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Isolation and Identification of Biofilm-Forming Marine Bacteria on Glass Surfaces in Dae-Ho Dike, Korea

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Bacterial strains were isolated from biofilms formed on glass slides submerged in seawater in Dae-Ho Dike. Eight strains showing fast attaching ability were selected and identified. Their exopolysaccharide (EPS)-producing ability and EPS properties were characterized. Based on Microlog System, 4 among the 8 strains were identified as *Micrococcus luteus* and the rest were *Bacillus thuringiensis, Bacillus megaterium, Staphylococcus saprophyticus* and *Agrobacterium vitis*. *A. vitis* was reidentified as *Sulfitobacter pontiacus* based on 16S rDNA sequence data. The amount of water-soluble EPS produced by the 8 strains ranged from 0.114 to 1.329 $g \cdot \Gamma^{-1}$ and the productivity was negatively correlated with the cell biomass. The molecular weight of the produced EPS ranged from 0.38 to 25.19×10^4 Da. Glucose and galactose were ubiquitous sugar components. Mannose, ribose, and xylose were also major sugar components. The molecular weight and composition of the EPS showed strain-specific variation.

***Key words*:** biofilm, marine bacteria, exopolysaccharide

Most marine environments contain only dilute substances that can be used for metabolism and growth. In contrast, natural surfaces tend to collect and concentrate nutrients by charge-charge or hydrophobic interactions (Beveridge *et al*., 1997). Bacterial colonization on abiotic materials such as suspended particles, metal surfaces and concrete or on biotic surfaces was thought to be one of the microbial survival strategies because it provides microorganisms with important advantages, including i) increased access to nutrients, ii) protection against toxins and antibiotics, iii) maintenance of extracellular enzyme activities and iv) shelter from predation (Dang and Lovell, 2000). For these reasons, surfaces in contact with water are rapidly colonized by bacteria.

Free-living or a planktonic mode of growth of microorganisms is usually observed in laboratory cultures. But this growth mode is infrequent in the natural environment and bacteria may seek out advantageous niches (Beveridge *et al*., 1997). Once planktonic cells meet such surfaces, they attach and eventually develop biofilms through growth and division (Beveridge *et al*., 1997). Although the attachment is initially reversible, it becomes stronger and irreversible with time. Although the chemical and biological processes are poorly understood, it is thought that the early process of biofilm formation occurs through a sequence of processes; i) it begins with the adsorption of organic and inorganic particles on the surface, ii) attachment of pioneer microorganisms, iii) growth and reproduction of primary colonizers, and then iv) maturation of the biofilm matrix (Dang and Lovell, 2000).

During the process of colonization on particular surfaces, bacteria overproduce extracellular polymeric substances (EPS; Geesey and White, 1990). These polymers, especially exopolysaccharide, designated as EPS frequently, are the materials which construct the biofilm matrix, serving as a multipurpose functional element for adhesion, immobilization of cells on the colonized surface, protection, recognition and facilitating spatial arrangement of different species within the biofilm (Allison *et al*., 1998).

In some respects, biofilm formation applies to human beings in the remediation process of wastewater, degradation of recalcitrant, aquaculture, etc. In other respects, biofilm processed on heat exchangers, pipelines, ship surfaces and other industrial devices causes serious problems and consumes large amounts of time and money removing it. Also, biofilm formed on implanted materials has been related to microbial diseases (Costerton *et al*., 1987). Therefore, control of biofilm formation has been an important topic of interest to date (Allison *et al*., 1998; Geesey *et al*. (eds.) 1994; Lindberg *et al*., 2001; Little, 1984). Most of the related studies concentrated on the process of biofilm succession or formation of extracellular polymers. There are a few cases estimating EPS-produc-

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ing activity from isolated bacteria.

In this study we performed i) isolation of bacteria from the initial stage of biofilm that formed on glass slides that had been exposed to natural seawater, ii) identification of selected bacterial strains, iii) estimation of EPS-producing ability and iv) characterization of the EPS produced by selected strains.

Materials and Methods

Preparation of biofilm on glass slide coupons

Glass slide coupons (25 mm in width, 75 mm in height, and 1 mm in thickness) were pre-cleaned with 1 N HCl and washed with filtrated Milli-Q water (Lee *et al*., 1999). The treated glass slides were held on an acrylic holder and exposed to natural seawater near Dae-Ho Dike in Daesan, Chungcheong-Nam Province, Korea. The coupons were periodically sampled at intervals up to 72 h. Afterwards, glass slides were rinsed with filtrated triple distilled water (TDW) to remove the attached bacteria. During the experiment the average water temperature was about 8.7°C (Lee *et al*., 1999).

Isolation of attached bacteria

The surfaces of the glass slides were scraped and suspended in 10 ml of filter-sterilized aged seawater using a cell scrapper (Corning). A dispersed and diluted sample was inoculated on ZoBell 2216e agar medium (peptone 5 g, yeast extract 1 g, $FePO_4$ 0.01 g, agar 15 g, distilled water 250 ml, aged seawater 750 ml, pH 7.2) and seawater based R2A agar medium (proteose peptone 0.5 g, yeast extract 0.5 g, casamino acid 0.5 g, glucose 0.5 g, soluble starch 0.5 g, sodium pyruvate 0.3 g, K_2HPO_4 0.3 g, $MgSO_4 \cdot 7H_2O$ 0.05 g, agar 15 g, distilled water 500 ml, aged seawater 500 ml, pH 7.2), respectively. The agar plates were incubated at 25°C for 3 days. Bacterial colonies showing different morphology were selected and purified on ZoBell 2216e agar plates. Among the isolated strains, bacterial strains showing superior film-forming ability were selected as follows. Pure cultured cell suspensions in filtered aged seawater (ASW) were incubated on a glass slide for 24 h in a 50 ml conical tube. Each slide was washed with TDW for washing out adsorbed bacteria. The number of attached bacteria on a glass slide was enumerated under a light microscope (Axioplan, Carl Zeiss) after staining with 0.3% methylene blue.

Biochemical characterization of attached bacteria

Biochemical characterization of selected bacterial strains was done with the Microlog System (Biolog Corp.). Morphology, Grams staining characteristics, production of oxidase and catalase were tested. Characterization was performed using the Biolog GN/GP Microplate Identification System, which was based on a single substrate utilization pattern.

Identification of attached bacteria based on 16S rDNA sequences

Selected strains were identified based on the partial sequence of 16S rDNA. The genomic DNA of each strain was purified with a Wizard Genomic DNA Purification Kit (Promega) after incubation in ZoBell 2216e broth for 1 day at 30°C. The primer sets 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1518R (5'-AAG GAG GTG ATC CAN CCR CA-3') were used for 16S rDNA amplification from the prepared genomic DNA template (Giovannoni, 1991). The PCR condition was as follows: 0.2 μM of primers, 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 0.1% of BSA (Boehringer Mannheim), 10 ng of template DNA, and 2.5 U of *Taq* DNA polymerase (Promega) prepared in a final 50 μl reaction mixture; then incubation at 95°C for 5 min, denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 2 min, 35 times and finally a 7 min extension at 72°C. The PCR products were purified using Wizard PCR Preps DNA Purification Kit (Promega) after confirming the size (1.5 kb) on agarose gel.

About 1,000 bases partial sequences of 16S rDNA were determined by ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems) and an automatic sequence analyzer system (model 377, Applied Biosystems). The primer set used for sequence determination consisted of 518r (5'-GTA TTA CCG CGG CTG CTG-3') and 338f (5'-ACT CCT ACG GGA GGC AGC-3').

Analyses of the 16S rDNA sequences were performed using SIMILARITY RANK from RDP (Maidak *et al*., 1999) and BLAST (Altschul *et al*., 1997). The partial 16S rDNA sequences (approximately 1,100 bp equivalent to *Escherichia coli* numbering 28 to 1,102) were manually aligned to pre-aligned 16S rDNA data from the RDP. The PHYLIP, ver 3.57c, was used to further analyze the sequence data (Felsenstein, 1993). DNADIST, performed with the Jukes-Cantor option, was employed to determine sequence similarities. FITCH was used to create a phylogenetic tree. The 16S rDNA sequences of this study are available through GenBank under accession numbers AY159882 to AY159889.

EPS producing ability

Bacterial strains grown on YMG agar medium (glucose 10 g, yeast extract 3 g, malt extract 3 g, peptone 5 g, distilled water 500 ml, ASW 500 ml, pH 7.0) were inoculated in YMG broth and pre-incubated at 25°C for 24 h. 200 μl of culture broth was inoculated into 50 ml of YMG broth and incubated at 25°C for 5 days at 120 rpm. Elimination of cells was followed by centrifugation (10,000×*g* for 20 min). The culture broth was mixed with 3 volumes of ethanol and after standing at 4°C for 24 h, it was cen-

trifuged (10,000×*g*, 4°C, 20 min). The weight of the precipitated EPS was measured after drying at 80°C for 3 days.

Characterization of EPS

To estimate the molecular weight of the polysaccharide, gel filtration chromatography was conducted with a Sephadex G-200 (Pharmacia, Uppsala, Sweden) column (1.5×60 cm). Samples (1 mg in 1 ml) were eluted with 0.4 M NaCl with a flow rate of 10 ml·h^{-1}. Fractions of 0.5 ml were collected and the total sugar content in each fraction was determined. The amount of total carbohydrate was measured by the method of anthrone-sulfuric acid (Daniels *et al*. 1994), using glucose as the standard. Blue dextran (Sigma, USA, MW 2,000 kDa, 464 kDa, 66.9 kDa and 37.5 kDa) was used as the molecular weight marker. Monosaccharide composition of the EPS was analyzed by TLC (Schaal, 1985) and samples were prepared by the method of Staneck and Roberts (1974). Polysaccharide samples (50 mg) were hydrolyzed with 4 ml of 2 N H_2SO_4 at 100°C for 2 h and were neutralized by adding 0.5g of $Ba(OH)_2$. After centrifugation (17,000×g, 4°C, 10 min), the supernatant was filtered through a 0.22 μm membrane filter, concentrated by evaporating under reduced pressure, and placed on TLC plates. Glucose, mannose, rhamnose, galactose, fructose, xylose, arabinose, fucose, raffinose, ribose and glucuronic acid were used as standards.

Results and Discussion

Isolation, selection and biochemical characterization of biofilm-forming bacteria

The bacterial numbers on glass slides exposed to seawater were increased with exposure times and reached 3.74×10^5 cells/mm^2/after 72 h and the increase rate was 4530/mm^{2}h (Lee *et al*., 1999). From these coupons, a total 139 strains grown on ZoBell agar and R2A agar medium were isolated. Among these strains, 8 strains, 98TH11316, 98TH11317, 98TH11318, 98TH11319, 98TH11320 98TH11321, 98TH11322 and 98TH11323 were selected based on their ability to attach to the glass slide surface (Fig. 1). The microcolonies that formed were divided into 3 different groups as shown in Figure. 1. Strains 98TH11318, 98TH11319, 98TH11321 and 98TH11322 showed a similar pattern. They aggregated and made small colonies on the surface. Strains 98TH11320 and 98TH11323 also made microcolonies but the shape was different from the first group. In contrast, strains 98TH11316 and 98TH11317 spread on the surface and did not make an aggregate or microcolony.

Biochemical characteristics of selected strains

Seven strains from the group of 8 selected strains were found to be Gram positive bacteria. Selected bacterial strains used 44 to 64% of the tested substrates (Table 1). Among the substrate groups, amino acids and aromatic chemicals were widely used by the Gram positive bacteria. Strain 98TH11323 distinctively used phosphorylated compounds but other strains could not use these compounds. Strain 98TH11323 could also use polymers. Strains 98TH11318, 98TH11319, 98TH11321 and 98TH11322 used carboxylic acids at comparatively high rates. The utilizing ability of carboxylic acids and 2,3-butanediol distinguished these strains from others. Strain 98TH11317 exhibited a lower capability of using polymers. Ross *et al*. (2001) reported that groundwater bacteria in the planktonic state could not use aromatic chemicals, but all our Gram-positive bacteria used them well. Contrary to the Gram-positive strains, the Gram negative strain used carbohydrates (24/28) well but did not utilize aromatic chemicals. Strain 98TH11320 showed the utilizing ability of a brominated chemical, which was poorly utilized by natural bacterial strains (Table 2).

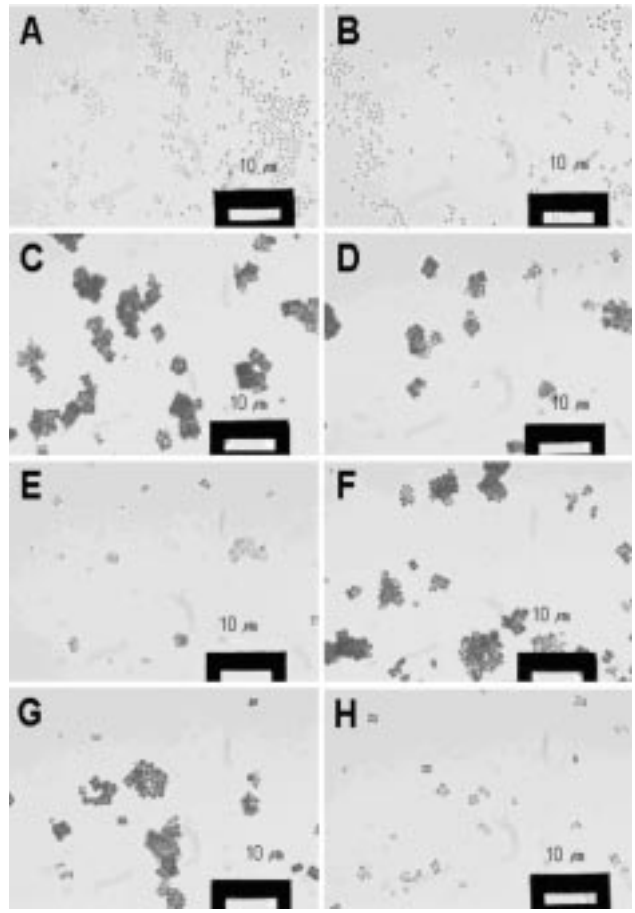

Fig. 1. Photographs of selected bacterial strains grown on the surface of glass slides. A; 98TH11316, B; 98TH11317, C; 98TH11318, D; 98TH11319, E; 98TH11320, F; 98TH11321, G; 98TH11322, H; 98TH11323.

Table 1. Substrate-group utilization of selected biofilm-forming Gram-positive bacteria by Biolog GP microplate

Substrate Groups and Numbers	98TH11316	98TH11317	98TH11318	98TH11319	98TH11321	98TH11322	98TH11323
Polymers 11	5	3	5	5	5	5	8
Carbohydrates and derivatives 39	24	22	12	14	10	13	14
Methyl esters 3	3	2	3	3	3	2	2
Carboxylic acids 17	7	6	11	11	11	11	6
Amines and amides 3	1	1	1	1	1	1	1
Amino acids and derivatives 8	7	7	6	6	6	7	7
Aromatic chemicals 5	5	5	5	5	4	5	5
Alcohols 2	1	1	2	2	2	2	1
Phosphorylated chemicals 7	0	0	0	0	0	0	7
Total 95	53	42	45	47	42	46	51

Table 2. Substrate-group utilization of biofilm-forming bacterial strain 98TH11320 by Biolog GN microplate

Substrate Groups and Numbers	98TH11320
Polymers 5	2
Carbohydrates and derivatives 28	24
Methyl esters 2	2
Carboxylic acids 24	13
Brominated chemical 1	1
Amines and amides 6	2
Amino acids and derivatives 20	11
Aromatic chemicals 4	1
Alcohols 2	1
Phosphorylated chemicals 3	0
Total 95	61

Identification of selected strains

Based on the Microlog Identification System, Strain 4 (a group showing a high rate of carboxylic acid utilization) were identified as *Micrococcus luteus* with the similarity level of 0.555 to 0.673 (Table 3). Other Gram-positive isolates were identified as *Bacillus megaterium, B. thuringiensis*, and *Staphylococcus saprophyticus*, respectively. These strains were usual members of the biofilm matrix. The Gram negative strain was identified as *Agrobacterium vitis* and showed the highest similarity level of 0.736 (Table 3). The identification results using the 16S rDNA sequence data matched well with the Microlog data for Gram positive strains (the similarities with those of corresponding type strains were more than 99%, Fig. 2). However, the 16S rDNA sequence of the Gram-negative strain showed higher similarities with *Sulfitobacter pontiacus* with a homology of 0.993. The homology of the 16S rDNA sequences between *A. vitis* and strain 98TH11320 was only 86.5%. In fact the Microlog System database did not include *S. pontiacus* in the list. *S. pontiacus* was first isolated from the Black Sea oxic/anoxic interface and identified as a member of α-proteobacteria (Sorokin, 1995). Its role was reoxidation of sulfite from an anoxic deep-water layer to sulfate. González *et al*. (1999) reported that many of the strains belonging to the *Roseobacter* group which include genus *Sulfitoobacte*r cleave the organic sulfur compounds such as DMS, DMSO, etc. The *Roseobacter* group composed 3 to 11% of bacterial clones from sediment, 2 to 24% from plant detritus and up to 26% from seawater (González *et al*. 1999). Thus it can be considered as one of the major groups in marine environments and the habitat is not restricted to the environment of oxic/anoxic interfaces.

EPS producing ability of selected bacterial strains

The dry cell weight of the 8 selected strains ranged from 0.391±0.156 (98TH11320) to 7.096±0.204 (98TH11319) $g \cdot l^{-1}$ and the dry weight of the EPS ranged from 0.114± 0.005 (98TH11321) to 1.329±0.191 (98TH11316) $g \cdot l^{-1}$ after 5 days incubation in YMG broth (Fig. 3). Strain 98TH11316 showed the highest EPS productivity, 2.606 (dried EPS weight/dried cell weight). The amounts of

Table 3. Comparison of the identification results of selected strains by the Microlog Identification System with those of 16S rDNA sequences

Strain	Microlog System (Similarity)	16S rDNA sequence (Similarity)
98TH11316	*Bacillus megaterium* (0.555)	*B. megaterium* (0.996)
98TH11317	*Staphylococcus saprophyticus* (0.673)	*S. saprophyticus* (0.998)
98TH11318	*Micrococcus luteus* (0.646)	*M. luteus* (0.994)
98TH11319	*M. luteus* (0.673)	*M. luteus* (0.997)
98TH11320	*Agrobacterium vitis* (0.736)	*Sulfitobacter pontiacus* (0.993)
98TH11321	*M. luteus* (0.555)	*M. luteus* (0.997)
98TH11322	*M. luteus* (0.555)	*M. luteus* (0.997)
98TH11323	*B. thuringiensis* (0.555)	B. thuringiensis (1.000)

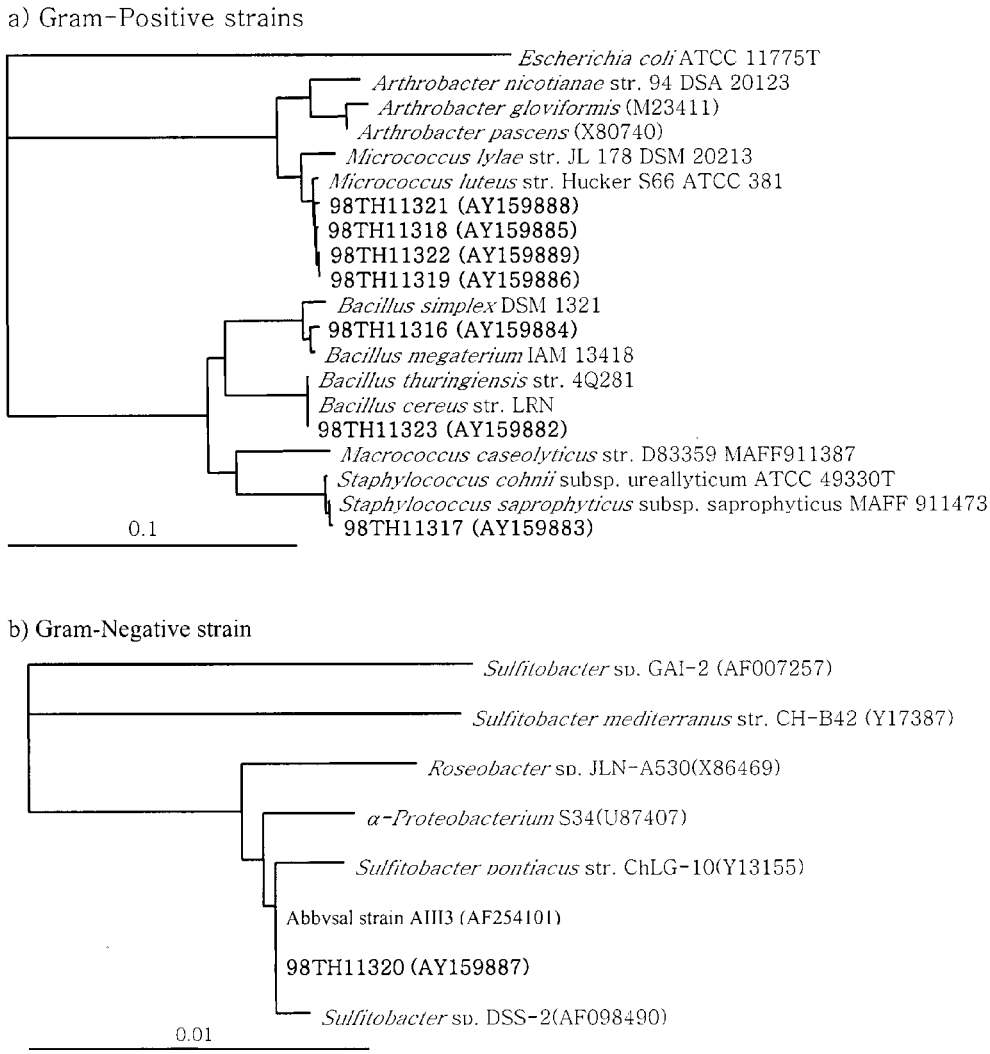

Fig. 2. Phylogenetic trees based on partial 16S rDNA gene sequence data (approx. 1,000 bp) showing the location of the isolated bacteria from glass slides.

EPS produced by the other 7 strains were less than half of the amount produced by strain 98TH11316. The EPS productivity of all selected strains was low compared with other strains (Ko *et al.*, 2000). One notable phenomenon was that strains showing superior cell growth showed lower EPS productivity. The average amount of EPS produced by 5 strains with a cell mass higher than 4.0 $g \cdot l^{-1}$ was 0.213 $g \cdot l^{-1}$. The average amount of EPS produced by the other 3 strains with less than 1.0 $g \cdot l^{-1}$ cell mass was 0.776 $g \cdot l^{-1}$. Consequently, cell growth and EPS productivity is negatively correlated ($r = -0.741$, $p = 0.035$). The difference in EPS productivity between the higher biomass group and the lower biomass group seemed to depend on their ability to use carbohydrate substrates. The lower biomass group utilized this group well but the higher biomass group showed a low utilization rate of this substrate group (Table 1). In this experiment, we measured only the water-soluble fraction of EPS. EPS could be differentiated into two types by its physical state. One is the cell-bound form and the other is a soluble form (Nielsen and Jahn, 1999). Bound-form EPS rather than cell mass seemed to compose a large portion of the centrifugally precipitated materials from high cell growth. Also there was the possibility of a correlation between the EPS form and the carbohydrates used, but it was not further studied. The majority (80%) of the strains showed superior cell growth belonging to *Micrococcus* sp.. From this result and microphotographs (Fig. 1) we assumed that these strains could grow well and exhibit good attaching ability to surfaces by producing cell-bound EPS but they produce a small amount of water-soluble EPS. Strain 98TH11323 showed similar properties with these strains.

Characteristics of EPS

The molecular weight and major sugar component of EPS are summarized in Table 4. All strains contained glucose as one of the major components. EPS from strain 98TH11316 is composed of four major sugar monomers

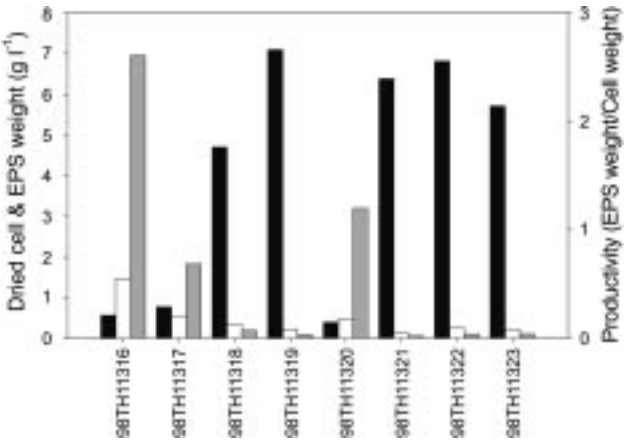

Fig. 3. Comparison of dried cell weight, dried EPS weight, and productivity of EPS by biofilm-forming marine bacteria on glass slides. ■, dry cell weight ($g \cdot l^{-1}$); □, dry EPS weight ($g \cdot l^{-1}$); ■ , productivity.

Table 4. Characteristics of EPS produced by the 8 selected strains

Strain	Molecular Weight of EPS ($\times 10^4$ Da)	Major Sugar Component of EPS
98TH11316	2.10	Glc, Man, Gal, Glc-a
98TH11317	1.20	Glc, Gal
98TH11318	12.72	Glc, Gal, Rib
98TH11319	0.64	Glc, Man, Gal
98TH11320	0.38	Glc, Gal, Xyl
98TH11321	25.19	Glc, Man, Gal
98TH11322	4.96	Glc, Gal, Rib
98TH11323	5.87	Glc, Xyl, Rib

Glc, Glucose; Gal, galactose; Man, mannose; Rib, ribose; Xyl, xylose; Glc-a, glucuronic acid

and it was the only one producing EPS containing glucuronic acid. The major sugar components of paper mill film bacteria were reported as glucose, mannose, galactose, rhamnose, xylose, and ribose. The sugar composition could be changed by the culture medium composition (Lindberg *et al*., 2001). The major sugar components of our strains were similar but the glucuronic acid was rare as a major sugar component. The EPS of strain 98TH11317 was composed of only two kinds of sugar monomers, glucose and galactose, and its molecular weight was 1,200 Da. It was the simplest of the isolated EPSs. The composition and molecular weight of EPS produced by the four *M. luteus* strains were different. Based on the composition they were divided into two groups, one with ribose and the other with mannose, but each had a different molecular weight. The bacterial strains belonging to *M. luteus* showed a higher rRNA sequence similarity (more than 99%) and showed different biochemical properties and produced different EPS in the same cultural condition. In many cases the types of EPS produced has been reported to be specific to a certain genus or species (Lindberg et al., 2001). Our result demonstrated that the characteristics of EPS and biochemical properties of biofilm-forming bacteria might be strain specific.

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