Asparagine degradation in *Rhizobium etli*

Alejandra Huerta-Zepeda, Socorro Durán, Gisela Du Pont
and Jorge Calderón

Author for correspondence: Jorge Calderón. Tel: +52 6 22 38 80. Fax: +52 5 50 00 48.

Departamento de
Biotecnología, Instituto de
Investigaciones
Biomédicas, Universidad
Nacional Autónoma de
México, Mexico DF 04510

The degradation of asparagine by *Rhizobium etli* involves asparaginase and aspartate ammonia-lyase (L-aspartase). The two enzymes were shown to be positively regulated by asparagine and negatively regulated by the carbon source. Asparaginase activity was not regulated by oxygen concentration or by nitrogen catabolite repression. Induction of both enzymes by asparagine enables *R. etli* to utilize asparagine as carbon source. Asparaginase may also be involved in maintaining the optimal balance between asparagine and aspartate. Aspartase was not involved in the utilization of aspartate or glutamate as carbon source. The presence of high levels of the two enzymes in *R. etli* bacteroids suggests that they may have a role in symbiosis between *R. etli* and *Phaseolus vulgaris*.

Keywords: *Rhizobium etli*, asparaginase, aspartase, catabolism, symbiosis

INTRODUCTION

In some organisms, L-asparagine can be utilized as sole carbon and nitrogen source through the action of two enzymes (Sun & Setlow, 1991). The first enzyme, asparaginase (EC 3.5.1.1), catalyses the hydrolysis of L-asparagine to L-aspartate and ammonium. The second enzyme, aspartase ammonia-lyase (L-aspartase; EC 4.3.1.1), catalyses the reversible deamination of L-aspartate to yield fumarate and ammonium.

Asparaginase activity has been studied in *Escherichia coli* and other Gram-negative bacteria such as *Salmonella enterica* (Jennings *et al.*, 1993), *Erwinia chrysanthemi* (Gilbert *et al.*, 1986) and *Vibrio proteus* (Sinha *et al.*, 1991), and in Gram-positive bacteria such as *Bacillus subtilis* (Sun & Setlow, 1991) and *Staphylococcus aureus* (Sobis & Mikucki, 1991; Rózalska & Mikucki, 1992). *E. coli* contains two L-asparaginase isozymes. L-Asparaginase I has a low affinity for asparagine, it is cytoplasmic and is thought to be constitutively produced (Jerlström *et al.*, 1989; Willis & Woolfolk, 1974); L-asparaginase II is a high-affinity enzyme secreted to the periplasm, and its expression is positively regulated by the cAMP receptor protein and by anaerobiosis via the product of the *fnr* gene (Cedar & Schwartz, 1967; Chesney, 1983; Ho *et al.*, 1970; Jerlström *et al.*, 1987; Russell & Yamasaki, 1978; Jennings & Beachman, 1993).

In *S. enterica*, an organism closely related to *E. coli*, L-asparaginase II is regulated by glucose levels in the medium, via the catabolite repressor protein, and by anaerobiosis. However, the anaerobic regulation of this

gene does not act via the FNR protein, contrasting with the case in *E. coli* (Jennings *et al.*, 1993). *B. subtilis* L-asparaginase and L-aspartase are encoded by the *ans* operon, which is subject to strong repression by a good nitrogen source, such as ammonium, and is also induced by asparagine and aspartate (Sun & Setlow, 1991; Atkinson & Fisher, 1991; Iijima *et al.*, 1977).

L-Aspartase activity has been studied in several bacteria, such as *E. coli*, *B. subtilis* (Sun & Setlow, 1991), *Pseudomonas fluorescens* (Tokushige, 1985; Miyamoto & Katsuki, 1992) and *Serratia marcescens* (Takagi & Kisumi, 1985). *E. coli* aspartase is activated by divalent ions (Rudolph & Fromm, 1971; Karsten & Viola, 1991) and regulated by catabolite repression and by anaerobiosis via the product of the *fnr* gene (Nishimura & Kisumi, 1984; Jerlström *et al.*, 1987).

Bacteria of the genus *Rhizobium* establish intracellular symbiosis with legumes in which the bacteroids fix atmospheric nitrogen and export ammonium to plant tissues. Carbon compounds produced by the plant are metabolized by the bacteria to supply the energy and reductant needed for nitrogen fixation. Identification and analysis of the bacterial and plant enzymes that are active in the nodule are important to understand the metabolic pathways used by both partners during symbiosis.

Kahn *et al.* (1985) proposed that the carbon supplied to bacteroids includes amino acids. Rastogi & Watson (1991) found that a *Rhizobium meliloti* mutant, unable to grow on aspartate as carbon source due to a deficiency of a novel atypical aspartate aminotransferase activity, does not fix

nitrogen, implying that aspartate is an essential substrate for bacteroids in the nodule (Watson & Rastogi, 1993).

Considering the importance that amino acid metabolism may have in the *Rhizobium* symbiosis, together with the finding that *Rhizobium etli* does not grow on aspartate but does grow on asparagine as carbon source, and that high asparaginase and aspartase activities have been found in *Rhizobium lupini* bacteroids (Kretovich *et al.*, 1981), we decided to study the degradation of asparagine in *R. etli*, a bacterium that establishes symbiosis with *Phaseolus vulgaris* (bean) (Segovia *et al.*, 1993).

In this report, we present evidence of an asparaginase–aspartase pathway for the degradation of asparagine in *R. etli* and the regulation of these enzymes. The enzymic activities of asparaginase and aspartase in bacteroids are also reported.

METHODS

Strains. The *Rhizobium etli* CFN42 wild-type strain used in this study was previously classified as *Rhizobium leguminosarum* biovar *phaseoli* (Segovia *et al.*, 1993).

Growth conditions. Batch cultures of *R. etli* were grown at 30 °C with shaking at 200 r.p.m. For growth on minimal medium (MM) (Beringer, 1974), cells previously grown overnight on a rich medium (PY) containing 0.5% (w/v) peptone, 0.3% (w/v) yeast extract and 7 mM CaCl₂ were washed and used as inoculum. The initial OD₅₄₀ of the medium was adjusted to 0.05. The nitrogen and carbon sources in MM were added at 10 mM, except for glycerol (2%, v/v). Microaerobic cultures were achieved by flushing a tightly stoppered Erlenmeyer flask containing 250 ml medium with nitrogen for 10 min and re-admitting air to give a 1% (v/v) oxygen concentration. Growth was monitored by measurement of the OD₅₄₀ and by protein determination using the Lowry method.

Determination of asparaginase activity. Cell extracts were prepared by sonicating whole cells in extraction buffer (100 mM KH₂PO₄, pH 7.6) with a Soniprep 150 ultrasonic disintegrator; homogenates were centrifuged for 5 min at 12000 r.p.m. (Beckman JA-20 fixed-angle rotor) at 4 °C. The assay measuring ammonium formation was performed in a 0.25 ml reaction mixture containing 30 mM KH₂PO₄, pH 7.6, 10 mM asparagine and 0.05 ml cell extract. Ammonium formation was measured as described elsewhere (Chaney & Marbach, 1962; Durán & Calderón, 1995). The assay measuring the formation of aspartate was performed in a 1 ml reaction mixture containing 30 mM KH₂PO₄, pH 7.6, 10 mM asparagine and 0.2 ml cell extract. Aspartate was determined with a System Gold Liquid Chromatographic system from Beckman and a Gilson Fluorometer model 121 after coupling with *o*-phthalaldehyde (Calderón *et al.*, 1985).

Determination of aspartase activity. Cell extracts were prepared by sonicating whole cells in extraction buffer (50 mM KH₂PO₄, 100 mM KCl, 1 mM EDTA, 5 mM 2-mercaptoethanol, pH 8) as described above. Assays for fumarate formation were carried out spectrophotometrically (Beckman DU 640 spectrophotometer) at 240 nm, the reaction mixture in a total volume of 1 ml containing 100 mM Tris/HCl, pH 8.8, 100 mM aspartate, 2 mM MgCl₂ and 0.025 ml cell extract (Tokushige, 1985). In the stoichiometry of aspartase, the assay mixture contained 30 mM KH₂PO₄, pH 8.8, instead of 100 mM Tris/HCl, pH 8.8, since Tris/HCl interfered with the determination of ammonium, although activity measured with phosphate buffer was 45% lower than with Tris/HCl buffer.

The assay for ammonium formation was performed in a 0.25 ml reaction mixture containing 30 mM KH₂PO₄, pH 8.8, 100 mM aspartate, 2 mM MgCl₂ and 0.05 ml cell extract.

Determination of aspartate aminotransferase activity. Cell extracts were prepared in extraction buffer (100 mM KH₂PO₄, pH 7.6) as described above. The homogenates were centrifuged for 2 min at room temperature in a microcentrifuge and dialysed overnight against extraction buffer plus 0.01 mM pyridoxal 5-phosphate at 4 °C. Oxaloacetate formation was determined spectrophotometrically as described elsewhere (Rej & Hørdér, 1987).

Determination of fumarase activity. Cell extracts were prepared in extraction buffer (100 mM KH₂PO₄, pH 7.6) as described above and the homogenates were centrifuged for 2 min at room temperature in a microcentrifuge. Fumarate formation was determined spectrophotometrically as described elsewhere (Stitt, 1984).

Determination of glutamine synthetase activity. Glutamine synthetase activity was assayed with cell extracts prepared by sonicating the cells in 10 mM imidazole hydrochloride and 0.5 mM EDTA (pH 7.0). It was measured by its synthetase activity as described by Bender *et al.* (1977).

Bacteroid isolation. Bacteroids from root nodules of *P. vulgaris* were isolated by utilizing self-generating Percoll gradients as described by Reibach *et al.* (1981). A crude extract was prepared by gently crushing 6 g nodules with a mortar and pestle in 15 ml grinding buffer (150 mM NaCl and 50 mM KH₂PO₄, pH 7.6). The crude homogenate was filtered through four layers of cheesecloth and the residue was washed with additional grinding buffer. The filtered crude extract was centrifuged for 5 min at 3000 r.p.m. (Beckman Instrument JA-20 fixed-angle rotor) at 4 °C, the supernatant was centrifuged again for 15 min at 15000 r.p.m. and the pellet was resuspended in 2 ml grinding buffer. A 50 ml polycarbonate centrifuge tube was filled with 24.5 ml Percoll concentrate, 3.5 ml 500 mM KH₂PO₄ plus 1.5 M NaCl, pH 7.6, and 7 ml H₂O, giving a final concentration of 70% Percoll. One millilitre of extract was layered on the top of the gradient. The tube was centrifuged for 50 min at 20000 r.p.m. The bacteroids were collected with a Pasteur pipette. Percoll was removed by diluting the fraction 1:10 with 150 mM NaCl and 50 mM KH₂PO₄, pH 7.6, and centrifuging for 20 min at 15000 r.p.m. The pellet was resuspended in 2 ml extraction buffer.

RESULTS AND DISCUSSION

Growth of *R. etli* on aspartate and asparagine as nitrogen and carbon source

R. etli grew very poorly on aspartate as nitrogen and carbon source, whereas on aspartate as nitrogen source and succinate as carbon source it grew as well as on ammonium plus succinate (Fig. 1). In contrast, on glutamate as nitrogen and carbon source, *R. etli* grew at half the rate observed with glutamate plus succinate. On asparagine as nitrogen and carbon source, *R. etli* grew almost as well as on asparagine plus succinate (Fig. 1). These results indicate that *R. etli* utilized aspartate, glutamate and asparagine as nitrogen sources, that aspartate is a poor carbon source and that asparagine is a good carbon source, in spite of the fact that in order to be utilized as carbon source, asparagine must first be degraded to aspartate.

Fig. 1. Growth curves of *R. etli* from cultures grown on: ammonium plus succinate (▼), peptone-yeast extract (Δ), asparagine plus succinate (▽), asparagine (●), aspartate plus succinate (■), aspartate (□), glutamate plus succinate (◇) and glutamate (▲).

Asparaginase

The assay conditions for measurement of asparaginase activity were optimized. It was shown that the amounts of aspartate (1.05 μmol) and ammonium (1.16 μmol) formed were similar.

Asparaginase activity was highest when *R. etli* was grown on asparagine as nitrogen and carbon source, and this activity decreased when *R. etli* was grown on asparagine plus another carbon source such as glycerol, glucose or succinate (Table 1). Asparaginase activity was similar when *R. etli* was grown on asparagine plus succinate in the absence or presence of ammonium, and low when bacteria were grown on a good inorganic nitrogen source such as ammonium or a poor nitrogen source such as nitrate. Asparaginase activity was low in cells grown on aspartate with or without succinate such as in ammonium plus succinate. When *R. etli* was grown on other amino acids such as glutamine, and glutamate with or without succinate, or on a rich PY medium, asparaginase activity was low (Table 1). When *R. etli* was grown on ammonium

plus succinate, asparaginase activity was similar during the different growth stages (data not shown). The amount of oxygen dissolved in the growth medium was found to be irrelevant to the asparaginase activity (Table 1).

This shows that asparaginase was: (a) positively regulated by its substrate asparagine as in *B. subtilis* (Sun & Setlow, 1991) and *V. proteus* (Sinha *et al.*, 1991), and (b) negatively regulated by the carbon source as shown by the reduction in the asparaginase activity when a carbon source such as glycerol, glucose or succinate was present in the growth medium in addition to asparagine. Similar findings have been reported for *E. coli* (Jennings & Beachman, 1993), *S. enterica* (Jennings *et al.*, 1993) and *Staph. aureus* (Rózalska & Mikucki, 1992).

R. etli asparaginase was not regulated by: (a) the amount of oxygen dissolved in the growth medium, in contrast to other bacteria such as *E. coli* (Jennings & Beachman, 1993), *S. enterica* (Jennings *et al.*, 1993) and *V. proteus* (Sinha *et al.*, 1991); (b) nitrogen catabolite repression, as shown by the inability of ammonium to reduce asparaginase activity and the low activity found when *R. etli* was grown on a poor nitrogen source (Table 1), in contrast with data for *B. subtilis* (Sun & Setlow, 1991), *Bacillus licheniformis* (Golden & Bernlohr, 1985) and *Saccharomyces cerevisiae* (Roon *et al.*, 1982). Our results suggest that *R. etli* asparaginase plays a catabolic role in the degradation of asparagine to carbon skeletons when this amino acid is present in the growth medium and may also play a role in maintaining the balance between asparagine and aspartate since some activity is present when *R. etli* is grown in medium without asparagine.

Aspartase

We found that magnesium was necessary in the assay mixture for optimal *R. etli* aspartase activity, giving a threefold stimulation. In the complete assay, the amounts of ammonium (1.14 μmol) and fumarate (1.07 μmol) formed were similar.

The influence of nutritional conditions on aspartase activity was examined. The highest aspartase activity was found when *R. etli* was grown on asparagine as nitrogen and carbon source, suggesting positive regulation as in *B. subtilis* (Sun & Setlow, 1991). This activity decreased when *R. etli* was grown on asparagine plus another carbon source such as glycerol, glucose or succinate (Table 1), suggesting negative regulation as in *E. coli* (Nishimura & Kisumi, 1984). Aspartase activity decreased when *R. etli* was grown on asparagine plus succinate in the presence of ammonium, suggesting repression by ammonium. Aspartase activity was low when bacteria were grown on a good inorganic nitrogen source, such as ammonium, or on a poor nitrogen source, such as nitrate. Aspartase activity was also low in cells grown on aspartate with or without succinate. When *R. etli* was grown on other amino acids such as glutamine, and glutamate with or without succinate, or on a rich PY medium, aspartase activity was low (Table 1). The amount of oxygen dissolved in the growth medium was found to be

Table 1. Asparaginase and aspartase activities of *R. etli* grown under different conditions

The activities were measured after 18 h growth. The numbers in parentheses represent the standard deviations.

Nitrogen/carbon source	Asparaginase		Aspartase	
	Specific activity [nmol ammonium min ⁻¹ (mg protein) ⁻¹]	Relative activity (%)	Specific activity [nmol fumarate min ⁻¹ (mg protein) ⁻¹]	Relative activity (%)
Asparagine	34.6 (6)	100	390.3 (50)	100
Asparagine/glycerol	14.0 (2)	40.5	32.3 (6)	8.7
Asparagine/glucose	12.2 (2)	35.3	26.8 (4)	6.9
Asparagine/succinate	16.0 (3)	46.2	261.0 (30)	60.4
Asparagine/ammonium/succinate	13.7 (3)	39.6	92.4 (15)	23.7
Ammonium/glycerol	3.7 (1)	10.7	8.4 (2)	2.15
Ammonium/glucose	6.0 (1)	17.3	1.5 (1)	0.4
Ammonium/succinate	4.5 (1)	13.0	16.2 (3)	4.15
Nitrate/succinate	5.5 (1)	15.9	10.0 (2)	2.6
Aspartate/succinate	4.1 (1)	11.8	8.1 (2)	2.1
Glutamine/succinate	2.5 (1)	7.2	12.0 (2)	3.1
Glutamate/succinate	3.5 (1)	10.1	7.8 (2)	2.0
Aspartate	4.7 (1)	13.6	30.4 (7)	7.8
Glutamine	3.7 (1)	10.7	12.1 (2)	3.1
Glutamate	3.4 (1)	9.8	16.3 (3)	4.2
Peptone-yeast extract	2.9 (1)	8.4	3.2 (1)	0.8
Asparagine/succinate, low O ₂	15.3 (2)	44.2	230.5 (25)	59.1
Ammonium/succinate, low O ₂	4.7 (1)	13.6	15.2 (3)	3.9

irrelevant to the aspartase activity (Table 1), in contrast with other bacteria such as *E. coli*.

Aspartate aminotransferase, fumarase and glutamine synthetase

The activity of aspartate aminotransferase, an enzyme involved in aspartate catabolism, was determined in *R. etli* crude extracts. This enzyme catalyses the reversible conversion of aspartate plus 2-oxoglutarate to glutamate plus oxaloacetate. We found that the levels of the enzyme were similar [310 nmol min⁻¹ (mg protein)⁻¹] when *R. etli* was grown on ammonium plus succinate, PY medium and asparagine or aspartate with or without succinate.

The activity of the Krebs cycle enzyme fumarate hydratase (fumarase), which catalyses the reversible conversion of fumarate to malate, was determined. The levels of this enzyme were similar when *R. etli* was grown on asparagine or aspartate plus succinate [550 nmol min⁻¹ (mg protein)⁻¹]. This activity was slightly lower when *R. etli* was grown on asparagine or aspartate [390 nmol min⁻¹ (mg protein)⁻¹].

The activity of the ammonium assimilation enzyme glutamine synthetase, which catalyses the synthesis of glutamine from ammonium, ATP and glutamate, was also determined. The level of this enzyme when *R. etli* was grown on asparagine plus succinate was similar to that under conditions of ammonium plus succinate [22 nmol min⁻¹ (mg protein)⁻¹]. Under conditions of aspartate plus succinate, glutamine synthetase activity was threefold

higher than with ammonium plus succinate. The levels of this enzyme under conditions of asparagine and aspartate were similar (sevenfold lower than on ammonium plus succinate).

The fact that *R. etli* does not grow on aspartate as nitrogen and carbon source despite containing aspartase is explained by the very low aspartase activity found under these conditions. The poor growth on aspartate is not due to lack of fumarase or glutamine synthetase since these enzyme activities were similar when *R. etli* was grown on asparagine or aspartate as nitrogen and carbon source. The higher glutamine synthetase activity found when grown on aspartate plus succinate, in comparison with asparagine plus succinate, indicates that aspartate is a poor nitrogen source, since glutamine synthetase is induced under conditions of nitrogen limitation (Bravo & Mora, 1988). The poor growth on aspartate as nitrogen and carbon source indicates an incapacity of enzymes otherwise capable of degrading aspartate under these conditions, such as aspartate aminotransferase, the enzyme responsible for the growth of *R. meliloti* under the same conditions (Rastogi & Watson, 1991; Watson & Rastogi, 1993). The low aspartase activity found on glutamate as nitrogen and carbon source indicates that glutamate must be degraded to carbon skeletons by another enzyme. It is interesting that the physiological role of aspartase is not to enable *R. etli* to utilize aspartate or glutamate as carbon source. Instead, aspartase is induced by asparagine along with asparaginase to enable *R. etli* to utilize asparagine as carbon source. This contrasts with *B. subtilis*, where

aspartase is induced by aspartate and enables *B. subtilis* to grow on aspartate as nitrogen and carbon source (Iijima *et al.*, 1977; Sun & Setlow, 1991), and with *E. coli*, where aspartase participates in two metabolic processes: the regeneration of oxaloacetate as amino acceptor for enabling growth on glutamate, and the formation of fumarate and succinate during anaerobic growth on glucose (Marcus & Halpern, 1969; Courtright & Henning, 1970)

Asparaginase and aspartase activities of bacteroids from *R. etli*

The role of asparagine-degrading enzymes in bacteroids was analysed by isolating *R. etli* bacteroids in a self-generating Percoll gradient (Reibach *et al.*, 1981) and assessing the asparagine-degrading enzyme activities. The nodules were harvested 28 d post-inoculation. The asparaginase activity in bacteroids [$38 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$] was as high as the activity found when *R. etli* was grown on asparagine as nitrogen and carbon source (Table 1). The aspartase activity was also high in bacteroids [$164 \text{ nmol min}^{-1} (\text{mg protein})^{-1}$].

These observations may indicate roles for these two enzymes in the symbiosis between *R. etli* and *P. vulgaris*. The degradation of asparagine by asparaginase and aspartase in bacteroids may also be important in other species of *Rhizobium*, such as in *R. lupini*, where levels of these enzymes have been found to be elevated (Kretovich *et al.*, 1981).

ACKNOWLEDGEMENTS

We are grateful to O. Ladron de Guevara, L. García and P. Padilla for amino acid determinations and to Isabel Pérez Montfort for critical review of the manuscript. This work was supported by DGAPA Grant IN203193 from the Universidad Nacional Autónoma de México and by the Consejo Nacional de Ciencia y Tecnología.

REFERENCES

- Atkinson, M. & Fisher, S. F. (1991). Identification of genes and gene products whose expression is activated during nitrogen-limited growth in *Bacillus subtilis*. *J Bacteriol* **173**, 23–27.
- Bender, R. A., Janssen, K. S., Resnick, A. D., Blumenberg, M., Foor, F. & Magasanik, B. (1977). Biochemical parameters of glutamine synthetase from *Klebsiella aerogenes*. *J Bacteriol* **129**, 1001–1009.
- Beringer, J. E. (1974). R factor transfer in *Rhizobium leguminosarum*. *J Gen Microbiol* **84**, 188–198.
- Bravo, A. & Mora, J. (1988). Ammonium assimilation in *Rhizobium phaseoli* by the glutamine synthetase-glutamate synthase pathway. *J Bacteriol* **170**, 980–984.
- Calderón, J., Morett, E. & Mora, J. (1985). ω -Amidase pathway in the degradation of glutamine in *Neurospora crassa*. *J Bacteriol* **161**, 807–809.
- Cedar, H. & Schwartz, J. H. (1967). Localization of two L-asparaginases in anaerobically grown *Escherichia coli*. *J Biol Chem* **242**, 3753–3755.
- Chaney, A. L. & Marbach, E. P. (1962). Modified reagents for the determination of urea and ammonia. *Clin Chem* **8**, 130–132.
- Chesney, R. H. (1983). *E. coli* L-asparaginase II production in the presence and absence of catabolite activation protein. *FEMS Microbiol Lett* **17**, 161–162.
- Courtright, J. B. & Henning, U. (1970). Malate dehydrogenase mutants of *Escherichia coli* K-12. *J Bacteriol* **102**, 722–728.
- Durán, S. & Calderón, J. (1995). Role of the glutamine transaminase- ω -amidase pathway and glutaminase in glutamine degradation in *Rhizobium etli*. *Microbiology* **141**, 589–595.
- Gilbert, H. J., Blazek, R., Bullman, H. M. S. & Minton, N. P. (1986). Cloning and expression of the *Erwinia chrysanthemi* asparaginase gene in *Escherichia coli* and *Erwinia carotovora*. *J Gen Microbiol* **132**, 151–160.
- Golden, K. J. & Bernlohr, R. W. (1985). Nitrogen catabolite repression of the L-asparaginase of *Bacillus licheniformis*. *J Bacteriol* **164**, 938–940.
- Ho, P. P. K., Milikin, E. B., Bobbit, J. L., Grinnan, E. L., Burk, P. J., Frank, B. H., Boeck, L. D. & Squires, R. W. (1970). Crystalline L-asparaginase from *Escherichia coli* B. I. Purification and chemical characterization. *J Biol Chem* **245**, 3708–3715.
- Iijima, T., Diesterhaft, M. D. & Freese, E. (1977). Sodium effect of growth on aspartate and genetic analysis of a *Bacillus subtilis* mutant with high aspartase activity. *J Bacteriol* **129**, 1440–1447.
- Jennings, M. P. & Beachman, I. R. (1993). Co-dependent positive regulation of the *ansB* promoter of *Escherichia coli* by CRP and the FNR protein: a molecular analysis. *Mol Microbiol* **9**, 155–164.
- Jennings, M. P., Scott, S. P. & Beachman, I. R. (1993). Regulation of the *asnB* gene of *Salmonella enterica*. *Mol Microbiol* **9**, 165–172.
- Jerlström, P. G., Liui, J. & Beachman, I. R. (1987). Regulation of *Escherichia coli* L-asparaginase II and L-aspartase by the *fnr* gene product. *FEMS Microbiol Lett* **41**, 127–130.
- Jerlström, P. G., Bezak, D. A., Jennings, M. P. & Beachman, I. R. (1989). Structure and expression in *Escherichia coli* K-12 of the asparaginase I-encoding *ansA* gene, and its flanking regions. *Gene* **78**, 37–46.
- Kahn, M. L., Kraus, J. & Somerville, J. E. (1985). A model of nutrient exchange in the *Rhizobium*-legume symbiosis. In *Nitrogen Fixation Research Progress*, pp. 193–199. Edited by H. Evans, P. Bottomley & W. E. Newton. New York: M. J. Nijhoff.
- Karsten, W. E. & Viola, R. E. (1991). Kinetics studies of L-aspartase from *Escherichia coli*: pH-dependent activity changes. *Arch Biochem Biophys* **287**, 60–67.
- Kretovich, V. L., Sidel'nikova, L. I., Ivanushkin, A. G. & Karayakina, T. I. (1981). Localization of aspartase, asparaginase, and glutaminase in intact bacteroids of *Rhizobium lupini*. *Prikl Biokhim Mikrobiol* **20**, 445–447.
- Marcus, M. & Halpern, Y. S. (1969). The metabolic pathway of glutamate in *Escherichia coli* K-12. *Biochim Biophys Acta* **177**, 314–320.
- Miyamoto, K. & Katsuki, H. (1992). Possible physiological roles of aspartase, NAD- and NADP-requiring glutamate dehydrogenase of *Pseudomonas fluorescens*. *J Biochem* **112**, 52–56.
- Nishimura, N. & Kisumi, M. (1984). Aspartase-hyperproducing mutants of *Escherichia coli* B. *Appl Environ Microbiol* **48**, 1072–1075.
- Rastogi, V. K. & Watson, R. J. (1991). Aspartate aminotransferase activity is required for aspartate catabolism and symbiotic nitrogen fixation in *Rhizobium meliloti*. *J Bacteriol* **173**, 2879–2887.
- Reibach, P. H., Mask, P. L. & Streeter, J. G. (1981). A rapid one-step method for the isolation of bacteroids from root nodules of soybean

plants, utilizing self-generating Percoll gradients. *Can J Microbiol* **27**, 491–495.

Rej, R. & Hørdér, M. (1987). Aspartate aminotransferase. In *Methods of Enzymatic Analysis*, 3rd edn, vol. 3, pp. 416–444. Edited by H. U. Bergmeyer. Weinheim: VCH.

Roon, R. J., Murdoch, M., Kunze, B. & Dunlop, P. C. (1982). Derepression of asparaginase II during exponential growth of *Saccharomyces cerevisiae* on ammonium ion. *Arch Biochem Biophys* **219**, 101–109.

Rózalska, M. & Mikucki, J. (1992). Staphylococcal L-asparaginase: catabolic repression of synthesis. *Acta Microbiol Pol* **41**, 145–150.

Rudolph, F. B. & Fromm, H. J. (1971). The purification and properties of aspartase from *Escherichia coli*. *Arch Biochem Biophys* **147**, 92–98.

Russell, L. V. & Yamasaki, H. (1978). The dependence of *Escherichia coli* asparaginase II formation on cyclic AMP and cyclic AMP receptor protein. *Can J Microbiol* **24**, 629–631.

Segovia, L., Young, P. W. & Martínez-Romero, E. (1993). Reclassification of American *Rhizobium leguminosarum* biovar phaseoli type I as *Rhizobium etli* sp. nov. *Int J Syst Bacteriol* **43**, 374–377.

Sinha, A., Manna, S., Roy, S. K. & Chakrabarty, S. L. (1991). Induction of L-asparaginase in *Vibrio proteus*. *Indian J Med Res* **93**, 289–292.

Sobis, M. & Mikucki, J. (1991). Staphylococcal L-asparaginase: enzyme kinetics. *Acta Microbiol Pol* **40**, 143–152.

Stitt, M. (1984). Fumarase. In *Methods of Enzymatic Analysis*, 3rd edn, vol. 4, pp. 359–362. Edited by H. U. Bergmeyer. Weinheim: Verlag Chemie.

Sun, D. & Setlow, P. (1991). Cloning, nucleotide sequence, and expression of the *Bacillus subtilis* *ans* operon, which codes for L-asparaginase and L-aspartase. *J Bacteriol* **173**, 3831–3845.

Takagi, T. & Kisumi, M. (1985). Isolation of a versatile *Serratia marcescens* mutant as a host and molecular cloning of the aspartase gene. *J Bacteriol* **161**, 1–6.

Tokushige, M. (1985). Aspartate amino-lyase. *Methods Enzymol* **17**, 618–627.

Watson, R. J. & Rastogi, V. K. (1993). Cloning and nucleotide sequencing of *Rhizobium meliloti* aminotransferase genes: an aspartate aminotransferase required for symbiotic nitrogen fixation is atypical. *J Bacteriol* **175**, 1919–1928.

Willis, R. C. & Woolfolk, C. A. (1974). Asparagine utilization in *Escherichia coli*. *J Bacteriol* **118**, 231–241.

Received 25 October 1995; revised 19 December 1995; accepted 22 December 1995.