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Bacterial Communities in the Initial Stage of Marine Biofilm Formation on Artificial Surfaces

Jin-Woo Lee[1], Ji-Hyun Nam[1], Yang-Hoon Kim[1], Kyu-Ho Lee[2], and Dong-Hun Lee[1]*

[1]*Department of Microbiology, Chungbuk National University, Chungbuk 361-763, Republic of Korea*
[2]*Department of Environmental Science, Hankuk University of Foreign Studies, Kyunggi-Do 449-791, Republic of Korea*

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Succession of bacterial communities during the first 36 h of biofilm formation in coastal water was investigated at 3~15 h intervals. Three kinds of surfaces (i.e., acryl, glass, and steel substratum) were submerged *in situ* at Sacheon harbor, Korea. Biofilms were harvested by scraping the surfaces, and the compositions of bacterial communities were analyzed by terminal restriction fragment length polymorphism (T-RFLP), and cloning and sequencing of 16S rRNA genes. While community structure based on T-RFLP analysis showed slight differences by substratum, dramatic changes were commonly observed for all substrata between 9 and 24 h. Identification of major populations by 16S rRNA gene sequences indicated that γ-*Proteobacteria* (*Pseudomonas*, *Acinetobacter*, *Alteromonas*, and uncultured γ-*Proteobacteria*) were predominant in the community during 0~9 h, while the ratio of α-*Proteobacteria* (*Loktanella*, *Methylobacterium*, *Pelagibacter*, and uncultured α-*Proteobacteria*) increased 2.6~4.8 folds during 24~36 h of the biofilm formation, emerging as the most predominant group. Previously, α-*Proteobacteria* were recognized as the pioneering organisms in marine biofilm formation. However, results of this study, which revealed the bacterial succession with finer temporal resolution, indicated some species of γ-*Proteobacteria* were more important as the pioneering population. Measures to control pioneering activities of these species can be useful in prevention of marine biofilm formation.

***Keywords*:** 16S rRNA, bacterial community, marine biofilm, succession, T-RFLP

A bacterial biofilm is defined as surface-associated bacterial communities enclosed in a polymeric matrix (Costerton *et al*., 1999). Biofilm forming or surface colonizing is an important mechanism for survival of marine bacteria in the oligotrophic environment, and provides a more favorable environment (Jefferson, 2004). Marine biofilm is composed of complex communities of microorganisms that interact with each other and their environment. A bacterial biofilm provides microorganisms with important advantages, including i) increased access to nutrients and eliminated waste matter, ii) protection against toxins and antibiotics, iii) maintenance of extracellular enzyme activities, and iv) shelter from predation (Dang and Lovell, 2000).

In some respects, biofilm is applied to human beings in the remediation process of wastewater, degradation of recalcitrant, and aquaculture. In other respects, biofilm formation on heat exchangers, pipelines, ship hulls, and other industrial devices causes serious problems, and consumes large amounts of time and money removing it (Lee *et al*., 2003). Especially, marine biofilm has negative effects on fishery and maritime industry by reducing efficiency in operation of vessels. Thus, antifouling paints or chemical treatments have been used for preventing of marine biofilms (Chambers *et al*., 2006). However, antifouling paint usually contains biocides or toxins and is well known to cause environmental pollution (Takeuchi *et al*., 2004).

Building a biofilm in seawater requires a series of discrete and regulated steps for mature structure (Stoodley *et al*., 2002). First, free-living bacteria in seawater interact with organic and inorganic particles on surface, and form the initial assemblage. Second, primary colonizing bacteria are accumulated in biofilm through growth and reproduction, and modify the surface characteristics of the substratum, rendering it suitable for subsequent colonization by secondary microorganisms. Third, constructed monolayer and other free-living bacteria interact with each other, and construct a primary biofilm community. Finally, a primary bacterial community in biofilm develops into a matured stage through competitive or synergistic interaction among the existing bacteria in biofilm and recruitment of new colonizing bacteria (Dang and Lovell, 2000).

Interestingly, previous studies reported different community compositions when different methods were employed, i.e., culture-dependent or culture-independent methods. The most abundant group isolated by plate culture methods was identified as γ-*Proteobacteria* such as *Pseudoalteromonas*, *Shewanella*, and *Vibrio* (Lee *et al*., 2003). In molecular biological analyses, α-*Proteobacteria* predominated in marine biofilms (Dang and Lovell, 2000; Jones *et al*., 2007). This discrepancy indicates that the species composition of bacterial communities in marine biofilms is largely unclear. Particularly, the initial stage of marine biofilm formation is

* To whom correspondence should be addressed.
(Tel) 82-43-261-3261; (Fax) 82-43-264-9600
(E-mail) donghun@chungbuk.ac.kr

least understood despite its importance in understanding the development of biofilm (Dang and Lovell, 2000; Dang *et al*., 2008). Furthermore, the composition of pioneering bacteria may be also affected by physicochemical properties of solid surfaces (Jones *et al*., 2007). Therefore, further studies on pioneer species, successional changes, and influence of substratum properties are required.

In the present study, we investigated the structure of pioneer communities of marine biofilms developed on three kinds of artificial surfaces (acryl, glass, and steel coupons) submerged in seawater. The composition of bacterial communities involved in pioneering activities during the first 36 h was analyzed by terminal restriction fragment length polymorphism (T-RFLP), and cloning and nucleotide sequencing of 16S rRNA genes at 3∼15 h intervals, which is the finest temporal resolution ever reported for marine biofilms.

Materials and Methods

Sampling of biofilm and seawater

Acryl and glass coupons (170×100×1.5 mm) were pre-cleaned with 2 N HCl and washed with sterilized water (Lee *et al*., 1999). Steel coupons (170×100×1.5 mm) were autoclaved. The three kinds of materials have different surface properties, e.g., hydrophobicity and electronegativity, and were recognized as typical substrata for marine biofilm formation, e.g., acryl and glass for aquariums and steel in vessels. Coupons were arranged on sterilized racks and submerged into seawater at a depth of 2 m next to a pier deck in Sacheon harbor (37°53'N, 128°49'E), located on the eastern shore of Korean Peninsula. The average value of water temperature and pH was 22℃ and 7.5, respectively. A subset of coupons were retrieved at intervals of 3, 9, 24, and 36 h, rinsed with seawater sterilized by filtration, and air-dried for 30 min. The microbial biomass on coupons were scraped by using a sterilized razor and put into 1.5 ml of sterilized microcentrifuge tube. The seawater at the same location was collected into an autoclaved polycarbonate bottle and its bacterial biomass was harvested by using Sterivex-GV filter (Millipore, USA).

DNA extraction

The genomic DNA of biofilm and seawater sample was extracted by using the bead mill homogenization method (Miller *et al*., 1999) and the freezing-thawing protocol (Rochelle *et al*., 1992), respectively. The DNA was purified by the phenol-chloroform extraction method (Sambrook and Russell, 2001). Nucleic acids were harvested by the addition of isopropanol (0.7 volume) and sodium acetate (final concentration of 0.3 M) and centrifugation at 14,000×g at 4℃ for 30 min. The genomic DNA was purified again using QIAamp DNA Micro Kit (QIAGEN, Germany) according to the manufacturer's instructions.

Amplification of 16S rRNA genes

The 16S rRNA genes were amplified by PCR using two universal primers: 27F; 5'-AGAGTTTGATCMTGGCTCAG-3' and 1492R; 5'-GGYTACCTTGTTACGACTT-3', in which the numbers were based on 16S rRNA gene of *E. coli* (Lane, 1991). The forward primer for the analysis of T-RFLP was labeled with 6-carboxyfluorescein at the 5'-end. PCR was carried out with 50 μl of reaction mixture containing 1× PCR buffer (100 mM Tris-HCl, 400 mM KCl, 1.5 mM $MgCl_2$, 500 μg/ml BSA, pH 8.3), 160 μM of dNTPs, 0.3 μM of each primer, 1.5 unit of *Taq* DNA polymerase (Genenmed, Korea), and 100∼150 ng of template DNA. The thermal cycling program was as follows: initial denaturation at 94℃ for 5 min; 35 cycles consisting of 95℃ for 1 min, 49℃ for 1 min, and 72℃ for 2 min; and a final extension step consisting of 72℃ for 10 min. The PCR products were electrophoresed in an agarose gel (1%) and purified with GENE ALL™ PCR purification kit (General Bio-system, Korea).

T-RFLP analysis

Purified PCR products of 16S rRNA gene were digested with 5 unit of restriction endonuclease, *Hha*I (TaKaRa, Japan), for 12 h at 37℃. Terminal restriction fragment (T-RF) patterns were obtained by electrophoresis on an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA). Cluster analysis and multi-dimensional scaling (MDS) analysis of T-RF profiles were achieved by GelCompar II program (Applied Maths, Belgium) and SPSS version 10 software (SPSS Inc., USA), respectively. Relative peak area of T-RF was used for the calculation of Pearson's correlation coefficients and a distance matrix. The T-RFs smaller than 60 bp or larger than 500 bp were excluded from the analysis.

Cloning and sequencing of 16S rRNA gene

The PCR products of 16S rRNA gene were ligated into pGEM-T vector (Promega, USA) and cloned with *E. coli* DH10B according to the manufacturer's instructions. The nucleotide sequences of the cloned 16S rRNA gene were determined using the ABI 3730XL DNA Analyzer (Applied Biosystems, USA), and identified by BLASTN and Classifier services of the National Center for Biotechnology Information (NCBI; http://ncbi.nlm.nih.gov) and Ribosomal Database Project (RDP; http://rdp.cme.msu.edu), respectively.

16S rRNA gene sequences of biofilms and reference strains were aligned with CLUSTAL X (version 1.83) program (Thompson *et al*., 1997). Phylogenetic trees were constructed with the programs of the PHYLIP package version 3.6 (Felsenstein, 2005). Evolutionary distance matrix was generated according to Jukes and Cantor (1969), and phylogenetic tree was inferred using the neighbor-joining method (Saitou and Nei, 1987). Neighbor-joining tree topology was evaluated by bootstrap analysis based on 1,000 resamplings (Felsenstein, 1985).

Results

Cluster analysis of T-RFLP

In order to investigate the change of bacterial communities, T-RF profiles of 16S rRNA genes were analyzed. As shown in Fig. 1, T-RFLP analysis revealed that the community structure of free-living bacteria was different from the microbial communities of biofilms. Pearson correlation coefficient calculated from the T-RF profiles of seawater and biofilm samples was 0.10. The samples taken after 24 h were assembled into a single cluster, with the exception of a glass sample retrieved at 36 h. Pearson correlation coeffi-

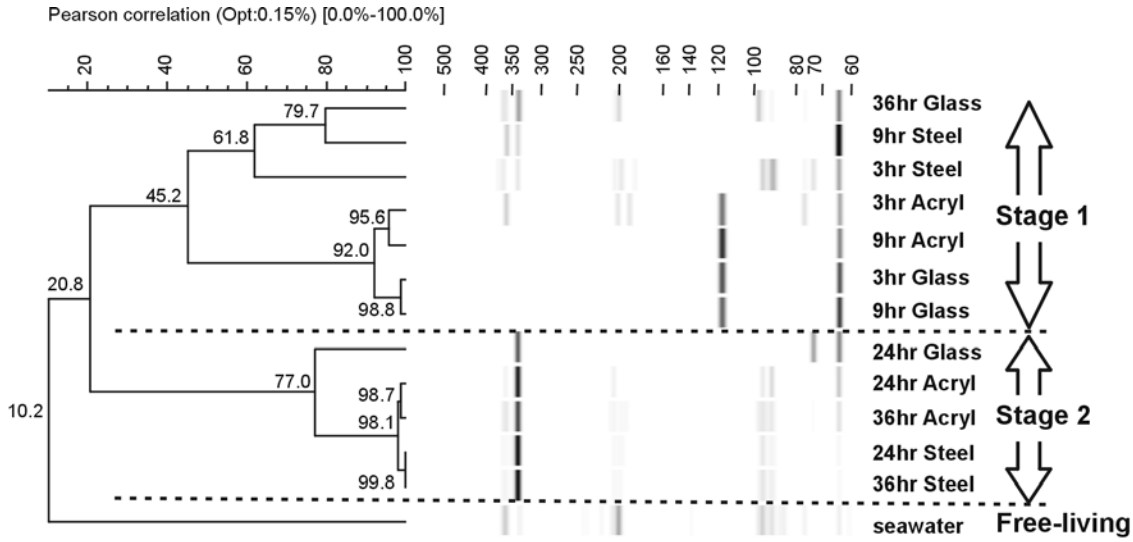

Fig. 1. Cluster analysis of *Hha*I T-RF profiles of marine biofilms on three artificial surfaces. The dendrogram was constructed by Pearson's correlation coefficient and UPGMA method. Numbers on the branch represent the similarity value of the samples belonging to the same cluster.

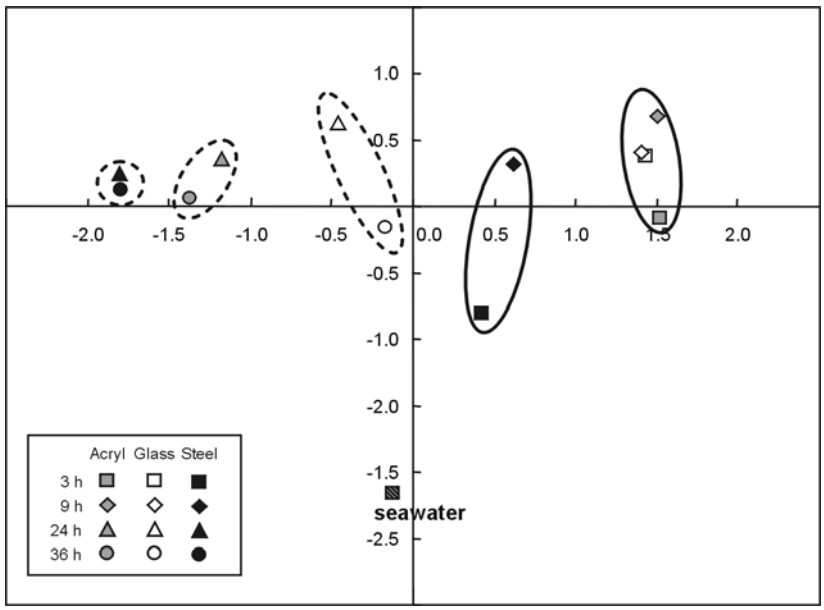

Fig. 2. MDS analysis of T-RF profiles of marine biofilms. MDS plot shows the change of bacterial communities of biofilm. The full- and dot-lined circles indicate the clusters of Stage 1 (0∼9 h) and Stage 2 (12∼36 h), respectively.

cient for these samples was larger than 0.77. Therefore, the T-RF profiling distinguished the bacterial populations in biofilms into two distinctive stages, i.e., Stage 1 and Stage 2. The clusters of Stage 1 and Stage 2 consisted of the samples taken at 3∼9 h and 24∼36 h, respectively. Especially, acryl and glass samples of Stage 1 showed a high coefficient of correlation, which was larger than 0.92.

T-RF profiles of Stage 1 and Stage 2 were also characterized by a distinct band. The bands of 118∼122 bp were observed as one characteristic band of Stage 1 with the exception of the steel samples. However, T-RFs of 90∼100 bp and 340∼350 bp were major bands of Stage 2. The band of 61∼66 bp was detected on the lanes of all samples, and revealed strong intensities in Stage 1.

MDS analysis of T-RFLP

MDS analysis based on T-RF profiles generated from the biofilms showed a typical succession of the bacterial populations (Fig. 2). Bacterial communities of Stage 1 and Stage 2 were clearly separated by Y-axis, and the free-living bacteria of seawater appeared on the bottom area of Y-axis. T-RF profiles of acryl and glass coupons taken at 3 and 9 h

revealed a single cluster, in which distance value was less than 0.77, while steel samples made another cluster. The late-stage clusters of acryl, glass, and steel samples retrieved at 24 and 36 h were different from each other, and distance values of three surfaces were 0.35, 0.88, and 0.12, respectively.

The cluster analysis and MDS analysis of T-RF profiles demonstrated that the structure of bacterial communities of biofilms developed from Stage 1 to Stage 2. The pioneering bacterial communities were established within 3 to 9 h, and subsequent successions were observed.

16S rRNA gene clone library analysis

In order to identify the major bacterial populations forming biofilms on hydrophobic and hydrophilic surfaces, 16S rRNA sequences of 65 and 71 clones were determined for acryl and steel samples, respectively. The nucleotide sequences obtained from the acryl samples were assigned to 6 different phyla including α-*Proteobacteria*, β-*Proteobacteria*, γ-*Proteobacteria*, *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*. The members of γ-*Proteobacteria* were especially predominant in Stage 1, as they occupied 31~53% of the clone libraries (Fig. 3). However, γ-*Proteobacteria* decreased gradually to 28% during the development of biofilm. Most of clones affiliated with γ-*Proteobacteria* were divided into 4 sub-clusters of Stage 1, which contained representative strains of *Acinetobacter junii* (X81664), *Pseudomonas synxantha* (AF267911), *Nevskia ramose* (AJ001010), and *Terrahaemophilus aromaticivorans* (AB098612) (Fig. 4). A small cluster composed of 4 clones related with *Oceanobacter kriegii* (AB006767) and *Colwellia psychrerythraea* (AF001375) was the cluster of Stage 2. *Pseudomonas* (25% at 9 h), *Acinetobacter* (16% at 3 h), and unknown group of γ-*Proteobacteria* (26% at 3 h) were the largest components of γ-*Proteobacteria*.

On the other hand, α-*Proteobacteria* was an important group of Stage 2. The proportion of α-*Proteobacteria* increased from 11% of the sample taken at 3 h to 44~50% during 24~36 h. Six clones of Stage 1 showed a close relationship with the strains of *Aurantimonas altamirensis* (DQ372921) or *Bradyrhizobium japonicum* (U69638). However, the ratio of these genera decreased and *Loktanella* and *Methylobacte-*

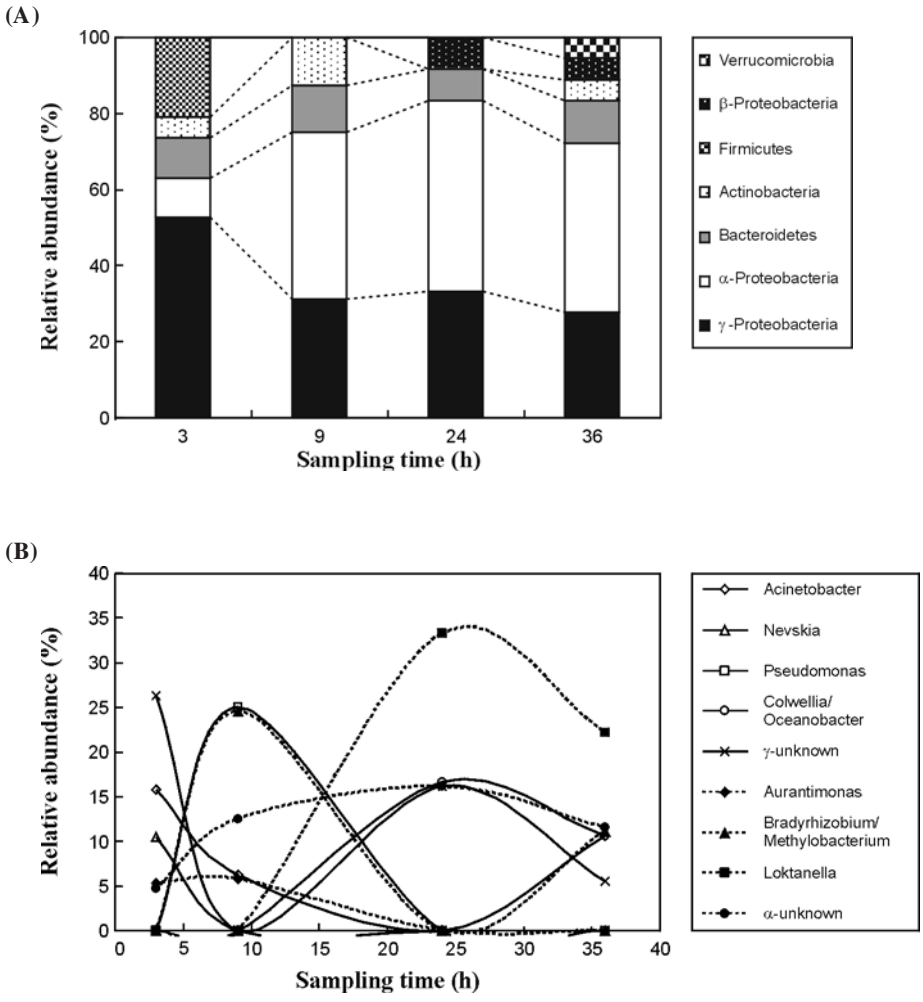

Fig. 3. Bacterial community structure of marine biofilms developed on acryl surface. The compositions of major phylum (A) and major genus belonging to α- and γ-*Proteobacteria* (B) are shown in the graph. The relative abundance was calculated as follows: $(n/N) \times 100$, where n is the number of clones belonging to the same phylum or genus and N is the total number of clones in a library.

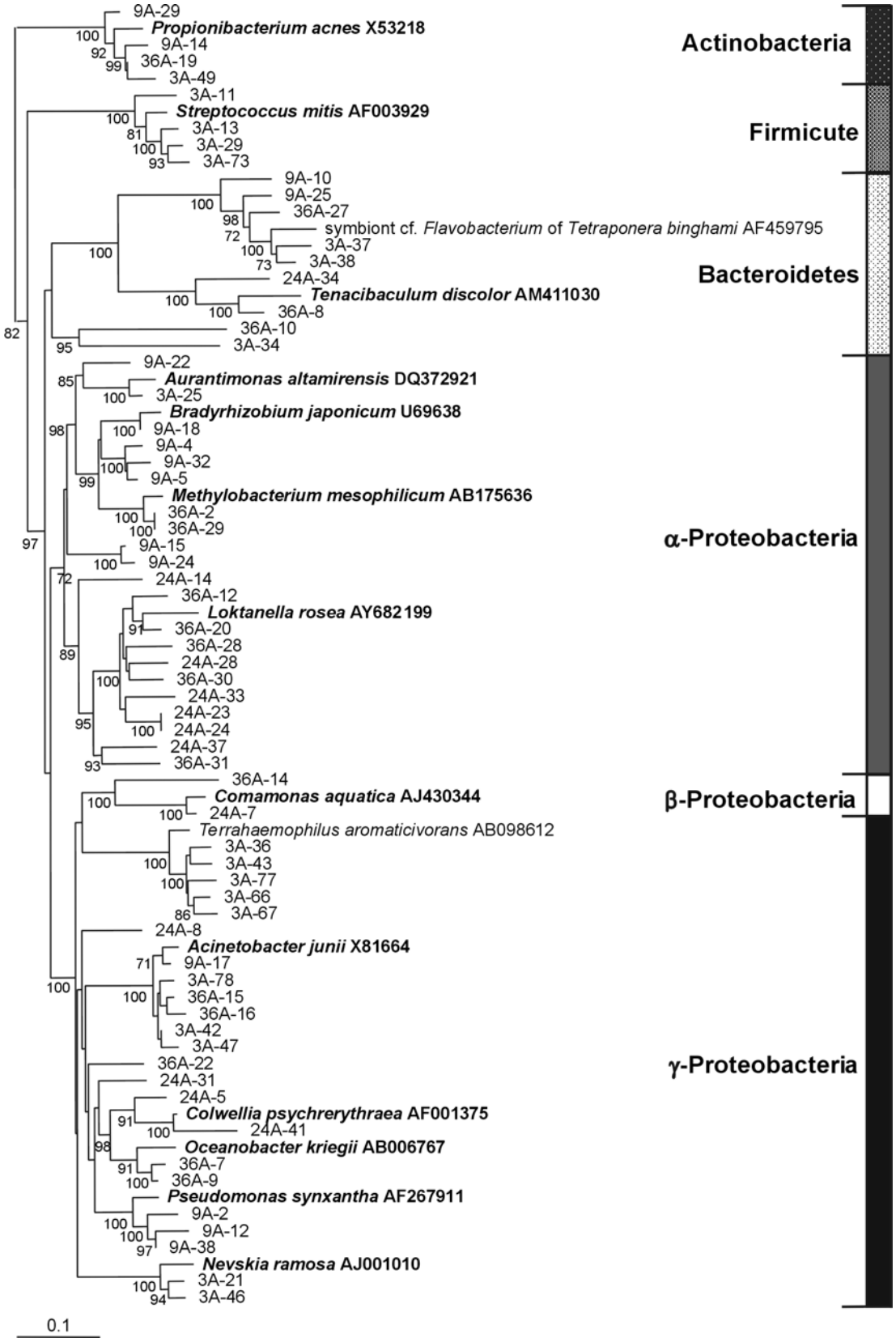

Fig. 4. Phylogenetic tree based on the partial sequences of 16S rRNA gene identified from the marine biofilms on acryl surface. The tree was constructed by using the neighbor-joining method and the sequences of *Aquifex pyrohilus* (M83548) as an outgroup. The names of type strains are bold-faced. The bootstrap values above 70% are shown at the internal nodes.

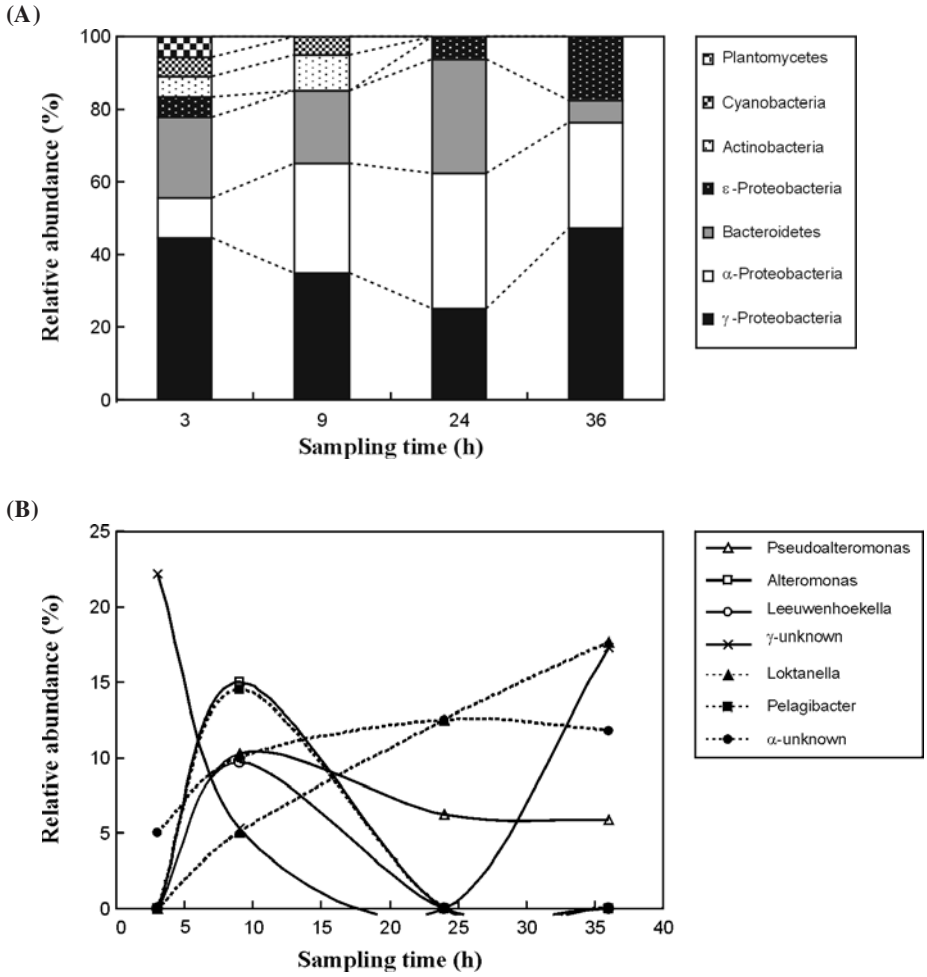

Fig. 5. Bacterial community structure of marine biofilms developed on steel surface. The compositions of major phylum (A) and major genus belonging to α- and γ-*Proteobacteria* (B) are shown in the graph. The relative abundance was calculated as follows: $(n/N) \times 100$, where n is the number of clones belonging to the same phylum or genus and N is the total number of clones in a library.

rium emerged to be major populations in Stage 2. Clones related with the strains of *Loktanella rosea* (AY682199) and *Methylobacterium mesophilicum* (AB175636) increased to 22-33% and 11% in Stage 2, respectively.

In the library of 16S rRNA genes amplified from biofilm samples on steel coupons, 7 different phyla were observed while γ-*Proteobacteria*, α-*Proteobacteria*, and *Bacteroidetes* being major groups (Fig. 5). Dynamics of α- and γ-*Proteobacteria* were similar for biofilms on acryl. The proportions of *α-proteobacteria* increased from 11% to 38%, while γ-*Proteobacteria* decreased from 44% to 25% for 24 h. *Loktanella* (12∼18%) was the same largest population in biofilms on steel coupons in Stage 2 as acryl coupons. However, populations at 3 h showed relatively even distribution of bacterial genera, and a predominant genus was not observed.

16S rRNA gene sequences identified from the steel surface showed relatively low similarity values (87∼99%) with entries in GenBank or RDP databases, and they were distributed over the phylogenetic tree irrespective of developmental stage of biofilms (Fig. 6). Clones related with *Loktanella* (18% at 36 h), *Arcobacter* (17% at 36 h), *Pelagibacter* (15% at 9 h), *Alteromonas* (15% at 9 h), *Pseudoalteromonas* (10% at 9 h), and unknown γ-*Proteobacteria* (22% at 3 h) were identified as members of large clusters.

Discussion

Previous studies reported that biofilm formation was initiated by attachment of specific groups of free-living bacteria in seawater (Dang and Lovell, 2000; Jones *et al*., 2007). This view was also supported by results from this study. The compositions of bacterial communities in biofilms sampled during 3∼36 h period clearly differed from that of the seawater in which the substrata of the biofilms were submerged. This difference can be explained by the hypothesis that pioneer populations are generally present in seawater with low abundances but have a rapid growth on substrata surfaces to make detectable differences in community composition. In addition, the continuous process of attachment of cells of the pioneering population present in seawater might enrich their biomass on the substrata surfaces.

Results in this study also demonstrated that the composi-

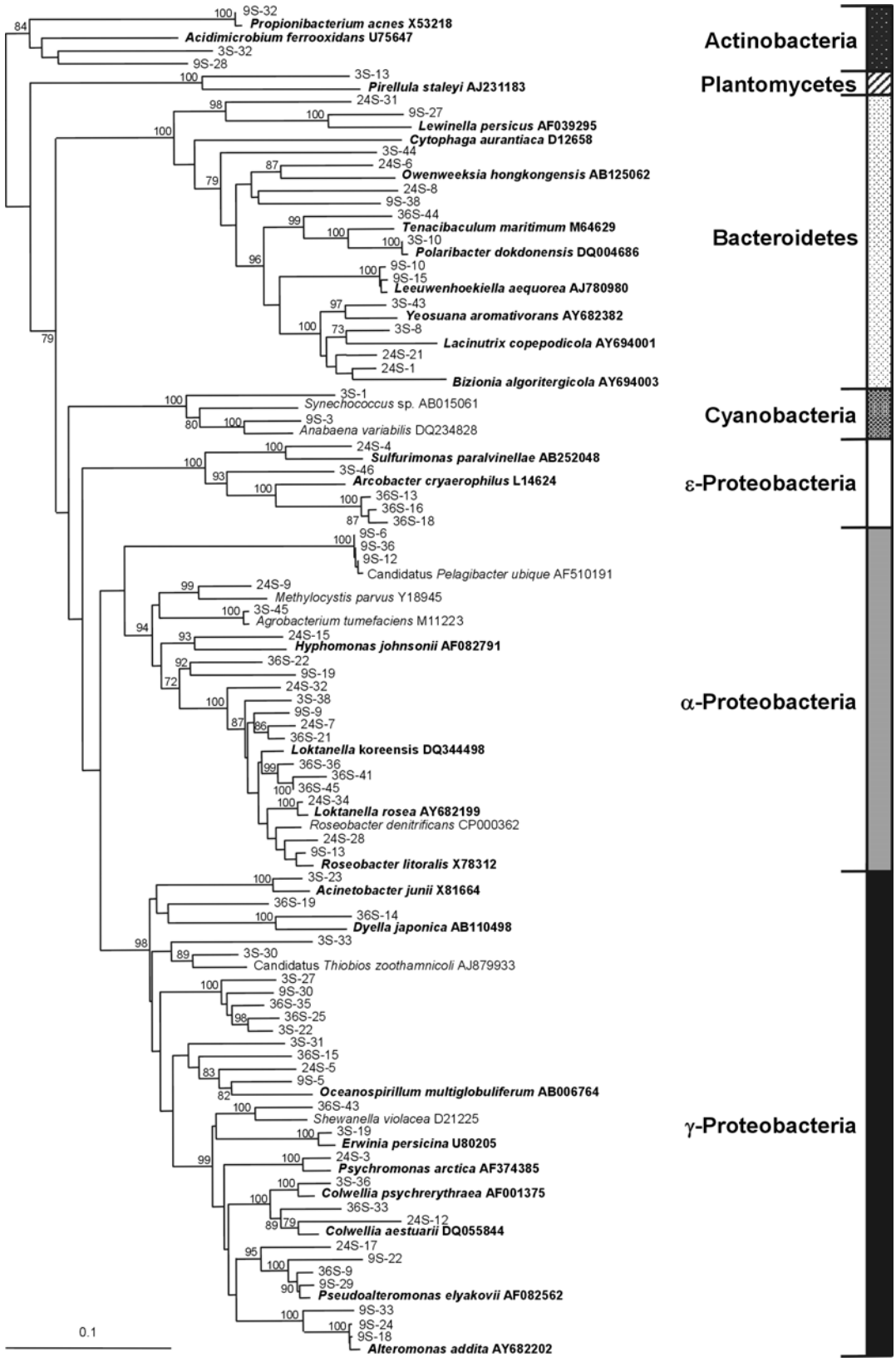

Fig. 6. Phylogenetic tree based on the partial sequences of 16S rRNA gene identified from the marine biofilms on steel surface. The tree was constructed by using the neighbor-joining method and the sequences of *Aquifex pyrohilus* (M83548) as an outgroup. The names of type strains are bold-faced. The bootstrap values above 70% are shown at the internal nodes.

tion of pioneering populations of bacteria developing on marine biofilms varies with both time course and surface type. As shown in Fig. 1 and 2, the bacterial communities of biofilm were clearly divided into two stages, i.e., Stage 1 during 0∼9 h and Stage 2 during 12∼36 h. Succession of bacterial community in biofilms is a well-known fact (Martiny *et al*., 2003; Lyautey *et al*., 2005; Siboni *et al*., 2007), and the mechanism of the succession was characterized as the sequence of pioneer-driven accumulation of biomass followed by enrichment of other groups (Dang and Lovell, 2000; Jones *et al*., 2007). However, the exact time frame for settlement of pioneer groups and subsequent recruitment of other groups were not known because previous studies on succession in marine biofilms were performed at >1-day intervals (Jones *et al*., 2007; Dang *et al*., 2008). Our result revealed that the pioneer community in marine biofilms settled within 3∼9 h and developed into later stages after 9 h of biofilm formation.

Related to our finding on the finer temporal scale of pioneering activity was the predominance of γ-*Proteobacteria* in pioneer community. This result may seemingly contradict to the previous view on major pioneer groups, i.e., α-*Proteobacteria* (Dang and Lovell, 2000; Dang and Lovell, 2002; Jones *et al*., 2007). However, the disagreement was due to difference in time frame defining pioneer community. Like previous reports, α-*Proteobacteria* predominated after 12 h (or a day) of biofilm formation. Predominance by γ-*Proteobacteria* during Stage 1 indicates that some species of γ-*Proteobacteria* are genuine pioneers of marine biofilm while α-*Proteobacteria* communities are secondary flora following these pioneers.

We also observed that marine biofilm communities were different as biofilms developed on different kinds of surfaces. MDS analysis of T-RFLP profiles showed distinct clusters of three surfaces, and the variation of communities was different from each other. Major genera identified from the 16S rRNA libraries of acryl and steel biofilms were also different from each other, with the exception of *Loktanella*. Embedding of bacterial cells in biofilm depends on several biotic and abiotic factors: cell-cell interaction, ecological effects, and surface properties (McLean *et al*., 2005; Webster and Negri, 2006). Physicochemical property of an artificial surface may be characterized by hydrophobicity, surface free energy, and electricity. It is known that microorganisms attach more rapidly and build more biofilms on a hydrophobic and nonpolar surface than hydrophilic materials (Cerca *et al*., 2005). Therefore, the evenness of the bacterial community in the biofilm samples taken at 3 h may indicate broadness of taxonomic spectrum of bacteria recruited on the hydrophilic surface of steel coupons.

Pioneering activity of marine bacteria is the starting point of the biofouling, which causes various nuisances in maritime activities and industries. The marine biofilms at their initial stage recruits other groups of bacteria and, in turn, various invertebrate fauna and flora. Therefore, studies on the dynamics of pioneering bacterial communities can provide information particularly useful for formulation of novel cost-effective measures for biofouling prevention.

Acknowledgements

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