Materials and Methods

Materials

1. Bacteria and maintenance of cultures.

The bacterial cultures used as references were as follow: Klebsiella oxytoca 1301; an isolate from rice grown in Japan, Pseudomonas sp. H8, P. KLH76, Azospirillum lipoferum 34H and A. lipoferum FS were isolated from the rice histosphere and soil of paddy fields at the International Rice Research Institute (IRRI), Philippines. The first strain was provided by Prof. Y. Hirota, Natl. Inst. Genet., Japan (NIG) and the latter 4 strains were presented to us by Dr. I. Watanabe Internatl. Rice Res. Inst., Philippines (IRRI).

The unknown bacterial strains; R15, R17 and R25 were isolated from the rice root and soil of paddy fields at the experimental site; Tapra and Rangsit, Thailand by P. Harinasut and J. Boonjawat, Chulalongkorn University, Thailand.

All unknown bacterial strains and K.oxytoca 1301 were maintained on rich agar medium. Reference isolated of N_2-fixing Pseudomonas and Azospirillum strains were maintained on tryptic soy agar and Difco Bacto-nutrient agar respectively. Before use, they were checked for purity by streaking on agar plates, and new single colony isolates were used.

2. Culture media and Cultivation

2.1 Rich medium (RM), (4)

Tryptone	10 g per l
Yeast extract	5 g per l
Sodium chloride, NaCl	10 g per l
Glucose	2 g per l
pH 7.0	
Noble agar	15 g per l (for solid medium)

2.2 Tryptic soy agar (TSA), (14)

Tryptic soy broth	1 g per l
Noble agar	15 g per l
pH 7.0	

2.3 Nitrogen-free medium (NF), (4)

Dipotassium hydrogen phosphate, K_2HPO_4	0.05 g per l
Potassium dihydrogen phosphate, KH_2PO_4	0.15 g per l
Calcium chloride, $CaCl_2 . 2H_2O$	0.01 g per l
Magnesium sulphate, $MgSO_4 . 7H_2O$	0.002 g per l
Sodium molybdate, $Na_2MoO_4 . 2H_2O$	0.002 g per l
Ferric chloride, $FeCl_3$	0.01 g per l
Sodium bicarbonate, $NaHCO_3$	0.01 g per l
Glucose	20.0 g per l
pH 7.0	

2.4 Glucose-yeast extract medium (GYE), (4)

Glucose	5.0 g per l
Yeast extract	0.1 g per l
Dipotassium hydrogen phosphate, K_2HPO_4	0.5 g per l

Ferrous sulphate, $FeSO_4 . 7H_2O$	0.04 g per l
Magnesium sulphate, $MgSO_4 . 7H_2O$	0.2 g per l
Sodium molybdate, $Na_2MoO_4 . 2H_2O$	0.0059 g per l
Calcium chloride, $CaCl_2 . 2H_2O$	0.2 g per l
Boric acid, H_3BO_3	0.15 mg per l
Zinc sulphate, $ZnSO_4 . 7H_2O$	0.11 mg per l
Cobalt sulphate, $CoSO_4 . 7H_2O$	0.07 mg per l
Cupric sulphate, $CuSO_4 . 5H_2O$	0.005 mg per l
Manganese chloride, $MnCl_2 . 4H_2O$	0.004 mg per l
pH 7.0	
Noble agar (for semisolid medium)	0.5 g per l

2.5 Malate-yeast extract medium (NFb), (16)

DL-malic acid	5.0 g per l
Yeast extract	0.05 g per l
Dipotassium hydrogen phosphate, K_2HPO_4	6.0 g per l
Potassium dihydrogen phosphate, KH_2PO_4	4.0 g per l
Magnesium sulphate, $MgSO_4 . 7H_2O$	0.2 g per l
Sodium chloride, NaCl	0.1 g per l
Calcium chloride, $CaCl_2 . 2H_2O$	0.02 g per l
Ferric chloride, $FeCl_3$	0.01 g per l
Sodium molybdate, $Na_2MoO_4 . 2H_2O$	0.002 g per l
Biotin	0.0001 g per l
pH 6.8	
Noble agar (for semisolid medium)	0.5 g per l

2.6 Malonate broth (29)

Yeast extract	1.0 g per l
Ammonium sulphate, $(NH_4)_2SO_4$	2.0 g per l

Dipotassium hydrogen phosphate, K_2HPO_4	0.6	g per l
Potassium dihydrogen phosphate, KH_2PO_4	0.4	g per l
Sodium chloride, NaCl	2.0	g per l
Sodium malonate	3.0	g per l
Glucose	0.25	g per l
Bromothymol blue	0.025	g per l

pH 7.0

2.7 Methyl Red-Voges-Proskauer (MRVP) broth, (29)

Peptone	7.0	g per l
Dipotassium hydrogen phosphate, K_2HPO_4	5.0	g per l
Glucose	5.0	g per l

pH 6.9

2.8 Tryptone-yeast extract medium (TY), (24)

Difco tryptone	5	g per l
Difco yeast extract	3	g per l

pH 7.0

2.9 Nutrient broth-yeast extract medium (NY), (24)

Difco nutrient broth	16	g per l
Difco yeast extract	5	g per l

pH 7.0

The glucose-yeast extract semisolid medium was used for enrichment and acetylene reduction test of N_2-fixing Pseudomonas. Isolates of Pseudomonas were cultivated on TSA for 5-7 days at 30°C, then subjected to acetylene reduction assay in semisolid GYE at 30°C.

A. lipoferum strains were cultivated on nutrient agar for 5-7 days at 30°C and in malate-yeast extract semisolid medium at 30°C under

stagnant condition. To produce non-N_2-fixing cultures, NH_4Cl (2.5 g/l) was added into liquid medium at 35°C.

As for unknown bacteria and K. oxytoca 1301, rich agar medium was used for purification and rich broth medium was used for enrichment at 35°C.whereas for C_2H_2 reduction assay, the nitrogen-free medium was used.

4. Chemicals

All chemicals used were laboratory grade, or otherwise specified.

3.1 For acetylene reduction activity (ARA) measurement

3.1.1 Gases

Compressed air	From the Military Science Department.
Hydrogen (H_2)	From the Military Science Department.
Ethylene standard (C_2H_4)	From Thai Industrial Gases Limited.
Nitrogen (N_2 OFN)	From Thai Industrial Gases Limited.
Acetylene (C_2H_2)	From Ratanachoke Engineering Limited.

3.1.2 Packing material

Porapak N (mesh 80-100)	From Water Associates, inc. USA.

3.2 Reagents for DNA isolation and Tm determination, (30)

Saline-EDTA: 0.15 M-NaCl plus 0.1 M-ethylenediaminetetraacetate (EDTA), pH 8.0

Sodium lauryl sulfate (SDS), 25 %

Sodium perchlorate, 5M

Chloroform-isoamyl alcohol, 24: 1 (V/V)

Ethyl alcohol, 95 %

Saline-citrate (1xssc): 0.15 M-NaCl plus 0.015 M-trisodium citrate, pH 7.0

0.1xssc: 0.015 M-NaCl plus 0.0015 M-trisodium citrate

1xssc: 1.5 M-NaCl plus 0.15 M-trisodium citrate

Ribonuclease, 2 mg/ml (crystalline, Armour) in 0.15 M-NaCl, pH 5.0

Acetate-EDTA: 3.0 M-sodium acetate plus 0.001 M-EDTA, pH 7.0

Isopropanol

3.3 Reagents for chromosomal DNA isolation, small scale, (31)

2X Pronase buffer: 20 mM-Tris, 20 mM-EDTA, 300 mM-NaCl

2X Lysis buffer: 4 mg/ml pronase in 4 % SDS

Phenol solution: saturated phenol in TE, pH 8.0 with 0.1 % 9-hydroxy-quinoline

TE buffer: 0.01 M-Tris, 0.001 M-EDTA, pH 7.4

Chloroform solution: 3 % isoamyl alcohol in chloroform

Ether

Stock 10 mg/ml RNase A

Absolute ethanol

70 % Ethanol

3.4 Reagents for detection of plasmid and agarose gel electrophoresis (27)

Lysing solution: 3 % SDS, 50 mM Tris-hydroxide (pH 12.6)

Phenol: Chloroform (50: 50, vol/vol)

E buffer: 40 mM Tris-acetate, 2 mM sodium EDTA

Tracking dye: 0.25 % bromocresol purple in 50 % glycerol 0.05 M Tris-acetate (pH 7.9)

Staining solution: 0.5 ug/ml ethidium bromide

DNA marker: λDNA

3.5 Reagents for enzyme restriction cut, (31)

10X High salt buffer: 100 mM-NaCl, 50 mM-Tris-HCl (pH 7.5)

10 mM-$MgCl_2$, 1 mM-DTT

Restriction endonuclease (Type II): EcoRI, provided by S. panyim

Tracking dye: 0.25 % bromocresol purple in 50 % glycerol 0.05 M Tris-acetate (pH 7.9)

Methods

1. Isolation of deoxyribonucleic acid from bacteria (30)

All operation was performed at room temperature except the RNase treatment, which was carried out at 37°C

Bacterial culture (400 ml) grown to logarithmic phase of their growth cycle were harvested by centrifugation at 7,700 xg for 30 min and washed once with 50 ml-saline-EDTA. About 2-3 g wet packed cells were collected by centrifugation, then suspended in a tatal volume of 25 ml-saline-EDTA. Cell lysis was performed by adding 2.0 ml of 25 % SDS and incubated in a 60°C water bath for 10 min then cooled to room temperature,

Sodium perchlorate (5M) was added to the final concentration of 1M to the viscous, lysed suspension and the whole mixture was shaken with an equal volume of chloroform-isoamyl alcohol in a ground-glass stoppered flask for 15 min. The mixture was separated into 3 layers by a 5-min centrifugation at 12,100 xg. The upper aqueous phase containing nucleic acids was carefully pipetted into another tube. The nucleic acids were then precipitated by gently layering approximately 2 vol. 95 %, ethyl alcohol on top of the aqueous phase. When these layers were gently mixed with a stirring rod, the nucleic acids spooled on the rod as a threadlike precipitate and was easily removed. The precipitate was drained free of excess alcohol by pressing the spooled rod against the vessel, and then transferred to approximately

10-15 ml of dilute saline-citrate buffer, pH 7.0 and gently removed from the stirring rod by swirling it back and forth. The solution was gently shaken or pipetted until dispersion was complete. The solution was adjusted to standard saline-citrate concentration by adding concentrated saline-citrate, shaken as before with an equal volume of chloroform-isoamyl alcohol for 10 min, centrifuged and the supernatant removed. It was then deproteinized repeatedly with chloroform-isoamyl alcohol as described, until only trace of protein was seen at the interface.

The supernatant obtained after the last in the series of deproteinizations (3-5 times), was precipitated with 95 % ethyl alcohol and dispersed in saline-citrate (about 0.5 to 0.75 final supernatant volume). Ribonuclease (2 mg/ml) was added to a final concentration of 50 ug/ml and the mixture incubated at 37^{o}C for 30 min. The digest was again subjected to a series of deproteinizations until there was little or no trace of protein visible at the interface after centrifugation.

The supernatant, after all treatment, was again precipitated with 95 % ethyl alcohol and the drained nucleic acids dissolved in 9.0 ml dilute saline-citrate. Into this solution, 1.0 ml of acetate-EDTA was added, rapidly stirred and 0.54 vol. isopropanol was added dropwise while on the vortex. The DNA was usually precipitated in a fibrous form while RNA or oligoribonucleotides and cellular or capsular polysaccharide were remained in the supernatant. The DNA precipitated was washed free of acetate and salt by gently stirring in progressively increasing concentration (70 to 95 %) of ethyl alcohol, and was then dissolved in dilute saline citrate.

Finally, the DNA solution was stored in a freezer at -20^{o}C, or in a refrigerator by adding 2 to 3 drops of chloroform into the tube.

2. Determination of DNA base compositions by melting temperature (Tm) method (22)

The solvent used for the DNA was dilute saline-citrate solution (0.1xssc solution, pH 7.0). The DNA solution was prepared at a concentration of approximately 20 ug/ml (assuming one unit of optical density at 260 nm for 50 ug of DNA), and transferred into a 1 ml-quartz cuvette having 1 cm. light path. Using 0.1xssc as blank, each sample of DNA solution was heated in the chamber of a recording Shimadzu UV spectrophotometer 240 equipped with graphic printer (PRI) in which the temperature was increased from 25 to 100°C by means of a thermostated circulating water bath using automatic program.

Usually in determining the Tm of each DNA solution, the temperature of the chamber was set initially at 25°C and raised quickly to about 5°C below the estimated onset of the melting region, when temperature equilibrium had been attained, the temperature was then raised slowly about 1°C at a time, allowing about 3-5 min for equilibration at each temperature. The optical density was read at 260 nm. A sharp increase in the absorbance usually occurred in the transition range during which the DNA denatured. When no further increase of A_{260} was observed on raising the temperature, the denaturation of DNA was considered to be completed.

The difference between the optical density (A_{260}) at each temperature and the A_{260} at 25°C (ΔA_{260}, %) were plotted versus the temperature of the solution. The temperature corresponding to 50 % the maximum increase in the optical density at 260 nm was designated as the Tm. The mole percent G+C content of native DNA was calculated using the equation of De ley (1970):

mol % G + C = 2.44 (Tm - 69.4°C), correct for 0.1xssc solution was done by using DNA from E. Coli B which has a Tm value of 90.5°C and a G + C composition of 50 % as a reference DNA.

Example: For the bacterial strain with a Tm value of 91.8°C;

mol % G + C = 2.44 (91.8 - 69.4 °C)

= 54.7 mol %

3. Determination of the reassociation rate by rapid start technique (24)

The DNA solution for reassociation was adjusted by 0.1xssc to an optical density at 260 nm in the range of 0.5 to 1.

The DNA sample was denatured by heating at 100°C for 5 min, and NaCl was added to make 5M concentration in order to boost the reassociation rate. The heat-denatured sample was injected directly into a cuvette held at the incubation temperature about 60-65°C already placed in the Shimadzu UV spectrophotometer and recorded the time required for the formation of double-stranded DNA molecule from the melted single strands of DNA.

At the end of the reassociation, the sample was again remelted. The maximum absorbance of remelted DNA should be the same as that of the previous melting.

4. Plasmid detection procedure (27)

Cells were grown in 3 ml of rich medium overnight at 30°C and pelleted by centrifugation (5,000 xg, 4°C, 7 min). The cell pellet was thoroughly suspended in 1 ml of E buffer. The cells were lysed by adding 2 ml of lysing solution, which was mixed by brief agitation. The solution was heated at 65°C in a water bath (0, 30, 60 min), and 2 volumes of phenol-chloroform solution (1: 1, vol/vol) were added.

The solution was emulsified by shaking briefly, and the emulsion was broken by centrifugation (5,000 xg , 15 min, 4°C). Avoiding the precipitate at the interface, the upper aqueous phase was transferred to a screw cap tube by using a pasteur pipette. Samples could be withdrawn directly for electrophoresis immediately. Usually 35 µl of sample was mixed on parafilm with 10 µl of 0.25 % bromocresol purple tracking dye (pH 7.9).

5. Isolation of long-chained chromosomal DNA from bacteria (small scale extraction), (31)

Cells were grown in 2 ml of rich medium overnight at 37°C and contrifuged at 7,000 xg for 2 min in a microcentrifuge tube. The supernatant was discarded and 100 µl of 2X pronase buffer was added, gently mixed followed with 100 µl of 2X lysis buffer. The suspension was mixed well but vigorous mechanical agitation was avoided. The suspension was incubated at 37°C for 15 min to achieve complete cells lysis. After that, 200 µl of phenol solution was added and mixed slowly by inverting the tube back and forth followed by 200 µl of chloroform solution and mixing as before. The tube was spun in a microcentrifuge at 7,000 xg for 3 min to separate the solution into two layers. The lower phase (phenol-chloroform layer) was removed and discarded. For complete removal of protein, the solution was extracted with phenol-chloroform at least three times.

After the last extraction, the upper phase was transferred into another 1.5 ml-microfuge tube. Contaminated phenol was removed from the solution by extraction with ether. RNase A was added to a final concentration of 100 µg/ml and the mixture was incubated at 37°C for 30 min. Again, the solution was extracted with phenol-chloroform and

ether for removal of RNase A. Chromosomal DNA was precipitated by adding sufficient 5M NaCl to make a final concentration of 0.1 M together with 2 vol. of absolute ethanol, and gently mixed. The tube was kept at -20°C about 60 min for complete precipitation of DNA, then centrifuged at 7,000 xg for 10 min. The precipitate was washed with 70 % ethanol, recentrifuged for 5 min, and let dried. The DNA precipitate was resuspended in 50 µl of TE and allowed to dissolve at 4°C overnight before subjected to agarose gel electrophoresis.

6. Analysis of plasmid pattern by agarose gel electrophoresis (31)

Agarose 0.7 g by weight was dissolved in 100 ml of E buffer by heating and let cooled to 60°C. The warm suspension was poured into a gel chamber leaving the slots for DNA loading, and allowed to cool down to room temperature. By complete solidification of agarose gel, E buffer was poured into the chamber. The DNA sample was mixed with the loading dye in the ratio 3 : 1 (DNA : loading dye) and was loaded into each slot of the agarose gel chamber. The chamber was attached to the power supply. allowed the current to run from the negative pole to the positive pole at 120 volts for 8-10 hours, or until the loading dye moved nearly to the other side of the gel. After turning off the power supply, the gel was removed from the chamber and stained with the staining solution (0.5 ug/ml ethidium bromide) for 15 min at room temperature and destained in distilled water for 30-60 min.

The DNA bands were observed by placing the gel under UV light. The concentration of DNA was estimated by comparing fluorescence intensity with the marker DNA.

7. DNA restriction cut by restriction endonuclease (31)

About 12 µl of nuclease-free distilled water was added into a microfuge tube to make the reaction mixture to a final volume of 20 µl, then 2 µl of 10X high salt buffer was added. After that, 5 µl, of DNA was added followed with 1 ul of the restriction enzyme EcoRI (approximately 1-2 units). Spun the microfuge tube in a microcentrifuge (Tomy-15 A) and incubated at 37°C for 60 min. The reaction was stopped by adding the tracking dye and subjected to further analysis by agarose gel electrophoresis.

8. Biochemical and Physiological tests

8.1 Malonate utilization (29)

A single colony from young agar slant was inoculated into a tube of malonate broth and incubated at 37°C for 48 hours. Observe the color change from green to deep blue for positive test.

8.2 Indole production (29)

Filter paper was cut into strips and soaked in a warm, saturated solution of oxalic acid. Crystals of the acid were formed on the strips upon cooling. The strips were dried thoroughly and inserted aseptically into the inoculated culture. The strip should not come into contact with the medium but rather should be bent so that it pressed against the wall of the tube and remained near the mouth when the cotton stopper or screw cap is replaced. The culture tube was incubated at 37°C for 48 hours. The paper strip became pink during growth of the culture.

8.3 Voges-Proskauer reaction (29)

The tube of MRVP broth was inoculated with a young agar slant or broth culture and incubated at 37°C for 48 hours. After incubation,

1 ml of culture was removed to another tube and 0.6 ml of 5 % (W/V) ∝- naphthol (dissolved in absolute ethanol) was added, mixed thoroughly, then 0.2 ml of 40 % aqueous KOH was added. After mixing well, the tube was incubated in slant position to increase the surface area of the medium. Strong red color began at the surface of the medium after 15 or 60 min of incubation.

8.4 Requirement for biotin (15)

Cultures grown in NFb + NH_4Cl (2.5 g/l) medium were inoculated into 25 ml of nutrient broth (Difco) and incubated at 37°C for 48 hours. The cells were harvested by centrifugation, at 7,700 xg for 15 min a and washed twice with 10 ml of sterile distilled water. The cells was resuspended in sterile distilled water to an optical density of 0.4 at 560 nm. One-tenth millilitre of this suspension was used to inoculated each 5 ml-volume of medium. (The medium used was NFb with 0.0001 g/l of biotin and NFb without biotin). The culture was incubated at 37°C for 48 hours in case where slight growth occured in the medium lacking biotin, compared with much heavier growth in the medium with biotin, a second serial transfer (0.1 ml) was made to the medium with and without biotin to confirm the requirement.

8.5 Sodium lauryl sulphate resistance (32)

The cultures were incubated in Difco nutrient broth supplemented with 5 or 10 % (W/V) SDS for 72 hours with rotary agitation on a shaker at room temperature. Growth was estimated in Klett units. Growth = > 100 Klett units; no growth = < 10 Klett units.

8.6 Salt tolerant (15)

A tube of NY or TY medium supplemented with 3 % or 5 % NaCl was inoculated with a single colony of a bacterial strain from an agar

slant and incubated at 37°C overnight. Growth was estimated from the turbidity of cultures comparing with normal growth in NY or TY medium without sodium chloride.

8.7 Drug resistance test (33)

Young culture of the test organisms was streaked on plated NY agar medium or TY agar medium containing the antibiotic. Antibiotics were tested at two-fold increasing concentrations starting from 6.25 µg/ml. Presence or absence of normal growth was recorded after 24-48 h of incubation at 30°C.

9. Growth pattern of bacteria

A colony of a bacterial strain from an agar slant was inoculated into 10 ml of culture medium, incubated 2-3 hours and then transferred to 200 ml of fresh medium (5 % inoculum). A 5 ml-aliquot was taken at various time intervals and measured the optical density at an appropiate wavelength.

During growth the bacterial culture was aerated by attaching a flask or tube to a rotary shaking platform or a rotating wheel. The volume of the vessel should be at least four times largerthan the volume of medium it contained so as to allow vigorous shaking.

For microaerophilic condition, 0.05 % of Difco noble agar was added into the medium and the culture was incubated by stagnant at appropiate incubation temperature.

10. Test for acetylene reduction activity (ARA)

Aerobic-culture, (4):

Single colony of a bacterial strain was inoculated into 5 ml

of NF + 10 % rich medium and incubated at 37°C. Cells were harvested in the midlog phase by centrifugation and then resuspended in NF medium. The culture was incubated aerobically at 30°C for 4 hours.

Microaerophilic-culture, (34):

ARA was carried out in 22 ml-tube containing 5 ml semisolid medium. The organisms were stab-inoculated into the medium and the cultures were incubated statically for 2 days at 30°C.

ARA assay:

Three replicate tubes were removed, cotton plugs were replaced with serum caps then 10 % of the head space air volume was replaced with acetylene. Great care was taken to avoid disturbing the zones of subsurface growth, since mixing caused cessation of ARA in case of microaerobic-culture (35). The tubes were again incubated for 24 hr at 30°C, after which, ethylene formed was measured in a Varien 3700 gaschromatograph, equipped with a flame ionization detector (FID) and using 30 ml/min of oxygen-free N_2 as carrier gas. Ethylene production per tube per 24 h was determined by comparing the peak height in cm with that of known amount of standard ethylene.

The aerobic heterotrophs were diluted and counted on TSA plates, incubated at 30°C for 2-3 days.

11. Cellular protein content determination (36)

After washing cells with 0.85 % NaCl, protein was extracted by mixing an one volume of cell suspension with equal volume of 1 N, NaOH and hydrolysed in boiling water bath for 5 min. After cooling, 1.0 ml of sample was mixed with 2.5 ml of carbonate-copper reagent (1 ml of 0.5 % $CuSO_4$, 1 ml of 1 % sodium potassium tartrate in 50 ml of 5 %

sodium carbonate) and let stand for 10 min at room temperature. Subsequently, 0.5 ml of phenol reagent (dilute 1 : 1) was instantly added, mixed and held at least for 30 min at room temperature. The absorbance was measured at 650 nm. Calibration curve was prepared by using bovine serum albumin (BSA) as standard protein.

In case of microaerobic condition, the protein content was determined by using the modified Lowry method (37) after solubilizing the cultures by adding 0.5 ml of 10 % SDS to 5 ml of semisolid culture medium and heating for 5 min at 100^{o}C. Sterile semisolid medium was used as blank, and standard BSA was prepared in semisolid medium. Protein determinations were performed in triplicate for each bacterial culture.