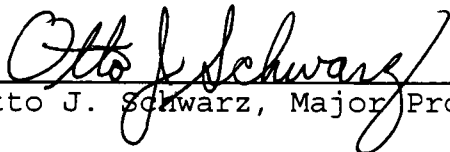


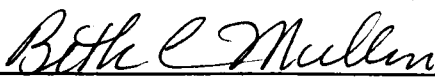
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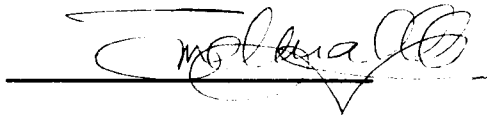
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PHYSIOLOGICAL STUDIES AND PHYLOGENETIC ANALYSIS OF A
ROOT-INDUCING BACTERIUM

A Thesis
Presented for the
Master of Science
Degree
The University of Tennessee, Knoxville

Venkataraman Sridhar

August 1994

DEDICATION

This thesis is dedicated to M. Narayanan 'Nadu' Menon.

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ABSTRACT

Studies have been carried out with the root-stimulating bacterium (RSB) isolated by Burns (1990) to better understand the mechanism of RSB-stimulated root induction in *in-vitro* cultures of hypocotylary explants of slash pine (*Pinus elliottii* Engelm.) and to understand the phylogenetic affiliations of the bacterium through the analysis of its 16S rRNA sequence. When inoculated with a culture of the bacterium, seedling explants of 10 half-sib genotypes of slash pine were found to root at different levels depending upon the genotype. Rooting percentages ranged from 8.33% through 66.67% showing a genotype-dependent variation in the level of root induction. A putative root-inducing signaling substance (RISS) was maximally extracted at a pH of 2.8 from RSB liquid culture medium. The extract at pH 2.8 induced a level of rooting in the slash pine explants that was not significantly different from the level of induction seen in explants inoculated with a living culture of RSB. The spectral profile of the methanol extract of the bacterium was found to be similar to the profile of carotenoid pigments. The 16S rRNA gene of the bacterium was amplified from its genomic DNA using the polymerase chain reaction with oligonucleotide primers that correspond to conserved eubacterial sequences that flank the majority of the 16S rRNA gene. The amplified product was cloned into the SmaI site of the vector pBluescript KS and

subsequently sequenced using the Sanger dideoxynucleotide sequencing technique. A phylogenetic analysis of the sequence was carried out using the sequence of 16S rRNA gene of 17 members of the α subdivision of *Proteobacteria* obtained from GenBank and the Ribosomal Database (Ribosomal Database Project). The analysis revealed that the bacterium is closely related to *Sphingomonas paucimobilis*, *Flavobacterium devorans* and *Caulobacter subvibrioides* in terms of the sequence of its 16S rDNA. The RSB matched most closely with *Caulobacter subvibrioides*. Comparative analyses of an alignment of the sequence of the four taxa revealed that the RSB differed from *Caulobacter subvibrioides* by only 45 nucleotides. A study of the segment of the 16S rRNA gene defined by Yabuuchi et al., (1990) revealed that the two taxa differed by only 4 mismatches suggesting that both might belong to the same genus.

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LIST OF ABBREVIATIONS

c.v.	Coefficient of variation
CIAA	Chloroform-isoamyl alcohol
CIP	Calf intestinal alkaline phosphatase
CTAB	Cetyl trimethyl ammonium bromide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
EDTA	Disodium ethylene diamine tetraacetic acid.2H ₂ O
GD medium	Gresshoff and Doy medium
GLM	General linear methods
HPLC	High performance liquid chromatography
IAA	Indole-3-acetic acid
IBA	Indole-3-butyric acid
ICA	Indole-3-carboxylic acid
IPTG	Isopropyl thiogalactoside
LB	Luria-Berthani medium
NAA	Naphthalene acetic acid
PAR	Photosynthetically active radiation
PCR	Polymerase chain reaction
rDNA	Ribosomal deoxyribonucleic acid
RDP	Ribosomal Database Project
REGWQ test	Ryan-Einot-Gabriel-Welsch Multiple Range Test
RISS	Root-inducing signalling substance
RNA	Ribonucleic acid
RNase	Ribonuclease
rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RSB	Root-stimulating bacterium
SDS	Sodium dodecyl (lauryl) sulfate
SSC	Sodium chloride sodium citrate
TAE	Tris-acetate EDTA
TBE	Tris-borate EDTA
TE	Tris-EDTA
TNE	Tris-EDTA with sodium chloride
UV	Ultraviolet
WP medium	Woody plant medium

CHAPTER I

INTRODUCTION

One of the greatest challenges facing plant biologists today is to meet the increasing demands for food, fuel and fiber. A major step in this direction is the improvement of existing crop and forest species and the development of new ones. In the case of forest species, there is a need for the rapid propagation of superior genotypes of woody plant species. A major stumbling block to the propagation of many woody species is the difficulty in rooting clonal explants. The microbial induction of adventitious rooting in woody species is currently being studied as an attractive method to root clonal woody explants.

This thesis describes an attempt to identify and further characterize a bacterium that induces adventitious rooting in slash pine explants. Experiments were performed in order to better understand the conditions necessary for the rooting of pine cuttings induced by a root stimulating bacterium (RSB) and to begin to investigate the physico-chemical basis for RSB root induction. To facilitate the identification of the bacterium, the entire base sequence of the 16S rRNA gene was determined and a phylogenetic analysis carried out. This investigation represents the beginning of the understanding of this novel bacterially stimulated rooting phenomenon.

Tissue culture is a technique which can be used to

increase the productivity of coniferous forests with the production of selected genotypes (Stein et al., 1990; Zobel, 1992; Mohammed and Vidaver, 1988). The cultivation and maintenance of a healthy stand of trees for reforestation and commercial cultivation calls for the selection of superior performing species. It is possible to introduce qualities such as superior wood, early maturity and disease resistance in the forest about 8 to 25 years earlier than by conventional seed cultivation techniques using tissue culture and other *in-vitro* culture techniques. These techniques could also introduce new genetic variability into the species and reduce barriers of crossability among species (Mohammed and Vidaver, 1988).

Towards this end, tissue culture has been applied to pine propagation through a variety of *in-vitro* techniques such as cotyledonary micropropagation, callus and suspension cultures and protoplast isolation and culture (Lesney et al., 1987). *In-vitro* techniques of vegetative propagation have also been applied to slash pine cultivation. Burns et al. (1991) reported the clonal propagation of slash pine through axillary bud proliferation induced by a pulse of 6-benzylaminopurine.

RSB was originally isolated from *in-vitro* cultures of explants obtained from slash pine seedlings. Slash pine (*Pinus elliottii* Engelm.) is widely cultivated in Australia, New Zealand, Colombia, Argentina and South Africa (Lesney et al., 1987). It is also one of the major tree species in the southern United States cultivated for its pulp, poles, lumber

and gum. The species is found from the coastal plains from South Carolina to Florida and as far west as Louisiana. 34 % of the forested land of Florida is covered with this species of pine which makes it the single most important tree species to the forestry industry in Florida.

One of the major hurdles in the vegetative propagation of conifers is the difficulty in rooting the clonal explants (Stein and Fortin, 1990; McAfee et al., 1993). The level of spontaneous rooting in *in-vitro* cultured conifers is very low (1-5%) (Mohammed and Vidaver, 1988) except in the case of radiata pine (*Pinus radiata* D. Don), in which spontaneous rooting has been observed at the rate of 36%. A variety of hormonal treatments have been carried out to overcome this major bottleneck in clonal propagation. Agar based media containing indole-3-acetic acid (IAA) or naphthalene acetic acid (NAA) (Boulay, 1979; Cheng and Voqui, 1977) or indole-3-butyric acid (Smith and Thorpe, 1975) have been used in order to root explants. The dosage of auxins has been optimized for a variety of systems. However the quality of roots produced in agar-based media is not always acceptable (Mohammed and Vidaver, 1988). *In-vitro* generated roots of white spruce [*Picea glauca* (Moench) Voss] are often nonfunctional and undergo a physiological break down when acclimatized (Toivonen, 1985). Gay (1990) found that roots produced in the presence of high concentrations of auxin supplied at the beginning of the culture period were thick and presented a

very limited elongation. The hormonal induction of aberrant roots has also been reported by Burns (1990) in slash pine.

Root induction by auxins

Auxins are known to improve root formation in cuttings of many species (Went and Thimann, 1937). Mixtures of synthetic substances with known auxin activity are frequently used for the propagation of ornamentals by cuttings (Wareing and Phillips, 1978). Naphthalene acetic acid (NAA) and/or indole-3-butyric acid (IBA) are used to induce root meristem differentiation (Minocha, 1980). Slankis (1973) reported that the addition of auxins to pine root organ cultures induced the development of short laterals and dichotomous branching similar to naturally occurring mycorrhizae.

Rooting of cultures has been done using agar based media containing auxins and nutrients. Such media provide a uniform distribution of the hormone and a good contact between the excised shoot and the substrate. This results in a more synchronized rooting. Rooting can also be achieved in soil-less media such as mixtures of peat, perlite, vermiculite or sand. However the difficulty in moisture regulation affects the maintenance of shoot health in these rooting media. White spruce rooted in agar-based media suffer from problems due to nonfunctional roots and are susceptible to damage during the transfer from the laboratory to the field (Toinoven, 1985). Agar probably interferes with gas exchange and inhibits the

differentiation of the vascular system and the development of root hairs (Mohammed and Vidaver, 1988).

For many coniferous species, the induction of rooting by auxins is inefficient and the establishment of rooted cuttings is difficult or impossible (Mott, 1981). In Douglas fir [*Pseudotsuga menziesii* (Mirb.) Franco], IAA produced only 1-2 roots per shoot and minimal callus at the shoot base, whereas NAA gave a better response (Boulay, 1979). In radiata pine optimal rooting was induced only if IBA was present during the first 4 days of the treatment (Smith and Thorpe, 1975). The level of rooting is also affected by the age of the donor material. In conifers, older explants are more recalcitrant in shoot and root development. Similarly, explants from juvenile flushes on the tree respond better to *in-vitro* treatments (Bonga, 1981) than do explants from mature flushes.

A continuous vascular connection between the shoot and root is necessary for the survival of the plantlet. Plantlets which develop large calli at the site of excision, often have roots formed within the calli which are not well connected to the vascular system. Such plantlets do not survive. (Thorpe, 1984). In Norway spruce [*Picea abies* (L.) Karst], black spruce [*Picea mariana* (Mill.) B.S.P.] and white spruce healthy plantlets have a minimal callus and a well connected root (Mohammed and Vidaver, 1988).

Studies are now underway to induce rhizogenesis by co-culturing pine tissues with microbes such as fungi and

bacteria. A majority of the research is focused on the induction of rhizogenesis by the inoculation of derooted pine seedling explants with *Agrobacterium rhizogenes*, a bacterium known to cause 'hairy root' symptoms in apple and other rosaceous species, (Strobel and Nachmias, 1988) and ectomycorrhizal fungi, such as *Hebeloma hiemale* Bres. (Gay, 1990), *Laccaria bicolor* (Maire) Orton and *Suillus cavipes* (Opat.) Smith & Thiers (Stein et al., 1990). In pine explants, rooting has also been induced by a root stimulating bacterium (RSB) initially identified as *Pseudomonas vesicularis* by Burns (1990). This bacterium was found to be non-pathogenic and to induce roots that were analogous to auxin-induced roots, but with a better morphology.

The major objective of this research was to carry out a phylogenetic analysis of the root stimulating bacterium using the sequence of its 16S ribosomal gene (rRNA) in order to establish the relationship of RSB to other known bacteria. In addition, experiments were performed to investigate the relationship of rooting efficiency and genotype of slash pine cuttings cultured under *in-vitro* conditions. Lastly, a bioassay of RSB culture medium and organic extracts was performed in order to first, attempt to separate the root stimulating principle from bacterial presence and then, to characterize its physico-chemical nature.

CHAPTER II

LITERATURE REVIEW

Adventitious root morphogenesis

Conifer shoots do not possess preformed root initials unlike other easily rooted woody plant species, and adventitious root development is generally preceded by the formation of callus (Mohammed and Vidaver, 1988). Roots initiated in this manner are called "wound roots". Within a few days in culture, tissue cultured shoots exhibit distinct morphological and histological changes. Cell division occurs near the shoot base in close association with the vascular cylinder, causing a swelling that leads to a rupture of the epidermis. In radiata pine (*Pinus radiata* D. Don) seedlings, a single target cell containing an intensely stained nucleus and many plastids was identified (Smith and Thorpe, 1975). The cytoplasm of this cell showed a darkening reaction when treated with dihydroxyphenylalanine (DOPA). This was an indication of melanin formation by the activity of polyphenol oxidase or tyrosinase. In this system it has been shown that rooting is correlated with the total phenol content in the tissue. Basipetally transported phenolics may be involved in the lignification of xylem cells for the future roots (Gaspar and Coumans, 1987). At this stage, in spruce tissues, some of the cells in the swollen region are differentiated into cambium-like cells that later develop into tracheid nests and

resin canals (Patel et al., 1986). Root primordia are produced in close association with the tracheid nests, which are in direct contact with the shoot vascular system. Thus the vascular system of the root is continuous with that of the shoot (Mohammed and Vidaver, 1988). The close association of tracheid nests with root primordia is very clear in *Pinus sylvestris* (Gronroos and Von Arnold, 1985). Plantlets of black and white spruce, Norway spruce and Douglas fir have a minimal callus and a well connected vascular system. Explants with a large calli often lack a well developed vascular system and differentiate roots which are not well connected to the shoot. Such plantlets suffer from a high degree of mortality during acclimatization, due to the inability to withstand water stresses (Mohammed and Vidaver, 1988).

Microbial induction of rhizogenesis

Induction of rooting by *Agrobacterium rhizogenes*

Agrobacterium rhizogenes, a gram negative soil bacterium causes the 'hairy root' disease in dicotyledonous plants (Zhan et al., 1990). It was first isolated from apple trees which expressed the 'hairy root' symptom. It was also found on other rosaceous plants. Riker (1930) studied the phenomenon and identified the causal agent as being distinct from *Agrobacterium tumefaciens*. In spite of the infection, no significant crop loss was associated with the pathogen. Riker suggested that the organism could be used to stimulate rooting

in certain plants. Since then, a variety of plant species have been rooted using *A. rhizogenes* (refer to Table II-1).

Mode of action of *A. rhizogenes*

A. rhizogenes has a defined region of DNA, called the T-DNA, on its root-inducing plasmid which is transferred to the plant cell during infection and is integrated into the host DNA. The T-DNA carries phytohormone genes which are expressed in the transformed host tissue and lead to the 'hairy root' symptom. Agropine strains of *A. rhizogenes* carry genes for auxin synthesis, but not cytokinin synthesis while mannopine strains carry neither. Both, however, carry phytohormone-like root-inducting *Rol* genes whose functions are not understood.

The T-DNA is flanked on one side by virulence (*vir*) genes which are instrumental in T-DNA transfer when triggered by small soluble phenolics such as those exuded by cultured or wounded plant tissue (Stachel et al., 1985). Agropine strains of *A. rhizogenes* contain 2 different transforming regions. One is on the left (TL) and the other on the right (TR). TR carries agropine and auxin biosynthetic (*aux*) genes while TL carries genes that impart to plant cells, the ability to differentiate roots. Thus mannopine and cucumopine strains which lack the *aux* gene cannot induce rooting under conditions of limited auxin (Failla et al., 1990). Carrot discs infected with *A. rhizogenes* express 'polarity' where the apical section (rich in auxin) develops hairy roots while the basal section

TABLE II-1

Woody Plants inoculated with *Agrobacterium rhizogenes* for the induction of rooting.

Tree	Common Name	Author
<i>Actinidia deliciosa</i>	Kiwi	Rugini <u>et al.</u> , 1991.
<i>Agathis australis</i>	Kauri pine	White and Lowell, 1984.
<i>Castanea sativa</i> Mill.	Chestnut	Rinallo and Mاتیotti, 1993.
<i>Corylus avellana</i> L.	Hazelnut	Bassil <u>et al.</u> , 1991.
<i>Eucalyptus</i>	Eucalyptus	McRae and Van Staden, 1993.
<i>Junglans</i>	Walnut	Dandekar <u>et al.</u> , 1988; Sutter and Luza, 1993.
<i>Larix laricina</i> (DuRoi) K.Koch	Larch	McAfee <u>et al.</u> , 1993.
<i>Larix x Eurolepis</i>	Hybrid larch	John, 1978.
<i>Malus pumila</i>	Apple	Patena <u>et al.</u> , 1988; Lambert and Tepfer, 1991; Sutter and Luza, 1993.
<i>Mangifera indica</i> L.	Mango	Strobel and Nachmias, 1988.
<i>Olea europaea</i> L.	Olive	Rugini, 1986.
<i>Persea americana</i> Mill.	Avocado	Strobel and Nachmias, 1988.
<i>Pinus monticola</i> Dougl.	Douglas fir	Proebsting <u>et al.</u> , 1987; McAfee <u>et al.</u> , 1993.
<i>Pinus banksiana</i> Lamb	Jack pine	McAfee <u>et al.</u> , 1993.
<i>Populus deltoides</i>	Poplar	Charest <u>et al.</u> , 1992.

TABLE II-1 (concluded)

Tree	Common Name	Author
<i>Prunus amygdalus</i> L.	Almonds	Strobel and Nachmias, 1988.
<i>Robinia pseudoacacia</i> L.	Black locust	Han <u>et al.</u> , 1993.
<i>Simmondsia chinensis</i> (Link) Schneider	Jojoba	Strobel and Nachmias, 1988.
<i>Vitis vinifera</i> L.	Grape	Strobel and Nachmias, 1988.

does not (Ryder et al., 1985).

Increased sensitivity of *Agrobacterium rhizogenes*-transformed plants to auxin

Shen et al. (1988) suggested that the response of *Agrobacterium rhizogenes*-transformed tissues included an increase in sensitivity of the tissue to exogenously applied auxin. They obtained 100 to 1000 times greater sensitivity of *A. rhizogenes*-induced roots to exogenous auxin compared to untransformed roots. Lambert and Tepfer (1992) obtained similar results. When a putative transformed material was transferred to rooting media containing 0.2 mg/l of auxin, necrosis was observed. The exact nature of this altered sensitivity is not known. Shen et al. (1988) hypothesize that the increased sensitivity is due to a modification in the auxin reception-transduction system in transformed roots. The modification could imply a greater number of receptors or more efficient ones. Thus auxin concentrations that limit the initiation of secondary meristems in normal tissues, are adequate for transformed roots, which exhibit the 'hairy root' phenomenon.

Agrobacterium rhizogenes and pines

Gymnosperms are transformed at a much lower frequency than most angiosperms, when infected with *A. rhizogenes* (Morris et al., 1989). Also the detection of opines, a

positive indication of transformation, is very difficult in conifers. Ellis et al. (1989) found gall and gall-like symptoms on 50% of the 4 species of conifers they infected with *A. tumefaciens*, but very few of the samples showed the presence of opines. Attempts at probing Southern blots of transgenic genomic DNA with the left T-DNA region of pRiA4b (an *A. rhizogenes* plasmid), did not reveal the presence of transferred DNA. McAfee et al. (1993) noted that the detection of a single copy gene integrated in the large pine genome may not be possible using the Southern blotting technique. Strobel et al. (1988) found similar results with transformed olives. Leyval and Berthelin (1989) suggested that the improved rooting of *Pinus sylvestris* co-cultured with *Agrobacterium* species is due to the secretion of organic acids such as malate, lactate, fumarate and citrate. The exact nature of the physiology of gymnosperm rooting induced by *A. rhizogenes* is still not clear.

Rhizogenesis induced by fungi

It was first hypothesized by Lindermann and Call (1977) that ectomycorrhizal fungi might secrete growth promoting substances that could enhance rooting in cuttings of vegetatively propagated woody ornamental plants. They reported an increase in the percentage of rooted cuttings and in root volume of the cuttings of bearberry (*Arctostaphylos uva-ursi* L. Spreng.) and huckleberry (*Vaccinium ovatum* Parsh.) when

inoculated with ectomycorrhizal fungi. Navratil and Rochon, (1981) reported similar findings on poplar cuttings, Branzanti et al. (1984) and Cristoferi et al. (1985) found similar results in fruit tree rootstocks. Gay (1981) showed that *Hebeloma hiemale* Bres. can induce adventitious rooting in *Pinus halepensis* Mill. hypocotyl explants in media supplemented with indole compounds.

Stein et al. (1990) studied the effect of *Laccaria bicolor* (Maire) Orton or *Suillus cavipes* (Opat.) Smith & Thiers on the rooting of *Picea mariana* (Mill.) B.S.P. and *Larix decidua* Mill. vegetative cuttings. The rooting was compared to that induced by various auxin treatments. Cuttings rooted by fungi had increased rooting percentages, number of adventitious roots per cutting and mean root length, especially in the case of *L. decidua*. There is good documentation of the secretion of growth promoting substances such as cytokinin-like and gibberellin-like chemicals, ethylene and auxins, especially indole-3-acetic acid (IAA) by ectomycorrhizal fungi (Stein et al., 1990). Navratil and Rochon (1981) suggested that the rooting of poplar cuttings induced by mycelia of *Pisolithus tinctorius* (Pers.) Coker & Couch was by the secretion of hormones by the fungus. Lindermann and Call (1977) suggested that these fungi might be secreting inhibitors of endogenous growth regulators. *Hebeloma hiemale* stimulates rooting in *Pinus halepensis* mill. hypocotyl cuttings in-vitro in the presence of suboptimal auxin

concentrations (Gay, 1981). While Branzanti et al. (1987) demonstrated that ectomycorrhizal fungi acted synergistically with exogenously applied auxins in the rooting of fruit rootstock cuttings. Gay (1990) was able to mimic the induction of rooting by *H. Hiemale* on derooted *Pinus halepensis* shoot hypocotyls using an ethyl acetate extract of fungal cultures supplemented with 1 mM tryptophan. He obtained a rooting percentage of 100% and each hypocotyl formed more than 13 roots while the average number of adventitious roots induced by the application of the crude filtrate (cell-free culture medium) was only 3.7. Further analysis of the extract by thin layer chromatography, followed by high performance liquid chromatography and mass spectrometry revealed the presence of IAA and indole-3-carboxylic acid (ICA). Since ICA is not biologically active (Gay 1990), it was concluded that the IAA present in the extract was responsible for the induction of rooting. Baraldi and Branzanti (1988) noticed a threefold increase in rooting of *Hebeloma sinapizans*-treated pear c.v. Bartlett microcuttings over auxin-treated controls. This variety of pear was incapable of spontaneous rooting. No significant difference was reported between the rate of rooting induced by the fungus as compared to that induced by the auxin in the medium.

Stein et al. (1990) reported that mature materials show a better response to fungal inoculation than juvenile materials. This is due to the high level of spontaneous

rooting seen in juvenile tissues. That the fungi induce rooting in tissues that do not respond to exogenous applications of hormones, indicates that other mechanisms apart from auxin secretion might be responsible for the induction of rooting.

Morphology of microbially induced roots

The site of root initiation depends on the organism and the specific strain of the organism. The A4 strain of *A. rhizogenes* initiates roots above and at the surface of the basal cut while strain R1000 induces a basal swelling from which roots emerge (McAfee et al., 1993). Co-cultivation of *P. banksiana* and *L. laricina* with *A. rhizogenes* strain A4 improved the quality of the new roots. The typical growth of non-geotropic 'hairy roots' associated with the infection of *A. rhizogenes* in some species (Tepfer, 1984; Spano et al., 1987) was not observed in any of the pine shoots treated with *A. rhizogenes*. Six months after transfer to the green house, the microbially rooted pines were similar to their respective controls (McAfee et al., 1993). In explants of *Larix laricina* (Du Roi) K. Koch rooted by cultures of *Laccaria bicolor* (Maire) Orton, and control explants rooted by auxin treatments, no callus was produced and rooting percentages were 93 and 100 respectively (Stein and Fortin, 1990). An upward distribution of roots was seen in fungal treatments along the hypocotyl, while auxin treated explants developed

roots only at the base of the hypocotyls. All NAA treatments (1, 10, and 50 μ M) developed substantial calli and the level of rooting never exceeded 10%. An IBA concentration of 1 μ M induced a slight swelling at the base of the cuttings whereas higher dosages (10 and 50 μ M) promoted the formation of calli. Hypocotyl explants cultured in auxin-supplemented media and inoculated with *L. bicolor* produced numerous root primordia on the hypocotyl axis without any callus tissue. At 10 μ M IBA large brownish calli were seen, from which numerous small roots emerged. However these did not elongate further. Basal necrosis and a withering of cuttings with a reddening of leaves was observed in hypocotyls treated with 50 μ M IBA (Stein and Fortin, 1990).

The root-stimulating bacterium (RSB)

Burns et al. (1990) isolated a bacterium which when co-cultured with *in-vitro* produced slash pine (*Pinus elliottii* Engelm.) microshoots of axillary origin or seedling-produced hypocotylary cuttings, initiates adventitious rooting. Schwarz et al. (1993) reported that the efficiency of rooting induced by the root stimulating bacteria (RSB) varies from 15% to 90% depending on physiological conditions, the age of the explant and the morphogenetic source of the explant. The root induction did not seem species specific as rhizogenesis has been demonstrated in *Pinus elliottii*, *Pinus strobus* and *Casuarina cunninghamiana* (Schwarz, personal communication). No

symptoms of pathology have been associated with the root stimulation and all roots initiated were geotropic, unlike 'hairy roots' induced by *A. rhizogenes*. The roots formed by treatment with RSB were normal in appearance and fully functional for transfer into soil (Burns, 1990). Also explants that bore roots had a minimal callus unlike untreated explants which developed prominent basal swellings. Attempts at the commercial identification of the RSB using classical nutritional and metabolic tests and detailed lipid profile analyses were unsuccessful.

Bacterial phylogeny and the 16S rRNA gene

The classification of organisms has traditionally been based on similarities in their morphological, developmental and nutritional characteristics. It is now clear that classification based on the traditional criteria does not necessarily correlate with evolutionary relationships as defined by the comparison of the nucleotide or amino acid sequence of their macromolecules (Lane et al., 1985). The 16S rRNA molecule is now widely recognized and used as a conservative macromolecule that allows phylogenetic placement of bacterial species (Woese, 1987).

"Of all natural systems, living matter is the one which, in the face of great transformation, preserves inscribed in its organization the greatest amount of its own past history"
Zuckerkandl and Pauling, 1965.

Genealogical relationships between diverse organisms can

be inferred from comparisons of the amino acid or nucleotide sequences of functionally equivalent biopolymers (Zuckerkindl and Pauling, 1965). Macromolecular sequences are preferred because they permit quantitative inference of relationships (Zuckerkindl and Pauling, 1965; Fitch and Margoliash, 1967). The comparison of amino acid sequences of cytochrome C, superoxide dismutase, ferredoxin, flavodoxins, azurins and plastocyanins have been used to determine phylogenetic relationships among bacteria (Jones and Krieg, 1984). Sometimes, in molecules such as the acyl carrier protein for the citrate-lyase system of *Klebsiella aerogenes* and the acyl carrier protein of the fatty acid synthetase system of *Escherichia coli*, the surviving similarities are so vague that even computer-based sequence comparisons are unable to validate relationships. While in other molecules, such as the diphtheria toxin fragment B and the human plasma lipid binding protein A1, similar sequences may appear in totally alien proteins as a result of mere chance, exon shuffling or, occasionally, by the convergent evolution of sequences with special properties (Doolittle, 1981). Ambler (1979) objected to the use of protein sequence comparisons due to the existence of non-congruence in protein evolutionary trees. He attributed this non-congruence to a) the comparison of 'paralogous' proteins; b) different rates of evolution in different branches of the phylogenetic tree; c) possibility of functional requirements limiting the evolution of the sequence

and therefore leading to convergence; and d) the possibility that the protein was present due to interspecific gene transfer.

The primary structure of the rRNAs are better suited for providing phylogenetic information than the primary structure of proteins. This is evident from their ubiquitous distribution and the presence of domains that change slowly in evolutionary time. The ribosome, the translation apparatus, is the most important organ of the cell and in characterizing its sequence, one is determining the extent to which and what features of, primitive forms of the translation apparatus, are reflected in the structure of its present day counterparts (Woese, Sogin and Sutton, 1974).

Ribosomes are ribonucleoprotein particles responsible for the translation of the genetic code. Unlike other cellular polymerases, they typically contain 50 to 60 percent RNA as an integral part of the structures (Noller, 1984). Typical rRNAs are those with a 1500-base small subunit and a 2900-base large subunit. Such rRNAs are found in eubacteria, archaebacteria, chloroplasts and plant mitochondria. Eukaryotic cytoplasmic and the remaining mitochondrial rRNAs are considered to be variants (Noller, 1984). Procaryotic ribosomes (70S) can be separated into 30S and 50S subunits by zonal centrifugation. The 30S subunit consists of a 16S rRNA to which 21 protein moieties bind to form a complex. The 50S subunit in turn consists of a 23S rRNA to which 34 protein moieties bind to

form a complex (Garrett and Wittmann, 1973). There are about 50 helical stalks in the 16S rRNA and about twice that number in the 23S rRNA (Woese, 1987). The 16S rRNA consists of approximately 1540 nucleotides while the 23S rRNA consists of approximately 2900 nucleotides (Noller, 1984).

Using RNase T1 oligonucleotide catalogs of 16S rRNA, Woese and his colleagues established a comprehensive outline of procaryotic phylogeny (Fox et al., 1980). With the development of DNA sequencing techniques, it became feasible to determine the nucleotide sequence of the 16S rRNA directly. Lane et al. (1985) designed three oligonucleotide primers, which provided access into 3 major 16S rRNA structural domains. The method called for the dideoxynucleotide terminated sequencing of rRNA from bulk cellular RNA preparations using reverse transcriptase and synthetic oligonucleotide primers complementary to the universally conserved 16S rRNA sites. Many difficulties were encountered using this technique since a) 'anomalies' were frequently sequenced (Lane et al., 1985); b) considerable amounts of rRNA were required; and c) the high concentration of RNases in bacteria made extracting intact rRNA molecules difficult. Bottger (1989) amplified rDNA from a preparation of total DNA using the polymerase chain reaction (PCR), using the primers described by Lane et al., (1985). He was able to sequence about 800 base-pairs of the 16S gene using this technique. Eden et al. (1991) amplified the entire 16S rRNA gene of

Aquaspirillum magnetotacticum using PCR with primers corresponding to positions 8-27 and positions 1510 to 1492 of the *Escherichia coli* 16S rRNA gene. Since then different laboratories have used different techniques to sequence the 16S rRNA gene.

Sequence data have been analyzed using many different algorithms. Of those commonly used are the distance matrix method, maximum parsimony method and cluster analysis (Woese, 1987). A variety of computer software is available commercially for carrying out the analysis. PAUP version 3.1 is a commonly used package (Swofford, 1991; O'Neill et al., 1992; Campbell et al., 1992).

Using 16S rRNA sequence information, Olsen (1987) developed a phylogenetic tree that describes the relative positions of eubacteria, archaebacteria and eukaryotes (refer to Figure II-1). This was done by compiling information from 24 representative organisms and establishing their relationships. This tree was not 'rooted' because the identity of the universal ancestor was not known. This classification changed the established concept of the living world being divided into the prokaryotes and the eukaryotes based on the level of cellular organization (Woese, 1987). Within the category of eubacteria, one would expect 2 major categories based on the gram staining procedure. Evidence from 16S sequences shows the assemblage of gram positive bacteria to be a coherent group while gram-negative bacteria can be

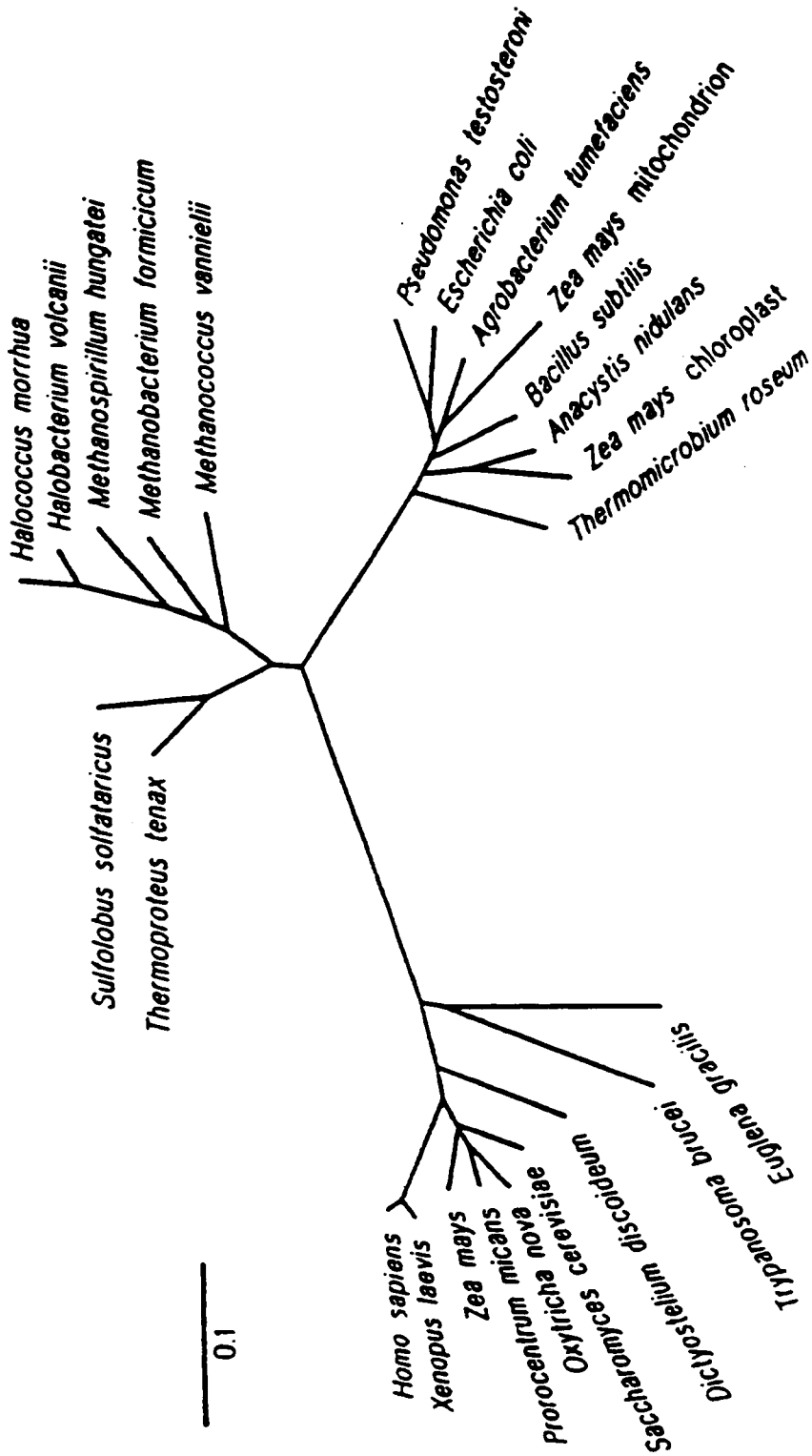
FIGURE II-1

A 16S rRNA sequence-based evolutionary tree.

This is an evolutionary tree based on the sequence of the 16S rRNA from a diverse collection of organisms and organelles consisting of *Sulfolobus solfataricus*, *Thermoproteus tenax*, *Halococcus morrhua*, *Halobacterium volcanii*, *Methanospirillum hungatei*, *Methanobacterium formicicum*, *Methanococcus vanniellii*, *Pseudomonas testosteroni*, *Escherichia coli*, *Agrobacterium tumefaciens*, *Zea mays* (mitochondrion), *Bacillus subtilis*, *Anacystis nidulans*, *Zea mays* (chloroplast), *Thermomicrobium roseum*, *Euglena gracilis*, *Trypanosoma brucei*, *Dictyostelium discoideum*, *Saccharomyces cerevisiae*, *Oxytricha nova*, *Protocentrum micans*, *Zea mays*, *Xenopus laevis* and *Homo sapiens*.

The length of each segment is proportional to the inferred number of fixed point mutations along that segment; the scale bar corresponds to 0.1 nucleotide substitutions per sequence position. The tree is unrooted. The tree was determined using the least squares, distance matrix method. This diagram was modified from Olsen (1987).

ARCHAEBACTERIA



EUBACTERIA

EUKARYOTES

considered as a cluster of 10 groups, each as distinct (in terms of ribonucleotide sequences) as the gram positive group (Woese, 1987).

The ease of the 16S rDNA sequencing technique, has now made the phylogenetic study of bacteria possible in most laboratories, with minimal equipment. Sequences of ribosomal RNA from various sources are being collected and maintained by the Ribosomal Database Project at the University of Illinois, Urbana-Champaign (Olsen et al., 1991). The most recent update of the database contains more than 2300 partial and complete sequences of 16S rRNA. The availability of the technology and the software needed for the analysis has made the sequencing of the 16S rRNA gene a standard technique in identifying and categorizing bacteria. This is especially true when standard phenotypical methods of analysis fail or the microbe cannot be cultured *in-vitro* for isolation and characterization (Campbell et al., 1992; Govindaswami et al., 1993).

CHAPTER III

ROOTING RESPONSE OF DIFFERENT GENOTYPES OF SLASH PINE SEEDLING EXPLANTS INOCULATED WITH RSB

Introduction

Genotypic differences have been observed in the rooting of cuttings from agronomic plants such as alfalfa (Haissig and Riemenschneider, 1988). Deuber (1940) noted differences between the rooting percentages of Norway spruce [*Picea abies* (L.) Kars.] cuttings originating from 38 clones of 26-years or 40-years old trees. Based on his studies on Norway spruce, Mergen (1960) concluded that rooting percentages and root volumes produced by Norway spruce cuttings were genetically controlled. Clonal differences in rooting ability have been noted for various gymnosperms including *Pinus strobus* L. (eastern white pine) (Kiang and Garrett, 1975), *Pinus echinata* Mill. (shortleaf pine) (Fancher and Tauer, 1981), several *Picea* species (spruce) (Rauter, 1971) and *Tsuga heterophylla* (Raf.) Sarg. (western hemlock) (Brix and Barker, 1975; Foster et al., 1984). Current evidence suggests that the capacity of cuttings to initiate roots is influenced by genetics (Haissig and Riemenschneider, 1988). There are no references, to date, that describe the differences in rooting ability of different genotypes of the same specie of pine under the influence of a microbial rooting stimulus. This experiment was set up to determine the rooting efficiency of 10 half-sib genotypes of

slash pine (*Pinus elliottii* Englem.) after inoculation with the root stimulating bacteria (RSB).

Materials and methods

Bacteria and cultures conditions

RSB was grown on agar-based WP medium (Lloyd and McCown, 1980) supplemented with 0.2% casein hydrolysate (Sigma Chemical Company, St. Louis, MO). A glycerol culture of RSB maintained at -80°C was used to streak the plates (Bowling, 1992). The plates were incubated at 27°C +/- 3°C for four days in a Percival single door incubator (Percival, Boone, IA). The culture plates were illuminated at 50 $\mu\text{moles quanta m}^{-2}\text{s}^{-1}$ PAR using Phillips™ F20t12/CW fluorescent lights at an 18 hour photoperiod.

Rooting media

Half strength Gresshoff and Doy medium (Gresshoff and Doy, 1972) as modified by Sommer et al., (1975) (Burns, 1990; McAfee et al., 1993) was used as the culture medium. The pH of the medium was adjusted to 5.5 using 0.1 N KOH prior to autoclaving. The composition of the medium is given in Appendix A. 20 ml of medium was dispensed into each culture tube (20 mm x 100 mm). All culture tubes were capped with Kaput™ caps. The tubes were autoclaved at 121°C, 1.27 kg/cm² for 20 minutes.

Seedlings

Ninety seeds each of 10 different half-sib genotypes of slash pine (*Pinus elliottii* Engelm.) provided by the USDA-Forest Service were sown in flats of a 50:50 peat perlite rooting mixture and watered as needed until they attained a height of approximately 8 cm (5 months). Genotypes designated 1-60, 36-62, 41-62, 96-61, 105-56, 122-56, 211-56, 259-55, 303-56 and 351-56 were used in the experiment. The seedlings were sprayed at 4-week intervals with Banrot TM at the rate of 0.5 teaspoon per gallon of spray fluid.

Sterilization and culture conditions

The hypocotylary explants were harvested and placed in culture by the method of Burns et al., (1991). The seedlings were derooted, dipped in 70% ethanol + 1 drop Tween^R-20 (FisherScientific, Fair Lawn, NJ) for 30 seconds, transferred to 2% commercial bleach for 5 minutes, followed by 70% ethanol for 30 seconds and rinsed in sterile distilled water for 30 minutes and air dried. Derooting was performed by cutting the seedlings at the base of the stem with a sharp scalpel. Control tubes were not inoculated while experimental tubes were inoculated with RSB by scraping the lawn of bacteria in the culture plates with the cut end of the stem and inserting the explant approximately 1-2 cm directly into the solidified culture medium. Culture tubes were capped and sealed with parafilm^R (American National CanTM, Greenwich, CT). The tubes

were incubated in a Sherer™ double door growth cabinet at 27+/- 3 °C and a light intensity of 50 $\mu\text{moles quanta m}^{-2}\text{s}^{-1}$ PAR with an 18 hour photoperiod for a period of nine weeks.

Experimental design and statistical treatment

The experiment was set up as a randomized complete block design with individual genotypes as the basis of blocking. The number of roots were counted manually. Rooting level was designated as 1 if roots were present and 0 if roots were absent. The results were analyzed using the general linear methods (GLM) subroutine available in the SAS™ package (SAS Institute Inc, Cary, NC). Analysis of variance was performed using the GLM procedure. Pair-wise comparisons of group means were performed using the Ryan-Einot-Gabriel-Welsch Multiple Range Test with a significance criterion (α) of 0.1.

Results and discussions

The one-way analysis of variance was performed on the data with the genotype as the source of variation. A [Pr>F] value of 0.0002 was obtained (refer to Table III-1). This implied that there was a significant difference in the response of the different genotypes to the RSB treatment. A high standard deviation within each genotype was detected based on the analysis of rooting data. This influenced the overall coefficient of variation (c.v. = 122.19 %). In order to account for this variation, the significance level was set

TABLE III-1

Analysis of variance* of genotypes for level of rooting per explant.

Source of variation	df	F	Pr > F
Genotype	10	3.78	0.0002

* Analysis of variance was performed on the level of rooting. The presence of rooting was scored as 1 while its absence 0.

df = degrees of freedom.

F = ratio of genotype mean square to error mean square.

Pr > F = significance probability value associated with the F value.

at 0.1 as opposed to 0.05 and the REGWQ test for mean separation was selected. This test controls the rate of maximum experiment-wise error and is significantly more conservative than the Duncan's multiple range test commonly used in most published analyses. Spontaneous rooting did not occur in the experiment as the untreated controls did not root. Depending on the genotype, the level of rooting ranged from 8.33% in genotype 1-60 to 66.67% in genotypes 41-62, 122-56 and 303-56 (refer to Table III-2). The mean number of roots ranged from 1 to 2 roots per rooted plantlet. Rooted seedlings had little or no visible callus at the base of the stem, while the roots seemed well connected and geotropic. Explants that did not root had well developed calli and no roots. Lateral branches were quite common in most of the roots.

These results are very much different from those obtained by Stein et al. (1990) in *Picea mariana* and *Larix laricina* (Stein et al., 1990) with a spontaneous rooting rate of 52% and 100% respectively. The seedlings were 4 weeks old in Stein et al.'s study, while the *Pinus elliotti* seedlings used in the bioassay were 5 months old. The difference in the level of spontaneous rooting may be attributed to the age of the donor material and conifer genus/species being studied. Both shoot production and rooting efficiency vary with conifers and in general are more difficult in explants derived from older plants (Mohammed and Vidaver, 1988). Comparatively, clones 41-62, 105-56 and 303-56 performed the best while clones 1-60 and

TABLE III-2

Percentage rooting per genotype and mean number of roots per rooted explant.

Genotype	n	Rooting %	Mean Number of Roots
Control	10	0.00 ^b	-
1-60	12	8.33 ^b	1.00
36-62	10	10.00 ^b	1.00
41-62	12	66.66 ^a	1.00
96-61	12	50.00 ^{ab}	1.16
105-56	12	66.66 ^a	1.87
122-56	12	50.00 ^{ab}	1.83
211-56	9	33.33 ^{ab}	2.00
259-55	11	54.54 ^{ab}	1.00
303-56	12	66.66 ^a	1.25
351-56	12	16.66 ^{ab}	1.00

Mean separations were performed on the level of rooting per genotype.

Genotype means with different superscripts are significantly different at 10 % level of error.

The [Pr > F] value for the test of rooting (rooting percentage) was 0.0002.

The post-hoc multiple mean separation procedure performed was the Ryan-Einot-Gabriel-Welsch Multiple Range Test (REGWQ).

n = number of explants treated.

36-62 were poor rooters. The observed variation in rooting efficiency was statistically shown to be genotype dependent (refer to Table III-2). Explants rooted with *Agrobacterium rhizogenes* often produce non-geotropic roots. However, such roots were not observed in RSB-induced adventitious roots. It was observed that all roots were induced at the site of excision rather than along the hypocotyl as reported by Stein and Fortin (1990) with reference to treatments of *Larix laricina* explants with *Laccaria bicolor*. The induction of rooting by RSB seems analogous to that induced by exogenously applied auxin (Burns, 1990).

It is thus clear that the induction of rooting in slash pines by RSB is genotype dependent, since the level of rooting varied from 8.33% to 66.67%. It seems likely that adventitious rooting is influenced by the genetics of the plant. The mechanism of rooting is possibly related to the activities of specific enzymes or specific levels of intermediary metabolites. The genes determining these enzyme systems might be subjected to mutations that lead to losses of rooting ability (Haissig and Riemenschneider, 1988) in different genotypes.

CHAPTER IV

ORGANIC EXTRACTION OF A ROOT-INDUCING FRACTION

Introduction

It is now well established that ectomycorrhizal fungi release indole-3-acetic acid (IAA), which enhances rooting in the host plant (Gay, 1990). Burns (1990) prepared an eluate of the bacterial (RSB) culture medium and incorporated it in the slash pine growth medium to assess the induction of rooting of hypocotylary explants in the absence of live bacteria. His results were not definitive due to the presence of a second contaminating organism of unknown identity. Gay (1990) used ethyl acetate extracts of a cell-free filtrate of a growing culture of *Hebeloma hiemale* in bioassays to test the possibility of microbe-free induction of rooting in derooted *Pinus halepensis* shoot hypocotyls. The following experiment describes an attempt to separate the root inducing principle from the live bacterium and to assay its root inducing activity.

Materials and methods

Bacterial cultures

RSB was grown in two 2 l flasks each containing 1 l of WP medium (Lloyd and McCown, 1980) supplemented with 0.2% casein hydrolysate (Sigma Chemical Company, St. Louis, MO). The pH of the medium was adjusted to 5.2 using 0.1 N KOH prior to

autoclaving. Each flask was inoculated with 200 μ l of a glycerol culture of RSB maintained at -80°C (Bowling, 1992). The flasks were incubated at $27^{\circ}\text{C} \pm 3^{\circ}\text{C}$ and grown under agitation at 200 rpm in a Percival single door incubator (Percival, Boone, IA). The culture was illuminated at 50 $\mu\text{moles quanta m}^{-2}\text{s}^{-1}$ PAR using PhillipsTM F20t12/CW fluorescent lights at an 18 hour photoperiod. The bacteria were grown for 5 days. The RSB liquid culture was stored at 4°C for further extractions. The glycerol culture was used to streak modified WP agar plates which were cultured without agitation under the conditions described above.

Organic partitioning and extraction

The 2 l culture was centrifuged two times at 10,000 rpm for 30 minutes, until the supernatant was visually clear and free of suspended particles. The medium was transferred to a 2 l flask and filtered through a sterile 0.2 μm nylon filter. To enhance the efficiency of extractions, the medium was divided into 4 portions of 450 ml each and extracted with 4 portions of ethyl acetate, each of 250 ml. After partitioning the organics and uncharged species into the organic phase, the 4 ethyl acetate extracts were pooled and washed with distilled water. The extract was dried with an excess of anhydrous sodium sulfate (Malinkrodt, Malinkrodt Inc., Paris, KY). The extracted medium was acidulated to a pH of 2.8 with 1 N HCl. The above extraction procedure was repeated with a fresh batch

of ethyl acetate. The two ethyl acetate extracts were then distilled separately under vacuum using a Rotavapor R model rotary evaporator (Brinkmann Instruments, Westbury, NY). After all the solvent was evaporated, the residue was dissolved in 2 ml of 100% ethanol and diluted with distilled water to a volume of 85 ml.

The extracts were stored under refrigeration. Three ethyl acetate extracts were used in the slash pine rooting bioassays; namely, 50 ml of the extract at pH 5.2, 50 ml of the extract at pH 2.8 and mixed extract consisting of 25 ml of each extract. The pH of the extracts was adjusted to 5.5 with 0.1 N KOH and the extracts were filter-sterilized using an autoclaved 0.2 μ m nylon filter.

Media

The rooting medium of choice was half strength Gresshoff and Doy medium (Gresshoff and Doy, 1972) as modified by Sommer et al., (1975) (Burns, 1990; McAfee et al., 1993). Half strength Gresshoff and Doy medium was prepared. The pH of the medium was adjusted to 5.5 using 0.1 N KOH prior to autoclaving. The composition of the medium is given in Appendix A. The medium was prepared in batches of 400 ml. Except for the controls, the concentration of the media nutrients and vitamins was adjusted so that 50 ml of the ethyl acetate extracts could be added to the medium after the autoclaving procedure. The medium was autoclaved separately at

121°C, 1.27 kg/cm² for 20 minutes along with the necessary glassware. The sterile media were cooled to about 45°C, supplemented with the organic extracts and dispensed into sterile culture tubes at 20 ml per tube (20 mm x 100 mm). Control media were dispensed directly into culture tubes. All culture tubes were capped with Kaput™ caps.

Seedlings

Ninety seeds of 5 half-sib genotypes of slash pine (*Pinus elliottii*, Engelm.) provided by the USDA-Forest Service were sown in flats of a 50:50 peat perlite rooting mixture and watered as needed until their average height was approximately 8 cm (5 months). The genotypes used were 41-62, 96-61, 105-56, 259-55 and 351-56. The seedlings were sprayed with Banrot™ at the rate of 0.5 teaspoon per gallon of water every 4 weeks. The hypocotylary explants were harvested and placed in culture by the method of Burns et al., (1991). The seedlings were derooted, dipped in 70% ethanol + 1 drop Tween^R-20 (FisherScientific, Fair Lawn, NJ) for 30 seconds, transferred to 2% commercial bleach for 5 minutes, followed by 70% ethanol for 30 seconds, rinsed in sterile distilled water for 30 minutes and air dried. Derooting was performed by cutting the seedlings at the base of the stem with a sharp scalpel. Explants were inserted into appropriate media tubes, capped and sealed with parafilm^R (American National Can™, Greenwich, CT). One set of the controls was inoculated with RSB by

scraping the lawn of bacteria in the culture plates with the cut end of the stem and inserting the explant approximately 1-2 cm directly into the solidified culture medium. Culture tubes were capped and sealed with parafilm^R. The tubes were incubated in a ShererTM double door growth cabinet at 27+/- 3 °C and a light intensity of 50 $\mu\text{moles quanta m}^{-2}\text{s}^{-1}$ PAR with an 18 hour photoperiod for a period of 9 weeks.

Experimental design and statistical treatment

The experiment was set up as a randomized complete block design with individual treatments as the basis of blocking. The number of roots were counted manually and the results analyzed using the general linear methods (GLM) subroutine available in the SASTM package (SAS Institute Inc, Cary, NC). Analysis of variance was performed using the GLM procedure. Pair-wise comparisons of group means were performed using the Ryan-Einot-Gabriel-Welsch Multiple Range Test (REGWQ test) with a significance criterion (α) of 0.1.

Results and discussion

This experiment was designed to determine which organic fraction contained the root initiation signaling substance (RISS). The extraction was carried out at two pH values i.e. 2.8 and 5.2.

The treatments were definitely significant at a level of 0.1 [Pr>F] = 0.0056 (refer to Table IV-1). This value

indicates a very strong deviation from the mean and therefore a significant effect of the treatment. However this value might be influenced by the high coefficient of variation (c.v. = 181.8). Thus an α value of 0.1 was selected as the error criterion and the REGWQ test for multiple mean comparisons was used as a suitable method to control the rate of maximum experiment-wise error (SAS/STAT guide for Personal Computers, SAS version 6.0). This test is significantly more conservative than Duncan's multiple range test, used in most analyses, and is sensitive to the variability of the material. Each treatment was applied to 4 explants from 5 genotypes. This step lead to a more reliable estimate of the treatment effect and a more conclusive result (refer to Table IV-2). It is to be noted that the 5 genotypes selected were the better rooting genotypes (refer to the previous chapter). While the live bacterium induced the highest level of rooting (45%), no spontaneous rooting occurred. Thus all induction of rooting can be positively attributed to the root induction treatment. Although the rate of rooting induced by the extract at pH 2.8 is lower than that induced by the live bacteria, the rates of rooting are not significantly different from each other. It appears as though the other 2 extracts were unable to bring about similar levels of root induction due to a lower concentration of the active principle or principles in these extracts. The mean number of roots per rooted explant ranges

TABLE IV-1

Analysis of variance* of the rooting response due to different extracts of the RSB culture medium

Source of Variation	df	F	Pr > F
Treatment	4	3.90	0.0056

df = degrees of freedom.

F = ratio of genotype mean square to error mean square.

Pr > F = significance probability value associated with the F value.

* Analysis of variance was performed on the level of rooting. The presence of rooting was scored as 1 while its absence 0.

TABLE IV-2

Percentage rooting per treatment, level of rooting as a percentage of the rooting induced by RSB, mean number of roots per rooted explant and mean length of the longest primary root per rooted explant.

Treatment	n	Rooting %	'Permax'	Rmean	Lmean
Control	20	0.00 ^c	0.00	-	-
Mixed pH	18	11.11 ^{bc}	27.43	1.00	36.50
RSB	20	45.00 ^a	100.00	1.80	14.77
pH 2.8	20	30.00 ^{ab}	66.67	1.33	33.16
pH 5.2	20	20.00 ^{abc}	44.45	1.00	25.25

All means were tested at a level of error of 10%. The [Pr>F] value for the test of rooting (rooting percentage) was 0.0056.

The post-hoc multiple mean separation procedure performed was the Ryan-Einot-Gabriel-Welsch Multiple Range Test (REGWQ).

Means with different superscripts are significantly different at a 10% level of error.

Permax is rate of rooting per live RSB-rooted explant expressed as a percentage.

Rmean = mean number of roots per rooted explant.

Lmean = mean length (mm) of longest primary root per rooted explant.

from 1 to 1.8 while the length of the longest primary root ranges from 14.7 to 36.5 mm.

The variable 'permax' indicates the level of rooting observed in each treatment expressed as a percentage of the level of rooting in the live RSB treatment and corrected for sample size. The extract at pH 2.8 approaches the efficiency of root induction by the live bacterium to the extent of 66.67%. Gay (1990) reported a 100% rooting induction by an ethyl acetate extract at pH 3.0 of a *Hebeloma hiemale* culture filtrate, with derooted explants that were obtained from 15 day old seedlings of *Pinus halepensis*. Considering that the *P. elliottii* explants were 5 months old when used in the bioassay, the level of root induction is quite high. The mean number of roots per rooted plant may also reflect the concentration of the putative RISS.

Upon purification, Gay (1990) reported that the root inducing principle in the ethyl acetate extract of *H. hiemale* was a mixture of indole-3-acetic acid (IAA) and indole carboxylic acid (ICA). ICA has no known biological activity. Considering the levels of rooting induced by the RSB extracts at pH 5.2 and the mixed extract, it seems apparent that the root inducing principle may have a carboxylic group that gets protonated under conditions of low pH and allowing its distribution into the organic phase. Further purification of the RSB-pH 2.8 ethyl acetate extract might reveal a rooting

principle analogous to IAA. It is of interest to note that the medium used to culture *H. hiemale* was supplemented with 1 mM tryptophan, the immediate precursor in most IAA biosynthetic pathways. The concentration of tryptophan in the WPM supplemented with 0.2% casein hydrolysate is 0.117 mM.

Further experimentation involving the production of organic extracts of RSB culture media supplemented with varying levels of tryptophan might prove enlightening.

CHAPTER V

PIGMENT ANALYSIS BY ABSORPTION SPECTROSCOPY OF METHANOL
EXTRACTS

Introduction

Pigment analysis through the use of spectral profiles of methanol extracts has been used by researchers to compare and contrast different types of bacteria. Holmes et al. (1977) differentiated *Pseudomonas* species from *Xanthomonas* species by comparing pigment absorbance spectra. Poindexter (1964) used methanol extracts of *Caulobacter* species to compare different strains of the genus. Fuerst et al. (1993) identified bacteriochlorophyll A peaks in the spectral profile of the methanol extract of *Porphyrobacter neustonensis*. Bally et al. (1990) carried out HPLC-based analyses and identified the characteristic pigment of *Pseudomonas paucimobilis* and *Flavobacterium capsulatum* to be the carotenoid pigment - nostoxanthin. Based on the use of spectral data by the above authors, the pigment absorption profile of the RSB was carried out.

Materials and methods

Bacterial cultures

RSB was grown in two 125 ml flasks each containing 50 ml of WP medium (Lloyd and McCown, 1980) supplemented with 0.2% casein hydrolysate (Sigma Chemical Company, St. Louis, MO).

Each flask was inoculated with 5 μ l of a glycerol culture of RSB maintained at -80°C . The flasks were incubated at $27^{\circ}\text{C} \pm 3^{\circ}\text{C}$ and grown under agitation at 200 rpm in a Percival single door incubator (Percival, Boone, IA). The culture was illuminated at 50 $\mu\text{moles quanta m}^{-2}\text{s}^{-1}$ PAR using Phillips™ F20t12/CW fluorescent lights at an 18 hour photoperiod. The bacteria were grown for 5 days.

Methanol extraction and spectrophotometry

The extraction procedure was modified from Poindexter, 1964. 70 ml of the culture was centrifuged at 10,000 rpm for 15 minutes. The supernatant was decanted and the cell pellet was resuspended in 3% NaCl aq. pH 5.2. The cells were centrifuged at 10,000 rpm for 15 minutes and the saline buffer decanted. The cells were resuspended in 100% methanol and incubated at room temperature for 30 minutes. The cells were then centrifuged at 10,000 rpm for 10 minutes and the cell debris discarded. The absorption spectrum of the methanol extract was determined between wavelengths of 200 to 800 nm on a Shimadzu UV-160 spectrophotometer.

Results and discussion

Two peaks were recorded at 475 nm and at 447 nm. A trough was observed at 465 nm (refer to Figure V-1 and V-2). There seems to be an inflection at approximately 420-430 nm. No peaks were seen at wavelengths longer than 600 nm (refer to

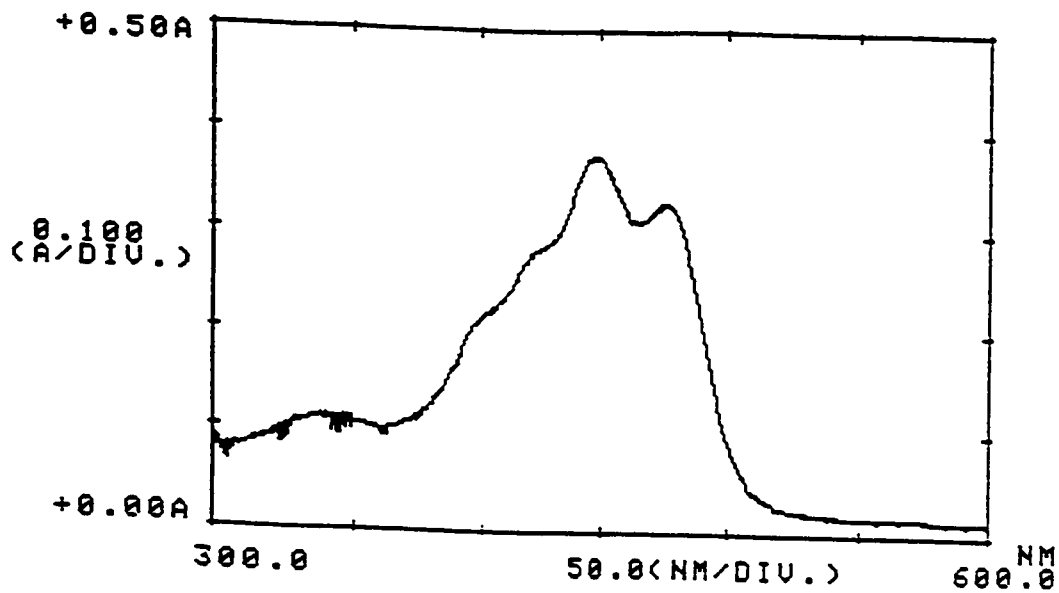


FIGURE V-1. Spectral profile of methanol extract
(300-600 nm)

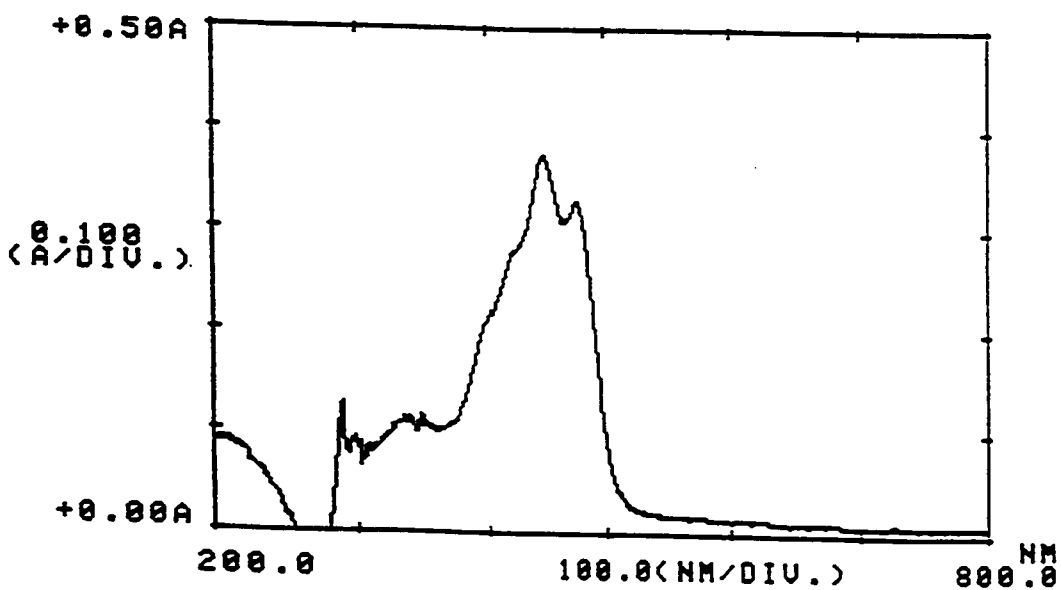


FIGURE V-2. Spectral profile of methanol extract
(200-800 nm)

Figure V-2). These values are the results of crude extractions and are only indicative of the general pigment profile. The presence of a specific pigment cannot be absolutely determined from this assay. The RSB profile is very similar to that obtained by Poindexter (1964) for *Caulobacter* strains CB10 and CB11. A comparison of the RSB pigment profile to that obtained by Poindexter (1964) from extracts of *Caulobacter* strains CB10 and CB11 suggests the presence of carotenoid pigments in the extract. The profiles of the pigment extracts of the *Caulobacter* strains peaked around 450 and 475 nm and troughed around 462 nm, suggesting the presence of carotenoid pigments in the extract. The resolution of the *Caulobacter* pigments on paper and column chromatograms revealed the presence of 4 to 10 components (Poindexter, 1964).

The possibility of some components actually being isomers or oxidation products of the native pigments was not ruled out (Poindexter, 1964). Holmes et al. (1977) studied the pigment profile of 10 strains of *P. paucimobilis*. They obtained peaks at 447-452 and 474-480 while an inflection was recorded at 420-430 nm. Though they did not mention a trough around 462 nm, the spectrum profile shows a trough at that approximate wavelength. Bally et al. (1990) used acetone instead of methanol as a solvent for pigment extraction. They compared the absorption spectra of acetone extracts of *P. paucimobilis* and *Flavobacteria capsulatum*. Both recorded an inflection at 428 nm and peaks at 452 and 480 nm respectively. HPLC

purification of the pigment revealed the presence of nostoxanthin, a carotenoid, previously found only in cyanobacteria and *P. paucimobilis*. They suggested the use of nostoxanthin as a marker for the yellow-pigmented, *Sphingomonas*-related group of non-photosynthetic bacteria. As stated earlier, Fuerst et al. (1993) identified bacteriochlorophyll A from methanol extracts of *Porphyrobacter neustonensis*, a bacterium closely related to *Erythrobacter longus*. The pigment peaked at 799-806 nm and 868-871 nm. Such peaks were not observed in RSB extracts.

These are the results of crude and preliminary experiments. The resolution of the pigment extract using different chromatographic methods could potentially reveal the presence of several pigments present in the extract and, if at all, that of key diagnostic pigments such as nostoxanthin.

CHAPTER VI

SEQUENCING AND PHYLOGENETIC ANALYSIS OF THE 16S RIBOSOMAL
RNA GENE OF THE RSB

Introduction

While studying bacterial endosymbionts associated with cytoplasmic incompatibility in insects, O'Neill et al. (1992) were unable to determine the relationship of different endosymbionts from different hosts due to the fastidious nature of the bacteria and therefore the inability to culture the bacteria outside the host. They sequenced the 16S rDNA of their bacterial strains and compared the sequences to determine the phylogenetic relationships of different endosymbionts to known bacteria. Govindaswami et al. (1993) characterized the phylogenetic placement of their dye-degrading bacterium using 16S rDNA sequences after attempts at identifying the strain using commercial identification methods were inconclusive. In recent years, the 16S rRNA sequence comparison has been used as a powerful tool to establish phylogenetic and evolutionary relationships among bacteria (Lane et al., 1985). Identification of the RSB through commercial techniques was inconclusive. Therefore, the 16S rRNA gene of the RSB was selectively amplified using the polymerase chain reaction and was subsequently cloned and sequenced. The sequence of the gene and its phylogenetic analysis is presented.

Materials and methods

Bacterial cultures and growth conditions

RSB was grown in a 125 ml flasks containing 30 ml of WP medium (Lloyd and McCown, 1980) supplemented with 0.2% casein hydrolysate (Sigma Chemical Company, St. Louis, MO). Each flask was inoculated with 5 μ l of a glycerol culture of RSB maintained at -80°C . The flasks were incubated for 5 days at $27^{\circ}\text{C} \pm 3^{\circ}\text{C}$ and grown under agitation at 200 rpm in a Percival single door incubator (Percival, Boone, IA). The culture was illuminated at 50 $\mu\text{moles quanta m}^{-2}\text{s}^{-1}$ PAR using Phillips™ F20t12/CW fluorescent lights at an 18 hour photoperiod.

Genomic DNA isolation

Genomic DNA was isolated from RSB using a procedure modified from Rogers et al. (1989). Cells were harvested from the liquid culture, washed twice with TNE (10 mM Tris-HCl, 100 mM NaCl and 1 mM EDTA, pH 8.0) and centrifuged. The packed cell volume was determined and the pellet resuspended in 5 to 10 volumes of TNE. The cells were resuspended in a tissue grinder, transferred to microfuge tubes and centrifuged for 30 seconds. The supernatant was decanted and the pellet resuspended in 200-400 μ l of 2X cetyl trimethyl ammonium bromide (CTAB) extraction buffer (2% CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, pH 8.0). The suspension was vortexed and incubated at 65°C for 60 minutes. An equal volume of 24:1 chloroform:isoamyl alcohol (CIAA) was added and the suspension

mixed gently by inverting the tubes several times. After centrifuging for 60 seconds, the aqueous phase was transferred to a fresh tube. The cell material at the interphase was pooled, washed once in 3 ml of TNE and resuspended, once again, in a tissue homogenizer. The extraction process was repeated as before. The aqueous phase after the CIAA wash was pooled with the previous preparation. 1/10 volume of CTAB precipitation buffer (10% CTAB, 0.7 M NaCl) preheated to 65°C was added to the pooled aqueous phase and mixed thoroughly. 1 volume of CIAA was added and mixed gently by inverting the tubes several times. The mixture was centrifuged for 1-4 minutes and the aqueous phase transferred to a fresh tube. DNA was precipitated from the aqueous phase by adding 2 volumes of cold absolute ethanol and centrifuging at 4°C. The supernatant was decanted and the pellet dried under vacuum. The pellet was then resuspended in 45 μ l of TE buffer (10 mM Tris-HCl, 1 mM EDTA at pH 8.0). RNA was hydrolyzed by treatment with 5 μ l of 100 μ g/ml RNase A (DNase-free pancreatic RNase, Sigma, Sigma Chemical Company, St. Louis, MO). After incubating the preparation at room temperature for 3 hours, an equal volume of CIAA was added. After mixing the contents of the tube gently, the tube was centrifuged for 5 minutes. The aqueous layer was transferred to a fresh tube and 5 μ l of 3 M sodium acetate pH 5.2, was added to the mixture. DNA was precipitated with absolute ethanol and resuspended in 50 μ l of TE buffer. This preparation was used as a stock of genomic DNA for all

subsequent reactions.

PCR amplification

A 10 ng/ μ l solution of genomic DNA was prepared from the original stock by reprecipitating the DNA and dissolving it in water. The polymerase chain reaction (PCR) (Mullis and Faloona, 1987; Saiki et al., 1985) was used to selectively amplify the section of DNA that encodes the 16S ribosomal gene. Amplification primers were synthesized by the University of Tennessee Molecular Biology Research Facility. The following primer sequences were synthesized on an Applied Biosystems model 381A synthesizer.

5' forward amplification primer:

5' - AGAGTTTGATCMTGGCTCAG - 3'

3' reverse amplification primer:

5' - GGTTACCTTGTTACGACTT - 3'

M = A or C

These two primers were selected from conserved regions of the eubacterial 16S rRNA. The forward primer corresponds to positions 8-27 of *Escherichia coli* 16S rRNA and the reverse primer corresponds to the complement of positions 1492 to 1510 (Eden et al., 1991). The PCR was performed as recommended in the GeneAmp^R kit (Perkin Elmer Cetus, Norwalk, CT) using Amplitaq^R DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) as the amplifying enzyme. Each reaction included 150 ng of the genomic DNA, 0.5 μ M of each amplification primer, 200 μ M each

of dATP, dCTP, dGTP, and dTTP, 10mM Tris-HCl pH 8.3, 50 mM KCl and 2.5 mM MgCl₂ in a volume of 99.5 μ l and 2.5 units of Taq DNA polymerase. A layer of mineral oil was added on top of the reaction mixture and the tubes were placed in a Perkin Elmer DNA thermal cycler. The amplification program used was as follows:

cycle 1: Denaturation at 95°C for 2 minutes, primer annealing at 40°C for 1 minute and chain extension at 72°C for 1 minute; cycles 2-30: Denaturation at 94°C for 1 minute, primer annealing at 40°C for 1 minute and chain extension at 72°C for 1 minute; each cycle was extended by 6 seconds at 72°C. A final extension was carried out after the last cycle at 72°C for 3 minutes and the reaction was held at a temperature of 4°C. After completing the amplification procedures, the aqueous phase was recovered from the reaction tubes and extracted once with an equal volume of CIAA to denature and separate the polymerase from the DNA preparation. The amplified DNAs were stored at -20°C for further use.

5 μ l of each amplification reaction was assessed qualitatively by electrophoresis through a 1.4 % agarose gel run at 100 V in a TBE buffer for 60 minutes. Following electrophoresis, the gel was stained with ethidium bromide and visualized on a UV transilluminator. The size of the product was estimated by comparison to the standard markers HaeIII-digested ϕ X174 DNA and HindIII-digested lambda phage DNA (Gibco BRL, Gaithersburg, MD).

The amplified fragment was used for a preliminary sequence analysis according to Böttger (1989), using the forward and reverse PCR primers as sequencing primers. The data were compared to sequence data available in the GenBank database.

Cloning

Insert

The PCR-amplified fragment was gel-purified and prepared for cloning by treatment with T4 polynucleotide kinase (Promega, Promega Corp., Madison, WI) and T4 DNA polymerase (New England Biolabs, Beverly, MA). Polynucleotide kinase treatment was necessary because the amplified fragments were generated from synthetic primers with hydroxylated 5' ends. This reaction was carried out by treating 480 ng of the purified PCR product with 1 μ l of polynucleotide kinase, 3 μ l of the 10X reaction buffer (provided with the enzyme), 2 μ l 10mM ATP (Sigma, Sigma Chemical Co, St. Louis, MO) in a 30 μ l reaction volume incubated at 37°C for 30 minutes. Overhangs at the 3' ends were filled in with T4 DNA polymerase in the presence of all 4 nucleotides to generate blunt ends.

Vector

pBluescript KS (Stratagene, Stratagene Cloning Systems, La Jolla, CA) was digested with the restriction enzyme, SmaI, and terminal phosphates were removed using calf intestinal

alkaline phosphatase (CIP) (New England Biolabs, Beverly, MA) according to company protocols in order to reduce self religation. The final reaction was extracted with an equal volume of TNE-equilibrated phenol-chloroform-isoamyl alcohol (50:24:1) and taken up in TE buffer.

Ligation

250 ng of linearized phosphatase-treated pBS-KS DNA was ligated overnight at 16°C to 400 ng of insert prepared as indicated above using T₄ DNA ligase (New England Biolabs, Beverly, MA) according to company protocols. The ligation product was electrophoresed on a 1% TAE-agarose gel to verify the ligation.

Transformation

NovaBlue competent cells (Novagen, Madison, WI) were transformed with 1 µl of the ligation reaction and spread on LB plates with ampicillin according to the company protocol. 100 µl of competent cells were mixed with 1 µl of the ligation reaction in pre-chilled 17 x 100 mm Falcon tubes and chilled on wet ice for 30 minutes. The cells were subsequently heat-shocked at 42 °C for 45 seconds and chilled on wet ice for 2 minutes. To each tube, 900 µl of SOC medium (provided in the Novagen pT7 Blue T-Vector kit) was added and the tubes were agitated for 1 hour at 37°C and 225 rpm. Aliquots of 200 µl were spread on 100 x 15 mm sterile polystyrene disposable

plates containing 20 ml of LB-agar with ampicillin (50 μ g/ml) and tetracycline (15 μ g/ml). The plates were spread with 50 μ l of a 20 mg/ml solution of bluogal (Gibco BRL, Gaithersburg, MD) in dimethyl formamide and 10 μ l of a 23.8 mg/ml aqueous solution of isopropyl thiogalactoside (Gibco BRL, Gaithersburg, MD) (IPTG). The plates were incubated overnight at 37°C. 20 white colonies were selected from these plates and streaked on fresh LB plates with ampicillin and tetracycline. After incubation overnight at 37°C, a single white colony was selected from each of these plates and was cultured in liquid LB medium (2 ml/vial) with ampicillin (100 μ g/ml) and tetracycline (15 μ g/ml) overnight at 37°C with agitation (225 rpm).

Isolation of plasmid DNA

DNA was extracted from the amplified colonies by the alkaline-lysis method, (Maniatis *et al.*, 1989). The overnight cultures were centrifuged for 30 seconds at 4°C, the supernatant was decanted and the cells resuspended in 100 μ l ice cold miniprep solution I (refer to Appendix B). After vortexing, 200 μ l of miniprep solution II (refer to Appendix B) was added and mixed by gentle inversions. 200 μ l ice cold miniprep solution III (refer to Appendix B) was then added and mixed by gentle inversions. The cell debris was removed by centrifugation at 4°C and the supernatant extracted with TNE-equilibrated phenol-chloroform-isoamyl alcohol (50:24:1). The

DNA pellet was precipitated with ethanol and taken up in 40 μ l TE buffer. RNA was hydrolyzed by treatment with 10 μ l of 100 μ g/ml RNase A (DNase-free pancreatic RNase, Sigma, Sigma Chemical Company, St. Louis, MO). 1 μ l of each DNA preparation was electrophoresed through a 1% TAE-agarose gel.

Two of the colonies picked (clones 7 and 20) appeared to contain plasmids with inserts of the correct size. The presence in the clone, of the proper amplification product was verified by Southern blotting and hybridization with a probe made from the purified-PCR product and by sequence analysis using the universal M13 -40 primer and the universal M13 reverse primer.

Southern hybridizations

DNA from clones 7 and 20 was electrophoresed through a 1% TAE-agarose gel and transferred to a nylon membrane according to the standard protocols (Amersham, Amersham International plc., UK). The 1% TAE-agarose gel was soaked in denaturing solution (1.5 M NaCl and 0.5 M NaOH) for 30 minutes and then rinsed and soaked for 2 hours in neutralizing solution (1.5 M NaCl, 0.5 M Tris-HCl, pH 7.2 and 1 mM EDTA) at 4°C to prevent the diffusion of bands in the gel. A sheet of nylon membrane was moistened with 2X SSC solution and placed on a stack of 3 layers of 3MM blotting paper and several layers of paper towels. The gel was blotted dry and placed on the nylon membrane. Bidirectional blotting was achieved by placing a

pre-soaked nylon membrane on the gel followed by a stack of 3 layers of 3MM blotting paper and several layers of paper towels. A weight of 0.5 kg was placed on the stacked membranes which were maintained overnight at 4°C. The membranes were then rinsed in 2X SSC and air-dried. The gels were stained with ethidium bromide and visualized on a transilluminator to assess the efficiency of the transfer. The membranes were then wrapped in plastic film and placed on a UV transilluminator for 5 minutes with the DNA-side facing the UV light, to bind the DNA to the membrane. The membranes were inserted into hybridization bags and soaked in 25 ml pre-hybridization solution containing 0.5 mg denatured salmon sperm DNA at 65°C for 4 hours with agitation. The salmon sperm DNA was denatured by heating in a boiling water bath for 5 minutes followed by snap cooling in wet ice.

This was followed by incubation in hybridization solution containing denatured ³²P-labelled probe (purified PCR-amplification of the 16S rRNA gene of RSB or pBS-KS vector) overnight at 65 °C with agitation. Following hybridization, the filters were washed twice with 50 ml 2X SSC at 65°C for 15 minutes. This was followed with a wash in 2X SSC, 0.1% SDS at 65 °C for 30 minutes and finally a wash in 0.1X SSC at 65°C for 10 minutes. The hybridization bags were partially drained and sealed. Kodak X-OMAT-AR™ films were exposed to the blots with two intensifying screens at -70°C for 19 hours.

Probes

Random primer labeling was used to generate radioactive probes (Random Primers DNA Labeling System, GIBCO BRL, Gaithersburg, MD). 15 ng of heat-denatured probe template DNA (purified PCR-amplification of the 16S rRNA gene of RSB or pBS-KS vector) in a volume of 10 μ l TE buffer, and added to a labelling cocktail consisting of 1 μ l dATP, 1 μ l dTTP, 1 μ l dGTP, 7.5 μ l random primer labelling buffer mixture, 2.5 μ l α^{32} P dCTP (3000 Ci/mmol, 10 mCi/ml, ICN Biochemicals Inc., Irvine, CA), 1.5 μ l water and 0.5 μ l Klenow enzyme. The mixture was incubated at room temperature for 1 hour and the reaction was stopped with 5 μ l of 'Stop' solution (provided in the kit).

Sequencing

Miniprep DNA was further purified with phenol/CIAA and precipitated with ethanol (Maniatis et al., 1989) until the A_{260}/A_{280} ratio was 1.99. The DNA was denatured by the alkali denaturation protocol recommended in the Sequenase Version 2.0 sequencing kit (United States Biochemical, Cleveland, OH). ^{35} S-dATP (1415 Ci/mmol, 12.5 mCi/ml, NEN, Du Pont, Wilmington, DE) was used as the radioactive label. The sequencing primers used were the M13/pUC primers which anneal to the vector sequences flanking the insert DNA. 3 μ g plasmid DNA was made up to a volume of 18 μ l and incubated at room temperature for 5 minutes with 2 μ l of 2 M NaOH/2 mM EDTA. The alkali-denatured

DNA was treated with 2 μ l 3 M sodium acetate, pH 5.2 and precipitated with ethanol. The DNA pellet was dissolved in 7 μ l of sterile distilled water. 2 μ l reaction buffer (200 mM Tris-HCl pH 7.5, 100 mM MgCl₂ and 250 mM NaCl) and 1 μ l primer (0.5 pmol/ μ l) solution was added to the DNA solution and the mixture heated to 65°C in a water bath. The reaction was allowed to cool to 35°C and placed on ice. 1 μ l dithiothreitol (0.1 M), 2 μ l labeling mixture (1.5 μ M dGTP, 1.5 μ M dCTP and 1.5 μ M dTTP), and 0.5 μ l ³⁵S-dATP were added to the reaction followed by 2 μ l of a 1:7 dilution of Sequenase^R, Version 2.0 with Sequenase enzyme dilution buffer (provided in the kit). The reaction was incubated at room temperature for 5 minutes. 3.5 μ l of the reaction was added to 2.5 μ l of each dideoxynucleotide solution at 37°C and incubated for 7 minutes. The elongation reaction was terminated by adding 4 μ l of the 'Stop' solution (provided in the kit) and stored at -5°C for further use.

The sequencing reactions were electrophoresed on a 6% polyacrylamide (19:1 acrylamide: bis-acrylamide), 48% urea gel using 0.4 mm spacers and sharkstooth combs on a BRL model SO electrophoresis apparatus at 1300-1600 volts for 90 minutes and 180 minutes depending on the number of nucleotides to be read. The gels were fixed in 4 l of 12% methanol, 10% acetic acid for 15 minutes and then transferred to Whatman 3MM paper and dried on a gel drier (Hoefer SE 1160) set at 80°C for 1 hour under vacuum. Kodak X-Omat AR X-ray films were overlaid

on the gels and exposed for 36 hours. Sequencing gels were read manually and proof-read several times until all ambiguities were resolved. Sequence data were entered in WordPerfect files on an IBM PC. The files were saved as DOS text files and transferred to the VAXcluster mainframe computer (University of Tennessee Computing Center) using the FTP package via an optical ethernet connection. Data files were converted into "UWGCG" (University of Wisconsin Genetics Computer Group) formats using the page editor on the VAX system and the REFORMAT program in the UWGCG package (Devereux et al., 1984). Overlapping sequences from different lanes of the sequencing gels were aligned using the LINEUP program and finally edited using the SEQED program, both part of the UWGCG package. A comparison of the sequences determined from the clones 7 and 20 and that of the PCR product showed that the clones carried the correct insert. However clone 20 had a mutation in the multiple cloning site and was not studied further.

Sequencing with progressive primers

Primers were selected by comparison of sequences with GenBank 16S rRNA sequences from *Caulobacter subvibrioides* (GenBank M83797), *Flavobacterium capsulatum* (GenBank M59296) and *Erythrobacter longus* (GenBank M59062) (refer to Table VI-1). A few primers were designed specifically to improve the resolution of certain spans of the gene.

TABLE VI-1

List of primers used for sequencing the RSB 16S rRNA gene.

Primer	Sequence of oligonucleotide			Size
UNIVF-40	5' -	GTT TTC CCA GTC ACG AC	-3'	17 *
PCRF	5' -	AGA GTT TGA TCM TGG CTC AG	-3'	20 *
PR115	5' -	CCG AAC CTG AGG GC	-3'	14
PF181	5' -	GCC TGA GGA TGA GCC CGC	-3'	18
PF308	5' -	GGA GGC AGC AGT GGG GAA	-3'	18
PF399	5' -	GCT CTT TTA CCC GGG	-3'	15
PR472	5' -	GWA TTA CCG CGG CKG CTG	-3'	18 *
SF482	5' -	CGG AGG GAG CTA GCG TTG	-3'	18
PR599	5' -	CCA CTC ACC TCT CCT GG	-3'	17
PF705	5' -	GCG AAA GCG TGG GGA GC	-3'	17
SR821	5' -	CCG TAC TCC CCA GGC GG	-3'	17
SF868	5' -	GGG GCC TGC ACA AGC GG	-3'	17
PR971	5' -	GCA CCT GTG TTC CAG TCC C	-3'	19
PF1111	5' -	CCG CCG GTG ATA AGC CG	-3'	17
SF1136	5' -	CGT CAA GTC CTC ATG GC	-3'	17
SR1149	5' -	CCC AGC GCG TAA GGG CC	-3	17
PR1233	5' -	CCG AAC TGA GAC GGC	-3'	15
PCRR	5' -	GGT TAC CTT GTT ACG ACT T	-3'	19 *
UNIVREV	5' -	AAC AGC TAT GAC CAT G	-3'	16 *

Glossary: A = Adenine, C = Cytosine, G = Guanine, T = Thymine, M = {AC}, K = {GT}, W = {AT}.

* Sequencing M13 primers, Sequenase Version 2.0

* Amplification conserved primer (Eden *et al.*, 1991)

* Conserved 16S rRNA sequence (Lane *et al.*, 1985)

All other primers were generated by comparison of sequences with GenBank 16S rRNA sequences from *Caulobacter subvibrioides* (GenBank M83797), *Flavobacterium capsulatum* (GenBank M59296) and *Erythrobacter longus* (GenBank M59062).

Analysis of data

The final sequence was proof-read several times. An rDNA sequence matrix was created with aligned 16S rDNA sequences obtained from the Ribosomal Database Project (RDP) (Olsen et al., 1991). RSB and other relevant 16S rDNA sequences obtained from GenBank, were manually aligned with the RDP sequences using the LINEUP program in the UWGCG package. All spaces were replaced with 'Xs'.

Phylogenetic analyses

The file containing the aligned sequence matrix was transferred from the VAX network to a Macintosh Centris computer at the Hodges Library microcomputer laboratory, using the Versaterm file transfer program. The files were formatted into the PAUP (Phylogenetic Analysis Using Parsimony, Version 3.0) format for further analysis (Swofford, 1991). The matrix consisted of 18 aligned 16S rDNA sequences including RSB (refer to Table VI-2). These were selected from GenBank and the RDP databases. Certain entries in GenBank and the RDP were incomplete or had ambiguous characters. Sectors of the matrix, which had missing or ambiguous characters, were excluded from the analysis. The heuristic search procedure in PAUP was used to generate the most parsimonious tree. This procedure was adopted considering its capacity to handle a data set of more than 17 taxa. Information from this tree was used to decide on specific branches to be studied and the taxa to be selected as

TABLE VI-2

Taxa used in the phylogenetic analysis.

Taxon	Accession number
<i>Caulobacter bacteroides</i>	GenBank M83796
<i>Caulobacter crescentus</i>	GenBank M83798
<i>Caulobacter</i> marine strain 6	GenBank M83811
<i>Caulobacter</i> marine strain 18	GenBank M83809
<i>Caulobacter</i> marine strain 24	GenBank M83810
<i>Caulobacter subvibrioides</i>	GenBank M83797
<i>Erythrobacter longus</i>	GenBank M59062
<i>Flavobacterium capsulatum</i>	GenBank M59296
<i>Flavobacterium devorans</i>	ATCC 10829
<i>Hyphomonas jannaschiana</i>	GenBank M83806
<i>Porphyrobacter neustonensis</i>	GenBank L01785
<i>Pseudomonas diminuta</i>	GenBank M59064
<i>Rhodospirillum salexigenes</i>	GenBank M59070
RSB	
<i>Sphingomonas capsulata</i>	ATCC 14666
<i>Sphingomonas paucimobilis</i>	GenBank D16144
<i>Zymomonas mobilis mobilis</i>	ATCC 10988
<i>Zymomonas mobilis pomaceae</i>	ATCC 29192

18 representative members of the relevant branches of the α subdivision of *Proteobacteria*, including RSB, whose 16S rDNA sequences were used in the phylogenetic analysis. These were selected from GenBank and the RDP.

outgroups. A clade containing RSB and other closely related species was selected for further study. The taxa in this clade were subjected to an exhaustive search procedure using *R. salexigenes* as an outgroup. This search revealed more accurate information on the phylogeny of the selected taxa. Trees generated under various conditions were saved as output files and retrieved later for analysis.

Evaluation of the best reconstruction

The best tree was evaluated based on the statistical support for each of its branches using the 'bootstrap' procedure (Felsenstein, 1985) available in the PAUP package. This procedure generates a new data set consisting of a modified version of the original matrix. Some characters from the original matrix are deleted while other characters are duplicated by sampling with replacement. Each 'new' data set is then evaluated just as the original data set and a 'majority-rule' consensus tree is built showing all of the monophyletic groupings that are present in a majority of the 'bootstrap' samples. A grouping present in at least 95% of the data sets is considered significant (Felsenstein, 1985).

Results

PCR amplification and cloning

PCR amplification of the genomic DNA preparation produced a single electrophoretically resolvable band of DNA having the

expected size of ≈ 1.5 kbp. The amplification procedure yielded 4 μ g of DNA per 100 μ l reaction.

There were no artifacts or contaminants detected in the reaction. Direct sequencing of the PCR products was found to be reliable when the amplification primers were used as sequencing primers. Direct sequencing of the PCR product was attempted with internal primers. However, the primers did not anneal very well with the template and sequencing was not reliable. The PCR product was therefore cloned into pBS-KS. The ligation of the insert into the vector was verified by Southern hybridization with probes for the vector made from the linearized vector (pBluescript KS digested with SmaI), while the probe for the insert was made from the gel-purified PCR product. Sequencing from the plasmid using the universal M13 -40 primer and the M13 reverse primer verified the identity of the insert.

Data analysis

The complete sequence of the RSB 16S rRNA gene is presented in Figure VI-1. The sequence of positions 1-250 and the complement of positions 1250-1446 of RSB 16S rDNA, obtained by directly sequencing the PCR product using the amplification primers as sequencing primers, were entered into the UWGCG package for sequence analysis. Preliminary estimates of taxonomic affinities of the RSB to other bacteria were made by searching for sequence similarities between the partial

FIGURE VI-1

1	AGAGTTTGAT	CATGGCTCAG	AATGAACGCT	GGCGGCATGC	CTAACACATG
51	<u>CAAGTCGAAC</u>	<u>GAAGGCTTCG</u>	<u>GCCTTAGTGG</u>	<u>CGCACGGGTG</u>	<u>GCTAACGCGT</u>
101	<u>GGGAATCTGC</u>	<u>CCTCAGGTTC</u>	<u>GGAATAACAG</u>	<u>CGAGAAATTG</u>	<u>CTGCTAATAC</u>
151	<u>CGGATGATAT</u>	<u>CGCGAGATCA</u>	<u>AAGATTTATC</u>	<u>GCCTGAGGAT</u>	<u>GAGCCCGCGT</u>
201	<u>AGGATTAGCT</u>	<u>AGTTGGTGTG</u>	<u>GTAAAGGCGC</u>	<u>ACCAAGGCGA</u>	<u>CGATCCTTAG</u>
251	<u>CTGGTCTGAG</u>	<u>AGGATGATCA</u>	<u>GCCACACTGG</u>	<u>GACTGAGACA</u>	<u>CGGCCAGAC</u>
301	<u>TCCTACGGGA</u>	<u>GGCAGCAGTG</u>	<u>GGGAATATTG</u>	<u>GACAATGGGC</u>	<u>GAAAGCCTGA</u>
351	<u>TCCAGCAATG</u>	<u>CCGCGTGAGT</u>	<u>GATGAAGGCC</u>	<u>TTAGGGTTGT</u>	<u>AAAGCTCTTT</u>
401	<u>TACCGGGGAT</u>	<u>GATAATGACA</u>	<u>GTACCGGGAG</u>	<u>AATAAGCTCC</u>	<u>GGCTAACTCC</u>
451	<u>GTGCCAGCAG</u>	<u>CCGCGGTAAT</u>	<u>ACGAGGGAG</u>	<u>CTAGCGTTAT</u>	<u>TCGGAATTAC</u>
501	<u>TGGGCGTAAA</u>	<u>GCGCACGTAG</u>	<u>GCGGCTTTGT</u>	<u>AAGTTAGAGG</u>	<u>TGAAAGCCTG</u>
551	<u>GAGCTCAACT</u>	<u>CCAGAATTGC</u>	<u>CTTTAAGACT</u>	<u>GCATCGCTTG</u>	<u>AATCCAGGAG</u>
601	<u>AGGTGAGTGG</u>	<u>AATTCGAGT</u>	<u>GTAGAGGTGA</u>	<u>AATTCGTAGA</u>	<u>TATTCGGAAG</u>
651	<u>AACACCAGTG</u>	<u>GCGAAGGCGG</u>	<u>CTCACTGGAC</u>	<u>TGGTATTGAC</u>	<u>GCTGAGGTGC</u>
701	<u>GAAAGCGTGG</u>	<u>GGAGCAAACA</u>	<u>GGATTAGATA</u>	<u>CCCTGGTAGT</u>	<u>CCACGCCGTA</u>
751	<u>AACGATGATA</u>	<u>ACTAGCTGTC</u>	<u>GGGGCTCTTA</u>	<u>GAGCTTCGGT</u>	<u>GGCGCACGTA</u>
801	<u>ACGCATTAAG</u>	<u>TTATCCGCCT</u>	<u>GGGGAGTACG</u>	<u>GCCGCAAGGT</u>	<u>TAAAACTCAA</u>
851	<u>ATGAATTGAC</u>	<u>GGGGGCCTGC</u>	<u>ACAAGCGGTG</u>	<u>GAGCATGTGG</u>	<u>TTTAATTCTGA</u>
901	<u>AGCAACGCGC</u>	<u>AGAACCCTAC</u>	<u>CAGCGTTTGA</u>	<u>CATGTCCGGA</u>	<u>CGATTTCCGG</u>
951	<u>AGACCGATCT</u>	<u>CTTCCTTCG</u>	<u>GGGACTGGAA</u>	<u>CACAGGTGCT</u>	<u>GCATGGCTGT</u>
1001	<u>CGTCAGCTCG</u>	<u>TGTCGTGAGA</u>	<u>TGTTGGGTTA</u>	<u>AGTCCCGCAA</u>	<u>CGAGCGCAAC</u>
1051	<u>CCTCGTCCTT</u>	<u>AGTTGCCATC</u>	<u>ATTTAGTTGG</u>	<u>GCACTCTAAG</u>	<u>GAAACCGCCG</u>
1101	<u>GTGATAAGCC</u>	<u>GGAGGAAGGT</u>	<u>GGGGATGACG</u>	<u>TCAAGTCCTC</u>	<u>ATGGCCCTTA</u>
1151	<u>CGCGCTGGGC</u>	<u>TACACACGTG</u>	<u>CTACAATGGC</u>	<u>GGTGACAGTG</u>	<u>GGCAGCAATC</u>
1201	<u>TCGCAAGGGT</u>	<u>GAGCTAATCT</u>	<u>CCAAAAGCCG</u>	<u>TCTCAGTTCTG</u>	<u>GATTGTTCTC</u>
1251	<u>TGCAACTCGA</u>	<u>GAGCATGAAG</u>	<u>GCGGAATCGC</u>	<u>TAGTAATCGC</u>	<u>GGATCAGCAT</u>
1301	<u>GCCGCGGTGA</u>	<u>ATACGTTCCC</u>	<u>AGGCCTTGTA</u>	<u>CACACCGCCC</u>	<u>GTCACACCAT</u>
1351	<u>GGGAGTTGGA</u>	<u>TTCACCCGAA</u>	<u>GGCAGTGCGC</u>	<u>TAACCGCAAG</u>	<u>GAGGCAGCTG</u>
1401	<u>ACCACGGTGG</u>	<u>GTTCAGCGAC</u>	<u>TGGGGTGAAG</u>	<u>TCGTAACAAG</u>	<u>GTAACC</u>

Figure VI-1. The nucleotide sequence of the 16S rDNA of the root-stimulating bacteria (RSB).

Position 1 on the sequence matches with position 8 of the nucleotide sequence of 16S rRNA from the *rrnB* cistron of *E. coli* (Brosius *et al.*, 1978).

Underlined positions indicate positions used in the phylogenetic analysis.

sequence of the 16S rDNA of RSB and the sequence of partial and complete 16S rDNAs deposited with GenBank. This search was performed using the FastA program in the UWGCG package which carries out a Pearson and Lipman (Pearson and Lipman, 1988) analysis of similarity. The sequence of bases 1-250 and the complement of 1250-1446 had a high homology (>89%) with the sequence of the 16S rRNA genes of *Caulobacter subvibrioides* (GenBank M83797), *Flavobacterium capsulatum* (GenBank M59296) and *Erythrobacter longus* (GenBank M59062). Internal sequencing primers were designed by comparing the homology within the sequences mentioned above and the sequence of pRSB16SR bases 1-250 and 1250-1446 where applicable. Data obtained by sequencing with the various primers were compiled. Primers were designed to cover sectors which were missed or did not yield satisfactory results.

Phylogenetic analysis

The analysis of the sequence obtained directly from the PCR product revealed that RSB is most closely related to *C. subvibrioides*, *F. capsulatum* and *E. longus* and therefore a member of the α subdivision of the *Proteobacteria* (purple bacteria) (homology is greater than 89%). The evolutionary affiliations of RSB within the subdivision were determined by parsimony analysis of the complete 16S rDNA data matrix using PAUP Version 3.0 for Macintosh. During the analysis, 475 bases were deleted from the 1601 bases in the aligned data matrix.

This step was necessary because many sequences deposited in GenBank are incomplete or include ambiguous data which could affect the outcome of the analysis. The heuristic search procedure was used to generate the most parsimonious tree and the bootstrap procedure was used to determine the statistical support for each branch of this tree. 'Exhaustive' searches were not performed on the entire matrix due to computing hardware limitations. Data matrices with more than 11 taxa could not be analyzed by an 'exhaustive' search. The tree generated by the bootstrap algorithm was identical to that provided by the RDP group except for the inclusion of RSB and other taxa not included in the RDP database but available in GenBank. The deletion of various taxa did not alter the general topology of the tree.

Based on the topology of the general tree (refer to Figure VI-2), *R. salexigenes* was selected as a suitable outgroup for further analyses. This taxon appears to belong to a sister group to the clade containing *C. subvibrioides*, *F. devorans*, *S. paucimobilis*, RSB, *Z. m. mobilis* and *Z. m. pomaceae*, *P. neustonensis*, *E. longus*, *S. capsulata* and *F. capsulatum*. Selection of *R. salexigenes* as an outgroup resolved the relationship between the 10 taxa better than other taxa (refer to Figure VI-3). When *H. jannaschiana* and *P. diminuta* were used as outgroups the length of the trees increased and the consistency index decreased (refer to Figures VI-4 and VI-5 respectively). This confirmed the

Figure VI-2. Single 50% Majority-Rule Consensus tree based on the maximum parsimony analysis of 16S rDNA sequence data of 18 members of the α subdivision of *Proteobacteria*.

This tree was developed using the heuristic algorithm of PAUP Version 3.0, yielding a tree with length of 777 steps, a consistency index of 0.595 and a rescaled consistency index of 0.431. Numbers above branches reflect branch lengths optimized to the consensus topology. Italicized numbers below the branches indicate the percentage of the original trees that support the group descending from that branch. Branches without numbers below are supported in all the original trees. This branch of the subdivision includes most of the sequenced *Caulobacter* sequences including *C. subvibrioides*, *C. crescentus*, *C. bacteroides* and many marine and fresh water strains of *Caulobacter*. Interspersed within this branch are two controversial *Flavobacterium* species, and two species of *Pseudomonas*. This branch contains genera which are morphologically divergent though closely related with respect to 16S rRNA sequences.

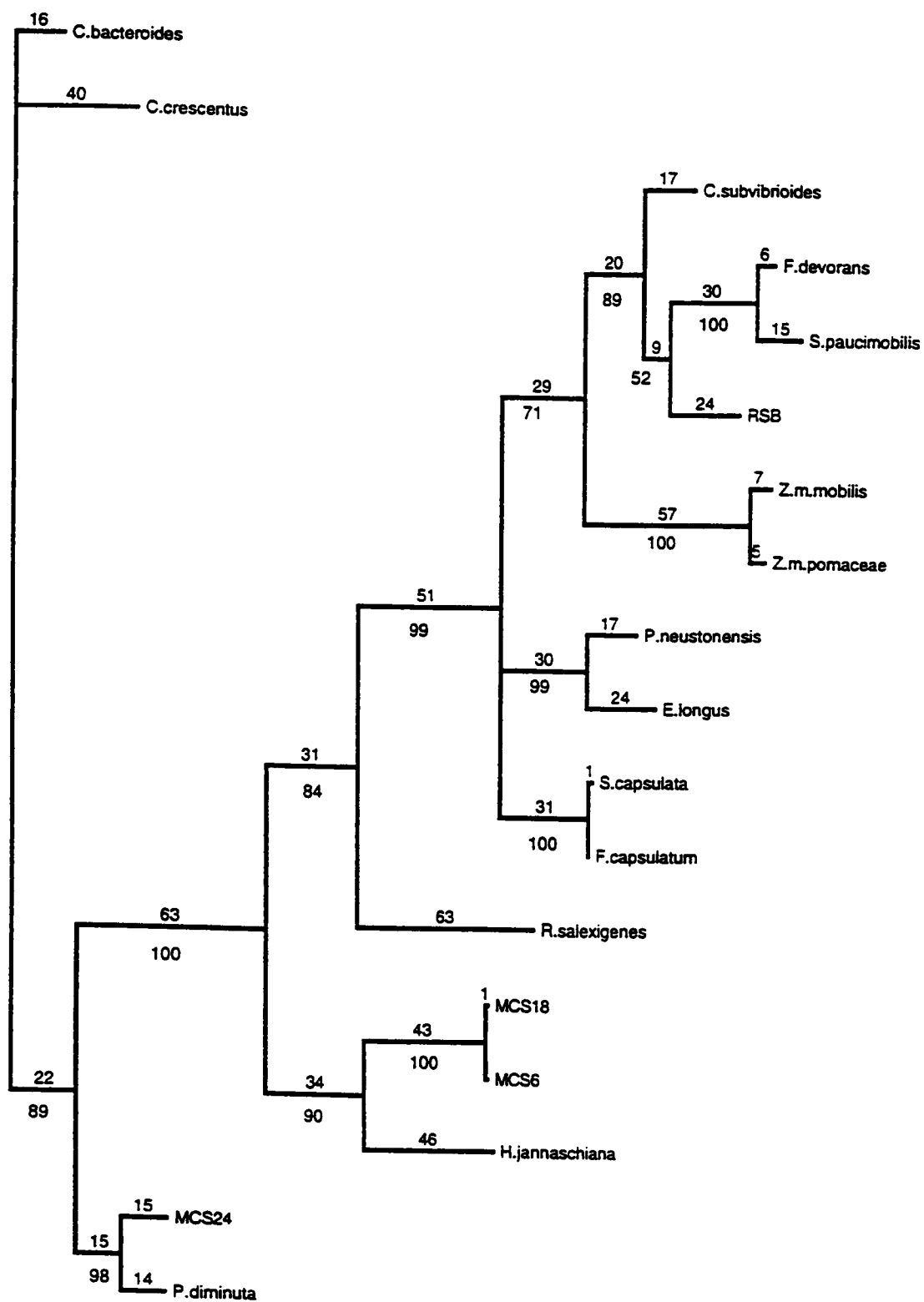


Figure VI-3. Single 50% Majority-Rule Consensus tree based on the maximum parsimony analysis of 16S rDNA sequence data of 11 members of the α subdivision of *Proteobacteria* with *R. salexigenes* defined as an outgroup.

This tree was developed using the heuristic algorithm of PAUP Version 3.0, yielding a tree with length of 427 steps, a consistency index of 0.717 and a rescaled consistency index of 0.493. Numbers above branches reflect branch lengths optimized to the consensus topology. Italicized numbers below the branches indicate the percentage of the original trees that support the group descending from that branch. Branches without numbers below are supported in all the original trees.

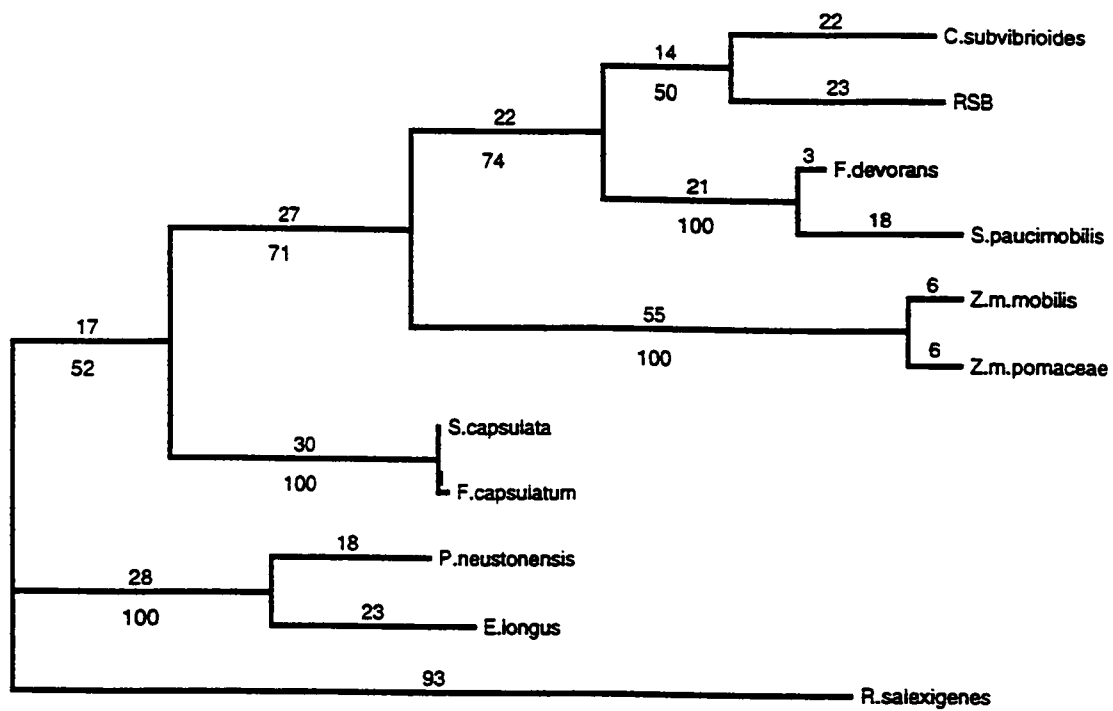


Figure VI-4. 50% Majority-Rule Consensus of 2 equally parsimonious trees based on the analysis of 16S rDNA sequence data of 11 members of the α subdivision of *Proteobacteria* with *H. jannaschiana* defined as an outgroup.

This tree was developed using the heuristic algorithm of PAUP Version 3.0, yielding a tree with length of 444 steps, a consistency index of 0.712 and a rescaled consistency index of 0.479. Numbers above branches reflect branch lengths optimized to the consensus topology. Italicized numbers below the branches indicate the percentage of the original trees that support the group descending from that branch. Branches without numbers below are supported in all the original trees.

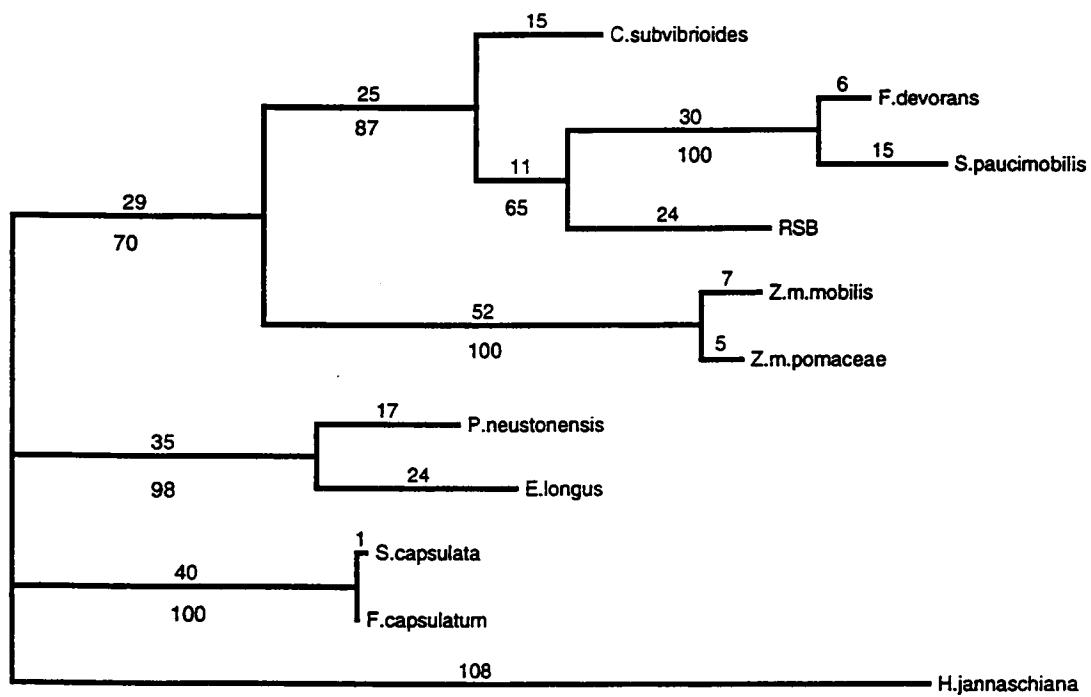


Figure VI-5. Single 50% Majority-Rule Consensus tree based on the maximum parsimony analysis of 16S rDNA sequence data of 11 members of the α subdivision of *Proteobacteria* with *P. diminuta* defined as an outgroup.

This tree was developed using the heuristic algorithm of PAUP Version 3.0, yielding a tree with length of 467 steps, a consistency index of 0.690 and a rescaled consistency index of 0.436. Numbers above branches reflect branch lengths optimized to the consensus topology. Italicized numbers below the branches indicate the percentage of the original trees that support the group descending from that branch. Branches without numbers below are supported in all the original trees.

validity of using *R. salexigenes* as an outgroup. The exhaustive search yielded 2 most parsimonious trees of 427 steps (refer to Figures VI-6 and VI-7 respectively). It was seen that only 79 trees could be generated shorter than 440 steps. This shows that the topology of the 10 selected taxa is quite robust. It is quite common for tree profiles to break up into 50 or more trees within 2 or 3 steps longer than that of the minimal tree. This tree had a consistency index of 0.717. This value is comparable to the index that Campbell and coworkers obtained (Campbell et al., 1992) while studying the phylogenetic relationships of symbiotic bacteria of weevils from 16S rDNA sequences.

RSB : Its position

The most parsimonious tree obtained by the exhaustive search places RSB closest to *C. subvibrioides*. This relationship is also evident from the distance table (refer to Table VI-3). The pair-wise mean distance between RSB and *C. subvibrioides* is 0.040 while the absolute distance is only 45 nucleotides (after correction). The absolute distance is the total number of nucleotide differences between 2 taxa considered. The mean distance is the average number of nucleotide differences per nucleotide of the total domain considered. The program is set up so that missing values are accounted for. Thus in cases where the sequence is incomplete, the mean distance is a better value to consider. In this

Figure VI-6. One of two most parsimonious trees obtained by an exhaustive search algorithm of PAUP Version 3.0 using *R. salexigenes* as an outgroup.

This tree has a length of 427 steps, a consistency index of 0.717 and a rescaled consistency index of 0.493.

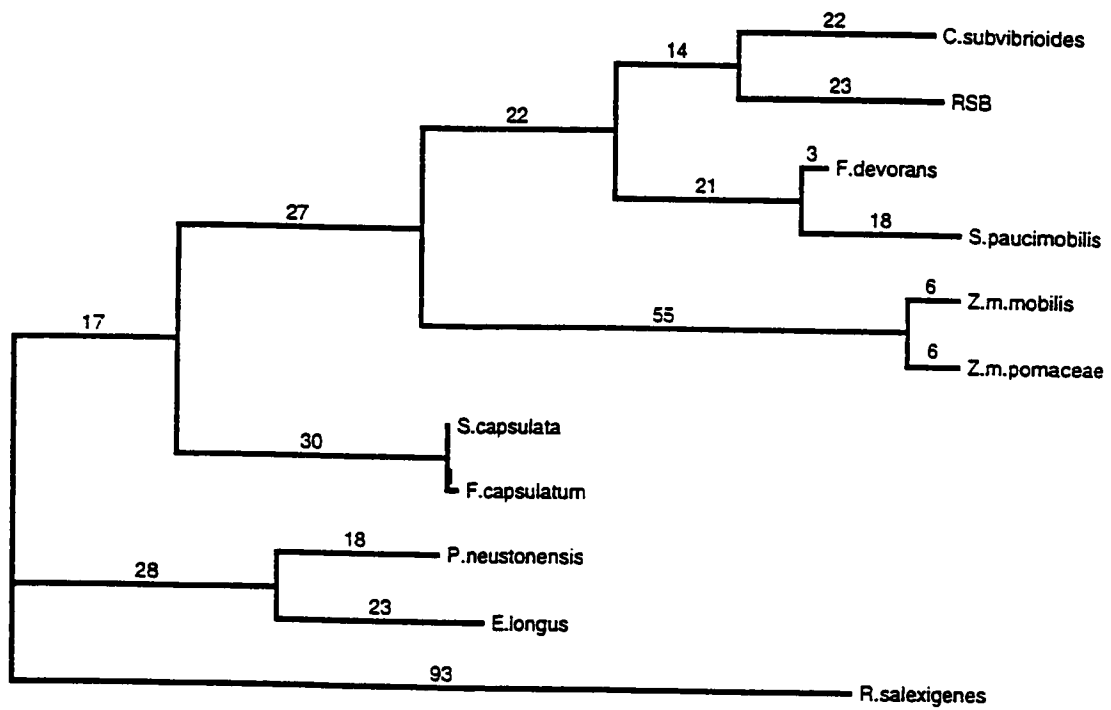


Figure VI-7. One of two most parsimonious trees obtained by an exhaustive search algorithm of PAUP Version 3.0 using *R. salexigenes* as an outgroup.

This tree has a length of 427 steps, a consistency index of 0.717 and a rescaled consistency index of 0.493.

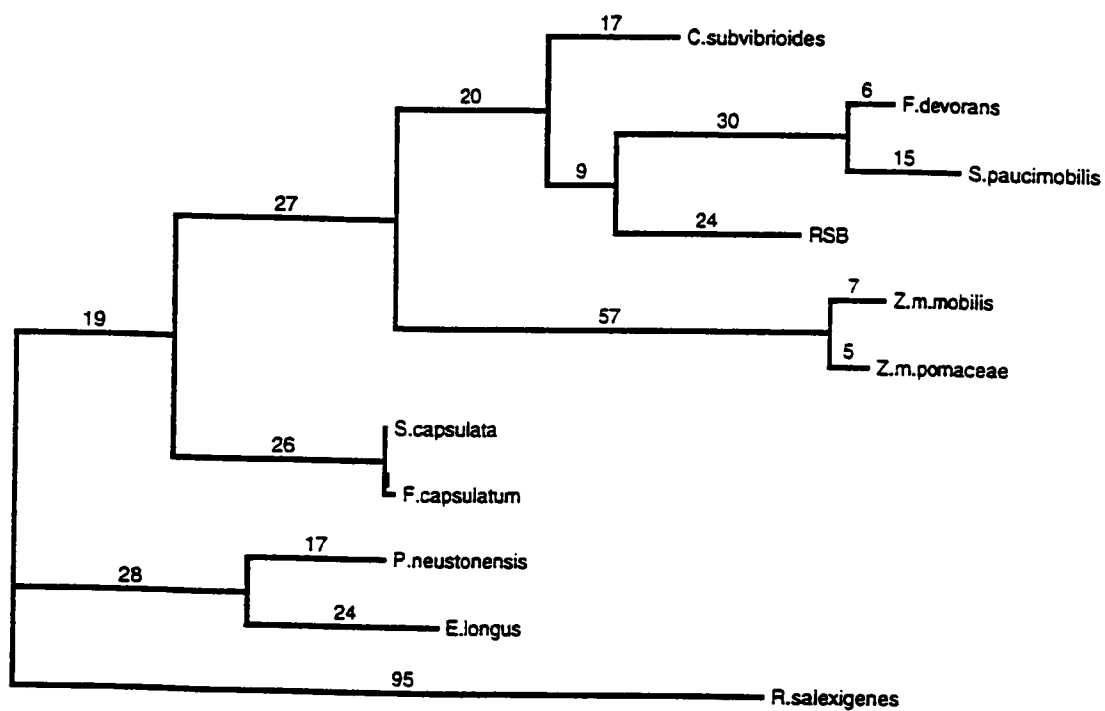


TABLE VI-3

Pairwise distances between closely related taxa

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
1 C.bacteroides	-	0.048	0.130	0.139	0.108	0.107	0.132	0.134	0.140	0.142	0.139	0.134	0.143	0.139	0.049	0.118	0.048	0.143
2 C.crescentus	54	-	0.137	0.146	0.120	0.118	0.145	0.144	0.149	0.152	0.150	0.147	0.147	0.150	0.072	0.127	0.067	0.149
3 C.subvibrioides	146	154	-	0.047	0.129	0.131	0.07	0.120	0.068	0.085	0.085	0.078	0.040	0.068	0.128	0.131	0.126	0.060
4 F.devorans	156	164	53	-	0.142	0.143	0.082	0.116	0.077	0.088	0.086	0.080	0.049	0.078	0.126	0.130	0.127	0.019
5 MCS18	121	135	145	160	-	0.002	0.137	0.131	0.140	0.134	0.138	0.137	0.143	0.139	0.121	0.081	0.111	0.154
6 MCS6	120	133	147	161	2	-	0.138	0.130	0.142	0.134	0.139	0.139	0.144	0.141	0.121	0.081	0.111	0.155
7 P.neustonensis	148	162	83	92	153	154	-	0.121	0.062	0.099	0.097	0.037	0.078	0.061	0.123	0.125	0.123	0.097
8 R.salexigenes	150	161	134	130	147	146	135	-	0.112	0.138	0.136	0.122	0.119	0.112	0.140	0.122	0.133	0.132
9 S.capsulata	157	167	76	87	157	159	70	126	-	0.098	0.096	0.072	0.073	0.001	0.134	0.121	0.138	0.092
10 Z.m.mobilis	159	171	96	99	150	151	111	155	110	-	0.011	0.092	0.092	0.096	0.139	0.138	0.142	0.098
11 Z.m.pomaceae	156	168	95	97	155	156	109	153	108	12	-	0.090	0.089	0.094	0.134	0.136	0.136	0.095
12 E.longus	150	165	88	90	154	156	41	137	81	103	101	-	0.082	0.070	0.125	0.124	0.129	0.093
13 R38	161	165	45	55	161	162	87	133	82	103	100	92	-	0.071	0.135	0.137	0.137	0.061
14 F.capsulatum	156	168	76	87	156	158	68	126	1	108	106	79	80	-	0.132	0.119	0.136	0.092
15 MCS24	55	81	143	141	135	135	137	156	150	155	150	140	151	148	-	0.121	0.026	0.131
16 H.jannaschiana	132	142	146	145	90	90	139	136	135	154	152	138	153	133	134	-	0.114	0.137
17 P.diminuta	54	75	141	143	125	125	138	149	155	159	153	145	154	153	29	127	-	0.131
18 S.paucimobilis	161	167	67	21	173	174	108	148	103	110	107	104	68	103	147	153	147	-

Numbers below diagonal indicate absolute distances between two taxa considered and numbers above the diagonal indicate mean distances (adjusted for missing data) between two taxa considered.

study, such effects have been eliminated by deleting all the missing characters from the data matrix. The corresponding mean distance values between RSB and *F. 'devorans'* and RSB and *S. paucimobilis* are 0.049 and 0.061, respectively. Thus considering the sequence data, the evidence points towards a closer relation between RSB and *C. subvibrioides*. The stability of the tree up to 13 steps longer than the most parsimonious topology indicates that the phylogeny suggested by the analysis is valid.

Comparative sequence analysis

Comparative sequence analysis of the 16S rRNA is said to be the best approach to arrive at both low and high levels of relationship among bacterial taxa (Yabuuchi et al., 1990). It is well known that certain domains in the gene cannot be considered for determining taxonomical affiliations because the nucleotides in these locations evolve at a faster rate than would be appropriate for determining differences at the level of genera. Positions 477-498 and 997-1044 of *Escherichia coli* are such domains. Yabuuchi et al. recommended the use of the span between positions 1220 and 1376 (1166-1321 in Figure VI-8, underlined) for taxonomical analysis as this domain does not evolve at such a fast rate. Using this alignment they suggested that a difference between two taxa of 15 bases might be a reasonable standard for the separation of 2 genera. The total number of mismatches in the above table is 127 and

RSB	1	AG AGTTT	GATCA	TGGCTCAGAA	TGAACGCTGG	40
<i>C.subvibrioides</i>		AACCTTGAG	AGTTT	NATCC	TGGCTCAGAA	CGAACGCTNG
<i>F.devorans</i>		NAAACTTGAG	AGTTT	GATCC	TGGCTCAGAA	CGAACGCTAG
<i>S.paucimobilis</i>				GAA	CGAACGCTGG	
RSB	41	CGGCATGCCT	AACACATGCA	AGTCGAACGA	AGGCTTCGGC	80
<i>C.subvibrioides</i>		CGGCATGCCT	AACACATGCA	AGTCGAACGA	GACCTTCGGG	
<i>F.devorans</i>		CGGCATGCCT	AACACATGCA	AGTCGAACGA	AGGCTTCGGC	
<i>S.paucimobilis</i>		CGGCATGCCT	AACACATGCA	AGTCGAACGA	AGGCTTCGGC	
RSB	81	CTTAGTGGCG	CACGGGTGGC	TAACGCGTGG	GAATCTGCCC	120
<i>C.subvibrioides</i>		TCTAGTGGCG	CACGGGTGGC	TRACGCGTGG	GAATCTGCCC	
<i>F.devorans</i>		CNNAGTGGCG	CACGGGTGGC	TAACGCGTGG	GAATCTGCCC	
<i>S.paucimobilis</i>		CTTAGTGGCG	CACGGGTGGC	TAACGCGTGG	GAATCTGCCC	
RSB	121	TCAGGTTTCGG	AATAACAGCG	AGAAATTGCT	GCTAATACCG	160
<i>C.subvibrioides</i>		TTGGGTTTCGG	AATAACTCGC	CGAAAGGCGT	GCTAATACCG	
<i>F.devorans</i>		TTAGGTTTCGG	AATAACAGCT	GGAAACGGCT	GCTAATACCG	
<i>S.paucimobilis</i>		TTAGGTTTCGG	AATAACAGCT	GGAAACGGCT	GCTAATACCG	
RSB	161	GATGATATCG	CGAGATCAAA	GATTTATCGC	CTGAGGATGA	200
<i>C.subvibrioides</i>		GATGATGTCG	TAAGACCAAA	GATTTATCGC	CCAGGGATGA	
<i>F.devorans</i>		GATGATATCG	CGAGATCAAA	GATTTATCGC	CTGAGGATGA	
<i>S.paucimobilis</i>		GATGATATCG	CGAGATCAAA	GATTTATCGC	CTGAGGATGA	
RSB	201	GCCCGCGTAG	GATTAGCTAG	TTGGTGTTGGT	AAAGGCGCAC	240
<i>C.subvibrioides</i>		GCCCGCGTAA	GATTAGCTAG	TTGGTGAGGT	AAAGGCTCAC	
<i>F.devorans</i>		GCCCGCGTTG	GATTAGGTAG	TTGGTGGGGT	AAAGGCCTAC	
<i>S.paucimobilis</i>		GCCCGCGTTG	GATTAGGTAG	TTGGTGGGGT	AAAGGCCTAC	

Figure VI-8. Alignment of RSB (RSB), *Caulobacter subvibrioides* (*C.subvibrioides*), *Flavobacterium devorans* (*F.devorans*) and *Sphingomonas paucimobilis* (*S. paucimobilis*).

Highlighted nucleotides indicate sites of mismatch. Underlined positions that is 1166 through 1321 [corresponding to positions 1220 through 1376 in the sequence described by Yabuuchi et al. (1990)] are those positions considered to be of phylogenetic importance by Yabuuchi et al.

	241			280
RSB	CAAGGCGACG	ATCCTTAGCT	GGTCTGAGAG	GATGATCAGC
<i>C.subvibrioides</i>	CAAGGCGACG	ATCCTTAGCT	GGTCTGAGAG	GATGATCAGC
<i>F.devorans</i>	CAAGCCGACG	ATCCATAGCT	AGTCTGAGAG	GATGATCAGC
<i>S.paucimobilis</i>	CAAGCCGACG	ATCCATAGCT	GGTCTGAGAG	GATGATCAGC
	281			320
RSB	CACACTGGGA	CTGAGACACG	GCCGAGACTC	CTACGGGAGG
<i>C.subvibrioides</i>	CACACTGGGA	CTGAGACACG	GCCGAGACTC	CTACGGGAGG
<i>F.devorans</i>	CACACTGGGA	CTGAGACACG	GCCNAGACTN	CNACGGGAGG
<i>S.paucimobilis</i>	CACACTGGGA	CTGAGACACG	GCCGAGACTC	CTACGGGAGG
	321			360
RSB	CAGCAGTGGG	GAATATTGGA	CAATGGGCGA	AAGCCTGATC
<i>C.subvibrioides</i>	CAGCAGTGGG	GAATATTGGA	CAATGGGCGA	AAGCCTGATC
<i>F.devorans</i>	CAGCAGTGGG	GAATATTGGA	CAATGGGCGA	AAGCCTGATC
<i>S.paucimobilis</i>	CAGCAGTGGG	GAATATTGGA	CAATGGGCGA	AAGCCTGATC
	361			400
RSB	CAGCAATGCC	GCGTGAGTGA	TGAAGGCCCT	AGGGTTGTAA
<i>C.subvibrioides</i>	CAGCAATGCC	GCGTGAGTGA	TGAAGGCCCT	AGGGTTGTAA
<i>F.devorans</i>	CAGCAATGCC	GCGTGAGTGA	TGAAGGCCNT	AGGGTTGTAA
<i>S.paucimobilis</i>	CAGCAATGCC	GCGTGAGTGA	TGAAGGCCCT	AGGGTTGTAA
	401			440
RSB	AGCTCTTTTA	CCCGGGATGA	TAATGACAGT	ACCGGGAGAA
<i>C.subvibrioides</i>	AGCTCTTTTA	CCCGGGATGA	TAATGACAGT	ACCGGGAGAA
<i>F.devorans</i>	AGCTCTTTTA	CCCGGGAA GA	TAATGACTGT	ACCGGGAGAA
<i>S.paucimobilis</i>	AGCTCTTTTA	CCCGGGAA GA	TAATGACTGT	ACCGGGAGAA
	441			480
RSB	TAAGCTCCGG	CTAACTCCGT	GCCAGCAGCC	GCGGTAATAC
<i>C.subvibrioides</i>	TAAGCTCCGG	CTAACTCCGT	GCCAGCAGCC	GCGGTAATAC
<i>F.devorans</i>	TAAGCCCCGG	CTAACTCCGT	GCCAGCAGCC	NCGGTAATAC
<i>S.paucimobilis</i>	TAAGCCCCGG	CTAACTCCGT	GCCAGCAGCC	GCGGTAATAC
	481			520
RSB	GGAGGGAGCT	AGCGTTATTC	GGAATTACTG	GGCGTAAAGC
<i>C.subvibrioides</i>	GGAGGGAGCT	AGCGTTGTTC	GGAATTACTG	GGCGTAAAGC
<i>F.devorans</i>	GGAGGGGGCN	AGCGTTGTTC	GGAATTACTG	GGCGTAAAGC
<i>S.paucimobilis</i>	GGAGGGGGCT	AGCGTTGTTC	GGAATTACTG	GGCGTAAAGC
	521			560
RSB	GCACGTAGGC	GGCTTTGTAA	GTTAGAGGTG	AAAGCCTGGA
<i>C.subvibrioides</i>	GCACGTAGGC	GGCTTTGTAA	GTCAGAGGTG	AAAGCCTGGA
<i>F.devorans</i>	GCACGTAGGC	GGCNTTGTAA	GTCAGAGGTG	AAAGCCTGGA
<i>S.paucimobilis</i>	GCACGTAGGC	GGCTTTGTAA	GTCAGAGGTG	AAAGCCTGGA

FIGURE VI-8 (continued)

	561			600
RSB	GCTCAACTCC	AGAAATGCCT	TTAAGACTGC	ATCGCTTGAA
<i>C.subvibrioides</i>	GCTCAACTCC	AGAACTGCCT	TTGAGACTGC	ATCGCTTGAA
<i>F.devorans</i>	GCTCAACTCC	AGAACTGCCT	TTGAGACTGC	ATCGCTTGAA
<i>S.paucimobilis</i>	GCTCAACTCC	AGAACTGCCT	TTGAGACTGC	ATCGCTTGAA
	601			640
RSB	TCCAGGAGAG	GTGAGTGGAA	TTCCGAGTGT	AGAGGTGAAA
<i>C.subvibrioides</i>	TCCAGGAGAG	GTGAGTGGAA	TTCCGAGTGT	AGAGGTGAAA
<i>F.devorans</i>	TCCAGGAGAG	GTGAGTGGAA	TTCCGAGTGT	AGAGGTGAAA
<i>S.paucimobilis</i>	TCCAGGAGAG	GTGAGTGGAA	TTCCGAGTGT	AGAGGTGAAA
	641			680
RSB	TTCGTAGATA	TTCGGAAGAA	CACCAGTGGC	GAAGGCGGCT
<i>C.subvibrioides</i>	TTCGTAGATA	TTCGGAAGAA	CACCAGTGGC	GAAGGCGGCT
<i>F.devorans</i>	TTCGTAGATA	TTCGGAAGAA	CACCAGTGGC	GAAGGCGGCT
<i>S.paucimobilis</i>	TTCGTAGATA	TTCGGAAGAA	CACCAGTGGC	GAAGGCGGCT
	681			720
RSB	CAC TGGACTG	GTATTGACGC	TGAGGTGCGA	AAGCGTGGGG
<i>C.subvibrioides</i>	CAC TGGACTG	GTATTGACGC	TGAGGTGCGA	AAGCGTGGGG
<i>F.devorans</i>	CAC TGGACTG	GTATTGACGC	TGAGGTGCGA	AAGCGTGGGG
<i>S.paucimobilis</i>	CAC TGGACTG	GTATTGACGC	TGAGGTGCGA	AAGCGTGGGG
	721			760
RSB	AGCAAACAGG	ATTAGATACC	CTGGTAGTCC	ACGCCGTAAA
<i>C.subvibrioides</i>	AGCAAACAGG	ATTAGATACC	CTGGTAGTCC	ACGCCGTAAA
<i>F.devorans</i>	AGCAAACAGG	ATTAGATACC	CTGGTAGTCC	ACGCCGTAAA
<i>S.paucimobilis</i>	AGCAAACAGG	ATTAGATACC	CTGGTAGTCC	ACGCCGTAAA
	761			800
RSB	CGATGATAAC	TAGCTGTCCG	GGCTCTTAGA	GCTTGGGTGG
<i>C.subvibrioides</i>	CGATGATAAC	TAGCTGTCCG	GGCACTTGGT	GCTTGGGTGG
<i>F.devorans</i>	CGATGATAAC	TAGCTGTCCG	GGCACTTGGT	GCTTGGGTGG
<i>S.paucimobilis</i>	CGATGATAAC	TAGCTGTCCG	GGCACTTGGT	GCTTGGGTGG
	801			840
RSB	CGCAGCTAAC	GCATTAAGTT	ATCCGCCTGG	GGAGTACGGC
<i>C.subvibrioides</i>	CGCAGCTAAC	GCATTAAGTT	ATCCGCCTGG	GGAGTACGGT
<i>F.devorans</i>	CGCAGCTAAC	GCATTAAGTT	ATCCGCCTGG	GGAGTACGGC
<i>S.paucimobilis</i>	CGCAGCTAAC	GCATTAAGTT	ATCCGCCTGG	GGAGTACGGC
	841			880
RSB	CGCAAGGTTA	AAACTCAAAT	GAATTGACGG	GGGCCTGCAC
<i>C.subvibrioides</i>	CGCAAGGTTA	AAACTCAAAG	GAATTGACGG	GGGCCTGCAC
<i>F.devorans</i>	CGCAAGGTTA	AAACTCAAAG	GAATTGACGG	GGGCCTGCAC
<i>S.paucimobilis</i>	CGCAAGGTTA	AAACTCAAAG	GAATTGACGG	GGGCCTGCAC

FIGURE VI-8 (continued)

	881			920
RSB	AAGCGGTGGA	GCATGTGGTT	TAATTCGAAG	CAACGCGCAG
<i>C.subvibrioides</i>	AAGCGGTGGA	GCATGTGGTT	TAATTCGAAG	NAACGCGCAG
<i>F.devorans</i>	AAGCGGTGGA	GCATGTGGTT	TAATTCGAAN	NAACGCGCAG
<i>S.paucimobilis</i>	AAGCGGTGGA	GCATGTGGTT	TAATTCGAAG	CAACGCGCAG
	921			960
RSB	AACCTTACCA	GCGTTTGACA	TGTCCGGACG	ATTTCCGGAG
<i>C.subvibrioides</i>	AACCTTACCA	GCGTTTGACA	TGTCCGGACG	ATTTCCAGAG
<i>F.devorans</i>	AACCTTACCA	GCGTTTGACA	TGGTAGGACG	ACTTCCAGAG
<i>S.paucimobilis</i>	AACCTTACCA	GCGTTTGACA	TGGTAGGACG	ACTTCCAGAG
	961			1000
RSB	ACCGATCTCT	TCCCTTCGGG	GACTGGAACA	CAGGTGCTGC
<i>C.subvibrioides</i>	ATGGATCTCT	TCCCTTCGGG	GACTGGAACA	CAGGTGCTGC
<i>F.devorans</i>	ATGGATTTCT	TCCCTTCGGG	GACCTACACA	CAGGTGCTGC
<i>S.paucimobilis</i>	ATGGATTTCT	TCCTTCGGG	GACCTACACA	CAGGTGCTGC
	1001			1040
RSB	ATGGCTGTCG	TCAGCTCGTG	TCGTGAGATG	TTGGGTTAAG
<i>C.subvibrioides</i>	ATGGCTGTCG	TNAGCTCGTG	TCGTGAGATG	TTGGGTTAAG
<i>F.devorans</i>	ATGGCTGTCG	TCAGCTCGTG	TCGTGAGATG	TTGGGTTAAG
<i>S.paucimobilis</i>	ATGGCTGTCG	TCAGCTCGTG	TCGTGAGATG	TTGGGTTAAG
	1041			1080
RSB	TCCCGCAACG	AGCGCAACCC	TCGTCCTTAG	TTGCCATCAT
<i>C.subvibrioides</i>	TCCCGCAACG	AGCGCAACCC	TCGCCNNTAG	TTACCATCAT
<i>F.devorans</i>	TCCCGCAACG	AGCGCAACCC	TCGCCNTTAG	TTACCATCAT
<i>S.paucimobilis</i>	TCCCGCAACG	AGCGCAACCC	TCGCCTTTAG	TTACCATCAT
	1081			1120
RSB	TTAGTTGGGC	ACTCTAAGGA	AAxCCGCCGG	TGATAAGCCG
<i>C.subvibrioides</i>	TTAGTTGGGG	ACTCTAAAGG	AAxCCGCCGG	TGATAAGCCG
<i>F.devorans</i>	TTGGTTGGGT	ACTCTAAAGG	AAxCCGCCGG	TGATAAGCNG
<i>S.paucimobilis</i>	TTGGTTGGGT	ACTCTAAAGG	ANACCGCCGG	TGATAAGCCG
	1121			1160
RSB	GAGGAAGGTG	GGGATGACGT	CAAGTCCTCA	TGGCCCTTAC
<i>C.subvibrioides</i>	GAGGAAGGTG	GGGATGACGT	CAAGTCCTCA	TGGCCCTTAC
<i>F.devorans</i>	GAGGAAGGTG	GGGATGACGT	CAAGTCCTCA	TGGCCCTTAC
<i>S.paucimobilis</i>	GAGGAAGGTG	GGGATGACGC	CAAGTCCTCA	TGGCCCTTAC
	1161			1200
RSB	GCGCTGGGCT	ACACACGTGC	TACAATGGCG	GTGACAGTGG
<i>C.subvibrioides</i>	GCGCTGGGCT	ACACACGTGC	TACAATGGCG	GTGACAGTGG
<i>F.devorans</i>	GCGCTGGGCT	ACACACGTGC	TACAATGGCA	ACTACAGTGG
<i>S.paucimobilis</i>	GCGCTGGGCT	ACACACGTGC	TACAATGGCA	ACTACAGTGG

FIGURE VI-8 (continued)

RSB	1201	<u>GCAGCAATCT</u>	<u>CGCAAGGGTG</u>	<u>AGCTAATCTC</u>	1240	<u>CAAAAGCCGT</u>
<i>C.subvibrioides</i>		<u>GCAGCAAACT</u>	<u>CGCGAGAGTG</u>	<u>CGCTAATCTC</u>		<u>CAAAAGCCGT</u>
<i>F.devorans</i>		<u>GCAGCGACCC</u>	<u>TGCGAGGGCG</u>	<u>AGCTAATCCC</u>		<u>CAAAAGTTGT</u>
<i>S.paucimobilis</i>		<u>GCAGCGACCC</u>	<u>TGCGAGGGCG</u>	<u>AGCTAATCCC</u>		<u>CAAAAGTTGT</u>
RSB	1241	<u>CTCAGTTCGG</u>	<u>ATTGTTCTCT</u>	<u>GCAACTCGAG</u>	1280	<u>AGCATGAAGG</u>
<i>C.subvibrioides</i>		<u>CTCAGTTCGG</u>	<u>ATTGTTCTCT</u>	<u>GCAACTCGAG</u>		<u>AGCATGAAGG</u>
<i>F.devorans</i>		<u>CTCAGTTCGG</u>	<u>ATTGTTCTCT</u>	<u>GCAACTCGAG</u>		<u>AGCATGAAGG</u>
<i>S.paucimobilis</i>		<u>CTCAGTTCGG</u>	<u>ATTGTTCTCT</u>	<u>GCAACTCGAG</u>		<u>AGCATGAAGG</u>
RSB	1281	<u>CGGAATCGCT</u>	<u>AGTAATCGCG</u>	<u>GATCAGCATG</u>	1320	<u>CCGCGGTGAA</u>
<i>C.subvibrioides</i>		<u>CGGAATCGCT</u>	<u>AGTAATCGCG</u>	<u>GATCAGCATG</u>		<u>CCGCGGTGAA</u>
<i>F.devorans</i>		<u>CGGAATCGCT</u>	<u>AGTAATCGCG</u>	<u>GATCAGCATG</u>		<u>CCGCGGTGAA</u>
<i>S.paucimobilis</i>		<u>CGGAATCGCT</u>	<u>AGTAATCGCG</u>	<u>GATCAGCATG</u>		<u>CCGCGGTGAA</u>
RSB	1321	<u>TACGTTCCCA</u>	<u>GGCCTTGTAC</u>	<u>ACACCGCCCG</u>	1360	<u>TCACACCATG</u>
<i>C.subvibrioides</i>		<u>TACGTTCCCA</u>	<u>GGCNTTGTAC</u>	<u>ACACCGCCCG</u>		<u>TCACACCATG</u>
<i>F.devorans</i>		<u>TACGTTCCCA</u>	<u>GGCTTTGTAC</u>	<u>ACACCGCNCG</u>		<u>TCACACCATG</u>
<i>S.paucimobilis</i>		<u>TACGTTCCCA</u>	<u>GGCCTTGTAC</u>	<u>ACACCGCCCG</u>		<u>TCACACCATG</u>
RSB	1361	<u>GGAGTTGGAT</u>	<u>TCACCCGAAG</u>	<u>GCAGTGCGCT</u>	1400	<u>AACxxCGCAA</u>
<i>C.subvibrioides</i>		<u>GGAGTTGGGT</u>	<u>TCACCCGAAG</u>	<u>GCGTTGCGCT</u>		<u>AACTxCGCAA</u>
<i>F.devorans</i>		<u>GGAGTTGGAT</u>	<u>TCACCCGAAG</u>	<u>GCGTTGCGCC</u>		<u>AACCTAGCAA</u>
<i>S.paucimobilis</i>		<u>GGAGTTGGAT</u>	<u>TCACCCGAAG</u>	<u>GCGTTGCGCC</u>		<u>AACCTAGCAA</u>
RSB	1401	<u>xxGGAGGCAG</u>	<u>CTGACCACGG</u>	<u>TGGGTTGAGC</u>	1440	<u>GACTGGGGTG</u>
<i>C.subvibrioides</i>		<u>xGAGAGGCAG</u>	<u>GCGACCACGG</u>	<u>TGGGCTTAGC</u>		
<i>F.devorans</i>		<u>TAGGAAGCAG</u>	<u>GCGACCACGG</u>	<u>TGGGTTGAGC</u>		<u>GACTGGGG..</u>
<i>S.paucimobilis</i>		<u>TAGGAAGCAG</u>	<u>GCGACCACGG</u>	<u>TGGGTTGAGC</u>		<u>GACTGGGGTG</u>
RSB	1441	<u>AAGTCGTAAC</u>	<u>AAGGTAACC</u>		1480	
<i>C.subvibrioides</i>						
<i>F.devorans</i>						
<i>S.paucimobilis</i>		<u>AAGTCGTAAC</u>	<u>AAGGTAGCCG</u>	<u>TAGGGGAACC</u>		<u>TGCGG</u>

FIGURE VI-8 (concluded)

within the predictive domain is less than 15 base mismatches. This suggests that the taxa considered are not different enough to belong to different genera (refer to Table VI-4). The number of base mismatches between RSB and *C. subvibrioides* is 4 bases while *C. subvibrioides* and *F. devorans* are 14 bases apart considering positions 1136 through 1321. RSB and *F. devorans* are 13 bases apart. This difference between the RSB and *C. subvibrioides*, in a domain that is very slow in evolution, shows that the two taxa are very closely related. Within the clade it can be said that RSB is more closely related to *C. subvibrioides* than to *F. devorans* or *S. paucimobilis*. Phylogenetically based probes are now commonly used for determinative and environmental studies. Radioactive probes can be synthesized homologous to particular target regions on the 16S gene and used to probe colony lifts (Stahl et al., 1992). However, this alignment (refer to Figure VI-8) shows that it will not be possible to synthesize probes based on the 16S rDNA sequence to identify RSB selectively from a mixed population of RSB, *C. subvibrioides* and Sphingomonads. Although there are a total of 127 mismatches, these positions are distributed throughout the gene. The 3' end of the gene has more mismatches than the 5' end and could be more suitable for the construction of specific probes.

TABLE VI-4

Comparison of nucleotide differences within the span defined by Yabuuchi et al. (1990) to be of taxonomic value and in the complete 16S data matrix.

	RSB	<i>F.</i> <i>devorans</i>	<i>S. pauci-</i> <i>mobilis</i>	<i>C. sub-</i> <i>vibrioides</i>
RSB	-	55	68	45
<i>F. devorans</i>	13	-	21	53
<i>S. paucimobilis</i>	13	0	-	67
<i>C. subvibrioides</i>	4	14	14	-

Numbers below the diagonal indicate the actual number nucleotide mismatches between the two taxa considered from position 1166 through 1321 [corresponding to positions 1220 through 1376 in the sequence described by Yabuuchi et al. (1990)]. Numbers above the diagonal indicate the total number of base mismatches considering the complete 16S rDNA data matrix used in the phylogenetic analysis.

Discussion

Use of 16S rDNA sequence data for phylogenetic analysis of bacteria

Eukaryotic organisms provide a multitude of morphological characters, which can serve as the basis for phylogenetic classification. Bacteria, on the other hand, have very simple morphologies. The lack of morphological characters to classify bacteria and develop phylogenies, necessitates the use of a different system to analyze them (Woese, 1987). In spite of the lack of suitable morphological characters of phylogenetic value, attempts at classifying bacteria based on morphology, have been made. In the light of molecular data, some of the genera determined by the classical system, now seem to be erroneous. Genera such as *Pseudomonas* can now be considered as a collection of at least five separate groups (Fox et al., 1977). "The sequencing of proteins and nucleic acids provides a new and more powerful approach to measuring evolutionary relationships and a new way of looking at them" (Wilson, 1977). Genotypic information, such as protein and nucleic acid sequence data, is superior to phenotypic information in that it is more readily and precisely interpreted and innately more informative of evolutionary relationships than phenotypic information is (Woese, 1987).

Ribosomal RNAs are useful as molecular chronometers (Woese, 1987). They show a high degree of functional constancy (Woese, 1982). rRNAs occur in all organisms and contain

domains which differ from one another in their rates of sequence mutation. This makes the measurement of phylogenetic relationships, among seemingly distant organisms possible. rRNAs are large molecules which consist of many domains. Each domain evolves at a particular rate. The 16S rRNA has 50 helical stems in its secondary structure while the 23S rRNA has twice that number. Woese (1987) suggested that the presence of so many domains makes rRNAs accurate chronometers. The ease of sequencing the rRNAs makes them all the more suitable for evolutionary studies (Woese, 1987).

Parsimony analysis

Unlike other methods of analysis, parsimony analysis treats differing positions among sequences individually (Felsenstein, 1982). It is based on the assumption that the correct tree is the one which requires the least number of mutations overall to describe the relationship of the organism to its common ancestor. Each branch in the tree and each segment between branching points is defined by specific changes that occur in some ancestral sequence on that branch (Woese, 1987). It is more suitable than other techniques, especially when the sequences compared include deletions and corrections for missing data.

The α subdivision of Proteobacteria

The phylum *Proteobacteria* derives its name from the

photosynthetic (purple) bacteria that it includes. Though the photosynthetic capacity has been lost several times in this group, the ancestor to the phylum should have been photosynthetic (Woese, 1987). Based on the structural difference and differences in base composition, *Proteobacteria* has been divided into four subdivisions - α , β , γ and δ . All the bacteria associated with and close to the RSB fall within the α subdivision. This is a very interesting subdivision with respect to evolution and association with plants. Photosynthetic and non-photosynthetic species are intimately juxtaposed suggesting a more or less continual evolution of the latter from the former (Woese et al., 1984). All species are gram-negative and there are several examples of aerobic metabolism in this subdivision. The two genera closely associated with plants - *Rhizobium* and *Agrobacterium* are present in this subdivision and form a tight cluster within subgroup α -2. This subdivision includes all *Caulobacter* species, some "high G+C content" *Flavobacterium* species such as *F. 'devorans'* and *F. capsulatum* (whose affiliation is in doubt), certain *Pseudomonads* and most *Sphingomonads*.

Description of the genera closely related to the RSB

The *Caulobacter* genus

Caulobacter species are predominantly aquatic bacteria found in a variety of fresh and marine environments. 16S rDNA analysis of *Caulobacter* collections show major branch points

separating marine strains of *Caulobacter* from fresh water strains (Stahl, 1992). The exception to this major cladistic clustering is *C. subvibrioides* which seems to be only peripherally related to the greater *caulobacter* assemblage (Stahl, 1992). It has all the morphological characters of a typical *Caulobacter*, such as the presence of a polar stalk contiguous with the cell membranes, a holdfast at the stalk distal tip and a single polar flagellum on the swarmer cell. All known subvibrioid isolates of *Caulobacter* able to utilize sugars for growth are classified as *C. subvibrioides*. However, based on 16S rDNA sequence information, it seems to be diverging from the main assemblage of *Caulobacter* species (Stahl et al., 1992).

The *Sphingomonas* genus

Based on 16S rRNA sequence homology, the presence of unique sphingolipids in cellular lipids and the major type of ubiquinone (Q10), Yabuuchi et al. (1990) proposed the genus *Sphingomonas* with *S. paucimobilis* as its type species. Within the genus were included *S. parapaucimobilis*, *S. yanoikuyae*, *S. adhesiva* and *S. capsulata*. This genus also includes species from genera such as *Pseudomonas* and *Flavobacterium*.

The identity of *Flavobacterium devorans* and *Flavobacterium capsulatum*, is being challenged. Yabuuchi and coworkers (Yabuuchi et al., 1979), suggested that *F. devorans* strain ATCC 10829 be renamed *Pseudomonas paucimobilis*, based

on its G+C content and its motility. Strain ATCC 10829 has a high concentration of 2-hydroxymyristic acid, palmitic acid and octadecenoic acid. Such a fatty acid profile is also seen in *P. paucimobilis*. This bacterium had been described previously by Holmes et al. (1977) as a yellow, polar monotrichous, non-fermentative, gram-negative rod associated with various human conditions. While *P. paucimobilis* is known to fix atmospheric nitrogen (Young, 1992), there are no references in the literature that suggest nitrogen fixation in either *Flavobacterium* or *Sphingomonas* genera. There are references to *F. devorans* strain ATCC 10829 as *Sphingomonas devorans* (Moore et al., 1993). rRNA cistron analysis places *F. capsulatum* in a separate rRNA superfamily that includes *Pseudomonas paucimobilis* as its nearest taxon (Bauwens and De Ley, 1981). Bally et al. (1990) suggest that *F. capsulatum* is a non-motile variant of *P. paucimobilis*.

S. paucimobilis was formerly known as *Pseudomonas paucimobilis* (Senoo et al., 1992). Among important physiological and environmental properties of *S. paucimobilis* and *P. paucimobilis* are a) the decomposition of γ -1,2,3,4,5,6-hexachlorocyclohexane (Nishiyama et al., 1992); b) the mineralization of dibenzo-p-dioxin (Moore et al., 1993) and c) the cleavage of the β -aryl ether linkage, commonly found in lignin (Masai et al., 1991).

It is clear that this is a genus that is not clearly defined in terms of distinct morphological characters but

includes members of *Pseudomonas* and *Flavobacterium* genera based on the presence of distinguishing cellular lipids, similarity in G+C content in their genome, rRNA:DNA hybridization similarities and the presence of nostoxanthin, a carotenoid pigment. The phylogenetic analysis of their 16S rDNA sequence seems to corroborate the classification. "Although *Sphingomonas* may be determined to be distinct taxonomically, there has not been, to date, detailed studies on the metabolism or the ecology of the genus" (Moore et al., 1993).

The *Zymomonas* genus

Zymomonas mobilis mobilis and *Zymomonas mobilis pomaceae* are defined by their "quantitative fermentation of glucose, fructose or sucrose-but no other sugar-to equimolar amounts of ethanol and CO₂" (Bergey's manual, 1984). They are flagellated and can tolerate a pH range of 3.5 to 7.5 (a property similar to RSB, which grows at a pH of 5.2). They are facultatively anaerobic, predominantly associated with the spoilage of beer and cider. *Zymomonas* is the only genus of bacteria found to be ethanol producing. There is only one specie in the genus and two subspecies).

The photosynthetic genera: *Erythrobacter* and *Porphyrobacter*

Erythrobacter longus is a marine photosynthetic rod while

Porphyrobacter neustonensis is a fresh-water photosynthetic rod. Both synthesize bacteriochlorophyll A under aerobic conditions. They differ in their nutritional requirements and in their biochemical reactions. They also differ quantitatively in the composition of their cellular fatty acids namely 3-hydroxy iso undecanoic acid and 2-hydroxy tetradecanoic acid (Fuerst et al., 1993). The presence of bacteriochlorophyll A in both of these, clearly distinguishes them from the other members of the clade.

Phylogenetic study of genus *Caulobacter* and closely related genera based on 16S rRNA affiliations

The *Caulobacter* assemblage as defined by 16S rRNA sequence homology, includes many non-*Caulobacters* (refer to Figure 6-C). *Rhodobacter capsulatum*, a photosynthetic, non-stalk forming bacterium is closely related to marine strains of *Caulobacter* such as MCS6, MCS18, marine *Hyphomonas* species, *Hyphomonas jannaschiana* and *Pseudomonas diminuta*. The close affiliation of *P. diminuta* with *Caulobacter* strains MCS17 and FWC14 is noteworthy. This suggests a relatively recent divergence of *P. diminuta* from the *Caulobacter* phenotype. Stahl et al. (1992) probed a sample of non-*Caulobacter* isolates from waste water treatment facilities with a probe for the gene encoding a *Caulobacter* flagellar filament protein. Of the 150 isolates screened, only one isolate hybridized with the probe. This isolate was characterized by a commercial

identification protocol as *P. vesicularis* (Biologue). *P. diminuta* and *P. vesicularis* form a distinctive branch of the *Pseudomonas* genus based on RNA homology (Stahl et al., 1992). The sequence of the 16S rRNA of *P. vesicularis* is not available in the database for analysis. A single polar flagellum is the only common characteristic between *P. vesicularis*, *P. diminuta* and the *Caulobacters* (Stahl et al., 1992). It is interesting to note that when Burns (1990) conducted his studies on the RSB, the bacterium was identified as *P. vesicularis* by a commercial agency. It suggested that these *Pseudomonads* are *Caulobacters* locked in the motile phase of the developmental cycle. Alternatively, the phenotype could be explained by a mutation that resulted in the loss of the genes that control the developmentally regulated functions of *Caulobacter* species (Stahl et al., 1992).

The presence of *R. capsulatus* within the assemblage of *Caulobacters* leads one expect photosynthetic *Caulobacters*, though none have been reported as yet. Although the marine *Hyphomonads* and *R. capsulatus* appear to descend from *Caulobacter* ancestors, it cannot be said with certainty that *C. subvibrioides* is ancestral to the branch of the α subdivision containing *Methylosinus trichosporium*, *Rhodopseudomonas acidophila*, *Agrobacterium tumefaciens*, *Rhodomicrobium vannielli* and the *Caulobacters*. This ambiguity in the interpretation of the early divergence of *C. subvibrioides* arises from the instability of the branching

order when a different group of organisms is used in the analysis (Stahl et al., 1992).

From phylogenetic studies of RSB and other closely related strains there seems to be a high degree of homology between the 16S rDNA sequences of *C. subvibrioides*, *S. paucimobilis*, *F. 'devorans'* and RSB (refer to Figures 6-D, 6-E, 6-F, 6-G and 6-H). The affiliation of *C. subvibrioides* seems closer to RSB and *S. paucimobilis* than to the other *Caulobacters*. However, the morphological characters of *C. subvibrioides* positively identify it as a *Caulobacter*. It remains to be verified if *C. subvibrioides* is a common ancestor to both the *Caulobacter* genus and the *Sphingomonas* genus. Studies of electron micrographs and phase-contrast micrographs of the RSB suggest a strong relationship with the *Caulobacters* (B. Ely, personal communication). The presence of the polar stalk ending in a bulbous holdfast and the observation of occasional bacterial rosettes are characters held in common with the *Caulobacters*. Homology of the 16S rRNA sequence with *C. subvibrioides* to the extent of 96.4% adds further proof of their very close phylogenetic relatedness.

CHAPTER VII

SUMMARY

The topology of the trees studied (refer to Figures VI-2,3,4,5,6 and 7) strongly suggests a close relationship between the RSB, *Sphingomonas paucimobilis*, *Flavobacterium devorans* and *Caulobacter subvibrioides*. The absolute distance between the strains is less than 70 nucleotides (refer to Table VI-4) in the corrected matrix of nucleotides. Using the criterion established by Yabuuchi et al. (1990), the total number of nucleotide differences between pairs of taxa within positions 1220-1376 [1136-1321 in the RSB alignment (refer to Figure VI-8)] is less than 15. This indicates that the 4 taxa considered are not distant enough to belong to different genera. However within the clade, RSB is closer to *C. subvibrioides* than the other two taxa considering nucleotide mismatch values and the relative placement on the tree (refer to Figure VI-6).

It is to be noted that the morphology of the RSB suggests a close relationship with the *Caulobacter* genus. From electron micrographs and phase contrast micrographs of the RSB, the bacterium appears to be stalked, have a bulbous holdfast and occasionally forms bacterial rosettes. These are characters typically found in members of the genus *Caulobacter*. Since a perfect match for the RSB's 16S sequence has not been found (homology greater than 99%), the identity of the RSB cannot be

inferred any further.

The pigment absorption profile of the RSB suggests the presence of carotenoid pigments in the extract. Such results have been obtained by Poindexter (1964) while characterizing different strains of *Caulobacter* species.

The rooting assays have clearly demonstrated that this bacterium is secreting a root inducing substance that is maximally extractable at a pH of 2.8 perhaps indicating the protonation of a putative RISS. This substance is able to induce rooting in woody species exhibiting little or no spontaneous rooting. In addition, the efficiency of root induction also depends on the genotype of the tree species.

The bacterium may be secreting an indole derivative. This substance may directly induce rooting or effect the explant's endogenous level of IAA resulting in an indirect enhancement of adventitious root production.

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APPENDICES

APPENDIX A

Media

Woody plant medium (WPM)

WPM macro	200 ml
MS micro	10 ml
GD iron	10 ml
Meso-inositol	0.1 g
Nicotinic acid (0.1 mg/ml)	5 ml
Pyridoxine-HCl (0.1 mg/ml)	5 ml
Thiamine HCl (1 mg/ml)	1 ml
Glycine (2 mg/ml)	1 ml
Sucrose	20 g
Casein hydrolysate	2 g

The solution was made up to 1 liter and the pH was standardized to 5.2. Agar (15 g) was added to the solution and autoclaved.

For a liquid culture medium, the same procedure was followed but for the agar which was excluded from the medium.

WPM stock solutions

WPM macro 5x (1 liter)

H ₂ O	300 ml
NH ₄ NO ₃	2.0 g
CaCl ₂ .2H ₂ O	0.48 g
MgSO ₄ .7H ₂ O	1.85 g
KH ₂ PO ₄	0.85 g
Ca(NO ₃) .4H ₂ O	2.78 g
K ₂ SO ₄	4.95 g
H ₂ O	upto 1 L

MS micro full strength (1 liter)

H ₂ O	300 ml
H ₃ BO ₃	1.69 g
MnSO ₄ .H ₂ O	1.69 g
ZnSO ₄ .7H ₂ O	0.86 g
KI	0.083 g
Na ₂ MoO ₄ .2H ₂ O	0.025 g
CuSO ₄ .5H ₂ O (2.5 mg/ml)	1.0 ml
CoCl ₂ .6H ₂ O (2.5 mg/ml)	1.0 ml
H ₂ O	upto 1 L

GD iron (1 liter)

FeSO ₄ .7H ₂ O	2.78 g
Na ₂ EDTA	3.73 g

The two components were dissolved in 500 ml of H₂O separately by stirring overnight on a magnetic stirrer. The two solutions

were then mixed.

Gresshoff and Doy medium (GD - Gresshoff and Doy, 1972)

H ₂ O	300 ml
GD macro	100 ml
GD micro	10 ml
GD iron	10 ml
Meso-inositol	0.01 g
Nicotinic acid (0.1 mg/ml)	1 ml
Pyridoxine-HCl (0.1 mg/ml)	1 ml
Thiamine hcl (1 mg/ml)	1 ml
Sucrose	20 g

The solution was made up to 1 liter and the pH was standardized to 5.5. The solution was heated till boiling and 10 g of agar was added. Charcoal (5 gm) added to the solution after the agar was well incorporated in the solution. The medium was dispensed into culture tubes and sterilized by autoclaving.

GD macro (2 liter)

KNO ₃	20 gm
CaCl ₂ .2H ₂ O	3.0 gm
MgSO ₄ .7H ₂ O	5.0 gm
(NH ₄) ₂ SO ₄	4.0 gm
NaH ₂ PO ₄ .H ₂ O	1.8 gm
Na ₂ HPO ₄	0.6 gm
KCl	6.0 gm

These salts were dissolved in 1800 ml of H₂O and the final volume brought up to 2 liters.

GD micro (1 liter)

H ₂ O	300 ml
H ₃ BO ₃	0.30 g
MnSO ₄ .H ₂ O	1.00 g
ZnSO ₄ .7H ₂ O	0.30 g
KI	0.075 g
Na ₂ MoO ₄ .2H ₂ O	0.025 g
CuSO ₄ .5H ₂ O (2.5 mg/ml)	10 ml
CoCl ₂ .6H ₂ O (2.5 mg/ml)	10 ml

The final volume brought up to 1 liter with H₂O.

Luria-Berthani medium (LB)**

Bacto-tryptone	10 g
Bacto-yeast	5 g
NaCl	10 g
H ₂ O	800 ml

The pH is then adjusted to 7.5 with NaOH and the volume made

up to 1 l. The medium is sterilized by autoclaving.

* An agar-based LB medium is prepared by including 15 gm of Bacto-agar in the recipe after adjusting the pH and prior to autoclaving.

* Antibiotics can be added to the medium after cooling the autoclaved medium (with or without agar) to 55 °C.

Ampicillin

Stock solution: A 20 mg/ml solution of ampicillin is prepared and sterilized by filtration. The solution is stored at -20 °C.

Liquid culture: 5 ml of the ampicillin solution was used per liter of LB medium.

Solid (agar-based) medium: 2.5 ml of the ampicillin solution was used per liter of LB medium.

Tetracycline

Stock solution: A 12.5 mg/ml solution of tetracycline hydrochloride is prepared in 50% ethanol. The solution is stored at -20 °C. This antibiotic is light sensitive and therefore stored in the dark.

Liquid culture: 1.2 ml of the tetracycline solution was used per liter of LB medium.

Solid (agar-based) medium: 1.2 ml of the tetracycline solution was used per liter of LB medium.

APPENDIX B

Solutions

Tris-borate EDTA (TBE)

Stock solution (10X) (1 liter)

Trizma base	54 g
Boric acid	27.5 g
0.5 M EDTA (PH 8.0)	20 ml

The final volume was brought up to 1 liter with H₂O.

1 M Tris-HCl, pH 8.0

Trizma base (121 g) was dissolved in 800 ml of water and the pH of the solution adjusted to 8.0 by adding approximately 42 ml of concentrated HCl. After final adjustments of the pH, the volume was made upto 1 l with H₂O, dispensed into aliquots and sterilized by autoclaving.

3 M NaOAc, pH 5.2

Sodium acetate.3H₂O (408.1 g) was dissolved in 800 ml of H₂O. The pH of the solution was adjusted to 5.2 with glacial acetic acid. The volume was then made upto to 1 l with H₂O and sterilized by autoclaving.

3 M NaOAc, pH 7.0

Sodium acetate.3H₂O (408.1 g) was dissolved in 800 ml of H₂O. The pH of the solution was adjusted to 7.0 with dilute acetic acid. The volume was then made upto to 1 l with H₂O and sterilized by autoclaving.

10% Sodium dodecyl sulfate (10% SDS)

Electrophoresis-grade SDS (100 g) was dissolved in 900 ml of H₂O by warming the solution up to 68°C. The pH was adjusted to 7.2 with a few drops of concentrated HCl and the volume made upto a liter.

20X SSC

Sodium chloride (175.3 g) and 88.2 g of sodium citrate was dissolved in 800 ml of H₂O. The pH was adjusted to 7.0 with a few drops of 10 N NaOH and the volume made upto a liter with H₂O. The solution was sterilized by autoclaving.

EDTA solution (0.5 M, pH 8.0)

Disodium ethylene diamine tetraacetic acid.2 H₂O (EDTA) (186.1 g) was dissolved in 800 ml was H₂O. The reagent was allowed to dissolve overnight by stirring the solution using a magnetic stirrer. The pH was adjusted to 8.0 with NaOH pellets and the volume made upto a liter. The solution was sterilized by autoclaving.

Tris EDTA (TE) (pH 8.0)

Tris-HCl (pH 8.0) (refer to the description) (1 ml) was added to 0.2 ml of 0.5 M EDTA (refer to the description) and the volume made upto 100 ml with H₂O.

TNE

Tris-HCl (pH 8.0) (refer to the description) (1 ml) was added to 0.2 ml of 0.5 M EDTA (refer to the description). Sodium chloride (0.596 g) was added to the solution and the volume made upto 100 ml with H₂O.

Denhardt's solution (50X) (DS)

Ficoll (5 g) was added to 5 g polyvinylpyrrolidone and 5 g of bovine serum albumin (BSA). The volume made upto 500 ml with H₂O. The solution was filter-sterilized.

Pre-hybridization solution (25 ml)

7.5 ml 20X SSC
2.5 ml 50X DS
1.25 ml 10% SDS
13.75 ml H₂O

Hybridization solution (25 ml)

7.5 ml 20X SSC
2.5 ml 50X DS
1.25 ml 10% SDS
13.75 ml H₂O

The denatured radioactive probe with heterologous DNA is added to the solution prior to hybridization.

Tris-Acetate-EDTA buffer (50X)

242 g Trizma base
57.1 ml glacial acetic acid
100 ml EDTA solution (0.5 M)

The volume is adjusted to 1 liter with H₂O

TAE-Agarose gel (1%)

30 ml TAE (1X)
0.3 g Agarose (Electrophoresis grade)

The solution is boiled for 2 minutes and poured into gel trays.

Miniprep solution I (200 ml)

50 mM glucose
25 mM Tris-HCl (pH 8.0)
10 mM EDTA (pH 8.0)

This solution is sterilized by autoclaving.

Miniprep solution II (100 ml)

0.2 N NaOH
1% SDS

Miniprep solution III (100 ml)

60 ml potassium acetate (5 M)
11.5 ml glacial acetic acid
28.5 ml H₂O

Phenol for extractions

Frozen phenol is thawed at 68 °C and extracted with 1.0 M Tris solution (pH 8.0). The extraction is continued several with a buffer consisting of 0.1 M Tris (pH 8.0) and 0.2% β-mercaptoethanol until the pH of the aqueous phase is > 7.6.

Chloroform: isoamyl alcohol (100 ml) (CIAA)

4 ml isoamyl alcohol
96 ml chloroform

Sequencing gel running buffer

17.28 g Trizma base
8.8 g boric acid
6.4 ml EDTA (0.5 M)
The volume is adjusted to 160 ml with H₂O.

Polyacrylamide sequencing gel

40.8 g urea (electrophoresis grade)
8.5 ml TBE (10X)
20 ml H₂O

These reagents are dissolved by warming the solution.
Then 12.75 ml polyacrylamide solution (38 g polyacrylamide +

2 g bis-acrylamide + 100 ml H₂O) is added to the mixture and the volume brought up to 85 ml. The mixture is then filtered and 0.725 ml of an aqueous solution of ammonium persulfate (0.072 g APS + 0.725 ml H₂O) is added to the mixture. The solution is degassed and 20 μ l of TEMED (N,N,N',N'-tetramethylethylene diamine) is added to the solution for polymerization.

APPENDIX C

Protocols

Section 1: Extraction of RSB genomic DNA

Genomic DNA Extraction Procedure modified from Roger et al. (1989).

1. Cells were harvested from 5 day 30 ml liquid culture.
2. The liquid culture was centrifuged at 10,000 rpm for 15 minutes in an 'Oakridge' tube. The medium was decanted and the cell pellet resuspended in 5 ml of TNE (10 mM Tris-HCl, 100 mM NaCl and 1 mM EDTA, pH 8.0) and centrifuged at 10,000 rpm for 10 minutes. The buffer was decanted and the washing procedure repeated.
3. The packed cell volume was determined and the pellet resuspended in 5 to 10 volumes of TNE.
4. The cells were homogenized in a tissue grinder, transferred to microfuge tubes and centrifuged for 30 seconds. The supernatant was decanted.
5. The pellet was resuspended in 200-400 μ l of 2X cetyl trimethyl ammonium bromide (CTAB) extraction buffer (2% CTAB, 100 mM Tris-HCl, 20 mM EDTA, 1.4 M NaCl, pH 8.0). The suspension was vortexed and incubated at 65°C for 60 minutes.
6. An equal volume of 24:1 chloroform:isoamyl alcohol (CIAA) was added to the suspension and mixed by gently by inverting the tubes several times.
7. After centrifuging for 60 seconds, the aqueous phase was transferred to a fresh tube.
8. The spent cell debris were pooled and washed once in 3 ml of TNE and homogenized, once again, in a tissue homogenizer. Extraction process was repeated as before (steps 4-7). The aqueous phase after the CIAA wash (step 7) was pooled with the previous preparation.
9. 1/10 volume of CTAB precipitation buffer (10% CTAB, 0.7 M NaCl) preheated to 65°C was added to the pooled aqueous phase and mixed thoroughly.
10. 1 volume of CIAA was added and mixed gently by inverting the tubes several times. The mixture was centrifuged for 1-4 minutes and the aqueous phase transferred to a fresh tube.
11. DNA was precipitated from the aqueous phase by adding 2

volumes of cold absolute ethanol and centrifuging at 4°C. The supernatant was decanted and the pellet dried under vacuum. The DNA pellet was rinsed with 200 µl of 70% ethanol and dried under vacuum.

12. The pellet was then resuspended in 45 µl of TE buffer (10 mM Tris-HCl, 1 mM EDTA at pH 8.0).

13. RNA was hydrolyzed by treatment with 5 µl of 100 µg/ml RNase A (DNase-free pancreatic RNase, Sigma, Sigma Chemical Company, St. Louis, MO). After incubating the preparation at room temperature for 3 hours.

14. An equal volume of CIAA was added to the RNase treated DNA. After mixing the contents of the tube gently, the tube was centrifuged for 5 minutes. The aqueous layer was transferred to a fresh tube.

15. 5 µl of 3 M sodium acetate pH 5.2 was added to the aqueous phase. DNA was precipitated from the aqueous phase by adding 2 volumes of cold absolute ethanol and centrifuging at 4°C. The supernatant was decanted and the pellet dried under vacuum. The DNA pellet was rinsed with 200 µl of 70% ethanol and dried under vacuum.

16. This preparation was used as a stock of genomic DNA for all subsequent reactions.

Section 2 : PCR amplification (Mullis and Faloona, 1987; Saiki et al., 1985)

1. A 10 ng/µl solution of genomic DNA was prepared from the original stock by reprecipitating the DNA and dissolving it in water. (DNA solutions in TE buffer cannot be used for amplification as EDTA interferes with polymerase reaction.)

The PCR was performed as recommended in the GeneAmp^R kit (Perkin Elmer Cetus, Norwalk, CT) using Amplitaq^R DNA polymerase (Perkin Elmer Cetus, Norwalk, CT) as the amplifying enzyme.

2. The composition of amplification reaction included

15 µl of genomic DNA (10 ng/µl)
5 µl of 10 µM forward amplification primer,
5 µl of 10 µM reverse amplification primer,
2 µl of 10 mM dATP
2 µl of 10 mM dCTP
2 µl of 10 mM dGTP
2 µl of 10 mM dTTP
10 µl of 10X Buffer (100 mM Tris-HCl pH 8.3, 500 mM KCl)

5 μ l of 25 mM MgCl₂
51.5 μ l distilled sterile water
0.5 μ l AmpliTaq DNA polymerase (5 units/ μ l).

3. A layer of mineral oil was added on top of the reaction mixture and the tube was placed in a Perkin Elmer DNA thermal cycler.

4. The amplification program used was as follows:

Cycle 1: Denaturation at 95°C for 2 minutes,
primer annealing at 40°C for 1 minute
chain extension at 72°C for 1 minute;

Cycles 2-30: Denaturation at 94°C for 1 minute,
primer annealing at 40°C for 1 minute
chain extension at 72°C for 1 minute;

Each cycle was extended by 6 seconds at 72°C.

A final extension was carried out after the last cycle at 72°C for 3 minutes and the reaction was held at a temperature of 4°C.

5. After completing the amplification procedure, the aqueous phase was recovered from the reaction tube and extracted once with an equal volume of CIAA to denature and separate the polymerase from the DNA preparation.

6. The amplified DNA was stored at -20°C for further use.

Section 3: Preparation of the insert

1. The PCR-amplified fragment was gel-purified.

2. The PCR product was treated with T4 polynucleotide kinase (Promega, Promega Corp., Madison, WI). Polynucleotide kinase treatment was necessary because the amplified fragments were generated from synthetic primers with hydroxylated 5' ends.

3. The following reaction was carried out

24 μ l of purified PCR product (20 ng/ μ l)
1 μ l of polynucleotide kinase,
3 μ l of the 10X reaction buffer (provided with the enzyme)
2 μ l 10mM ATP (Sigma, Sigma Chemical Co, St. Louis, MO).

4. The mixture was incubated at 37°C for 30 minutes.

5. The mixture was incubated at 70°C for 20 minutes.

This step denatured the enzyme for further treatments.

6. 3 μ l sodium acetate 3M, pH 5.2 was added to the mixture followed by 2 volumes of 100% ethanol. The DNA was precipitated by centrifugation and rinsed with 60 μ l of 70% ethanol.

7. The DNA pellet was dissolved in 10 μ l TE buffer.

8. The following reaction was carried out

10 μ l Insert DNA in TE (from step 7)
2 μ l New England Biolabs (NEB) Buffer 2
1 μ l 2 mM dNTPs
1 μ l Bovine serum albumin (1 mg/ml)
5 μ l Distilled sterile water
1 μ l T4 DNA polymerase (New England Biolabs, Beverly, MA)

9. The mixture was incubated at 12°C for 20 minutes

10. 5 μ l of 50 mM EDTA was added to the mixture.

11. The mixture was incubated at 70°C for 20 minutes

12. The DNA was precipitated by adding 2.5 μ l of 3 M sodium acetate pH 7.0 to the reaction, followed by 2 volumes of 100% ethanol and centrifugation.

13. The final pellet was dissolved in 10 μ l of TE buffer.

Section 4: Preparation of the vector.

1. Restriction digestion of vector with SmaI.

10 μ l of 500 ng/ μ l pBluescript KS DNA (Stratagene, Stratagene
Cloning Systems, La Jolla, CA)
2 μ l react 4 buffer (Bethesda Research Laboratories) (BRL)
6 μ l sterile distilled water
2 μ l SmaI restriction enzyme (BRL)

2. The mixture was incubated at 30°C for 60 minutes.

3. The linearized vector was treated with calf intestinal phosphatase by the following method.

20 μ l reaction (from step 2)
3 μ l 10X Calf intestinal phosphatase buffer (New England
Biolabs, Beverly, MA)
6 μ l Distilled sterile water
1 μ l Calf intestinal phosphatase enzyme

4. The mixture was incubated at 37°C for 60 minute.
5. 4 μ l of 50 mM EDTA was added to the mixture.
6. The mixture was incubated at 65°C for 10 minute.
7. The final reaction was extracted with an equal volume of TNE-equilibrated phenol-chloroform-isoamyl alcohol (50:24:1) and taken up in 10 μ l of TE buffer. (Note: DNA was precipitated with 3M sodium acetate pH 7.0).

Section 5: Ligation

1 μ l (250 ng) of linearized phosphatase-treated pBluescript-KS DNA (from section 4, step 7) was ligated overnight at 16°C to 10 μ l (400 ng) of insert prepared as indicated above (Section 3, step 13) using T₄ DNA ligase (New England Biolabs, Beverly, MA) according to company protocols.

1. The following ligation reaction was carried out.

1 μ l linearized vector DNA
10 μ l insert DNA
3 μ l Distilled sterile water

2. The mixture was incubated at 70°C for 3 minutes and chilled on ice.

3. 2 μ l of 10X ligase buffer (NEB) and 4 μ l T₄ DNA ligase was added to the reaction.

4. The mixture was incubated at 16°C overnight.

VITA

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