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I am submitting herewith a dissertation written by Anjana Rajen Sharma entitled "Characterization of root stimulating bacterium and its root stimulating abilities." I have examined the final electronic copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Botany.

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CHARACTERIZATION OF ROOT STIMULATING BACTERIUM AND ITS ROOT STIMULATING ABILITIES

A Dissertation
Presented for the
Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Anjana R. Sharma
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DEDICATION

This thesis is dedicated to my family

for their love, support and encouragement.

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ABSTRACT

In this study, the characterization of a novel root stimulating bacterium (RSB) is reported and the root stimulating ability of RSB is compared to *Sphingomonas* and other related bacteria. A morphological examination of RSB was conducted. Nutritional and biochemical characterization was performed in order to obtain a profile of RSB. Pigment analysis indicated the presence of nostoxanthin, a pigment commonly found in *Sphingomonas* bacteria. Lipid analysis of RSB revealed the presence of sphingolipids in addition to other fatty acids suggesting similarity with other *Sphingomonas* bacteria. Pulse-field gel electrophoresis of RSB and *Sphingomonas echinoides* showed a high level of difference in the DNA of these two organisms suggesting difference at least at the strain level.

The root stimulating ability of RSB, *Sphingomonas* and other related bacteria were evaluated using mung bean bioassays. Results of these bioassays suggest that the spent medium used for culturing RSB and *Sphingopyxis macrogoltabida* contain acidic and neutral compounds that contribute to their promotion of adventitious root formation whereas the spent medium used to culture *Novosphingobium capsulata, Sphingomonas echinoides* and *Sphingomonas paucimobilis* contains root stimulating components of acidic nature. Thin layer chromatography of acidic fractions obtained from RSB, *Sphingomonas echinoides, Sphingomonas paucimobilis, Sphingopyxis macrogoltabida* and *Novosphingobium capsulata* spent growth medium showed Rf values close to those for the standards indolelactic acid (ILA) and indoleacetic acid (IAA).

Based on all these tests, RSB most closely resembles bacteria of the *Sphingomonas* group and the rooting ability observed for RSB is characteristic of some of the *Sphingomonas* and related bacteria.

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ABBREVIATIONS

ANOVA	analysis of variance
BSTFA	bis(trimethylsilyl)trifluoroacetamide
CHEF	contour-clamped homogeneous electric field
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ETC	electron transport chain
FA	ferulic acid
GC	gas chromatography
GLM	general linear model
HPLC	high-performance liquid chromatography
IAA	indole-3-acetic acid
IAM	indole acetamide
IBA	indole butyric acid
ILA	indole lactic acid
IPA	indole propionic acid
MS	mass spectrometry
mWPM	modified woody plant medium
NA	nutrient agar
NAA	naphthaleneacetic acid
PFGE	pulse-field gel electrophoresis
PHA	poly hydroxyalkanoic acid
PHB	poly-β-hydroxy butyrate
PLFA	polar lipid fatty acid
RBD	randomized block design
rRNA	ribosomal ribonucleic acid
RSB	root stimulating bacterium
TLC	thin layer chromatography
WPM	woody plant medium
WRC's	wounding related compounds

CHAPTER 1

INTRODUCTION

Root stimulating bacterium (RSB) was discovered as a pine (*Pinus elliottii*) culture medium contaminant. Interest in this bacterium arose as a result of its ability to stimulate adventitious rooting in difficult-to-root cuttings and in vitro cultured plants. The roots stimulated by this bacterium resembled natural roots produced by plants and were not like the short, stubby roots sometimes formed upon external hormone treatment. In addition, this bacterium was non-pathogenic to the plants tested. An extract obtained from the spent medium in which RSB was cultured was capable of inducing rooting in several different groups of plants. This indicated that the bacterium was producing a compound/s that stimulated adventitious rooting and that the presence of the bacterium itself was not required for rooting activity. The rooting ability of RSB is thus chemically induced and hence different from that observed with *Agrobacterium* where genetic exchange is involved. RSB was isolated and its root stimulating abilities were partially characterized and patented (Patent # 5,629,468).

The first of the two main objectives of this research was to accumulate information about RSB that would facilitate its identification. The following steps were taken in order to achieve the objective of characterizing the bacterium:

A. Morphological analysis of RSB using transmission electron microscopy,
B. Study of culture characteristics and utilization of amino acids permitting growth of RSB cells,

C. Biochemical analyses including enzymatic activity and carbon source utilization of RSB,

D. Pigment analysis,

E. Lipid composition of RSB cells, and

F. Pulse field gel electrophoresis (PFGE) to compare RSB and *Sphingomonas echinoides.*

Preliminary experiments showed that in addition to RSB culture medium extracts, *S. echinoides* culture medium extracts were also capable of stimulating rooting in mung bean (*Vigna radiata*) seedlings. The second objective of this investigation was to determine if this rooting ability was specific for the bacteria mentioned above or if it was of a more widespread occurrence among *Sphingomonas* bacterial species. The following steps were taken to achieve this objective:

A. Selection of bioassay for rooting experiments,

B. Selection of bacterial species to be used in rooting experiments,

C. Preparation of bacterial extracts,

D. Testing extracts using the mung bean bioassay, and

E. Test for the presence of indoles.

CHAPTER 2

LITERATURE REVIEW

A. Root stimulating bacterium -

RSB was isolated as a contaminant from in vitro cultured slash pine (*Pinus elliottii* Engelm.) seedling explants because of its ability to stimulate a greater degree of rooting than was normally observed in hypocotyls cuttings of slash pine seedlings (Burns and Schwarz, 1996). Initial attempts to identify the bacteria through application of standardized testing methods failed as it would not grow on the media normally used in these diagnostic tests. RSB grows well on modified woody plant medium (mWPM) (Burns and Schwarz, 1996) and liquid potato medium (Patent # 5,629,468). Good growth has also been observed in tryptic soy broth/agar and Voges-Proskauer medium after lowering the pH of the medium to 5.5 (Carole Dabney-Smith – personal communication). RSB grows well when cultured between 20 and 35°C with an optimum growth temperature of 27 – 30°C (Patent # 5,629,468). RSB cells are Gram-negative rods and the colonies formed are yellow, circular and convex with a smooth margin. RSB cells are 0.65 – 0.7 μm in length, stalked, having a holdfast and occasionally forming rosettes (Figure 1). A general pigment profile by Venkataraman (1994) based on absorption spectra suggests the presence of carotenoid pigment/s. Based on literature review of pigments in other related organisms, Venkataraman proposed that the carotenoid could possibly be nostoxanthin. The sequence of 16S rRNA gene of RSB and a phylogenetic analysis with 17 other sequences of members of α subdivision of proteobacteria suggests close similarity of RSB to *Brevundimonas subvibrioides* (previously known as

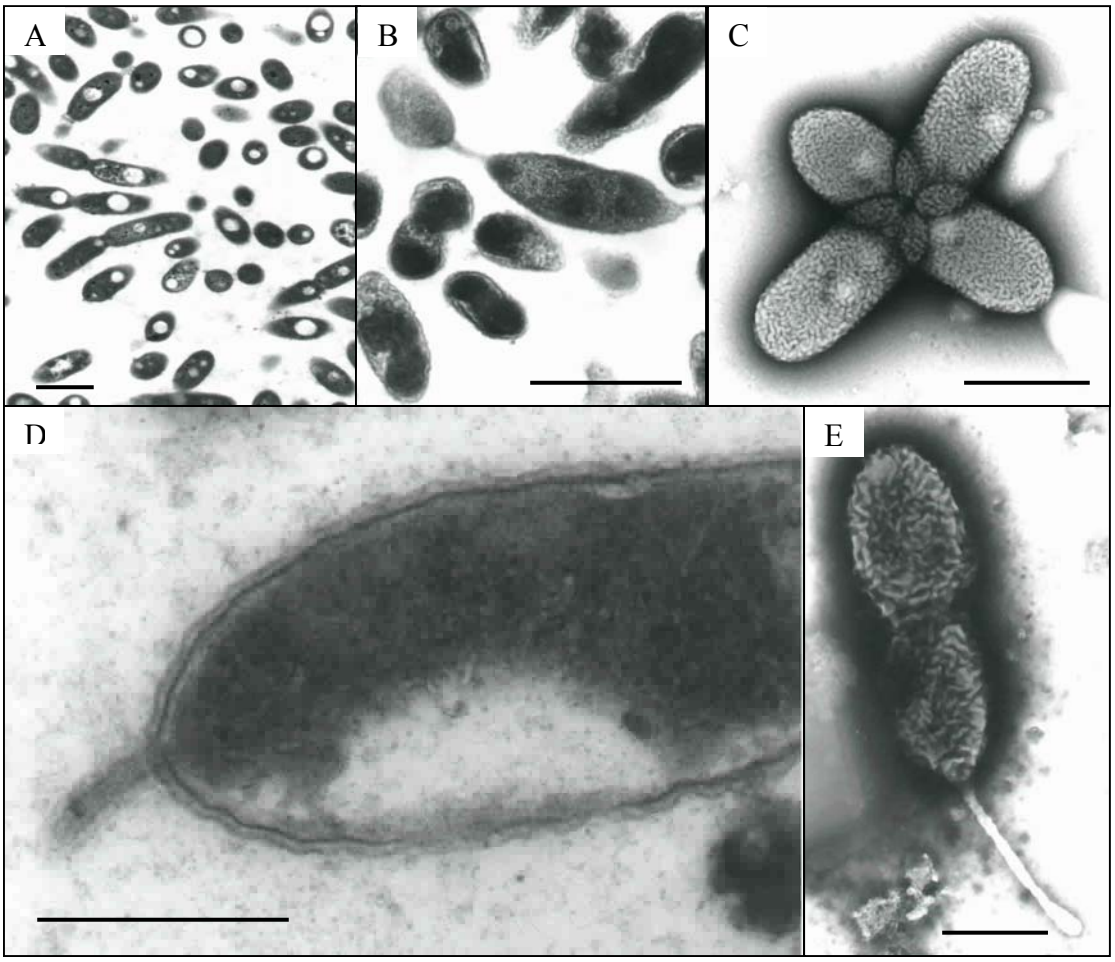


Figure 1. Electron micrographs of RSB. (A) Thin section of RSB. Bar = 1µm. (B) Thick section of RSB. Bar = 1µm. (C) Negative staining of rosette formation of RSB cells in Woody plant medium. Bar = 1µm. (D) Section of RSB cell showing holdfast. Bar = 0.5µm. (E) Negative staining of RSB cell showing holdfast. Bar = 0.5µm. Photographs by Dr. J. Dunlap, University of Tennessee, Knoxville.

Caulobacter subvibrioides), *Flavobacterium devorans* and *Sphingomonas paucimobilis* (Venkataraman 1994).

RSB and extracts prepared from the RSB culture medium have stimulated rooting in difficult-to-root and in vitro cultured plants such as *Pinus elliottii* (Burns and Schwarz 1996), dogwoods (Sharma 1999) and bamboo (Schwarz, Personal communication).

B. Characterization of bacteria -

Methodologies for characterization of bacteria have involved the study of several phenotypic properties such as morphology, structure, cultivation, nutrition, biochemistry, metabolism, pathogenicity, antigenic properties and ecology (Krieg 1994). A common problem associated with this approach is that different test methods are chosen depending on the organism. This can make comparisons difficult at times. Another complicating factor is that classifications based on phenotypic similarities do not always correlate with phylogenetic relationships (relationships based on the ancestry of organisms) (Krieg 1994). In recent years, microbial taxonomists have considered nucleic acid hybridization and sequencing studies as better and more rational ways to designate species and determine relationships between different organisms (Towner 1993). The 16S rRNA molecule is now widely recognized and used in the phylogenetic placement of bacterial species (Woese 1987). Increasing use of such molecular techniques have led to revisions among several groups of organisms. For example, based on studies of the 16S rRNA gene sequences, the species of the previously recognized genus Sphingomonas were subdivided into four new genera: *Sphingomonas sensu stricto*, *Sphingobium*, *Novosphingobium* and *Sphingopyxis* (Takeuchi 2001). A few chemotaxonomic and

phenotypic differences were observed among them. However, these characters alone could not be used to differentiate between the groups and the final classification of these organisms involved a combined approach using phylogenetic, chemotaxonomic and phenotypic approaches.

The review by Busse et al. (1996) provides a good description of the recommended methods for identification of bacteria. They emphasize the importance of a combined approach for identification of bacterial isolates that includes cultural characterization, morphological studies, physiological characterization, biochemical tests, chemotaxonomic analysis, genotypic studies and phylogenetic analysis.

Wayne et al. (1987) have provided a molecular definition of a species, where in a species should include strains with approximately 70% or greater DNA-DNA relatedness, and with 5°C or less divergence values (ΔT_m). However, they suggest giving a formal name to only those species that can be differentiated by phenotypic properties. Murray et al. (1990) point out that defining genera and species purely on the basis of phylogenetic data is impracticable. They suggest initially characterizing genera on the basis of phenotypic properties. Stackebrandt and Goebel (1994) also strongly discourage the sole use of 16S rDNA sequence in affiliating an isolate to a known species – even in cases of 99.5% sequence similarity.

At present, the approach for prokaryotic taxonomy consists of obtaining the 16S rDNA sequence and using it to determine the phylogenetic position of an isolate relative to its nearest phylogenetic neighbor. This position is then used to determine the appropriate methods to be used in identifying the isolate as belonging to a previously described taxon or as a guide in the description of a species (Stackebrandt and Pukall

1999). DNA-DNA hybridization values are considered very important for delineation of bacterial species.

Thus, an integration of phenotypic and phylogenetic data seems to be the most satisfactory approach to bacterial classification.

C. The genus *Sphingomonas* -

The genus *Sphingomonas* was erected by Yabuuchi et al. (1990) belongs to the α-4 subclass of proteobacteria and includes *Sphingomonas paucimobilis* as the type species. Since its creation, several bacteria from diverse environments have been included in this group. This genus includes strictly aerobic, Gram-negative rod shaped bacteria that have a G+C content of 61-67% and contain a unique sphingolipid replacing the lipopolysaccharide of Gram-negative bacteria. Bacteria placed under this genus contain ubiquinone-10 (Q-10), show the presence of 2-hydroxymyristic acid (14:0 2-OH), absence of 3-hydroxy fatty acids, spermidine or sym-homospermidine as the major polyamine and nostoxanthin carotenoid pigment. The distribution of Sphingomonads is widespread as bacteria belonging to this group have been isolated from soil, river water, deep-sea sediments, hospital supplies, corroding copper pipes, the rhizosphere and plant surfaces (Laskin and White 1999; White 1996). These bacteria have been known for their capacity to degrade environmental pollutants (Leung et al. 1999) and are sources of valuable biopolymers (Ashtaputre and Shah 1995; Pollock, 1993; White et al. 1996).

Later, Takeuchi et al. (2001) divided the formerly known *Sphingomonas* group that contained approximately 30 established and 2 invalidated species into 4 new genera: *Sphingomonas sensu stricto* (type species - *Sphingomonas paucimobilis*), *Sphingobium*

(type species - *Sphingobium yanoikuyae*), *Novosphingobium* (type species – *Novosphingobium capsulata*) and *Sphingopyxis* (type species - *Sphingopyxis macrogoltabida*) (Table 1). This proposal was based on phylogenetic analysis of 16S rRNA gene, fatty acid profiles, polyamine patterns and other chemotaxonomic and phenotypic analyses.

D. Pulse-field gel electrophoresis -

PFGE, first described by Schwartz and Cantor (1984), involved short pulses of alternating electric fields that were perpendicular to each other and allowed separation of large DNA molecules between 50 and 2000 kb. An improvement to this technique that is commonly used now was made by Chu et al. (1986) who devised the contour-clamped homogeneous electric field (CHEF) system where electric field was reoriented at 120° angles resulting in clearer, more reproducible bands and straight lanes of DNA bands.

The PFGE technique involves three main steps: (i) Preparation of unsheared DNA in agarose blocks – A bacterial suspension made from cultures in mid- to late-log phase of growth is mixed with an agarose solution and pipetted into molds to form plugs of bacterial cells in agarose. The agarose protects the DNA from being sheared during the extraction process. The bacterial cell wall is then lysed with lysozyme and proteinase K solutions. (ii) Digestion with rare cutting enzymes – Based on the approximate G+C % values of bacterial DNA, rare cutting restriction endonucleases are chosen. Plugs with high molecular weight DNA are treated with these enzymes overnight. (iii) Electrophoresis – DNA fragments are separated electrophoretically.

Table 1: Characteristics of the genera *Sphingomonas, Sphingobium, Novosphingobium* and *Sphingopyxis.* Data compiled from Takeuchi et al. (2001)

	Genera			
Characteristics	***Sphingomonas***	***Sphingobium***	***Novosphingobium***	***Sphingopyxis***
Gram reaction	Negative	Negative	Negative	Positive
Spore production	-	-	-	-
Shape	Rods	Rods	Rods	Rods
LxB (um)	0.3 - 0.8 x 1.0 - 1.9	0.3 - 0.7 x 1.0 - 3.5	0.3 - 0.5 x 1.0 - 3.0	0.3 - 0.5 x 0.9
Motility	Motile/Non-motile	Motile/Non-motile	Motile/Non-motile	Motile/Non-motile
Colony color	Yellow or off-white	Yellow or whitish-brown	Yellow or whitish-brown	Yellow or whitish-brown
Atmospheric growth conditions	Strictly aerobic and chemo-organotrophic	Strictly aerobic and chemo-organotrophic	Strictly aerobic and chemo-organotrophic	Strictly aerobic and chemo-organotrophic
Catalase rxn.	Positive	Positive	Positive	Positive
Nitrate reduction	mostly +ve with a few exceptions	Negative	Positive	Negative
Respiratory quinone	Q-10	Q-10	Q-10	Q-10
Fatty acids	Major FA - 18:1, 16:0 & /or 17:1	Major FA - 18:1, minor - 16:0	Major FA - 18:1, minor - 16:0	Major FA - 18:1, 16:0 & 16:1
Major 2-hydroxy fatty acids	2-OH 14:0 or 2-OH 15:0	2-OH 14:0	2-OH 14:0	2-OH 14:0, 2-OH 15:0 &/or 2-OH 16:0
Glycosphingolipids	Present	Present	Present	Present
Polyamine	Homospermidine	Spermidine	Spermidine	Spermidine

Table 1: Continued.

	Genera			
Characteristics	***Sphingomonas***	***Sphingobium***	***Novosphingobium***	***Sphingopyxis***
G+C content (mol %)	62 - 68	62 - 67	62 - 67	63 - 65
Characteristic 16S rRNA signatures at positions	52:359 (C:G), 134 (G), 593 (G), 987:1218 (G:C) and 990:1215 (U:G)	52:359 (U:A), 134 (G), 593 (U), 987:1218 (A:U) and 990:1215 (U:G)	52:359 (U:A), 134 (G), 593 (U), 987:1218 (A:U) and 990:1215 (U:G)	52:359 (C:G), 134 (G), 593 (U), 987:1218 (G:C) and 990:1215 (U:G)
Habitat	Soil and clinical specimens	Soil, pentachlorophenol-contaminated soil and clinical specimes	Soil, roots of rose tree, clinical specimens, stocked distilled water, coastal plain sediments and fluidized-bed reactor	Soil and underground water
Organisms included in this group	*Sphingomonas adhaesiva, S. assacharolytica, S. echinoides, S. mali, S. parapaucimobilis, S. paucimobilis, S. pruni, S. sanguinis and S. trueperi*	*Sphingobium chlorophenolicum, S. herbicidovorans, S. yanoikuyae*	*Novosphingobium aromaticivorum, N. capsulatum, N. roseae, N. stygiae, N. subarcticum, N. subterraneae*	*Sphingopyxis macrogoltabida, S. terrae*
Type species	*Sphingomonas paucimobilis*	*Sphingobium yanoikuyae*	*Novosphingobium capsulatum*	*Sphingopyxis macrogoltabida*

Parameters affecting the separation of DNA fragments using PFGE are agarose concentration, DNA quality, DNA concentration, switching times, electric field strength, temperature and ionic strength of buffer (Kaufmann and Pitt 1994).

Schwartz and Cantor (1984) have described the detailed mechanism of PFGE. The pores formed in the agarose gel are smaller than the coiled DNA molecules. To facilitate the movement of DNA through the gel the coils must elongate parallel to the electric field. The electric field in one direction is shut off and another field is generated at a different angle. The DNA then reorients in order to move through the gel. This allows separation of larger DNA fragments.

The PFGE technique has been used to separate yeast chromosomes (Chu et al. 1986; Schwartz and Cantor 1984), estimate genome size and construct chromosome maps (Thong et al. 1997; Wagner et al. 1998) and to determine genomic diversity of strains of pathogenic bacteria (Anthony et al. 2002; Saunders et al. 1997). PFGE was chosen as a method for detecting differences at the DNA level between RSB and *Sphingomonas echinoides* in this research project.

E. Review of adventitious root formation -

Roots that arise from sites in the plant other than their normal sites in the embryo or the primary root are defined as adventitious. Adventitious root formation is an important step in vegetative propagation that is widely used in agriculture, forestry and horticulture (Hartmann et al. 1990). Methods to improve rooting and understand the rooting process are of great importance since the difficulty of rooting is still the major obstacle to successful propagation. Adventitious roots develop from already

differentiated cells that dedifferentiate to initiate a new root meristem. Not all cells are found to be competent for the formation of adventitious roots. Depending on the plant, different cell types give rise to adventitious roots. However in general adventitious roots typically arise from parenchymatous cells around or close to the vascular bundles.

In auxin treated cuttings, auxin enters the plant through the cut surface (Kenney et al. 1969) and is taken up in the cells by pH trapping (Rubery and Sheldrake 1973) and influx carriers (Delbarre et al. 1996). The dedifferentiated cells, that are competent, can respond to the auxin that is taken up. The cells from which the root primordia originate are usually between or adjacent to the vascular bundles (Blakesley et al. 1991, Hartmann et al. 1990, Klerk et al. 1999) and show the accumulation of starch in the initial induction phase (Klerk et al. 1999). The cells then divide to form a meristemoid of about 30 cells with the concomitant loss of starch grains. After this stage, auxin is no longer required and that it can be inhibitory for further growth. The meristemoids develop into dome shaped root primordia and then into roots during the differentiation phase.

Various researchers divide the rooting process into different phases based either on physiology (Eriksen 1974, Gasper et al. 1992, 1994, 1997, Klerk et al. 1999, Smith and Thorpe 1975) or histology (Jasik and Klerk 1997, Sircar and Chatterjee 1973). Nag et al. (2001) have divided the rooting process into three interdependent physiological phases called induction (0-24 h), initiation (24-72 h) and expression (after 72 h). The inductive phase consists of the biochemical events preceding the initiation of the cell division that lead to the formation of root primordial. Auxins have been known to be involved in the stimulation of adventitious root formation (Thimann 1936; Torrey 1950, 1956; Wrightman et al. 1980; Gasper et al 1994). Changes in polyamine and peroxidase

contents within the plant have also been observed during the root inductive phase (Gasper et al 1994, Nag et al. 2001). An increase in the endogenous level of free indole-3 acetic acid (IAA) is seen in the inductive phase of rooting followed by a decrease in IAA levels during the initiative and expressive phases. The study of peroxidase activity by Nag et al. (2001) showed low activity during the root inductive phase and higher activity during the initiative phase. Thus the curves for IAA and peroxidases appear to be the reverse of one another. Studies on rooting of poplar shoots in vitro have shown an increase in the level of putrescine, close to when an increase in the IAA level is observed. IAA and putrescine are known to control cell division cycles and may be required to initiate cell divisions at the end of the rooting inductive phase however the exact relationship between auxin and polyamine content is not clearly understood yet.

Levels of endogenous phenolics have also been shown to increase in cuttings during rooting, which is consistent with the observations that phenolics accumulate as a result of wounding. Of the various phenolic compounds tested by Klerk et al. (1999), ferulic acid (FA) was the most active compound and it strongly enhanced rooting in the presence of IAA but not in the presence of 1-naphthaleneacetic acid (NAA). Since IAA can be oxidized, but NAA cannot, Klerk et al. (1999) suggest that FA may be acting as an inhibitor of auxin oxidation.

When a plant tissue is wounded, cell compartments are destroyed and catabolic enzymes capable of degrading cell walls and membranes are released (Klerk et al. 1999). The breakdown products of these cell structures are referred to as wounding-related compounds (WRCs) play an important role in the dedifferentiation phase of rooting (Van der Krieken et al. 1997). WRC's possibly increase the uptake of auxin or reduce the

conjugation or oxidation of applied auxin. Klerk et al. (1999) suggest that WRC's possibly enhance the competence of the tissue to respond to plant hormones.

Auxin stimulated rooting, in some cases, is thought to result from stimulated ethylene production by the treated tissue. In rice plants, submergence in water acts as the primary signal leading to an accumulation of ethylene in the plant through both increased ethylene biosynthesis and physical entrapment as a result of low rate of diffusion of ethylene in water. Alternately ethylene may enhance sensitivity to auxin (Liu and Reid 1992, Visser et al. 1996).

Application of cytokinin has an inhibitory effect on root formation, however a small amount of cytokinin along with higher auxin concentrations may be necessary for rooting (Davis and Haissig, 1990). Gibberellic acid has been widely reported to (with a few exceptions) inhibit adventitious root formation in cuttings (Davis and Haissig, 1990). Abscisic acid has shown varying responses ranging from inhibition, to no known effect, to promotion of adventitious rooting (Davis and Haissig, 1990).

Besides efforts to identify the chemical nature of compounds that stimulate adventitious root formation, recent molecular work has resulted in the identification and characterization of genes that are expressed during lateral and adventitious root formation. A hydroxyproline-rich glycoprotein (HRGPnt3) is expressed transiently in a subset of cells that are actively involved in root initiation (Vera et al. 1994). [HRGPnt3 is a cell wall protein and it may modify the structure of the cell wall of those cells that penetrate the other cell layers before emergence]. The Lateral root primordium 1 (LRP1) gene expression was observed in lateral and adventitious root primordia in *Arabidopsis* and may be involved in initiating primordium development (Smith and Fedoroff 1995).

LRP1 gene expression is activated during the very early stages of root primordium development and is turned off before the emergence of lateral roots from the parent root. Thus LRP1 and HRGPnt3 genes can be useful molecular markers for studying the early stages of lateral and adventitious root primordium development. Similarly, the root meristemless (*rml*) mutants of *Arabidopsis* are characterized by growth arrest of lateral and adventitious roots at a size of less than 2 mm, and the *RML* genes can thus be considered as markers for late lateral and adventitious root development including emergence of the root primordium (Cheng et al. 1995).

Before cell division, a high expression of gene cdc2 coding for A-type cyclin (cyclins are proteins that complex with and activate protein kinases or cyclin dependent kinases) was observed in areas of active cell division where lateral or adventitious roots are formed (Hemerly et al. 1993). Light, wounding and hormones such as auxin and cytokinin can induce cdc2 expression and they may thereby induce competence to divide.

In an effort to better understand the rooting process, Ermel et al. (2000) have combined histological studies with gene expression patterns in walnut cotyledon explants. The expression of gene LRP was induced early on between 0 and 48 hours after auxin treatment. Expression of γ-TIP (Tonoplast induced protein) gradually increased from 48-96 hours after auxin exposure and then declined while the chalcone synthase gene (CHS) showed gradually increasing expression from 0-120 hrs. γ-TIP and CHS are also known to be activated during primary root development.

F. Bacterial stimulated rooting -

Bacterial stimulated rooting has also been observed in many plants. Several authors have reported successful rooting using *Agrobacterium rhizogenes* in different plant species such as almond (Damiano et al. 1998), apple (Sutter and Luza 1993), *Eucalyptus* (MacRae and Van Staden 1993), Kiwi (Rugini et al. 1991), *Larix* (McAfee et al. 1993), *Pinus* (McAFee et al. 1993, Magnussen et al. 1994, Mihalievic et al. 1996, Tzfira et al. 1996) and walnut (Caboni et al. 1996). Root induction is due to the integration and subsequent expression of a portion of the bacterial DNA (T-DNA) from the Ri (Root Inducing) plasmid in the plant genome (Damiano and Monticelli, 1998). Four loci, involved in root formation, have been identified in the T-DNA, and called root loci (*rol*) A, B, C, D (Spena et al. 1987). Besides *A. rhizogenes*, *A. tumefaciens* is also commonly used to transform plants (Han et al. 1997).

A large number of bacteria are known to produce plant growth regulating substances, mainly of the gibberellin and auxin type (Arshad and Frankenberger, 1998; Brown, 1972). Bacteria producing root-promoting substances have been isolated from the rhizosphere and identified as belonging to the genera *Azotobacter, Nocardia, Arthrobacter, Flavobacterium, Brevibacterium and Pseudomonas* (Brown, 1972, Brown and Burlingham (1968). Interaction between bacteria, protozoans and plants has also been suggested (Clarholm, 1985; Bonkowski and Brandt, 2002) for the root stimulating process and the mechanism involved is known as the 'microbial loop in soil'. According to this mechanism, soil bacteria obtain some of the carbon present in their biomass from root exudates and grow. Protozoans prey on the bacteria and release nitrogen which becomes available for plant growth. Protozoans liberate tryptophan, a precursor of IAA,

from preyed microbial cells and this further stimulates growth of IAA producing bacteria. Release of IAA from soil bacteria increases root growth which again stimulates bacterial and protozoan growth.

CHAPTER 3

MATERIALS AND METHODS

A. Source of bacterial strains and cultivation-

RSB glycerol cultures stored at –80°C (ATCC accession number 55580) were used. *Sphingomonas echinoides* (ATCC 14820) and *Sphingopyxis macrogoltabida* (ATCC 51380) cultures were obtained from the American Type Culture Collection (Manassas, VA, USA) as freeze-dried samples. Glycerol cultures of *Sphingomonas paucimobilis*, *Sphingobium yanoikuyae* and *Novosphingobium capsulata* were provided by Julia Gouffon from the Bacterial collection at the Center for Biomarker Analysis, University of Tennessee, Knoxville and were stored at –80°C. Freeze dried samples were revived by resuspending in nutrient broth (NB) according to the instructions provided by ATCC and stored as glycerol cultures at –80°C. All bacteria except *S. yanoikuyae* were subsequently cultured in mWPM at 30°C. *S. yanoikuyae* failed to grow well in mWPM and was therefore cultured in tryptic soy broth/agar at 37°C. For lipid analysis, RSB and *S. echinoides* were also cultured on nutrient agar (NA) at 30°C.

Modified WPM was prepared by combining 2.3 g/L of Lloyd and McCown's woody plant medium basal salts (Phytotechnology laboratories Inc., Shawnee Mission, KS) with 20 g/L sucrose, 0.1 g/L sucrose, 2 g/L casein hydrolysate, 0.5 mg/L nicotinic acid, 0.5 mg/L pyridoxine, 1 mg/L thiamine HCl, 2 mg/L glycine and 15 g/L bacto-agar (Difco laboratories, Detroit, MI). The pH of the medium was adjusted to 5.2 prior to autoclaving.

B. Microscopic examination of RSB -

The fine structure of the RSB was examined with an electron microscope (Hitachi H-800) using both negative staining of entire cells and glutaraldehyde and osmium tetroxide (OsO_4) fixation of thin sections (Appendix 1 & 3).

(i) Preparation of bacterial cultures used in microscopic examination:

RSB cells used in microscopic analysis were obtained from glycerol stocks and were cultured in Erlenmeyer flasks containing mWPM in the dark at 30°C on a shaker at 100 rpm for either 48 h or 96 h.

(ii) Negative staining:

A drop of the bacterial suspension in mWPM was placed on a formvar and carbon coated copper grid for one min followed by staining with 2% uranyl acetate (pH 4.5). A detailed description of the procedure is given in Appendix 1.

(iii) Thin sections of RSB cells:

A detailed description of glutaraldehyde and OsO_4 fixation of cells for preparation of thin sections is given in Appendix 3. Cell pellets were obtained by centrifuging bacterial suspension at 2000 rpm for 10 min. The cells were fixed in 3% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and rinsed in phosphate buffer (0.2 M). Post fixation of cells was carried out with 2 % OsO_4 in 0.1 M phosphate buffer (pH 7.2) for one hour at room temperature. The cells were then dehydrated in an increasing series of acetone

solutions (50, 70, 90, 100, 100 % and absolute acetone) and infiltrated and embedded in molds filled with Spurrs epoxy resin. Thin sections were cut with a Reichert OMU3 microtome (Reichert, Vienna, Austria). The thin sections were stained with saturated uranyl acetate in 50% ethanol for 30 min and post stained with lead citrate for 5 min. The cells were examined under a Hitachi H 800 (Hitachi High Technologies America, Pleasanton, CA) transmission electron microscope operating at 75 kV.

C. Nutritional and biochemical characterization -

(i) Amino acid requirement for growth:

A 2 d old culture of RSB cells was grown in petri dishes containing mWPM. Cells were scraped from the agar surface and used to prepare a suspension of bacterial cells in mWPM lacking additional glycine or casein hydrolysate and having an absorbance of 1.5 at λ =600 nm. One ml of this bacterial suspension was used as inoculum per 50 ml of medium amended with individual amino acids.

D-amino acids and cystine used in the experiment were obtained from Sigma (Sigma-Aldrich Inc., St. Louis, MO). Twenty-four flasks containing 50 ml mWPM lacking additional glycine and casein hydrolysate were prepared. Individual amino acids were added to each flask to get a concentration of 1.6 mM. All amino acids except cysteine and glutamine were added before autoclaving media. Cysteine was prepared just prior to use and both cysteine and glutamine were filter sterilized and added to cooled media. One ml of RSB cell suspension in distilled water having an OD of 1.5 at λ=600 was added to each flask containing 50 ml of medium. The flasks were incubated in the

dark at 30°C on a shaker at 100 rpm for 72 h. Absorbance at λ =600 nm was measured on a Hitachi U2000 spectrophotometer (Hitachi Instruments Inc., Buford, GA).

(ii) Biochemical analyses:

Preliminary physiological and biochemical characterization of RSB was attempted using the kit API 20NE (bioMerieux Inc., MO, U.S.A.), however no results were obtained from this kit as the bacterium failed to grow on the test kit media. Similar problems were also encountered earlier when RSB cultures were sent to various laboratories for biochemical analysis.

RSB was tested with the BIOLOG system (Biolog, CA, USA.) for utilization or oxidation of 95 different carbon sources. However, RSB failed to grow on the recommended medium and was cultured on mWPM before testing using the Biolog system. This deviation from the recommended protocol resulted in several intermediate reactions that could not be clearly distinguished as positive or negative responses.

(a) Tests for enzymatic reactions:

A study of 19 enzymatic reactions was carried out using the API ZYM test kit (bioMerieux Inc., MO, U.S.A.) as per instructions provided by the supplier. The API ZYM test is a semi-quantitative method consisting of a strip with 20 microwells (cupules), the base of which contains the enzymatic substrate and its buffer. The cupules were inoculated with a dense suspension (McFarland turbidity of 5-6) of RSB in sterile distilled water. *Sphingomonas echinoides* was also tested similarly. The metabolic end

products produced after an incubation period of 24 h were visually detected through colored reactions upon addition of the reagents ZymA and ZymB and recorded.

(b) Tests for carbohydrate metabolism:

The test kit API 50 CH (bioMerieux Inc., MO, U.S.A.) was used to test for carbohydrate metabolism of RSB and *Sphingomonas echinoides*. This test kit consists of 50 microtubes each containing a defined amount of dehydrated substrate. One ml of bacterial suspension in water was prepared to a turbidity level equivalent to McFarland standard 4 and was added to 10 ml of CHBE medium (bioMerieux Inc., MO, U.S.A.) and mixed well. This suspension was pipetted into the microtubes and incubated for 24 - 96 h. No growth or color change reaction was observed in any of the reaction tubes for both RSB as well as *S. echinoides* after 24 and 48 hours. However, at 96 h results were observed and recorded.

(c) Oxidase test:

The oxidase test was performed by rubbing a loop containing freshly cultured RSB/*S. echinoides* cells on a filter paper moistened with 0.5 ml of a 1% solution of tetramethyl-ρ-phenylene diamine dihydrochloride (Smibert 1994). For the next few seconds, the filter paper was observed for development of blue-purple color.

(d) Catalase test:

Catalase activity was tested using a 3% hydrogen peroxide solution (Smibert 1994). Freshly cultured RSB/*S. echinoides* cells were smeared in the center of a glass

slide. Three-four drops of 3% hydrogen peroxide were placed over the bacterial cells and covered with a slide cover. The slides were observed for the evolution of gas bubbles under the cover glass during the next 1-2 min.

D. Pigment analysis -

(i) Pigment extraction:

Pigments were extracted using the method of Saito et al. (1994) with slight modifications (See protocol in Appendix 5). RSB and *S. echinoides* cells were grown in 1 L mWPM at 30°C in the dark at 100 rpm for 5 d. Cells were collected by centrifugation at 16, 000 g (10,000 rpm) for 15 mins at 4°C. To remove saponifiable material, 10N KOH was added to the cells and the suspension was charged with N_2 gas and stirred for 24 h at room temperature. The suspension was neutralized to a pH of 7.0 with conc. HCl in an ice bath. Two volumes of acetone were added to the suspension and incubated at room temperature for one hour. Supernatant was collected after centrifugation at 25,000 g (14,000 rpm) and 4°C for 10 min. An equal volume of ethyl ether was added to the supernatant. Distilled water was added to allow separation into two layers. The ether layer (top) containing the pigments was dried using a rotary evaporator and the pigments were resuspended in 10-15 ml of benzene. The pigments were stored at 0°C in a closed vial charged with N_2 gas.

(ii) Spectrometry:

Absorption spectra of RSB pigments in different solvents like benzene, ethanol and methanol were obtained with a Beckman DU Series 600 (Beckman Instruments Inc., Fullerton, CA) spectrophotometer.

(iii) Thin-layer chromatography (TLC):

Thin layer chromatography was performed on 20 x 20 cm silica gel GF 250 μm TLC plates (Fisher Scientific, Pittsburgh, PA). Pigments dissolved in benzene were spotted on the plates and developed with acetone:benzene (36:64; v/v). Rf values were calculated and the pigment spots were eluted separately with benzene, ethanol and methanol.

(iv) High performance liquid chromatography (HPLC):

Pigments extracted from RSB and *S. echinoides* were tested with HPLC using the method of Bally et al. (1990). The instruments used were Waters 2690 separations module (Waters Corporation, Milford, MA) with a Waters 2487 Dual λ absorbance detector. The column used was a Waters C_{18} column with 4 μm particle size. Pigments were run in a 90:10 Methanol, water solvent and absorbance measured at 452 and 480 nm.

(v) Mass spectrometry (MS):

Pigment samples were analyzed using Electron Ionization Mass Spectrometry at the MS facility in Department of Chemistry (University of Tennessee, Knoxville). The instrument used was ZAB-EQ (Micromass, Originally VG-Analytical, Manchester, U.K).

E. Lipid analysis -

(i) Polar lipid fatty acid (PLFA) extraction:

Total lipids from lyophilized cells of RSB and *Sphingomonas echinoides*, cultured on mWPM plates at 30°C in the dark for 5 d, were extracted using a modified Bligh-Dyer procedure (White et al. 1979) (See Appendix 7 for a detailed protocol). Silicic acic column chromatography was used to separate lipids into neutral lipids, glycolipids and polar lipids (Guckert et al. 1985). Polar lipids were transesterified into methyl esters by alkaline methanolysis for Gas chromatography (GC) analysis (Guckert et al. 1985).

(ii) PLFA analysis:

The instrument used was a Hewlett Packard (HP) 5890 series II GC equipped with a HP 5973 series mass selective detector (Hewlett-Packard Co, Palo Alto, CA, USA). A fused-silica column (Rtx®-1; Restek Corporation, Bellefonte, PA, USA) was used. Fatty acid methyl esters were identified by comparing peak retention times with known standards. They were also confirmed by the mass spectra obtained for each of those peaks.

Analysis of variance was performed using the GLM procedure with STATISTICA software (Statsoft Inc., Tulsa, OK, USA). The differences in the mean proportion of PLFA between various treatments were tested using Tukey's Honest-Significant-Difference procedure.

(iii) Sphingolipid extraction:

Sphingolipid extraction was performed as described by Leung et al. (1999) (See Appendix 8 for a detailed protocol). Total lipid of the RSB and *S. echinoides* samples cultured in either mWPM or NA was extracted using the modified Bligh and Dyer procedure (White et al. 1979). The lipid extract was dried under a stream of nitrogen gas at room temperature, redissolved in 1ml of 3 M hydrochloric acid and heated at 100°C for 3 h. The acid hydrolyzed sample was cooled to room temperature and made strongly alkaline by the addition of KOH. The digest was extracted with chloroform, dried under N_2 and derivatized using 100 μl bis(trimethylsilyl)trifluoroacetamide (BSTFA) at 60°C for 30 min. Samples were dried under nitrogen and resuspended in 40 μl of hexane and shot (injection volume = 1 μl) immediately into the GC-MS.

(iv) Sphingolipid analysis:

The instrument used for sphingolipid analysis was the same as that used to analyze PLFA. The instrument used was a Hewlett Packard (HP) 5890 series II GC equipped with a HP 5973 series mass selective detector (Hewlett-Packard Co, Palo Alto, CA, USA). A fused-silica column (Rtx®-1; Restek Corporation, Bellefonte, PA, USA)

was used. Sphingolipids were identified by comparing sample mass spectral profiles with profiles of known standards.

F. Pulse-field gel electrophoresis -

PFGE was performed as described in Appendix 9 & 10. Preparation of unsheared DNA in agarose blocks and cell lysis was carried out according to the protocol of Riethman et al. (1997). RSB and *S. echinoides* cells grown in mWPM for 3 d were collected by centrifugation at 6000 rpm and 4°C for 10 min. Cell pellet was washed once in 3 ml of NaCl/Tris (pH 7.2)/EDTA and resuspended in 1 ml of NaCl/Tris (pH 7.2)/EDTA. This cell suspension and 2% agarose were mixed in a 1:10 ratio and poured into molds (Bio Rad laboratories, CA, USA) with the help of a 3 ml syringe. Plugs were removed from the mold and stored in 50 mM EDTA at 4-5°C. The plugs to be digested were prepared for cell wall lysis by placing them in eppendorf tubes with 1 ml of bacterial cell lysis solution and incubated for 24 hours at 37°C. The cell lysis solution was discarded and the plugs were placed in 1 ml of Proteinase-K solution and incubated at 50°C for 24 hours. Following Proteinase-K treatment, the plugs were rinsed in 2-3 ml of 50 mM EDTA for 30 min. The plugs were washed three times in 2-3 ml of TE containing 1 mM PMSF for 30 min each.

Digestion of DNA and PFGE was carried out according to the protocol of Lou Tisa, University of New Hampshire, USA; personal communication with minor modifications by Dr. B. Mullin, University of Tennessee, Knoxville. Plugs were cut into 1/3rd along the width and washed in distilled water for 1 h with gentle shaking in order to remove EDTA. These plugs were then equilibrated in restriction enzyme buffer for 30

min. Plugs were incubated in digest buffer 2 times for 15 min each. Fresh digest buffer was then added along with 50 U of the restriction enzyme and incubated at 37°C for 24 h. A second sample set of RSB and *S. echinoides* plugs was digested for an additional 24 h with fresh 50 U of restriction enzyme.

Selection of restriction enzymes for PFGE was based on percent G + C content of 65.8 % reported for *S. echinoides* (Owen and Jackman, 1982). Due to the high G + C content of *S. echinoides*, enzymes that recognize A + T-rich sequences were tested. The enzymes used included *Xba*I (5′-T↓CTAGA-3′), *Dra*I (5′-TTT↓AAA-3′), *Spe*I (5′-A↓CTAGT-3′) and *Nde*I (5′-CA↓TATG-3′). Restriction enzymes were obtained from Fisher Scientific, NJ, USA.

After digestion with restriction enzymes, the plugs were incubated in ESP (EDTA/sodium dodecyl sulfate/proteinase-K) at 50°C for 2 h followed by two washes in PFGE buffer. Markers used for PFGE were lambda DNA concatemers and yeast chromosome markers (BioRad Laboratories, CA, USA). PFGE was carried out using the contour-clamped homogeneous electric field (CHEF) method on a CHEF DR®-II system (BioRad Laboratories, CA, USA). The gel was run using a 1% agarose gel and 0.5X TBE buffer for 19-20 hours at 14°C and 200 V with a pulse time of 40 seconds. The gel was stained with 200 ml of 0.5 X TE containing 10 µl of 10 mg/ml ethidium bromide in the dark for 15 min followed by destaining in distilled water for 10 min. The gel was observed under UV light and the image was digitally captured using the AlphaImager 2200 (Alpha Innotech Corp., San Leandro, CA).

G. Rationale for selection of bacteria to test the spread of bacterial stimulated rooting activity -

The 16S rRNA sequence (Venkataraman 1994) was used to search for other closely matching sequences in Genbank database using BLAST® (Basic Local Alignment Search Tool) that was accessed through the National Center for Biotechnology Information (NCBI) website www.ncbi.nlm.gov (Bethesda, MD, USA). The RSB sequence showed the highest (97%) sequence similarity with the 16S rRNA sequence of *S. echinoides* and most of the 25 closest matches were members of the *Sphingomonas* group. Sequences from the 25 closest matches were compared using the program ClustalX 1.8 (Thompson et al. 1997). The phylogenetic tree obtained (Figure 2) showed close resemblance to the tree obtained by Takeuchi et al (2001).

Takeuchi et al. (2001) examined complete 16S rRNA gene sequences of the 30 established and 2 invalidated species placed in the *Sphingomonas* group and proposed a reclassification scheme for these organisms. The phylogenetic tree obtained by them showed 4 main clusters that are now recognized as 4 new genera - *Sphingomonas sensu stricto* (type species - *Sphingomonas paucimobilis*), *Sphingobium* (type species - *Sphingobium yanoikuyae*), *Novosphingobium* (type species – *Novosphingobium capsulata*) and *Sphingopyxis* (type species - *Sphingopyxis macrogoltabida*).

To test how widespread the bacterial stimulated rooting activity is, the type species of each of the newly formed genera along with *Sphingomonas echinoides*, the organism that RSB most closely resembles, were included in this study.

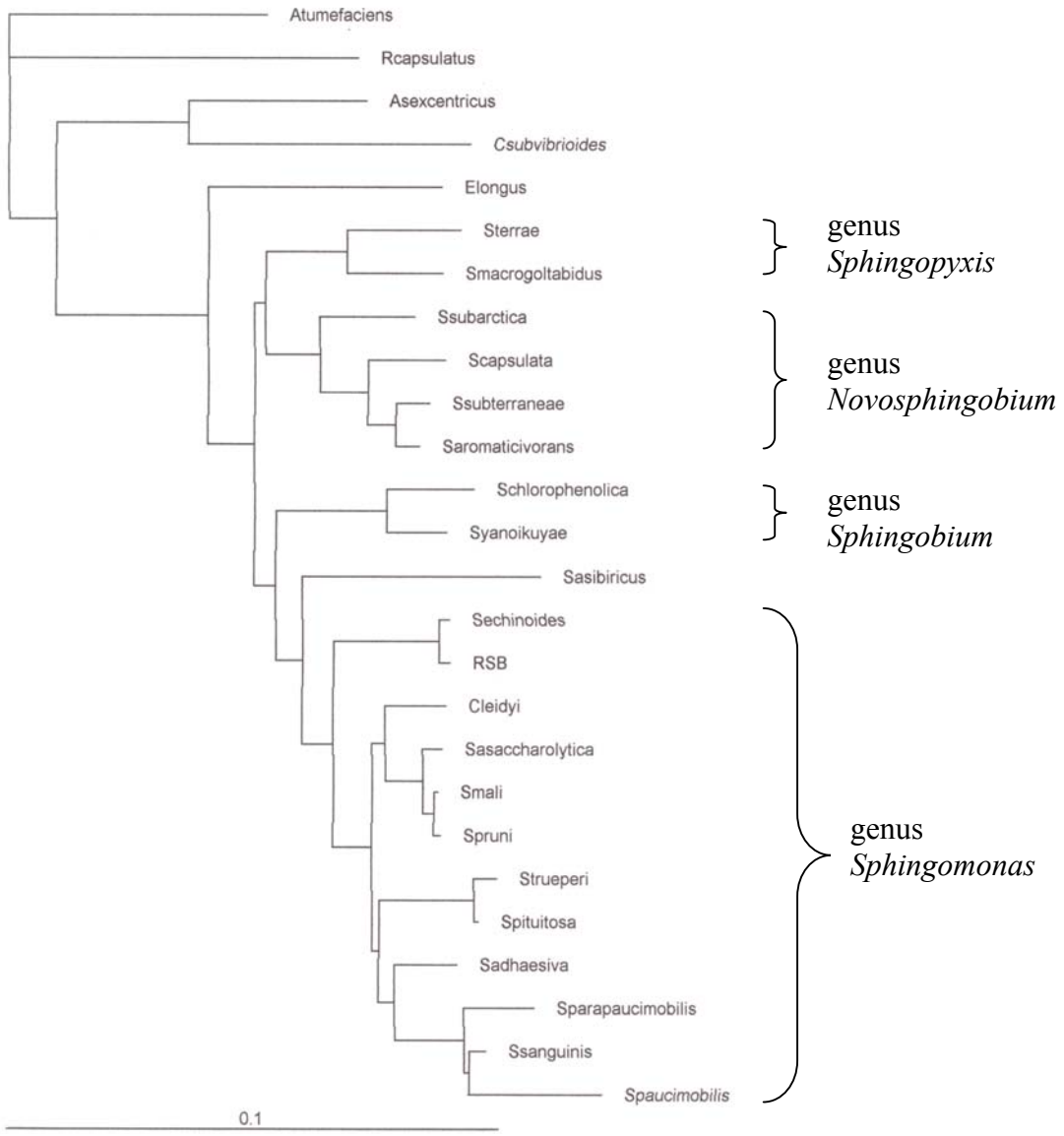


Figure 2. Phylogenetic tree obtained on comparing RSB 16S rRNA sequence with sequences of other *Sphingomonas* species using ClustalX (1.8). Reference for genus designations – Takeuchi et al. 2001.

H. Preparation of bacterial culture medium extracts -

A 5 d old culture of RSB in mWPM at 30°C was centrifuged to separate the cells from the culture filtrate. The cells were discarded and the filtrate adjusted to a pH of 8 followed by addition of ethyl acetate. This mixture was stirred for 45 minutes and the two phases were separated. The ethyl acetate fraction was rotary evaporated and the dried contents were resuspended in 95 % ethanol and stored in the freezer. The pH of the water fraction was adjusted to 3 and re-extracted using the same procedure as described above.

Using the same protocol, extracts were obtained at pH 3 and pH 8 for *Sphingomonas echinoides, Sphingomonas paucimobilis, Sphingopyxis macrogoltabida,* and *Novosphingobium capsulata* cultured in 1 L of mWPM for 5 d at 30°C and 100 rpm in the dark. *Sphingobium yanoikuyae* did not grow well in mWPM and therefore was cultured in Tryptic soy broth at 37°C and 100 rpm in the dark.

A small volume of the culture medium from which the extracts were prepared was serial diluted and plated for obtaining colony forming units (CFU's) in the culture medium. The CFU's were used to adjust the concentration of the bacterial extracts while performing the bioassays.

I. Mung bean bioassays -

Samples included in the mung bean bioassay were pH 3 and pH 8 culture medium extracts from RSB, *Sphingomonas echinoides, Sphingopyxis macrogoltabida, Sphingomonas paucimobilis, Novosphingobium capsulata and Sphingobium yanoikuyae*.

Controls included in the bioassay were pH 3 and pH 8 extracts obtained from mWPM and Tryptic soy broth.

The plant material consisted of 8 d old seedlings that were grown in soil in a growth chamber with 16/8 hour light-dark photoperiod, at 24-27°C and under 335 $\mu mol.m^{-2}.sec^{-1}$ light. The seeds were surface sterilized for 15 min in 10% bleach and rinsed thoroughly before planting.

Bacterial extracts were dried under a stream of N_2 gas and contents resuspended in distilled water which was adjusted to a pH of 5.5. The cell count obtained from the culture medium obtained previously was used to estimate the amount of distilled water required for the dilutions. This was done so that all the different bacterial treatments would be obtained from the same number of bacterial cells since at this time we are not able to adjust the concentration based on the quantity of the rooting factor.

An appropriate amount of culture medium extract was estimated based on the individual cell counts for each bacterium tested. The extract was added to test tubes containing 4 ml of distilled water. Seedlings were cut 3 cm below the cotyledonary node and placed in the test tubes containing the test solution. Test tubes were incubated at 24°C under $\mu mol.m^{-2}.sec^{-1}$ light with a 16/8 h light-dark photoperiod. After 48 hours of pulse treatment the seedlings were transferred to new tubes containing distilled water. Data on number of roots formed was collected on the 10th day after treatment began.

The experimental design used for the rooting experiments was a two factorial randomized block design (RBD) with 10 replicates per treatment. Transformation of the root counts was done to normalize the data. This conversion was done as follows:

Transformed number = Log_{10}(Number of roots + 1). Data processing involved using analysis of variance (ANOVA) from the GLM (general linear model) procedure in SAS (SAS Institute Inc., 1999).

J. Test for the presence of indoles -

The bacterial culture medium extracts in 95% ethanol were examined for indoles using TLC. Samples included pH 3 and pH 8 fractions of culture medium extracts for RSB, *Sphingomonas echinoides, Sphingomonas paucimobilis, Sphingopyxis macrogoltabida, Sphingobium yanoikuyae* and *Novosphingobium* capsulata. Standards included in the study were indolepyruvic acid, indolebutyric acid, indoleacetic acid, indoleacetamide, indolelactic acid, indolecarboxaldehyde and N-acetyl-trytophan. Twenty five µl of each sample was applied to a 20 x 20 cm Silica Gel GF 250 µm TLC plate (Fisher Scientific, Pittsburgh, PA). The plates were developed in propanol:water:28% ammonium hydroxide (8:1:1). After the plates were air-dried, the indoles were located by spraying with Ehrlich's reagent (1 g ρ- Dimethylamino benzaldehyde in 95 ml water + 20 ml conc. HCl) and Rf values were calculated.

CHAPTER 4

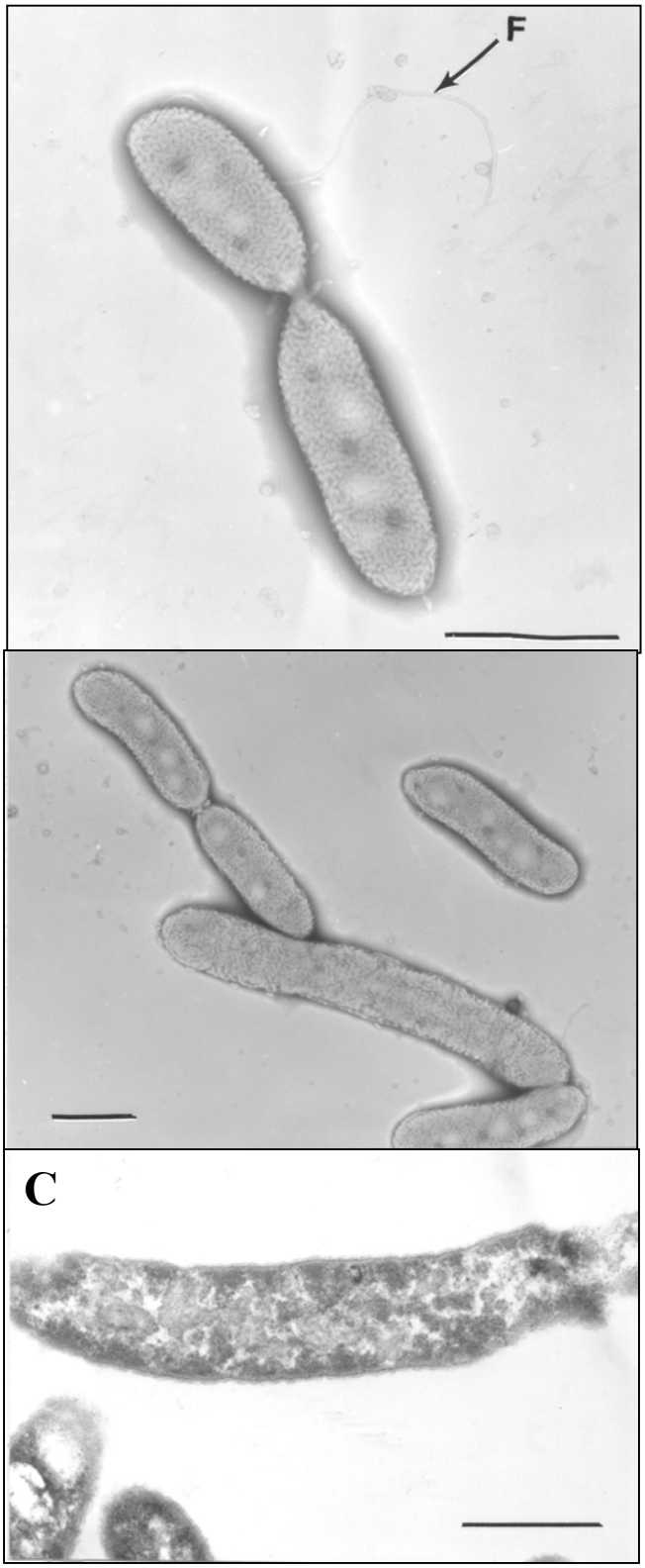
RESULTS AND DISCUSSION

A. Morphology of RSB -

The cells appeared rod-shaped (Figure 3) and were 0.6 – 0.8 μm wide. The length of the cells was highly variable ranging from 0.7 – 5.5 μm. Motile cells had a single polar flagellum. Sections of RSB cells showed a thin cell wall characteristic of Gram-negative cells. There was no major difference in the appearance of the cells between the young (48 h) and old (96 h) cultures except that most of the cells in the young (48 h) culture were undergoing cell division. Previous electron micrographs of RSB (Schwarz, Personal communication) have shown RSB possessing a hold-fast structure and the capability to form rosette-like structures when cultured in WPM and potato yeast extract. These features were not observed in this study possibly since RSB was only cultured in mWPM. Such rosettes (cell aggregates) have been observed in *Sphingomonas echinoides* in liquid cultures and in agar media and are observed as small flocs in the broth (Denner, et al. 1999).

There were several light and dark staining circular areas within the bacterial cells that resembled the poly-β-hydroxy butyrate and polyphosphate storage areas observed by Denner et al. (2001) in *Sphingomonas pituitosa*. However, further work needs to be done for positive confirmation. Several bacteria of the genus *Pseudomonas* are known to accumulate poly-β-hydroxybutyric acid as storage material (Schroll et al. 1996). PHB is a kind of poly-hydroxyalkanoic acid (PHA) and is commercially used as a biosynthetic polymer. PHB and other PHAs are produced by some bacteria as storage materials for

Figure 3. Transmission Electron Microscope Images of RSB cells. (A) Negative stain of a dividing RSB cell showing a single polar flagella. F=flagella. Bar = 1μm. (B) Negative stain of RSB cells of varying lengths. Bar = 1 μm. (C) Thin section of RSB cell demonstrating its rod shape and granular cytoplasmic contents. Bar = 1 μm.


F
C

carbon and energy and are usually produced when there is abundant carbon in the medium when other macronutrients are limiting (Kolibachuk et al. 1999). Polymers prepared from PHB have properties similar to polypropylene or poly(ethylene terephthalate) and are considered valuable since their degradation byproducts are more eco-friendly mainly consisting of CO_2 and H_2O (Schroll et al. 1996).

B. Nutritional and biochemical characterization -

(i) Amino acid requirement for growth:

RSB grew well in mWPM [amended with 2 g/L casein hydrolysate and 2 mg/L glycine (0.53 mM)] whereas poor growth was observed when casein hydrolysate and glycine were left out of the culture medium (Figure 4). Total glycine content in mWPM is calculated to be approximately 0.53 mM. Good growth of RSB was observed in flasks amended with 1.6 mM of the amino acids D-glycine, D-Asparagine and D-Glutamine. Growth was poor or absent when mWPM was substituted with the other 18 amino acids. Thus glycine, asparagine and glutamine seem to be the preferred amino acids for growth of RSB cells.

Bacteria require the addition of one or more amino acids for their growth. This is due to the inability of the bacteria to synthesize certain de novo certain amino acids that are required for their survival. Glutamine and asparagines are the preferred nitrogen sources in laboratory culture of yeast (S*accharomyces cerevisiae*) and these amino acids are utilized before other non-preferred sources of nitrogen (Chen and Kaiser 2002; Magasanik and Kaiser 2002).

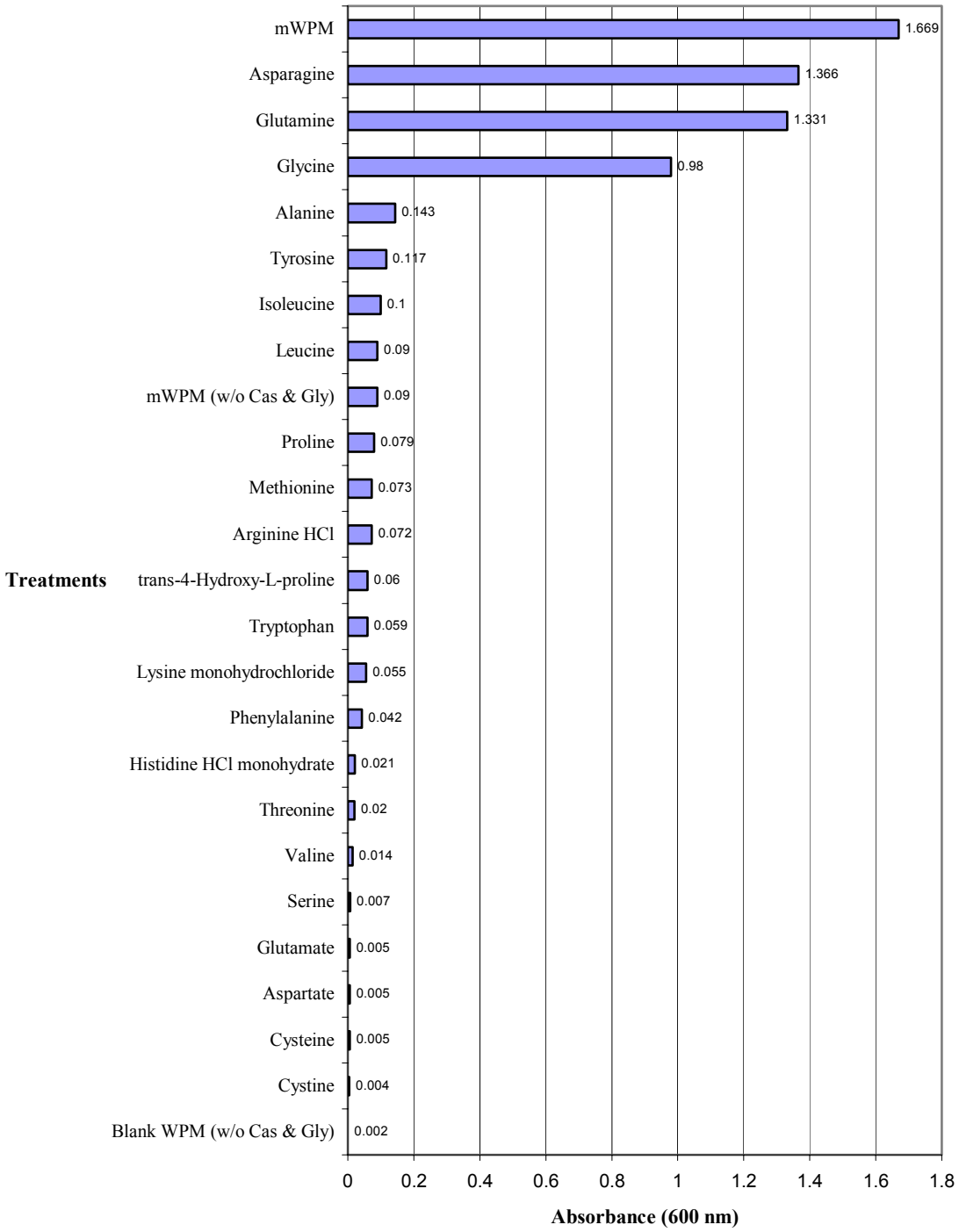


Figure 4. Effect of amino acid supplements in mWPM on growth of RSB cells.

Amino acids are taken up into the cell by several different amino acid permeases. The amino acid transport system in Cyanobacteria has been studied in detail (Montesinos, et al. 1997). It consists of three transport systems- one for basic amino acids and glutamine, one specific for neutral amino acids and one specific for glutamic acid and glutamine. In *Synechocystis* species Nat permease is responsible for transport of neutral amino acids and histidine and its NatA and NatB components are similar to BraF and BraC components of the amino acid transport system of *Pseudomonas aeruginosa.* Bgt is responsible for transport of basic amino acids and glutamine and GltS and Gtr are involved with glutamate uptake.

Glutamine is synthesized from glutamate with the help of the enzyme glutamine synthetase and organisms lacking this enzyme would therefore require glutamine for their growth (Magasanik and Kaiser 2002). Glutamate and glutamine are essential for synthesis of other nitrogenous compounds required by the organisms. No growth was observed when glutamate was added to the culture medium. However, good growth was observed when the culture medium was amended with glutamine. Therefore it may be that RSB lacks the enzyme glutamine synthetase or it could be a result of the inability of the organism to take up glutamate for the medium.

Asparagine is synthesized from aspartate via two pathways, one of which is ammonia dependent and the other one that is glutamine dependent (Heeke and Schuster 1989). Casein hydrolysate used in these experiments lacked asparagines, but contained aspartate and glutamine. Thus, it is possible that when casein is added to the medium, RSB is able to synthesize asparagines, but not able to do so when only aspartate is provided in the medium as was the case in this experiment.

(ii) Biochemical analysis –

(a) Tests for enzymatic reactions:

Activity of 19 different enzymes was tested using the API ZYM test kit (bioMerieux Inc., MO, USA). Activity was visually detected through colored reactions and recorded as positive or negative. As shown in Table 2, RSB and *Sphinogomonas echinoides* showed positive reactions for the enzymes alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase and β-glucosidase. Negative activity was observed in RSB and *Sphingomonas echinoides* cells for the enzymes β-glucuronidase, N-acetyl- β-glucosaminidase, α-mannosidase and α-fucosidase. Thus RSB does not differ from *Sphingomonas echinoides* in the activity of the enzymes tested above possibly indicating a close phylogenetic relationship.

(b) Tests for carbohydrate metabolism:

Metabolic patterns obtained using the API 50 CH test kit or BIOLOG system (Biolog, CA, USA) are commonly used in the identification of organisms. RSB was tested with the BIOLOG system (Biolog, CA, USA) for utilization or oxidation of various carbon sources. RSB was cultured on mWPM before testing because of the failure of RSB to grow on the recommended medium. No results were obtained from this test.

Table 2: Results of enzyme assay of RSB and *Sphingomonas echinoides*.

Test results given in the table were read after 24 h and at 30°C.
+, Positive reaction; -, negative reaction.

Enzyme assayed for	RSB	*S. echinoides*
Control	-	-
Alkaline phosphatase	+	+
Esterase	+	+
Esterase Lipase	+	+
Lipase	+	+
Leucine arylamidase	+	+
Valine arylamidase	+	+
Cystine arylamidase	+	+
Trypsin	+	+
α-chymotrypsin	+	+
Acid phosphatase	+	+
Naphthol-AS-BI-phosphohydrolase	+	+
α -galactosidase	+	+
β-galactosidase	+	+
β -glucuronidase	-	-
α -glucosidase	+	+
β -glucosidase	+	+
N-acetyl- β -glucosaminidase	-	-
α -mannosidase	-	-
α -fucosidase	-	-

Alternately, carbohydrate metabolism experiments were attempted for RSB and *Sphingomonas echinoides* using the API 50 CH test kit (bioMerieux Inc., MO, USA). Results were recorded after 96 h of incubation at 30°C. Each cupule of the test strip contained a single compound that served as the carbon source for the growth of the organism and assimilation (conversion of nutrients to cell mass) is indicated by growth of the organism in the cupule (API 50 CH instruction manual, bioMerieux Inc, MO, USA). As shown in Table 3, the following compounds are assimilated by RSB and by S*phingomonas echinoides*: L-arabinose, D-xylose, galactose, glucose, fructose, mannose, cellobiose, maltose, sucrose and trehalose. The carbohydrate metabolism pattern for RSB differed from *Sphingomonas echinoides* for only a few compounds. RSB was found to be capable of assimilating N-acetyl-glucosamine, arbutin and 5-keto-gluconate in addition to the above mentioned compounds utilized by both organisms.

Oxidation of a particular compound was observed as a color change in the cupule portion of the test strip and resulted from aerobic acid production, an energy yielding process (API 50 CH instruction manual, bioMerieux Inc, MO, USA). RSB and *Sphingomonas echinoides* tested positive for acid production from galactose, glucose, fructose, mannose, esculin and D-fucose. In addition, RSB also tested positive for acid production from L-arabinose, D-xylose, N-acetyl-glucosamine, arbutin and cellobiose. *Sphingomonas echinoides* showed a weak positive reaction for acid production from sucrose while RSB remained negative for the same reaction. These carbon metabolism results form a biochemical profile that can be useful for future identification and comparison of bacteria.

Table 3: Results of carbohydrate metabolism of RSB and *Sphingomonas echinoides.*

Test results given in the table were read after 96 h and at 30°C.
+, Positive reaction; -, negative reaction; (+), weak positive reaction.

	Compound	Assimilation		Acid production	
		RSB	*S. echinoides*	RSB	*S. echinoides*
1	Glycerol	-	-	-	-
2	Erythritol	-	-	-	-
3	D Arabinose	-	-	-	-
4	L Arabinose	+	(+)	+	-
5	Ribose	-	-	-	-
6	D Xylose	+	(+)	+	-
7	L Xylose	-	-	-	-
8	Adonitol	-	-	-	-
9	β-Methyl-D-Xyloside	-	-	-	-
10	Galactose	+	(+)	+	+
11	Glucose	+	+	+	+
12	Fructose	(+)	+	+	+
13	Mannose	(+)	(+)	+	(+)
14	Sorbose	-	-	-	-
15	Rhamnose	-	-	-	-
16	Dulcitol	-	-	-	-
17	Inositol	-	-	-	-
18	Mannitol	-	-	-	-
19	Sorbitol	-	-	-	-
20	α-Methyl-D-Mannoside	-	-	-	-
21	α-Methyl-D-Glucoside	-	-	-	-
22	N-Acetyl-Glucosamine	+	-	+	-
23	Amygdalin	-	-	-	-
24	Arbutin	+	-	(+)	-
25	Esculin	-	-	+	+
26	Salicin	-	-	-	-
27	Cellobiose	+	+	(+)	-
28	Maltose	+	+	-	-
29	Lactose	-	-	-	-
30	Melibiose	-	-	-	-
31	Sucrose	+	+	-	(+)
32	Trehalose	+	+	-	-
33	Inulin	-	-	-	-
34	Melizitose	-	-	-	-
35	Raffinose	-	-	-	-
36	Starch	-	-	-	-
37	Glycogen	-	-	-	-
38	Xylitol	-	-	-	-
39	Gentiobiose	+	+	-	-
40	D Turanose	-	-	-	-

Table 3: continued.

	Compound	Assimilation		Acid production	
		RSB	*S. echinoides*	RSB	*S. echinoides*
41	D Lyxose	-	-	-	-
42	D Tagatose	-	-	-	-
43	D Fucose	-	-	+	+
44	L Fucose	-	-	-	-
45	D Arabitol	-	-	-	-
46	L Arabitol	-	-	-	-
47	Gluconate	-	-	-	-
48	2-keto-gluconate	-	-	-	-
49	5-keto-Gluconate	(+)	-	-	-

(c) Oxidase test:

Cytochrome oxidase is an enzyme found in some bacteria that transfers electrons to oxygen serving as the final electron acceptor in some electron transport chains (ETC). The enzyme oxidizes the artificial electron acceptor tetramethyl-ρ-phenylene diamine that forms the basis for the oxidase test (Madigan et al. 1997). RSB grown on mWPM tested oxidase negative (no development of blue-purple color) while upon culture on Sabourauds glucose agar, it tested oxidase positive. *Sphingomonas echinoides* was oxidase positive when cultured on mWPM. This oxidase positive reaction is a somewhat unique trait not commonly seen among *Sphingomonas* bacteria (Denner et al 2001). Among other Sphingomonads, *Sphingomonas echinoides* (Denner et al. 1999) and *Sphingomonas pituitosa* (Denner et al. 2001) have been reported to be oxidase positive. Thus, the oxidase negative reaction in mWPM, can be used to distinguish RSB from *Sphingomonas echinoides*.

(d) Catalase test:

Toxic hydrogen peroxide is produced as a byproduct of several enzymatic reactions. Some bacteria can convert this toxic hydrogen peroxide into diatomic oxygen and water with the help of the enzyme catalase (Madigan et al. 1997). Both RSB and *S. echinoides* tested catalase positive that was observed by the formation of air bubbles upon addition of 3% hydrogen peroxide to fresh bacterial cultures. Denner et al. (1999) have also reported *Sphingomonas echinoides* to be catalase positive.

$$H_2O_2 + H_2O_2 \xrightarrow{\text{catalase}} 2H_2O + O_2$$ (From Madigan et al. 1997)

C. Pigment analysis -

The absorption maxima of an extract of RSB pigments in methanol, ethanol and benzene were obtained (Table 4, Figure 5). The RSB pigment spectrum in methanol showed λ_{max} (423), 446, 472, λ_{max} (423), 447, 474 in ethanol and λ_{max} (430), 460, 487 in benzene. *Sphingomonas echinoides* absorption spectra had λ_{max} (423), 448, 475 in methanol and λ_{max} (424), 450, 478 in ethanol. These spectra indicate that the pigment found in both bacterial extracts is most probably a carotenoid (Bally et al. 1990). The migration pattern and Rf values obtained from TLC of pigments from RSB and *Sphingomonas echinoides* were similar (Table 5) showing two faint bands of lower polarity preceeding the main pigment band with Rf value of 0.57 for RSB and 0.58 for *Sphingomonas echinoides*. Jenkins et al. (1979) have also reported two faint bands proceeding the main band for nostoxanthin [(2R,3R,2′R,3′R)-β,β-carotene-2,3,2′,3′-tetrol] from *Sphingomonas* (*Pseudomonas*) *paucimobilis.*

The pigment was further characterized using HPLC. Absorption was measured at λ=452 nm and λ=480 nm which are wavelengths close to the peaks seen in the absorption spectra for the two bacterial extracts. Pigment peaks from both organisms had similar retention times (Rt) (Table 6) with the main pigment peak measured at 452 nm and 480 nm having a retention time of 9.68 min for RSB (Figure 6) and 9.65 min for *Sphingomonas echinoides* (Figure 7). The overlay showing HPLC spectra of RSB and *Sphingomonas echinoides* pigment at 452 nm shows similar peak Rt at 9.653 min (Figure 8).

Table 4: Absorbance maxima of RSB and *Sphingomonas echinoides* pigments in methanol, ethanol and benzene.

Solvent	**RSB**	***Sphingomonas echinoides***
Methanol	(423), 446, 472	(423), 448, 475
Ethanol	(423), 447, 474	(424), 450, 478
Benzene	(430), 460, 487	-

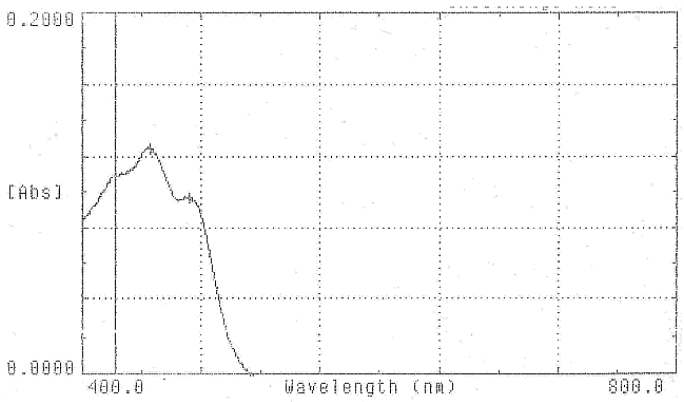
Table 5: Rf values from TLC of RSB and *Sphingomonas echinoides* pigments

Band #	**RSB**	***Sphingomonas echinoides***
1	0.57	0.56
2	0.54	0.53
3	0.49	0.48

Table 6: HPLC peak retention times for RSB and *Sphingomonas echinoides* pigments.

Organism	Wavelength (nm)	Peak number	Peak retention time
RSB	452	1	2.075
		2	5.457
		3	9.681
S. echinoides	452	1	2.061
		2	9.653
RSB	480	1	2.071
		2	4.267
		3	5.2
		4	9.681
S. echinoides	480	1	2.061
		2	2.216
		3	4.159
		4	9.647

(A)



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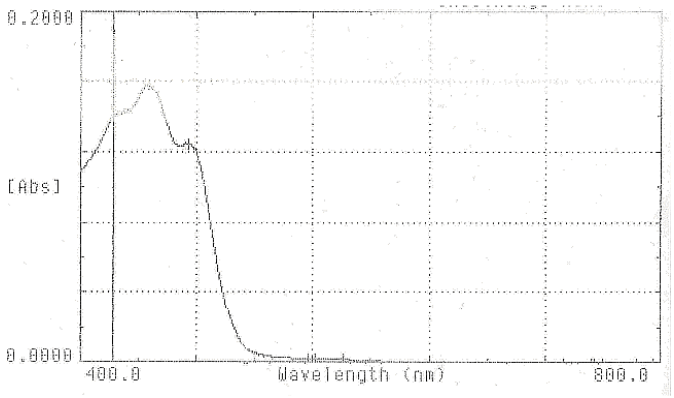

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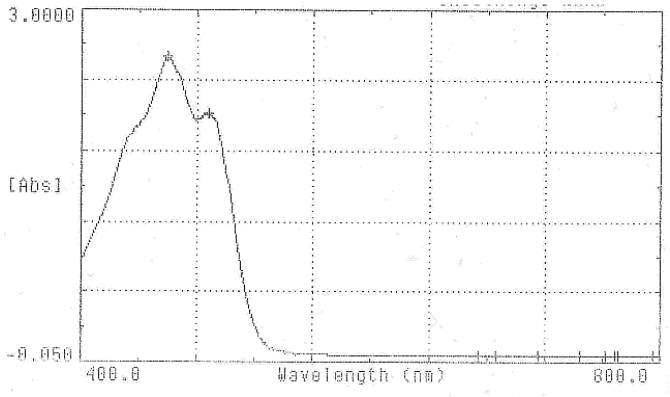
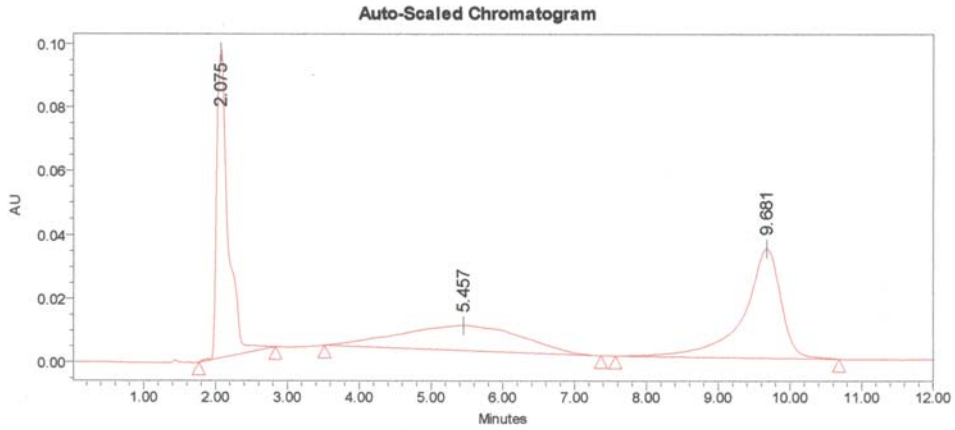


Figure 5. Absorption spectra of RSB pigments in (A), methanol; (B), ethanol; (C) benzene.

(A)



(B)

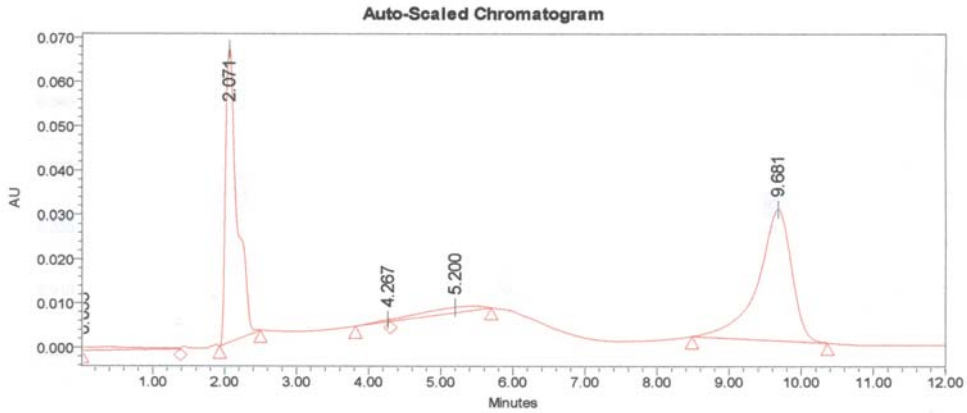
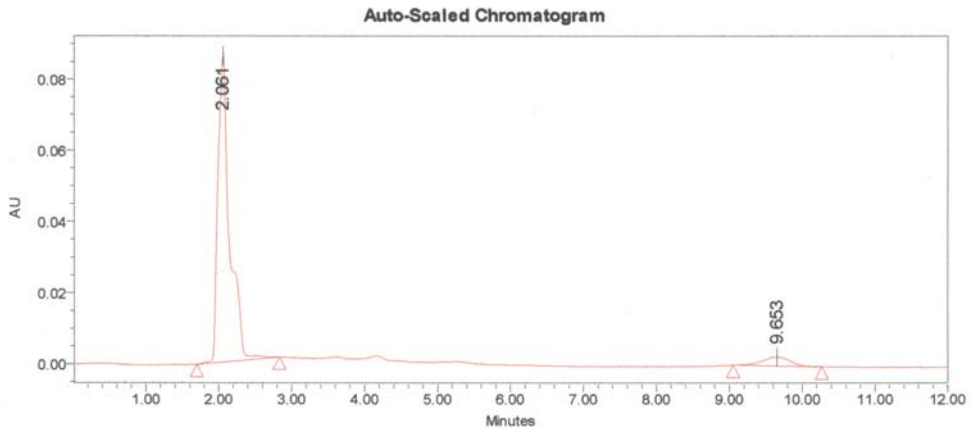


Figure 6. HPLC spectra of RSB pigments at (A) 452 nm and (B) 480 nm.

(A)



(B)

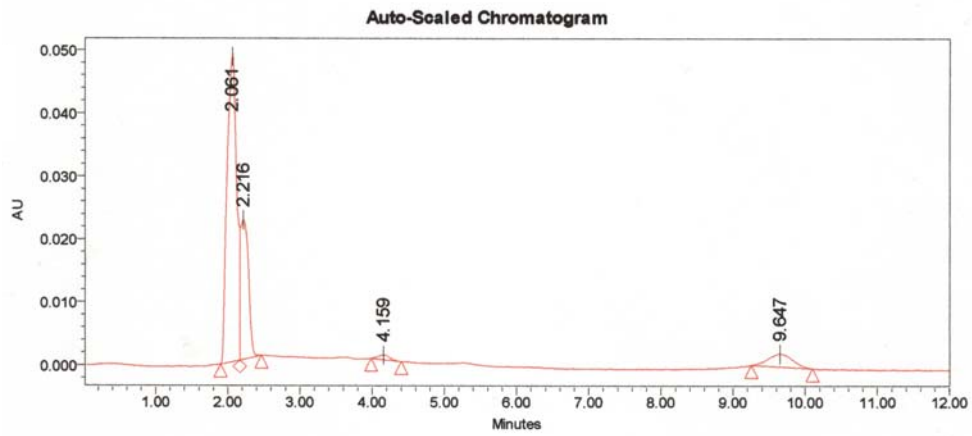


Figure 7. HPLC spectra of *S. echinoides* pigments at (A) 452 nm and (B) 480 nm.

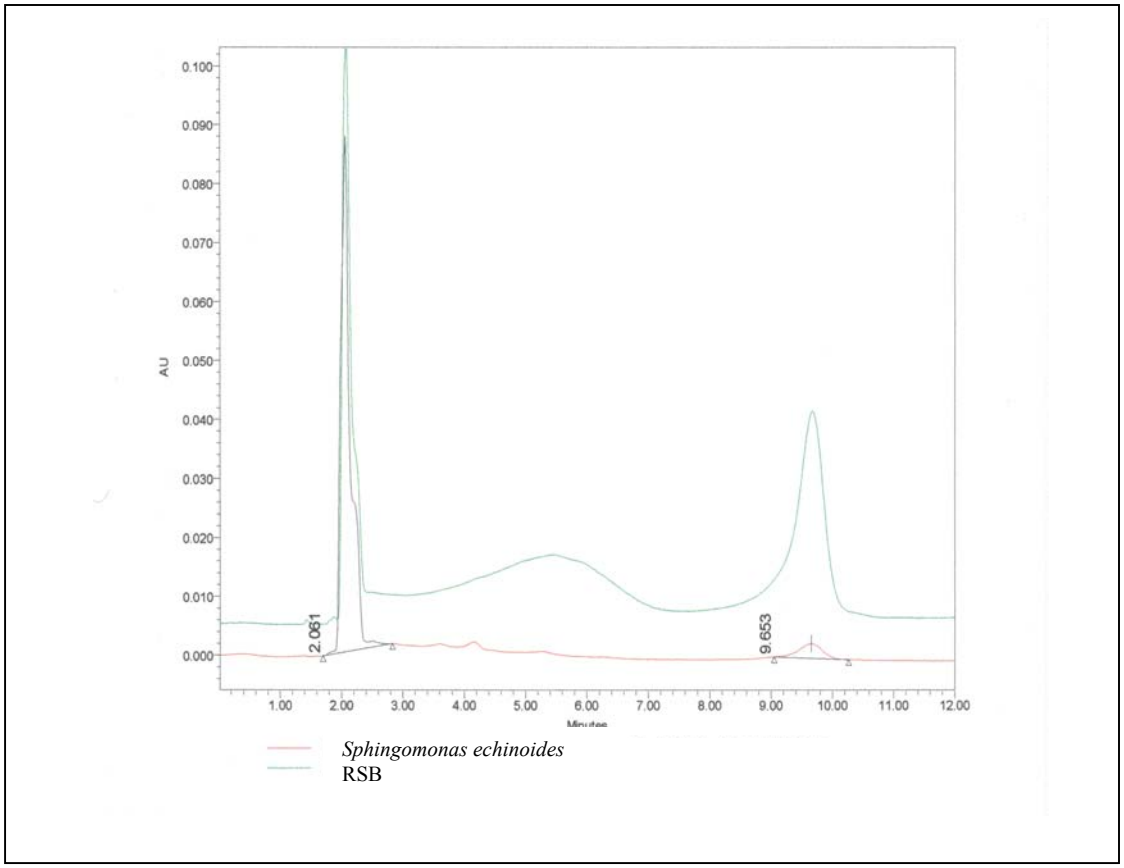


Figure 8. Overlay of HPLC chromatograms for RSB and *Sphingomonas echinoides* pigments at 452 nm.

Jenkins et al. (1979) have identified the yellow pigment of *Pseudomonas echinoides* (Now classified as *Sphingomonas echinoides* (Denner, E. B. M, et al 1999)) using chromatography, mass spectrometry, NMR analysis and CD spectra, as the carotenoid nostoxanthin ($C_{40}H_{56}O_4$) (Figure 9). Nostoxanthin is commonly found in cyanobacteria and the *Sphingomonas* group of bacteria (Jenkins et al. 1979). Based on our results and comparison with *S. echinoides* pigments, the RSB pigment is most probably nostoxanthin.

In a further attempt at characterization, pigments extracted from RSB and *Sphingomonas echinoides* were also analyzed with Electron Ionization Mass Spectrometry. The mass spectral results do not match those obtained by Jenkins at al. (1979) for nostoxanthin extracted from *Pseudomonas* (*Sphingomonas*) *paucimobilis*. RSB and *Sphingomonas echinoides* pigments were identical and had two components. Component 1 showed a major peak of mass 453 and component 2 had a major peak of mass 503. The second component was much smaller than the first. These results did not show a peak of mass 600 as expected for nostoxanthin (Jenkins et al., 1979). This could be the result of denaturation of the pigments during the extraction process despite efforts at keeping all steps of the extraction and subsequent pigment extract storage under minimal oxygen exposure. However, the fragmentation patterns of both pigment samples were identical, further pointing towards the presence of nostoxanthin in RSB.

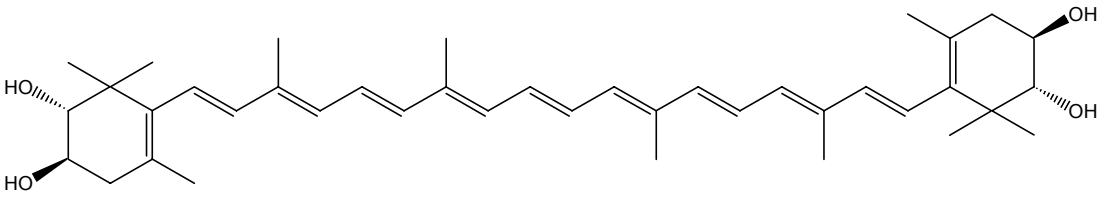


Figure 9. Structure of Nostoxanthin ($C_{40}H_{56}O_4$). (Ref: Pfander et al. 1987)

D. Lipid analysis -

Lipid studies are widely used in bacterial chemotaxonomy. The *Sphingomonas* group of organisms is characterized by the absence of 3-hydroxy acids and the presence of 2-hydroxytetradecanoic acids and octadecanoic acids. Another characteristic feature of the *Sphingomonas* group is the presence of sphingolipids. Members of the *Sphingomonas* group also differ from other Gram-negative bacteria as they do not contain lipopolysaccharide (Kawasaki, et al. 1994).

Fatty acids are usually designated as X:YωZc/t where X represents the carbon chain length, Y represents the number of double bonds, Z represents the number of carbon atoms between the methyl end of the molecule and the unsaturated bond. The c and t represent the cis and trans forms. The following results are presented using this nomenclature.

(i) PLFA analysis:

The profile obtained after mild alkaline methanolysis of RSB and *Sphigomonas echinoides*, both cultured on mWPM, showed the presence of methyl esters of fatty acids, 16:0 (hexadecanoic acid), branched 18:1, 16:1ω7c, 16:1ω7t and 18:1ω7c (Table 7) and trace amounts of the fatty acids 18:0 and 18:1ω7t. Analysis of fatty acid methyl esters was carried out on NA in addition to mWPM in order to verify our results with those reported for *S. echinoides* cultured in NA by Rowe et al. (2000). When cultured on NA, RSB and *S. echinoides* showed the presence of fatty acids 16:0, 18:0, branched 18:1, 16:1ω7c, 18:1ω7t and trace amounts of 16:1ω7t and 18:1ω7t. We did not detect

Table 7: Percent major polar lipid fatty acids composition of RSB and *Sphingomonas echinoides* cultured on mWPM and NA.

	mWPM						NA					
Fatty acids (Categorized)	**RSB**			***S. echinoides***			**RSB**			***S. echinoides***		
	Sample 1	**Sample 2**	**Average**	**Sample 1**	**Sample 2**	**Average**	**Sample 1**	**Sample 2**	**Average**	**Sample 1**	**Sample 2**	**Average**
Normal Saturated												
16:0	24.89	24.78	24.84*	21.96	22.67	22.32*	25.56	25.11	25.34*	22.47	22.16	22.32*
18:0	TR	TR	TR	TR	TR	TR	1.66	1.21	1.44	1.36	1.27	1.32
Mid branched monoenoics												
br18:1	6.75	7.18	6.97	9.62	8.41	9.02	8.13	9.23	8.68	8.96	8.75	8.86
Monoenoics												
16:1w7c	2.35	2.37	2.36*	1.81	1.58	1.70*	1.54	1.84	1.69*	1.08	1.21	1.15*
16:1w7t	2.73	2.92	2.83*	1.4	1.28	1.34*	TR	TR	TR	TR	TR	TR
18:1w7c	60.48	59.98	60.23	61.02	62.4	61.71	59.52	58.32	58.92*	61.61	61.95	61.78*
18:1w7t	TR	TR	TR	TR	TR	TR	TR	TR	TR	1.2	1.26	1.23

TR – Trace amounts (Less than 1%)
* - Statistically significant differences

2-hydroxytetradecanoic acids in our analysis, as expected for *S. echinoides*, since the procedure we used involved alkaline methanolyis. As described by Rowe (2000) for *S. echinoides*, 2-hydroxytetradecanoic acids can only be detected using a method involving acid methanolysis probably because of the presence of an amide linkage. Other fatty acids observed in the experiments correlated with those observed by Rowe et al. (2000).

The same fatty acids were identified for both RSB and *S. echinoides*. However, the relative percentages of the fatty acids were significant differences statistically between the two organisms. When cultured on mWPM, significant differences were observed between RSB and *S. echinoides* for fatty acids 16:1ω7c, 16:1ω7t and 16:0. When RSB and *S. echinoides* was cultured on NA, a significant difference was observed for the fatty acids 16:0, 16:1ω7c and 18:1ω7c.

Although differences in fatty acids were observed between RSB and *S. echinoides,* both organisms have lipid profiles that resemble other *Sphingomonas* species (Denner et al. 1999; 2001; Rowe et al. 2000). Based on these studies, the lipid content of RSB seems to closely resemble bacteria belonging to the *Sphingomonas* group.

(ii) Sphingolipid analysis:

RSB and *Sphingomonas echinoides* showed the presence of Sphingolipids (Table 8) when cultured on NA. The sphingolipids were identified by their signature MS fragments (Leung et al. 1999) with m/z 73, 103, 116, 132. RSB and *Sphingomonas echinoides* cultured on NA showed the presence of sphinganine bases 18:0, 20:1, 21:0. RSB when cultured on mWPM also showed the presence of the sphinganine base 16:0 in addition to those observed upon culture in NA. The concentration of sphingolipids

Table 8: Percentage sphingolipid composition of RSB and *Sphingomonas echinoides* cultured on mWPM and NA.

	NA		**mWPM**
Sphingolipids	**RSB**	***S. echinoides***	**RSB**
18:0	33.95	23.12	41.7
20:1	8.21	27.25	23.19
21:1	57.84	49.44	34.05
16:0	0	0	1.06
19:0	0	TR	0

TR – Trace amounts (Less than 1%)

Above numbers are average percentages of two replicate samples per treatment.

extracted from *Sphingomonas paucimobilis* was too low in our samples and could not be identified. The presence of Sphingolipids is considered an identifying character of the *Sphingomonas* and related genera (Denner et al. 1999). Thus, RSB fits well among the *Sphingomonas* bacteria.

E. Pulse-field gel electrophoresis -

Due to the high G+C content of *S. echinoides*, enzymes that recognize A+T-rich sequences were used for digestion of DNA obtained from RSB and *S. echinoides* for PFGE analysis. The enzymes used included *Xba*I, *Spe*I, *Dra*I and *Nde*I. *Xba*I and *Spe*I cleaved the DNA into a convenient number of fragments to be analyzed by PFGE (Figure 10). *Dra*I resulted in a few bands that were not well resolved possibly as a result of incomplete digestion. *Nde*I produced several small sized fragments of low molecular weight that ran close to the bottom of the gel making it difficult to compare the banding pattern between the two organisms tested. There was no difference in banding pattern between RSB samples treated with restriction enzyme *Xba*I for 24 h (Lane 2, Figure 10) and RSB samples treated twice for 24 h each (Lane 3, Figure 10) with *Xba*I ruling out the possibility of difference in banding pattern due to incomplete digestion. A similar observation was made for *S. echinoides* DNA samples (Lanes 4 and 5, Figure 10).

Completely different banding patterns were observed for RSB and *S. echinoides* DNA samples when digested with *Xba*I. A similar observation was made for both samples when digested with *Spe*I showing only one band in common indicating a high level of difference in the DNA sequence of both organisms.

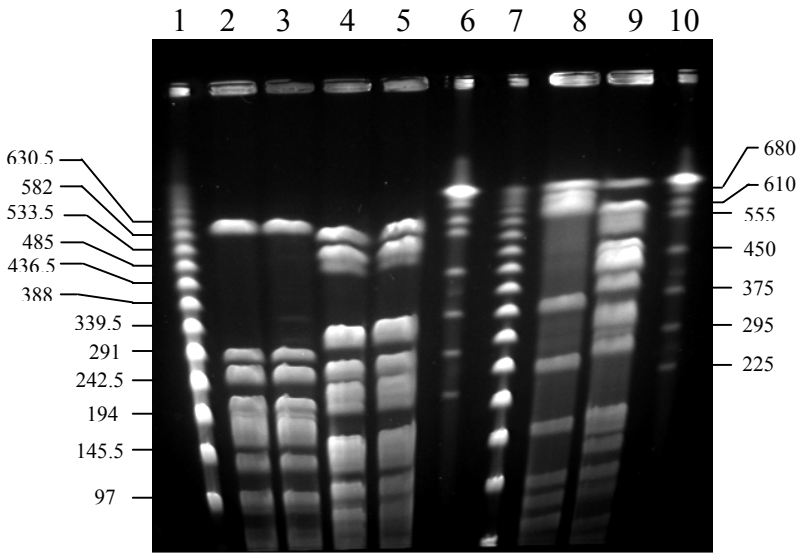


Figure 10. PFGE of RSB and *Sphingomonas echinoides* DNA digestion with *XbaI* and *SpeI*. Lanes: 1 and 7 lambda DNA concatemers; 6 and 10, yeast chromosome markers; 2, RSB 24 h *Xba*I digestion; 3, RSB 2 x 24 h *Xba*I digestion; 4, *S. echinoides* 24 h *Xba*I digestion; 5, *S. echinoides* 2 x 24 h *Xba*I digestion; 8, RSB 24 h *Spe*I digestion; 9, *S. echinoides* 24 h *Spe*I digestion.

The PFGE technique has been used to separate yeast chromosomes (Chu et al. 1986; Schwartz and Cantor 1984), estimate genome size and construct chromosome maps (Thong et al. 1997; Wagner et al. 1998) and to determine genomic diversity of strains of pathogenic bacteria (Anthony et al. 2002; Saunders et al. 1997).

PFGE can be used for resolving differences at the strain level (Belkum et al. 1998; Gillis, et al. 1993). Based on the high level difference in banding pattern observed between RSB and *Sphingomonas echinoides* we identify these two organisms as different strains.

F. Mung bean bioassay –

This study was conducted in order to determine whether the rooting activity observed for RSB was specific to RSB or if it was more widespread among the *Sphingomonas* and related bacteria. Based on similarity of the 16 S rRNA sequence of RSB to *S. echinoides* and the recent reclassification among members of the former genera *Sphingomonas* into 4 new genera (Takeuchi et al. 2001) (Table 1), we decided to include type species of the 4 newly formed genera along with *S. echinoides* in our bioassay studies.

Rooting bioassays are suitable for detecting physiological activity (Heuser 1988). In order to analyze and assess the ability of the bacterial culture medium extracts to induce adventitious rooting it was necessary to have a reliable and short bioassay. The mung bean (*Vigna radiata*) bioassay, originally developed by Hess (1957) was chosen for the experiments. This bioassay has gone through several modifications by different authors (Blazich and Heuser 1979, Heuser 1988). It is one of the simplest rooting

bioassays having a considerable degree of replication for statistically satisfactory results (Heuser 1988).

Samples included in the mung bean bioassay were pH 3 and pH 8 culture medium extracts from RSB, *Sphingomonas echinoides, Sphingopyxis macrogoltabida, Sphingomonas paucimobilis, Novosphingobium capsulata and Sphingobium yanoikuyae*. Controls included in the bioassay were pH 3 and pH 8 extracts obtained from mWPM and tryptic soy broth (TSB). The experiment was performed twice and the data was pooled together before analysis. Transformation of the root counts was done to normalize the data. This conversion was done as follows: Transformed number = Log_{10}(Number of roots + 1). A higher transformed number (TN) indicates higher rooting ability. This TN is used in the discussion of the rooting bioassay results.

The results of pH 3 & 8 fractions (Table 9; Figure 11 & 12) obtained from RSB, *Sphingomonas echinoides, Sphingopyxis macrogoltabida, Sphingomonas paucimobilis and Novosphingobium capsulata* cultures were compared with control mWPM since these bacteria were cultured on mWPM before extraction. The result of pH 3 & 8 fractions obtained from *Sphingobium yanoikuyae* were compared with control TSB since *S. yanoikuyae* showed poor growth when cultured in mWPM and was cultured on TSB as recommended by ATCC.

(i) Mung bean bioassay results of pH 3 fractions:

The mWPM pH 3 control treatment had a TN of 1.33. Results obtained for pH 3 bacterial culture medium extracts can be divided into three main groups showing

Table 9: Results of testing bacterial media extracts using the mung bean bioassay

Treatment	Media extract pH	Mean number of Roots	Mean of Log_{10}(# Roots + 1)*
mWPM	3	23.350	1.332^{BC}
	8	26.300	1.384^{ABC}
RSB	3	37.830	1.551^{AB}
	8	31.875	1.486^{AB}
Sphingomonas echinoides	3	29.550	1.439^{ABC}
	8	29.050	1.441^{ABC}
Sphingopyxis macrogoltabida	3	27.600	1.440^{ABC}
	8	34.000	1.457^{AB}
Sphingomonas paucimobilis	3	41.500	1.595^{A}
	8	27.850	1.387^{ABC}
Novosphingobium capsulata	3	28.150	1.451^{AB}
	8	17.100	1.211^{C}
Sphingobium yanoikuyae	3	27.000	1.431^{ABC}
	8	26.850	1.433^{ABC}
Tryptic soy broth	3	25.550	1.394^{ABC}
	8	24.950	1.390^{ABC}

* -Numerical values followed by the same letter are not significantly different at the 0.05 level of significance. Mean separation was performed on the transformed data.

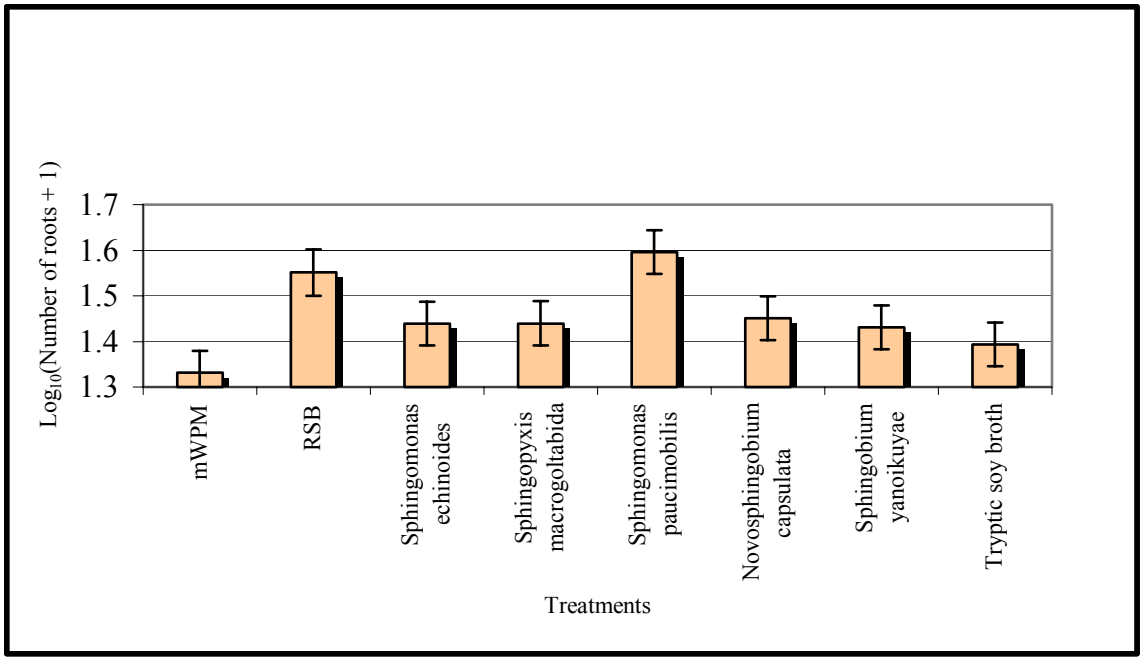


Figure 11. Effect of bacterial pH 3 culture media extracts on Log_{10} (Number of roots + 1) of mung bean seedlings. Error bars show standard error of the mean

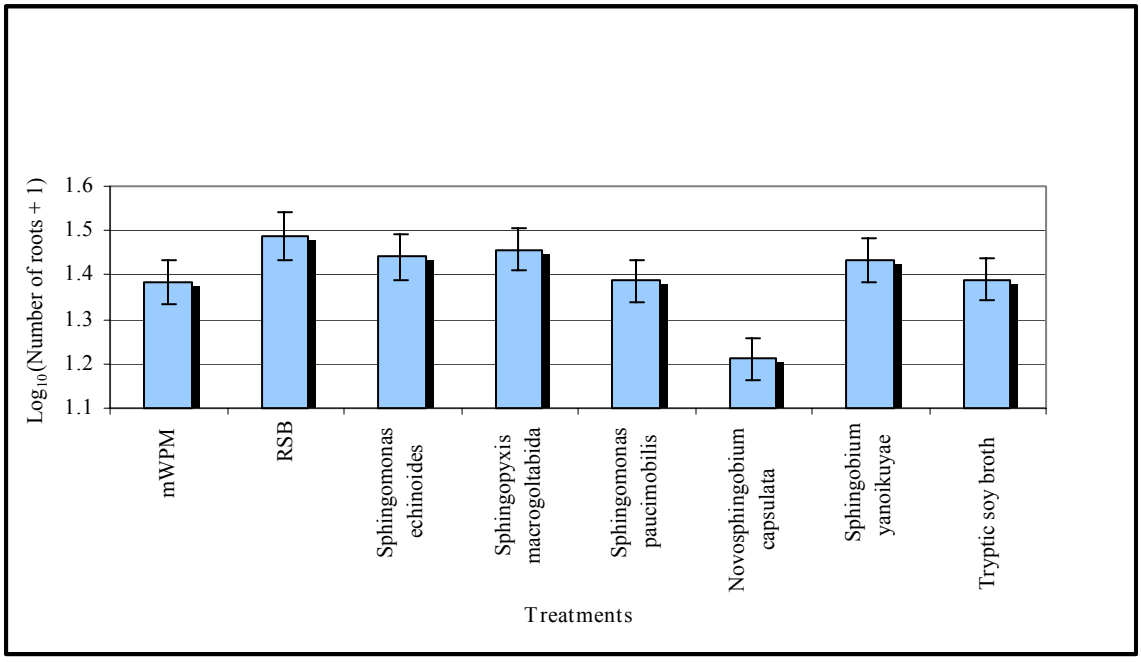


Figure 12. Effect of bacterial pH 8 culture media extracts on Log_{10} (Number of roots + 1) of mung bean seedlings. Error bars show standard error of the mean.

statistically significant differences in levels of rooting. Maximum rooting activity was observed in *Sphingomonas paucimobilis* with a TN of 1.60. The second group consists of RSB and *Novosphingobium capsulata* with a TN of 1.55 and 1.45 respectively. The third group includes *Sphingomonas echinoides* and *Sphingopyxis macrogoltabida* with a TN 1.44 each.

Rooting effect of *Sphingobium yanoikuyae* pH 3 fraction resulted in a TN of 1.43 and it did not differ significantly from its control TSB which had a TN of 1.39.

(ii) Mung bean bioassay results of pH 8 fractions:

The mWPM pH 8 control treatment had a TN of 1.38. RSB and *Sphingopyxis macrogoltabida* showed significantly greater rooting with a TN of 1.49 and 1.46 respectively. *Sphingomonas echinoides* and *Sphingomonas paucimobilis* pH 8 fractions did not differ in their rooting ability from the control treatment and had a TN of 1.44 and 1.39. *Novosphingobium capsulata* pH 8 fraction was found to inhibit rooting in mung bean seedlings.

The rooting ability of pH 8 fraction of *Sphingobium yanoikuyae* did not differ from the control TSB activity.

It appears that organisms in the *Sphingomonas* and *Novosphingobium* group contain acidic component/s that contribute to the rooting activity while bacteria in the *Sphingopyxis* group have acidic as well as neutral components that contribute to the rooting activity. The genus *Sphingobium* does not show any significant rooting potential. RSB shows the presence of acidic and neutral compound/s that significantly improves rooting in mung bean cuttings.

Based on the bioassay results, it can be said that the root stimulating ability is not restricted to RSB alone but is widespread among *Sphingomonas* and related bacteria. The genera *Sphingomonas, Novosphingobium* and *Sphingopyxis* should be added to the list of bacteria (Arshad and Frankenberger, 1998; Brown, 1972) capable of producing root promoting substances.

G. Test for the presence of indoles -

Culture medium extracts of pH 3 and pH 8 from RSB, *Sphingomonas echinoides, Sphingomonas paucimobilis, Sphingopyxis macrogoltabida, Sphingobium yanoikuyae* and *Novosphingobium capsulata* were chromatographed using TLC. The controls used were extracts obtained from uninoculated mWPM and TSB media. After chromatography, the TLC plates were sprayed with Ehrlich's reagent that is used for identification of indole compounds. Colored spots were observed upon spraying and the Rf values were recorded (Table 10).

The pH 8 bacterial fractions showed no colored spots. The pH 3 fractions obtained from RSB, *Sphingomonas echinoides, Sphingomonas paucimobilis Sphingopyxis macrogoltabida* and *Novosphingobium capsulata* showed purple staining spots that had Rf values close to those for the standards indolelactic acid (ILA) and indoleacetic acid (IAA). However, positive confirmation on the identity of these spots as ILA or IAA could not be made due to a small difference in their Rf values.

Indolepyruvic acid (IPA) was found to breakdown when chromatographed into 5 spots. A similar observation for IPA was made by Kaper and Veldstra (1958) but the

Table 10: TLC results of indole standards and bacterial extracts (pH 3).

Sample	Band #	Rf value	Color
Standards			
IPA	1	0.67	Purple
	2	0.59	Brown
	3	0.53	Blue/green
	4	0.50	Purple
	5	0.17	Yellow/brown
IBA	1	0.73	Purple
IAA	1	0.66	Purple
IAM	1	0.91	Purple
ILA	1	0.65	Purple
NAcTrypt	1	0.71	Purple
Bacterial samples (pH 3)			
mWPM	No bands	-	-
RSB	1	0.63	Purple
Se	1	0.63	Purple
Sp	1	0.63	Purple
Sm	1	0.66	Purple
Nc	1	0.67	Purple
Sy	1	0.83	Purple
	2	0.66	Purple
	3	0.47	Brown
	4	0.43	Purple
	5	0.40	Brown
TSB	1	0.83	Purple
	2	0.68	Purple
	3	0.49	Purple
	4	0.43	Purple

solvent system used was different and hence comparisons using Rf values could not be made. *Sphingobium yanoikuyae*, cultured in TSB, and the control TSB fraction resulted in the same spots positive for indoles. Hence, a positive confirmation of indoles produced by *Sphingobium yanoikuyae* without the media effect could not be made. Several bacteria are known to produce IAA that is required for the pathogenicity of that organism (Fett, et al. 1987). Fett, et al. (1987) also suggest that IAA may be required by pathogenic bacteria for survival on plant surfaces. Two pathways are known for the synthesis of IAA from tryptophan. The indolepyruvate pathway is more common among bacteria and plants (Fett, et al. 1987). The first step in this pathway is the conversion of tryptophan to IPA. The second step involves conversion of IPA to indoleacetaldehyde (IAAld) with ILA as the byproduct. ILA has also been shown to have auxin-like activity on wheat coleoptites (Fett, et al. 1997). In the third step, IAAld is converted to IAA. The second pathway involves the conversion of trytophan to IAA via the formation of indoleacetamide (IAM). No spots for IAM were detected in the chromatograms. However spots that could possibly be IAA or ILA were observed. Thus it is possible that IAA is synthesized among the bacteria tested via the IPA pathway. Future experiments using GC-MS are suggested for positive confirmation of these indoles.

H. Summary -

Electron microscope examination indicates that RSB, a Gram-negative bacterium is a straight rod measuring 0.6-0.8 × 0.7-5.5 μm, motile or non-motile, with motile cells having a single polar flagellum and several inclusion bodies (possibly poly hydroxy butyrate and polyphosphate) and occasionally shows the presence of a holdfast. RSB

requires asparagine, glutamine, or glycine in the medium for growth. When cultured on mWPM, RSB cells are oxidase negative and catalase positive. RSB is capable of producing the enzymes alkaline phosphatase, esterase, esterase lipase, lipase, leucine arylamidase, valine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, α-glucosidase, and β-glucosidase. RSB is capable of assimilating L-arabinose, D-xylose, galactose, glucose, fructose, mannose, cellobiose, maltose, sucrose and trehalose, N-acetyl-glucosamine, arbutin, gentiobiose, and 5-keto-gluconate. RSB tested positive for acid production from galactose, glucose, fructose, mannose, esculin and D-fucose, L-arabinose, D-xylose, N-acetyl-glucosamine, arbutin and cellobiose. Pigment isolates from RSB and *Sphingomonas echinoides* produced similar chromatographic, spectral and mass spectrographic analysis indicating the presence of nostoxanthin in both species. RSB possibly produces the pigment nostoxanthin. The major fatty acids are 16:0 and 18:1. Sphingolipids were also found to be present showing another characteristic similar to the *Sphingomonas* bacteria. From the PFGE results we can say that RSB differs from *Sphingomonas echinoides* at least at the strain level.

Based on the mung bean bioassay results for testing root-promoting ability of bacteria, it appears that *Sphingomonas paucimobilis, Sphingomonas echinoides,* and *Novosphingobium capsulata* contain acidic component/s that contribute to the rooting activity, while RSB and *Sphingopyxis macrogoltabida* have both acidic and neutral components that contribute to the rooting activity. The genus *Sphingobium* does not show any significant rooting potential. Based on TLC results, the rooting activity could be a result of the ability of these bacteria to release indolelactic acid (ILA) and/or

indoleacetic acid (IAA) and other indole containing compounds into the culture medium. Thus we can add *Sphingomonas, Novosphingobium* and *Sphingopyxis* to the list of bacterial genera capable of producing plant growth regulating substances.

Suggested future work includes extraction and GC analysis for confirming production of poly-β-hydroxy butyrate by RSB. Confirmation can also be obtained by staining with Sudan black B and Safranine (Trigiano R., personal communication). Cells can be observed under the microscope and PHB granules would appear black if present. DNA-DNA hybridization experiments of RSB and *Sphingomonas echinoides* is also suggested for identification of RSB at the species level. A 70% or greater DNA-DNA relatedness and 5°C or less divergence value (ΔTm) would indicate that RSB be considered a new species. In addition to the preliminary test for indoles included in this study a GC analysis for quantitative identification of indoles is suggested.

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APPENDICES

Appendix 1. Protocol for negative staining of RSB cells (Dr. J. Dunlap, University of Tennessee, personal communication)

1. Pick up a formvar and carbon coated copper grid with forceps and place one drop of the bacterial suspension on the grid.

2. Let settle for one minute.

3. Carefully wick away any excess liquid with a piece of filter paper touching the edge of the grid.

4. Add one drop of the stain (2% Uranyl acetate pH 4.5) on the grid.

5. Dry excess stain with filter paper. (Grid should have an even, shiny appearance)

6. Let it air dry and observe under the transmission electron microscope.

Notes:

Do not let the grid dry out at any step during the process except at step 6.

Make sure that the bacteria and stain get only on one side of the grid.

Appendix 2. Solutions for glutaraldehyde & osmium tetroxide fixation
(Dr. J. Dunlap, University of Tennessee, personal communication)

Buffer stock solutions:

Solution A – 5.36 g $Na_2HPO_4.7H_2O$ in 100 ml glass distilled water. (Refrigerate)
Solution B – 2.76 g $NaH_2PO_4H_2O$ in 100 ml glass distilled water (Refrigerate)

Phosphate buffer (0.2 M)

Mix 15 ml of solution A (above) with 3 ml of Solution B (above). If necessary, adjust pH to 7.2 with either A or B. This buffer should be made up fresh each time and kept in an ice bath or refrigerator until time for use.

3% Glutaraldehyde

Mix 1 ml of 25% glutaraldehyde with 3.2 ml of glass distilled water. This gives 4.2 ml of 6% glutaraldehyde.

Mix the 6% glutaraldehyde and the 0.2 M phosphate buffer together in a 1:1 ratio. This gives 3% glutaraldehyde in 0.1 M phosphate buffer at pH 7.2

[Note: Glutaraldehyde purchased in bulk quantities is usually too acid (pH is below 3). It should be washed in barium carbonate and/or activated charcoal to raise the pH to 7 and remove yellowish impurities.]

4% Osmium tetroxide (OsO_4)

Mix 0.5 g OsO_4 in 12.5 ml of glass distilled water. Seal tightly and store in freezer. Thaw gently before use.

2% Osmium tetroxide in 0.1 M phosphate buffer (pH 7.2)

Mix 4% OsO_4 and 0.2 M phosphate buffer in 1:1 ratio.

Spurrs mixture

2.5 g VCD (Vinyl cyclohexene Dioxide)
1.5 g DER (DER 736 Epoxy resin)
6.5 g NSA (Nonenyl succinic anhydride modified)
0.1 g DMAE (2-(Dimethylamino) ethanol

Mix above contents thoroughly with an applicator stick, without creating air bubbles, in a disposable container.

Appendix 3. Protocol for glutaraldehyde & osmium tetroxide fixation of RSB cells (Dr. J. Dunlap, University of Tennessee, personal communication)

1. Centrifuge bacterial suspension at 2000 rpm for 10 min. to collect cells and discard the supernatant. Repeat this step once more.

2. Add fixative (3% glutaraldehyde in 0.1 M phosphate buffer) to the cell pellet, tap gently to loosen pellet and wait one hour. Centrifuge again and discard the fixative.

3. Rinse bacterial cells in phosphate buffer 3 times for 15 minutes each.

4. Post fix cells with 2% OsO_4 in 0.1 M phosphate buffer (pH 7.2) for one hour at room temperature.

5. Dehydrate samples through a graded acetone series:

50% acetone	15 min.
70% acetone	15 min.
90% acetone	15 min.
100% acetone	15 min. x 2 changes
Absolute acetone	15 min.

6. Fill embedding molds with Spurrs mixture and put samples into mold or BEEM™ capsules.

7. Place in the oven at 60°C for 2 days.

8. Cut thin sections using ultramicrotome (Reichert OMU3, Reichert, Vienna, Austria).

9. Collect cut sections on uncoated 400 mesh copper grids.

10. Stain sections with saturated uranlyacetate in 50% ethanol for 30 min.

11. Post stain with lead citrate for 5 min.

12. Observe under the transmission electron microscope (Hitachi H800, Hitachi High Technologies America, Pleasanton, CA)

Appendix 4. Composition of reagents for biochemical analysis using API ZYM and API 50CH test kits (Biomerieux Inc., MO, USA)

ZYM A (Biomerieux Inc.):

25 g Tris-hydroxymethyl-aminomethane
11 ml Hydrochloric acid (37%)
10 g Sodium lauryl sulfate
100 ml Water

ZYM B (Biomerieux Inc.):

0.35 g Fast Blue BB
100 ml 2-methoxyethanol

API 50CHB/E medium (Biomerieux Inc.):

2 g Ammonium sulfate
0.5 g Yeast extract
1 g Tryptone
0.18 g Phenol red
10 ml Mineral base (Cohen-Bazire)
1000 ml Phosphate buffer (pH 7.8)

Appendix 5. Protocol for pigment extraction (From Saito, T. et al. 1994 with minor modifications)

1. Inoculate 1 L of mWPM with RSB. Incubate cultures at 30°C in the dark at 100 rpm for 5 d.

2. Harvest the cells by centrifuging at 16,000 g (10,000 rpm) and 4°C for 15 min.

3. Add 25 ml of 10 N KOH to the cells and charge with N_2 gas. Stir this suspension overnight at room temperature.

4. After 24 h adjust the pH of the suspension to 7.0 with conc. HCl in an ice bath.

5. Add 2 volumes of absolute acetone and incubate at room temperature for 1 h with occasional stirring.

6. Centrifuge at 25,000 g (14,000 rpm) and 4°C for 10 min. Discard the pellet and measure volume of supernatant.

7. Add equal volume of ethyl ether to the supernatant and transfer contents to a separatory funnel.

8. Add water slowly to separate the two layers and move pigments slowly to the top (ether) layer.

9. Discard the lower acetone/water layer. If the lower layer still contains pigments then repeat the ethyl ether extraction (steps 7 and 8) once more until a clear acetone/water layer is obtained.

10. Rotary evaporate the top ether layer and collect contents in 10-15 ml of benzene. Store pigments in a closed container charged with N_2 gas at 0°C.

Appendix 6. Preparation of reagents used for lipid extraction

Phosphate buffer (pH 7.4)

Dissolve 8.7 g Potassium phosphate Dibasic ($K_2HPO_4.3H_2O$) in 995 ml of distilled water. Adjust pH to 7.4 with 3 M HCl. Filter using a 0.2 µm filter. Add 50 ml of chloroform. Close bottle, shake gently and allow to extract overnight.

Methanolic KOH

Dissolve 0.28 g KOH in 25 ml of methanol.

Appendix 7. Protocol for extraction & analysis of PLFA

(A) Harvesting cells for extraction

1. Grow RSB and *Sphingomonas echinoides* cells on mWPM plates at 30°C in the dark for 5 days (until late log or early stationary phase). Also incubate one mWPM plate under the same conditions to be used as a control.

2. Label eppendorf tubes for each treatment and add 1.5 ml of HEPES buffer to each tube.

3. Scrape bacterial cells from the surface of the plate (being careful not to get any agar) and suspend them in 50 mM HEPES (*N*-2-Hydroxyethylpiperazine-*N′*-2-ethanesulfonic acid) buffer (pH 7.0) in eppendorf tubes.

4. Subject the control plate to the same number of scrapings as for the plates containing the bacteria.

5. Centrifuge cells at 10,000 rpm for 5 mins.

6. Decant the supernatant.

7. Lyophilize for 24 h and perform lipid extraction. Tubes can also be stored in the freezer (-40 to -80°C) for extraction at another time.

(B) Extraction (Ref. White et al. 1979)

[Note-All glassware used in lipid extraction was washed with phosphate free soap + microsoap and rinsed several times in water and deionized water. Glassware was wrapped in foil and muffled in an oven at ~450°C for 4-5 h.]

1. Weigh each of the lyophilized cell samples individually and transfer to labeled test tubes.

2. To each test tube add the following (First phase of modified Bligh/Dyer extraction –phosphate buffer:methanol:chloroform in the ratio of 1.6:4:2)

Phosphate buffer-	4 ml
Methanol-	10 ml
Chloroform-	5 ml

[Roughly the ratio used was 1 ml of chloroform for every 2 mg of cells]

3. Vortex tubes (~ 5 Seconds) and burp tubes (Open and close lid to release pressure)

4. Sonicate tubes for 2 min.

5. Let stand for 2-3 h.

6. To each tube add (to split phase)

Chloroform-	5 ml
Water-	5 ml

7. Vortex tubes (~ 5 seconds) and centrifuge at 2000 rpm for 15 min.

8. Remove tubes from centrifuge and let stand for 15 min.

9. With a Pasteur pipette transfer contents of lower phase into another clean and labeled test tube.

10. Dry under a stream of N_2 gas with test tubes placed in a water bath ≤ 37°C.

(C) Silicic acid column chromatography (Ref. Guckert et al. 1985)

1. With a clean tweezer pick up and place Pasteur pipette (containing glass wool and muffled at ~450°C for 4-5 h.) in a rack.

2. Wet the glass wool with a few drops of 20 mM ammonium acetate in methanol. Discard carefully any waste that drips from the Pasteur pipette.

3. To the Pasteur pipette column add a slurry of 0.5 g silicic acid (activated at 100°C for 1 h.) in 5 ml of 20 mM ammonium acetate in methanol. (Make sure there are no air bubbles in the column.)

4. Wash the column with 5 ml of acetone.

5. Wash the column with 5 ml of chloroform.

6. With a clean Hamilton syringe add 150-200 µl of chloroform to the dried sample from step 10 above.

7. Using a Pasteur pipette, add sample to the silicic acid column. Wash the test tube containing the sample twice with 150-200 µl of chloroform and add any remaining sample to the column.

8. Place a clean test tube under the column and elute with 5 ml of chloroform. Label eluent as neutral lipids (NL).

9. Next place another clean test tube under the column and elute with 5 ml of acetone. Lable this fraction as glycolipids (GL) + pigments.

10. Place another clean test tube under the column and elute with 10 ml of methanol. This is the fraction containing the polar lipids (PL).

11. Dry all three fractions (NL, GL and PL) under a stream of N_2 gas and store at -20°C.

(D) Preparation of fatty acid methyl esters (FAME) (Ref. Guckert et al. 1985)

(Use polar lipid samples for preparation of FAME.)

1. Remove Polar lipid (PL) samples from freezer and thaw for a few minutes.

2. To each PL sample add 0.5 ml methanol and 0.5 ml chloroform.

3. Add 1 ml of methanolic KOH to each sample and vortex for a few seconds.

4. Incubate tubes at 60°C for 30 min.

5. Add 2 ml of hexane to each tube and vortex 30 seconds.

6. Centrifuge at ~2000 rpm for 5 min.

7. Pipette the top layer to another clean test tube and label FAME. Repeat steps 5 and 6 and collect any remaining FAME.

8. Dry tubes labeled FAME under a stream of N_2 gas.

9. Add 500 µl of hexane to each test tube and transfer lipids to GC vials. Repeat twice with 500 µl each time.

10. Dry samples in vials under a stream of N_2 gas.

11. Resuspend dry lipid sample in 300 µl hexane. Use this sample to inject into the GC-MS.

Temperature program used for GC analysis:
Initial temperature = 60°C. Hold for 2 min.
Rate = 10°C/min until 150°C.

Rate = 3°C/min until 312°C.
Total run time = 65 min.

Internal standard used was 50 pmol/µl of 21:0

Appendix 8. Protocol for extraction & analysis of sphingolipids (Ref. Leung et al. 1999)

1. Harvest cells as described for PLFA extraction.

2. Follow extraction steps 1-9 as described in protocol for PLFA extraction.

3. Reextract by adding 5 ml of chloroform, vortexing briefly, burping tubes and centrifuging at 2000 rpm for 15 mins. Let stand 15 mins and collect lower phase.

4. Repeat extraction (step 3 above) once more and pool all lower phase fractions together in one test tube.

5. Dry chloroform under a stream of N_2.

6. Add 1 ml of 3 N HCl to each sample tube and heat tubes at 100°C for 3 h.

7. Let tubes cool down to room temperature. Add 2 pellets of KOH to each tube and shake tube to dissolve KOH.

8. Add 2 ml of chloroform to each tube.

9. Vortex briefly and centrifuge at 2000 rpm for 5 min.

10. With a Pasteur pipette collect the lower phase (chloroform) in another set of tubes.

11. Repeat extraction with 2 ml of chloroform two more times (steps 8-10).

12. Dry chloroform under N_2.

    (Tubes can be stored at –20°C at this point. Follow remaining steps just before injecting samples into the GC-MS.)

13. To each tube add 100 µl of bis(trimethylsilyl)trifluoroacetamide (BSTFA) and heat at 60°C for 30 min.

14. Dry under N_2.

15. Resuspend in 40-300 µl hexane.

16. Analyze by GC-MS. (Sample volume injected = 1 µl)

Appendix 9. Solutions for obtaining high molecular weight DNA & for PFGE

1 M Tris-HCl (pH 7.2)

12.11 g of Tris [Tris(hydroxymethyly) aminomethane] was dissolved in 50 ml of water. pH of the solution was adjusted to 7.2 using 1 N HCl. After adjusting the pH, the volume was made up to 100 ml with water. The solution was filtered using a 0.45 μm filter and sterilized by autoclaving.

0.5 M EDTA (pH 8.0)

9.306 g of EDTA (disodium ethylene diamine tetraacetic acid) was dissolved in 25 ml of water. 3 N NaOH was used to adjust the pH to 8.0. After adjusting the pH, the volume was made up to 50 ml with water. The solution was filtered using a 0.45 μm filter and sterilized by autoclaving.

5M NaCl

14.61 g of NaCl (Sodium chloride) was dissolved in 30 ml of water. The final volume was adjusted to 50 ml with water. The solution was sterilized by autoclaving.

NaCl/Tris HCl (pH 7.2)/EDTA (pH 8.0)

4 ml of 5 M NaCl was mixed with 1 ml of Tris HCl (pH 7.2), 20 ml of 0.5 M EDTA (pH 8.0) and 75 ml of water. The solution was filtered using a 0.45 μm filter and sterilized by autoclaving.

Bacterial cell lysis solution (10 ml)

0.1ml 1 M Tris HCl (pH 7.2)
0.1ml 5 M NaCl
2 ml 0.5 M EDTA (pH 8.0)
20 mg Sodium deoxycholate (Deoxycholic acid sodium salt)
50 mg *N*-lauroylsarcosine sodium salt
7.8 ml water

The above components were combined and the solution was filter sterilized using a 0.22 μm filter and stored at room temperature.

10 mg lysozyme was added to this filtered solution (10 ml) just before use.

Bacterial cell proteinase K solution

0.1 g *N*-lauroylsarcosine sodium salt was added to 10 ml of 0.5 M EDTA (pH 8.0). The solution was warmed slightly to dissolve *N*-lauroylsarcosine sodium salt. The solution was filter sterilized using a 0.22 µm filter and stored at room temperature. 0.25 ml of 20 mg/ml Proteinase K solution was added to this filtered solution just before use.

TE (Tris EDTA) Buffer (pH 8.0)

1 ml of 1 M Tris HCl (pH 8.0) was added to 0.2 ml of 0.5 M EDTA (pH 8.0) and the volume was adjusted to 100 ml with water. The solution was filtered using a 0.45 µm filter and sterilized by autoclaving.

10 mM PMSF

17.4 mg PMSF (phenyl methyl sulfonyl fluoride) was dissolved in 10 ml of isopropanol and stored at 0°C.

TE containing 1 mM PMSF

1.4 ml of 10 mM PMSF was added to 12.6 ml of TE [10 mM Tris HCl (pH 8.0)/ 1 mM EDTA (pH 8.0)]

5X TBE (Tris borate EDTA) buffer

Dissolve 24.2 g Tris base and 12.4 g Boric acid in 800 ml distilled water. Add 2 ml 0.5 M EDTA. Adjust the volume to 1 L with distilled water.

Restriction enzyme buffer

180 µl dH_2O
20 µl 10X Restriction enzyme buffer (Supplied with the restriction enzyme)
Combine above components and mix well.

Digest buffer

3.6 ml dH_2O
400 µl 10X buffer (Supplied with the restriction enzyme)

2 µl 1 M DTT (Do not add if present in 10X buffer)
40 µl 10 mg/ml BSA (1 mg/ml final)
Combine above components and mix well.

ESP

1 g SDS (Sodium dodecyl sulfate) in 100 ml 0.5 M EDTA (pH 9.0)
1 mg/ml Proteinase-K
Mix well, aliquot and store at –20°C.

PFGE Buffer

1800 ml dH_2O
200 ml 5X TBE
20 ml 10 mM Thiourea
Combine above components and mix well.

Appendix 10. Protocol for PFGE

(A) Preparation of unsheared DNA in agarose blocks (Ref. Riethman et al. 1997)

1. Grow bacteria (RSB and *Sphingomonas echinoides*) in 15 ml of mWPM for 3 d.

2. Centrifuge bacterial suspension at 6000 rpm and 4°C for 10 min.

3. Discard the supernatant and resuspend each cell pellet in 3 ml of NaCl/Tris (pH 7.2)/EDTA.

4. Centrifuge bacterial suspension at 6000 rpm and 4°C for 10 min.

5. Discard the supernatant and resuspend cell pellet in 1 ml of NaCl/Tris (pH 7.2)/EDTA by pipetting up and down several times. Keep the cell suspension in a water bath at 38°C.

6. Prepare a solution of 2% SeaPlaque GTG Agarose in water and place in a water bath at 43°C.

7. Mix cell suspension and 2% agarose in the ratio of 1:10 (for RSB and *S.* echinoides) by pipetting up and down quickly and immediately pour the mixture with a 3 ml syringe into molds carefully avoiding the formation of air bubbles. Wait for 30 mins or until the mixture solidifies in the mold.

8. Remove plugs from the mold into a tube. Pour enough 50 mM EDTA to cover plugs completely and store plugs at 4-5°C.

(B) Cell lysis (Ref. Riethman et al. 1997)

1. Place 3 plugs in an eppendorf tube. Add 1 ml (approx. 3-4 volumes) of bacterial cell lysis solution. Incubate tubes at 37°C overnight.

2. Discard cell lysis solution and add 1 ml (approx. 3-4 volumes) of Bacterial cell Proteinase-K solution to each tube containing 3 plugs and incubate tubes at 50°C overnight.

3. Discard the proteinase-K solution and wash plugs in 2-3 ml (approx. 10 volumes) of 50 mM EDTA for 30 mins. (These plugs can be stored in fresh 50 mM EDTA at 4-5°C.)

4. Wash plugs 3 times in 2-3 ml (approx. 10 volumes) of TE containing 1 mM PMSF (Phenylmethyl sulfonyl fluoride) for 30 min each.

(C) Digestion of DNA with restriction enzymes

1. Cut the plug into 1/3rd along width.
2. Wash cut plugs in distilled water for 1 h. with gentle shaking to remove EDTA.
3. Equilibrate plugs in restriction enzyme buffer for 30 min.
4. Discard restriction enzyme buffer and incubate plugs in digest buffer 2 times for 15 min. each.
5. Discard digestion buffer and add 200 µl of fresh digestion buffer
6. Add 50 U of restriction enzyme. Mix well and incubate tubes with plugs at 37°C overnight.

(D) Pulse field gel electrophoresis

1. Remove plug from digestion buffer and incubate in ESP at 50°C for 2 h.
2. Wash plugs in PFGE buffer 2 times for 15 min. each. Also wash the marker plugs in PFGE buffer twice for 15 min. each.
3. Prepare gel for PFGE by combining
   90 ml dH_2O
   10 ml 5X TBE buffer
   1 g PFGE agarose
   1 ml of 10 mM Thiourea
   Pour a small amount of PFGE buffer over the gel, after it solidifies, to keep the gel from drying out.
4. With the help of sterile spatulas insert plug slices into the wells of the PFGE gel.
5. Seal plugs in wells with 0.8% Low melting temperature agarose in 0.5X TBE.
6. Set up the PFGE apparatus such that the PFGE buffer can circulate through a cold (12-14°C) water bath.
7. Place gel in the gel box making sure that there are no air-bubbles under the gel.

8. Pour enough (approx. 2 L) cool PFGE buffer over the gel (gel should be 1-2 mm below the buffer level).

9. Set the parameters (run time, pulse time, voltage) and run the gel.

10. Stain gel with 200 ml of 0.5X TBE containing 10 µl of 10 mg/ml ethidium bromide in the dark for 15 mins.

11. Rinse the gel twice in water and destain in water for 10 mins.

12. Observe and photograph under UV light.

Appendix 11. Protocol for preparation of bacterial growth medium extracts

1. Grow bacteria in 1 L mWPM for 5 d in the dark at 30°C on a shaker at 100 rpm.

2. Centrifuge bacterial culture at 10,000 rpm and 4°C for 15 min.

3. Collect and measure the volume of the supernatant. Discard cell pellet.

4. Adjust pH of the supernatant to 8.0.

5. Add ½ volume of ethyl acetate to the supernatant. Cover and stir very slowly at room temperature for 45 min.

6. Pour contents into a separatory funnel and pipette out the top ethyl acetate layer (pH 8 fraction) into an evaporating flask. Do NOT discard the lower layer.

7. Dry the pH 8 fraction using a rotary evaporator and collect with 20 ml of 95% ethanol. Label and store at 0°C.

8. Measure volume of the water fraction (lower layer from step 6) and adjust the pH to 3.0.

9. Add ½ volume of ethyl acetate to the pH 3.0 water fraction. Cover and stir very slowly at room temperature for 45 min.

10. Pour contents into a separatory funnel and pipette out the top ethyl acetate layer (pH 3 fraction) into an evaporating flask. Discard the lower water fraction.

11. Dry the pH 3 fraction using a rotary evaporator and collect with 20 ml of 95% ethanol. Label and store at 0°C.

Appendix 12. Mung bean bioassay

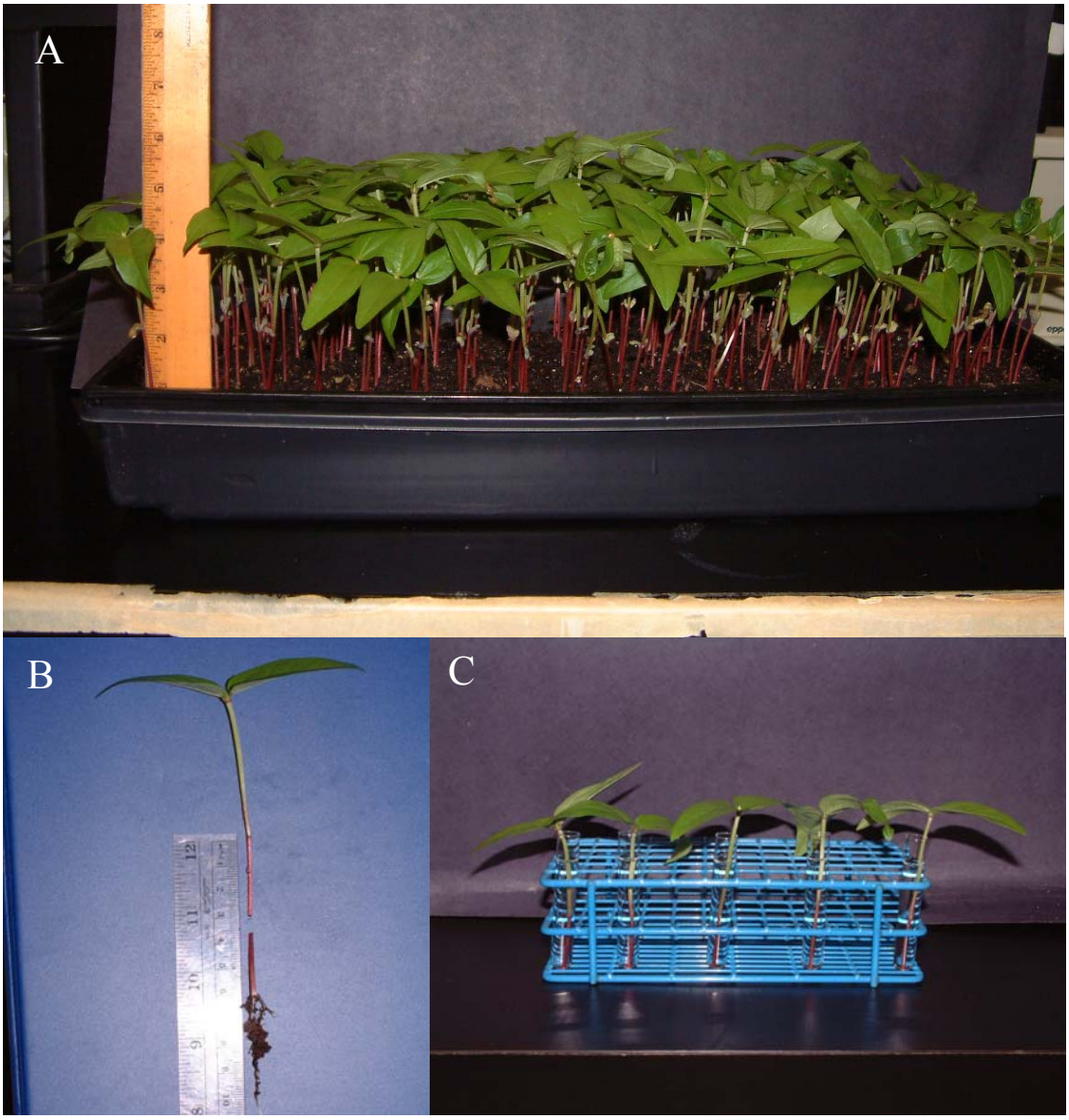

Figure 13. Picture showing mung bean bioassay. (A) Eight day old mung bean seedlings. (B) Mung bean seedling cut three centimeters below the cotyledonary node. (C) Seedlings in test tubes containing bacterial extracts.

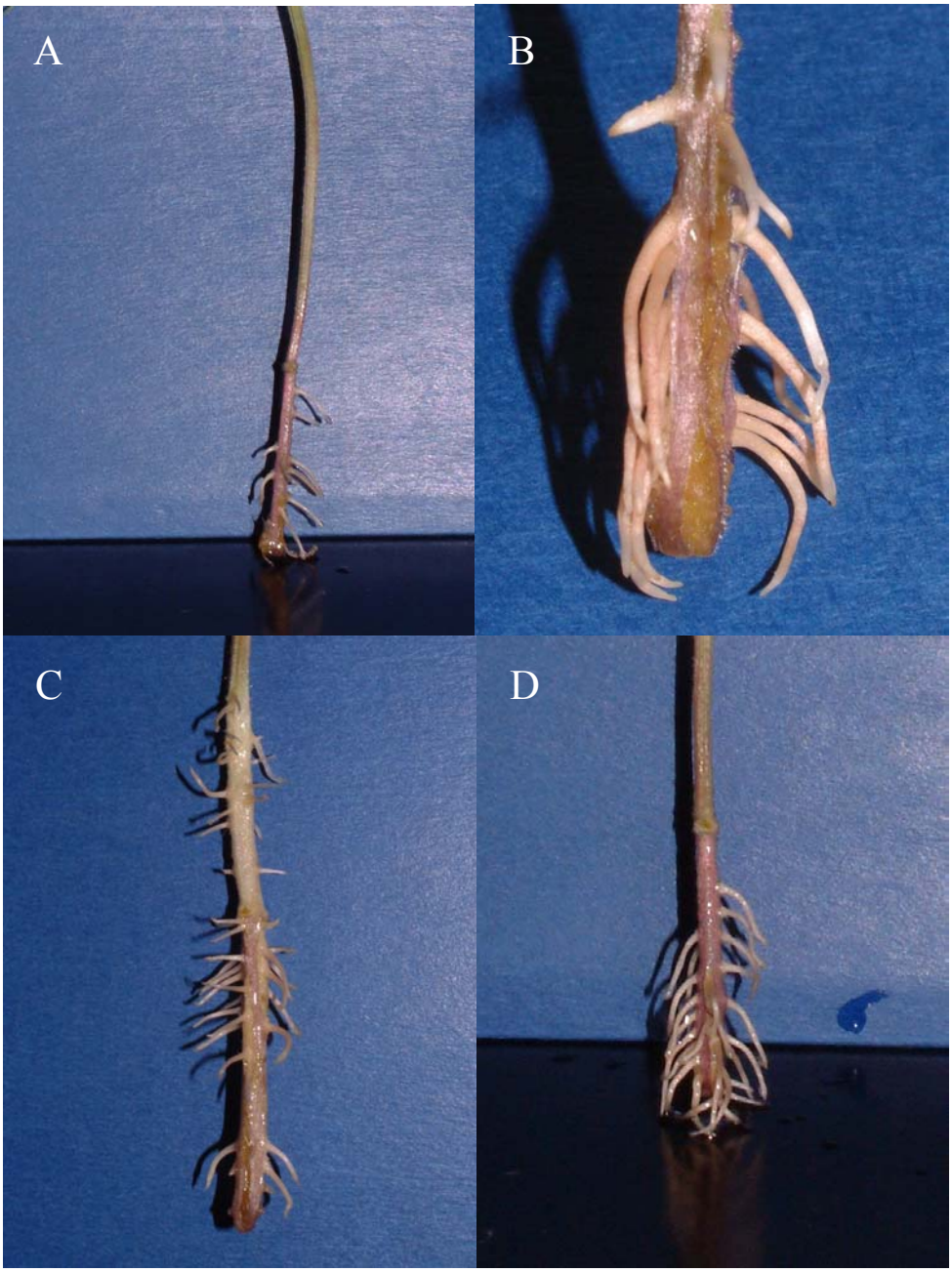

Figure 14. Rooted mung bean seedlings

VITA

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