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Title	A First Marine Vibrio Biocatalyst to Produce Ethanol from Alginate, which is a Rich Polysaccharide in Brown Macroalgal Biomass
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- 32 **Running title**
- 33 A First Vibrio Biocatalyst to Produce Ethanol from Alginate

35 Abstract

The use of un-utilized feedstock and seawater for material and/or energy production using marine 36 microbial catalysts is one potential option towards contributing to the development of a more 37 38 sustainable society. Ethanol production from alginate, which is an oxidized polysaccharide present in 39 brown seaweed, is extremely difficult due to the imbalance of reducing power in the microbial cells. 40 Production of ethanol by such means has so far been unsuccessful using marine microbial biocatalysts. To produce ethanol from alginate, an alternative pathway consisting of a pyruvate decarboxylase gene 41 (pdc) and an alcohol dehydrogenase II gene (adhII) derived from Zymomonas mobilis strain ZM4 42 was implemented into a metabolically engineered bacterium, Vibrio halioticoli, which is a 43 44 representative marine alginate decomposer. No ethanol from alginate was produced in the wild-type 45 V. halioticoli, however, the engineered V. halioticoli harboring the pdc and adhII operon (Pet operon), 46 designated to the V. halioticoli (Pet), was able to produce 880 mg/L ethanol in maximum from 1.5% alginate for 72 h. The Pet operon also worked on the other marine alginolytic vibrios for ethanol 47 production from alginate. This is the first case of ethanol production from alginate using marine 48 49 bacterial biocatalysts under seawater-based media.

50

51• Keywords: vibrio, ethanol, metabolic engineering, seaweed, marine
52

53 INTRODUCTION

54 Sustainable energy production and supply are two of the critical global issues in developing a more sustainable society. To combat the negative effects on both the environment and human society 55 56 due to the use of fossil fuels, the development of alternative renewable energy has been undertaken since the late 1970's after the so-called 'oil shock' era [1-3]. In particular, the last four decades could 57 58 be defined as the era of biomass energy revolution; there have been a number of technical innovations 59 in the production of ethanol, hydrogen and methane using biomass as feedstock [1-2,4-7]. Among 60 these, bioethanol is the only fuel which is commercially available as a realistic gasoline (transportation fuel) alternative. Generally, biomass energy uses land-based biomass, such as corn, 61 sugar cane and switch grass, as types of feedstock. This supply of biomass has caused a controversy 62 in the food vs energy debate (FvE) [e.g. 8,9]. To overcome these limitations, there have been many 63 64 attempts to use marine biomass as a feedstock for bioethanol production [8-15] because marine biomass has many advantages not only in solving the FvE issue but also in its rapid growth, lack of 65 lignin, fewer fertilization uses, and soft texture capable of being milled and crushed by simple 66 mechanical operations [11-12,15]. The uses of marine biomass also could contribute to balancing CO₂ 67 68 in the ocean to sustain Blue Carbon [16]. Traditional aquaculture has been capable of strengthening 69 regional seaweed production in Japan, Chile, China, Indonesia, the Philippines, South Korea, however, bioethanol production from marine biomass from alginate is extremely difficult due to the absence of 70 71 traditionally matured biotechnologies in alcohol fermentation or in energy production using seaweed 72 as feedstock [9-12,15].

Alginate is a major component of brown algae showing viscous features [10]. The structure is constructed by linear block copolymers of uronic acids; β -D-mannuronate (M) and α -L-guluronate (G). They are arranged in homopolymeric and heteropolymeric forms; poly-mannuronate (polyM) and poly-guluronate (polyG) are for homo- and MG random, respectively [15,17]. The high oxidative state of the polyuronate structure also forces the use of the Entner-Doudoroff pathway following the TCA cycle in bacteria [10,12,18], which causes lower levels of alcohol production due to reducing 79 power and energy imbalances. Nevertheless, there have also been many reports on alginate-degrading microorganisms isolated from terrestrial and marine environments [19-21], however, up to now it has 80 not been reported that wild-type strains of alginate assimilating bacteria could produce ethanol. In 81 2011, successful ethanol production from alginate by genetically engineered Sphingomonas sp. A1 82 was reported [10], and also by E. coli [12]. The Sphingomonas sp. A1 implemented Pet-operon and 83 84 deleted *ldh* gene can ingest "intact" alginate through the super-channel and produced ethanol at a 85 maximum 13 g/L from 5% (w/v) alginate as a substrate after 3-days culture under aerobic conditions 86 [10]. Genetically engineered E. coli implemented with genes encoding alginate degradation, transport, and metabolism derived from V. splendidus and the production of ethanol (Pet)-operon produced 20 87 88 g/L ethanol from an artificially prepared substrate mixture consisting of alginate, mannitol, and 89 glucose at a ratio of 5:8:1, which is determined in a wild seaweed, Sacharina japonica [12]. However, during the culture with seaweed, salt from both raw and dried seaweed caused lower cell growth and 90 91 ethanol production in these bacteria from terrestrial origins because these bacteria show a low 92 tolerance to salinity. Therefore, a better microbial biocatalyst more tolerant to salinity is needed rather 93 than terrestrial bacteria in order to achieve efficient ethanol production from marine biomass.

94 Vibrio halitoticoli is a facultatively anaerobic, non-motile, alginolytic marine bacterium, 95 isolated from Haliotis discus hannai, and produces ethanol from mannitol [19]. Nine more related species have been described, and they form a monophyletic clade, Halioticoli [22-23]. The 96 fermentation pathway was predicted to be similar to that of E. coli on the basis of genome-wide 97 metabolic pathway reconstruction [23-24]. Ethanol production from alginate has never been reported 98 99 using the wild-type of this bacterium, nevertheless acetic acid production from alginate in this 100 bacterium has been achieved [25]. As mentioned above, the major reasons why the bacterium did not 101 produce ethanol were due to an imbalance of reductive power in alginate metabolisms [10,12,23,26]. 102 A sugar alcohol, mannitol, generates two molecules of NADH, but alginate generates only one 103 molecule of NADH during metabolism [24].

104

The aims of this study are to create a metabolically engineered V. halioticoli to compensate

105 for the reducing power and produce ethanol from alginate. To achieve this purpose, we tried to 106 introduce two genes; pyruvate decarboxylase (pdc) gene and alcohol dehydrogenase II (adhII) gene 107 both derived from Z. mobilis strain ZM4 [6] (Fig. S1). Z. mobilis, which is used in the tropics in the production of pulque and alcoholic palm wines, using the Entner-Doudoroff (ED) pathway to 108 109 metabolize glucose, which results in only one mole of ATP being produced per one mole of glucose. This bacterium was isolated from locally brewed alcohol and possesses the ability to ferment alcohol, 110 and is currently utilized commercially in the production of tequila [18]. The Pdc catalyzes the 111 112 irreversible conversion of pyruvate to acetaldehyde, in which NADH is not required. The Pdc has the lowest Km value: 4.4×10^{-3} M at pH 6.0 [27]. AdhII catalyzes the reversible reaction of acetaldehyde 113 to ethanol. The optimum pH of AdhII is pH 5.0 for acetaldehyde reduction and pH 9.5 for ethanol 114 115 oxidation, respectively [27]. Those genes were arranged in tandem to create a synthetic operon, which is called the Pet (production of ethanol) operon, and it has already been confirmed that the expression 116 117 of the Pet operon enables ethanol production from glucose in E. coli [6]. We also expect that the expression of the Pet operon saves reducing powers in the cells, and these excess reducing powers 118 might be used for ethanol production in V. halioticoli [6,23-24]. Here we report the first creation of 119 120 new marine microbial biocatalysts based on alginolytic marine vibrios including V. halioticoli 121 implemented with the Pet operon resulting in ethanol production from alginate.

122

123 MATERIALS AND METHODS

124 Bacterial Strains and the Culture

E. coli strains were grown in LB agar medium at 37°C. *V. halioticoli* IAM 14596^T was grown
on ZoBell 2216E agar medium supplemented with 0.5% sodium alginate at 25°C [19]. In addition to *V. halioticoli*, seven alginolytic marine vibrios belonging to the Halioticoli and Splendiduds clades
[22-23] were also used in this study. They were grown on the same medium used for the *V. halioticoli*.
The *Z. mobilis* strain ZM4 possessing *pdc* and *adhII* genes was grown in YG agar medium at 30°C
[18,28,29].

132 Construction of Recombinant Vibrio halioticoli

133	The	pdc	and	adhII	genes	were	amplified	by	PCR	using	primers	1
134	(CGCGCATC	GCGCA	ATGA	GTTAT	ACTGC	Г)			and			2
135	(GTGTCTAC	GAAAA	ACTAC	GAGGAG	GCTTGT	TAACA	AGGC),	а	nd	prim	iers	3
136	(ACGGCATO	GCGGG	GTGA	GGTTAT	AGCTA	ΓG)			and			4

137 (GGCTCTAGAGAAAACCGTTTTCCTGTTTTG) from a chromosomal DNA of Z. mobilis ZM4 as 138 a template, respectively. Single PCR product was digested using SphI and XbaI and ligated into a SphI/XbaI-treated pVSV208 [Cm^r, rfp] vector [30]. Both PCR product and vector were purified after 139 140 an agarose gel electrophoresis. Each gene was located under the control of *lac* promoter of pVSV208. The resultant plasmid was first transformed into E. coli DH5 α λ -pir by electroporation using 141 142 MicroPulser according to the protocol provided by the manufacturer (BioRad, Hercules, CA), and 143 then the recombinant plasmid harboring pdc or adhII was transferred to wild-type V. halioticoli by a triparental mating with *E. coli* strain CC118 λ -*pir* containing pEVS104 (Km^r, *oriT*) as a helper [31]. 144 Before the triparental mating, E. coli colonies showing higher Pdc activity were selected based on 145 146 growth on LB agar plate supplemented with 2% glucose for 3 days at 37°C. Bigger colonies were 147 reported to show higher Pdc activity than normal because of increased substrate utilization rates and resultant improved cell growth [6,32]. Recombinant V. halioticoli cells were further screened on a 148 149 TCBS plate at 25°C based on higher growth rates, and the selected colonies were purified on a ZoBell 2216E agar plate supplemented with 0.5% alginate and 5 µg/mL chloramphenicol at 25°C. Finally, 150 151 positive recombinant *V. halioticoli* possessing *pdc* or *adhII* genes were confirmed using a colony PCR. 152 Enzyme activities of Pdc and AdhII were measured in the PCR positive vibrios manually (see the 153 following section Enzyme Assay for Pdc and AdhII). Pdc and AdhII expressing recombinant V. halioticoli strains were named as V. halioticoli (pVIP208) and the V. halioticoli (pVIA208), 154155 respectively. The recombinant plasmids were extracted and purified from those recombinant V. halioticoli cells using Pure Yield kit (Promega, Madison, WI) for further steps to obtain the Pet 156

157 (production of ethanol) operon.

158 To obtain a plasmid containing the Pet operon containing pdc and adhII gene cassettes, an overlap PCR was performed [33-34] (Fig. S2). The overlap PCR has been widely used in site-directed 159 mutagenesis or in splicing DNA fragments. The overlap PCR method consists of two rounds of PCRs. 160 161 two target fragments are amplified using primers In the first round, 1 and 5 (GTTGAAGAAGCCATAGCTATAACCTCACCCAAACTAGAGGAGCTTGTTGAACAGGCTTA 162 2 163 CG) and primers and 6 164 (CGTAAGCCTGTTAACAAGCTCCTCTAGTTTGGGTGAGGTTATAGCTATGGCTTCTTCAAC), which added overlapped sequences of about 25 bp to join two genes, and pVIA208 and pVIP208 165 as templates, respectively, and then purified. In the second-round PCR, two PCR products were 166 167 annealed by the overlapped sequences using a thermal cycler, and then further PCR using specific primers 1 and 4 was performed to obtain the PET operon. PCR was performed using pfu DNA 168 169 polymerase (Promega) under the following conditions: 94°C for 1 min, 5 cycles (94°C for 1 min, 55°C for 30 s, and 72°C for 4 min). The overlap PCR product was digested with SphI and XbaI, and 170 then ligated into SphI/XbaI-treated pVSV208. The resultant plasmid was introduced into wild-type V. 171172halioticoli by triparental mating in the same way as described above. The Pdc and AdhII activities of 173 the selected colonies were measured (see the section below on Enzyme Assay for Pdc and AdhII) and confirmed sequences of inserted genes. Recombinant vibrios other than V. halioticoli were also 174175 created using the same methodology.

176

177 Enzyme Assay for Pdc and AdhII

V. halioticoli cells were cultured in 100 mL flasks containing 50 mL of ZoBell 2216E broth at 25°C with shaking (130 rpm). The cultured cells were collected by centrifugation (6,200 x g, 4°C, Allegra 21R, Beckman, California), and washed once with 50 mM MES buffer (pH 7.5), suspended in the same buffer, and then finally treated with the addition of a couple of drops of chloroform. After centrifugation at 19,000 x g for 2 min. at 4°C, the supernatant was used as a crude enzyme fraction. 183 The Pdc assay was performed by monitoring the pyruvate-dependent reduction of NAD with alcohol dehydrogenase as a coupling enzyme [28]. In the first reaction, pyruvic acid is reduced to 184 acetaldehyde, and the acetaldehyde is further reduced to ethanol accompanied by the NAD reduction 185 in the second step. These reactions were performed at room temperature at pH 6.0. AdhII assay was 186 performed by monitoring the ethanol reduction of NAD [29]. The reactions were performed at room 187 temperature at pH 10.8. Increases or decreases in absorbance at 340 nm were continuously monitored 188 189 using a spectrophotometer (Ultrospec2000, Pharmacia Biotech, Little Chalfont, UK). One unit of Pdc 190 or AdhII activity was defined as 1 mmol/min change of substrate. Protein concentration was measured 191 by spectrophotometer (Ultrospec2000, Pharmacia Biotech) at the absorbance of 280 nm. Specific 192 activities were measured in triplicate samples.

193

194 **Ethanol Production**

195 Recombinant strains prepared in this study were pre-cultured in a 300 mL flask containing 100 mL of the ZoBell 2216E broth supplemented with 5 µg/mL chloramphenicol at 25°C for 24 h. 196 A total of 1 mL pre-cultured strain was inoculated in 99 mL of ZoBell 2216E broth supplemented 197 198 with substrates (1.5% alginate or 1.5% mannitol), 1.5% glutathione, 0.1 M MOPS (Dojindo Lab., 199 Kumamoto, Japan) and 5 µg/mL chloramphenicol at pH 7.5, and a batch culture for ethanol production was carried out. pH controller (DT-1023P, Tokyo) was used to maintain a pH of 7.5 by 200the addition of filter-sterilized 5 M NaOH. The batch culture was basically carried out at 20°C for 201 72 hours, and was stirred continuously using a magnetic bar and a magnetic stirrer (RO 10 power 202IKAMAG, IKA, Staufen, Germany) at 100 rpm for 72 h. Subsamples (1 mL) were taken using a 1 203 204mL syringe every 24 h under aseptic conditions.

205

206 Analytical Methods

Ethanol production was measured using gas chromatography (GC2014, Shimadzu, Kyoto,
Japan) with HP-B ALC column (Agilent, Santaclala, CA, USA). Organic acids were measured

using high-performance liquid chromatography (Shimadzu, Kyoto, Japan) with tandem Shim-pack
SCR-101H column system according to the manufacturer's protocol (Shimadzu). Cell growth was
monitored using a spectrophotometer (Ultrospec2000, Pharmacia Biotech) at 620 nm as OD620.
Quantification of alginate was performed with the phenol-sulfuric acid method, using sodium

214

213

215 Sequencing of *pdc* and *adhII* genes

alginate as the standard [35].

Recombinant *V. halioticoli* was cultured in 1,000 mL flask containing 400 mL ZoBell 2216E
broth containing 5 µg/mL chloramphenicol for 24h. Plasmid extraction was performed using Pure
Yield Plasmid Miniprep System (Promega, Madison, WI). Extracted plasmid was purified using
DNeasy Blood & Tissue Kit (QIAGEN, Valencia, USA) and concentrated with ethanol precipitation.
DNA sequencing was performed using a BigDye Terminator v3.1 Cycle Sequencing Kit and Genetic
Analyzer 3130xl according to the protocol provide by the manufacturer (Applied Biosystems, Foster
City, CA, USA).

223

224 Statistics

Bayes statistics was used for test whether null hypothesis (H₀), which is the effect size=0, was more probably occurred or not than the alternative hypothesis (H₁) based on bayes factor (BF₁₀) using JASP software version 0.17.1 (https://jasp-stats.org/).

228

229 **RESULTS and DISCUSSION**

230 Metabolic engineering has opened new doorways in material production using mainly model

231 microorganisms such as *E. coli*, *Saccharomyces* and so on [6-7,12-13,15]. In this study, we have

successfully engineered alginolytic marine vibrios, and have produced ethanol from alginate, which

is a highly oxidized polysaccharide contained in brown macroalgal biomass. To our knowledge, this

234 is the first report of successful ethanol production from alginate using recombinant marine bacteria.

235 Three types of recombinant V. halioticoli strains, harboring adhII, pdc, and the Pet operon 236 (adhII-pdc gene cassette) were constructed. These recombinants were named as the V. halioticoli (pVIA208), the V. halioticoli (pVIP208) and the V. halioticoli (Pet), respectively. Specific activities 237 of Pdc or Adh in a wild-type V. halioticoli were 0.002 U/mg protein and 0.008 U/mg protein, 238 respectively, however, the specific activities of Adh in the recombinant V. halioticoli (pVIA208) and 239 Pdc in the recombinant V. halioticoli (pVIP208) increased to 0.017 U/mg and 1.07 U/mg, respectively 240 241 (Fig. 1). The Adh activity remained at low levels in the V. halioticoli (pVIA208), but the Pdc activity 242 was significantly higher than that of the wild type. Furthermore, both Pdc and Adh activities greatly increased in the recombinant V. halioticoli (Pet) compared to those of the recombinant V. halioticoli 243 implemented by single gene, reaching 7.8 U/mg protein and 2.8 U/mg protein, respectively (Fig. 1). 244These specific activities of Pdc and Adh in the V. halioticoli (Pet) increased 3,459- and 355-folds, 245respectively. More Pdc activity than AdhII activity was also measures in E. coli (Pet) [6], so similar 246 247 trends of the Pet operon under control of lac promoter were observed in this marine vibrio.

Nevertheless, no apparent ethanol peak was detected in the culture supernatant of wild-type 248 V. halioticoli in a culture, apparent and reproducible ethanol production was measured in that of 249 250recombinant V. halioticoli (Pet) (Fig. 2AB). The ethanol production reached 880 mg/ L from 15 g/L 251 alginate as a substrate up to 72 h maximum (612±237 SD mg/L in average in triplicate samples). Almost all the alginate, which corresponded to 99.999% substrates, was consumed, and only 0.2 252253mg/L alginate remained, during the culture period. The conversion efficiency of ethanol was 1%. Levels of organic acid production between the engineered and wild was strains were unlikely to be 254255 different, but the recombinant V. halioticoli (Pet) produced slightly higher lactate (120 mg/L) than 256that (60 mg/L) of the wild-type V. halioticoli (Fig. 2C). The growth of the recombinant V. halioticoli 257 was at the same level to that of wild-type, in particular, until those stationary phases (data not shown). In preliminary experiments, more ethanol production by the engineered V. halioticoli (Pet) 258259 was observed at pH 7.5 and 20°C.

Seven more recombinant alginolytic vibrios (Pet) were also constructed in this study (Fig. 3). The recombinant *V. rarus* RW22^T (Pet) produced ethanol at 656 mg/L, followed by the recombinant *V. pelagius* (Pet) (628 mg/L), *V. halioticoli* (Pet), and the other strains (*V. comitans, V. ezurae, V. gallicus, V. inusitatus*, and *V. neonatus*) (below 400 mg/L ethanol). Apparent ethanol productions by recombinant vibrios from alginate rather than those of wild type strains were strongly supported based on Bayes factors (BF₁₀=5483.440) (Fig. 3).

266 The Pet operon, consisting of pdc and adhII genes cassette form Z. mobilis ZM4, 267 developed by Ingram et al. (1987), is a powerful tool to create ethanologenic E. coli [6]. The Pet operon was widely used to engineer alginolytic terrestrial microbes to be homoethanologenic 268269 because of the imbalance of reducing powers between the oxidized substrate, alginate, and ethanol, 270even under aerobic conditions in alginolytic Sphingomonas sp. A1, and in a dual engineered alginolytic E. coli [12] and a yeast [13,14]. In addition, it is proved that the Pet operon also worked 271 272 well in not only the alginolytic V. halioticoli cells but also in the other alginolytic marine vibrios and was homoethanologenic. We tried to use the other pdc and adhII homologus genes from the 273 274other Z. mobilis strain, but only genes from ZM4 show positive effects on ethanol production from 275alginate in this marine vibrio (data not shown), so pdc and adhII genes from Z. mobilis ZM4 are 276 essential for creating ethanologenic vibrios. Unfortunately, engineered terrestrial microbes such as E. coli, Sphingomonas, and yeast do not tolerate saline and/or marine culture conditions, so the 277278success of ethanol production using engineered marine vibrios under marine culture conditions extends the uses of Pet operon in marine biofuel production. Considering the limitations of 279280freshwater use in this current global warming era, those engineered vibrios could contribute to 281 sustainable biofuel production using seawater-based media minimizing the need for freshwater in 282 achieving the future Carbon Neutral World.

In the engineered *V. halioticoli* (Pet), more lactate was likely to be produced than in the wild type (Fig. 2C), which suggests unexpectedly greater reducing powers of the engineered vibrio cells leaked in pyruvate node to lactate during the alginate metabolism. Lactate leakages were also

286 observed in five more marine vibrio recombinants. Such leakage of reducing powers from a pyruvate node to lactate has been frequently reported in E. coli [12, 36], so the construction of ldh 287 knockout mutants in these vibrios could be effective in bioethanol production using alginate as a 288 289 feedstock. In the engineered Sphingomonas sp. strain A1, the functioning of the Pet pathway was 290 successful under aerobic conditions with vigorous shaking [11]. These aerobic culture conditions could repress the expression of *pfl* gene encoding pyruvate-formate lyase, which causes the 291 292 reduction of carbon flux to formate and acetate production. With the *ldh* knock out and optimum 293 expression of Pet pathway in those engineered marine vibrios, more ethanol production from alginate will be examined in further studies. 294

295 Even in preliminary experiments, the imbalance of reducing equivalents by alginate was 296 improved to direct more ethanol productions by mannitol-supplementation in the engineered V. halioticoli (Pet), and the recombinant could produce over 3,000 mg/L ethanol from 50 g/L seaweed 297 298 powder. V. halioticoli prefers polyG block structure in alginate substrate, so polyM and/or MG random block structures are left degraded [37]. Engineered alginolytic marine vibrios belonging to 299 300 the Halioticoli clade [22,23] possess similar alginolytic assimilation pathways, but only those 301 belonging to the Splendidus clade possess a polyM specific enzyme system. Combination cultures 302 using both type of alginolytic marine vibrios or in the quest for better strains showing no substrate specificity in alginate metabolism could be further ways in increasing ethanol production and 303 304 decreasing residual substrates.

305

306 CONCLUSION

We succeeded in producing ethanol using highly oxidized alginate and seaweed as substrate and feedstock, respectively, with metabolically engineered marine vibrios in a seawater-based medium. This study may contribute to establishing a platform for efficient ethanol production from marine biomass, and ultimately towards a more sustainable society. Using the metabolic engineering platform based on marine microbial biocatalysts, new types of material productions and material recycling, e.

312	g. in the plastics industry, are also expected.
313	
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325	YI, TS; Writing -review and editing: TS, CJ, HK, and SM; Resources: TS. All authors contributed
326	to the article and approved the submitted version.
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329	References
330	1.Stephanopoulos G (2007) Challenges in engineering microbes for biofuels production. Science
331	315:801-804. 10.1126/science.1139612
332	
333	2. Alper H, Stephanopoulos G (2009) Engineering for biofuels: exploiting innate microbial capacity
334	or importing biosynthetic potential? Nat Rev Microbiol 7:715-723.
335	https://doi.org/10.1038/nrmicro2186
336	
337	3. Dürre P, Richard T (2011) Microbial energy conversion revisited. Curr Opin Biotechnol 22:309-
338	311. doi: 10.1016/j.copbio.2011.04.021
339	
340	4. Pohland FG (1968) Thermal energy interchange during anaerobic methane fermentation of waste
341	organic substrates. Appl Microbiol 16:1518-1523. https://doi.org/10.1128/am.16.10.1518-
342	1523.1968
343	
344	5. Schauer NL, Ferry JG (1980) Metabolism of formate in Methanobacterium formicicum. J
345	Bacteriol 142:800-807. https://doi.org/10.1128/jb.142.3.800-807.1980
346	
347	6. Ingram LO, Conway T, Clark DP, Sewell GW, Preston JF (1987) Genetic engineering of ethanol
348	production in Escherichia coli. Appl Environ Microbiol 53:2420-2425. 10.1128/aem.53.10.2420-
349	2425.1987
350	
351	7. Trinh CT, Unrean P, Srienc F (2008) Minimal Escherichia coli cell for the most efficient
352	production of ethanol from hexoses and pentoses. Appl Environ Microbiol 74:3634–3643.
353	https://doi.org/10.1128/AEM.02708-07
354	

355	8. Borines MG, de Leon RL, McHenry MP (2011) Bioethanol production from farming non-food
356	macroalgae in Pacific island nations: Chemical constituents, bioethanol yields, and prospective
357	species in the Philippines. Renew Sustain Energy Rev 15:4432-4435.
358	https://doi.org/10.1016/j.rser.2011.07.109
359	
360	9. Zhu L (2013) Where food and energy compete. United Nation Chronicle.
361	https://www.un.org/en/chronicle/article/where-food-and-energy-compete. 27 June 2013 article
362	
363	10. Roesijadi G, Jones SB, Snowden-Swan LJ, Zhu Y (2010) Macroalgae as a biomass feedstock: A
364	preliminary analysis. United States: N. p., 2010. Web. doi:10.2172/1006310
365	
366	11. Takeda H, Yoneyama F, Kawai S, Hashimoto W, Murat K (2011) Bioethanol production from
367	marine biomass alginate by metabolically engineered bacteria. Energy Environ Sci 4:2575.
368	https://doi.org/10.1039/c1ee01236c
369	
370	12. Wargacki AJ, Leonard E, Win MN et al. (2012) An engineered microbial platform for direct
371	biofuel production from brown macroalgae. Science 335:308-313.
372	https://doi.org/10.1126/science.1214547
373	
374	13. Enquist-Newman M, Faust AM, Bravo DD et al. (2014) Efficient ethanol production from
375	brown macroalgae sugars by a synthetic yeast platform. Nature. 505:239-243. doi:
376	10.1038/nature12771
377	
378	14. Takagi T, Sasaki Y, Motone K et al. (2017) Construction of bioengineered yeast platform for
379	direct bioethanol production from alginate and mannitol. Appl Microbiol Biotechnol 101:6627-
380	6636. doi: 10.1007/s00253-017-8418-y

382	15. Sasaki Y, Yoshikuni Y (2022) Metabolic engineering for valorization of macroalgae biomass.
383	Metab Eng 71:42-61. 10.1016/j.ymben.2022.01.005
384	
385	16. Macreadie PI, Anton A, Raven JA (2019) The future of Blue Carbon science. Nat Commun
386	10:3998. doi: 10.1038/s41467-019-11693-w
387	
388	17. Gacesa P (1998) Bacterial alginate biosynthesis - recent progress and future prospects.
389	Microbiology 144:1133-1143. https://doi.org/10.1099/00221287-144-5-1133
390	
391	18. Seo J-S, Chong H, Park HS, et al (2005) The genome sequence of the ethanologenic bacterium
392	Zymomonas mobilis ZM4. Nat Biotechnol 23:63-68. https://doi.org/10.1038/nbt1045
393	
394	19. Sawabe T, Sugimura I, Ohtsuka M, Ezura Y (1998) Vibrio halioticoli sp. nov., a non-motile
395	alginolytic marine bacterium isolated from the gut of the abalone Haliotis discus hannai. Int J Syst
396	Bacteriol 48:573-580. https://doi.org/10.1099/00207713-48-2-573
397	
398	20. Wong TY, Preston LA, Schiller NL (2000) ALGINATE LYASE: review of major sources and
399	enzyme characteristics, structure-function analysis, biological roles, and applications. Annu Rev
400	Microbiol 54:289-340. doi: 10.1146/annurev.micro.54.1.289
401	
402	21. Barzkar N, Sheng R, Sohail M, Jahromi ST, Babich O, Sukhikh S, Nahavandi R (2022) Alginate
403	Lyases from Marine Bacteria: An Enzyme Ocean for Sustainable Future. Molecules 27:3375. doi:
404	10.3390/molecules27113375
405	
406	22. Jiang C, Tanaka M, Nishikawa S, Mino S, Romalde JL, Thompson FL, Gomez-Gil B, Sawabe T 16

407	(2022) Vibrio	Clade 3.0: new	Vibrionaceae	evolutionary	v units using	genome-based	approach.
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408 Current Microbiology (2022) 79:10. https://doi.org/10.1007/s00284-021-02725-0

409

- 410 23. Jiang C, Mino S, Sawabe T (2022) Genomic analyses of Halioticoli clade species in
- 411 *Vibrionaceae* reveal genome expansion with more carbohydrate metabolism genes during symbiotic
- 412 to planktonic lifestyle transition. Front. Mar. Sci., 9:84498323.
- 413 https://doi.org/10.3389/fmars.2022.844983

414

- 415 24. Bock A, Sawers G (1996) Fermentation. In: Frederick C. Neidhardt (ed) Escherichia coli and
- 416 Salmonella: Cellular and Molecular Biology, 2nd edition. ASM Press, Washington, D.C., pp 262–

417 282

418

- 419 25. Sawabe T, Setoguchi N, Inoue S, Tanak R, Ootubo M, Yoshimizu M, Ezura Y (2003) Acetic acid
- 420 production of *Vibrio halioticoli* from alginate: a possible role for establishment of abalone–V.
- 421 *halioticoli* association. Aquaculture 219:671–679. https://doi.org/10.1016/S0044-8486(02)00618-X
 422
- 423 26. Alam KY, Clark DP (1989) Anaerobic fermentation balance of *Escherichia coli* as observed by
- 424 in vivo nuclear magnetic resonance spectroscopy. J Bacteriol 171:6213–6217.

425 https://doi.org/10.1128/jb.171.11.6213-6217.1989

426

- 427 27. Hoppner TC, Doelle HW (1983) Purification and kinetic characteristics of pyruvate
- 428 decarboxylase and ethanol dehydrogenase from Zymomonas mobilis in relation to ethanol
- 429 production. Eur J Appl Microbiol Biotechnol 17:152–157. https://doi.org/10.1007/BF00505880

- 431 28. Conway T, Osman YA, Konnan JI, Hoffmann EM, Ingram LO (1987) Promoter and nucleotide
- 432 sequences of the *Zymomonas mobilis* pyruvate decarboxylase. J Bacteriol 169:949–954.

433 https://doi.org/10.1128/jb.169.3.949-954.1987

434

29. Conway T, Sewell GW, Osman YA, Ingram LO (1987) Cloning and sequencing of the alcohol 435 dehydrogenase II gene from Zymomonas mobilis. J Bacteriol 169:2591-2597. doi: 436 10.1128/jb.169.6.2591-2597.1987 437 438 439 30. Dunn AK, Millikan DS, Adin DM, et al (2006) New rfp - and pES213-derived tools for 440 analyzing symbiotic Vibrio fischeri reveal patterns of infection and lux expression in situ. Appl Environ Microbiol 72:802-810. https://doi.org/10.1128/AEM.72.1.802-810.2006 441 442 31. Stabb EV, Ruby EG (2002) RP4-based plasmids for conjugation between Escherichia coli and 443 444 members of the vibrionaceae. Method Enzymol 358:413-426 445 32. Huerta-Beristain G, Utrilla J, Hernández-Chávez G et al (2008) Specific ethanol production rate 446 in ethanologenic Escherichia coli strain KO11 is limited by pyruvate decarboxylase. Microb 447448 Physiol 15:55-64. https://doi.org/10.1159/000111993 449 33. Bryksin A V., Matsumura I (2010) Overlap extension PCR cloning: a simple and reliable way to 450451 create recombinant plasmids. Biotechniques 48:463-465. https://doi.org/10.2144/000113418 452453 34. Xiao Y-H, Pei Y (2011) Asymmetric overlap extension PCR method for site-directed 454mutagenesis. Methods Mol Biol 687:277-682. doi: 10.1007/978-1-60761-944-4 20 455 35. DuBois M, Gilles KA, Hamilton JK, Rebers RA, Smith F (1956) Colorimetric method for 456457 determination of sugars and related substances. Anal Chem 28:350-356. https://doi.org/10.1021/ac60111a017 458

460	36. Donnelly MI, Millard CS, Clark DP, Chen MJ, Rathke JW (1998) A novel fermentation pathway
461	in an Escherichia coli mutant producing succinic acid, acetic acid, and ethanol. Appl Biochem
462	Biotechnol 70-72:187-198. https://doi.org/10.1007/BF02920135
463	
464	37. Sawabe T, Oda Y, Shiomi Y, Ezura Y. (1995) Alginate degradation by bacteria isolated from the
465	gut of sea urchins and abalones. Microb Ecol 30:193-202. doi: 10.1007/BF00172574
466	
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468	Figure legends
469	Fig. 1. Expression of AdhII (A) and Pdc (B) activities in the engineered Vibrio halioticoli.
470	Faint endogenous activities were measured in wild type. Bayes T-test with independent samples was
471	performed using the JASP version 0.17.1 and activities of Pet > those of <i>adh</i> or <i>pdc</i> only was set as
472	an alternative hypothesis (H1), respectively. Alternative hypothesis was more likely to be occurred
473	by 53 and 8 folds, respectively (N=3). The error bar indicates standard error.
474	
475	Fig. 2. Detection of ethanol in Vibrio halioticoli (Pet) after being cultured with alginate and the
476	fermentation profiles. A: wild type, B: V. halioticoli (Pet). The arrowhead indicates the peak of
477	ethanol. C: fermentation profiles.
478	
479	Fig. 3. Developments of ethanol-producing marine vibrio catalysts implemented by the Pet operon.
480	X indicates mean in the box plot. Bayes T-test with independent samples was performed using the
481	JASP version 0.17.1 and ethanol production of Pet-implemented vibrios > those of wild type was
482	set as an alternative hypothesis (H1). The alternative hypothesis was 5,486-folds more likely to be

occurred (N=11).

485 Supplementary Figures

486 Fig. S1. New pathways in the engineered *Vibrio halioticoli*.

487

488 Fig. S2. Outline of overlap PCR methodology to create Pet operon.