

Research Note

The *dnaJ* (*hsp40*) Locus in *Rhizobium leguminosarum* bv. *phaseoli* Is Required for the Establishment of an Effective Symbiosis with *Phaseolus vulgaris*

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P121R25 is a Tn5-induced mutant of the effective *Rhizobium leguminosarum* bv. *phaseoli* strain P121R that is unable to use glutamate as the sole carbon and nitrogen source and is defective in symbiotic nitrogen fixation. Enzymatic analysis showed that three enzymes implicated in glutamate metabolism (glutamate dehydrogenase, 2-oxoglutarate dehydrogenase, and glutamate synthase) were affected by this mutation. Sequencing of the chromosomal locus bordering the Tn5 in P121R25 indicated the presence of the *dnaK* and *dnaJ* genes in an arrangement similar to that described in *R. leguminosarum* bv. *viciae* (GenBank accession number Y14649). The mutation was located in the *dnaJ* (*hsp40*) gene.

Additional keywords: glutamate catabolism.

The symbiosis between the soil bacteria genus *Rhizobium* and legumes culminates in a new morphological structure, the nodule, where rhizobial bacteroids use plant photosynthates to fuel biological nitrogen fixation (Vance and Heichel 1991). It is generally accepted that C₄-dicarboxylic acids are the major carbon sources supplied to the bacteroids to support nitrogen fixation (Finan et al. 1983; Streeter 1987), an idea that is supported by the phenotypes of many mutants altered in dicarboxylic acid transport and utilization (Arwas et al. 1985; Lafontaine et al. 1989). The possibility that amino acids, specially glutamate, may be transported from the plant cytosol into the bacteroids to serve as carbon and energy sources for nitrogen fixation has been suggested (Donald and Ludwig 1984; O'Gara and Shanmugam 1976) and later developed into a model (Kahn et al. 1985). Much evidence suggests an important role of glutamate and other amino acids in the symbiosis. In fact, glutamate and related amino acids have been detected in high concentrations in cytosol and in *Bradyrhizobium ja-*

ponicum bacteroids (Streeter 1987). Furthermore, some mutants defective in the degradation of glutamate or aspartate as the sole carbon and nitrogen sources are defective in symbiotic nitrogen fixation (Fitzmaurice and O'Gara, 1993; Watson and Rastogi 1993; Labidi et al. 1996). Little is known about the genes implicated in glutamate catabolism in *R. leguminosarum* bv. *phaseoli* and their importance in symbiosis.

Strain P121R is a stable derivative of the very effective wild-type strain of *R. leguminosarum* bv. *phaseoli* P121, resistant to rifampicin at 50 µg ml⁻¹, and P121R25 is a glutamate catabolism, Tn5-induced mutant unable to use glutamate as the sole carbon and nitrogen sources (Table 1). To clone the wild-type locus, total DNA from mutant P121R25 was completely digested with *EcoRI* (which does not cut in the Tn5) and ligated into pUC19. Plasmids containing Tn5 with P121R25 DNA fragments were selected using ampicillin and kanamycin. The insert was digested with *EcoRI* and *XhoI* and three fragments (two of which contained Tn5-chromosomal DNA) were subcloned in pUC21 and sequenced using the T7 sequencing kit (Amersham Pharmacia Biotech, Uppsala, Sweden). The University of Wisconsin Genetics Computer Group Program (Madison, WI, U.S.A.) was used for analysis of nucleic acid data; the software packages Seqed, Find, Gap, Bestfit, Translate, Map, and Assemble were routinely used (Devereux et al. 1984). Three fragments of interest were isolated, two of which contained Tn5 and *Rhizobium* sequences and a third (fragment III) contained only a *Rhizobium* DNA (Fig. 1). Two sequences, pP121R25I and pP121R25II, encoded for Tn5 extremities starting at the *XhoI* site. Fragment I showed 93% homology with the *dnak* gene of *R. leguminosarum* (accession number Y14649, GenBank and EMBL). The Tn5 was inserted at nucleotide position 2,255 of the partial *dnaJ* zone (Fig. 2). The remaining 679 bp of fragment II showed 99.6% homology with *dnaJ* of locus Y14649. Fragment III showed 95% similarity to the *dnaK* of *R. leguminosarum*.

Total DNA from strain P121R was isolated as described by Laberge et al. (1989), partially digested with *Sau3A*, and puri-

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Nucleotide and/or amino acid sequence data have been deposited at the GenBank database as accession number Y14649.

fied on a sucrose gradient (Maniatis et al. 1982). Fragments of approximately 23 kb were ligated to *Bam*HI-digested cosmid pLAFR3 and packaged into Lambda phage heads (Promega 1991; Maniatis et al. 1982). Aliquots of the packaging mixture were used to infect *Escherichia coli* strain LE392. Tetracycline-resistant colonies were tested for the presence of inserts. DNA library amplification was performed as described by Stanley et al. (1987). Cosmids were transferred from strain LE392 to mutant P121R25 by triparental mating using pRK600 as a helper plasmid (Glazebrook and Walker 1991). The complemented mutants were screened on γ -aminobutyrate (GABA) as a sole carbon and nitrogen source supplemented with rifampicin (25 μ g/ml), kanamycin (50 μ g/ml), and tetracycline (10 μ g/ml). Cosmids were isolated from the complemented mutant, transformed into *E. coli* (DH5), reisolated, and digested with *Eco*RI. The inserts of cosmids complementing P121R25 were subcloned in pUC21 and their extremities sequenced according to Sanger et al. (1977). Six cosmids were able to complement P121R25 for its ability to use glutamate as sole carbon and ni-

trogen sources. Glutamate plays a central role in ammonia assimilation and amino acids biosynthesis; therefore, it is possible that the mutation could be complemented by many different loci on the chromosome. Therefore, we screened the cosmid bank to isolate cosmids that complement P121R25 for its ability to use GABA. This amino acid is catabolized via the glutamate catabolic pathways. Using GABA as a substrate, mutant P121R25 was complemented by only two cosmids. One complemented mutant, P121R25(pC11), had the same phenotype as the wild strain P121R grown on BM₁-GABA or glutamate medium (Labidi et al. 1996). The cosmid pC11, which complemented the P121R25 mutant for its ability to grow on GABA, was completely digested with *Eco*RI, and the resulting fragments were cloned into pUC21. The 210 bp of fragment number 6 (pC11.6) showed 97% homology with *dnaK* of locus Y14649.

A comparison of key enzyme activities on wild-type, mutant, and complemented strains was carried out. Cells at midexponential phase were collected by centrifugation (10 min at 10,000 \times g at 4°C), washed twice in a wash medium

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>Rhizobium leguminosarum</i> bv. <i>phaseoli</i>		
P121R	Wild-type parent; Fix ⁺ Rif ^r	Labidi et al. 1996
P121R25	P121R glu ⁻ mutant, symbiotically defective, unable to catabolize glutamate, contains Tn5; Fix ⁻ Km ^r Rif ^r	Labidi et al. 1996
<i>Escherichia coli</i>		
DH5 α	Host strain; endA1 hsdR17supE44 thi-1 recA1 gyrA96 relA1 (argF-lacZYA)U169 Φ 80dlacZ Δ M15	J. Jessee, Bethesda Laboratories
LE 392	SupF58	Promega
MT616	Carries helper plasmid, pRK600 (pRK2013::Tn 9, Cm ^r)	Finan et al. 1986
Plasmids		
pLAFR3	Cosmid cloning vehicle, Tc ^r	Friedman et al. 1982
pUC19; pUC21	Cloning vector, Ap ^r	Norrande et al. 1983
pP121R25Tn5	pUC19 carrying <i>Eco</i> RI P121R25 mutant DNA fragment containing the Tn5; Ap ^r , Km ^r	This study
pP121R25I	pUC21 carrying <i>Xho</i> I fragment I from P121R25 mutant DNA fragment containing the Tn5; Ap ^r	This study
pP121R25II	pUC21 carrying <i>Xho</i> I fragment II from P121R25 mutant DNA fragment containing the Tn5; Ap ^r	This study
pP121R25III	pUC21 carrying <i>Xho</i> I fragment III from P121R25 mutant DNA fragment containing the Tn5; Ap ^r	This study
pC11	pLAFR3 containing a <i>Sau</i> 3AI fragment(18 to 30 kb) from P121R DNA that complements the P121R25 Glu ⁻ phenotype	This study
pC11.6	pUC21 carrying <i>Eco</i> RI fragment of PC11	This study

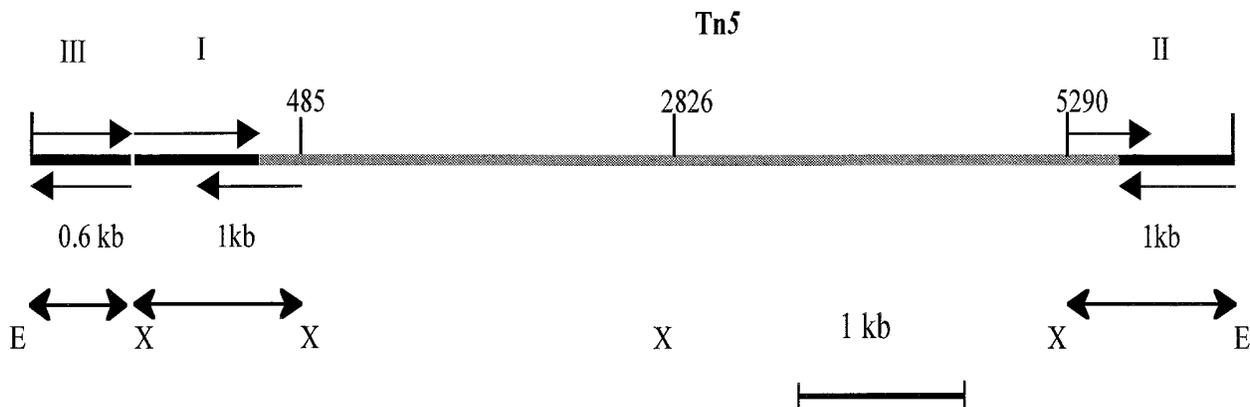


Fig. 1. Map of the Tn5 insertion region in mutant pP121R25 of *Rhizobium leguminosarum* bv. *phaseoli* showing the hybrid DNA fragments I and II and the bacterial DNA fragment III. The hatched line is the Tn5 and the normal line is the bacterial DNA. Arrows indicate the direction of sequencing. Numbers indicate the positions of restriction sites of *Xho*I on the Tn5. Restriction sites: E = *Eco*RI and X = *Xho*I.

(Salminen and Streeter 1990) containing 40 mM succinate and the following salts (grams per liter): $\text{CaCl}_2 \times 2\text{H}_2\text{O}$ (0.74), NaCl (1.0), $\text{MgSO}_4 \times 7\text{H}_2\text{O}$ (1.0), and K_2SO_4 (1.50), and concentrated 100-fold by suspension in this medium. The cell suspension was sonicated at 0°C as described by Salminen and Streeter (1990) with the following modification: cells were exposed to 8×20 s of sonication (pulse mode 50) with a 1-min interval, on ice. The cellular debris were centrifuged (10 min at $14,000 \times g$), and the supernatant was desalted on Bio-Rad 10 DG (desalting gel) columns (Bio-Rad Laboratories, Richmond, CA, U.S.A.) with 0.01 M sodium phosphate buffer (pH 7.5). The eluate was then used in enzyme assays and its protein content was determined according to Lowry et al. (1951). Total glutamine synthetase (GS, EC 2.6.1.2.) activity was determined by a semibiosynthetic assay as described by Fuchs and Keister (1980). Glutamate dehydrogenase (GDH, EC 1.4.1.3) and glutamate synthase (GOGAT, EC 2.6.1.53) activities were measured by following NADH oxidization (340 nm) at 30°C (Duke and Ham 1976). The 2-oxoglutarate dehydrogenase (OGDH, EC 1.2.4.2) assay was based on the method described by Salminen and Streeter (1990). Succinic semialdehyde dehydrogenase (SSDH, EC

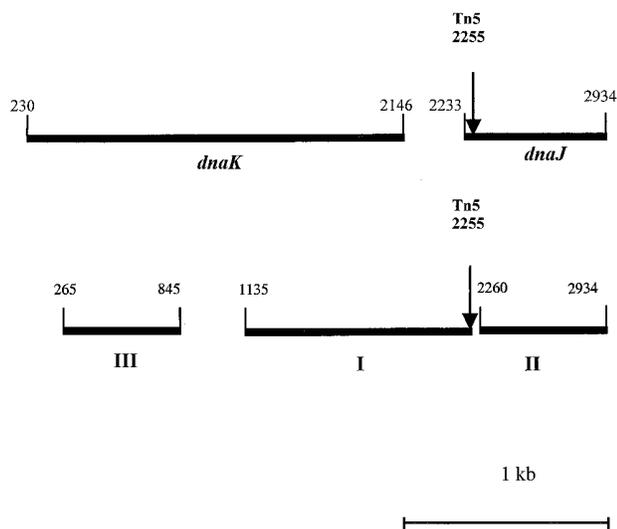


Fig. 2. Localization of fragments I, II, and III on locus Y14649 containing *dnaJ* and *dnaK* of *Rhizobium leguminosarum*.

Table 2. Activities of some enzymes involved in glutamate catabolism in *Rhizobium leguminosarum* bv. *phaseoli* strain P121R, its glutamate-negative mutant P121R25, and the complemented mutant P121R25(pC11)

Enzymes ^a	nmol per min per mg of protein ^b		
	P121R	P121R25	P121R25(pC11)
GDH	6.27 (0.4)	3.90 (0.5)	5.71 (0.5)
OGDH	10.24 (0.7)	4.06 (0.5)	9.87 (0.2)
GOGAT	5.45 (0.1)	2.41 (0.6)	4.74 (0.5)
GS	0.14 (0.01)	0.14 (0.01)	0.14 (0.002)
SSDH	8.45 (1.1)	6.98 (1.5)	9.46 (1.1)
GAAT	59.13 (7.0)	33.90 (1.5)	47.83 (3.1)

^a GDH = Glutamate dehydrogenase, OGDH = 2-oxoglutarate dehydrogenase, GOGAT = glutamate synthase, GS = glutamine synthetase, SSDH = succinic semialdehyde dehydrogenase, and GAAT = γ -aminobutyrate aminotransferase.

^b Values are the mean of three independent replications. Values in parentheses are the standard error.

1.2.1.16) activity was measured as described by Miller et al. (1991). Gamma-aminobutyrate aminotransferase (GAAT, EC 1.6.1.19) activity was determined with a colorimetric reaction according to Yonaha and Toyama (1980). Glutamate decarboxylase (GAD, EC 4.1.1.15) activity was measured by the method of Fonda (1985) as modified by Vézina et al. (1988). The bacteria used to measure GAD activity were grown in a minimal media containing L-glutamate as carbon source (Labidi et al. 1996) instead of proline because GAD was shown in *E. coli* to be induced by glutamate (Fonda 1985). Enzymatic analysis showed a significant reduction of the activities in the mutant P121R25 in comparison to the wild strain (Table 2). The observed decreases were 38% for GDH, 56% for GOGAT, and 60% for OGDH. These results show that the mutation affected the metabolism of glutamate and suggest that GDH and OGDH activities are probably involved in glutamate catabolism in *R. leguminosarum* bv. *phaseoli*. No GAD activity was detected in strain P121R, indicating that the GABA shunt is not a major pathway of glutamate catabolism as observed in *R. meliloti* (Fitzmaurice and O'Gara 1993). Salminen and Streeter (1990) also previously reported the absence of GAD in *B. japonicum*. Confirmation of the absence of GAD activity in strain P121R was also obtained by using different levels of pH buffer, varying from 4.0 to 7.5, as mentioned by Salminen and Streeter (1990) and Fonda (1985). The use of different concentrations (1 to 10^{-4} units) of GAD standards indicated that our test is sensitive to 10^{-2} units of enzyme. On the other hand, the two last enzymes in the GABA shunt (GABA aminotransferase and succinic semialdehyde dehydrogenase) have normal activities. This suggests that the GABA could be taken up from the outside by the free-living bacteria and from the nodular cytosol by the bacteroids. Kouchi and Fukai (1988) also suggested this possibility. In fact, many reports indicate the presence of high levels of GABA in the nodule cytosol (Miller et al. 1991; Streeter 1987; Ta et al. 1988; Vance and Heichel 1991).

The activity of three enzymes implicated in glutamate metabolism (GDH, OGDH, and GOGAT) returned to their wild-type levels in the Nod⁺ transconjugant P121R25(pC11), which contains the *dnaJ* (*hsp40*) gene. This observation is similar to the results obtained by Ogawa and Long (1995) on the *R. meliloti* mutant affected in the *groELc* gene coding for a heat shock protein 60 homolog (HSP60). In the same way, Krishnan and Pueppke (1991) have found an *R. fredii* mutant affected in the *nolC* gene unable to nodulate soybean. The *nolC* gene shares homology with a heat shock gene. Further work is required to determine how the two chaperones, HSP40 and HSP70, affect glutamate metabolism and symbiotic nitrogen fixation in *R. leguminosarum* bv. *phaseoli*.

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LITERATURE CITED

- Arwas, R., McKay, I. A., Rowney, F. R. P., Dilworth, M. J., and Glenn, A. R. 1985. Properties of organic acid utilization mutants of *Rhizobium leguminosarum* strain 300. *J. Gen. Microbiol.* 131:2056-2066.
- Devereux, J., Haerberli, P., and Smithies, O. 1984. A comprehensive set

- of sequence analysis programs for the VAX. *Nucleic Acid Res.* 12:387-395.
- Donald, R. G. K., and Ludwig, R. A. 1984. *Rhizobium* sp. strain ORS 571 ammonium assimilation and nitrogen fixation. *J. Bacteriol.* 158:1144-1151.
- Duke, S. H., and Ham, G. E. 1976. The effect of nitrogen addition on N₂-fixation and on glutamate dehydrogenase and glutamate synthase activities in nodules and roots of soybeans inoculated with various strains of *Rhizobium japonicum*. *Plant Cell Physiol.* 17:1037-1044.
- Finan, T. M., Kunkel, B., De Vos, G. F., and Signer, E. R. 1986. Second symbiotic megaplasmid in *Rhizobium meliloti* carrying exopolysaccharide and thiamine synthesis genes. *J. Bacteriol.* 167:66-72.
- Finan, T. M., Wood, J. M., and Jordan, D. C. 1983. Symbiotic properties of C₄-dicarboxylic acid transport mutants of *Rhizobium leguminosarum*. *J. Bacteriol.* 154:1403-1413.
- Fitzmaurice, A. M., and O'Gara, F. 1993. A *Rhizobium meliloti* mutant lacking a functional γ -aminobutyrate (GABA) bypass, is defective in glutamate catabolism and symbiotic nitrogen fixation. *FEMS Microbiol. Lett.* 109:195-202.
- Fonda, M. L. 1985. Glutamate decarboxylase from bacteria. *Methods Enzymol.* 113:11-16.
- Friedman, A. M., Long, S. R., Brown, S. E., Buikema, W. J., and Ausubel, F. M. 1982. Construction of a broad host range cosmid cloning vector and its use in the genetic analysis of *Rhizobium* mutants. *Gene* 18:289-296.
- Fuchs, R. L., and Keister, D. L. 1980. Identification of two glutamine synthetases in *Agrobacterium*. *J. Bacteriol.* 141:996-998.
- Glazebrook, J., and Walker, G. C. 1991. Genetic techniques in *Rhizobium meliloti*. *Methods Enzymol.* 204:398-418.
- Kahn, M. L., Kraus, J., and Sommerville, J. E. 1985. A model of nutrient exchange in the *Rhizobium*-legume symbiosis. Pages 193-199 in: *Nitrogen Fixation Research Progress*. H. J. Evans, P. J. Bottomley, and W. E. Newton, eds. Martinus Nijhoff, Dordrech, The Netherlands.
- Kouchi, H., and Fukai, K. 1988. Uptake and metabolism of aspartate and glutamate by soybean nodule bacteroids. Page 561 in: *Nitrogen Fixation: A Hundred Years After*. H. Bothe, F. J. de Bruijn, and W. E. Newton, eds. Gustav Fischer, Stuttgart, Germany.
- Krishnan, H. B., and Pueppke, S. G. 1991. *nolC*, a *Rhizobium fredii* gene involved in cultivar-specific nodulation of soybean, shares homology with heat-shock gene. *Mol. Microbiol.* 5:737-745.
- Laberge, S., Gagnon, Y., Bordeleau, L. M., and Lapointe, J. 1989. Cloning and sequencing of the *glT* gene, encoding the glutamyl-tRNA synthetase of *Rhizobium meliloti* A2. *J. Bacteriol.* 171:3926-3932.
- Labidi, M., Lalande, R., Laberge, S., and Antoun, H. 1996. Effect of glutamate transport and catabolism on symbiotic effectiveness in *Rhizobium leguminosarum* bv. *phaseoli*. *Plant Soil* 182:51-58.
- Lafontaine, P. J., Lafrenière, C., and Antoun, H. 1989. Some properties of carbohydrate and C₄-dicarboxylic acid utilization negative mutants of *Rhizobium leguminosarum* bv. *phaseoli* strain P121. *Plant Soil* 120:195-201.
- Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Maniatis, T., Fritsch, E. F., and Sambrook, J. 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, U.S.A.
- Miller, R. W., McRae, D. G., and Joy, K. 1991. Glutamate and γ -aminobutyrate metabolism in isolated *Rhizobium meliloti* bacteroids. *Mol. Plant-Microbe Interact.* 4:37-45.
- Norrander, J., Kempe, T., and Messing, J. 1983. Construction of improved M13 vectors using oligonucleotide-directed mutagenesis. *Gene* 26:101-106.
- O'Gara, F., and Shanmugam, K. 1976. Regulation of nitrogen fixation by *Rhizobium* export of fixed nitrogen as NH₄⁺. *Biochim. Biophys. Acta* 437:313-321.
- Ogawa, J., and Long, S. R. 1995. The *Rhizobium meliloti groELc* locus is required for regulation of early *nod* genes by the transcription activator NodD. *Gene Dev.* 9:714-729.
- Promega, 1991. *Promega Protocols and Applications Guide*. 2nd ed. Promega Corp., Madison, WI, U.S.A.
- Salminen, S. O., and Streeter, J. G. 1990. Factors contributing to the accumulation of glutamate in *Bradyrhizobium japonicum* bacteroids under microaerobic conditions. *J. Gen. Microbiol.* 136:2119-2126.
- Sanger, F., Nicklen, S., and Coulson, A. R. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. U.S.A.* 74:5463-5467.
- Stanley, J., Dowling, D. N., Stucker, M., and Broughton, W. J. 1987. Screening cosmid libraries for chromosomal genes: An alternative interspecific hybridization method. *FEMS Microbiol. Lett.* 48:25-30.
- Streeter, J. G. 1987. Carbohydrate, organic acid, and amino acid composition of bacteroids and cytosol from soybean nodules. *Plant Physiol.* 85:768-773.
- Ta, T. C., Macdowall, F. D. H., Faris, M. A., and Joy, K. W. 1988. Metabolism of nitrogen fixed by root nodules of alfalfa (*Medicago sativa* L.): I. Utilization of [¹⁴C,¹⁵N] glutamate and [¹⁴C,¹⁵N] glutamine in the synthesis of γ -aminobutyrate. *Biochem. Cell. Biol.* 66:1342-1348.
- Vance, C. P., and Heichel, G. H. 1991. Carbon in nitrogen fixation: Limitation or exquisite adaptation. *Annu. Rev. Plant Physiol.* 42:373-392.
- Vézina, L. P., Margolis, H. P., McAfee, B. J., and Delaney, S. 1988. Changes in the activity of enzymes involved with primary nitrogen metabolism due to ectomycorrhizal symbiosis on jack pine seedlings. *Physiol. Plant.* 75:55-62.
- Watson, R. J., and 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.
- Yonaha, K., and Toyama, S. 1980. γ -Aminobutyrate: γ -Ketoglutarate aminotransferase from *Pseudomonas* sp. F.126: Purification, crystallisation, and enzymologic properties. *Arch. Biochem. Biophys.* 200:156-164.