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Isolation of the Agarolytic Bacterium *Vibrio cyclotrophicus* DAG-130 from Abalone Gut

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We isolated 1,916 strains of bacteria from gut and feces of abalone. The most active agarolytic bacterium, DAG-130, was identified from the gut of the abalone *Haliotis gigantea*. Of the bacteria harbored by both *H. discus hannai* and *H. gigantea*, 59% were agarolytic. There was no significant difference in the number of agarolytic bacteria isolated from abalone fed on the seaweeds *Gelidium amansii*, *Laminaria japonica*, or *Undaria pinnatifida*. Of the agarolytic bacteria, 72% were isolated from the guts of all sources tested while 43% came from the feces. The strain DAG-130 showed 100% identity with the bacterium *Vibrio cyclotrophicus* based on phylogenetic analysis of 16S rDNA. The bacterium produced monomers and oligomers from the agar substrate.

Key word: Abalone, Agar, Agarolytic bacterium, *Vibrio cyclotrophicus*

Introduction

A number of studies have investigated the interactions between bacteria and their invertebrate hosts. Since many species of abalone feed largely on kelp, which is deficient in many essential nutrients, it is possible that gut bacteria play an integral role in the digestion of complex algal polysaccharides (Erasmus et al., 1997). Agar is a renewable complex polysaccharide composed of agarose and agaropectin produced by some marine red algae such as *Gelidium latifolium, G. amansii, Gracilaria verrucosa,* and others (Duckworth and Yaphe, 1971). Agar is used in the preparation of microbial growth media, candy, agar jelly, and agarose for gel electrophoresis and immunoassays. Agar degradation can be used as a potent supply of biomass, as a source of oligosaccharides, in the purification of useful substances from seaweed, in protoplast production, and in digesting electrophoresis gels (Li et al., 2003). Some researchers have investigated the possibility of obtaining agar-hydrolyzing enzymes from bacteria (Ohta et al., 2005; Lee et al., 2007). The agarolytic enzyme α-agarase produces agaroologosaccharides, and β-agarase produces neoagarooligosaccharides (Kato, 2000). Most agarolytic bacteria have been isolated from marine environments, but little is known about the distribution of agarolytic bacteria derived from marine invertebrate hosts. The objectives of this study were to survey the distribution of agarolytic bacteria from the gut and feces of herbivorous abalone, select the most active agarolytic strain, and identify the bacterium by phylogenetic analysis.

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Materials and Methods

Abalone and seaweed

The abalone species *Haliotis discus hannai* produced by Wando Aquaculture Farms was obtained from a local market. *H. gigantea* was collected at Sungsanpo, Jeju Island, Korea. The animals were kept in a seawater aquarium at 18°C and fully fed three different diets, i.e., *Gelidium amansii*, *Laminaria japonica*, and *Undaria pinnatifida*, separately.

Isolation of bacteria

Bacteria were isolated from the gut and feces of abalone fed each type of seaweed. They were incubated at 20°C for 3 d on agar medium plates (0.05% yeast extract, 0.2% peptone, and 2% agar in filtered sea water at pH 7.8). Lugol's solution (2 g KI and 1 g I_2 in 200 mL water) was poured on the agar medium

after 3 d of culture. Bacterial colonies that formed a clear zone on the agar were counted as agarolytic bacteria and further purified by the same plating method. The bacterium DAG-130, which produced the largest clear zone among 1,916 strains, was selected for further analysis.

Scanning electron microscopy

The strain DAG-130 was grown on agar plates as described above at 20°C for 3 days and suspended in a solution containing 2% paraformaldehyde and 2% glutaraldehyde. The cells were incubated at room temperature for 2 hrs. Phosphate-buffered saline (PBS; 0.1M phosphate buffer, pH 7.4 in saline) was used to wash out the fixative solution before the cells were mixed with 2% OsO_4 at room temperature for 30 min. The cells were washed twice with buffer and dehydrated through a series of different concentrations of ethanol (50%, 60%, 70%, 80%, 90%, and 100%). Finally, the cells were stained using 3-methylbutyl acetate before they were observed with a scanning electron microscope at 30 kV (JEOL JSM-6700F, Tokyo, Japan).

Isolation of bacterial DNA

Cultured cells in exponential growth phase were harvested by centrifugation, and the supernatant was discarded. Total DNA was extracted following the procedure described by Magarvey et al. (2004) with some modifications. Cultures were grown in 15 mL liquid medium (0.05% yeast extract and 0.2% peptone in filtered sea water, pH 7.8) for 2 days and then centrifuged at 12,800×g for 5 min. The pellet was resuspended in 700 μL of TE (10 mM Tris-HCl and 1 mM EDTA, pH 7.5) containing lysozyme (20 mg/mL) and RNase A (20 mg/mL), and incubated at 37°C for 1 hr. Following incubation, 150 μL of 0.5 M EDTA, 150 μL of TE containing Proteinase K (5 mg/mL), and 100 μL of 10% SDS were added, and the mixture was incubated at 37°C for another hour. The tube was mixed by inversion after the addition of 100 μL of 5 M NaCl and heated in a 65°C water bath for 10 min. Cellular debris was removed by centrifugation at 12,800×g for 5 min, and the supernatant solution was transferred to a new microtube. Proteins and lipids were removed by the addition of 0.3 volumes of phenol-chloroform and centrifuged at 12,800×g for 5 min. DNA in the aqueous phase was precipitated with an equal volume of 100% ethanol and kept for 15 min at -20°C; it was then centrifuged at 12,800×g for 5 min to remove ethanol. Finally, the DNA was dissolved in 200 μL TE for immediate use or storage at -20°C.

16S rDNA amplification and sequencing

The 16S rRNA gene was amplified from genomic DNA obtained from bacterial cultures by PCR with the universal primers 27F (5'-AGAGTTTGATCC-TGGCTCAG-3') and 1492R (5'-GGATACCTTGTT-ACGACTT-3') (Yoon et al., 1996). The reaction mixture (final volume, 20 μL) contained 50 ng DNA, PCR buffer [10 mM KCl, 10 mM $(NH_4)_2SO_4$, 20 mM Tris-HCl, 2 mM $MgSO_4$, 0.1% Triton X-100, pH 8.8], 10 mM deoxyribonucleotide triphosphate mixture, 10 pmol of each primer, and 0.3 U of *Taq* DNA polymerase. PCR conditions consisted of an initial denaturetion at 94°C for 5 min; 30 cycles at 94°C for 30 sec, 55°C for 30 sec, and 72°C for 90 sec; and a final 7-min extension at 72°C. The amplification products were examined by 1% agarose gel electrophoresis and purified with a QIAquick PCR cleanup kit using the protocol suggested by the supplier (Qiagen Inc., Chatsworth, CA). The nearly complete 16S rRNA gene was sequenced using the PCR products directly as sequencing templates with the same primers that were used for PCR (SolGent Co., Ltd., Daejeon, Korea).

Data analysis

Molecular taxonomy was determined based on the 16S rDNA sequence and phylogenetic analysis of related organisms. Sequences were edited and manipulated using MEGA3 (Kumar et al., 2004). For sequence comparisons, multiple sequence alignments were performed using sequences identified in the present study and 17 Vibrionaceae species retrieved from DDBJ/EMBL/GenBank, using ClustalX (Thompson et al., 1994). *Pseudomonas damselae* (GenBank accession # AB032015), *Grimontia hollisae* (AJ-514909), and *Salinivibrio costicola* (X95527) were used as outgroups. Genetic distance values were calculated using the aligned DNA sequences according to the two-parameter distance method of Kimura (1980). A phylogenetic tree was inferred by use of the neighbor-joining algorithm (Saito and Nei, 1987) in MEGA3 with bootstrap analysis from 1,000 bootstrap replications. Manipulation and tree editing were done using the program TreeView.

Thin layer chromatography

Quantitative analysis of final products was performed using thin layer chromatography (TLC). The bacteria were cultured on the above agar medium for 3 days at 20°C, and then the clear zone after Lugol application was removed and frozen overnight. After thawing the agar pieces, the supernatant was collected by centrifugation at 12,800×g for 5 min. The

condensed exudate was applied to a TLC plate (Whatman, Germany) and developed in isopropanol-water (4:1). Carbohydrates were stained by spraying with aniline-diphenylamine and heating for 5 min at 80°C (Dawson et al., 1986).

Results

Bacteria from gut and feces of abalone

In total, 1,916 strains of bacteria were isolated from the gut and feces of 15 abalones. Of the bacteria, 59% showed strong agarolytic activity (Table 1). Of the bacteria isolated from *H. discus hannai*, 58% showed agarolytic activity; similarly, 60% of the bacteria from *H. gigantea* showed agarolytic activity. When *Gelidium amansii*, *Laminaria japonica*, and *Undaria pinnatifida* were fed separately to abalone for at least 1 week, the number of agarolytic bacteria isolated was 57%, 58%, and 61%, respectively. There were no significant differences in the number of agarolytic bacteria between the two abalone species and among the three seaweed diets tested. Of the agarolytic bacteria, 72% were isolated from the gut, while 43% were from the feces. Moreover, 78% of the gut bacteria of *H. gigantea* fed *U. pinnatifida* were agarolytic. The most active agarolytic strain, DAG-130, was selected and used for further experiments.

Shape of the bacterium DAG-130

Shape and size of the DAG-130 cells were examined by scanning electron microscopy (Fig. 1). Most cells appeared rod-shaped, with a few cells being curved. Most cells were motile during growth phase. Cells possessed either one or two polar or subpolar flagella. Exponential-phase cells measured 0.5-0.7 μm×1.2-1.5 μm.

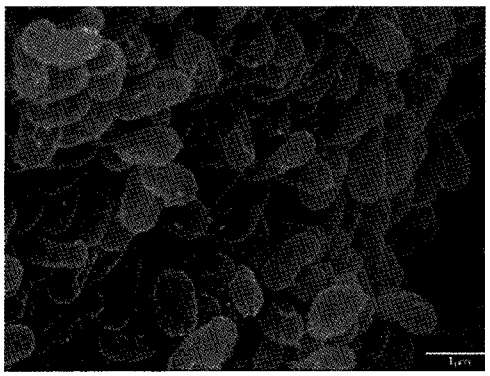

Fig. 1. Scanning electron micrograph of the strain DAG-130. Bar indicates 1 μm.

Phylogenetic analysis

A 1,494-base pair (bp) product was produced by PCR (data not shown). The complete 16S rDNA sequence (1,446 bp) from the 40th to 1,485th base was identified by comparison to the sequence of *Vibrio parahaemolyticus* (GenBank accession # DQ-026024) (Fig. 2). A fragment of 1,381 bp from the 47th to 1,427th base was compared to the 16S rDNA sequences of 20 strains obtained from the EMBL rDNA database. Fig. 3 shows the inferred relative phylogenetic relationships of strain DAG-130 to other species belonging to the Vibrionaceae produced using the BlastN algorithm. The 16S rDNA sequences of DAG-130 and *V. cyslitrophicus* (GenBank accession # DQ481610) were 100% identical.

Agarolytic activity

Vibrio cyclotrophicus strain DAG-130 was grown on agar plates for 3 days at 20°C. The clear zone identified after Lugol application was removed and the exudate applied to TLC plates. Carbohydrates stained by aniline-diphenylamine appeared in the lane

Table 1. Number of bacteria isolated from the gut and feces of abalones which fed different seaweed species

Abalone species	Seaweed fed	Isolation source	Agarolytic bacteria / Total bacteria tested	Rate (%)
Haliotis discus hannai	*Gelidium amansii*	Gut	78 / 110	71
		Feces	40 / 79	51
	Laminaria japonica	Gut	80 / 132	61
		Feces	42 / 100	42
	Undaria pinnatifida	Gut	158 / 210	75
		Feces	69 / 173	40
Haliotis gigantea	*Gelidium amansii*	Gut	52 / 73	71
		Feces	50 / 122	41
	Laminaria japonica	Gut	212 / 285	74
		Feces	86 / 202	43
	Undaria pinnatifida	Gut	201 / 257	78
		Feces	69 / 173	40

5'-AGAGTTTGATCATGGCTCAGATTGAACGCTGGCGGCAGG**CCTAACACATGCAAGTCGAGCGGAAACGACA**
CTAACAATCCTTCGGGTGCGTTAATGGGCGTCGAGCGGCGGACGGGTGAGTAATGCCTAGGAAATTGCCTTGA
TGTGGGGGATAACCATTGGAAACGATGGCTAATACCGCATGATGCCTACGGGCCAAAGAGGGGGACCTTCGGG
CCTCTCGCGTCAAGATATGCCTAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCGACGATCCC
TAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTG
GGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAA
GTACTTTCAGTTGTGAGGAAGGGTGTGTAGTTAATAGCTGCGCATCTTGACGTTAGCAACAGAAGAAGCACCG
GCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCGAGCGTTAATCGGAATTACTGGGCGTAAAGCGC
ATGCAGGTGGTTCATTAAGTCAGATGTGAAAGCCCGGGGCTCAACCTCGGAACTGCATTTGAAACTGGTGAAC
TAGAGTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGTGAAATGCGTAGAGATCTGAAGGAATACCAGTG
GCGAAGGCGGCCCCCTGGACAGATACTGACACTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCC
TGGTAGTCCACGCCGTAAACGATGTCTACTTGGAGGTTGTGGCCTTGAGCCGTGGCTTTCGGAGCTAACGCGT
TAAGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGT
GGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAAGCCAGCGGAGA
CGCAGGTGTGCCTTCGGGAGCTCTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGG
TTAAGTCCCGCAACGAGCGCAACCCTTATCCTTGTTTGCCAGCGAGTCATGTCGGGAACTCCAGGGAGACTGC
CGGTGATAAACCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCTACACACGTGC
TACAATGGCGCATACAGAGGGCAGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGTGCGTCGTAGTCCGGAT
TGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGTGAATACGT
TCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAGTGGGCTGCAAAAGAAGTGGGTAGTTTAACCTTTC
GGGGAGGACGCTCACCACAAGTCGTAACAAGGTATCC-3'

Fig. 2. 16S rDNA sequence (1,494 base) of the *Vibrio cyclotrophicus* DAG-130. The strain was isolated from gut of the abalone *Haliotis gigantea*. PCR primers are underlined. Complete 16S rDNA compared with *V. parahaemolyticus* (accession # DQ026024) is described by bold letters. Sequence of *V. cyslitrophicus* (accession # DQ481610) is in italic letters.

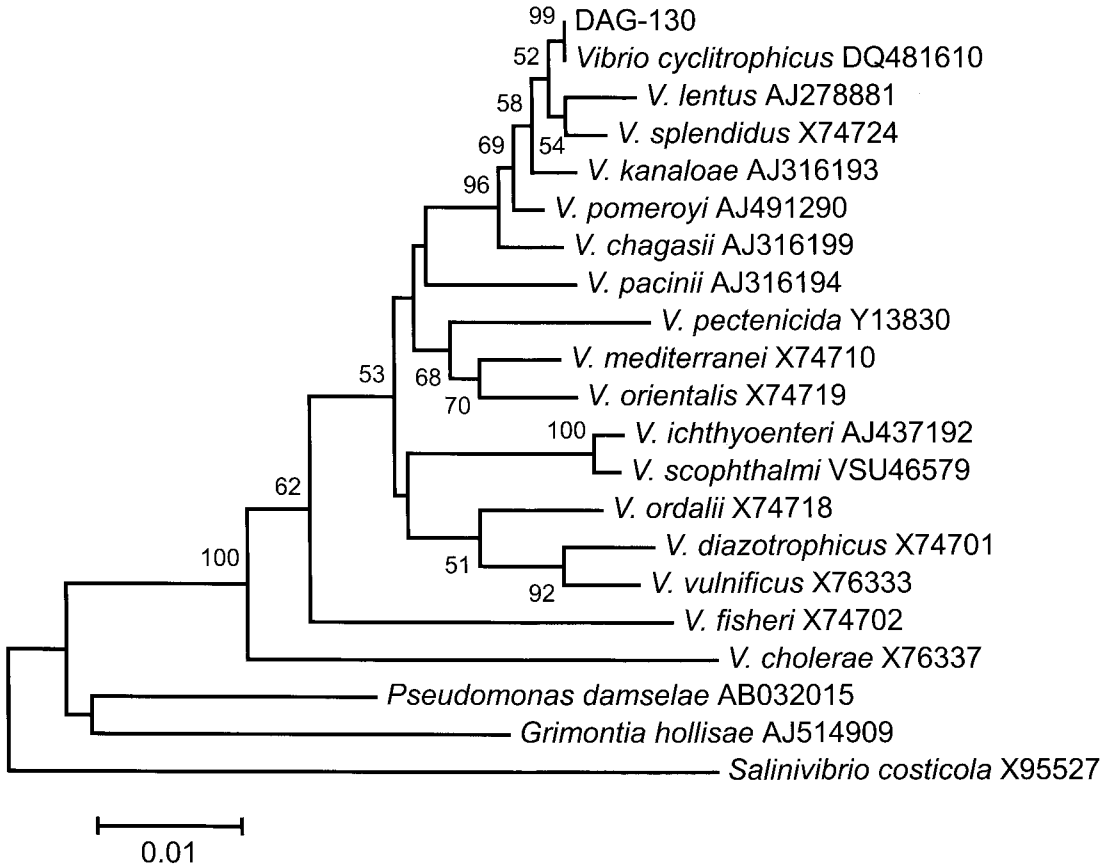

Fig. 3. Phylogenetic dendrogram of the *V. cyclotrophicus* strain DAG-130 with the most closely related *Vibrio* species based on 16S rDNA sequences (1,381 bp), and constructed by the neighbour-joining method (pairwise deletion, Kimura two-parameter). Numbers at nodes indicate level of bootstrap support (1,000 replicates).

spotted with exudates from the clear zone formed by the bacterium (Fig. 4). The products were assumed to contain monomers and oligomers of agar, although their identities remain to be confirmed.

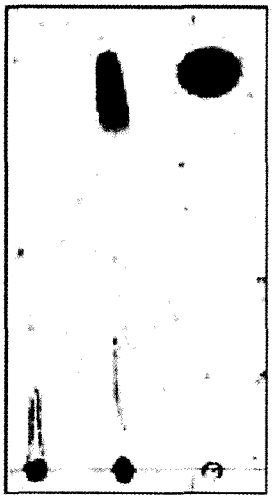

Fig. 4. TLC of agarolytic products by the *V. cyclotrophicus* strain DAG-130. A, exudate from non-clear zones of the agar medium. B, exudate from clear zones formed by the bacterium on the agar medium. C, D-galactose.

Discussion

Agar, a complex polysaccharide present in the cell walls of some red algae, can be degraded by several bacterial strains isolated from marine environments as well as other sources. Agarolytic bacteria are ubiquitous in coastal and estuarine regions; however, they are not exclusively autochthonous to the marine environment, since some reports have shown that they also occur in freshwater, sewage, and soil (Agbo and Moss, 1979). Most herbivorous abalones consume seaweed as their sole diet; thus the animals or the gut-dwelling bacteria must be able to digest the cell wall components. In this experiment, we found that 72% of the total number of bacteria isolated from the guts of abalone were agarolytic. Meanwhile, 43% of the bacteria isolated from feces showed agarolytic activity. The agarolytic bacteria use agarose and agaropectin as convenient carbon and energy sources. Some agarolytic bacteria have been assigned to the genera *Alteromonas*, *Cytophaga*, *Streptomyces*, *Pseudomonas*, and *Vibrio*, among others (Vera et al., 1998; Zhang and Sum, 2007). Several marine agarolytic strains of *Vibrio*-like species are capable of fixing nitrogen for anaerobic growth using agar as the carbon and energy sources (Shieh et al., 1988). *Alterococcus agarolyticus*, the only thermophilic agarolytic species, is a marine facultatively anaerobic, fermentative, Gram-negative coccus that grows between 38 and 58°C with optimal growth at about 48°C (Shieh and Jean, 1998). No other agarolytic bacteria have been reported to grow at temperatures of 45°C or higher (Jean et al., 2006). Most agarolytic bacteria produce β-agarase enzyme. Recently, a *Thalassomonas* strain of agarolytic bacterium that produces a novel α-agarase was isolated from the sediment off Noma Point, Japan (Ohta et al., 2005). When neoagarohexaose is used as a substrate, α-agarase produces agaropentose and agarotriose, while β-agarase produces neoagarotetraose and neoagarobiose. Agarase applications play an important role in many areas of industry and scientific research, such as generating simple neo-oligosaccharides from complex polysaccharides, liberating DNA and other embedded molecules from agarose, and extracting bioactive or medicinal compounds from algae and seaweed. These applications all depend on the natural functionality of purified agarases (Zhang and Sum, 2007).

In this report, we have described a marine bacterial species, *V. cyclotrophicus,* as an agarolytic bacterium isolated from abalone gut. The bacterium is facultatively anaerobic, requires at least 1-7.5% NaCl for growth, is catalase- and oxidase-positive, and uses some amino acids, carbohydrates, organic acids and sugar alcohols for growth. The bacterium can also use several two- and three-ring polycyclic aromatic hydrocarbons as substrates (Hedlund and Staley, 2001).

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