Running Title: alginate metabolic pathway in bacterial consortium

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

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Abstract

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.

(Introduction)

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, 2, 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, 5, 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, 9, 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, 14, 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

1 alginate lyase (10, 11). These monomers are eventually metabolized to pyruvate and
2 glyceraldehydes-3-phosphate, which are key intermediates of the glycolytic pathway
3 (10, 11). On the other hand, to date, research on the anaerobic degradation of alginate by
4 bacterial communities has been limited, despite its potential importance in fermentative
5 production of valuable chemical compounds, such as short-chain fatty acids (16, 17),
6 and biofuels such as methane (18, 19). Methane production by anaerobic digestion of
7 macroalgae is a promising algal bioenergy option (18); it is known that the rate of
8 degradation (hydrolysis and acidogenesis) of polysaccharides is one of the rate-limiting
9 factors (20). In the case of brown algae, it was reported that the methane fermentation
10 rate is sometimes restricted by the degradation capacity of alginate (21). However little
11 is known regarding the mechanisms of alginate degradation by bacterial consortia.
12 Indeed, Seon *et al.* (17) reported one microbial community structure degrading alginate
13 to volatile fatty acids in an anaerobic condition. However, the information regarding the
14 pathway of anaerobic alginate degradation by microbial consortium has not been
15 clarified.

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

21

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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 KH₂PO₄, 7.0 g of K₂HPO₄, 1.0 g of (NH₄)₂SO₄, 0.12 g of Na₂MoO₄·2H₂O, 0.039 g of Fe(NH₄)₂SO₄·6H₂O, 0.029 g of Co(NO₃)₂·6H₂O, 0.021 g of CaCl₂·2H₂O, 0.25 g of MgSO₄·7H₂O, 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

1 RSpak KC-811 column (Showa Denko, Kanagawa, Japan), and a guard column (Shodex
2 RSpak KC-G; Showa Denko) at 60 °C. Ultrapure water containing 0.1% (v/v)
3 phosphoric acid was used as the mobile phase at a flow rate of 0.7 mL/min. Crotonate
4 was used as an internal standard.

5
6 **Quantification of alginate** We measured alginate concentration using a modified
7 carbazole sulfuric acid method (23) and sodium alginate as standard. For this procedure,
8 we added 0.06 ml of 4 M sulfuric acid solution to 0.5 ml of culture suspension and
9 adjusted to pH 7.5 using KOH. We subsequently added 0.06 ml of 1 M borate buffer
10 (adjusted to pH 8.4 using KOH) and mixed it vigorously. The solution was kept in
11 boiling water for 6.5 min after adding 3 ml of H₂SO₄ and cooled on ice for 2 min.
12 Furthermore, after adding 0.125 ml of 0.2% carbazole solution and vigorously mixing
13 the solution; it was boiled for 10 min before measuring absorbance at 525 nm using a
14 spectrophotometer.

15
16 **Metagenome and 16S rRNA gene analyses based on high-throughput sequencing**
17 **data** Whole cells were harvested from the bacterial consortium when OD₆₆₀ reached
18 0.4–0.5. Total genomic DNA was extracted using NucleoSpin Tissue (Macherey-Nagel,
19 Düren, Germany). A paired-end library of the whole genome was generated for
20 metagenome sequencing by Hokkaido system science Co. Ltd. (Sapporo, Japan) using
21 Illumina HiSeq 2000. High-throughput sequencing of 16S rRNA gene and analysis was
22 performed using Roche GS FLX+, Hokkaido system science, in which the 16S rRNA
23 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-RFLP (restriction fragment length polymorphism) and T(terminal)-RFLP 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 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).

Alginate lyase assays The bacterial consortium was cultured in basal medium supplemented with alginate. Cells were harvested by centrifugation (20,400 ×*g* for 10

1 min at 4°C) at OD₆₀₀ = 0.3–0.5. Harvested cells were washed and suspended in 50 mM
2 phosphate buffer (pH 6.8) and subsequently sonicated using Branson Digital sonifier
3 (Branson Ultrasonics, Corp., Danbury, CT) with 2.0 s ‘On’ and 2.0 s ‘Off’ pulses (total
4 10 min) at 20% power amplitude. Cell-free extracts were obtained by centrifuging the
5 sonicated cells (20,400 ×g for 30 min at 4°C). The protein concentrations of the
6 cell-free extracts were determined using the Bradford method. Alginate lyase activity
7 was measured following the method reported by Park *et al.* (2012) with slight
8 modifications. In brief, the cell-free extracts were added to a reaction solution
9 containing 0.1% alginate and 50 mM phosphate buffer (pH6.8). Enzyme activity was
10 monitored by measuring the increase in A₂₃₅ at 30°C. One unit was defined as the
11 amount of enzyme required to achieve an increase in A₂₃₅ of 0.1 per minute.

12

13 RESULTS AND DISCUSSION

14

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

21 Figure 1 shows a typical time course of the batch culture of the bacterial
22 consortium grown on alginate at 30°C. Alginate concentration decreased concomitantly
23 with growth (OD₆₆₀). When alginate was supplied as a the sole carbon source, the
24 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 CO₂ (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 names for genus, family and class were obtained, respectively. A total of 11,024 reads were used for community analyses.

Table 1 shows the results of the taxonomic classification of the bacterial consortium using 16S rRNA gene. These results revealed that the bacterial consortium was constituted by at least three genera of bacteria, *Clostridium*, *Dysgonomonas*, and *Citrobacter*. In particular, the BLAST search identified that the most abundant strains, representing 39.9% and 44.0% of the total bacteria in the consortium, were related to *Clostridiaceae* bacterium SK082 and *D. capnocytophagoides*, respectively.

Clostridiaceae bacterium SK082 was identified as belonging to an unclassified *Clostridiaceae* species. The anaerobic microorganism AN-C16-KBRB isolated from bovine rumen was also closely related to *Clostridiaceae* bacterium SK082, with a 16S rRNA gene sequence similarity of 98% (24). AN-C16-KBRB can degrade cellulose, which possesses a chemical structure similar to alginate in addition to presenting bifunctional endo-/exo-type cellulase CelEdx16 (25, 26). For these reasons, *Clostridium*

1 is thought to play an important role in the degradation of polymers such as alginate. *D.*
2 *capnocytophagoides* has been classified as a group DF-3 organism (dysgonic fermenter
3 3) by the Centers for Disease Control and Prevention (CDC) (27). The biochemical
4 profile of *D. capnocytophagoides* appears to be similar to that of *Capnocytophaga*; in
5 addition, *Dysgonomonas* spp. form a genetic cluster together with *Bacteroides forsythus*
6 and *Bacteroides distasonis*, based on 16S rRNA gene sequences (28, 29). However,
7 alginate metabolism by *Dysgonomonas* has not been reported.

9 **16S rRNA gene cloning analysis and community dynamics of the bacterial**

10 **consortium** As mentioned above, taxonomic classification based on high-throughput
11 sequencing data reveals that the consortium is mainly composed by two dominant
12 strains related to *Clostridiaceae* bacterium SK082 and *D. capnocytophagoides*.
13 However, in both cases, fragment length was not long enough to allow for the
14 identification of the specific strain. To this end, we conducted cloning and detailed
15 sequence analysis of the 16S rRNA gene. 16S rRNA gene fragments derived from the
16 bacterial consortium were ligated into *Sma*I digested pUC19 and cloned. The clones
17 obtained were classified using PCR–RFLP analysis (Fig. 2A). Three taxonomic units
18 were obtained based on the RFLP patterns, designated HUA-1, HUA-2, and HUA-3.
19 Based on the level of sequence similarity for 16S rRNA gene sequence (1400 bp),
20 HUA-1 (accession no. LC020431), HUA-2 (LC020432) and HUA-3 (LC020433)
21 showed to be related to *Clostridiaceae* bacterium SK082 (99%, AB298754), *D.*
22 *capnocytophagoides* (95%, AB548674), and *Citrobacter freundii* (98%, AB680314),
23 respectively. Therefore, we obtained the 16S rRNA gene sequence for the two dominant
24 strains (HUA-1 and HUA-2) and one minor strain (HUA-3) shown in Table 1.

1 We subsequently analyzed the band patterns derived from bacterial consortium
2 obtained following PCR-RFLP of 16S rRNA gene (Figure 2B). The intensity of the
3 band corresponding with HUA-3 was considerably lower than those of HUA-1 and
4 HUA-2.

5 To elucidate the dynamics of the bacterial consortium during alginate degradation,
6 we used T-RFLP as shown in Figure 3. In an initial cultivation phase (0 - 0.5 days), the
7 abundances of HUA-1 were higher than that of HUA-2; however, after 1 day of
8 cultivation, HUA-1 and HUA-2 abundances stabilized at similar levels. On the other
9 hand, the abundance ratio of HUA-3 remained below 1% during cultivation. These
10 results suggested that both HUA-1 and HUA-2 play important pivotal roles in the
11 degradation of alginate.

12 To analyze HUA-1 and HUA-2 strains in more detail, we attempted to isolate these
13 strains using the roll tube method (30) with agar medium containing alginate, which is
14 similar to the medium used to subculture the bacterial consortium except the presence of
15 agar. A 16S rRNA gene analysis, following isolation, revealed that all the small colonies
16 formed belong to the HUA-3 strain. Although all the isolated strains identified as
17 HUA-3 were cultured in a liquid medium containing alginate, none of them were
18 confirmed to consume alginate (data not shown). The culture medium also contained
19 yeast extract, which could have served HUA-3 as carbon source instead of alginate.
20 Despite HUA-1 and HUA-2 were dominant strains in this consortium and its abundance
21 reached 83.9 % in total as shown Table 1, these strains could not be isolated with agar
22 medium containing alginate as a sole carbon source. Indeed, the growth of anaerobic
23 alginate-degrading microorganisms on the agar medium may not be straightforward.
24 However, the bacterial community structure and its alginate degradation ability have

1 now been maintained for more than 5 years. Hence, these results suggest that alginate
2 metabolism in this consortium could have arisen as a consequence of anaerobic bacterial
3 interactions like symbiosis, although the type of symbiosis such as cooperation,
4 commensalism, or other interactions (31) is unknown.

5
6 **Predicted alginate metabolic pathway in the bacterial consortium** The
7 possibility of a symbiotic metabolism of alginate in the bacterial consortium also gives
8 rise to the possibility of a unique alginate metabolic pathway. The first step of alginate
9 metabolism in bacteria is usually the breakdown of alginate into monomers through the
10 action of alginate lyase (11, 32). To confirm the presence of alginate lyase in the
11 consortium, we assessed alginate lyase activity. The level of enzymatic activity detected
12 in cell-free extracts was 2.3 ± 0.3 U/mg protein. We did not detect alginate lyase activity
13 in the supernatant derived from the culture medium. These results indicated the
14 presence of an alginate degradation pathway mediated by alginate lyase in the bacterial
15 consortium. To investigate whether the bacterial consortium possesses all of genes
16 known to be involved in alginate metabolism, we performed a metagenome analysis of
17 the bacterial consortium by high-throughput sequencing techniques using Illumina
18 HiSeq 2000.

19 A total of 32,37,966 reads were obtained by high-throughput sequencing. As the
20 first step of assembly, read data was assembled using program velet. Then, Meta Velet
21 which is extension assemble program of velet was used for meta genome assembly. We
22 used the bacterial Genome Annotation Pipeline (MiGAP) to annotate assembly data,
23 and the annotated gene sequences were translated into amino acid sequences using

1 Genetyx software. A blastp search on DDBJ was subsequently conducted using the
2 translated amino acid sequences.

3 Figure 4 shows a previously reported metabolic pathway to convert alginate into
4 acetate in bacteria (10, 11). Usually, alginate is break down into monomers through the
5 action of alginate lyases. Alginate monomers are then spontaneously converted into
6 4-deoxy-L-erythro-hexoseulose uronic acid (DEH), which is subsequently converted
7 into 2-keto-3-deoxy-D-gluconic acid (KDG) and 2-keto-3-deoxy-6-phosphogluconic
8 acid (KDPG) by KDG reductase and KDG kinase, respectively. Furthermore, KDPG is
9 degraded into Glyceraldehyde-3-phosphate (G3P) and pyruvate by KDPG aldolase. G3P
10 is converted into pyruvate through the glycolytic pathway mediated by G3P
11 dehydrogenase, phosphoglycerate kinase, phosphoglycerate mutase, and pyruvate
12 kinase. Acetate is eventually produced from pyruvate through acetyl-CoA and acetyl
13 phosphate through the reactions mediated by phosphotransacetylase and acetate kinase.
14 The acetate production from G3P or pyruvate is typical metabolism in bacteria. Hence
15 degradation of alginate to G3P and pyruvate by alginate lyase, KDG reductase, KDG
16 kinase, and KDPG aldolase are more important steps in alginate degradation.

17 Metagenomic analysis revealed that the bacterial consortium possessed 10 putative
18 alginate lyase genes (see Table S1), 9 putative KDG reductase genes (see Table S2), 6
19 putative KDG kinase genes (see Table S3) and 6 of putative KDPG aldolase genes (see
20 Table S4). Putative alginate lyase, KDG kinase, and KDPG aldolase genes were also
21 annotated using MiGAP. Both alginate lyase and heparinase belong to polysaccharide
22 lyase families (33). The amino acid sequence of alginate lyase is known to be highly
23 similar to that of heparinase II/III family proteins; in particular, exo-type alginate lyases
24 possess a domain in the C-terminal region found in heparinase II/III family (34). For

1 this reason, putative heparinase II/III are also included as alginate lyase candidates in
2 Supplemental Table 1.

3 Putative KDG reductase genes could not be annotated by MiGAP; therefore, we
4 performed a local blast X using Genetyx software for known KDG reductase gene
5 (A1-R) derived *Sphingomonas* sp. A1 (35). This search resulted in 9 putative genes. The
6 amino acid sequence of the putative KDG reductase showed 28%–32% identity with the
7 A1-R amino acid sequence. An additional BLAST search on the DDBJ server showed a
8 high level of identity between the putative KDG reductase and known short-chain
9 dehydrogenase/reductase (SDR) superfamily proteins. In fact, the A1-R gene belongs to
10 the SDR superfamily of proteins. Figure 5 shows a multiple sequence alignment of
11 A1-R and putative KDG reductases. Putative KDG reductases possess highly conserved
12 sequence motifs characteristic of the SDR superfamily of proteins (35), such as a
13 glycine-rich pattern in the cofactor binding Rossmann fold region
14 (Thr-Gly-X-X-X-Gly-X-Gly) indicated in the figure by black arrows and a catalytic
15 triad (Thy, Lys, and Ser) indicated by white arrows. Hence putative 9 proteins were
16 considered to be KDG reductase. The enzymes involved in conversion from pyruvate to
17 acetate were annotated by MiGAP and shown in Table 2.

18 In relation to the alginate to pyruvate pathway, the genes associating with
19 *Clostridium* were the most abundant ones, as shown supplemental tables. Total 14 genes
20 associated with *Clostridium* were confirmed. Among them, these are 6 putative alginate
21 lyase genes (see Table S1), 4 putative KDG reductase genes (see Table S2), 3 putative
22 KDG kinase genes (see Table S3) and 1 of putative KDPG aldolase genes (see Table S4).
23 From the taxonomic classification of 16S rRNA gene based on high-throughput
24 sequencing data, *Clostridium* was the dominant genus in the consortium. Since

1 abundance of HUA-1 reaches 94.5% in the strains classified as *Clostridium*, HUA-1
2 may possess some of these genes involved in alginate metabolism. Genes related to
3 *Citrobacter*, comprising a series of minor strains in the consortium, were not found
4 upstream of the alginate to pyruvate pathway; however, the downstream pathway
5 contains several genes (KDG kinase and DKPG aldolase) belonging to *Citrobacter*.
6 Thus, our results suggested that *Citrobacter* may be involved in the downstream section
7 of the alginate degradation pathway. On the other hand, only one gene associated with
8 *Dysgonomonas* was confirmed, despite a *Dysgonomonas* was dominant in the
9 consortium. It has not been reported whether *Dysgonomonas* can degrade alginate.
10 Therefore, contribution to alginate degradation of *Dysgonomonas* is unknown.

11 To the best of our knowledge, the pathway of anaerobic alginate degradation by
12 microbial consortium has not been previously reported. In this study, metagenome
13 analysis revealed that the bacterial consortium studied here possessed all candidate
14 genes involved in the predicted metabolic pathway of the alginate to acetate. These
15 novel findings aid the further analysis of anaerobic alginate degradation by bacterial
16 consortia. However, anaerobic degradation mechanisms of alginate by bacterial
17 consortia are not still understood. Therefore, further efforts are required to isolate and
18 characterize the key bacteria from this consortium.

19

20

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21

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Figure legends

FIG. 1. Bacterial growth and alginate consumption in cultures of the bacterial consortium. Each experiment was performed in triplicate. Values represent the mean $\pm$ 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 (circle) and alginate consumption (triangle).

FIG. 2. (A) 16S rRNA gene analysis of the clones by PCR-RFLP and (B) PCR-RFLP analysis of the bacterial consortium. (A) Lane 1: DNA size marker; Lane 2: PCR-RFLP HUA-3 band; Lane 3, 5, 6, 8, 9, and 10: HUA-1 bands; Lane 4 and 7: HUA-2 bands. (B) Lane 1: DNA size marker; Lane 2: PCR-RFLP bands for the bacterial consortium; Lane 3: HUA-1 bands; Lane 4: HUA-2 bands; Lane 5: HUA-3 bands

FIG. 3. Community structure of the bacterial consortium revealed by T-RFLP. Black and white bars represent the abundance of HUA-1 (black bar) and HUA-2 (white bar), respectively, in the bacterial consortium.

FIG. 4. Predicted alginate metabolism pathway in the bacterial consortium. 1: alginate lyase; 2: KDG reductase; 3: KDG kinase; 4: KDPG aldolase; 5: G3P dehydrogenase; 6: phosphoglycerate kinase; 7: phosphoglycerate mutase; 8: pyruvate kinase; 9: pyruvate dehydrogenase; 10: phosphotransacetylase; 11: acetate kinase. DEH: 4-deoxy-L-erythro-hexoseulose uronic acid; KDG: 2-keto-3-deoxy-D-gluconic acid; KDPG: 2-keto-3-deoxy-6-phosphogluconic acid; G3P: Glyceraldehyde-3-phosphate.

1

2 **FIG. 5.** Multiple sequence alignment of A1-R and candidate KDG reductase genes
3 present in the bacterial consortium. Numbers 2–1 to 2–9 coincide with the numbers in
4 Table 3. Solid arrow shows the motif of the cofactor binding Rossmann fold region
5 (Thr-Gly-X-X-X-Gly-X-Gly) from the SDR super family of proteins. Open arrows
6 indicate the catalytic triad (Thy, Lys, and Ser).

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1 Highlights

- 2 • Alginate-degrading bacterial consortium was obtained from sand on the beach.
- 3 • This consortium was mainly composed of two dominant genera.
- 4 • This consortium possessed all the candidate genes involved in the alginate
- 5 metabolic pathway.

6

7

Table 1. Taxonomic classification of the species in the consortium, based on 16S rRNA gene sequence

Related genera	Related strains	Designation	Similarity (%)	Accession no.	Abundance (%)	Total abundance (%)
<i>Dysgonomonas</i>	<i>D. capnocytophagoides</i>	HUA-2	94	AB548674	44.0	44.0
<i>Clostridium</i>	Clostridiaceae bacterium SK082	HUA-1	99	AB298754	39.9	42.2
	<i>C. saccharolyticum</i> WM1		99	CP002109	1.7	
	<i>Clostridium</i> sp. AP81		99	AB273730	0.3	
	<i>Clostridium</i> sp. K11		98	AB610562	0.1	
	<i>Clostridium</i> sp. A4-27		98	AB238848	0.1	
	Other 6 strains				0.1	
<i>Citrobacter</i>	<i>C. murlinae</i>	HUA-3	99	JN092600	6.1	11.7
	<i>C. freundii</i>		99	AB680314	3.1	
	<i>Citrobacter</i> sp. 40		99	HQ399664	2.0	
	<i>Citrobacter</i> sp. NG2(2010)		99	GU566312	0.4	
	<i>Citrobacter</i> sp. Binzhou CLT		98	AF530068	0.1	
Other genera	<i>Pantoea</i> sp. LM25(2011)		98	HQ891940	0.7	2.1
	Methanotrophic bacterium H4		99	HQ424981	0.4	
	<i>Enterobacter aerogenes</i> KCTC2190		98	CP002824	0.3	
	<i>Klebsiella</i> sp. LB-2		99	DQ831003	0.3	
	<i>Serratia</i> spl. E98a		99	JF513189	0.2	
	<i>Raoultella ornithinolytica</i>		99	HM231274	0.1	
	<i>Desulfotomaculum</i> sp. CYP1		99	DQ479411	0.1	

Table 2. Candidate genes encoding enzymes involved in acetate production from glyceraldehyde-3-phosphate

Enzymes	Numbers of ORFs
Glyceraldehyde-3-phosphate dehydrogenase	9
Phosphoglycerate kinase	4
Phosphoglycerate mutase	14
Pyruvate kinase	6
Pyruvate dehydrogenase	5
Phosphotransacetylase	5
Acetate kinase	6

Figure 1, Kita et al.

Fig. 2, Kita et al.

Fig. 3, Kita et al.

Fig. 4, Kita et al.

A1-R	1	-----MFPDLKGRVLT	↓↓	TGSSQGI	↓↓	GLATARLFARAGAKVGLHGRKAPANIDE	47
2-1	1	-----MRAVITGGTSGI		GLSTAIELGKAGYDIIITGIS		-HEDGKS	39
2-2	1	-----MLDGKIAVVTGASRGI		GRAIALRLGLEGATVIVNYNGSEEKALQ			44
2-3	1	-----MKLKGKVAVVTGGARDL		GREISLKLAKGAKVVVNYFDKIEEAE			45
2-4	1	-----MHIDLTGKKALVTGASRGL		GRAIALSLAKAGADVITYEKSAEKAQA			47
2-5	1	MVPKPVFNPNYIGTGKLDKAALVTGGDSGI		GRAVSLAFAGEGADISIVYYDEHEDAQI			60
2-6	1	-----MNRNVLTGASRGI		GAAIALAFAGKADNVGINYLFDQEGAEN			42
2-7	1	-----VDQQILKGKYAIVTGGSDGI		GLAIKVFAGNGANIVLIA-RNEKKLEQ			47
2-8	1	-----MLDGKIAVVTGAARGI		GRAIAVKLAGQGATVIINNGSDSKAME			44
2-9	1	-----MKLLEGKTAIITGAARGI		GKAIKIAKYAQEGANIAFTDLAVDDNAKQ			46
A1-R	48	TIASMRADGGDAFFAADLATSEACQQLVDFVAKFGG-		IDVLIINNAGG--		LVGRKPLPE	104
2-1	40	ALETLSHEINSEFIYSDSMNEEDVNRCFEKISQTYDS-		LDVLVNNVGG--		LGGQRQFEE	96
2-2	45	VVKEIEEKGGKAEAIACNVSDYEASGIFLEGVIKKYGK-		LDILINNAGI--		TRD-NLLMK	100
2-3	46	TKSLIETVGGECIIVQGDMTKATDVKKLFDAGKEAFGSKLD		ILVNVVGG--		LVGRKQITE	103
2-4	48	VANEITALGRHGEAIQANSANAQAIQNAVNHITQSLGG-		LDILVNNAGI--		ARG-GPLES	103
2-5	61	TKSYIEAQGRKCILISGDVDRDQLFCQNAVSETVSAFGG-		LDVLVNNAGV--		QFPQKGIED	117
2-6	43	TCMAAREMGVKAEMYQADVGNAKEDDEEMFAAFLOEFGK-		IDVLIINNACGGLKIPEGGFES			101
2-7	48	VKQDLSVYGVGIYTFADLGVLPVKTISEKILRTIPK-		VDILINNAGI--		GRFIPFKE	103
2-8	45	VVKEIEEKGGTAEAIQCNSVDYESVGAFLGVIKKYGK-		LDILVNNAGI--		TRD-NLLMK	100
2-9	47	TEKEIAALGVKVAYASNAADFDDTHKVVDIAIVADFGK-		VDILVNNAGI--		TRD-GLMMR	102
A1-R	105	IDDTFYDAVMDANIRSVVMTTKFALPHLAAAASGQTS		SAVISTGSIAGHTG-		GGPGAGL	163
2-1	97	METSFMRKVMALNFDSAFFASRAAIPLL-----		KKGNHPSIINYSTIAVTS-		GGPGAGI	150
2-2	101	MSEEEFDAVINTNLKGVFNCMKHVSQR-----		IKQRGGRIINISS-		VSGIL-GNAGQAN	153
2-3	104	QDEAWYDFLMDVNMRSWCWTREAVPYI-----		TEGG--SIINFSSLAARDG-		GGPGASM	155
2-4	104	LSLEDIDALINVNIRGVVVAIQALPHL-----		PEGGRIINIGSCLANRV-		AQPGIAV	155
2-5	118	ISVEQMVLTFEVNFFGIFIMTKMALPYL-----		KDGS--TIINTTSITAYVG-		NEQLID-	168
2-6	102	MPLTYWDEMMAINISAAAYCSSYAVKDM-----		VEKKTAGSIVNISSVH		SVVTYVKKRFTF	157
2-7	104	TSEEALDLHINLNIRAPYFLTQYLLESL-----		IESKGNVINISSYFSHRMLS		GRDSTA	157
2-8	101	MSEEDFDAVINTNLKGVFNCMKHVARQM-----		IKQKSGRIINISS-		VSGVL-GNAGQAN	153
2-9	103	MTEQQWDLVINVNLSAFNFIHAITPVM-----		MKQKSGSIINMSS-		VVGLS-GNAGQTN	155
A1-R	164	YGAAKAFLHNHVHKNWVDFHTKDGVRFNIVSPGTVDI		-----		AFHADKTQ-DVRDRISN	215
2-1	151	YAASKAAIEGVTRALAKDLAEYGIRVNAVSPGTIDT		-----		AFHSATKR-EILESWE	202
2-2	154	YCAAKAGVIGMTKSFARESASRCITVNAIAPGYVQT		-----		DMTDVLS-DVKEATLD	205
2-3	156	YATAKAAMVTFTRSMAGELGPKGIRVNALAPGTIAT		-----		SFHDRFNTPENREKMS	208
2-4	156	YAMTKSALNSLTRGLARDLGPRGITVNLVHPGPTDS		-----		DMNPADGE-QADSQR-Q	206
2-5	169	YSSTKGAIVSFTRAMAHSLVSKGIRVNAVAPGPIWT		-----		PLQPSSWPADYIPTFGS	221
2-6	158	YCAAKAGLNLTKTMGVVDINHGIHVNNAVAPGFIST		-----		SATTRYNQ-EELRAFLR	209
2-7	158	YSLTKGAIDSFTKSLAFEMGKGVNVNAIAPGSIQTP		QQLNLNIEKLTPHEQERF		REMIQT	217
2-8	154	YCAAKAGVIGITKSFAREVASRGITVNAVAPGFIHT		-----		DMTDVLS-DVKAASLE	205
2-9	156	YSASKAGMIGLAKSIAKEIGSRGIRANCICPGFIIT		-----		EMTGALSD-DVKKQWAE	207
A1-R	216	GIFPMGRFGTAEEEMAPAFLEFFASHLASGYITGQVLD		INGGQYKH-----			258
2-1	203	GILMKRLGEPSEVATVIGFLASE-KASFITGEIIQING		QGVFI-----			244
2-2	206	QIFPMKRFGTPEDIAAAAVFLASD-PAGYITGQVISV		DGGMAM-----			246
2-3	209	VYPLRREGDAGDVADLVAFLAAD-DSDYITGTNIDIN		GGIFFS-----			250
2-4	207	LIATGHYGTPELVAAAVTFLASP-AAGQISGTGLD		VGGGLNA-----			247
2-5	222	DTPMKRAGQVELAPTIVYVLASN-DSSFVTGQVLH		VGGQSMQS-----			264
2-6	210	KIPAGRLGKVEDITPLVLFLADPEKSGFTVGQTFV		VGGQSIDGTIDYMLQE		EMKGEDKA	269
2-7	218	IYPLGKIGEADVAQAALFLASD-QAKWITGSIMS		VGGGLTTN-----			259
2-8	206	QIPLKRFSGAEDIAGTVAFLASE-EAGYITGQVISV		DGGMAM-----			246
2-9	208	QIPLRRGTPEDVANVAVFLGSD-LSSYVSGQAIPV		CGGMNM-----			248

Fig. 5, Kita et al.