sequence had the same similarity value, only the accession number of the first sequence is given.

a similarity level with database sequences in the range of 91 to 97%; the remaining 20.4% were less than 90% similar.

Clones (47) were analyzed from final stage sample, and were distributed among three phyla: *Bacteroidetes, Firmicutes*, and *Proteobacteria*. Twenty-two clones (46.8%) had sequences that exhibited similarity to those from cultured phylotypes: MBFOS-06 (4 clones) with *Salegentibacter* sp., MBFOS-03 (3 clones) with Iodide-oxidizing bacterium, MBFOS-04 (2 clones) with *Peptostreptococcaceae* bacterium, MBFOS-01 (2 clones) with *Brumimicrobium mesophilum*, MBFOS-05 (2 clones) with *Bacillus smithii*, MBFOS-8 (2 clones) with *Bacillus polygonumi*, MBFOS-11 (2 clones) with *Bacillus* sp., MBFOS-12 (3 clones) with *Flavobacteriaceae bacterium*, MBFOS-14 (2 clones) with *Planomicrobium* sp. Twenty-five clones (53.2%) had sequences with similarity to those from uncultured phylotypes: MBFOS-02, 09, 10, and 13 (23 clones) with uncultured bacterium, MBFOS-

07 (2 clones) with uncultured compost bacterium. Among all clones, uncultured bacterium (MBFOS-10) occurred most frequently (8 clones); similar to the high temperature stage sample clone MBFOS-10 was most closely related to uncultured bacterium AKIW500 (DQ129377). Approximately 91.5% of the sequences in this library exhibited similarity to database sequences in the range of 91 to 99%; the remaining 8.5% were less than 90% similar (table).

Phylogenetic placement of bacteria based on metagenomic analysis. Phylogenetic placement of unculturable bacteria revealed that most of the sequences from initial stage sample were placed within the *Proteobacteria* (41%) and *Bacteroidetes* (36.4%), the remaining 22.6% bacteria were under the phyla *Firmicutes* (9.1%), *Planctomycetes* (6.8%), *Actinobacteria* (2.3%), *Verrucomicrobia* (2.2%) and unclassified bacteria (2.2%) (Fig. 3A). For the high temperature stage sample, most of the sequences were placed within the *Firmicutes* phylum (81.4%), 18.6% were under the phylum *Bacteroidetes*, and the remaining 9.3% were unclassified bacteria (Fig. 3B). In the final stage sample, 66% sequences were also placed within the *Firmicutes* phylum; the remaining 34% were under the phyla *Bacteroidetes* (23.4%) and *Proteobacteria* (10.6%) (Fig. 3C). Bacterial diversity was highest in the initial stage sample (7 phyla), but it was less in the high temperature (3 phyla) and final stage samples (3 phyla) (Fig. 4). *Bacteroidetes* and *Firmicutes* species were present at all three stages. However, in the initial stage sample, 42.22% of the metagenomic bacteria were classified under *Proteobacteria* and 35.56% belonged to *Bacteroidetes*. However, in the high temperature and final stage samples, the greatest distribution of bacteria was within *Firmicutes* phylum, as observed by metagenomic analyses. Moreover, metagenomic bacteria belonging to the *Firmicutes* phylum dramatically increased from the initial stage sample (8.8%) to the high temperature stage sample (78.6%) and made up 69.7% of the bacteria in the final stage sample as assessed by metagenomics.

DISCUSSION

We found that the temperature of stored oyster shell changes over time, from a starting temperature of about 17°C, to a high of about 60°C, and then back to 16°C, and this change was unaffected by ambient temperatures. The high temperature stage took place during the spring, suggesting that oyster shell organic matter decomposition by microorganisms is the cause of the temperature rise. When that process is complete, the material returns to the ambient temperature. In the high temperature stage, only thermophilic bacteria can survive, and these are presumed to degrade the oyster shell. It has been suggested that temperature, and the availability of specific substrates, are key factors in the selection of microbial communities [25].

In this study, the microstructure of the oyster shell was analyzed by FE-SEM. The crystal structure and nanograin of the oyster shell surface became gradually blunted and smaller from the initial stage sample to the final stage sample. This blunting may have been due to degradation of the shell by microorganisms. Lee and Choi [26] found an organic membrane in oyster shell at the interface between the myostracum (aragonite) and folia (calcite), which was composed of a chitin-like macromolecule. Some chitinase enzymes have optimal activity at high temperature, for example, Zhang et al. [27] found that the optimum temperature for chitinase activity of *Stenotrophomonas maltophilia* strain C3 was 45 to 50°C. We suggest that the high temperature stage promotes the chitinase activity of the bacterial community that we have observed on the shell surface (by FE-SEM and by 16S rDNA analysis), resulting in the digestion of the chitin-like compound of oyster shell. Chitinase activities are clearly important for understanding the ecology of chitin-degrading bacteria in aquatic systems [16]. Likewise, they might be important for the microbial ecology in waste storage environments.

Through metagenomic analyses we observed the highest diversity of bacterial species in the initial stage. *Bacteroidetes* and *Firmicutes* species were present in all three stages. However, in the initial stage sample, the greatest distribution of bacteria was *Proteobacteria* (41.0%), followed by *Bacteroidetes* (36.4%). *Firmicutes* had the greatest species representation in the high temperature and final stage samples (81.4 and 66.0%, respectively). These bacteria were dramatically increased after the initial stage sample. Results showed that the diversity of uncultivable bacterial species decreased after the initial stage during oyster shell fermentation in open-air storage. Only specific bacterial strains showed the ability to survive in the high temperature.

The oyster shell is a hard tissue consisting of calcium carbonate and organic matrices; the organic matrices are believed to play important roles in shell formation [7]. Previous studies of the nacreous layer of oyster shells showed that the major components of organic matrices are chitin, and proteins such as the Asp-rich calcium-binding proteins, nacrein, MSI60, and N16. Various biominerals, such as the exoskeletons of crustaceans and the nacreous layer of seashells, are thought to contain chitin [8]. Many bacteria, belonging to the genera: *Bacillus, Clostridium, Serratia, Streptomyces* or *Xanthomonas*, are capable of degrading chitin [10]. The first step in chitin degradation, which is primarily done by microbes [28], is the hydrolysis of the glycosidic bonds between *N*-acetyl-D-glucosamine residues by chitinases. The capacity to degrade chitin is widespread among taxonomic groups of prokaryotes, including: gliding bacteria, *Vibrios, Photobacterium* spp., enteric bacteria, Actinomycetes, *Bacilli, Clostridia* [29]. Moreover, some uncultivable bacteria that were more prevalent in the high temper-

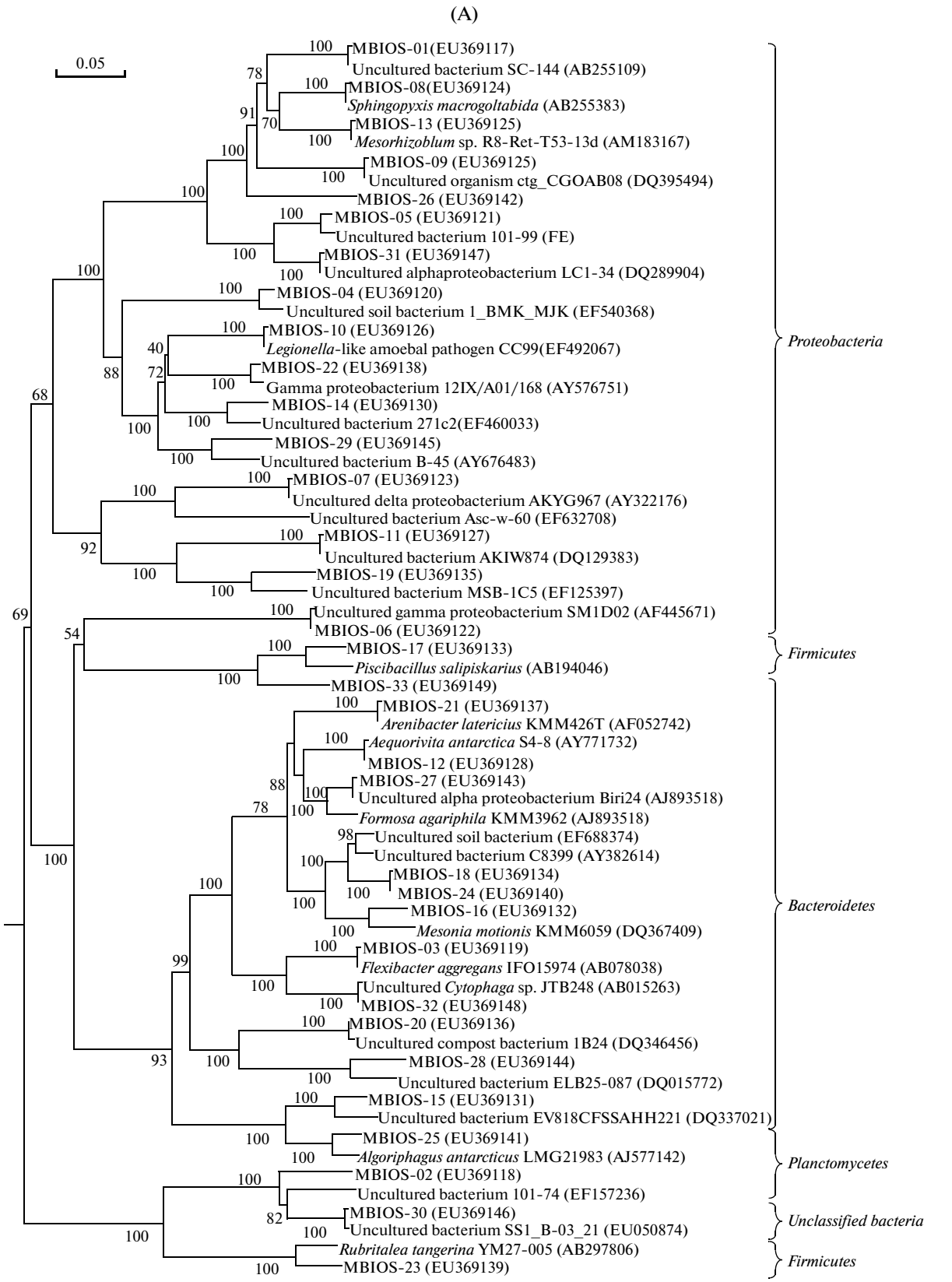

Fig. 3. Phylogenetic placement of 16S rDNA sequences from the metagenomic bacteria collected from oyster shells at initial stage (A), high temperature stage (B), and final stage sample (C). Numbers above each node indicate percentage of confidence levels generated from 1 000 bootstrap trees. The scale bar is in fixed nucleotide substitutions per sequence position. Phylogenetic analysis was performed using neighbor-joining methods. Bootstrap analysis was carried out using data resampled 1 000 times using the DNAMAN analysis system.

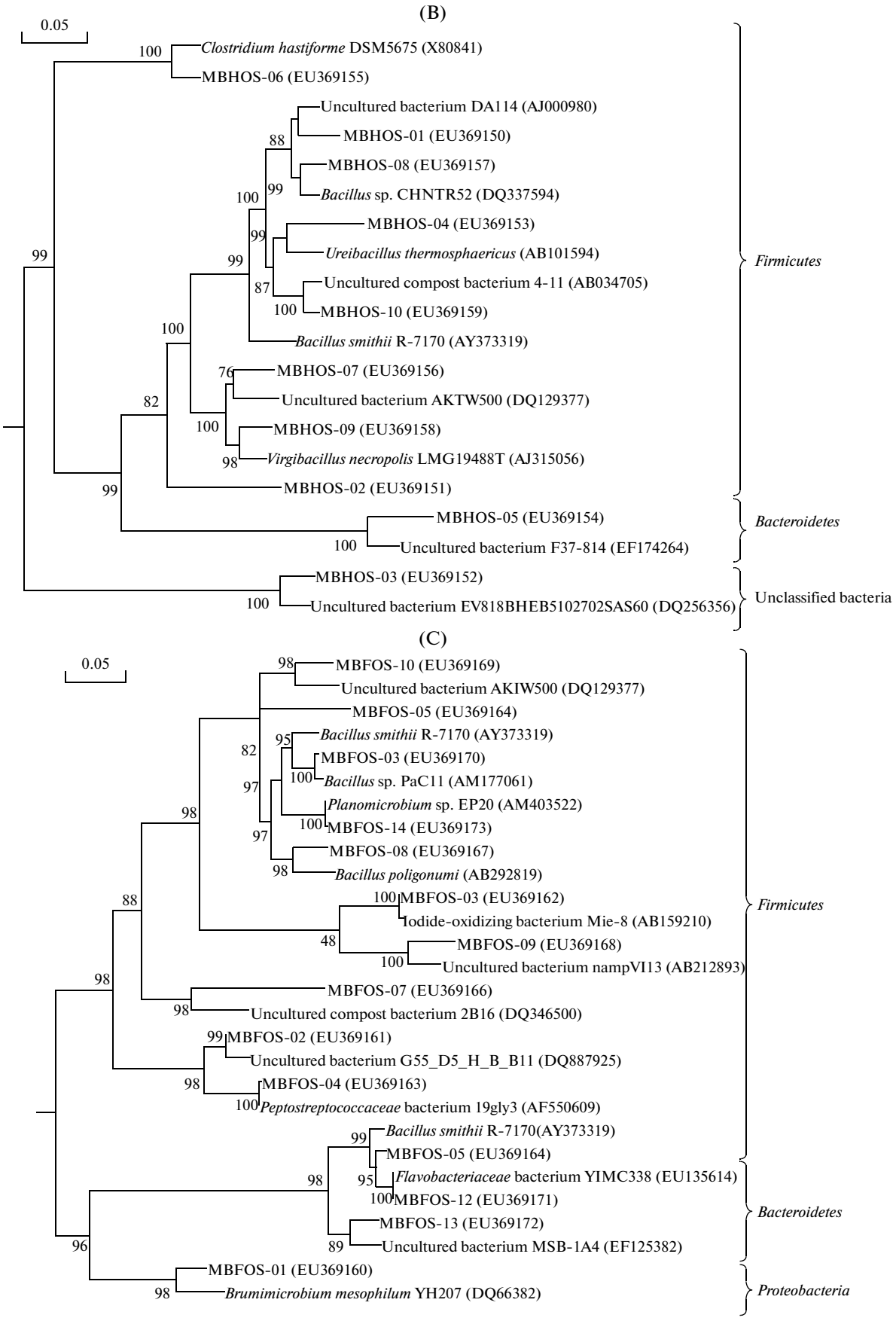
(B)
0.05
Clostridium hastiforme DSM5675 (X80841)
MBHOS-06 (EU369155)
Uncultured bacterium DA114 (AJ000980)
MBHOS-01 (EU369150)
MBHOS-08 (EU369157)
Bacillus sp. CHNTR52 (DQ337594)
MBHOS-04 (EU369153)
Ureibacillus thermosphaericus (AB101594)
Uncultured compost bacterium 4-11 (AB034705)
MBHOS-10 (EU369159)
Bacillus smithii R-7170 (AY373319)
MBHOS-07 (EU369156)
Uncultured bacterium AKTW500 (DQ129377)
MBHOS-09 (EU369158)
Virgibacillus necropolis LMG19488T (AJ315056)
MBHOS-02 (EU369151)
MBHOS-05 (EU369154)
Uncultured bacterium F37-814 (EF174264)
MBHOS-03 (EU369152)
Uncultured bacterium EV818BHEB5102702SAS60 (DQ256356)
Firmicutes
Bacteroidetes
Unclassified bacteria
(C)
0.05
MBFOS-10 (EU369169)
Uncultured bacterium AKIW500 (DQ129377)
MBFOS-05 (EU369164)
Bacillus smithii R-7170 (AY373319)
MBFOS-03 (EU369170)
Bacillus sp. PaC11 (AM177061)
Planomicrobium sp. EP20 (AM403522)
MBFOS-14 (EU369173)
MBFOS-08 (EU369167)
Bacillus poligonumi (AB292819)
MBFOS-03 (EU369162)
Iodide-oxidizing bacterium Mie-8 (AB159210)
MBFOS-09 (EU369168)
Uncultured bacterium nampVI13 (AB212893)
MBFOS-07 (EU369166)
Uncultured compost bacterium 2B16 (DQ346500)
MBFOS-02 (EU369161)
Uncultured bacterium G55_D5_H_B_B11 (DQ887925)
MBFOS-04 (EU369163)
Peptostreptococcaceae bacterium 19gly3 (AF550609)
Bacillus smithii R-7170(AY373319)
MBFOS-05 (EU369164)
Flavobacteriaceae bacterium YIMC338 (EU135614)
MBFOS-12 (EU369171)
MBFOS-13 (EU369172)
Uncultured bacterium MSB-1A4 (EF125382)
MBFOS-01 (EU369160)
Brumimicrobium mesophilum YH207 (DQ66382)
Firmicutes
Bacteroidetes
Proteobacteria

Fig. 3. Contd.

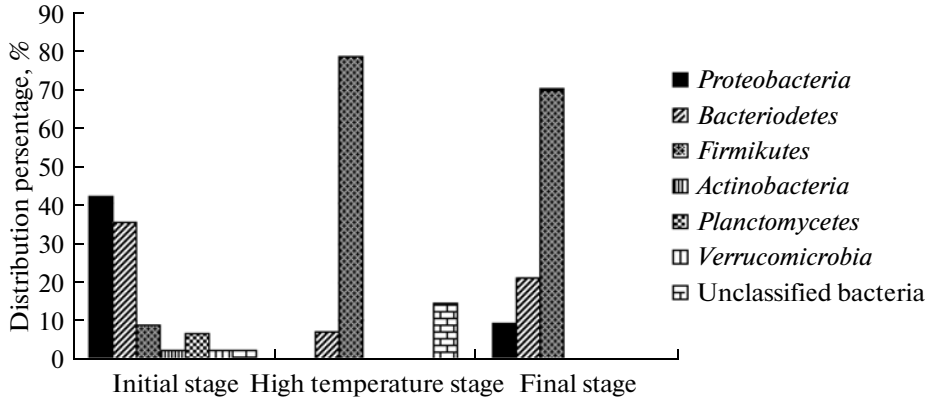

Fig. 4. Comparison of bacterial distribution by 16S rDNA sequence analysis of unculturable bacteria in oyster shells at three stages. Percentage of microcosm in each of the three stages is shown.

ature stage were assumed to be capable of producing chitinase and degrading oyster shell. In some cases, high temperature (70°C) is optimum for chitinase activity [30]. The study of uncultured chitin-degrading bacteria and their chitinases will provide a better understanding of chitin degradation in the sea [16] as well as in the storage conditions of oyster shells.

In conclusion, metagenomic analyses revealed that bacteria belonging to the phylum *Firmicutes* were predominant in the high temperature and final stages. These bacteria apparently survive in high temperature conditions and may play an important role in biodegradation of oyster shells. Further research needs to be carried out to identify chitinase encoding genes from bacteria identified by metagenomic analyses that can degrade oyster shell for industrial use.

ACKNOWLEDGMENTS

This research was financially supported by the Ministry of Education, Science Technology (MEST) and Korea Institute for Advancement of Technology (KIAT) through the Human Resource Training Project for Regional Innovation. Shah Md. Asraful Islam is supported by scholarships from the BK21 Program, Ministry of Education and Human Resources Development, Republic of Korea.

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