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Culture and Identification of Bacteria from Marine Biofilms

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We isolated and cultured bacteria that inhabited marine biofilms, and identified them by phylogenetic analysis using 16S rDNA sequences. In the marine environment, biofilms cover most subtidal and intertidal solid surfaces such as rocks, ships, loops, marine animals, and algae. The bacteria in most biofilms are embedded in extracellular polymeric substances that comprise mainly of exopolysaccharides. The exopolysaccharides are excreted from multiple bacterial species; therefore, biofilms are a good source for screening exopolysaccharide-producing bacteria. Thirty-one strains were cultured, and a total of 17 unique strains were identified. Phylogenetic analysis using 16S rDNA sequences indicated that the 17 strains belonged to α-Proteobacteria (*Ochrobactrum anthropi*, *Paracoccus carotinifaciens*); γ-Proteobacteria (*Pseudoalteromonas agarovorans*, *P. piscicida*, *Pseudomonas aeruginosa*, *Shewanella baltica*, *Vibrio parahaemolyticus*, *V. pomeroyi*); CFB group bacteria (*Cytophaga latercula*, *Tenacibaculum mesophilum*); high GC, Gram-positive bacteria (*Arthrobacter nicotianae*, *Brevibacterium casei*, *B. epidermidis*, *Tsukamurella inchonensis*); and low GC, Gram-positive bacteria (*Bacillus macroides*, *Staphylococcus haemolyticus*, *S. warneri*).

Key words: biofilm, α-Proteobacteria, γ-Proteobacteria, CFB group bacteria, Gram-positive bacteria

Bacteria can adhere to natural or artificial surfaces and form sessile multicellular communities known as biofilms (Dalton and March, 1998). The natural and artificial surfaces covered by biofilms include cells and tissues of organisms, soils, sediments, pore in glaciers, thermal vent, pipelines, heat exchangers, separation membranes, and filters. In the marine environment, biofilms cover most subtidal and intertidal solid surfaces such as rocks, ships, loops, marine animals, and algae.

The bacteria in most biofilms are embedded in extra cellular polymeric substances (Lawrence *et al.*, 1991) that offer a microniche with stable arrangements and a certain degree of homeostasis (Wimpenny, 2000). These extra cellular polymeric substances can sequester nutrients from the environment as part of a general microbial strategy for survival under oligotrophic conditions (Wolfaardt *et al.*, 1998; Decho, 2000). Extracellular polymeric substances also have the potential to physically prevent access of certain antimicrobial agents by acting as an ion exchanger; thereby restricting diffusion of compounds into the biofilm (Gilbert *et al.*, 1997). In addition, they can provide protection from a variety of environmental stresses such as UV radiation, pH shifts, osmotic shock, and desiccation (Mayer, 1999).

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The composition and characteristics of the extracellular polymeric substances have been identified from studies on certain model organisms such as *Pseudomonas aeruginosa* (Wingender *et al.*, 2001). Extracellular polymeric substances comprise mainly of exopolysaccharides (40-95%), protein (1-60%), nucleic acids (1-10%), and lipids (1-40%) (Davey and O'Toole 2000; Flemming and Wingender 2001). Exopolysaccharides, the main component, are excreted from multiple bacterial species, which make biofilms a good source for screening exopolysaccharide-producing bacteria. The bacterial species forming the biofilm communities are unknown for most natural biofilms, and members of the exopolysaccharide-producing bacteria have hardly been cultured or identified (Davey and O'Toole, 2000).

In this study, we isolated and cultured bacteria that inhabited marine biofilms, and identified them by phylogenetic analysis using 16S rDNA sequences. This is the first step to screening marine bacteria that produce exopolysaccharides - a substance with a promising prospect for industrial and medical use.

Materials and Methods

Sample collection and culture condition

The biofilms were collected from a buoy-type concrete pier in Mokpo Harbor on November 18, 2002. The biofilms were scraped off from 5 different locations, and, in total, 10 ml of biofilm was put into a sterile 50 ml conical

tube. An additional 10 ml of sterilized seawater was added to the conical tube, which was kept in an icebox and transferred to the laboratory. After vortexing the solution for 5 min, 1 ml was diluted in 9 ml of sterilized seawater. After a series of dilutions, 100 μl of diluents was spread on ZoBell 2216e agar plates. The plates were incubated at 25°C for 1 week, and bacterial colonies showing different morphological characteristics were transferred onto fresh ZoBell 2216e agar plates. The purified isolates were then cultured in ZoBell 2216e media and stored at -70°C in a fresh medium that contained 10% (v/v) sterile glycerol.

DNA extraction and PCR amplification

The genomic DNA was extracted from 1 ml of isolate cultured in the ZoBell 2216e buffer using the AccuPrep genomic DNA Extraction kit (Bioneer, Korea). From the genomic DNA, nearly full-length 16S rDNA sequences were amplified by PCR using primers 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1522R (5'-AAG GAG GTT ATC CAN CCR CA-3'). The PCR mixture consisted of 5 μl of 10× PCR buffer (final concentrations: 100 mM KCl, 20 mM Tris-HCl pH 8.0), 2.5 mM of $MgCl_2$, 2.5 mM of each dNTP, 1 μl of each primer, 1 μl of the template DNA, and 5.0 units of *Taq* polymerase (TaKaRa, Japan) for a total volume of 50 μl. The thermal cycling program used was as follows: initial denaturation at 95°C for 5 min; 35 cycles consisting of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min; and a final extension step consisting of 72°C for 7 min. Amplified PCR products were analyzed by agarose gel electrophoresis, purified

Table 1. List of bacterial species isolated from marine natural biofilms

Group	Strain No.	Colony				Closest match	Similarity (%)
		Color	Form	Elevation	Margin		
α-Proteobacteria	13737*	ivory	circular	convex	entire	*Ochrobactrum anthropi*	100.00
α-Proteobacteria	13733*	orange	circular	convex	entire	*Paracoccus carotinifaciens*	100.00
γ-Proteobacteria	13703*	yellow	circular	convex	entire	*Pseudoalteromonas piscicida*	100.00
γ-Proteobacteria	13704	yellow	circular	raised	entire	*Pseudoalteromonas piscicida*	100.00
γ-Proteobacteria	13687*	cream	circular	flat	erose	*Pseudoalteromonas agarovorans*	100.00
γ-Proteobacteria	13684	ivory	circular	flat	entire	*Pseudoalteromonas agarovorans*	100.00
γ-Proteobacteria	13716*	yellow	irregular	raised	undulate	*Pseudomonas aeruginosa*	99.13
γ-Proteobacteria	13723*	yellow	circular	convex	entire	*Shewanella baltica*	100.00
γ-Proteobacteria	13719	cream	circular	raised	entire	*Shewanella baltica*	100.00
γ-Proteobacteria	13705*	ivory	irregular	raised	erose	*Vibrio parahaemolyticus*	100.00
γ-Proteobacteria	13706	ivory	irregular	raised	lobate	*Vibrio parahaemolyticus*	100.00
γ-Proteobacteria	13721*	ivory	circular	raised	entire	*Vibrio pomeroyi*	99.71
γ-Proteobacteria	13732	ivory	irregular	raised	undulate	*Vibrio pomeroyi*	99.71
CFB	13731*	yellow	circular	raised	entire	*Cytophaga latercula*	97.97
CFB	13685*	orange	circular	convex	entire	*Tenacibaculum mesophilum*	99.71
CFB	13683	fluorescent	irregular	flat	lobate	*Tenacibaculum mesophilum*	99.71
CFB	13688	yellow	irregular	flat	undulate	*Tenacibaculum mesophilum*	99.71
CFB	13692	fluorescent	irregular	flat	lobate	*Tenacibaculum mesophilum*	99.71
CFB	13701	yellow	irregular	raised	lobate	*Tenacibaculum mesophilum*	99.71
CFB	13712	yellow	circular	flat	entire	*Tenacibaculum mesophilum*	99.71
High GC, Gram +	13707*	yellow	circular	convex	entire	*Arthrobacter nicotianae*	97.40
High GC, Gram +	13710	yellow	circular	convex	entire	*Arthrobacter nicotianae*	97.40
High GC, Gram +	13720	cream	circular	convex	entire	*Arthrobacter nicotianae*	97.40
High GC, Gram +	13729*	white	circular	convex	entire	*Brevibacterium casei*	100.00
High GC, Gram +	13730	ivory	circular	convex	entire	*Brevibacterium casei*	100.00
High GC, Gram +	13686*	yellow	irregular	convex	erose	*Brevibacterium epidermidis*	99.13
High GC, Gram +	13689	white	irregular	flat	erose	*Brevibacterium epidermidis*	99.13
High GC, Gram +	13690*	orange	irregular	flat	erose	*Tsukamurella paurometabola*	98.55
Low GC, Gram +	13724*	yellow	filamentous	raised	erose	*Bacillus macroides*	100.00
Low GC, Gram +	13717*	white	circular	convex	entire	*Staphylococcus haemolyticus*	100.00
Low GC, Gram +	13691*	white	circular	convex	entire	*Staphylococcus warneri*	100.00

*The 17 strains that were finally identified.

with High Pure PCR Product Purification Kit (Roche, Germany), and sequenced using an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, USA).

Sequence Analysis

The sequencing was performed using primer 518R (5'-GTA TTA CCG CGG CTG CTG G-3'), and sequences of the 16S rDNA between 362-484 bp (average 451 bp) were submitted to the Advanced BLAST search program of the NCBI to identify whether they aligned with closely related organisms. The related sequences were preliminarily aligned with the default settings of CLUSTAL X (Thompson *et al.*, 1997), and complete sequence alignments were performed using PHYDIT (Chun, 1995) and manual comparison to secondary structures. The phylogenetic analysis was performed with PHYLIP (Felsenstein, 1993), and phylogenetic trees were inferred using the neighbor-joining method (Saitou & Nei, 1987).

Results and Discussion

Thirty-one strains of bacteria were cultured (Table 1). Among them, 16S rDNA sequences of 14 strains were identical with other strains sequences and 17 unique strains were identified (Fig. 1). Phylogenetic analysis using 16S rDNA indicated that the 17 strains belonged to α-Proteobacteria (*Ochrobactrum anthropi*, *Paracoccus carotinifaciens*); γ-Proteobacteria (*Pseudoalteromonas agarovorans*, *P. piscicida*, *Pseudomonas aeruginosa*, *Shewanella baltica*, *Vibrio parahaemolyticus*, *V. pomeroyi*); CFB group bacteria (*Cytophaga latercula*, *Tenacibaculum mesophilum*); high GC, Gram-positive bacteria (*Arthrobacter nicotianae*, *Brevibacterium casei*, *B. epidermidis*, *Tsukamurella inchonensis*); and low GC, Gram-positive bacteria (*Bacillus macroides*, *Staphylococcus haemolyticus*, *S. warneri*).

α-Proteobacteria

The 16S rDNA sequence of strain 13737 showed 100% homology to that of *Ochrobactrum anthropi* (Table 1). *Ochrobactrum anthropi* has been primarily reported from human clinical specimens (Holmes *et al.*, 1988) and was isolated from soil and wheat roots (Lebuhn *et al.*, 2000). Since the homology of the16S rDNA sequences between *O. anthropi* and *O. intermedium* exceeded 98.0% (Lebuhn *et al.*, 2000), a full sequence comparison and further taxonomic studies are needed to identify strain 13737.

Strain 13733 showed 100% homology to a soil bacterium *Paracoccus carotinifaciens* that produces astaxanthin and forms circular and orange colonies (Tsubokura *et al.*, 1999). The colony type of strain 13733 is identical with that of *P. carotinifaciens*; thus strain 13733 is supposed to belong to species *P. carotinifaciens*.

γ-Proteobacteria

Strains 13703 and 13704 showed 100% homology to *Pseudoalteromonas piscicida*, a bacterium isolated from red tide seawater (Venkateswaran *et al.*, 2000). Strains 13687 and 13684 showed 100% homology to *Pseudoalteromonas agarovorans*, an agarolytic marine isolate (Romanenko *et al.*, 2003). Since the 16S rDNA sequence of *P. agarovorans* is 99.9% identical to *P. atlantica P. distincta, P. elyakovii P. espejiana* and *P. haloplaktis* subsp. *Haloplaktis* (Romanenko *et al.*, 2003), full sequence comparison and further taxonomic studies are needed to identify strains 13687 and 13684. Strain 13716 showed 99.13% homology to *Pseudomonas aeruginosa*. Strains 13723 and 13719 showed 100% resemblance to *Shewanella baltica*, which is isolated from Baltic Sea (Ziemke *et al.*, 1998). Strains 13705 and 13706 showed 100% homology to *Vibrio parahaemolyticus*, a bacterium isolated from aquatic environments (West *et al.*, 1986). Strains 137021 and 13732 showed 99.71% homology to *Vibrio pomeroyi*, which is isolated from bivalve larvae and forms circular colonies that are beige in color (Thompson *et al.*, 2003).

CFB group bacteria

Strain 13731 showed 97.97% homology to *Cytophaga latercula*, a bacterium that is isolated from seawater aquarium outflow (Nakagawa and Yamasato, 1993). Strains 13685, 13683, 13688, 13692, 13701, and 13712 showed 99.71% homology to *Tenacibaculum mesophilum*, which forms circular and yellow colonies and is isolated from a marine sponge (Suzuki *et al.*, 2001). The six strains showed different colony types from each other; thus requiring further taxonomic study.

High GC, Gram positive bacteria

Strains 13707, 13710 and 13720 showed 97.40% homology in sequence to *Arthrobacter nicotianae*. A strain of *A. nicotianae* from the oil-polluted Arabian Gulf has been reported to utilize hydrocarbon (Radwan *et al.*, 2001). Strains 13729 and 13730 showed 100% homology with *Brevibacterium casei*, which is isolated from cheese (Brennan *et al.*, 2002). Strains 13686 and 13689 showed 99.13% homology to *Brevibacterium epidermidis*. A strain of *B. epidermidis* has been reported to oxidize cyclic alcohols and ketones (Brzostowicz *et al.*, 2002). Strain 13690 showed 98.55% homology to *Tsukamurella paurometabola*.

Low GC, Gram positive bacteria

Strain 13724 showed 100% homology to *Bacillus macroides*. Strain 13717 showed 100% homology to *Staphylococcus haemolyticus*, a pathogen resistant to multiple antibiotics (Tabe *et al.*, 2001). Strain 13691 showed 100% homology to *Staphylococcus warneri*.

Most of the strains cultured in this study belong to either α-Proteobacteria, γ-Proteobacteria, CFB, or gram-positive bacteria (Table 1; Fig. 1). α-Proteobacteria, γ-Proteo bacteria and CFB group bacteria are dominant groups in

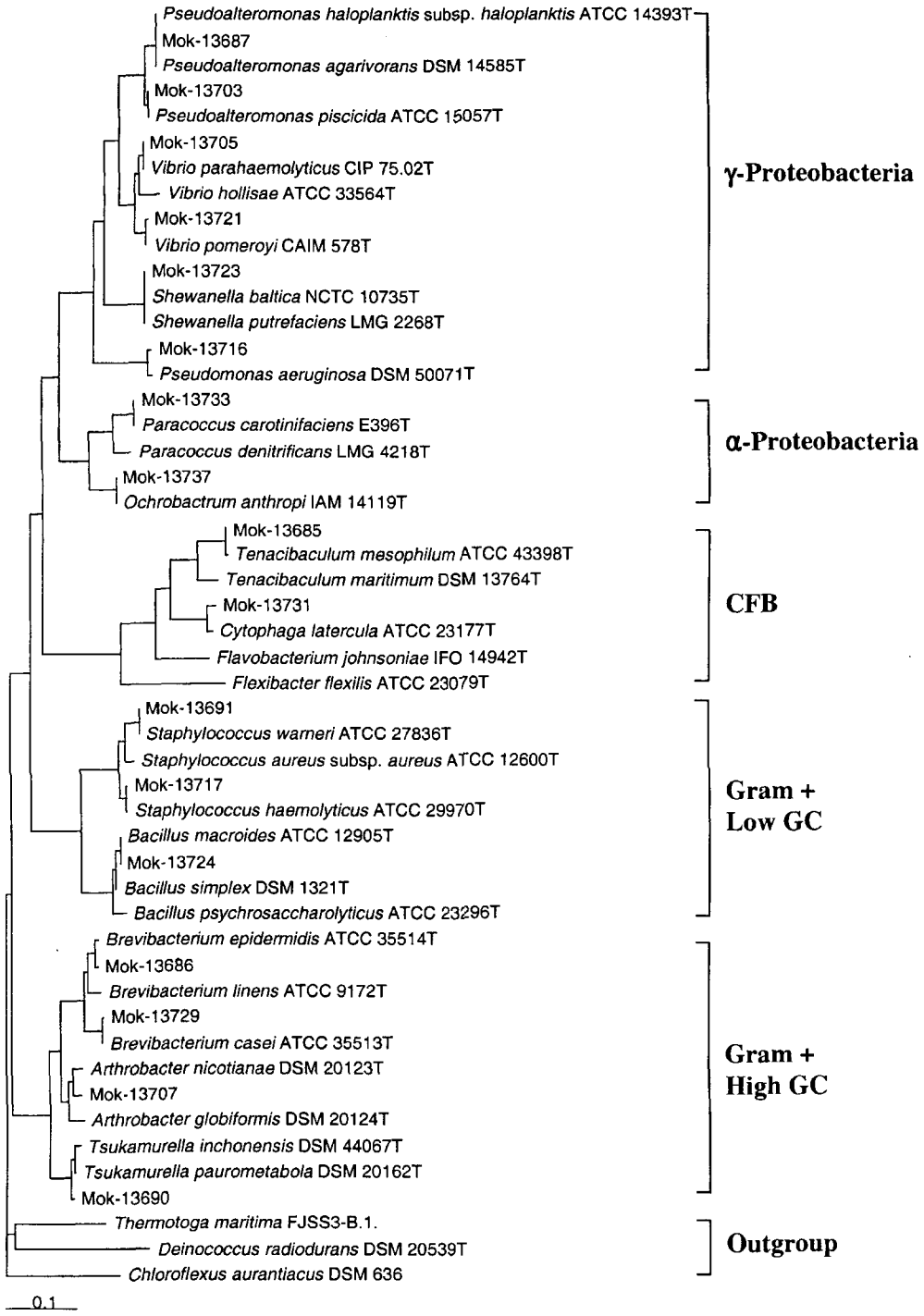

Fig. 1. Phylogenetic position of 17 bacterial isolates from marine biofilms. 16S rDNA sequences (average 451 bp) were aligned based on secondary structures and a best fit neighbor-joining tree was constructed. *Arthrobacter globiformis* DSM 20124T, M23411; *A. nicotianae* DSM 20123T, X80739; *Bacillus psychrosaccharolyticus* ATCC 23296T, X60635; *B. simplex* DSM 1321T, X60638; *B. macroides*, X70312; *Brevibacterium casei* ATCC35513T , X76564; *B. epidermidis* ATCC35514T, X76565; *B. linens* ATCC9172T, X77451; *Cytophaga latercula* ATCC 23177T, M58769; *Flavobacterium johnsoniae* IFO 14942, D12664; *Flexibacter flexilis* ATCC 23079T, M62794; *F. maritimus* ATCC 43398T, M64629; *Ochrobactrum anthropi* IAM 14119T, D12794; *Paracoccus carotinifaciens* E396T, AB006899; *P. denitrificans* LMG 4218T, X69159; *Pseudoalteromonas agarivorans* DSM 14585T, AJ417594; *P. haloplanktis* subsp. *haloplanktis* ATCC 14393T, X67024; *P. piscicida* ATCC 15057T, X82215; *Pseudomonas aeruginosa* DSM 50071T, X06684; *Shewanella baltica* NCTC 10735T, AJ000214; *S. putrefaciens* LMG 2268T, X81623; *Staphylococcus aureus* subsp. *aureus* ATCC 12600T, L37597; *S. haemolyticus* ATCC 29970T, X66100; *S. warneri*, Z26903; *Tenacibaculum maritimum* DSM 13764T, AB078057; *T. mesophilum* ATCC 43398T, AB032501; *Tsukamurella inchonensis* DSM 44067T, X85955; *T. paurometabola* DSM 20162T, Z46751; *Vibrio hollisae* ATCC 33564T, X56583; *V. parahaemolyticus* CIP 75.02 T, X74720; *V. pomeroyi* CAIM 578T, AJ491290.

the marine environment (Kelly and Chistoserdov, 200) and have been reported from various marine biofilms. α-Proteobacteria, γ-Proteobacteria and CFB group bacteria are dominant in dead coral surfaces (Frias-Lopez *et al.*, 2002) and are associated with tubes of a worm living in the deep-sea hyperthermal vent. In addition, α-Proteobacteria, γ-Proteobacteria, CFB, and gram-positive bacteria are dominant in bacteria attached to shrimp and are isolated from hydrocarbon-degrading biofilms (Lau *et al.*, 2002; Stach and Burns, 2002). Several species such as *Arthrobacter nicotianae*, *Cytophaga laterculai*, *Pseudoalteromonas agarovorans*, *P. piscicida*, *Shewanella baltica*, *Tenacibaculum mesophilum*, and *Vibrio pomeroyi* were reported to have been isolated from seawater or marine organisms (Nakagawa and Yamasato, 1993; Ziemke *et al.*, 1998; Venkateswaran *et al.*, 2000; Radwan *et al.*, 2001; Suzuki *et al.*, 2001; Romanenko *et al.*, 2003; Thompson *et al.*, 2003).

Bacteria forming raised colonies are expected to produce abundant exopolysaccharides. Thus, further studies on exopolysaccharides are needed for strains 13705, 13716, 13721, 13724 and 13731. Strains forming convex colonies are also putative exopolysaccharides-producing bacteria. This study provides an initial step in deciphering the bacterial diversity of marine biofilms, and in screening exopolysaccharides-producing bacteria.

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