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An Attempt to Isolate Nodule-Specific cDNA Clones from *Alnus incana*

By

Tim Gleeson

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UNIVERSITY OF OTTAWA

ABSTRACT

Plants from several orders are capable of forming symbiotic associations with nitrogen-fixing bacteria. Following infection with the specific bacterial symbiont, root nodules develop within which reduction of gaseous nitrogen by the bacterial enzyme nitrogenase occurs. The development of the nodule and the establishment of the symbiotic state must proceed by following a regulated developmental pathway requiring the coordinate expression of both bacterial and plant genes. These plant genes are referred to as nodulins and their protein products as nodulins.

The symbiotic association between nitrogen-fixing non-legumes, termed actinorhizal plants, and bacteria of the genus *Frankia* has, at the molecular level, received little attention. A molecular understanding of the genes involved in nitrogen-fixation in an actinorhizal plant would provide a basis for the analysis of a non-legume symbiotic system and may help in the improvement and/or creation of new nitrogen-fixing plants by genetic engineering.

Here we report on the attempt to isolate nodule-specific cDNA clones from a cDNA library that was derived from an actinorhizal species, *Alnus incana*, nodule poly A+ enriched RNA. mRNA corresponding to these cDNA clones could be detected in RNA isolated from the actinorhizal nodule but, could not be detected in RNA isolated from the root. The pattern of expression of four genes corresponding to the cDNA clones was investigated at various

time points following infection. It appears the nodulin clones could be divided into two categories of expression; one showing a maximal expression prior to the onset of nitrogen fixation, and the other expressing a maximal level after the onset of nitrogen fixation.

Sequence characterization of these clones eventually revealed that the cDNA clones were ribosomal in nature of both plant and bacterial origin. However, further analysis revealed that one cDNA clone of bacterial origin had a unique 105 bp region. PCR analysis suggests that it may be possible to survey various bacterial species for the presence or absence of this insert in order to classify and investigate the evolution of 23S rRNA within this region of the gene.

DEDICATION

This thesis is dedicated to my wife Alice,

my daughters Tara and Angela and my family

for their unwavering support and encouragement.

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Most importantly, I wish to thank my parents for enduring and encouraging me throughout this project. As well a special thanks to my wife Alice for her love, support and understanding provided throughout this project, and my daughters, Tara and Angela who have kept life very interesting.

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LIST OF ABBREVIATIONS

ATP:	adenosine triphosphate
bp:	base pair(s)
c.c.:	cubic centimetre, millilitre
cDNA:	complimentary DNA; DNA copy
dATP:	deoxyadenosine triphosphate
dCTP:	deoxycytosine triphosphate
DNA:	deoxyribonucleic acid
E. coli:	*Escherichia coli*
EDTA:	ethylene-diaminetetra-acetic acid
kb:	kilobase pairs
MOPS:	morpholino propanesulphonic acid
mRNA:	messenger RNA
O.D.:	optical density
RNA:	ribonucleic acid
SDS:	sodium dodecyl sulphate
UV:	ultraviolet light

INTRODUCTION

Following infection of plants from several taxonomic orders with a specific bacterial symbiont, root nodules develop within which reduction of dinitrogen by the bacterial enzyme nitrogenase occurs. The development of the nodule and the establishment of the symbiotic state must proceed by following a regulated, developmental pathway requiring the coordinate expression of both bacterial and plant genes. These plant specific genes are referred to as nodulin genes and their protein products as nodulins (Verma *et al* 1986). In this chapter, several aspects of nodulation in legumes will be discussed as will aspects of actinorhizal nodulation with emphasis on the present lack of knowledge, at the molecular level, of nodulation processes in actinorhizal plants.

1.1 Nodule Initiation and Development in Legumes

The formation of nodules in legumes is the result of infection with either *Rhizobium* or *Bradyrhizobium* spp. (Postgate, 1982). Recognition of the legume host by *Rhizobium* or *Bradyrhizobium* is the initial step towards nodule development. (For sake of simplicity, hereafter both *Rhizobium* and *Bradyrhizobium* are referred to as *Rhizobium*). The initial stages of the infection process involve the induction of *Rhizobium* nodulation genes which is mediated by specific plant flavonoid compounds found in root exudates (Downie and Johnston, 1988; Rolfe and Gresshoff, 1988; Long, 1989). *Rhizobium* entry into the plant is achieved by invading

the legume root hairs (root hairs are slender tubular extensions of root epidermal cells). Attachment of *Rhizobia* to root hairs is thought to be mediated by plant lectins bound to root hair surfaces which interact with specific *Rhizobium* cell surface components (Diaz *et al.*, 1989; Dazzo and Gardiol, 1984). Curling or contortion of the root hair is the first visible sign of infection. The *Rhizobia* become entrapped in the curl of the root hair which is accompanied by an induction of cortical cell division adjacent to the root hair cell (Newcomb, 1981; Calvert *et al.*, 1984). The *Rhizobia* proliferate within the infection thread and the thread passes from the root hair cell to the newly dividing cortical cells where branching of the infection thread results in the penetration of many of the dividing cortical cells (Turgeon and Bauer, 1982). At the tips of the infection threads, *Rhizobia* are released into the cytoplasm of dividing cells by a process resembling endocytosis. This leads to the enclosure of the bacteria by the infection thread membrane, which at this stage becomes known as the peribacteroid membrane (Newcomb, 1981: Sutton, 1983). Within the peribacteroid membrane, the *Rhizobia* continue to divide virtually filling the cytoplasm with bacteria. The membrane increases in rough proportion. In the soybean nodule, as many as eight *Rhizobia* may be surrounded by one peribacteroid membrane. Within the peribacteroid membrane, the *Rhizobia* are known as bacteroids which are morphologically different from the free-living *Rhizobia* (Sutton *et al.*, 1981).

In the maturing nodule, cortical cell division generally ceases and subsequent increases in the size of the nodule are due to cell enlargement. The increase in the volume of numerous infected cells causes the nodule to expand radially resulting in a spherically shaped structure. About half of the nodule cells remain uninfected and these cells are smaller and structurally different from the adjacent infected cells but play an important role in nodule metabolism.

1.2 Nodulins in Legumes

For the past few years, research into nodulin gene structure and function has focused upon the relatively well-studied symbioses formed between legumes and *Rhizobium* (Govers *et al.* 1987a; Vance *et al.* 1988; Verma and Delauney 1988). Classical genetic experiments have indicated that specific plant genes are involved in nodule formation and effectiveness (Holl and LaRue, 1976; Caldwell and Vest, 1977; Nutman, 1981). The host appears to influence the symbiosis at several stages including recognition of the *Rhizobium* strain, the time of nodule appearance, nodule abundance and size, intracellular organization and the level of nitrogen fixation (Nutman, 1981; Gresshoff and Delves, 1986; Vance *et al.*, 1988). There are very likely numerous nodulins involved in the symbiosis. For instance; nodulins expressed during the early stages of infection may include proteins required for infection thread synthesis and cellular division. As the nodule develops,

an increase in the levels of components involved in the synthesis of various structural components will occur, eg. peribacteroid membrane. There will also occur an increase in enzymes involved in carbon metabolism and nitrogen assimilation. In addition, proteins such as leghaemoglobin which are involved in facilitating nitrogen fixation will be induced.

Research effort has resulted in the construction of cDNA libraries from nodule mRNA, the use of nodule-specific cDNA probes to isolate and characterize several nodulin clones (Fuller *et al.* 1983; Sengupta-Gopalan *et al.* 1986; Campos *et al.* 1987; Gloudemans *et al.* 1987; Govers *et al.* 1987b) and the determination of their sequences. The cDNA clones have in turn been used to isolate nodulin genes, several of which have been analyzed in order to identify DNA motifs which may be involved in nodule-specific gene regulation (Stougaard *et al.* 1987; Jensen *et al.* 1988; Jorgensen *et al.* 1988).

1.2.1 Leghaemoglobin

The best studied and characterized nodulin is leghaemoglobin (Wittenberg *et al.* 1974) found in all legume nodules. It is a very abundant protein found in the inner region of the nodule. Its function is to transport oxygen to the bacteroid at a concentration which is harmless to its nitrogenase (discussed later) (Postgate, 1987). In soybean, leghaemoglobins are encoded by a small-multi gene family. Four major leghaemoglobin species, Lba, Lbc_1, Lbc_2, Lbc_3, are contained in soybean nodules, differing

slightly in amino acid sequence (Sievers *et al.*, 1978; Fuchsman and Appleby, 1979). The leghaemoglobin gene family also includes two pseudogenes and two truncated genes (Brisson and Verma, 1982; Bojsen *et al.*, 1983; Wiborg *et al.*, 1983). The genes are found as clusters (Lee *et al.*, 1983; Bojsen *et al.*, 1983). One cluster contains Lba, Lbc_1, Lbc_3, and a pseudogene. A second cluster contains the Lbc_2 and the second pseudogene. The two truncated genes are located at two independent loci (Verma and Nadler, 1984).

1.2.2 Glutamine Synthetase

Ammonia produced by nitrogen fixation within the bacteroid is excreted into the plant cytoplasm for assimilation by the host. Glutamine synthetase is the major enzyme responsible for the first step in the assimilation of ammonia (Miflin and Cullimore, 1984). A nodule-specific form of glutamine synthetase from *Phaseolus vulgaris* (Cullimore *et al.* 1984) and *Medicago sativa* (Dunn *et al.* 1988) has been isolated. The corresponding cDNA has been isolated and used to determine that this glutamine synthetase is expressed in the nodule (Cullimore *et al.*, 1984) and not in the root. A recent report, however, has indicated that this form of glutamine synthetase is expressed at low levels in stems, petioles and green cotyledons (Bennett *et al.*, 1989) and thus is not entirely nodule-specific.

1.2.3 Uricase II

Uricase II (nodulin-35) in the soybean *Glycine max* (Bergmann *et al.*, 1983), has been identified as a nodule-specific ferm of uricase. Uricase is an important enzyme for the production of ureides and catalyses the conversion of uric acid to allanatoin (Shubert, 1986) during ammonia assimilation.

1.2.4 Sucrose Synthetase

A nodule-specific cDNA corresponding to nodulin-100 has been isolated from *Glycine max*, characterized by Thummler and Verma (1987) and was found to encode sucrose synthetase. This enzyme is involved in carbon metabolism and is detected at elevated levels in nodules (Morell and Copeland, 1985).

1.2.5 Peribacteroid Membrane Nodulins

It has been proven possible to use antibodies to determine the subcellular distribution of nodulins such as nodulin-24 (Fortin *et al.* 1985; Katinakis and Verma 1985) and nodulin-26 (Fortin *et al.* 1987; Sandal and Marcker, 1988) and nodulin-23 (Mauro *et al.*, 1985). These nodulins appear to be peribacteroid membrane nodulins. Since the peribacteroid membrane segregates the bacteroids from the host cytoplasm it may have nodule-specific functions that involve the exchange of metabolites between the host

and the bacteroid. With the area of the peribacteroid membrane in an infected soybean cell 20-40 fold higher than the area of the plasma membrane in the same cell (Verma *et al.*, 1978), it is reasonable to assume that soybean nodules would contain elevated levels of enzymes involved in peribacteroid membrane synthesis as well as structural components of the membrane itself. Characterization of nodulin-26 has indicated that it is a transmembrane protein (Fortin *et al.*, 1987) and it has been proposed by Sandal and Marcker (1988) that nodulin-26 may be involved in transporting small molecules by forming small channels in the membrane.

1.2.6 "Nodulin-A" Family

A small number of soybean nodulins have been found to be members of an abundantly transcribed gene family named the "Nodulin-A" family by Verma and Delauney, 1988. Six members of this family have been identified: nodulin-20, nodulin-22, nodulin-23, nodulin-26b, nodulin-27, and nodulin-44 (Sengupta-Gopalan *et al.*, 1986; Jacobs *et al.*, 1987; Sandal *et al.*, 1988). Immunoprecipitation of the translation products of hybrid-selected mRNAs with antisera against proteins from the peribacteroid membrane and against soluble proteins have suggested that nodulin-23 is located in the peribacteroid membrane and nodulin-27 is located in the cytosol (Jacobs *et al.*, 1987). The function of these nodulins is unknown, however, primary structure analysis by Sandal *et al.*, 1987 has indicated

putative metal binding domains in the proteins of this gene family and therefore metal binding may be important in their function.

1.2.7 Early Nodulins

Several nodulins have been detected early in soybean nodule development when the nodule is being formed and are transiently expressed (Gloudemans *et al.*, 1987). Induction of these nodulins does not require the presence of bacteria inside the root tissue, which is in contrast to the previously discussed "late" nodulins. In 1987 Franssen *et al.*, reported the characterization of a cDNA (ENOD2) corresponding to a 1.2 kb mRNA species detected in early nodule development. To identify the early nodulin encoded by the cDNA clone, mRNA was hybrid selected with the clone and translated *in vitro*. Two nodulins with an apparent molecular weight of 75 000 were produced and both polypeptides referred to as nodulin-75. It has been demonstrated that the ENOD2 gene is specifically expressed in the inner cortex of the soybean and pea nodule and it has been suggested that the ENOD2 nodulin has a role in the characteristic morphology of the inner cortex and the function of this tissue as barrier for oxygen diffusion into the root nodule (van de Wiel *et al.*, 1990). Nodulin-75 may be conserved among legumes since mRNA homologous to the nodulin-75 cDNA sequence has been detected in infected tissue from pea (Govers *et al.*, 1987), vetch (Moerman *et al.*, 1987) and *Sesbania rostrata* (Strittmatter *et al.*, 1989).

Other early nodulin genes involved in nodule morphogenesis which have been characterized are ENOD13 and ENOD55 (Franssen *et al.*, 1988). An early nodulin gene involved in the infection process in pea has recently been isolated. ENOD12 is a proline-rich protein which is believed to be involved in cell wall synthesis (Scheres *et al.*, 1990).

In combination with traditional biochemical approaches the cloning of nodulin genes has lead to important insights into the development of the symbiotic state. However these insights have been primarily limited to a few plants within the family *Leguminoseae*.

1.3 The Actinorhizal Plant

In contrast to the situation which exists in the legumes, the important symbiotic associations between nitrogen-fixing non-legumes, now termed actinorhizal plants, and bacteria of the genus *Frankia* have received little attention. This was due to the belief that these plants were of little agricultural interest. It was only in the 1950's when their potential for forestry was recognized, that studies really began on the symbiotic nitrogen-fixing associations formed by actinorhizal plants. However, fundamental knowledge concerning this type of symbiosis is still very fragmentary, when compared to the legume-*Rhizobium* situation.

Approximately 220 nodule-bearing species, grouped into 23 different genera from 8 plant orders, have been recorded in which nitrogen-fixing

associations occur (Dawson 1983; Newcomb and Wood 1987). The ability to understand and control the mode of intercellular infection adds to the possibility that the actinorhizal type of symbiosis can be extended to other plants.

1.3.1 Actinorhizal Plant Distribution

Most actinorhizal plants occur in temperate regions or high altitude zones in the tropics. The major exception to this generalization are genera from the family Casuarina, which occur in the coastal areas of the Indian Ocean and in the tropical western Pacific and a number of tropical and subtropical *Myrica* species, some of which are large trees. Actinorhizal plants can be found in forest, swamp, riparian, shrub, prairie and desert ecosystems. They also appear to become more prevalent in colder climates as they fill the niche dominated by woody legumes in the tropics (Dawson, 1986).

In general, actinorhizal plants are found on nitrogen-poor sites such as sandy and gravelly soils, raw mineral soils, and wet soils. They frequently occur as pioneer vegetation at early stages of succession following disturbances such as fire, landslides, volcanic eruptions and flooding eg. colonization by red alder (*Alnus rubra* Bong.) of mud flows covered with ash resulting from the eruption of Mount St. Helens in the state of Washington, USA (Dawson, 1986).

This ability to reclaim devastated lands by introducing nitrogen into the poor soils generally results in the displacement of the actinorhizal plants by successional plants requiring richer soil. However, many actinorhizal plants have the capacity to persist as a dominant species or as a component of more stable plant communities. Examples include *Cercocarpus* species that occur interspersed with ponderosa pines in the southwestern United States and stands of *Casuarina glauca* Sieb. ex Spreng. that dominate coastal swamps in southeastern Australia.

1.3.2 Alders

Perhaps the most important actinorhizal genus is *Alnus* or the alders. These actinorhizal plants occupy diverse geographical and ecological habitats, are generally woody perennials and are believed to be more common in temperate forests than woody legumes (Dawson, 1986; Moiroud and Gianinazzi-Pearson, 1984). Alder taxonomy is structured as follows:

Division	Spermatophyta
Class	Angiospermae
Subclass	Dicotyledoneae
Order	Fagales
Family	Betuloideae
Geneus	Alnus

Approximately 35 species and subspecies of *Alnus* occur in temperate and cool climates of the northern hemisphere and in the highlands of Central and Southern America. Many alder species produce medium size

trees. They have been widely planted outside their native ranges, where they usually become nodulated by *Frankia* present in the soil.

Alder species are usually associated with moist environments but some alders, such as green alder (*Alnus viridis* ssp. *crispa* (Ait.) Turrill) of North America, are more characteristically found on dry sites.

The associations formed between the tree *Alnus* and its bacterial endophyte *Frankia* are able to fix nitrogen at rates of 60-209 kg/ha/yr (Dawson, 1983), which is comparable to legume systems where reported rates of 100-400 kg/ha/yr for a good stand of clover have been reported (Lewis, 1986). Alders make the largest contribution of all actinorhizal plants to land reclamation, soil regeneration, fertilization and stabilization, and could play a major role in forestry by providing fixed nitrogen to the soil and thus stimulating the growth of trees (Dawson, 1983). Thickets of *Alnus crispa* 5 years old and 1.5m tall were calculated to add 156 kg nitrogen/ha/yr to the ecosystem by leaf fall alone (Lawrence, 1958).

The economic value of *Alnus glutinosa* in European forestry for interplanting with *Pinus sylvestris*, *Picea abies*, and *Populus* spp. has long been recognized (Stone, 1955; Virtanen, 1957; Redko, 1958; Van der Meiden, 1960. *Populus trichocarpa* saplings associated with *Alnus crispa* weighed 22.5 times more than those of equal age in areas lacking alders (Lawerence, 1958). Leaf litters of *Alnus incana* and *Alnus glutinosa* were found to be superior to leaf mulches of other trees for the growth of *Pinus sylvestris*

(Mikola, 1958). Farmers in the highlands of Guatemala recognize that scattered alder trees allowed to remain in fields of maize increase crop yields (Dawson, 1979). In unfertilized plots of the Netherlands the leaf nitrogen content of apple leaves was negatively correlated with distance of the apple trees from *Alnus glutinosa* hedges (Delver and Post, 1968). Trees of the following genera have also exhibited increased growth in association with *Alnus* spp.: *Fraxinus, Liquidambar, Liriodendron, Platanus, Juglans, Populus* and *Pseudotsuga* (Tarrant, 1968; Friedrich and Dawson, 1984). Several other uses of *Alnus* spp. by man for improved crop yields, erosion control and soil improvement are covered by Dawson (1986). Therefore actinorhizal plants are potentially of important economic value for forestry and land reclamation and deserve close consideration as providers of fixed nitrogen in forest systems, as well as disturbed and exposed sites. However, presently virtually no molecular information about the plant, plant genes and nitrogen-fixation is available. A molecular understanding of these aspects could greatly improve the potential for use of these plants beyond those mentioned above.

1.4 The Endophyte

In 1964, electron microscope studies of *Alnus* and *Myrica* nodules revealed that the actinorhizal endophyte is a prokaryote, closely related to the Actinomycetes (Becking *et al.*, 1964; Silver, 1964; Gardner,

1965; Goiroud and Gianinazzi-Pearson, 1984) and in 1970 Becking introduced into this class the genus *Frankia*, which groups all the endophytes of actinorhizal plants. It was not until eight years later that the first isolation and *in vitro* culture of a *Frankia* strain (HFPCpI1) and the successful reinfection of the host plant, *Comptonia*, was reported (Callaham *et al.*, 1978a and 1978b) and verified (Lalonde, 1978). There have since been many reports of endophyte isolation from different nitrogen-fixing non-legumes (Moiroud and Gianinazzi-Pearson, 1984). All *Frankia* isolated to date are gram positive, filamentous, branching prokaryotes (Lechevalier, 1984).

Even though an organism can be identified as belonging to the genus *Frankia*, considerable morphological and physiological variation occurs within the genus, making species characterization a difficult problem. Thus no species names are used, rather strains have been designated by a combination of numbers and letters (Lechevalier 1986).

Physiologically, *Frankia* can be separated into two groups. Type "A" strains are genetically heterogenous, aerobic, grow rapidly in the presence of carbohydrates and are not infective on their host plants. Type "B" strains are serologically, genetically and chemically related, are less tolerant of O_2, take up very little carbohydrate and are infective (able to form nodules) and effective (able to fix nitrogen) on the host plant of origin (Lechevalier and Lechevalier, 1984). DNA-DNA hybridization studies have shown that "B"

organisms are 67.4 to 94.1% similar, while "A" isolates have less than 39% homology with "B" organisms, and less than 33% homology with other "A"-type organisms. The "A" group is therefore genetically and physiologically heterogeneous (An *et al.*, 1985).

The isolation of pure *Frankia* cultures has facilitated *in vitro* cross-inoculation studies and studies of nodule morphogenesis both of which would have been difficult using collected material. This has led to investigation of the genetics of *Frankia* (Normand and Lalonde 1986), the infection process(es) and nodule structure (Newcomb and Wood 1987), nodule physiology (Tjepkema *et al.* 1986), and attempts to characterize nodule-specific proteins (Moiroud and Gianiazzi-Pearson 1984). Nodule development in actinorhizal plants is more complex than in leguminous plants (Newcomb and Wood 1987) requiring the formation of both a pre-nodule and the nodule itself. Wide variations in nodule development and morphology are observed between nodulated species which make comparisons between experimental systems difficult.

1.5 Host Plant-Endophyte Interactions

The development of root nodules involves a complex sequence of cell-cell interactions which are regulated by environmental factors and the genomes of at least two organisms, the host plant and the endophyte (Moiroud and Gianinazzi-Pearson, 1984). The sequence of events must

involve a certain amount of recognition between the two symbionts.

1.5.1 Root Hair Infection

Although nothing is known about the recognition phenomena that exist in actinorhizal symbioses, it is reasonable to think that they may be similar to those reported in *Rhizobium*-legume associations (Bauer, 1981,; Dazzo and Gardiol, 1984; Diaz *et al.*, 1989) where plant lectins bound to root hair surfaces interact with specific *Rhizobium* cell surface components in order to mediate attachment. It has been suggested that bacterial binding to root hairs may be mediated by fibrillar material including mucigel (Berry and Torrey, 1983), lectin-binding macromolecule (Chaboud and Lalonde, 1983) the bacterial glycocalyx (Costerton *et al.*, 1981) or a combination of these. Chauboud and Lalonde (1982) have localized certain specific sugar residues on the outer walls of hyphae, vesicles and sporangia of *in vitro Frankia* cultures but whether these compounds are involved in the interaction between host and endophyte is still unknown. The sequence of development can be altered or terminated prematurely at any developmental stage, including deformation of growing root hairs, invasion of the deformed root hair, encapsulation of the infective hyphal filaments, stimulation of mitoses resulting in the formation of a prenodule, and establishment of a symbiotic relationship within the infected host cells.

In the presence of the host plant, the endophyte seems to proliferate in the rhizosphere surrounding the elongating region of the root and this is accompanied, as in *Rhizobium*-legume interactions, by root hair deformation (Figure 1a). Penetration of the root hair by the endophyte proceeds by hyphal growth and occurs at the root hair tip where it is most strongly curved (Figure 1b). This region of the root hair tends to have a cell wall that appears to have an altered arrangement of microfibrils (Berry *et al.*, 1986). After penetration, the host cell wall and plasmalemma invaginate to surround the endophyte. While this activity occurs in the infected root hair the adjacent root hairs rapidly degenerate. As the endophyte enters the root hair it becomes separated from the host plasmalemma by an encapsulating material, probably consisting of cell wall material from the host since at this time a large amount of wall material is being synthesized by the host. The hyphae then continue to grow towards the base of the root hair through the host cell wall into the adjacent cortical cells, which are stimulated to undergo numerous divisions. The result of this activity, is a slight thickening of one side of the root giving rise to what is known as a pre-nodule which is essential for nodule formation to occur in actinorhizal plants (Figure 1c). The pre-nodule contains both infected and uninfected cells. The uninfected cells contain numerous starch granules in amyloplasts and phenolic deposits in vacuoles. It should be mentioned that the pre-nodule does not fix nitrogen and remains in a disorganized state thus never

giving rise directly to the true nodule. The events leading from pre-nodule formation to the formation of a true nodule depend upon whether the nodule is an *Alnus* type or *Myrica* type. These nodule types result from morphological differences and are named for the plants where they were first observed.

1.5.2 *Alnus* Type Nodules

In the *Alnus* type the root primordia originate from the pericycle and develop avoiding the infected root cortical cells thus giving rise to a true nodule. When a primordium is sufficiently developed, the cortical parenchyma cells become infected by hyphae of the endophyte coming from the pre-nodule (Figure 1d). The nodule lobe grows through the epidermis, elongates and the meristem divides repeatedly to give a nodule (Figure 1e).

1.5.3 *Myrica* Type Nodules

In the *Myrica* type of nodule, three stages of root nodule development succeed follow the pre-nodule stage; nodule lobe formation, a transitional phase of arrested growth and nodule root development. Several root primordia originate at the same time as pre-nodule formation and develop slowly to form a cluster of nodule lobes. The cortical cells of these lobes are then invaded by the actinorhizal endophyte. Growth becomes

FIGURE 1: *ALNUS* TYPE NODULE FORMATION

The infection process presented here for the formation of an alder nodule has been derived from Tjepkema *et al.*, 1984; Newcomb and Wood, 1987; and Bidwell, 1979.

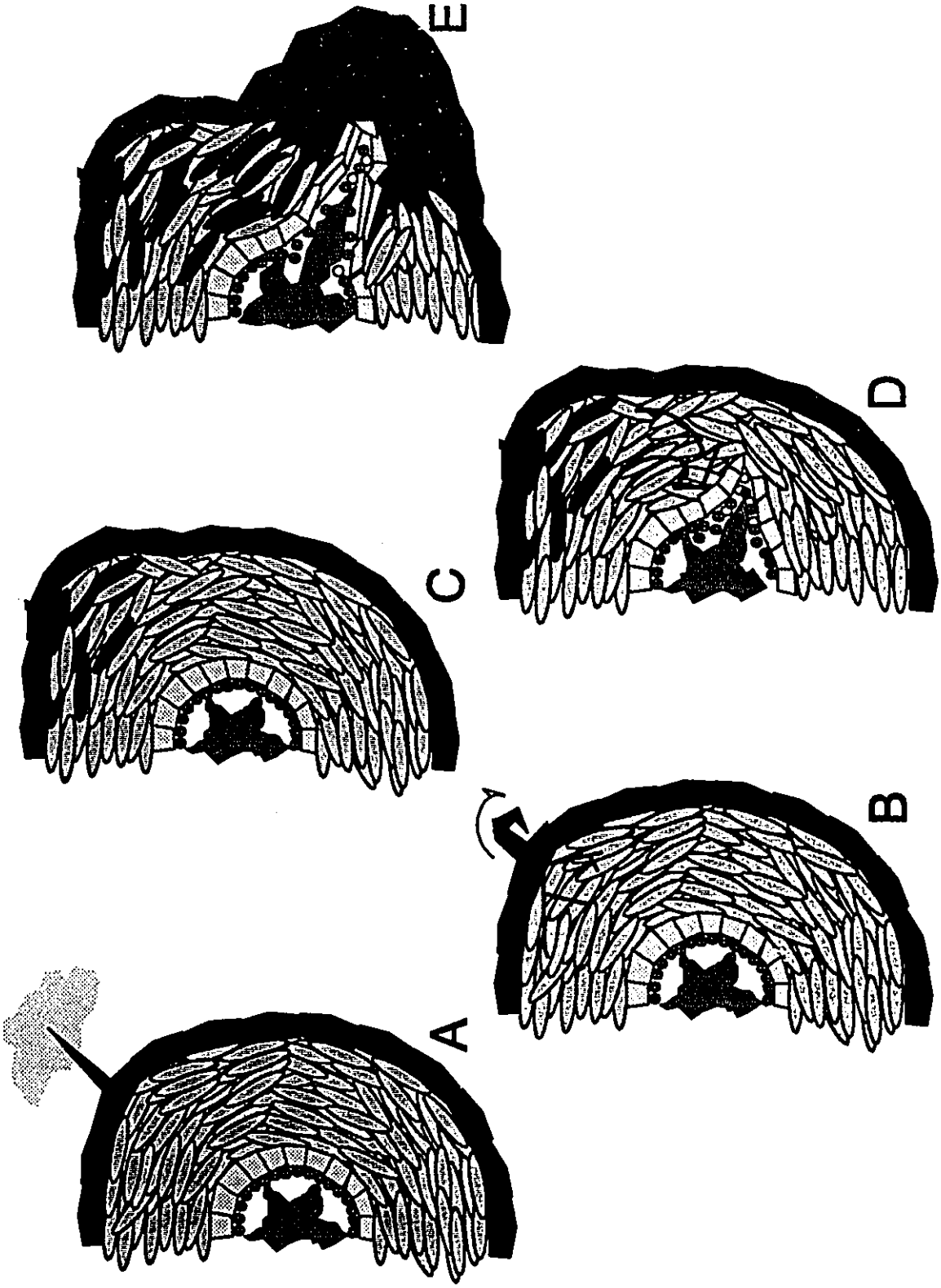
a. Endophyte surround the root hair which is then accompanied by root hair deformation.

b. Pentration of the root hair, by the endophyte, occurs at the root hair tip and proceeds by hypal growth. The hyphae continue to grow towards the root hair base, through the host cell wall and into the cortical cells.

c. The cortical cells undergo numerous divisions resulting in a thickening of one side of the root resulting in a pre-nodule.

d. Cortical parenchyma cells become infected by hyphae of the endophyte originating from the pre-nodule.

e. Growth of the nodule lobe continues eventually giving rise to a nodule.


E
D
C
B
A

arrested for a few days to several weeks. The nodule then forms but the endophyte stays within the nodule lobe. In both types of nodule formation, infection by hyphal penetration is achieved by localized chemical degradation of the host cell wall.

1.5.4 Intercellular Penetration

Until recently the only mode of entry considered for *Frankia* was via root hair infection. A second mechanism, intercellular penetration, has been identified (Miller and Baker, 1986) and has only been observed in *Elaeagnaceae*. This process differs greatly from that of root hair infection, where the bacterium becomes an intracellular endophyte, in that the advance of the bacterium is almost entirely intercellular. Root hairs are few on the roots of *Elaeagnus* and are not involved in the infection process. *Frankia* colonize the root surface and hyphal penetration through the middle lamella between two epidermal cells occurs by enzymatic digestion, resulting in entry to the intercellular spaces of the root. Early in the infection a nodule primordium is initiated from the pericycle of the vascular bundle but no pre-nodule is formed. Intercellular spaces of the cortex are colonized towards the nodule. The hyphae then penetrate intercellularly through the protoperiderm and then colonize the intercellular spaces of the nodule cortex. They then invade the individual cells and become encapsulated endophyte resulting in a nitrogen-fixing nodule. Miller and

Baker (1986) showed that the same strain of *Frankia* will infect two different species of actinorhizal plants, *Elaeagnus* and *Myrica*, but the infection process will be by intercellular penetration for *Elaeagnus* and by root hair infection for *Myrica*. It is also known that the presence of pre-nodules is not always followed by nodule formation and that the endophyte is strictly limited to a well-defined zone in the host tissues (Perradin *et al.*, 1983). These observations indicate that the host plant somehow controls the endophyte development and thus the occurrence of nitrogen-fixation.

1.5.5 Nitrogen Fixation

Nitrogen fixing activity appears to be localized within the vesicles of the *Frankia* endophyte and the process of nitrogen reduction to ammonia is catalyzed by the enzyme nitrogenase. The process is irreversibly inactivated by the presence of oxygen. It has been established that oxygen uncompetitively inhibits substrate reduction and ATP hydrolysis catalyzed by nitrogenase in *Azotobacter* (Wong and Burris, 1972). The pO_2 in the nodule is high (Tjepkema, 1983), close to atmospheric pressure. There is evidence that a leghaemoglobin-like pigment, which protects nitrogenases in legume nodules from oxygen by having an affinity for oxygen, exists in the actinorhizal nodule (Tjepkema, 1983). DNA sequences hybridizing to soybean leghemoglobin genes have also been detected (Hattori and Johnson, 1985). There is also evidence that the

structure of the vesicle envelope may be analogous to those of heterocysts of nitrogen-fixing cyanobacteria. The complex envelope is multi-layered certain layers of which contain lipids, thus creating a physical barrier for gas exchange (Torrey and Callaham, 1982). Therefore either or both mechanisms may function.

Fixed nitrogen in the form of ammonium is assimilated by a glutamine synthase/glutamate synthatase enzyme system in the host but the form of fixed nitrogen transported to other parts of the host plant is not known. Nitrogen fixation requires great amounts of energy. At 52 days after germination, seedlings of *Alnus* used 11.6% of gross photosynthate for nodule respiration and 4.1% for nodule mass (Tjepkema *et al.*, 1986). The endophyte obtains its carbon source from the host. The exact form is unknown; however, it is believed that for *Alnus* it may be in the form of lipids and saturated and unsaturated fatty acids (Maudinas *et al.*, 1982). Another source of energy for the endophyte may come from hydrogen which is produced during nitrogen fixation as protons that are then reduced to give hydrogen. Hydrogen production reduces the yield of nitrogen-fixation but the energy loss may be recuperated in part by the endophyte with a very effective hydrogenase thus the recycling hydrogen formed. This enzyme can also oxidize atmospheric hydrogen from soil thus increasing the energy source of the endophyte.

1.6 Objectives of this Work

Our knowledge of the molecular events leading to the development of the symbiotic state is rudimentary. Thus, a molecular understanding of the genes involved in nitrogen-fixation in an actinorhizal plant may provide not only a basis for the analysis of a specific non-legume symbiotic system but also for comparative studies between different actinorhizal nitrogen-fixing associations. Such an analysis could be extended to include evolutionary relationships between the development of nitrogen fixation in legumes and non-legumes. It is conceivable that this information could be useful in the improvement and/or creation of new nitrogen-fixing plants by genetic engineering. It is from this point we have started our work on the isolation of nodulin genes from an actinorhizal species.

The alder, *Alnus incana*, was chosen for the study because it is a widely distributed species and the potential exists for the recovery of genetically diverse plants from different habitats. A cDNA library was constructed using poly A^+ RNA from nitrogen-fixing nodules. By using differential hybridization we had hoped to isolate cDNA plasmid clones containing sequences which specifically hybridize to nodule RNA and not to root RNA. These cDNA plasmid clones would be subjected to further analysis eg. northern analysis, slot blot analysis, and sequenced in order to determine their uniqueness as nodule-specific clones. It was also hoped

that any genes which may be isolated from the two symbionts, would be useful for analysis of symbiosis or to identify and monitor symbionts by employing restriction fragment length polymorphism (RFLP). This would be useful not only for increasing our understanding of the symbiotic association in the studied system but, by RFLP analysis, a system for the typing of *Frankia* species and their monitoring in the soil would be useful for further studies of varying symbiotic partners. Unfortunately, our goals for this work were not realized. We were not successful in isolating cDNA nodulin clones but did however, uncover what may prove to be a useful marker for *Frankia*.

MATERIALS AND METHODS

2.1 Growth and Maintenance of Alders

2.1.1 Growing *Frankia* ACN 14a

Frankia strain ACN 14a was obtained form M. Lalonde, Laval University, Quebec. QmodB culture medium (Normand et al., 1983) was prepared, autoclaved and incubated at 30°C for at least three days to ensure sterility.

From an original inoculum, 10mls of *Frankia* cells were transferred into 500ml of fresh medium. Cultures were left standing and allowed to grow 30-60 days, with occasional swirling to provide aeration.

2.1.2 Sprouting Alders

Alnus incana seeds were purchased from F.W. Schumacher, Sandwich Mass., USA. Surface sterilization of the seeds was accomplished with 20% Javex followed by rinsing with distilled water. The seeds were spread evenly over a wet bed of vermiculite and covered with a thin layer of dry vermiculite in plastic trays previously sterilized at 65°C for two hours. The sprouts were set at 20°C under VitaLites (Duro-test) with an 18 hour light/6 hour dark schedule. Sprouts were kept moist by misting.

2.1.3 Planting and Inoculation of Alders

After two weeks, the sprouts were transplanted to 10cm X 10cm pots filled with and Perlite watered in half-strength nitrogen-free

watering solution (Cutting and Schulman, 1969). *Frankia* inoculum was passed through an 18 gauge needle to form a homogeneous suspension. Alders were inoculated with the *Frankia* suspension by the addition of 40ml per pot at the base of the plants. A one time addition of NH_4NO_3 to a final concentration of 1mM was administered in order to increase plant survival. Control plants received watering solution supplemented with 1mM NH_4NO_3 as the nitrogen source. Plants were grown at 20°C with the same light schedule as the sprouts for up to 14 weeks.

2.2 Harvesting Tissue

Root and nodule tissues were collected at 2, 3, 4, and 14 weeks after inoculation. Perlite was removed from the roots by a quick rinse in distilled water. Tissues were frozen in liquid nitrogen and stored at -80°C. Nodules from 14 week plants were hand picked individually with forceps. Roots of 2, 3 and 4 week old plants were cut from the stem.

Control plants were harvested at 14 weeks and the root tissue was treated as described for the nodule tissue. Tissue was stored for a maximum of 30 days.

2.3 Acetylene Reduction Assay

Acetylene reduction is a measure of nitrogen fixation. The assay used in this thesis was based on the work by Hardy *et al.*, 1968. For

each assay three plants were used. Their roots were severed at the plant base and placed inside a 100 c.c. bottle sealed with a rubber top. Three c.c. of acetylene were introduced into the bottle by injection and 1 c.c. samples were taken at 0, 45, and 90 minutes after the introduction of acetylene. The samples were injected into a gas chromatograph (Carle Instruments GC9700 Basic Galf Chromatograph). Calculations of the acetylene reduction activity were done based on the graph of a standard injected into the machine.

2.4 DNA Isolation

2.4.1 Isolation of Plasmid DNA

2.4.1.1 Small Scale

Small scale preparation of plasmid DNA was done by the alkaline lysis method of Birnboim and Doly, 1979. The resulting DNA was used for restriction analysis, differential hybridization analysis or sequencing after being further purified by the Geneclean method (Bio/Can Scientific).

2.4.1.2 Large Scale

Large scale preparation of plasmid DNA was carried out using cesium chloride-ethidium bromide gradients as described in Maniatis *et al.*, 1982, except that a 50VTi rotor (Beckman) centrifuged at 44,000 rpm for 16-

18 hrs at 20°C was used. After butanol extraction the plasmid DNA was precipitated with the addition of 3 volumes of TE*[1] and 10 volumes of cold 95% ethanol.

2.4.2 Isolation of DNA Fragments

Restriction digestions were electrophoresed on a 0.8% agarose gel, with Lambda DNA digested with HindIII as a standard. Fragments were isolated from the gel with Geneclean following the manufacturer's (BIO 101) instructions. The amount of DNA was estimated by electrophoresing 10% of the sample on a 0.8% agarose gel stained with ethidium bromide using Lambda DNA digested with HindIII as control.

2.4.3 Polymerase Chain Reaction

Polymerase Chain Reactions were done using the GeneAmp DNA Amplification Reagent Kit protocol (Perkin Elmer Cetus). Reactions were initiated with 3ng of plasmid. This routinely yielded 5µg of double stranded insert. The yield was determined by A_{260} and electrophoresis of 5-10% of a sample through a 0.8% agarose gel run in TAE buffer* (Maniatis *et al.*, 1982), after staining with ethidium bromide and visualization by UV light.

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[1] *Indicates found either in the list of abbriviations or in the Buffer section.

2.5 RNA Isolation

2.5.1 Isolation of Total RNA

Four procedures for total RNA isolation from alder nodules were tried. In all cases RNA sterile techniques were observed. RNA yield was determined by A_{260} readings and visualized on 1.5% agarose denaturing gels containing formaldehyde as described in Maniatis *et al.*, 1982.

The first attempt followed the guanidinium thiocyanate procedure as described by Chirgwin *et al.*, 1979.

Then the LiCl/Urea procedure as described by Auffray and Rougeon, 1980 was attempted.

The third isolation procedure was a hybrid of the two preceding protocols. It involved an initial extraction of the homogenized tissue with guanidinium isothiocyanate and an overnight precipitation (Chirgwin *et al.*, 1979). A second extraction with LiCl/Urea (Auffray and Rougeon, 1980) was performed overnight followed by Sevag' extraction and ethanol precipitation. Finally the RNA pellet was collected, washed, dried and resuspended.

The final RNA isolation procedure used was according to the procedure of Logemann *et al.*, 1987. This procedure involved an extraction with 8M guanidine hydrochloride of the powdered tissue. The extractant is pelleted and the aqueous layer extracted with phenol/chloroform/isoamyl alcohol' followed by precipitation with 95% ethanol and 1M acetic acid overnight at -20°C. The precipitated RNA is pelleted and washed twice

with 3M sodium acetate pH5.2 and subsequently dissolved in sterile water. The following modifications were then performed. An extraction with phenol/chloroform and precipitation with 1/10 volume of 3M sodium acetate pH5.2, and 2.5 volumes of cold 95% ethanol.

2.5.2 Isolation of Poly A$^+$ RNA

2.5.2.1 Oligo dT Cellulose

Typically 100µg of total RNA was added to an oligo dT cellulose column and poly A$^+$ RNA isolated as described in Maniatis *et al.*, 1982. A yield of 3-5% was usually observed as determined by A_{260} readings.

2.5.2.2 Hybond map

250µg of total RNA in 200µl of water were applied to 4cm^2 of Amersham Hybond map paper. The samples were treated as described in Amersham's Hybond map protocol booklet. Yields of 5% of total RNA were usually observed as determined by A_{260} readings.

2.6 cDNA Synthesis

2.6.1 Preparationof ds-cDNA

4-6µg of poly A$^+$ RNA was used for cDNA synthesis. Preparation of ds-cDNA was according to the procedure of Rutledge *et al.*, 1988 without fractionation of the cDNA over a Sepharose 4B column.

Double stranded-cDNA yields were typically in the range of 20-30%.

2.6.2 Molecular Cloning of ds-cDNA

The vector pGem-4B (Promega) was digested with SmaI to yield blunt ends and treated with calf intestinal alkaline phosphatase (Boehringer Mannhiem). EDTA* and SDS* were added to 20 mM and 0.5% respectively and the reaction heated at 65° for 10 minutes in order to inactivate the enzyme. The vector was tested and used without further purification. Ligation mixtures containing equimolar ratios of vector:ds-cDNA were incubated overnight at 4°C and allowed to warm slowly to 18°C. The ligation mixture was then introduced into competent *E. coli* DH5α (BRL) cells. A control of 1ng of pGEM-4B was also added to verify transformation efficiency which was usually 5 X 10^6 colonies/μg vector.

2.7 Labelling of Probes

2.7.1 Random Primer Extension

The DNA inserts of the cDNA clones, described in DNA isolation section 2.4.2, were labelled with α^{32}P dCTP by the random priming method (Feinberg and Volgelstein, 1984) using the Multiprime kit supplied by Amersham. The labelled inserts were then purified over a Sephadex G50M (Pharmacia) spun column as described in Maniatis *et al.*, 1982. The probe specific activity ranged from 5 X 10^8 - 2 X 10^9 counts per minute per

µg insert.

2.7.2 Nick Translation

Plasmid DNA was labelled with α^{32}P dCTP by the nick translation method (Kelly *et al.*, 1970) using a kit supplied by Amersham. Labelled plasmid was then purified using the glass bead method of the Geneclean kit supplied by BIO 101 Inc. The probe specific activity ranged from 8 X 10^8 - 3 X 10^9 counts per minute per µg plasmid.

2.7.3 End Labelling

RNA was hydrolyzed in alkali to a length of approximately 200 bases, precipitated and resuspended in water (Schleif and Wensink, 1981). The RNA was labelled with γ^{32}P ATP using T4 polynucleotide kinase (Maniatis *et al.*, 1982). The labelled RNA was purified over a Sephadex G50M (Pharmacia) spun column as described in Maniatis *et al.*, 1982. The specific activity of the probe ranged from 7 X 10^8 - 3 X 10^9 counts per minute per µg labelled RNA.

2.8 Agarose Gel Electrophoresis

2.8.1 Gel Electrophoresis of DNA

Restriction fragments of DNA were separated by electrophoresis through horizontal agarose slab gels. The concentration of

the gels varied between 0.8-1.2% depending on the size range of the fragments to be resolved. The electrophoresis buffer contained 40mM Tris-acetate, 2mM EDTA' pH 8.0 (Maniatis *et al.*, 1982). Lambda HindIII digests or pBR322 TaqI digests were used as size markers.

2.8.2 Gel Electrophoresis of RNA

RNA was separated by electrophoresis through 1.5% agarose gels containing formaldehyde (Lehrach *et al.*, 1977). RNA samples for electrophoresis were prepared as described in Maniatis *et al.* (1982). The gels contained 20 mM MOPS' (pH7.0), 5 mM sodium acetate, 1 mM EDTA, 2.2 M formaldehyde. The running buffer contained 20 mM MOPS (pH 7.0), 5 mM sodium acetate, 1 mM EDTA and 1.1 M formaldehyde. Following electrophoresis, the gels were washed with 4-5 changes of water for 2 hours and two changes of 0.1 M ammonium acetate for one hour. The gel was stained with 0.5 µg/ml of ethidium bromide in 0.1 M ammonium acetate for 45 minutes. The gels were destained for 2 hours with 2-3 changes of 0.1 M ammonium acetate and then photographed. *E. coli* 23S, 16S and 5S rRNAs were used as size standards.

2.9 Transfers

2.9.1 Southern Transfers

DNA in an agarose gel, was denatured in 1.5M NaCl / 0.5M

NaOH and neutralized in 3M sodium acetate as described in the ICN protocol handbook. DNA was allowed to transfer onto a Biotrans membrane overnight by capillary action with 20X SSC as the transfer buffer (Southern, 1975). Transfer was verified by uv illumination of the gel after the overnight procedure. Following transfer, the DNA was crosslinked to the membrane by UV illumination (Black Ray lamp, Ultra-Violet Products Inc. San Gabriel, CA) for 5 minutes.

2.9.2 Northern Transfers

RNA samples were electrophoresed on denaturing agarose gels as described. After a one hour wash of the gel in distilled water, the RNA was allowed to transfer onto a Biotrans membrane as described for Southern transfers. The RNA was UV crosslinked to the membrane and the gel, following transfer, was stained (Maniatis *et al.*, 1982) to verify the transfer of the RNA.

2.9.3 Slot Blots

Hybond-N was cut to the size of the S&S Milifold apparatus (Schleicher & Schuell Inc.). It was prewetted with 2X SSC and assembled into the Milifold apparatus as per manufacturers instructions.

2.9.3.1 RNA Preparation

RNA samples were prepared by adding three volumes of a denaturing solution containing 500µl formamide (deionized)

162µl formaldehyde (37%)

100µl 10X MOPS buffer

and incubated for 5 minutes at 65°C. Four volumes of 20X SSC were then added and the samples were applied to the slots under vacuum. The membrane was then baked at 80°C for two hours under vacuum.

2.9.3.2 DNA Preparation

DNA samples were heat denatured by boiling for two minutes and then quickcooled on ice. One volume of 20X SSC was then added and the sample applied to the slots under vacuum. Once transfer was complete the membrane was treated for five minutes on Whatman 3MM paper soaked with 1.5M NaCl and 0.5M NaOH to ensure the denatured state of the DNA. This was followed by neutralization for five minutes by laying the membrane on Whatman 3MM paper soaked with 3M NaAc pH5.5. The membrane was then baked at 80°C for two hours under vacuum.

2.10 Hybridization

The hybridization protocols presented here are those for ICN Biotrans membrane recommended by (ICN Biomedicals, Inc.).

2.10.1 Southern Hybridization

Membranes were prehybridized with 4ml of hybridization solution per 100cm^2 membrane and hybridized with 2ml of hybridization solution per 100cm^2 membrane. Hybridization solution: 5X Denhardt's*, 5X SSPE*, 0.2% SDS*, 500µg/ml denatured non-homologous DNA. Prehybridization was done for one hour at 65°C, and hybridization was done overnight also at 65°C.

Blots were washed with a volume of 250ml/100cm^2 membrane of wash buffer (5mM sodium phosphate pH7, 1mM EDTA, 0.2% SDS) 3 X 30 minutes at room temperature. The membranes were then exposed to Kodak XAR-5 film, with the aid of Quanta III intensifying screens.

2.10.2 Northern and Slot Blot Hybridization

Membranes were prehybridized with 4ml of hybridization solution per 100cm^2 membrane and hybridized with 2ml of hybridization solution per 100cm^2 membrane. Hybridization solution: 5X Denhardt's, 5X SSC, 50mM sodium phosphate pH7, 0.1% SDS, 250µg/ml denatured non-homologous DNA and 50% (v/v) deionized formamide. Prehybridization was done for one hour at 42°C, and hybridization was done overnight also at 42°C.

After hybridization the membranes were washed four times in 2X SSC, 0.1% SDS, at 250ml/100cm^2 membrane, with vigorous agitation at

room temperature. The final washes were done in 0.1X SSC, 0.1% SDS twice for 30 minutes at 50°C. The membranes were then exposed to Kodak XAR-5 film, with the aid of Quanta III intensifying screens.

2.11 Rehybridization

Membranes were stripped of probe with a one hour wash at 65°C in 50% formamide (non-deionized), 10mM sodium phosphate pH 7, at 100ml/100cm^2 followed by 15 minutes at room temperature in 2X SSC, 0.1% SDS at 250ml/100cm^2 membrane. The efficiency of the stripping was checked by an overnight exposure to Kodak XAR-5 film with the aid of Quanta III intensifying screens. On the occasions when the hybridization was strong, it was necessary to repeat this process before the probe was completely removed. Care was taken not to let the membranes dry between round of hybridization, washing and stripping.

2.12 Scanning of Slot Blots

Once the slot blots had been exposed, the autoradiographs were then scanned with a LKB Ultrascan XL densitometer. From the plotted scans of the control samples and the test samples, it was then possible to quantitate the test samples.

2.13 Sequencing

2.13.1 Phage DNA DIGE (DIrect Gel Electrophoresis)

M13mp19 and/or M13mp18 (Messing,1983) phage supernatants were electrophoresed on a 0.8% TBE* (Maniatis *et al.*, 1982) agarose gel to verify approximate insert size and hybridization to the proper probes.

2.13.2 Preparation of DNA

Small scale preparation of phage DNA was done by PEG*/NaCl precipitation, according to the M13 cloning and sequencing handbook from Amersham, except that L broth* was used for growth of bacteria instead of 2YT. The phage DNA prepared was then used as single-stranded template for sequencing.

2.13.3 Template Orientation

Complementation tests (C-tests) were performed with the phage DNA to determine the relative orientation of the clones to help in sequencing. Briefly, 1.3µl of each DNA tested was added to 15.4µl of water and 2µl of 20X SSC, and incubated at 65°C for 90 minutes. The samples were electrophoresed on a 0.8% agarose TBE* gel following the addition of 3µl of M13 loading buffer and 1 µl of 2% SDS.

2.13.4 DNA Deletions

Sequential deletion of DNA samples followed by double-stranded sequencing eliminates the need for subcloning. Serial deletions were performed using the Erase-a-base kit from Promega. The use of either the pGEM-7Zf(-) or pGEM-7Zf(+) vector allowed for the resulting deletion clones to be sequenced either by double stranded sequencing or single stranded sequencing following the use of a rescue phage.

2.13.5 Sequencing Reactions

Sequencing reactions were done according to the dideoxy chain termination method (Sanger *et al.*, 1977) using the Amersham M13 sequencing protocol or the United States Biochemical sequenase kit. 7 µl of the prepared M13 phage template was sufficient to obtain a clear sequence. α ^{32}P-dATP or α ^{35}S-dATP (Amersham) was used to label the DNA. Usually, 350-400 nucleotides could be read from a double loading. Double-stranded sequencing was also done from pGEM-7Zf subclones, using the procedure outlined in the Sequenase protocol. The reactions were electrophoresed on a 7% acrylamide' vertical gel in 0.5X TBE buffer. Two runs were executed, the first for 5 hours and the second for 1.5 hours. The gels were run at 2100V, 190 mA and 55 watts. Gels were dried under vacuum at 80°C and exposed overnight to x-ray film.

2.13.6 Computer Analysis

Primary sequence analysis with pAnod4, was carried out using PC/Gene 6.5 software and the EMBL July 1991 databank. Sequence comparison used the FSTNSCAN program which uses the Lipman-Pearson FASTN algorithm (Lipman and Pearson, 1985), which finds and scores regions that are similar to a reference sequence. With a k-tuple setting of 4, the program makes a table of quadruplet nucleotides versus their positions in the reference sequence and then checks each quadruplet in the test sequence against the table. For each match, the difference in position (the offset) is noted and a score is calculated. The final score for an offset is the scoring factor (two) times the k-tuple value times number of matches, minus the number of mismatches of nucleotides for that offset. Sequences are then aligned with the reference sequence and this optimal alignment is then presented.

2.14 PCR Amplificaton

PCR amplifications were performed using 1 X standard PCR reaction buffer (BIO/CAN Scientific), 200µM dNTPs, 2.5 units Taq polymerase (BIO/CAN Scientific), 20 pmoles of each primer and 0.5 µg of *Frankia* or *E. coli* DNA in a reaction volume of 50µl. The primers used for the amplification were dATGACCAAGGTTCCTG (5' primer, base pair #23-39) and dTGTCGGTTTAGGGTACG (3' primer, base pair #428-412). DNA was

amplified for 35 cycles of 94°C for 2 minutes, 54°C for 1 minute and 72°C for 2 minutes using a Perkin Elmer Cetus DNA thermal Cycler and the products were electrophoreses on a 1.5% agarose gel.

2.15 Bacterial Strains and Relevant Phenotypes

JM101 *lac*α *mcr*A$^+$, *mcr*B?, *hsd* (r_k^+, m_k^+), *sup*E, Δ(*lac-pro*AB), [F', *tra*D36, *pro*AB$^+$, *lac*I^q *lac*ZΔM15]

DH5α φ80d*lac*ZΔM15, Δ(*lac*ZYA-*arg*F)U169, *deo*R, *rec*A1, *end*A1, *hsd*R17(r_k^-, m_k^+), *sup*E44, λ$^-$, *thi*-1, *gyr*A96, *rel*A1

DH5αF' F', φ80d*lac*ZΔM15, Δ(*lac*ZYA-*arg*F)U169, *deo*R, *rec*A1, *end*A1, *hsd*R17(r_k^-, m_k^+), *sup*E44, λ$^-$, *thi*-1, *gyr*A96, *rel*A1

HB101 F', *mcr*B, *mrr*, *hsd*S20(r_B^-, m_B^+), *rec*A13, *sup*E44, *ara*14, *gal*K2, *lac*Y1, *pro*A2, *rps*L20(Sm^r), *xy*15, λ$^-$, *leu*, *mtl*1

References: Maniatis *et al.*, 1982; Yanisch-Perron *et al.*, 1985; Hanahan, 1983.

2.16 Buffers and Other Solutions

Buffers and solutions presented here are from Maniatis *et al.*, 1982, unless otherwise indicated.

A. 7% acrylaminde gel for sequencing: 33.6 g urea, 37 ml water, 14 ml 40% bis/acrylamide (20:1), 4 ml 10X TBE, 56 µl TEMED, 560 µl ammonium persulfate (0.1 mg/ml).

B. 50X Denhardt's solution: 1% (m/v) Ficoll (400 000 M. Wt.), 1% (w/v) polyvinylpyrolidone (360 000 M. Wt.), 1% (w/v) bovine serum albumin, (Denhardt, 1966).

C. L Broth: (per l) 10 g tryptone, 5 g yeast extract, 5 g NaCl, pH 7.2.

D. 20X TAE: (per l) 96.8 g Tris Base, 32 g sodium acetate, 7.4 g disodium EDTA. pH 8.2 with the addition of glacial acetic acid.

E. 10X TBE: (per l) 108 g Tris base, 55 g boric acid, 8.5 g disodium EDTA, pH 8.3.

F. TE Buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

G. 20X SSPE: 3.6 M NaCl, 0.2 M sodium phosphate, pH 8.3, 0.02 M EDTA (ICN Biomedicals, Inc.).

H. 20X SSC: 3 M NaCl, 0.3 M sodium citrate, pH 7.0.

I. Phenol/Chloroform/Isoamyl Alcohol: solid phenol was melted at 65°C, 8-hydroxyquinoline was added to 0.1% (w/v), and the phenol was equilibrated with 1 M Tris pH 8.0 until the pH of the aqueous phase was ~ 7.9. An equal volume of 24:1 chloroform:isoamyl alcohol was added to the phenol.

J. 10X MOPS buffer: 0.2 M MOPS, 0.05 M sodium acetate, 0.01 M EDTA, pH 7.0 with NaOH (Lehrach *et al.*, 1977).

RESULTS

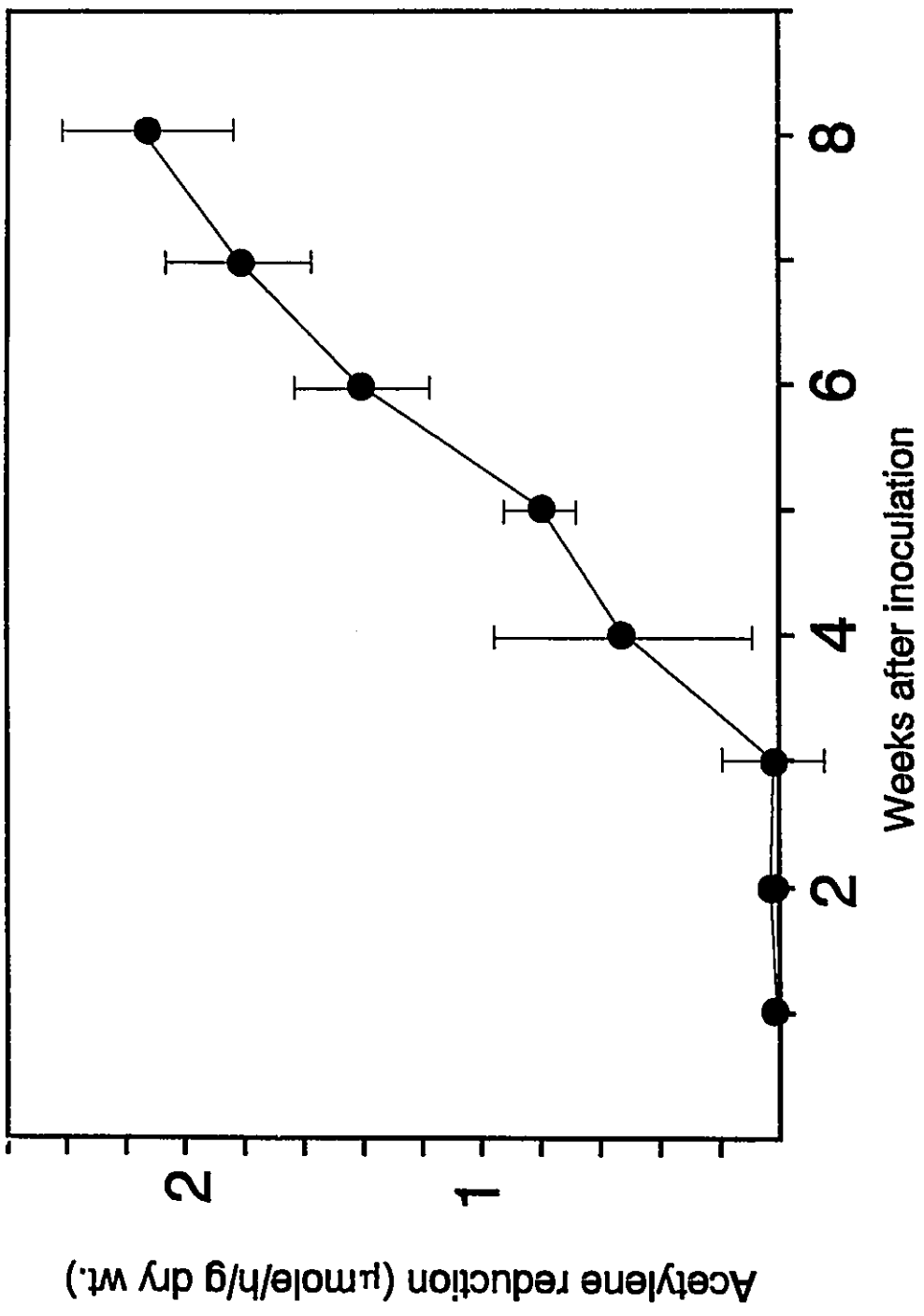
3.1 Nitrogen Fixation

Acetylene Reduction Measured by Acetylene Reduction Assay

In order to ensure that active symbiosis is occurring in our system and is comparable to other healthy plant values, acetylene reduction assays were performed. Inoculated plant samples were grown to cover a 14 week period allowing for weekly sampling. For each weekly sample, three time point samples of 0, 45 and 90 minutes after acetylene addition were taken. Fifteen plants were harvested for each time point and tested as five sets of three plants. One set of 3 plants of uninoculated roots and the acetylene alone for each of the time points were used as controls (in Material and Methods). The reduction of acetylene expressed in µmole/hour/g dry wt. was calculated for each time point and plotted (Figure 2). Previous studies with pure *Frankia* isolates indicated that nitrogen fixation in *Alnus glutinosa* commenced at about 10 days after inoculation based on acetylene reduction assay (Burggraaf and Shipton 1983). In legumes it was found that nitrogen fixation in the symbiotic state began 10-12 days after inoculation (Fuller and Verma 1984). Our findings indicate that nitrogen fixation in our system takes twice as long to commence, compared to the *Frankia* isolate and legume symbiotic state. This may be due to a slower penetration time by the *Frankia* strain used for inoculation thus resulting in a delay in the infection period. There may also be

FIGURE 2: ACETYLENE REDUCTION IN ALDER NODULES

Calculated acetylyene reduction activity for weekly time points were performed in order to determine the time at which nitrogen fixation commences.


Acetylene reduction (µmole/h/g dry wt.)
1
2
2
4
6
8
Weeks after inoculation

differences in the growth conditions and strain differences may also influence the values. Our final reduction value of 2.1 µmole/hour/g dry wt., measured at fourteen weeks, fell within the range for those previously reported which varied from 0.5-15 µmole/hour/g dry wt. in *Alnus crispa* and *Alnus rugosa* to 20-64 µmole/hour/g dry wt. in *Alnus glutinosa* (Normand and Lalonde, 1982; Perinet and Lalonde, 1983). We therefore concluded that our system was actively fixing nitrogen and that functional symbiosis between *Alnus incana* and *Frankia* ACN 14a had been established.

It was determined that nitrogen fixation commences between 3 and 4 weeks after inoculation with *Frankia*. From this it was decided that plant tissue from 2, 3, 4 and 14 week inoculated plants would be used in this study.

These times represent; prior to fixation - 2 weeks

development of fixation - 3 and 4 weeks

active fixation - 14 weeks

3.2 RNA Isolation

3.2.1 Isolation of Alder Nodule RNA

No previous attempt to isolated RNA from any actinorhizal species had been reported. This being the case we had to try different procedures in order to obtain suitable RNA. We defined suitable RNA as having clear, sharp rRNA bands when total RNA is electrophoresed on a

FIGURE 3: GEL ELECTROPHORESIS OF ALDER NODULE RNA SAMPLES

Various protocols were performed in an effort to extract RNA from Alder nodules. Approximately 9µg of RNA, as determined by absorption at 260nm, was applied to each track. Samples were processed for gel electrophoresis in the presence of formaldehyde as described in Materials and Methods.

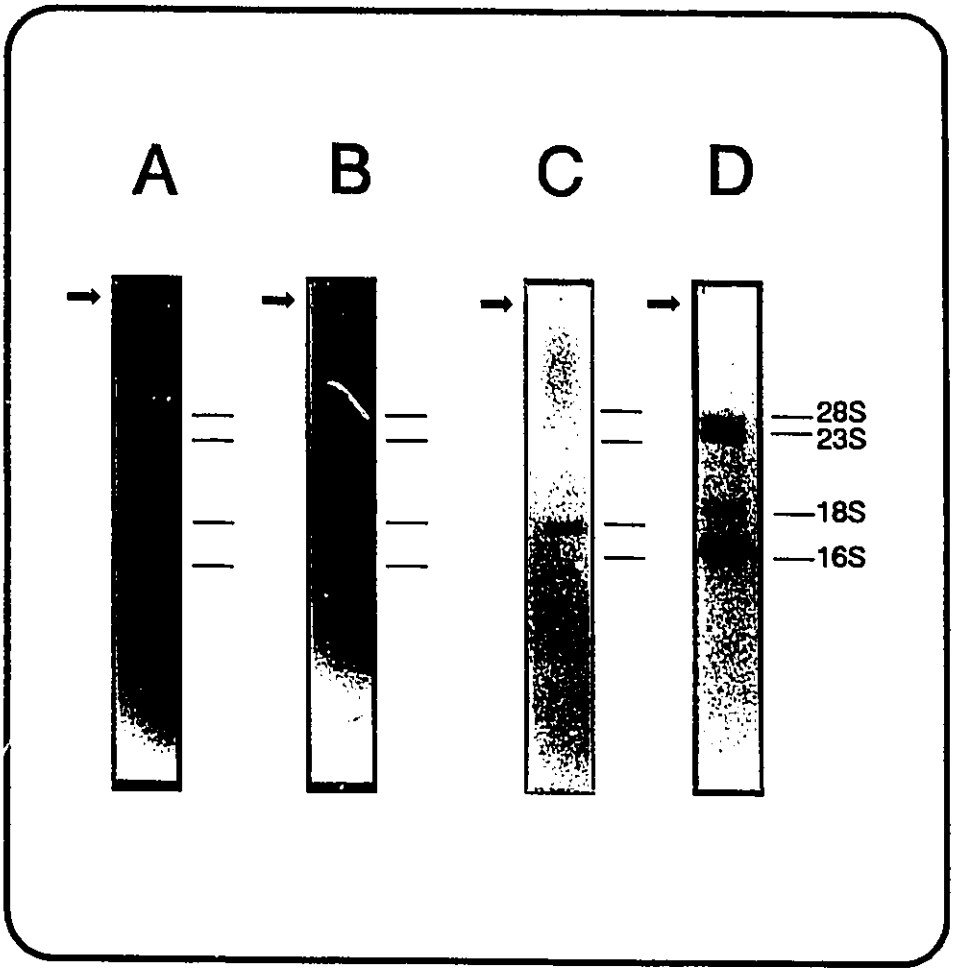
A. RNA extracted by method I (Chirgwin *et al.*, 1979)

B. RNA extracted by method II (Auffray and Rougeon, 1980)

C. RNA extracted by hybrid of I and II (see Materials and Methods)

D. RNA extracted by method IV (Logemann *et al.*, 1987)

Note: Arrow indicates origin of gel.


A
B
C
D
28S
23S
18S
16S

denaturing gel. The four procedures used in this work were described in the Materials and Methods section for the isolation of nodule RNA and the results are summarized in Table II. The initial procedure attempted (Chirgwin *et al.*, 1979) had proven to be very successful with soybean nodules and was thus used first. This procedure however, proved to be unsuccessful for alder nodules (Figure 3a). Instead of observing rRNA bands on a denaturing gel, the RNA was degraded appearing as a low molecular weight smear on the gel. The products of these preparations were also heavily pigmented at the end of the procedure. This pigment, which was orange in colour, may be due to the presence of plant phenolics (Dr. C. Nozzolillo, personal communication) which may inhibit the extraction process or subsequent purification steps or cDNA synthesis, and were thus judged necessary to be removed. After several attempts this protocol was abandoned and the protocol of Auffray and Rougeon, 1980 was tried. In the past this protocol had achieved minor success in our laboratory when used for the isolation of alder RNA. However, attempts to isolate nodule RNA met with virtually no success. Again, as with the first procedure, no rRNA bands were observed, instead a smear was present on the gel (Figure 3b). The occurrence of the orange pigmentation was, however, drastically reduced in the LiCl/Urea solution but was still visible. At this stage the established protocols had proven themselves to be of little use for RNA extraction from woody tissue such as alder nodules; however,

certain aspects of each protocol appeared to be of use. In the guanidine thiocyanate procedure (Chirgwin et al., 1979), guanidine thiocyanate has the advantage of being a very effective denaturant of ribonuclease (Von Hippel and Wang, 1964; Castellino and Barker, 1968). In the LiCl/Urea procedure (Auffray and Rougeon, 1980), the high salt and urea concentrations help to inhibit ribonucleases and RNAs are selectively precipitated while contaminants such as DNA, polysaccharides and proteins remain in solution. Also a reduction in the contaminating orange pigmentation was observed. Thus a hybrid protocol of these two protocols was attempted (see Materials and Methods). The result was RNA with reduced orange pigmentation and a yield of 14-20µg of total RNA per gram of tissue. As seen in Figure 3c, a visible band of rRNA migrating at 16S is present, as well, a small smear at 23S and above is visible.

At this time we also discovered that unlike soybean, the age of the alder nodule appeared to affect the quantity and quality of the RNA. Since the recovery of nodule material per plant is very low, we had been storing nodules at -80°C in order to accumulate sufficient nodule mass for the comparison of various procedures. But as seen in Table I (harvest date 08-07-87), storage of the tissue at -80°C for periods greater than one month appeared to result in the gradual decrease in RNA yield and/or quality. Therefore, harvesting of nodule tissue was quickly followed by extraction of RNA in order to avoid this RNA degradation.

The fourth procedure, which was published during the course of the experiments, was incorporated because the time frame for the isolation of RNA could be cut in half, from four days to two days. Not only was the time saving achieved but also an improvement of both yield and apparent quality of the RNA was also achieved as see in Figure 3d. Here the rRNA bands are intact and there is no suggestion of degraded product. It was now possible to isolate total nodule RNA within two days with yields ranging between 7-35µg for gram of tissue. A summary of the attempts to isolate RNA from alder nodules by the four methods is given in Table II.

From the above results it is apparent that extraction of alder nodule RNA was difficult. The first two methods used (Chirgwin *et al.*, 1979; Auffray and Rougeon, 1980) proved to be unsuccessful for RNA isolation from alder nodules. The third method, a hybrid of the first two, yielded useable RNA but the quantity was low. The fourth method yielded RNA of both useable quantity and quality. It is this method which was used for all further RNA isoaltions from alder nodules. The yields, although reasonable, do not come near to the total RNA yields that have been obtained in our laboratory for soybean nodules which are in the range of 200-500 µg of total RNA per gram of tissue from actively fixing plants. It should also be noted that for the harvest of 14 week old nodules, we required on average 250-300 plants to recover 1g of nodule tissue. Therefore each mg of total alder RNA requires the processing of 6,000 to 15,000 plants. The apparent difficulties

TABLE I: RNA ISOLATION FROM ALDER NODULES

The history of RNA extraction from alder nodules is presented. The changes in techniques resulted in improved RNA yields as seen from the table. Techniques are as follows:

1. RNA extracted by method I (Chirgwin *et al.*, 1979)
2. RNA extracted by method II (Auffray and Rougeon, 1980)
3. RNA extracted by hybrid of I and II (see Materials and Methods)
4. RNA extracted by method IV (Logemann *et al.*, 1987)

DATE NODULES PICKED	AMOUNT NODULE (g)	DATE RNA EXTRACTION	YIELD		QUALITY
			µg TOTAL RNA	µg POLY A⁺/g TISSUE	
TECHNIQUE 1					
27-10-86	4	18-02-87	-	-	DEGRADED RNA
		24-02-87	-	-	DEGRADED RNA
	3	26-05-87	-	-	DEGRADED RNA
11-12-86	2.75	05-03-87	-	-	DEGRADED RNA
	3	26-05-87	-	-	DEGRADED RNA
TECHNIQUE 2					
11-12-86	5	01-06-87	-	-	SMEAR + BANDS
15-06-87	5	18-06-87	-	-	DEGRADED RNA
1986	5	18-06-87	-	-	DEGRADED RNA
TECHNIQUE 3					
08-07-87	5	13-07-87	100	0.784	INTACT RNA
	5	11-08-87	80	0.776	INTACT RNA
	5	12-08-87	70		INTACT RNA
	10	18-08-87	25	0.110	SLIGHT SMEAR

	5	20-08-87	86	0.480	SMEAR
	25	31-08-87	-	-	NOTHING
TECHNIQUE 4					
10-09-87	10	23-09-87	395	0.800	INTACT RNA
	10	13-10-87	485	1.060	INTACT RNA
06-01-88	10	14-01-88	250	0.890	INTACT RNA
05-02-88	10	23-02-88	200	0.850	INTACT RNA
	5	21-03-88	2	-	NOTHING
28-03-88	5	30-03-88	100	1.000	INTACT RNA
		05-04-88	150		
		11-04-88	243		
		12-04-88	297		
05-05-88	7	11-05-88	380	0.900	INTACT RNA
18-07-88	30	21-07-88	1230	0.800	INTACT RNA

TABLE II: RNA ISOLATION SUMMARY

RNA isolation from Alder nodules presented in Table I are summarized. This illustrates the yield efficiency changes as techniques were changed. The techniques are as follows:

1. RNA extracted by method I (Chirgwin *et al.*, 1979)
2. RNA extracted by method II (Auffray and Rougeon, 1980)
3. RNA extracted by hybrid of I and II (see Materials and Methods)
4. RNA extracted by method IV (Logemann *et al.*, 1987)

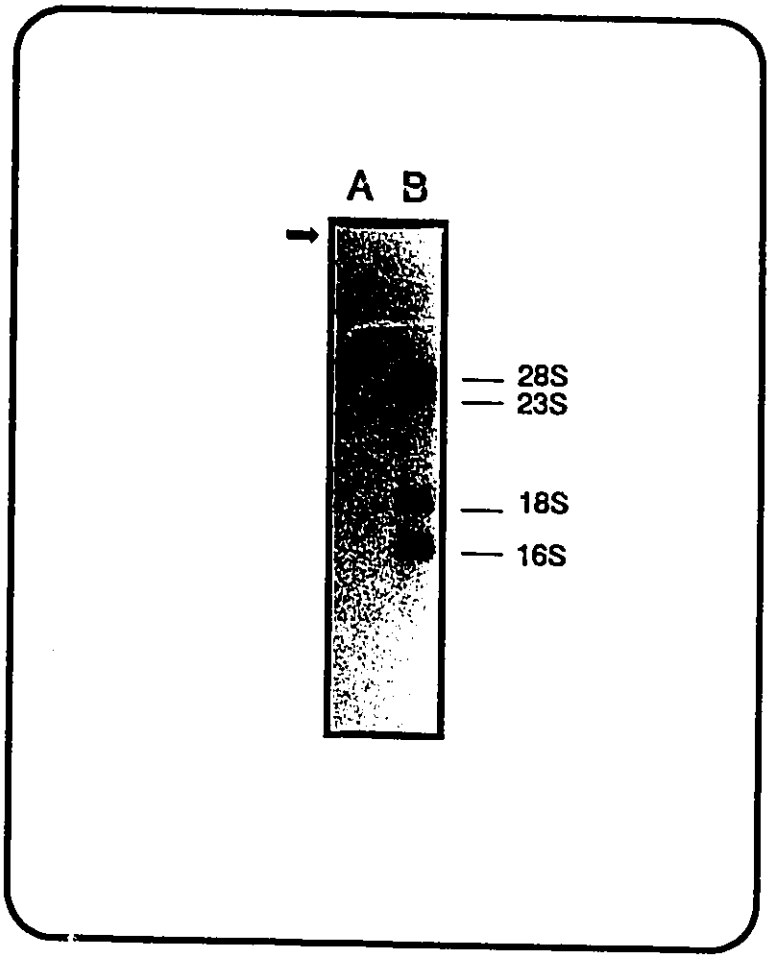
TECHNIQUE	NO. OF ATTEMPTS	NO OF PLANTS	NODULE WT. (g)	µg RNA
1	5	4,400	16	degraded
2	3	3,750	15	degraded
3	6	12,500	50	361
4	11	22,500	102	3,700

FIGURE 4: GEL ELECTROPHORESIS OF POLY A$^+$ RNA ISOLATED FROM ALDER NODULES

Total RNA isolated on 23-09-87 from 14 week old nodules by method 4 (refer to Figure 3d and Table I) was used for oligo dT cellulose isolation of poly A$^+$ nodule RNA. Purified poly A$^+$ nodule RNA was viewed on a 1.5% denaturing gel in order to check for any ribosomal contamination.

Lane A. 2µg of PolyA$^+$ RNA

Lane B. 6µg of total RNA


A B
28S
23S
18S
16S

in isolating RNA from such a recalcitrant tissue and the large numbers of plants required, placed severe time limits on our attempts to make cDNA and screen cDNA libraries.

3.2.2 Isolation of Poly A$^+$ RNA

Two methods for the isolation of poly A$^+$ RNA were used, each with their own application. Oligo-dT cellulose was used to isolate poly A$^+$ RNA from total RNA isolated on 23-09-87, by method four, of 14 week old alder nodules for use in cDNA synthesis. This method of isolation yielded mRNA with no visible rRNA bands (Figure 4) but a faint smear, which did not reproduce in the photograph, indicates high molecular weight mRNA. This procedure for obtaining mRNA for the use of cDNA production was recommended by Dr. M. Tenniswood (University of Ottawa, Department of Biochemistry). For other applications such as probe preparation and northern analysis, the less difficult and much faster method using hybond-map paper from Amersham was substituted. Typically the resulting mRNA yield was 3-5% of the total for both procedures. Soybean yields of 1-2% of the total RNA as poly A$^+$ suggests that some rRNA contamination may still be present.

3.3 cDNA

3.3.1 cDNA Library Construction

The vector pGEM-4B (Promega) has several features which were found to be attractive for our use in the construction of the cDNA library. The vector codes for the lacZ α-peptide within which a unique multiple cloning site is contained. This configuration allows for the colour selection in the presence of IPTG (inducer) and X-gal (indicator) for recombinants. The multiple cloning site has several useful restriction sites which can be exploited for future subcloning into both plasmid and phage vectors. pGEM-4B also contains two promoter sequences for T7 and SP6 RNA polymerases, one on each side of the multiple cloning site. These can be used for the production of RNA *in vitro* and also allow for sequencing from either end using double stranded sequencing and complementary primers. The plasmid was digested with SmaI and dephosphorylated with calf intestine alkaline phosphatase (Boehringer Mannheim) in order to promote the insertion of the cDNA and to reduce the possibility of plasmid self ligation. Transformed DH5α (Bethesda Research Laboratories) colonies were selected by the acquisition of ampicillin resistance. Initial attempts at colony hybridization were unsuccessful due to high backgrounds. Therefore plasmid DNA was isolated from 900 ampicillin resistant, lac$^-$ colonies and after digesting with Eco RI and Bam HI, in order to excise the cloned inserts, these plasmid DNA digests were run on an 0.8% agarose gel. The

DNA was then transferred to ICN Biotrans membrane and screened by differential hybridization (see Materials and Methods) using end-labelled RNA.

3.3.2 Screening of the Nodule cDNA library

Initially, plasmid DNA from 900 colonies was screened by sequential hybridization with ^{32}P end-labelled root poly A$^+$ RNA followed by nodule poly A$^+$ RNA. This technique as described in the Materials and Methods is known as differential hybridization and is useful for screening a large number of clones. From soybean cDNA library screening (S. Gottlob-McHugh, Ph.D thesis), it was expected that 0.5% of the clones would be nodule-specific. The initial screening suggested that 14 or 1.6% of the clones were potential nodulin cDNA clones, as judged by hybridization to the nodule-specific probe only or more strongly with the nodule-specific probe than the root specific probe. Although the number of potential clones obtained was higher than the expected number, we felt that our attempt here was still relevant given that this was the initial screening and a different symbiotic system was being analyzed. The remaining clones appeared to hybridize with equal intensities to both probes, although some "root-specific" sequences could be detected. These apparent root specific clones were not investigated further. The designations pAnod 1, pAnod 2 etc. referring to an Alder nodulin clone, were given to the 14 plasmids

containing potential nodulin cDNA inserts. The average insert size for the cDNA library was 1,500bp with a range of 187-2,000bp (Table III). From previous work in legumes (S. Gottlob-McHugh, Ph.D. thesis) similar work yielded inserts ranging from 500-900 bp in size.

3.4 Expression of Nodulin Genes in Alder Root Nodules

In order to determine whether or not these 14 potential clones were indeed nodule-specific, northern analysis was used. Northern analysis also allowed for the investigation of gene expression following infection with *Frankia*. Five separate blots were prepared using 2, 3, 4 and 14 week total nodule RNA and total root RNA as the control. At least two separate probings with each clone were carried out on separate membranes. High stringency wash conditions were applied to the membranes using 0.1X SSC and 0.1% SDS at 50°C. From this analysis it was determined that 8 of the 14 clones could be potentially classified as nodule-specific. The properties of these clones have been summarized in Table III.

pAnod 5, pAnod 6, pAnod 7 and pAnod 8 hybridize to 2 RNAs measuring 2110 and 1650 nucleotides. Their pattern of expression is also similar exhibiting an increase over time (Figure 5c). As a result they have been tentatively classified together as clones derived from the same RNA species. Further analysis was carried out using pAnod 5 as the representative clone since it was available to us prior to pAnod 6 or 7.

TABLE III: CHARACTERIZATION OF POTENTIAL NODULIN CLONES FROM *A. incana*

Fourteen original potential nodulin clones were screened by northern analysis as described in the Materials and Methods. Results of these screenings are summarized in this Table. NNS-Not Nodule Specific

CLONE	INSERT (bp)	BLOT DATES	mRNA (n)
Anod 1	1500	21-11-88 26-11-88	3350
Anod 2	800	21-11-88 26-11-88	3600; 2000
Anod 3	175	21-11-88 27-11-88	NNS
Anod 4	2000	20-08-88 14-11-88	3350
Anod 5	190	20-08-88 14-11-88	2110; 1650
Anod 6	330	21-09-88 21-10-88	2150; 1650
Anod 7	330	21-09-88 21-10-88	2110; 1650
Anod 8	250	26-09-88 02-11-88	2125; 1650
Anod 9	575	23-09-88 27-10-88	NNS
Anod 10	525	02-11-88 10-11-88	NNS
Anod 11	400	02-11-88 16-11-88	NNS
Anod 12	400	15-09-88 10-11-88	NNS
Anod 13	325	10-11-88 16-11-88	3500
Anod 14	425	15-09-88 10-11-88	NNS

FIGURE 5: EXPRESSION AS INVESTIGATED BY NORTHERN ANALYSIS OF POTENTIAL NODULIN CLONES

Northern transfers were probed with each potential nodulin clone as described in the Material and Methods. Message sizes for four candidates were determined.

A. pAnod 2

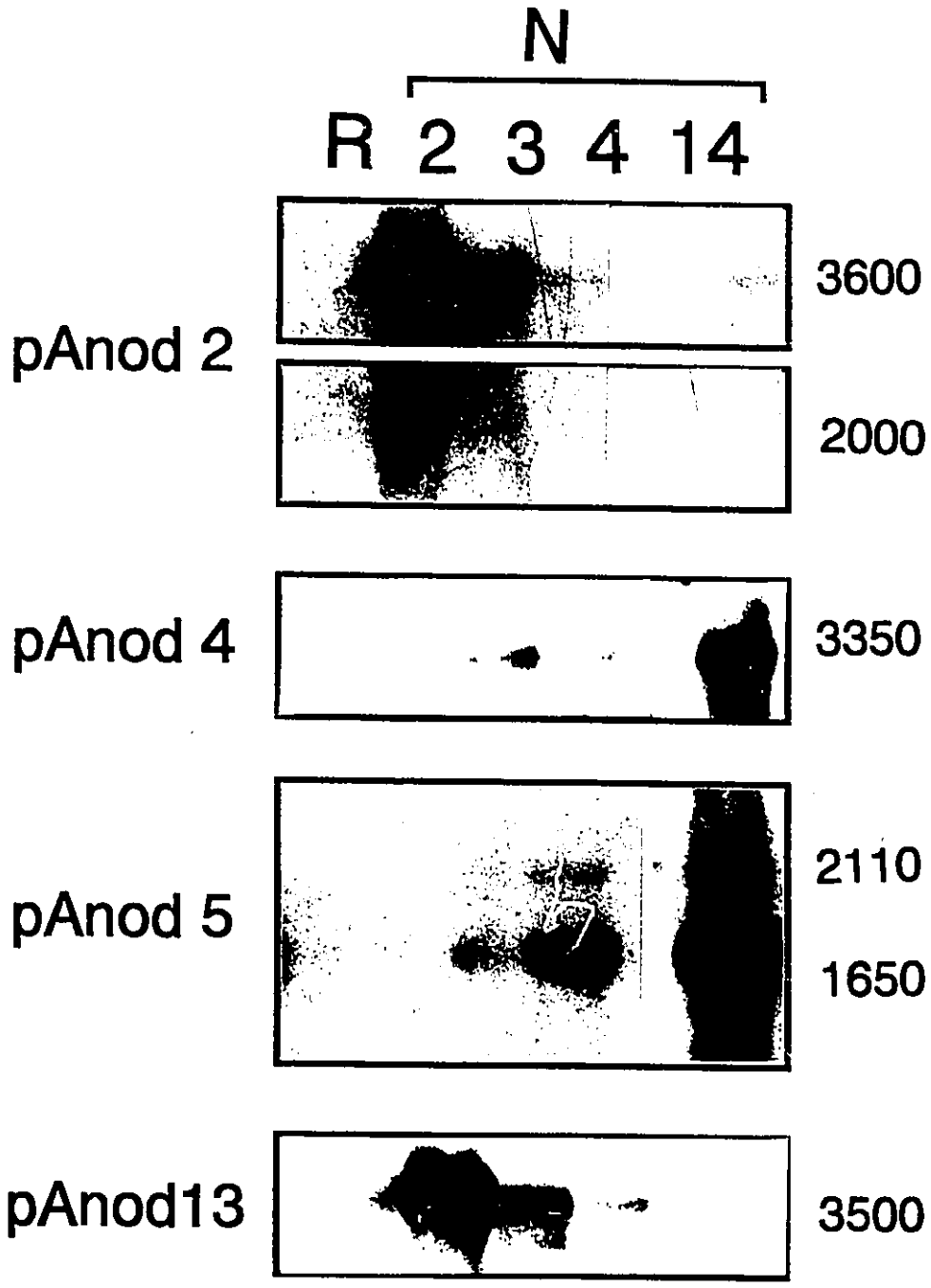
B. pAnod 4

C. pAnod 5

D. pAnod 13

R root RNA

N nodule RNA isolated from 2, 3, 4 and 14 week old nodules


N
R 2 3 4 14
pAnod 2
3600
2000
pAnod 4
3350
pAnod 5
2110
1650
pAnod13
3500

Similarly pAnod 1 and pAnod 4 detect an RNA of the same size, 3350 nucleotides, and showed a similar pattern of expression of an increase in signal strength over time (Figure 5b). In this case further analysis was carried out using pAnod4 as the representative clone since it appeared to contain the larger insert.

pAnod 2 hybridizes uniquely to 2 RNAs 3600 and 2000 nucleotides in size. The pattern of expression of pAnod2, exhibited an initial increase prior to the onset of fixation followed by a signal decrease as fixation was turned on (Figure 5a).

An eighth clone, pAnod 13, showed a similar expression pattern to that of pAnod2 but hybridized to a single RNA of 3500 nucleotides and showed root expression (Figure 5d). The level of root expressions observed in pAnod 13 was low when compared to the increased level observed after inoculation. This change in expression levels was deemed sufficient to classify pAnod 13 as a potential nodulin clone. However, further work on this clone was not carried out since it came to light a while after the other three.

It appears at this stage that four different cDNA clones which are expressed in a nodule-specific fashion have been isolated.

3.5 Categorization of the Nodulin Clones

From the Northern analysis, it appears the nodulin clones can

be divided into two categories based on the pattern of expression of the RNA (Figure 6). The first group includes pAnod 4 and pAnod 5. These cDNAs have a molecular phenotype characterized by an increase in hybridization signal with the age of the nodule. They show an expected increase in expression as fixation is activated as observed in "late" legume nodulins. The second group contains pAnod 2 and perhaps pAnod 13, which show an increase in hybridization intensity two weeks after inoculation and prior to the detection of symbiotic activity at three weeks with a subsequent decrease over time. This gene is most likely expressed at a low level in the root of the plant and is activated upon infection. This gene may be compared to the early nodulin genes detected in legumes at the time of infection or the onset of nodule development. The transient expression observed in these genes may be related to the similar expression which we have observed.

3.6 Slot Blot Analysis

Slot blots of the three potential nodulins, pAnod 2, pAnod 4, pAnod 5, were performed (see Materials and Methods), in order to better quantitate and illustrate the expression patterns of the clones. This was done by densitometric scans of slot blots. The results of the these scans have been plotted in Figure 7. From Figure 7, the two categories of expression observed with the northern analysis are observed.

FIGURE 6: EXPRESSION OF POTENTIAL NODULIN CLONES

Northern blots were scanned by densitometric means and the data plotted.

A. pAnod 2

B. pAnod 4

C. pAnod 5

D. pAnod 13

Note: Nodule RNA from 2, 3, 4 and 14 week old nodules. Abscissa in arbitrary units

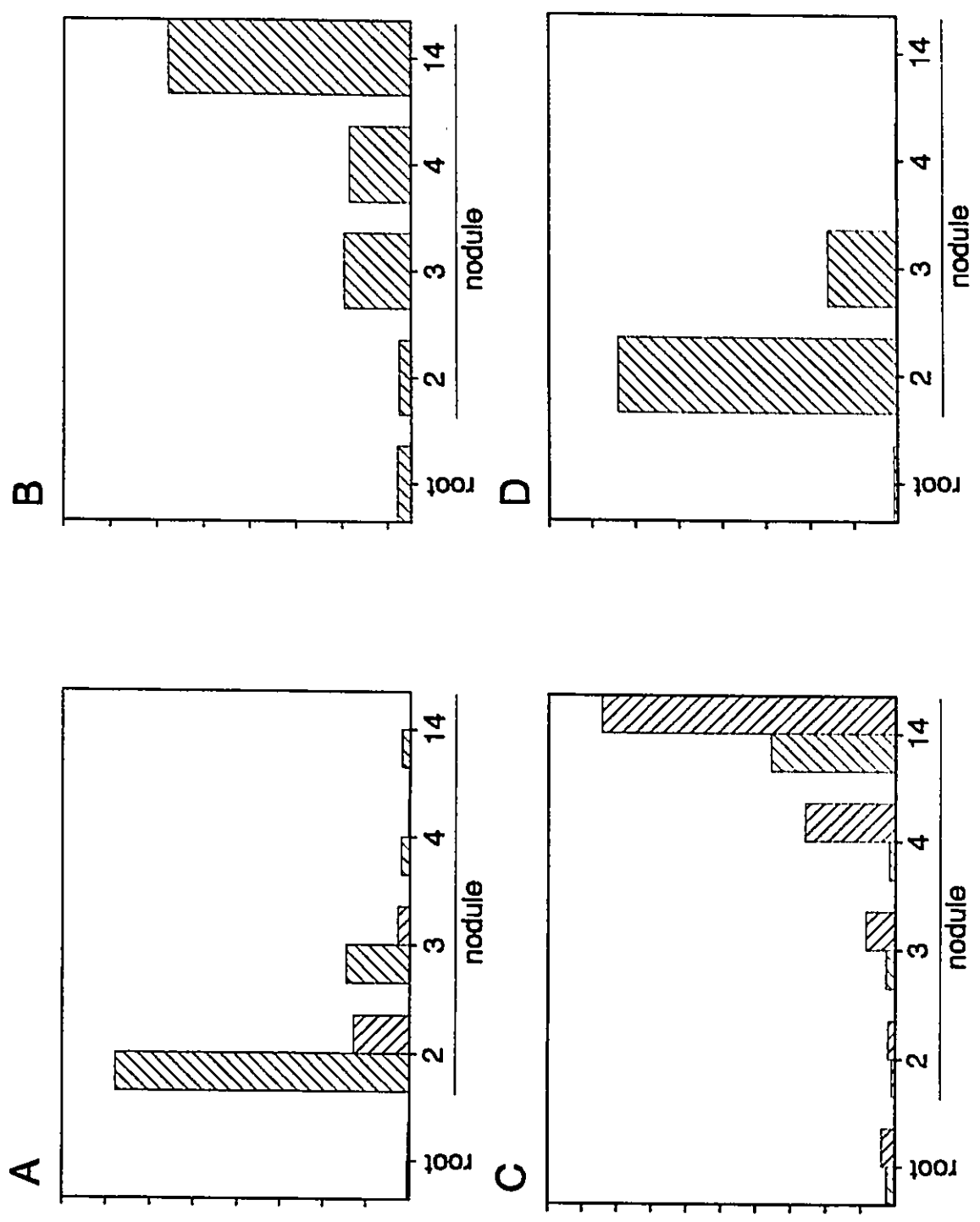
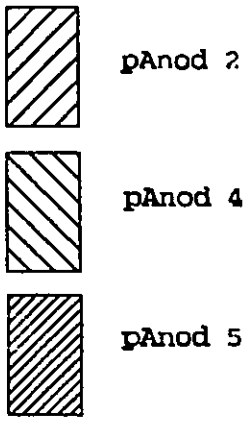

A
B
C
D
root
2
3
4
14
nodule

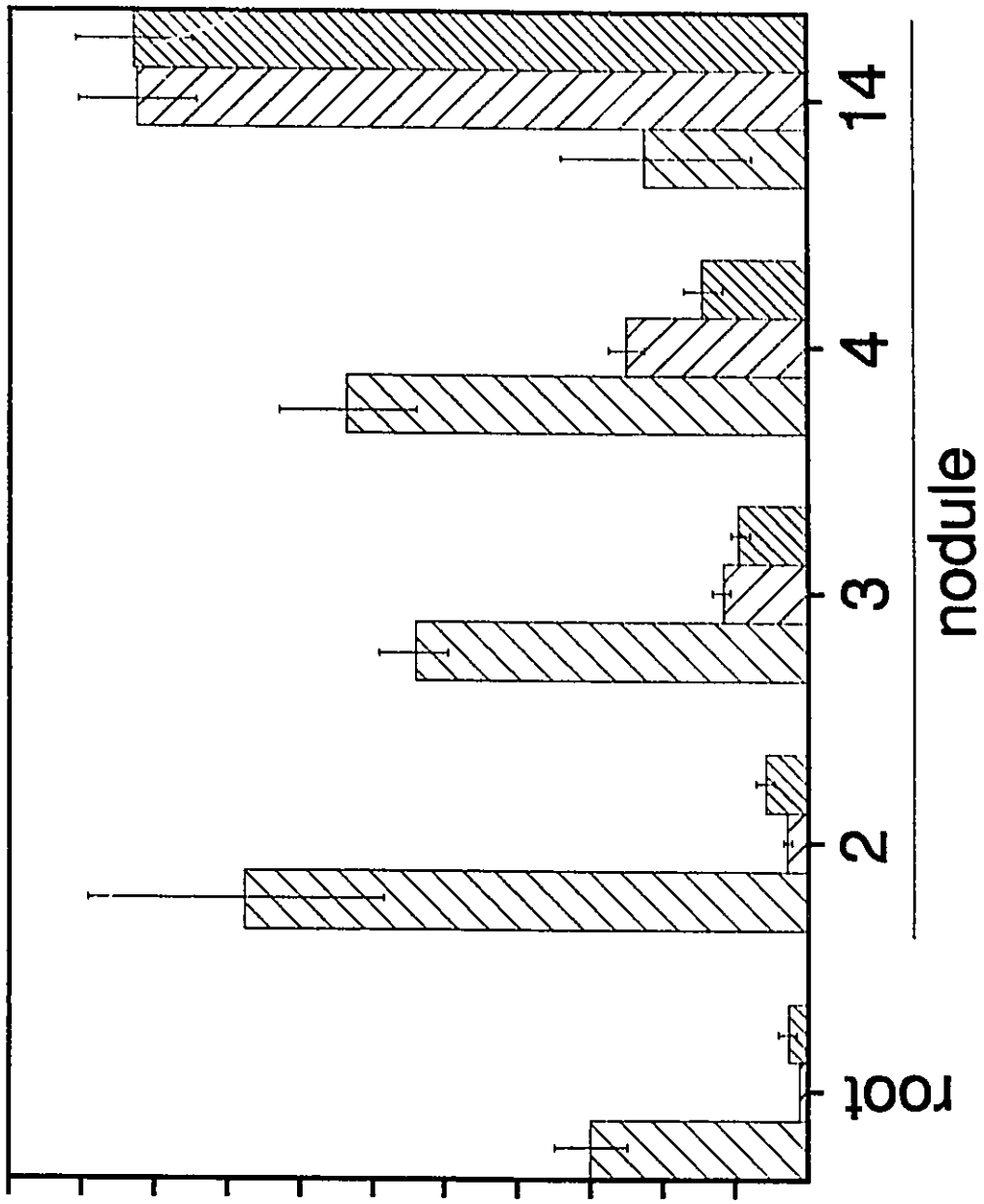
FIGURE 7: EXPRESSION OF POTENTIAL NODULE-SPECIFIC mRNAs AS MEASURED BY SLOT BLOT ANALYSIS

As described in this work, three of the potential nodulin clones were probed by slot blot analysis and the results densitometrically scanned.



Note: Nodule RNA from 2, 3, 4 and 14 week old nodules.

Abscissa in arbitrary units


root
2
3
4
14
nodule

3.7 Sequencing Analysis

The final step in the process of determining the validity of the isolated nodulin clones was to sequence the cloned inserts and perform a data bank search. Sequencing of the clones was done as described in the Materials and Methods with both strands of the inserts being sequenced. The size of the cloned inserts were found to be 613 bp for pAnod 2, 187 bp for pAnod 5 and 1718 bp for pAnod 4. These sequences were subjected to comparison of the EMBL databank of Microgenie 4.0. The parameters for the comparison were a minimum of 15 nucleotide matches in a homology with a minimum of 80% homology. This comparison proved negative in that no regions of homology to the databank were detected.

At this stage of the project Northern analysis had indicated three nodulin genes. Two of the genes exhibit an increase in the hybridization signal with the age of the nodule indicating genes potentially involved in the metabolism of nitrogen fixation. The third gene exhibited an initial increase in the hybridization signal prior to the onset of nitrogen fixation and then a decrease in hybridization signal as nitrogen fixation was established. This indicates a possible involvement with the early stages of nodule development preceding the onset of nitrogen fixation. These observations were reinforced by slot blot analysis followed by densitometric scanning. Because of these observations and the negative results from the databank searches of plant/organelle DNA/RNA sequences, these genes were

FIGURE 8: SOUTHERN ANALYSIS

Alder DNA and *Frankia* DNA were used for southern analysis and probed with either pAnod 2 insert or pAnod 4 insert as described in Materials and Methods.

A. Probe pAnod 2 insert:

Lane 1. 5µg Alder DNA cut with Eco RI

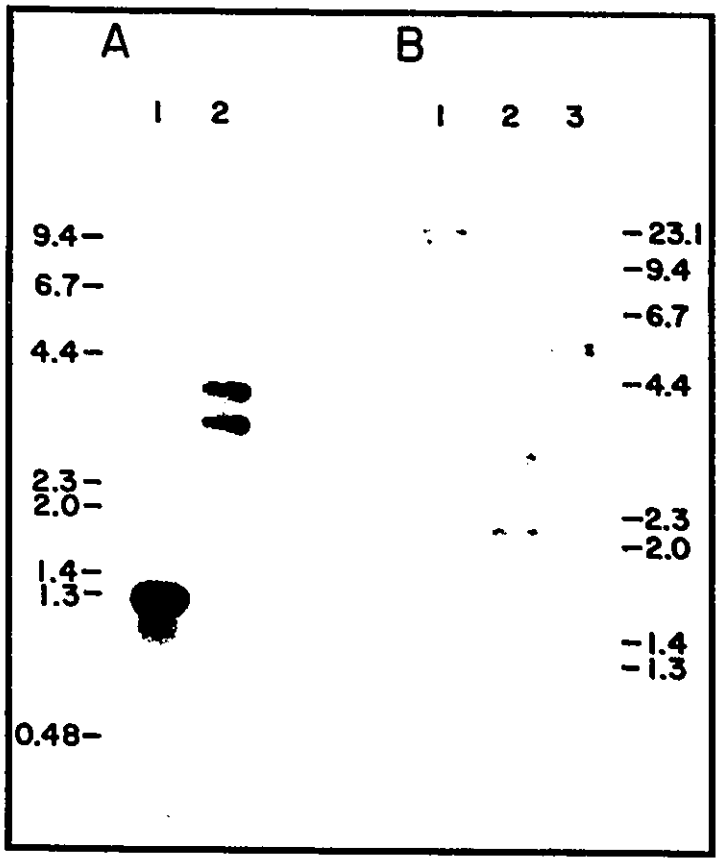
Lane 2. 5µg Alder DNA cut with Bam HI

B. Probe pAnod 4 insert:

Lane 1. 100ng *Frankia* ACN14A DNA cut with Eco RI

Lane 2. 100ng *Frankia* ACN14A DNA cut with Bam HI

Lane 3. 100ng *Frankia* ACN14A DNA cut with Sst I


A
1 2
9.4
6.7
4.4
2.3
2.0
1.4
1.3
0.48
B
1 2 3
23.1
9.4
6.7
4.4
2.3
2.0
1.4
1.3

believed to be true nodulin genes.

A more extensive databank search using the NRC Wisconsin package, demonstrated that the cDNA sequences were ribosomal in nature. pAnod 4 and pAnod 5 showed a high degree of homology to bacterial 23S and 16S ribosomal RNA genes respectively, whereas pAnod 2 was found to be homologous to plant 25S ribosomal RNA genes. It was determined at this time that the Microgenie program used had been unknowingly damaged prior to these searches, thus yielding the negative results that it did.

3.8 Southern Analysis

Southern blots were performed using alder DNA and *Frankia* DNA and each probed with pAnod 2 and pAnod 4 (Figure 8).

Probing the alder Southern with 2 X 10^6 cpm/ml of pAnod 2 followed by an exposure of 6hr. results in a 1.2kb band when an Eco RI digest is performed and two bands of 4.0 and 3.4 kb when a Bam HI digest is performed (Figure 8a). A longer exposure gives rise to an increase in the number of bands. These extra bands may be due to either polymorphism or cross hybridization to mitochondrial or chloroplast sequences. It should be noted that no hybridization was observed to *Frankia DNA* even after two days of exposure. Therefore as suggested by the computer search, pAnod 2 contains 613 bp of Alder 25S rRNA sequence.

When pAnod 4 is used as a probe against *Frankia* DNA a series of

bands are seen as indicated in Figure 8b. Probing Alder DNA results in bands with sizes different from those observed in Figure 8a of pAnod2 (data not shown). These sizes (4.7 and 0.7 kb with Eco RI digestion and 5.8 kb with Bam HI digestion) are similar to those observed by M. Levesque (MSc. thesis) for isolated alder chloroplast clones and thus represent cross-hybridization to chloroplast 23S rRNA genes. Therefore pAnod 4 contains 1,718 bp of *Frankia* 23S rRNA sequence.

3.9 Secondary Structure Comparison

Our analyses have indicated that the clones which we have isolated and characterized are rRNA clones of plant (pAnod 2) and bacterial (pAnod 4) in origin. rRNA gene sequences have been extensively used to investigate the evolutionary relationships between species. Therefore the sequence information gained in the previous section was analyzed from this point of view.

3.9.1 pAnod 4 Sequence Analysis

Both the primary sequences and secondary structures were compared between *Frankia* and *E. coli* large rRNAs. The sequence of pAnod 4 matched with *E. coli* sequence and exhibited about 75% homology over a region of 1,617 bp (Figure 9a.) from nucleotide 1282 to nucleotide 2898 of the *E. coli* sequence. This amount of sequence was sufficient to do a

secondary structure comparison. Mismatches included base deletions and base additions with respect to the *E. coli* sequence. As seen in Figure 10, many of these mismatches should not affect the secondary structure with respect to that of *E. coli* 23S rRNA. Several compensatory base changes eg. G-C → A-T occurred which likewise should not directly affect the secondary structure of the *E. coli* sequence. As a result of the above mentioned changes, most structural changes would occur at variable regions (Appendix I) of *E. coli* 23S rRNA. The one big change that would occur is at the site of a 105 bp insertion at nucleotide 1405 of the *E. coli* sequence while at the same time a 11 nucleotide deletion, 1405-1415, occurs at the same site in the *E. coli* sequence (Figures 9 and 10). It is assumed that this stem of the *Frankia* structure would differ significantly from that of *E. coli*. It should be noted that this site occurs immediately 5' to variable region 10 of *E. coli* (Appendix I).

The occurence of this insertion at this site in other bacterial species was investigated using the July 1991 update databank of EMBL. A total of 18 bacterial species (Appendix II), with respect to 23S RNA, were available for the search. From this search it was indicated that this insertion was not present in any other bacterial species. Even the closely related actinomycete, *Streptomyces* species did not show any homolgy to this region. This suggests that a unique sequence for *Frankia* may be present and could potentially be used as a marker in *Frankia*. This idea could be tested by

FIGURE 9: PRIMARY SEQUENCE COMPARISON

The determined nucleotide sequence of the potential nodulin clones were compared for homology as described in this work. Mismatches are indicated as *.

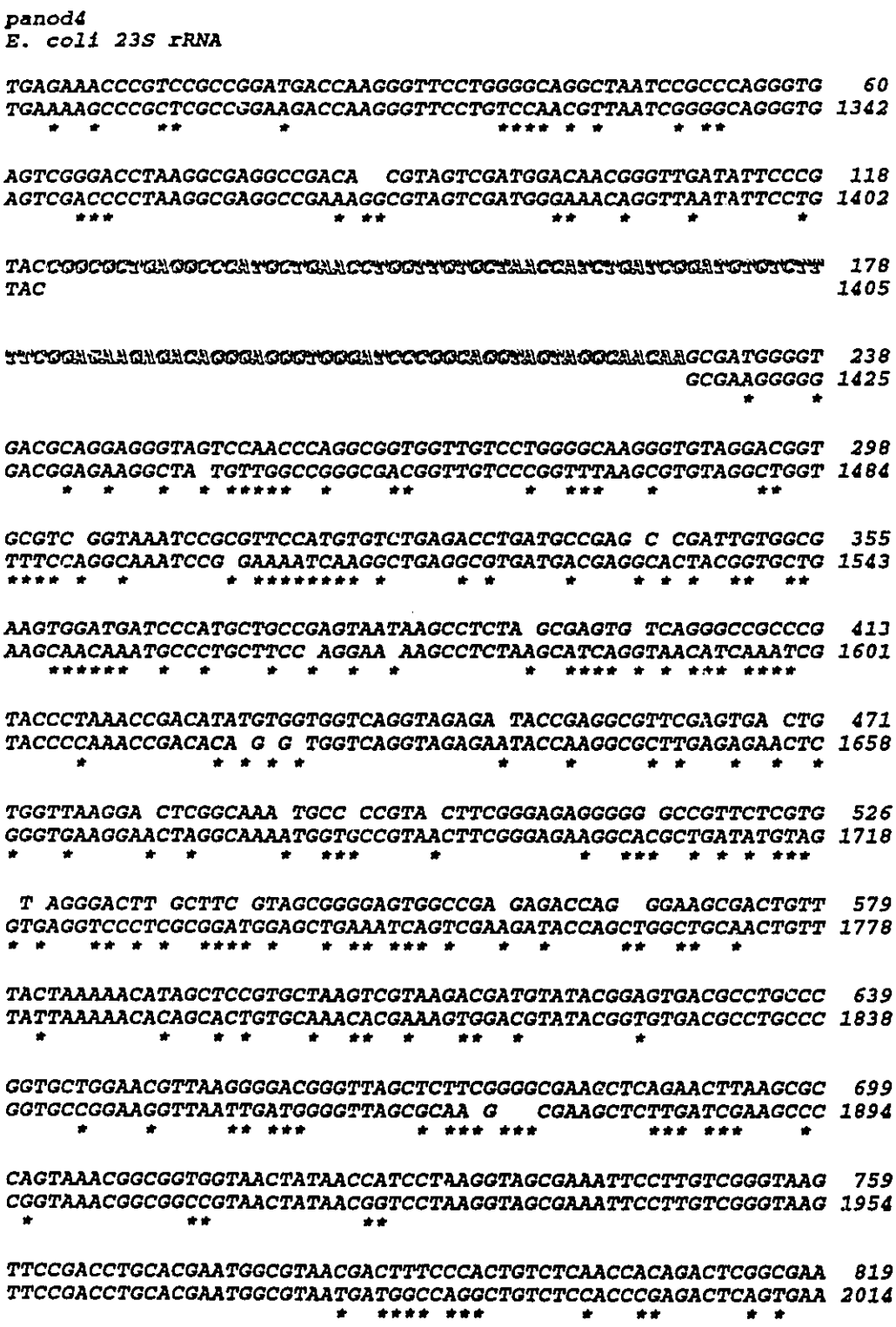
A. Comparison of pAnod 4 with *E. coli* 23s rRNA

Note: Extra sequence from pAnod 4 is indicated in shadowed type

B. Comparison of pAnod 2 with *Citrus limon* 26S rRNA

C. Comparison of pAnod 5 with *Streptomyces* 16S rRNA

A



```
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ATTGAACTCGCTGTGAAGATGCAGTGTACCCGCGGCAAGACGGAAAGACCCCGTGAACCT 2074
    * * ****  *       ****   *       *                 *  *

TTACTATAGCTTGATATTGGTGTTCGGTTCGGCTTGTGTAGGATAGGTGGGAGACTGTGA  938
TTACTATAGCTTGACACTGAACATTGAGCCTTGATGTGTAGGATAGGTGGGAGGCTTTGA 2134
               * *  **** * *** ****                 *  *

AGCTGGGACGCCAGTTCTGG TGGAGTCATTGTTGAAATACCACTCTGGTCGTACTGGAT  997
AGTGTGGACGCCAGT CTGCATGGAGCCGACCTTGAAATACCACCCTT TAATGTTTGAT 2192
  ***          *   **     * ****             *  ** ** ** *

GT CTAACCTCGGTCCGTGATCCGGTACAGGGACAGTGTCTGGTGGGTAGTTTAACTGGG 1056
GTTCTAACGTTGACCCGTAATCCGGGTTGCGGACAGTGTCTGGTGGGTAGTTTGACTGGG 2252
  *     * * **    *       *****                 *

GCGGTTGCCTCCTAAAGAGTAACGGAGGCGCCCAAAGGTTCCCTCAGCCTGGTTGGCAAT 1116
GCGGTCTCCTCCTAAAGAGTAACGGAGGAGCACGAAGGTTGGCTAATCCTGGTCGGACAT 2312
     **                    *  * *      **  * *       *  **

CAGGTGTTGAGTGCAATGGCACAAGGGAGCTTGACTGTGAGACAGACATGTCGAGCAGGT 1176
CAGGAGGTTAGTGCAATGGCATAAGCCAGCTTGACTGCGAGCGTGACGGCGCGAGCAGGT 2372
    * * *              *   **           *   ***   ****

GCGAAAGCAGGGCATAGTGATCCGGCGGTGGCTTGTGGAAGCGCCGTCGCTCAACGGATA 1236
GCGAAAGCAGGTCATAGTGATCCGGTGGTTCTGAATGGAAGGGCCATCGCTCAACGGATA 2432
           *                *   ******      *   *

AAAGGTACCCCGGGGATAACAGGCTGATCTTGCCCAAGAGTCCATATCGACGGCAAGGTT 1296
AAAGGTACTCCGGGGATAACAGGCTGATACCGCCCAAGAGTTCATATCGACGGCGGTGTT 2492
         *                 ***          *            ***

TGGCACCTCGATGTCGGCTCGTCGCATCCTGGGGCTGGAGTAGGTCCCAAGGGT TGGCT 1355
TGGCACCTCGATGTCGGCTCATCACATCCTGGGGCTGAAGTAGGTCCCAAGGGTATGGCT 2552
                  *  *              *                *

GTTCGCCCATTAAAGCGGTACGCGAGCTGGGTTTAGAACGTCGCGAGA AGTTCGGTCCC 1414
GTTCGCCATTTAAAGTGGTACGCGAGCTGGGTTTAGAACGTCGTGAGACAGTTCGGTCCC 2612
        **       *                                *   *

TATCCGCCGCAGGCCGCAGGAGACTTGAGAAGGGCTGTCCCTAGTACGAGAGGACCGGGA 1474
TATCTGCCGTGGGC GCTGGAGAACTGAGGGGGGCTGCTCCTAGTACGAGAGGACCGGAG 2671
    *    **   *  *     **    **       **                  **

CGGACGAACCTCTGGTGTGCCAGTTGTTCTGCCAAGGGCATGGCTGGTTGGCTACGTTCG 1534
TGGACGCATCACTGGTGTTCGGGTTGTCATGCCAATGGCACTGCCCGGTAGCTAAATGCG 2731
*     * * *        * **     **       *    **  ** * *    ** *

GAAGGGATAACCGCTGAAAGCATCTAAGCGGGAAGCCTGCTTCGAGATGAGGTCTCC    1591
GAAGAGATAAGTGCTGAAAGCATCTAAGCACGAAACTTGCCCCGAGATGAGTTCTCCCTG 2791
    *     **                  **   * *   **          *     ***

ACAGGGTA GCCTGGTAAGGCCCCCGACTAGATGATCGGGTTGATAGGCCGGAGGTGGAA 1650
ACCCTTTAAGGGTCCTGAAGGAACGTTGAAGACGACGACGTTGATAGGCCGGGTGTGTAA 2851
  ****  * ** ** * * *** *****   *  ****                ** *

GTGCGGTGACGCATGGAGATGACCGGTACTAATAGGCCGAG GGCTT              1696
GCGCAGCGATGCGTTGAGCTAACCGGTACTAATGAACCGTGAGGCTT              2898
 *  * *  * * *  * *              ***  * *
```

B

panod2
Citrus limon 26S rRNA

```
GGCAAGTGC AGGCCCC  TGAGTAGGAGGGCGCGGCGGTC CTGCAAAACCTGGGGCGC   56
GGCAAGTGCCAGGCCCCGATGAGTAGGAGGGGCCGGCGGTCGCCGCAAAACCCGGGGCGC 1417
          *       **            **        * *       *

G  CA GGGCGGACGGGCCATCGGTGCAGATCTTGGTGGTAGTAGCAAATATTCAAATGA  113
GAGCCCGGGCGGAGCGGCCGTCGGTGCAGATCTTGGTGGTAGTAGCAAATATTCAAATGA 1477
 ** **        **    *

GAACTTTGAAGGCCGAAGAGGGGAAAGGTTCCATGTGAACGGCACTTGCACACTGGGTTA  173
GAACTTTGAAGGCCGAAGAGGGGAAAGGTTCCATGTGAACGGCACTTGCACA TGGGTTA 1536
                                                      *

GTCGATGGTAAGAGACGGGGGAAGCCTGTCTGATAGCGTGTCTGCAC AGCTTCGAAAGG  232
GTCGATCCTAAGAGACGGGGGAAGCCCGTCCGACAGCGCCACCGCGCGAGCTTCGAAAGG 1596
      **                   *   *  *    *** *  * *

GAATCGCGTTAAAATTCCTGAACCGGGACGTGCCGGTTGACGGCAACGTTAGGGAGTCAG  292
GAATCGGGTTAAAATTCCTGAACCGGGACGCGGCGGCTGACGGCAACGTTAGGGAGTCCG 1656
      *                          *     *                  *

GAGACGTCGGCGGGG C TCGGGAAGAGTTATCTTTTTCTGTTTAACAGCCTACC ACCC  345
GAGACGTCGGCGGGGGCCTCGGGAAGAGTTATCTTTT CTGTTTAACGGCCTGCCCACCC 1715
                 * *                    *         *    * *

TG AAA GCCTCAGCCGGAGGTAGGGTCCAGCGGC G AAGAGCACCGCACTTCGCGTGG  405
TGGAAACGGCTCAGCCGGAGGTAGGGTCCAGCGGCCGGAAGAGCACCGCACGTCGCGCGG 1775
  *   * *                             * *             *     *

TGTCCGGTGC CCC  GGCGGCCCTTGAAAATCCGGAGGACCGAG                 447
TGTCCGGTGCGCCCCCGGCGGCCCTTGAAAATCCGGAGGACCGAG                1820
          *   **
```

C

panod5
Streptomyces lividans 16S rRNA

```
TTAACACATGCAAGTCGAGCGAGGGGC   TTCGGCTCC  T AGCGGCGAACGGGTGAG  54
TTAACACATGCAAGTCGAACGATGAACCACTTCGGTGGGGATTAGTGGCGAACGGGTGAG  97
                    *   * ** ***     ****** *  *

TAACACGTGGGCAACCTGCCCCGAGCTCTGGAATAACCTCGGGAAACCGGGGCTAATGCC 114
TAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAACGGGGTCTAATACC 168
               *      ****      * *  * * *      *   *     *

GGATA TGACGCTACCGGGCATCTG GTGGTGTGGAAAGATTTATCGGCTCGGGATGGGC 172
GGATACTGACCCTCGCAGGCATCTGCGAGGT TCGAAAGCTCCGGCGGTGAAGGATGAGC 227
     *    *  ** *         * *   * *     * ****   ****     *

CCGCGGCCTATCAG                                               188
CCGCGGCCTATCAG                                               241
```

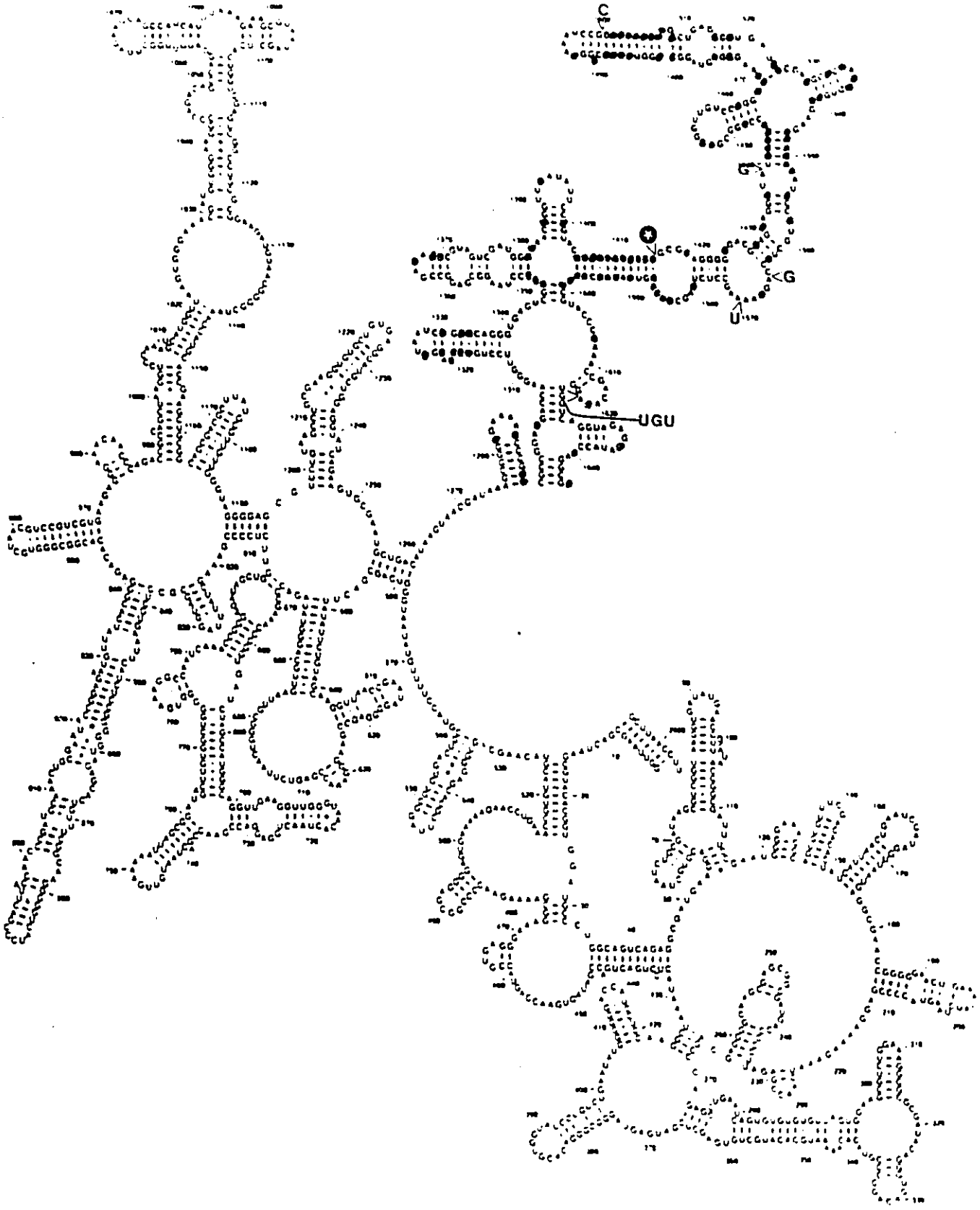
FIGURE 10: SECONDARY STRUCTURE COMPARISON

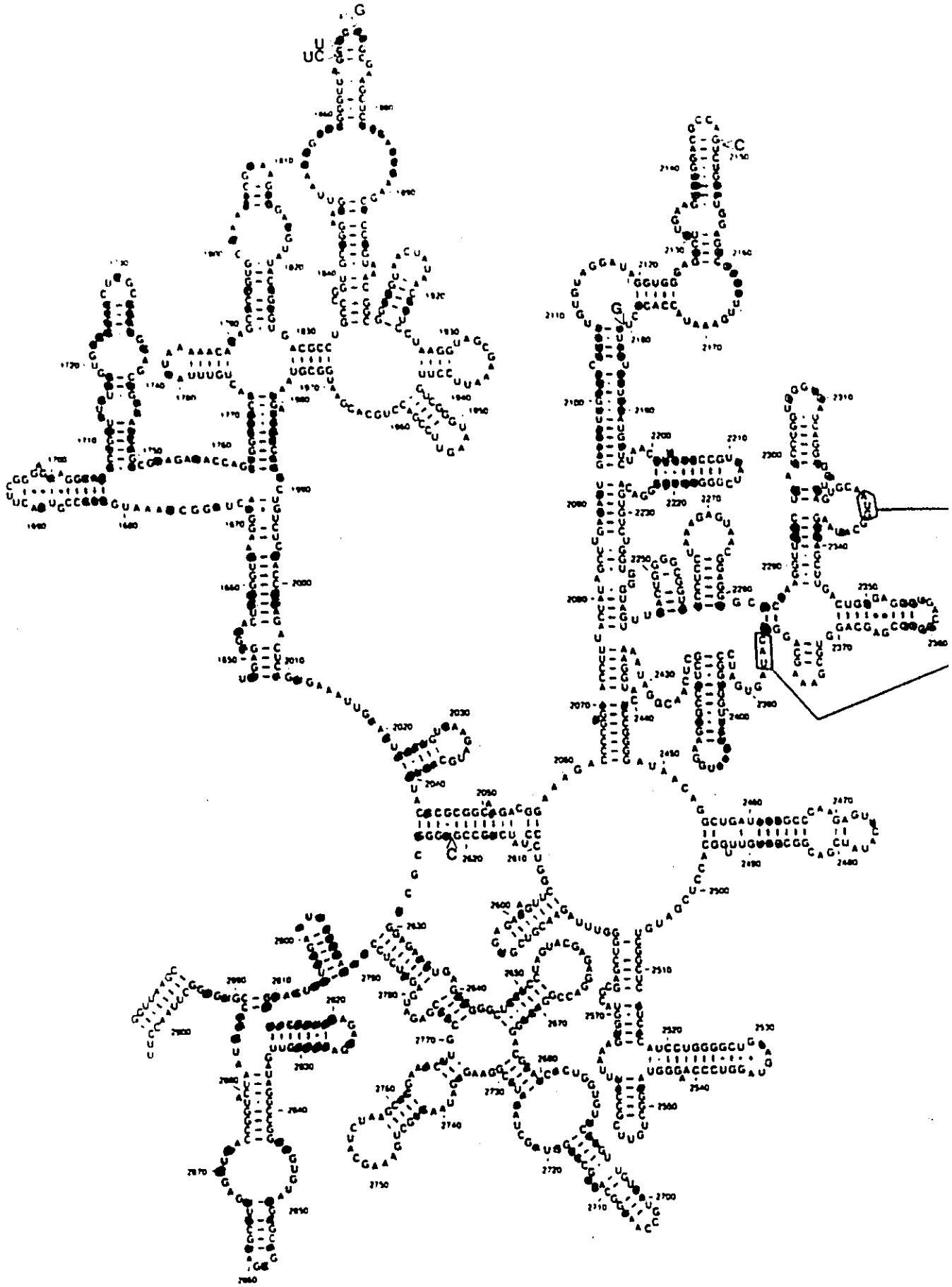
pAnod 4 was laid over the secondary structure of *E. coli* 23S rRNA (Gutell and Fox, 1988) as described in this work. The changes to the *E. coli.* sequence are indicated as follows:

blue	-nucleotide change
green	-compensatory change (includes G-U to G-C)
red	-deletion
orange	-no change
>	-insertion

note that ✪ indicates a 105 bp. insertion

[Figure on overleaf]


C
G
G
U
UGU



using a PCR-based approach. This may be done by PCR amplifying the region containing the insert and comparing it to the PCR product of various bacterial species for the absence or presence of the insert.

As a result of the changes depicted above, it is believed that the secondary structure for pAnod 4 would deviate from that of *E. coli* but we are not sure to what extent since the software required for such analysis was not available to us and a full length clone was not obtained.

3.9.2 pAnod 2 and pAnod 5 Sequence Analysis

pAnod 2 exhibited greater than 85% homology to various plant 25S rRNA genes over a region of 467 nucleotides (Figure 9b). However, this high homology was found to occur over too short of a region to provide sufficient information for secondary structure comparison.

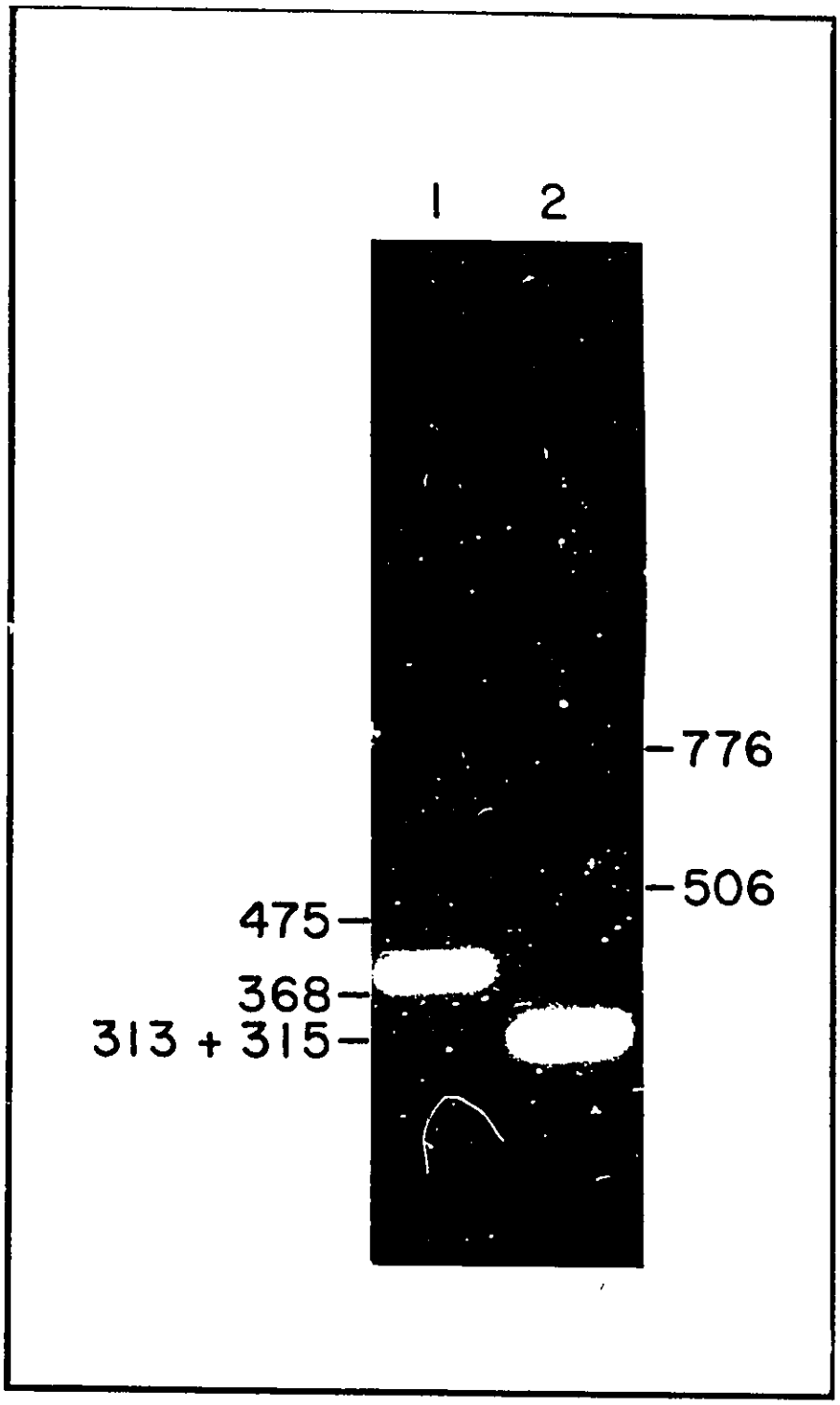
Primary sequence analysis of pAnod 5 revealed a 65-85% homology to various bacterial 16S rRNA genes over regions of 85-187 nucleotides (Figure 9c). Since this clone is only 187 bp overall and the regions of homology are short, further secondary structure comparison was not warranted.

FIGURE 11: PCR AMPLIFICATION

PCR amplifications were performed on *E. coli* and *Frankia* DNA as described in the Materials and Methods. Following PCR amplification, the products were electrophoresed on a 1.5% agarose gel.

Lane 1. *Frankia* PCR product

Lane 2. *E. coli* PCR product


1
2
776
506
475
368
313 + 315

3.10 Results of PCR Amplification

As suggested previously, PCR may be useful for determining which bacterial species contain the additional sequences found in the 23S rRNA gene of *Frankia* between nucleotides 121 and 228 and also whether the presence or absence of this insert may be useful for bacterial classification. In order to test this hypothesis two primers were designed by searching for regions of homology between *Frankia* and *E. coli* sequences (see Materials and Methods for the sequence and position of primers).

Following PCR amplification, bands with sizes of approximately 400 bp for *Frankia* and 300 bp for *E. coli* were observed (Figure 11). These values compare well to the sizes predicted from sequencing. The difference in size is due to the insert in *Frankia* as seen from the sequence comparison in Figure 9a. This result suggests that it should be possible to survey various bacterial species for the presence or absence of the insert, to use the information for the purpose of classification and to investigate the evolution of 23S rRNA within this region of the gene. Recently, Stackebrandt *et. al.* (1991), reported that a region of the 23S rRNA in *Streptomyces* had a high potential for species definition. They found that this region had a high degree of variation making it an ideal target for diagnostic probes and selective PCR primers.

DISCUSSION

In summary, a cDNA library made from alder nodule poly A+ mRNA was constructed in the vector pGem4B. By screening replicate Southern transfers of digested plasmid DNA, through the use of a differential screening technique, clones thought to be nodule-specific were isolated. These DNAs were used to investigate RNA expression following infection of *Alnus incana* with *Frankia* isolate AcN14a. Three distinct patterns of expression were observed suggesting the isolation of three different clones each corresponding to a highly expressed message. However, sequencing of the cDNA inserts demonstrated unequivocally that the clones contain regions of rRNA. Although these findings are disappointing, there are certain positive aspects of this work. Also it is necessary to provide explanations for the results obtained.

The major hurdle to be overcome at the beginning of this work was the isolation of RNA from alder nodules. Unlike legume nodules, alder nodules present several additional challenges for the isolation of RNA. The extraction process was made difficult by the woody properties of the nodule and breaking open of the cells demanded a great deal of physical effort. Once the nodule cells were broken the presence of coloured compounds, probably phenolics, interfered with subsequent analyses. These compounds gave an orange colour to the RNA preparations and resulted in the RNA being "trapped" at the top

of the gel during electrophoresis. RNA migration through the gel was strongly affected. We were surprised to discover that fast freezing of nodule tissue in liquid nitrogen and storage at -80°C did not prevent degradation of the RNA observed when tissue was stored for greater than one month. This is in contrast to legume nodules and contributed to several unsuccessful attempts in the beginning. As indicated in the Results section, several attempts using different techniques were required before the successful isolation of alder nodule RNA was possible. In must be remembered that 250-300 infected plants were required in order to recover 1g of nodule tissue for RNA extraction. Therefore several thousand plants were grown during this study in order to successfully isolate total RNA by the method of Logemann *et al.* (see Figure 3 and Table II).

The isolation from a cDNA library of clones containing rRNA sequences may indicate that the polyA+ mRNA was contaminated with rRNAs. If so, then the ability of the oligo-dT cellulose column to purify polyA+ mRNA away from rRNAs was not perfect. Contamination of the polyA+ RNA with rRNA may be possible if the rRNA contains an A-rich region with sufficient homology to oligo-dT cellulose. The transcription of rRNA by reverse transcriptase with the oligo-dT primer used for cDNA synthesis could then occur using the same regions of homology, thus yielding ribosomal cDNA. It has been

demonstrated that this situation is possible (Sullivan *et al.*, 1980). In the future it may be possible to eliminate any contribution of rRNA by pre-hybridizing the filters with a 500 fold or greater excess of unlabelled rRNA (Sullivan *et al.*, 1980). A second possibility for the source of the contamination is the presence of DNA fragments in the RNA preparation. In the future it may be possible to eliminated this DNA by treating the RNA preparations with RNAse free DNAse.

Knowing that the clones are ribosomal in nature, it is still necessary to address the results obtained by Northern transfer. We observed two patterns of expression. The first showed an increase in signal intensity over time (pAnod4 and pAnod5) while the second showed a sharp increase followed by a decrease in signal over time (pAnod2). Initially these patterns were interpreted as indicating a late and early nodulin genes respectively.

pAnod4 and pAnod5 were both found to be bacterial in origin. As seen in Figure 5b, the size of the hybridizing signal for pAnod4 is 3,350 bases which is slightly larger than the size of the *E. coli* 23S rRNA (2,904 bases) as expected from the sequence data. In this analysis a constant amount of RNA was added to each track. Therefore the increase in signal with time observed for pAnod4 would indicate an increase in the bacterial RNA content of the nodules with time. This might be expected to occur as infection leads to proliferation of the

bacterial symbiont (Newcomb and Wood, 1987) resulting in an increase in the proportion of the nodule RNA being of bacterial origin. Although this explanation seems possible, there is no direct evidence for it in any actinorhizal system. In Figure 5c pAnod5 hybridizes strongly to an RNA with a size of 1,650 bases which is expected for a bacterial 16S rRNA (*E. coli* 16S RNA is 1,542 bases). The argument given above for pAnod4 would also apply to pAnod5. A second, weaker, signal at 2,110 nucleotides may represent cross-hybridization to the alder mitochondrial 18S rRNA (Soybean 18S RNA is 1,807 bases). Perhaps the increased expression observed relates to increased mitochondrial numbers in infected nodule cells (Newcomb and Wood, 1987).

pAnod2 was found to be of plant origin. As indicated in Figure 5a, strong hybridization to a band at 3,600 bases is observed. This would represent hybridization with the 25S rRNA of the host plant. pAnod 13 also hybridizes with a single species of 3,500 nucleotides (Figure 5d) and, although not sequenced, may represent an additional 25S rRNA clone. A second, weaker, band with a size of 2,000 bases was observed and may represent cross-hybridization to 18S rRNA. The pattern of expression observed for these clones showed an initial increase from root to 2 week samples followed by a decrease over time. As the bacterial component of the RNA increases with time, the plant

content as a percentage of the RNA may decrease over time. We postulate that it is this change in proportional content which is observed in Northern analysis since an equal amount of total RNA isolated from each time point was loaded onto the gel. However the initial increase would indicate a massive increase in rRNA expression in the plant in order to account for the strong response. This is hard to explain since rRNA represents approximately 90% of the total RNA in the cell (Lewin, 1990) and a massive turn on of these genes should not be observed.

The cDNAs which were isolated and characterized have been shown not to be nodule-specific; however, such cDNAs may have been cloned and are present in our cDNA library. The cDNAs investigated in this study represented clones which showed the largest difference in signal following differential hybridization. True nodule-specific clones may have exhibited a less dramatic change in signal and were therefore not picked for further analysis. This remains to be proved.

CONCLUSION

There remains a great deal of information yet to be discovered about the symbiotic association between Alder and its endophyte *Frankia*. The strategy used in this thesis in an effort to uncover some of the mysteries of this symbiotic association, though not successful, is believed to be sound and worth further attempts. It is unfortunate that what promised to be an exciting result did not occur.

We have however, developed a means to reproducibly isolate quality RNA in resonable amounts from alder nodules. We have also isolated a new block of *Frankia* sequence which may be useful for systematic work on *Frankia* and other bacterial species by a PCR-based approach.

If the expertise and experience obtained in this thesis are applied, the challenge of looking for a nodule-specific gene may once again be undertaken. Then it may be possible to acquire a molecular understanding of the genes involved in nitrogen fixation in actinorhizal plants and their regulation, which may help in the improvement and/or creation of new nitrogen-fixing plants by genetic engineering. This possibility was made more feasible by the report of the transformation of alder (Mackay et al. 1988). Isolation of nodulin genes from alder would represent the first step towards this possibility.

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APPENDIX I

SEQUENCE HOMOLOGY BETWEEN pAnod4 AND VARIOUS BACTERIAL SPECIES

Primary sequence homology between pAnod4 and 23S rRNA of 18 different bacterial species was determined as described in Materials and Methods. The 105 bp insertion of pAnod4 (see text) was excluded for this analysis in order to obtain more representative values for the various 23S rRNA homologies.

* -only a partial sequence for *Mycoplasma* 23S rRNA was available.

ORGANISM	POSITION	% HOMOLOGY
Micrococcus luteus	1490-3087	81.4
Escherichia coli	1290-2888	79.3
*Mycoplasma (PG50)**	2-866	78.5
Bacillus subtilis	3522-5123	77.3
Bacillus stearothermophilus	1323-2922	76.9
Pseudomonas aeruginosa	1289-2887	75.1
Leptospira interrogans	1373-2952	74.7
Pseudomonas cepacia	1271-2872	72.1
Ruminobacter amylophilus	1268-2861	71.6
Anacystis nidulans	1541-3120	71.4
Pivelíula marina	1293-2876	71.4
Flavobacterium odoratum	1298-2719	70.6
Rhizobium sphaeroides	4185-5769	70.1
Flavobacterium flexilis	1295-2815	70.0
Rhodobacter capsulatis	1305-2878	69.6
Thermus thermophilus	1328-2907	69.5
Methanococcus vannielii	1365-2956	61.9
Desulfurococcus mobilis	1484-3068	60.0

APPENDIX II

LOCATION AND SIZE OF VARIABLE REGIONS IN *E. coli* 23S rRNA

Coordinates for variable regions, according to the *E. coli* 23S rRNA sequence (Raue, 1988).

VARIABLE REGION	COORDINATES	NUMBER OF NUCLEOTIDES
V1	131-176	46
V2	271-365	95
V3	533-560	28
V4	636-655	20
V5	845-847	3
V6	931-933	3
V7	1020-1029	10
V8	1164-1185	22
V9	1371-1373	3
V10	1416-1419	4
V11	1521-1542	22
V12	1579-1586	8
V13	1707-1751	45
V14	1835-1905	71
V15	2127-2161	35
V16	2197-2226	30
V17	2626-2629	4
V18	2789-2810	22