Journal of Applied Bacteriology 1991, **70**, 66–70

Ammonium and glutamate assimilation by tryptophan-catabolic variants of *Bradyrhizobium japonicum* USDA 26

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Accepted 28 May 1990

Paper number: 3304/02/90

T. KANESHIRO. 1991. Tryptophan-catabolic variants, tan 4b and 18ac, of *Bradyrhizobium japonicum* USDA 26 were isolated from enrichment cultures in nitrogen (N)-limited media containing either ammonium or glutamate. Presence of exogenous tryptophan (*trp*) in the medium led to sparse restricted growth and elicited selective growth of tan-coloured variants over that of parental USDA 26. A 36% increase in cellular uptake-accumulation of the ammonia analogue [^{14}C]-methylamine was found with *trp*-induced tan 18ac cells over that measured with either ammonium-induced tan 18ac or *trp*-induced tan 4b. The assimilation patterns of uniformly labelled [^{14}C]-glutamate also differed when tan 18ac was compared with tan 4b. These studies of tan variants isolated from enrichment cultures suggest that bradyrhizobial populations can be manipulated by changing the N sources that limit their growth.

INTRODUCTION

Soil conditions that lead to effective, competitive, and stable *Bradyrhizobium japonicum* inoculants are not known but could depend on the available nitrogen (N) sources. Kucey *et al.* (1989) conclude that nitrogenous fertilization of up to 50 kg/ha improves both vigorous seedling growth and subsequent utilization of fixed dinitrogen by soybeans. When soil lack indigenous *B. japonicum*, inoculant-strains without competitors produce nodulated soybeans in which symbiotic dinitrogen fixation correlates significantly with crop yield. Inoculants occupy fewer nodules and are not effective, however, when indigenous bradyrhizobia of the soil are more competitive and form more stable symbionts (Vest *et al.* 1973; Schmidt & Roberts 1985).

This work supports the generalization that N compounds of the soil are limiting factors for legume productivity (Postgate 1987) and, consequently, controlling factors that could affect stability and competitiveness of inoculants. For example, strains from different subspecies of *B. japonicum*, USDA 110 and 26 (Kaneshiro & Kurtzman 1982; Kaneshiro & Nicholson 1990), differed in their fixed N requirements for asymbiotic growth. Ammonium (NH_3) and glutamate (*glu*) served as differential N sources to separate the two subspecies. This difference suggested that the appropriate N form in a growth of soil environment may provide competitive advantage and stability to such strains. Tryptophan (*trp*)-catabolic variants of *B. japonicum* USDA 26 that accumulate indolyl-3-pyruvic (IPA) and indolyl-3-acetic acids (Kaneshiro *et al.* 1983) also grow selectively in the presence of exogenous *trp* as N source. An orange-tan pigmentation of colonies and enrichment cultures (tan), produced by spontaneous decomposition of IPA (Paris & Magasanik 1981), was a convenient marker for the detection of selective growth. For this report, a novel variant of USDA 26 that utilized *trp* as sole N source, tan 18ac, was isolated by *trp* enrichment culture, in which NH_3 was a limiting N source. Tan coloured colonies were then selected from dilution plates of nutrient agar containing *trp*.

Evidence is presented that the N source for bradyrhizobial growth can alter the uptake-assimilation pattern of N substrates. Since bradyrhizobia are slow-growing microbes with doubling times of 9 to 17 h, the uptake-assimilations of the NH_3 analogue [^{14}C]-methylamine (Kleiner 1981) and [^{14}C]-glu are conveniently followed within 1–3 h periods. Tan variants of *B. japonicum* USDA 26 (Kaneshiro & Nicholson 1990), belonging to subspecies II category (Hollis *et al.* 1981) isolated from *glu*-mediated (tan 4b) and NH_3-mediated (tan 18ac) enrichment cultures, are used to evaluate NH_3, *glu* and *trp*-induced cellular growth. The consequences of such induced growth are

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characterized by the uptake and assimilation of methylamine (MA) and *glu*.

MATERIALS AND METHODS

Strains and media

Bradyrhizobium japonicum strains USDA 26 (Beltsville Culture Collection, Beltsville, MD) and tan 4b, synonymous to NRRL L-259 and B-14075 respectively, were previously characterized by symbiosis and N-assimilation (Kaneshiro & Nicholson 1990). Strain tan 18ac was isolated from an NH_3-limited enrichment culture containing *trp* (0·3 g/l) and assigned the NRRL designation B-14371. The cultures were maintained on an agar medium containing yeast extract, mannitol, gluconate, and soil extract. Aerobic starter cultures were then grown in liquid medium containing (g/l): glutamic acid, 1; and yeast extract, 0·3, as N sources, which have been described earlier (Kaneshiro & Kurtzman 1982).

Induced growth by fixed nitrogen sources

Aerobic growth of strains was induced at 25°C by using either (g/l): L-glutamic acid, 1; NH_4Cl, 0·37; or L-*trp*, 0·3 as N source in a basal medium. The basal medium adjusted to pH 6·7 contained (g/l): potassium D-gluconate, 10; D-mannitol, 3; KH_2PO_4, 6; $MgSO_4 . 7H_2O$, 0·2; $CaCl_2 . 2H_2O$, 0·08; and a (Fe-Mo) solution containing 33·5 mg ferric citrate $. 5H_2O$ and 7 mg $Na_2MoO_4 . 2H_2O$. Where designated, α-ketoglutaric acid (1 g/l) and limiting N sources (g/l) were added to detect growth attributed to the added N nutrient: NH_4Cl, 0·08; and glutamic acid, 0·44. Turbidimetric growth in $Klett_{66}$ units ranged from 70 to 270, proportional to 0·1 and 0·7 mg cellular protein/ml, respectively (Kaneshiro & Nicholson 1990).

Uptake-accumulation of nitrogen sources

Limited aerobic growth (25°C for 4 d) induced by *glu*, NH_3 or *trp* ranged from 170–200 Klett units, equivalent to approximately 0·5 mg cellular protein/ml. These cultures were used without further pretreatment (Kleiner 1981) for uptake-accumulation experiments. Cellular uptake of uniformly labelled [^{14}C]-glu and [^{14}C]-MA containing the same specific activity ($1·4 \times 10^6$ cpm/μmol substrate in 10 ml cultures) was measured in 1 ml samples taken over a 3 h period. Cells collected on Millipore membrane filters were assayed by liquid scintillation counting to compare radioisotope retention. Radiolabelled MA accumulations measured NH_3 transport into cells (Kleiner 1981; Newton & Tyler 1989). Cellular uptake of [^{14}C]-trp was also determined in 10 ml cultures which contained $7·5 \times 10^5$ cpm/μmol *trp*. Appropriately induced cultures of USDA 26, tan 4b and tan 18ac were compared to determine N assimilation patterns.

Chemicals

Inorganic chemicals were reagent grade and were used without further purification. All organic chemicals were purchased from Sigma Chemical Co., St. Louis, MO. Both [2′-^{14}C]-L-tryptophan (49 μCi/μmol) and [^{14}C]-methylamine hydrochloride (54 μCi/μmol) were purchased from Research Products International Corp., Mt. Prospect, IL., and uniformly labelled [^{14}C]-glutamic acid (200 μCi/μmol) was obtained from International Chemical and Nuclear Division, Irvine, CA.

RESULTS

Enrichment with ammonium for tryptophan-catabolic variants

Tan 18ac was isolated after selective enrichment of a USDA 26 culture in medium containing combined NH_3 (30 mg/l NH_4Cl) and *trp* (0·3 g/l) as N sources. Earlier, tan 4b was isolated by a similar enrichment technique (Kaneshiro *et al.* 1983) as a tan-coloured colony on agar that contained the combined N-sources *glu* and *trp* (0·3 g/l each). Of 25 random tan colonies selected from the

Table 1 Fixed-nitrogen nutrients required for the aerobic growth* of *Bradyrhizobium japonicum* USDA 26 and its tryptophan-catabolic (tan) variants

	$Klett_{66}$ turbidity of strains		
Nitrogen† source added	*USDA 26*	*Tan 4b*	*Tan 18ac*
None	10	20	10
Ammonium chloride	140	160	150
Tryptophan	10	60	50
Combined ammonium-tryptophan	140	150	200
Glutamate	160	180	180

* Inoculum-cultures USDA 26 and tan 4b were induced by combining tryptophan (0·3 g/l) and ammonium chloride (80 mg/l) as N sources (See Kaneshiro *et al.* 1983). The novel tan 18ac culture was induced with trytophan as sole N source in the growth medium before inoculation. Inoculated cultures were then aerated on a rotary shaker for 4 or 5 d at 25°C.

† N source of a liquid basal medium supporting limited growth (mg/l): 1·5 mmol/l ammonium chloride, 80; 1 mmol/l L-tryptophan, 200; 3 mmol/l L-glutamic acid, 441; or combined ammonium-tryptophan (2·5 mmol/l total). All media except those containing glutamate also contained 3·3 mmol/l α-ketoglutarate as an additional C source.

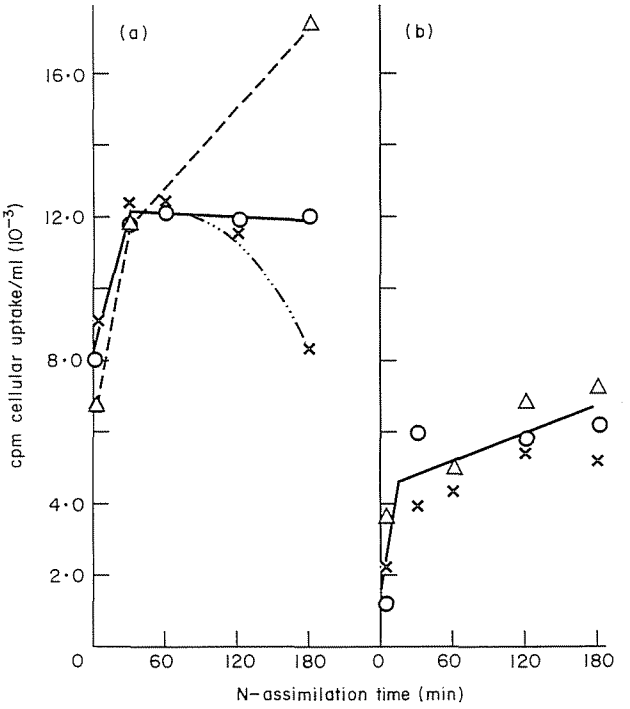

Fig. 1 Cellular ^{14}C uptake of glutamate (a) and methylamine (b) by glutamate-induced aerobic cultures of *Bradyrhizobium japonicum* USDA 26 (○), tan 4b variant (×), and tan 18ac variant (△). Uptake of nitrogenous substrates (mean ± range × 10^3 cpm/ml) calculated from sets of 2 to 17 measurements at 0, 0·5–2, 3 h to determine reproducibility of each curve. Glutamate uptake by USDA 26 and tan 4b (Curve a), 8·9 ± 1·4, 11·8 ± 2·9, 10·9 ± 2·5, respectively; and tan 18ac, 6·0 ± 0·7, 12·5 ± 0·7, 14·5 ± 2·9. Methylamine uptake by the three strains (Curve b), 1·9 ± 1·6, 4·2 ± 2·3, 6·2 ± 1·1.

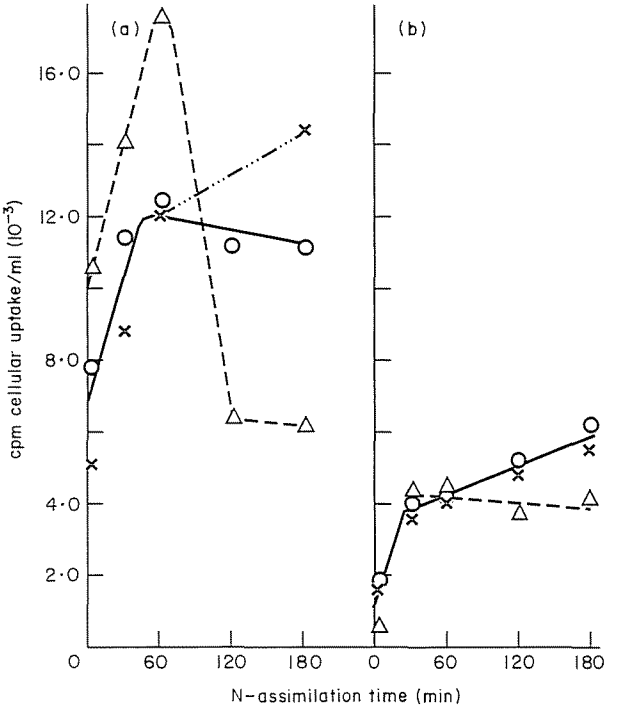

Fig. 2 Cellular ^{14}C uptake of glutamate (a) and methylamine (b) by ammonium-induced *Bradyrhizobium* cultures of USDA 26 (○), tan 4b (×), and tan 18ac (△). Reproducibility determined from uptake of substrates (mean ± range × 10^3 cpm/ml). Glutamate uptake (3 to 10 measurements at 0, 0·5–1, and 2–3 h) by USDA 26 and tan 4b (Curve a), 6·9 ± 1·8, 10·9 ± 2·9, 11·2 ± 3·2; and tan 18ac, 8·1 ± 2.6, 13·2 ± 4·4, 8·8 ± 4·0. Methylamine uptake (5 to 19 measurements at 0, 0·5–2, and 3 h) by the three strains (Curve b), 0·9 ± 0·9, 4·8 ± 2·4, 5·9 ± 2·3.

NH_3-enrichment medium, tan 18ac and five others appeared to grow better than *glu*-induced tan 4b when *trp* was the sole N source in a basal agar medium.

In liquid medium, both tan 18ac and tan 4b grew sparingly (Table 1) and elicited large amounts of coloured pigment, similar to that generated when unstable IPA is produced (Kaneshiro *et al.* 1983). Unlike parental USDA 26 cultures (Table 1), the tan variants appeared to assimilate the amino group of *trp* for moderate cellular growth. Consequently, a selective advantage of tan variants over USDA 26 was apparent when *trp* was the sole N source.

Glutamate and ammonium-induced growth

The uptake and assimilation patterns of *glu* and NH_3 were determined in order to differentiate tan 18ac and tan 4b (Figs 1–3). *Glu*-induced cells (Figs 1a and 1b) assimilated both *glu* and MA. *Glu* appeared to be the N source preferred by all three strains. However, tan 18ac accumulated more *glu* in cells than the others after incubation for 1 h. Uptake-assimilation of MA indicated that the three strains accumulated approximately 5·0 × 10^3 cpm or 3·6% of the MA within 1 h. In comparison, these *glu*-induced strains (Klett 200 or 0·45 mg cellular protein/ml) were capable of accumulating 8·6% of *glu* of the same specific activity (1 μmol and 1·4 × 10^6 cpm per 10 ml) as MA.

As with *glu*-induced cells, NH_3-induced cells (Fig. 2a and 2b) took up and accumulated most of their N sources within 1 h. Also, NH_3-induced tan 18ac appeared to utilize both the N and C of [^{14}C]-glu metabolically, as indicated by a sudden decrease of intracellular ^{14}C after incubation for 1 h. If the metabolic fate of *glu* carbons (>1 h incubations) is disregarded, it may be concluded that both *glu* and NH_3 elicited induced cells with similar uptake-accumulation patterns among the three strains.

Tryptophan-induced growth

Tan 18ac differed from tan 4b (Fig. 3 and Table 1) only when aerobic cellular growth was induced with *trp* (0·3 g/l) in the presence of limiting NH_3 (80 mg/l NH_4Cl). In short,

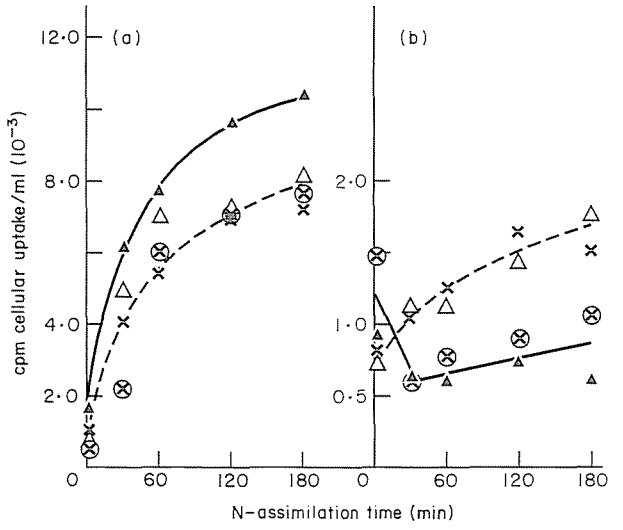

Fig. 3 Cellular ^{14}C uptake of methylamine (a) and L-tryptophan (b) after adding 200 mg/l tryptophan and 50 mg/l NH_4Cl as combined N sources to induce aerobic growth of *Bradyrhizobium japonicum* tan 4b (⊗) and tan 18ac (▲) at 25°C (Klett turbidity 170–200). As controls, ammonium-induced cultures devoid of exogenous tryptophan (×, △) were also measured. Uptake of substrates (mean ± range × 10^3 cpm/ml) calculated from sets of 2 to 12 measurements at 0, 0·5–2, and 3 h to determine reproducibility. Methylamine uptake by controls and tan 4b (Curve a), 0·8 ± 0·3, 5·5 ± 2·3, 7·5 ± 0·7; and tan 18ac, 1·3 ± 0·6, 7·4 ± 2·1, 10·4. Tryptophan uptake by controls (Curve b), 0·8 ± 0·1, 1·3 ± 0·3, 1·6 ± 0·1; and tan 4b and tan 18ac, 0·9 ± 0·6, 0·8 ± 0·5, 1·0 ± 0·4.

the quantity of MA accumulated by tan cells may differ slightly (Fig. 3a), but the general pattern of MA uptake-assimilation (Figs. 1b, 2b) is the same. The *trp*-induced tan 18ac cells accumulated 36% more [^{14}C]-MA at 1 h compared with either *trp*-induced tan 4b or NH_3-induced control cells. However, both tan 18ac and tan 4b cells appeared to process [^{14}C]-trp similarly (Fig. 3b), as indicated by the sudden decrease of ^{14}C radioactivity within 0·5 h. As evident from data in Table 1 and Fig. 3, limited additions of NH_3 to *trp* elicited additional growth of tan 18ac. Accordingly, NH_3 arising from deamination of *trp* appeared to be utilized for tan 18ac growth in addition to that engendered by NH_4Cl.

DISCUSSION

An enrichment medium may be considered to resemble a soil environment in that each offers an environmental niche which selects and controls the types of bacteria that grow best under the imposed conditions. Where fixed N compounds in soils are limiting factors for plant growth (Postgate 1987; Kucey *et at.* 1989), the limited source of N may also control bradyrhizobial populations by selection of specific types. For example, strains USDA 110 and 123 (Schmidt & Roberts 1985) of the category I subspecies (Hollis *et al.* 1981) may compete directly for *glu* as an organic N source for growth. By contrast, strains USDA 26 and its derived tan variants of the category II subspecies grow readily on either inorganic NH_3 or organic *glu* (Kaneshiro & Nicholson 1990) and can thus occupy a different niche.

Enrichment media with *glu* and NH_3 were used initially as differential N sources to establish discrete N dependency in order to isolate *trp*-catabolic variants 4b and 18ac, respectively. Generally, tan coloured variants were found to deaminate the amino group of *trp* to produce extracellular IPA (Kaneshiro *et al.* 1983). In addition, the tan variants utilized *trp* as N source only sparingly, thereby giving them a selective advantage over the parental type in enrichment cultures. When enrichment growth was mediated by NH_3 and *trp* in combination, however, such variants as tan 18ac were subsequently isolated on agar media that utilized *trp* as sole N source. These variants resembled *Klebsiella aerogenes* Tut^+ mutants that utilize *trp* as sole N source and α-ketoglutarate as a C source for growth (Paris & Magasanik 1981). However, *trp* as sole N source in a liquid basal medium (Table 1) elicited only restricted growth of the tan variants.

A previous description of *glu* and NH_3-induced cultures (Kaneshiro & Nicholson 1990) indicated that strains belonging to different *B. japonicum* subspecies, USDA 110 and 26, assimilated [^{14}C]-glu and [^{14}C]-MA consistent with their N requirements for growth. Ammonia-induced USDA 110 cultures did not grow on NH_3 as sole N source and did not accumulate MA. In the present work, *glu* and NH_3-induced tan variants as well as parental USDA 26 (Figs 1 and 2) all displayed similar uptake-accumulation of both *glu* and MA within 1 h. Beyond incubation for 1 h, tan 18ac was unlike tan 4b and USDA 26 by displaying a complex response, either assimilating (Fig. 1a) or catabolizing (Fig. 2a) the uniformly labelled [^{14}C]-glu. Although this later uptake after 1 h requires clarification, the results suggest that metabolism of *glu* might be regulated differently in tan 18ac and tan 4b cells.

Both *trp*-induced tan 4b and *trp*-induced tan 18ac displayed similar uptake-assimilations of N substrates. Evidently, the slight differences in 'NH_3' accumulations (Fig. 3a) were one of quantity rather than a reflection of the deamination pattern of *trp* (Fig. 3b). The difference elicited by *trp*-induced cultures could not explain the restricted growth with *trp* (Table 1) but may be related to the way NH_3 is transported into cells (Ludwig 1978; Kleiner 1981). Requirement of both *glu* and NH_3 for bradyrhizobial N metabolism has been described by Ludwig (1978, 1984) and O'Gara & Shanmugam (1976). The underlying mechanisms

are not apparent but our results suggest that the tan variants utilize N groups of NH_3, *glu* and *trp* through different regulated pathways. Accordingly, uptake-accumulation of N substrates may shift the bradyrhizobial (USDA 26) population depending on the available N source. The different uptake responses of the tan 4b and 18ac strains from enrichment cultures suggest the further possibility of such population shifts in the soil.

ACKNOWLEDGEMENTS

The author wishes to acknowledge helpful discussions with M.E. Slodki.

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