CHAPTER II

MATERIALS AND METHODS

A. MATERIALS

1. Bacterials Strains

The nitrogen-fixing bacterial strains of *Azospirillum spp.* and *Azotobacter spp.* used in this study were obtained from Mr. Settha Siripin and Dr. Nantakorn Boonkerd, Department of Agriculture, Ministry of Agriculture and Co-operative. All of these bacterial strains were isolated from sugar cane or corn roots or from various sources of Thai soil as summarized in Table 2.

Five strains of *Escherichia coli* were used, firstly *E.coli* K_{12}JC 5466 (Spc^R, *trp*$^-$, *his*$^-$, *rec* A_{46}) which harbours the plasmid pRD1 (Km^R, $Carb^R$, Tc^R, *rfb*$^+$, *gnd*$^+$, *his*$^+$, *nif*$^+$, *xhi* A^+) (Dixon *et al.*, 1976), secondly *E. coli* HB_{101} (leu, thi, lac Y, hsd R, end A, rec A, rps L_{20} (str^R), pro, gal K_2, xyl-5, mtl-1, sup E_{44}, ara-14, lam^-) which harbours the plasmid pSA30 (Tc^R, *nif* HDKY) (Cannon *et al*, 1979), thirdly *E.coli* HB_{101} (F^-, had S_{20}(r_B^-, m_B^-), rec A_{13}, ara - 14, pro A_2, lac Y_1, gal K_2, rps L_{20}(Sm^r), xyl - 5, mtl - 1, sup E_{44}, λ^-) which harbours the plasmid pBR322 (Ap^R, Tc^R) (Bolivar *et al*, 1977), fourly, *E. coli* 5K (res K^-, mod K^+, thr, leu B_1, ton A, sup E, λ^5) which harbours the plasmid pCK3 (Km^R, Tc^R, *nif* A) (Kennedy and Drummond, 198) and fively, *E. coli* C_{600} (F^-, thi-1, leu B_6, lac Y^+, ton A_{21}, sup E_{44}, λ^-) which does not harbours any plasmid. *Klebsiella pneumoniae* M_5a_1 (*nif*$^+$) were also used in this study.

Table 2. Strains used for localization and characterization

Bacterial strains	Source of Soil	references
Azotobacter vinelandii KT1	Tamoung, Kanchanaburi	Siripin, 1986
Azotobacter vinelandii KT2	Tamoung, Kanchanaburi	Siripin, 1986
Azotobacter chroococcum KT	Tamoung, Kanchanaburi	Siripin, 1986
Azotobacter chroococcum NP	Pakchong,Nakornratchasima	Boonkerd and this thesis
Azotobacter paspali B	Bangkok	Boonkerd
Azospirillum lipoferum SpMRAI	Bangkok	Boonkerd
Azospirillum brasilense A2	Bangkok	Boonkerd
Azospirillum spp. A12	Bangkok	Boonkerd
Azospirillum brasilense Sp7	Rio de Janeiro, Brazil	Tarrand, 1978

2. Chemicals

All chemicals were obtained commercially and were of reagent grade or better.

Restriction endonucleases : EcoRI, BamHI, Bgl II, Hind III and Sma I were purchased from Bethesda Research Laboratories, Inc.(BRL). Restriction endonucleases : Pst I, Sal I, Xho I and Hae III; DNA polymerase I and the radioactive α^{32} P-dATP were obtained from Amersham International Limited.

Deoxyadenosine 5-triphosphate, deoxycytidine 5-triphosphate, deoxyguanosine 5-triphosphate, deoxythymidine 5-triphosphate, dithiotreitol, polyvinylpyrrolidone (MW.40,000), bovine serum albumin (RIA grade), sodium dodecyl sulfate, calf thymus DNA, agarose (type-II), low-melting agarose (type-VII), lysozyme, deoxyribonuclease I, pronase, ribonuclease A, phenol and tris-(hydroxymethyl)-aminomethane were products of Sigma Chemical Company. Sephadex G-50 (fine) and Ficol-400 were obtained from Pharmacia Fine Chemicals. DEAE-Cellulose paper, Whatman 3 MM paper and GF/A paper were obtained from Whatman. Nitrocellulose paper was the product of Biorad Laboratories.

3. Bacterial media

3.1 Luria-Bertani medium (LB) (Maniatis et al, 1982)

tryptone	10 gm per 1.
Yeast extract	5 gm per 1.
Sodium Chloride	10 gm per 1.

The medium was adjusted to pH 7.0 with 1N NaOH, autoclaved at 121 °C for 15 min. In order to make a solid medium 15 gm of bacto agar was added into 1 litre of liquid medium.

3.2 Burk's medium

Glucose	5 gm per 1.
$MgSO_4.7H_2O$	0.2 gm per 1.
$FeSO_4$	5 gm per 1.
$CaCl_2.2H_2O$	8.53 gm per 1.
$Na_2MoO_4.2H_2O$	0.24 gm per 1.
K_2HPO_4	0.4 gm per 1.
KH_2PO_4	0.2 gm per 1.

The medium was adjusted to pH7.1 with 1N NaOH. In order to make a solid medium, 15 gm. of bacto agar was added into 1 litre of liquid medium.

3.3 Rich medium (RM ; modified Burk's medium) (Robson et al, 1984).

Glucose	5 gm per l.
$MgSO_4.7H_2O$	0.2 gm per l.
$FeSO_4$	5 gm per l.
$CaCl_2.2H_2O$	8.53 gm per l.
$Na_2MoO_4.2H_2O$	0.24 gm per l.
K_2HPO_4	0.4 gm per l.
KH_2PO_4	0.2 gm per l.
Nutrient broth	2 gm per l.
Yeast extract	0.1 gm per l.

The medium was adjusted to pH7.1 with 1N NaOH. In order to make a solid medium, 15 gm of bacto agar was added into 1 litre of liquid medium.

3.4 NF semi-solid medium

K_2HPO_4	6.0 gm per l.
KH_2PO_4	4.0 gm per l.
$MgSO_4.7H_2O$	0.2 gm per l.
NaCl	0.1 gm per l.
$CaCl_2$	0.02 gm per l.
$FeCl_3$	0.01 gm per l.
$NaMoO_4.2H_2O$	0.002 gm per l.
DL-Malic acid	5.0 gm per l.
Yeast extract	0.02 gm per l.

The medium was adjusted to pH 6.8 with 4N.NaOH, then 0.5 gm of Bacto agar was added into 1 litre of medium.

4. Instruments

Autocal pH meter, model PHM 83 (Radiometer Ltd., Denmark).

Beckman refrigerated centrifuge, model J-21 C (Beckman Instruments Inc.,California, U.S.A.).

Gilson pipetman (Rainin Instrument Company, Inc., France).

High-speed microcentrifuge, model MC-15 A (Tomy Seiko Co.,Ltd., Japan).

Mupid, mini-gel electrophoresis system (Maruzen oil Biochemical Co.,Ltd., Japan).

Packard PRIAS Tris-Carb Liquid Scintillation Spectrometer, model PL (Packard Instrument Company, Inc., U.S.A.).

Power supply, model EPS 3200 (Qualitech Instruments Co.,Ltd., Thailand).

Spectronic 20 (Bauch and Lomb, Inc., Ltd., U.S.A.).

Spectronic 2000 (Bauch and Lomb, Inc., Ltd., U.S.A.).

Thermolyne dri-bath, model DB 17610-26 (.Thermolyne Corporation, U.S.A.).

Top bench centrifuge, Minor 35 (MSE, Ltd., England).

Top bench centrifuge, Super minor (MSE, Ltd., England).

Top bench centrifuge, Kokusan H-18 (KOKUSAN ENSINDI. Ltd.,Japan).

UV-transilluminator, model TS-20 (UVP, Inc., U.S.A.).

Vocuum oven, type VT 5042 EX (Heraeus)

Water bath, model 0128 T (Scientific Instrument Development and Service Center, Facalty of Science C.U., Thailand).

B. METHODS

1. Growth condition of bacterial strains.

All the Azospirillum spp. were grown in Luria-Bertani (LB) medium with shaking at 30°C.

Azotobacter chroococcum KT, Azotobacter chroococcum NP and Azotobacter vinelandii KT2 were grown in RM medium with shaking at 30°C whereas Azotobacter vinelandii KT1 and Azotobacter paspali B were grown in LB medium with shaking at 30°C.

All E.coli strains were grown in LB medium with shaking at 37°C.

For maintenance of bacterial strains, 0.5 ml of bacterial culture was mixed with 0.5 ml of steriled glycerol. The bacterial suspension in 50 % glycerol was kept at -20°C for years.

2. Determination of plasmid DNA from Azospirillum spp. and Azotobacter spp.

Three available methods of plasmid extraction were utilized to examine a number of plasmid content and to search for the better extracter procedure. These are modified Eckhardt lysate electrophoresis (Eckhardt, 1978 and Nirunsuksiri, 1984), large plasmid isolation (Hansen and Olsen, 1978) and rapid alkaline extraction (Bindoim and Doly, 1979).

2.1 Modified Eckhardt lysate electrophoresis (Eckhardt, 1978 and Nirunsuksiri, 1984).

Bacterial strains were grown in 50 ml flask containing 10 ml broth medium for overnight at room temperature with vigorous shaking. The cell were harvested by centrifugation of 1 ml culture in a 1.5 ml microfuge tube in microcentrifuge at 7,000 rpm for 10 min. Cells were washed once with PEM (5 mM K_2HPO_4, 0.1 mM EDTA, 0.5 mM $MgCl_2$, pH 8.0).

The cell pettet was kept at -20 °C for 15 min, then thawed at room temperature in order to weaken the cell walls and immediately suspended in 25 µl of solution A (10 % sucrose, 25 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.2 µg/ml RNase, 5 mg/ml lysozyme). After this lysozyme treatment, spheroplasts would be generated.

A 0.5-0.7 % agarose gel plate was prepared to contain 2 slots for each array, one was behind the other. Twenty-five microlitres of spheroplast suspension were pipetted into the front slot, then 50 µl of solution B (5 % sucrose, 4 % SDS) was pipetted into the concomitant back slot and electrophoresis buffer (TB : 89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.3) was poured into the chamber until the gel was slightly submerged underneath the buffer level. Electrophoresis was begun immediately. The voltage was constant at 15 volt for 1-2 hrs, followed by a change to 70-80 volt and electrophoreased for 3-5 hrs.

2.2 Large plasmids isolation (Hansen and Olsen, 1978).

The bacterial culture was grown to log phase in 250 ml flask containing 40 ml broth medium at 30 °C with vigorous shaking. After the cells were harvested by centrifugation at 8,000 rpm, 4 °C for 20 min in Beckman (J-21 C) centrifuge using JA-10 rotor, these washed once with PEM (5 mM K_2HPO_4, 0.1 mM EDTA, 0.5 mM $MgCl_2$, pH 8.0). The washed cells were resuspended by mixing in Vortex mixer at maximum speed in 1.35 ml of 25 % sucrose and 0.05 M Tris-HCl, pH 8.0 at ambient temperature. All mixing steps from this point through polyethylene glycol 6000(PEG 6000) addition were done by gentle inversion of the centrifuge tube at the frequency lower than 20 times per min. First, 0.1 ml of 10 mg/ml lysozyme in 0.25 M Tris-HCl, pH 8.0 was added and mixed by four inversions, and the tubes were put in an ice-water bath

for 5 min. Then, 0.5 ml of 0.25 M EDTA, pH 8.0 was added and mixed by five inversions, and chilled for 5 min more in ice-bath. After the addition of 0.5 ml of sodium dodecyl sulfate (20 % W/V in TE), eight cycles of a heat pulse and mixing were performed (one cycle was 15 sec in a 55 °C water bath, and then five inversions during 15 sec after being removed from the water-bath), a clear viscous solution of lysed cells was obtained. At ambient temperature, 0.5 ml of 3 N NaOH (freshly prepared) was added, immediately followed by 3 min of inversion (at 20 inversions per min). Then 1.0 ml of 2 M Tris-HCl, pH 7.0 was added, immediately followed by 1 min of inversion. After the addition of 0.65 ml of SDS (20 % W/V in TE), 1.25 ml of 5 M NaCl was added immediately followed by 20 inversions. Then an appearance of white floc-like material should be obtained. The tubes were chilled in an ice-water bath and refrigerated (4 °C) for at least 6 hrs. Centrifugation was performed at 15,000 rpm, 4 °C for 30 min in Beckman (J-21 C) centrifuge using JA-20 rotor,then the large white pellet of salt-precipitated chromosome-membrane complexes was discarded. The volume of the supernatant was measured (usually about .48 ml for 40 ml of culture input) and decanted into the chilled tubes in ice-water bath, then 0.313 volume of PEG 6000 (42 % W/V in 0.01 M sodium phosphate buffer, pH 7.0) was added to make 10 % final concentration. A slightly cloud solution would be obtained after mixing. After regrigerated (4 °C) for at least 6 hrs the pallet of crude plasmid(s) was collected by a centrifugation at 2,500 rpm, 4 °C for 10 min in Top bench centrifuge. The pallet was resuspended in 0.15 ml of cold solution of 0.05 M Tris-HCl, pH 8.0, 0.05 M NaCl and 5 mM EDTA, and kept in an ice-water bath. Samples of 30 µl of crude plasmid(s) preparation were mixed with 10 µl of tracking dye solution (0.07 % bromophenol blue, 0.7 % SDS, 33 %

glycerol) and subjected to 0.7 % agarose gel electrophoresis in TB buffer (89 mM Tris-HCl, 89 mM boric acid, 2 mM EDTA, pH 8.3).

2.3 Rapid alkaline extraction (Binboim and Doly, 1979)

Bacterial strains were grown overnight in 50 ml flask containing 10 ml broth medium at 30 °C with vigorous shaking. The cell pellet were harvested by centrifugation at maximum speed for 15 min in Top bench centrifuge. Cells were washed once in PEM (5 mM K_2HPO_4, 0.1 mM EDTA, 0.5 mM $MgCl_2$, pH 8.0) and then throughly resuspended in 1 ml of solution containing 5 mg/ml lysozyme in 5 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0. The mixture was left at 0 °C for 30 min, follow by the addition of 2 ml of freshly prepared 0.1 % SDS in 0.2 N NaOH. The lysate was neutralized by adding 1.5 ml of 3 M sodium acetate, pH 4.8. The solution mixture was mixed by gently inverting the tube for several times. The mixture was further left on ice for 30 min before centrifugation at maximum speed for 30 min in Top bench centrifuge. The plasmid DNA was recovered from the supernatant by adding 2 volumes of absolute ethanol and kept at -20 °C for overnight.

3. Isolation and purification of chromosomal DNA from Azospirillum spp. and Azotobacter spp. (modified from Rodriquez, 1983).

The bacterial culture was grown to log phase in 500 ml flask containing 100 ml medium with vigorous shaking. After the cells were harvested by centrifugation at 8,000 rpm, 4 °C for 20 min in Beckman (J-21 C) centrifuge using JA-10 rotor then washed once with PEM (5 mM K_2HPO_4, 0.1 mM EDTA, 0.5 mM $MgCl_2$, pH 8.0). The cell pellet was frozen at -70 °C for 10 min and then thawed in warm water. The cells were resuspened in 2 ml of SET buffer (20 % sucrose, 50 mM Tris-HCl, pH 7.6, 50 mM EDTA) and put on ice. Cell lysis was performed by adding 0.2 ml

of 5 mg/ml lysozyme plus 0.1 ml of 10 mg/ml RNase (previously preheated to inhibit DNase activities at 80°C for 10 min). The mixture was incubated on ice for 15 min. The cell suspension was poured into a 25 ml screw-cap tube and 50 µl of 25 % SDS was added. The mixture was incubated at 37°C with gentle shaking for 3 to 6 hrs. After the addition of 300 µl of 2 mg/ml Pronase and 1.5 ml of 3 % isoamyl alcohol in chloroform further incubation at 37°C with gentle shaking was performed for overnight. One mililitre of sterile distilled water and 2 volumes (10 ml) of 3 % isoamyl alcohol in chloroform were added into the mixture. The cap was tightened and the tube was inverted by gently for 5 min. The mixture was poured into a centrifuge tube, after contrifugation at 5,000 rpm for 30 min in MSE superminor centrifuge to separate the phases. The aqueous (upper) phase was removed in a steriled 25 ml screw-cap tub a wide-mouth pasture pipette. The chloroform / isoamly alcohol extraction was repeated twice. The chromosomal DNA was precipitated from the solution by adding 200 µl of 5 M NaCl and 2 volumes of ice-cold absolute ethanol. The content was mixed gently but thoroughly and placed at -20°C for 10 min. Fibrous strands of precipitated DNA was spooled out with a glass rod and dissolved in 3 ml of TEN (10 mM Tris-HCl, pH 7.6, 1 mM EDTA and 10 mM NaCl). The further purification was performed by the addition of 0.5 volume of 7.5 M ammonium acetate, pH 7.5 and 2 volume of ice-cold absolute ethanol. After gently mixed and incubated at -20°C for at least 5 min, then the pure DNA was recovered by centrifugation. The DNA pellet was redissolved in 3 ml of TEN again and stored at 4°C.

4. Chromosomal DNA digestion

The class of enzymes known as restriction endonucleases are isolated chiefly from microorganisms. They cleave both strands of DNA at the sites internal to the molecule after recognizing specific nucleotide sequences on the molecule. Because a restriction enzyme cleavage pattern is specific for a given DNA and enzyme, the restriction fragments, when isolated on a preparative scale, represent a homogeneous population of DNA molecules. Some restriction enzymes and their recognition sequences are shown in table 3.

Table 3. Examples of type II restriction endonucleases.

Enzyme	Isolated from	Recognition sequence
BamHI	Bacillus amyloliquefaciens H	G▼G A T C C
EcoRI	Escherichia coli RY13	G▼A A T T C
Hind III	Haemophilus influenzae Rd	A▼A G C T T
Pst I	Providencia stuartii	C T C G A▼G
Sma I	Serratia marcescens	C C C▼G G G

Purified chromosomal DNA of Azotobacter and Azospirillum were digested by various restriction endonucleases. The digestion was performed in 100 µl reaction mixture containing 4 µg of purified chromosomal DNA, 30-80 units of restriction endonuclease and appropiate buffer. The reaction mixture was incubated at 37°C for 2 hrs. After phenolization and ethanol precipitation (see section 13.3 and 13.2), the precipitated DNA fragments were recovered by centrifugation. The DNA pellet was dissolved in 30 µl of TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA). The pattern of DNA fragments obtained from digestion was was analyzed by agarose gel electrophoresis.

Three types of appropiate buffer for restriction endonuclease

digestion were used, low salt buffer (10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$ and 1 mM DTT), medium salt buffer (10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM DTT) and high salt buffer (10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$ and 1 mM DTT). Low salt buffer was used for the digestion of restriction endonuclease; Bgl II. Medium salt buffer was used for the digestion of restriction endonuclease; Bam HI, Hind III and Pst I whereas high salt buffer was used for the digestion of restriction endonuclease; EcoRI and Sal I. Because the enzyme Sma I will not work well in any of the above buffers, a separate buffer was be made up, which was 20 mM KCl, 10 mM Tris-HCl (pH 8.0), 10 mM $MgCl_2$, 1 mM DTT.

5. Large scale preparation of pSA30 (Birboim and Doly, 1979).

E. coli HB101 harbouring plasmid pSA30 was grown in 250 ml LB-broth containing 25 µg/ml tetracyclin in 1 litre flask at 37 °C with vigorous shaking. The log phase cell culture (OD600 = 0.6) was harvested by centrifugation at 8,000 rpm, 4 °C for 20 min in Beckman (J-21 C) centrifuge using JA-10 rotor and then thoroughly suspended in 5 ml of solution containing 5 mg/ml lysozyme in 5 mM glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0. The mixture was left on ice for 30 min, followed by the addition of 10 ml of freshly prepared 0.1 % SDS in 0.2 N NaOH. The lysate was subsequently neutralized by adding 7.5 ml of 3 M sodium acetate, pH 4.8. The solution mixture was mixed by gently inverting the tube several times. The mixture was further left on ice for 1 hr for complete chromosomal DNA precipitation before centrifigation at 17,000 rpm, 4 °C for 30 min in Beckman (J-21 C) centrifuge using JA-20 rotor. The plasmid DNA was recovered from the supernatant by adding 2 volumes of absolute ethanol and kept at -20 °C for overnight. After the DNA pellet was obtained by centrifugation at 12,000 rpm for 30 min

in JA-20 rotor in Beckman (J-21 C) centrifuge, the DNA pellet was redissolved in TE buffer (10 mM Tris-HCl, pH 8.0 and 1 mM EDTA) and stored at 4 °C.

6. Purification of supercoiled form of plasmic (Dretzen et al, 1981 and Nirunsuksiri, 1984).

The crude plasmid obtained from rapid alkaline extraction was always contaminated with residue of small moledular weight RNA and sometimes with chromosomal DNA, further purification of the plasmid was performed by recover only the supercoiled plasmid from agarose gel. The process can be divided into three steps as follows.

a). Preparation of DEAE-cellulose paper.

Strips of DEAE-cellulose paper were cut to appropriate size according to the cross-sectional area of the agarose gel. The strips were soaked in 2.5 M NaCl for several hours and then washed many times in sterile distilled water and stored in 1 mM EDTA at 4 °C until used.

b). Agarose gel electrophoresis and transfer of DNA to DEAE-cellulose paper.

Plasmid DNA was separated on 0.7 % agarose gel electroploresis in nuclease-free TB buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) for 2 hrs. The buffer were poured out and the gel slot containing DNA markers was carefully cut from the whole gel plate and stained in 2.5 μg/ml ethidium bromide for 30 min. The DNA band to be purified were located by UV light and needles were inserted into the gel to mark the DNA band positions. This gel was returned to the gel plate, two thin lines above and below the DNA band to be recovered

in a sandwich pattern was introduced in the gel by a steriled surgical blade. Pieces of DEAE-cellulose paper, prepared as described in (a), were carefully inserted into these thin slots. The paper above the band protected against any contamination by higher molecular weight DNA, whereas the paper below the band was used to trap the DNA. The gel was squeezed firmly against the paper to eliminate air-bubbles, then the gel was sealed with melted agarose and electrophoresis buffer was poured into the chamber until the gel was slightly submerged underneath the buffer level. Electrophoresis was continued until the DNA had entered the DEAE-cellulose paper strip (about 1 hr.). This was verified by observation under UV illuminator.

c). Elution of DNA from DEAE-cellulose paper.

The DEAE-cellulose paper was removed from the gel and washed with 10 ml of cold sterile distilled water, then drained and blotted dry with Whatman 3 MM paper. The DEAE-cellulose paper was chopped into small pieces before placing in a siliconized glass tube. Elution buffer containing 20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 50 mM arginine (free base) and 1.5 M NaCl was added at a volume of 500 μl per 50 mm^2 of the DEAE-cellulose paper. The tube was vortexed and incubated at 37 $^{\circ}$C for 2 hrs with occasional agitation. Then the mixture was transfered to a 1.5 ml microfuge tube containing a plug of siliconized glass wool which served to trap the paper filter and a hole was made at the bottom of the plastic microfuge tube. This tube was then placed in the mounth of a glass tube (13x100 mm) such that the lip of the microfuge tube was rested securely on the lip of the glass tube. After centrifugation for 1 min at maximum speed (MSE centrifugation), the eluate was extracted with 3 volumes of n-butanol and the DNA was precipitated with 2 volumes of absolute ethanol, at

-20 °C overnight, the DNA pellet was centrifuged at 14,000 rpm for 15 min, and dissolved in appropriate volume of 10 mM Tris-HCl, pH 8.0 and 1 mM EDTA AND then stored at 4 °C.

7. Characterization of pSA30

To assure that the plasmid used in this study is pSA30, the plasmid was characterized by the size of DNA fragments after restriction endonuclease (Bam HI, EcoRI, Hind III and Sal I) digestion.

The experiment was performed by digesting 1 μg of the suspected plasmid DNA with 2-3 units of BamHI or EcoRI or Hind III or Sal I in 20 μl reaction mixture containing the appropriate buffer. The reaction was carried out at 37 °C for 2 hr. The size of DNA fragments obtained from the digestion were determined by electrophoretic mobilily on 0.7 % agarose gel electrophoresis in TB buffer (89 mM Tris, 89 mM boric acid and 2.5 mM EDTA, pH 8.3) using λ DNA fragments generated by cutting λ DNA with Hind III as a standard.

8. Preparation of some DNA fragment from pSA30.

To assure that the region of homology between the pSA30 and DNA from nitrogen-fixing bacteria in this study was nif structural gene inserted pSA30 plasmid. So, hybridization should be performed by vector DNA; pACYC184 as a probe. The preparation of pACYC184 fragment from pSA30 plasmid could be performed as followed.

The pSA30 was prepared as described in section 5, then 1 μg of pSA30 was digested with 2 units of EcoRI in 20 μl reaction mixture containing 10 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM $MgCl_2$ and 1 mM DTT. The reaction was incubated at 37 °C for 2 h. After EcoRI digestion of pSA30, pACYC184 fragment (4.0 kb) and nif structural gene

fragment (6.9 kb) were obtained. These DNA fragments were separated on low-melting temperature agarose gel electrophoresis and then the pACYC184 fragment was recovered from the gel as described in section 9.2.

9. Preparation of *nif* DNA fragments as a probe.

The region of homology between the *Klebsiella pneumoniae nif* structural gene and DNA from *Azotobacter* and *Azospirillum* was localized more precisely by hybridized DNA from these with purified ^{32}P-labelled restriction fragments A1, A2 and A3 derived from *nif* strutural gene inserted pSA30 plasmid (figure 3).

According to the restriction map of pSA30 (see figure 3), fragment A1 (5.76-kb) which carry part of *nif* K, fragment A2 (1.44 kb) which carry part of *nif* D and fragment A3 (3.7-kb) which carry *nif* HD would be obtained after digested the pSA30 with BamHI and Hind III. This DNA fragments were separated on low-melting temperature agarose gel electrophoresis. The process for preparation of *nif* fragments could be divided into three steps as followed.

9.1 Digestion of pSA30 with BamHI and HindIII

The pSA30 was prepared as described in section 5, then 7 μg of pSA30 was digested with 10 units of Bam HI and 10 units of HindIII in 40 μl reaction mixture containing 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM $MgCl_2$ and 1 mM DTT. The reaction was incubated at 37°C for 2 h. Then the DNA fragments were separated on low-melting temperature agarose gel electrophoresis.

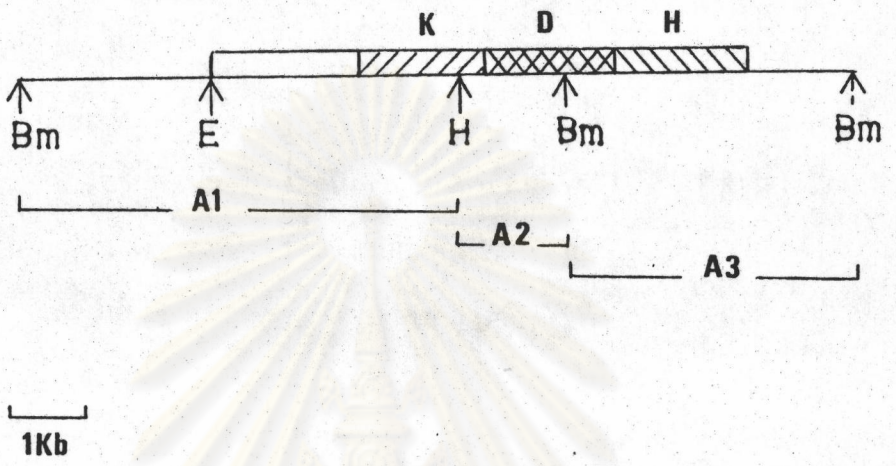

Figure 3. Restriction map of pSA30 and nif structural gene fragments.

The sketch showns the position of nif structural gene fragments obtained from restriction enzyme digestion of pSA30.

Endonuclease clevage sites are indicated below each map by : E, EcoRI ; H, Hind III and Bm, Bam HI.

9.2 Recovery of DNA from low-melting temperature agarose gel electrophoresis(modified from Maniatis et al, 1982).

The 0.7 % low-melting-temperature agarose (type VII, sigma) prepared in electrophoresis buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3) was dissolved by heating to 70°C. This solution was poured into a hole of conventional agarose gel plate (type II, sigma), as a supporter, until the level was equal to the supporter gel. After the low-melting-temperature agarose gel was completely set (4 h at room temperature) the comb was carefully remove then the DNA sample was loaded and electrophoresis was carried out at room temperature as described in section 13.1. After voltage was supplied at 50 volt for 5 h, the gel slot containing DNA markers was carefully cut from the whole gel plate and stained in 2.5 µg/ml ethidium bromide for 30 min. The DNA band to be further purified were located by UV light and needle were inserted into the gel to mark the DNA band positions. This gel was returned to the gel plate. The desired segments of the gel were cut out and about 5 volumes of 20 mM Tris-Hcl (pH 8.0) and 1 mM EDTA were added before heat for 20 min at 65°C to melt the gel. At room temperature, the melted gel was extracted with an equal volume of phenol (equilibrated with 0.1 M Tris-HCl, pH 8.0 and containing 0.1 % hydroxyquinoline and 0.2 % betamercaptoethanol), the aqueous phase was recovered by centrifugation and reextracted with 3 % isoamyl alcohol in chloroform before extracted with water-saturated ether. After ethanol precipitation in the present of 0.2 M NaCl, the precipitated DNA was dissolved in appropriate volume of 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

10. Labeling of DNA probes by "nick-translation " (Rigby, 1979 and Maniatis et al, 1982)

In vitro labelling of purified plasmid pSA30 or nif DNA fragments by nick translation was performed by using the modified procedure described by Rigby (1977). In principle, 3′-hydroxyl terminus is introduced into the DNA deplex by DNase I. Nucleotide on the 5′-phosphate side of the nick is removed by the 5′-exonuclease activity of E. coli DNA polymerase. New deoxy nucleotide triphosphates including (α- ^{32}p) -dATP will be inserted by the polymerase activity of the DNA polymerase to replace the one removed by exonuclease.

The reaction mixture (25 µl) contained 200 ng of DNA, 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 50 µg/ml nuclease-free BSA fraction V, 1 mM DTT, dCTP and dTTP at a final concentration of 10 µM of each nonradioactive dNTPs, 50 µCi of (α- ^{32}p) dATP, 1 pg of DNase I and 3 Unit of E. coli polymerase I.

The reaction was carried out at 16°C until maximum incorporation of the radioactive into the DNA was obtained. The progress of the reaction was followed by determining the accumulation of acid insoluble radioactivity with time. Measuring of the incorporation of (α- ^{32}p) -dATP into acid-insoluable product was done by sampling 1 µl aliquot of the mixture and spotted on the GF/A paper previously soaked in 10 % TCA. The filter was washed with 20 ml of 5 % TCA and 95 % ethanol respectively; then dried and placed into a scintillation vial containing 5 ml of distilled water and counted for the incorporation of ^{32}p into the DNA. To measure the total counts of radioactive added into the incubation mixture, another 1 µl aliquot was spotted onto the untreated GF/A paper and counted as described above.

After the maximum incorporation of ^{32}P-dATP into the DNA was obtained, the reaction was stopped by addition of an equal volume of 0.25 M EDTA and heat at 70°C for 10 min. The labeled DNA was separated from labeled deoxynucleotides by passing through Sephadex G-50 (fine) in a 1 ml steriled disposable plastic syring which was packed by centrifugation at 2,500 rpm for 2 min in a top bench centrifuge (Kokusan), then the column was transfered (disposable plastic syring) to a glass tube (15x100 mm) containing a microfuge tube with out the lip in the bottom. After the reaction mixture was applied to the column and was recentrifuge at 2,500 rpm for 2 min in top bench centrifuge (Kokusan).

To assure that the labelled DNA obtained from nick-translation is a native form of pSA30, the agarose gel electrophoresis pattern was determine. About 10,000 cpm of ^{32}P-pSA30 and 200 ng of crude pSA30 were subjected to 1 % agarose gel electrophoresis in Tris-borate system. The gel plate containing standard DNA marker and crude pSA30 was stained with ethidium bromide whereas the slot containing ^{32}P-pSA30 was blotted dry and then sealed in plastic bag and autoradiographed (as described in section 13.4) for 2 hr. The ethidium bromide staining pattern was compared to that of autoradiographic pattern.

11. Southern blot hybridization

In principle, Southern blot was prepared by fixing DNA fragments that have been electrophoretically separated on agarose gel onto nitrocellulose filter, the DNA are immobilized in the filter in exact replicas of their gel separations. The nitrocellulose filter was then prehybridized and hybridized with the labelled pSA30 probe in a sealed pastic bag. After hybridization process, the nitrocellulose filter was washed to remove the non-hybridized labelled probe. The hybrid formed was determined by Autoradiographic method.

11.1 Southern blotting

The modified method of Southern (1975) was used in this study. The restriction fragments from various species of Azotobacter and Azopirillum, which had been fractionated in agarose gels, were transferred onto nitrocellulose filter. This process can be divided into two steps :-

a). Treatment of the gel

After electrophoresis, the 0.7 - 0.8 % agarose gel (9x10x0.5 cm) was stained with ethidium bromide (2.5 µg/ml) and photographed. The gel was gently shaken in a plastic box containing 200 ml of 0.25 M HCl at room temperature for 10 min. This was performed twice, and the gel was then rinsed with sterile water immediately. The gel was transferred to a box containing 200 ml solution of 0.5 M NaOH and 1.5 M NaCl, and shaken for 10 min. The solution was poured off, a fresh solution was added and further incubated for 10 min. The gel was neutralized with 1 M Tris-MCl, pH 8 in 1.5 M NaCl for 5 min, then treated with fresh solution for 15 min.

b). Blot transfer step

After treatment, the agarose gel would be carefully laid on top of a stack of Whatman paper (3 MM; cut to exactly the same size as the gel and 1 cm high) which partially immersed in the 20xSSC (3.0 M NaCl and 0.3 M Tri-sodium citrate, pH 7.0). A piece of nitrocellulose filter (cut about 1 mm larger than the gel on all size and previously soaked in water and equilibrated in 20xSSC for 30 min before used) was carefully laid on top of the gel, all air-bubbles trapped between the nitrocellulose filter and the gel were carefully removed. At the top of this was a stack of Whatman 3 MM paper (1 cm

high) and a stack of paper towels (7 cm high). A polyethylene sheet (Saran wrap) was used to cover the blotting set to prevent evaporation of the solution. A plastic box (12x15 cm) containing 200 ml water was placed on top of the stack, so that a flow of 20xSSC (3 M NaCl and 0.3 M Tri-sodium citrate, pH 7.0) from the reservoir through the gel and the nitrocellulose filter can be proceeded. The transfer of DNA fragments was allowed to proceed for 12-14 hrs.

After the blot was complete, the nitrocellulose was soaked in 1xSSC (0.15 M NaCl and 15 mM tri-sodium citrate pH 7.0) for 5 min. and then blotted dry between a sandwich of Whatman paper (3 MM). The DNA fragments were fixed on the nitrocellulose filter by baking at 80°C for 2 hrs in vacuum oven. The filter was kept in sealed-plastic bag at 4°C until used. The dried gel was transferred from the blotting set to a box containing 2.5 µg/ml ethidium bromide to check whether the transfer was completed or not.

11.2 Hybridization (Maniatis et al, 1982 and Tirawanchai, 1983)

11.2.1 Pre-hybridization

The bloted nitrocellulose filter was placed in a plastic bag containing 4 ml of pre-hybridization solution, made up of 5xSSC (0.75 M NaCl and 75 mM Tri-sodium citrate, pH 7.0), 5X Denhardt's solution (0.1 % Ficoll, 0.1 % polyvinylpyrrolidone, 0.1 % BSA fraction V), 5 mM EDTA, 20 mM Tris-HCl, pH 8.0, 50 % formamide and 100 µg/ml denatured calf thymus DNA (denatured by heating at 100°C in thermolyne dri-bath for 10 min, followed by rapid cooling on ice). Air bubbles in the bag were removed as much as possible. The bag was sealed and incubated at 37°C for 16-18 hrs without shaking.

11.2.2 Hybridization

One edge of the bag was cut off and the prehybridization solution was squeezed out. Four mililitres of hybridization solution containing 5xSSC (0.75 M NaCl and 75 mM tri-sodium citrate, pH 7.0), 5x Denhardt's solution (0.1 % Ficoll, 0.1 % polyvinylpyrrolidone, 0.1 % BSA fraction V), 5 mM Tris-HCl, pH 8.0, 50 % formamide, 100 µg/ml denatured calf thymus DNA and 500 µl of denatured radioactive probe (specific activity $6\text{-}7\text{x}10^6$ cpm/µg) ; were denatured by heating at 100°C for 10 min then rapid cooling on ice,and then were added to nitrocellulose filter. Air bubbles were removed and radioactive probe were spreaded in the bag. The bag was sealed and incubated at 37°C for 48 hrs without shaking.

11.3 Washing off the non-hybridized probe from the nitrocellulose filter

The hybridization solution containing the radioactive probe was removed into a siliconized glass tube (this solution could be reused, by stored at 4°C and heat denature before used). The nitrocellulose filter was removed from the bag into a plastic box and subsequently nonstringently washed with two changes of 3xSSC (0.45 M NaCl and mM tri-sodium citrate, pH 7.0) for 30 min interval at room temperature with shaking. The filter was then washed with two changes of a nonstringent washing solution containing 3xSSC (0.45 M NaCl and 45 mM tri-sodium citrate, pH 7.0), 1 mM EDTA and 0.1 % SDS for 30 min interval at 50°C with shaking. And then the nitrocellulose filter was washed with two changes of a stringent washing solution (0.1xSSC, 0.1 % SDS and 1 mM EDTA) for 30 min interval at 50°C with shaking. The nitrollulose filter was blotted dry between sandwiches of 3 MM Whatman

papers and then sealed in plastic bag and autoradiographed as described in section 13.4.

In order to use this nitrocellulose filter for rehybridization with a new probe, the filter was washed twice in a stringent washing solution comprising of 0.1xSSC, 0.1 % SDS and 1 mM EDTA at 60 °C for 30 min. Then the filter can be rehybridized with a new probe after washing in 10 mM Tris-HCl, pH 7.5 and 0.1 % SDS solution at 80°C for 1 hr, then hybridization can be proceeded without pre-hybridization.

12. Dot blot hybridization

In principle, DNA dot blot was prepared by fixing denatured chromosomal DNA onto nitrocellulose filter which was then prehybridized and hybridized with labelled probe in a sealed plastic bag and then the filter was washed to remove the non-hybridized probe. The hybrid formed was measured by following the radioactivity of the remaining labelled DNA probe.

For dot blot hybridization, ten microlitre of 500 ng of DNA of Azotobacter and Azospirillum were treated with an equal volume of 0.5 M HCl and left at room temperature for 10 min. Ten microlitre of 0.6 N NaoH was added and stand on ice for 10 min. Finally 10 µl of 2 M ammonium acetate was added into the mixture which was then boiled at 100 °C for 10 min in thermolyne dri-bath, followed by rapid cooling in ice. The concentration of DNA was now 12.5 ng/µl. The treated DNA, which final concentration of 25 ng/ul were prepared from 10 ul of 1 µg of chromosomal DNA as previously described. Then, 250 ng (20 µl of 12.5 ng/µl) and 200 ng (20 µl of 25 ng/µl) of denatured DNA solution were spotted on the 20xSSC buffersturated nitrocellulose filter as shown in the diagram below. The DNA fragments were fixed

on the nitrocellulose filter by baking at 80 °C for 2 h. The nitrocellulose filter was then prehybridized, hybridized and washed as described in section 11.2 and 11.3. After the autoradiography was performed as described in section 13.4, the filter was cut into even pieces of 1x1 in^2, then immersed in a vial containing 5 ml of Bray's solution (6 % napathalene, 0.4 % PPO, 0.02 % POPOP, 10 % methanol and 2 % ethylene glycol in dioxan) and counted for radioactivity.

13. General Techniques

13.1 Standard agarose gel electrophoresis

The analysis of plasmids and restriction endonuclease fragments were performed in submarine horizontal gel electrophoresis of 0.6-0.8 % agarose prepared in Tris-borate buffer solution (TB ; 89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH 8.3). The gel was 100x80x5 mm in size. Usually, four parts of DNA sample was mixed with one part of tacking dye containing 40 % Ficoll 400, 5 mM EDTA and 0.1 % bromphenol blue and was then loaded into a sample well of the gel. Electrophoresis was carried out for 3-5 hrs at 80 volt.

After electrophoresis, the gel was stained by submerging in 2.5 μg/ml of ethidium bromide for 20 min to visaulize the DNA bands. The gel was subsequently destained by submerging in distilled water to remove excessive unbound ethidium bromid from agarose gel. The pattern of DNA band was observed and photographed under UV light (UV transilluminator-UVP) on Kodax Tri-X film. The molecular size or molecular weight of the unknown DNA was compared to that of the standard DNA of the same molecular form. Restriction fragments of λ DNA and pBR322 were used as standard size maker.

13.2 Precipitation of DNA

DNA could be precipitated out of the solution by addition of 2 volumes of cold absolute ethanol in the presence of 0.1 M NaCl, at -20 °C for overnight. After centrifugation, the dried pellet was dissolved in and appropriate volume of TE buffer (10 mM Tris-HCl, pH 8.0 , 1 mM EDTA).

13.3 Phenolization of DNA

The standard way to remove proteins from nucleic acid solution is to extract once with equal volume of phenol (equilibrated with 0.1 M Tris-HCl, pH 8.0 and containing 0.1 % hydroxyquinoline and 0.2 % β-mercaptoethanol) in a polypropylene tube or siliconized glass tube with a plastic cap. The content of the tube was gently mixed, then centrifuged for 1 min at 12,000 rpm in a microcentrifuge or 15 min at 3,000 rpm in Top bench centrifuge. The aqueous solution was removed to a new tube. The DNA solution was mixed with an equal volume of 3 % isoamyl alcohol in chloroform and centrifuged as above. The aqueous solution was removed to another new tube for further extraction with water-saturated ether.

13.4 Autoradiography

The blotted-dry nitrocellulose filter in a sealed plastic bag was exposed to Fuji RX 100 X-ray film, between two intensifying screens. The cassette was incubated at -70 °C for appropriate time.