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Bacterial community structure and predicted alginate metabolic pathway in an alginate-degrading bacterial consortium

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Methane fermentation is one of the effective approaches for utilization of brown algae; however, this process is limited by the microbial capability to degrade alginate, a main polysaccharide found in these algae. Despite its potential, little is known about anaerobic microbial degradation of alginate. Here we constructed a bacterial consortium able to anaerobically degrade alginate. Taxonomic classification of 16S rRNA gene, based on high-throughput sequencing data, revealed that this consortium included two dominant strains, designated HUA-1 and HUA-2; these strains were related to *Clostridiaceae* bacterium SK082 (99%) and *Dysgonomonas capnocytophagoides* (95%), respectively. Alginate lyase activity and metagenomic analyses, based on high-throughput sequencing data, revealed that this bacterial consortium possessed putative genes related to a predicted alginate metabolic pathway. However, HUA-1 and 2 did not grow on agar medium with alginate by using roll-tube method, suggesting the existence of bacterial interactions like symbiosis for anaerobic alginate degradation.

[**Key words:** Alginate; Consortium; Symbiosis; *Dysgonomonas*; *Clostridium*]

Land biomass including both agricultural crops and lignocellulosic components has been identified as a renewable energy resource, although several highly energetic crops, such as sweet potato or corn, are in direct competition with food production. Other plant materials, such as straw or remains from forest clearing tend to be rich in lignin, and are consequently less efficient as energy sources because of the high costs associated with converting this biomass into fermentative sugars (1–3). On the other hand, abundant elements of marine ecosystems, such as seaweed, are hardly utilized as a food resource and possess low or no lignin content, making them a suitable alternative to land biomass for energy generation (2,4–6). Therefore, seaweed has been receiving increasing attention as third-generation biomass (7). Among marine seaweed biomass, brown algae have been highlighted as the most promising feedstock as a renewable resource (8–10).

Alginate is a linear polysaccharide composed by two uronic acids, β-D-mannuronate (M) and α-L-guluronate (G) covalently (1–4)-linked in different sequences (11,12). Alginate is a major component of brown algae and represents at up to 40% of the organism's dry weight, depending on the species (4,13). As a consequence, comprehensive understanding of alginate metabolism is essential to ensure efficient biorefinery procedures to exploit this group as an energy source; however, several microorganisms are known to metabolize alginate (13–15), through an alginate degradation pathway mediated by alginate lyase. This enzyme catalyzes the depolymerization of endo-type alginate into oligomers via an endolytic β-elimination reaction (10,11). The resultant oligomers are further degraded into unsaturated monomers through the action of an exolytic enzyme known as exotype alginate lyase (10,11). These monomers are eventually metabolized to pyruvate and glyceraldehyde-3-phosphate, which are key intermediates of the glycolytic pathway (10,11). On the other hand, to date, research on the anaerobic degradation of alginate by bacterial communities has been limited, despite its potential importance in fermentative production of valuable chemical compounds, such as short-chain fatty acids (16,17), and biofuels such as methane (18,19). Methane production by anaerobic digestion of macroalgae is a promising algal bioenergy option (18); it is known that the rate of degradation (hydrolysis and acidogenesis) of polysaccharides is one of the rate-limiting factors (20). In the case of brown algae, it was reported that the methane fermentation rate is sometimes restricted by the degradation capacity of alginate (21). However, little is known regarding the mechanisms of alginate degradation by bacterial consortia. Indeed, Seon et al. (17) reported one microbial community

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structure degrading alginate to volatile fatty acids in an anaerobic condition. However, the information regarding the pathway of anaerobic alginate degradation by microbial consortium has not been clarified.

In this study, we established a bacterial consortium able to anaerobically degrade alginate. This bacterial consortium has now been cultured for >5 years while maintaining its alginate degradation ability. Here we describe not only the community structure of this bacterial consortium but also its predicted alginate metabolic pathway by metagenome analysis.

MATERIALS AND METHODS

Growth media and culture conditions Since its establishment, the consortium have been cultured at 30°C under anoxic conditions in a basal medium (pH = 6.8) containing 5.5 g of KH_2PO_4, 7.0 g of K_2HPO_4, 1.0 g of $(NH_4)_2SO_4$, 0.12 g of $Na_2MoO_4 \cdot 2H_2O$, 0.039 g of $Fe(NH_4)_2SO_4 \cdot 6H_2O$, 0.029 g of $Co(NO_3)_2 \cdot 6H_2O$, 0.021 g of $CaCl_2 \cdot 2H_2O$, 0.25 g of $MgSO_4 \cdot 7H_2O$, 0.1 g of yeast extract, 0.1 g of tryptone, 10 ml of trace elements, 10 ml of Wolfe's vitamin solution (22), and 1.0 mg of resazurin per liter of deionized water. Anoxic conditions were generated by using titanium (III) citrate solution as reducing agent. The basal medium was supplemented with 5.0 g/l of alginate or fructose as the sole carbon source. *Escherichia coli* HST08 (Takara Bio Inc., Shiga, Japan) was used for plasmid construction and 16S rRNA gene clone analysis. *E. coli* strains were cultured at 37°C in LB medium supplemented with appropriate antibiotics.

Enrichment of bacterial consortium Sand sample was collected from the beach on the gulf of Hiroshima, Japan. Approximately 1 g wet weight of sand sample was inoculated into 10 ml of basal medium and vigorously shaken. The suspensions (1 ml) were cultured for 48 h in 9.0 ml basal medium supplemented with 5.0 g/l alginate at 30°C. The sample where alginate consumption could be confirmed was further subcultured in basal medium with alginate.

Quantification of culture products Organic substances in culture medium were quantified by high-performance liquid chromatography (LC-2000 Plus HPLC; Jasco, Tokyo, Japan) equipped with a refractive index detector (RI-2031 Plus; Jasco), Shodex RSpak KC-811 column (Showa Denko, Kanagawa, Japan), and a guard column (Shodex RSpak KC-G; Showa Denko) at 60°C. Ultrapure water containing 0.1% (v/v) phosphoric acid was used as the mobile phase at a flow rate of 0.7 mL/min. Crotonate was used as an internal standard.

Quantification of alginate We measured alginate concentration using a modified carbazole sulfuric acid method (23) and sodium alginate as standard. For this procedure, we added 0.06 ml of 4 M sulfuric acid solution to 0.5 ml of culture suspension and adjusted to pH 7.5 using KOH. We subsequently added 0.06 ml of 1 M borate buffer (adjusted to pH 8.4 using KOH) and mixed it vigorously. The solution was kept in boiling water for 6.5 min after adding 3 ml of H_2SO_4 and cooled on ice for 2 min. Furthermore, after adding 0.125 ml of 0.2% carbazole solution and vigorously mixing the solution; it was boiled for 10 min before measuring absorbance at 525 nm using a spectrophotometer.

Metagenome and 16S rRNA gene analyses based on high-throughput sequencing data Whole cells were harvested from the bacterial consortium when OD_{660} reached 0.4–0.5. Total genomic DNA was extracted using NucleoSpin Tissue (Macherey-Nagel, Düren, Germany). A paired-end library of the whole genome was generated for metagenome sequencing by Hokkaido system science Co. Ltd. (Sapporo, Japan) using Illumina HiSeq 2000. High-throughput sequencing of 16S rRNA gene and analysis was performed using Roche GS FLX+, Hokkaido system science, in which the 16S rRNA gene fragments were amplified using PCR performed in KOD FX Neo (Toyobo, Tokyo, Japan) with the tagged primer set Bact27f (5′-AGAGTTTGATCCTGGCTCAG) and Bact519r (5′-GWATTACCGCGGCKGCTG).

16S rRNA gene cloning analysis Genomic DNA from the bacterial consortium was extracted using NucleoSpin Tissue. 16S rRNA gene fragments were amplified using PCR performed in a KOD FX Neo with the primer set Bact27f and Bact1492r (5′-TACGGYTACCTTGTTACGACTT). The PCR fragments obtained were subsequently ligated into *Sma*I-digested pUC19, and the products were used to transform *E. coli* HST08. The 16S rRNA gene -harboring plasmids were extracted from *E. coli* transformants and sequenced by Eurofins Genomics (Tokyo, Japan). Nucleotide sequence similarities were determined using a BLAST search on DDBJ (http://www.ddbj.nig.ac.jp/) and Genetyx software (Genetyx, Tokyo, Japan).

PCR–restriction fragment length polymorphism and terminal-restriction fragment length polymorphism 16S rRNA gene fragments from the bacterial consortium or the cloned plasmids was amplified by PCR performed in KOD FX Neo with the primer set Bact27f and Bact1492r and purified using MagExtractor-PCR&Gel Clean up (Toyobo). For terminal-restriction fragment length polymorphism (T-RFLP), we used dye-labeled Bact27f primers. For PCR-RFLP, purified 16S rRNA gene fragments were digested with *Mbo*I and separated by electrophoreses in 2% agarose gel. For T-RFLP, 16S rRNA gene fragments were digested with *Hha*I and analyzed in a CEQ-2000 DNA sequencer (Beckman Coulter, Inc., Brea, CA, USA).

Alginate lyase assays The bacterial consortium was cultured in basal medium supplemented with alginate. Cells were harvested by centrifugation (20,400 ×*g* for 10 min at 4°C) at OD600 = 0.3–0.5. Harvested cells were washed and suspended in 50 mM phosphate buffer (pH 6.8) and subsequently sonicated using Branson Digital sonifier (Branson Ultrasonics, Corp., Danbury, CT, USA) with 2.0 s 'On' and 2.0 s 'Off' pulses (total 10 min) at 20% power amplitude. Cell-free extracts were obtained by centrifuging the sonicated cells (20,400 ×*g* for 30 min at 4°C). The protein concentrations of the cell-free extracts were determined using the Bradford method. Alginate lyase activity was measured following the method reported by Park et al. (24) with slight modifications. In brief, the cell-free extracts were added to a reaction solution containing 0.1% alginate and 50 mM phosphate buffer (pH 6.8). Enzyme activity was monitored by measuring the increase in A_{235} at 30°C. One unit was defined as the amount of enzyme required to achieve an increase in A_{235} of 0.1 per minute.

RESULTS AND DISCUSSION

Enrichment culture of bacterial consortium Bacterial consortium that could anaerobically degrade alginate was obtained from sand from the beach on Hiroshima Gulf, Hiroshima, Japan. Subculture of the bacterial consortium was repeated using the basal medium supplemented with alginate as the sole carbon source. Although this bacterial consortium has been subcultured for >5 years, the level of alginate consumption activity remained stable (data not shown).

Fig. 1 shows a typical time course of the batch culture of the bacterial consortium grown on alginate at 30°C. Alginate concentration decreased concomitantly with growth (OD_{660}). When alginate was supplied as a the sole carbon source, the bacterial consortium consumed 4.82 ± 0.13 g/l of alginate and mainly produced acetate (23.5 ± 0.18 mM) after culturing for 5 days. In addition, small amounts of formate (1.89 ± 0.11 mM), propionate (1.54 ± 0.08 mM), ethanol (0.65 ± 0.09 mM), and CO_2 (14.0 ± 1.09 mM) were produced.

Taxonomic classification of 16S rRNA gene based on high-throughput sequencing data To reveal the community structure of the bacterial consortium, we sequenced 16S rRNA gene by high-throughput sequencing techniques using Roche GS FLX+ to obtain a total of 12,690 reads with an average length of 462 base. All reads of 250 base or longer were used as input reads. Homology search of input reads was performed using BLAST against the 16S rRNA gene database from DDBJ. Taxonomy ID and

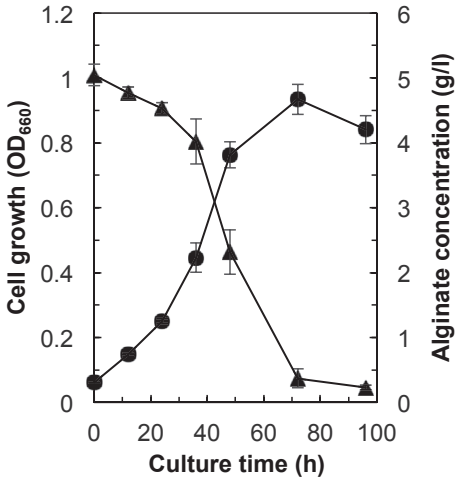

FIG. 1. Bacterial growth and alginate consumption in cultures of the bacterial consortium. Each experiment was performed in triplicate. Values represent the mean ± standard deviation for each point. Basal medium supplemented with 5.0 g/l of alginate as the sole carbon source and 50 mM of phosphate buffer (pH 6.8) was used as the growth medium. Time course of growth (circles) and alginate consumption (triangles).