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Isolation and Characterization of Plant Growth-Promoting Rhizobacteria

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I. INTRODUCTION

Biotechnology has opened up new possibilities concerning the application of beneficial bacteria to the soil for the promotion of plant growth and the biological control of soil-borne pathogens.[1-3] Since the large scale release of genetically engineered bacteria to the environment faces a number of regulatory hurdles, the need to isolate and select superior, naturally occurring rhizosphere bacteria continues to be of interest. Apart from rhizobia symbionts, the rhizosphere-associated beneficial bacteria consist of the following genera: (1) *Pseudomonas* and *Bacillus*, which antagonize pathogenic or deleterious microorganisms (biological control) and (2) bacteria that enhance plant growth directly such as *Azospirillum*, *Herbaspirillum*, *Enterobacter*, *Acetobacter*, *Azotobater*, and *Pseudomonas*, as well as many unidentified rhizosphere isolates.

The nutritional and environmental requirements of these bacteria are very diverse, and hence there is no general method that can be used to isolate all species of Plant Growth-Promoting Rhizobacteria (PGPR). Accordingly, a variety of methods have been developed, primarily within the last two decades.

II. HABITAT

Generally, there are no specific sites where PGPR can be found since all plant roots are associated with numerous species of microorganisms, beneficial as well as pathogenic. However, some strategies have been established:

1. The best place to search for a biological control agent is in the same ecological niche as the target pathogen.[3-4]
2. Wild ancestors of crop plants which have evolved with PGPR can be isolated and used later to inoculate cultivated plants (M. Feldman, unpublished).
3. When a target plant is introduced into the soil, low numbers of native PGPR in the soil can be enriched in the rhizosphere to form large populations for recovery (Y. Bashan, unpublished).
4. Potential PGPR for biocontrol are selected on the basis of their siderophore or antibiotic production *in vitro*.[2]
5. Of the thousands of bacteria isolated randomly from the plant rhizosphere of diverse habitats, many are being selected for growth promotion without regard to taxonomy or the mechanisms involved. This type of approach is common to industrial R&D programs.[2]

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III. ISOLATION

Endorhizosphere bacteria (bacteria colonizing the root interior) are distinct from root surface isolates.5 Thus, different strategies are followed for the isolation of each of these two groups.

A. ROOT SURFACE BACTERIA

1. To equalize osmotic pressure, roots are first soaked for 10 min in sterile phosphate-buffered saline (PBS) [10 *mM* K_2PO_4-KH_2PO_4, 0.14 *M* NaCl, pH 7.2], then chopped into small pieces (3 cm), inoculated on the selected enrichment medium or media (described later), and incubated at 25 to 30°C for 1 to 2 weeks until visible growth is apparent.[6]
2. For the isolation of root surface bacteria with root adhesion capacity, the roots must first be washed thoroughly with running tap water, then with sterile distilled water and finally soaked in PBS. The selected enrichment medium is then inoculated with these roots and incubated at 25 to 30°C until visible growth is apparent.
3. Root surface bacteria can be isolated by shaking small pieces of roots for 10 min on a mechanical gyrator shaker in PBS or phosphate buffer-peptone containing, per liter: peptone, 1.0 g; K_2HPO_4, 1.21 g; KH_2PO_4, 0.34 g.[7] Optional alternative diluents are: PBS plus 0.025% Tween 20, 0.01% Tween 40,6 or 0.1% tryptic soy broth (TSB).[8-9] Diluted samples are then inoculated on the appropriate medium at the selected temperature until growth is visible.

B. ENDORIZOSPHERE BACTERIA

The roots are surface-sterilized by soaking in 95% (v/v) ethanol and 0.1% (w/v) acidified $HgCl_2$ for 1 min, respectively, and then washed (a minimum of ten times) with sterile tap water.[10] Alternate disinfection procedures include (1) soaking roots in 70% ethanol for 5 min, in 6.25% sodium hypochlorite for 10 min, followed by several rinses in sterile distilled water[7] or (2) soaking in 10% H_2O_2 for 15 sec and then rinsing three times with 0.1 *M* $MgSO_4$.[5]

Plant material is then suspended in 0.05 *M* PBS or 0.1 *M* MgSO4 and ground with a mortar and pestle, or homogenized by a high speed shaft for 1 to 3 min (100 ml of PBS for each 5 g fresh weight of roots). The slurry may be filtered through sterile cotton wool.[11] Diluted or undiluted aliquots are then prepared for inoculation onto an enrichment medium.

An alternative isolation method is to aseptically spread the sterilized roots on nutrient agar supplemented with glycerol (1%) to verify the surface sterility of the roots. Roots are cut lengthwise, placed onto the selected enrichment medium, preferably supplemented with glucose (since glucose-utilizing, acid-producing bacteria seem to dominate in the endorhizosphere), and incubated at the selected temperature until growth is visibly detected.[7] After the incubation period, 0.1 ml of the growth medium is spread onto chosen selective, solid media and incubated again at the same temperature. Isolates must be restreaked several times on the same solid medium until purity is obtained.

C. DIAZOTROPHIC PLANT GROWTH-PROMOTING RHIZOBACTERIA

1. Spermosphere Model for Isolating Diazotrophs from the Rhizosphere

The spermosphere model[12] consists of a seed germinated in the dark which is releasing exudates in a medium free of carbon and nitrogen. This is then inoculated with soil dilutions and incubated under an acetylene atmosphere. The system is appealing for two reasons: (1) the germinating seedling provides the bacteria with the most useful carbon sources they encounter in the soil, thus avoiding bias in the carbon nutrition and (2) the growing seedling

consumes any nitrogen made available by the diazotrophs, keeping the medium nitrogen-free and highly selective.

1. 10 g of rhizosphere soil (roots and the soil that adheres to them) is macerated in a mortar and pestle and diluted to 100 ml in PBS within 2 h of sampling. Additional dilutions of the slurry, in PBS, should be made.
2. Seeds are disinfected by any standard method (designated for a particular plant species).
3. Disinfected seeds are placed on the medium surface of 5 ml of semisolid (0.3% agar) N-free, C-free medium in a 35 ml test tube (described below). The spermosphere tubes are kept in the dark at 28°C (or any other desired temperature) for a week. Tubes with a side arm containing 2 ml 1 *N* NaOH, which serves as a CO_2 trap, may be used when necessary.
4. When coleoptiles are 1 cm high or more, the spermosphere assemblies are inoculated with 0.5 ml aliquots of soil dilutions obtained from Step #1. Earlier inoculation frequently results in seedling death. A delay in inoculation also allows for the identification of seed contamination from insufficient disinfection.
5. The number of diazotrophs is calculated by the Most Probable Number method (MPN).[13] The estimation is based on the numbers (ten replicate tubes for each dilution) of nitrogenase-positive tubes detected by the acetylene reduction assay.[14]
6. The contents of ten tubes of the highest dilution at which all tubes are ethylene positive, are pooled, homogenized, serially diluted (10^{-5} to 10^{-9}) and plated on N-free solid medium[15-16] in flat (120 ml or more) serum bottles and incubated under 1 % acetylene for 4 to 8 days. (Pooling and homogenization avoid confusing problems arising from the possible transfer of a small piece of root to a high dilution tube.) Colonies which develop should be picked individually, purified on N-free medium, and tested for nitrogen fixation as described above. In some instances, it is necessary to partially hydrolyze the capsular material using 0.5 or 0.1 *N* NaOH to remove any contaminants.

Note: This technique does not give a complete survey of nitrogen-fixing bacteria in the rhizosphere but only delineates the numbers and the nature of the most abundant diazotrophs.

2. Semisolid Enrichment Cultures of Root Pieces for Isolation of Microaerophilic Diazotrophic Bacteria

Semisolid, N-free media are the key to the isolation of a number of root-associated microaerophilic diazotrophs.[17,18] Techniques using these media are very simple and useful when there is an abundance of diazotrophs associated with roots. Pure cultures can be obtained with only a few purification steps and without much difficulty. This technique was used in the discovery of four *Azospirillum* strains, *Campilobacter nitrofigilis*, *Herbaspirillum seropedicae*, some diazotrophic *Pseudomonas*, *Acetobacter diazotrophicus*, and *Bacillus azotofixans*.

a. Procedure #1

Intact root pieces (0.5 to 1.0 cm) are placed into semisolid (0.05% agar or less) NFb, OAB, or BL media (described below) and incubated without shaking for 2 to 5 days at 25 to 35°C. (At lower incubation temperatures, the incubation time should be extended.) Following this incubation, a white bacterial pellicle is formed 2 to 10 mm below the surface. An assessment of the ability of the enriched culture to fix nitrogen should be done by the acetylene reduction technique.[14] (Special care should be taken not to disturb the pellicle because this immediately stops nitrogenase activity.) Almost pure cultures can be obtained after one to three subcultures onto the same medium followed by streaking out of 24-hour-old cultures on solid agar plates consisting of the same medium.[18]

b. Procedure #2 (for isolation of the diazotroph Acetobacter associated with sugarcane)

Roots and stems of sugarcane are washed with tap water, macerated in a blender, and serial dilutions are prepared in a 5% sucrose solution and incubated in semisolid medium (described below). Continue as in Procedure #1 above.19

3. Nonselective Media for Isolation of Rhizosphere Bacteria in General

The TSA medium described below recovers a wide range of aerobic and facultatively anaerobic gram-negative and gram-positive bacteria.9 However, it is advisable to try the isolation procedure simultaneously with different isolation media since a nonselective medium will only allow the recovery of a small percentage of the existing PGPR population.

1. 1/10 strength TSBA medium (tryptic soy broth agar) for heterotrophic bacteria contains tryptic soy broth, 3 g and agar, 15 g.[9] A more diverse population of bacteria can be isolated from soil and other environmental samples by using a more diluted (1/100 strength; 0.3 g TSBA) medium .[4]
2. TSA medium contains the following (g/1): meat extract, 3.0; yeast extract, 3.0; peptone from casein, 15.0; peptone from meat, 5.0; lactose, 10.0; sucrose, 10.0; glucose, 1.0; $NH_3^+Fe^{3+}$ citrate, 0.5; NaCl, 5.0; sodium thiosulfate, 0.5; phenol red, 0.024; agar, 12.0; distilled water, 1000 ml; pH 7.4.[20] The elimination of gram-positive bacteria in 1/10 strength TSA is done by adding 2 µg/l of crystal violet[4] or 1.2 g/1 of sodium lauroyl sarcosine (SLS) to S1 medium (described in Section III.C.5).

4. Media for the Isolation of Different PGPR

The following media can be used for recovery of most common PGPR populations:

1. Selective medium for the isolation of pseudomonads[20] is based on TSA medium supple-mented with (µg/ml): basic fuchsin, 9; nitrofurantoin, 10; nalidixic acid, 23; (mg/ml): cycloheximide, 0.9; TTC (triphenyltetrazolium-chloride), 1.4; the TSA base is supplied with basic fuchsin and TTC before autoclaving. Nalidixic acid and nitrofurantoin are sterilized by filtering through 0.45 µm membrane filters and added aseptically to the sterilized TSA medium. Cycloheximide is either sterilized by filtering before addition to the sterile medium, or by adding directly as the nonsterile powder.
2. Coryneforms and other gram-positive bacteria are isolated on D-2 medium[22] in which potassium dichromate (50 mg/1) and cycloheximide (100 mg/1) are incorporated to enhance selectivity, or on methyl-red agar for gram-positive bacteria .[23]
3. King's B medium for pseudomonads contains (g/1): proteose peptone no. 3 (Difco), 20; glycerol, 10 ml; asparagine, 2.25; K_2HPO_4, 1.5; $MgSO_4 \cdot 7H_2O$, 1.5; agar, 15 [21] With an incubation temperature of 30°C, the results can be assessed after 48 h.

Note: All the above media can be supplemented with benomyl, 20 mg/ml (Benlate, 50%, W.P. Dupont, USA) or Nystatin and actidione (50 mg of each per liter) to reduce fungal growth.[7,9]

5. Media for the Isolation of Fluorescent Pseudomonads

The fluorescent pseudomonads (*P. putida* and *P. fluorescens*) are a large group found in the rhizosphere of various crop plants, which can be isolated easily on the following media.

1. Modified King's B medium supplemented with the antibiotics (mg/1): chloramphenicol, 5; cycloheximide, 75; novobiocin, 45; and penicillin G, 75,000 units.[24]
   Note: Resistance to the recommended antibiotics is not unique to the pseudomonads.
   Note: The original King's B medium is currently accepted as a diagnostic medium for

the detection of fluorescence; however, it is not particularly suitable for the isolation of these bacteria because it is relatively unselective.

2. D4 medium[22] contains (g/1): glycerol, 10.0 ml; sucrose, 10.0; casein hydrolysate, 1 g; NH_4Cl, 5.0 g; sodium dodecyl sulfate, 0.6 g (to eliminate nonpseudomonads); Na_2HPO_4 (anhydrous), 1.5 g; agar, 15 g. This medium is commonly used for Gram-negative bacteria; however, many other Gram-negative bacteria can grow on it, and the fluorescence cannot be observed.
3. Medium S1[8] contains (g/1): agar, 18; sucrose, 10; glycerol, 10 ml; casamino acids (Difco), 5.0; $NaHCO_3$, 1.0; $MgSO_4 \cdot 7H_2O$, 1.0; K_2HPO_4, 2.3; sodium lauroyl sarcosine (SLS), 1.2; and 20 mg of trimethoprim (Sigma), (5-[(3,4,5- trimethoxyphenyl) methyl]-2,4-pyrimidinediamine). The final pH of the medium is 7.4 to 7.6.
   Note: Trimethoprim is added after the medium has been autoclaved and cooled.
   Note: This medium has several advantages over other media used for the isolation of fluorescent pseudomonads; it consistently provides high selectivity and good recovery of fluorescent pseudomonads with samples obtained from a variety of habitats. Fluorescence can be observed during the initial isolation.

6. Medium for the Spermosphere Model

***Solution A*:** (mg/1): H_3BO_3, 750; $ZnSO_4 \cdot 7H_2O$, 550; $CoSO_4 \cdot 7H_2O$, 350; $CuSO_4 \cdot 4H_2O$, 22; $MnC1_2 \cdot 4H_2O$, 10; distilled water 1000 ml.

Solution B (g/1): $FeSO_4 \cdot 7H_20$, 0.8; $MgSO_4 \cdot 7H_20$, 4.0; Na_2MoO_4 $2H_2O$, 0.118; $CaCl_2 \cdot 2H_2O$, 4.0; EDTA, 0.8; solution A, 4 ml; distilled water 1000 ml.

***Final basal solution*:** (g/1): KH_2PO_4, 1.8; K_2HPO_4, 2.7; solution B, 50 ml; Nobel agar, 5; distilled water, 1000 ml; pH adjusted to 6.8 using KOH; sterilization by autoclaving.

The N-free medium used to grow the bacteria from the spermosphere model's contains (g/1): yeast extract as starter, 0.1; starch, 5; glucose, 5; mannitol, 5; malic acid, 3.5; plus the basal medium listed above,[25] or the following combined carbon medium.

7. Combined Carbon Medium for Isolation of Diazotrophs

The combined carbon medium was designed to incorporate common factors of various N-free media since the basal composition of these media is very similar. Mannitol was included to support the growth of *Azotobacter* sp., biotin, and p-aminobenzoic acid for *Bacillus* sp. Yeast extract was included to supply miscellaneous organic growth factors and may supply 'starter' nitrogen that promotes growth without inhibiting acetylene reduction.

Solution A: K_2HPO_4, 0.8 g; KH_2P0_4, 0.2 g; NaCl, 0.1 g; $Na_2FeEDTA$, 28 mg; $Na_2MoO_4 \cdot 2H_2O$, 25 mg; yeast extract, 100 mg; mannitol, 5 g; sucrose, 5 g; sodium lactate, 0.5 m1 (60%, v/v) distilled water, 900 ml.

Solution B (g): $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.06; distilled water, 100 ml.

The solutions should be autoclaved separately, cooled, and mixed. To this new solution, filter-sterilized biotin (5 µg/1) and p-aminobenzoic acid (10 µg/l) should be added and the final pH adjusted to 7.0.

8. Media for the Isolation of *Azospirillum*

The most commonly used medium for the isolation of *Azospirillum* is semisolid NFb medium. [18,41] Several useful modifications of this medium have been developed and are indicated below.

a. NFb Medium (g/l)

DL-malic acid, 5; KOH, 4; K_2HPO_4, 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; NaCl, 0.1; $FeSO_4 \cdot 7H_2O$, 0.5; (mg/l): $NaMoO_4 \cdot 2H_2O$, 2; $MnSO_4 \cdot H_2O$, 10; 0.5% alcoholic solution (or dissolved in 0.2 *N* KOH) of bromothymol blue, 2 ml; agar, 0.0175 to 0.5%; 1000 ml distilled

water, pH 6.8.[18] When this medium is supplemented with low concentrations (0.002 to 0.005%) of yeast extract, it still eliminates the growth of contaminants and permits colony formation under aerobic conditions.[17] In cases where malic acid-KOH inhibits growth, they can be replaced with 10 g/1 sodium succinate[26] or 0.5% sucrose. Bromothymol blue can be replaced by 0.001 % bromocresol purple .[27]

b. OAB Medium

This modification is more suitable for *Azospirillum* growth than for isolation procedures .[28] It is not highly selective for this genus, but it provides increased buffering capacity over the original medium, microelements, a limited amount of NH_4Cl to initiate aerobic growth, and a small amount of yeast extract to shorten the lag phase and aid vigorous growth. It can be used as a liquid, semisolid (0.05% agar), or solid medium. For optimal growth of *Azospirillum* in liquid medium, the culture should be maintained at a constant pO_2 of 0.005 to 0.007 atm under an atmosphere of a mixture of N_2 and air.

Solution A: (g/l) DL-malic acid, 5; NaOH, 3; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2$, 0.02; NaCl, 0.1; NH_4Cl, 1; yeast extract, 0.1; $FeCl_3$, 0.01; (mg/1) $NaMoO_4 \cdot 2H_2O$, 2; $MnSO_4$, 2.1; H_3BO_3, 2.8; $Cu(NO_3)_2 \cdot 3H_2O$, 0.04; $ZnSO_4 \cdot 7H_2O$, 0.24; 900 ml distilled water.

Solution B: (g/1) K_2HPO_4, 6; KH_2PO_4, 4; 100 ml distilled water.

After autoclaving and cooling, the two solutions should be mixed. The medium pH is 6.8.

Note: $FeCl_3$ can be replaced by 10 ml of Fe(III)-EDTA (0.66%, w/v, in water). This medium can also be supplemented with 10 ml of a vitamin solution to enhance its isolation ability for heterotrophic microaerophilic nitrogen-fixing bacteria. The vitamin solution contains: D-biotin (200 mg), calcium pantothenate (40 mg), myoinositol (200 mg), niacinamide (40 mg), p-aminobenzoic acid (20 mg), pyridoxine hydrochloride (40 mg), riboflavin (20 mg), thiamine dichloride (4 mg), in 10 ml and is sterilized by filtration .[29]

9. Semiselective Media for *Azospirillum*

a. Congo red-NFb

This medium is basically NFb medium supplemented with 15 ml/1 medium of 1:400 aqueous solution of Congo-red, autoclaved separately and added just before using.[30]

Note: This medium permits the recognition of *Azospirillum* colonies on plates and facilitates the isolation of pure cultures since the colonies appear dark red or scarlet with typical colony characteristics, whereas many soil bacteria do not absorb Congo-red.

b. BL and BLCR Media

These two semiselective media[31] are based on OAB medium. BL medium is OAB medium supplemented with (mg/l) streptomycin sulfate, 200; cycloheximide, 250; sodium deoxycholate, 200; and 2,3,5-triphenyltetrazolium chloride, 15. BLCR is BL medium supplemented with an aqueous solution of Congo-red (approximately 1 ml of a 1 mg/ml solution per liter).

Note: These media are very suitable for the isolation of *Azospirillum* from the rhizosphere since the colonies are easily recognizable, especially on BLCR medium. However, some strains of *A. brasilense* failed to grow on this medium,[32] and the growth of *Azospirillum* on BLCR medium is significantly slower compared to the original OAB medium (about 10 days incubation time).

10. Medium for Isolation of Halophilic Diazotrophs

(g/1): DL-malic acid, 5; KOH, 4.8; $MgSO_4 \cdot 7H_2O$, 0.25; $CaCl_2$, 0.22; NaCl, 1.2; Na_2SO_4, 2.4; $NaHCO_3$, 0.5; K_2SO_4, 0.17; Na_2CO_3, 0.09; Fe (III)-EDTA, 0.077; K_2HPO_4, 0.13; yeast extract, 0.02;(mg/1): biotin,0.1; $Na_2MoO_4 \cdot 2H_2O$,2; $MnCl_2 \cdot 4H_2O$,0.2; H_3BO_3, 0.2; $CuCl_2$ $2H_2O$, 0.02; $ZnCl_2$, 0.15; agar 2 to 8; 1000 ml distilled water.[29,33] The medium pH is 8.5. Malic acid,

KOH, and agar are dissolved in one half of the total volume and autoclaved. The salt fraction is sterilized by filtration after dissolving the ingredients in one half of the total volume and discarding the precipitate after centrifugation of the medium. Slight modifications in concentrations are also possible.[33]

11. Medium for Isolation of Halophilic Rhizosphere Bacteria

Rhizosphere bacteria from xerophytic plants in hypersaline soils (5.0 to 10.7% NaCl) can be isolated by changing the total salt concentration of the medium to 9, 50, 100, 200, and 250 g/1 of total salts used.

The basic medium[34] contains 200 g/1 of total salt content and is composed of (g/1): NaCl 158.9; $MgCl_2$, 13.8; $MgSO_4$, 20.9; $CaCl_2$, 1.5; KCl, 4.2; $NaHCO_3$, 0.2; NaBr, 0.5.

These basal salt mixtures can be supplemented with one of the three following media, final concentration (g/1): (1) Peptone P (Oxoid), 10. (2) Yeast extract, 10; Proteose Peptone (Difco), 5; glucose, 1. (3) Yeast extract, 5; glucose, 1; with added soil extract. The soil extract is prepared by autoclaving equal volumes of garden soil and the corresponding salt solution; after decanting, the extract is filtered through paper and the other nutrients are added to the filtrate. pH values are adjusted to 7.5 with KOH.

12. Medium for the Isolation of Diazotrophic Acetobacter from Sugarcane

This semisolid medium[19] is based on NFb medium, with modifications for this acid tolerant species.

Solution A: (g/1) cane sugar (or sucrose or glucose), 100; $MgSO_4 \cdot 7H_2O$, 0.2; $CaCl_2 \cdot 2H_2O$, 0.02; $FeCl_3 \cdot 6H_2O$, 0.01; agar, 2.2; (mg/1) Na,,$MoO_4 \cdot 2H_2O$, 2; 0.5% solution (dissolved in 0.2 *N* KOH) of bromothymol blue, 5 ml; 900 ml distilled water.

Solution B: (g/1) K_2HPO_4, 0.2; KH_2PO_4, 0.6; 100 ml distilled water.

Note: It is advisable to autoclave the two solutions separately and mix them after they are cool. Then, the medium is acidified with acetic acid to pH 4.5. For the purification of isolates, the medium is supplemented with 0.02 g yeast-extract and 15 g agar. The isolation can be improved with the addition of 1% cane juice. The colonies appear dark orange.

13. Medium for Isolation of Marine Beneficial Diazotrophs (HGB)

HGB medium is based on OAB medium[28] with several modifications to suit marine bacteria and is practical for diazotrophic vibrios[70]

Solution A (g/890 ml distilled water): DL-malic acid, 5; NaOH, 3; $MgSO_4 \cdot 7H_2O$, 3; $CaCl_2$, 0.02; NaCl, 20; yeast extract, 0.1.

Solution B (stock solution, g/500 ml distilled water): $FeCl_3$, 0.5; $Na_2MoO_4 \cdot 2H_2O$, 0.1; $MnSO_4$, 0.105; H_3BO_3, 0.14; $CuCl_2 \cdot 2H2O$, 0.0014; $ZnSO_4 \cdot 7H_2O$, 0.012.

Solution C: 100 ml of PBS 0.39 M, pH 7.6.

Ten ml of Solution B are added to Solution A and autoclaved. After cooling, this solution is mixed with the buffer, Solution C, which should be autoclaved separately.

14. Media for the Isolation of Bacillus

Bacillus spp. are selected by heat-treating dilutions at 100°C for 15 min prior to plating on 1/10 TSA medium.[4]

15. Medium for the Isolation of Azotobacter

An efficient N-free medium for the isolation of *Azotobacter*[35] is based on soil extract and on mannitol as carbon source.

***Composition* (g/1):** KH_2PO_4, 1.0; $MgSO_4 \cdot 7H_2O$; 0.2; NaCl, 0.2; $FeSO_4 \cdot 7H_2O$, 0.005; soil extract. 100 ml; tap water, 900 ml: agar 15; and mannitol, 20 . The PH is adjusted

to 7.6 with NaOH prior to autoclaving. Mannitol and $FeSO_4$ are sterilized separately and added to the rest of the medium when cool. The soil extract is prepared as follows.[36] Non-earth material is discarded from the soil samples, and the soil is pulverized aseptically. Pulverized soil (10g) is shaken in 90 ml of sterile distilled water for 15 min. One milliliter of this suspension is diluted in 9 ml of 0.85% NaCl, and 1.0 ml of this is plated onto the N-free medium. The plates are then incubated at 26°C for 4 to 5 days.

IV. CHARACTERIZATION

A. *IN VITRO* TESTS FOR ANTIMICROBIAL ACTIVITY OF RHIZOSPHERE BACTERIA

Selection of PGPR can sometimes be based on bacterial antagonism toward plant pathogens. The major problem in identifying microbial isolates for use as biocontrol agents is the need to screen large numbers of cultures. There are no reports of useful characteristics associated with pathogen repression that could be used to screen isolates from a collection, thereby reducing the numbers that have to be evaluated by bioassay.[9]

1. Agar Assays

(1) The bacterial isolates are inoculated onto the center of a potato dextrose agar plate and incubated at 28°C. Bacterial growth is terminated after 4 days by exposure to chloroform vapors. Spores of the fungal pathogen are suspended in sterile, distilled water and sprayed over the bacterial growth plates. Zones of inhibition are measured after 36 h of growth at 28°C.[37] (2) Spore suspensions of fungal pathogens are obtained by flooding sporulating culture growth on plates with sterile 0.05% Tween 40. Spore suspensions (0.5 ml) are then plated on the same agar medium used to isolate the rhizobacteria and allowed to dry for 2 to 3 h. Rhizobacteria isolates are stabbed in quadrants of agar plates containing the fungal pathogen and incubated (for 7 days, as in the case of *Fusarium oxysporum* at 27°C). Antibiotic production against the pathogen is observed as a zone of growth inhibition around the agar stabs of the rhizobacterial isolates.6

Note: While many studies have utilized an agar assay to determine pathogen repression by PGPR isolates, this practice is limited by the absence of a plant which can greatly affect the ability of an amended bacterium to survive, colonize, and repress pathogenic fungi.

2. The 'in planta' Assay

The 'in planta' assay is more representative of conditions to which the amended bacteria will be exposed once field trials are initiated.[9] The following is one example of the numerous 'in planta' assays for disease biocontrol.

The fungal pathogen is cultured on potato dextrose agar for 72 h; then one plate is macerated in 100 ml of 0.01 *M* potassium phosphate buffer (pH 6.8). The homogenate is used as inoculum for 4 kg of a sterile mixture of sand and vermiculite (1:1, v/v), amended with calcium carbonate at a rate of 10 g/kg, and packed in sterile glass test tubes (200 mm x 25 mm). The tubes are filled to a depth of 15 cm and moistened with 18 ml of half-strength Hoagland's nutrient solution. Surface-sterilized seeds are placed on the soil surface and inoculated with approximately 50 µl of 10^7 cfu/ml of the tested PGPR suspension. The seeds are then covered with an additional 2.0 cm layer of infected `soil mixture', and an additional 3 ml of nutrient solution are added. Reference PGPR strains, as well as untreated seeds planted in pathogen-inoculated and in noninfected mixtures of sand and vermiculite, are used as positive controls. A strain is considered a promising biocontrol agent if it performs as well as one of the reference strains.

3. Antagonistic Activity of PGPR Regulated by Siderophores

The majority of fluorescent pseudomonads are siderophore producers. These siderophores efficiently deplete iron from the environment, making it less available to certain competing microorganisms including plant pathogens.[6] The antagonistic activity of pseudomonad PGPRs can be tested by measuring their ability to inhibit the growth of *Erwinia agricola* and *F. oxysporum* on low-iron media such as SR medium and SR-Fe^{3+} (20 μg of $FeCl_3$/ml) media[24] Presumably, rhizobacteria which are able to inhibit the test microorganism on SR but not on SR-Fe^{3+} produce extracellular iron-chelating siderophores.

B. CHARACTERIZATION AT THE SPECIES LEVEL

To facilitate species characterization of PGPR, two distinct approaches can be taken: (1) isolating different strains of known species, and (2) attempting to isolate new species of bacteria, which is usually a time-consuming process. In some instances, it is difficult to choose between these two approaches because one does not always know in advance which species will be detected. However, choosing between these two possibilities is important since it can reduce the amount of labor and financial investment in cases where the researcher only needs better strains than he/she already has.

1. Primary Screening of New Isolates

This screening should be done according to morphological, physiological, nutritional, and biochemical characteristics in pure culture, with the guiding principle that more tests are better than fewer. Many such tables, lists, and tests have been published. For example, although outdated for several species, the *Bergey's Manual for Systematic Bacteriology*[38] should be consulted, at least for the genus description. The sources for species description data are the following: For *Azospirillum* ,[33,39-43] for *Herbaspirillum*,[44] and for *Acetobacter*.[19] As for pseudomonads, many species of *Pseudomonas* isolated from the field are very heterogenous, vaguely defined, and often fail to fit precisely into established taxonomic subdivisions.[45] Members of the genus *Pseudomonas* can be classified into different groups based on (1) phenotypic characteristics,[46] (2) their cultural and biochemical characters,[47,48] (3) rRNA-DNA homology,[49] and (4) composition of ubiquinone and cellular fatty acids with special reference to hydroxy fatty acids.[50-52]

C. DNA AND RNA HOMOLOGY

To relate a new strain to a known species after completion of the screening steps, DNA and RNA homology tests are reliable and very common tools. These tests are not specific to PGPR. Therefore, any general procedure of DNA and RNA homology can be used to characterize new species of PGPR such as the methods of Johnson[53] used to characterize strains of *Azospirillum*.[54,55] The main limitation of these methods is the fundamental requirement for well-defined reference strains from known culture collections for comparison with newly isolated strains.

D. PROTEIN PROFILE ANALYSES (FINGERPRINTING)

These powerful methods are based on one- or two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Soluble or total proteins of the tested isolates are compared with the corresponding proteins of reference strains, and the comparisons cannot only adequately differentiate reference strains but in many cases are sensitive at the species level. Large numbers of isolates can be characterized and compared in a relatively short period of time. Two-dimensional (2D)-total-protein analysis is suitable for the characterization of even very closely-related strains; strains that produce identical 2D fingerprints are highly related, if not identical.[56,57] These techniques prove useful for patenting procedures.

Each method is divided into two steps; the protein extraction step is crucial.

1. Soluble Protein Extraction

Soluble proteins can be obtained by strong sonication of a bacterial suspension.[58,59] The proteins present in the slurry are concentrated by mixing with acetone at a 1:5 (v/v) ratio. After centrifugation at 15,000 x g, the supernatant is discarded and the remaining acetone is evaporated from the pellet under vacuum. The resulting pellet is suspended in 62 m*M* Tris-HCl buffer (pH 6.8) supplemented with 2.3% (w/v) SDS and 5% (v/v) β-mercaptoethanol. These samples are boiled for 3 min prior to electrophoresis. Other soluble protein extraction methods described primarily for *Bradyrhizobium*[60] and *Bacillus*[61] were successfully used for the characterization of *Azospirillum*.[62,63]

2. Total Protein Extraction

***Method #1*:**[56] Pellets of washed, stationary-phase cells (in phosphate saline buffer, pH 7.2) are suspended in 0.75 ml extraction buffer containing: 0.7 *M* sucrose, 0.5 M Tris, 30 m*M* HCl, 50 m*M* EDTA, 0.1 *M* KCl, and 40 m*M* dithiothreitol, and incubated for 15 *min* at ambient temperature. An equal volume of phenol (saturated with 50 m*M* Tris-HCl, pH 8.0) is then added. The mixture is maintained under continuous shaking. Subsequent to phase separation by centrifugation, the phenol phase is recovered and re-extracted twice with an equal volume of the extraction buffer. Proteins are precipitated from the phenol phase by the addition of 5 volumes of 0.1 *M* ammonium acetate dissolved in methanol and incubation of this mixture for several hours at -20°C. The precipitate is washed twice with cold ammonium acetate solution and finally with cold 80% acetone. The pellet is air-dried and dissolved in 75 µl of lysis buffer consisting of 9.8 *M* urea, 2% (v/v) Nonidet P-40 (LKB), 100 m*M* dithiothreitol, and 2% (v/v) of a mixture of pH 5 to 7 and pH 3.5 to 10 ampholytes (LKB) at a 5:1 ratio. Samples are stored at -80°C.

***Method #2*:**[64] Each tested isolate is transferred into one well in each of two 48-well tissue culture plates (Costar) containing 500 µl of TSB (tryptic soy broth). The bacteria are incubated in the wells at 28°C for exactly 48 h. Then, glycerol is added to the fully grown cultures (final concentration, 25% [v/v]). One plate is stored at-70°C, and the other is used for further strain characterization.

The plates containing the fully grown isolates are centrifuged for 30 min at 300 x g (fixed angle) by using a special swinging adaptor. The supernatants are discarded. Each pellet is preincubated for 15 min at 37°C in 10 µl of a 1 mg/ml lysozyme solution. Total cellular proteins are extracted by boiling each pellet at 95°C for 10 min in 50 µl of sample-buffer mixture (2.5% SDS, 0.125 *M* β-mercaptoethanol, 150 m*M* Tris [pH 8.8], 4 m*M* EDTA, 0.75 *M* sucrose, 0.075% bromophenol blue) and sonicating for 10 sec. After the protein solutions have been cooled on ice, 7 µl of a 0.5 *M* iodoacetamide solution is added to each well.

3. One-Dimensional Soluble Protein Profile Analysis

Samples are subjected to one-dimensional SDS-PAGE by using 12‰ resolving and 5% stacking *gels*.[60,63] A 10 µl *sample is* loaded on *gels containing* 50 to 100 µl of protein per well, as estimated by the Bradford procedure.[65] After standard electrophoresis, the gels are stained for 1 h in 0.2% (w/v) Coomassie Brilliant Blue R-250, rinsed overnight in 7% acetic acid, and destained in a solution containing absolute methanol-glacial acetic acid-water (9:2:9, v/v/v). After photographing, the gel is scanned with a gel scanner at the narrowest slit width to give maximum resolution.

The photographs of the fingerprints are compared and sorted in fingerprint types (FPTs), which are numbered sequentially. FPTs are identified by classical biochemical tests in combination with commercial identification kits.[64] This type of characterization can also be based on cell envelope protein patterns as well as by the analysis of lipopolysaccharides.[5]

4. 2D Total Protein Profile Analysis

The first dimension of 2D gel electrophoresis, isoelectric focusing (IEF), is carried out on IEF rod gels (8 x 0.1 cm) containing 9.8 *M* urea, 2% (v/v) Nonidet P-40, 6.4% (v/v) ampholytes pH 5 to 7, 1.3% (v/v) ampholytes pH 3.5 to 10, 0.23% *NN*'-methylenebisacrylamide, and 4% acrylamide. Following prefocusing (15 min at 200 V, 30 min at 300 V, and 60 min at 400 V), 10 µl samples are loaded at the basic end of the gels and overlaid with 10 µl of a solution containing 8 *M* urea, 1% (v/v) ampholytes, 5% (v/v) Nonidet P-40, and 100 m*M* dithiothreitol. The upper (cathode) buffer consists of 20 m*M* NaOH; the lower (anode) buffer consists of 10 m*M* H_3PO_4. Focusing to equilibrium is conducted for 20 h at 400 V.

The second dimension (SDS-PAGE) is carried out in slab gels (5%o stacking gel, 15% separating gel, 0.1 % SDS). The IEF gel rods are extruded directly onto the stacking gels and covered with equilibration buffer (60 m*M* Tris-HCl, pH 6.8, 2% SDS, 100 m*M* dithiothreitol, 10% glycerol, 0.002% bromophenol blue). Low molecular weight range standards should be used. After equilibration for 10 min, gels are run at 15 mA, stained with Coomassie Brilliant Blue R-250, and dehydrated on a slab gel dryer. Results are analyzed from photographs taken of the gels.[56]

E. STRAIN IDENTIFICATION BY RFLP ANALYSIS

The technique of restriction fragment length polymorphism (RFLP) analysis is particularly useful for specific strain identification.[67] With this procedure, the total genomic DNA is isolated and cleaved with one or a number of endonucleases. The ensuing genomic DNA fragments are size-separated by agarose gel electrophoresis and probed with a cloned DNA fragment. Specificity is determined by both the probe-target sequence and restriction endonuclease digestion patterns. Probe-target sequences are often repetitive, thereby increasing detection sensitivity. Frequently used restriction enzymes have a 6-by (base pair) recognition site. These two factors, (1) repetitive target sequences and (2) very short enzyme recognition site, essentially eliminate strain variation due to sequence losses or rearrangement. They also generate an RFLP profile that, when compared with a strain's RFLP 'standard' profile, confirms or refutes strain identity.

1. Genomic DNA extraction. Pellets from 20 ml of stationary-phase cells are suspended in 50 m*M* Tris, pH 8.0, containing 20% sucrose, treated with 1 mg/ml lysozyme after the addition of EDTA to a final concentration of 25 m*M*, and lyzed in 0.5% SDS (added as 10%). Following digestion with RNase A (40 µg/ml) and proteinase K (20 µg/ml), DNA is banded in $CsCl_2$ gradients in the presence of ethidium bromide, precipitated with isopropanol, washed with 70% ethanol, and lyophilized. Pellets are suspended in distilled H_2O, and DNA concentrations are determined by absorption at 260 nm.
2. Restriction digests and probes. 3 µg of genomic DNA are incubated with 15 units of the appropriate restriction enzyme (e.g., *Eco*RI, *Pst*I or *Pvu*II) in a total volume of 28 µl for 2 h. A plasmid probe (pAM141) is labeled using either the Oligo Labeling Kit (No. 27-9250-01, Pharmacia) or the DNA Labeling and Detection Kit (nonradioactive, No. 1093-625, Boehringer Mannheim). Prehybridization and hybridization are done in 50% formamide, 5% dextran sulfate, 5%o blocking agent (Boehringer Mannheim), 5X SSC (1X SSC = 0.15 *M* NaCl plus 0.015 *M* sodium citrate), 0.1% N-lauroylsarcosine, and 0.02%o SDS at 42°C. Blots are hybridized for at least 6 h at 42°C and washed at room temperature for 2X 5 min in 2X SSC, 0.1% SDS, and at 68°C for 2X 15 min in 0.1%o SSC, 0.1% SDS.
3. Electrophoresis and blots. Restricted samples of genomic DNA are subjected to elec-trophoresis in 1 % agarose[68] and vacuum blotted to GeneScreen Plus (NEN). Size markers are produced by cutting phage lambda DNA (BRL) with both *Hind*III and *Bgl*II.

F. STRAIN IDENTIFICATION BY GAS CHROMATOGRAPHIC ANALYSIS

The method of identifying bacterial strains by gas chromatographic analysis[69] of cellular fatty acids is very useful and very accurate and can identify strains at the species level. However, an individual researcher needs to have easy access to a major bacterial culture collection. In addition, manual analysis of gas chromatograph patterns is extremely laborious, especially when there is no primary clue as to the identification of the genus in question. Computer software, which screens aerobic and clinical bacterial libraries, is commercially available. However, unless a laboratory is set up to do this on a routine basis, commercial identification services using this technique are recommended.

V. STORAGE

Isolates can be stored for short periods on 1/10 TSBA slants (OAB[28] or the combined carbon medium[16] for diazotrophs) at 2°C. For prolonged periods, isolates can be stored in 30% glycerol solutions at -15 and -70°C .[37] For indefinite storage, isolates should be lyophilized to dryness by any conventional lyophilization technique.

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REFERENCES

1. **Bashan, Y. and Levanony, H.,** Current status of *Azospirillum* inoculation technology: *Azospirillum* as a challenge for agriculture, *Can. J. Microbiol.,* 36, 591, 1990.
2. **Kloepper, J. W., Lifshitz, R., and Schroth, M. N.,** *Pseudomonas* inoculants to benefit plant production, in ISI *Atlas of Science: Animal and Plant Sciences*, 60, 1988.
3. **Kloepper, J. W., Lifshitz, R., and Zablotowicz, R. M.,** Free-living bacterial inocula for enhancing crop productivity, *Trends Biotechnol*, 7, 39, 1989.
4. **Tipping, E. M., Onofriechuk, E. E., Zablotowicz, J. W., Kloepper, J. W., and Lifshitz, R.,** Screening of bacteria isolated from peat for biocontrol of *Pythium ultimum*, in *Proceedings Symposium Peat and Peatlands*, Vol. II, Overend, R. P. and Juglum, J. K., Eds., Canadian Society of Peat and Peatlands, St. Foy, Quebec, 1989.
5. **Van Peer, R., Punte, H. L., De Weger, L. A., and Schippers, B.,** Characterization of root surface and endorhizosphere Pseudomonads in relation to their colonization of roots, *Appl. Environ. Microbiol.,* 56, 2462, 1990.
6. **Kremer, R. J., Begonia, M. F., Stanley, L., and Lanham, E. T.,** Characterization of rhizobacteria associated with weed seedlings, *Appl. Environ. Microbiol.,* 56, 1649, 1990.
7. **Lalande, R., Bissonnette, N., Coutlée, D., and Antoun, H., Identification** of rhizobacteria from maize and determination of their plant-growth promoting potential, *Plant Soil,* 115, 7, 1989.
8. **Gould, W. D., Hagedorn, C., Bardinelli, T. R., and Zablotowicz, R. M.,** New selective media for enumeration and recovery of fluorescent pseudomonads from various habitats, *Appl. Environ. Microbiol.,* 49, 28, 1985.
9. **Hagedorn, C., Gould, W. D., and Bardinelli, T. R.,** Rhizobacteria of cotton and their repression of seedling disease pathogens, *Appl. Environ. Microbial.,* 55, 2793, 1989.
10. **De Freitas, J. R. and Germida, J. J.,** Plant growth promoting rhizobacteria for winter wheat, *Can. J. Microbiol.,* 36, 265, 1990.
11. **Lindberg, T. and Granhall,** U., Isolation and characterization of dinitrogen-fixing bacteria from the rhizosphere of temperate cereals and forage grasses, *Appl. Environ. Microbiol.,* 48, 683, 1984.
12. **Thomas-Bauzon, D., Weinhard, P., Villecourt, P., and Balandreau, J.,** The spermosphere model. I. Its use in growing, counting, and isolating N_2 fixing bacteria from the rhizosphere of rice, *Can. J. Microbiol.,* 28, 922, 1982.

13. **Postgate, J. R.,** Viable counts and viability, in *Methods in Microbiology,* Vol. 1, Norris, F. N. and Ribbons, D. W., Eds., Academic Press, New York, 1969, 611.
14. **Hardy, R. W., Burns, R. C., and Holsten, R. D.,** Application of the acetylene-ethylene assay for measurement of nitrogen-fixation, *Soil Biol. Biochem.,* 5, 47, 1973.
15. **Omar, A. M. N., Richard, C., Weinhard, P., and Balandreau, J.,** Using the spermosphere model technique to describe the dominant nitrogen-fixing microflora associated with wetland rice in two Egyptian soils, *Biol. Fertil. Soils,* 7, 158, 1989.
16. Rennie, R. J., A single medium for the isolation of acetylene-reducing (dinitrogen-fixing) bacteria from soils, *Can. J. Microbiol.,* 27, 8, 1981.
17. **Döbereiner, J.,** Isolation and identification of root associated diazotrophs, *Plant Soil,* 110, 207, 1988.
18. **Döbereiner, J. and Day, J. M.,** Associative symbioses in tropical grasses: characterization of microorganisms and dinitrogen-fixing sites, in *Proc. 1 st Int. Symp. Nitrogen Fixation,* Newton, W. E. and Nyman, C. J., Eds., Washington State University Press, Pullman, U.S.A., 1976, 518.
19. **Cavalcante, V. A. and Döbereiner, J.,** Anew acid-tolerant nitrogen-fixing bacterium associated with sugarcane, *Plant Soil,* 108, 23, 1988.
20. **Stolp, H. and Gadkari, D.,** Nonpathogenic members of the Genus *Pseudomonas,* in *The Prokaryotes, Vol.* I, 2nd ed., Starr, M. P., Stolp, H., Trüper, H. G., Balows, A., and Schlegel, H. G., Eds., Springer-Verlag, New York, 1986, 722.
21. **King, E. D., Ward, M. K., and Raney, D.** E., Two simple media for the demonstration of pyocyanin and fluorescin, *J. Lab. Clin. Med.,* 44, 301, 1954.
22. **Kado, C. I. and Heskett, M. G.,** Selective media for isolation of *Agrobacterium, Corynebacterium, Erwinia, Pseudomonas,* and *Xanthomonas, Phytopathology,* 60, 969, 1970.
23. **Hagedorn, C. and Holt, J. G.,** Ecology of soil arthrobacters in Clarion-Webster toposequences of Iowa, *Appl. Microbiol.,* 29, 211, 1975.
24. **Sands, D. C. and Rovira, A. D.,** Isolation of fluorescent pseudomonads with a selective medium, *Appl. Microbiol.,* 20, 513, 1970.
25. **Berge, O., Heulin, T., and Balandreau, J.,** Diversity of diazotroph populations in the rhizosphere of maize (*Zea mays* L.) growing on different French soils, *Biol. Fertil. Soils, 11,* 210, 1991.
26. **Tyler, M. E., Milam, J R., Smith, R. L., Schank, S. C., and Zuberer, D.** A., Isolation of *Azospirillum* from diverse geographic regions, *Can. J. Microbiol.,* 25, 693, 1979.
27. **New, P. B. and Kennedy, I. R.,** Regional distribution and pH sensitivity of *Azospirillum* associated with wheat roots in eastern Australia, *Microb. Ecol.,* 17, 299, 1989.
28. **Okon, Y., Albrecht, S. L., and Burris, R. H.,** Methods for growing *Spirillum lipoferum* and for counting it in pure culture and in association with plants, *Appl. Environ. Microbiol.,* 33, 85, 1977.
29. **Reinhold, B., Hurek, T., Niemann, E: G., and Fendrik, L,** Close association of *Azospirillum* and diazotrophic rods with different root zones of Kallar grass, *Appl. Environ. Microbiol.,* 52, 520, 1986.
30. **Rodríguez Cáceres, E. A.,** Improved medium for isolation of *Azospirillum* spp., *Appl. Environ. Microbiol.,* 44, 990, 1982.
31. **Bashan, Y. and Levanony, H.,** An improved selection technique and medium for the isolation and enumeration of *Azospirillum brasilense, Can. J. Microbiol.,* 31, 947, 1985.
32. **Horemans, S., Demarsin, S., Neuray, J., and Vlassak, K.,** Suitability of the BLCR medium for isolating *Azospirillum brasilense, Can. J. Microbiol.,* 33, 806, 1987.
33. **Reinhold, B., Hurek, T., Fendrik, I., Pot, B., Gillis, M., Kersters, K., Thielemans, S., and de Ley, J.,** *Azospirillum halopraeferens* sp. nov., a nitrogen fixing organism associated with roots of Kallar grass (*Leptochloa fusca* (L.) Kunth), *lnt. J. Syst. Bacteriol.,* 37, 43, 1987.
34. **Quesada, E., Ventosa, A., Rodriguez-Valera, F., and Ramos-Cormenzana, A.**, Types and properties of some bacteria isolated from hypersaline soils, *J. Appl. Bacterial.,* 53, 155, 1982.
35. **Kole, M. M., Page, W. J., and Altosaar, I.,** Distribution of *Azotobacter* in Eastern Canadian soils and in association with plant rhizospheres, *Can. J. Microbiol.,* 34, 815, 1988.
36. **Page, W. J.,** Sodium-dependent growth of *Azotobacter chroococcum, Appl. Environ. Microbiol.,* 51, 510, 1986.
37. **Juhnke, M. E., Mathre, D. E., and Sands, D.** C., Identification and characterization of rhizospherecompetent bacteria of wheat, *Appl. Environ. Microbiol.,* 53, 2793, 1987.
38. *Bergey's Manual for Systematic Bacteriology,* Williams & Wilkins, U.S.A.
39. **Bashan, Y., Singh, M., and Levanony, Y.,** Contribution of *Azospirillum brasilense* Cd to growth of tomato seedlings is not through nitrogen fixation, *Can. J. Bot.,* 67, 2429, 1989.
40. **Bashan, Y., Mitiku, G., Whitmoyer, R. E., and Levanony, H.,** Evidence that fibrillar anchoring is essential for *Azospirillum brasilense* Cd attachment to sand, *Plant Soil,* 132, 73, 1991.
41. **Döbereiner, J., Marriel, I. E., and Nery, M.,** Ecological distribution of *Spirillum lipoferum* Beijerinck, *Can. J. Microbiol.,* 22, 1464, 1976.

42. **Magalhães, F. M. M. and Döbereiner, J.,** [Occurrence of *Azospirillum amazonense* in some amazonian ecosystems], *Rev. Microbiol., São Paulo*, 15, 246, 1984 (in Portugese).
43. **Tarrand, J. J., Krieg, N. R., and Döbereiner, J.,** A taxonomic study of the *Spirillum lipoferum* group, with descriptions of a new genus, *Azospirillum* gen. nov. and two species, *Azospirillum lipoferum* (Beijerinck) comb. nov. and *Azospirillum brasilense sp.* nov., *Can. J. Microbiol.*, 24, 967, 1978.
44. **Baldani, J. L, Baldani, V. L. D., Seldin, L., and Döbereiner, J.,** Characterization of *Herbaspirillum seropedicae* gen. nov., sp. nov., a root-associated nitrogen-fixing bacterium, *Int. J. Syst. Bacterial.*, 36, 86, 1986.
45. **Lifshitz, R., Kloepper, J. W., Scher, F. M., Tipping, E. M., and Laliberté, M., Nitrogen-fixing** pseudomonads isolated from roots of plants grown in the Canadian high arctic, *Appl. Environ. Microbiol.*, 51, 251, 1986.
46. **Stanier, R. Y., Palleroni, N. J., and Doudoroff, M.,** The aerobic pseudomonads: a taxonomic study, *J. Gen. Microbial.*, 43, 159, 1966.
47. **Hugh, R. and Gilardi, G. L.,** *Pseudomonas*, in *Manual of Clinical Microbiology*, 2nd ed., American Society for Microbiology, Washington, D.C., 250, 1974.
48. **Holding, A. J. and Collee, J. G.,** Routine biochemical tests, in *Methods in Microbiology*, Vol. 6A, Norris J. R. and Ribbons, D. W., Eds., Academic Press, New York, 1971, 1.
49. **Palleroni, N., Kunisawa, J. R., and Contopoulou, R.,** Nucleic acid homologies in the genus *Pseudomonas*, *Int. J. Syst. Bacteriol.*, 23, 333, 1973.
50. **Ikimoto, S., Kuraishi, H., Komagata, K., Azuma, M., Suto, T., and Murooka, H.,** Cellular fatty acid composition in *Pseudomonas* species, *J. Gen. Appl. Microbiol.*, 24, 199, 1978.
51. **Oyaizu, H. and Komagata, K.,** Grouping of *Pseudomonas* species on the basis of cellular fatty acid composition and their quinone system with special reference to the existence of 3-hydroxy fatty acids, *J. Gen. Appl. Microbiol.*, 29, 17, 1983.
52. **Watanabe, L, Rolando S. O., Ladha, J. K., Katayama-Fujimura, Y., and Kuraishi, H.,** A new nitrogenfixing pseudomonad: *Pseudomonas diazotrophicus* sp. nov. isolated from the root of wetland rice, *Can. J. Microbial.*, 33, 670, 1987.
53. **Johnson, J. L.,** Genetic characterization, in *Manual of Methods for General for General Bacteriology*, Gerhardt, P., Murray, R. G. E., Costilow, R. N., Nester, E. W., Wood, W. A., Krieg, N. R., and Phillips, G. B., Eds., American Society for Microbiology, Washington, D.C., 1981, 450.
54. **Falk, E. C., Döbereiner, J., Johnson, J. L., and Krieg, N. R.,** Deoxyribonucleic acid homology of *Azospirillum amazonense* Magalhães et al. 1984 and emendation of the description of the genus *Azospirillum*, *Int. J. Syst. Bacteriol.*, 35, 117, 1985.
55. **Falk, E. C., Johnson, J. L., Baldani, V. L. D., Döbereiner, J., and Kreig, N. R.,** Deoxyribonucleic and ribonucleic acid homology studies of the genera *Azospirillum* and *Congtomeromonas*, *Int. J. Syst. Bacterial.*, 36, 80, 1986.
56. **De Mot, R. and Vanderleyden, J.,** Application of two-dimensional protein analysis for strain fingerprinting and mutant analysis of *Azospirillum* species, *Can. J. Microbiol.*, 35, 960, 1989.
57. **Lambert, B., Meire, P., Joos, H., Lens, P., and Swings, J.,** Fast-growing, aerobic, heterotrophic bacteria from the rhizosphere of young sugar beet plants, *Appl. Environ. Microbiol.*, 56, 3375, 1990.
58. **Bally, R., Givaudan, A., Bernillon, J., Heulin, T., Balandreau, J., and Bardin, R.,** Numerical taxonomic study of three N_2-fixing yellow-pigmented bacteria related to *Pseudomonas paucimobilis*, *Can. J. Microbiol.*, 36, 850, 1990.
59. **Kersters, K. and De Ley, J.,** Identification and grouping of bacteria by numerical analysis of their electrophoretic protein patterns, J. *Gen. Microbial.*, 87, 333, 1975.
60. **Kamicker, B. J. and Brill, W. J.,** Identification of *Bradyrhizobium japonicum* nodule isolates from Wisconsin soybean farms, *Appl. Environ. Microbiol.*, 51, 487, 1986.
61. **Shivakumar, A. G., Gundling, G. J., Benson, T. A., Casuto, D., Miller, M. F., and Spear, B. B., Vegetative** expression of the delta-endotoxin genes of *Bacillus thuringiensis*, sub-species Kurstaki in *Bacillus subtilis*, *J. Bacterial.*, 166, 194, 1986.
62. **Bilal, R., Rasul, G., Qureshi, J. A., and Malik, K.** A., Characterization of *Azospirillum* and related diazotrophs associated with roots of plants growing in saline soils, *World* J. *Microbiol. Biotechnol.*, 6, 46, 1990.
63. **Sundaram, S., Arunakumari, A., and Klucas, R. V.,** Characterization of azospirilla isolated from seeds and roots of turf grass, *Can. J. Microbiol.*, 34, 212, 1988.
64. **Lambert, B., Leyns, F., Van Rooyen, F., Gosselé, F., Papon, Y., and Swings, J.,** Rhizobacteria of maize and their antifungal activities, *Appl. Environ. Microbiol., 53, 1866*, 1987.
65. **Bradford, M.,** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal. Biochem.*, 72, 248.
66. **O'Farrell, P. H.,** High resolution two-dimensional electrophoresis of proteins, J. *Biol. Chem.*, 250, 4007,

67. **Brown, G., Khan, Z., and Lifshitz, R.,** Plant growth promoting rhizobacteria: strain identification by restriction fragment length polymorphisms, *Can. J. Microbiol.*, 36, 242, 1990.
68. **Maniatis, T., Fritsch, E. F., and Sambrook, J.,** Molecular cloning: a laboratory manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.
69. **Sasser, M.,** Identification of bacteria through fatty acid analysis, in *Methods in Phytobacteriology*, Klement, Z., Rudolph, K., and Sands, D. C., Eds., AKademiai Kiado, Budapest, 1990, 199.
70. **Holguin, G., Guzman, M. A., and Bashan, Y.,** Two new nitrogen-fixing bacteria from the rhizosphere of mangrove trees: their isolation, identification and in vitro interaction with rhizosphere *Staphylococcus* sp., *FEMS Microbiol. Ecol.*, 101, 207, 1992.