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FORMATE-DEPENDENT GROWTH IN *BRADYRHIZOBIUM JAPONICUM*

By

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ABSTRACT

FORMATE-DEPENDENT GROWTH BY BRADYRHIZOBIUM JAPONICUM

By

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Bradyrhizobium japonicum is capable of growth on formate, deriving energy from the oxidation of formate by formate dehydrogenase and assimilating carbon as CO_2 via the reductive pentose phosphate cycle, which involves the activities of ribulose-1,5-bisphosphate carboxylase and phosphoribulokinase. Enzymes of formate-dependent growth are subject to catabolite repression in that the presence of glutamate or xylose partially repressed the induction of ribulosebisphosphate carboxylase and phosphoribulokinase activities. Xylose but not glutamate repressed formate dehydrogenase activity, indicating that the regulation of energy acquisition can be separated from that of carbon assimilation. Cyclic AMP accumulated to high levels in cultures growing on formate but not in cultures growing on glutamate or xylose. Addition of xylose to formate medium blocked the accumulation of cAMP but did not prevent induction of enzymes of formate-dependent growth indicating

that increased cAMP accumulation is not essential for their induction. However, a role for cAMP in regulation of formate-dependent growth is not precluded.

To further investigate the regulation of enzymes of formate-dependent growth, genes essential for such growth were isolated by complementation of B. japonicum mutants with a cosmid gene library of B. japonicum DNA. Three related cosmids containing 18.7 kbp of B. japonicum DNA in common were identified as able to restore formate-dependent growth capability to mutants lacking either ribulosebisphosphate carboxylase or both ribulosebisphosphate carboxylase and phosphoribulokinase activities. To further localize the complementing DNA a series of four deletions spanning a total of 16.1 kbp were generated on plasmids in vitro and introduced into the B. japonicum chromosome by homologous recombination. Each resulting deletion mutant lacked ribulosebisphosphate carboxylase activity and immunologically-detectable protein. Three also lacked phosphoribulokinase activity. Two other mutants in which the in vitro-generated plasmid had cointegrated into the chromosome also prevented induction of ribulosebisphosphate carboxylase activity and protein and also phosphoribulokinase activity. The data are consistent with the identified genes comprising an operon including the structural genes encoding phosphoribulokinase and the large subunit of ribulosebisphosphate carboxylase and the operon being transcribed from the former to the latter.

to Mary Lou, of course

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CHAPTER 1

INTRODUCTION

Biological nitrogen fixation by symbioses between leguminous plants and bacteria of the genera Bradyrhizobium and Rhizobium contributes ecologically and agriculturally significant amounts of fixed nitrogen to the biosphere. Nitrogenase, the enzyme complex which catalyzes the biological reduction of atmospheric nitrogen to ammonia, has the apparently inherent property of also reducing protons to form H_2, resulting in a diversion of 25-30% of the electrons passed to nitrogenase away from ammonia production and into H_2 synthesis. The evolution of H_2 by legume root nodules is considered a wasteful use of plant photosynthate (Schubert and Evans 1976), which ultimately is limiting to symbiotic nitrogen fixation (Hardy and Havelka 1976). Nodules from a small proportion of legumes do not evolve hydrogen, apparently due to the activity of a bacterially encoded H_2-oxidizing system which recycles the H_2 evolved by nitrogenase (Eisbrenner and Evans 1983). An uptake hydrogenase was first demonstrated in 1941 in pea nodules incited by Rhizobium leguminosarum (Phelps and Wilson 1941). Twenty-six years later this was rediscovered by Dixon (1967,1972). Since the rediscovery an uptake hydrogenase activity also has been detected in soybean nodules and the

activity has been shown to be associated with B. japonicum bacteroids (Schubert et al. 1977,1978).

The induction of hydrogenase activity in free-living (i.e. ex planta) B. japonicum (Maier et al. 1978a) quickly led to the demonstration that the energy derived from H_2 oxidation could be coupled to CO_2 assimilation, allowing B. japonicum to grow autotrophically (Hanus et al. 1979; Lepo et al. 1980). A number of other bacteria from diverse taxa are capable of aerobically oxidizing H_2 and coupling that energy to CO_2 fixation (Bowien and Schlegel 1981). A smaller number of bacteria can assimilate CO_2 with energy derived from the oxidation of formate by formate dehydrogenase (FDH). These bacteria include a number of H_2-oxidizing autotrophs such as Alcaligenes eutrophus (Friedrich et al. 1979), Pseudomonas oxalaticus (Quayle and Keech 1959a,b) and Paracoccus denitrificans (van Verseveldt and Stouthamer 1978). Recently both B. japonicum and Rhizobium meliloti were shown to be capable of formate-dependent growth (Manian and O'Gara 1982a,b), although R. meliloti lacks hydrogenase and cannot grow autotrophically.

The formate dehydrogenase of B. japonicum is a soluble NAD-dependent enzyme (Manian and O'Gara 1982a). Both P. oxalaticus and A. eutrophus possess a similar enzyme as well as a second NAD-independent membrane-bound isozyme (Johnson et al. 1964; Friedrich et al. 1979; Dijkhuizen et al. 1980; Hopner et al. 1982). As stated above, during autotrophic growth energy is obtained from the oxidation of H_2. The B. japonicum hydrogenase is a membrane-bound, nickel-containing

iron-sulphur dimer of subunits of 63 and 30 kilodaltons (kD) (Harker et al. 1984,1985; Stults et al. 1984; Arp 1985). Similarly, the membrane-bound hydrogenase of A. eutrophus is a nickel-containing (B. Friedrich et al. 1981) dimer of 67 and 30 kD subunits (Schink and Schlegel 1979) which is immunologically related to that of B. japonicum (Schink and Schlegel 1980; Arp et al. 1985). Genes essential for hydrogenase expression have been isolated from B. japonicum although their characterization is still incomplete (Cantrell et al. 1983; Haugland et al. 1984; Hom et al. 1985; Lambert et al. 1985).

In all aerobic H_2-oxidizers, including B. japonicum, CO_2 is assimilated by the reductive pentose phosphate (RPP) cycle during autotrophic as well as formate-dependent growth (Lepo et al. 1980; Bowien and Schlegel 1981). During formate-dependent growth, however, the assimilated CO_2 is not necessarily derived from formate oxidation (Bowien and Schlegel 1981). The actual fixation of CO_2 is accomplished by the carboxylation of ribulose-1,5-bisphosphate (RUBP) in a reaction catalyzed by ribulose bisphosphate carboxylase-oxygenase (RUBISCO). This reaction is unique to the RPP cycle, as is the final step in the cyclic regeneration of RUBP, the phosphorylation of ribulose-5-phosphate by phosphoribulokinase (PRK). Both these reactions are essentially irreversible, giving directionality to the cycle. The bacterial RPP cycle is the same as that found in higher plants (Bassham 1979).

RUBISCO is probably the most abundant protein in the

biosphere and has been subject to a great deal of study (Miziorko and Lorimer 1983). The higher plant enzyme is a heterohexadecamer composed of 8 larger (approximately 55 kD) and 8 smaller (14-20 kD) subunits. A number of bacterial enzymes including those of B. japonicum (Purohit et al. 1982) and A. eutrophus (Bowien et al. 1976,1980; Bowien and Mayer 1978) are similarly composed of larger and smaller subunits. These heterohexadecameric enzymes are collectively referred to as Form I enzymes, although there is heterogeneity among them. Crystallographic data suggest that the arrangement of small subunits on the large subunit core differs between the tobacco and A. eutrophus enzymes (Bowien et al. 1980; Miziorko and Lorimer 1983). This difference in quaternary structure is presumably reflected in differences in the primary amino acid sequences of the proteins and hence in the nucleotide sequences of the genes. Consequently, although numerous large subunit genes from plants and cyanobacteria have been isolated and their nucleotide sequences have been determined and found to be conserved (e.g. McIntosh et al. 1980; Curtis and Hazelkorn 1983), probes from these genes have not been useful for the isolation of bacterial large subunit genes. A number of bacterial RUBISCOs, called Form II enzymes, do not contain smaller subunits. The Rhodospirillum rubrum enzyme is a homodimer of 56 kD subunits (Schloss et al. 1979) and Rhodopseudomonas sphaeroides and Rhodopseudomonas capsulata have Form II enzymes which are hexamers of 55 kD subunits and also have Form I RUBISCOs (Gibson and

Tabita 1977a,b,c). Immunological and peptide mapping data confirm that the Form I and Form II large subunit proteins are different (Gibson and Tabita 1977a,b,c,1985) and although genes for several Form II enzymes have been cloned (Somerville and Somerville 1984; Quivey and Tabita 1984; Muller et al. 1985), these genes also have not facilitated the identification of a bacterial Form I gene. Recently genes encoding a Form I RUBISCO have been cloned and shown to be reiterated on the chromosome and on a large plasmid in A. eutrophus (Andersen and Wilke-Douglas 1984) but more detailed information is not yet available.

Phosphoribulokinase (PRK) is a second enzyme unique to the RPP cycle. PRK has been purified from A. eutrophus and has been shown to be an octamer of identical 33 kD subunits (Siebert et al. 1981; Siebert and Bowien 1984). Similar data have been obtained from members of the Rhodospirillaceae (Tabita 1980; Rippel and Bowien 1984). Genes for PRK were recently identified in A. eutrophus and, like RUBISCO, were found to be reiterated both on the chromosome and on a large plasmid (Klintworth et al. 1985). In higher plants this enzyme is important in light regulation of photosynthetic CO_2 flow (Latzko and Kelly 1979; Flugge et al. 1982). In prokaryotes this enzyme is subject to both positive and negative allosteric regulation (Latzko and Kelly 1979; Ohmann 1979) and in A. eutrophus an inactivation/reactivation mechanism has been described (Leadbeater et al. 1982; Leadbeater and Bowien 1984).

Because H_2, formate and CO_2 are major endproducts of microbial fermentation in the soil, the ability to utilize these substrates for growth might confer a competitive advantage. B. japonicum must persist in the soil for lengthy periods in the absence of its legume host and persistence is presumably related to its competitiveness with other soil organisms for available nutrients. A given B. japonicum strain must then compete with other strains of B. japonicum for nodulation sites when roots of the appropriate host reappear, and inoculum size, dictated by persistence, is a major component of competitiveness at this level (Bushby 1982). In the soil environment the concomitant use of H_2, formate, CO_2 and heterotrophic substrates, i.e. mixotrophy, probably predominates among facultative autotrophs such as B. japonicum (Bowien and Schlegel 1981), making the regulation of the various enzymes involved in order to optimize growth and energy conservation vitally important.

Assimilation of CO_2 by the RPP cycle is energetically expensive. The synthesis of cell material from 1 mol CO_2 uses 7.9 mol ATP, in contrast to a requirement of 2.9 mol ATP to convert 1 mol carbohydrate carbon to cell material (Ohmann 1979). Not surprisingly then, enzymes involved in CO_2 assimilation and oxidation of heterotrophic substrates are tightly regulated. In general, the presence of heterotrophic substrates represses activities of RUBISCO and PRK as well as hydrogenase. However, mixotrophy, the concomitant use of inorganic and organic compounds as energy

and/or carbon sources, has been shown in a number of hydrogen bacteria (Bowien and Schlegel 1981). Usually this mixotrophy involves oxidation of H_2 as an ancillary energy source during oxidation of the heterotrophic substrate while carbon is primarily derived from the heterotrophic substrate. However, both hydrogenase and RUBISCO have been detected in the presence of certain heterotrophic substrates in A. eutrophus, albeit at levels reduced from those found in autotrophically-growing cells (Stukus and DeCicco 1970; C.G. Friedrich et al. 1981). Bowien and Schlegel (1981) refer to growth by formate oxidation and CO_2 assimilation as an unusual sort of mixotrophy with the organic substrate, formate, serving as the energy source. Again, due to the relative expense of CO_2 assimilation, in the presence of heterotrophic carbon substrates enzymes of formate-dependent growth are repressed, although they are still detectable at reduced levels in A. eutrophus (C.G. Friedrich et al. 1981; Im and Friedrich 1983), P. oxalaticus (Blackmore and Quayle 1968; Dijkhuizen et al. 1978; Dijkhuizen and Harder 1979a,b,1984) and in B. japonicum (Simpson et al. 1979; Manian and O'Gara 1982a; Manian et al. 1982).

In many organisms during growth on mixtures of substrates, one substrate, usually that which supports most rapid growth, is preferentially utilized and the activities of enzymes associated with the utilization of the "poorer" carbon substrates are repressed. This is commonly referred to as catabolite repression. The best-known example of this is the "glucose effect" in enteric bacteria. The presence

of glucose represses, to varying degrees, the utilization of a variety of other carbon substrates. It was subsequently shown that the addition of glucose resulted in a rapid lowering of the intracellular cAMP concentration (Makman and Sutherland 1965) and that the addition of cAMP could at least partially overcome the glucose effects (Ullmann et al. 1969). The effect of cAMP was shown to be exerted via interaction with a protein factor, the cAMP receptor protein (CRP), which is a DNA-binding protein (Emmer et al. 1970; Zubay et al. 1970). It is now accepted that the cAMP-CRP complex acts as a positive regulatory factor for initiation and termination of transcription (Ullmann and Danchin 1983). Many genes in Escherichia coli are under the control of the cAMP-CRP complex and the observed hierarchy of responsiveness can be attributed to differential affinities of these genes' promoters for the complex. In addition to the many examples of positive regulation by the cAMP-CRP complex, this complex also exerts negative regulation, most notably on the expression of the genes encoding adenylate cyclase (cya; Aiba 1985; Kawamukai et al. 1985), which synthesizes cAMP, and CRP (crp; Aiba 1983), which have been cloned and characterized from E. coli and other organisms (Ullmann and Danchin 1983).

Although the role of the cAMP-CRP complex in the expression of many genes is well established, several lines of evidence suggest that catabolite repression is not modulated exclusively through the complex. Most convincingly, catabolite repression has been demonstrated in

mutants lacking adenylate cyclase (Dessein et al. 1978) or lacking CRP (Guidi-Rontani et al. 1980). Ullmann et al. (1976) described and partially purified a water soluble, heat stable compound of low molecular weight called catabolite modulator factor which exerts strong repression on catabolite-sensitive operons but not on catabolite-insensitive operons. Because of these observations Ullmann and Danchin (1983) have suggested that variations in cAMP levels respond to, rather than cause, catabolite repression. In their view, cAMP behaves as an alarmone (Stephens et al. 1975) whose concentration responds to cellular carbon and energy status. The observations that intracellular cAMP increases in response to carbon deprivation (Makman and Sutherland 1965) or upon transfer to anaerobiosis (Phillips et al. 1978) are consistent with this hypothesis.

How the level of cAMP is controlled is not clear. Rates of synthesis by adenylate cyclase, of hydrolysis by phosphodiesterase and of efflux from the cells (in E. coli up to 99.9% of the cAMP synthesized is excreted (Matin and Matin 1982) are all potential regulatory steps. Ullmann and Danchin (1983) suggest that rate of synthesis is primarily responsible for variation in cAMP levels.

Most of the study of cAMP has been in enteric bacteria. However, cAMP has been detected in a wide range of bacterial taxa (Botsford 1981; Biville and Guiso 1985), including Bradyrhizobium (Lim and Shanmugam 1979; Ching et al. 1981; Maier and Merberg 1982; Merberg et al. 1983; Guerinot and Chelm 1985; McGetrick et al. 1985) and Rhizobium (McGetrick

et al. 1985). In most species studied levels of cAMP appeared to be regulated with culture age (Lim and Shanmugam 1979; Biville and Guiso 1985; McGetrick et al. 1985) or with carbon source (Lim and Shanmugam 1979). Pseudomonas is a notable exception. Although cAMP has been detected, intracellular levels remain constant with culture age (Biville and Guiso 1985) and during growth on diverse carbon substrates (Siegel et al. 1977; Phillips and Mulfinger 1981). Pseudomonas displays catabolite repression of several functions by succinate which cannot be overcome by exogenous cAMP (Siegel et al. 1977; Phillips and Mulfinger 1981). Similarly, although cAMP has been detected in Bacillus during oxygen limitation (Mach et al. 1984) and during sporulation (Biville and Guiso 1985), catabolite repression occurs in conditions under which cAMP cannot be detected (Setlow 1973). Clearly then, although catabolite repression in E. coli involves cAMP, it is not exclusively modulated by cAMP, and other bacteria display catabolite repression without any apparent involvement of cAMP.

A variety of catabolite-repression-like phenomena have been described in Bradyrhizobium and Rhizobium. As in Pseudomonas, the best catabolite repressors appear to be dicarboxylic acids which have been shown to repress B-galactosidase activity (Ucker and Signer 1978), arabinose utilization (Ferrenbach and Lepo 1985), glucose, fructose and mannose transport (Arias et al. 1982; deVries et al. 1982; Hornez et al. 1984), aromatic utilization (Dilworth et al. 1983; Rohm and Werner 1985), hexose catabolism (Stowers

and Elkan 1985), hydrogenase activity (Lim and Shanmugam 1979; Maier et al. 1979; Merberg et al. 1983; McGetrick et al. 1985) and CO_2 assimilation (Manian and O'Gara 1982a; McGetrick et al. 1985). Exogenous cAMP failed to overcome repression of B-galactosidase activity in R. meliloti (Ucker and Signer 1978) and repression of CO_2 assimilation in R. meliloti and B. japonicum (McGetrick et al. 1985). The only demonstrated effects of cAMP have been in Bradyrhizobium, where cAMP relieves the repression of hydrogenase by malate (Lim and Shanmugam 1979; McGetrick et al. 1985) and the repression of arabinose utilization by succinate (Ferrenbach and Lepo 1985). In addition, cAMP has been shown to repress activities of enzymes of ammonia assimilation in B. japonicum (Upchurch and Elkan 1978) and also to derepress nitrogenase activity in Bradyrhizobium sp. (cowpea) (Pankhurst 1981).

Genes encoding adenylate cyclases have been isolated from B. japonicum (Guerinot and Chelm 1984) and R. meliloti (Kiely and O'Gara 1983) on the basis of their ability to complement E. coli mutants lacking adenylate cyclase activity. Both these genes appear to be unique within their respective genomes and they do not hybridize with each other (Kiely and O'Gara 1983; Guerinot and Chelm 1984; Guerinot and Chelm, personal communication). The R. meliloti gene seems to function in B. japonicum where its presence will partially overcome the malate repression of hydrogenase activity when present in extra copies (McGetrick et al. 1985). A B. japonicum mutant in which the cya region has

been deleted still has wild type or perhaps slightly reduced levels of cAMP and no apparent phenotype has been detected (Guerinot and Chelm 1985). This may indicate that B. japonicum has isozymes of adenylate cyclase (Guerinot and Chelm 1986b). B. japonicum has isozymes of glutamine synthetase which are encoded by different genes (Darrow and Knotts 1977; Carlson et al. 1985). Bacterial isozymes of adenylate cyclase have been demonstrated in Myxococcus xanthus (Devi and McCurdy 1984).

The purpose of the research presented in this thesis is to determine the molecular mechanisms of regulation of formate-dependent growth in Bradyrhizobium japonicum. As outlined above, the regulation of synthesis of enzymes involved in the utilization of formate in an efficient fashion probably contributes to the competitiveness of the organism in the soil. It is hoped that the mechanisms elucidated will have general significance in understanding gene regulation in B. japonicum. Such an understanding is an essential prerequisite for manipulation of the symbiosis with soybean, a goal of major agronomic importance. Elucidating the mechanisms of control of carbon and energy utilization during growth on mixed substrates could have direct application to increasing nodule hydrogenase activity which has been shown to influence yield (Schubert and Evans 1976; Schubert et al. 1978).

I have combined physiological and molecular genetic approaches. In the second chapter I describe the induction of enzymes of formate-dependent growth during the transition

from heterotrophic growth on substrates such as glutamate or xylose to mixotrophic growth on formate or on mixtures of formate and heterotrophic substrates. The role of cAMP in the catabolite repression observed is explored. In the third chapter I have used this physiological information to design experiments to identify and isolate genes essential for the expression of enzymes of formate-dependent growth. Genes so identified will be useful in several ways. The structural genes for enzymes of formate-dependent growth can be used as molecular probes to monitor transcriptional regulation of these enzyme activities. The analysis of regulatory genes will contribute to understanding the molecular mechanisms of regulation of enzymes of formate-dependent growth.

CHAPTER 2

Formate-Dependent Growth in Bradyrhizobium japonicum.

Introduction

Bradyrhizobium japonicum, when not in association with soybean roots, must persist in the soil and compete with other soil inhabitants for carbon and energy sources. Formate, H_2 and CO_2 are common endproducts of microbial fermentation in the soil, and the ability to utilize these substrates presumably would be advantageous. An uptake hydrogenase was demonstrated in Rhizobium leguminosarum-induced nodules on pea roots (Phelps and Wilson 1941; Dixon 1967,1972) and in B. japonicum-induced nodules on soybean roots (Schubert et al. 1977, 1978). The demonstration of this uptake hydrogenase activity in free-living B. japonicum (Maier et al. 1978a) allowed the development of conditions for autotrophic growth in B. japonicum (Hanus et al. 1979; Lepo et al. 1980). The assimilation of CO_2 was by ribulose-1,5-bisphosphate carboxylase (RUBISCO), a key enzyme of the reductive pentose phosphate (RPP) cycle (Lepo et al. 1980). The ability to support CO_2 assimilation with H_2 oxidation is widespread amongst bacteria of diverse taxa (Bowien and Schlegel 1981). A number of aerobic hydrogen-oxidizers including Pseudomonas oxalaticus (Quayle and Keech 1959a,b)

Paracoccus denitrificans (van Verseveld and Stouthamer 1978) and Alcaligenes eutrophus (Friedrich et al. 1979) are also able to support CO_2 assimilation with energy derived from the oxidation of formate. Formate-dependent growth has been shown in B. japonicum and Rhizobium meliloti, with energy derived from the activity of a soluble, NAD-dependent formate dehydrogenase (FDH) and CO_2 assimilated by RUBISCO (Manian and O'Gara 1982a,b).

CO_2 assimilation by RUBISCO is energetically expensive (Ohmann 1979) and in general, heterotrophic growth substrates repress formation of enzymes of formate- and H_2/CO_2-dependent growth (Schlegel and Eberhardt 1972; Bowien and Schlegel 1981). However mixotrophy, the concomitant utilization of inorganic and organic compounds as energy and/or carbon sources, has been demonstrated in a number of species and is probably of ecological importance for facultative chemolithoautotrophs such as A. eutrophus and B. japonicum (Bowien and Schlegel 1981). In A. eutrophus hydrogenase and RUBISCO are synthesized during growth on fructose or alanine, which only partially repress the formation of key enzymes of lithoautotrophic metabolism. Other substrates such as lactate, pyruvate or acetate completely repress the synthesis of these enzymes (Stukus and DeCicco 1970; C.G. Friedrich et al. 1981). Enzymes of formate-dependent growth, specifically FDH and RUBISCO, have been detected during heterotrophic growth on certain organic substrates in B. japonicum (Manian and O'Gara 1982a), A. eutrophus (Friedrich et al. 1979; Im and Friedrich 1983),

P. oxalaticus (Blackmore and Quayle 1968; Dijkhuizen et al. 1978; Dijkhuizen and Harder 1979a,b,1984) and Thiobacillus (Smith et al. 1980; Wood and Kelly 1981).

The repression of enzymes of formate-dependent growth to varying degrees by different carbon substrates suggests that the regulatory mechanism might be similar to that of catabolite repression as it is understood in enteric bacteria (Botsford 1981; Ullmann and Danchin 1983). In E. coli the presence of glucose results in lowered cAMP levels (Makman and Sutherland 1965). Cyclic AMP in conjunction with its receptor protein is required as a positive regulatory factor for the expression of genes encoding enzymes for the catabolism of poorer carbon substrates such as lactose and maltose (Ullmann and Danchin 1983). In B. japonicum cAMP was found to relieve the catabolite repressing effect of malate on hydrogenase activity (Lim and Shanmugam 1979; McGetrick et al. 1985). However, exogenous cAMP did not relieve malate repression of RUBISCO in B. japonicum and R. meliloti (McGetrick et al. 1985). It is thought that cAMP is not involved in regulation of hydrogenase in A. eutrophus (Friedrich 1982). In Pseudomonas catabolite repression does not seem to involve cAMP (Siegel et al. 1977; Phillips and Mulfinger 1981).

In the present study we have investigated the induction of the activities of three key enzymes of formate-dependent growth, FDH, RUBISCO and phosphoribulokinase (PRK) in B. japonicum. We have measured enzyme activities and protein accumulation during transitions from heterotrophic growth to

growth on formate or on mixtures of formate and heterotrophic substrates. We also have determined levels of cAMP during these transitions and during growth on formate or on heterotrophic substrates.

Materials and Methods

Bacterial Growth. All experiments used a stable small-colony derivative of Bradyrhizobium japonicum USDA strain 3I1b110 (Kuykendall and Elkan 1976; Guerinot and Chelm 1986a). Cultures were grown in a defined mineral medium containing the following (per liter): 3-(N-morpholino)-propanesulphonate (MOPS), 10.47 g; $(NH_4)_2SO_4$, 0.33 g; KH_2PO_4, 0.3 g; Na_2HPO_4, 0.3 g; $MgSO_4 \cdot 7\ H_2O$, 0.12 g; $CaCl_2$, 50 mg; H_3BO_4, 10 mg; $ZnSO_4 \cdot 2\ H_2O$, 0.1 mg; $CuSO_4 \cdot 5\ H_2O$, 0.5 mg; $MnCl_2 \cdot 4\ H_2O$, 0.5 mg; $Na_2MoO_4 \cdot 2\ H_2O$, 0.1 mg; $FeCl_3$, 1.0 mg; $NiCl_2$, 1.19 mg; riboflavin, 20 ug; biotin, 12 ug; thiamine·HCl, 80 ug; inositol, 48 ug; p-aminobenzoic acid, 8 ug; nicotinic acid, 50 ug; calcium pantothenate, 80 ug; cyanocobalamin, 0.1 ug. The pH was adjusted to 6.8 with NaOH before autoclaving. The $CaCl_2$ and vitamins were sterilized by filtration and added aseptically after autoclaving. Sterile solutions of formate, glutamate and xylose were added to give a final concentration of 0.15% (w/v). For experiments in which cells were switched from growth on one carbon substrate to growth on a second substrate, cultures were grown to an optical density (420 nm) of 0.3, harvested by centrifugation, washed in minimal

medium base without carbon substrate and then resuspended in the second medium. Where indicated, rifampicin (200 ug/mL; U.S. Biochemical Corp., Cleveland OH) and chloramphenicol (400 ug/mL; Sigma, St. Louis MO) were added to block transcription and translation, respectively. All cultures were grown at 30°C.

Preparation of Cell-free Extracts. Cell pellets were resuspended in the buffers indicated below and ruptured by passage 3 times through a French pressure cell at 844 kg cm^{-2} at 4°C. The extracts were centrifuged at 20,000 x g for 30 min at 4°C and the supernatants were used for enzyme assays. For PRK and RUBISCO assays cells were resuspended in R/P buffer, with the following composition: 0.1 M Tris (pH 8.0), 20 mM $MgCl_2$, 20 mM $NaHCO_3$, 10 mM 2-mercaptoethanol (2-ME), 0.4 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). Cells for FDH assays were resuspended in F buffer, with the following composition: 0.2 M $NaPO_4$ (pH 7.0), 10 mM 2-ME, 0.4 mM DTT and 0.5 mM PMSF.

Assays. RUBISCO was assayed by measuring the ribulose-1,5-bisphosphate (RUBP; Sigma, St. Louis MO) dependent incorporation of $^{14}CO_2$ into acid-stable products (Wishnick and Lane 1971). $NaH^{14}CO_3$ (Amersham, Arlington Heights IL) was added directly to crude extract (or to crude extract diluted in R/P buffer) to yield a specific activity of 1 mCi/mmol and the sample was activated for 30 min at 30°C.

The reaction was initiated by adding RUBP to give 400 uM. A duplicate of each sample was assayed without RUBP. Reactions were terminated after 30 min at 30°C by adding 2 volumes of 6 N acetic acid. The sample was transferred to a scintillation vial, the sample tube was washed with 4 volumes of 6 N acetic acid which was combined with the sample in the scintillation vial and unreacted $^{14}CO_2$ was volatilized by drying the sample at 100°C. The sample was redissolved in 0.4 mL of 0.5 N HCl, scintillation cocktail (Safety Solve; Research Products Intl., Mount Prospect IL) was added and the residual radioactivity was determined in a Beckman LS6800 scintillation counter. For determining the heat sensitivity of RUBISCO, crude extract was added to preheated tubes and incubated at the indicated temperatures for varying times after which the samples were rapidly cooled in ice-water. The samples were then assayed normally at 30°C.

PRK was assayed by coupling the reaction to RUBISCO and monitoring the ribulose-5-phosphate (R5P; Sigma) dependent incorporation of $^{14}CO_2$ into acid-stable products (Siebert et al. 1981). To crude extract, or to crude extract diluted with R/P buffer were added B-NADH (to give 2 mM), ATP (to give 2 mM), $NaH^{14}CO_3$ (to give a specific activity of 1 mCi/mmol) and excess activated Rhodospirillum rubrum RUBISCO (prepared by the procedure of Somerville and Somerville 1984). The reaction was initiated by the addition of R5P to give 1.15 mM, incubated at 30°C, terminated, processed and analysed as described for RUBISCO.

FDH was assayed in crude extracts incubated at 30°C by measuring the formate-dependent reduction of NAD spectrophotometrically at 340 nm (Johnson et al. 1964) using a Perkin Elmer Lambda 7 spectrophotometer. The reaction mix contained $NaPO_4$ at 0.1 M, NAD at 50 mM and sodium formate at 50 mM.

Protein was measured by the procedure of Lowry et al. (1951) using bovine serum albumin (BSA) as a standard.

To measure cAMP, extracts were prepared by boiling 2 mL samples from bacterial cultures for 10 min, centrifuging at 7700 x g for 10 min at 4°C to remove bacterial debris and lyophilizing the supernatant (Guerinot and Chelm 1984). The dried residue was suspended in 0.5 mL of 50 mM sodium acetate (pH 6.2) and cAMP was determined by radioimmunoassay (New England Nuclear Corp., Boston, MA). Samples were tested for susceptibilty to phosphodiesterase (3',5'-cyclic nucleotide-nucleotidohydrolase; Boehringer-Mannheim, Indianapolis IN) by incubation with 0.005 U of phosphodiesterase for 20 min at 25°C at pH 6.8 in the presence of Mg^{2+} (1.5 mM) and DTT (2 mM) (Butcher 1974). Reactions were terminated by incubation for 10 min at 90°C and residual cAMP was determined by radioimmunoassay. These conditions resulted in complete hydrolysis of 5 pmol of exogenous cAMP.

Protein Electrophoresis and Immuno-Blotting. Gradient (10-15%) SDS-polyacrylamide gel electrophoresis (Laemmli 1970) was performed on RUBISCO crude extracts. Proteins were

electroblotted to cellulose nitrate (Millipore HA, 0.45 um; Millipore Corp, Bedford MA) for 4 h at 1.5 A (Towbin et al. 1979). Non-specific antibody binding was blocked by incubation of the cellulose nitrate transfer first in W buffer (20 mM Tris (pH 7.4), 0.9% (w/v) NaCl) plus 3% (w/v) BSA and then in W buffer plus 0.05% (v/v) Tween-20. The blocked transfer was incubated overnight at 4°C with antiserum prepared against purified B. japonicum RUBISCO holoenzyme (Purohit et al. 1982) diluted 1:100 in W buffer plus 1% (w/v) BSA, 0.01% (w/v) NaN_3 and 0.05% (v/v) Tween-20. The transfer was washed in W buffer plus 0.05% Tween-20 and then incubated for 3 h at room temperature with alkaline phosphatase conjugated to Staphylococcus aureus protein A (Sigma) diluted 1:1000 in the antibody solution minus NaN_3. The transfer was washed in a solution of 100 mM Tris (pH 7.5), 100 mM NaCl, 2 mM $MgCl_2$ and 0.05% Tween-20 and then in a solution of 100 mM Tris (pH 9.5), 100 mM NaCl and 5 mM $MgCl_2$. Color was developed by incubation of the cellulose nitrate transfer at room temperature in a solution 100 mM Tris (pH 9.5), 100 mM NaCl and 5 mM $MgCl_2$ to which nitro-blue tetrazolium (0.33 mg/mL; Sigma) and 5-bromo-4-chloro-3-indoyl phosphate (0.17 mg/mL; Sigma) had been added. The reaction was stopped by replacing the color development solution with a solution of 10 mM Tris (pH 7.5) and 1 mM ethylenediaminetetraacetic acid (EDTA).

Results

Induction of Enzymes of Formate-dependent Growth. Initial experiments were designed to determine the time course of induction of FDH, PRK and RUBISCO activities following transfer of cells growing logarithmically on glutamate to a formate based medium. All 3 enzyme activities were induced co-ordinately and were detectable within 10 h of transfer (Figure 1). Growth, as monitored by optical density and protein concentration, was completely blocked by rifampicin, an inhibitor of transcription, or chloramphenicol, an inhibitor of translation. Induction of all three enzyme activities was also blocked, indicating a requirement for de novo transcription and translation for their induction (Figure 1). Accumulation of the large subunit of RUBISCO, as determined immunologically by immuno-blotting, followed the same time course of induction (Figure 2) as did enzyme activity, indicating that the induction of RUBISCO activity was a direct result of protein accumulation. The small subunit of RUBISCO either did not react well with the antibody or else did not transfer well to the cellulose nitrate and was never detected in our experiments.

Regulation of Enzymes of Formate-dependent Growth. Induction of FDH, PRK and RUBISCO was dependent on the presence of formate. Cells grown on either glutamate or xylose did not have detectable activities of any of these

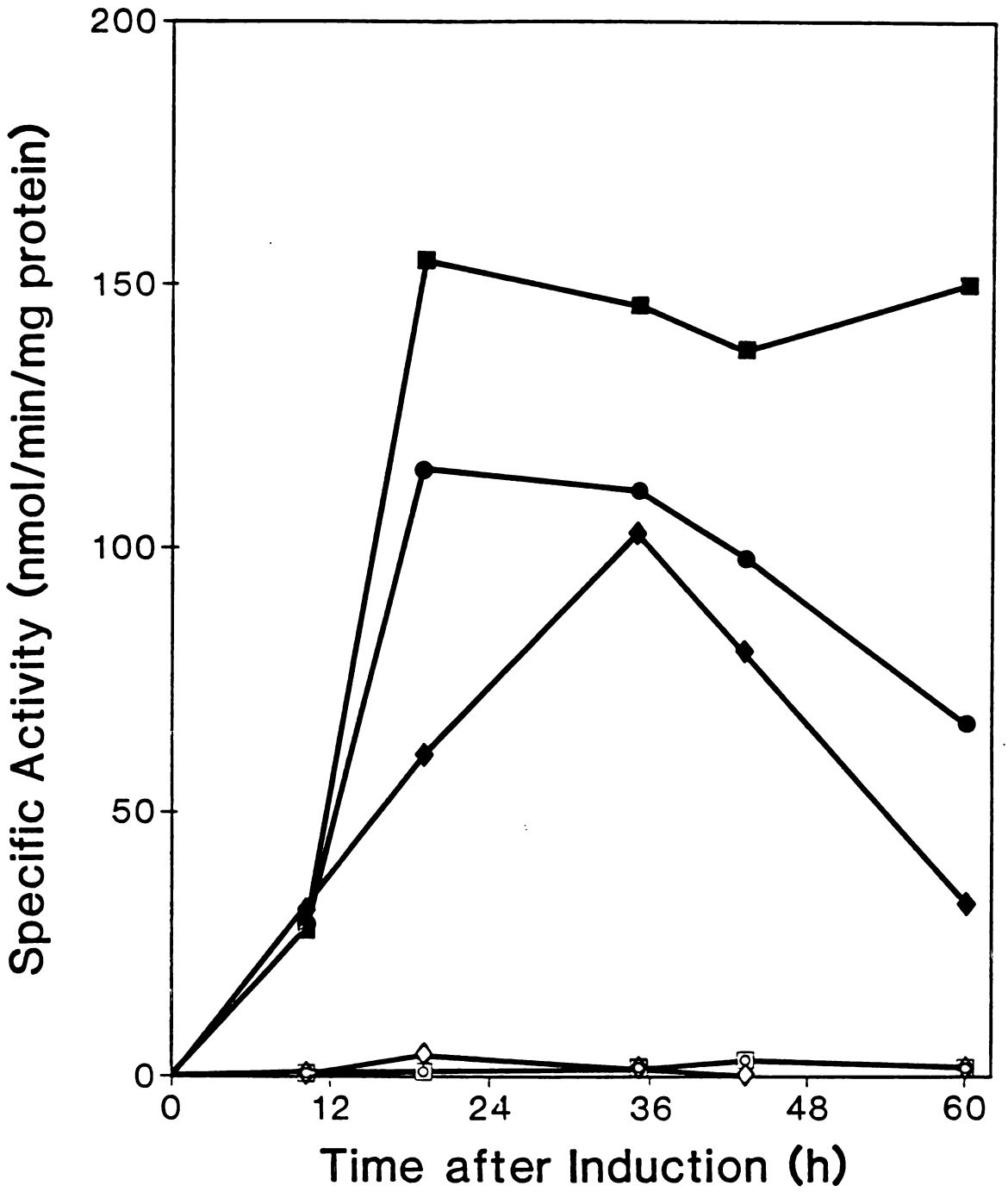
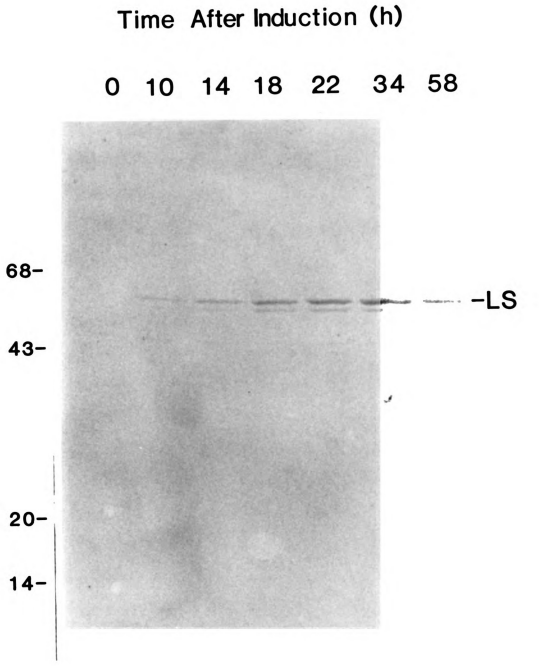


Figure 1. Enzymes of formate-dependent growth following transfer of cells from growth on glutamate to growth on formate at T=0 h. Symbols: triangles, formate dehydrogenase; circles, ribulose-1,5-bisphosphate carboxylase; squares, phosphoribulokinase. Closed symbols are in the absence of and open symbols are in the presence of chloramphenicol (400 ug/mL).

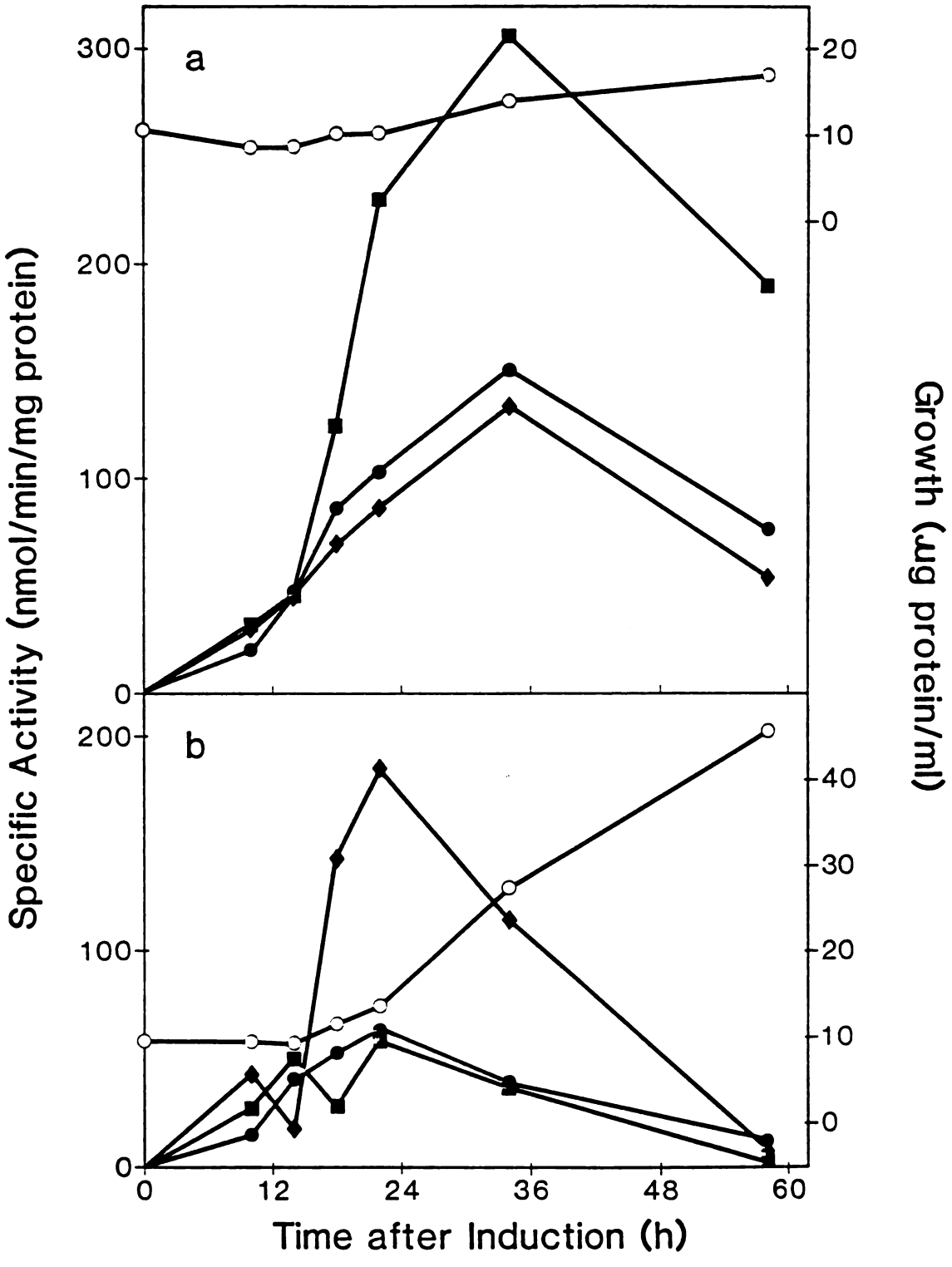
Figure 2. Accumulation of the large subunit (LS) of ribulose-1,5-bisphosphate carboxylase (RUBISCO) following transfer from growth on glutamate to growth on formate at T=0 h. LS was visualized by probing immuno-blots of crude cell-free extracts of B. japonicum with antiserum directed against purified B. japonicum RUBISCO.


Time After Induction (h)
0 10 14 18 22 34 58
68-
43-
20-
14-
-LS

enzymes (Figures 3 and 4). Furthermore, cells growing on glutamate and transferred to a minimal medium lacking formate or any other carbon/energy source did not induce FDH, PRK or RUBISCO activities. Maximum activities of FDH, PRK and RUBISCO detected in the absence of any carbon/energy source were 3.5%, 0.4% and 0.5%, respectively, of the levels induced in the presence of formate. Similarly, A. eutrophus lacking an energy substrate, as following the exhaustion of a heterotrophic substrate, did not induce RUBISCO (Friedrich 1982). When glutamate-grown B. japonicum cells were transferred to either formate or to a formate-glutamate mixture, growth initially ceased for 14-18 h and then resumed at a rate characteristic of formate alone or of glutamate alone, respectively (Figure 3). FDH, PRK and RUBISCO activities were all detected within 10 h of transfer to either medium. Activities were maximal 36 or 24 h after transfer to formate or to formate-glutamate, respectively, and then declined as cultures approached stationary phase. FDH was induced to a similar level in the formate-glutamate mixture as it was in the formate medium. However, PRK and RUBISCO activities were both substantially reduced by the addition of glutamate to the formate medium, suggesting that glutamate was repressing the CO_2-assimilating pathway but not the energy-acquiring pathway.

When cells growing logarithmically on xylose were transferred to media containing formate or formate-xylose growth initially ceased and then resumed after 12-14 h at rates characteristic of formate or xylose alone,

Figure 3. Growth and enzymes of formate-dependent growth following transfer from growth on glutamate to growth on either formate (Panel A) or a mixture of formate and glutamate (Panel B) at T=0 h. Symbols: triangles, formate dehydrogenase; closed circles, ribulose-1,5-bisphosphate carboxylase; squares, phosphoribulokinase; open circles, growth.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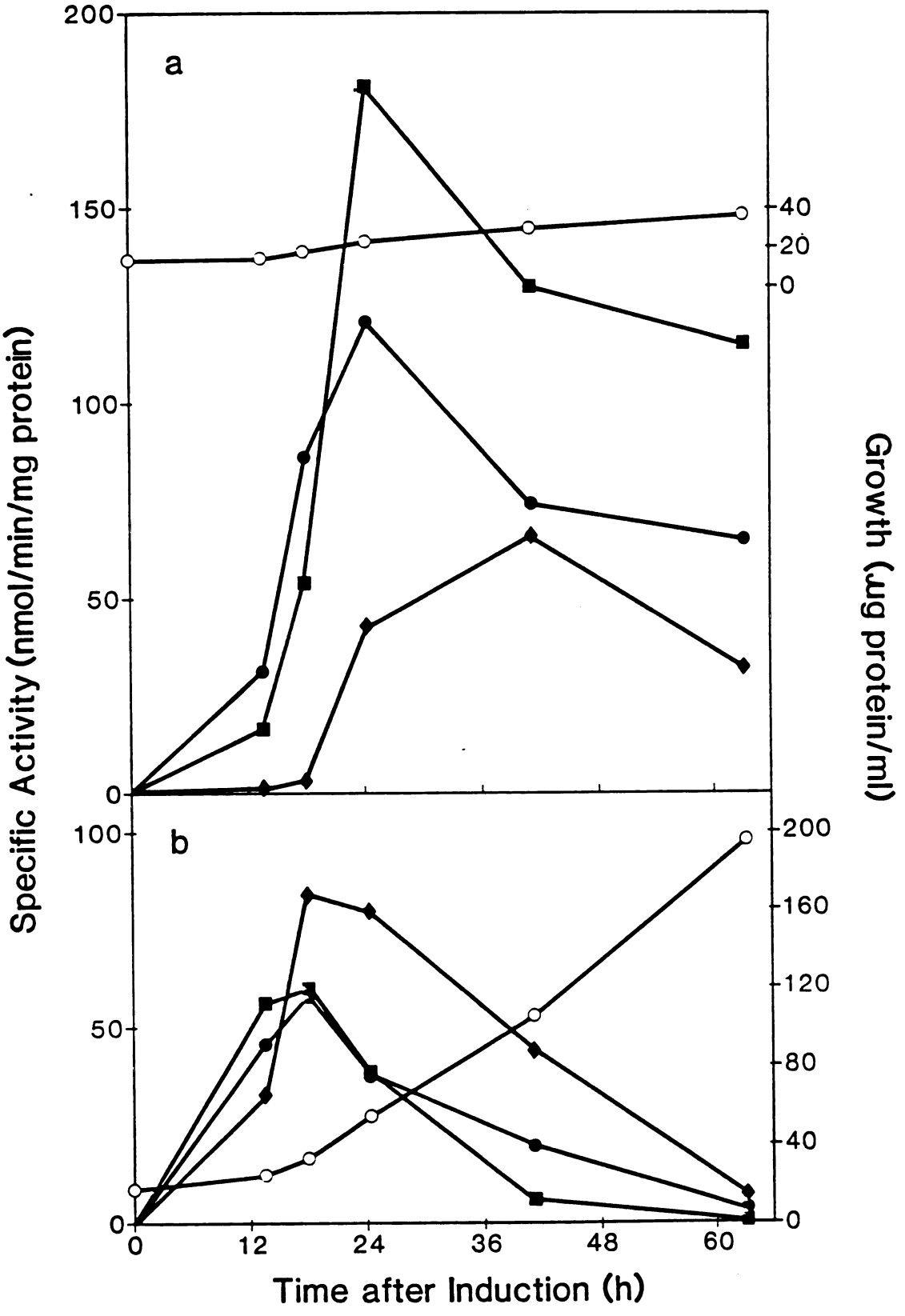
36
48
60
Specific Activity (nmol/min/mg protein)
Growth (µg protein/ml)
Time after Induction (h)

Figure 4. Growth and enzymes of formate-dependent growth following transfer from growth on xylose to growth on either formate (Panel A) or a mixture of formate and xylose (Panel B) at T=0 h. Symbols: triangles, formate dehydrogenase; closed circles, ribulose-1,5-bisphosphate carboxylase; squares, phosphoribulokinase; open circles, growth.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12
24
36
48
60
Specific Activity (nmol/min/mg protein)
Growth (µg protein/ml)
Time after Induction (h)

respectively (Figure 4). PRK and RUBISCO activities were co-ordinately induced and were detectable within 12 h. Addition of xylose to the formate medium resulted in substantially lowered activities of PRK and RUBISCO. In these respects xylose-grown cells behaved similarly to glutamate-grown cells. However, the induction of FDH was significantly different in the xylose experiment than in the glutamate experiment. When xylose-grown cells were transferred to formate medium the induction of FDH was delayed until 18 h after transfer and maximal activity was only 30% that after transfer from glutamate to formate. When xylose-grown cells were transferred to a formate-xylose medium the delay in FDH induction was not observed. However, maximal activity was still only 50% of that in cells transferred from glutamate to formate-glutamate. Thus both glutamate and xylose repress CO_2 assimilation whereas xylose but not glutamate seems to repress energy assimilation by FDH.

Cyclic AMP Levels During Growth Under Various Carbon Regimes. Both glutamate and xylose supported growth of B. japonicum at faster rates and to higher final densities than did formate (Figure 5). During growth on either glutamate or xylose the level of cAMP in the culture remained low (approximately 10 pmol/mg protein) and relatively constant. In contrast, cAMP accumulated to very high levels in cultures growing on formate (Figure 5). Measurements of cAMP levels in cultures grown on xylose and transferred to

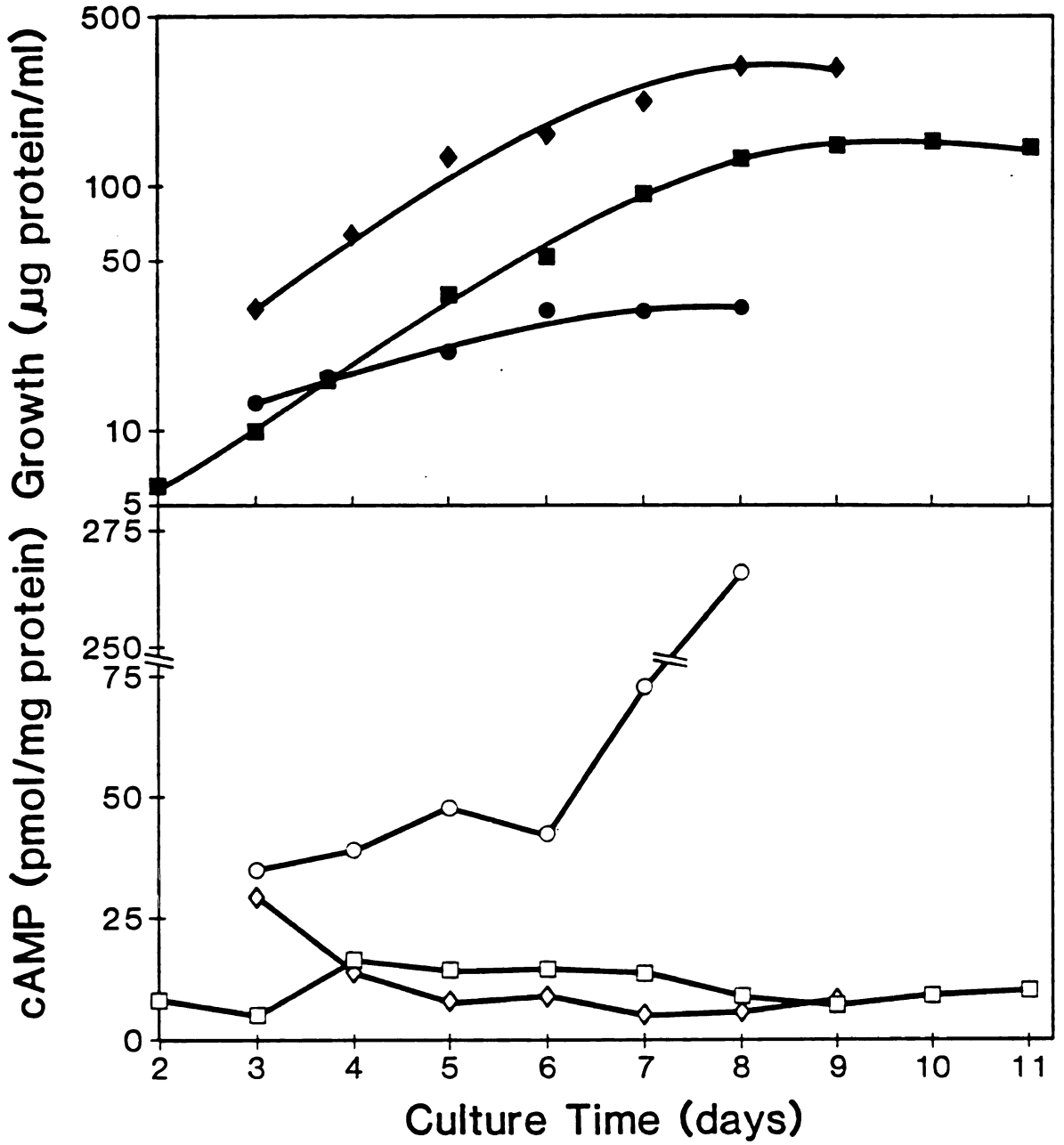


Figure 5. Growth (solid symbols) and cAMP levels (open symbols) in cultures of B. japonicum growing on formate (circles), glutamate (squares) or xylose (diamonds).

either formate or to a mixture of formate and xylose were consistent with this. Cultures transferred to formate alone began to accumulate cAMP whereas cultures transferred to the mixture did not accumulate cAMP (Figure 6). Similarly, cultures transferred from glutamate to formate began to accumulate cAMP. This accumulation was prevented by addition of either chloramphenicol (Figure 7) or rifampicin (data not shown). Treatment with phosphodiesterase reduced detectable cAMP by >90% (data not shown).

Characterization of RUBISCO and PRK Activities in Crude Extracts of Formate-grown Cells of B. japonicum. We investigated a number of characteristics of RUBISCO and PRK activities in crude cell-free extracts. For both enzymes, incorporation of $^{14}CO_2$ proceeded linearly for at least 40 min (Figure 8). PRK activity was dependent on the addition of NADH to the reaction mix. In the presence of NADH (2mM) activity increased 200 fold from 0.23 nmol/mg protein/min to 39.7 nmol/mg protein/min (means of duplicate assays). Catalytic activity of RUBISCO is known to require activation involving addition of CO_2 and Mg^{2+} to the native enzyme (Lorimer et al. 1976; Miziorko and Lorimer 1983). We found that when RUBISCO extracts were prepared in the presence of 20 mM Mg^{2+} and 20 mM HCO_3^- RUBISCO was fully activated and incubation with CO_2 and Mg^{2+} for up to a further 60 min at 30°C did not affect activity (Table 1). In crude extracts RUBISCO showed a broad pH optimum from 7.0-8.0 (Figure 9) and was extremely heat stable. Activity was unaffected by

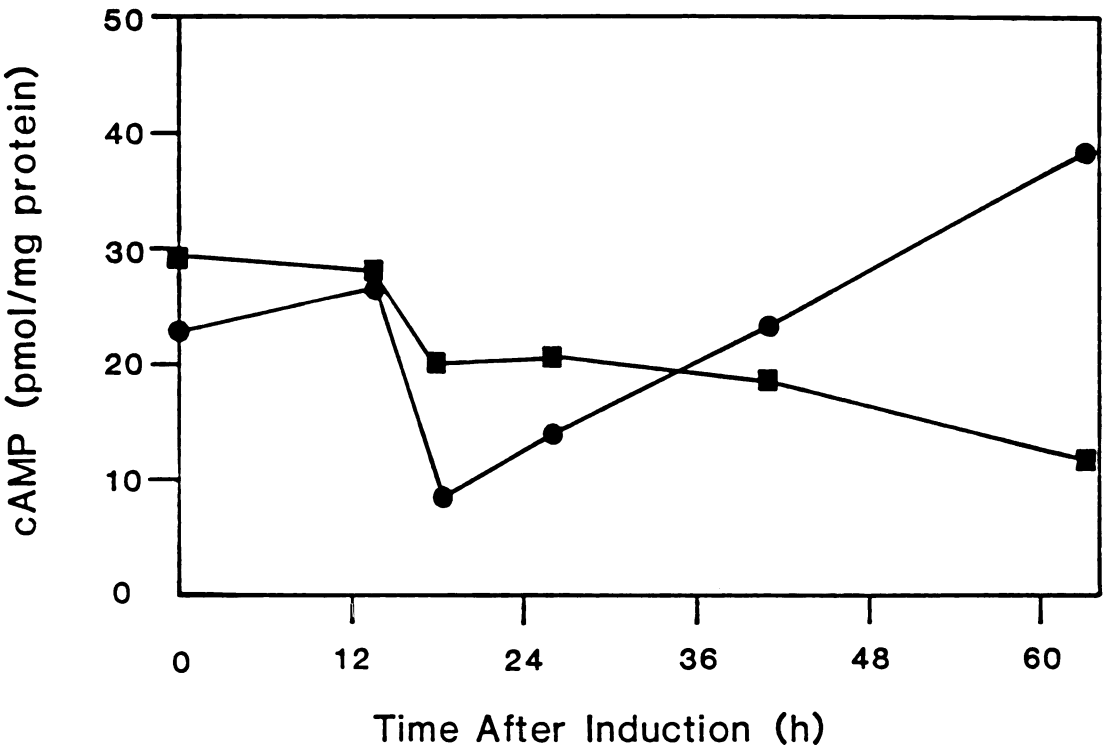


Figure 6. Cyclic AMP levels in cultures transferred at T=0 h from growth on xylose to growth on either formate (circles) or a mixture of formate and xylose (squares).

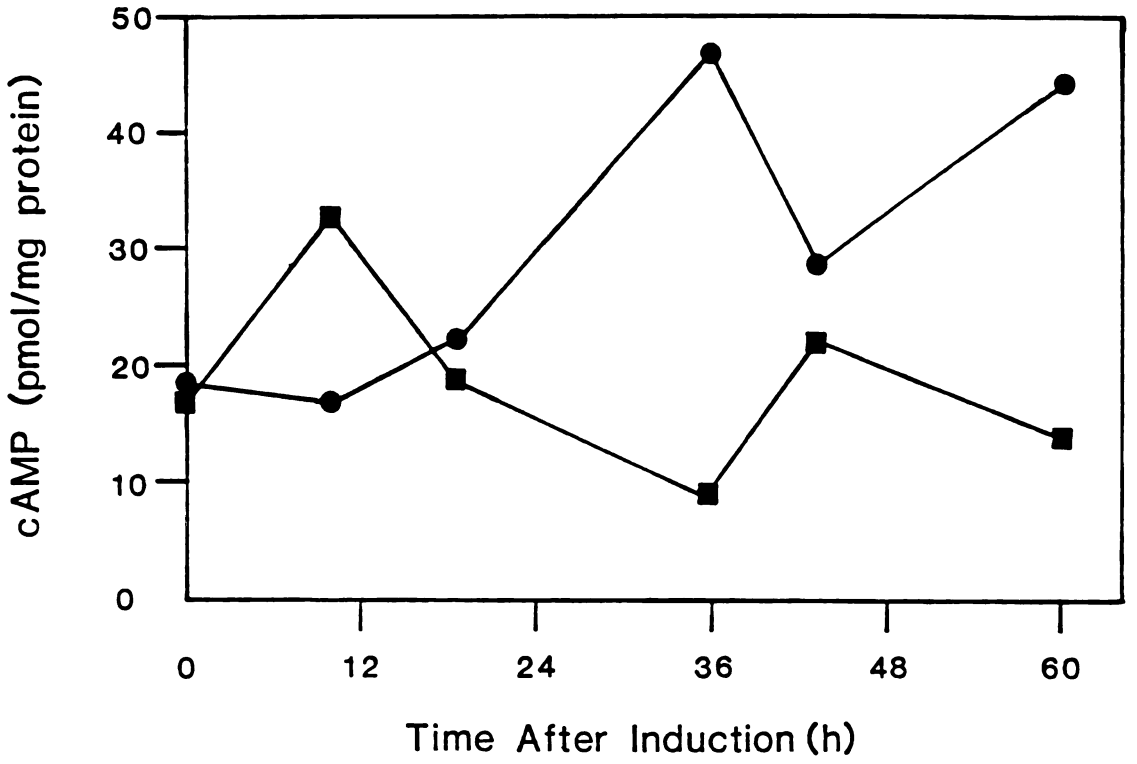


Figure 7. Effect of chloramphenicol on cAMP levels in cultures transferred at T=0 h from growth on glutamate to growth on formate. Symbols: circles, no chloramphenicol; squares, plus chloramphenicol (400 ug/mL).

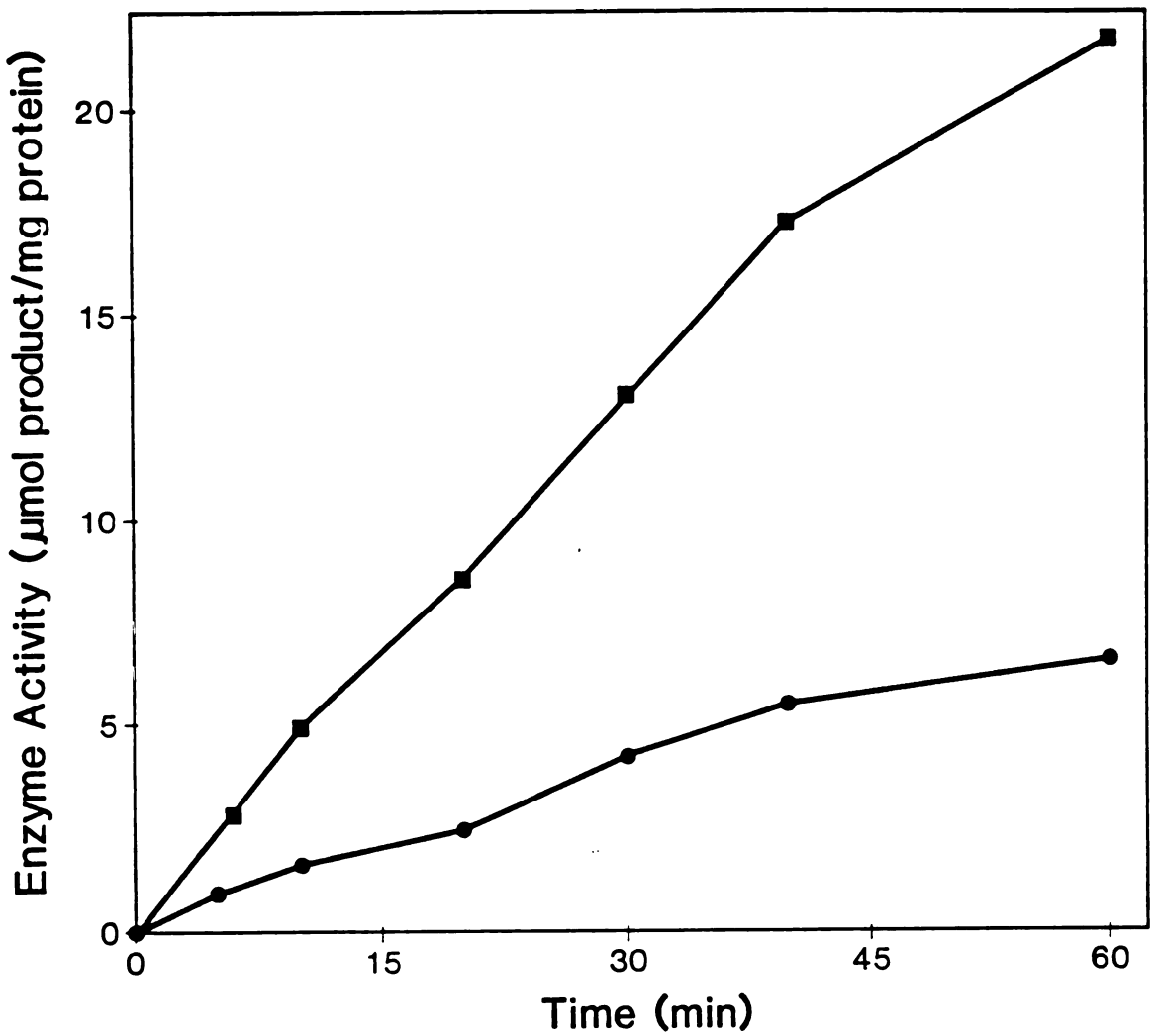


Figure 8. Time course of incorporation of $^{14}CO_2$ into acid-stable products by ribulose-1,5-bisphosphate carboxylase (circles) or phosphoribulokinase (squares) in crude cell-free extracts of cultures of B. japonicum grown on glutamate and transferred to formate for 30 h.

Table 1. Effect of time of activation in the presence of Mg^{2+} (20 mM) and HCO_3^- (20 mM) on ribulose-1,5-bisphosphate carboxylase activity in crude cell-free extracts of B. japonicum.

Time of Activation (min)	Specific Activity (nmol/mg protein/min)
0	69.8
5	77.6
10	76.6
15	73.7
20	75.7
25	73.5
30	70.6
45	69.3
60	72.2

Table 2. Inhibition of activity of ribulose-1,5-bisphosphate carboxylase in crude cell-free extracts by phosphogluconate.

Phosphogluconate (mM)	Specific Activity (nmol/mg protein/min)	Inhibition (%)
0	73.8	-
0.2	63.2	14.4
0.5	52.1	29.4
1.0	44.3	39.9
2.0	31.7	57.0
5.0	19.4	73.7
10.0	12.4	83.2

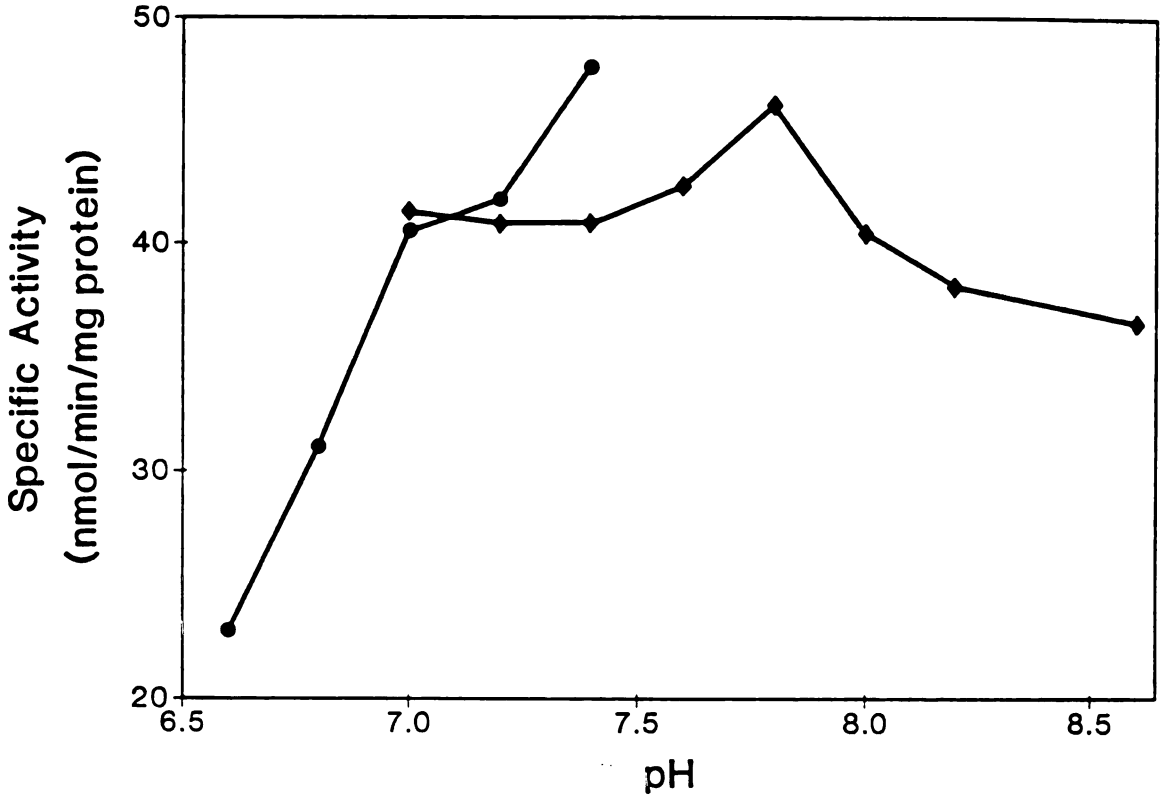


Figure 9. Effect of pH on ribulose-1,5-bisphosphate carboxylase activity in crude cell-free extracts of B. japonicum grown on glutamate and transferred to formate for 30 h. Circles indicate extracts buffered with imidazole and diamonds indicate extracts buffered with Tris.

incubation at 60°C for 60 min. At 65°C RUBISCO activity decayed with a half life of 18 min (Figure 10). As was shown by Purohit et al. (1982), RUBISCO activity was inhibited by phosphogluconate (Table 2). PRK and RUBISCO activities in these crude extracts were stable for several months when stored at -70°C, and samples could be thawed and frozen repeatedly without appreciable loss of activity. In contrast, FDH activity in crude extracts prepared as described in Materials and Methods was not stable and extracts lost >50% activity when stored overnight either at 4°C or at -70°C.

Discussion

The current level of understanding of the mechanisms of regulation of enzymes of formate- and H_2/CO_2-dependent growth is incomplete. In B. japonicum and R. meliloti FDH, hydrogenase, RUBISCO and PRK are all subject to catabolite repression in that their activities are reduced to varying degrees in the presence of various organic carbon substrates (Simpson et al. 1979; Manian et al. 1982,1984; Graham et al. 1984; McGetrick et al. 1985). The role, if any, of cAMP in regulation of FDH, PRK and RUBISCO in B. japonicum has not yet been elucidated. In B. japonicum exogenous cAMP has been shown to relieve the succinate repression of arabinose utilization (Ferrenbach and Lepo 1985) and the malate repression of hydrogenase (Lim and Shanmugam 1979;

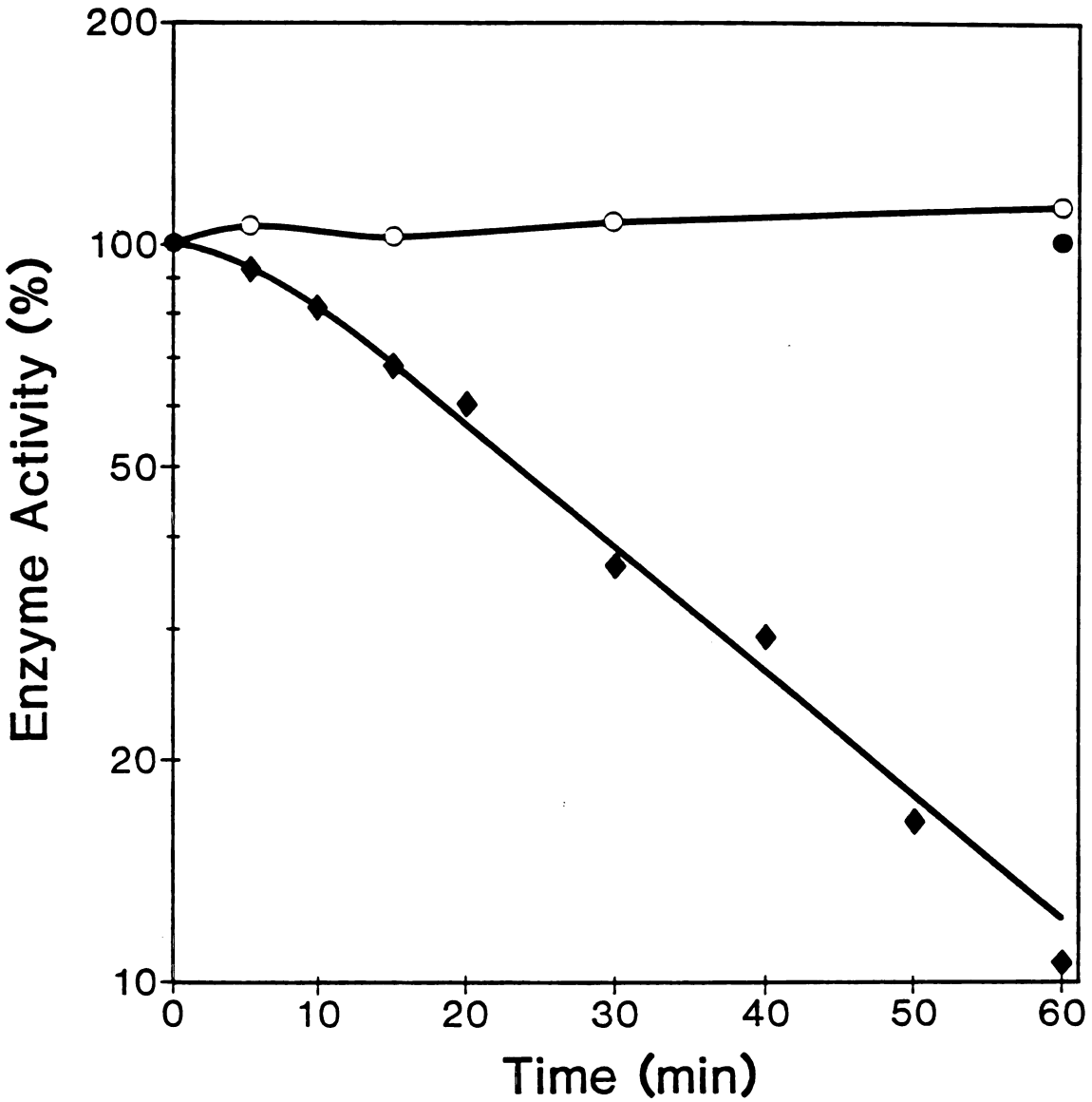


Figure 10. Effect of incubation at 30°C (closed circles), 60°C (open circles) or 65°C (diamonds) on ribulose-1,5-bisphosphate carboxylase activity in crude cell-free extracts of B. japonicum grown on glutamate and transferred to formate medium for 30 h.

McGetrick et al. 1985). The malate repression of hydrogenase activity could also be alleviated by increasing intracellular cAMP levels in B. japonicum by increasing the copy number of the cya gene, which encodes adenylate cyclase. This was carried out by placing the cloned R. meliloti cya gene on a plasmid in the B. japonicum cells (McGetrick et al. 1985). However, mutants hypersensitive to repression of hydrogenase by O_2 and carbon substrates did not have reduced cAMP levels (Maier and Merberg 1982) and relatively small increases were detected in mutants constitutive for hydrogenase expression (Merberg et al. 1983). Both these studies, however, reported only single time point measurements of cAMP. We observed that cultures growing on formate alone accumulated high levels of cAMP, and that this accumulation was blocked by the presence of inhibitors of transcription and translation. However, FDH, RUBISCO and PRK were all induced in conditions under which cAMP was not accumulating (in cells transferred from xylose to formate plus xylose). Thus the accumulation of cAMP does not appear to be essential for induction of these enzyme activities. Furthermore, the addition of cAMP did not overcome the repression of malate on RUBISCO activity in B. japonicum and R. meliloti (McGetrick et al. 1985). Apparently cAMP levels per se are not controlling expression of FDH, PRK and RUBISCO in B. japonicum. However, cAMP was detectable in all these cultures and so might be involved but not be limiting. Catabolite repression in Escherichia coli does not seem to be uniquely dependent on levels of

cAMP (Botsford 1981; Ullmann and Danchin 1983). Mutants lacking cAMP or CRP still displayed catabolite repression (Dessein et al. 1978; Guidi-Rontani et al. 1980). The cAMP-CRP complex may actually be a response to catabolite repression rather than a modulator of it (Ullmann and Danchin 1983). Genes encoding adenylate cyclase, which catalyses cAMP synthesis, have been identified and isolated from B. japonicum (Guerinot and Chelm 1984) and R. meliloti (Kiely and O'Gara 1983). These genes should facilitate generation of mutants defective in cAMP synthesis and metabolism. Such mutants will be useful in elucidating the role of cAMP in catabolite repression and in gene regulation in general in B. japonicum.

It was originally thought that hydrogenase and RUBISCO were coordinately regulated (Hanus et al. 1979). However, it was later found that hydrogenase activity was expressed in bacteroids while RUBISCO activity was not (Simpson et al. 1979). Manian and O'Gara (1982a) demonstrated the induction of RUBISCO activity in the absence of hydrogenase activity during formate-dependent growth, and cAMP has been shown to alleviate the malate repression of hydrogenase activity (Lim and Shanmugam 1979; McGetrick et al. 1985) but not of RUBISCO activity (McGetrick et al. 1985). Clearly the regulation of hydrogenase is separable from that of RUBISCO. Since FDH and hydrogenase activities are involved in energy acquisition while RUBISCO and PRK activities are involved in carbon acquisition it seems reasonable that their regulation would be separable, responding to energy status and

carbon availability, respectively. Indeed, this seems to be the case in the choice between autotrophy and heterotrophy in A. eutrophus, where hydrogenase is derepressed in response to limitation of electron donors whereas RUBISCO is derepressed in response to carbon starvation (C.G. Friedrich et al. 1981; Friedrich 1982). However, there are clearly common elements to the systems regulating hydrogenase and RUBISCO, since H_2-uptake constitutive (Hup^c) mutants of B. japonicum express RUBISCO as well as hydrogenase activity under heterotrophic conditions which completely repress both activities in wild type cells (Merberg and Maier 1984).

In B. japonicum FDH seems to be subject to dual levels of control. Induction requires formate. Catabolite repression by preferred carbon substrates such as succinate, malate (Manian and O'Gara 1982a; Manian et al. 1982,1984) and xylose (this study) is superimposed. In the present study glutamate did not repress formate-induced FDH activity and so apparently is a weak catabolite repressor of FDH. However, Manian et al. (1982) reported that glutamate repressed FDH induction. The reason for this discrepancy is not clear; it might result from strain differences. The present study clearly demonstrates that the time after induction of sampling will influence the results since enzyme activities increase to a peak and then decline, and it is possible that the repression reported by Manian et al. (1982) resulted from sampling during the period of declining activity. The addition of glutamate to the formate medium did accelerate the induction and decline in FDH activity

(Figure 3).

Under conditions investigated to date, RUBISCO and PRK activities in B. japonicum appear to be co-regulated, as has been reported in A. eutrophus (B. Friedrich et al. 1979; C.G. Friedrich et al. 1981). The presence of either glutamate or xylose repressed the induction of PRK and RUBISCO equally. Both are induced in the presence of formate or of H_2 and CO_2 and have not been detected in their absence. However, in A. eutrophus RUBISCO has been induced in the absence of either formate or H_2/CO_2 during growth on succinate when succinate was limiting (in continuous cultures) or when succinate transport was partially repressed (C.G. Friedrich et al. 1981; Friedrich 1982), implying that neither formate nor H_2 nor CO_2 are inducers.

In A. eutrophus it was observed that PRK was less active in heterotrophically-growing than in autotrophically-growing cells (Leadbeater et al. 1982) and it was subsequently shown that PRK was reversibly inactivated and reactivated in response to organic carbon supply (Leadbeater and Bowien 1984). It is thought that PRK activity can constitute a rate-limiting and hence regulatory step in A. eutrophus. The data presented herein suggest that PRK activity is in excess over RUBISCO activity in B. japonicum but further investigation is required to determine if the *in vitro* activities reported accurately reflect the *in vivo* levels, and whether PRK activity can regulate CO_2 assimilation.

In A. eutrophus it has been shown that genes encoding PRK and RUBISCO are present in at least two copies, with at least one copy of each located on the chromosome and another of each located on a megaplasmid (Andersen and Wilke-Douglas 1984; Klintworth et al. 1985). Loss of the megaplasmid does not affect formate-dependent growth but does prevent the induction of PRK and RUBISCO in the presence of heterotrophic substrates such as fructose (Behki et al. 1983; Bowien et al. 1984). It is not yet known if this results from the loss of a regulatory function on the plasmid or rather represents differential control of chromosomal and plasmid-borne genes where the plasmid-borne genes are less sensitive to catabolite repression. Neither PRK nor RUBISCO genes have yet been identified in B. japonicum, although a locus essential for their expression has been identified by mutation and cloned by complementation (Chapter 3). This genetic approach should contribute to our understanding of the regulation of formate-dependent growth.

CHAPTER 3

Isolation of Genes Essential for Formate-dependent Growth by Bradyrhizobium japonicum

Introduction

Bradyrhizobium japonicum is capable of assimilating carbon for growth entirely as CO_2 via the reductive pentose phosphate (RPP) cycle (Lepo et al. 1980). Two key enzymes of that cycle are phosphoribulokinase (PRK) and ribulose-1,5-bisphosphate carboxylase-oxygenase (RUBISCO). CO_2 assimilation can be driven by energy derived either from formate by an NAD-linked soluble formate dehydrogenase (FDH) (Manian and O'Gara 1982a) or from H_2 by a membrane-bound uptake hydrogenase (Simpson et al. 1979). CO_2 assimilation by the RPP cycle is energetically expensive (Ohmann 1979). Consequently the induction of PRK and RUBISCO activities is subject to catabolite repression, with the availability of heterotrophic substrates such as glutamate, xylose and malate (McGetrick et al. 1985; Chapter 2) resulting in a lowered level of expression. However, both PRK and RUBISCO as well as FDH are induced during growth on mixtures of formate with glutamate or xylose (Chapter 2). Similarly, enzymes of formate-dependent growth have been detected

during heterotrophic growth on organic substrates in Pseudomonas oxalaticus (Blackmore and Quayle 1968; Dijkhuizen et al. 1978; Dijkhuizen and Harder 1979a,b,1984) and in Alcaligenes eutrophus (C.G. Friedrich et al. 1981; Im and Friedrich 1983). Friedrich (1982) has proposed that enzymes involved in energy acquisition, such as hydrogenase and formate dehydrogenase, are regulated in response to energy status while enzymes of CO_2 assimilation respond to carbon availability. A. eutrophus carries at least two copies each of the genes encoding PRK and RUBISCO. These genes are each present on both the chromosome and a megaplasmid (Andersen and Wilke-Douglas 1984; Klintworth et al. 1985). Induction in the presence of hetrotrophic substrates is dependent upon plasmid-encoded functions (Behki et al. 1983; Bowien et al. 1984).

However, in none of these systems is the molecular mechanism of regulation of CO_2 assimilation and energy acquisition during autotrophic growth understood. In B. japonicum, cAMP has been shown to alleviate repression of hydrogenase by malate (Lim and Shanmugam 1979; McGetrick et al. 1985). cAMP accumulates during formate-dependent growth but not during growth on glutamate or xylose or on a mixture of formate and xylose, yet FDH, PRK and RUBISCO activities are all induced on the xylose/formate mixture as well as on formate (Chapter 2). Furthermore, exogenous cAMP does not affect catabolite repression of RUBISCO by malate in B. japonicum and R. meliloti (McGetrick et al. 1985). Thus, although cAMP seems to be involved in regulation of

hydrogenase, the role of cAMP, if any, in regulating induction of enzymes of formate-dependent growth in B. japonicum is not clear. In A. eutrophus cAMP is not thought to be involved in the regulation of the choice between autotrophy and heterotrophy (Friedrich 1982).

We have shown that de novo transcription and translation are necessary for the induction of RUBISCO, PRK and FDH activities in B. japonicum (Chapter 2). The identification and isolation of genes involved in formate-dependent growth will allow detailed analysis of the molecular mechanisms involved in their regulation. Toward that end we have generated a series of B. japonicum mutants defective in formate-dependent growth and have used a gene library of B. japonicum DNA to complement several of the mutants which are deficient in RUBISCO and PRK activities. We have identified a locus essential for RUBISCO and PRK activities and have generated stable chromosomal deletions of this DNA region.

Materials and Methods

Strains. B. japonicum strain 110 is a stable small colony derivative of B. japonicum strain USDA 3I1b110 (Guerinot and Chelm 1986a). B. japonicum strain SR is a $Sm^rKm^rHup^+$ derivative of strain USDA DES122 (Maier et al. 1978b). B. japonicum strains SR122 and SR173 are independent RUBISCO deficient mutants derived from strain SR by ethyl methane

sulfonate (EMS) treatment (Maier 1981). The Escherichia coli strains ED8654 (galK, galT, trpR, metB, hsdR, supE, supF, lacY) (Borck et al. 1976) and HB101 (hsdS, r^-_B, m^-_B, recA, ara, proA, lacY, galK, rpsL, xyl, mtl, supE) (Boyer and Roulland-Dussoix 1969) were used for routine plasmid construction and maintenance. The library of B. japonicum 110 DNA in the cosmid vector pLAFR1 (Friedman et al. 1982) was described by Adams et al. (1984). pRK2013 is a helper plasmid carrying the conjugal transfer genes of RK2 (Ditta et al. 1980). pDS4101 is a derivative of ColK which mobilizes pBR322 derivatives (Wolk et al. 1984). pKC7 carries the nptII gene encoding kanamycin resistance (Km^r) (Rao and Rogers 1979).

Media and Growth Conditions. B. japonicum was grown at 30°C in YEX medium (Adams et al. 1984) or in minimal media (Chapter 2) with either formate (FM), glutamate (GM), or xylose (XM) added as sterile supplements to give 0.15% (w/v). For plates, FM medium was solidified with 1.5% (w/v) Noble agar (Difco, Detroit MI). In some experiments tetracycline (Tc; U.S. Biochemical Corp., Cleveland OH) was added at 100 ug/mL for YEX plates or 50 ug/mL for FM broth. Exconjugates from cosmid matings were plated on XM plates containing ampicillin (25 ug/mL; Sigma, St. Louis MO) and tetracycline (100 ug/mL) or used to inoculate FM broth without drugs. Exconjugates from gene replacement matings were plated on YEGG plates (Guerinot and Chelm 1986a) containing rifampicin (50 ug/mL; U.S. Biochemical Corp.) and

kanamycin (100 ug/mL; Sigma). E. coli strains were routinely grown at 37°C in LB (Miller 1972).

Mutagenesis and Carbenicillin Enrichment. The EMS mutagenesis procedure was based on those described by Meade and Signer (1977) and Maier (1981). B. japonicum 110 was grown to late log phase in YE medium and a total of 50 mL of culture was harvested by centrifugation and resuspended in 0.5 mL of 0.2 M Tris (pH 7.6) containing 0.85% NaCl. EMS (20 uL; Sigma) was added and the suspension was incubated at 30°C for 2 h with shaking. To terminate the mutagenesis 1 mL of 20 mM $Na_2S_2O_3$ was added. The cells were then harvested by centrifugation and washed twice with 20 mM $Na_2S_2O_3$. This procedure resulted in approximately 99% killing. The mutagenized cells were used to inoculate 10 mL of XM broth and grown non-selectively for 24 h at 30°C. The culture was harvested by centrifugation, washed with minimal medium base (no carbon substrate) and the cells were inoculated into FM broth. After growth for 48 h carbenicillin was added to give 5 mg/mL culture. After a further 48 h the cells were harvested by centrifugation, washed in minimal medium base and resuspended in XM broth. Such a carbenicillin enrichment procedure decreased viable cell numbers of the parent B. japonicum 110 400-fold compared to untreated cells (data not shown). The cells were grown in XM broth for 4 days, harvested, washed, inoculated into FM broth and the carbenicillin enrichment was repeated. Survivors from the second carbenicillin

enrichment were plated onto XM medium. Colonies were replicated onto FM and XM media and grown for 14 days. Colonies which failed to grow or which grew poorly on FM plates were retested for the ability to grow in FM broth.

Induction and Assay of Enzymes of Formate-dependent Growth. We have previously described conditions under which formate dehydrogenase (FDH), ribulose-bisphosphate carboxylase (RUBISCO) and phosphoribulokinase (PRK) activities are induced in cells previously grown heterotrophically on glutamate (Chapter 2). Briefly, cells were grown in GM broth to an optical density (420 nm) of 0.3, harvested by centrifugation, washed in minimal medium lacking a carbon substrate and resuspended in FM broth. After 30 h, by which time induction was approximately maximal in the wild type parent strain B. japonicum 110, cells were harvested by centifugation. Cell-free extracts were prepared and enzyme activities were assayed as previously described (Chapter 2).

DNA and Protein Methods. Routine DNA procedures were carried out as described by Adams and Chelm (1984). The triparental mating system of Ditta et al. (1980) was used for the conjugal transfer of cosmids. Cells were mixed in the ratio (mL culture x OD_{600}) of 1.8 B. japonicum recipient to 1 ED8654/cosmid to 1 HB101/pRK2013, pelleted, washed once in 5% LB, resuspended in 5% LB and spread onto YEX plates. After 4 days at 30°C the cells were resuspended in 0.01% Tween-80 and dilutions were plated onto XM plates containing

Tc (100 ug/mL) to select for the cosmids and Ap (25 ug/mL) to counter-select against E. coli donor and helper cells. To recover cosmids from B. japonicum, total DNA was extracted and used to transform E. coli to tetracycline resistance (Tc^r) (Hom et al. 1985). SDS-polyacrylamide gel electrophoresis and immuno-blotting were performed as previously described (Chapter 2). Antiserum against purified B. japonicum RUBISCO was prepared by Purohit et al. (1982).

Gene-Directed Mutagenesis. Gene-directed mutagenesis was as described by Guerinot and Chelm (1986a). Plasmids in which regions of B. japonicum DNA had been deleted and replaced with the nptII gene from pKC7 were constructed using standard recombinant DNA techniques. The resultant plasmids pBJ183, pBJ184, pBJ193 and pBJ194 were independently transformed into E. coli HB101 carrying pDS4101 for use as donor strains in conjugal matings with B. japonicum. For matings, cells were mixed in the ratio (mL culture x OD_{600}) of 1.8 B. japonicum 110 recipient to 1 HB101/deletion plasmid/pDS4101 to 1 HB101/pRK2013. The cells were pelleted, washed once in 5% LB, resuspended in 5% LB and spread onto a YEX plate. After 4 days at 30°C the cells were resuspended in 0.01% Tween-80 and dilutions were plated onto YEGG plates containing 100 ug/mL kanamycin to select for exconjugates which had stably integrated the nptII gene, and 50 ug/mL rifampicin to counterselect against E. coli donor and helper cells. Exconjugates which were stably Km^r

were screened by colony hybridization (Grunstein and Hogness 1975) for the presence of nptII sequences and for the presence/absence of vector sequences. The DNA structure of these deletion strains was determined by hybridization analysis of restriction endonuclease digests of total genomic DNA (Southern 1975).

Plant Tests. The symbiotic phenotype of one B. japonicum deletion mutant, CRM35, which was unable to grow on formate was determined as described by Guerinot and Chelm (1986a). Soybean seeds (Glycine max (L.) Merr. cv. Amsoy 71) were surface-sterilized by soaking in 1% NaOCl (a 20% v/v solution of commercial bleach in water) for 5 min followed by soaking in 3% H_2O_2 for 30 min. After 4 x 15 min rinses in sterile distilled H_2O, the seeds were soaked for 60 min in a late log phase culture of the appropriate B. japonicum strain or in sterile YEX medium for uninoculated controls. Seeds were planted in modified Leonard jars (Vincent 1970) using the N-free medium of Johnson et al. (1966). The plants were grown with a 16 h daily light period at a fluence rate of 300 $umol \cdot m^{-2} \cdot s^{-1}$, day/night temperatures of 28/25°C, and a relative humidity of 60%. Plants were harvested 28 days after planting. Nodules were excised and the C_2H_2 reduction assay was used to estimate nitrogenase activity (Hardy et al. 1968). Bacteria were reisolated from surface-sterilized nodules and checked for genetic markers to assess contamination. Randomly chosen nodules were immersed in 95% ethanol for 30 s and then in 3% H_2O_2 for 4

min. After rinsing 6 times with distilled H_2O the nodules were aseptically crushed in 1 mL of a sterile solution of 0.01% Tween-80. Serial dilutions were plated onto non-selective YEX medium plates and 100 randomly chosen colonies were screened for kanamycin resistance.

Results

Isolation and Characterization of Mutants Unable to Grow on Formate. Survivors of EMS mutagenesis were subjected to 2 cycles of carbenicillin enrichment under formate-dependent growth conditions and then plated on XM plates to exclude auxotrophs. Approximately 2.0×10^4 individual colonies were replicated and screened for inability to grow on formate. Over 200 potential mutants displaying reduced growth on FM plates were rescreened for growth in FM broth, which allowed better discrimination of the mutant phenotype. Twenty mutants were clearly unable to grow on formate and were retained for further study.

The 20 mutants defective in formate-dependent growth, as well as 2 other mutants, SR122 and SR173, identified as unable to grow autotrophically (Maier 1981), were analysed for the induction of FDH, RUBISCO and PRK activities after a shift to inducing conditions. The mutants were divided into several classes on the basis of inducible activities of FDH, RUBISCO and PRK. One mutant, CRM15, did not induce

significant activities of either RUBISCO or PRK. FDH activity was induced, but was reduced to a level of 36% relative to wild type. Our data confirmed that SR122 and SR173 were defective in RUBISCO (Maier 1981). However, both strains induced essentially wild type levels of PRK activity and thus differ from CRM15. None of the other mutants were totally deficient in any of the 3 enzyme activities. The results are summarized in Table 1.

The mutants were screened for the presence of immunologically identifiable large subunit of RUBISCO by immuno-blotting. The 3 mutants which lacked RUBISCO activity, CRM15, SR122 and SR173, also clearly lacked the large subunit of RUBISCO (Figure 1). None of the other mutants showed detectable alterations in the mobility of the large subunit of RUBISCO.

We have previously shown that B. japonicum RUBISCO activity in crude extracts is stable at 60°C and decays at 65°C with a half-life of 18 min (Chapter 2). We therefore tested each of the mutants which expressed RUBISCO activity for altered heat sensitivity of RUBISCO, which could indicate a structural alteration in the protein. However, none of the mutants differed from wild type in their RUBISCO stability at 65°C.

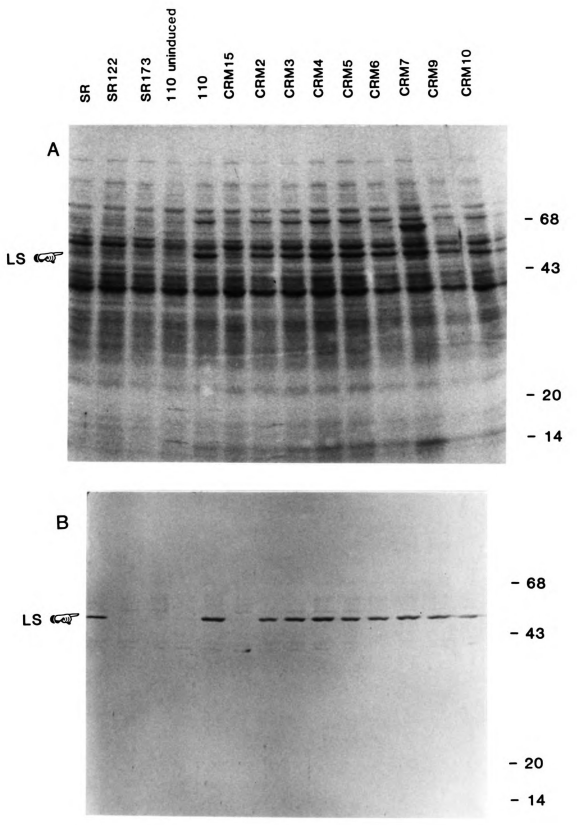
By analysis of protein patterns in one-dimensional SDS-polyacrylamide gels one mutant, CRM7, was distinguished by the appearance of a single, apparently novel polypeptide of molecular weight 66 kilodaltons (kD; Figure 1). This polypeptide does not cross-react with anti-serum against

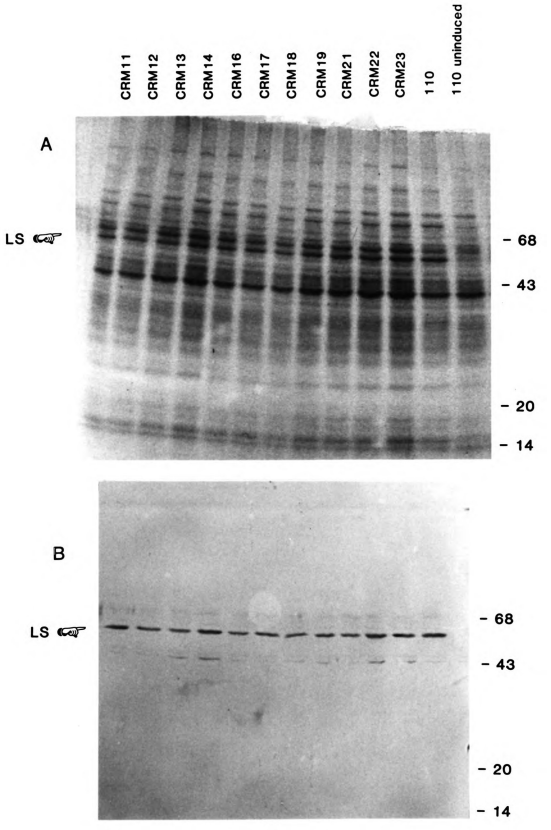
Table 1. Characteristics of Bradyrhizobium japonicum strains defective in formate-dependent growth.

Strain	Enzyme Activity[a] %WT[b]			Presence of RUBISCO Antigen[c]
	RUBISCO	PRK	FDH	
110-WT	100	100	100	+
CRM 2	40	60	45	+
CRM 3	60	60	89	+
CRM 4	97	158	64	+
CRM 5	68	93	49	+
CRM 6	88	105	54	+
CRM 7	97	97	111	+
CRM 9	76	57	42	+
CRM 10	48	37	48	+
CRM 11	56	42	68	+
CRM 12	41	31	47	+
CRM 13	33	53	74	+
CRM 14	61	49	53	+
CRM 15	3	3	36	-
CRM 16	37	40	30	+
CRM 17	48	39	44	+
CRM 18	40	43	59	+
CRM 19	66	54	38	+
CRM 21	46	54	58	+
CRM 22	49	40	52	+
CRM 23	52	40	50	+
SR-WT	100	100	100	+
SR 122	2	77	66	-
SR 173	2	79	117	-

a. Assays performed on crude cell-free extracts prepared from cells grown on glutamate and induced on formate as described in Materials and Methods.
b. WT = wild type
c. Assayed by Immuno-blots as described in Materials and Methods.

Figure 1a,b. Polypeptide patterns of crude cell-free extracts prepared from B. japonicum strains induced for formate-dependent growth as described in Materials and Methods. A: SDS-polyacrylamide gel stained with Coomassie Blue. B: Immuno-blot of a duplicate of the gel in A probed with antiserum against purified B. japonicum RUBISCO. LS indicates the large subunit of RUBISCO. Molecular mass standards (kD) are indicated.


SR
SR122
SR173
110 uninduced
110
CRM15
CRM2
CRM3
CRM4
CRM5
CRM6
CRM7
CRM9
CRM10
A
LS
- 68
- 43
- 20
- 14
B
LS
- 68
- 43
- 20
- 14


CRM11
CRM12
CRM13
CRM14
CRM16
CRM17
CRM18
CRM19
CRM21
CRM22
CRM23
110
110 uninduced
A
LS
- 68
- 43
- 20
- 14
B
LS
- 68
- 43
- 20
- 14

B. japonicum RUBISCO (Figure 1). CRM7 induced RUBISCO and PRK activities to apparently normal levels and induced FDH activity to 66% of wild type (Table 1). The nature of this novel polypeptide is not known.

Complementation of the RUBISCO⁻ Mutants. A cosmid gene library prepared from *B. japonicum* 110 DNA (Adams et al. 1984) was conjugally transferred *en masse* into each of the 3 mutants (CRM15, SR122 and SR173) unable to induce RUBISCO activity. Preliminary experiments indicated that simultaneous selection for the restoration of the ability to grow on formate and expression of vector-encoded tetracycline resistance yielded no exconjugates. We therefore selected singly either for drug resistance or for formate-dependent growth and then screened for the second phenotype. In one set of experiments dilutions of the mating mixture were plated on YEX medium containing Tc (100 ug/mL). Tetracycline resistant exconjugates, obtained at a frequency of 10^{-2}/recipient, were then either replicated onto FM plates or else were pooled and used to inoculate FM broth. The FM broth culture was grown to late log phase and dilutions were plated onto FM medium containing Tc (50 ug/mL). Tetracycline resistant colonies on the FM plates were retested individually for growth in FM broth and for resistance to tetracycline. In a second series of experiments, dilutions of the mating mixture were used directly to inoculate FM broth. The cultures were grown to late log phase and dilutions were plated onto YEX

medium containing tetracycline. Tetracycline resistant colonies were retested individually for growth in FM broth. A total of 5 stable Tc^r exconjugates of CRM15, 10 of SR122 and 9 of SR173 were identified as able to grow on formate.

Analysis of Cosmids Able to Restore Formate-Dependent Growth. Total DNA independently extracted from 4 of the CRM15 exconjugates and from 3 exconjugates each of SR122 and SR173 was used to transform *E. coli* ED8654 to tetracycline resistance. Cosmid DNA was then isolated from the *E. coli* transformants, digested with restriction endonuclease EcoRI and DNA restriction fragments were separated by agarose gel electrophoresis. Three distinct but related EcoRI restriction patterns were detected among the 10 cosmids. Restriction endonuclease mapping with several enzymes suggested that the *B. japonicum* DNA inserts of the 3 cosmids, named pCRM6, pCRM7 and pCRM20, overlapped over much of their lengths (Figure 2).

To confirm that restoration of the ability to grow on formate was due to the *B. japonicum* DNA inserts in the cosmids, each of the 3 cosmids as well as the vector, pLAFR1, was conjugally transferred into each of the 3 mutants, and Tc^r exconjugates were selected. Ten randomly chosen Tc^r exconjugates from each of the matings were then tested for their ability to grow in FM broth. Each of the mutant exconjugates carrying either pCRM6, pCRM7 or pCRM20 was able to grow on formate, but none of the mutants carrying pLAFR1 could grow on formate (Table 2). The cosmid

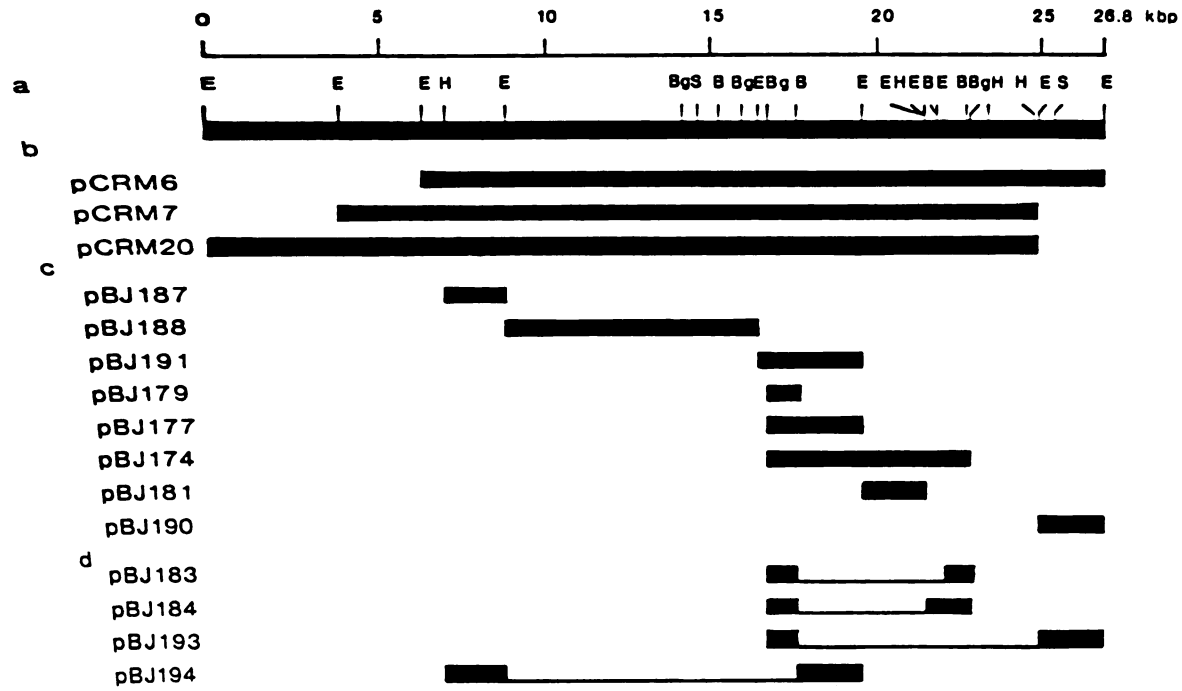


Figure 2. (a) Restriction endonuclease map of the B. japonicum 110 chromosomal region identified as capable of complementing mutants lacking RUBISCO and PRK activities. Restriction sites are BamHI (B), BglII (Bg), EcoRI (E) HindIII (H) and SmaI (S). (b) B. japonicum 110 DNA inserts of cosmids identified by complementation of mutants lacking RUBISCO and PRK activities. (c) Inserts of plasmid subclones used to construct plasmids for site-directed mutaganesis and of plasmids used as hybridization probes. (d) Inserts of plasmids used for site-directed mutagenesis. Regions of B. japonicum 110 DNA deleted and replaced by the nptII gene are indicated by narrow lines. Flanking regions of B. japonicum 110 DNA included to allow integration into the chromosome by homologous recombination are indicated by broad lines.

Table 2. Complementation of B. japonicum mutants lacking ribulose bisphosphate carboxylase (RUBISCO) activity[a].

Mutant	Complementing Cosmid	RUBISCO$^+$/Tc^r
CRM15	pCRM6	10/10
	pCRM7	10/10
	pCRM20	10/10
	pLAFR1	0/9
SR122	pCRM6	10/10
	pCRM7	9/10
	pCRM20	10/10
	pLAFR1	0/9
SR173	pCRM6	9/9
	pCRM7	7/7
	pCRM20	6/7
	pLAFR1	0/10

[a] Each of the 3 cosmids as well as the vector, pLAFR1, was independently transferred by conjugation into each of the 3 mutants. Randomly selected Tc^r exconjugates were then screened for RUBISCO activity as determined by the ability to grow on formate as a sole carbon/energy source. Data are presented as the number of exconjugates expressing RUBISCO activity per total number of Tc^r exconjugates tested.

vector pLAFR1 shows a high incidence of spontaneous loss from B. japonicum when grown under non-selective conditions (Cantrell et al. 1983; Haugland et al. 1984; Hom et al. 1985). Mutants complemented with each of the 3 cosmids were grown non-selectively for several generations and strains spontaneously cured of the cosmids were identified by sensitivity to tetracycline. In each instance the loss of the cosmid was accompanied by simultaneous loss of the ability to grow on formate. This 1:1 cosegregation of the drug resistance marker with the phenotypic restoration of formate-dependent growth ability and the dependence of complementation on the presence of a specific region of B. japonicum DNA in the cosmids indicates that the B. japonicum DNA common to the 3 cosmids pCRM6, pCRM7 and pCRM20 contains the wild type DNA sequences which were mutated in the 3 independently-derived mutants.

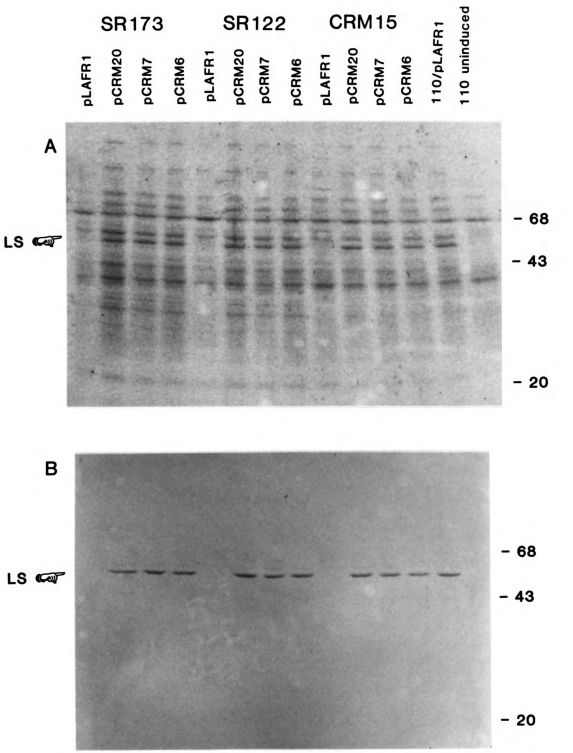
RUBISCO and PRK Activities in Complemented Mutant Strains. RUBISCO and PRK activities were determined in crude extracts of the mutants and of the mutants carrying the complementing cosmids or the cosmid vector. In each case the presence of the complementing cosmid but not the vector alone resulted in restoration of wild type enzyme activities (Table 3). Similarly. the presence of the complementing cosmids but not of the vector restored the presence of an apparently unaltered RUBISCO large subunit as detected on immuno-blots (Figure 3).

Table 3. Ribulose bisphosphate carboxylase (RUBISCO) and phosphoribulokinase (PRK) activities of exconjugates of mutants lacking RUBISCO activity.

		Enzyme Specific Activity (nmol/mg protein/min)	
Strain	Cosmid	RUBISCO	PRK
110-WT[a]	pLAFR1	152	169
CRM15	-	2	2
	pCRM6	127	134
	pCRM7	146	138
	pCRM20	171	159
	pLAFR1	0	<1
SR122	-	1	64
	pCRM6	129	138
	pCRM7	138	196
	pCRM20	146	133
	pLAFR1	3	160
SR173	-	1	65
	pCRM6	124	138
	pCRM7	146	193
	pCRM20	102	139
	pLAFR1	0	118

[a]. WT = wild type.

Figure 3. Complementation of mutants lacking RUBISCO activity: polypeptide patterns of crude cell-free extracts prepared from mutants carrying each of the 3 complementing cosmids or the vector, pLAFR1, which had been induced for formate-dependent growth as described in Materials and Methods. A: SDS-polyacrylamide gel stained with Coomassie Blue. B: Immuno-blot of a duplicate of the gel in (A) probed with antiserum prepared against purified B. japonicum RUBISCO. LS indicates the large subunit of RUBISCO. Molecular mass standards (kD) are indicated.


SR173
SR122
CRM15
pLAFR1
pCRM20
pCRM7
pCRM6
pLAFR1
pCRM20
pCRM7
pCRM6
pLAFR1
pCRM20
pCRM7
pCRM6
110/pLAFR1
110 uninduced
A
LS
- 68
- 43
- 20
B
LS
- 68
- 43
- 20

Analysis of the DNA Region Required for Formate-dependent Growth. The DNA region common to pCRM6, pCRM7 and pCRM20 is 18.7 kbp in size (Figure 2). We sought to further localize the region(s) responsible for restoration of formate-dependent growth. The strategy we employed was to generate a series of stable, defined chromosomal deletions spanning the region defined by the cosmid inserts. A total of 4 deletions spanning a total of 16.1 kbp of DNA were generated in vitro from a series of subclones of the cosmid inserts which had been prepared in high copy number E. coli plasmid vectors by standard recombinant DNA techniques (see Figure 2). The strategies employed to generate the 4 deletion-bearing plasmids pBJ183, pBJ184, pBJ193 and pBJ194 are presented in Figure 4. In each deletion a different region of B. japonicum DNA had been removed and replaced with a 1.8 kbp EcoRI/BamHI (or, for pBJ184, a 1.8 kbp HindIII/BamHI) restriction fragment isolated from pKC7 containing the nptII gene such that the nptII gene was flanked by colinearly oriented regions of B. japonicum DNA. These in vitro constructed deletions were used to replace the corresponding B. japonicum chromosomal regions by the gene replacement technique described by Guerinot and Chelm (1986a). To verify that the gene replacements and resultant deletions were as expected (Figure 5), total DNA was extracted from presumptive mutant strains CRM35 and CRM43 (derived from pBJ183), CRM44 and CRM48 (from pBJ184), CRM52 (from pBJ193) and CRM55 (from pBJ194) and cellulose nitrate transfers of restriction endonuclease digests were hybridized to various

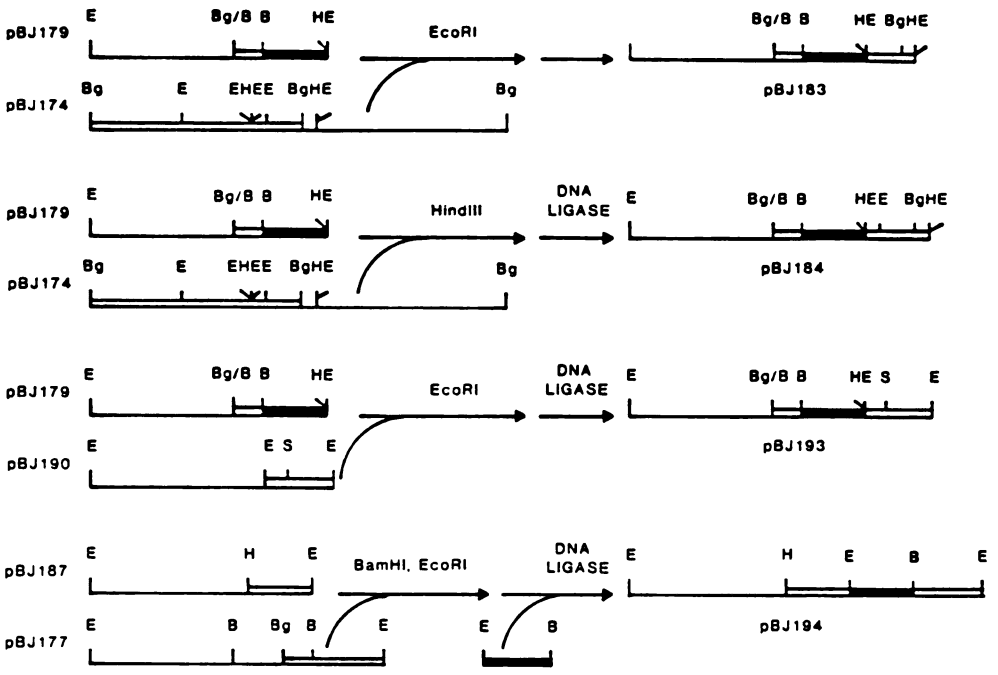


Figure 4. Strategies used to generate the deletion-bearing plasmids which were used for site-directed mutagenesis. Restriction sites are BamHI (B), BglII (Bg), EcoRI (E), HindIII (H) and SmaI (S). Narrow lines indicate vector DNA, open boxes indicate B. japonicum DNA and solid boxes indicate nptII gene DNA.

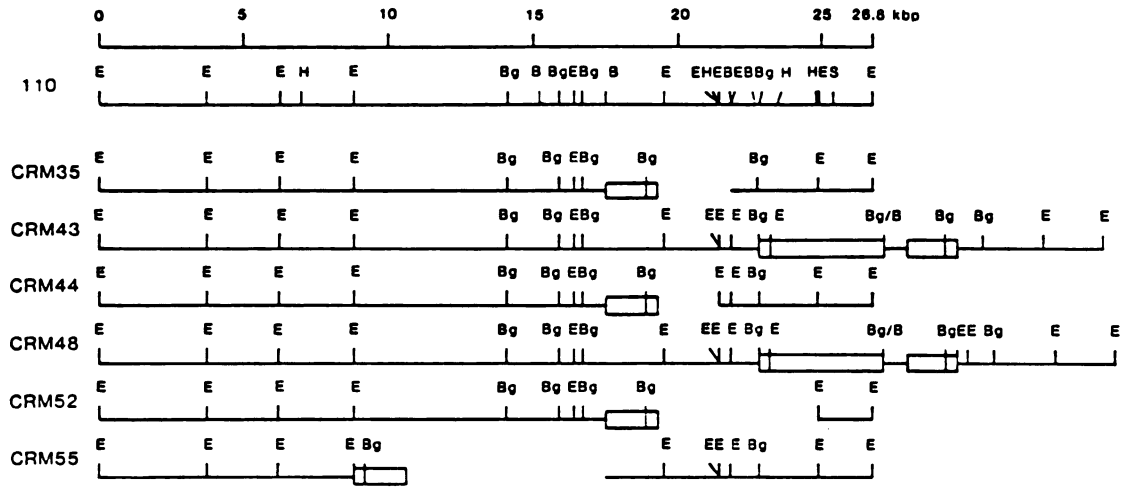


Figure 5. Restriction endonuclease maps of the chromosomal DNA regions of the B. japonicum strains generated by gene-directed mutagenesis and characterized as unable to grow on formate. CRM35 is a double recombinant and CRM43 is a single recombinant derived from pBJ183. CRM44 is a double recombinant and CRM48 is a single recombinant derived from pBJ184. CRM52 and CRM55 are double recombinants derived from pBJ193 and pBJ194, respectively. The map of the parental strain (110) is included for reference. Narrow lines represent B. japonicum DNA. Open boxes indicate DNA derived from pKC7. Deletions of B. japonicum DNA are indicated by spaces. Restriction sites are BamHI (B), BglII (Bg), EcoRI (E), HindIII (H) and SmaI (S).

DNA probes (Figure 6). As expected, a probe of pBJ181 did not hybridize to DNA prepared from CRM35, CRM44 or CRM52 (Figure 6, lanes aa, bb and dd, respectively) but hybridized to a 1.9 kbp EcoRI fragment from parental (strain 110, lane cc) DNA. Similarly, a probe of pBJ188 failed to hybridize to DNA from CRM55 (lane ee) but hybridized to a 7.6 kbp EcoRI fragment of strain 110 (lane ff) DNA. The nptII-containing plasmid pKC7 hybridized to fragments of predicted lengths in the 4 deletion mutants (compare maps of Figure 5 with Figure 6 lanes a-h) but did not hybridize to 110 (Figure 6, lanes i and j) DNA. When probed with pBJ191, which contains a 1.9 kbp EcoRI insert which should have been altered in each of the gene replacement strains, restriction fragments with the expected altered mobilities were observed for each of the deletion mutants (compare maps of Figure 5 with Figure 6,lanes k-r).

Each of the 4 constructed deletion-bearing strains was unable to grow on formate as a sole carbon substrate. When cell-free extracts were prepared from mutants induced for enzymes of formate-dependent growth by transfer from growth on glutamate to FM broth, none of the mutants induced RUBISCO activity (Table 4) and all lacked the large subunit of RUBISCO (Figure 7). Mutants CRM35, CRM44 and CRM52 failed to induce PRK activity. Thus these 3 deletion mutants mimic the phenotype of the EMS-generated mutant CRM15. However, CRM55 induced wild-type activity of PRK (Table 4) and therefore is analagous to SR122 and SR173. These data indicate that the DNA region required for

Figure 6. Hybridization analyses of B. japonicum genomic DNA. BglII (lanes a,c,e,g,i,k,m,o,q,s,u,w,y) or EcoRI (lanes b,d,f,h,j,l,n,p,r,t,v,x,z,aa-ff) digests of total genomic DNA from strains 110 (lanes i,j,s,t,u,v,cc,ff), CRM35 (lanes a,b,k,l,aa), CRM43 (lanes w,x), CRM44 (lanes c,d,m,n,bb), CRM48 (lanes y,z), CRM52 (lanes e,f,o,p,dd) or CRM55 (lanes g,h,q,r,ee) were transferred to cellulose nitrate and hybridized to the following radiolabelled DNA probes: pKC7 (lanes a-j), pBJ191 (lanes k-z), pBJ181 (lanes aa-dd) and pBJ188 (lanes ee,ff).

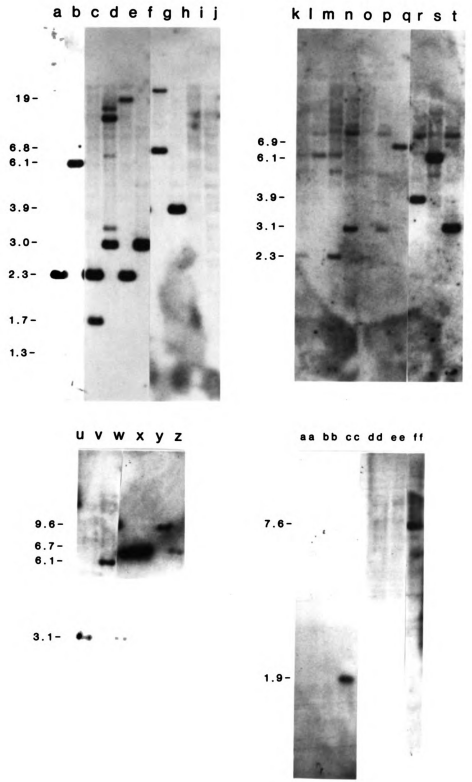

a b c d e f g h i j
19-
6.8-
6.1-
3.9-
3.0-
2.3-
1.7-
1.3-
k l m n o p q r s t
6.9-
6.1-
3.9-
3.1-
2.3-
u v w x y z
9.6-
6.7-
6.1-
3.1-
aa bb cc dd ee ff
7.6-
1.9-

Table 4. Activities of enzymes of formate-dependent growth in gene-directed deletion mutants of B. japonicum induced by transfer from growth on glutamate to growth on either formate alone (F) or on a mixture of formate plus glutamate (FG).

	Enzyme Specific Activity (nmol/mg protein/min)					
	RUBISCO		PRK		FDH	
Strain	F	FG	F	FG	F	FG
110-WT[a]	90	41	143	74	110	37
701[b]	130	nd[c]	179	nd	33	nd
CRM35	0	0	0	<1	2	3
CRM43	3	nd	8	nd	nd	nd
CRM44	0	<1	0	<1	6	12
CRM48	2	nd	5	nd	nd	nd
CRM52	0	0	0	<1	2	8
CRM55	<1	<1	222	172	2	51

a. WT = wild type.
b. 701 is a Km^r strain in which the nifDK region has been deleted and replaced with the nptII gene, and represents a control in this expreiment.
c. nd = not determined.

Figure 7. Immuno-blot of crude cell-free extracts prepared from the indicated deletion-bearing strains of B. japonicum which had been induced for formate-dependent growth as described in Materials and Methods. The cellulose nitrate transfer was probed with antiserum prepared against purified B. japonicum RUBISCO. LS indicates the large subunit of RUBISCO. Molecular mass standards (kD) are indicated.

110
CRM35
CRM43
CRM44
CRM48
CRM52
CRM55
110
68-
43-
20-
14-
-LS

induction of RUBISCO activity spans the BamHI site at 17.5 kbp which marks the right-hand (as drawn in Figures 2, 4 and 5) limit of the deletion in CRM55 and the left-hand limit of the deletions in CRM35, CRM44 and CRM52. The region of DNA essential for the induction of PRK activity must lie entirely to the right of the BamHI (17.5 kbp) site since CRM55 was unaffected in induction of PRK activity.

We also investigated the phenotypes of strains arising from single recombination events in each of the gene replacement crosses. A single recombination event results in the cointegration of the entire plasmid into the chromosome. The B. japonicum DNA present in the plasmid is thus present in two copies in the cointegrates. If the region of DNA where the crossover occurs lies within an operon, then the event is mutagenic because the single recombination event results in the generation of two incomplete copies of the operon (Williams and Szalay 1983). Such events seem to have occurred in the gene replacements with pBJ183, generating CRM43 and with pBJ184, generating CRM48 as indicated by the following results. Both these mutants are unable to grow on formate and both strains fail to induce either PRK or RUBISCO activity. The hybridization patterns observed when a cellulose nitrate transfer of DNA from these strains digested with restriction endonucleases BglII or EcoRI and run on agarose gels was probed with pBJ191 (Figure 6, lanes u-x) were consistent with single crossovers having occurred in the right-hand flanking sequences (compare Figure 6 with maps in Figure 5). This

implies that the 5' end of the operon must lie to the right of the EcoRI site at 21.8 kbp, which marks the right-hand limit of the deletion in CRM35 (the left-most limit of the right-hand flanking DNA in pBJ183) and quite possibly lies to the right of the BglII site at 22.8 kbp which marks the right-hand limit of the flanking sequences in both pBJ183 and pBJ184.

The RUBISCO and PRK phenotypes of the deletion mutants were similar to those of the EMS-generated mutants. However, the 4 deletion mutants were unable to induce FDH whereas FDH activities of the EMS-derived mutants were relatively unaffected. This implied that the deletions interrupted a locus essential for FDH induction. However, the analysis described above suggested that the break point between the deletion of CRM55 and the deletions of the other 3 was involved in RUBISCO and not in PRK induction. Thus the FDH results imply that there are two non-contiguous regions required for FDH induction, one deleted in CRM55 and the other affected in CRM35, CRM44 and CRM52 or, alternatively, that induction of FDH activity requires expression of RUBISCO and PRK activity. Since FDH is involved in energy acquisition, perhaps the absence of a sink for the reducing power it generates might repress its induction. We therefore repeated the induction experiment, this time adding glutamate to the formate broth, hypothesizing that reducing power generated by FDH activity might be utilized in glutamate catabolism, alleviating the repression of FDH. We have previously shown that FDH

induction in FM broth is not repressed by the presence of glutamate (Chapter 2). Indeed, although induction of RUBISCO and PRK activities in the deletion mutants was unaffected by the presence of glutamate, FDH activity was induced in CRM55 to wild type levels when glutamate was added to FM broth. CRM35, CRM44 and CRM52 induced low levels of FDH in the presence of formate and glutamate (Table 4). Thus it seems that the DNA region deleted in CRM55 is not directly essential for induction of FDH activity, but rather exerts a secondary effect through the primary effect on RUBISCO activity.

The presence of pCRM6 but not pLAFR1 restored the ability to grow on formate to each of the deletion-bearing strains CRM35, CRM44, CRM52 and CRM55, suggesting that the phenotypes of these deletions resulted from simple loss of function and the deletions were not resulting in dominant or cis effects.

Symbiotic Performance of CRM35. Soybeans inoculated with CRM35 ($PRK^-RUBISCO^-$) formed fully effective nodules. Plants inoculated with the mutant did not differ from those inoculated with wild type B. japonicum 110 on the basis of nodule acetylene reduction, shoot or root dry weight, nodule wet weight and nodule number (Table 5). When bacteria were reisolated from nodules incited by CRM35 all retained the kanamycin resistance of CRM35 and so were not contaminants. Similarly, Maier (1981) and Manian et al. (1984) reported that $RUBISCO^-$ B. japonicum strains effectively nodulated

Table 5. Symbiotic performance of a deletion mutant of *B. japonicum* unable to induce ribulose bisphosphate carboxylase and phosphoribulokinase activities.[a]

Inoculum	Shoot Dry Weight (g/plant)	Root Dry Weight (g/plant)	Nodule Number (/plant)	Nodule Wet Weight (g/plant)	Acetylene Reduction (nmol/g nodule/h)
none	0.7 (.4)	0.9 (.1)	0	0	0
110	2.7 (.3)	0.7 (.1)	71 (3)	1.2 (.1)	2.0 (.7)
CRM35	2.5 (4)	0.8 (.3)	80 (15)	1.2 (.2)	2.8 (.5)

[a] Data are presented as means with standard deviations in parentheses for 3 plants of each treatment.

soybeans. RUBISCO activity has not been detected in B. japonicum bacteroids (Simpson et al. 1979; Purohit et al. 1982). It seems that RUBISCO and PRK activities are not required for effective nodulation.

Discussion

We have identified a series of three recombinant cosmids with overlapping inserts of B. japonicum 110 DNA which contain a gene or genes essential for expression of PRK and RUBISCO activities during formate-dependent growth. The cosmids were identified by their ability to restore formate-dependent growth capability to 3 independently-derived mutants, two of which lacked RUBISCO activity and one which lacked both RUBISCO and PRK activities. Exconjugates of the mutants carrying the cosmids had wild-type levels of enzyme activities and contained apparently normal RUBISCO large subunit when induced for formate-dependent growth. Restoration of the wild-type phenotype was by complementation and not by recombination as indicated by several results. The cosmids could be recovered by transformation into E. coli and reconjugated back into the B. japonicum mutant strains resulting in 100% restoration of the ability to grow on formate. In addition, the exconjugates lost the ability to grow on formate when cured of the cosmids.

The cloned gene or genes could either represent structural genes for RUBISCO and PRK or a regulatory gene or

genes essential for expression of RUBISCO and PRK. Mutants in this DNA region have been found with two different phenotypes. EMS mutants SR122 and SR173 and deletion mutant CRM55 are $PRK^{+}RUBISCO^{-}$ and EMS mutant CRM15 and deletion mutants CRM35, CRM44 and CRM52 are $PRK^{-}RUBISCO^{-}$. If the locus is a single regulatory gene, then these phenotypes would suggest it is a positive regulator required for expression, since a loss of function results in a failure to turn on PRK and RUBISCO. It is unlikely, however, that different point and deletion mutations in a single regulatory gene would selectively prevent expression of only one of two coordinately regulated genes.

The observed mutant phenotypes are consistent with the locus encoding the structural genes for PRK (prk) and the large subunit of RUBISCO (rbcL). If these two genes were in an operon transcribed from prk to rbcL, a point mutation in rbcL could generate the $PRK^{+}RUBISCO^{-}$ phenotype of SR122 and SR173. A point mutation in the promoter/operator region or in the PRK-coding region could produce the $PRK^{-}RUBISCO^{-}$ phenotype of CRM15, if all transcription were prevented in the former case or if, in the latter case, the mutation in the PRK-coding region were polar and prevented downstream transcription or translation of rbcL. Such an operon structure implies transcription from right to left (as drawn in Figures 2 and 3) with prk lying entirely to the right of the BamHI site (at 17.5 kbp) and the RUBISCO large subunit coding sequence spanning the BamHI site since deletion mutant CRM55, which is deleted to the left of

the BamHI site is $PRK^{+}RUBISCO^{-}$ and the deletions in CRM35, CRM44 and CRM52, which are $PRK^{-}RUBISCO^{-}$, are entirely to the right of the BamHI site. The phenotypes of the two insertion strains, CRM43 and CRM48, resulting from single crossover events during the gene replacement matings with pBJ183 and pBJ184 are also consistent with the model of an operon transcribing prk and then rbcL from right to left. For a mutagenic event to arise from such a cointegration, the crossover must occur within the operon so that the resulting duplication generates two incomplete copies of the operon. The recombination events leading to strains CRM43 and CRM48 occurred in the right-hand flanking sequences, indicating that the 5' end of the operon lies to the right of the EcoRI site (at 21.8 kbp) which marks the right-hand limit of the deletion in CRM35. Transcription from the promoter is interrupted by the inserted plasmid DNA. Although a complete copy of the DNA lies to the left of the insertion, it is separated from its promoter by the insertion and thus cannot be transcribed. The promoter of the operon therefore lies within the 3.1 kbp region of DNA lying between that EcoRI site (21.8 kbp) and the EcoRI site at 24.9 kbp which marks the right-hand extent of cosmids pCRM7 and pCRM20. Thus the operon extends at least 4.3 kbp, completely spanning the region of DNA deleted in CRM35. This provides more than sufficient coding capability to account for PRK [a 33 kD protein in other bacteria (Tabita 1980; Siebert et al. 1981; Rippel and Bowien 1984), thus requiring approximately 0.8 kbp of coding sequence] and for

the large subunit of RUBISCO [a 55 kD protein in B. japonicum (Purohit et al. 1982) which would require approximately 1.4 kbp of coding sequence]. There would also be sufficient coding sequence available for the small subunit of RUBISCO [14 kD in B. japonicum (Purohit et al. 1982), implying 0.4 kbp of DNA]. In the cyanobacteria there are examples of large and small subunits being cotranscribed (Shinozaki and Sugiura 1983,1985; Nierzwicki-Bauer et al. 1984). It is also possible that the operon includes other genes encoding other enzymes of the reductive pentose phosphate pathway which might be coregulated with PRK and RUBISCO, or genes encoding functions associated with FDH activity.

CHAPTER 4

Discussion

Attempts to manipulate and improve the symbiosis between legumes and Bradyrhizobium and Rhizobium almost certainly will benefit from increased understanding of the global regulation mechanisms employed by both partners in the symbiosis. At present, mechanisms of global regulation governing the induction and repression of groups of genes in B. japonicum are not well understood. In the soil mixotrophic growth on mixtures of compounds such as formate, CO_2, H_2 and heterotrophic substrates is likely to contribute to the competitive success of the organism. Competition for these substrates is dependent on optimal regulation of systems of energy and carbon acquisition so as to maximize growth and persistence. I have initiated a study of the regulatory mechanisms controlling formate-dependent growth. Development from the free-living state into the bacteroid involves a series of environmental changes in some ways analogous to medium shifts. Hopefully insights derived from these studies on the regulation of formate-dependent growth will contribute to the analysis of regulation of the processes involved in nodulation and nitrogen fixation.

The identification and characterization of genes essential for formate-dependent growth would greatly

facilitate the analysis of regulation of that growth. The strategy adopted was to obtain such genes by complementation of mutants characterized as defective in formate-dependent growth with a gene library of B. japonicum DNA. The analysis of B. japonicum mutants unable to grow on formate required some understanding of the physiology of that growth. Therefore the time course of induction of enzymes of formate-dependent growth and the effect of the presence of heterotrophic substrates such as glutamate and xylose on the induction were determined. RUBISCO and PRK activities appeared to be coordinately regulated, and were partially repressed by additional glutamate or xylose. In contrast, FDH activity was unaffected by the addition of glutamate to formate media. When cells were transferred from xylose to formate media, induction of FDH was delayed relative to induction of RUBISCO and PRK. Thus it seems that FDH is regulated differently than the enzymes of carbon assimilation. Although it was thought originally that hydrogenase and RUBISCO activities were coordinately regulated in B. japonicum (Hanus et al. 1979), the demonstration of RUBISCO activity in the absence of hydrogenase activity during formate-dependent growth (Manian and O'Gara 1982a) clearly indicated that RUBISCO and hydrogenase could be independently regulated. This had been previously indicated by the detection of hydrogenase activity but not RUBISCO activity in bacteroids (Simpson et al. 1979). However, it appears that there are common elements involved in the regulation of hydrogenase and

RUBISCO since hydrogenase constitutive (Hup^C) mutants also express RUBISCO constitutively (Merberg and Maier 1984). It should be noted that as bacteroids these Hup^C mutants overexpress hydrogenase but do not express RUBISCO (Merberg and Maier 1983,1984). Maier (1981) described a series of Hup^- mutants of B. japonicum and noticed that these mutants induced RUBISCO at low levels, but he cautioned that this result could have been due to the energy-starved condition of the cells, which might have prevented full derepression of RUBISCO. Indeed, physiological studies of A. eutrophus demonstrated that RUBISCO activity was derepressed in response to carbon limitation, but only under conditions of energy availability while hydrogenase activity was derepressed in response to energy limitation (Friedrich 1982). Similar results have been obtained with P. oxalaticus (Dijkhuizen and Harder 1984). It seems that enzymes of carbon assimilation and energy aquisition are also subject to independent regulation in B. japonicum.

The role of cAMP in the regulation of FDH, RUBISCO and PRK activities remains ambiguous. Cyclic AMP accumulates during autotrophic growth (Lim and Shanmugam 1979) and during growth on formate (this study). Cyclic AMP definitely can affect the regulation of hydrogenase since the repression of hydrogenase activity by malate was overcome by cAMP (Lim and Shanmugam 1979; McGetrick et al. 1985). However, the repression of RUBISCO activity by malate was not alleviated by cAMP (McGetrick et al. 1985). In this study I showed that the addition of xylose blocked

the accumulation of cAMP during growth with formate. Under these conditions RUBISCO, PRK and FDH were all induced, although at reduced levels. Thus an increased accumulation of cAMP is not required for induction of these enzymes. However, cAMP was always detectable and so might not have been limiting and a role for cAMP in expression of RUBISCO and PRK is not precluded.

Twenty strains of B. japonicum unable to grow on formate were identified among the survivors of EMS treatment. The induction conditions determined in the physiological studies were used to partially characterize the phenotypes of these mutants. The phenotype of only one strain, CRM15, was clear. This strain failed to induce either RUBISCO or PRK activity under appropriate conditions. The defects of the other 19 mutants were more subtle and are not yet understood. One strain, CRM7, overexpressed an apparently novel polypeptide whose nature is unclear. The remaining 18 strains have no obvious defects. They all express the three enzyme activities when assayed in vitro. Many of the 18 induce FDH, RUBISCO and PRK activities at reduced levels and the inability to grow on formate could result directly from this, or the reduced levels detected could be the secondary results of primary lesions affecting other physiological aspects. This situation would be analogous to the low RUBISCO activities observed in Maier's (1981) Hup^- mutants as discussed above. Among the 18 mutants one could expect to find lesions in loci essential for the other enzymes of formate-dependent growth such as

other enzymes of the RPP cycle or components involved in electron transport from FDH. In vivo CO_2 assimilation by these strains has not been determined. The in vitro assay employed involves activation of RUBISCO in conditions of high Mg^{2+} and high CO_2 concentrations which probably do not reflect the conditions found in vivo. In Arabadopsis thaliana, mutants incapable of in vivo activation have been identified (Somerville et al. 1982): these mutants lack a soluble chloroplast protein composed of two polypeptides which is required for in vivo activation, but not for in vitro activation with high Mg^{2+} and CO_2 (Salvucci et al. 1986). Defects in the activation of RUBISCO might not have been detected by the method employed in the present study. Clearly, further characterization of these mutants is needed.

I have concentrated on mutants of B. japonicum unable to assimilate CO_2 by the RPP cycle. Strain CRM15 clearly lacks both RUBISCO and PRK activities and two strains described by Maier (1981) lack RUBISCO activity but induce wild type levels of PRK activity. A single region of B. japonicum DNA at least 4.6 kbp in length is capable of restoring a wild-type phenotype to both classes of mutants. At present it is not certain if this DNA encodes regulatory proteins essential for the expression of RUBISCO and PRK or if the DNA encodes the structural proteins for PRK, the large subunit of RUBISCO and, possibly, the small subunit of RUBISCO. The region could also encode both structural and regulatory genes. As far is as known, RUBISCO and PRK

activities in B. japonicum are coregulated, implying a common mode of regulation. Several mutants, one (CRM15) generated by EMS treatment and four (CRM35, CRM43, CRM44 and CRM48) generated by gene-directed deletion mutagenesis, lack both RUBISCO and PRK activities. Other EMS-generated (SR122 and SR173) and deletion-bearing (CRM55) mutants lack RUBISCO activity but have wild type levels of PRK activity. It is unlikely that different lesions in a regulatory locus will result in selective expression of one of two commonly regulated enzymes. The suggested model of an operon in which structural genes encoding PRK (prk) and the large subunit of RUBISCO (rbcL) are cotranscribed from prk to rbcL is consistent with all available data. Although there are no data on the B. japonicum rbcS gene, which encodes the small subunit of RUBISCO, in other prokaryotes with Form I RUBISCOs rbcL and rbcS are cotranscribed (see for examples, Shinozaki and Sugiura 1983,1985; Nierzwicki-Bauer et al. 1984). In those systems it is not known if the prk gene is linked to the rbcLS operon. In A. eutrophus, genes encoding RUBISCO and PRK have been identified and isolated (Andersen and Wilke-Douglas 1984; Klintworth et al. 1985), but it is not known if the loci are closely linked. According to the operon model suggested above, the lesions in SR122 and SR173 would be downstream of the prk coding sequence and affect rbcL. The lesion in CRM15 affects both genes. It could be either in the promoter region, preventing the transcription or translation of the operon, or in the prk sequence such that a polar effect was exerted, preventing the

expression of rbcL as well as resulting in a non-functional PRK. The deletion in CRM55 which affects only RUBISCO activity is consistent with a deletion into the 3' end of the operon extending into the rbcL gene but not into the prk gene. The deletions in CRM35, CRM44 and CRM52 would delete at least part of both genes. In the case of the deletion in CRM52, which extends to the end of the insert of two of the classes of cosmids (pCRM7 and pCRM20) identified as able to complement the mutations, the entire 5' end of the operon is almost certainly removed. The single crossover mutants CRM43 and CRM48 are also consistent with the operon model. For the integration of a plasmid to be mutagenic the integration must occur at a site within the locus such that although the entire DNA region is present with some duplication, the operon is interrupted by vector sequences. That is, the 5' region is separated from the 3' region by heterologous DNA such that the expression of the 3' region of the operon, distal to the insertion, is blocked. This analysis further allows the 5' end of the operon to be localized between the boundary of the deleted DNA in the replacement plasmids (pBJ183 and pBJ184) used to construct the single crossover mutants (CRM43 and CRM48) and the boundary defined by the insert DNA in the complementing cosmids (pCRM7 and pCRM20).

Similar approaches have been applied to other systems in an effort to isolate the genes encoding bacterial Form I RUBISCOs. In the hydrogen/methanol autotroph Xanthobacter sp., Lehmicke and Lidstrom (1985) obtained mutants lacking

RUBISCO (but unaffected in PRK activity) and have isolated a 3.2 kbp DNA sequence which will complement the mutant phenotype. This sequence is unlinked to sequences complementing H_2-oxidation mutants. It is not yet known whether the isolated DNA encodes structural or regulatory proteins.

In A. eutrophus sequences of both plasmid and chromosomal origin have been isolated which complement mutants lacking RUBISCO activity (Andersen and Wilke-Douglas 1984). In a separate study the amino acid sequence of the amino terminus of PRK was determined and used to design a synthetic oligonucleotide hybridization probe which was used to isolate genes encoding PRK (Klintworth et al. 1985). prk genes were found in both plasmid and chromosomal locations. Interestingly, plasmid-cured strains are capable of formate-dependent growth but fail to express RUBISCO and PRK activities in the presence of heterotrophic substrates (Behki et al. 1983; Bowien et al. 1984). It is not known if the chromosomal genes are more sensitive to catabolite repression than the plasmid-borne genes or if some regulatory function essential to expression of PRK and RUBISCO in the presence of heterotrophic substrates lies on the plasmid. At least some genes essential for H_2 oxidation are found only on the plasmid.

Weaver and Tabita (1983) described an interesting mutant of R. sphaeroides incapable of photoautotrophic growth which also failed to increase levels of Form I RUBISCO when grown photoheterotrophically on butyrate or

under CO_2 limitation, conditions which normally result in a 4-8 fold increase in Form I RUBISCO in wild type. They have isolated a 2.7 kbp DNA fragment which restores photoautotrophic growth capability (Weaver and Tabita 1985). However, complementation is complicated in that it seems to require recombination, and complemented strains still fail to derepress Form I RUBISCO to normal levels. The nature of the gene(s) encoded by the DNA fragment remains to be determined.

Thus in several systems genetic analysis of CO_2 assimilation by the RPP cycle is beginning. Particularly in B. japonicum there is also considerable interest in the genetics of hydrogen uptake (Cantrell et al. 1983; Haugland et al. 1984; Hom et al. 1985; Lambert et al. 1985). Substantial progress towards understanding the regulation of these metabolic systems can be anticipated.

In conclusion, I have examined the physiology of the induction of enzymes of formate-dependent growth and have used this information to evaluate mutants defective in formate-dependent growth. Two classes of mutants which fail to induce either RUBISCO or both RUBISCO and PRK activities have been studied in more detail than the other mutants which all express, to varying degrees, RUBISCO, PRK and FDH activities. I have identified and begun to characterize a region of DNA which will complement the mutants which fail to express RUBISCO and PRK. It is likely that this DNA region contains an operon which includes the structural genes encoding PRK and the large subunit of RUBISCO,

although definitive proof is still lacking. Efforts to provide such proof will focus on expression of the cloned DNA *in vitro* and analysis of the resultant protein products on the basis of molecular size, immunological cross-reactivity with antiserum against purified *B. japonicum* RUBISCO and *in vitro* enzyme activity. Additionally, the nucleotide sequence of the region will be determined. The polypeptide products predicted by analysis of the nucleotide sequence wil be inspected for homology to known proteins. The nucleotide sequence will also greatly facilitate analysis of the protein products of the region. Promoter regions identified will be studied in detail. Transcriptional and translational fusions of these genes to easily assayed markers such as B-galactosidase can provide the basis of a selection system by which to identify mutations which prevent induction of PRK and RUBISCO, thus allowing the molecular analysis of their regulation. These global regulatory mechanisms may truly contribute to an understanding of the symbiotic development of *Bradyrhizobium*.

It is not yet known if any of the other mutants identified in this study can be complemented by the isolated DNA region. Among these mutants it is likely there are some with defects elsewhere in the RPP cycle of CO_2 fixation or in the pathway of energy acquisition by FDH. These mutants merit further investigation.

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APPENDIX

The Nucleotide Sequence of the *Bradyrhizobium japonicum* Gene Encoding 5-Aminolevulinic Acid Synthase (*hemA*)

Introduction

Leghemoglobin is a myoglobin-like hemoprotein which is found in nitrogen-fixing nodules. Leghemoglobin functions to allow high oxygen flux at low oxygen tension which supports oxidative metabolism of bacteria while protecting the oxygen-sensitive nitrogenase from inactivation (Appleby 1984). The apoprotein of leghemoglobin is encoded in the plant nuclear genome (Sullivan et al. 1981; Jensen et al. 1981). The bacterial fraction from nodules incorporates specific precursors into heme more rapidly than the plant fraction (Cutting and Schulman 1969; Godfrey and Dilworth 1971). The first and last enzymes in the pathway of heme biosynthesis, 5-aminolevulinic acid synthase (ALAS; Nadler and Avissar 1977) and ferrochelatase (Porra 1975) are predominantly associated with the bacterial rather than with the plant fraction and bacterial ALAS activity and heme content increase in parallel in developing soybean nodules (Nadler and Avissar 1977). However, 5-aminolevulinic acid dehydratase (ALAD) is found in both plant and bacterial

fractions of the nodule (Godfrey et al. 1975; Nadler and Avissar 1977) and plants can synthesize heme (Granick and Beale 1978). It is possible that there is a coordinate effort between plant and bacteria to synthesize heme in the nodule (Godfrey et al. 1975) like that observed in animal cells where different biosynthetic steps are localized in the cytoplasm and in the mitochondria (Sano and Granick 1961).

Genes, termed hemA, encoding ALAS have been isolated from both Rhizobium meliloti (Leong et al. 1982) and Bradyrhizobium japonicum (Guerinot and Chelm 1986). Although mutants of R. meliloti lacking ALAS activity formed alfalfa nodules which lacked leghemoglobin and were ineffective (Leong et al. 1982), mutants of B. japonicum deleted for hemA which were ALA auxotrophs in culture formed effective nodules containing leghemoglobin on soybeans (Guerinot and Chelm 1986). This latter result shows that B. japonicum ALAS activity cannot be the sole source of ALA for heme synthesis in soybean nodules. However, it does not preclude an alternative bacterial pathway expressed in nodules but not in the free-living state. Whether the difference in results between the B. japonicum/soybean and R. meliloti/alfalfa symbioses indicates differences in the ability of the plant to contribute to leghemoglobin heme biosynthesis is not yet clear.

Further investigation of heme biosynthesis is warranted, and to this end we have determined the complete

nucleotide sequence of the region of B. japonicum DNA encoding ALAS and its flanking DNA.

Materials and Methods

Plasmids, Bacterial Strains and Media. The B. japonicum hemA gene was cloned into pBR322 as a 4.2 kbp PstI fragment to yield pBJ110 (Guerinot and Chelm 1986). The Escherichia coli strains ED8654 (galK, galT, trpR, metB, hsdR, supE, lacY) (Borck et al 1976) and HB101 [hsdS, (r_B^-, m_B^-), recA, ara, proA, lacY, galK, rpsL, xyl, mtl, supE] (Boyer and Roulland-Dussoix 1969) were used for routine plasmid construction and maintenance. E. coli strains were routinely grown on LB (Miller 1972).

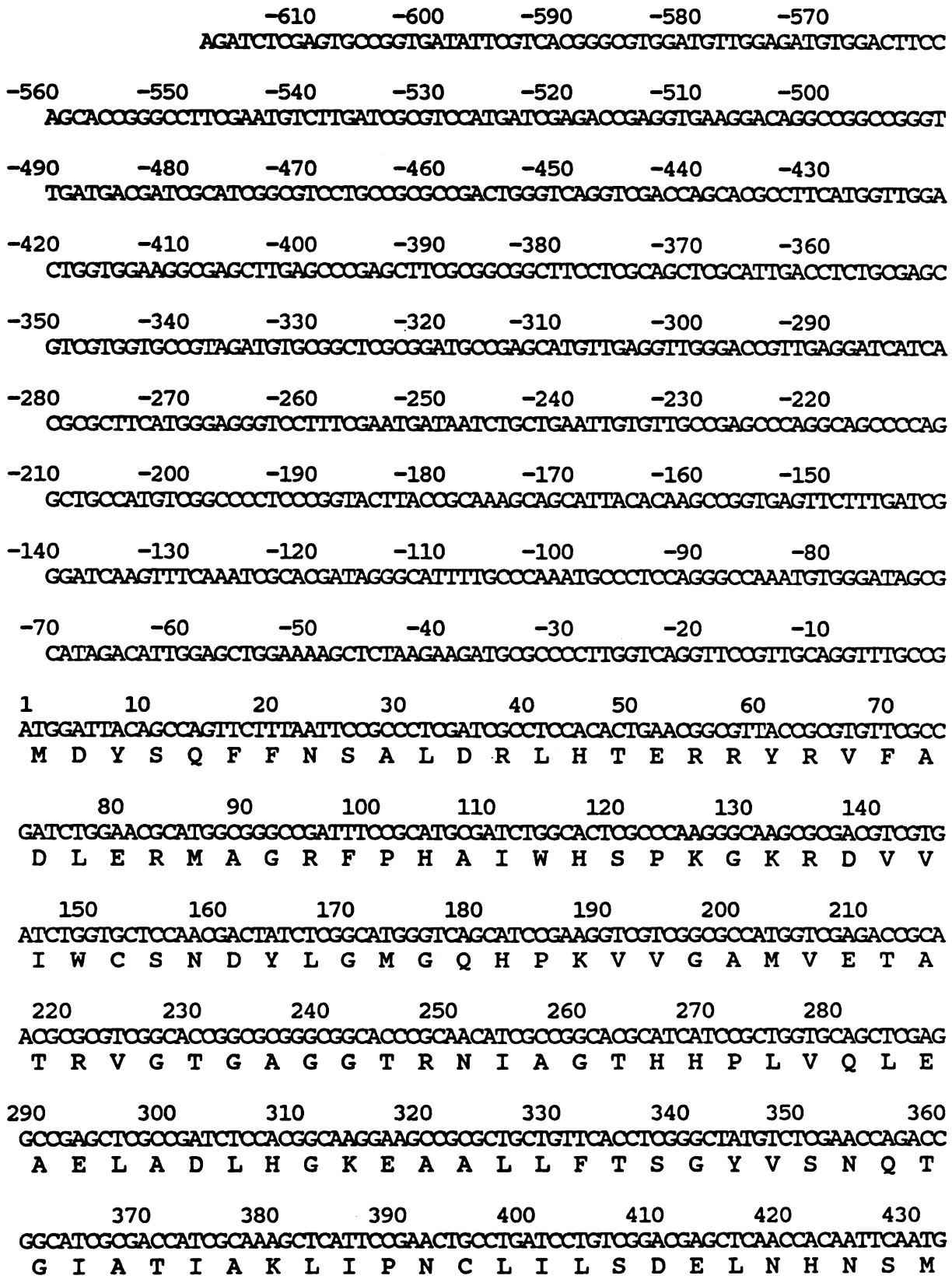
DNA Techniques. Relaxed replicon plasmid DNA was isolated by the method of Clewell and Helinski (1972). DNA restriction endonuclease fragments were end-labelled and isolated by separation and elution from polyacrylamide gels as described by Adams and Chelm (1984). Nucleotide sequence was determined by the method of Maxam and Gilbert (1980). The computer programs of Pustell and Kafatos (1984) and Lipman and Pearson (1985) were used for analysis of nucleotide and amino acid sequences.

Results and Discussion

The sequence of a 2453 nucleotide BglII-PvuI fragment from pBJ110 (Guerinot and Chelm 1986) was determined with contiguous overlapping readings for both strands and is shown in Figure 1. The nucleotide sequence contains an extensive open reading frame from an ATG at nucleotide 1 to nucleotide 1227. This sequence predicts a protein of 408 amino acids with a molecular weight of 44599 daltons. The direction of transcription of this open reading frame is consistent with the direction predicted from hybridization with DNA probes homologous to either the 5' or 3' regions of the R. meliloti hemA gene region (Guerinot and Chelm 1986).

Several lines of evidence indicate that this open reading frame encodes ALAS. There are no other open reading frames of sufficient coding capacity in either direction in the sequenceed region. The ALA auxotroph constructed by Guerinot and Chelm (1986) was deleted from the XhoI site at nucleotide 283 to an EcoRI site 2.1 kb downstream from the XhoI site (i.e nucleotide 2400). Finally, both the nucleotide and predicted amino acid sequences of this open reading frame show striking homology to the partial sequences of the R. meliloti hemA gene and protein (Leong et al. 1985) and also to the complete sequences determined for the chicken embryonic liver hemA gene (Borthwick et al. 1985). A comparison of the nucleotide sequences of

Figure 1.


-610 -600 -590 -580 -570
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-560 -550 -540 -530 -520 -510 -500
AGCACCGGGCCTTCGAATGTCTTGATCGCGTCCATGATCGAGACCGAGGTGAAGGACAGGCCGGCCGGGT
-490 -480 -470 -460 -450 -440 -430
TGATGACGATCGCATCGGCGTCCTGCCGCGCCGACTGGGTCAGGTCGACCAGCACGCCTTCATGGTTGGA
-420 -410 -400 -390 -380 -370 -360
CTGGTGGAAGGCGAGCTTGAGCCCGAGCTTCGCGGCGGCTTCCTCGCAGCTCGCATTGACCTCTGCGAGC
-350 -340 -330 -320 -310 -300 -290
GTCGTGGTGCCGTAGATGTGCGGCTCGCGGATGCCGAGCATGTTGAGGTTGGGACCGTTGAGGATCATCA
-280 -270 -260 -250 -240 -230 -220
CGCGCTTCATGGGAGGGTCCTTTCGAATGATAATCTGCTGAATTGTGTTGCCGAGCCCAGGCAGCCCCAG
-210 -200 -190 -180 -170 -160 -150
GCTGCCATGTCGGCCCCTCCCGGTACTTACCGCAAAGCAGCATTACACAAGCCGGTGAGTTCTTTGATCG
-140 -130 -120 -110 -100 -90 -80
GGATCAAGTTTCAAATCGCACGATAGGGCATTTTGCCCAAATGCCCTCCAGGGCCAAATGTGGGATAGCG
-70 -60 -50 -40 -30 -20 -10
CATAGACATTGGAGCTGGAAAAGCTCTAAGAAGATGCGCCCCTTGGTCAGGTTCCGTTGCAGGTTTGCCG
1 10 20 30 40 50 60 70
ATGGATTACAGCCAGTTCTTTAATTCCGCCCTCGATCGCCTCCACACTGAACGGCGTTACCGCGGTGTTCGCC
M D Y S Q F F N S A L D R L H T E R R Y R V F A
80 90 100 110 120 130 140
GATCTGGAACGCATGGCGGGCCGATTTCCGCATCGCGATCTGGCACTCGCCCAAGGGCAAGCGCGACGTCGTG
D L E R M A G R F P H A I W H S P K G K R D V V
150 160 170 180 190 200 210
ATCTGGTGCTCCAACGACTATCTCGGCATGGGTCAGCATCCGAAGGTCGTCGGCGCCATGGTCGAGACCGCA
I W C S N D Y L G M G Q H P K V V G A M V E T A
220 230 240 250 260 270 280
ACGCGCGTCGGCACCGGCGCGGGCGGCACCCGCAACATCGCCGGCACGCATCATCCGCTGGTGCAGCTCGAG
T R V G T G A G G T R N I A G T H H P L V Q L E
290 300 310 320 330 340 350 360
GCCGAGCTCGCCGATCTCCACGGCAAGGAAGCCGCGCTGCTGTTCACCTCGGGCTATGTCTCGAACCAGACC
A E L A D L H G K E A A L L F T S G Y V S N Q T
370 380 390 400 410 420 430
GGCATCGCGACCATCGCAAAGCTCATTCCGAACTGCCTGATCCTGTCGGACGAGCTCAACCACAATTCAATG
G I A T I A K L I P N C L I L S D E L N H N S M

Figure 1 continued.

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     440       450       460       470       480       490       500
ATCGAGGGCATCCGCCAGTCCGGCTGCGAGCGGCAAGTGTTCCGCCACAACGATCTGCCGACCTCGAAGCG
 I  E  G  I  R  Q  S  G  C  E  R  Q  V  F  R  H  N  D  L  A  D  L  E  A

   510       520       530       540       550       560       570
CTGTTGAAGGCGGCGGGTGCGAACCGGCCGAAGCTGATCGCCTGCGAGAGCCTCTATTCCATGGACGGCGAC
 L  L  K  A  A  G  A  N  R  P  K  L  I  A  C  E  S  L  Y  S  M  D  G  D

 580       590       600       610       620       630       640
GTCGCTCCGCTCGCCAAGATCTGCGATCTGCCGAGAAATATAACGCGATGACCTATGTCGACGAAGTCCAC
 V  A  P  L  A  K  I  C  D  L  A  E  K  Y  N  A  M  T  Y  V  D  E  V  H

650       660       670       680       690       700       710       720
GCGGTCGGCATGTACGGCCCGCGCGGCGGCGGCATCGCCGAGCGTGACGGCGTCATGCATCGCATCGACATT
 A  V  G  M  Y  G  P  R  G  G  G  I  A  E  R  D  G  V  M  H  R  I  D  I

       730       740       750       760       770       780       790
CTCGAAGGCACGCTGGCCAAGGCGTTCGGCTGCCTCGGCGGCTACATCGCCGCCAACGGCCGGATCATCGAC
 L  E  G  T  L  A  K  A  F  G  C  L  G  G  Y  I  A  A  N  G  R  I  I  D

     800       810       820       830       840       850       860
GCCGTGCGCTCCTATGCGCCGGGCTTCATCTTCACCACCGCGCTGCCGCCGGCGATCTGCTCGGCCGCGACC
 A  V  R  S  Y  A  P  G  F  I  F  T  T  A  L  P  P  A  I  C  S  A  A  T

   870       880       890       900       910       920       930
GCCGCGATCAAGCACCTGAAGACCTCGAGCTGGGAGCGCGAGCGCCACCAGGACCGCGCCGCCCGCGTCAAG
 A  A  I  K  H  L  K  T  S  S  W  E  R  E  R  H  Q  D  R  A  A  R  V  K

 940       950       960       970       980       990      1000
GCGATCCTCAACGCCGCCGGTCTCCCGGTGATGTCGAGCGACACCCACATCGTGCCGCTGTTCATCGGCGAT
 A  I  L  N  A  A  G  L  P  V  M  S  S  D  T  H  I  V  P  L  F  I  G  D

1010      1020      1030      1040      1050      1060      1070      1080
GCCGAGAAGTGCAAGCAGGCCTCCGACCTGCTGCTCGAAGAGCACGGCATCTACATCCAGCCGATCAACTAT
 A  E  K  C  K  Q  A  S  D  L  L  L  E  E  H  G  I  Y  I  Q  P  I  N  Y

      1090      1100      1110      1120      1130      1140      1150
CCGACCGTCGCCAAGGGCTCCGAGCGCCTGCGCATCACGCCCTCGCCCTATCACGATGACGGCCTGATCGAT
 P  T  V  A  K  G  S  E  R  L  R  I  T  P  S  P  Y  H  D  D  G  L  I  D

    1160      1170      1180      1190      1200      1210      1220
CAGCTGCCGAAGCCCTGTTGCAAGTGTGGGACCGCCTCGGCCTGCCGCTCAAGCAAAAGTCGCTGGCGGCG
 Q  L  A  E  A  L  L  Q  V  W  D  R  L  G  L  P  L  K  Q  K  S  L  A  A

  1230      1240      1250      1260      1270      1280      1290
GAGTAGGTTTTTCTTCGCCTCTCCCCGCTTGCGGGGAGAGGCCGGATCGCATCGCCAGGTGCGATCCGGGTG
 E  -

1300      1310      1320      1330      1340      1350      1360
AGGGGGAGTCTCCGCGAGTCCAGCTGCCACCGTCCCCGCGGAGAGCCCCCCTCACCCCACCCTCTCCCCGCA

       1380      1390      1400      1410      1420      1430      1440
AGCGGGGCGAGGGAGAGCAGGCAGCTCGCCCTACGGCTTAACGACGACGCCAGTTCCCGCCCCTTCCGGGCA
```

Figure 1 continued.

```
      1450       1460       1470       1480       1490       1500       1510
TTTCGCTGTCGCCTCGCCTCGTCCAACGCGCTAGATTTGCAGGGAAAATGAGACCGGGCACCGTCGCGCC

      1520       1530       1540       1550       1560       1570       1580
CAGGGAGAAGCACGCTGCCATGCTGCACGACTGGGGCGTGATTGCCGCCGCCTTTGGCTATATCGGCTC

      1590       1600       1610       1620       1630       1640       1650
CCTGTTCCTGGTGGCGAGCCACGGCGACCGCCGCTCGCCGGCCGGGCCAGGCCGCGCCTCCGGCCTGATC

      1660       160       1680       1690       1700       1710       1720
TATCCGCTGTCGCTGGCGATCTACTGCACCTCCTGGACCTTCTTCGGCTCGGTTGGCTTCGCCACCCGCA

      1730       1740       1750       1760       1770       1780     1790
CCTCGACCGACTTCCTCGCCATCTATGTCGGCCCGATCCTGGTGATCGGCCTCGGTGCCGGCGTGGCTGC

      1800       1810       1820       1830
GCCGCGTGATCCAGCTCGCCAAGGCCCACAACATCACCTCGATCG
```

Figure 1. Nucleotide sequence of the Bradyrhizobium japonicum hemA gene region and predicted amino acid sequence of 5-aminolevulinic acid synthase. The nucleotide sequence is numbered with the hemA translation start as 1. Only the non-coding DNA strand is shown.

B. japonicum and chicken embryonic liver hemA genes is shown as a homology matrix in Figure 2. This matrix was generated with the analysis program of Pustell and Kafatos (1984) set such that each letter in the matrix represents homology of at least 57% over 41 nucleotides. We used the program of Lipman and Pearson (1985) to compare the predicted amino acid sequence of the B. japonicum ALAS with those of R. meliloti (Leong et al. 1985) and chicken embryonic liver (Borthwick et al. 1985). There is 53% identity of the B. japonicum and R. meliloti proteins over the 76 amino acids of sequence available for R. meliloti while the B. japonicum and chicken proteins show 48.8% identity over the entire length of the B. japonicum protein (Figure 3). The similarities between the B. japonicum and R. meliloti and chicken ALAS amino acid sequences are highly significant. Using the RDF program of Lipman and Pearson (1985) with ktup=1 and 1000 randomized sequences the similarity scores of the B. japonicum sequence were 232 and 1094, with z values of 32.4 and 134.9 to the sequences from R. meliloti and chicken embryonic liver, respectively. A z value of greater than 10 is considered significant.

The high degree of amino acid sequence conservation of ALAS between such evolutionarily distant organisms as Bradyrhizobium and chicken suggests major structural constraints for this enzymatic activity. The homology between the B. japonicum and chicken sequences did not extend to the amino terminal 191 residues of the chicken

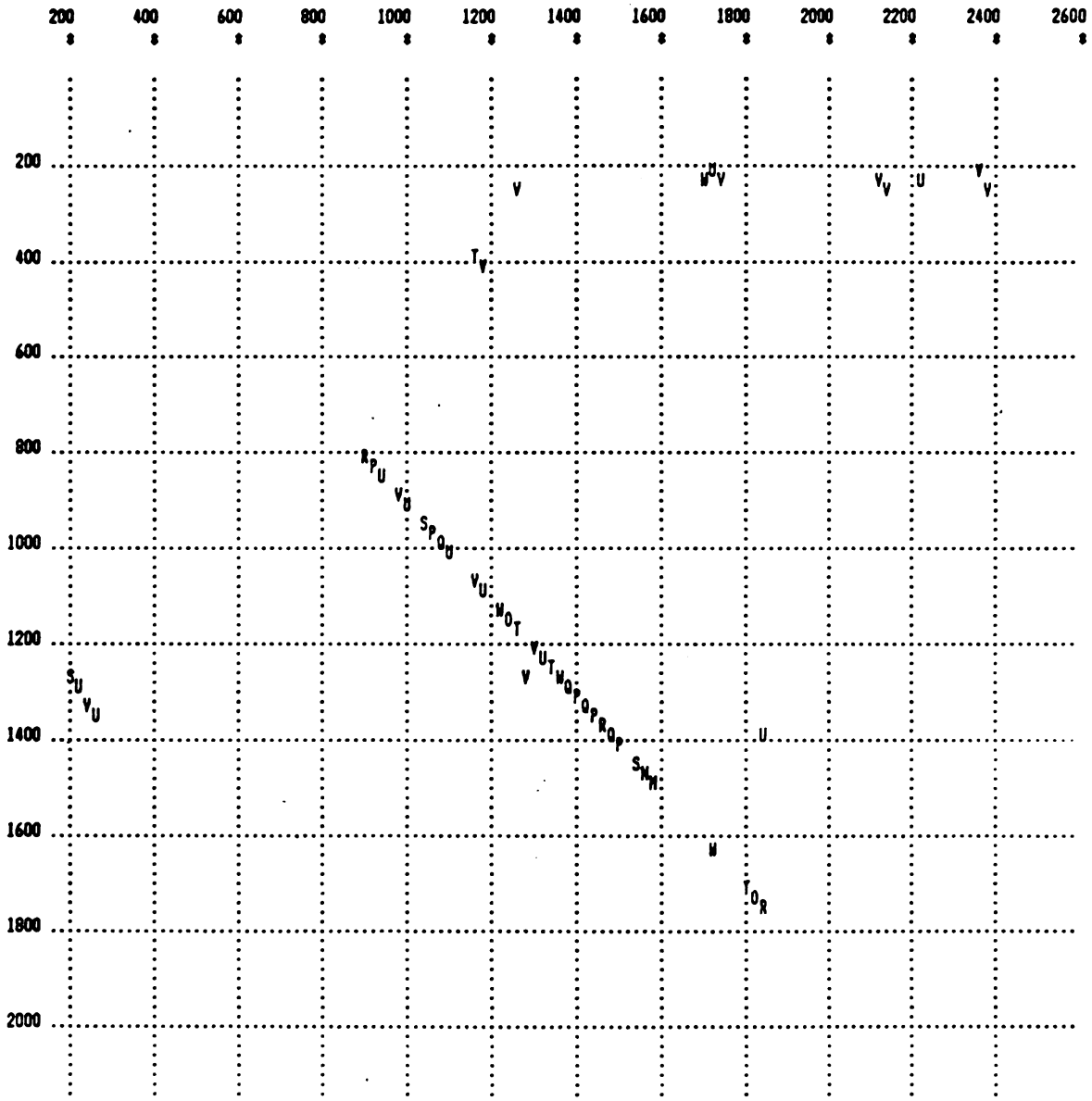

Figure 2. DNA homology matrix comparing B. japonicum hemA (X-axis) to chicken embryonic liver hemA (Y-axis) generated with the Pustell and Kafatos (1984) sequence analysis program using the following parameters: range = 20, scale factor = 0.99, minimum value = 57 and compression = 20. The scores for individual points in the matrix are encoded into letters with increments of 2%: A=100%, B=98-99% and so on to W=56-57%.

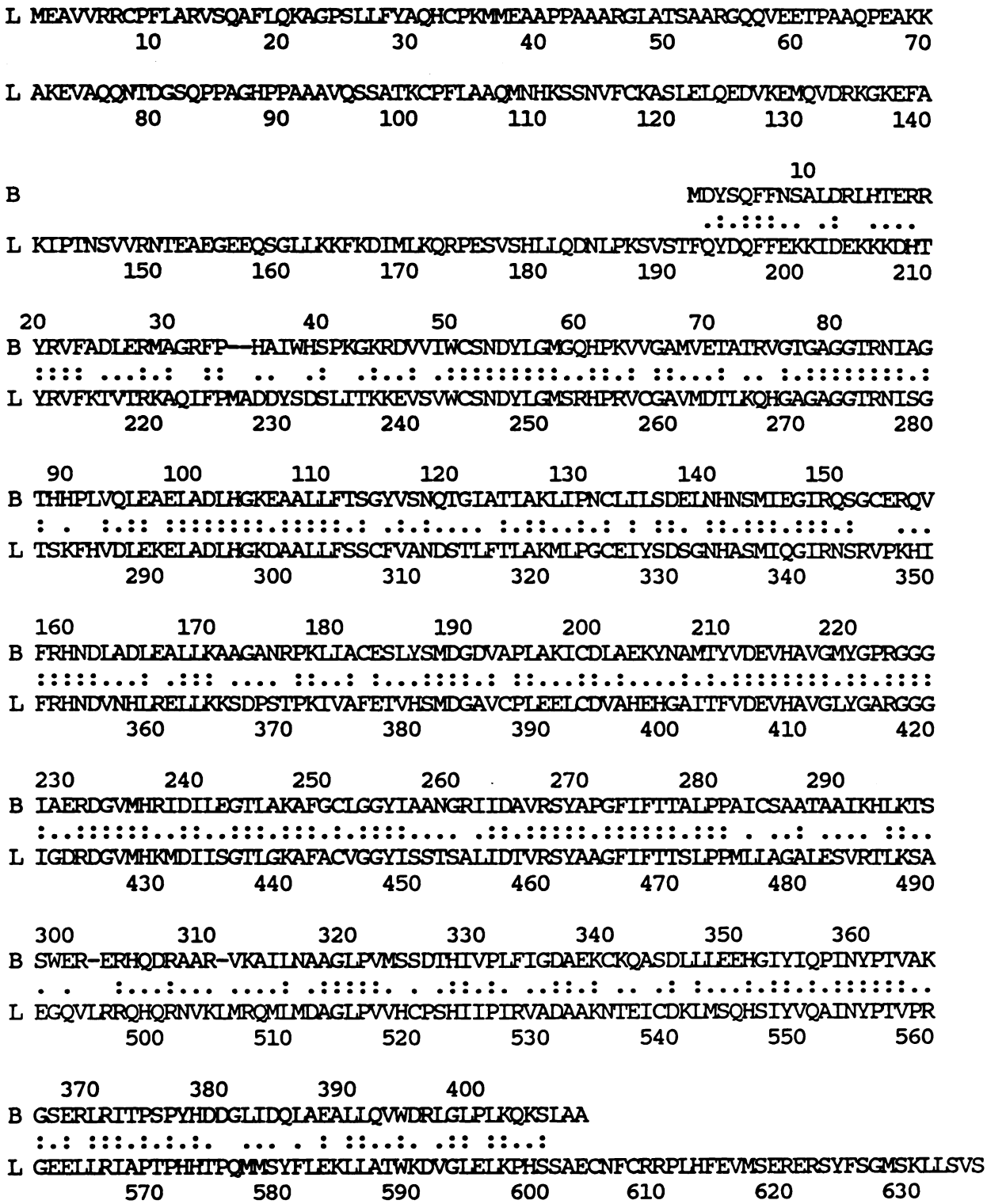

Figure 3. Alignment of the amino acid sequences of the B. japonicum (B) and chicken embryonic liver (L) _-aminolevulinic acid synthases, generated with the sequence analysis program of Lipman and Pearson (1985). : indicates identical residues and . indicates conservative substitution.

protein. This region of the chicken protein includes a highly basic mitochondrial presequence of 56 amino acids (Borthwick et al. 1985) which would not be needed in B. japonicum. The function of the remaining 135 residues of the chicken embryonic liver protein which have no counterpart in the B. japonicum protein is not known.

Studies currently under way are aimed at identifying the promoter(s) of this gene. We are interested in the regulation of expression of the B. japonicum hemA gene under both ex planta and symbiotic conditions.

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