CHAPTER II

MATERIALS AND METHODS

2.1 Instruments

Name	Company	Country
Autoclave: Model HA-30	Hirayama	Japan
Autopipette: Pipetman; P20, P100, P1000	Gilson	France
BioChiller™2000	Fotodyne Inc.	USA
Camera: Pantex Super A		
Centrifuge, refrigerated centrifuge:	Beckman Instrument Inc.	Japan
Model J-21C		
Centrifuge, refrigerator microcentrifuge	Kubota	Japan
Geiger counter:		USA
Gene Pulser™	Bio-Rad	USA
GS GENE LINKER™ UV CHAMBER	Bio-Rad	USA
High speed microcentrifuge: HA-30	Memmert Gmb	Germany
Hybridization oven	Bio-Rad	USA
Incubator: BM600	Memert Gmb	Germany
pH/Ion meter: PHM 95	Radiometer	Denmark
Spectrophotometer: UV-240	Shimadzu	Japan
Spectrophotometer:DU series 650	Beckman	USA
UV transluminator:	San Gabrial	USA
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2.2 Supplies

Name	Company	Country
Filter Paper: HA 0.45 μM pore size	Millipore Corporation	USA
Nylon Transfer Membrane	Bio-Rad	USA
NENSORB^{TM} 20 Nucleic Acid Purification Cartridges	DuPont	USA
Whatman 3MM paper	Whatman International Ltd.	England
Whatman #42 filter paper	Whatman International Ltd.	England
X-ray film: X-Omat XK-1	Eastman Kodak Company	USA

2.3 Chemicals

Name	Company	Country
Agarose	Seakem	USA
Ampicillin	Sigma	USA
[α-^{32}P]dATP	Amersham	USA
[γ-^{32}P]ATP	Amersham	USA
Betaine aldehyde chloride	Sigma	USA
Choline chloride	Sigma	USA
Chloramphenicol	Sigma	USA
Lambda (λ) DNA	Biolabs	USA
Glycine betaine	Sigma	USA

The other common chemicals were obtained from Fluka or Sigma

2.4 Kit

Name	Company	Country
Nick translation kit	Amersham	USA
QIAquick Gel Extraction Kit	QIAGEN	Germany

2.5 Enzymes and restriction enzymes

Name	Company	Country
Lysozyme	Sigma	USA
Proteinase K	Sigma	USA
RNase	Sigma	USA
T_4 DNA ligase	Pharmacia	Sweden
T_4 polynucleotide kinase	Biolabs	USA
Restriction endonuclease, *Eco*RI, *Bam*HI, *Hin*dIII, *Nde*I, *Pst*I, *Sau*3AI and *Xba*I	Biolabs	USA

2.6 Bacterial strains and plasmids

Aphanothece halophytica was isolated from solar lake in Israel. The organism was kindly provided by Dr. T. Takabe of Nagoya University, Japan.

Eshcherichia coli JM109 with genotype, F' *traD36 proA*$^+$ *proB*$^+$ *lacI*^{q}*lacZΔM15/recA1 endA1 gyrA96* (Nal^r) *thi hsdR17*($r_k^- m_k^+$) *supE44 relA1 Δ(lac-proAB) mcrA*, was used as a host for transformation in a general cloning procedure.

E. coli DH5α with genotype, φ80d *lacZ*ΔM15 *recA*1 *endA*1 *gyrA*96 *thi*-1 *hsdR*17($r_k^- m_k^+$) *supE*44 *relA*1 *deoR* Δ*(lacZYA-argF)*U169, was used as a host for harboring pUC18 and pJB007.

Recombinant plasmid, pJB007, was a clone of *gbsAB* genes from *Bacillus subtilis* in pHSG575 (Boch et al., 1996) and used as a probe for hybridization. This plasmid was kindly donated by Dr. E. Bremer of University of Marburg, Germany.

pUC18/*Bam*HI/BAP(Pharmacia) was used as a vector for cloning and transformation into *E. coli*.

2.7 Extraction and purification of chromosomal DNA from *A. halophytica*

The salt tolerance cyanobacterium *A. halophytica* was grown at 30°C in Turk Island Salt Solution plus modified BG_{11} medium (Appendix 3) containing 0.5 M NaCl, under continuous illumination with fluorescent white light. Ten-day old culture in mid-exponential growth was harvested by centrifugation at 3500 rpm for 10 minutes at 4°C, washed once with SET buffer (20% sucrose, 50 mM EDTA and 50 mM Tris-HCl pH 7.6). The cell pellet was frozen at -70°C for 10 minutes, thawed at 65°C before put on ice and resuspended in 2 ml of SET buffer Then 200 µl of lysozyme (5 mg/ml in TEN buffer) and 100 µl of DNase-free RNase (10 mg/ml in 0.1 M sodium acetate pH 7.4 and 0.3 mM EDTA) were added to the suspension. The mixture was incubate for 30 minutes at 37°C under gentle shaking. Subsequently, 50 µl of 25% SDS was added to the suspension. After an incubation at 37°C for 3-6 hours, 300 µl of 2 mg/ml proteinase K was added and further incubated for 30 minutes. The mixture was extracted once with an equal volume of chloroform/isoamyl alcohol (24:1), incubated at 37°C with gentle shaking for 10-16 hours and centrifuged at 3500 rpm for 20 minutes. The aqueous layer was collected and reextracted twice with an equal volume of chloroform/isoamyl alcohol (24:1). High molecular weight DNA was precipitated by adding 200 µl of 5 M NaCl and 2 volume of ice-cold absolute ethanol and chilled at -20°C for 10 minutes. The DNA was collected by centrifugation at 5000 rpm for 10 minutes and washed with 70%

ethanol to remove excess salts. The DNA was allowed to air-dried, resuspended in an appropriate volume of TE buffer (10 mM Tris-HCl pH 8.0 and 1 mM EDTA) and stored at 4°C until further needed. The purity of the DNA was checked by measuring the ratio of A_{260}/A_{280} which should be greater than 1.8 for pure DNA.

2.8 Agarose gel electrophoresis

To measure the size and the amount of DNA in the sample, 0.7% agarose gel in 1X TBE buffer (89 mM Tris-HCl, 89 mM boric acid and 2.5 mM EDTA pH 8.3) was used. The DNA sample was mixed with 1/3 volume of loading dye (0.25% bromphenol blue, 40% ficoll400 and 0.1% SDS) before loading into the well of gel which was submerged in the TBE buffer in an electrophoretic chamber. An appropriate amount of λ/*Hin*dIII was also loaded to the gel to serve as a DNA marker. Generally, the gel was run at 100 volts until bromphenol blue migrated to the other edge of the gel, which was approximately 2 hours. After electrophoresis, the gel was stained with 2.5 μg/ml ethidium bromide solution for 5 minutes and subsequently destained with large volume of water for 10-15 minutes to leach out unbound ethidium bromide. The DNA bands were visualized under UV light and photographed through a red filter with Kodak Tri-X pan400 film. The concentration and molecular weight of DNA samples were estimated by comparing with the intensity and relative mobility of λ/*Hin*dIII. The red fluorescent intensity of the standard DNA bands (500 ng λ/*Hin*dIII) of 23.1, 9.4, 6.6, 4.4, 2.3, 2.0 and 0.6 kb correspond to 240, 98, 67, 45, 24, 21 and 6 ng of DNA, respectively.

2.9 Preparation of *Sau*3AI partially digested chromosomal DNA

2.9.1 Trial digestion

To optimize the condition for partial digestion, 3 microfuge tubes were prepared, each containing 0.5 µg of *A. halophytica* chromosomal DNA, 1X *Sau*3AI buffer (100 mM NaCl,10 mM Bis Tris Propane-HCl, 10 mM $MgCl_2$, and 1 mM dithiothreitol, pH 7.0). 0.2, 0.5, and 1.0 units of *Sau*3AI were added to each of the 3 tubes, respectively, and incubated at 37°C. 2.5 µl aliquot from each tube was removed after 5, 10, 15, 20, 25, 30, 35, 40 and 45 minutes of incubation and the reaction was stopped by adding 1/3 volume of tracking dye. Digested DNA were subjected to electrophoresis in 0.7% agarose gel along with λ/*Hin*dIII until bromphenol blue tracking dye reached the bottom of the gel. The gel was stained in 2.5 µg/ml of ethidium bromide for 5 minutes and visualized by UV light. The duration of digestion and enzyme concentration that gave DNA with maximum intensity in the 3-6 kb region was chosen to proceed to scale-up.

2.9.2 Scale-up digestion

The optimal digestion was scale-up 100 times. Five hundred nanograms of chromosomal DNA was digested with 100 units of *Sau*3AI. The reaction was stopped by heating at 70°C for 10 minutes. An aliquot of the digested DNA was run on a 0.7% agarose gel to verify the size distribution of the DNA fragments. Partially digested DNA was extracted with 1 ml phenol/chloroform/isoamyl alcohol (25:24:1), mixed by inversion and centrifuged at 3000 rpm for 10 minutes to separate the aqueous phase. The DNA was precipitated by adding 100 µl of 5 M NaCl and 1.0 ml ice-cold absolute ethanol, mixing by inversion and incubating on ice for 10 minutes.

The precipitate was collected by centrifugation at 3000 rpm for 10 minutes at 4°C. The air-dried DNA was resuspended in 480 µl of TE buffer.

2.10 Ligation

The pUC18, digested with *Bam*HI and dephosphorylated by bacterial alkaline phosphatase (Pharmacia), was used as vector. The ligation mixture contained 50 ng of vector, 100 ng of the *Sau*3AI digested DNA, 1X ligation buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP and 25 µg/ml bovine serum albumin) and 2 units of T_4 DNA ligase in a total volume of 20 µl. The reaction was incubated at 16°C for 12-16 hours and stopped by heating at 70°C for 10 minutes.

2.11 Transformation of *E. coli* by electroporation (Dower et.al., 1988)

2.11.1 Preparation of competent cells

A single colony of *E. coli* was inoculated to 50 ml of LB medium and incubated at 37°C overnight with shaking. Twenty five millilitres of the overnight culture was diluted with 500 ml of LB medium and incubated at 37°C with vigorous shaking for 2-3 hours until the OD_{600} reached 0.5-0.7. The culture was transferred to sterile ice-cold 250 ml centrifuge bottle and chilled on ice for 20 minutes. After centrifugation at 4000 rpm for 10 minutes at 4°C, cell pellet was resuspended in 200 ml of ice-cold sterile water, mixed and recentrifuged as described above. The resulting pellet was further washed with 40 ml of ice-cold water, followed by 20 ml of ice-cold 10% glycerol and resuspended in a final volume of 1.5-2.0 ml ice-cold 10% glycerol. This cell suspension was dispensed in 40 µl aliquots into 1.5 ml microcentrifuge tubes and stored at -70°C. The cell was competent for at least 6 months under these conditions.

2.11.2 Electroporation

Forty microlitres of the competent cells was thawed on ice prior to the addition of 1 µl of the ligation mixture. The transformation mixture was mixed by pipeting up and down once and then electroporated in a chilled 0.2 cm electroporation cuvette. The Gene pulser® apparatus was set at an exponential decay pulse of 2.5 kV, 25 µF capacitance and 200 Ω resistance. After electroporation, 1 ml of LB medium was immediately added to the cuvette and the entire mixture was transferred to a new tube. The cell suspension was incubated at 37°C for 1 hour with gentle shaking.

2.12 Screening of transformed cells containing *bet* genes by phenotypic test

2.12.1 Effect of NaCl concentration on growth of *E. coli*

To determine the NaCl concentration suitable for phenotypic test, M63 medium with 22 mM glucose was used as the minimal growth medium. A single colony of *E. coli* JM109 was inoculated in 50 ml of M63 medium and incubated at 37°C with shaking overnight. The overnight culture was centrifuged at 3500 rpm for 10 minutes. The cell was collected and diluted in M63 medium and M63 medium containing 0.4, 0.6, 0.8, 1.0 and 1.2 M NaCl to the optical density at 420 of 0.1. The culture medium were incubated at 37°C with shaking at 200 rpm. Growth was monitored by measuring the optical density at 420 nm with a spectrophotometer. The minimum NaCl concentration that suppressed growth of the cells was chosen for further testing.

2.12.2 Effect of exogenous choline and glycine betaine on growth of *E. coli*

After centrifugation of the overnight culture, cells were dilluted in M63 medium containing 0.7 M NaCl and M63 medium containing 0.7 M NaCl plus 1 mM choline or glycine betaine to the optical density at 420 of 0.1. The cell suspension was incubated at 37°C with shaking at 200 rpm. Growth was monitored by measuring the optical density at 420 nm with a spectrophotometer. With choline and glycine betaine, cells with the *bet* genotype should be able to grow normally.

2.12.3 Bet phenotypic test

After transformation of the recombinant plasmids into *E. coli* cells by electroporation, 1 ml LB was added and incubated at 37°C for 1 hour. Then 9 ml of M63 medium and 100 µg/ml ampicillin were added and incubated at 37°C overnight. Subsequently, 100 µl of cell suspension was spread on the M63 agar plate containing 0.7 M NaCl, 1 mM choline and 100 µg/ml ampicillin and incubated at 37°C for 3 days. Transformants harboring the *bet* clone should be able to grow in this medium.

2.13 Preparation of synthetic oligonucleotide probe

2.13.1 Oligonucleotide design and synthesis

The oligonucleotide probe was designed by comparing the published nucleotide sequences of *betB* gene from various bacteria; from bacteria; *E. coli* (Lamark et al., 1991), *Bacillus subtilis* (Pocard et al., 1996) *and Rhizobium meliloti* (Pocard et al., 1997), and plants ; *Sorghum bicolor* (Wood et al., 1996), *Hordeum vulgare* (Ishitani et al., 1995), *Atriplex hortensis* (Xiao et al., 1995), *Spinacia oleracea* (Weretilnyk and Hanson, 1990), *Beta vulgaris* (McCue and Hanson, 1992) and *Aramanthus hypochondriacus* (Ligaria and Ituttiaga, 1997), were compared by

using ClustralX (1.64b). The sequences were aligned by using a ClustalX(1.64b) program. The highly homologous sequence was selected. The oligonucleotide, to be used as a probe for the detection of *betB* gene from *A. halophytica*, was synthesized commercially from Bioservice Unit, Thailand.

2.13.2 5′ end-labeling with [γ-^{32}P]ATP using T_4 polynucleotide kinase

Ten pmol of the oligonucleotide was end-labeled in a 10 µl reaction volume containing 50 µCi of [γ-^{32}P]ATP, 10 units of T_4 polynucleotide kinase and 1X T_4 polynucleotide kinase buffer (70 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$ and 5 mM dithiothreitol). The reaction mixture was incubated at 37°C for 30 minutes and then stopped by incubating at 70°C for 10 minutes.

2.14 Screening of transformed cells containing *betB* gene by colony hybridization

2.14.1 Colony blotting

The transformant colonies which grew on LB plate containing 100 µg/ml ampicillin were transferred to new plates by steriled toothpick and incubated until the colonies were approximately 1 mm in diameter. A piece of Whatman filter paper #42 which was previously marked with a pencil and pre-cut to produce 3 asymmetry sites was carefully laid onto the agar surface until it was completely wet. Before lifting the filter, the orientation of the filter was marked on the plate with a marker pen. The filters were processed by placing on Whatman 3MM paper saturated with denaturing solution (0.5 N NaOH and 1.5 M NaCl) for 7 minutes. The filter paper containing lysed colonies was transferred to 3MM paper saturated with neutralizing solution (1 M Tris-HCL pH 7.6 and 1.5 M NaCl) for 3 minutes and then repeated with fresh

neutralizing solution. The neutralized paper was rinsed briefly in 2X SSC and then air-dried on a sheet of filter paper. The DNA was fixed to the paper by baking at 80°C for 2 hours. The filter paper could be used immediately or stored between sheets of Whatman 3MM paper in a sealed plastic bag at 4°C.

2.14.2 Colony hybridization

Prehybridization and hybridization were performed in Hybaid bottle to maximize user safety and minimize probe volumes. Prehybridization was carried out by incubating the membrane in a prehybridization buffer (10X Denhardt' reagent, 6X SSC and 0.5% SDS). Salmon sperm DNA was denatured by boiling for 5 minutes and then added to the prehybridization buffer to a final concentration of 50 μg/ml. The membrane was prehybridized for at least 1 hour at 37°C. After prehybridization, the prehybridization buffer was removed and the membrane was washed twice with prehybridization buffer. The purified labeled probe was added to the prehybridization buffer in the bottle. Hybridization was incubated at 30°C overnight. The hybridized filter was washed twice with washing solution (2X SSC and 0.1% SDS) for 30 minutes at 37°C. After washing, the membrane was air-dried for 15 minutes, wrapped in Saran wrap and autoradiographed at -70°C in a cassette with an intensifying screen for approximately 2 hours. If the radioactivity was high, higher temperature wash was required.

2.14.3 Screening of positive colony

Positive clones were identified by aligning the master plate on the autoradiograph. Colonies which gave strong hybridization signal were picked up from the master plate with steriled toothpick and regrew in 3 ml of LB medium

containing 100 μg/ml ampicillin at 37°C overnight. The remaining of the culture was subjected to plasmid preparation.

2.15 Alkaline extraction of plasmid DNA

A single colony of *E. coli* harboring recombinant plasmid was grown in 1.5 ml of LB broth (1.0% tryptone, 0.5% yeast extract and 1.0% NaCl) containing 100 μg/ml ampicillin, at 37°C for overnight with shaking. The cells were harvested by centrifugation at 4000 rpm for 10 minutes at 4°C and suspended in 100 μl of solutionI (50 mM glucose, 25 mM Tris-HCl pH 8.0 and 10 mM EDTA) by vigorous vortexing. After 5 minutes incubation at room temperature, the cells were lysed by the addition of 200 μl of freshly prepared solutionII (0.2 N NaOH and 1% SDS), mixed by gentle inversion and incubated on ice for 10 minutes. The cell lysate was neutralized by gently mixing with 150 μl of 3 M sodium acetate pH 4.8 followed by 10 minutes incubation on ice. The mixture was centrifuged at 8000 rpm for 10 minutes at 4°C. The clear lysate was collected, extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1), and once with chloroform/isoamyl alcohol (24:1). Subsequently, the plasmid was precipitated by adding 2 volumes of ice-cold absolute ethanol, mixed by inversion serveral times before incubating at -70°C for 10 minutes and then centrifuged for 10 minutes at 10000 rpm at 4°C. The plasmid was washed with 70% ethanol and recollected by centrifugation for 1 minute at room temperature. Finally, the air-dried pellet was dissolved in 20 μl TE buffer (10 mM Tris-HCl pH 8.0 and 1mM EDTA) and stored at 4°C.

2.16 Restriction endonuclease analysis of the recombinant plasmid

Recombinant plasmids obtained from the screening of *betB* gene by colony hybridization, were analyzed by restriction enzyme digestion. In order to isolate the

inserted DNA fragment from the vector, recombinant plasmids were digested with *Hin*dIII and *Eco*RI. The reaction was carried out in a 20 µl reaction mixture containing 500 ng plasmids, 10 units of *Hin*dIII and *Eco*RI and 1X NEBuffer 2 (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol pH 7.9). After incubation at 37°C for 2 hours, the reaction mixture was stopped by adding 4 µl of tracking dye and analyzed by agarose gel electrophoresis.

2.17 Nucleic acid transfer by Southern blotting

2.17.1 Restriction digestion of chromosomal DNA

A. halophytica chromosomal DNA was digested separately with 5 restriction endonucleases, *Bam*HI, *Eco*RI, *Hin*dIII, *Pst*I, and *Xba*I. Each reaction consisted of 4 µg chromosomal DNA, 1X restriction buffer, 10 units restriction enzyme and sterile distilled water to the final volume of 40 µl. After digestion, the DNA was size-fractionated by agarose gel electrophoresis. The DNA fragments in the agarose gel were treated to ensure efficient transfer and to generate single-stranded DNA suitable for hybridization. The gel was placed in 0.25 M HCl for 10 minutes at room temperature with gentle shaking to break the DNA into smaller fragments suitable for transfer. Then, the gel was rinsed with sterile distilled water and placed in a denaturing solution (0.5 N NaOH and 1.5 M NaCl) for at least 30 minutes and rinsed with water before placing in a neutralizing solution (0.5 M Tris-HCl pH 7.4 and 1.5 M NaCl) for 30 minutes. The DNA fragments were then transferred to nylon membrane by Southern blotting or the Hybaid Vacu-Aid vacuum blotting apparatus.

2.17.2 Southern blotting

Two sheets of Whatman 3MM™ filter paper cut to the same width and slightly wider than the gel were pre-wet in 10X SSC and placed across the raised support with the ends submerged in the buffer reservior. This serves as a wick for the flow of the buffer through the gel. The gel was inverted and placed centrally on the filter paper. A piece of nylon membrane cut to the size of the gel and marked for the orientation, was placed on the surface of the gel. The membrane was covered with two sheets of Whatman 3MM™ paper cut to the size just larger than the gel. A stack of absorbent paper towels was placed on the top of the filter paper and weighed down with a 1 kg weight. After overnight blotting, the position of the wells were marked on the hybridization membrane with a pencil. The membrane was then rinsed briefly in 2X SSC and air dried on a sheet of dry filter paper. The DNA was fixed to the hybridization membrane either by baking at 80°C for 2 hours or by UV crosslinking.

2.17.3 Vacuum blotting

A sheet of Whatman 3MM™ filter paper 0.5-1.0 cm larger than the gel was pre-wet in 10X SSC (1.5 M NaCl and 150 mM sodium citrate) before placing onto the porous screen. The nylon membrane was cut to the same size or slightly larger than the gel, pre-wet in 10X SSC and positioned on the filter paper. Then, the rubber mask was per-wet in distilled water and positioned over the membrane. The rubber mask should have a template cut such that the window was 2-5 mm smaller than the gel or enough to provide for a good seal. The gel was transferred carefully into position over the opening in the rubber mask in contact with the membrane. The cover lid was placed on the base of the unit. The locking levers were turned into position, and vacuum of 5 mm of Hg was applied. Subsequently, the transfer buffer 10X SSC was poured gently on to the surface of the gel. The meniscus formed by the gel should

contain sufficient buffer for complete transfer. The transfer was allowed to proceed for approximately one and half hours. When the transfer was completed, the pump was turned off and the remaining transfer buffer was removed. The apparatus was dismantled, the membrane was removed and placed in 2X SSC for 5 minutes at room temperature with gentle shaking. The membrane was dried at room temperature on a piece of filter paper for 15-30 minutes. Finally, the DNA was fixed to the nylon membrane by baking at 80°C for 2 hours or by UV crosslinking.

2.18 Preparation of *gbsA* probe

2.18.1 Digestion of pJB007 with *Nde*I and *Pst*I

The recombinant plasmid pJB007 was digested with 2 restriction enzymes, *Nde*I and *Pst*I. The digestion was carried out in 50 µl reaction which consisted of 5 µg of pJB007, 1X NEBuffer 4, 10 units *Nde*I, and sterile distilled water. After incubation at 37°C for 3-5 hours, 10 units *Pst*I and 1X NEBuffer 3 were added to the digested reaction and incubated at 37°C for 3-5 hours. Then, a portion of the sample (0.3 µg of pJB007) was analyzed for the completion of the digestion by agarose gel electrophoresis.

From the restriction map of pJB007 in Appendix 2, five DNA fragments, approximately 0.56, 0.92, 1.21, 2.61 and 3.04 kb, were generated by *Nde*I and *Pst*I digestion. 0.92 kb DNA fragment was the partial part of *gbsA* gene as shown in Fig. 2.1.

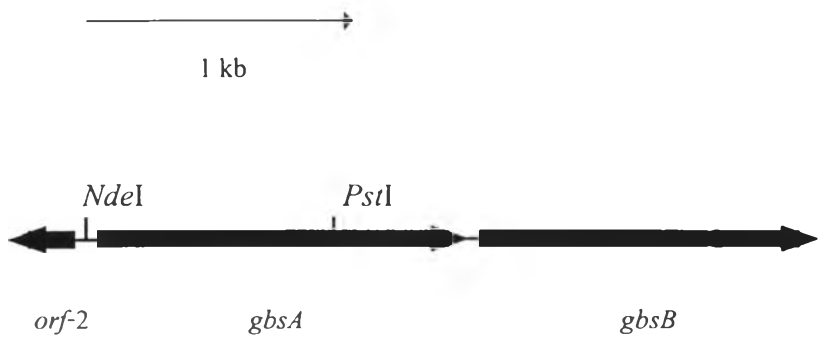

Figure 2.1 *gbsAB* containing insert in pJB007

2.18.2 DNA fragment elution

The 0.92 kb fragment was recovered from the agarose gel by using QIAquick Gel Extraction Kit. After electrophoresis, the desired DNA fragment was excised as gel slice from the 1.2% agarose gel with a clean, sharp scalpel. The gel slice was put in a microcentrifuge tube and weighed. Three volumes of Buffer QX1 was added to 1 volume of gel (100 mg ~ 100 µl) and incubated at 50°C for 10 minutes with mixing by vortexing every 2-3 minutes to help dissolve the gel. After the gel slice was dissolved completely, the color of the mixture should be yellow similar to Buffer QX1. If the color of the mixture was orange or violet, 10 µl of 3 M sodium acetate pH 5.0 was added and mixed. The color should turn to yellow. A QIAquick spin column was placed in a provided 2-ml collection tube. The sample was applied to the QIAquick column and centrifuged at 12000 rpm for 1 minute. The flow-through was discarded. The column was washed by adding 0.75 ml of Buffer PE and centrifuged for 1 minute. After the flow-through was discarded, the QIAquick column was placed into a clean 1.5-ml microfuge tube. To elute DNA, 30 µl of elution buffer was added to the center of the QIAquick column, let stand for 1 minute and then centrifuged for 1 minute.

2.18.3 Probe labeling by nick translation

The 0.92 kb *gbsA* probe was labeled by using nick translation kit (Amersham). The DNA was diluted to a concentration of 2-50 ng/µl in TE buffer. Fifty nanograms of the DNA sample was placed into a clean microcentrifuge tube. 10 µl of the nucleotide buffer solution (300 µM each of dCTP, dGTP and dTTP in Tris-HCl pH 7.8, mercaptoethanol and $MgCl_2$), 33 pmol of [α-^{32}P]dATP, 5 µl of enzyme solution (0.5 units/µl DNA polymerase I and 10 pg/µl DNase I in Tris-HCl pH 7.5, $MgCl_2$, glycerol and bovine serum albumin) and sterile distilled water were added for the final reaction volume of 50 µl. The reaction mixture was mixed gently by pipetting up and down, and the tube was capped before spinning for a few seconds to bring the content to the bottom of the tube. The reaction was incubated at 15°C for 1 hour and stopped by the addition of 5 µl of 0.2 M EDTA pH 8.0.

2.18.4 Purification of labeled probe

The labeled probe was purified with the NENSORB^{TM} 20 nucleic acid purification cartridge (DuPont). Firstly, the column was clamped to a secure support (Fig. 2.2) and rinsed with 2 ml of absolute methanol. The disposable plastic syringe was filled with air and attached to the adapter. Constant gentle pressure was applied to push methanol slowly through the cartridge. Secondly, 2 ml of reagentA (10 mM triethylamide, 1 mM EDTA and 0.1 M Tris-HCl pH 7.7) was loaded into the column and pushed through the column with gentle pressure. Then, 10 µl of the labeling reaction and 3 ml of reagentA were mixed and carefully loaded directly to the top of the column bed by using micropipette. Unbound material was eluted with water. The radioactive-labeled oligonucleotide was quantitively eluted with 1 ml of 20% n-propanol. The effluent of 200 µl (9-11 drops) fractions was collected in eppendorf tubes for using in hybridization.

2.19 Screening of *betB* gene by Southern blot hybridization

Prehybridization and hybridization were performed in Hybaid bottle to maximize user safety and minimize probe volumes. Prehybridization was carried out by incubating the membrane in prehybridization buffer (10X Denhardt' reagent, 6X SSC and 0.5% SDS). Salmon sperm DNA was denatured by boiling for 5 minutes and then added to the prehybridization buffer to a final concentration of 50 μg/ml. The membrane was prehybridized for at least 1 hour at 37°C. After prehybridization, the prehybridization buffer was removed and the membrane was washed twice with prehybridization buffer. The purified labeled probe was denatured by heating at 95-100°C for 5 minutes then chilled on ice and added to the prehybridization buffer in the bottle. Hybridization was incubated at 30°C overnight. The hybridized filter was washed twice with washing solution (6X SSC and 0.5% SDS) for 30 minutes at 45°C. After washing, the membrane was air-dried for 15 minutes, wrapped in Saran wrap and autoradiographed at -70°C in a cassette with an intensifying screen for approximately 2 hours. If the radioactivity was high, higher temperature wash was required.