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2 Glucomannan Utilization Operon of *Bacillus subtilis*

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Abstract

We have characterized the glucomannan utilization operon (*gmuBACDREFG,* formerly *ydhMNOPQRST*) of *Bacillus subtilis*. Transcription of the operon is induced by konjac glucomannan and requires the last mannanase gene (*gmuG*). Cellobiose and mannobiose, possible degradation products of glucomannan by GmuG, are strong inducers of transcription. We show that an internal regulator gene (*gmuR*) encodes a repressor of the operon, as disruption of this gene enhances transcription of the operon in the absence of inducers. The expression of the glucomannan utilizing operon of *B. subtilis* is thus induced by degraded glucomannan products, and repressed by an internal repressor.

1. Introduction

A cluster of eight open reading frames (ORFs, *gmuBACDREFG,* formerly *ydhMNOPQRST*) coding for proteins with similarity to mannanase and related enzymes involved in the degradation and utilization of mannan was found by the *Bacillus subtilis* genome project [1-2]. Mannanase genes have been isolated from *Bacillus* species by several groups, but without further characterization [3-4]. Until now, no reports have appeared, which describe the mannan utilization operon *of B. subtilis.* It seems that *B. subtilis* kept in modern laboratories has never been fed with mannan for a scientific purpose.

The first three ORFs (*gmuB, gmuA,* and *gmuC* or *ydhM, ydhN* and *ydhO*) of the above cluster code for proteins homologous to the enzymes IIB, IIA, IIC of lactose-class phosphotransferase systems (PTSs). The Gmu-PTS seems to be specific for oligo-α-mannoside and its components strongly resemble the proteins of the cellobiose-specific Lic-PTS of *B. subtilis* (about 40% identity). The fourth ORF (*gmuD* or *ydhP*) codes for a protein with similarity to

phospho-β-glucosidase. The fifth ORF (*gmuR* or *ydhQ*) codes for a DNA-binding protein with DNA binding domain belonging to the GntR family, more specifically to a subgroup containing a small molecule-binding UTRA (UbiC transcription regulator-associated) domain [5]. The sixth and the seventh ORF (*gmuE* and *gmuF* or *ydhR* and *ydhS*) code for proteins homologous to fructokinase and mannose-6-phosphate isomerase. The last ORF (*gmuG* or *ydhT*) codes for a protein homologous to secretory mannan endo-1,4-β-mannanase with a signal sequence at the N-terminus [1].

As a soil bacterium, *B. subtilis* must be able to degrade different kinds of plant derived saccharides including sucrose, amylose, levan, and lichenan [6]. These are degraded by extracellular enzymes, levansucrase (*sacB*), amylase (*amyE*), levanase (*sacC*), and lichenase (*licS*). In this communication we describe the gene organization of the glucomannan utilization (*gmu*) operon of *B.subtilis,* which is located at about 55 degrees on the chromosome and consists of eight ORFs utilized for digestion, incorporation, and phosphorylation of glucomannan,

a β-D-1,4-linked linear polymer of glucose and mannose with one acetyl group on every 9-19 sugar units [7].

2. Materials and methods

2.1. Bacterial strains and plasmids used

All strains used were derivatives of *B. subtilis* Marburg 168 *trpC2* [8]. BSU71 (*trpC2* P_{gmuB}::pMUTIN2) carried the pMUTIN2 plasmid inserted downstream of the *gmuB* promoter. BSU73 (*trpC2 gmuB*::pMUTIN2), BSU75 (*trpC2 gmuA*::pMUTIN2), BSU77 (*trpC2 gmuC*::pMUTIN2), BSU81 (*trpC2 gmuR*::pMUTIN2) and BSU87 (*trpC2 gmuG*::pMUTIN2) were insertional disruptants of ORFs of the *gmuBACDREFG* operon with pMUTIN2 (*erm* in *B.subtilis*) [9]. An insertionally disrupted *ccpA* gene with neomycin cassette was introduced by transformation of BSU71, BSU81 and BSU87 to Neo^R with DNA from FU402 (*trpC2 ccpA::neo*) provided by Y. Fujita. This gave BSU72 (*trpC2* P_{gmuB}::pMUTIN2 *ccpA::neo*), BSU82(*trpC2 gmuR*::pMUTIN2 *ccpA::neo*), and BSU88(*trpC2 gmuG*::pMUTIN2 *ccpA::neo*).

2.2 Media, reagents, enzymes and LacZ activity

Cells of *B. subtilis* or *Escherichia coli* were grown in Luria-Bertani broth. Reagents and enzymes were purchased from TaKaRa (Kyoto, Japan), Wako (Osaka, Japan), Sigma (St.Louis, Mo.). Konjac glucomannnan was purchased from Wako. The LacZ activity was measured as in [10].

2.3. DNA manipulation and transformation

Plasmid DNA purification, PCR amplification of DNA fragments, digestion of DNA with restriction enzyme, and DNA ligation were carried out as described elsewhere [8]. Transformation of *B.subtilis* strain 168 and *E. coli* strain C600 was carried out as described in [8].

2.4. Disruption of ORFs

For disruption of the ORFs, a primer pair with *Hin*dIII or *Bam*HI site was used for PCR amplification of a portion of each ORF. These primers were custom-made (Espec Oligo Service, Tsukuba, Japan). Disruption with the integrative plasmid pMUTIN2 was carried out as described elsewhere [8-9]. The 5'-proximal internal portion of each ORF was cloned into the pMUTIN2 plasmid and the resulting recombinant plasmid was used to disrupt each ORF. Primer pairs were

5'-GCCGAAGCTTAAAAAAATATTACTCG/

5'-CGCGGATCCGTTGTATTGATCTGCTT for *gmuB*, 5'-GCCGAAGCTTGAACAG ATGAAAATCAC/ 5'-CGCGGATCCAGCTGTTGCTTCTCCGC for *gmuA*,

5'-GCCGAAGCTTCTCCACGGCGACAATGG/

5'-CGCGGATCCGTTGATAATGATCTGTC for *gmuC*,

5'-GCCGAAGCTTAAATGAAATGAGAAATA/ 5'-CG CGGATCCGGCGGCCACTTCCTCAG for *gmuR*, and 5'-GCCGAAGCTTAAAAA CAGTGATGAACT/ 5'-CGCGGATCCCAACTCTTGAAGTCCGT for *gmuG*. The resulting disrupted ORFs carried the transcriptionally fused *E.coli lacZ* gene to the disrupted gene. For promoter cloning, primers

5’-GCCGAAGCTTAGCTGAATCCCATAAAAATAATTCC and

5’-CGCGGATCCCAACTGCTATCCCCCCTGTTATTAAAACG were used.

2.5. Determination of the start site of transcription of the putative gmu operon

Total RNA was extracted from cells of the wild type strain 168 grown in LB medium in the presence of 0.1% cellobiose. The primer extension was carried out as described elsewhere [11] with digoxigenin labeled primer DNA (5’-ACAGCCCAAATCTCCGCTTCC) and reverse transcriptase (Invitrogen Super Script^{TM} II RNase H^{-} Reverse Transcriptase).

2.6. Northern analysis of transcription of the putative gmu operon

Northern analysis was performed with DIG-labeled *gmuG* RNA probe as described elsewhere [8], using BSU87(*gmuG*::pMUTIN2) DNA as a PCR template.

3. Results and discussion

3.1. Glucomannan induced the transcription of the putative gmu operon of B.subtilis

In order to observe the induction of transcription of the putative *gmuBACDREFG* operon, we constructed a fusion of the *gmuB* promoter P_{gmuB} to the *lacZ* gene with the *spoVG* SD sequence. The strain BSU71 carried P_{gmuB}-*lacZ* and the whole putative *gmu* operon downstream of the inserted pMUTIN. We found that the LacZ activity of the P_{gmuB}-*lacZ* construct on the integrated pMUTIN plasmid was strongly induced upon addition of glucomannan from konjac flour but not by yeast mannan which is a α-D-1,6-linked linear polymer of mannose with side chains of mannose (Fig. 1A-1,2 and Fig.1C). To our surprise, the β-D-1,4-linked linear polymer of carboxy methyl glucose (CM cellulose) induced only very weak activity (BSU71, Fig.1A-3). Transcription of *lacZ* on the pMUTIN plasmid used to disrupt the last *gmuG* gene was not

induced by glucomannan, mannan, nor by CM cellulose (BSU87, Fig.1B-1,2,3 and Fig.1C).

However, prolonged incubation with the nutrient agar permitted LacZ expression even by the *gmuG* disrupted strain. This activity may be due to the presence of cellulase such as BglC (SubtiList, http://genolist.pasteur.fr/SubtiList/).

3.2. Cellobiose or mannobiose induced, and glucose repressed the transcription of the putative gmu operon of B.subtilis

Cellobiose (Glcβ1-4Glc), which induces the putative *gmuBACDREFG* operon in microarray assays (Y.Fujita, personal communication), induced LacZ activity even in the above *gmuG*-disrupted strain (BSU87) (Fig.1B-5, Fig. 1C and Table 1), as well as in the P_{gmuB}-*lacZ* strain (BSU71) (Fig.1A-5, Fig. 1C and Table 1). Furthermore mannobiose (Manβ1-4Man) was an inducer of the above putative

operon (Fig.1A,B-4). These results indicate that exogenous glucomannan and cellulose were digested by the putative secretory GmuG, β-mannanase, and that oligosaccharide products such as cellobiose and mannobiose were incorporated by the GmuBAC transporter and induced the putative *gmuBACDREFG* operon. In fact, few monosaccharides were found in the GmuG dependent degradation products of glucomannan but some oligosaccharides found in the GmuG dependent degradation products of glucomannan have been shown to induce the above operon (T.Nakajima, personal communication).

Glucose addition strongly repressed transcription induction by cellobiose of the putative *gmuBACDREFG* operon (Table 1). Repression of the putative *B.subtilis gmu* operon was greatly relieved by the *ccpA* mutation (Table 1), both in the Gmu^+ (BSU72) and the $GmuG^-$ (BSU88) strain.

Glucose causes repression of gene expression via CcpA (catabolite control protein A) through the catabolite fructose-biphosphate (FBP) [6]. FBP stimulates HPr kinase/phosphatase-catalized phosphorylation of HPr (histidine-containing phosphoprotein) or Crh (catabolite repression HPr-like protein). Phosphorylated

HPr (HPr-P) or Crh (Crh-P) binds to CcpA. This interaction is also stimulated by FBP. The HPr-P/CcpA or Crh-P/CcpA complex binds to the operator, *cre* (catabolite responsive element). A CcpA target sequence (*cre*) was found in the ORF *gmuC* (TTGGAAGCGGTATCA), but it is one for which glucose repression is not strong [12]. Another *cre* candidate sequence ATGTAAGCGTTTTAA (suggested by Y.Fujita), covering the transcription initiation site G, may also contribute to catabolite repression.

Even without cellobiose addition, the Gmu^+ strain (BSU71) showed ten times stronger LacZ activity than the $GmuG^-$ strain (BSU87)(Table 1). This may be due to some glucomannan-like polysaccharides in the yeast extract contained in LB broth.

3.3. The predicted internal regulatory gene coding for a repressor of the putative operon

The fifth gene, *gmuR,* seems to code for a protein homologous to a helix-turn-helix protein of the GntR family, a regulatory DNA binding protein belonging to a subgroup containing a small molecule-binding UTRA domain [5]. Disruption of *gmuR* resulted in an elevated transcription of the whole putative operon even without cellobiose (Table 1). Addition of IPTG to restore downstream ORF expression did not affect derepressed operon induction in *gmuR* disruptants. Furthermore, introduction of cloned *gmuR* gene on a plasmid suppressed the derepressed operon induction (data not shown). It appears thus that the presumed GmuR protein is a repressor of the putative operon. We note, however, that induction of operon expression by cellobiose in strain BSU71 (P_{gmuB}-*lacZ*) or BSU87 (*gmuG::lacZ*) was stronger than in BSU81 (*gmuR::lacZ*). Therefore, the repressor protein seems to be required for full induction of the operon by cellobiose. Repression by glucose and derepression when *ccpA* was disrupted was also observed in the *gmuR* disruptant strain (Table 1), although the repression was not strong. It seems that wild type GmuR may play a role in

glucose repression, similar to what was observed for *tre* operons [13-15], where glucose-6-P binds the repressor protein and acts as an anti-inducer.

3.4. Detection of a transcript covering the whole gmu operon

To detect transcripts covering whole ORFs of the putative operon, RNA was extracted from cells grown in the presence of cellobiose and subjected to Northern analysis with a *gmuG* probe. As shown in Fig. 2, a transcript of ca. 8.0kb length was detected with the *gmuG* probe. The number of nucleotides from the initiation codon of *gmuB* to the end codon of *gmuG* is 7237 bases. The length of the detected *gmuBACDREFG* RNA is thus longer. This transcript was found thick in cells grown in the presence of cellobiose. We conclude that *gmuBACDREFG* constitutes an operon. Two additional bands of 2kb and 1kb were also detected. We did not investigate the origin of these bands, which may be the products of RNA processing or transcribed from minor promoters.

3.5. Transcription initiation site of the gmu operon

The transcription start site was determined by primer extension as shown in Fig. 3. Strong signals were detected only in the cells grown in the presence of 0.1% cellobiose. The transcription initiation site G was 31 bases upstream of the start codon TTG of the first gene and was preceded by the -35 and -10 sequences TATAGACA and TATAAT separated by 16 bases. This is similar to the consensus SigA recognizing promoter sequence TTGACA-N_{17}-TATAAT [16].

3.6. Effect of the gmuBAC PTS genes disruption on the expression of the gmuBACDREFG operon

Insertional inactivation of any one of *gmuB*, *gumA* or *gmuC* resulted in derepressed expression of the *gmu* operon without inducer because of reduced expression of the downstream repressor gene *gmuR* (Table 2). Addition of IPTG

to restore downstream *gmuR* expression reduced derepressed expression. Disruption by pMUTIN of any one of the *gmuB-gmuA-gmuC* PTS genes did not impede induction of the operon by cellobiose addition. This may be due to incorporation of cellobiose through other PTS systems like the lichenan specific phosphotransferase system LicBAC of *B.subtilis* [17-18], where LicB, LicA and LicC are homologous to GmuB, GmuA and GmuC. In fact, the lichenan utilization operon, consisting of *licB-licA-licC* PTS genes and the *licH* gene encoding 6-phospho-β-glucosidase, is inducible by cellobiose as well as lichenan. Unlike *gmu* operon, which has the internal negative regulator GmuR, as described in this study, this operon is preceded by a regulator gene *licR*, which encodes a positive regulator of the *lic* operon. LicR requires phosphorylation by the general PTS proteins E1 and HPr to be functional [18].

However, addition of IPTG did not greatly reduce greatly *gmu* operon expression in the *gmuA*::pMUTIN strain and cellobiose addition restored the expression. It thus seems that inactivation of *gmuA* results in inactivation of repression. This would seem to suggest that the repressor GmuR requires

activation by GmuA through phosphorylation or phosphorylation-dependent interaction. However, ~~I~~introduction of a multicopy plasmid carrying the cloned *gmuR* gene repressed derepressed *gmu* operon expression in the *gmuA* disruptant (data not shown), suggesting that large amounts of GmuR proteins function as repressor even without phosphorylation.

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Table 1 Induction and repression of the putative *gmu* operon

Strains	LacZ activity (MU)		
		+ cellobiose	
	none	- Gc	+ Gc
BSU71 (P_{gmuB}-*lacZ*)	11.5±2.9	98.7±7.8	10.2±0.8
BSU72 (P_{gmuB}-*lacZ ccpA::neo*)	10.2±3.0	109.4±7.1	104.2±6.4
BSU81 (*gmuR-lacZ*)	57.4±10.4	53.7±9.6	23.9±0.3
BSU82 (*gmuR-lacZ ccpA::neo*)	87.4±2.4	90.6±10.3	80.6±11.3
BSU87 (*gmuG-lacZ*)	1.0±0.8	83.7±26.3	2.3±1.1
BSU88 (*gmuG-lacZ ccpA::neo*)	1.2±0.9	106.3±22.3	98.4±18.0
In the presence of 1mM IPTG*			
BSU81 (*gmuR-lacZ*)	96.6±1.1	66.4±7.1	38.0±3.8
BSU82 (*gmuR-lacZ ccpA::neo*)	121.4±32.9	134.1±18.1	138.3±21.6

Cells in exponential growth phase in LB broth accepted cellobiose (0.1%) or cellobiose (0.1%) and glucose (Gc, 0.1%) for 60min. LacZ activity indicated is the average value with standard deviation of two independent experiments.

* IPTG was added from the start of growth.

Table 2

Derepression of expression of the putative *gmu* operon expression in *gmuA* disruptant

Strain	LacZ activity (MU)		
		+ IPTG	
	none	-Cel	+Cel
BSU73 (*gmuB-lacZ*)	62.1±12.1	0.6±0.2	152.5±20.5
BSU75 (*gmuA-lacZ*)	71.5±11.1	38.9±8.6	170.0±20.4
BSU77 (*gmuC-lacZ*)	58.2±6.2	1.2±0.2	112.2±34.4

Cells in exponential growth phase in LB broth containing IPTG (1mM) or IPTG (1mM) and cellobiose (Cel, 0.1%) were examined for LacZ activity. LacZ activity indicated is the average value with standard deviation of two independent experiments.

Legends for Figures

Fig. 1. Induction of the putative *gmu* operon of *B. subtilis* by konjac glucomannan. A and B, A portion of the culture of the strain BSU71 (P_{gmuB}::pMUTIN2)(A) or the strain BSU87 (*gmuG*::pMUTIN2)(B) was placed on LB agar containing erythromycin (0.3 μg/ml) and X-gal (0.01%). Konjac glucomannan flour (#1, 16mg, solid), yeast mannan (#2, 3.5mg, solid), CM cellulose (#3, 5mg, solid), mannobiose (#4, 20μl, 10%) and cellobiose (10%)(#5, 20μl, 10%) were placed on the center of each agar. The agar plate was incubated overnight at 37℃. Blue areas indicate LacZ activity.

C**,** Solid konjac glucomannan (30mg) was added to 30ml of cell culture in exponential growth at 37℃ in LB medium. Cellobiose was used at a concentration of 0.1%. A portion of the sample was taken at the indicated time and LacZ activity was determined. Filled symbols, BSU71(P_{gmuB}::pMUTIN2); open symbols, BSU87(*gmuG*::pMUTIN2). Circle, konjac glucomannan; square, cellobiose.

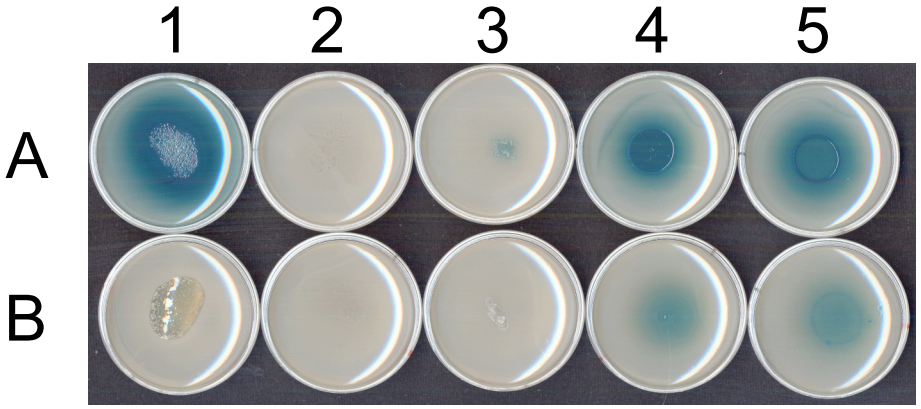
Fig. 2 . Northern analysis of the putative *gmu* operon.

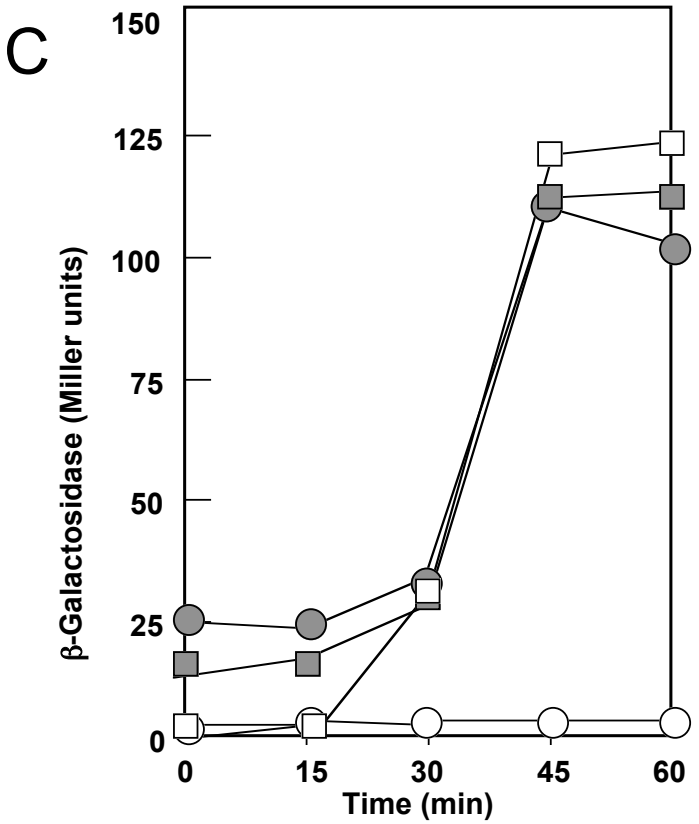
RNAs extracted from wild type cells grown with or without cellobiose (0.1%) were subjected to electrophoresis. The sample was hybridized with a DIG labeled *gmuG* probe. Lane 1, no inducer; lane 2, 30 min after addition of inducer; lane 3, 60 min after addition of inducer. Thin arrows indicate the ladder of single strand RNA markers at 9, 6, 5, 4, 3, 2.5, 2, 1.5, 1, 0.5kb (RNA Millennium^{TM} Size Marker, Ambion Inc., Austin Texas). The thick black arrow indicates the *gmu* operon RNA. Thick white arrows indicate 23S and 16S rRNAs.

Fig. 3. Transcription initiation site and promoter region of the putative *gmu* operon. A.The DIG labeled primer complementary to the region 87 bases downstream of the initiation codon of the *gmuB* was extended by using a template RNA extracted from the wild type strain 168 grown in LB broth without cellobiose (lane 1), with cellobiose (0.1%) for 30 min (lane 2) and 60 min (lane 3). B. Promoter region of the putative *gmu* operon. Sequences complementary to the primer used in Fig.3A are underlined.

Fig. 4. A model of the glucomannan utilization operon of *B.subtilis.* Partial digestion of glucomannan by the secretory GmuG mannanase (β-1,4-D-mannanase) results in the production of oligo-glucomannans (indicated with a single asterisk). These are taken up by the oligo-β-mannoside specific PTS system II composed of GmuBAC. Incorporated oligo-glucomannans (indicated with two asterisks), such as cellobiose or mannobiose, function as inducers of the operon and are further processed by GmuD (phospho-β-glucosidase), GmuE (fructokinase) and GmuF (mannose-6-phosphate isomerase) enzymes. The operon is negatively regulated by an internal repressor GmuR and the carbon catabolite control protein CcpA. Open rectangles indicate ORFs. Direction of transcription of ORFs on the horizontal line is from left to right. That of ORFs under the line is from right to left.

Fig. 1


1
2
3
4
5
A
B


C
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125
100
75
50
25
0
β-Galactosidase (Miller units)
0
15
30
45
60
Time (min)

Fig. 2

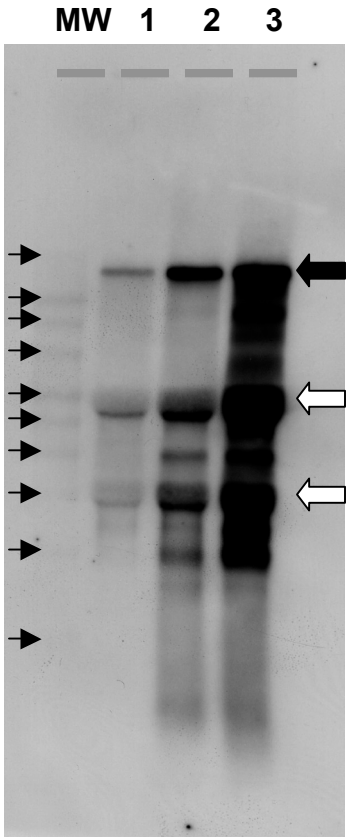
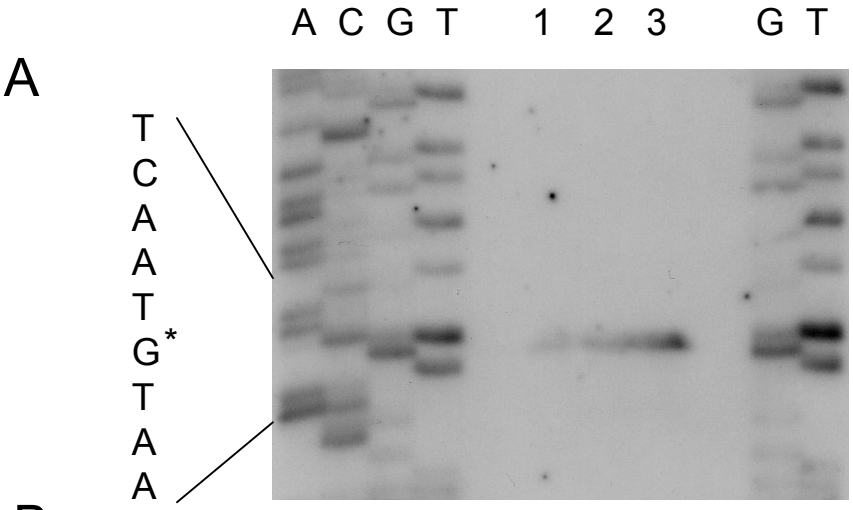


Fig. 3

A



B

CTGCAATCGTAGAAGCTGCAAGCAGGAAAACTTTGAAATTCATAACAAACTCCTTTACT

TAAATGTTTTGATAAATAAAAAAAATCCTGATTACAAAAAATGTCATAAACAAATTT

ydhL

TGTAATCAGGATTTTACGGTTCCTGGTAGACACCCTCAAACCATATTATTGAGGTTATA

CAAGTGATAATAGCTATTTAATTGATTCGTTTCCGTTGATGAGTGTACCACATTATGAA

TGAATCTTCCATAGAAAAATTTATAAAACGGTGAACCGGAAAAAAATATGATTATATTT

ATTTTATAAAAGTATAGACATTTAAAATTAAATGACTATAATAATCAATG*TAAGCGTT

-35 -10

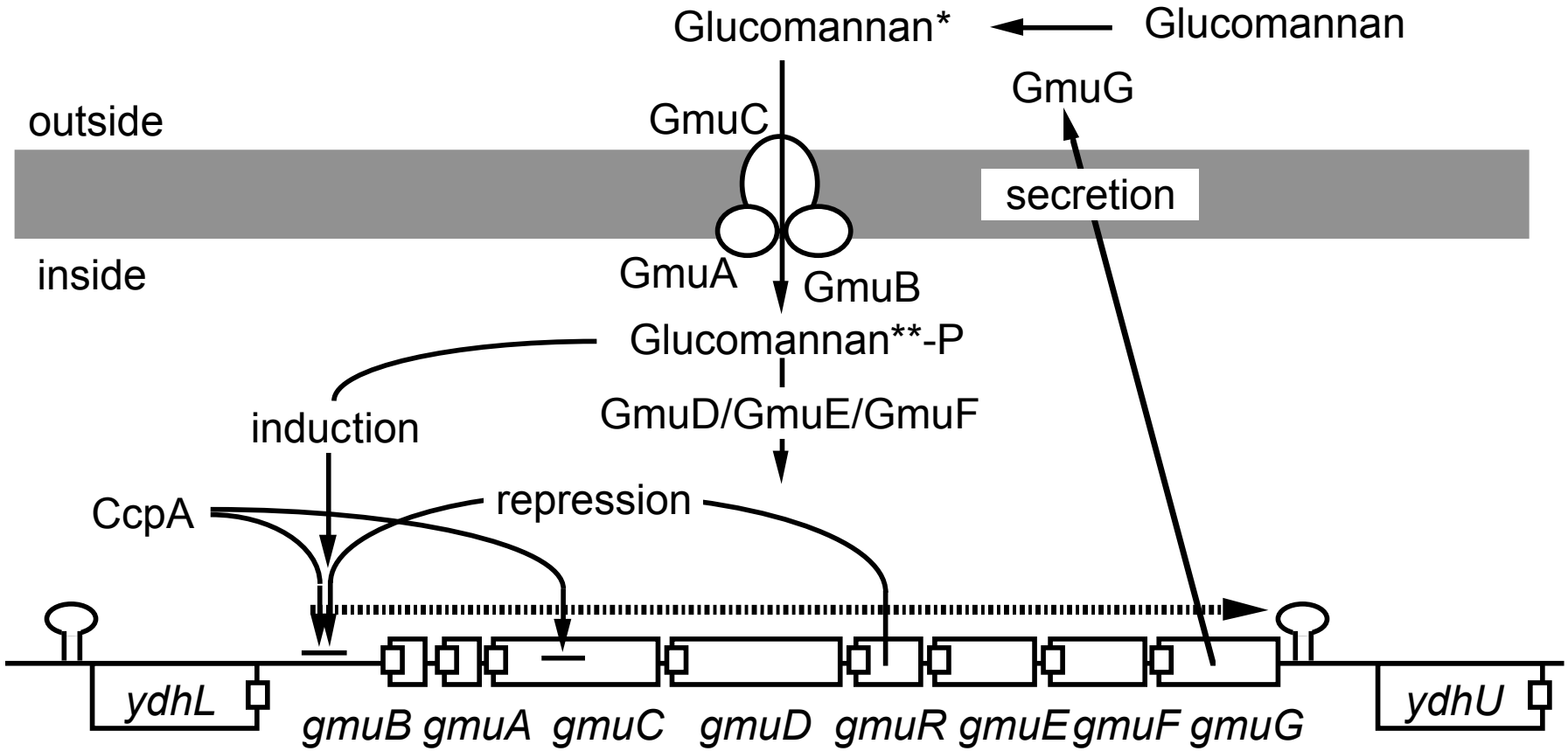
gmuB

TTAATAACAGGGGGGATAGCAGTTGAAAAAAATATTACTCGCGTGCAGTTCAGGAATGT

CTACCAGTTTATTGGTGACAAAAATGAAGGAATACGCACAGTCTATCGGTGAGGAAGCG

GAGATTTGGGCTGTTGGCCAGGATAAAGCAAAAGAAGACATGAGAAAAGCGGATGCCGT

Fig. 4


Glucomannan*
Glucomannan
GmuG
outside
GmuC
secretion
inside
GmuA
GmuB
Glucomannan**-P
GmuD/GmuE/GmuF
induction
repression
CcpA
ydhL
gmuB gmuA gmuC gmuD gmuR gmuE gmuF gmuG
ydhU