Cloning, sequencing and expression of *galE*: knock-out mutant is defective in both LPS synthesis and nodulation of soybean
Cloning, sequencing and expression of \textit{galE}: knock-out mutant is defective in both LPS synthesis and nodulation of soybean.
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ABSTRACT

The enzyme UDP-galactose 4-epimerase (GalE) is involved in one of the major steps of galactose metabolism in bacteria. GalE mediates the incorporation of galactose in extracellular polysaccharide materials such as the O-side chain of lipopolysaccharide (LPS). In this study, we describe the cloning and characterization of the galE gene from Brdayrhizobium japonicum, a soybean endosymbiont. Nucleotide sequence analysis of the subcloned DNA identified the galE gene: Comparison of the deduced amino acid sequence of B. japonicum GalE with published data showed significant homology with the GalE of Azospirillum brasilense (68%), Aquifex aeolicus (68%), and Synechocystis sp. (66%). Functional identity was achieved by the complementation of a galE mutant Escherchua coli PL2 galE with the subcloned genes in galactose toxicity test. Galactose is toxic for galE-negative bacteria because it results in elevated levels of metabolic intermediates. Galactose epimerase activity of the complemented strain was essentially identical to that of the wild type E. coli DH5α. In vivo expression study showed that a 36 kDa protein was expressed from the complementing plasmids.

To study the role of galE gene in B. japonicum LPS biosynthesis,
the *galE* gene was inactivated in chromosome by double cross-over homologous recombination where *galE*-knockout fragment replaced the *galE* gene in *B. japonicum*. A *galE* knock-out mutant strain of *B. japonicum* was found to be far more hydrophobic than the wild type strain based on the cell surface hydrophobicity (CSH). To confirm the inactivation of the *galE* gene in chromosomal DNA, genomic Southern blot hybridization was performed. The digested with *EcoRI* and *HindIII* from the *galE*-inactivated strain HS11 showed an increased DNA size that is characterized by a 1.3kb higher DNA size than that from the parental strain 61A101C. A standard plant infection test using the wild type and *galE* defective *B. japonicum* strains showed that the *galE* gene is indeed involved in nodulation process of *B. japonicum* with its soybean host plant.
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1. Introduction

Nitrogen is an essential plant nutrient. It is the nutrient that is most commonly deficient, contributing to reduced agricultural yields throughout the world. Synthetic nitrogen use has grown from 3 million to 80 million tons over the last 40 years. This increase occurred in both developed and developing countries. The current annual worldwide expenditure for fertilizer nitrogen exceeds $20 billion an amount comparable to that for synthetic chemical pesticides. Modern industrial production of fertilizer nitrogen demands large inputs of energy in the form of natural gas, a finite natural resource; fertilizer constitutes a major energy cost in the production of a high-yield corn or rice crop. Molecular nitrogen or dinitrogen (N2) makes up four-fifths of the atmosphere but is metabolically unavailable directly to higher plants or animals. It is available to some species of microorganism through Biological Nitrogen Fixation (BNF) in which atmospheric nitrogen is converted to ammonia by the enzyme nitrogenase. Associative nitrogen-fixing microorganisms are those diazotrophs that live in close proximity to plant roots (that is, in the rhizosphere or within plants) and can obtain energy materials from the plants.

BNF requires energy. Those microbes that fix nitrogen independent of other organisms are called free living. The free-living diazotrophs require a chemical energy source if nonphotosynthetic, whereas the photosynthetic
diazotrophs utilize light energy. The free-living diazotrophs contribute little fixed nitrogen to agricultural crops, thus reducing the need for expensive and environmentally damaging fertilizer additions. They may make a modest contribution of fixed nitrogen to agriculture and forestry, but quantification of their potential has not been established. Some of these strains are more efficient at obtaining nitrogen than others, and some are more environmentally tolerant than others. The symbiosis between legumes and the nitrogen-fixing rhizobia occurs within nodules mainly on the root and in a few cases on the stem. The plant supplies energy materials to the diazotrophs, which in turn reduce atmospheric nitrogen to ammonia. This ammonia is transferred from the bacteria to the plant to meet the plant’s nutritional nitrogen needs for the synthesis of proteins, enzymes, nucleic acids, chlorophyll, and so forth.

To obtain this nitrogen from the atmosphere, these leguminous plants maintain a relation with a bacteria called *Bradyrhizobium japonicum*. *B. japonicum* is a gram negative, rod-shaped, nitrogen-fixing bacterium which develops a symbiosis with the soybean plant *Glycine max*. Among plant-associated bacteria, it can be legitimately argued that *B. japonicum*, the nitrogen-fixing symbiont of soybean, is one of the most agriculturally important microorganisms (Puvanesarajah et al. 1987). The establishment of nitrogen-fixing nodules in the *B. japonicum* symbiosis is a complex multistep interaction between microsymbiont and its specific host plant (Priefer. 1989). Early events involve recognition and deformation of curling of root hair cells. Cortical cell division, leading to the formation of a unique organ, the nodule
is induced probably by diffusible bacteria is initiated by penetration of the root hair cell wall and the formation of an infection thread in which the bacteria are carried towards the dividing root cortex cells. They remain separated from the host cytoplasm by peribacteroid membranes and differentiate into bacteroids able to reduce atmospheric nitrogen. This complex development process obviously requires continuous signal exchange between plant and bacterial cells. Undoubtedly, bacterial cell surface components play an important role in this specific interaction; particularly extracellular polysaccharides (EPSs) and lipopolysaccharides (LPSs) have been hypothesized to be involved in the symbiotic process (Halverson and Stacey. 1986). In *B. japonicum*, the lipopolysaccharides (LPS) are likely to be involved in the infection process and consists of lipid A, a core oligosaccharide and an O-antigenic side chain. Therefore, rhizobial LPS has been suggested to be involved in specific recognition and attachment of bacteria to the root hair cells of compatible host plants, mediated by the binding of lectins as a lectin receptor. They are major Differences in LPS composition were described between nodulating and nonnodulating strains of *Rhizobium leguminosarum bv. trifolii* (previously called *R. trifoli*) (Priefer. 1989). Similarly, the structure difference in the LPS of a rfaF\(^{-}\) mutant of a slow-growing *B. japonicum* strain was observed (So et al. 2000).

In the case of the slow-growing which is *B. japonicum*, we identify and characterize of the *galE* gene in LPS biosynthesis by *B. japonicum*, Subsequent informatics studies on the cloned ORFs regions revealed *galE* gene which is similar to other *galE* gene strain. To study the role of the
galE gene in LPS biosynthesis in *B. japonicum* and to identify *galE* gene which is necessary for soybean nodulation, a *galE* gene knockout mutant was constructed from the strain of *B. japonicum* 61A101C by antibiotic cassette, kanamycin. Using the mutant thus obtained, they were characterized by morphological, genetic, and molecular means. The mutant was used in detail to determine its possible symbiotic function.
Materials and Methods

2.1 Plant material, bacterial strains and plasmids

The soybean cultivar *Glycine max* cv *Essex* was used as plant material. *Escherichia coli* DH5α (F-F80dlacZDM15 endA1 recA1 hsdR17 supE44 thi-1 λ-) was used as a primary host for the transformation and preparation of plasmids (Sambrook et al. 1989). For the galactose toxicity test and enzyme assay, *E. coli* PL2 (galE28, l-, relA1, spoT1, thi-1) strain was used. *E. coli* BL21 (DE3) (F', hsdS, gal, /λ cI857, ind1, sam7, nin5, lacUV5-T7 gene1) strain was used for expression of T7 promoter system (Studier and Moffatt. 1986). This strain contains a single copy of the gene for T7 RNA polymerase in the chromosome under control the inducible lacUV5 promoter. *Bradyrhizobium japonicum* 61A101C (Wild type) was used as a recipient for the construction of galE-knockout mutant. The bacterial strains and plasmids used in this study are described in Table 1.

2.2 Media

*E. coli* strains were routinely grown in Luria–Bertani (LB) medium. LB medium contained 10g of Bacto-trypton, 5g of Bacto–yeast extract, 1ml of 1N NaOH, and 5g of Sodium chloride per liter. *E. coli* harboring plasmid was
grown in LB medium supplemented with 50µg of ampicillin per ml (Sambrook et al. 1989). M9 minimal medium was used for the growing of *E. coli* PL2 containing the expression plasmids, pGEM-3Zf(+), subcloned pKM series which contained 200µl of 5X M9 Salt (54g of disodium phosphate, 14g of potassium phosphate, 2.5g of sodium chloride, and 5.0g of ammonium chloride per liter), 2µl of 1M magnesium chloride, 1µl of 0.1M calcium chloride, and 20µl of 20% galactose or glucose as carbon source per liter. *B. japonicum* wild type strain 61A101C and galE-knockout mutant were all cultivated in an AMA broth (10g of mannitol, 1g of bacto yeast extract, 0.2g of magnesium sulfate, and 0.2g of sodium chloride per liter of deionized water) (So. 1991). For nodulation assay, Nitrogen-free Plant Nutrient Solution (PNS) which contained 15mg of NaFeEDTA, 23mg of K₂SO₄, 0.79g of KCl, 0.11g of NaH₂PO₄·H₂O, 0.17g of Na₂HPO₄, 1µl of Na₂HPO₄, 1.0% sucrose, and 1µl of Micronutrient Solution (3.73g of KCl, 1.55g of H₃BO₃, 0.85g of MnSO₄·H₂O, 0.13g of CuSO₄·5H₂O, 0.018g of (NH₄)₆Mo₇O₂₄·4H₂O, and 0.58g of ZnSO₄·7H₂O per liter) was used (Wacek and Brill. 1976).

2.3 Enzymes, chemicals and others.

All restriction enzymes and other DNA modifying enzymes were purchased from TaKaRa Co. (Korea). *E. coli* Klenow fragment was from Boeringer-Mannheim, Germany) for making digoxigenin-labelled pKM5 probe. UDP galactose, NAD, and UDP glucose dehydrogenase, which was used for
UDP galactose 4-epimerase assay. IPTG (isopropyl-β-D-thiogalactopyranoside-1,2-dioxane), X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), and antibiotics were obtained from Sigma.

Antibiotics were added to the medium to the final concentration for galE-knockout mutant, 100μg/ml of kanamycin, 200μg/ml of ampicillin, and for E. coli, 50μg/ml of ampicillin, 50μg/ml of kanamycin.

### 2.4 Transformation

#### 2.4.1 Transformation of E. coli

Plasmid DNA was introduced into E. coli essentially as described by Sambrook et al (1989), with minor modifications. Fifty milliliters of LB broth was inoculated with 0.5ml of an overnight grown culture of an appropriate E. coli strain and incubated at 37°C with vigorous shaking to an O.D₆₀₀ = 0.5. The cells were harvested and washed 3 times with sterile distilled water prior to being resuspended in 10% glycerol at 50 fold concentration. For electroporation, the Gene Pluser Apparatus (Invitrogen, Gene PulserIII, USA) was used with a 0.1cm cuvette supplied by the manufacture. The pulse was applied at 7,500V, 70μF, 50Ω for E. coli. For The competent E. coli cells were transformed with plasmid DNA. Transformed cells were grown at SOC medium at 37°C for 30 min. The cells were plated on LB agar plate containing antibiotics, 50μg/ml of ampicillin.
Table 1. Bacterial strains and plasmids

<table>
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<tr>
<th>Strains</th>
<th>Characteristics</th>
<th>References or source</th>
</tr>
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<tr>
<td><strong>B. japonicum</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>61A101C</td>
<td>Wild type</td>
<td>Nitragin, Wiscosin</td>
</tr>
<tr>
<td>HS11</td>
<td>61A101C galE':Km, Nod--</td>
<td>Stacey et al. 1991</td>
</tr>
<tr>
<td><strong>E.coli</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DH5α</td>
<td>F-P80dlacZDM15 endA1 recA1 hsdR17(rk-mk-) supE44</td>
<td></td>
</tr>
<tr>
<td>PL2</td>
<td>galE28, l-- relA1, spoT1, thi-1</td>
<td>Sambrook et al. 1989</td>
</tr>
<tr>
<td>DE3</td>
<td>F', hsdS36gal, /λ cl'857, ind1, sarn1, nin5, lacUV5-17 gene1</td>
<td>Studier and Moffatt. 1986</td>
</tr>
<tr>
<td><strong>S. typhimurium LT2</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>G30</td>
<td>galE(-)854</td>
<td></td>
</tr>
<tr>
<td>TN1117</td>
<td>galE(-) zbi812::Tn10</td>
<td></td>
</tr>
<tr>
<td>LB5010</td>
<td>metA22 metE551 ilv-452 leu-3121 trpC2 xyl-404</td>
<td></td>
</tr>
<tr>
<td></td>
<td>galE856 hsdL6</td>
<td></td>
</tr>
<tr>
<td>SA3297</td>
<td>hsdSA29 hsdSB121 rpsL120 H1-b H2-c,n,x fla-66</td>
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<tr>
<td></td>
<td>nlm(-) Fel-2(-) hsdLT6 hsdSA29 hsdSB121 metA22</td>
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<td></td>
<td>metE551 trpC2 ilv-452 leu-3121</td>
<td><strong>S. typhimurium Genetic Stock Center</strong></td>
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<td>zxx-3011::Tn10(del16 del17) trpC2 metA22 metE551</td>
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<tr>
<td></td>
<td>his-6165 ilv-452 GalE496 H1-b H2-c,n,x nml(-)</td>
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</tr>
<tr>
<td></td>
<td>(cured of Fels2) fla-66 rpsL 120 xyl-404</td>
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<td></td>
<td>GalE496 hsdL6 hsdSA29 (del) malB/ F112 (E.coli genes metB-malB-lamB-pyrB)</td>
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<tr>
<td><strong>R. leguminosarum</strong></td>
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<td>RBL5835</td>
<td>RBL5523,exo7::Tn5</td>
<td>Cremers et al. 1990</td>
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<tr>
<td>RBL5809</td>
<td>RBL5523,exo8::Tn5</td>
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<tr>
<td>RBL5811</td>
<td>RBL5523,exo52::Tn5</td>
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<tr>
<td>Plasmids</td>
<td>Characteristics</td>
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<td>--------------</td>
<td>------------------------------------------------------</td>
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</tr>
<tr>
<td>pHC79</td>
<td>Broad host range vector for gram-negative bacteria, Te, Ap</td>
<td>So et al. 1987</td>
</tr>
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<td>pHK34</td>
<td>ptac11 clone carrying the 5.5-kb EcoRI LPS fragment, Ap</td>
<td>So, 1991</td>
</tr>
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<td>pHC79 clone carrying the 1.9-kb SalI fragment, Ap</td>
<td>This study</td>
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<td>pHC524:km</td>
<td>pHC524 containing galE-knock, Kmr, Ap</td>
<td>This study</td>
</tr>
<tr>
<td>pGEM-3zf(+)</td>
<td>Ap'</td>
<td>Promega</td>
</tr>
<tr>
<td>p34S-Km</td>
<td>p34S carrying the antibiotic cassette, Kmr, Kmr'</td>
<td>Yonsei Univ.</td>
</tr>
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<td>This study</td>
</tr>
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<td>This study</td>
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<td>pKM4</td>
<td>5.5-kb BamHI fragment of pKM2 in pGEM-3zf(+), Ap</td>
<td>This study</td>
</tr>
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</tr>
<tr>
<td>pKM6</td>
<td>pGEM-3zf(+) clone carrying the 1.9-kb SalI fragment, Ap</td>
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<tr>
<td>pKM7</td>
<td>pGEM-3zf(+) clone carrying the 0.75-kb SalI fragment, Ap</td>
<td>This study</td>
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</table>
2.4.2 Transformation of *B. japonicum*

Transformation of *B. japonicum* with plasmid DNA was done by the competent cell which was prepared by the method described *E. coli* transformation. The pulse was applied at 10,000V, 50μF, 50Ω for *B. japonicum*. Recipient strain was inoculated in 500μl of AMA broth and cultured for 24hr at 30°C with shaking. Transformed cells were spread on the AMA agar plate containing appropriate antibiotic (Km: 50μg/ml).

2.5 Preparation of DNA

2.5.1 Small-scale plasmid DNA preparation from *E. coli*

The rapid plasmid isolation method of Sambrook et al.(1989) was used to isolate recombinant plasmid DNA as small scale from *E. coli*. Small scale purification of recombinant plasmid was done as follows: A 3ml of bacterial culture was harvested, and cell pellet was suspend in 100μl of 50mM glucose, 25mM Tris–Cl, and 10mM EDTA. The mixture was stirred on ice for 10 min, followed by adding 200μl of 0.2N NaOH and 1% SDS. The suspension was kept again on ice until glear lysate was formed, then 150μl of 5M potassium acetate was added placed on ice for an another 10min. The mixture was then centrifuged at 15,000rpm for 15min at 4°C and 5μl of RNase was added into the supernatant. The mixture was stood at 37°C for
30min. and the same volume of phenol/chloroform/isoamylalcohol (25:24:1) was added into the mixture. After the supernatant was removed into a fresh microtube, 800µl of absolutely ethanol was added into the supernatant. The mixture was centrifuged at 15,000rpm for 15min. After the pellet was dried and dissolved in 50µl of TE buffer (10mM Tris–Cl, 1mM EDTA, pH8.0).

2.5.2 Preparation of chromosomal DNA from *B. japonicum*

Isolation of *B. japonicum* chromosomal DNA was performed by the method (So et al. 1987) with minor modifications. 10ml of *B. japonicum* cell which was grown at 27°C to late log phase of growth was harvested and resuspended with TE buffer (50mM Tris–Cl, 20mM EDTA, pH8.0). After 80 µl of lysozyme was added, the mixture was incubated at 37°C for 1hr and 60 µl of 10% SDS was added throughly into mixed supernatant for cell lysis. After 2hr, the lysate was extracted twice with 600µl of phenol and once with chloroform. Aqueous phase was separated by centrifugation at 15,000rpm for 5min at 4°C and transferred to a new microtube. DNA was precipitated by adding of 3M sodium-acetate (pH4.8) and 360µl of isopropanol. Isolated DNA was resuspended in 500µl of TE buffer and 10µl of RNase was added to the dissolved DNA solution. This solution was incubated at 37°C for 2hr. After RNA and residual protein were removed, DNA solution was treated with phenol/chloroform/isoamylalcohol (25:24:1) and DNA subsequently was precipitated with two volume of absolute ethanol and
followed by washing with 70% ethanol. DNA pellet was dried and resuspended in 100µl of TE buffer.

2.6 Electrophoresis of DNA

DNA was analyzed by electrophoresis on an agarose gel using TAE buffer (0.04M Tris-acetate, 1mM EDTA) (Sambrook et al. 1989). 1µg/µl EtBr solution was added on a 0.7%(W/V) agarose gel and gels photographed with transmitted UV light using a CCD camera.

2.7 DNA sequence analysis

The DNA/protein analysis, the database of the degree of similarity of DNA/protein, the various proteins that DNA is encoding and characteristics expression of protein were analyzed by using on-line server through the internet that predicts the secondary structure of protein similarity of DNA/protein and amino acid sequence using the Anthe program. The Blastx and Blocks were used to search databases for protein amino acid sequences similar to those obtained by translating the DNA sequence. Similar sequence were aligned by using the Pileup program of the GCG. Percent identity and homology were calculated by using the GCG program Bestfit with a gap weight of 3.0 and a gap length of 0.1. The information of amino acid sequence was obtained by searching the following addresses. BLAST
search(http://www.ncbi.nlm.nih.gov) and ExPASy Molecular Biology Server(http://www.expasy.hcuge.ch) has information on the comparison and analysis of amino acid sequence and of its homology.

2.8 Subcloning of *galE* gene from pHK34

Approximately 10μg of each pGEM-3zf(+) and pHK34 from *E. coli* were digested with different restriction enzymes. After inactivation of restriction endonuclease by heat-shock at 65°C for 10 min, the fragments were diluted to approximately 0.05μg of DNA per μl and ligated overnight. The ligation mixture was introduced *E. coli* DH5α by transformation and tested for the presence of β-galactosidase activity by patching on LB medium (supplemented with ampicillin) containing 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal; 100μg/ml) with isopropyl-β-D-thio galactopyranoside-1,2-dioxane (IPTG; 200 μg/ml). Colonies producing a white color on plates were selected. To confirm the identification of transformants, all other DNA was isolated and digested according to the methods of Sambrook et al (1989).

2.9 Complementation test

2.9.1 Galactose toxicity test
Bacteria from an overnight culture were inoculated 1:100 in minimal media containing 1% glycerol. Once the cultures began growing, they were split in half and either galactose or glucose was added to a final concentration of 1%. The A600 of each culture was then measured over a 24hr period (Pierson and Carlson. 1996).

2.9.2 Enzyme assay

Bacteria were grown to the late log phase in minimal medium containing as the carbon source. Cultures were then introduced for 1hr with 1% galactose. Extracts of the cultures were made by solution in 15mM phosphate buffer 15mM Na2HPO4, 10mM KH2PO4). The protein concentration of extracts was determined with a Bradford assay (Bollag and Edelstein. 1991). Enzyme activity was assayed by a modification of the method wilson and (Wilson and Hogness. 1966). Assays were performed in a 1ml reaction volume containing 100µg of protein, 0.5mM NAD, 0.25mM UDP galactose, and 0.025U of UDP galactose dehydrogenase (all from sigma). The change in A340 was measured over a 6 min period. An A340 change of 1U/min corresponds to the formation of 162 nmol of NADH. Enzyme units are expressed as nanomoles of NADH formed per minute per milligram of protein (Pierson and Carlson. 1996).

2.10 Construction of B. japonicum galE-knockout mutant
Figure 1. Physical map of *galE* gene region. Top line represent the *EcoRI* restriction enzyme map of the chromosomal gene region cloned from pHK34. Subclones of a plasmid pGEM-3zf(+) clone are designed by pKM series.
orfX  Heptosyl synthetase

rfaF  LPS-Heptosyl transferase

lpcC  LPS-mannosyl transferase

rfaD  ADP-Heptose-epimerase

galE  UDP-galactose 4-epimerase

Figure 2. Physical map of the LPS-gene region.
CTGCGGCCAGGCTTGTTTCTCTGATCCGACACGAAAGCTCTCCTTCTCCTTACGCATACATGAGTCTCC

GCGGGGCGAAACCCCTCTTTACAGCTCTCCGCAGCTAGCAGGAGACAGCTCTATACGACATTGAAGAT
SD

GGGCGAATCCGGCTATATCGAGATCCATCGCTTCTATGACCACCCGCGAGGCGGCCCTTGGTATCGAC

GAGYIGSHMVHALVIAGESVYYVVIDNLSGT

TTTCGCTCCTCGGTAACGGCTGGCTTCGCTCCTTCGATCCGACGGCGAGACCTACTGTCGAGGCTGAC

FGSAFLPEGVPVLFLFIDAGDADENLVEGVIQAH

KpnI

ATGACACATATGCATTTGGGCTCCCTCTTCTACTCGAGATGCGGAGTCCCGCTCAGCTACTAGCAACAAC

IDSIIIEFAGSVDVVPDSMRDPLGLGYYRNNTMT

ACGGCCTACCTCCTCAGAGCCTGCCTTGTTGCTGCTCGTCTCGCGAAGCCGGCGCCGCTTCTAGGCAAC

TGSILNAAVTKGGGVSRFIFSSSTAAAVYGNPD

GCTCGGCTCTTCTCTGACAGCAGACGGCGGCTATCGAAGCAGTAAATGACAGCTACGCTTGACACGAG

VPVPRIESAPTTRSLPSYGSSKLMTEIMLHDAV

TCGGCTCAGCTGATACGCTACGCGATGCTGCCTCCTGCTCTACGGCAGCTGCGCTGCGCTTGGCCACAC

GSAHGMSSYVYVLRYFRVAGADPKGRVGLATTG

GCGACCCATCTCGCAGACGAAGCTCGTCTCTACCCGTCGCGTCGCTGCTGCTGCTGCGCTGCTGCTGCT

AATHLLKIENAAATGQRKIAKLDFVDGFTDYPGT

GCGACGCTCGAATCTCGAAACCGGCTCGTCTCGCAGCGCGATGCGGAGGGCGCGCTGCGCTTGGCCACAC
GSCIRHDIFHIHVSDDLYVEAHRAALSYSYLRAGGS

GTGACCGCTGAATTCCGGTTATGCCTGCCGCTATTTCTGTGGCAGCCAATGCGCGCTGCGCGCTGCTGAG

 جميل

VTLCNCYGRGRGYSVYLETIEAVRRRVSRRRNFAV

BamHI

GCGCTCGCGCGCGGGCGGCAGACATCATAGACATGTGCGGAGACGACAGAAAGGATCTCTGCTCTGACTGAC

AGCGCGCGGCTTCAAYAARRPGDIDMTMYMVADMHTTRSLRLLDWDTPR

GACGATCCTCGAAGGCAACGGAACGACGACGCTCGCTCGAGGAGGATCGCTTGCGGGCGGCGCTCGCGGCGG

ADDLETIASHALAWREKLFHRAGSRQAES

GCTTAAACAAACAAACCAGGGTTATCAGAGTTTTGCTCTTTGAAAAAGGGGGCAAGGGGGCAAGGAGTCTCGGCAACCTGAGGGCGGCG

A*

GCTGCGCGCTGAATCTCAGACGCGCTGCGCTGAGTTTCAAGAAAAATTACGAGAATACGTATAGGCGGCGTCTTGAGGCGATCGATTGGCGGC

1440

GTCATGCGAACAGGGGATTACCTATGCGGGCGCTTCTGTGCGCTGCGCTGCGGCTGTAAGCGGCGCGCGCGGCGAC

Stal

Solf 1480

CTACGCCGCTCGCCAGGCTATCAGGACCGCTATATGCGAC

Figure 3. Nucleotide sequence and the deduced amino acid residues of the galE gene from B. japonicum strain 61A101C. A putative Shine-Dalgarno (SD) ribosome-binding site, possible promoter sequence (-10 and -35), and specific enzyme sites are underlined.

, putative ρ-independent termination, *, stop codon.
2.10.1 Construction of shuttle vector pHC524::Km

The pHC79 plasmid which was used in *B. japonicum* was digested with *SalI* enzyme and the vector was treated by heat-blocking at 65°C for 10min. This cosmid vector was ligated with 1.9kb DNA fragment which was previously obtained from pKM5 plasmid by treatment with the same enzyme. The resulting plasmid was pHC524. Shuttle vector, pHC524::Km was constructed as follows: 1.3kb DNA fragment which was a kanamycin resistance gene cassette obtained from plasmid p34S-Km (From Yon-Sei, Dennis and Zylstra. 1998) by digestion with *KpnI* was inserted into a 1.9kb internal *KpnI* site containing the *galE* gene pHC524 which had been digested with the same enzyme. The overall schemes for the construction of shuttle vector was shown in Figure 4.

2.10.2 Introduction of homologous recombinant mutation

The pHC524::Km plasmid containing *galE*-knockout mutant gene, was transformed into *B. japonicum* 61A101C. Transformants were selected on an AMA agar plate containing 50μg of kanamycin per ml at 30°C. Kanamycin resistant colonies were transferred and grown in AMA medium containing 50 μg of kanamycin per ml.
Figure 4. Construction of pH524::km
2.10.3 Southern blot hybridization

A probe specific for galE gene was isolated by digestion of plasmid pKM5 with SalI, followed by gel elution of a 1.9kb fragment to the galE gene. The probe was labeled by using a nonradioactive digoxigenin-dUTP labeling system (Boehringer Mannheim, Germany). Isolated chromosomal DNA from each B. japonicum 61A101C and galE-knockout mutant was digested with EcoRI or HindIII, subjected to electrophoresis, and capillary transferred onto a nylon membrane with subsequent chemiluminescent detection as recommended by the supplier. The developed filter was stripped of the probe according to the supplier's instructions and prehybridized and hybridized to filters under high-stringency conditions in hybridization solution at 65°C. The hybridized probes were immunodetected with anti-digoxigenin-AP, Fab fragments and were then visualized with the colorimetric substrates NBT/X-phosphate as in the DIG DNA Labelling and Detection kit (Boehringer Mannheim, Germany).

2.10.4 Assay for the cell surface hydrophobicity (CSH)

The cells were harvested by centrifugation at 5,000 xg, and resuspended in a PBS buffer (per liter of deionized water; 22.2 g K2HPO4·3H2O, 7.26 g KH2PO4, pH 7.0) to give O.D.600 = 1.0. The CSH was determined by measuring the characters of the adhesion to n-hexadecane, as described by
(Aono and Kobayashi. 1997). Briefly, the bacterial suspensions were mixed with n-hexadecane as indicated and vortexed at room temperature for 2hr. To determine the characters of the cells, washed samples of the bacterial suspensions (5mL) were mixed with 5mL of n-hexadecane, and the mixture was maintained at room temperature for 2hr. as described above (Park and So. 2000).

2.10.5 Assay for the cell pellet patterns

After cells were cultured for 5 days, each cell was transferred into microtube. The cells were harvested by centrifugation at 10,000 rpm, for 5 min for identifying the cell pellet pattern (Park and So. 2000).

2.11 LPS profile assay

2.11.1 Isolation of LPS

Wild type 61A101C and Mutant type HS11 grown on liquid medium were harvested and suspended in 10mL of phosphate-buffered saline, pH7.2. A portion (15mL) of this suspension was centrifuged at 15,000 rpm for 5 min. The pellets were solubilized in 50μL of lysing buffer containing 2% SDS, 4% 2-mercaptoethanol blue. Lysates were heated at 100°C for 10 min. For protein digestion, 25μg of proteinase K (PK) solubilized in 10μL of lysing
buffer was added to each boiled lysate and incubated at 60°C for 60 min. (Hitchcock and Brown. 1983)

2.11.2 SDS–PAGE

The 4% stacking gel and the 12% separating gel did not contain SDS. Electrophoresis was done at 50mA of constant current with Tris-glycine (pH8.3) plus 0.1% SDS buffer for approximately 1.5h.

2.11.3 Ag–LPS staining procedure

The followed procedure was used for Ag–LPS staining: (i) overnight fixation in 200mℓ of 25% (vol/vol) isopropanol in 7% (vol/vol) acetic acid; (ii) 5-min oxidation in 150mℓ of distilled water with 1.05g of periodic acid and 4 mℓ of 25% (vol/vol) isopropanol in 7% (vol/vol) acetic acid (solution made up just before use); (iii) eight 30-min washes, each time with 200mℓ of distilled water; (iv) 10-min silver staining in a solution consisting of 0.1N NaOH (28 mℓ), concentrated (29.4%) ammonium hydroxide (1mℓ), 20% (wt/vol) silver nitrate (5mℓ), and distilled water (115mℓ) (make up solution just before use and stir constantly while making); (v) four 10-min washes, each time with 200mℓ of distilled water; (vi) 10 to 20 min of developing in 250mℓ of developer solution (citric acid [50mg], 37% formaldehyde [0.5mℓ], distilled water [amount sufficient to make 1 liter of solution]; made up just before use) at
an optimal temperature of 25°C (if solution cools, staining of protein as well as LPS will occur); (vii) 1h in a stop bath (200 ml of distilled water plus 10 ml of 7% [vol/vol] acetic acid); (viii) final wash with 200 ml of distilled water and then storage (gel may be stored in a zip-lock plastic bag with a small amount of water to prevent desiccation). Use of concentrated (29.4%) ammonium hydroxide is essential for the preferential staining of LPS. To maintain the quality of this reagent, we place small amounts of ammonium hydroxide from a freshly opened bottle into small (25 to 50 ml) bottles with caps that can be tightly sealed. Loss of "strength" of the reagent results in persistence of the brown precipitate in the staining solution. Addition of more ammonium hydroxide will dissolve the precipitate; however, preferential staining of LPS will not result (Hitchcock and Brown, 1983).

2.12 Nodulation assay

2.12.1 Seed sterilization and germination

Seeds (*Glycine max*. cv, *Essex*) are surface-sterilized by adding seeds to immersion distilled water containing Sodium Hypochloride (20% v/v) and shaking vigorously for 10 min (Whatley and Spiess, 1977). After removing and rinsing three times with sterilize distilled water, seeds were supplemented with 0.01N HCl for an additional 10 min. Rinse seeds three times with sterile distilled water for periods of 1 min and rinsed three times
with sterilize distilled water. To synchronize the germination process, seeds were placed in petri dishes containing 5mℓ of sterile distilled water. The seeds were followed by incubation at 28°C for 2 days. Seed sterilization of soybean and radish resulted in 100% sterility and 80% germination (Matthysse and McMahan. 1998).

2.12.2 Inoculation of plants and plant growth

When the soybean roots were 1 to 2cm long, the plants were then placed in soil in 35-mℓ serum vials which had a hole pierced in the tip. The soil was a vermiculite with plant nutrient solution (Wacek and Brill. 1976) supplemented 1% sucrose which had been sterilized by heating it in an autoclave in covered foil and stored for a week before use according to the procedure. Bacterial inoculum cultures in late log phase (O.D.₅₃₀, 1.0; the actual concentration of viable bacteria was determined in each inoculum culture by plate counts with the overlay procedure) were grown on an fresh AMA agar plain plate and with 50µg/mℓ of Kanamycin, respectively. After the suspension cells were prepared, the soybean seedlings were inoculated with 1mℓ of a prepared suspension containing 10⁶ CFU/mℓ cells which were the wild type and the knock-out mutant, respectively. Each vial was sealed with sterile whirlpak bags and the plants were maintained for 35 days in a climate-controlled growth chamber (28°C, 70% relative humidity, 12h of daylight) to allow the plants to grow.
2.12.3 Sampling of roots

At different times after inoculation the plants and soil were removed from the vials, the soil was carefully separated from the roots and the roots were gently shaken in washing water. The number of nodule was counted (Deshmane and Stacey. 1989).
Result

3.1 DNA sequence analysis

Nucleotide sequence examination of the 5.5kb insert of pHK34 demonstrated the presence of four open reading frames (ORFs). The relative locations of these ORFs are shown in Figure 2. Two of the ORFs corresponded well with the demonstrated locations of genes which encode the ADP-L-Glycero-D-Manno-Heptose-6-Epimerase and ADP-Heptose-LPS-Heptosyltransferase, and these ORFs were named rfaD and rfaF, respectively (Kim and So. 2000). Another ORF corresponded with the predicted location of gene which encodes the UDP-galactose 4-epimerase, based on the ability of subcloned plasmids to complement strain E. coli PL2. This ORF was named galE. The nucleotide sequence of the galE gene was determined and deposited in the Gene bank (accession number AF253311). Analysis of the sequence revealed galE gene of 1,099 bases, starting with ATG codon at position 1597-1600 and terminating with the TAA stop codon at position 586-589. The ORF and its deduced amino acid sequence are shown in Figure 3. Analysis of the galE gene predicted that it encodes a peptide consisting of a 337 amino acid translation product with a molecular weight of 36Kd, which is the same size as that from the recently published A. aeolicus galE gene sequence. The putative promoter sequence of galE showed a
typical -10 (TACGAT) and a -35 (GGGAGAGCT), and this galE is preceded by a possible ribosome binding site (GGGAGAGCT) complementary to the 3′ end of the 16S rRNA (3′-UUCCUCCA-5′) of B. japonicum. The galE gene identified is predicted to encode a protein with a high degree of homology with the product of the galE gene, UDP-galactose 4-epimerase. This homology extends over the entire length of the protein. Of the 6 amino acids that are shown by the crystal structure of E. coli UDP-galactose 4-epimerase to be within the binding βαβ fold for the fingerprint sequence cofactor (Holden et al. 1996). It is also present 5 bases from the initial ATG codon of galE. As the deduced amino acid sequences were compared to the BLAST database, the galE protein of B. japonicum showed homology of 54% with Azospirillum brasilense (Ab) (De Troch et al. 144), 50% with 50% Aquifex aeolicus (Aa) (Deckert et al. 1998), and 48% with Synechocystis sp. (Ss) (Kaneko et al. 1996).

3.2 Nucleotide sequence accession number

The nucleotide sequence data of the galE gene have been submitted to the NCBI/Gene Bank accession number of the 1.15kb fragment from pHK34 is AF253311.
MT---------VLVTGGAGYIGSHMHVALVDAGESVVIDNLDSTGFSALPGEVLPFIG 50
MTDQTAASP--VLVTGGAGYIGSHMHVALTDAGIPAVTDDLSAGRREAIPAAVPVLEG 58
MKK----------VLVTGGAGYIGSHVKALESGKGYVLIYDNLSTGNEWAVLYG-KLVKA 50
MT-QS--SP-PKILVTGAGYIGSSVRRQVLGEAGYSIVYVDNCSTGF-PSSILYQQLVIG 55
                  *                        .                   .
                    DADGNLVEGVIAGHIDSIIHFAGSVVVPSDARPPLGyyRNMTMGSSLLNAAVKGGS 110
DGSAEILDVMDRVDVMHFAFSIVIVPESVKLPDGYRMNNTANSLTLGACLRAID 118
DLADKETLRRVFEEFKPDAMVHFAAYIVPESEKVPLKYRNYNVIINLENLEVMQEGFN 110
DLADTERLHQQFHEHIEIALVMHFAGSILVPESLIHPLNYANNTSNTSLIRCCQIFGVN 115
                   .                   **                     .
RFIFSSTAAYGNPVDRVPFPFA-PTRLPSGGSKLMEIMLHDVA-SAHGMFYVLLRY 168
KVFSSTAAYGAPESVPREDAPA-PTVPINYPGSKLMTEQMLRAG-AAHGLRSLVIYR 176
KFVFSSAAAYVGRIPSVEKDA-PLNPINYPGETKAVTERLRLDNKSNKDNYVLSLYR 169
RLIFSSTAAYGNSSNPIS-EAIPCPINYPGYRSLASWEEIYQDA-KSSALQVIYR 174
                  *                       ***                    .
FNVAGADPKGRVGLATTGATHHKAVE-AATGG-RAKIDVFDTYQDGDSCIRDFH 226
FNVAGADPKGRTQATPVATHLIDKAQALL--GGPPPLAIFGDYQDTPGCTIDYIH 234
FNVAGADPKGRKIFAYFNPETHLLIIRAVKAA--GEFDRLEIYGDYQDTPGCTIDYIH 227
FNVAGADPKGRELQGKSTTHVLRVSCDAILNLK--SLDFIGTDFTPRGTYSRDIY 232
                  ***                    *                       .
SDLVEAHRALSYSRAGGQSVTSNLNGYGRGSVLTEIAVRRVSMLNFAVAYAARPPGD 286
SDLADAHVLALLHHRLRGGSLLMNCGYGRGASVREVVRLEVESGQVFATPAFDRAPGDP 294
TLDAEAHILALLEYFSGGKSEVLNCYGHGSVLTVNAVKKVTGDFKVEAPFREGPD 287
EDLAKAHLDALFYNLEEINGESEQLNLNGYGGGYSSVREVTDRAKIADVFDFLVERTERLDP 292
                  *                       ***                    *
MTMVADTTIRSSLLDWTTRRDPDDFFLDHIALWESWKLFRERAGSQAESA* 338
PQLVAGADRIRQQLGWFPKHDRLDGVSRALSWRL--------------------- 331
PALVADNKIKKIRVLNWEKYDDLFIIKTAWEVKK---------------------- 323
ASVIACADSIRQVRSLTPKNNLDILRUALBIK------------------------ 328
                   .                   *                        *

Figure 5. Multiple a.a. sequence alignment of UDP galactose 4-epimerase of
Bradyrhizobium japonicum(Bj), Azospirillum brasiliense(Ab), Aquifex aeolicus(Aa),
and Synechocystis sp (Ss). Asterisks indicate identity, dots indicate conservative
changes. Individual sequence identity was found to be Ab, 54%; Aa, 50%; Ss, 48%.
Table 2. Characteristics of *galE* proteins of gram-negative bacteria

<table>
<thead>
<tr>
<th>Organism</th>
<th><em>n</em></th>
<th>Nonpolar (%)</th>
<th>Polar (%)</th>
<th>Acidic (%)</th>
<th>Basic (%)</th>
<th>M.W</th>
<th><em>pI</em></th>
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</thead>
<tbody>
<tr>
<td><em>H. japonicum</em></td>
<td>337</td>
<td>48.4</td>
<td>32.3</td>
<td>10.7</td>
<td>8.6</td>
<td>36,067</td>
<td>5.80</td>
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<tr>
<td><em>A. brasilense</em></td>
<td>331</td>
<td>52.9</td>
<td>25.9</td>
<td>11.2</td>
<td>10.0</td>
<td>35,173</td>
<td>6.20</td>
</tr>
<tr>
<td><em>A. aeolicus</em></td>
<td>323</td>
<td>45.4</td>
<td>28.2</td>
<td>14.0</td>
<td>12.4</td>
<td>36,122</td>
<td>5.80</td>
</tr>
<tr>
<td><em>Synechocystis sp.</em></td>
<td>326</td>
<td>45.2</td>
<td>36.4</td>
<td>10.1</td>
<td>8.3</td>
<td>35,767</td>
<td>5.76</td>
</tr>
</tbody>
</table>

*n.* Number of amino acid

*pI.* Isoelectric point
Figure 6. Secondary structure prediction of amino acid of the galE gene in B. japonicum.
Figure 7. Analysis of the amino acid sequence of the \textit{B. japonicum galE} gene. Hydropathy index computation was done with the Anthe pro 2000 (ver. 5.0) analysis program.
Table 3. UDP-binding βαβ fold fingerprint sequence of various GalE protein

<table>
<thead>
<tr>
<th>Protein (Peptide)</th>
<th>Sequence&lt;sup&gt;b&lt;/sup&gt;</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDPHE&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4-VTGGAGYIGSHMV</td>
<td>This study</td>
</tr>
<tr>
<td>UDPHE&lt;sup&gt;d&lt;/sup&gt;</td>
<td>4-VTGGSGYIGSHTC</td>
<td>Thoden et al. 1996</td>
</tr>
<tr>
<td>UDPHE&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4-VLGGAGYIGSHTV</td>
<td>Bettenbrock and Alpert 1998</td>
</tr>
<tr>
<td>UDPHE&lt;sup&gt;f&lt;/sup&gt;</td>
<td>5-VTGGAGYIGSHVV</td>
<td>Deckert et al. 1998</td>
</tr>
<tr>
<td>UDPHE&lt;sup&gt;g&lt;/sup&gt;</td>
<td>10-VTGGAGYIGSSVV</td>
<td>Hirosawa et al. 1996</td>
</tr>
<tr>
<td>UDPHE&lt;sup&gt;h&lt;/sup&gt;</td>
<td>12-VTGGAGYIGSHVL</td>
<td>Troch et al. 1994</td>
</tr>
<tr>
<td></td>
<td>* * * GXGXXG</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> UDPHE, UDP-glucose-4-epimerase.
<sup>b</sup> The number preceding the sequence is the residue number of the acid shown relative to the amino terminus.
<sup>c</sup> From *B. japonicum*.
<sup>d</sup> From *E. coli*.
<sup>e</sup> From *L. casei*.
<sup>f</sup> From *Aquifex aeolicus*.
<sup>g</sup> From *Synechocytis sp*.
<sup>h</sup> From *Azospirillum brasilense*.
3.3 Subcloning *galE* gene region

Various recombinant plasmids were constructed by subcloning the restriction fragments of pHK34. Clone pHK34 contains a 5.5kb insert fragment which complements the LPS defect in four different LPS mutant *B. japonicum* (So. 1991). Subclone pKM1 was constructed by digestion of pHK34 with the restriction enzyme *PstI*. A 3.5kb fragment was gel purified and then introduced into pGEM-3zf(+) (Promega), which had been digested with *PstI* (see figures 8 and 9). pKM2 has the same 3.2kb fragment as pKM1 subcloned in pGEM-3zf(+) in the opposite orientation (see figures 8 and 9). PKM3 was constructed by selfligation pKM1 which had been digested with *BamH I*, deletion in the site 2.2kb *BamH I* fragment (see figures 10 and 11). PKM4 was constructed by the same way as pKM3. As a result, 1.3kb deleted recombinant plasmid was obtained (sees figure 10 and 11). pKM1 plasmid was digested with *SalI* restriction enzyme and 1.8kb DNA fragment was eluted by the method Sambrook et al. (1989). The obtained 1.8kb DNA fragment was ligated with pGEM-3zf(+) which was previously digested with *SalI*. The resulting plasmid was named pKM5 and pKM6 contains the same 1.8kb DNA fragment as pKM5 subcloned in pGEM-3zf(+) in the opposite orientation. PKM7 was constructed with a 0.7kb the smaller fragment which was a rest of digested pKM1 by *SalI* restriction enzyme (see figures 12 and 13). The overall schemes for the subcloning of *galE* gene is depicted (see figure 1).
Figure 8. Construction of pKM1 and pKM2 plasmids from pHK34 plasmid containing 3.5Kb PstI fragment
Figure 9.  *Bam*HI Restriction patterns of pKM1 and pKM2 plasmids containing 3.5kb fragment
Figure 10. Construction of pKM3 and pKM4 plasmids containing deficient galE gene
Figure 11. Different Restriction patterns of constructed pKM3 and pKM4 plasmids containing deficient *galE* gene
Figure 12. Construction of pKM5, pKM6, and pKM7 plasmids from pHK34 containing each salI fragment
Figure 13. *Bam*HI Restriction of the constructed pKM5, pKM6, and pKM7 plasmids.
3.4 Complementation test

UDP-galactose is toxic to bacteria at high concentrations. galE mutant cannot convert UDP-galactose to UDP-glucose. Thus, addition of galactose to galE mutant and incomplemented E. coli PL2 is lethal because of the accumulation of excess UDP-galactose (Adhya. 1987). Plasmid pHK34, containing the putative galE gene, was introduced into E. coli PL2, containing the galE28 allele. This strain was then examined for growth on media containing either 1% glucose or 1% galactose (see figure 14). Growth of PL2 was inhibited on media containing galactose. Both pHK34 and subcloned plasmids containing galE gene as a control complemented the growth defect on galactose. However, the growth rate of the PL2 strain with the complemented plasmids in media containing galactose was the same as that in media containing glucose. As galE mutants do not produce UDP-galactose, which serves as the galactose donor for LPS biosynthesis, the enzyme of these same strains were examined to determine normal galE gene production to E. coli. Taken together, these data suggest that the B. japonicum galE gene has UDP-galactose 4-epimerase activity, at least when subcloned into E. coli (see figures 15 and Table 4). To demonstrate definitively this activity of the subcloned gene in E. coli, a UDP-galactose 4-epimerase assay was performed. The UDP-galactose 4-epimerase activity of the extracts was determined by the method of Wilson and Hongress (1966).
3.5 Construction of \textit{galE}-knockout mutant

To understand the role of the \textit{galE} gene LPS biosynthesis, a \textit{galE}-knockout \textit{B. japonicum} strains was constructed and its LPS character was examined. The insert, 1.9kb DNA \textit{SalI} fragment from pKM5 was ligated with the cosmid vector pHC79 by treatment with the same enzyme. To determine and confirm whether it contains the \textit{galE} gene, the pHC524 from DH5\textalpha{} was digested with \textit{SalI}, and resolved on 0.7% agarose gel. Identical bands of 1.9kb band 6.6kb were shown in the pHC524. DNA sequence analysis showed that the full length of \textit{galE} gene was partly located on. The \textit{galE} gene was inactivated by inserting a kanamycin gene cassette into the internal \textit{KpnI} site in the recombinant plasmid, pHC524 at position 1,370bp. The resulting mutant plasmid, pHC524::Km was the introduced into \textit{B. japonicum} 61A101C by electroporation. One of the kanamycin resistant \textit{B. japonicum} colonies was selected and Homologous recombination designated \textit{galE}-knockout mutant, \textit{B. japonicum} HS11 (see figure 22). The inactivated gene in chromosome was expected by double crossing-over which was exchanging \textit{galE}-knockout fragment and \textit{galE} gene. To confirm the inactivation of the \textit{galE} gene on the chromosomal DNA, southern blot hybridization was carried out. The digested with \textit{EcoRI} and \textit{HindIII} from the \textit{galE}-inactivated strain HS11 showed an increased DNA size that is characterized by a 1.3kb higher DNA size than that from the parental strain 61A101C. 1.9kb \textit{SalI} fragment isolated from the pKM5
was labeled with digoxygenin, and used as a probe. Fragments digested with \textit{EcoRI} and \textit{HindIII}.

3.6 Assay for cell surface hydrophobicity

To demonstrate whether this \textit{galE} mutant strain can be used to elucidate the role of the carbohydrate moiety of LPS and demonstrate that the change in the surface hydrophobicity of \textit{B. japonicum} is correlated with an LPS defect, in particular, the loss of the O-antigenic part (see figures 17 and 18). The knockout mutant cells tended to aggregate at stages of growth, thereby prompting the present study on the physicochemical properties of the mutant cell surface including the CSH (Park and So. 2000). It has been previously reported that bacteria with hydrophobic cell surfaces tend to adhere to one another, resulting in the formation of clumps. In this study, therefore, the overall cell surface hydrophobicity was investigated by measuring the cell partition with hydrocarbon as described in Materials and Methods. Figure 17 illustrates the partitioning patterns of \textit{B. japonicum} 61A101C with n-hexadecane. When mixed with n-hexadecane, the \textit{galE}-knockout mutant cells were immediately nearly completely removed from the aqueous phase layer, whereas the wild type cells remained unaffected in the aqueous phase. One additional observation concerning the \textit{galE}-knockout mutant of \textit{B. japonicum} was that after centrifugation it formed a cell pellet quite discernable from that of the wild type.
Figure 14. Galactose sensitivity assay. The growth of *E. coli* wild-type DH5α and *galE* mutants strain PL2 containing the different recombinant plasmids on the region of *galE* was examined in minimal media containing 1% glucose (A) and 1% galactose (B).
Figure 15. Linearity of the UDP-galactose 4-epimerase assay with length of time. Assay conditions were described in Materials and Methods. The rate of UDP-glucose production were lined against the enzyme concentration.
Table 4. UDP galactose 4-epimerase assays of an *E. coli* galE mutant containing different clones.

<table>
<thead>
<tr>
<th>Strains&lt;sup&gt;a&lt;/sup&gt;</th>
<th>UDP galactose 4-epimerase activity&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>DH5α</td>
<td>14.9</td>
</tr>
<tr>
<td>PL2</td>
<td>1.35</td>
</tr>
<tr>
<td>PL2/pGEM-3zf(+)</td>
<td>1.35</td>
</tr>
<tr>
<td>PL2/pKM1</td>
<td>8.91</td>
</tr>
<tr>
<td>PL2/pKM2</td>
<td>10.9</td>
</tr>
<tr>
<td>PL2/pKM3</td>
<td>1.35</td>
</tr>
<tr>
<td>PL2/pKM4</td>
<td>1.10</td>
</tr>
<tr>
<td>PL2/pKM5</td>
<td>7.00</td>
</tr>
<tr>
<td>PL2/pKM6</td>
<td>7.94</td>
</tr>
<tr>
<td>PL2/pKM7</td>
<td>1.62</td>
</tr>
</tbody>
</table>

a. Bacteria was grown in minimal media glycerol and subjected to 1h of induction in the presence of galactose.

b. Activity is defined as nanomoles of NADH formed per minute per milligram of protein.
Figure 16. Hybridization of wild 61A101C and galE–knockout mutant, HS11 containing km cassette. A. EcoRI and HindIII restriction enzyme of the galE gene region of 61A101C and HS11 and the precise locations of Km cassette (▼) insertion in the 1.9 kb KpnI fragment. B. Lane 1 and 2: the picture of HindIII digests of HS11 (galE–knockout mutant) and 61A101C (wild type). Lane 3 and 4: EcoRI digests of genomic DNA from HS11 and 61A101C and probed with galE DNA fragment (→).
E; EcoRI, H; HindIII, K; KpnI, SI; Sall.
3.7 LPS profile assay

LPS obtained by procedure was subjected to SDS-PAGE and visualized by the sensitive silver staining method of Hitchcock and Brown. As shown in Figure 17, wild type *B. japonicum* LPS displayed some degree of size as evidenced by the various bands located in the lower to upper region of the gel. When *galE*-knockout mutant was examined, band corresponding to the O-antigenic side chain were absent (see figure 17). The result clearly demonstrated that in the *galE*-knockout mutant strain of *B. japonicum* LPS was missing so that HS11 appeared hydrophobicity.

3.8 Nodulation assay

To determine the *galE* gene responsible for the role of the LPS synthesis and the symbiotic function encoded in this region. *B. japonicum* wild type 61A101C and *galE*-knockout mutant HS11 were tested for the ability to nodulate soybean cultivar Essex. The percentage of nodules per plant induced by *galE*-knockout mutant failed to produce nodules even after prolonged time periods. However, the percentage of nodules per plant 35 days after inoculation was more than 95% for wild type *B. japonicum* 61A101C (see figure 20).
Figure 17. Cell Surface Hydrophobicity test. Partitioning of cells of *B. japonicum* wild type 61A101C (Left) and *galE*-knockout mutant HS11 strain (Right) to n-hexadecane.
Figure 18. Cell pellet patterns formed by 61A101C and *galE*-knockout mutants after centrifugation. One and half milliliter of 6-days old
Figure 19. Detection of lipopolysaccharides by periodic acid silver stain. Samples of 1µg were analyzed on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Lane1 and 3, *B. japonicum* wild type 61A101C; lane 2 and 4, *galE*–knockout mutant HS11.
Figure 20. Nodulation phenotype of HS11. A homogenate and the wild type strain 61A101C were tested on *G. max* cv. Essex for their nodulation phenotype. Nodulation kinetic is shown as percentage of nodulated plants.
Discussion

A gene cluster involved in *B. japonicum* LPS production has been cloned analyzed. Sequencing of a 5,500 bp DNA fragment revealed the presence of 4 open reading frames, which showed similarities to LPS synthesis genes in other gram negative bacteria. In a previous study, we have characterized two ORFs which were found to be *rfaF* and *rfaD*. The *rfaF* gene is known to encode heptosyltransferase II that transfers a second heptose to the inner core of lipopolysaccharide. The cloned *B. japonicum* ORF was able to functionally complement a *rfaF* mutant of *Salmonella typhimurium* SL3789 (So et al. 2000). In this study, we have further characterized an additional ORF having a significant sequence homology with *galE* genes of a number of other organisms. A search of the databases with amino acid sequence of the ORF showed the polypeptide as a potential UDP-galactose 4-epimerase. The homologies were *A. brasilense* (68%), *A. aeolicus* (68%), and *Synechocystis* sp. (66%). The putative *B. japonicum galE* gene was introduced into UDP-galactose 4-epimerase deficient *E. coli* strain PL2 for complementation. With the introduced *B. japonicum galE* gene, *E. coli* PL2 were transformed from a Gal-sensitive to a Gal-tolerant. Analysis of cell extracts from strain PL2 and the transformant confirmed the presence of UDP-galactose 4-epimerase activity in the Gal-tolerant transformants.

It is known that in some microorganisms UDP-galactose can be
*, intermediates in the Leloir pathway; ▲, intermediates in the De Ley–Douderoff pathway; ■, intermediates in the Enter–Douderoff pathway.

Figure 21. Metabolic pathway for the conversion of glucose and galactose. Abbreviation used: G6PDH, glucose-6-phosphate dehydrogenase; KDG, 2-keto-3-deoxygalactonate; KDPG, 2-keto-3-deoxy-6-phosphogalactonate; AcCoA, acetyl coenzyme A; GK, glucokinase; GalK, galactokinase; GDH, glucose dehydrogenase; GalDH, galactose dehydrogenase; UG4'E, UDP-glucose 4'-epimerase; GalT, galactose-1-phosphate uridyl transferase.
Figure 22. Putative LPS structure of B. japonicum.
Figure 23. Recombination scheme following transformation of a mutated clone into the wild-type strain. Black arrow: double crossover resulting in the formation of cassette exchange. Normal arrows: single crossover resulting in the formation of a co-integrate. DNA is drawn as thick line; the thin line represent vector DNA. The speck and wave lines designate chromosomal DNA. Antibiotic cassette insertions are a vacant thick squares.
synthesized from galactose-1-P by the Leloir pathway (see figure 21), as well as by the more common epimerization of UDP-galactose to UDP-glucose (Potter and Lo. 1996). The galE gene product, UDP-galactose 4-epimerase, plays a role in the biosynthesis of the carbohydrate portion of LPS in which Uridine disphosphogalactose (UDP-Gal) is a precursor. UDP-Gal is also involved in galactose metabolism when galactose is the sole carbon available. The galE-knockout mutant in B. japonicum produced truncated-LPS molecules that lack O-polysaccharide. As a consequence, it seems reasonable to assume that the non-sugar substitutions at the galactose residues are missing. These non-sugar modifications of the galactosyl residue therefore could have a very subtle influence on the attachment of the bacterial cells to the root hair surface, which is an essential prerequisite for bacterial invasion and nodule induction (Puvanesarajah et al. 1987 and Carlson et al. 1987).

Whatever functions the bacterial cell surface polysaccharides may have in the infection process, they are ultimately dependent on the chemical structure of B. japonicum. Only a few reports exist in the literature that describe the cell surface compositions of Rhizobium species (Carlson, R. W. 1982). In contrast, there are published reports on the purification or the composition of LPS from slow-growing B. japonicum. B. japonicum is reported to have five different sugars (Puvanesarajah et al. 1987), including galactose, as components of its LPS, raising the possibility that the galE gene in involved in the
synthesis of the nucleotide sugar donor of one of these five other sugars (Pierson and Carlson, 1996). To study the role of the galE gene in LPS biosynthesis, the galE-knockout mutation was introduced into the B. japonicum galE gene by homologous recombination. This result led to the suggestion that the mutant HS11 is somehow defective in its cell surface. The galE-knockout mutant HS11 genotype was confirmed by Southern analysis. These results are consistent with the loss of galactose in the carbohydrate moiety of LPS, leading to a low-molecular weight, rough-form LPS without O-side chain in B. japonicum (Brink et al. 1990). The purified LPS from each strain was resolved on 12% SDS-PAGE and stained with silver nitrate as described (Hitchcock and Brown, 1983). As shown in Figure 19, the purified LPS from the galE-knockout mutant strain showed an altered profile that is characterized by a lower molecular weigh than that from the wild type. After structural characterization of the LPS from the wild type 61A101C and the mutant strain HS11 has been done, plant interaction test shows that two are different in nodulation.

Our results show that the nonnodulating mutant strain HS11 is a rough and hydrophobic mutant lacking a complete LPS moiety. Thus, it appears that a galE gene in a complete LPS biosynthesis should be necessary for the ability to infection and nodulate soybeans.

This study resulted in the complementation of a galE gene and the construction of a galE-knockout mutant that would be useful tools for structural and functional studies of B. japonicum LPS.
References


감사의 글

새로운 세계에 대한 두려움과 설레임으로 시작한 2년간의 대학원생활은 하루하루를 보내는 것이 아니라 제가 가진 열정과 주위의 따뜻한 격려로 부족한 나의 능력을 채워 가는 과정이었습니다. 이 자리에 있기를 많은 도움을 주신 모든 분들께 감사의 글로 마음을 전하고자 합니다.

특히 부족한 저를 깜짝 받아주시고, 학문적인 큰 가르침과 끝없는 지도와 노력을 아끼지 않으시고 이끌어주시신 지도교수님 이신 소 재성 교수님에게 글은 감사를 드립니다. 학문적인 가르침 외에 삶에 대해 던져주신 헤 병기 교수님, 헤 태현 교수님, 구 온모 교수님, 김 은기 교수님, 김 동일 교수님, 윤 현식 교수님, 이 척균 교수님 생물공학과 교수님들께 여러 숙이 깊은 감사를 드립니다.

지난 4년간의 대학시절에 여러 가지 조언과 압력을 제시해 주시면 김 은희 교수님, 이 종수 교수님, 곽 한식 교수님, 곽 인영 교수님, 김 학근 교수님 모든 분께 이 자리를 빌어 감사의 말씀을 전하고자 합니다. 많은 관심과 도움을 준 무배 서울대학교 박사과정 문영이, 생명공학연구소 영희에게 늘 고맙게 생각하고 있습니다.

세네기 때의 낙설움을 모두 잃게 베편 입학동기이며 졸업동인이 자본한 수환이, 옥심이 앞서지만 열심인 지숙이, 착한 성환이, 친구 같은 영범이, 깔끔한 세원이, 힘들 때나 기쁨 때도 늘 같이 혼란 경원이, 언제나 열굴에 웃음이 가득한 상옥이, 언제나 진지한 영미, 왕일드 하지만 매번 어린 세경이, 착하다 착한 광용이 모두들에게 고맙다는 말을 이 글로 대신하고자 합니다. 생물공학과의 말대로 공정 방의 우주를 항아리 가시는 잘 하실 거라 믿고, 찰분한 성호, 진희 언제나 한결같이 친착한 동기 세포 방의 상윤이, 몸 건강하게 병역을 마감하기를 바라고, 책을 좋아하는 규학, 언제나 밝은 미소인 병혁, 지숙, 윤정, 꺾기의 열심인 환경 방의 백석이, 진수, 승학이, 늘 즐거움을 선사해 주는 정숙이, 어려운 가운데
거다란 버팀목이 되어주는 식품 방의 기용이영, 미국 연수기간 동안 먹어가고 깨워진 원규, 식품방의 삼림을 잘 이끌어 가는 지연이, 총대학원의 임원이며 시스템 방의 일꾼인 용민이, 원호, 천지, 귀연 준 세대기 경영 (호중이, 호익이, 대원이), 반 유전방 인이며 반응방인 많은 일도 적혀있으나가는 정은이, 현 원우의 회장인 보현, 착진 현호, 많은 시간을 두고 대학을 나누지 못했지만 놀란가 맞추주는 제어 방의 복사과정 정문이, 고품종 낙종이, 아 쉽게도 루학을 한 온영이, 이렇게 많은 분들과 함께 했던 대학원 생활은 저에게 소중한 기억이 되고 간직 할 것입니다.

그리고 제가 2년 동안 음달았던 유전자 공학 실험실의 실험원들 모두에게 감사함을 표현하고자 합니다. 1년 반 동안 별같이 지내온 현규, 언제나 나에게 보여줬던 계획성과 몰입하게 처리하는 자세는 나에게 거다란 귀감이 되게 하였습니다. 부족한 선택을 위해 항상 뒤에서 도움을 주고 힘이 되어 주었던 동직인 지철이, 앞으로의 실험실 방향으로 잔영이 많은 섬유, 유전 방의 삼림以致으로 성실한 재상이, 세대기 은택이, 수영이, 태연이, 주현이, 모두들 서로로서 도와가며 좋은 결실을 맺길 바라고, 가장 오랜시간 동안 헌신한 지식으로 나를 놀라게 하는 친동생 같은 원인이 KAIST에 가서도 잘하길...., 이름에 걸맞게 부자연한 작은 경민이, 실험실과 늘 같이 했던 상호영, 성근이 모든 분께 감사드립니다.

졸업하신 선배, 후배 그리고 동기들께도 감사드립니다. 공부라는 평생으로 자주 연확하 지 못한 프리마 91 동기, 영운, 준호, 홵백, 경영, 상진, 명문 모두들에게 감사하며 많은 분들께 다시 한번 머리 숙여 감사의 드리며 됩니다.

마지막으로, 형으로써 대신해 모든 일들을 아무런 불평 없이 나의 자리를 챡워준 경호에게 고마움 마음과 무엇보다도 절을 믿고 따뜻한 눈으로 봉주시고 저의 든든한 후원자가 되어주신 부모님께 말로 표현하지 못할 감사를 드립니다.

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