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ISOLATION AND IDENTIFICATION OF NIF GENE HOMOLOGUE OF LOCALLY ISOLATED AZORHIZOBIUM STRAINS FROM STEM NODULES OF SESBANIA ROSTRATA

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By

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LIST OF ABBREVIATIONS

Symbol	Description
h	hour
N	nitrogen
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
rRNA	ribosomal RNA
SSU	small subunit
nif	nitrogen fixation
YL	yeast extract-lactate
YM	yeast extract-mannitol
GM	glutamate medium
PA	peptone medium
CCC	covalently closed circular
OC	open circular
Mdal	megadalton
SDS	sodium dodecyl sulphate
CFU	colony forming unit

EDTA	ethylenediamine tetraacetic acid
C_2H_4	acetylene
TE8	Tris-EDTA pH8
ARA	acetylene reduction assay
IAR	intrinsic antibiotic resistance

Abstract of thesis submitted to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science.

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Faculty : Agriculture

Characterisation of UPMR 43, UPMR 44 and X was conducted. Morphological studies of colonies showed that all strains gave a relatively large gummy, watery, translucent or whitely opaque colour on a modified glutamate agar. Intrinsic antibiotic resistance (IAR) patterns of all strains indicated that they were resistant to ampicillin of up to 50 mg/l but susceptible to streptomycin, tetracycline and kanamycin at concentrations of 25, 15 and 10 mg/l, respectively. The growth characteristics of UPMR43 and UPMR44 (in a modified glutamate medium (GM)

and peptone medium (PA)) was studied using shake flask method. UPMR43 grew better in GM compared to PA but the reverse was true for UPMR44. PA significantly reduced the cell concentration and affected growth characteristic. The generation times for both strains in both media were less than 6 h indicating that they were fast growers. The banding pattern of total DNA of three strains digested with *Eco*RI showed that they were very similar.

A large plasmid, subject to confirmation was detected in UPMR44 using Hirsch method and one small plasmid in UPMR43 using Casse method. A 13.5 kb *Bam*HI and a 7.0 kb *Eco*RI DNA fragment from total DNA extracts of all local strains were detected in Southern blot experiments using probes carrying the *nif*HDK genes of *Klebsiella pneumoniae* and *Azotobacter vinelandii*. The results showed that the *nif* genes of the *Azorhizobium* strains were detectable using the heterologous *nif* probes from *K. pneumoniae* and *A. vinelandii*. The similar results obtained from colony morphology, generation time, IAR patterns, total DNA restriction patterns and DNA-DNA hybridisation of the three strains studied suggests that they may be the same strain. The obtained results also showed that local strains have a similar characteristics with *A. caulinodans* ORS571. Strain X may be considered a unique strain since *S. rostrata* was easily infected although it is not native to Malaysia. It is postulated that this strain may have an alternative host plant.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan Ijazah Master Sains.

PEMENCILAN DAN MENGENALPASTI GEN *NIF* HOMOLOG DARIPADA STRAIN *AZORHIZOBIUM* TEMPATAN DIASINGKAN DARIPADA NODUL BATANG *SESBANIA ROSTRATA*

OLEH

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APRIL 1997

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Kajian pencirian lanjutan ke atas strain UPMR43, UPMR44 dan X telah dijalankan. Morfologi koloni bagi semua strain adalah melekat, berair, lutcahaya atau warna putih legap pada agar glutamat yang telah diubahsuai. Corak kerintangan antibiotik dalaman menunjukkan semua strain rintang kepada ampisilin sehingga 50 mg/l tetapi tidak rintang kepada streptomisin 25 mg/l, tetrasiklin 15 mg/l dan kanamisin 10 mg/. Kesan dua jenis media, medium glutamat yang telah diubahsuai

(GM) dan medium pepton (PA) ke atas ciri pertumbuhan bagi strain UPMR43 dan UPMR44 telah dikaji dengan menggunakan eksperimen kelalang goncang. UPMR43 tumbuh lebih baik dalam medium GM daripada PA tetapi berlawanan dengan UPMR44. Medium PA berupaya merendahkan kepekatan sel dan mempengaruhi ciri pertumbuhan strain. Masa generasi atau masa sel membahagi dua bagi kedua-dua strain dan pada dua media yang berbeza adalah kurang daripada enam jam. Ini menunjukkan bahawa kedua-dua strain adalah strain petumbuh cepat. Corak jalur bagi keseluruhan DNA untuk ketiga-tiga strain yang dihadamkan dengan *Eco*RI menunjukkan ketiga-tiga strain mempunyai corak jalur yang sama.

Kemungkinan satu plasmid yang besar telah dapat dikesan pada UPMR44 yang dipencilkan dengan menggunakan kaedah Hirsch dan satu plasmid kecil pada UPMR43 yang dipencilkan dengan kaedah Casse. Fragmen berukuran 13.5 kb dan 7.0 kb telah berjaya dikesan daripada ektrak DNA keseluruhan yang dihadamkan secara berasingan dengan *Bam*HI dan *Eco*RI pada ketiga-tiga strain melalui eksperimen Southern blot dengan menggunakan prob gen *nif* daripada *Klebsiella pneumoniae* dan *Azotobacter vinelandii*. Keputusan menunjukkan bahawa gen *nif* pada *Azorhizobium* boleh dikesan menggunakan prob *nif* heterolog daripada *K. pneumoniae* dan *A. vinelandii*. Keputusan yang sama diperolehi daripada ujian morfologi koloni, masa generasi, corak IAR, corak jaluran DNA keseluruhan dan

hibridisi DNA-DNA ke atas ketiga-tiga strain menunjukkan kemungkinan mereka adalah merupakan satu strain yang sama. Hasil kajian juga mendapati bahawa strain tempatan mempunyai ciri yang hampir sama dengan *A. caulinodans* ORS571. Strain X dianggap sebagai strain unik kerana *S. rostrata* mudah diinfeksi walaupun ia bukan berasal dari Malaysia. Adalah dipostulasikan bahawa kemungkinan strain X mempunyai perumah alternatif.

CHAPTER I

INTRODUCTION

The discovery of stem nodulation of *Sesbania rostrata*, a tropical legume, by Dreyfus and Dommergues (1981) prompted interest in the study of stem-nodulating legumes and their microsymbionts. *S. rostrata* was first found colonizing waterlogged soils in the Sahel region of West Africa and has an ability to form nitrogen-fixing nodules on both the roots and stems. The most studied bacterial strain, ORS 571 from *S. rostrata* stem nodules resembles the fast growing *Rhizobium* species in its generation time, but its capacity for nonsymbiotic nitrogen fixation, the size and shape of nodules it forms are more typical of the slow growing *Bradyrhizobium* (Dreyfus et al., 1984). Because of their uniqueness, Dreyfus et al. (1988) assigned these bacteria to a new genus and species, *Azorhizobium caulinodans*.

A. caulinodans strain ORS 571 displayed unusual features due to its ability to form effective nitrogen-fixing nodules on both stems and roots of the tropical legume, *S. rostrata* (Dreyfus and Dommergues, 1981). Apart from that, ORS 571 strain was also unique among N_2-fixing bacteria because it grew rapidly at the expense of NH_4^+ or N_2 as the sole source of nitrogen and fixes N_2 in the free-living

state as well (Dreyfus et al., 1983). *A. caulinodans* in association with *S. rostrata* could fix more than 200 kg N ha^{-1} in 50 days (Rinaudo et al., 1983).

Unlike other fast-growing rhizobia which showed the presence of symbiotic megaplasmids (Corbin et al., 1983; Downie et al., 1984), no plasmid has been successfully isolated from ORS 571 strain. In fact, the *nif* gene of ORS571 has been shown to be located on the chromosomal DNA, despite it being a fast grower. Large plasmids known as megaplasmids of molecular weight of up to 400 X 10^6 dalton in several strains of fast growing rhizobia have been described (Nuti et al., 1977; Denarie et al., 1981; Casse et al., 1979). These megaplasmids have been proven to bear genes controlling symbiotic properties such as host specificity and nodule information (Beynon et al., 1980 and Hooykaas et al., 1981) and nitrogen fixation (Nuti et al., 1977; Prakash et al., 1981 and Krol et al., 1982).

To date, two *Azorhizobium* strains, UPMR 43 and 44 (Shamsuddin, unpublished) have been isolated from sandy tin tailings, UPM. These strains have been shown to cause stem nodulation in *S. rostrata*. This new discovery prompted the study on the existence of *nif* gene that is responsible for nitrogen fixation in these strains. As an approach to identify *nif* gene in these bacteria, a research project was carried out with the following objectives:

(1) To isolate *Azorhizobium* from stem nodules of *S. rostrata* grown in paddy-field.

(2) To characterise the locally isolated *Azorhizobium* strains.

(3) To determine the presence and to isolate plasmid from these strains.

(4) To identify the existence of *nif* gene by using *nif* gene probes of *Klebsiella pneumoniae* and *Azotobacter vinelandii* .

CHAPTER II

LITERATURE REVIEW

Nitrogen-Fixing Bacteria

Nitrogen fixation is the reduction process of gaseous nitrogen (dinitrogen, N_2) to ammonia. Biological nitrogen fixation is apparently carried out only by certain prokaryotes (called *diazotrophs*), including various cyanobacteria, members of the Azotobacteriaceae, Methylococcaceae, Rhizobiaceae, Rhodospirillaceae, Actinomycetaceae and some *Bacillus* and *Clostridium* species. Diazotrophic organisms may be free-living in soil or water but many occur in mutualistic association with other organisms (which benefit from the supply of fixed nitrogen). Some *Azotobacter* species fix nitrogen in soils and some in association with the roots of the grass *Paspalum notatum* whereas *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* species fix nitrogen in leguminous roots and stem nodules.

Taxonomy

The determination of exact taxonomic position of root- and stem-nodulating strains from *S. rostrata* is currently undergoing. However Sprent (1989) has suggested a possible taxonomic relationships between these strains and other rhizobia.

Formerly, bacteria which form nitrogen-fixing nodules on leguminous plants have been divided into two genera, *Rhizobium* and *Bradyrhizobium* (Jordan, 1984). The genus *Rhizobium*, the fast-growing bacteria are comprised of four species; *Rhizobium leguminosarum*, *Rhizobium meliloti*, *Rhizobium loti and Rhizobium fredii*. They form nodules on roots of leguminous plants in temperate zones. However, the *species R. fredii* has been placed in the new genus *Sinorhizobium* (Scholla and Elkan, 1984). The other genus, *Bradyrhizobium* comprises only of one well-defined species, *Bradyrhizobium japonicum* which is a slow grower and forms nodules on roots of tropical leguminous plants and some temperate leguminous plants.

Bacteria that are able to produce N_2-fixing nodules both on the roots and on the stems of *S. rostrata* has been discovered by Dreyfus and Dommergues (1981). Two types of strains are associated with *S. rostrata* as described below (Dreyfus et al., 1984) : (i) Stem-nodulating strains which fix atmospheric N_2 in culture and grow at the expense of this fixed N_2 (Dreyfus et al., 1983) and always nodulate both stems and roots of *S. rostrata* (Gebhardt et al., 1984), referred to strain ORS571, and (ii) Some strains do not fix N_2 in culture and generally nodulate the roots of *S. rostrata* only, named root nodulating strains (Dreyfus et al., 1986).

Based on the results of deoxyribonucleic acid (DNA)-ribosomal ribonucleic acid (rRNA) hybridisations (Jarvis et al., 1986), two *Sesbania* root-nodulating strains, ORS22 and ORS51 are closely related to genus *Rhizobium*. Inversely, stem and root-nodulating strains ORS571 is genotypically a member of

Rhodopseudomonas palustris- B. japonicum rRNA branch in rRNA superfamily IV sensu (De Ley, 1978). ORS571 strain is closely related to *Xanthobacter* but they are phenotypically and genotypically different from the *Rhizobium* and *Bradyrhizobium* genus to deserve a separate generic rank (Dreyfus et al., 1988) (Figure 1).

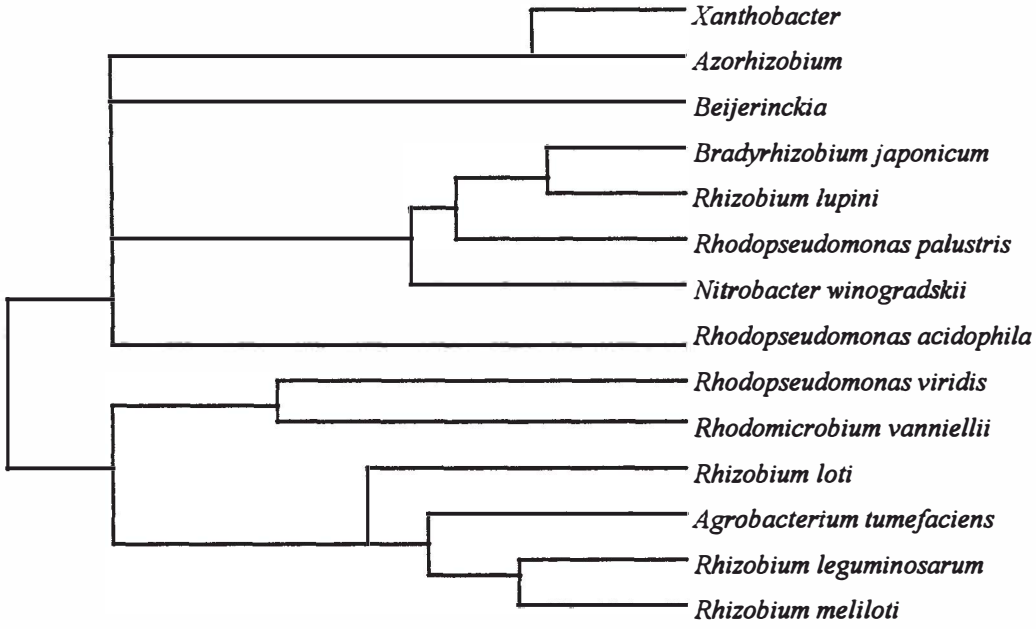

Figure 1 : Possible evolutionary/taxonomic relationships amongst bacteria related to rhizobia (After Sprent, 1989)

Therefore, a new genus, *Azorhizobium* is proposed with one species, *Azorhizobium caulinodans*. The type strain is strain ORS571. Although a second species has been recognized by DNA-DNA hybridisation assays (Rinaudo et al., 1991), the species cannot be named until they can be differentiated by phenotypic tests. The important features of *Rhizobium*, *Bradyrhizobium* and *Azorhizobium* are listed in Table 1. Recently, the potential of using sequences of the small subunit ribosomal RNA (SSU or 16S rRNA) as a good phylogenetic tool has been reviewed

(Young and Haukka, 1996). The SSU data support the well-established subdivision of rhizobia into three genera: *Rhizobium*, *Bradyrhizobium* and *Azorhizobium*. Table 2 gives a list of the names of *Rhizobium* species currently in use.

Table 1

Some relevant features of rhizobial genera

Feature	*Rhizobium*	*Bradyrhizobium*	*Azorhizobium*
Flagella on			
liquid medium	0	0	1 lateral
solid medium	peritrichous	1 polar or sub-polar	peritrichous
N_2 fixation *ex planta*	rare	some strains	generic character
Growth on N_2 fixed ex planta	0	0	generic character
Growth rate in culture	usually fast	usually slow	fast
Location of *nod* and *nif* genes	mainly plasmid	mainly chromosomal	probably chromosomal
Host specificity range	usually narrow	often broad	only one species so far identified

Source : (Sprent and Sprent, 1990)

Table 2

Taxonomic classification of rhizobia

Recognized Genera	Typical species
Rhizobium	*R. leguminosarum* *R. tropici* *R. etli*
Sinorhizobium	*S. meliloti* *S. fredii* *S. saheli* *S. teranga*
Mesorhizobium	[*Rhizobium*] *loti* [*Rhizobium*] *huakuii* [*Rhizobium*] *ciceri* [*Rhizobium*] *tianshanense* [*Rhizobium*] *mediterranium*
Another genus	[*Rhizobium*] *galegae*
Bradyrhizobium	*B. japonicum* *B. elkanii* *B. iaoningense*
Azorhizobium	*A. caulinodans*

(Source : Young and Haukka, 1996)

Characteristics of *Azorhizobium caulinodans* Strain ORS571

Azorhizobium caulinodans cells are Gram negative, small rods that are 0.5 to 0.6 by 1.5 to 2.5 μm and motile. The cells have peritrichous flagella on solid medium and one lateral flagellum in liquid medium. The morphology of their colonies on agar are circular and have a creamy colour. They are obligately aerobic, fix atmospheric N_2 under microaerobic conditions and grow well on N_2 with vitamins present in a

nitrogen-free medium whereas *Rhizobium* and *Bradyrhizobium* could not. Besides the vitamins, nicotinic acid is required for N_2 fixation under microaerobic conditions. The nitrogenase activity is 30 nmol of C_2H_2 produced per mg of protein per min which is 3 to 6 times better than that for *Bradyrhizobium*. They differ from *Bradyrhizobium* by their fast growing characteristic; their generation times are 3 and 5 h in yeast extract-lactate (YL) (Dreyfus et al., 1983) and yeast extract-mannitol (YM) (Vincent, 1970) medium, respectively.

A. caulinodans also do not assimilate sugars except glucose but assimilate alcohols such as 1,2-propanediol and 2,3-butanediol unlike *Rhizobium* and *Bradyrhizobium*. Organic acids such as fumarate, glutamate, malate, α-ketoglutarate and citrate are the preferred carbon substrates for both NH_4^+ and N_2-dependent growth. Growth occurs from 12 to 43°C and good growth at pH range of 5.5 to 7.8 . *Azorhizobium* strains are tolerant to carbenicillin but sensitive to nalidixic acid whereas *Rhizobium* strains show inverse responses (Robertson et al., 1995).

Stem Nodulation

To date, there are three genera of stem-nodulating legumes known; *Sesbania*, *Aeschynomene* and *Neptunia*. Their predetermined nodulation site can be observed up to 2 m above the water level in 3 meter tall plants (Dreyfus and Dommergues, 1981). These plants can also form nodules on both stems and roots at the same time. The stem nodulation site of *S. rostrata* have two consistently unique characteristics: