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THE ISOLATION AND CHARACTERIZATION OF BRADYRHIZOBIUM JAPONICUM DNA SEQUENCES THAT ARE TRANSCRIBED SPECIFICALLY IN BACTEROIDS

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ISOLATION AND CHARACTERIZATION OF *BRADYRHIZOBIUM JAPONICUM* DNA SEQUENCES THAT ARE EXPRESSED SPECIFICALLY IN BACTEROIDS

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ABSTRACT

ISOLATION AND CHARACTERIZATION OF BRADYRHIZOBIUM JAPONICUM DNA SEQUENCES THAT ARE EXPRESSED SPECIFICALLY IN BACTEROIDS

By

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The formation of nitrogen-fixing nodules on the roots of soybean (Glycine max) after infection by the Gram negative bacterium Bradyrhizobium japonicum is a complex process which involves differentiation by both partners in the symbiosis. This interaction is a developmental process, known to require the induction of gene transcription in both organisms. A method was developed using differential hybridization techniques which permits the isolation of sequences from B. japonicum which are expressed specifically in bacteroids, the differentiated form of the bacteria found in nodules. Several sequences exhibiting bacteroid-specific expression were examined by deletion analysis for effects on the development of the symbiosis. Seven derivatives of wild-type B. japonicum strain 110d which had deletions of sequences exhibiting bacteroid-specific expression were constructed. In all cases the mutant bacterial strains were

fully competent for symbiosis as indicated by the ability to form nodules and to reduce exogenously supplied acetylene to ethylene. The growth rates of the deletion derivatives and the parental strain were found to be identical in both rich and minimal culture media. Expression of the bacteroid-specific sequences under various physiological conditions was examined and transcription of two sequences was shown to be induced by lowered oxygen tension. The DNA sequence of one of the regions exhibiting bacteroid-specific expression was determined and two divergent transcriptional initiation sites were localized by S1 nuclease protection analysis. The DNA sequence of the promoter regions of these two sequences does not resemble that of B. japonicum genes known to be involved in the symbiosis and thus defines a new type of symbiotically-regulated promoter.

for my wife, Pat, my son Thomas and my parents

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CHAPTER 1

Introduction

Biological nitrogen fixation is estimated to account for more than fifty percent of annual global nitrogen fixation (Hardy and Havelka, 1975; Burris, 1980). The ever-increasing demand for nitrogenous fertilizer and the high cost of industrial nitrogen fixation have prompted investigations aimed at improving the efficiency of the biological process (Hardy, 1980). Due to its direct impact on world agriculture, the nitrogen-fixing symbiotic association between leguminous plants and bacteria of the genera Rhizobium and Bradyrhizobium has been the focus of much research. An understanding of the molecular mechanisms by which the plant and bacterial symbiotic partners form a productive association is a necessary component of efforts to increase the efficiency of biological nitrogen fixation.

The development of an effective nitrogen-fixing symbiosis between the Gram negative soil bacterium Bradyrhizobium japonicum and its plant host, Glycine max (soybean), is a complex process involving the differentiation of both organisms. Studies have revealed a series of morphological, physiological and biochemical changes which take place during the development of root nodules, the specialized bacteria-containing "organelles" within which nitrogen fixation occurs. The attachment of

bacterial cells to the soybean root is the earliest event in the establishment of the symbiosis which exhibits specificity (Turgeon and Bauer, 1982; Dazzo and Gardiol, 1984). In the presence of the bacteria, growth in emerging root hairs is arrested at the tip causing a deformation of the hairs into the characteristic "shepherd's crook", effectively surrounding some sites of bacterial attachment (Turgeon and Bauer, 1985). The entrapped bacteria then directly penetrate the plant cell wall by a mechanism which is not well understood. Although the bacteria seem to partially degrade the host cell wall, they do not come into direct contact with the host cytoplasm but cause instead an invagination of the plant cell wall which elongates to form a tubular structure known as the infection thread (Turgeon and Bauer, 1985). The bacterial cells multiply within the infection thread which extends through the epidermal cell and ramifies into the cortical cell layers of the root. The mechanism by which infection thread formation occurs is of interest since the structure is not seen in invasion of legumes by pathogenic bacteria.

Another early response of the host plant is the dedifferentiation of certain cells in the inner cortex of the root. Studies on the R. meliloti/alfalfa symbiosis have shown that within 21-24 hours after inoculation, well prior to the arrival of the infection thread, a group of cortical cells begins to divide actively (Dudley et al., 1987).

When the infection thread reaches the meristematic region, some of the bacteria are released into the plant cytoplasm enclosed by the peribacteroid membrane which is of host origin (Brewin et al., 1985). The cells of most bacterial species now begin to visibly differentiate, increasing in size and DNA content (Bisseling et al., 1977; van den Bos et al., 1978). Among the physiological changes which have been documented are a decrease in the thickness of the bacterial cell wall (MacKenzie et al., 1973; van Brussel et al., 1977), a rapid increase in the amount and activity of nitrogenase (van den Bos et al., 1978), a decrease in protein synthesis (Bisseling et al., 1979), and the elaboration of a novel set of cytochromes (Appleby, 1969a,b). The accumulation of large amounts (up to 50% of bacteroid dry weight) of the storage polymer poly-β -hydroxybutyrate is perhaps the most conspicuous change visible microscopically (Klucas and Evans, 1968; Wong and Evans, 1980). The above-mentioned biochemical, physiological and morphological alterations during the development of the mature bacteroid-containing nodule led to efforts to identify both plant and bacterial genes which are involved in this process.

Solution hybridization studies by Auger et al. (1979; 1981) comparing mRNA populations from Bradyhizobium-infected and uninfected soybean roots demonstrated the existence of 20-40 nodule-specific transcripts. The protein products of such messages, termed nodulins, have been examined for a

number of legumes (e.g. Legocki and Verma, 1979, 1980; Bisseling et al., 1983, 1984; Lang-Unnasch and Ausubel, 1985, Lang-Unnasch et al., 1986; Mellor et al., 1986; Sengupta-Gopalan et al., 1986; Verma et al., 1986; Moerman et al., 1987). The biochemical functions of a few nodulins have been determined and include enzymes involved in nitrogen metabolism (Bergmann et al., 1983; Triplett, 1985; Sengupta-Gopalan and Pitas, 1986), carbohydrate metabolism (Thummler and Verma, 1987), membrane synthesis (Mellor et al., 1986), and control of oxygen tension (Appleby, 1984). Although some additional nodulins are thought to play a role in maintenance of nodule structure (Franssen et al., 1987), the majority of the nodulins have no known function (Verma et al., 1986). Since techniques have not yet been devised for re-introducing into plants specific mutations generated in vitro in cloned plant genes, it is not known whether any of the nodulins are in fact necessary or essential for the establishment of the symbiosis.

Although the number of plant genes induced specifically in root nodules is known, a good estimate of the number of bacterial genes involved in the symbiosis has yet to be obtained. Nonetheless, molecular genetic techniques have provided much more precise information on the nature of bacterial mutations which affect the symbiosis. Naturally occurring and chemically-induced ineffective strains were initially used in the development of a genetic system for rhizobia (summarized in Vincent, 1980). The demonstration

by Beringer et al. (1978) that the transposable element Tn5 could be transferred to and would function in rhizobia led a number of groups to use random transposon mutagenesis for the isolation of metabolic and symbiotic mutants (e.g. Buchanan-Wollaston et al., 1980; Cen et al., 1982; Rolfe et al., 1980; Duncan, 1981; Meade et al., 1982; Hom et al., 1984; Rostas et al., 1984; Chua et al., 1985; Applebaum et al., 1985; Regensburger et al., 1986; Wilson et al., 1987). The Tn5-mutagenized populations were then screened for the desired mutant strains by a variety of techniques.

The most intensively studied mutant strains have been those which were unable to fix nitrogen (Fix^-) or were unable to form root nodules (Nod^-). The nif genes are a subset of the fix genes which have homologues in the well-studied free-living diazotroph Klebsiella pneumoniae. Considerable sequence similarity exists between the nif genes in K. pneumoniae (reviewed in Dixon, 1984) and their homologues in Rhizobium and Bradyrhizobium. This similarity permitted the use of cloned K. pneumoniae nif genes as hybridization probes to identify the structural genes encoding nitrogenase (e.g. Ruvkun and Ausubel, 1980; Hennecke, 1981; Corbin et al., 1982), as well as other nif genes (e.g. Fuhrmann et al., 1985; Ebeling et al., 1987) and a nitrogen fixation specific regulatory gene (e.g. Szeto et al., 1984; Buikema et al., 1985; Fischer et al., 1986).

Nodulation (nod) genes were isolated by direct complementation of Nod^- mutants (Long et al., 1982; Downie

et al., 1983; Schofield et al., 1984; Kondorosi et al., 1984). The discovery that nod genes and other genes essential for nitrogen fixation (nif and fix genes) tended to be clustered (Corbin et al., 1982; Long et al., 1982) resulted in the use of site-directed mutagenesis (e.g. Ruvkun and Ausubel, 1981) of cloned DNA fragments to identify other genes involved in the symbiosis. Many groups studying a wide variety of rhizobia and bradyrhizobia have now used cloned genes as heterologous hybridization probes followed by site-directed mutagenesis to identify and characterize numerous nif, fix and nod loci (reviewed in Ausubel, 1984b; Ausubel et al., 1985; Downie and Johnston, 1986; and Kondorosi and Kondorosi, 1986).

Leigh et al. (1985) screened a population of transposon-mutagenized R. meliloti strains with the fluorescent stain Calcofluor to detect alterations in the production of extracellular polysaccharides (EPS). Finan et al. (1985) screened a similarly mutagenized collection of Rhizobium strains for loss of reactivity with a monoclonal antibody specific for the rhizobial cell surface and for insensitivity to a series of phages which infect Rhizobium. The mutant strains identified in these experiments failed to produce EPS and, when inoculated on plants, produced ineffective nodules which contained no bacteria. These observations indicate that exopolysaccharides play an important role in the development of the symbiosis and

demonstrated that plant and bacterial differentiation could be effectively uncoupled (Finan *et al.*, 1985).

Physiological experiments indicated that the bacterial microsymbiont provided the heme moeity of leghemoglobin, the abundant nodulin involved in maintenance of low oxygen tension (Nadler and Avissar, 1977). This notion that heme metabolism might play an important role in the development of the symbiosis was confirmed by Leong *et al.* (1982) who isolated the *R. meliloti hemA* gene, which catalyses the first committed step in heme biosynthesis. Strains carrying mutations at this locus produced ineffective nodules on alfalfa. Since rhizobia are obligate aerobes, mutations in components of the electron transport chain might well be expected to affect symbiotic competence. Guerinot and Chelm (1986) demonstrated, however, that a deletion in the *hemA* gene in *B. japonicum* produced 5-aminolevulinic acid (ALA) auxotrophy, yet the bacterial enzyme was not essential for the formation of effective nodules. These results may reflect host-dependent differences in the availability of plant ALA to the bacterium or may indicate the presence of a second as yet undescribed bacterial gene responsible for ALA synthesis. Recently, O'Brian *et al.* (1987) screened transposon-mutagenized *B. japonicum* on plates containing Nadi reagent (Marrs and Gest, 1973) to detect mutants with altered cytochrome oxidase activity. One cytochrome aa_3 mutant was obtained (O'Brian and Maier, 1987) as were other mutant strains which formed ineffective nodules and may

contain an insertion in a gene affecting heme biosynthesis (O'Brian et al., 1987).

Hybridization of cloned genes from the Agrobacterium tumefaciens chromosomal virulence region (chv genes) to R. meliloti gene banks identified regions of R. meliloti DNA that exhibited homology to these probes. These regions were then cloned and the corresponding chromosomal copies deleted from the R. meliloti genome (Dylan et al., 1986). Interestingly, these deletion strains produced ineffective nodules suggesting that Agrobacterium and Rhizobium possess structurally related genes which are necessary for the establishment of infection whether it ultimately leads to pathogenesis or to symbiosis.

An additional class of bacterial symbiotic mutants was discovered during investigations into the carbon metabolism of Rhizobium. Although mutations in a number of bacterial genes involved in carbon metabolism had no effect on the development of symbiosis (Ronson and Primrose, 1979; Duncan, 1981; Glenn et al., 1984) some mutants unable to transport C_4-dicarboxylic acids (dct mutants) in R. trifolii (Ronson et al., 1981), R. leguminosarum (Finan et al., 1983; Ronson et al., 1984; Arwas et al., 1985) and R. meliloti (Bolton et al., 1986; Engelke et al., 1987) formed ineffective nodules. Since carbon availability may limit nitrogen fixation (Hardy, 1975; Guerinot and Chelm, 1987), this carbon transport system may play a pivotal role in determining the efficiency of this process. The induction of the dctA gene

by growth on dicarboxylic acids was exploited by Ronson et al. (1987) to screen a Tn5-mutagenized population of R. meliloti cells for mutants unable to activate expression of a dctA-lacZ gene fusion. These mutants were shown to contain Tn5 insertions in the regulatory gene ntrA and to produce ineffective nodules on alfalfa. The ntrA gene product (NtrA) is a sigma factor (Hirshman et al., 1985) which confers specificity on core RNA polymerase for a particular set of promoters. These promoters also require activation by one of a group of regulatory proteins related to the nitrogen regulatory protein NtrC (Nixon et al., 1986; Ronson et al, 1987).

A final class of symbiotic mutants was discovered in studies of auxotrophic mutants of Rhizobium and Bradyrhizobium. Strains carrying mutations at some steps in presumed pathways for amino acid biosynthesis are symbiotically defective while strains carrying mutations at other steps in the same pathway or in other pathways are fully competent for symbiosis (e.g. Denarie et al., 1976; Pain, 1979; Wells and Kuykendall, 1983; Hom et al., 1984; Sadowsky et al., 1986). The mechanisms by which auxotrophy affects the development of the symbiosis are unknown.

As the review presented above indicates, a considerable number of genes essential for effective symbiosis have been identified. The complexity of the process is reflected in the variety of metabolic pathways which, when interrupted, produce a symbiotic phenotype. Despite the fact that in a

few instances the biochemical functions of some of these gene products are well characterized (e.g. the nitrogenase subunits, Brooks et al., 1985), little is known about how plant and bacterial gene products interact to promote or disrupt the development of the symbiosis. Several types of experiments have been undertaken which attempt to address this question. In one group of experiments the expression of nodule-specific plant genes (nodulins) has been examined when the infecting rhizobial strains carry particular mutations. Although there is significant variation in nodulin gene expression when different host plant/bacterial mutant combinations are compared, nodules incited by most Nif$^-$ and Fix$^-$ strains contain the majority of the nodulin gene products seen in wild-type nodules (Lang-Unnasch and Ausubel, 1985; Govers et al., 1985; Gloudemans et al., 1987). Ultrastructural analysis of of nodules incited by Nif$^-$ and Fix$^-$ strains supports this observation (Hirsch and Smith, 1987). Even the Exo$^-$ mutants which form neither infection threads nor bacteroids (Finan et al., 1985) incite nodules which contain six of the eleven nodulins examined (Lang-Unnasch et al., 1986). Only mutations in the nod genes appear to completely eliminate nodulin gene expression. Interpretation of experiments involving two interacting organisms is at best difficult, especially given the large numbers of genes being studied. Nonetheless, it has been possible to deduce an approximate temporal framework for the interaction of some bacterial and plant

gene products (Govers et al., 1985; Moerman et al., 1987; Morrison and Verma, 1987; Gloudemans et al., 1987; Govers et al., 1987; Lullien et al., 1987).

A second type of experimental approach utilized in examination of plant/bacterial interaction during symbiosis evolved from the observation that extracts from plants can affect bacterial gene expression (Mulligan and Long, 1985). The expression of the nodABC operon was shown to increase markedly when both a functional nodD gene was present and exudate from plant roots or seeds was added to the culture medium (Mulligan and Long, 1985; Rossen et al., 1985; Innes et al., 1985). The active compounds from several plant exudates have been purified and identified as substituted flavones, isoflavones or flavanones (Peters et al., 1986; Redmond et al., 1986; Firmin et al., 1986; Kosslak et al., 1987). Although these compounds are present in many families of plants and cannot therefore account for the specificity seen in the Bradyrhizobium and Rhizobium/legume interactions, it is the first example of communication between the two symbionts to be characterized at the molecular level.

The identification of the nodule-specific plant genes was based on their differential transcription when Rhizobium-infected and uninfected roots were compared (Auger and Verma, 1981). In contrast, most of the bacterial genes involved in the symbiosis were isolated by complementation of mutant strains. The transcription of symbiotically

important bacterial genes has been examined directly in the case of the nif genes (Corbin et al., 1983). The low levels of expression of nod genes and fix genes makes the transcripts difficult to detect and these genes have been monitored primarily by the use of lacZ fusions (Jacobs et al., 1985; Aguilar et al., 1985). Two recent reports by Renalier et al. (1987) and David et al. (1987) describe an analysis of transcription of the entire R. meliloti sym plasmid, yet there are no reports in the literature identifying symbiotically important bacterial genes solely on the basis of their nodule-specific transcription.

The major focus of this thesis project was the development of a method for isolating bacterial genes which are transcribed specifically in nitrogen-fixing soybean nodules incited by B. japonicum. Differential hybridization techniques (Sargent, 1986) have been used to isolate transcriptionally-regulated genes in a variety of eukaryotic organisms including Saccharomyces cerevisiae (St. John and Davis, 1979), Xenopus laevis (Sargent and Dawid, 1983), Aspergillus nidulans (Zimmerman et al., 1980) and Avena sativa (Hershey et al., 1984). The regulated genes were identified solely on the basis of differential transcription in distinct cell types or developmental stages. Since this approach had not been used previously to study development in prokaryotes, it was adopted in order to isolate new classes of genes involved in the B. japonicum/soybean symbiosis which might not have been found using the methods

employed by other researchers. It was anticipated that the highly transcribed *nif* genes would be among those isolated by such a method. It was also expected that additional symbiotically induced genes would be identified which are essential for growth and thus can not be mutated or are non-essential for development and therefore have no discernible phenotype when mutated. The promoter regions from such genes might well lead to the identification of regulatory mechanisms important to the development of the *Bradyrhizobium japonicum*/soybean symbiosis.

CHAPTER 2

Isolation of *Bradyrhizobium japonicum* DNA Sequences Transcribed Specifically in Bacteroids

Introduction

One key to understanding the genetic regulation of development in biological systems is the identification of genes which play a role in the developmental process. Two general approaches have been employed in the search for such genes: the isolation of mutants exhibiting a defect in the developmental program and the identification of genes whose expression is altered during development. The first method can detect only those genes which are dispensable for normal vegetative growth and which provide a detectable phenotype when mutated. The second method is less restrictive in that it can also identify essential genes and genes which are developmentally regulated but have no readily apparent mutant phenotype.

The study of development in prokaryotic systems has traditionally depended primarily on the isolation of phenotypic mutants (Dworkin, 1985). In *Bacillus subtilis* chemical mutagenesis has been the major tool used to identify more than fifty genetic loci which specifically affect endospore formation (reviewed in Losick and Youngman, 1984) and several of the genes corresponding to these loci

have been cloned (Dubnau et al., 1981; Ferrari et al., 1982; Shimotsu et al., 1983). Some understanding of the genetic control of the Rhizobium/legume symbiosis has also been achieved, principally through the use of random transposon mutagenesis (reviewed in Rolfe and Shine, 1984). The development of gene fusion techniques (Casadaban and Cohen, 1979) extended the use of transposon mutagenesis to allow the identification of genes which are transcribed only under specific conditions. Fusion techniques have proven useful for a wide variety of organisms including Bacillus subtilis (Perkins and Youngman, 1986), Myxococcus xanthus (Kroos and Kaiser, 1984), Caulobacter crescentus (Bellofatto et al., 1984), Agrobacterium tumefaciens (Stachel et al., 1985) and Saccharomyces cerevisiae (Ruby et al., 1983). Gene fusion techniques were also employed to isolate sets of DNA-damage-induced (din) genes in S. cerevisiae (Ruby and Szostak, 1985) and B. subtilis (Love et al., 1985) which were analogous to those isolated earlier from E. coli (Kenyon and Walker, 1980). Although both chemical and transposon mutagenesis have proven to be useful in the study of development, neither technique can be used to identify non-auxotrophic genes which are essential for growth of a haploid organism.

Another general method for identifying developmentally regulated genes involves differential hybridization techniques (St. John and Davis, 1979) which compare mRNA populations from physically separable cell types or

developmental stages. Radioactively-labelled probes prepared from these mRNAs have been used to screen genomic or cDNA libraries for sequences which have altered levels of expression. This approach has been successfully employed in the study of differentiation in Saccharomyces cerevisiae (Clancy et al., 1983), Aspergillus nidulans (Zimmerman et al., 1980), and Xenopus laevis (Sargent and Dawid, 1983).

The lack of an efficient method for purifying mRNA from bacteria has limited the use of of differential hybridization in the study of development in prokaryotes. The principal problem encountered when hybridization probes are prepared from total bacterial RNA is the high level of background hybridization. This background hybridization is probably due to homology between the stable RNA (mostly rRNA) of the organism being investigated and the rRNA of the E. coli cells being used to propagate the library. The experiments described in this chapter were designed to determine whether removal of sequences derived from reverse transcription of rRNA (referred to hereafter as rcDNA) from cDNA probes made from total bacterial RNA would make these probes suitable for differential hybridization experiments. Experiments described here demonstrate that when depleted of rcDNA sequences, such probes can be used to identify genes which are transcribed at different levels in two bacterial cell types. The model system compares gene expression levels in free-living cells of Bradyrhizobium japonicum to that in purified bacteroids, a highly differentiated form of

the soybean microsymbiont isolated from nitrogen-fixing nodules.

Materials and Methods

Chemicals and Enzymes. Hydroxylapatite and Bio-gel P-60 were purchased from Bio-Rad (Richmond, CA); reverse transcriptase from Life Sciences, Inc., (St. Petersburg, FL); lysozyme, ribonulcease A and deoxyribonuclease I from Sigma Chemical Co. (St. Louis, MO); deoxyribonucleic acid (isolated from salmon testes) from Pharmacia P-L Biochemicals (Piscataway, NJ); formamide GR from EM Science (Gibbstown, NJ) and bovine serum albumin, Faction V from U.S. Bichemical Corp. (Cleveland, OH). These products were used according to the specifications of the manufacturer.

Bacterial Strains. The Escherichia coli K-12 strain ED8654 (gal met hsd_K supE supF) (Borck et al., 1976) was used for routine plasmid construction, maintenance and purification. The lambda phage library was propagated on E. coli strain K802 (lacY met $hsdR_K$ galK supE). BJ110d, a small colony isolate of Bradyrhizobium japonicum USDA 3I1b110 (Kuykendall and Elkan, 1976; Meyer and Pueppke, 1980), was used in all experiments.

Plasmids and Phage. Plasmid pRJ676-1 contains 2.1 kb of upstream sequence and the 5' portion of the coding region

for nifD, one of the nitrogenase structural genes (Adams and Chelm, 1984). Plasmid pBJ53A contains the entire coding region for glutamine synthetase, glnA, (Carlson et al., 1985). Plasmid pBJ110 contains the entire coding region for 5-amino levulinic acid synthase hemA (Guerinot and Chelm, 1986). Plasmid pRNA404 contains the entire ribosomal RNA operon from Anacystis nidulans (Golden and Sherman, 1983). Plasmid pLAFRI is a wide host range cosmid vector (Friedman et al., 1982). The B. japonicum genomic library in bacteriophage lambda was constructed as described (Carlson et al., 1985). Total B. japonicum genomic DNA, partially digested by restriction endonuclease MboI, was ligated with lambda cloning vector BF101 DNA (Maniatis et al., 1982) which had been digested with restriction endonuclease BamHI. After ligation, the DNAs were packaged in vitro into lambda phage particles as described (Hohn, 1979) and plated on E. coli strain K802. 1.4 x 10^5 plaques were obtained. Assuming that the B. japonicum genome is no larger than 10^5 kb and that the minimum insert size in the recombinant phage is 6 kb, the complete genome should be represented with a probability of 0.99 by 7,700 phage. The B. japonicum genomic library in cosmid vector pLAFR1 was constructed as described (Adams et al., 1984). Total B. japonicum genomic DNA, partially digested with restriction endonuclease EcoRI, was ligated with EcoRI-cleaved, phosphatase-treated pLAFR1 DNA at a ratio of 1:5 (Maniatis et al., 1982). After ligation, the DNAs were packaged in vitro into lambda phage

particles as described (Hohn, 1979) and E. coli strain ED8654 was infected. Tetracycline resistant transductants were replicated in an ordered array in 96-well polycarbonate microtiter dishes containing LB medium with 15% glycerol (v/v), incubated at 37°C for 24 h and frozen at -80°C. For screening, the cosmid-containing isolates were replica plated onto cellulose nitrate filters which had been placed on agar-solidified media. Plates were incubated at 37°C.

Bacterial Growth Conditions. B. japonicum was grown at 30°C in yeast extract broth as described (Adams et al., 1984) except that mannitol was substituted for xylose. E. coli strain K802 was grown at 37°C in TB medium (Miller, 1972). E. coli strain ED8654 was grown in LB medium (Davis et al. 1980) at 37°C.

Nodule Bacteria Fractionation. The total B. japonicum population from frozen 5-6 week-old soybean nodules was isolated as described by Adams and Chelm (1984). Three developmental fractions representing bacteroids, transforming bacteria and nodule bacteria were isolated on a discontinuous sucrose gradient as described by Ching et al. (1977) scaled up for use in a Beckman 14 Ti zonal rotor as described by Carlson et al. (1985). In each of two separate experiments, 150 grams of nodules were macerated and the bacterial cells subjected to two rounds of sucrose gradient purification. This procedure yielded an average of 1.0, 1.1

and 3.5 grams of cells in the bacteroid, transforming bacteria and nodule bacteria fractions respectively, which in turn yielded an average of 0.7, 1.2 and 5.1 mg of total RNA.

RNA Methods Total bacterial RNA was isolated as described by Adams and Chelm (1984). Discontinuous CsCl gradients consisting of a 5.0 ml cushion of 5.6 M CsCl overlayed with no more than 13.4 ml of cell homogenate was subjected to centrifugation at 18°C in a Beckman SW-28 rotor at 22,500 rpm for 36-40 hours. Gels for northern hybridizations were prepared as described (Maniatis et al., 1982). RNA (1 ug) was size fractionated by electrophoresis on a denaturing 0.9% agarose gel made using buffer containing 6% (v/v) formaldehyde; 20 mM sodium 3-[N-morpholino]propanesulfonic acid (MOPS), pH 7.0; 5 mM sodium acetate; and 1 mM (ethylenedinitrilo)-tetraacetic acid (EDTA). The RNA samples were dissolved in gel buffer which also contained 60% (v/v) formamide and 5% (v/v) glycerol. The samples were heated to 55°C for 15 minutes prior to loading on the gel which was subjected to electrophoresis at 100 volts (35 mA) for four hours. During electrophoresis the gel buffer was recirculated at a rate of 5 ml/min using a peristaltic pump. After electrophoresis, a portion of the gel was stained with ethidium bromide for visualization of the RNA. The RNA in the remaining portion of the gel was transferred onto Biodyne membrane (Pall Ultrafine Filtration Corp., Glen

Cove, N.Y.) by the method of Southern (1975) using 20X SSC buffer (1 X SSC = 0.15 M NaCl, 0.015 M sodium citrate [pH 7]). The filter was dried at 80°C *in vacuo* for 60 min and hybridized with radioactively labelled probes under the following conditions which were specified by the manufacturer. Hybridization buffer contained 50% formamide (v/v), 5X SSC, 1X Denhardt's solution (0.02% Ficoll [w/v], 0.02% polyvinylpyrrolidone [w/v], and 0.02% bovine serum albumin Pentax Fraction V [w/v] in water), 30 mM Tris (pH 7.5), 0.1% sodium lauryl sulfate (SDS), 1 mM EDTA, and 100 ug/ml denatured salmon sperm DNA. The filter was pre-hybridized with hybridization buffer (0.1 ml/cm^2) for four h at 37°C. Fresh buffer and approximately 3 x 10^6 cpm of ^{32}P-labelled DNA probe was added and hybridized for 16-20 h at 37°C. After hybridization, the filters were washed in 200 ml of 2X SSC, 0.1% SDS at room temperature for twenty min with four changes of buffer followed by washing in 200 ml of 0.1X SSC, 0.1% SDS at 42°C for 1 h with four changes of buffer. The radioactively labelled probes adhering to the filters were then visualized by autoradiography.

DNA Methods. Isolation and purification of recombinant lambda phage and extraction of phage DNA were performed as described (Davis *et al.*, 1980). Plasmid DNA was isolated from *E. coli* by CsCl-ethidium bromide centrifugation (Clewell and Helinski, 1972). Total genomic DNA from *B. japonicum* was isolated by a modification of the method of

Marmur and Doty (1962). Five ml of a culture of B. japonicum grown in the presence of 10 mM KNO_3 was made to 0.1% (w/v) with N-lauroylsarcosine, mixed briefly by vortexing and the cells pelleted by centrifugation. The cell pellet was resuspended in a solution of 25% sucrose (w/v) to which was added 200 ul of a 10 mg/ml solution of lysozyme in water. After incubation for 3 min at room temperature, 3 ml of 25% sucrose (w/v) 185 ul of 0.2 M EDTA were added. The cells were lysed by the addition with gently mixing of 0.5 ml of a 10% (w/v) solution of N-lauroylsarcosine and RNA was hydrolysed by the simultaneous addition of 1 ul of a 10 mg/ml solution of ribonuclease A in water. After cell lysis was complete as indicated by the clearing of the solution, the lysate was extracted with 4 ml of phenol which had been redistilled and equilibrated with water and 4 ml of chloroform. After mixing the aqueous and organic phases were separated by centrifugation at 3,000 x g for 10 min at 4°C. The aqueous phase was retained and re-extracted with phenol/chloroform as outlined above until a whitish interface was no longer visible between the two phases. The aqueous phase was then extracted twice with 4.0 ml of chloroform alone and then the nucleic acids precipitated by the addition of one tenth volume of 3 M sodium acetate (pH 5.2) and two volumes of 100% ethyl alcohol. The DNA pellets were resuspended in an appropriate volume of 10 mM Tris (pH 8) and 1 mM EDTA. DNA was transferred to cellulose nitrate filters from agarose

gels by the method of Southern (1975), from phage lambda plaques by the method of Benton and Davis (1977) and from E. coli or B. japonicum colonies by the method of Grunstein and Hogness (1975). Hybridization of radioactively-labelled probes to the DNA affixed to the filters, washing of the filters and autoradiography was performed as described (Adams and Chelm, 1984; Adams et al., 1984). Before hybridization, filters were incubated for 1 h at 65°C in 5X Denhardt's solution, 5X SSPE (1X SSPE = 0.18 M NaCl, 10 mM $NaPO_4$ [pH 7.7], 1 mM EDTA), and 100 ug/ml sheared and denatured salmon sperm DNA. ^{32}P-labelled hybridization probes were hybridized with the filters for 20 to 24 h at 65°C in 5X SSPE, 1.5X Denhardt's solution and 100 ug/ml salmon sperm DNA. After hybridization, filters were washed for 30 min at room temperature in 200 ml of 2X SSPE containing 0.1% SDS (w/v) with one change of buffer followed by washing for 30 min at room temperature in 200 ml of 0.1X SSPE containing 0.1% SDS (w/v) with one change of buffer. The isolation of DNA fragments for use as hybridization probes has been described (Adams and Chelm, 1984).

cDNA Synthesis. The DNA polymerase isolated from RNA tumor virus particles is able to efficiently synthesize complementary DNA (cDNA) if a suitable primer is hydrogen bonded to an RNA (or DNA) template (Taylor et al., 1976). Random oligonucleotide primers were prepared by DNase I digestion of salmon sperm DNA as described elsewhere

(Maniatis et al., 1982). RNA (1 ug) and 125 ug of primer DNA were combined in 12 ul of water, heated to 95°C for 10 minutes and then allowed to cool to room temperature. The final reaction conditions for cDNA synthesis in a 25 ul volume were: 50 mM Tris-HCl (pH 8.0), 1 mM dithioerythritol (DTE), 5 mM $MgCl_2$, 40 mM KCl, 1 mM each of dATP, dGTP, dTTP and 50 uM dCTP. 50 uCi of ^{32}P-labelled dCTP (15.17 TBq/mmole, Amersham Corp., Arlington Heights, IL.) and 30 units of reverse transcriptase (Life Sciences, St. Petersburg, FL.) were then added and the reaction incubated at 37°C for 60 min. The synthesis reaction was stopped by the addition of 2 ul of 0.5 M EDTA (pH 8.0). The template RNA in the RNA:DNA hybrids resulting from this reaction was hydrolysed by the addition of 2.5 ul of 3.0 M KOH followed by incubation at 37°C for 18 h. Unincorporated and newly hydrolysed nucleotides were removed by chromatography on a 2 ml Bio-gel P-60 column equilibrated in 10 mM Tris-HCl (pH 8.0), 1mM EDTA. A cDNA synthesis reaction typically yielded approximately 0.5 ug of ^{32}P-labelled cDNA having a specific activity of 5 x 10^7 cpm/ug. When sized by electrophoresis on 5% acrylamide-8 M urea gels, the cDNA had an apparent size range of 50 to 400 nucleotides. To assess the ability of the cDNA to efficiently hybridize with RNA, RNA-driven solution hybridization experiments were conducted as described by Chelm and Hallick (1976). Approximately 85% of the cDNA hybridized with an apparent $Rot_{1/2}$ of 0.003

indicating that the cDNA represented primarily the abundant, stable RNA species.

Solution Hybridization and Competition Hybridization. Approximately 0.5 ug of radioactively-labelled cDNA (3-4 x 10^7 cpm) was hybridized with 100 ug of pBJ142 plasmid DNA which had been sheared by four ten-second bursts at 50 watts on a Sonifier Cell Disruptor equipped with a microtip (Heat Systems-Ultrasonics, Inc., Plainview, NY). A sample of the sheared plasmid DNA was size fractionated on an agarose gel and had an apparent modal size of 500 bp. The sheared plasmid DNA and the cDNA in water were heated to 95°C in a tightly capped microfuge tube for ten minutes, then placed at 74°C in buffer containing 0.3 M NaCl, 5 mM Tris-HCl (pH 7.4), and 1 mM EDTA as described by Chelm et al. (1977). The temperature used in the hybridizations (74°C) was 25°C below the melting temperature (Tm) of the B. japonicum DNA as estimated by the method of Bishop (1972) using a guanine plus cytosine (G+C) content of 64% as determined by Elkan and Usanis (1971). (Tm = 16.6 logM + 0.41[G+C] + 81.5 where M = the concentration of salt in the reaction mixture). The hybridization reaction was allowed to proceed to a Cot of 1 (molar nucleotides - seconds) in order to permit the radioactively-labelled rcDNA sequences to hybridize to complete kinetic termination with the excess unlabelled rDNA plasmid DNA. In a typical solution hybridization reaction, the driver rDNA plasmid DNA was present at a concentration

of 450 ug/ml of which only 31.5% (7.5 kb of one DNA strand from 11.9 kb of double-stranded plasmid) is effective driver DNA. This effective driver DNA concentration (142 ug/ml) is equivalent to 4.3×10^{-4} molar nucleotides (1 ug/ml DNA = 3.03×10^{-6} M nucleotides). To achieve a Cot = 1 (Co = nucleic acid concentration in molar nucleotides, t = time in seconds), the nucleic acids must be hybridized for $1/4.3 \times 10^{-4}$ seconds = 38.8 min. Double-stranded nucleic acids were then removed by chromatography on a 1.0 ml hydroxylapatite column at 60°C. The column was washed six times with 1.0 ml aliquots of 0.12 M sodium phosphate (pH 6.8) at 60°C to elute the single-stranded nucleic acids. Aliquots of each fraction were analysed by liquid scintillation counting and the peak ^{32}P-containing fractions were pooled.

For lambda library screening experiments, rDNA plasmid DNA was used to block the hybridization of any rcDNA sequences remaining in the probes with the E. coli rDNA or rRNA had been present in the plaques and was transferred to the cellulose nitrate filters. The pBJ142 plasmid DNA was sheared and denatured as outlined above and included at 50 ug/ml in both the pre-hybridization and the hybridization solutions.

Results

Isolation and Characterization of the rDNA Genes of B. japonicum. A genomic library of B. japonicum DNA was screened for recombinant phage carrying the rDNA genes by plaque hybridization. The filters were probed using 5 x 10^6 CPM of cDNA synthesized using total RNA isolated from purified bacteroids as template. As expected, the probe hybridized to all plaques since E. coli rDNA is always present. However, four individual plaques gave signals which were well above this constant background (Fig. 1). Restriction endonuclease digests of phage DNA purified from these plaques revealed two common EcoRI fragments and several different EcoRI fragments. One phage (LrD1) was further characterized by hybridization of bacteroid cDNA to Southern blots of phage DNA digested with various restriction enzymes to better localize the region containing the rDNA genes. The region hybridizing to the probe was found to reside on a 7.6 kb BamHl fragment. This fragment was subsequently subcloned into the BamHl site of plasmid pBR322 resulting in plasmid pBJ142.

To verify that the cloned DNA actually contained rDNA sequences, hybridization probes were prepared by nick-translation of fragments of the plasmid pRNA404 (Golden and Sherman, 1983) which contains a copy of the rDNA operon from Anacystis nidulans. These probes permitted

Figure 1. Genomic library of *B. japonicum* 110d in lambda vector BF101 probed with 5 x 10^6 CPM of cDNA synthesized from bacteroid RNA. Filter A is from the initial screening of the library and Filter B is from a re-screening for plaque purification. Arrows point to plaques containing the rDNA operon.

Figure 2. Northern blot of total *B. japonicum* 110d RNA (1 ug and 0.5 ug in adjacent lanes) probed with (A) the 1.3 kb BglII fragment of plasmid pBJ142 which contains a portion of the 16S RNA coding region or (B) the 1.4 kb PstI fragment of plasmid pBJ142 which contains a portion of the 23S RNA coding region. (See Fig. 3 for restriction map of plasmid pBJ142.)

Figure 3. Restriction map of the *B. japonicum* rDNA operon cloned on a 7.5 Kb BamH1 fragment inserted into cloning vector pBR322 to make plasmid pBJ142. A = AvaI, B = BamH1, Bg = BglII, E = EcoR1, K = Kpn1, P = Pst1, Rv = EcoRV, S = Sph1, X = Xho1. No restriction sites for Cla1, Sal1, or Xba1.

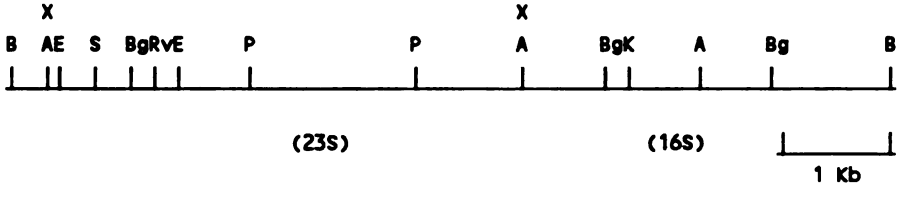
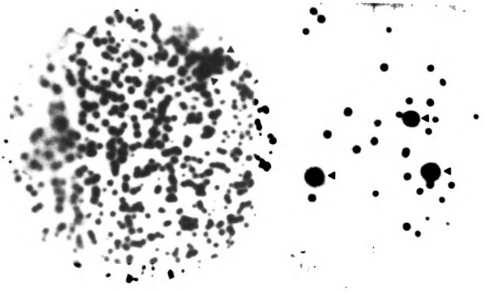
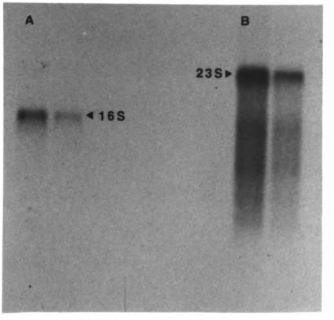


Figure 4. Determination of *B. japonicum* rDNA operon copy number. Total genomic DNA of *B. japonicum* digested with various restriction enzymes and probed with 5 x 10^6 cpm of randomly primed cDNA synthesized using RNA isolated from *B. japonicum* cells grown in culture. Arrows are placed beside bands expected to be internal to the rDNA operon. Bg = BglII, E = EcoRI, Rv = EcoRV, P = PstI.

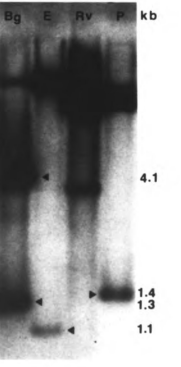
1.



2.



4.



localization of 16s and 23s RNA homologies within the B. japonicum clone. The DNA fragments corresponding to these two B. japonicum regions were then labelled and used to probe northern blots of total B. japonicum RNA (Fig. 2). These results confirmed that pBJ142 contains rDNA coding for 16s and 23s RNA. A restriction map is shown in Figure 3.

To determine the rDNA operon copy number of B. japonicum, total genomic DNA from B. japonicum was digested with restriction enzymes known to cut within the rDNA coding region. These digests were blotted onto cellulose nitrate filters by the method of Southern (1975) and probed with plasmid pBJ142 labelled with ^{32}P by nick translation. The existence of multiple rDNA operons would result in multiple restriction fragments hybridizing to the rDNA probe by virtue of the differences in DNA sequence flanking the rDNA operon(s). Only rDNA containing fragments of predicted sizes were detected indicating that B. japonicum contains a single rDNA operon (Fig. 4).

Preparation of rcDNA-depleted cDNA Probes. To assess the efficiency with which a particular protocol removed rcDNA sequences from random-primed cDNA, probes prepared by different methods were hybridized to digests of plasmids containing known B. japonicum genes which had been transferred to cellulose nitrate filters by the Southern transfer method. The three genes utilized were: (i) nifH, which encodes component II of nitrogenase (Adams and Chelm,

1984), (ii) the rDNA genes of B. japonicum, (iii) either hemA which encodes 5-amino levulinic acid synthase (Guerinot and Chelm, 1986) or glnA which encodes glutamine synthetase (Carlson et al., 1985). The nifH gene is known to be expressed at high levels and the hemA and glnA genes at low levels in bacteroids (Adams and Chelm, in press; J. Somerville, personal communication). Various protocols were compared for their ability to reduce the hybridization signal from the rDNA plasmid (pBJ142) to a level below that seen from the other known genes, indicating that the probe had been depleted of rcDNA sequences.

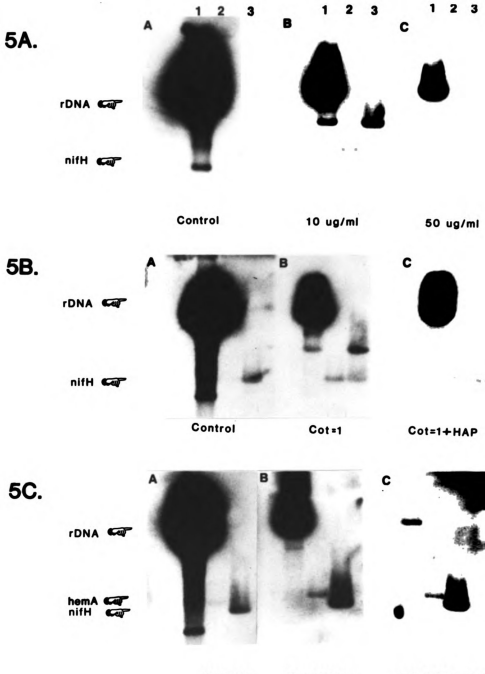
Three different methods for removing rcDNA sequences from the cDNA pool were devised. In one method, the rDNA plasmid (pBJ142) was sheared by sonication, denatured by heating and included as a competitor in both the pre-hybridization and hybridization solutions at 10 ug/ml or 50 ug/ml (Fig. 5A). The second method involved the hybridization of cDNA, in solution, to an excess of sheared and denatured pBJ142 (rDNA) plasmid to a Cot of 1. Since the $Rot_{1/2}$ of the fast-reacting component of the cDNA (presumably the rcDNA) is 0.003, these conditions permitted the rcDNA sequences to hybridize to complete kinetic termination. This entire reaction mix was then used as probe or, alternatively, aliquots were chromatographed on an hydroxylapatite (HAP) column to remove the double-stranded rDNA:rcDNA hybrids. When HAP column chromatography was performed, 10-15% of the radioactive label eluted in the

Methods for lowering the hybridization signal from rcDNA.

Figure 5A. Method 1. Restriction endonuclease digests of plasmids containing the rDNA (lane 1), glnA (lane 2) and nifH (lane 3) genes hybridized with cDNA. The control filter in panel A was hybridized with 5 x 10^6 cpm of randomly primed cDNA. The filters in panels B and C were hybridized in an identical manner, but with 10 and 50 ug/ml, respectively, of competitor rDNA plasmid DNA included in the pre-hybridization and the hybridization solutions.

Figure 5B. Method 2. Restriction endonuclease digests of plasmids containing the rDNA (lane 1), glnA (lane 2), and nifH (lane 3) genes hybridized with cDNA. The control filter in panel A was hybridized with 5 x 10^6 cpm of unfractionated cDNA. The filter in panel B was hybridized with 5 x 10^6 cpm of cDNA which had been previously hybridized with sheared and denatured pBJ142 DNA to a Cot = 1. In panel C the filter was hybridized with the single-stranded fraction (7 x 10^5 cpm) of 5 x 10^6 cpm of cDNA which had been previously hybridized to plasmid pBJ142 DNA to a Cot = 1 and then fractionated on a hydroxylapatite (HAP) column.

Figure 5C. Method 3. Restriction endonuclease digests of plasmids containing the rDNA (lane 1), hemA (lane 2), and nifH (lane 3) genes hybridized with cDNA. The control filter in panel A was hybridized with 5 x 10^6 cpm of unfractionated cDNA. The filter in panel B was hybridized with cDNA which had been previously hybridized in solution with rDNA plasmid DNA and chromatographed on HAP as in panel C of Figure 5B. The filter in panel C was hybridized with cDNA treated as in panel B, but with the addition of 50 ug/ml of competitor rDNA plasmid DNA in the pre-hybridization and the hybridization solutions. The glnA gene was replaced by the hemA gene in this figure since the latter gave a more readily detectable signal. In Figure 5C only, the autoradiograms in panels B and C were exposed six-fold longer than that in panel A.


5A.
A
1 2 3
B
1 2 3
C
1 2 3
rDNA
nifH
Control
10 ug/ml
50 ug/ml
5B.
A
B
C
rDNA
nifH
Control
Cot=1
Cot=1+HAP
5C.
A
B
C
rDNA
hemA
nifH
Control
Cot=1+HAP
Cot=1+HAP+50 ug/ml

low-salt, single-stranded fraction and was used as probe (Fig. 5B). Finally, since neither of the first two methods lowered the hybridization signal to the rDNA plasmid to an acceptable level, the three techniques of solution hybridization, HAP chromatography and competition solution hybridization were performed sequentially. This final approach did succeed in lowering the hybridization signal from the rDNA plasmid in Southern blot analyses to below that of the nifH signal and near that of the low abundance hemA signal (Fig. 5C, panel c). A method has thus been developed which allows the detection of relatively low level gene expression using randomly primed cDNA prepared from total bacterial RNA. Artifactual hybridization bands are visible in several lanes in Figure 5 (e.g. Fig. 5B, panel B, lanes 2 and 3). Hybridization in solution of rcDNA with sheared, denatured plasmid pBJ142 DNA produces some double-stranded hybrids which also contain single-stranded plasmid pBR322-derived sequences. The hybridization of these vector sequences (and the attached, labelled rcDNA sequences) to vector sequences on the filters produces the artifactual signals. Removal of the labelled hybrids by HAP chromatography results in removal of the artifactual signals (e.g. Fig. 5B, panel C, lane 3).

Screening the Phage Library for Bacteroid-Specific Transcription. The B. japonicum genomic library was screened for recombinant phage carrying B. japonicum DNA

sequences expressed specifically in bacteroids. Approximately 8,000 phage were screened by plaque hybridization. The probe used was cDNA synthesized using total bacteroid RNA as template. The background hybridization signal resulting from high levels of rcDNA was reduced by enriching for mRNA by sequential solution hybridization, HAP column chromatography and competition hybridization as described above. Thirty phage were isolated which gave signals above background. These thirty phage were dotted in an ordered arrays onto a TB medium solidified with agar and seeded with E. coli strain K802, incubated overnight at 37°C, and then transferred to cellulose nitrate filters. Plaque hybridization was performed on these filters using cDNA synthesized with either total RNA from purified bacteroids or total RNA from a pure culture of B. japonicum 110d grown in rich medium (YEM) as template. Of the 30 phage analysed, 16 appeared to contain sequences which were expressed at higher levels in bacteroids than in free living cells. An analysis of six recombinant phage in which four give stronger signals when probed with cDNA prepared from bacteroids than with cDNA prepared from cells grown in culture is presented in Figure 6.

To further examine the expression of these sequences, purified DNA was prepared from each of the 16 retained phage, digested with appropriate restriction enzymes, size fractionated by electrophoresis on agarose gels and then

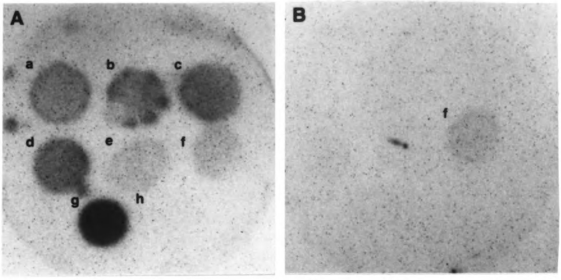


Figure 6. Rescreening of recombinant bacteriophage containing B. japonicum sequences which are expressed specifically in bacteroids. Arrays of recombinant phage were dotted onto TB medium which had been solidified with agar and seeded with E. coli strain K802, the plates incubated at 37°C overnight and DNA from the resultant plaques transferred to cellulose nitrate. Two filters were prepared from the same array of phage. Filter A was probed with mRNA-enriched cDNA synthesized using B. japonicum RNA isolated from purified bacteroids as template and filter B was probed with mRNA-enriched cDNA prepared from RNA isolated from B. japonicum cells grown in rich medium. The plaques labelled a - f were produced by recombinant lambda clones B16, B17, B22, B24, B26 and B27. Control plaques g and h were produced by recombinant phage NH-1 which contains the B. japonicum nifH gene and by lambda cloning vector BF101. The first four recombinant phage in the array presented above (B16, B17, B22 and B24) were classified as containing sequences induced in bacteroids and the latter two (B26 and B27) were not further characterized. (See Table 1 for a list of phage and corresponding cosmids).

transferred onto cellulose nitrate. Duplicate filters were probed with equal amounts of cDNA synthesized from either bacteroid RNA or RNA from free-living cells. The cDNA probes had been depleted of rcDNA sequences by solution hybridization and HAP column chromatography. The results confirmed that the sixteen phage analysed contained sequences which are expressed at higher levels in bacteroids than in free living cells. An analysis of seven recombinant phage which contain sequences transcribed at higher levels in bacteroids than in cells grown in culture is presented in Figure 7.

Identification of Cosmids Containing Sequences Expressed Specifically in B. japonicum Bacteroids. To facilitate comparisons between the B. japonicum sequences carried on the recombinant phage and previously cloned genes, DNA from each of the 16 phage described above was labelled by nick-translation and the labelled phage pooled in groups of four or five. These pools were used to probe an ordered B. japonicum genomic library constructed in the wide host range cosmid vector pLAFR1. Thirty-nine cosmids which gave positive hybridization signals were then replated in an ordered array and colony hybridizations were performed using each of the individual nick-translated phage singly as probe. Ten groups of cosmids were thus identified which contained B. japonicum sequences homologous to the phage inserts (Table 1). All thirty-nine cosmids were also

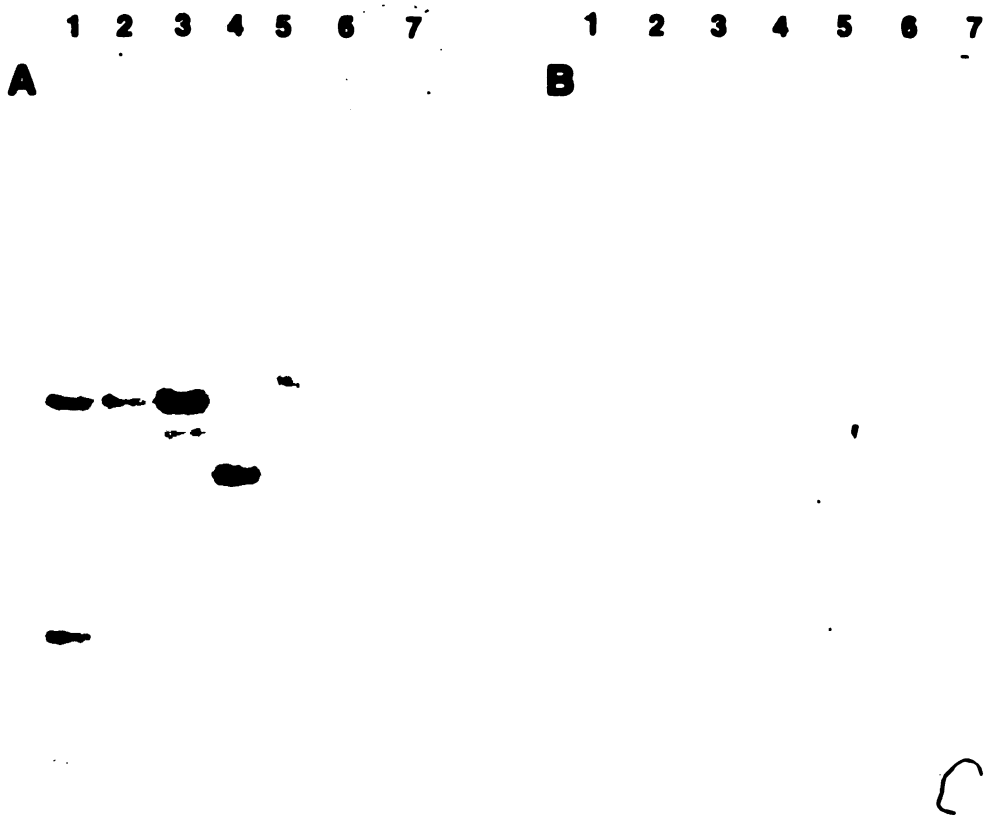


Figure 7. Restriction endonuclease digests of DNA purified from seven recombinant bacteriophage was size-fractionated on an agarose gel and transferred to cellulose nitrate. The resultant filters were probed with 5 x 10^5 cpm of mRNA-enriched cDNA synthesized using RNA isolated from either (A) purified B. japonicum bacteroids or (B) B. japonicum cells grown in culture. Lanes 1-7 on each filter contain DNA from recombinant bacteriophage B15, B16, B17, B19, B20, B22 and B24. (See Table 1 for a list of phage and corresponding cosmids).

Table 1. List of recombinant lambda phage containing B. japonicum sequences that are expressed specifically in bacteroids and the corresponding members of the cosmid library of B. japonicum containing these same sequences. The underlined cosmids were selected as representative of each group.

Phage #	Corresponding Cosmid(s)	Plasmid Subclones
B1	7-39, 7-75	
B2	11-78	
B5	4-51, 5-35, 7-41 8-10, 10-91	pBJ273
B6	15-75	
B9	7-39 (same as B1)	
B15	none	pBJ270
B16	2-56, 3-61, 13-36, 15-68	pBJ214 pBJ216
B17	none	
B18	same as B16	
B19	14-8	pBJ227
B20	same as B5	
B21	8-17, 8-62	
B22	1-20	
B24	4-14, 5-14, 6-94, 7-36, 7-92 8-11, 8-68, 8-76, 10-90, 12-66 (contain previously identified nifA and fixA genes)	
B25	12-37	
B30	same as B24	

Number of recombinant phage containing sequences exhibiting bacteroid-specific expression = 16
Number of unique phage = 12
Number of cosmid groups identified by unique phage = 10

screened by colony hybridization using plasmids containing previously identified *nif* genes (Hennecke, 1981; Adams *et al.*, 1984) and *fix* genes (Lamb and Hennecke, 1986). One of the ten groups of cosmids carrying sequences transcribed at high levels in bacteroids was shown to contain the *fix* genes, while the remaining nine groups appear to define new genes or operons which are expressed at higher levels in bacteroids than in cells grown in rich medium.

Discussion

Differential hybridization is a powerful technique which has been used successfully to isolate developmentally regulated genes in *Saccharomyces cerevisiae* (Clancy *et al.*, 1983), *Aspergillus nidulans* (Zimmerman *et al.*, 1980) and *Xenopus laevis* (Sargent and Dawid, 1983). Experiments described in this paper extend the use of this technique to the prokaryotic partner in the *Bradyrhizobium japonicum*/soybean symbiosis. Differential hybridization has traditionally been used primarily in eukaryotic systems where techniques exist for separating messenger RNA from rRNA and other stable RNA species (Aviv and Leder, 1972). The hybridization kinetics of the cDNA synthesized in our experiments to total bacterial RNA (85% of labelled cDNA hybridizing with a $rot_{1/2}$ = 0.003) confirm that the products synthesized consist mostly of sequences homologous to

abundant stable RNA species. Removal of the sequences homologous to the rDNA operon (rcDNA) by sequential solution hybridization, HAP chromatography and competition hybridization was sufficient to permit hybridization signals from highly transcribed mRNAs to be visualized above background. This result suggests that the rDNA homology between B. japonicum and E. coli can account for most of the high level of background hybridization which has previously prevented the use of differential hybridization.

In a recent report Gianni and Galizzi (1986) described the isolation of three genes expressed preferentially during Bacillus subtilis spore outgrowth. These investigators used excess vegetative RNA to compete out background hybridization when a lambda phage library was probed with end-labelled RNA from outgrowing spores. This method provides an example of how differential hybridization has been used to isolate genes which are expressed only under specific conditions in prokaryotes. To examine the possible use of this methodology for investigating the B. japonicum/soybean symbiosis, the hybridization signals produced by probes prepared by end-labelling RNA were compared with those produced by the cDNA method outlined above and were seen to be distinctly lower. Improvements in the sensitivity of these methods, however, should allow the identification of developmentally regulated genes.

We have isolated ten groups of cosmids which contain B. japonicum sequences which are transcribed at higher levels

in nitrogen-fixing bacteroids than in cells grown in culture. One group contains previously identified genes for nitrogen fixation (Lamb and Hennecke, 1986), but the remaining eight groups represent transcribed regions whose functions are as yet unknown. In B. subtilis, mutational analysis has identified more that fifty loci which are necessary for endospore formation (reviewed in Youngman et al., 1985). Since the differentiation of B. japonicum bacterial cells into nitrogen-fixing bacteroids presumably involves a large number of genes, the highly transcribed sequences isolated in this study probably represent only a small subset of the total number of genes which are transcritionally regulated during development. A better estimate of the number of genes involved in B. japonicum bacteroid development may be derived if additional methods can be found which further reduce the background hybridization signal resulting from rcDNA sequences.

The methods utilized to obtain these differentially expressed DNA sequences permits the isolation of both essential genes and genes which have no obvious phenotype when mutated. To analyze the function of these sequences genetically, B. japonicum strains were constructed in which several of the regions containing the differentially expressed sequences were individually deleted from the B. japonicum chromosome. In the following chapter the properties of these deletion derivatives are described.

CHAPTER 3

Genetic Analysis of *Bradyrhizobium japonicum* DNA Sequences That Are Transcribed Specifically in Bacteroids

Introduction

Although natural variants and chemically-induced mutants have been used in genetic analyses of the *Bradyrhizobium japonicum*/soybean symbiosis (Vincent, 1980), the most widely employed technique for identifying genes required for symbiosis has been transposon mutagenesis (Berg, 1977; Rolfe and Shine, 1984; de Bruijn and Lupski, 1984; Ditta, 1985). In Chapter 2 an alternative method for isolating genes specifically expressed in bacteroids was described. Differential hybridization was employed to identify sequences which are expressed specifically in *B. japonicum* bacteroids. This approach made the assumption that the rate of transcription of at least some symbiotically important genes changes during the developmental process. Examples of both repression and derepression of gene expression during development have been reported in a number of different eukaryotic systems (e.g. Firtel, 1972; Zimmerman *et al.*, 1980; Putzer *et al.*, 1984; Kurtz and Lindquist, 1984; Kopachik *et al.*, 1985).

Auger and Verma (1981) demonstrated that in the case of the *Rhizobium*/legume symbiosis, a set of plant genes (termed

nodulins) were specifically induced in developing nodules and were not present in uninfected roots. Regarding the bacterial partner of this symbiosis, it has been demonstrated that the genes encoding the subunits of the nitrogenase enzyme are highly induced during nodule development (Corbin et al., 1982). Studies in which western blots of total protein from bacteroids and free-living cells were probed with polyclonal antibodies raised against bacteroids demonstrated that a number of additional gene products are specifically induced in bacteroids (Verma et al., 1986). The likely importance of developmentally regulated gene expression prompted efforts to adapt differential hybridization techniques for use in studying the Bradyrhizobium japonicum/soybean symbiosis. Techniques which permit the deletion of particular DNA sequences were employed to examine the effect of removing from the B. japonicum genome several regions of DNA which are transcribed at high levels specifically in bacteroids.

Materials and Methods

Bacterial Strains. The E. coli strains are described in Chapter 2. BJ2101 is a derivative of BJ110d from which the nifA-like gene has been deleted (Adams, 1986). BJ271 is a derivative of BJ110d with an insertion of the neomycin

phosphotransferase gene (nptII) in the ntrBC operon (G. Martin and B.K. Chelm, unpublished).

Plasmids. The cosmid library of BJ110d DNA in vector pLAFR1 was described by Adams et al. (1984). Plasmid pRK2013 is a helper plasmid derived from RK2 (Ditta et al., 1980) which provides conjugal transfer functions. pDS4101 is a derivative of the ColK plasmid which helps mobilize pBR322 derivatives (Wolk et al., 1984). pRL161, pRL170 and pRL425 are positive selection cloning vectors (Elhai and Wolk, submitted) and pKC7 is a cloning vector which contains the nptII gene, which encodes kanamycin resistance (Rao and Rogers, 1979). The following plasmids contain B. japonicum DNA sequences: pBJ33 contains the nifH gene which encodes one of the subunits of nitrogenase (Adams et al., 1984); pBJ53A contains the glnA gene encoding glutamine synthetase I (Carlson et al., 1985); pBJ110d contains the hemA gene encoding 5-amino levulinic acid synthase (Guerinot and Chelm, 1986); pBJ196A contains the glnII gene encoding glutamine synthetase II (Carlson and Chelm, 1986); pBJ220 contains the recA gene (P. Hall, unpublished results). Plasmid JKI contains a 5.4kb HindIII fragment which carries the E. coli dnaK gene and plasmid pJK5 contains an EcoRI-ClaI internal fragment of the E. coli dnaK gene; both plasmids were a gift of C. Georgopoulos. The plasmid pDNAK1 contains a PvuI-EcoR1 internal fragment from the E. coli dnaK gene subcloned from plasmid JKI.

Bacterial Growth Conditions. B. japonicum was grown at 30°C in YEM (0.04% yeast extract [w/v], 1% mannitol [w/v], 3 mM K_2PO_4, 0.8 mM $MgSO_4$, 1.1 mM NaCl) or in YEGG (YEM with 0.5% gluconate and 0.1% glutamate replacing the mannitol). The minimal medium was described by Manian and O'Gara (1982) and contained xylose at 0.3% (w/v). For the low oxygen experiments, B. japonicum strain BJ110d was grown in YEM or YEM plus 10mM KNO_3 in 10 l fermenters sparged with a mixture of 0.2% oxygen (v/v) and 99.8% nitrogen (v/v) as described (Adams and Chelm, in press). For the nitrogen limitation experiments, BJ110d was grown in minimal medium with 1 mM NH_4Cl as sole nitrogen source as described (Carlson et al., 1987). For the heat shock experiments, BJ110d was grown in YEM at 30°C to an optical density of 0.3 at 420 nm at which time the cultures were shifted to 37°C and shaken vigorously for the designated lengths of time (5, 10, 30 or 60 min). The heat-shocked cells were then rapidly cooled in an ice-water bath, collected by centrifugation and frozen at -80°C.

DNA and RNA Methods. The methods for Southern transfer and colony hybridizations and for the isolation of plasmid and total genomic DNA were described in Chapter 2. The isolation of total bacterial RNA and cDNA synthesis were performed as described in Chapter 2. The cDNA used for probing Southern blots was depleted of rcDNA sequences by solution hybridization as described in Chapter 2, but since

the probes were hybridized to purified DNA, competitor rDNA plasmid DNA was omitted from the hybridization solutions. Slot blots of RNA samples were performed by the method of Schloss et al. (1984). The RNA samples were denatured by heating to 65°C in a buffer consisting of 50% v/v formamide, 6% v/v formaldehyde, and 20 mM $NaPO_4$ (pH 7.7) and then chilled on ice. The denatured RNA was diluted in cold phosphate buffer (10X SSC [1X SSC = 0.15 M NaCl, 0.015 M sodium citrate], 3% v/v formaldehyde, 20 mM $NaPO_4$ [pH 7.7]) and aliquots were transferred to cellulose nitrate using a Minifold II Slot-Blotter (Schleicher & Schuell, Keene, N.H.). The filters were then baked in vacuo at 80°C for two hours and probed with nick-translated DNA probes as described (Schloss et al., 1984).

Gene-directed Mutagenesis. Gene-directed mutagenesis was performed as described by Guerinot and Chelm (1986). Seven different plasmids containing B. japonicum DNA inserts in which the central portion had been deleted and replaced by the nptII gene were constructed in E. coli using standard recombinant DNA methods (Maniatis et al., 1982). The restriction fragments containing the nptII gene were isolated from plasmids pKC7, pRL161 or pRL170, depending on the restriction site requirements. Donor strains for conjugal matings were obtained by transforming the deletion-containing plasmids (pBJ241, pBJ242, pBJ243, pBJ261, pBJ281, pBJ282, pBJ283) individually into E. coli

strain HB101 which contained plasmid pDS4101. For the tri-parental matings, cells were mixed at a ratio of approximately 1 x 10^8 B. japonicum recipients to 5 x 10^7 HB101 donors to 5 x 10^7 HB101/pRK2013 helpers. The mating mix was spread on YEM plates and incubated for 4 days at 30°C. The cells were then resuspended in 5.0 ml of 0.01% Tween-80 and 200 ul aliquots were plated on YEGG agar containing kanamycin (150 ug/ml) and chloramphenicol (30 ug/ml), the latter to counterselect against the E. coli donor and helper cells. Kanamycin resistant colonies were then screened by colony hybridization for the absence of vector (pBR322) sequences. Since the deletion construction plasmids cannot replicate in B. japonicum, kanamycin resistant strains arise only when the plasmid containing the nptII gene integrates into the B. japonicum chromosome. An homologous double recombination event involving the flanking sequences on both sides of the nptII gene (see Fig. 2) effectively deletes a region of the B. japonicum chromosome and replaces it with the kanamycin resistance gene.

Plant Tests and Acetylene Reduction Assays. The ability of the mutated B. japonicum strains to form an effective symbiosis with soybeans (Glycine max, cv. Amsoy) as determined by nodule formation and nitrogen fixation was assessed as described (Guerinot and Chelm, 1986). Surface sterilized soybean seeds were inoculated with one of the B. japonicum 110d deletion derivatives, with wild-type BJ110d

or were left uninoculated. The seeds were then planted in modified Leonard jars (Vincent, 1970) using the nitrogen-free medium of Johnson et al. (1966) and incubated in a growth chamber under conditions described by Guerinot and Chelm (1986). After twenty-eight days the nodules were picked and their ability to reduce acetylene to ethylene was measured by the method of Hardy et al. (1968). Six to eight nodules from each plant were also surface sterilized and the resident bacteria were extracted and checked for phenotype as described by Guerinot and Chelm (1986).

Extraction of Poly-β-hydroxybutyrate (PHB) From B. japonicum Bacteroids. PHB is a storage polymer which can account for up to 50% of the dry weight of B. japonicum bacteroids (Klucas and Evans, 1968). PHB was extracted from frozen B. japonicum nodules by the method of Wong and Evans (1971) with the following modifications. Approximately 500 mg of nodules were ground in 5 ml of cold 0.05M Tris (pH 8.4) using a mortar and pestle. The homogenate was squeezed through four layers of cheesecloth into a 15 ml Corex centrifuge tube and subjected to centrifugation at 300 x g for 10 min at 4°C. The supernatant was transferred carefully to a clean 15 ml Corex centrifuge tube and recentrifuged under the same conditions. The supernatant was transferred again to a clean tube and the cells collected by spinning at 8,000 x g for 10 min at 4°C. The cell pellet was washed twice by being resuspended in 5.0 ml

cold (4°C) sterile water and again collected by centrifugation. The cell pellet was resuspended finally in 1.0 ml H_2O and transferred to a pre-weighed microcentrifuge tube (each microcentrifuge tube was heated to 85°C for 30 minutes, cooled to room temperature and weighed to +/- 0.00005 mg on a Mettler model H10 balance). The cells were pelleted again by centrifugation in a Fisher Micro-Centrifuge (model 235C) at 4°C for 30 sec and the supernatant carefully removed and discarded. The cell pellets were dried to a constant weight at 85°C (usually for 12-16 h). Commercial bleach was added (200 ul/mg of cell pellet) and cell hydrolysis allowed to proceed at room temperature overnight. The lipid granules which remained after cell hydrolysis were pelleted by centrifugation in a microcentrifuge for 10 minutes at room temperature. The supernatant was removed and discarded and the pellets were washed sequentially with 500 ul of sterile H_2O and 500 ul of acetone and collected by centrifugation in a microcentrifuge for 30 seconds at room temperature. The pellets were dissolved in 1.0 ml of $CHCl_3$ at 65°C for 10 minutes (with intermittant vortexing) in the tightly capped microcentrifuge tube and then cooled to room temperature. The concentration of the dissolved PHB was then determined.

Determination of the Concentration of PHB Dissolved in $CHCl_3$. The concentration of the PHB dissolved in $CHCl_3$ was determined by the method of Law and Slepecky (1961). This

assay is based on the fact that crotonic acid, the hydrolysis product of PHB, has an extinction coefficient at 235nm of 1.55×10^4 M^{-1} cm^{-1}. PHB solution (25 ul of the PHB solution in chloroform) was allowed to dry down in a 16 x 150 mm culture tube at room temperature and then 10.0 ml of concentrated H_2SO_4 was added. The tube was topped with a marble and incubated at 100°C for ten min. After cooling to room temperature, the optical density of the solution at 235 nm was measured. From these results the total dry weight of PHB extracted per mg of dry weight of bacteroids was calculated. As a control, known amounts of commercial PHB were dissolved in $CHCl_3$ and the concentration measured by the protocol outlined above.

Electron and Light Microscopy. Nodules were harvested from roots of four week old soybean plants, rinsed in sterile deionized water, and thin (1 mm) cross-sectional slices were hand cut with a razor blade. Sections of the slices were immediately fixed in 4% glutaraldehyde, 0.15 M sodium cacodylate (pH 7.2) for 2 h *in vacuo* at room temperature and then incubated at 4°C overnight. Following fixation, the tissue was rinsed for three 1 hr periods at room temperature with Buffer A (Maupin and Pollard, 1983) containing 0.2% (w/v) tannic acid and then for 15 min in Buffer A only. Postfixation was in 1% (w/v) osmium tetroxide in Buffer A for 1 hr at room temperature. The tissue was then dehydrated through a graded ethanol series, cleared in

propylene oxide and embedded in VCD/HSXA ultra low viscosity medium (Ladd Research Industries, Burlington, VT). Thin sections were cut on an LKB Ultrotome III with a Dupont diamond knife. Sections were subsequently stained with uranyl acetate and lead citrate and examined using a Philips EM300 electron microscope. For light microscopy, 2-3 um thick sections were stained with 0.5% toluidine blue.

Results

Identification, Mapping, and Subcloning of Sequences Transcribed Specifically in Bacteroids. Nine cosmids containing sequences that are specifically transcribed in bacteroids were digested with the restriction endonuclease EcoRI, size fractionated on agarose gels and transferred to cellulose nitrate. The DNA bound to the filters was then hybridized with cDNA synthesized using total RNA from bacteroids or from free-living cells grown on YEM. The cDNA probes had been enriched for mRNA by the protocol outlined in Chapter 2. Four DNA fragments from three different cosmids (pRJcos3-61, pRJcos4-51 and pRJcos14-8) which produced the best differential signals (Fig. 1; Chapter 2, Table 1) were subcloned into appropriate plasmid vectors, restriction mapped with a variety of restriction endonucleases (Fig. 2) and probed again with the same cDNAs to further localize the regions which were actively transcribed in bacteroids. Recombinant lambda bacteriophage

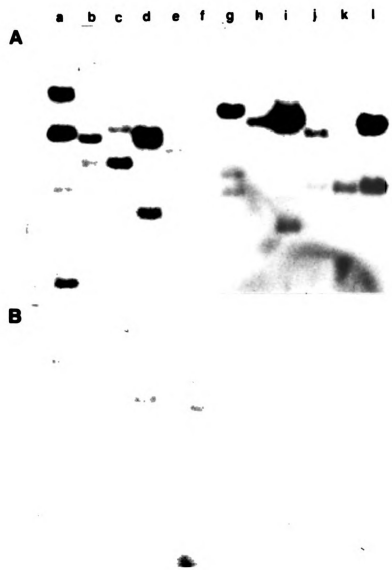
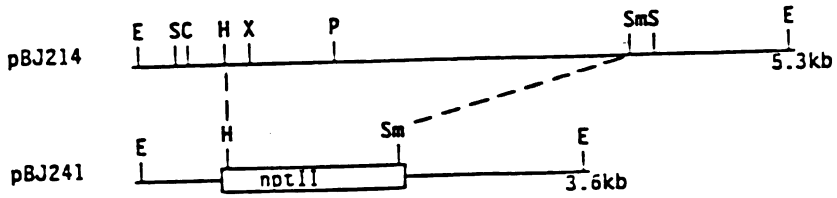
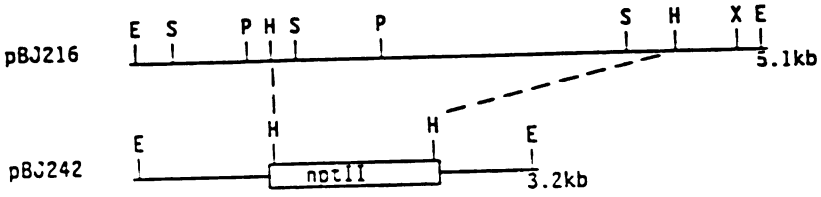
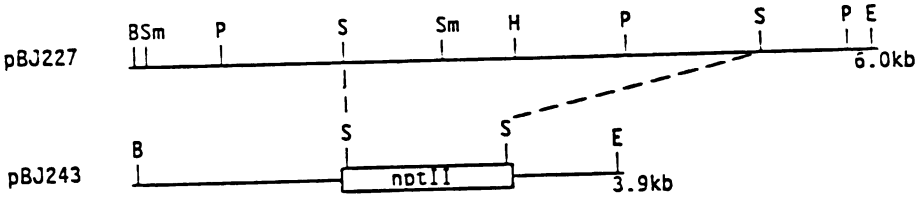


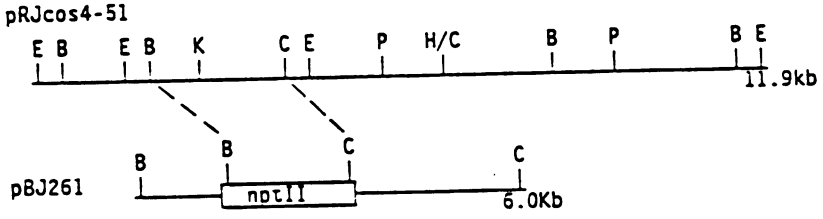
Figure 1. Cosmids containing sequences exhibiting bacteroid-specific expression. DNA from nine cosmids which contain sequences transcribed at high levels in bacteroids was digested with restriction enzymes and transferred onto cellulose nitrate. The filters were hybridized with equal amounts (2×10^6 cpm) of cDNA prepared using either RNA isolated from bacteroids (panel A) or from cells grown in culture (panel B). Lane a = cosmid pRJcos2-63 which contains the nitrogenase structural genes (*nifDK*); lanes b-j are cosmids isolated in this study = pRJcos 1-20, 3-61, 4-51, 7-39, 8-62, 11-78, 12-37, 14-8, and 15-75; lane k = pRJcos7-36 and lane l = pRJcos10-90 both of which contain genes necessary for nitrogen fixation (*fix* genes).

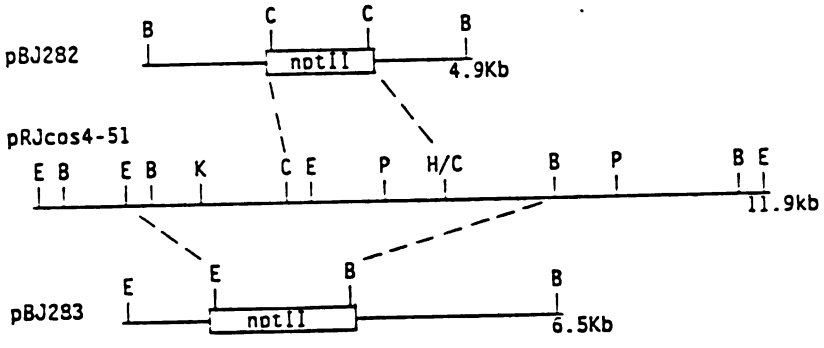
Figure 2. Restriction maps of five regions of the B. japonicum genome (pBJ214, pBJ216, pBJ227, pRJcos4-51, pBJ270) which contain sequences which are specifically transcribed in bacteroids. Dashed lines connect the restriction maps to diagrams of contructions used to generate seven deletions strains in which the transcribed regions were replaced by the neomycin phosphotransferase (nptII) gene. B = BamHI, C = ClaI, E = EcoRI, H = HindIII, K = KpnI, P = PstI, S = SalI, Sm = SmaI, St = SstI, X = XhoI.

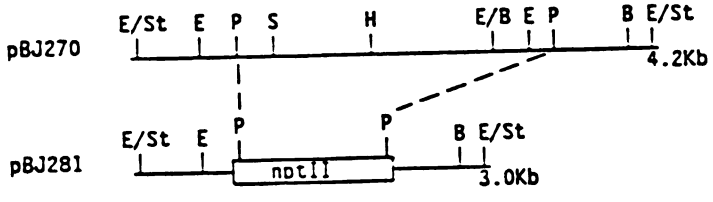

pBJ214
E SC H X P SmS E
5.3kb
pBJ241
E H Sm E
nptII
3.6kb


pBJ216
E S P H S P S H X E
5.1kb
pBJ242
E H H E
nptII
3.2kb


pBJ227
BSm P S Sm H P S P E
6.0kb
pBJ243
B S S E
nptII
3.9kb


pRJcos4-51
E B E B K C E P H/C B P B E
11.9kb
pBJ261
B B C C
nptII
6.0Kb


pBJ282
B C C B
nptII
4.9Kb
pRJcos4-51
E B E B K C E P H/C B P B E
11.9kb
pBJ283
E E B B
nptII
6.5Kb

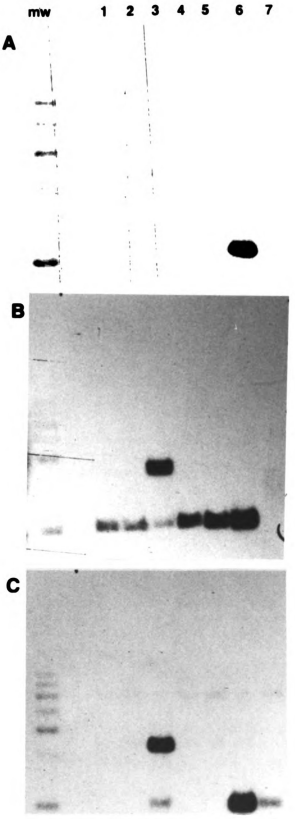

pBJ270
E/St E P S H E/B E P B E/St
4.2Kb
pBJ281
E/St E P P B E/St
nptII
3.0Kb

B15, which contained sequences specifically transcribed in bacteroids, showed no homology to any member of the cosmid library (Chapter 2, Table 1) and so represented a fifth differentially expressed region of the B. japonicum genome. A 4 kb SstI fragment of DNA was subcloned directly from this phage to create plasmid pBJ270, which was subsequently mapped and analysed as described above (Fig. 2).

Construction of Deletion Strains. Stable deletion derivatives of BJ110d were constructed in order to examine the effect of deleting sequences from the B. japonicum genome which are highly transcribed specifically in bacteroids. Seven regions of the B. japonicum genomic DNA, ranging in size from 2.0 to 6.3 kilobases, were replaced in vitro by DNA fragments containing the nptII gene which encodes neomycin phosphotransferase II (Kan^r). In each construct the nptII gene was flanked by colinearly oriented regions of B. japonicum genomic DNA of at least 500 bp in length in order to provide adequate substrates for homologous recombination. The corresponding chromosomal regions were then replaced by the deletion constructions (depicted schematically in Figure 2) using the tri-parental mating method described by Guerinot and Chelm (1986). To confirm that the double crossover recombination event necessary to produce the deletion had occurred, total genomic DNA from several isolates of each derivative was prepared. The DNA was digested with appropriate restriction

enzymes, size fractionated on agarose gels and then transferred to cellulose nitrate. The resulting filters were hybridized with nick-translated plasmids containing vector pBR322 sequences only, vector pBR322 sequences and the nptII gene, or vector pBR322 sequences and DNA from the B. japonicum regions which had been deleted. The results for deletion strain BJ261 are presented in Fig. 3 as an example. As expected, the nick-translated vector failed to hybridize the filters except in lanes where DNA from a strain resulting from a single crossover event (which results in the incorporation of vector sequences into the genome) was included as a control (Figure 3A, lane 6). Also as expected, the nick-translated probe containing the deleted region hybridized to DNA from the single crossover strain and BJ110d (Fig. 3C, lanes 6 and 7), but not to DNA from double crossover derivative strains. The probe containing the nptII gene hybridized to the DNA of both the mutant and the single crossover derivative strain resulting in bands of the predicted size (Figure 3B, lanes 1,2,4,5 and 6). The results for the hybridization of all three probes to the DNA from the strain in lane 3 are anomalous and the strain may have arisen by insertion of the deletion-bearing plasmid into the B. japonicum genome by non-homologous recombination. Results similar to those for the correctly constructed BJ261 deletion derivative strains (e.g. those in Fig. 3, lanes 1,2,4 and 5) were obtained for all the other mutant constructions except for strain BJ227 in which the

Figure 3. Verification of structure of B. japonicum deletion derivative BJ261. Plasmid pBJ261 (see Figure 2) was conjugated into wild-type strain BJ110d in which it is unable to replicate. Kanamycin resistant colonies were screened by colony hybridization for the absence of vector sequences as an indication that a double crossover recombination event had occurred. Total genomic DNA (2 ug) isolated from five presumptive deletion derivatives (lanes 1-5), one single crossover derivative (lane 6) and wild-type B. japonicum 110d (lane 7) was digested with restriction endonucleases, size fractionated on agarose gels and transferred onto cellulose nitrate filters. Three identically prepared filters were hybridized with nick-translated DNA of vector pBR322 (panel A), pKC7 (panel B) and plasmid pBJ172 which contains the region being deleted from the chromosome (panel C). The lack of homology to pBR322 or to the deleted region probe in lanes 1,2,4, and 5 confirm that the target and vector sequences are not present. The presence of sequences homologous to plasmid pKC7 indicates that the nptII gene has been inserted into the chromosome in the Kan^r strains (panel B, lanes 1-6) and is absent in BJ110d (panel B, lane 7). The derivative in lane 3 may have arisen by non-homologous recombination. All three probes hybridize to the DNA of the single crossover in lane 6 and only the deleted region probe hybridizes to BJ110d DNA as expected. The lane marked MW contains molecular weight standards.

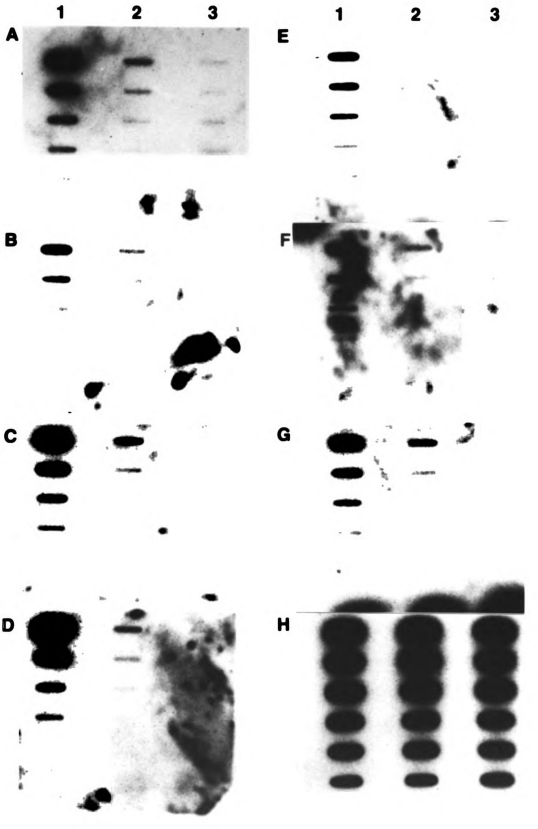

mw
1
2
3
4
5
6
7
A
B
C

deleted region hybridized to numerous fragments in the genomic digests. This latter result may indicate that a repeated sequence analogous to those described by Kaluza *et al*. (1985) and Hahn and Hennecke (1987) may be present in the deleted region of BJ227.

Verification of Bacteroid-specific Induction of Deleted Regions. In order to confirm that the apparent induction of the bacteroid-specific sequences was not an artifact of the cDNA synthesis and fractionation, RNA from bacteroids and cells grown in culture was transferred to cellulose nitrate filters using a slot blot apparatus. The filters were probed with nick-translated clones of the regions which had been deleted in each of five of the constructed strains. In all cases, the RNA corresponding to each deleted region is present in greater amounts when bacteroids (Fig. 4, lane A) are compared to cells growing exponentially in culture (Fig. 4, lane C) or cells in stationary phase in culture (Fig. 4, lane B). The expression of a gene known to be induced in bacteroids (*nifH*, Fig. 4, panel A) and one known to have similar levels of expression in all three cell types (*glnA*, Fig. 4, panel B) were monitored as controls. Comparison of signals seen in Figure 4 (panel A, lanes 1 and 2) suggest that RNA homologous to the *nifH* gene is present at approximately 20 to 30-fold higher levels in *B. japonicum* bacteroids than in cells grown to stationary phase in culture. Under similar conditions, the *glnA* gene shows a

Figure 4. Slot blots of B. japonicum RNA probed with nick-translated plasmids containing control genes or one of the sequences replaced in the deletion constructions. Lane 1 = bacteroid RNA, lane 2 = stationary culture (YEM) RNA, and lane 3 = late exponential culture (YEM) RNA. In panels A to G, RNA from each source was loaded in a vertical array of six slots with (from top to bottom) 3.0, 1.0, 0.3, 0.1, 0.03 and 0.01 ug of RNA. In panel H the RNA concentrations were lowered to cover the range 0.3 to 0.001 ug in order to obtain more readily quantifiable signals.

The probes used were: panel A = pBJ33 (nifH), B = pBJ49 (glnA), C = pBJ214, D = pBJ216, E = pBJ227, F = pBJ281, G = pBJ285, H = pBJ142 (rDNA).


1
2
3
A
B
C
D
1
2
3
E
F
G
H

three to five-fold induction (Fig. 4, panel B). The bacteroid-specific sequences isolated in this study all showed between ten and thirty-fold induction (Fig. 4, panels C,D,E,F, and G). The filter probed with the nick-translated rDNA plasmid (Fig. 4, panel H) demonstrates that equal amounts of total RNA were present in each sample.

Characterization of Deletion Strains. At least six individual soybean plants were inoculated with each of the deletion mutant strains and grown in nitrogen-free medium in modified Leonard jars. After four weeks, the plants were examined for appearance, nodule formation and the ability of excised nodules from each plant to reduce acetylene to ethylene. In all cases the mutant B. japonicum strains were similar to the wild-type strain BJ110d (Table 1). To ensure that the phenotype seen on plants inoculated with deletion strains was not due to contamination by wild-type B. japonicum, six nodules from each plant were crushed and the antibiotic resistance phenotype of the resident bacteria was determined. All of the bacteria isolated from "mutant" nodules from each plant were resistant to kanamycin, and all of the bacteria isolated from control plants inoculated with BJ110d were sensitive to kanamycin indicating that the "mutant" nodules indeed contained only deletion-bearing bacteria.

The mutant strains were further examined by comparison with BJ110d for growth on rich medium (YEGG) and on minimal

Table 1. Acetylene reduction by excised nodules of B. japonicum deletion strains and wild-type strain BJ110d. Nodules were picked from the roots of four-week-old soybean plants grown in Leonard jars. The amount of acetylene reduced is presented as a percentage of the amount reduced by wild-type strain BJ110d.

Strain	Acetylene reduced relative to wild-type (%)
BJ241	108
BJ242	83
BJ243	133
BJ261	103
BJ281	105
BJ282	107
BJ283	100

BJ110d reduced acetylene to ethylene at a rate of 5.6 ± 0.4 umoles/g of nodules/h.

medium amended with 0.3% xylose. The growth of each of the mutant strains was identical to that of wild-type BJ110d under both conditions (Fig. 5). The ultrastructure of nodules incited by each mutant strain was examined by electron microscopy. No significant differences were observed when each of the mutant strains was compared to wild-type BJ110d (Fig. 6). Bacteria purified from nodules incited by four of the mutant strains (BJ214, BJ216, BJ227 and BJ261) were also assayed for levels of the storage polymer poly-β-hydroxybutyrate and found to contain large amounts (Table 2). Although there is some variability in the data, since PHB is being actively accumulated it was expected that deletion of a gene involved in PHB synthesis would result in a marked decrease in PHB levels. The high levels of PHB detected was in agreement with evidence from the electron micrographs (Fig. 6).

Expression of the Bacteroid-specific Sequences Under Various Physiological Conditions. Since deletion of the bacteroid-specific sequences from the genome of BJ110d had no apparent effect on nodulation, nitrogen fixation, or growth in culture, the expression of the sequences was examined under a variety of conditions in an attempt to determine how the expression of these presumptive genes was regulated. In each of the experiments described below, cosmid or plasmid DNAs were digested with appropriate restriction endonucleases, the fragments size fractionated

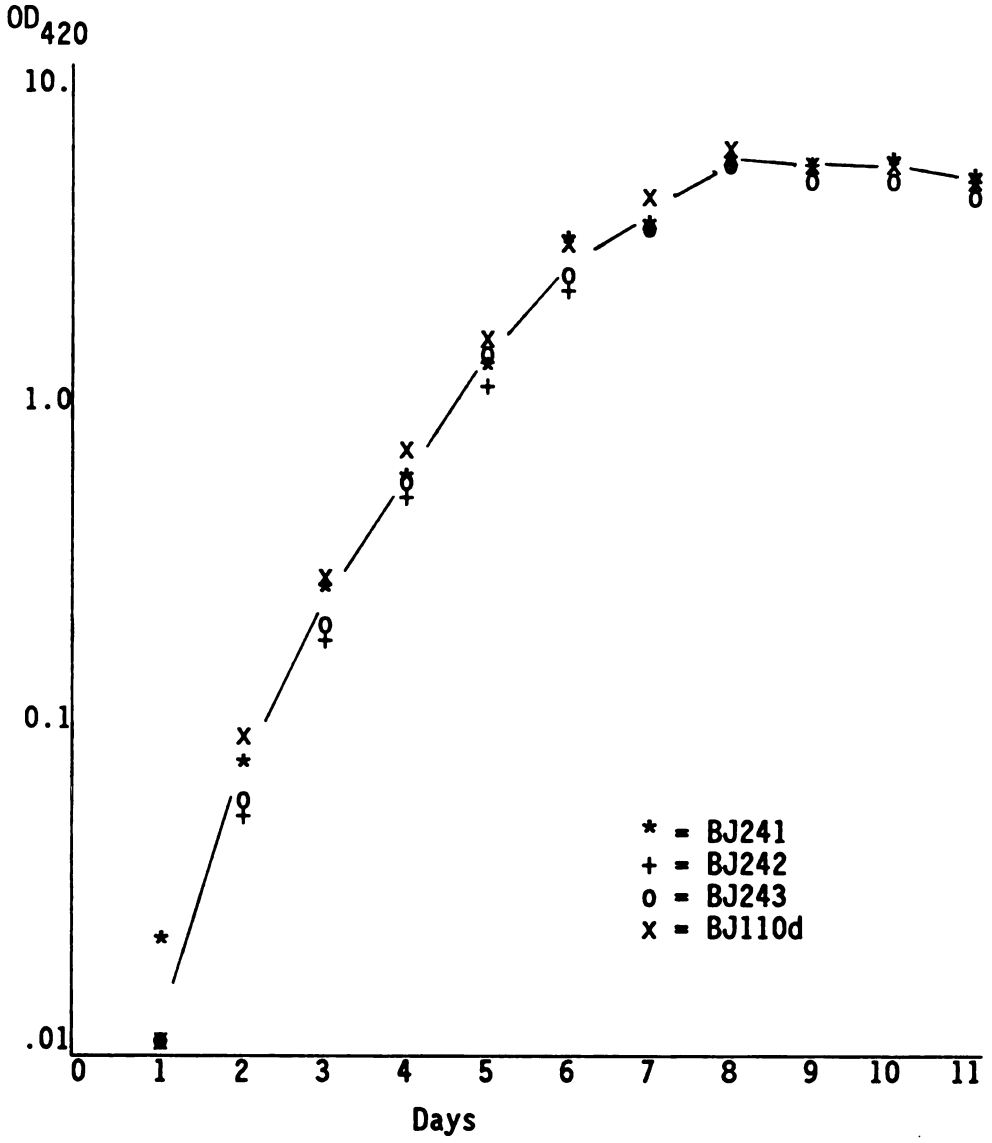


Figure 5a. Growth curve of B. japonicum deletion strains in minimal medium with 0.3% xylose as the carbon source. Growth was monitored by the change in optical density at 420 nanometers.

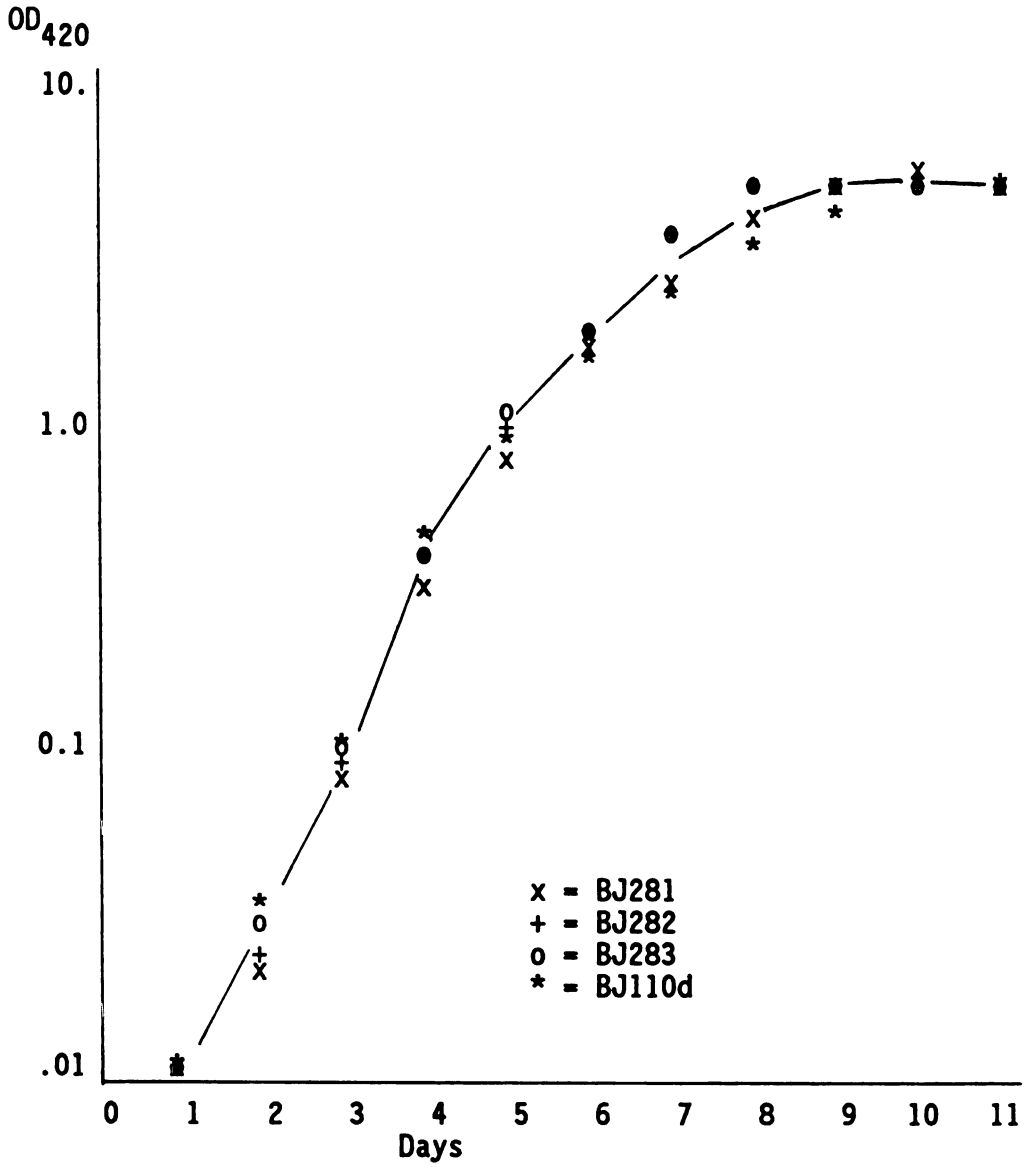


Figure 5b. Growth curve of B. japonicum deletion strains in minimal medium with 0.3% xylose as the carbon source. Growth was monitored by the change in optical density at 420 nanometers.

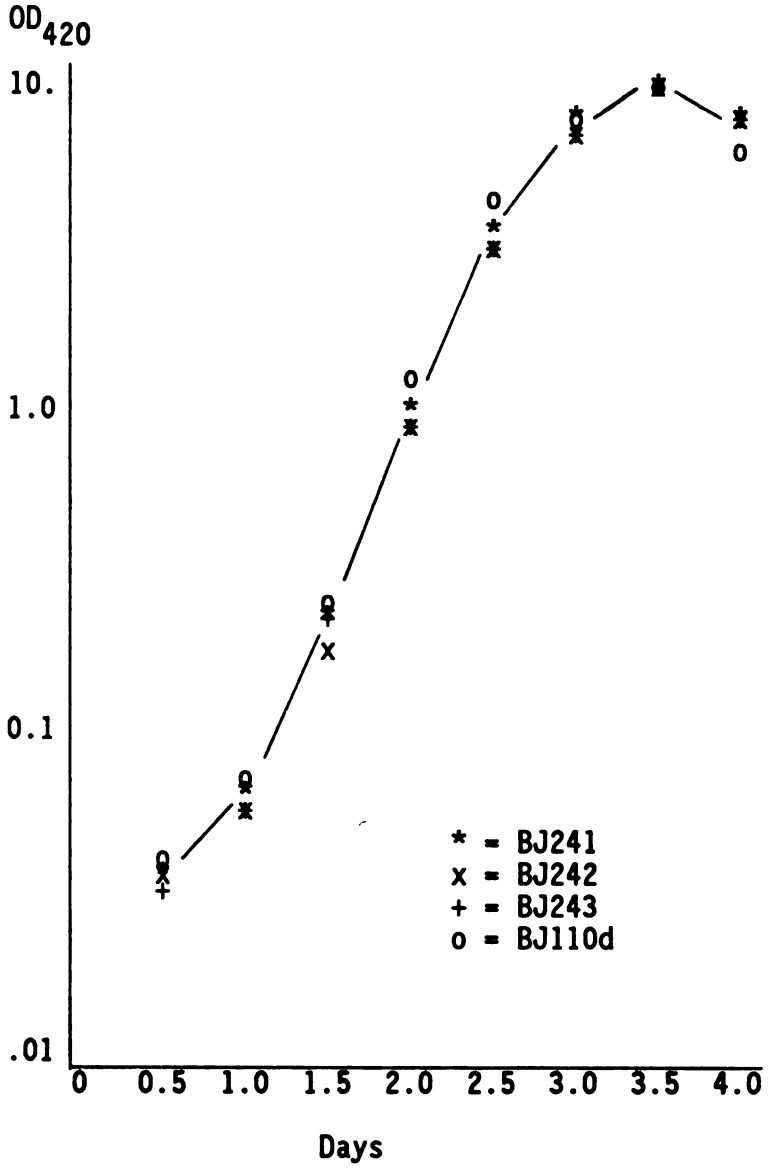


Figure 5c. Growth of B. japonicum deletion strains in rich medium (YEGG). Growth was monitored by the change in optical density at 420 nanometers.

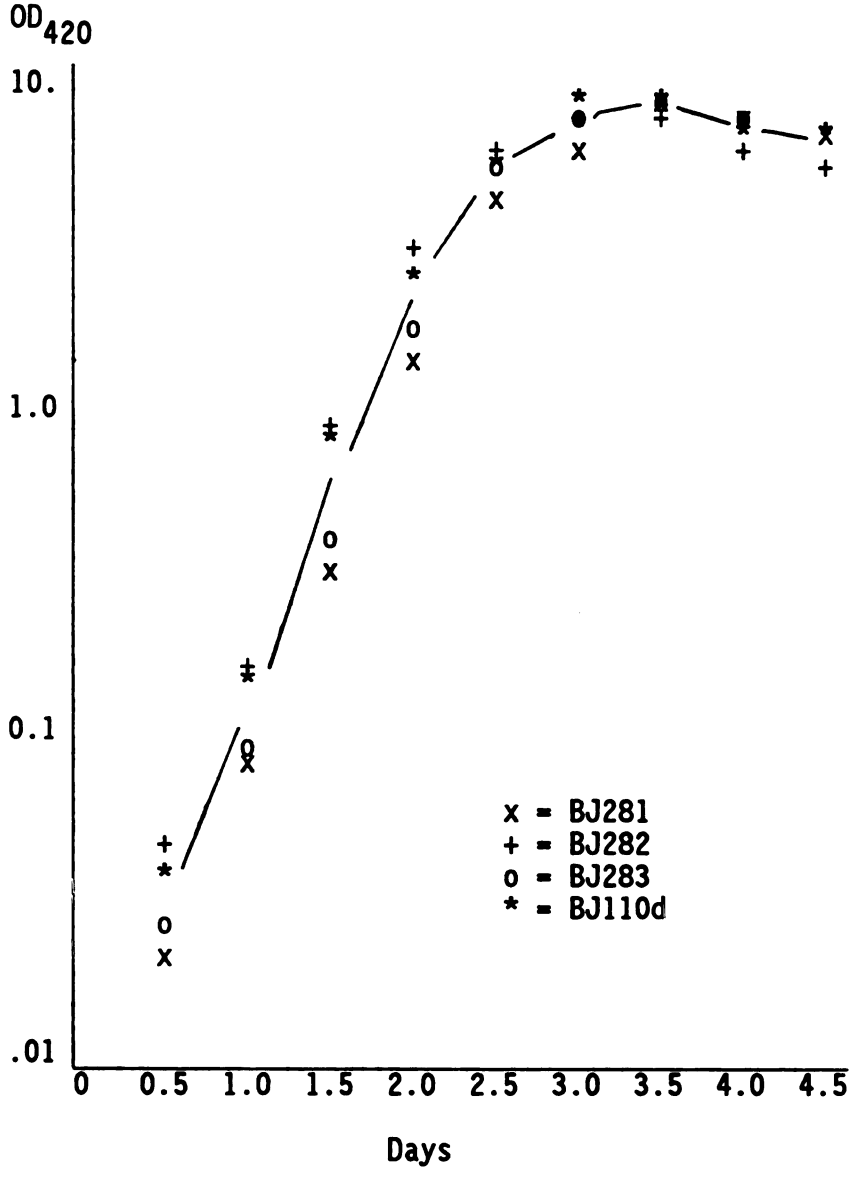


Figure 5d. Growth of B. japonicum deletion strains in rich medium (YEGG). Growth was monitored by the change in optical density at 420 nanometers.

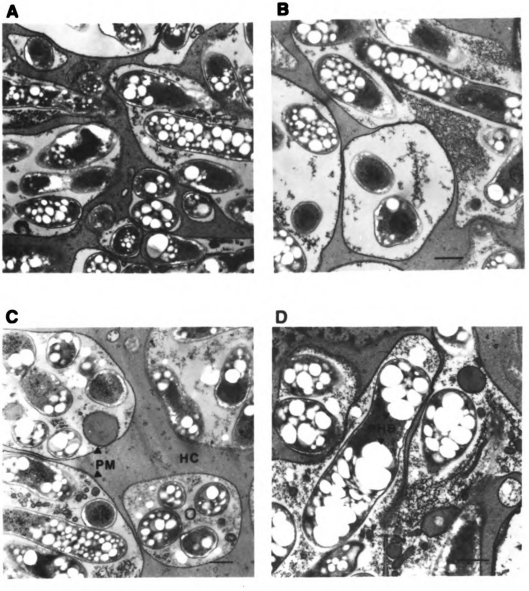


Figure 6. Transmission electron micrographs of sections of nodules incited by B. japonicum deletion strains and wild-type strain BJ110d. Representative cross-sections of deletion strains BJ261 (panel A), BJ282 (panel B), BJ283 (panel C), and wildtype BJ110d (panel D) are at 12-15,000 magnifications. HC = host cytoplasm, PM = peribacteroid membrane, PHB = poly-β-hydroxybutyrate granules. Bar equals 0.67 um.

Table 2. Poly-β-hydroxybutyrate content of B. japonicum nodules incited by deletion strains BJ241, BJ242, and BJ243 and wild-type strain BJ110d. The results of two separate experiments (designated as -1, or -2) are presented below.

Strain	OD_{235}	Ave.	[PHB]	Total ug/ml	mg of mg PHB	% PHB cells	dry wt.
BJ241-1	.396		.461	2.56	1.02	1.8	56.9
	.527						
BJ241-2	.718		.682	3.79	1.51	2.2	68.6
	.595						
	.733						
BJ242-1	.258		.279	1.55	.62	0.9	68.9
	.300						
BJ242-2	.449		.478	2.65	1.06	2.8	37.9
	.496						
	.488						
BJ243-1	.426		.451	2.50	1.00	2.8	35.8
	.441						
	.488						
BJ243-2	.355		.373	2.07	.83	2.3	36.0
	.370						
	.396						
BJ110d-1	.597		.672	3.72	1.49	3.1	48.1
	.747						
BJ110d-2	.768		.731	4.05	1.62	3.7	43.8
	.705						
	.719						
Blanks-1	.016	s.d.	.012				

by agarose gel electrophoresis and then transferred to cellulose nitrate filters. The DNA bound to the filters was subsequently hybridized with cDNA made from total RNA extracted from B. japonicum cells of various genotypes grown under particular cultural conditions or isolated from soybean nodules.

In order to examine the possibility that the low level of expression of the bacteroid-specific regions seen in cells grown on YEM was due to the particular stage in the growth curve at which the cells were harvested, samples of cells were collected at middle and late logarithmic phase as well as at three points in stationary phase. cDNA was prepared from the RNA extracted from each sample and from each of the three cell types isolated from the nodules of 5-week-old soybean plants. As shown in Fig. 7, with the exception of sequences on cosmid pRJcos8-62 which are highly expressed in each cell type, each cosmid contains one or more bands which produce a greater signal when probed with bacteroid cDNA when compared to cDNA prepared from cultured cells. The sequences from cosmids 3-61, 4-51, and 14-8 which were selected for analysis are not expressed appreciably at any phase in the growth curve yet are highly expressed in bacteroids. Possible carbon source effects were also examined and cDNA was prepared from cells grown on minimal medium containing either xylose or formate as the sole source of carbon. Neither growth on the "good" carbon source (xylose) nor on the "poor" carbon source (formate)

Figure 7. Effect of developmental stage and growth stage on the expression of B. japonicum DNA sequences that are transcribed at high levels in bacteroids. DNA from nine cosmids was digested with restriction endonucleases, size fractionated by agarose gel electrophoresis and transferred onto cellulose nitrate filters. The filters were probed with random primed cDNA prepared using RNA from eight different B. japonicum cell types (A-H). A = bacteroids; B = transforming bacteria; C = nodule bacteria; D - H = cells grown in culture (YEM) and harvested at OD_{600} of 0.5 (D), 0.8 (E), 2.5 (F), 3.5 (G) and 3.7 (H). The B. japonicum cell types A, B, and C were isolated from 5-week old soybean root nodules. Arrows in panel A indicate the bands from particular cosmids which showed differential hybridization signals when filters were hybridized with cDNA from symbiotic cell types (panels A, B, or C) as compared with cDNA from cells grown in culture (panels D, E, F, G, or H).

1-20
3-61
4-51
7-39
8-62
11-78
12-37
14-8
15-75
A
B
C
D
E
F
G
H

appeared to induce transcription of the bacteroid-specific regions (Figure 8).

Although Rhizobium and Bradyrhizobium species are obligate aerobes, it is a well known that the oxygen tension in nodules is maintained at low levels, presumably through the action of leghemoglobin, an oxygen-binding plant protein which is specifically induced in nodules (Appleby, 1984). This abundant molecule is thought to provide the high oxygen flux necessary for the actively respiring rhizobia while maintaining a free oxygen tension low enough to prevent irreversible inactivation of nitrogenase (Mortenson and Thornley, 1979). Several reports that some Bradyrhizobium strains can be induced to fix nitrogen in free-living microaerophilic culture (Keister, 1975: Pagan et al., 1975; Kurz and Larue, 1975; McComb et al., 1975; Tjepkema and Evans, 1975) led to further studies of the effect of low oxygen tension on the transcription of genes involved in the symbiosis (Ditta et al., 1987; Adams and Chelm, in press). In order to determine whether the level of free oxygen might be involved in the transcriptional regulation of the sequences isolated in this study, cDNA was prepared using total RNA (provided by T. Adams) isolated from B. japonicum cells grown in YEM with and without 10mM KNO_3 in fermenters sparged with nitrogen containing 0.1% O_2 (microaerobic conditions). Transcripts homologous to pBJ227 and pBJ270 were apparently induced under these conditions (Fig. 9). Although the signals obtained were below levels seen in

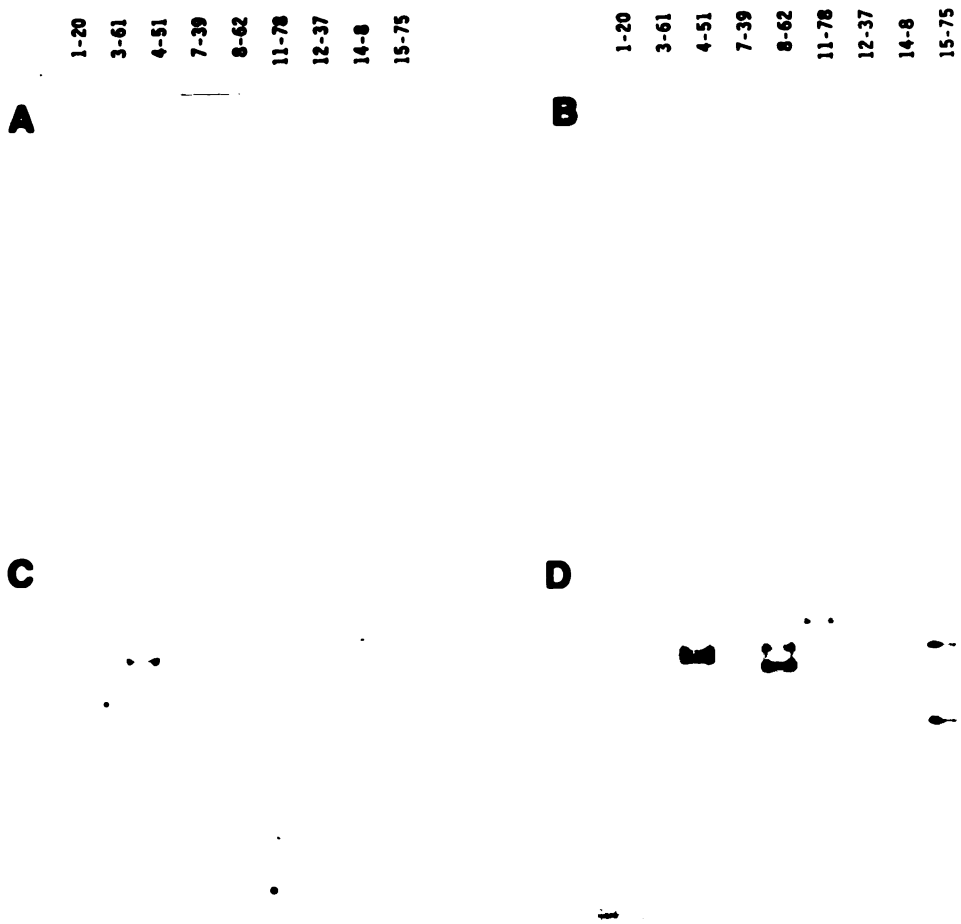


Figure 8. DNA from nine cosmids identified as containing sequences which are transcribed at high levels in bacteroids was digested with restriction enzymes and blotted onto cellulose nitrate. The filters were probed with random primed cDNA synthesized using RNA isolated from B. japonicum cells grown under the following conditions: (A) minimal medium with 0.3% formate as the carbon source; (B) minimal medium with 0.3% xylose as the carbon source; (C) YEM medium at 0.2% oxygen; YEM with 10mM KNO_3 at 0.2% oxygen. The control filter showing hybridization patterns for cDNA synthesized using bacteroid RNA is included in Figure 7.

bacteroids (compare Fig. 9A, lanes 4 and 6 to Figs. 9C and 9D, lanes 4 and 6), they were similar to those for pBJ33 (nifH) (Fig. 9, lane 1). This result suggests that transcripts originating from these two sequences may be regulated in a manner similar to nif genes, at least with regard to their response to oxygen levels.

Another global control mechanism for gene regulation is the ntr system which is associated specifically with nitrogen metabolism. Initially identified and studied in the Enterobacteriaceae, the system employs two positive regulatory proteins (ntrA and ntrC) to activate numerous genes involved in nitrogen assimilation (reviewed in Magasanik, 1982). In Klebsiella pneumoniae nitrogen fixation genes are activated specifically by the ntrA and the nifA gene products (Ausubel, 1984a; Gussin et al., 1986). The regulation of symbiotic nitrogen fixation is similar to that in free-living diazotrophs, but differs in that the ntrC gene product is not necessary for activation of the nifA gene (Szeto et al., 1987). To determine if the nifA gene product might be involved in the transcriptional regulation of the sequences isolated in this study, RNA was prepared from B. japonicum cells extracted from nodules incited by strain BJ2101 in which the nifA gene had been deleted (Adams et al., manuscript in preparation). Complementary DNA (cDNA) synthesized from total nodule RNA isolated from BJ2101 and BJ110d was hybridized to filters to which digests of plasmids containing the bacteroid-specific

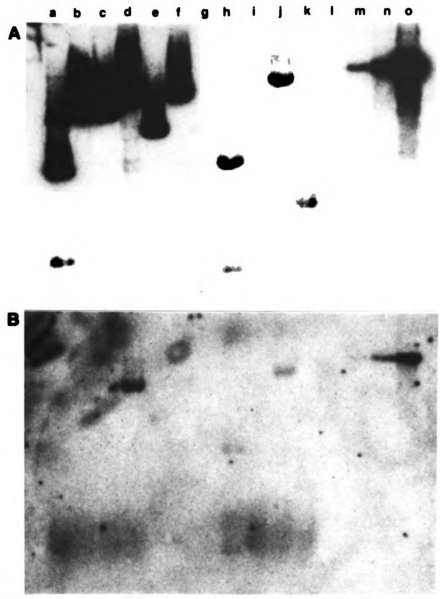


Figure 10. Expression of bacteroid-specific sequences in two-week-old nodules incited by BJ2101, a nifA deletion strain of B. japonicum. Restriction endonuclease digests of plasmid DNAs were size fractionated on agarose gels, transferred onto cellulose nitrate and hybridized with cDNA synthesized using RNA isolated from nodules incited by wild-type strain BJ110d (panel A) or nifA deletion strain BJ2101 (panel B). The plasmids contained the bacteroid-specific sequences isolated in this study (lanes b-f = pBJ214, pBJ216, pBJ227, pBJ270, pBJ273) or various control genes (lane a = nifH, lane g = glnA, lane h = hemA, lane i = recA, lane j = pBJ294 which contains a sequence expressed in all cell types, lane k = glnII, and lanes l-o = .0001, .001, .01, .1 ug of pBJ142 which contains the rDNA operon).

sequences had been transferred. Little or no signal was detected on the filters probed with the nifA deletion strain cDNA (Fig. 10, panel B). Sequences homologous to pBJ227 were present in small amounts as were sequences homologous to various control genes (hemA, glnA, rDNA). From the low level of these signals it is apparent that much less intact RNA was obtained from the nodules incited by the nifA deletion strain (BJ2101). The nodules incited by BJ2101 do not develop normally and the nif gene transcripts are not detectable (Adams and Chelm, in press; Fisher et al., 1986). Although this experiment does not provide evidence for a direct involvement of the nifA gene product in the transcriptional activation of the bacteroid-specific sequences, it suggests that a regulatory mechanism or pathway may be shared.

Two sets of experiments were conducted to examine whether the global nitrogen regulatory (ntr) genes affect the expression of the bacteroid-specific sequences. In their studies of glutamine synthetase II regulation, Carlson et al. (1987) grew cultures of BJ110d with a limited supply of fixed nitrogen and took samples at various times. As expected, the depletion of available nitrogen resulted in the cessation of growth and the concomitant transcriptional activation of GSII. RNA extracted from these same cultures (a gift of T. Carlson) was used to prepare cDNA probe which was hybridized to cellulose nitrate filters similar to those used in the preceding experiments. The low signal levels

obtained indicate that induction of the ntr system alone is insufficient to promote induction of the bacteroid-specific sequences isolated in this study (Fig. 11). In addition, when cDNA prepared from total RNA (a gift of G. Martin) extracted from nodules incited by an ntrC$^-$ strain of BJ110d (G. Martin, unpublished) was used as probe, all of the bacteroid-specific sequences were expressed at levels approximating those seen in wild-type nodules (Fig. 12, lanes 2-6). As a control for this experiment, digests of plasmid pBJ196A which contains an ntrC-regulated gene (GSII) (Carlson et al., 1987) were included (Fig. 12, lanes 7,8) and the lack of a hybridization signal to this DNA confirms that the ntrC gene product was indeed inactivated (Fig. 12, lanes 7,8).

Genes involved in the high temperature response (the heat shock response) are known to be activated by a number of diverse environmental stimuli (Neidhardt et al., 1984). Although physiological conditions in soybean nodules are not known to provide any of the characterized heat-shock response inducing stimuli, the environment inside the plant root differs greatly from the soil environment. It seemed possible, therefore, that stresses imposed by the change in environment might result in induction of stress-inducible systems (e.g. heat-shock genes) and that the induction of the bacteroid-specific sequences might be due to physiological stresses encountered during nodule development. This could explain why the highly expressed

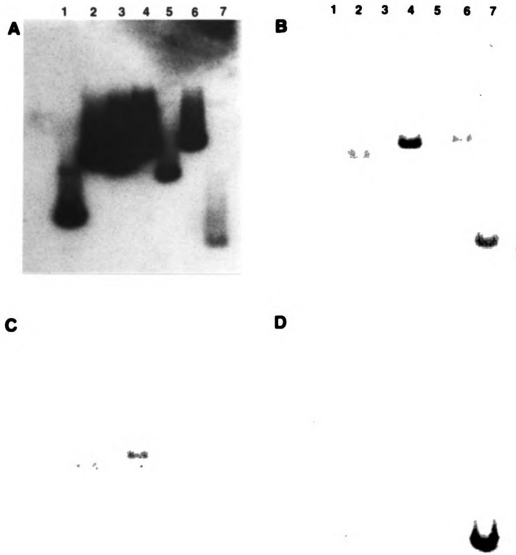


Figure 11. Expression of the bacteroid-specific sequences in B. japonicum cultures grown under nitrogen-limiting conditions. Restriction endonuclease digests of plasmid DNAs were size fractionated on agarose gels and transferred onto cellulose nitrate filters. The filters were hybridized with cDNA synthesized using RNA isolated from B. japonicum bacteroids (panel A) or from aerobic cultures in which the supply of fixed nitrogen was progressively depleted (panels B, C, and D). The plasmids contained nifH (lane 1), the bacteroid-specific sequences isolated in this study (lanes 2-6 = pBJ214, pBJ216, pBJ227, pBJ270, pBJ273) or glnII (lane 7).

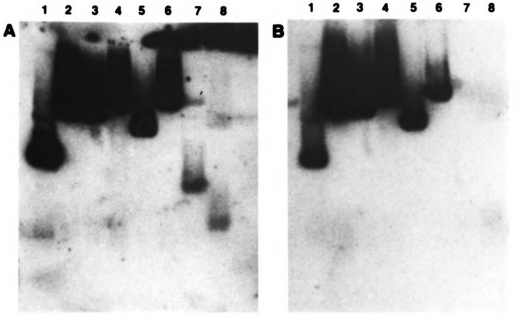


Figure 12. Expression of the bacteroid-specific sequences in a B. japonicum ntrC insertion strain. Restriction endonuclease digests of plasmid DNAs were size fractionated on agarose gels and transferred onto cellulose nitrate filters. The filters were hybridized with cDNA made using RNA isolated from nodules incited by wild-type strain BJ110d (panel A) or ntrC insertion strain BJ262 (panel B). The plasmids contained nifH (lane 1), the bacteroid-specific sequences isolated in this study (lanes 2-6 = pBJ214, pBJ216, pBJ227, pBJ270, and pBJ273), or glnII (lanes 7 and 8).

bacteroid-specific sequences are not essential for effective symbiosis. To test this hypothesis, cDNA was synthesized from total RNA isolated from heat-shocked cultures of BJ110d. Hybridization of this cDNA to cellulose nitrate filters containing restriction digests of the cloned regions of bacteroid-specific sequences indicated that none of the presumptive genes are induced by these treatments (Fig. 13). Hybridization of total B. japonicum genomic DNA with heterologous HSP70 (= dnaK, a well characterized heat shock gene) probes derived from either E. coli (Georgeopolous et al., 1982) or Arabadopsis thaliana (Wu et al, manuscript in preparation) did not detect a homologous sequence in B. japonicum which could serve as a positive control for heat-shock induction. Fortunately, the lack of an appropriate control for heat induction was obviated by the fortuitous induction in B. japonicum of the transcription of a sequence which is also contained on one of the plasmids used as a molecular weight marker. This sequence from B. japonicum is not transcribed at detectable levels in bacteroids (Fig. 13, panel A, lane 1), but its transcription is induced when B. japonicum cells are shifted from 30°C to 37°C for 10 min (Fig. 13, panel C, lane 1).

Discussion

An underlying assumption in this effort to isolate genes which are transcribed specifically in Bradyrhizobium

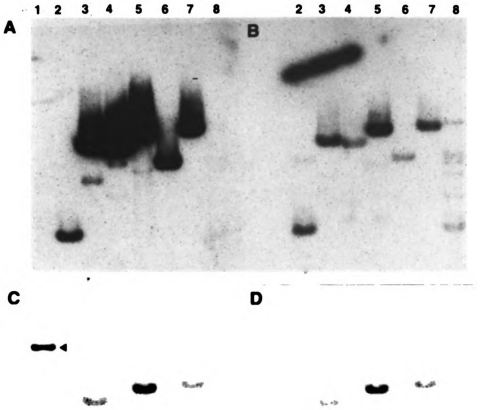


Figure 13. Expression of the bacteroid-specific sequences in heat-shocked B. japonicum cells. Restriction endonuclease digests of plasmid DNAs were size fractionated on agarose gels and transferred onto cellulose nitrate filters. The filters were hybridized with cDNA which was prepared using RNA isolated from nodules incited by wild-type B.japonicum 110d (panel A), or from B. japonicum cells which had been heat-shocked at 37°C for 0 (panel B), 10 (panel C) or 30 (panel D) minutes. The plasmids contained glnII (lane 2), the bacteroid-specific sequences isolated in this study (lanes 3-7 = pBJ214, pBJ216, pBJ227, pBJ270, and pBJ273), and the E. coli dnaK gene (lane 8). Lane 1 in panels A and C contains a ladder of plasmids used as molecular weight standards and the arrow in panel C indicates the plasmid which contains a sequence which is heat-shock inducible.

japonicum bacteroids was the notion that such genes would have functions necessary for the proper development of the B. japonicum/soybean symbiosis. Although genes which are induced specifically during development have been identified in a number of different eukaryotic systems (e.g. Firtel, 1972; Zimmerman et al., 1980; Putzer et al., 1984; Kurtz and Lindquist, 1984; Kopachik et al., 1985), there have been no reports of the effect on development of mutations in these genes. The prokaryotic system in which the relationship between transcriptional regulation and development has been most carefully examined is fruiting body formation in Myxococcus xanthus (Kaiser et al., 1979). Using a transposon Tn5-derived promoter probe (Tn5 lac) which upon integration into the chromosome fuses lacZ expression to exogenous promoters (Kroos and Kaiser, 1984), Kroos et. al. (1986) examined 2374 Tn5 lac insertion-containing M. xanthus strains for both development-specific expression and for developmental phenotype. Thirty-six strains were identified which had significantly increased levels of β-galactosidase activity when placed under conditions known to induce fruiting body formation. Only three of these M. xanthus strains (and eight of the original 2374) also caused abnormal fruiting body development, apparently indicating that far fewer genes are essential for development than are regulated during development (Kroos et al., 1986).

In Rhizobium and Bradyrhizobium the best characterized example of developmentally regulated genes are the nifH,D,K

genes which encode the subunits of nitrogenase (Krol et al., 1980; Prakash et al., 1982; Corbin et al., 1982). The nif genes are induced at high levels specifically in bacteroids (Corbin et al., 1982) and a mutation in any one of the three structural genes prevents the reduction of dinitrogen to ammonia (Ruvkun and Ausubel, 1981). The bacteroid-specific sequences isolated and analysed in this study are all expressed at levels approximately equal to those seen for the nif genes (Fig. 1). The results of the deletion analysis indicate that none of these highly transcribed regions is necessary for nodulation or nitrogen fixation. The simplest explanation for these results is that the B. japonicum genome contains an additional copy or copies of these sequences. Examples of gene duplication have been reported in Rhizobium (Renalier et al., 1987; Honma et al., 1985; Quinto et al., 1982), but with the exception of the sequence contained on plasmid pBJ227, hybridization of nick-translated plasmid DNA from the deleted regions to total B. japonicum genomic DNA did not reveal the existence of other copies of the cloned regions (e.g. Fig. 3, panel C). It is possible, however, that the functions carried out by the products of these regions are duplicated by other non-homologous regions of the genome. An example of such a functional duplication in B. japonicum was described by Carlson et al. (1985, 1986) who isolated two separate genes encoding glutamine synthetase. Although the two gene products perform the same enzymatic function, the two

proteins differ in physical properties and mode of regulation (Carlson et al., 1987). One gene (glnA) has homology to the corresponding gene in E. coli while the second gene (glnII) has homology to the corresponding gene in eukaryotes, but they are not homologous to each other at the DNA sequence level (Carlson and Chelm, 1986). Mutational analysis revealed that deletion of either gene alone did not result in glutamine auxotrophy and did not affect symbiotic competence, but that a strain carrying deletions in both genes required glutamine for growth and formed ineffective nodules (Carlson et al., 1987). If the highly transcribed bacteroid-specific sequences isolated in this study were functionally duplicated by non-homologous sequences located elsewhere in the B. japonicum genome, then the lack of a symbiotic phenotype in nodules incited by the deletion strains would be expected.

A second possible explanation for the failure of the B. japonicum deletion strains to produce a symbiotic phenotype is that the gene products encoded by the deleted regions are not involved in the processes of nodulation or nitrogen fixation but are active at a later stage of the interaction (e.g. senescence). The determination of the poly-β-hydroxybutyrate content of nodules incited by the deletion strains was an effort to examine this possibility, since PHB is accumulated during nodule development and hydrolysed during nodule senescence (Wong and Evans, 1971). No evidence was found, however, for altered levels of PHB

accumulation in the deletion strains (Table 2). It would seem unlikely, moreover, that all of the isolated sequences would fall into the "late gene" category. The fact that the bacteroid-specific sequences were actively transcribed in nodules harvested at two (Fig. 10, panel A), four (Fig. 9, panel A) and five (Fig. 7, panel A) weeks after infection also argues against this explanation.

Examination of the expression of the bacteroid-specific sequences in B. japonicum wild-type cells grown under microaerobic conditions (0.2 % O_2 +/- 10 mM KNO_3) provides insight into the mechanism by which two of the sequences are regulated. As was previously demonstrated for the nif genes (Adams and Chelm, in press), lowered oxygen tension is sufficient to induce transcription of the sequences contained on plasmids pBJ227 and PBJ270 (Fig. 9, lanes 5,6). The control of free oxygen levels in soybean root nodules is presumed to be mediated by leghemoglobin, the apo-protein portion of which is not synthesized until the nodule structure has begun forming, approximately ten days after infection (Gloudemans et al., 1987). If active leghemoglobin is necessary for the expression of oxygen-regulated genes in B. japonicum, then the gene products encoded by these genes are most likely utilized, like nitrogenase, after nodule development is well underway. There is no evidence, however, that the bacteroid-specific sequences are regulated differentially among the three developmental cell types isolated from nodules (Fig. 7).

Only one of the bacteroid-specific sequences is transcribed at a detectable level in nodules incited by BJ2101, the nifA deletion strain of B. japonicum (Fig. 10, panel B). Given the pleiotropic nature of this mutation (Adams, 1986; Fisher et al., 1986) it was important that in total RNA extracted from two week old nodules incited by BJ2101, transcripts hybridizing to the hemA gene, plasmid pBJ294 (a B. japonicum sequence expressed in all cell types examined) and the rDNA plasmid were all present, albeit at only approximately 10% of the levels seen in bacteroids (Figure 10, panels A and B). This apparently low level of intact RNA is in agreement with the observation that, as visualized by the electron microscope, B. japonicum cells which reach the developing nodules neither undergo release into the plant cells nor differentiate into bacteroids (Adams, 1986). While the failure to detect transcription of four of the bacteroid-specific sequences in BJ2101-incited nodules does not provide evidence for the direct activation of the bacteroid-specific sequences by the nifA gene product, interruption of the regulatory pathway for nif gene activation prevents the transcription of these four sequences. This experiment also divides the bacteroid-specific sequences into at least two classes, since the sequences carried on pBJ227 do not require the nifA gene product for their expression.

The level of expression of the bacteroid-specific sequences in B. japonicum nodules incited by the ntrC

insertion strain does not differ from those seen in nodules incited by wild-type strain BJ110d (Fig. 12). This result was anticipated since ntrC deletion strains of both R. meliloti (Szeto et al., 1987) and B. japonicum (G. Martin, unpublished results) are not altered in symbiotic competence. The failure of heat shock to induce expression of the bacteroid-specific sequences indicates that the increased transcription in nodules is not due to heat-shock related physiological stresses imposed by the nodule environment.

In summary, the role in development of the B. japonicum/soybean symbiosis of the highly transcribed sequences isolated in this study is not readily apparent. The bacteroid-specific expression of these transcripts would lead to the assumption that they are involved in some aspect of metabolism related to nitrogen fixation or assimilation. The most probable explanation for the unaltered symbiotic competence of the strains specifically deleted for these regions is that functional but non-homologous duplications exist elsewhere in the B. japonicum genome. Despite this functional redundancy, however, determination of the fine structure of the promotors for these transcripts should provide some insight into transcriptonal regulatory mechanisms employed in the development of the B. japonicum/soybean symbiosis.

Chapter 4

Fine Structure Analysis of Two Bacteroid-Specific Promoters in *Bradyrhizobium japonicum*

Introduction

The regulation of developmental processes which involve the interaction of two organisms is complex (VanEtten and Kistler, 1984; Long, 1984). In the *Bradyrhizobium* and *Rhizobium*/legume symbioses there is evidence that some amount of developmental control is exerted at the level of transcription (Verma *et al.*, 1986). Elucidation of the regulatory mechanisms by which the levels of certain transcripts are modulated during nodule development may provide important insights into the overall regulation of symbiotic development. One approach to this problem is the physical characterization of the promoter regions of genes which are regulated during development. Analysis of promoter structure might suggest possible mechanisms by which promoter activity is modulated.

A number of bacterial genes which are essential for the *Bradyrhizobium* and *Rhizobium*/legume symbioses have been identified. Three classes of genes have been characterized at the molecular level: (i) The *nif* genes which encode the nitrogenase enzyme complex. (ii) The *nod* genes which affect the ability of the bacterial cells to invade the host plant.

(iii) The *fix* genes which are essential for the process of nitrogen fixation. The analysis of *nif*, *nod*, and *fix* gene promoters has revealed several regions of conserved DNA sequence which have been implicated in transcriptional control. In *Rhizobium* and *Bradyrhizobium* as well as in the free-living diazotroph *Klebsiella pneumoniae*, many genes involved in nitrogen assimilation are positively regulated by the *ntrA* gene product in combination with either the *ntrC* or the *nifA* gene product (reviewed in Ausubel, 1984a). The promoters of genes which are regulated by the level of available nitrogen generally contain two conserved sequences centered at -24 bp and -12 bp upstream from the transcription initiation site (Ausubel, 1984a; Johnston and Downie, 1984). Activator sequences which may interact with the *nifA* gene product have been identified further upstream of these conserved sequences (Buck *et al.*, 1986). The promoters of genes essential for nodulation contain a 47 bp conserved sequence approximately 200-240 bp upstream of the translational start codon (Rostas *et al.*, 1986; Spaink *et al.*, 1987). Disruption or mutation of the conserved regions of these *nif*, *fix*, and *nod* promoter regions has been shown to reduce or abolish the ability of the bacteria to form an effective symbiosis with the host plant (Gussin *et al.*, 1986; Rostas *et al.*, 1986). Although the importance of the conserved regions in transcriptional regulation has been demonstrated genetically, the underlying molecular mechanisms are not well understood.

Determination of the transcription initiation sites of additional genes which are transcriptionally regulated during the development of the B. japonicum/soybean symbiosis is a first step in the characterization of the molecular basis for this regulation. Partial DNA sequence of two divergently transcribed bacteroid-specific sequences has been obtained and the promoter regions and transcription initiation sites localized.

Materials and Methods

Bacterial Strains and Plasmids. E. coli strain JM83 was used as the host for clones constructed in plasmid vector pUC8 (Vieira and Messing, 1982). E. coli strain JM103 (Messing et al., 1981) was used as the host for clones constructed in the M13 phage mp8 and mp9 (Messing and Vieira, 1982) or in M13 mp18 and mp19 (Yannish-Perron et al., 1985). All other bacterial strains and plasmids used were described in Chapters 2 and 3.

DNA Sequence Analysis. A 1000 bp PstI-HindIII B. japonicum DNA fragment from plasmid pBJ216 was cloned into vector pUC8 to make plasmid pBJ296. This plasmid was linearized by digestion with restriction endonuclease HindIII or PstI to expose one end of the B. japonicum DNA insert. A set of progressively larger deletions from either end of the DNA

insert was generated using exonuclease BAL-31 as described by Poncz *et al*. (1982). The action of this nuclease results in predominantly blunt-ended fragments. The overlapping variable length inserts were separated from vector sequences by digestion with the appropriate restriction endonuclease (either *Pst*I or *Hin*dIII) and then ligated into the M13 vector mp19 which had been previously digested with restriction endonucleases *Hin*cII (which produces a blunt end) and either *Pst*I or *Hin*dIII. The products of the ligation reaction were used to transform *E. coli* strain JM103 and the transformants plated on LB plates in 3 ml LB top agar in the presence of 1.3 mM isopropyl-beta-D-thiogalactopyranoside (IPTG) and 5-bromo-4-chloro-3-indolyl-beta-galactoside (X-gal, 40 ul of 2% w/v in dimethylformamide). In the presence of these compounds, phage containing inserts will produce colorless plaques while phage in which no fragment has been cloned will produce blue plaques (Messing, 1983). M13 phage from colorless plaques were picked with sterile toothpicks and used to inoculate 2 ml of L broth containing 50 ul of a stationary phase culture of *E. coli* strain JM103. After incubation with shaking for 5 h at 37°C, the cells in 1.5 ml of each culture were collected by centrifugation for 1 min in a microcentrifuge at room temperature. The supernatants were removed and saved for later purification of single-stranded DNA. Plasmid DNA from the bacterial cell pellet was isolated by the method of Holmes and Quigley

(1981), digested with appropriate restriction endonucleases and size fractionated on agarose gels. After analysis of the plasmid digests, a series of nested M13 clones was selected which contained progressively smaller B. japonicum DNA inserts differing in size by approximately 200 bp. Single-stranded phage DNA suitable for use in DNA sequence determination was purified by published methods from phage particles present in the corresponding mini-lysate supernatants (Messing, 1983).

The nucleotide sequence of the B. japonicum DNA inserts contained in the nested M13 clones described here was determined by the dideoxy chain termination method of Sanger et al. (1977) as described in the manual published by Amersham (1983). The template and primer DNAs were combined in a 10 ul reaction which contained: 0.5 - 1.0 ug of M13 template DNA, 0.2 pmoles of universal primer DNA (dGTAAAACGACGGCCAGT, available from Bethesda Research Laboratories [BRL], Gaithersburg, MD.), and 20 uCi of [alpha^{32}P]-dCTP (400 Ci/mmole, Amersham Corp., Arlington Heights, IL) in 1X Klenow reaction buffer (6 mM Tris.HCl [pH 7.5], 1 mM dithiothreitol, 50 mM NaCl, 9 mM $MgCl_2$). The reaction mixture was heated to 55°C for 10 min and then allowed to return slowly to room temperature to permit annealing of the primer to the template DNA. Following the addition of 0.5 units of the Klenow fragment of DNA polymerase I (BRL, Gaithersberg, MD), four 2 ul aliquots were removed and primer extension reactions initiated in

each aliquot by the addition of 2 ul of a solution containing the four deoxynucleotides plus one each of the dideoxynucleotides as described (Amersham Corp., 1983). To destabilize DNA secondary structure during gel electrophoresis, thereby reducing "compression" of bands which complicates sequence analysis, 7-deaza-2'-deoxyguanosine-5'-triphosphate (c^7dGTP) was substituted for deoxyguanosine (Barr et al., 1986) at the concentration recommended by the manufacturer (Boehringer Mannheim, Indianapolis, IN). After incubation for 20 min at room temperature, the reaction was completed by addition of 2 ul of a solution containing all four deoxynucleotides at a concentration of 0.5 mM and further incubating for 15 min at room temperature. The reaction was then stopped by the addition of 5 ul of loading dye (0.1% bromophenol blue, 0.1% xylene cyanole, 95% formamide).

The DNA fragments in the stopped primer extension sequencing reactions were denatured by immersion in water at 95°C for 5 min. Aliquots (2 ul) were size fractionated by electrophoresis on 41 cm long denaturing gels made with wedge-shaped (0.4 mm to 1.2 mm) spacers. The gels contained 6-8% acrylamide (depending on the desired conditions), 50% w/v urea in TBE buffer (89 mM Tris-base, 112 mM boric acid, 2.5 mM EDTA [pH 8.3]). Electrophoresis was carried out at 1100-1400 volts for 4.5, 8 or 11 h in order to resolve fragments of increasing molecular weight (chain length). The gels were then fixed by immersion in a solution of 10%

methanol and 10% glacial acetic acid in water for 20 min. Fixed gels were then dried down onto a sheet of chromatography paper (Whatman, 3MM) at 80°C, *in vacuo*, for 60 min using a Bio-Rad Model SE1125B Slab Gel Dryer. The labelled fragments on the gel were visualized by autoradiography for 12 to 24 h at room temperature using Kodak X-Omat AR film.

Nuclease S1 Protection Analysis. Uniformly labelled single-stranded DNA probes were synthesized by primer extension as described by Holben *et al*., (1988). The 215 bp *Acc*I-*Sty*I and the 272 bp *Sty*I-*Sal*I fragments isolated from plasmid pBJ296 were used to prepare end-labelled single-stranded DNA probes as described by Adams and Chelm (1984). Both types of probes were used in S1 nuclease protection experiments to determine the direction of transcription and the approximate location of the RNA transcription initiation site. S1 protection analysis was carried out by the method of Berk and Sharp (1977) as adapted by Adams and Chelm (1984), except that RNA/DNA hybridizations were performed at 58°C. Single-stranded DNA probes for precise determination of the transcription initiation site were synthesized using gene-specific oligonucleotide primers as described by Adams and Chelm (1988) and Carlson *et al*. (1987). The sequences of the two primers used are: 5' -TCATCGCGGTCTACGAG (Oligonucleotide #1) and 5' -GCAAGGACGTAATATGC (Oligonucleotide #2). The

single-stranded template DNA was derived from the 1000 bp PstI-HindIII fragment from plasmid pBJ296 cloned into phage vectors M13 mp8 and M13 mp9. DNA sequence comparisons were performed using the MicroGenie software system (Beckman, Palo Alto, CA).

Results

Initial S1 nuclease protection experiments using uniformly labelled single-stranded DNA probes indicated that total RNA isolated from soybean nodules incited by B. japonicum strain 110d protected portions of both strands of the DNA present on plasmid pBJ296 (Fig. 1, lanes 2 and 7). The probes were specifically protected from S1 nuclease digestion by total RNA isolated from bacteroids. Conversely, no protection was detected when the probes were hybridized with total RNA isolated from cultured cells of B. japonicum grown aerobically in YEM medium (Fig. 1, lanes 3 and 8) or microaerobically in YEM medium with 10 mM KNO_3 (Fig. 1, lanes 4 and 9). These results are in agreement with the results of experiments presented in Chapter 3 in which the bacteroid-specific expression of the same region of DNA was examined using mRNA-enriched cDNA probes (Chapter 3, Figs. 7 and 8).

The DNA sequence of the 1000 bp B. japonicum PstI-HindIII fragment contained in plasmid pBJ296 was determined (Fig. 2 and Fig.3). The transcription initiation

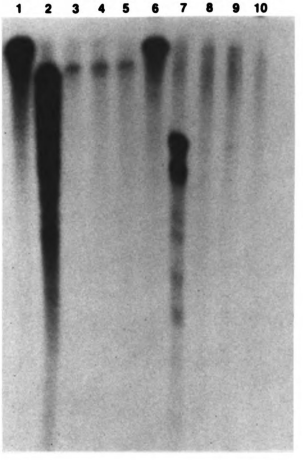


Figure 1. Nuclease S1 protection analysis of two divergently transcribed B. japonicum sequences which exhibit bacteroid-specific expression. Total RNA (10 ug) purified from B. japonicum 110d was hybridized with two uniformly-labelled probes synthesized using single-stranded templates derived from the SalI-PstI fragment of plasmid pBJ296 cloned into M13 phage vectors mp18 and mp19. The RNA was isolated from root nodule bacteria (lanes 2 and 7), cells grown in culture (lanes 3 and 8), or cells grown microaerobically on YEM with 10 mM KNO_3 (lanes 4 and 9). Lanes 1 and 6 contain probe which was not treated with S1 nuclease. Lanes 5 and 10 contain probe which was hybridized without RNA and then treated with S1 nuclease.

Figure 2. The nucleotide sequence of the B. japonicum 1000 bp PstI-HindIII DNA fragment from plasmid pBJ296. The transcription initiation sites for the two symbiotically-regulated promoters are marked by asterisks. Refer to the diagram in Fig. 3 for the direction of transcription.

```
                                                          50
CTGCAGGGAC AGAGCGCTGC CCCTGTGTGT CCGCGGGGAG ATAAGCGCCG

                                                         100
AGCCAAGTTC TGCTAAGTTC AACTGGCGGG CGGGTAGGGA CAGCGCCTGC

                                                         150
TCCAGCGCAA CCAAACGGCA CCGGGTCGCG CCCGCACTGC TTCCGATCAA

                                                         200
TCATCAGCAA TACTTTGGAA CGGGCAAGAA GACTGCGTGG CTGAATCCCC

                                                         250
TCCACCCTTA TCTTCGTGCC ATTCTCTCCG CCAGGCGTGC CGACGAGCGA

                                                         300
CGCCGGCAGC GTCGCCCATT TTTCAACGAG GTACTCCTCA TCGCGGTCTA

                                                         350
CGAGGGTCTC GGATAACTTT CCAGCGGCTT TCTATCGTGC TCCGCCTCGA

                                                         400
TTGACTCTGC CCACATACTT GCGTCGCTCA TGTGCGATTG GCATGTTTTC

                                                         450
TTGTTCATGC AACGTGCATG TGTCTGGTCG TGCTGGTGTC TCCAATTCAA

      *                                                  500
GGCAAAAACT AGCTTAGAGA GTCGCCGACG AGGAGGGGAG CCACCGATTT

                                                  *      550
TGGTAGGTGC CCCTTGGTAC TTCTCGAAGA TACAACTTTG TAGCCCTTTA

                                                         600
GAATTGGAGC CTCTTGGGAG CAAAGGTGGC CAGCCCCAGT CTTTGCGACC

                                                         650
GGGTTGGCCA CCAGCGCTCG CGACATCATG CCCACAGGGC AGCTACAGGC

                                                         700
CAACGAATGC ATATATTCGA GCGGCATATT ACGTCCTTGC GGCCGCAAGC

                                                         750
GCTTGCGGTG CTTGCAGCCA ATCGGGTCCG CGGCCCCCCA TCGGTCTCTG
```

Figure 2 continued.

```
                                                              800
GGACTATCAG ATCGCAGGGC GGCCACATTT AACGTCGACG AAGTTCTGGC

                                                              850
GATGCTGGCC ATACTCGATT GCGTGGAACC CAACCTGCGG CCGAAAAGCG

                                                              900
CGACAGATCG CTGCGCGCAT ACGCGCGCCT CTAGAGGGGC CGCATGGGGG

                                                              950
GGGCAACCAG TTCGGGTAGG ATGTCTGTGA GACCATGAGG TGTGAGCTTT

                                                             1000
CAGTAGGTTG TCCATCCGGT CCTGAACGAG GAAGCAACCT GCCGAAGCTT
```

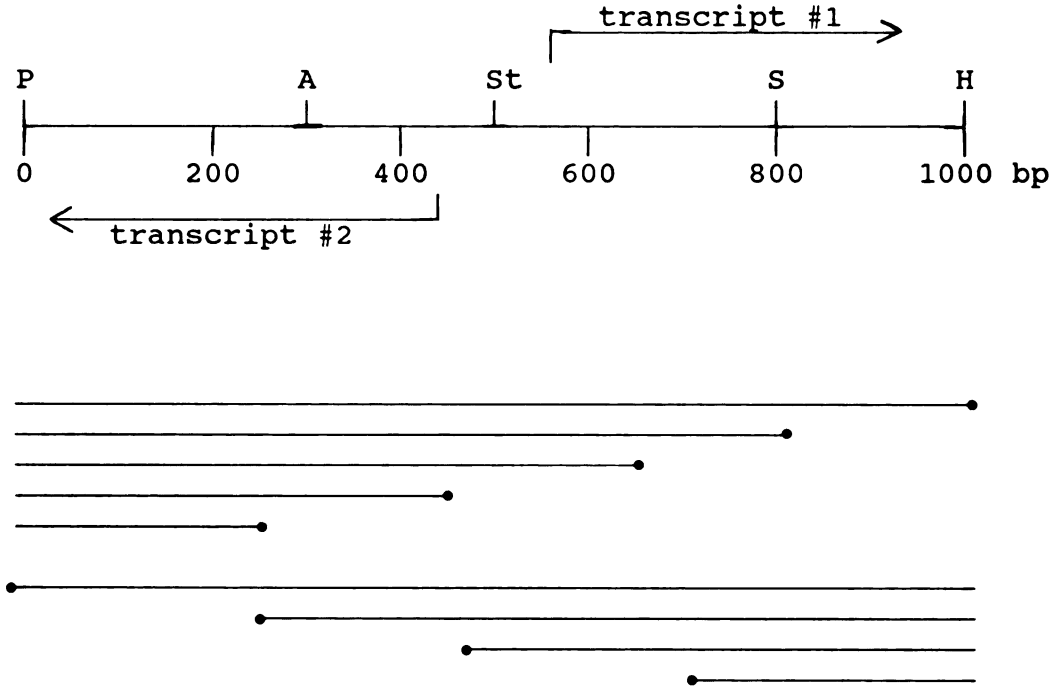


Figure 3. Restriction map of and sequencing strategy for the 1000 bp PstI-HindIII fragment of B. japonicum DNA contained in plasmid pBJ296. The approximate locations of the transcription initiation sites for two divergent, symbiotically regulated transcripts are indicated. P = PstI, A = AccI, St = StyI, S = SalI and H = HindIII. Beneath the restriction map is a schematic representation of the series of nested deletion fragments cloned in M13 mp18 or mp19 which were subjected to DNA sequence analysis. The sequence was determined from the end of the fragment designated with a dot.

sites for the two bacteroid-specific sequences transcribed from plasmid pBJ296 were localized by nuclease S1 protection analysis using end-labelled DNA fragments as probes (Fig. 4). The transcription initiation sites were then precisely identified by comparison of the protected portion of end-labelled oligonucleotide-generated probes to a DNA sequence ladder corresponding to the same region of DNA (Fig. 5). Appropriate regions of the DNA sequences of the two B. japonicum promoters were compared with the conserved regions of the E. coli consensus promoter (Hawley and McClure, 1983) and some degree of similarity was found (Fig. 6). DNA sequence comparisons of the two promoters with that of the promoters of the B. japonicum nifDK, hemA, glnA and glnII genes as well as the entire Genbank data base did not identify any regions of DNA with significant similarities. The amino acid sequences of two polypeptides which could be predicted from the DNA sequence of the two transcribed regions were also compared with the sequences of proteins recorded in the Genbank data base and no significant simlarities were found.

Discussion

The bacteroid-specific expression of B. japonicum sequences present on the recombinant phage B16 was demonstrated initially by differential plaque hybridization

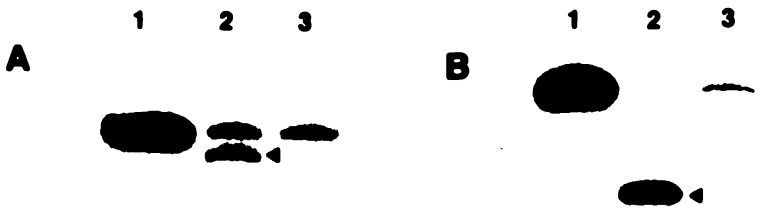


Figure 4. Nuclease S1 protection analysis of two divergently transcribed B. japonicum sequences which exhibit bacteroid-specific expression. Total bacterial RNA (10 ug) purified from root nodules incited by B. japonicum strain 110d was hybridized with end-labelled, strand-separated DNA probes prepared from the 272 bp StyI-SalI (panel A) and the 215 bp AccI-StyI (panel B) fragments from plasmid pBJ296. Lane 1 contains DNA probe which was not digested with S1 nuclease. Lane 2 contains DNA probe which was hybridized with RNA and then subjected to digestion with S1 nuclease. Lane 3 contains DNA probe which was placed under hybridization conditions without added RNA and then subjected to digestion with S1 nuclease. The band in lane 2 indicated by the arrow is the probe DNA partially protected by RNA. The band which migrates at the same size as the untreated probe DNA in both lanes 2 and 3 represents full length protection of the probe by DNA:DNA hybridization.

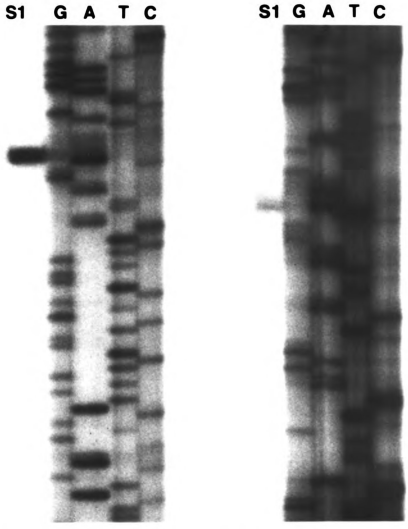


Figure 5. Precise localization of the transcription initiation sites for two divergently transcribed symbiotically-regulated sequences in B. japonicum. Single-stranded DNA probes were generated by primer extension of two specific end-labelled 17 bp oligonucleotides (see Materials and Methods). These probes were hybridized with total bacterial RNA (10 ug) isolated from root nodules incited by B. japonicum strain 110d and then subjected to digestion by S1 nuclease. The protected DNA fragments were accurately sized by subjecting them to electrophoresis next to a DNA sequencing ladder which had been produced by primer extension of the same oligonucleotide.

Figure 6. DNA sequence of the promoter regions of two B. japonicum symbiotically-regulated sequences. The transcription initiation sites are labelled above the nucleotide(s) with an asterisk. The regions exhibiting similarity to the E. coli promoter consensus sequences at -10 nt and -35 nt upstream of the transcription initiation sites are underlined. A nine base pair sequence which is centered at position -97 nt in transcript #1 and at position -100 nt in transcript #2 is underlined with asterisks.

Transcript #1

```
    -100       -90       -80       -70       -60
     TCTCCAATTCAAGGCAAAAACTAGCTTAGAGAGTCGCCGACGAGGAGGGGA
      *********

 -50       -40       -30       -20       -10         *
  GCCACCGATTTTGGTAGGTGCCCCTTGGTACTTCTCGAAGATACAACTTTGTAG
```

Transcript #2

```
    -100       -90       -80       -70       -60
  GCTCCAATTCTAAAGGGCTACAAAGTTGTATCTTCGAGAAGTACCAAGGGGCAC
   *********

  -50       -40       -30       -20       -10         *
   CTACCAAAATCGGTGGCTCCCCTCCTCGTCGGGACTCTCTAAGCTAGTTTTTG
```

E. coli consensus promoter.

```
                                                       *
   --------------TTGAC-----------------TATAAT------CA
```

Ntr consensus promoter.

```
   ----------------------CTGGCAC-----TTGCA----------*
```

experiments (Chapter 2, Fig. 6). Randomly primed cDNA synthesized using total RNA from root nodules or from cells grown in culture was hybridized to purified DNA from the recombinant phage B16 which had been digested with restriction endonucleases, size fractionated on agarose gels and transferred to cellulose nitrate. The difference in the hybridization signal levels produced by the cDNA probes synthesized from nodule bacteria and cultured cell RNA compares favorably with the results from the plaque hybridization experiments (Chapter 2, Fig. 7). The data obtained from the S1 nuclease protection experiments eliminates the possibility that the cDNA which hybridized to recombinant phage B16 was transcribed from elsewhere in the genome since protection of a single-stranded DNA probe from digestion by the nuclease can occur only by precise (i.e. 100% homologous) pairing of the DNA probe with RNA. Therefore, the single protected bands seen in Fig. 2 could only arise by protection of each of the two DNA probes by RNA transcripts from those regions. These experiments also confirmed that transcripts from the two regions examined are present at significantly higher levels in B. japonicum cells isolated from soybean root nodules than in B. japonicum cells grown in culture either aerobically or microaerobically (Fig. 1).

Since experiments described in this chapter confirmed that the DNA region contained on plasmid pBJ216 and subclone pBJ296 is transcribed specifically in bacteroids, the DNA

sequence of the two symbiotically-regulated promoter regions present on the plasmid was determined and the transcription initiation sites precisely localized. The regions 12 nucleotides and 24 nucleotides upstream of each of the transcription initiation sites exhibit little similarity to known nitrogen-regulated promoters from B. japonicum (Fig. 5; Carlson et al., 1987). This result is not surprising since transcription from this region was not induced in cultures in which nitrogen had been depleted (Chapter 3, Fig. 11).

The significance of the similarity between the E. coli consensus promoter (Hawley and McClure, 1983) and the two symbiotically regulated B. japonicum promoters is unclear. The glnA gene promoter is the only other characterized B. japonicum promoter which has similarity to the E. coli promoter consensus sequence. Carlson et al. (1985) found that the glnA gene, unlike the sequences isolated in this study, was transcribed at a relatively constant rate in all cell types and under all experimental conditions examined. It seems unlikely, therefore, that the same promoter sequences would be responsible for both types of regulation.

A sequence of potential importance in the transcriptional regulation of these regions, however, is the 9 bp sequence which is centered at -97 and -100 nucleotides, respectively, from transcription initiation site in each promoter (Fig. 5). Because these regions are transcribed divergently and have only 83 bp between the initiation

sites, the reverse complement of the 9 bp conserved sequence is also present either seven (transcript #1) or nine (transcript #2) base pairs after transcription initiation site. Further experimentation is necessary to determine if and how this sequence is involved in bacteroid-specific transcriptional regulation and whether it functions upstream or downstream of the transcription initiation sites.

CHAPTER 5

Discussion

A developmental process has been defined as a complex, ordered series of events which results in a new morphological or biochemical condition (Dworkin, 1985). Genetic analyses have been applied to such developmental processes in a variety of experimental systems, the most thoroughly examined of which are sporulation in Bacillus subtilis (reviewed by Losick et al., 1986) and Saccharomyces cerevisiae (reviewed by Esposito and Klapholtz, 1981), fruiting body formation in Myxococcus xanthus and Dictyostelium discoideum (reviewed by Kaiser, 1986), and conidiation in Aspergillus nidulans (Timberlake, 1980). In prokaryotic systems the genetic analyses have been based primarily on the characterization of mutants exhibiting impaired development (reviewed by Dworkin, 1985). Transposon mutagenesis techniques have greatly facilitated the isolation of such mutants and the cloning of the corresponding genes (e.g. de Bruijn et al., 1986). In most eukaryotic systems, however, the lack of similar methods for the identification and isolation of genes has led to the use of differential hybridization techniques (Sargent, 1986) to study development. These techniques are more general than mutagenesis in that they can identify genes which are essential for growth of the organism and genes which are

developmentally regulated but produce no phenotype when mutated. Despite the apparent utility of this approach, differential hybridization has been employed only infrequently to study prokaryotic development (e.g. Gianni and Galizzi, 1986). The major focus of this thesis project was the adaptation of differential hybridization techniques to permit the isolation of developmentally regulated genes from the bacterial partner in the Bradyrhizobium japonicum/soybean symbiosis.

Differential hybridization was developed initially as a method for isolation of inducible genes in S. cerevisiae (St. John and Davis, 1979). Complementary DNA (cDNA) probes were prepared from RNA isolated from S. cerevisiae cells grown in the presence or absence of galactose. Libraries of S. cerevisiae DNA were then screened for recombinant phage which contained sequences whose transcription was induced specifically by the addition of galactose. The RNA used for the cDNA synthesis had been enriched for messenger RNA (mRNA) by oligothymidylic acid-cellulose (oligo-dT) chromatography (Aviv and Leder, 1972), a technique which utilizes the hybridization of long stretches of adenylic acid residues (poly-[A]) present on the 3' end of many eukaroytic mRNAs with the oligo-(dT)-cellulose to separate mRNA from ribosomal, transfer and other stable RNA species. This mRNA enrichment step is essential for the reduction of background hybridization signals which would otherwise mask the signal differences produced by regulated genes. There have been a number of reports that poly-(A)-containing RNA

can be isolated from a variety of bacterial species including E. coli (Nakasato et al., 1975; Srinivasan et al., 1975), Bacillus brevis (Sarkar et al., 1978), B. subtilis (Kerjan and Szulmajster, 1980; Gopalakrishna and Sarkar, 1982), Rhodospirillum rubrum (Majumdar and McFadden, 1984) and Methanococcus vannielii (Brown and Reeve, 1985). Efforts to isolate poly-(A)-containing RNA from B. japonicum were unsuccessful. Alternative methods for obtaining mRNA-enriched probes were therefore developed.

Synthesis of randomly primed cDNA from total bacterial RNA is an efficient way to generate single-stranded DNA probes of high specific activity (Taylor et al., 1976). The use of template RNA which has not been enriched for mRNA, however, results predominantly in labelled stable RNA species since these account for more than 90% of the total cellular RNA (Ingraham et al., 1983). The stable RNA template molecules often have considerable sequence similarity to the corresponding genes which encode stable RNAs in E. coli. As a result, cDNA probes synthesized from total bacterial RNA using random primers produce strong background signals when hybridized to either phage or cosmid libraries made in E. coli due to this heterologous hybridization to E. coli stable RNA genes (e.g. Chapter 2, Fig. 1). In the experiments reported here, the background hybridization signal produced in plaque filter hybridizations by the cDNA complementary to rRNA (rcDNA) was reduced by a combination of techniques. The cDNA probes were first hybridized in solution to an excess of unlabelled

plasmid DNA which contained the B. japonicum rDNA operon. The resultant rcRNA:DNA hybrids were removed by hydroxylapatite (HAP) chromatography and the single-stranded fractions retained for use as a hybridization probe. An additional reduction in the background signal level was achieved by including 50 ug/ml of sheared, denatured rDNA plasmid DNA in the pre-hybridization and hybridization solutions when probing B. japonicum genomic libraries propagated in E. coli. In combination, these techniques permitted visualization of some hybridization signals above background levels. The strong signals resulted from the hybridization of the rcDNA-depleted cDNA probe with plaques produced by recombinant phage which contained B. japonicum DNA encoding mRNAs that were abundant in nitrogen-fixing soybean root nodules. These experiments demonstrate that cDNA probes suitable for use in differential screening of phage libraries can be synthesized from total bacterial RNA and used for isolating bacterial genes expressed at high levels in particular cell types or developmental stages.

One limitation of the differential hybridization methods described in this thesis is the level of sensitivity achieved. If the sensitivity of detection could be improved, the method might also be used to identify genes expressed differentially, but at low levels. One obvious approach to this problem would be to further lower the background hybridization signals obtained in plaque hybridization experiments. The reduction in background signal obtained thus far was achieved solely by removal of, or competition

with, rDNA sequences. If additional B. japonicum genes are identified which have considerable sequence similarity to genes in E. coli, such sequences could also be removed from the cDNA probes. This approach might well succeed since the overall sequence similarity between the two organisms is low (Heberlein et al., 1967) and even the highly conserved E. coli dnaK gene (the homologue to the heat shock gene HSP70) fails to hybridize to B. japonicum genomic DNA under conditions of low stringency (see Chapter 3).

The identification of differentially transcribed genes might also be facilitated if the B. japonicum DNA libraries were constructed using smaller DNA fragment sizes. The phage library in vector BF101 had 6 to 10 kb inserts and thus each recombinant phage contained sufficient B. japonicum DNA to encode several transcripts. The presence of a constitutively transcribed gene (or genes) on a segment of DNA could mask a differential hybridizational signal from an adjacent regulated gene, particularly if the regulated gene were expressed at comparatively low levels. The technical problems involved with handling the increased numbers of phage necessary for screening small-insert libraries (approximately 45,000 recombinant phage for library of 1 kb fragments from B. japonicum) would not be a significant obstacle to such an approach. In fact, the potential improvement in sensitivity might well be worth the time necessary to synthesize and screen a small-insert library.

The identification of developmentally regulated genes by differential hybridization has both advantages and disadvantages when compared with methods previously used for isolating such genes. Two limitations of traditional mutational analyses are that the genes of interest must be dispensible for the growth of the organism and that such genes must produce a detectable phenotype when mutated. These limitations are avoided if genes are identified solely on the basis of their transcriptional regulation. Thus, one advantage of differential hybridization is that the technique permits the identification and isolation of any gene which is transcribed at detectably different rates in any two cell types or developmental stages. Several classes of differentially regulated genes might be isolated which would not be identified by traditional mutagenesis. None of the genes isolated in this study were essential for growth, although only five distinct regions were examined. The developmentally regulated sequences which were isolated, however, could not have been identified by chemical or transposon mutagenesis since they produced no symbiotic phenotype when mutated.

One disadvantage of the technique described above is the amount of time and effort required to characterize a differentially transcribed region of DNA once the recombinant phage containing it has been identified. The region must be subcloned into plasmid vectors, mapped with restriction endonucleases and the transcribed portions localized more precisely by probing fragments of plasmids

generated by restriction endonuclease digestion with rcDNA-depleted cDNA. The transcribed regions can then deleted and replaced by a segment of DNA containing an antibiotic resistance marker gene. These constructions may then be introduced into B. japonicum by conjugation and recombination into the chromosome and the strains resulting from double-crossover events identified. With a relatively slow-growing bacterium such as B. japonicum this entire process can take several weeks. The examination of large numbers of transcribed regions would thus be very time consuming.

This problem has been overcome in several microbial developmental systems by the construction of "promoter probe transposons". The promoter probes are transposons which have a promoterless reporter gene (e.g. the lacZ gene) inserted near one end. An insertion of the transposon into an actively transcribed gene in the correct orientation causes the reporter gene to be transcribed and creates a strain with an easily scorable phenotype. Such promoter probe transposons have been successfully used in Myxococcus xanthus (Kroos and Kaiser, 1984), Caulobacter crescentus (Bellofatto et al., 1984) and Bacillus subtilis (Perkins and Youngman, 1986) and have permitted the isolation of genes which are expressed specifically during particular stages of development in these organisms. Genes identified by this method must be inessential for growth since they are mutated by the insertion of the transposon, but the technique does permit the screening of large numbers of insertionally

mutated strains for developmentally regulated genes. The presence of an endogenous beta-galactosidase (the lacZ gene product) activity in soybean root cells and the sensitivity of plant cells to the antibiotic kanamycin have been obstacles to the use of available promoter probes for analysis of the B. japonicum/soybean symbiosis. A fast-growing Rhizobium fredii strain was mutagenized using a mini-mu phage (Mu-dI [Kan, lac]) which had been integrated into a plasmid which could be transferred to, but would not replicate in, Rhizobium (Olson et al., 1985). The kanamycin resistant transconjugants were analysed for lacZ expression in the presence of soybean root exudate, root extract, nodule extract, and low oxygen tension. Several genes were identified which were transcribed at higher levels under these "symbiotic-like" conditions (Olson et al., 1985). The direct analysis of B. japonicum or R. fredii gene expression during the development of the symbiosis may be possible using the luxAB gene from Vibrio harveyii (Baldwin et al., 1984) which has been shown to function in B. japonicum (Legocki et al., 1986). The E. coli beta-glucuronidase gene (GUS) has also been developed as a gene-fusion marker (Jefferson et al., 1986) and most higher plant species have been shown to lack this activity (Jefferson et al., 1987). If a promoter probe transposon could be devised utilizing either of these reporter genes, the identification of bacterial genes which are regulated during soybean root nodule development would be greatly facilitated.

Although it may be possible to improve methods for isolating developmentally regulated genes from B japonicum, perhaps the most important and intriguing observation resulting from this analysis of five regions containing such genes was the fact that deletion of the regions did not affect symbiotic competence. Similar results have also been obtained in studies of development in M. xanthus and B. subtilis in which only a small percentage of the promoter probe transposon insertions in genes which are transcribed specifically during development were found to be essential for development (Kroos and Kaiser, 1986; P. Youngman, personal communication). The fact that deletion or disruption of genes which appear to be transcribed specifically during development does not perturb the process may be explained in several ways: i) developmental processes may consist of many branching pathways so that the loss of any one pathway can be compensated for by others. So little is known about the biochemical functions of genes activated during development that it will require much more research before useful models can be advanced. ii) there may be considerable redundancy of function between developmentally regulated genes and thus mutational analysis will be difficult since a single mutation will not eliminate a particular phenotype. iii) the methods used for identifying the functions of the deleted genes may not be sensitive enough to detect small changes (i.e. lowered efficiency rather than complete loss of function). In any case, it is clear that deletion analysis alone will be

insufficient as a method for identifying the functions of genes isolated by virtue of their transcriptional regulation.

The results of the experiments described in this thesis suggest several possible avenues for future research into the molecular mechanisms of developmental regulation. First, additional promoter regions should be identified, the DNA sequences obtained, and the transcription initiation sites localized. Comparative sequence analysis of these promoter regions might reveal the presence of conserved blocks of DNA sequence which are involved in regulation. Secondly, direct evidence for the involvement of particular portions of the promoter region in transcriptional regulation might be obtained either by deletion analysis or by oligonucleotide-directed mutagenesis. These types of experiments are most conveniently carried out using promoter-reporter gene fusions which have an easily detectable phenotype. Promoter fusions could also be used in a third type of experiment in which the expression of the gene fusion is assayed in randomly mutagenized bacterial strains. Second-site mutations which alter the level of expression of the promoter-reporter gene fusion product might result from mutated regulatory genes. Lastly, a search for regulatory proteins which interact with the promoter regions could be undertaken. The binding of DNA by proteins from cell extracts can be detected by filter binding, electrophoretic mobility shift, or nuclease protection experiments (Hennighausen and Lubon, 1986).

Proteins which bind specifically to regulated promoters would be candidates for purification and inclusion in *in vitro* transcription/translation systems to analyse expression from developmentally regulated promoters. The results from these four general types of experiments either singly or in concert may provide a overview of how developmentally regulated promoters function in the *B. japonicum*.

The results obtained during this thesis project are but a small step toward an understanding of the molecular mechanisms which regulate bacterial gene expression during the development of nitrogen-fixing nodules in the *Bradyrhizobium japonicum*/soybean symbiosis. The isolation and characterization of promoters of *B. japonicum* genes which are regulated during symbiotic development should, however, provide a starting point for research on the more difficult and complex question of how the expression of such promoters and genes is controlled.

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