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Title: Production of polyhydroxybutyrate and alginate from glycerol by *Azotobacter vinelandii* under nitrogen-free conditions

Running title: Production of biopolymers from glycerol

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ABSTRACT

Glycerol is an interesting feedstock for biomaterials such as biofuels and bioplastics because of its abundance as a by-product during biodiesel production. Here we demonstrate glycerol metabolism in the nitrogen-fixing species *Azotobacter vinelandii* through metabolomics and nitrogen-free bacterial production of biopolymers, such as poly-D-3-hydroxybutyrate (PHB) and alginate, from glycerol. Glycerol-3-phosphate was accumulated in *A. vinelandii* cells grown on glycerol to the exponential phase, and its level drastically decreased in the cells grown to the stationary growth phase. *A. vinelandii* also overexpressed the glycerol-3-phosphate dehydrogenase gene when it was grown on glycerol. These results indicate that glycerol was first converted to glycerol-3-phosphate by glycerol kinase. Other molecules with industrial interests, such as lactic acid and amino acids including γ-aminobutyric acid, have also been accumulated in the bacterial cells grown on glycerol. Transmission electron microscopy revealed that glycerol-grown *A. vinelandii* stored PHB within the cells. The PHB production level reached 33% per dry cell weight in nitrogen-free glycerol medium. When grown on glycerol, alginate-overproducing mutants generated through chemical mutagenesis produced two-fold the amount of alginate from glycerol than the parental wild-type strain. To the best of our knowledge, this is the first report on bacterial production of biopolymers from glycerol without addition of any nitrogen source.

INTRODUCTION

Azotobacter vinelandii is a free living, nitrogen-fixing bacterium.[1,2] One of the remarkable characteristics of this species is that it can grow sufficiently in a nitrogen-free minimal medium.[3] In *A. vinelandii*, nitrogenases prerequisite for nitrogen fixation catalyze the reduction of nitrogen to ammonia using a large amount of energy derived from ATP hydrolysis.[4,5] In the recently determined genome sequence of *A. vinelandii*, three different types of nitrogenases have been identified.[2] In addition, it has been established that *A. vinelandii* has the potential to produce industrially useful biopolymers, including extracellular alginate and intracellular poly-D-3-hydroxybutyrate (PHB).[6-9] Alginate is a linear polysaccharide consisting of (1-4)-β-D-mannuronic acid and α-L-guluronic acid. Commercially available alginate is classically derived from seaweed, although two bacterial genera, *Pseudomonas* and *Azotobacter*, are expected to be potential alginate producers.[8] PHB belongs to the polyhydroxyalkanoate (PHA) family of polyesters, and many bacterial species accumulate PHAs as intracellular granules for energy storage.[10] PHAs are also promising alternatives to plastics because of their biodegradability, biocompatibility, and thermoplasticity.[6,11] Hence, *A. vinelandii* is considered to be an attractive bacterium for production of two industrially useful biopolymers, alginate and PHB, in the absence of nitrogen sources.[6,9,12-14]

The biosynthetic pathways for alginate and PHB production in *A. vinelandii* have previously been reviewed by Galindo *et al*.[6] As shown in Fig. 1, alginate is synthesized from fructose 6-phosphate by many enzymes encoded by the *alg* cluster,[8,15] whereas PHB is synthesized in three steps from acetyl-CoA and three *phb* genes are essential for synthesis.[16] The regulatory mechanisms for the production of these biopolymers have been analyzed.[16-25] Thus, the biopolymers are expected to be produced from excess

and/or unused resource by *A. vinelandii*.

A. vinelandii is known to assimilate various carbon sources. Sucrose, glucose, fructose, mannose, sorbitol, mannitol, glycerol, gluconate, and acetate can all be used as the sole carbon source for cell growth.[26,27] Furthermore, some of them are also used for biopolymer production.[26] However, little information exists on bacterial glycerol metabolism including biopolymer production in a nitrogen-free environment. Glycerol is generated as a major by-product during biodiesel production, and its efficient utilization is now sought in various areas of food, pharmaceutical, agricultural, and environmental research. Moreover, microorganism-mediated conversion of glycerol to other valuable materials is being developed worldwide. Production of hydrogen, ethanol, butanol, 1,3-propanediol, propionic acid, and PHAs has previously been undertaken using bacteria.[28,29]

To the best of our knowledge, no report exists on glycerol utilization in a nitrogen-free environment. Moreover, glycerol metabolism and production of biopolymers from glycerol in *A. vinelandii* remain to be clarified. The present study involves metabolomics-based identification of the glycerol metabolic pathway in *A. vinelandii* and the bacterial production of biopolymers from glycerol in a nitrogen-free environment.

RESULTS AND DISCUSSION

Sucrose and glycerol metabolism. Metabolites in *A. vinelandii* cells grown on sucrose or glycerol were analyzed by a capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) (Tables 1 and 2). Small amounts of glucose-, fructose-, and glycerol-related metabolites, but not glycerol-3-phosphate accumulated in the cells

(Table 1). Considerable amounts (400 pmol/OD_{600} ml) of glycerol-3-phosphate were detected in the glycerol-grown cells collected at the exponential growth phase, whereas its level drastically decreased (82 pmol/OD_{600} ml) in cells collected at the stationary growth phase. Moreover, the dihydroxyacetone phosphate level in the glycerol-grown cells was much higher than that in the sucrose-grown cells. These results suggest that glycerol was first catabolized to glycerol-3-phosphate, then to dihydroxyacetone phosphate, and finally converged into the glycolytic pathway. Because cell growth on glycerol was slower than that on sucrose as described later, accumulation of glycerol-3-phosphate was considered to be a rate-limiting step.

In the recently determined genome sequence of *A. vinelandii* strain DJ,[2] putative genes for glycerol uptake and degradation were found at a locus containing four genes, *glpF*, *glpK*, *glpR*, and *glpD*. GlpF and GlpR are annotated as putative glycerol-uptake and -repressor proteins, respectively, whereas GlpK and GlpD are annotated as glycerol kinase and glycerol-3-phosphate dehydrogenase, respectively. Although GlpK and GlpD are classified into a group involved in phospholipid metabolism according to genome annotation, metabolomics in the present study demonstrated that glycerol and glycerol-3-phosphate were substrates of GlpK and GlpD, respectively, and that both enzymes were necessary for glycerol metabolism.

Two pathways were postulated for the conversion of glycerol to dihydroxyacetone phosphate. One is through glycerol-3-phosphate and the other through dihidroxyaceton. A number of microorganisms can use glycerol as the sole carbon source through dihydroxyacetone.[29-31] Furthermore, the genome sequence of *A. vinelandii* revealed the presence of glycerol dehydrogenase- and dihydroxyacetone kinase-like genes (Fig. 1). Hence, a dehydrogenase for glycerol and one for glycerol-3-phosphate were assayed to

118 determine the main glycerol assimilation pathway. However, no activity of either
119 enzyme was detected in the bacterial cell extract, possibly due to their low protein
120 expression level. Hence, transcription levels of the genes of the two dehydrogenases
121 were monitored by quantitative PCR (Fig. 2). As shown in Fig. 2A, the total RNA of
122 bacteria grown in all conditions was extracted without degradation. In the case of the
123 glycerol-3-phosphate dehydrogenase gene (Fig. 2B left), the cells grown to the
124 exponential growth phase showed higher gene expression than those grown to the
125 stationary growth phase. In bacteria grown to the exponential growth phase, gene
126 expression in the glycerol medium was 38-fold higher than that of bacteria grown in the
127 sucrose medium. In contrast, the glycerol dehydrogenase gene was transcribed at basal
128 levels in bacteria grown to the exponential growth phase in media (Fig. 2B right). The
129 expression of glycerol dehydrogenase in all cases tested was extremely low, but near the
130 detectable limit for our experimental conditions (data not shown). These metabolomic
131 and quantitative PCR results demonstrate that the glycerol-3-phosphate pathway is
132 predominant in *A. vinelandii*.

133 Sucrose metabolites, such as fructose 6-phosphate, mannose 6-phosphate, mannose
134 1-phosphate, and GDP mannose, were detected even in glycerol-grown *A. vinelandii*
135 (Table 1). This result indicates that gluconeogenesis, which involves the conversion of
136 fructose 1,6-biphosphate to fructose 6-phosphate, occurred in the bacteria. In addition,
137 occurance of gluconeogenesis in *A. vinelandii* demonstrates its potential for producing
138 alginate concomitantly with PHB from glycerol, which is described later.

139 No conspicuous rate-limiting metabolites were identified between fructose
140 1,6-biphosphate and acetyl CoA (Table 1). However, accumulation of pyruvic acid (42.5
141 pmol/OD_{600} ml) was observed in glycerol-grown cells collected at the exponential

142 growth phase. Pyruvic acid is an important precursor for many metabolites, including
143 ethanol, acetyl CoA, and lactic acid.[32] In the present study, *A. vinelandii* produced large
144 amounts of lactic acid in all cases, especially in glycerol-grown cells (Table 2), and
145 levels of several amino acids accumulated in glycerol-grown cells were higher than
146 those in sucrose-grown cells. Both glutamine and glutamic acid were considerably
147 produced in all cases, especially in glycerol-grown cells collected at the exponential
148 growth phase. Arginine, lysine, and proline are synthesized from glutamic acid in most
149 bacteria. A similar accumulation profile of these amino acids was observed in *A.*
150 *vinelandii*, as shown in Table 2. Thus, *A. vinelandii* demonstrated the potential for
151 amino acid production under nitrogen-free conditions. Furthermore, γ-aminobutyric acid
152 (GABA), a neurotransmitter of clinical interest,[33] was stored in the cells grown to the
153 exponential growth phase in sucrose and glycerol. Because bacteria-produced GABA is
154 known as a molecule of intracellular pH management due to decarboxylation of
155 glutamic acid,[34] this molecule is suggested to play an important role in the
156 neutralization of organic acids accumulated in the cells.
157 Metabolomic analysis performed in the present study supported the role of the
158 metabolic pathway predicted previously, as shown in Fig. 1. In addition, based on
159 results of the present study, *A. vinelandii* is promising as a potential producer of many
160 useful materials, such as amino acids, GABA, lactic acid, and biopolymers, in a
161 nitrogen-free environment.
162
163 **PHB production.** PHB accumulation was investigated in wild-type (WT) *A. vinelandii*
164 grown on sucrose or glycerol. In addition, the mutant *ΔalgD* cells, which have a
165 disrupted alginate synthetic gene *algD*, were also subjected to the PHB assay because

166 PHB and alginate productions are thought to be competitive. First, intracellular PHB
167 granules were monitored by transmission electron microscopy (TEM) (Fig. 3). Similar
168 to many PHB-producing bacteria, WT cells produced white and globular PHB granules,
169 which were found in both sucrose- and glycerol-grown cells. On the other hand,
170 globular PHB granules were scarcely observed and apparently degraded in *ΔalgD* cells.
171 The time course of cell growth and PHB production are shown in Fig. 4. In the
172 sucrose medium, WT and *ΔalgD* cells showed similar growth profiles (Fig. 4A). Cell
173 growth of both strains exceeded an OD_{600} of 14. However, the lag phase of both strains
174 grown on glycerol was longer than that on sucrose. WT growth reached an OD_{600} of
175 6.12, whereas that of *ΔalgD* reached an OD_{600} of 16.8.
176 Figure 4B indicates the time course of intracellular PHB accumulation (per dry cell).
177 The intracellular PHB level in WT cells grown on sucrose as well as glycerol reached
178 approximately 33%. Interestingly, the PHB level of *ΔalgD* cells grown on either source
179 initially increased, but subsequently decreased. As shown in Fig. 4A, PHB was
180 degraded in *ΔalgD* cells. *A. vinelandii* is known to convert from vegetative cells to cysts
181 under unfavorable environments for growth, and PHB has been observed in the
182 cyst-forming cells as a probable energy and carbon storage material.[14] Although the
183 reasons for PHB degradation have yet to be determined, the necessity for PHB might
184 waver due to the lack of alginate production following cyst formation. Production of
185 PHB and alginate is closely regulated in *A. vinelandii*.[10] The results obtained herein may
186 provide valuable hints on the relationships among alginate, PHB, and cyst formation.
187 Although *A. vinelandii* grown on glucose together with nitrogen sources synthesizes
188 much PHB (74% per dry cell),[35] and some bacteria such as *Burkholderia cepacia*,[36]
189 *Chelatococcus daeguensis*,[37] *Cupriavidus necator* (formerly *Ralstonia eutropha*),[38,39]

Paracoccus denitrificans,[40] *Pseudomonas oleovorans*,[41] and *Zobellella denitrificans*,[42] have also been known to produce PHB from glycerol with nitrogen sources, the bacterial production of PHB from glycerol without a nitrogen source contributes to green chemistry.

Alginate production. The alginate production level was determined in *A. vinelandii* grown on sucrose or glycerol. The sucrose-grown cells obtained at the stationary phase produced alginate at 0.3–0.4 mg/ml, whereas those grown in the glycerol medium produced enhanced levels of approximately 0.5 mg/ml. To elevate the alginate production level, a random NTG-treated mutation library was used to screen alginate-overproducing mutants. Unlike for PHB, it was easy to select alginate-overproducing mutants because these mutants seemed to form high mucoid colonies. More than 100 mutants were isolated as high mucoid colonies in comparison with WT colonies.

One of the mutants (MT1) was subjected to TEM analysis after preparation of cell thin section (Fig. 5A). Recently, *A. vinelandii* cells were demonstrated to be equipped with a special secretion system for alginate through formation of cell-surface blebs. These blebs containing alginate are formed on the bacterial cell surface and are subsequently released around the cells.[43] MT1 cells formed a large number of blebs on the cell surface (Fig. 5A). On the other hand, few blebs were observed on WT cells (Fig. 5B). Thus, WT cells were subjected to scanning electron microscopy (SEM) analysis. A few blebs were observed on the cell surface (Fig. 5C). The time course of alginate secretion for WT and MT1 cells grown on glycerol is characterized in Fig. 6. Cell growth (3.97 at OD_{600}) of MT1 at 96 h was lower than that (6.64 at OD_{600}) of WT (Fig.

6A). In contrast, alginate secretion by MT1 cells (0.87 mg/ml) was higher than that by WT cells (0.52 mg/ml) (Fig. 6B). This result indicates that MT1 cells have the potential for more alginate production through improvement in their growth conditions.

In conclusion, this is the first report on glycerol metabolism in *A. vinelandii* analyzed through the metabolomic approach. The results indicate that *A. vinelandii* grown on glycerol, but in the absence of any nitrogen source, may be useful for producing many substances, including amino acids and biopolymers (especially PHB and alginate), with industrial interests.

MATERIALS AND METHODS

Bacteria and culture conditions. The bacterial strains used in the present study are listed in Table 3. Cells of WT *A. vinelandii* ATCC 12837 and an alginate-deficient mutant with a disruption of *algD* (*ΔalgD*)[43] were grown aerobically in a minimal glycerol medium, i.e., modified Burk's medium (G-MB; 20 mg/ml glycerol, 200 μg/ml NaCl, 50 μg/ml $CaSO_4$, 200 μg/ml $MgSO_4$ $7H_2O$, 2.9 μg/ml Na_2MoO_4 $2H_2O$, 27 μg/ml $FeCl_3$, 0.66 mg/ml K_2HPO_4, and 0.16 mg/ml KH_2PO_4) at 30°C with agitation of 120 strokes per min. Sucrose (20 mg/ml) was also used for a carbon source instead of glycerol in G-MB (S-MB).

Metabolome analysis. Metabolome analysis was supported by Human Metabolome Technologies (Tsuruoka, Japan). Precultured WT cells were inoculated in 50 ml fresh S-MB or G-MB, and grown to the exponential or stationary growth phase. To reach the stationary growth phase, cells were grown in each medium for 84 h. To reach the exponential growth phase, cells were grown in S-MB and G-MB for 40 and 60 h

(approximately 1.2 at OD_{600}), respectively. The cells were harvested by centrifugation (5700 ×*g*, 4°C, 5 min), and were washed twice with 10 ml pure water. Each cell pellet was homogenized in 2 ml methanol. After homogenization, chloroform (1.6 ml) was added to 1.6 ml of cell extract, and the mixture was well agitated. Aqueous and chloroform layers were separated by centrifugation (2300 ×*g*, 4°C, 5 min), and the aqueous layer was subjected to ultrafiltration (9100 ×*g*, 4°C, 120 min) using the Ultrafree-MC UFC3 LCC (molecular weight cut-off, 5000; Millipore, Bedford, MA). The filtrate was dried and resolved in 50 μl of pure water. Metabolites were identified and quantified using CE-TOFMS system (Agilent Technologies, Santa Clara, CA).

Enzyme assay. Dehydrogenases for glycerol and glycerol-3-phosphate were assayed according to a previously described method.[44] Briefly, WT cells were inoculated in 50 ml of fresh S-MB or G-MB, and cultured to the exponential or stationary growth phase. The cells were washed twice with 10 mM potassium phosphate buffer (pH 7.0), and resuspended in 2 ml of the same buffer. The cells were ultrasonically disrupted at 4°C and 9 kHz for 10 min (Insonator model 201M; Kubota, Tokyo, Japan). Insoluble substances were removed by centrifugation (20,000 ×*g*, 4°C, 15 min), and the supernatant was used as the cell extract for analysis. The cell extract (20 μl) was mixed with 10 mM (final concentration) potassium phosphate buffer (pH 7.0), 5 mM substrate (glycerol or glycerol-3-phosphate), and 0.5 mM coenzyme (NAD^+ or $NADP^+$). The change in the absorbance of the reaction mixture at a wavelength of 340 nm was monitored at 30°C for 10 min.

Quantitative PCR. Total RNA extraction, *in vitro* cDNA synthesis, and quantitative

PCR were performed to monitor the expression levels of specific genes in the bacterial samples. *A. vinelandii* grown in S-MB or G-MB was harvested at the exponential and stationary growth phases. Total RNA was extracted using the standard hot phenol method. DNA degradation and subsequent RNA purification were performed using the RNase-Free DNase Set (Qiagen, Tokyo, Japan) and an RNeasy Mini Kit (Qiagen), respectively. cDNA, synthesized from a 200 ng RNA sample as using the ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions, was used as a template. Quantitative PCR was performed using the SYBR Premix Ex Taq GC (Takara Bio, Shiga, Japan) and the LineGene instrument (Toyobo).

Electron microscopy. TEM and SEM analyses were entrusted to Tokai Electron Eicroscopy Analysis Co. (Nagoya, Japan). *A. vinelandii* was grown in S-MB or G-MB media (30 ml of working volume) for 72 h. In the case of TEM analysis, the culture and fixing solution A (2% paraformaldehyde, 2% glutaraldehyde, and 0.1 M potassium phosphate buffer, pH 7.4) were mixed in a ratio of 1:1 (total 1 ml), and stored at 4°C for 60 min. Cell pellets were collected by centrifugation (5000 ×*g*, 4°C, 5 min). Fixing solution B (2% osmium tetroxide and 0.1 M potassium phosphate buffer, pH 7.4) was added to each cell pellet and agitated. Preparation of an ultrathin section and TEM analysis using a JEM-1200EX instrument (JEOL, Tokyo, Japan) were carried out as described previously.[43] In the case of SEM analysis, the bacterial cells were prefixed at 4°C for 1 h by mixing the culture with an equal volume of fixative consisting of 4% paraformaldehyde and 4% glutaraldehyde in 100 mM cacodylate buffer (pH 7.4). After centrifugation, the bacterial cells were fixed at 4°C for 24 h with 2% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), additionally fixed at 4°C for 2 h with 1% tannic acid

in 0.1 M cacodylate buffer (pH 7.4), and washed at 4°C with the same buffer. The fixed cells were treated at 4°C for 3 h with 2% OsO_4 in 0.1 M cacodylate buffer (pH 7.4) and dehydrated in a series of ethanol (50%, 70%, 90%, and 100%, each 30 min). After dehydration, the cells were continuously dehydrated with 100% ethanol at room temperature overnight. The cells were substituted into tert-butyl alcohol at room temperature, followed by freeze drying under vacuum. The cells were coated with a thin layer (60 nm) of osmium by an osmium plasma coater (NL-OPC80NS, Nippon Laser & Electronics Laboratory, Nagoya, Japan). The cells were observed using a scanning electron microscope (JSM-6340F, JEOL, Tokyo, Japan) at an acceleration voltage of 5.0 kV.

PHB detection. Intracellular PHB was detected according to a previously described procedure with slight modification.[45,46] Dried cells were treated at 100°C for 140 min with 1 ml chloroform containing 0.5% (w/v) benzoic acid as an internal standard and 1 ml methanol containing 3% sulfuric acid. After addition of 1 ml distilled water, each solution was agitated for 1 min and centrifuged (1000 ×*g*, 4°C, 5 min). The lower organic solvent layer of each sample was subjected to gas chromatography (GC) analysis using a GC-2014 instrument (Shimadzu, Kyoto, Japan) and a DB-5 column (30 m × 0.25 mm × 0.25 μm; Agilent Technologies). Helium was used as the carrier gas. Detector and injector temperatures were set to 275 and 230°C, respectively. Initial and end temperatures were set to 60 and 200°C, respectively, with a gradient of 8°C/min. An authentic sample of PHB (Sigma, St. Louis, MO) was also treated as described above and was used for the identification and quantitative determination of PHB.

Isolation of alginate-overproducing mutant. *A. vinelandii* was grown in G-MB to the exponential growth phase (approximately 0.8 at OD_{600}), and the harvested cells obtained by centrifugation were washed with MB buffer (i.e., MB without a carbon source). To create random mutation in the species, the cells were treated with 50 μg/ml *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine (NTG; Sigma) at 30°C for 30 min. The NTG solution was removed from the sample by centrifugation, and the cells were incubated overnight in S-MB. After colony formation on an S-MB plate, the highly mucoid cells were selected as candidates for alginate-overproducing mutants.

Alginate assay. Alginate was assayed according to the method of Knutson and Jeanes.[47] Briefly, the culture broth (200 μl) was mixed with 0.5 M EDTA (12 μl) and 5 M NaCl (4 μl). Cells were removed from the mixture by centrifugation (7000 ×*g*, room temperature, 5 min). The supernatant (87.5 μl) was mixed with an ice-chilled mixture of sulfuric acid (732.5 μl) and boric acid solution (17.5 μl; 45 mM KOH and 1 M boric acid) as well as 0.1% (w/v) carbazol (25 μl). The mixture was incubated at 55°C for 30 min, and its absorbance 530 nm was subsequently measured. The alginate concentration in the culture broth was determined based on the calibration using seaweed alginate as a standard.

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FIGURE LEGENDS

Figure 1. Putative synthetic pathway for PHB and alginate from glycerol in *A. vinelandii*. The genes involved in glycerol metabolism are also indicated.

Figure 2. Gene expression of glycerol-3-phosphate and glycerol dehydrogenases. (A) Total RNA from *A. vinelandii* grown in G-MB or S-MB to the exponential and stationary growth phases. (B) Relative gene expression of glycerol-3-phosphate (left) and glycerol dehydrogenases (right). For quantitative PCR, cDNA synthesized from was used as a template in all samples. The expression levels of both genes in S-MB at the stationary phase were standardized as the relative expression of 1.

Figure 3. PHB granules in *A. vinelandii* cells revealed by TEM. Bacteria were cultured for 72 h in S-MB or G-MB as indicated.

Figure 4. Time course of PHB production by *A. vinelandii*. (A) Cell growth. (B) PHB accumulation in dry cells. Open circles, WT grown on sucrose; closed circles, WT grown on glycerol; open triangles, *ΔalgD* grown on sucrose; closed triangles, *ΔalgD* grown on glycerol.

Figure 5. Alginate-overproducing mutants of *A. vinelandii*. (A) TEM observation of MT1 cells grown on glycerol. (B) TEM observation of WT cells grown on glycerol. (C) SEM observation of WT cells grown on glycerol. Panels A′ B′, and C′ are magnified views of the regions of interest indicated in panels A, B, and C, respectively.

Figure 6. Alginate production from glycerol-grown *A. vinelandii*. (A) Cell growth. (B) Extracellular alginate. Circles, WT; triangles, alginate-overproducing mutant.

Figure 1.

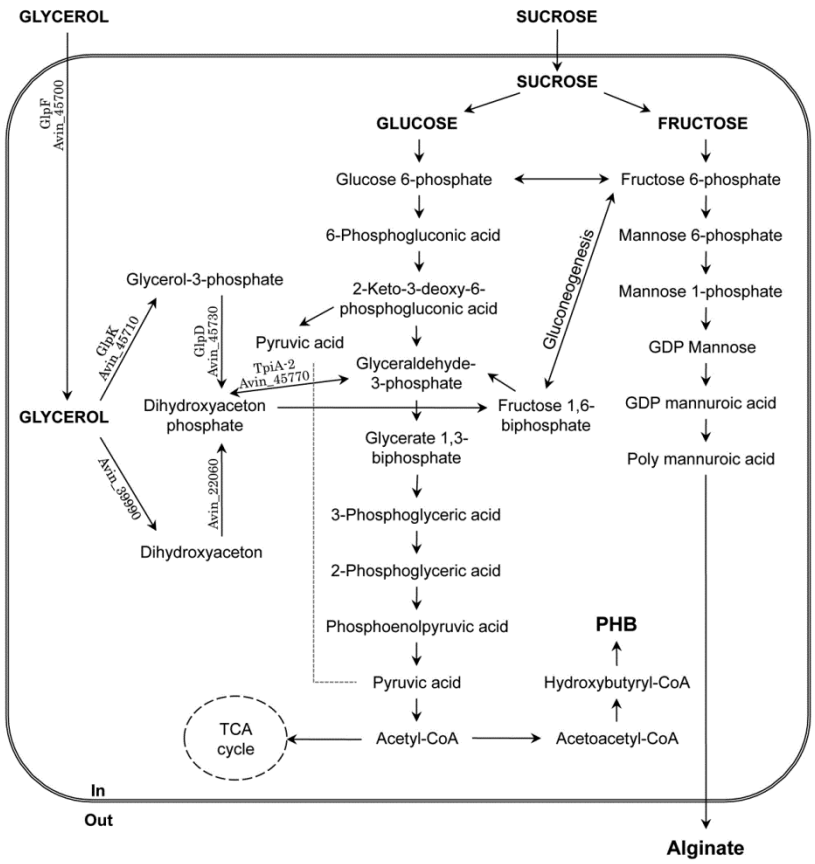


Figure 2.

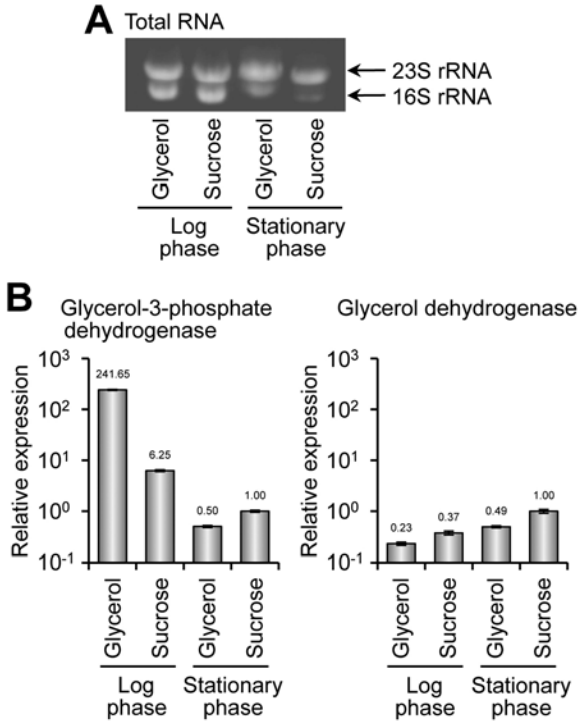
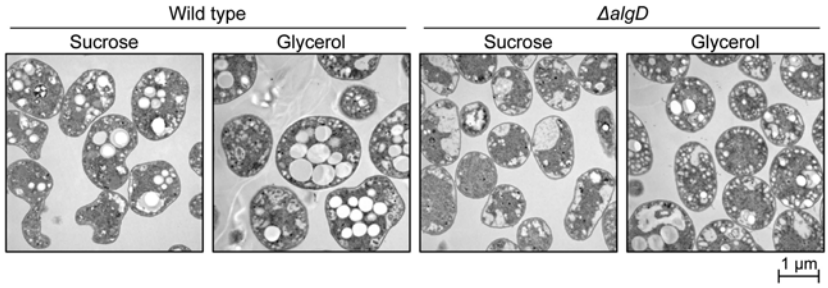


Figure 3.



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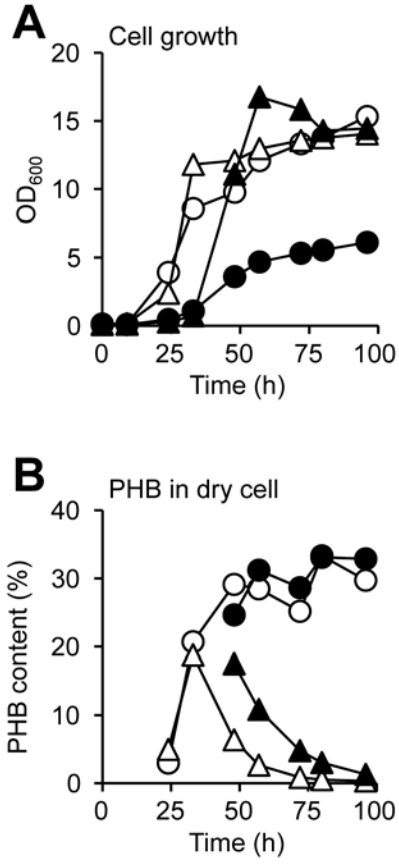

Figure 5.

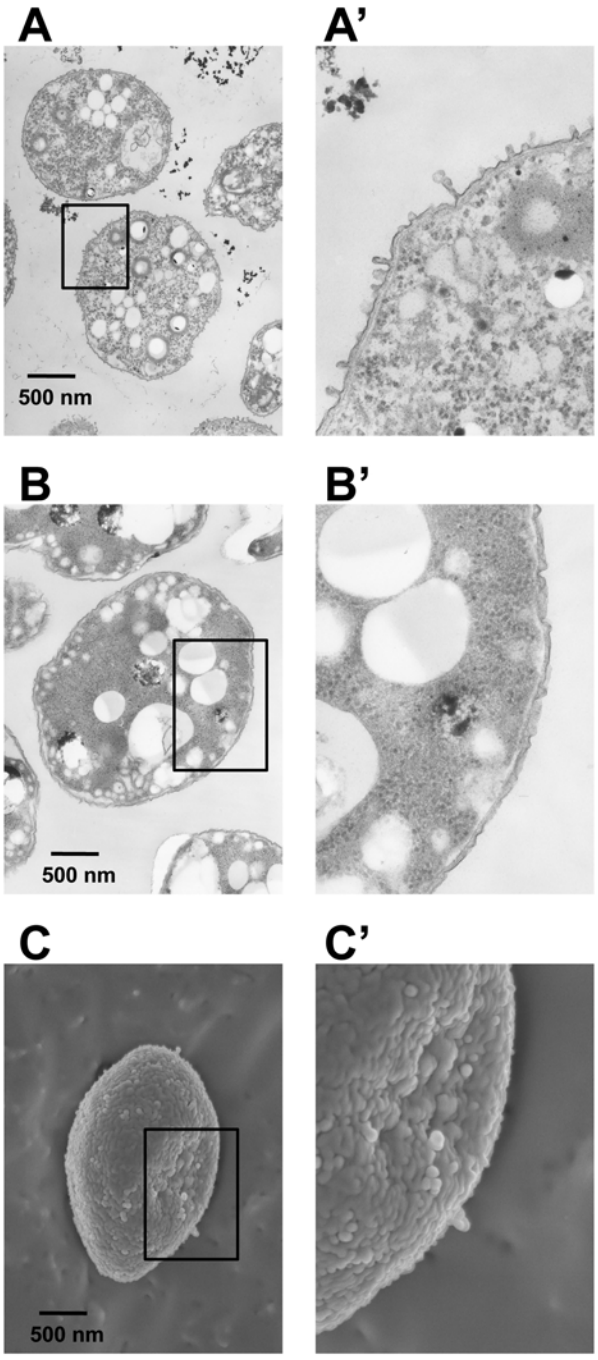

Figure 6.

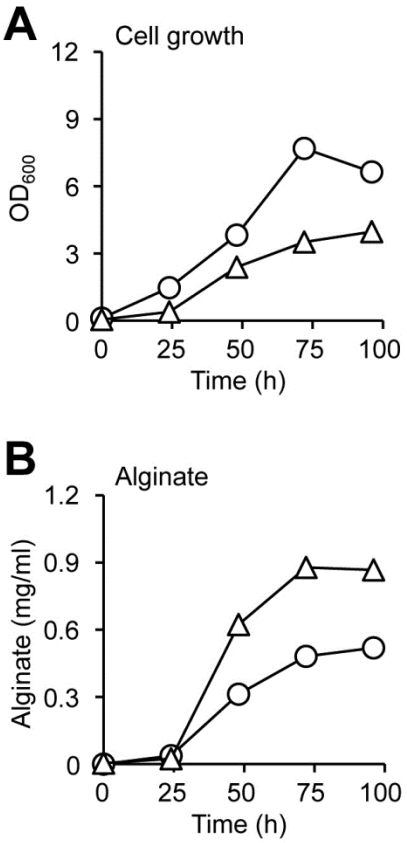

Table 1. Intracellular metabolites determined through metabolomics.

Metabolite	Concentration (pmol/OD_{600} ml)			
	Sucrose		Glycerol	
	Log phase	Stationary phase	Log phase	Stationary phase
Glucose 6-phosphate[*1]	18.7 (±4.2)	22.5 (±29.3)	22.6 (±5.2)	61.0 (±25.4)
6-Phosphogluconic acid	U. L.	U. L.	U. L.	U. L.
2-Keto-3-deoxy-6-phosphogluconic acid	N. D.	N. D.	N. D.	N. D.
Fructose 6-phosphate[*2]	8.3 (±1.7)	8.2 (±11.6)	13.9 (±6.3)	29.4 (±13.1)
Mannose 6-phosphate[*1]	18.7 (±4.2)	22.5 (±29.3)	22.6 (±5.2)	61.0 (±25.4)
Mannose 1-phosphate[*2]	8.3 (±1.7)	8.2 (±11.6)	13.9 (±6.3)	29.4 (±13.1)
GDP Mannose	0.3 (±0.1)	1.0 (±0.2)	2.1 (±0.6)	5.5 (±1.3)
GDP Mannuroic acid	N. D.	N. D.	N. D.	N. D.
Glycerol-3-phosphate	11.0 (±4.4)	4.6 (±3.1)	400.3 (±186.4)	82.0 (±61.9)
Dihydroxyacetone phosphate	0.8 (±0.2)	2.0 (±1.9)	12.6 (±5.3)	8.5 (±2.5)
Fructose 1,6-biphosphate	0.5 (±0.1)	1.3 (±0.1)		2.3 (±0.4)
Glyceraldehyde-3-phosphate	2.4 (±0.4)	1.7 (±1.0)	6.2 (±2.0)	7.6 (±0.6)
Glycerate 1,3-biphosphate	N. D.	N. D.	N. D.	N. D.
3-Phosphoglyceric acid	0.8 (±0.2)	5.0 (±4.1)	1.4 (±0.1)	10.3 (±5.5)
2-Phosphoglyceric acid	N. D.	N. D.	N. D.	N. D.
Phosphoenolpyruvic acid	U. L.	7.3 (±0.1)	U. L.	14.4 (±4.0)
Pyruvic acid	11.5 (±3.7)	9.7 (±3.7)	42.5 (±24.8)	12.7 (±14.2)
Acetyl CoA	2.5 (±1.5)	4.8 (±3.8)	2.3 (±0.2)	9.1 (±4.6)

U. L., undetectable level; N. D., not detectable under this experimental condition. [*1], summation of glucose 6-phosphate and mannose 6-phosphate; [*2], summation of fructose 6-phosphate and mannose 1-phosphate. Data are averages (± SD) of three experiments.

Table 2. Intracellular amino acids and other organic acids.

	Concentration (pmol/OD_{600} ml)			
	Sucrose		Glycerol	
Metabolite	Log phase	Stationary phase	Log phase	Stationary phase
Ala	121.9 (±18.1)	95.0 (±61.3)	220.9 (±88.1)	114.1 (±35.9)
Arg	55.3 (±13.4)	19.7 (±26.0)	51.6 (±26.0)	93.7 (±16.4)
Asn	1.7 (±1.1)	U. L.	1.9 (±1.4)	5.9 (±7.4)
Asp	14.2 (±4.1)	4.9 (±3.8)	24.5 (±4.1)	24.3 (±28.1)
Cys	U. L.	U. L.	U. L.	U. L.
Gln	134.3 (±8.8)	146.3 (±180.5)	217.1 (±158.1)	184.1 (±73.3)
Glu	85.7 (±25.4)	75.4 (±112.6)	368.6 (±127.6)	134.3 (±106.9)
Gly	45.5 (±22.1)	9.5 (±5.4)	65.1 (±9.3)	70.3 (±84.6)
His	4.4 (±1.6)	1.4 (±0.8)	4.3 (±1.9)	13.3 (±12.8)
Ile	18.8 (±5.1)	1.1 (±0.5)	30.3 (±13.3)	8.9 (±11.7)
Leu	45.1 (±11.5)	1.7 (±0.7)	76.8 (±45.6)	13.0 (±17.3)
Lys	47.4 (±7.6)	13.1 (±12.9)	69.1 (±21.1)	83.5 (±32.1)
Met	7.8 (±1.8)	0.3 (±0.1)	8.5 (±5.9)	2.3 (±2.4)
Phe	7.5 (±2.9)	1.0 (±0.3)	14.9 (±8.1)	7.8 (±7.4)
Pro	15.4 (±3.3)	4.1 (±4.2)	28.2 (±5.7)	31.3 (±12.8)
Ser	58.5 (±36.7)	14.3 (±9.7)	68.1 (±23.7)	115.2 (±137.9)
Thr	39.3 (±9.8)	4.3 (±3.1)	65.0 (±25.8)	34.9 (±29.8)
Trp	2.0 (±1.0)	0.4 (±0.3)	2.0 (±1.0)	0.8 (±0.8)
Tyr	22.0 (±5.2)	3.5 (±3.2)	44.3 (±29.7)	20.7 (±10.3)
Val	121.6 (±37.6)	3.4 (±1.6)	168.8 (±100.8)	22.8 (±24.4)
γ-Aminobutyric acid (GABA)	113.9 (±39.6)	0.8 (±0.2)	238.9 (±278.8)	1.7 (±2.1)
Lactic acid	501.0 (±61.1)	530.5 (±309.1)	1239.3 (±507.3)	719.2 (±646.8)

U. L., undetectable level. Data are averages (± SD) of three experiments.

Table 3. Bacterial strains used in the present study.

Bacterial strains (abbreviation)	Characteristics or sequences	References
Azotobacter vinelandii ATCC 12837 (WT)	Wild type strain	ATCC
A. vinelandii ΔalgD (*ΔalgD*)	Alginate-deficient strain with an insertion of the tetracycline resistance gene in chromosomal *algD*.	43
A.vinelandii MT1 (MT1)	Alginate-overproducing strain derivatized from WT by NTG treatment.	Present study

ATCC, American Type Culture Collection.