Article title: Phosphate enhances levan production in the endophytic bacterium *Gluconacetobacter diazotrophicus* Pal5

Running head: Phosphate enhances levan production

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Abstract

Gluconacetobacter diazotrophicus is a gram-negative and endophytic nitrogen-fixing bacterium that has several beneficial effects in host plants; thus, utilization of this bacterium as a biofertilizer in agriculture may be possible. *G. diazotrophicus* synthesizes levan, a D-fructofuranosyl polymer with β-(2→6) linkages, as an exopolysaccharide and the synthesized levan improves the stress tolerance of the bacterium. In this study, we found that phosphate enhances levan production by *G. diazotrophicus* Pal5, a wild type strain that showed a stronger mucous phenotype on solid medium containing 28 mM phosphate than on solid medium containing 7 mM phosphate. A *G. diazotrophicus* Pal5 levansucrase disruptant showed only a weak mucous phenotype regardless of the phosphate concentration, indicating that the mucous phenotype observed on 28 mM phosphate medium was caused by levan. To our knowledge, this is the first report of the effect of a high concentration of phosphate on exopolysaccharide production.

Introduction

Due to the increasing cost of chemical nitrogenous fertilizers and concerns about contamination of soil and water, there is a need to reduce usage of chemical fertilizer [1]. Plant-associated microorganisms containing nitrogenases have attracted attention as alternative biofertilizers [2], since nitrogenase catalyzes a reaction referred to as biological nitrogen fixation, in which atmospheric nitrogen is converted to ammonia.

Plant-associated nitrogen-fixing microorganisms reside in the internal parts of plant and the rhizosphere, providing host plants with nitrogenous compounds [3], while the host plants supply the microorganisms with nutrients such as carbon sources, organic acids, and amino acids. One such plant-associated microorganism, *Gluconacetobacter diazotrophicus*, is a gram-negative, obligate aerobic, and endophytic nitrogen-fixing bacterium that was originally isolated from sugarcane [4]. This bacterium has also been isolated from natural *Ipomoea batatas* (sweet potato) [5], *Coffea arabica L.* (coffee) [6], *Pennisetum purpureum* (cameroon grass) [7], and *Ananas comosus* (pineapple) [8]. As well as serving as a nitrogen source for the host plant, *G. diazotrophicus* produces phytohormones such as indole acetic acid and gibberellic acid [9-11], and antimicrobial compounds against phytopathogenic *Xanthomonas albilineans* [12]. *G. diazotrophicus* can also solubilize insoluble metals *in vitro* [13, 14]. Thus, utilization of *G. diazotrophicus* as a biofertilizer in agriculture may allow reduced use of chemical fertilizers.

G. diazotrophicus cells mainly inhabit the host plant and the survival rate is very low when the bacterium is inoculated artificially in soil [15]. Thus, use of *G. diazotrophicus* as a biofertilizer requires a study of its physiological properties, including how this bacterium responds to extracellular compounds in the environment. In this study, we unexpectedly found mucous growth of *G. diazotrophicus* Pal5 on a solid medium with a high concentration of phosphate. We show that the mucous trait is caused by production of levan, a linear fructose polymer, that is enhanced by a high concentration of phosphate.

Results

A high mucous phenotype of *G. diazotrophicus* Pal5 at a high phosphate concentration

In growth experiments on solid media such as LGI-P, C2-NaCl, Dygs, Y & P, and Y & P-NaCl, the *G. diazotrophicus* Pal5 strain showed a stronger mucous phenotype on solid Y & P and Y & P-NaCl medium than on solid LGI-P medium (data not shown). LGI-P [16] is the medium used for *G. diazotrophicus* (Table 1), Y & P medium has been used for *E. coli* carrying *nif* clusters of *Klebsiella pneumonia* [17], and Y & P-NaCl medium is Y & P medium

that lacks NaCl and thiamine (Table 1).

To identify the ingredient(s) in the medium that led to the highly mucous phenotype, we initially showed that each ingredient specific to LGI-P medium ($FeCl_3$, $CaCl_2$, biotin, and pyridoxal; Table 1) had no effect on the highly mucous trait of *G. diazotrophicus* Pal5, excluding the possibility that these ingredients caused the highly mucous phenotype. We then focused on the phosphate concentrations in the LGI-P and Y & P-NaCl media, which are 6 mM and 50 mM, respectively (Table 1). To check the effect of this concentration, growth of the Pal5 strain was examined on solid low P medium (Table 1), in which the 6.25 g/L Na_2HPO_4 in Y & P-NaCl medium was reduced to 0.20 g/L (final phosphate, 7.0 mM). The mucous trait of the cells was significantly reduced on solid low P medium (Fig. 1), suggesting that a high concentration of Na_2HPO_4 caused the highly mucous phenotype of the Pal5 strain.

The Pal5 strain still exhibited a highly mucous phenotype on solid high P medium (Table 1), in which the 6.25 g/L Na_2HPO_4 in Y & P-NaCl medium was reduced to 3.13 g/L (final phosphate, 28 mM). The Pal5 strain also had a mucous trait regardless of use of a sodium or potassium salt, since the strain showed a highly mucous phenotype on medium in which 6.25 g/L Na_2HPO_4 in Y & P-NaCl medium was replaced by 6.25 g/L K_2HPO_4 (data not shown). The highly mucous phenotype of the Pal5 strain also occurred on solid high P medium with adjustment of its normal pH 7.5 to 6.3; whereas the less mucous phenotype occurred on solid low P medium with adjustment of its normal pH 6.3 to 7.5 (data not shown). These findings indicate that the pH of the medium was not involved in the highly mucous phenotype. To examine the effect of a high concentration of Na^+, *G. diazotrophicus* Pal5 was grown on a medium containing 7 mM phosphate and 45 mM Na^+ (Table 1, Fig. 2). No mucous phenotype occurred with this medium, indicating that a high concentration of Na^+ was not associated with the mucous phenotype. Collectively, these data show that a high P concentration (>28 mM) enhances the highly mucous phenotype of *G. diazotrophicus* Pal5.

Enhanced levan production by *G. diazotrophicus* Pal5 at a high phosphate concentration

To identify the components in the mucous material, a *G. diazotrophicus gumD* disruptant (MK4004) strain was constructed. The *gumD* gene codes for a protein that is probably responsible for the first step in extracellular polysaccharide (EPS) production. The amount of EPS produced by the *gumD* disruptant of the Pal5 strain in liquid LGI-based medium with 20 g/L sucrose is reduced by approximately 50% compared to that of wild type (WT) [18]. However, the *gumD* disruptant still formed highly mucous colonies on solid high P medium,

similarly to the WT strain (Fig. 1).

A *G. diazotrophicus lsdA* disruptant was grown in the same way. The *lsdA* gene codes for levansucrase, an extracellular fructosyltransferase that catalyzes synthesis of levan from sucrose. Levan is a linear fructose polymer with β-(2→6) links and more than 100 fructosyl residues. The mucous trait of the *lsdA* disruptant strain was substantially lower than that of the WT strain on solid high P medium (Fig. 1), suggesting that the mucous material is levan.

To confirm that the mucous material was levan, mucous colonies of Pal5 cells on solid high P medium and less mucous colonies on solid low P medium were hydrolyzed and analyzed by HPLC. Authentic samples of hydrolyzed levan, sucrose, and fructose all gave a levan peak at a retention time of 15.3 min (Fig. S1, Fig. 3). The levan peak was higher in hydrolyzed mucous colonies from high P medium than from hydrolyzed less mucous colonies from low P medium (Fig. 3, Fig. S1). Moreover, thin layer chromatography (TLC) analysis that specifically detects levan, fructose, and fructosyl derivatives demonstrated that hydrolyzed mucous colonies from high P medium contain higher amounts of fructose and fructosyl derivatives than hydrolyzed less mucous colonies from low P medium (Fig. S2). Hydrolyzed less mucous colonies of *lsdA* disruptant cells on solid high P medium showed a much smaller levan peak than those of WT or *gumD* disruptant cells on solid high P medium on HPLC analysis. These data confirm that the mucous material is levan and that a high concentration of phosphate (>28 mM) enhances production of levan by Pal5 cells on a solid medium.

To examine whether a high concentration of phosphate (>28 mM) enhances production of levan by Pal5 cells in a liquid medium, Pal5 cells were grown aerobically in liquid high and low P media, and dried EPS (including levan) in the supernatant was weighed, hydrolyzed, and analyzed. The amounts of dried EPS from the two cultures were about the same: 35 ± 2 mg (n = 4; from 25 ml supernatant of high P medium) and 33 ± 6 mg (n = 4; from 25 ml supernatant of low P medium), and gave levan peaks of the same height (data not shown). This indicates that enhanced production of levan by phosphate is a phenotype specific to a solid medium, and does not occur in a liquid medium.

Sugarcane juices were prepared from sugarcanes a1, a2, a3, b1, b2, b3, c1, c2, and c3 (Table 4). Bacteria were isolated from each sugarcane juice and were tentatively identified as *Pantoea dispersa* A1, *Pantoea dispersa* A2, *Klebsiella pneumonia* A3, *Klebsiella variicola* B1, *Klebsiella variicola* B2, *Klebsiella variicola* B3, *Gluconacetobacter diazotrophicus* C2, and *Pantoea agglomerans* C3, respectively, based on the rDNA sequences (Fig. S3). The

phosphate concentrations in the sugarcane juices were 2.8-10.8 mM (Table 4). All isolated bacteria showed a stronger mucous phenotype on solid high P medium than on solid low P medium (Fig. 4). However, these highly mucous phenotypes were not attributable to levan alone, since levan peaks were not observed in A3 cells and were very weak in other cells (Fig. S4).

Tolerance to hydrogen peroxide

To examine whether the tolerance of *G. diazotrophicus* Pal5 to reactive oxygen species (ROS) is improved by levan, we cultivated *G. diazotrophicus* Pal5 WT and its *lsdA* disruptant on solid media containing hydrogen peroxide (H_2O_2) and 0, 3, 10, and 28 mM phosphate (Fig. 5), since the phosphate concentrations of sugarcane juices were determined to be 2.8 to 10.8 mM (Table 4). The *lsdA* disruptant exhibited lesser growth and lesser mucous growth on solid media with 28 mM phosphate and "physiological" phosphate concentrations (3 and 10 mM) in the absence and presence of hydrogen peroxide. Notably, the *lsdA* disruptant showed particularly marked reduction in growth in the presence of hydrogen peroxide and the phosphate concentration had no effect on tolerance (Fig. 5). HPLC analysis confirmed that *G. diazotrophicus* Pal5 synthesizes levan in the presence of 3, 10 and 28 mM phosphate (Fig. S1B). Collectively, these data suggest that levan is important for ROS resistance of *G. diazotrophicus* Pal5 and that a high concentration (28 mM) of phosphate has no effect on ROS resistance.

Discussion

Utilization of *G. diazotrophicus* strains as biofertilizer in agriculture requires an improved understanding of their physiological properties, including how this bacterium responds to extracellular compounds in the environment. Here, we found that *G. diazotrophicus* forms highly mucous colonies and produces a higher amount of levan on a solid medium with a high concentration of phosphate (>28 mM).

Production of levan by *G. diazotrophicus* strains may occur due to the ability of this bacterium to assimilate sucrose [19-21]. In particular, Arrieta et al. found that the *G. diazotrophicus* SRT4 strain forms mucous colonies on solid sucrose-containing LGIE medium [20], although the amount of mucus was not described. LGIE medium is a LGI-based medium that contains (in g/L) tryptone, 1; yeast extract, 0.2; sucrose 50; and glycerol, 10; plus LGI salts [4] (LGI-P, Table 1) [16] including 6 mM phosphate, but not glucose, ammonium

sulfate, biotin, and pyridoxal. Previous studies of *G. diazotrophicus* [4] [19, 22] have used this LGI-based medium. Thus, the effects of a high phosphate concentration on production of levan have not been examined, since the LGI-based medium contains only 6 mM phosphate.

Disruption of levansucrase genes in other several bacteria impairs their behavior in association with plants or animals. A disruption of the *Paenibacillus polymyxa* levansucrase gene impaired its ability to aggregate soil in the wheat rhizosphere [23]. Levansucrase mutants of fireblight pathogen *Erwinia amylovora* caused retarded development of necrotic symptoms on inoculated pear seedlings [24]. The extracellular fructosyltransferase-deficient strain of *Streptococcus mutans* was less pathogenic compared with the wild-type strain [25]. In the case of *G. diazotrophicus*, levan improves stress tolerances, desiccation, osmotic pressure, and NaCl stress of this bacterium [22]. ROS resistance is also important for *G. diazotrophicus* Pal5 in colonization of plants because plants generate superoxide against pathogens as a defense mechanism [26]. Thus, a *G. diazotrophicus* disruptant strain in which ROS-detoxifying genes are destroyed is unable to colonize plant roots efficiently [27]. Use of an *lsdA* disruptant in this study suggested that levan itself is significant for tolerance of hydrogen peroxide, and *G. diazotrophicus* Pal5 was found to synthesize levan on a solid medium containing "physiological" concentrations (3 and 10 mM) of phosphate. This suggests that levan may facilitate this bacterium to colonize and reside in plants by improving tolerance to desiccation, osmotic pressure, and NaCl stress [22], and increasing tolerance to ROS, although the physiological role of the highly mucous phenotype of Pal5 caused by a high concentration of phosphate remains unclear. With regard to the structures of the EPS produced by *G. diazotrophicus*, other than levan, little has been elucidated. It has been recently reported that *G. diazotrophicus* Pal5 produced the EPS that has 4-*O*-substituted units of β-glucose, 3-*O*-substituted units of β-galactose and 2-*O*-substituted units of α-mannose in the liquid medium containing mannitol [28].

Finally, our finding that synthesis of levan by *G. diazotrophicus* Pal5 is increased on a solid medium containing a high concentration of phosphate may be important for industrial production of levan. Levan is currently synthesized on an industrial scale using bacterium such as *Bacillus* species [29]. Hypocholesterolemic [30] and cosmeceutical [31] effects of levan have been reported and application of levan is likely in a variety of industrial fields, including food, cosmetics, and medicine.

Materials and Methods

202 Bacterial strains and growth conditions

203 *G. diazotrophicus* Pal5 ATCC49037 (American Type Culture Collection) was grown at
204 30°C in C2-NaCl, Dygs [32], LGI-P [16], Y & P-NaCl, or high and low P media. C2-NaCl
205 medium is C2 medium [33] that lacks NaCl and contains (in g/L) tryptone, 10; glucose, 15;
206 yeast extract, 5; pH 6.5. Y & P-NaCl medium is Y & P medium [17] that lacks NaCl and
207 thiamine. The compositions of Y & P-NaCl, LGI-P, high P, and low P media are shown in
208 Table 1. Solid medium was made by adding agar at 15 g/L. *Escherichia coli* DH5α was used
209 as the cloning host and was grown at 37°C in LB medium, which contains (in g/L) tryptone,
210 10; yeast extract, 5; NaCl, 10; pH 7.2.
211
212 Construction of plasmids and recombinant *G. diazotrophicus* Pal5 strains

213 Primers and plasmids are shown in Tables 2 and 3. pKTY320-kan::gumD was
214 constructed as follows. *gumD* (1521 nt) was amplified by PCR with primers 1 and 2, using *G.*
215 *diazotrophicus* Pal5 genomic DNA as a template. The PCR product was inserted into
216 HincII-treated pUC119, yielding pUC119-gumD. Using this plasmid as a template, inverse
217 PCR was conducted with primers 3 and 4. Km^r fragment, obtained by SalI digestion of
218 pUC4K, was inserted by in-fusion (Clontech) into the amplified DNA fragment, resulting in
219 pUC119-kan::gumD. Using this plasmid as a template, the Km^r-inserted *gumD* was amplified
220 by PCR with primers 5 and 6. The amplified fragment was inserted by in-fusion into
221 HincII-treated pKTY320, giving pKTY320-kan::gumD.

222 pKTY320-kan::lsdA was similarly constructed. A DNA fragment containing a part of
223 *lsdA* (1460 nt: from nt 247 to 1706 in a 1755 nt full length *lsdA*) was amplified by PCR with
224 primers 7 and 8, using the Pal5 genomic DNA as a template. The PCR product was inserted
225 into HincII-treated pUC118, yielding pUC118-lsdA-2BamHI. Using this plasmid as a
226 template, this DNA fragment was amplified by PCR with primers 9 and 10. The PCR product
227 was digested with XmaI/HindIII and ligated into XmaI/HindIII-treated pUC118, resulting in
228 pUC118-lsdA-1BamHI. The Km^r fragment obtained by BamHI digestion of pUC4K was
229 inserted into the BamHI site of pUC118-lsdA-1BamHI to give pUC118::kan-lsdA. Using this
230 plasmid as a template, the Km^r-inserted *lsdA* was amplified by PCR with primers 11 and 12.
231 The amplified fragment was inserted by in-fusion into HincII-treated pKTY320, yielding
232 pKTY320-kan::lsdA.

233 *G. diazotrophicus* Pal5 mutant strains (MK4004, *gumD*::Km^r in Pal5; MK4384,
234 *lsdA*::Km^r in Pal5) were made by insertional mutagenesis [33]. pKTY320-kan::gumD or

pKTY320-kan::lsdA was introduced into the *G. diazotrophicus* Pal5 strain by electroporation. The applied pulse conditions were 10.0 kV/cm and 5 msec using Bio-Rad Gene Pulser Xcell (Bio-Rad). Competent cells (100 µl) were mixed with 100 ng of plasmid DNA. Transformants were selected on solid Dygs medium containing kanamycin (200 µg/ml).

HPLC and TLC analyses of *G. diazotrophicus* Pal5 cells

G. diazotrophicus Pal5 cells for HPLC analysis were grown on membranes as follows. Pal5 cells were grown in C2-NaCl liquid medium aerobically at 30°C overnight and collected by centrifugation at 10,000×g for 2 min. The cells were washed 3 times with 0.9% (w/v) NaCl and diluted to OD_{600} of 1.0. The cells in 50 µl of this suspension were put on a membrane (diameter 13 mm, pore size 0.22 µm) that was placed on solid medium and incubated at 30°C for 1 week. Colonies of the cells together with mucous materials that grew on the membrane were collected with a sterilized spatula into an Eppendorf tube and weighed.

For HPLC analysis, collected and weighed cells were suspended in 4% (w/v) H_2SO_4 to reach 14% (w/w). Authentic samples of levan (L8647 from *Erwinia herbicola*; Sigma-Aldrich), sucrose, fructose, and glucose were dissolved in 4% (w/v) H_2SO_4 to reach 0.5%, 2%, 1%, and 1% (w/w), respectively. The suspensions were hydrolyzed at 121°C for 1 h and centrifuged at 20,000×g for 5 min at 4°C. The supernatant was filtered (pore size 0.2 µm) and analyzed by HPLC using an Aminex HPX-87H column (300×87 mm; Bio-Rad), a RID-10A detector (Shimazu, Kyoto, Japan), an effluent of filtered and degassed 0.01N H_2SO_4, a flow rate of 0.6 ml/min, and a column temperature of 65.0°C.

For TLC analysis, collected and weighed cells were suspended in 3% (v/v) trichloroacetic acid (TCA) to reach 14% (w/w). Authentic sample of levan was dissolved in 3% (v/v) TCA to reach 0.5% (w/v). Authentic samples of levan, fructose, and glucose were also dissolved in water to reach 0.5% (w/w). The suspensions in 3% (v/v) TCA were hydrolyzed at 55°C for 15 min [34, 35] and centrifuged at 20,000×g for 5 min at 4°C. The supernatant (5 µl) and other authentic sample (5 µl) were spotted on TLC silica gel 60 F_{254} (Merck KGaA) and developed in a solvent system consisting of butanol–acetate–water (3:3:2 v/v/v). Levan, fructose, and fructosyl derivatives were specifically detected using resorcinol and thiourea as described [34].

Isolation of levan from liquid culture

G. diazotrophicus Pal5 cells were precultured in liquid C2-NaCl medium, washed with 0.9% NaCl as above, and inoculated to OD_{600} of 0.1 in 30 ml of fresh liquid high P medium in a 300 ml Erlenmeyer flask. Cells were cultivated at 150 strokes per min at 30°C for 48 h and supernatant was obtained by centrifugation at 20,000 g for 5 min. Levan was isolated from the supernatant as described previously [19]. Briefly, levan was precipitated with 2 volumes of ethanol 99.5%, collected with centrifugation at 20,000 g for 20 min, washed once with 66% ethanol, and freeze-dried. The dried material was weighed and a portion was dissolved in 4% (w/v) H_2SO_4 to reach 1% (w/w), hydrolyzed, and analyzed by HPLC as above.

Determination of phosphate concentration in sugarcane juice

Sugarcanes grown in Okinawa were purchased from Ryuka Shoji (Okinawa, Japan), Hatsuhino (Okinawa, Japan), and Ryukyu farm (Okinawa, Japan). Phosphate concentrations were determined in three sugarcanes from each company (a1, a2, a3, b1, b2, b3, c1, c2 and c3) using a previously described method [36].

Isolation of bacteria from sugarcane juice

Sugarcane juice (1 µl) was streaked on solid high P medium containing 5 ml of 0.5% bromothymol blue in 0.2 N KOH in 1 L of medium. Each colony was purified by streaking twice on solid low P medium. Genomic DNA was isolated from bacteria and the 16S rDNA gene was amplified using primers 13 and 14 (Table 2). Sequence analysis of the amplified fragment was conducted using primers 13, 14, 15 and 16. rDNA sequences were analyzed by BLAST search [37] using the NCBI 16S ribosomal RNA sequence database. Bacteria were tentatively identified based on the highest max score.

Tolerance to hydrogen peroxide

G. diazotrophicus Pal5 was precultured in C2-NaCl, washed 3 times with 0.9% NaCl, and diluted to OD_{600} of 1.0. The cell suspension (2 µl) was spotted on solid media containing Na_2HPO_4 as a sole phosphate source. The pH was adjusted to 5.0 to match that of sugarcane juice.

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418 **Table 1.** Composition of LGI-P, Y&P-NaCl, high P, and low P media

	LGI-P (g/L)	Y & P-NaCl (g/L)	High P medium (g/L)	Low P medium (g/L)
Sucrose	-	20	20	20
Glucose	5	-	-	-
KH_2PO_4	0.6	0.75	0.75	0.75
K_2HPO_4	0.2	-	-	-
Na_2HPO_4	-	6.25	3.13	0.20
$(NH_4)_2SO_4$	0.13	0.13	0.13	0.13
$FeSO_4 \cdot 7H_2O$	-	0.025	0.025	0.025
$FeCl_3 \cdot 6H_2O$	0.01	-	-	-
$CaCl_2 \cdot 2H_2O$	0.02	-	-	-
$Na_2MoO_4 \cdot 2H_2O$	0.021	0.025	0.025	0.025
$MgSO_4 \cdot 7H_2O$	0.2	0.2	0.2	0.2
Biotin	0.0001	-	-	-
Pyridoxal	0.0002	-	-	-
pH	6.3	7.8	7.5	6.3
Phosphate (mM)	6	50	28	7
Na^+ (mM)	0.16	88.2	44.2	3.0

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420

421 **Table 2.** Primers used in the study [a]

Primer	Sequence	Note
Primer 1	ACAAGCTT*CGCTTGCCCGAT GGAGATC*	Sequence in italics corresponds to position -3 to 16 of 1521 nt of *gumD* (1,521 nt).
Primer 2	CAGAATTC*GCGTCAGAAGG CATGACGGC*	Sequence in italics corresponds to position +3 to 1505 of *gumD*.
Primer 3	CCCCTGCAGGTCGAC*GCAT CATCCCATCGTGCCGT*	Sequence in italics corresponds to 411 to 430 of *gumD*.
Primer 4	CCCCCCCCTGCAGGT*AGCAT GTACAGGACGATGAC*	Sequence in italics corresponds to 410 to 391 of *gumD*.
Primer 5	TTATCCCGTGTTGAC*TTGCC CGATGGAGATCTTCG*	Sequence in italics corresponds to position 1 to 20 of *gumD*.
Primer 6	TTGCCCGGCGTCAAC*TCAG AAGGCATGACGGCTGA*	Sequence in italics corresponds to position 1502 to 1521 of *gumD*.
Primer 7	GATCCTCTAGAGTC*GACTTT ACCGCCCGCTGGACAC*	Sequence in italics corresponds to position 247 to 268 of *lsdA* (1755 nt).
Primer 8	GCATGCCTGCAGGTCGTGG ACGCCTGCGCCGCCAGAC	Sequence in italics corresponds to position 1685 to 1706 of *lsdA*.
Primer 9	CCC**CCCGGGG**GGGACTTT ACCGCCCGCTGGACAC	XmaI site is in bold.
Primer 10	GTAAAACGACGGCCAGT	M13 forward Primer
Primer 11	TTATCCCGTGTTGAC*GACTT TACCGCCCGCTGGACAC*	Sequence in italics corresponds to position 247 to 268 of *lsdA*.
Primer 12	TTGCCCGGCGTCAAC*GTGG ACGCCTGCGCCGCCAGAC*	Sequence in italics corresponds to position 1685 to 1706 of *lsdA*.
Primer 13	GAGTTTGATCCTGGCTCAG	For amplification and sequencing of 16S rDNA.
Primer 14	GGCTACCTTGTTACGA	For amplification and sequencing of 16S rDNA.
Primer 15	TACCAGGGTATCTAATCC	For sequencing of 16S rDNA.
Primer 16	GGCTACCTTGTTACGA	For sequencing of 16S rDNA.

422 [a] Underlined sequences are for in-fusion. Primers 1 to 6 have been described previously [18].

423

424 **Table 3.** Plasmids used in the study

Plasmid	Note	Reference
pUC119	Amp^r ColE1 replicon	Takara
pUC118	Amp^r ColE1 replicon	Takara
pUC4K	Amp^r Km^r ColE1 replicon	GE healthcare
pKTY320	Amp^r Cm^r Mob p15A replicon	Kimbara *et al.* 1989
pUC119-gumD	*gumD* in HincII site of pUC119	This study
pUC118-lsdA-2BamH1	*lsdA* in HincII site of pUC118	This study
pUC118-lsdA-1BamH1	*lsdA* in XmaI/HindIII sites of pUC118	This study
pUC119-kan::gumD	Km^r-inserted *gumD* in pUC119	This study
pUC118-kan::lsdA	Km^r-inserted *lsdA* in pUC118	This study
pKTY320-kan::gumD	Km^r-inserted *gumD* in HincII site pKTY320	This study
pKTY320-kan::lsdA	Km^r-inserted *lsdA* in HincII site pKTY320	This study

425

Table 4. Concentration of phosphate in sugarcane juice

Company	Ryuka Shoji			Hatsuhino			Ryukyu Farm		
Sugarcane	a1	a2	a3	b1	b2	b3	c1	c2	c3
Phosphate (mM)	5.4	9.6	8.0	2.9	3.0	2.8	10.8	5.7	6.2

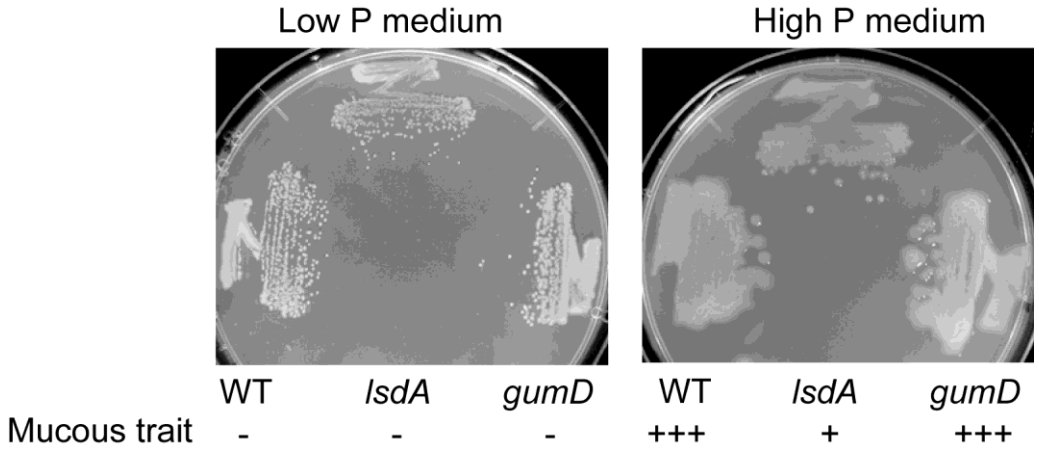
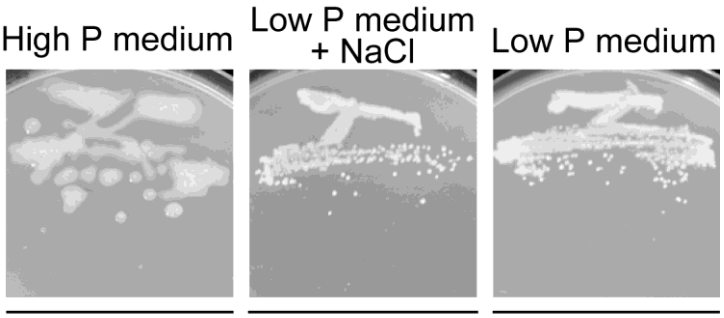


Fig. 1. *G. diazotrophicus* Pal5 WT, *gumD* mutant, and *lsdA* mutant strains grown on high or low P solid medium. Plus (+++) indicates the strongest mucous trait, plus (+) is moderate, and minus (-) is the weakest. Cells were grown for 1 week at 30°C.



	High P medium	Low P medium + NaCl	Low P medium
Total PO_4^{3-} (mM)	28.0	7.0	7.0
Total Na^+ (mM)	44.2	44.2	3.0
Mucous trait	+++	-	-

Fig. 2. *G. diazotrophicus* Pal 5 WT grown on high P, low P, and low P medium + NaCl containing the same concentration of Na^+ as that of the high P medium. Cells were grown for 1 week at 30°C.

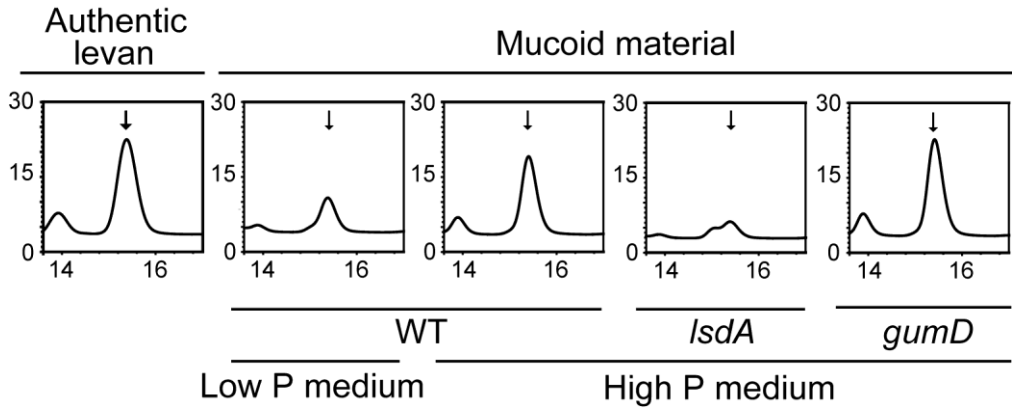


Fig. 3. HPLC analysis of mucous materials. Authentic levan and mucous material collected from colonies of WT, *gumD*, and *lsdA* cells grown for 1 week at 30°C on the indicated solid media were hydrolyzed and analyzed. The vertical axis of the chromatograph shows the relative peak level. The retention time (min) is shown on the horizontal axis. Arrows show the levan peak (15.3 min).

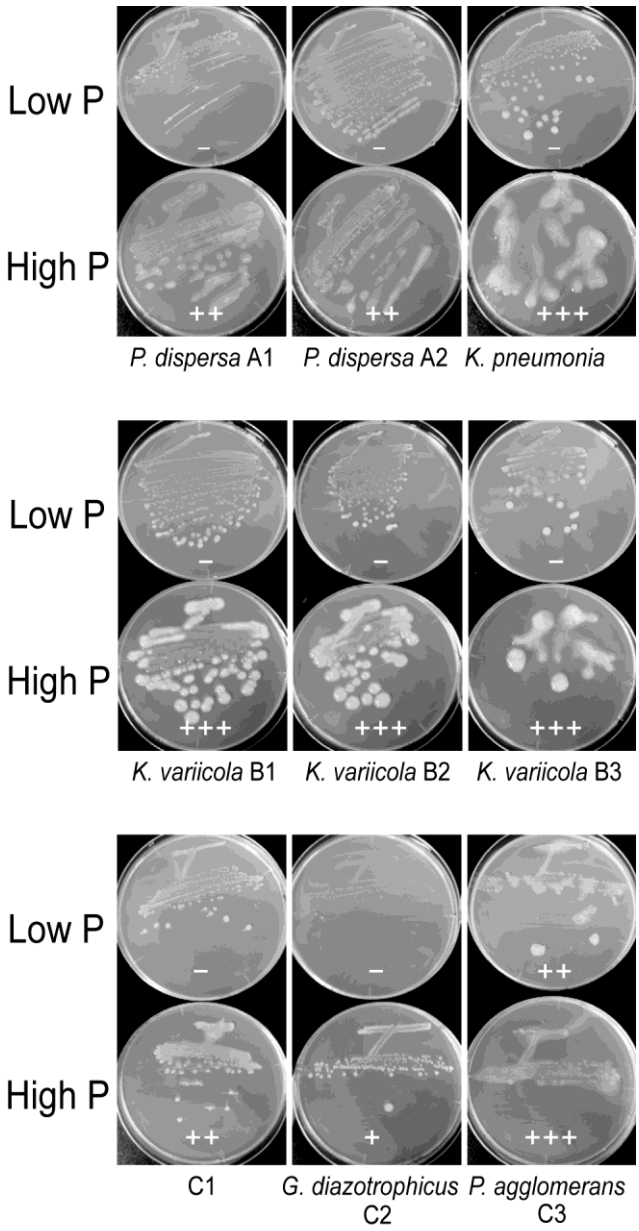
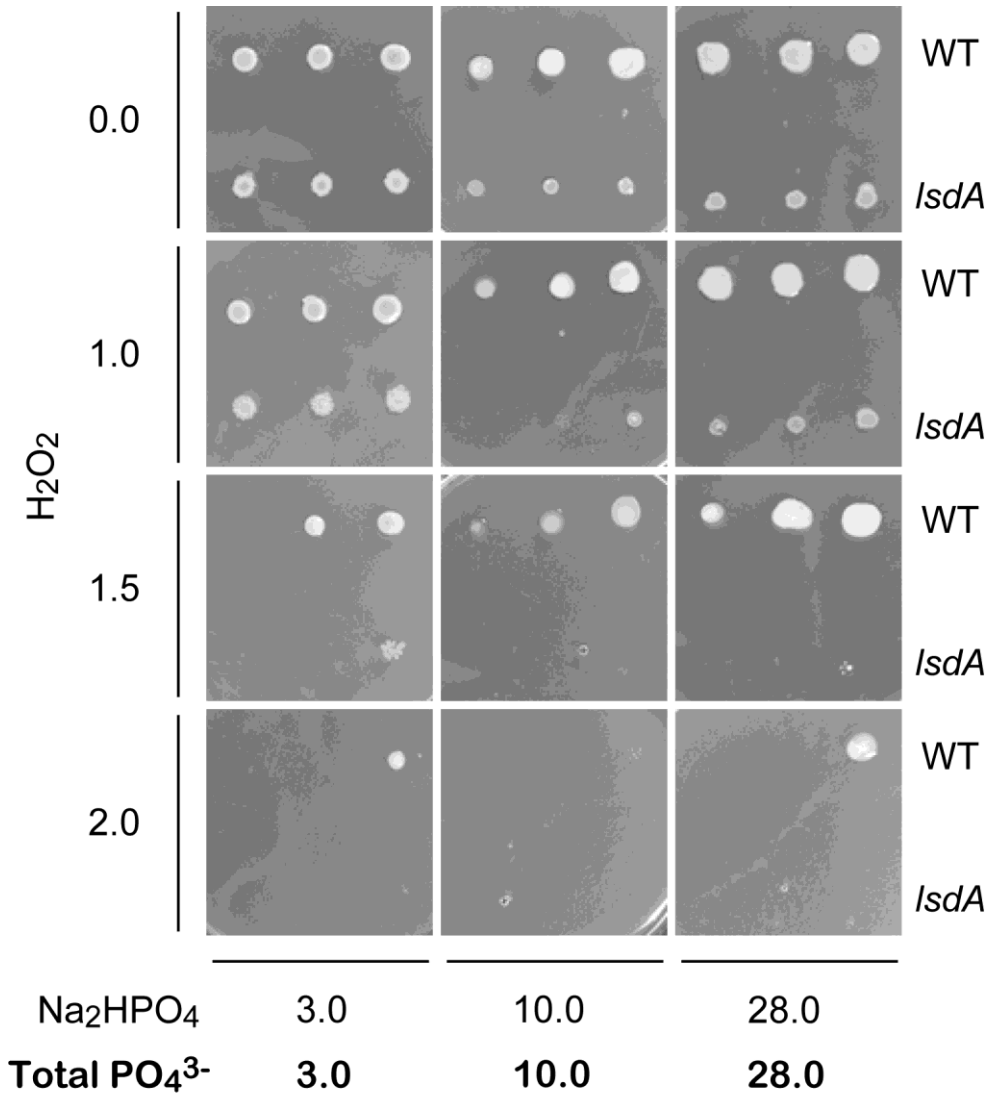


Fig. 4. Growth on high P or low P medium of bacteria isolated from sugarcane juice. Plus (+++) indicates the strongest mucous trait, plus (+) is moderate, and minus (-) is the weakest. Cells were grown for 1 week at 30°C. Bacteria were tentatively identified based on the rDNA sequences.



462

463 **Fig. 5.** Growth of *G. diazotrophicus* Pal5 WT and *lsdA* disruptant on solid media containing
464 hydrogen peroxide (H_2O_2). Three spotted cells were diluted tenfold from OD_{600} of 1.0 to 0.01
465 from right to left.

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Supplemental Figures

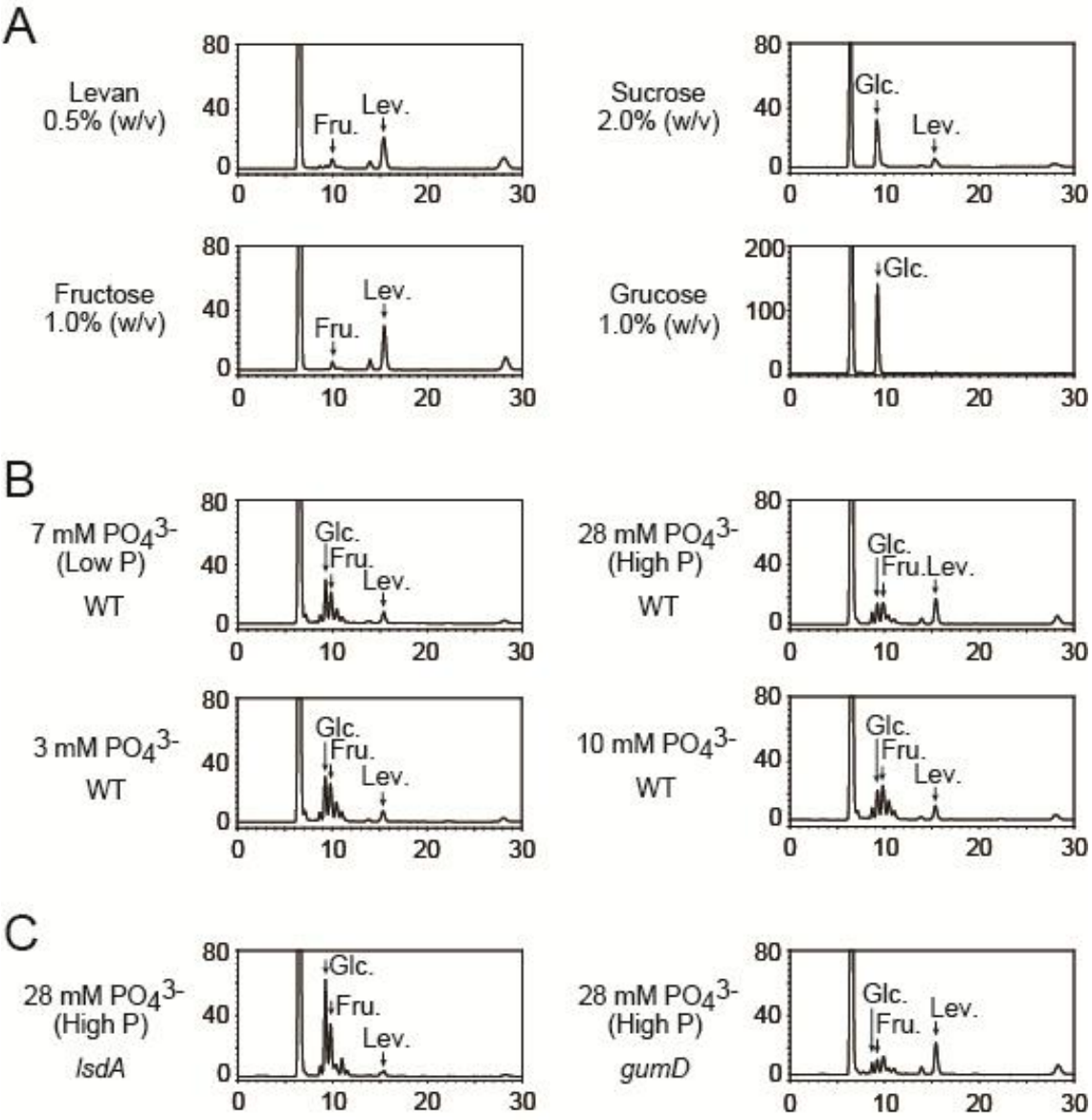

Fig. S1 Results of HPLC analysis. The vertical axis of the chromatograph shows the relative peak level. The retention time (min) is shown on the horizontal axis. Cells were grown for 1 week at 30°C. A, Chromatographs of authentic samples. B, Chromatographs of *G. diazotrophicus* wild type (WT) cells grown on solid media containing 3, 7, 10, or 28 mM phosphate (PO_4^{3-}). C, Chromatographs of *G. diazotrophicus* disruptants grown on solid high P medium. Lev. shows the levan peak.

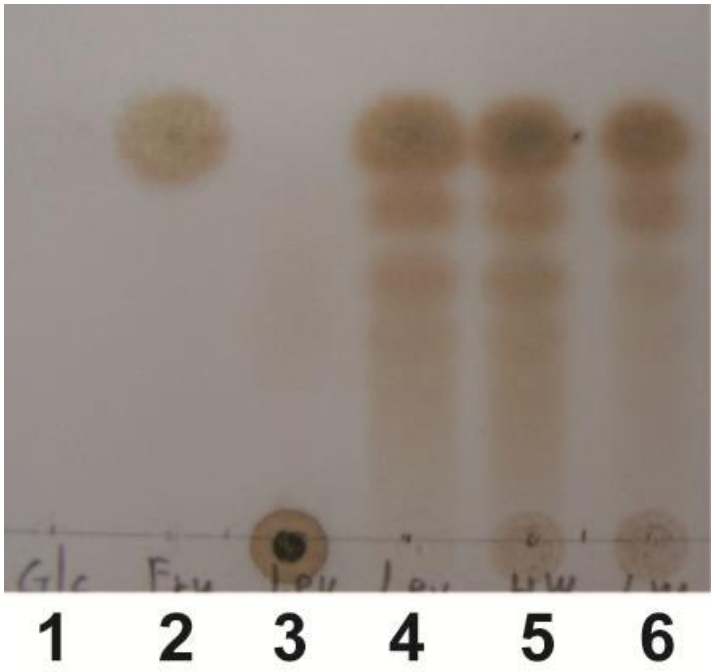

Fig. S2 TLC analysis that specifically detects levan, fructose, and fructosyl derivatives. Sample preparation and analysis was conducted as described in Materials and Methods. Lanes 1 – 3: 0.5% (w/v in water) authentic samples without hydrolysis; 1, glucose, 2, fructose, 3, levan. Lane 4: hydrolyzed 0.5% (w/v) levan in 3% (v/v) TCA at 55°C for 15 min. Lanes 5 and 6: 14% (w/v) cells hydrolyzed in 3% (v/v) TCA at 55°C for 15 min; 5, cells from high P medium (lane 5) and low P medium (lane 6).

rDNA sequence of *Pantoea dispersa* A1

TCGAACGGCAGCACAGAAGAGCTTGCTCTTTGGGTGGCGAGTGGCGGACGGGTGAGTAATGTCT
GGGAAACTGCCCGATGGAGGGGGATAACTACTGGAAACGGTAGCTAATACCGCATAACGTCGCAA
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rDNA sequence of *Pantoea dispersa* A2

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rDNA sequence of *Klebsiella pneumonia* A3

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rDNA sequence of *Klebsiella variicola* B1

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AGCTTAACCTTCGGGAGGGCG

rDNA sequence of *Klevsiella variicola* B2

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rDNA sequence of *Klevsiella variicola* B3

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rDNA sequence of *Gluconacetobacter diazotrophicus C2*

GTCGCACGAACCTTTCGGGGTTAGTGGCGGACGGGTGAGTAACGCGTAGGGATCTGTCCATGGG
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CCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGCGTTGCTCGGAATGACTG
GGCGTAAAGGGCGCGTAGGCGGTTTGGACAGTCAGATGTGAAATTCCTGGGCTTAACCTGGGGG
CTGCATTTGATACGTACAGACTAGAGTGTGAGAGAGGGTTGTGGAATTCCCAGTGTAGAGGTGAA
ATTCGTAGATATTGGGAAGAACACCGGTGGCGAAGGCGGCAACCTGGCTCATAACTGACGCTGAG
GCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGTGTG
CTGGATGTTGGGTGGCTTAGCCCCTCAGTGTCGTAGTTAACGCGATAAGCACACCGCCTGGGGAG
TACGGCCGCAAGGTTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGG
TTTAATTCGAAGCAACGCGCAGAACCTTACCAGGGCTTGACATGGGGAGGCTGCAGTCAGAGATG
GCTGTTTCCCGCAAGGGACCTCCTGCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGA
TGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCGCCTTTAGTTGCCAGCATGATTGGGTGGGCA
CTCTAAAGGAACTGCCGGTGACAAGCCGGAGGAAGGTGGGGATGACGTCAAGTCCTCATGGCCC
TTATGTCCTGGGCTACACACGTGCTACAATGGCGGTGACAGTGGGAAGCCAGGCAGCGATGCCGA
GCTGATCTCAAAAAGCCGTCTCAGTTCGGATTGCACTCTGCAACTCGAGTGCATGAAGGTGGAAT
CGCTAGTAATCGCGGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGT
CACACCATGGGAGTTGGTTTGACCTTAAGCCGGTGAGCGAACCCAGCAATGGGGCGCAGCCGAC

rDNA sequence of *Pantoea agglomerans C3*

GCAAGTcgGACGGTAGCACAGAGGAGCTTGCTCCTCGGGTGACGAGTGGCGGACGGGTGAGTAAT
GTCTGGGGATCTGCCCGATAGAGGGGGATAACCACTGGAAACGGTGGCTAATACCGCATAACGTC
GCAAGACCAAAGAGGGGGACCTTCGGGCCTCTCACTATCGGATGAACCCAGATGGGATTAGCTAG
TAGGCGGGGTAACGGCCCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCACA
CTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGC
GCAAGCCTGATGCAGCCATGCCGCGTGTATGAAGAAGGCCTTCGGGTTGTAAAGTACTTTCAGCG
GGGAGGAAGGCGATGTGGTTAATAACCGTGTCGATTGACGTTACCCGCAGAAGAAGCACCGGCTA
ACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAA
GCGCACGCAGGCGGTCTGTTAAGTCAGATGTGAAATCCCCGGGCTTAACCTGGGAACTGCATTTG
AAACTGGCAGGCTTGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGA
GATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAA
GCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTTGGAGG
TTGTTCCCTTGAGGAGTGGCTTCCGGAGCTAACGCGTTAAGTCGACCGCCTGGGGAGTACGGCCG
CAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCG
ATGCAACGCGAAGAACCTTACCTACTCTTGACATCCACGGAATTTGGCAGAGATGCCTTAGTGCCT
TCGGGAACCGTGAGACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAG
TCCCGCAACGAGCGCAACCCTTATCCTTTGTTGCCAGCGATTCGGTCGGGAACTCAAAGGAGACT
GCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGAGTAGGGCT
ACACACGTGCTACAATGGCGCATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAA
GTGCGTCGTAGTCCGGATCGGAGTCTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGT
GGATCAGAATGCCACGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGAG
TGGGTTGCAAAAGAAGTAGGTAGCTTAACCTTCGGGAGGGCGCTTACCA

Fig. S3. rDNA sequences of bacteria isolated from sugarcane juices. Sequences analyzed by both forward and reverse primers are shown in uppercase letters, and that analyzed with only one primer pair is in lowercase letters.

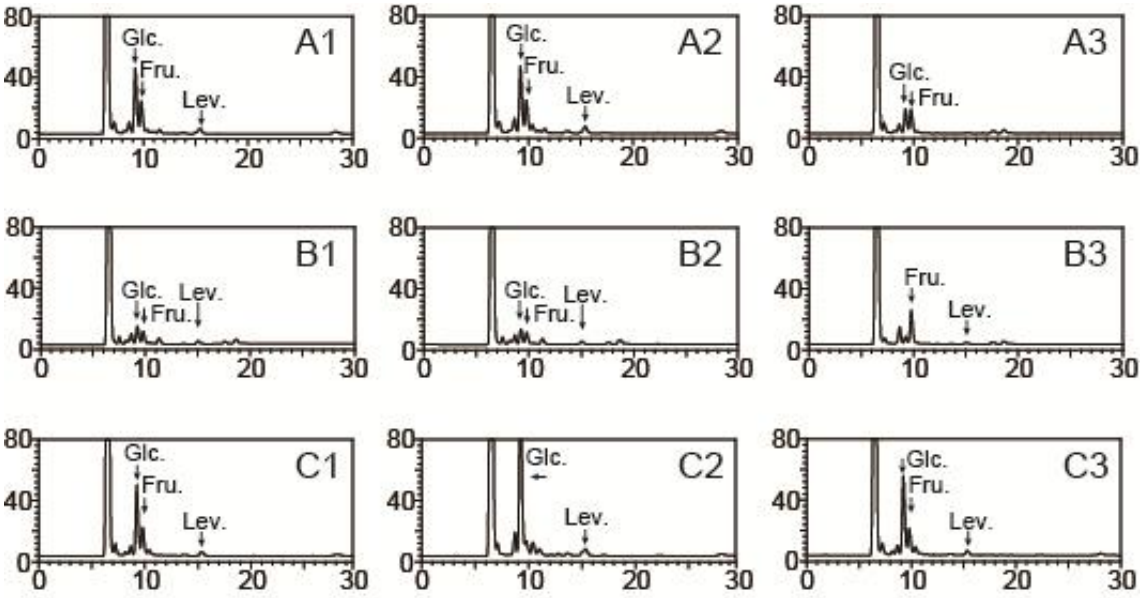

Fig. S4 Results of HPLC analysis of bacterial cells from sugarcane juice. The vertical axis of the chromatograph shows the relative peak level. The retention time (min) is shown on the horizontal axis. Cells were grown for 1 week at 30°C. The levan peak was not detected in A3.