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Active Transport of Amino Acids in *Thiobacillus thioparus* Is a Low-Affinity Process

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A method for the isolation of amino acid auxotrophs of *Thiobacillus thioparus* is described. Characterization of a leucine auxotroph indicated that leucine biosynthesis in *T. thioparus* was not different from that of heterotrophic bacteria. *T. thioparus* cells accumulated amino acids via an active mechanism. K_t values of amino acid transport were between 15 and 330 μM, and V_{max} values were 200 to 350 pmol min^{-1} mg of $protein^{-1}$. Amino acid transport was carried out by a limited number of systems, each responsible for the uptake of several amino acids. Amino acid auxotrophs of *T. thioparus* exhibited transport and growth properties similar to those of transport-deficient mutants of heterotrophs which lost the high-affinity, but retained the low-affinity, amino acid transport systems.

Obligately chemolithotrophic thiobacilli respond with reluctance to the presence of organic material in that organic compounds fail to enhance the growth rate, and their contribution to growth extent is minor. One theory proposed to explain this phenomenon is that these organisms lack active transport mechanisms for organic compounds (51). However, it was shown in several cases that these bacteria take up organic material and incorporate it into various cell components (10, 21-23, 52, 53). Even at relatively high concentrations, organic matter does not contribute more than 10% of the newly synthesized cell carbon, provided that the organism is not limited by its specific inorganic energy source (36). Most studies of organic matter assimilation by obligately chemolithotrophic thiobacilli do not distinguish clearly between transport phenomena and the incorporation of organic matter into macromolecules (22-24, 52, 53), except in a single case where it was shown that membrane vesicles and whole cells of *Thiobacillus neapolitanus* carry out active transport of amino acids (37). It was demonstrated in this study that active transport systems for amino acids existed in another obligate chemolithotroph, *T. thioparus* ATCC 8158. We suggest that, in contrast to analogous systems in heterotrophs (e.g., *Escherichia coli*), amino acid transport in *T. thioparus* is a low-affinity process.

MATERIALS AND METHODS

Bacterial strains. All mutant strains are derivatives of *T. thioparus* ATCC 8158. Strain SN is resistant to streptomycin and nalidixic acid. Strains SNL, SNP, SNH, and SNT are, respectively, leucine, proline, histidine, and tryptophan auxotrophs derived from strain SN.

Media. The mineral medium designated ATM (autotrophic *Thiobacillus* medium) was a modification of the medium described by Brierley and Brierley (6) and contained the following salts dissolved in 100 mM potassium phosphate buffer (pH 7.1): 100 mM NaCl, 30 mM NH_4Cl, 1 mM $MgSO_4$, 50 μM $CaCl_2$, 10 μM H_3BO_3, 10 μM $FeSO_4$, 10 μM $MnCl_2$, 1 μM $ZnSO_4$, 1 μM $CoCl_2$, 1 μM $CuSO_4$, and 1 μM $NaMoO_4$. The energy source was $Na_2S_2O_3$ at 10 mM. Salt solutions were autoclaved, and thiosulfate was filter sterilized. We prepared ATM agar by making the above in 1.5% Difco agar, raising thiosulfate to 50 mM, and adjusting the pH to 6.8. We then added the appropriate amino acid (60 μg/ml) for the growth of auxotrophs.

Conditions of culture. Stocks were routinely transferred at 2- to 3-week intervals on ATM agar and were stored at 4°C. Single colonies were inoculated into ATM and shaken at 30°C until a titer of 1×10^7 to 3×10^7 colony-forming units (CFU) per ml was reached. Cultures were then diluted to 10^5 CFU/ml in ATM and shaken at 30°C. Such cultures were used in growth experiments. Fourteen-liter cultures were aerated by sparging sterile air at 30°C. Growth was monitored by direct microscopy, by the viable count technique, and by thiosulfate consumption. Cultures were routinely checked for contamination by heterotrophs by streaking nutrient agar plates.

Isolation of mutants. Antibiotic-resistant mutants were isolated by plating 10^9 CFU of ATCC 8158 onto ATM agar containing (in micrograms per milliliter): nalidixic acid, 20; streptomycin, 1; rifampin, 10; or kanamycin, 5. Antibiotic-resistant mutants appeared after 5 days of incubation at 30°C at frequencies of 5×10^{-9}, 1×10^{-9}, 50×10^{-9} and 1×10^{-9}, respectively. A streptomycin-resistant mutant was further selected from the nalidixic acid-resistant strain and was designated strain SN.

The mutagenesis procedure used here was a modification of the method of Adelberg et al. (1). About 5 $\times 10^9$ CFU in the exponential growth phase were concentrated in ATM without thiosulfate (pH 6.0) and incubated with 100 μg of 1-methyl-3-nitro-1-nitrosoguanidine per ml at 30°C for 15 min, washed in ATM, diluted 500-fold in ATM containing the appropriate supplements, and grown to a titer of 2×10^7 to 3×10^7 CFU/ml. Treatment with 1-methyl-3-nitro-1-nitrosoguanidine elevated antibiotic-resistant mutant frequencies by 160- to 2,000-fold.

Typical growth curves of strains ATCC 8158 and SN consist of an exponential phase and a death phase without a stationary phase. For an undetermined reason, 100 μg of uracil or 200 μg of thymidine per ml postpones death at the maximal growth extent by 40 to 50 h, thus creating a stationary-like phase in which nongrowing cells can survive exposure to penicillin. Thus, 100 μg of uracil per ml was incorporated in all media used for penicillin selections.

Mutagen-surviving cells were allowed to grow in ATM containing the desired amino acid (50 μg/ml) to 2×10^7 to 3×10^7 CFU/ml. The cells were washed and suspended in ATM without the requirement, were shaken for 1 h at 30°C, and were exposed to 2,000 U of penicillin G per ml for 12 h. The surviving fraction (1 to 10%) was washed in ATM, diluted 50-fold in ATM containing the requirement, and grown to 10^7 to 10^8 CFU/ml. Cultures were then diluted 50-fold in the same medium and grown to 2×10^7 CFU/ml before a second round of penicillin selection was applied. Leu$^-$, Trp$^-$, Pro$^-$, and His$^-$ auxotrophs appeared at frequencies of 0.5 to 5% after three or four rounds of selection, respectively.

Harvesting of cells and preparation of extracts. We harvested cultures of ≤1 liter by centrifugation. Larger cultures were harvested by filtration onto Whatman no. 1 paper impregnated with 0.3 to 0.5 g kieselguhr (E. Merck AG, Darmstadt, Germany) per cm^2. Elemental sulfur was separated by repeated centrifugation at 30 to 50 $\times$ *g* for 2 min. Cell pellets were quick-frozen at −70°C, mixed with alumina (Alcoa A-305, 2.5 g of alumina per pellet), and ground at 0°C with gradual addition of the appropriate buffer. DNase (25 μg/ml) was added, followed by incubation at room temperature for 20 min and centrifugation at 30,000 $\times$ *g* for 20 min at 4°C. The supernatant was frozen and stored at −70°C. Extracts for the measurement of α-isopropylmalate (α-IPM) synthetase, β-IPM isomerase, and β-IPM dehydrogenase were prepared in 50 mM potassium phosphate buffers at pH 8.5, 7.0, and 7.4, respectively.

Preparation of cell suspensions for the assay of amino acid uptake. Washed, sulfur-free cells were suspended in ATM without thiosulfate or supplements to a concentration of 0.1 to 1 mg of protein per ml and were starved by shaking at 30°C for 1 to 3 h. Protein synthesis was then arrested by exposure to 100 μg of rifampin per ml for 25 to 30 min. A sample was then made 20 mM in thiosulfate, and the rate of thiosulfate oxidation was determined.

Those suspensions which oxidized thiosulfate at a rate higher than 500 nmol min^{-1} mg of protein^{-1} were used in transport experiments. We assayed transport by adding thiosulfate to a concentration of 20 mM, shaking for 1 min, and adding labeled amino acids. Incubation was continued with shaking, and 0.5-ml samples were withdrawn every 60 s onto nitrocellulose membrane filters. They were quickly washed with 7.5 to 15 ml of ice-cold ATM, dried, and counted as described below.

Paper chromatography of hot-water extracts after amino acid uptake. Rifampin-treated cells were allowed to take up labeled amino acids for 5 min. The cells were washed three times in ATM, suspended in distilled water, incubated for 15 to 20 min at 100°C, and centrifuged. The supernatants were chromatographed on Whatman no. 3 paper in an *n*-propanol-water (7:3) solvent system. Chromatograms were stained with ninhydrin. Radioactive chromatograms were sliced and counted as described below.

Enzyme and other assays. Partial purification of α-IPM synthetase was carried out as described by Kohlhaw and Leary (28). α-IPM synthetase was assayed by the method of Kohlhaw and Leary (28). β-IPM isomerase was assayed by a modification of the method of Gross (12). Reaction mixtures contained 420 nmol of β-IPM and 100 to 500 μg of protein in 1.2 ml of 100 mM potassium phosphate buffer (pH 7.0). Incubation was at 27°C, and absorbance at 235 nm was continuously monitored against a reaction mixture without β-IPM. β-IPM dehydrogenase was assayed according to Parsons and Burns (39). Protein was determined according to Lowry et al. (32). Thiosulfate was determined as described by Roy and Trundinger (49). Protein synthesis was determined by incorporation of radioactively labeled amino acids into hot trichloroacetic acid-precipitable material. Radioactivity measurements were carried out in toluene containing 5 g of PPO (2,5-diphenyloxazole) and 0.3 of dimethyl POPOP [1,4-bis-(5-phenyloxazolyl)benzene] per liter in a Packard Tri-Carb 3380 scintillation spectrometer.

Chemicals. Streptomycin sulfate and penicillin G were from Teva Pharmaceutical Co., Israel. Rifampin was from Abic Ltd., Israel. Kanamycin, nalidixic acid, amino acids, purines, and pyrimidines were from Sigma Chemical Co., St. Louis, Mo. Dimethyl citraconate was from Eastman Kodak Co., Rochester, N.Y., and 1-methyl-3-nitro-1-nitrosoguanidine from Aldrich Chemical Co., Milwaukee, Wis. Dicyclohexylcarbodiimide (DCCD) and carbonyl cyanide *m*-chlorophenylhydrazone were from Calbiochem, La Jolla, Calif. All minerals were obtained from E. Merck AG. Radioactive chemicals were from the Radiochemical Centre, Amersham, England. β-IPM sample was kindly provided by J. Guardiola of the National Institute of Genetics, Naples, Italy.

RESULTS

Reversion frequencies of auxotrophs. Prototrophic revertant colonies of strains SNL, SNT, SNP, and SNH appeared on minimal ATM plates after 3 days of incubation at frequencies of 5×10^{-8}, 1×10^{-7}, 2×10^{-8} and 7×10^{-8}, respectively. This indicates that in each case auxotrophy was achieved by a single mutational event.

Characterization of the genetic lesion in strain SNL. Strain SNL grows at a normal rate

(0.32 generation per h) and to a normal extent (2×10^8 CFU/ml) in ATM containing 50 μg of leucine or 50 μg of α-ketoisocaproate per ml. Growth on 50 μg/ml of α-ketoisovalerate, dimethyl citraconate, or β-IPM was extremely slow (0.03 generation per h) and was comparable to that obtained with minimal ATM. Measurements of enzymes involved in leucine biosynthesis indicated that, except for β-IPM isomerase, mutant cells contained specific activities comparable to or higher than those found in the parent SN strain: α-IPM synthetase, 865 and 775 pmol min^{-1} mg of $protein^{-1}$; β-IPM dehydrogenase, 311 and 2,990 pmol min^{-1} mg of $protein^{-1}$; and β-IPM isomerase, 413 and 0.8 nmol min^{-1} mg of $protein^{-1}$ in strains SN and SNL, respectively. α-IPM synthetase activity was 49 and 76% inhibited by 0.1 and 0.5 mM leucine, respectively. These results show that the pathway of leucine synthesis in *T. thioparus* is identical to that found in many heterotrophs. Similar situations have been found for phenylalanine synthesis in *T. neapolitanus* (23, 25, 26) and for tryptophan synthesis in the obligate autotroph *Agmenellum quadruplicatum* (18).

Relationship between required concentration of amino acid and growth rate of auxotrophs. Except for strain SNL, all auxotrophs died quickly when deprived of their specific amino acid requirement under otherwise permissive growth conditions. A similar "tryptophanless death" phenomenon was reported for a Trp^- Arg^- mutant of *Bacillus subtilis* upon deprivation of tryptophan (35).

Similarly to strain SNL, all auxotrophs grew normally when provided with 50 μg of the required amino acids per ml. However, at intermediate concentrations, a dependence of the growth rate on the amino acid concentration was always observed (Fig. 1). In fact, the smallest concentration of required amino acid needed to support growth at an optimal rate was, depending on the strain, 15- to 30-fold higher than the one needed by that strain to fully satisfy its maximal demand for growth, as judged by the amounts of radioactive amino acids incorporated into protein during growth.

Characteristics of amino acid transport in strain SN. Treatment of SN cells with rifampin abolished incorporation of label into hot trichloroacetic acid-precipitable material, whereas uptake of amino acids was slightly affected. The absence of thiosulfate or the presence of cyanide, the uncoupler carbonyl cyanide *m*-chlorophenylhydrazone, or the ATPase inhibitor DCCD reduced transport (Fig. 2). In view of the low levels of uptake, it could be argued that the small amount of energy required for

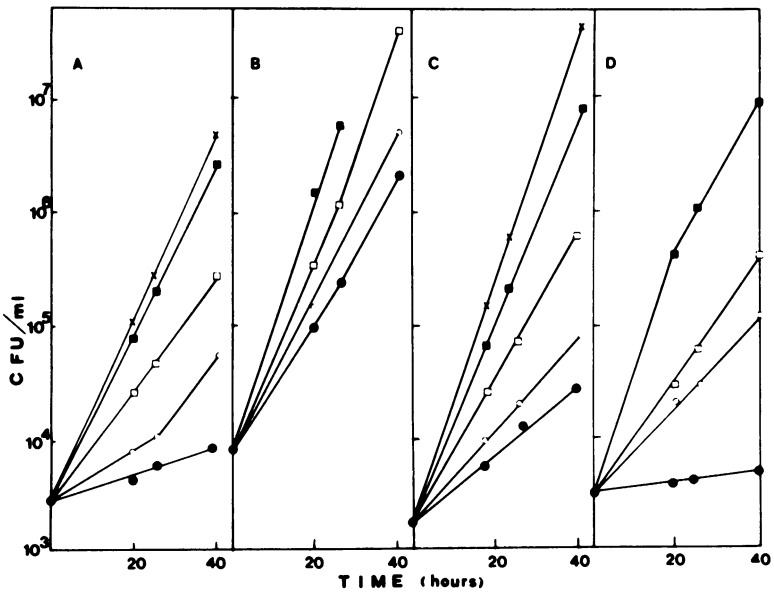

FIG. 1. *Dependence of growth rate on exogenous amino acid concentration during culture of auxotrophic strains of T. thioparus. The values shown pertain to liquid ATM cultures shaken at 30°C in the presence of the following micromolar amounts of the appropriate amino acid. A, strain SNP, proline:* (●) *8.7,* (○) *17.4,* (□) *34.8,* (■) *87, and* (×) *174. B, strain SNT, tryptophan:* (●) *4.9,* (○) *9.8;* (□) *24.5, and* (■) *49. C, strain SNL, leucine:* (●) *7.6;* (○) *15.5;* (□) *38.1,* (■) *76, and* (×) *152. D, strain SNH, histidine:* (●) *19.3;* (○) *51.5;* (□) *64.4, and* (■) *96.7.*

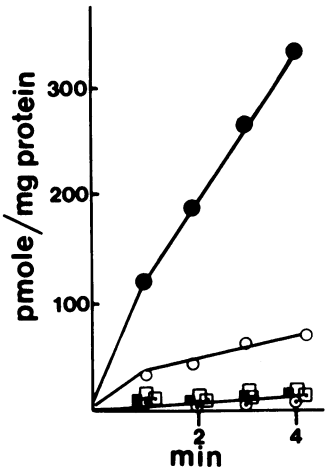

FIG. 2. *Energetics of leucine transport in strain SN. Reaction mixtures contained 780 μg of cell protein per ml and 33 μM [^{14}C]leucine (311 Ci/mol). (●) Addition of thiosulfate, (●) addition of thiosulfate and hot trichloroacetic acid-precipitable material; (○) uptake in the absence of thiosulfate, (■) addition of thiosulfate and 25 μM carbonyl cyanide m-chlorophenylhydrazone (□) addition of thiosulfate and 2.3 mM DCCD, (⊡) addition of thiosulfate and 10 mM cyanide.*

such transport could possibly be supplied by limited oxidation of organic matter which otherwise would be insufficient for growth or for detection. No uptake was observed after 30 min of incubation of the cells in the presence of 1 mg of glucose, ribose, galactose, mannose, α-ketoglutarate, fumarate, malate, succinate, oxaloacetate, glycerol, or β-hydroxybutyrate per ml (data not shown).

Leucine transport was found to be a low-affinity process. K_t was 500-fold higher and V_{max} was 500-fold lower than the values measured for leucine transport in *Escherichia coli* B under identical conditions. (K_t = 21 μM versus 38 nM and V_{max} = 175 pmol versus 50 nmol min^{-1} mg of protein^{-1} for *T. thioparus* and *E. coli*, respectively) (Fig. 3). The uptake of tryptophan, proline, or histidine was stimulated by thiosulfate and manifested kinetic parameters similar to those measured for leucine (Table 1). There is a possibility, however, that amino acid pools in the prototrophic strain SN cannot be controlled from without, and, thus, the kinetic parameters measured actually reflect the saturation of the internal cell volume by endogenously produced amino acids, preventing the entry of additional molecules from without. The use of amino acid auxotrophs of *T. thioparus* in which amino acid pools are determined by exogenously administered amino acids eliminated the problem of endogenous amino acid production.

Amino acid transport in auxotrophic strains. (i) Energetics of amino acid transport. To deplete amino acid pools in auxotrophic strains, mutant cells were starved for their appropriate requirement before exposure to labeled amino acids. Uptake experiments with such cell suspensions treated with rifampin showed that the transport of amino acids was dependent on temperature and that the transported amino acids were not incorporated into protein (Fig. 4).

Transport of all amino acids was abolished in the presence of cyanide or carbonyl cyanide *m-*

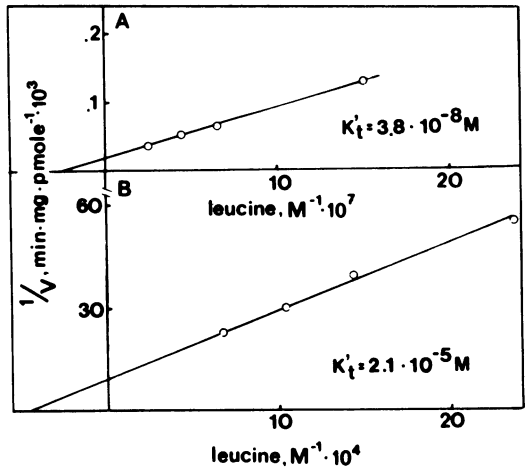

FIG. 3. *Apparent K'_t values for leucine transport in strain SN and E. coli B Leu$^-$. (A) E. coli B was grown in ATM containing 10 mM thiosulfate, 30 μg of leucine per ml, and 5 mg of glucose per ml, washed, and harvested as in the text. Reaction mixtures contained 10 μg of cell protein per ml, 10 mM thiosulfate, 5 mg of glucose per ml, 100 μg of rifampin per ml, and [^{14}C]leucine (20 to 500 nmol, 311 Ci/mol). (B) Leucine transport in strain SN. Mixtures contained 780 μg of cell protein per ml and [^{14}C]leucine (5 to 20 μM, 311 Ci/mol).*

TABLE 1. *Amino acid transport in strain SN*[a]

Amino acid	V_{max} (pmol min^{-1} mg of protein^{-1})	K_t' (10^{-5} M)	Uptake in presence of $S_2O_3^{2-}$ [b]
Leucine	175	2.1	0.21
Tryptophan	200	1.5	0.40
Proline	500	3.5	0.16
Histidine	350	1.5	0.43

[a] Reaction mixtures contained 890 μg of cell protein per ml, 20 mM thiosulfate (when added), and ^{14}C-labeled amino acids: leucine (6 to 200 μM), tryptophan (10 to 300 μM), histidine (10 to 300 μM), and proline (10 to 400 μM).

[b] pmol/mg of protein^{-1} 4 min^{-1}.

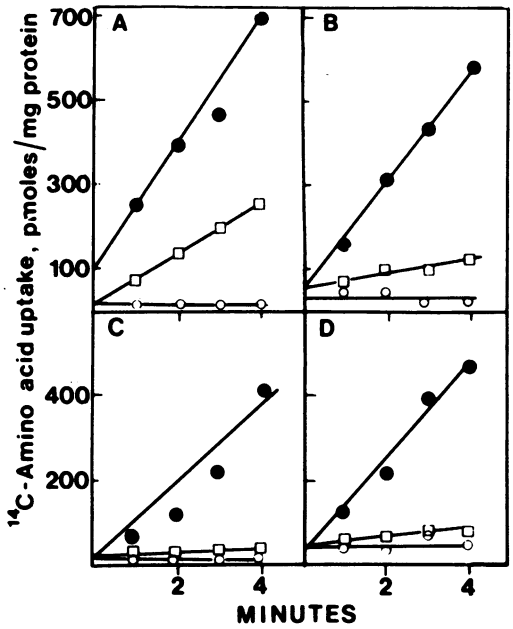

FIG. 4. *Effect of temperature on amino acid transport in auxotrophic strains of T. thioparus. Transport of 0°C was carried out after 15 min of preincubation at 0°C and samples were withdrawn when the cells were shaken at 0°C. Mixtures contained 1,000 μg of protein per ml. (●) Transport at 30°C, (□) transport at 0°C, (○) hot trichloroacetic acid-precipitable material at 30°C. (A) Leucine transport in strain SNL, [^{14}C]leucine at 20.7 μM, 290 Ci/mol; (B) tryptophan transport in strain SNT, [^{14}C]tryptophan at 24.2 μM, 54 Ci/mol; (C) proline transport in strain SNP, [^{14}C] proline at 20.8 μM, 270 Ci/mol; and (D) histidine transport in strain SNH, [^{14}C]histidine at 20.6 μM, 324 Ci/mol.*

chlorophenylhydrazone and was reduced in the absence of thiosulfate (Fig. 5). The ATPase inhibitor DCCD inhibited the transport of leucine and tryptophan but did not affect histidine transport and stimulated proline transport (Fig. 5). An accumulation of amino acids against concentration gradients was found in every case. Based on cell volume and specific radioactivity, internal amino acid concentrations were calculated to be 5- to 20-fold higher than the external concentration (data not shown). Chromatography of hot-water extracts of cell suspensions after uptake in the absence of protein synthesis indicated that, once taken up, amino acid remained inside the cells without further metabolism (Table 2).

(ii) Kinetics of amino acid transport. Kinetic parameters of amino acid transport in auxotrophic strains were found similar to those measured for the prototrophic strain SN (Table 3).

Since suspensions of amino acid auxotrophs starved for their specific amino acid requirement were used, it can be argued that, in this case, high internal pools of amino acids could not possibly affect transport rates, and it thus appears that low-affinity amino acid transport is a general property of wild-type *T. thioparus*.

Relationship between low-affinity transport and auxotrophic mutant growth rate. Optimal growth rates of amino acid auxotrophs of *T. thioparus* were obtained at amino acid concentrations that were 15- to 30-fold higher than the amounts sufficient to satisfy their biosynthetic requirement. At intermediate concentrations (in the range between biosynthetic and optimal growth demands), growth rates were

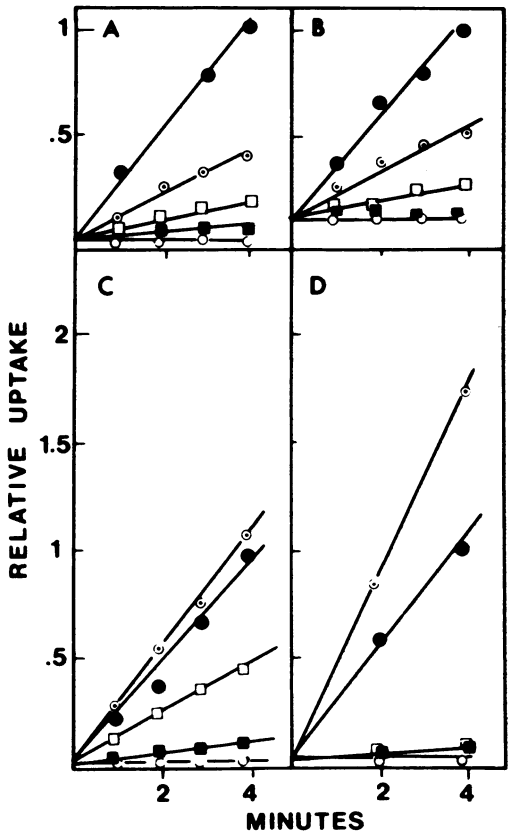

FIG. 5. *Energetics of amino acid transport in auxotrophs. (A) Leucine transport in SNL. 350 μg of cell protein per ml, 10.8 μM [^{14}C]leucine, 311 Ci/mol; (B) tryptophan transport in SNT, 1 mg of cell protein per ml, 18 μM [^{14}C]tryptophan, 54 Ci/mol; (C) histidine transort in SNH, 935 μg of cell protein per ml, 20 μM [^{14}C]histidine, 324 Ci/mol; (D) proline transport in SNP, 222 μg of cell protein per ml, 31 μM [^{14}C]proline, 270 Ci/mol. (○) 10 mM cyanide, (◎) 2.3 mM DCCD, (■) 25 μM carbonyl cyanide m-chlorophenylhydrazone, (□) without thiosulfate, and (●) with thiosulfate.*

TABLE 2. *Chromatography of amino acids extracted after transport in resting cell suspensions of T. thioparus auxotrophs*[a]

Amino acid	Transport in strain	Treatment[b]	R_f
[^{14}C]leucine		Standards	0.81
[^{14}C]leucine		Processed	0.82
[^{14}C]leucine	SNL	Extracted	0.85
[^{14}C]tryptophan		Standards	0.59
[^{14}C]tryptophan		Processed	0.58
[^{14}C]tryptophan	SNT	Extracted	0.57
[^{14}C]proline		Standards	0.44
[^{14}C]proline		Processed	0.43
[^{14}C]proline	SNP	Extracted	0.41
[^{14}C]histidine		Standards	0.17
[^{14}C]histidine		Processed	0.13
[^{14}C]histidine	SNH	Extracted	0.10

[a] Amino acid uptake, extractions, and chromatography were monitored according to the text.

[b] Standards, ^{14}C-amino standards; processed, ^{14}C-labeled amino acids diluted in ATM containing 20 mM thiosulfate, boiled for 15 min, and chromatographed to detect any nonspecific changes which might occur during boiling of amino acids with thiosulfate; and extracted, hot-water extracts of cell suspensions after transport.

proportional to the amino acid concentrations. To investigate the effect of other amino acids on mutant growth, threshold concentrations of the requirements were determined. Threshold concentrations were defined as the minimal amino acid concentrations which enabled mutant strains to grow at a rate of at least 0.3 generation per h. Thus, threshold concentrations for histidine, proline, leucine, and tryptophan were 123, 70, 150, and 11 μM, respectively.

Growth inhibition of mutant strains by competitor amino acids. Similarly to other thiobacilli (36), *T. thioparus* was inhibited by certain amino acids. The prototrophic strain was inhibited by 1 mM cysteine, methionine, phenylalanine, serine, or threonine (data not shown). Auxotrophic strains were found to be sensitive to the above mentioned amino acids and to some otherwise inert amino acids when grown at threshold concentrations of their specific amino acid requirements in the presence of a 10- to 20-fold excess of another amino acid (Fig. 6). Strain SNL was inhibited by alanine, glycine, isoleucine, tryptophan, and valine; strain SNT was inhibited by alanine, glycine, isoleucine, leucine, and valine; strain SNH was inhibited by alanine, glycine, isoleucine, and tryptophan (and to a lesser extent by arginine and proline); and strain SNP was inhibited by glutamate and valine. Inhibition of growth by competing amino acids depended on concentration and could be overcome by increasing the concentration of the required amino acids (data not shown).

These findings suggest that amino acids which are inhibitory for an amino acid auxotroph but not for the parent strain compete for the transport system specific for the required amino acids. A similar phenomenon has been shown in *Neurospora crassa* (34).

Specificity of amino acid transport systems. The above results suggested that several amino acid transport systems exist in *T. thioparus*; those inert amino acids which inhibited the growth of amino acid auxotrophs also inhibited uptake of the specific amino acid requirement (Table 4). Depression of the uptake of an amino acid by another amino acid was concentration dependent, and the kinetics were compatible with the model for competitive inhibition (Fig. 7).

There are, however, some discrepancies between uptake and growth data. Proline transport was not affected by glutamate, whereas growth was markedly depressed. In other cases, the degree of growth inhibition did not correlate with the degree of transport inhibition. In view of the hypothesis (36) that obligate chemolithotrophs are usually prone to organic matter imbalance, abnormal relative concentrations of amino acids might not affect growth in the same way that they affect transport.

It appears that amino acids were taken up by *T. thioparus* via a limited number of transport systems. One system was responsible for the uptake of leucine, isoleucine, valine, alanine, glycine, histidine, and tryptophan. Similar broad specificity systems exist in *T. neapolitanus* (19) and *Chlorobium* sp. (26). Proline was taken up by a different system, and histidine may be cotransported, in addition to the leucine system, via a second system specific for arginine.

TABLE 3. *Kinetic parameters of amino acid transport in auxotrophic strains of T. thioparus*

Strain	K_t' (10^{-5} M)	V_{max} (pmol min^{-1} mg of protein^{-1})
SNL[a]	1.6	200
SNT[b]	1.8	200
SNP[c]	5.9	250
SNH[d]	2.1	350

[a] Leucine transport: 350 μg of cell protein per ml and 6 to 200 μM leucine.

[b] Tryptophan transport: 1 mg of cell protein per ml and 10 to 300 μM tryptophan.

[c] Proline transport: 220 μg of cell protein per ml and 10 to 400 μM proline.

[d] Histidine transport: 260 μg of cell protein per ml and 10 to 300 μM histidine.

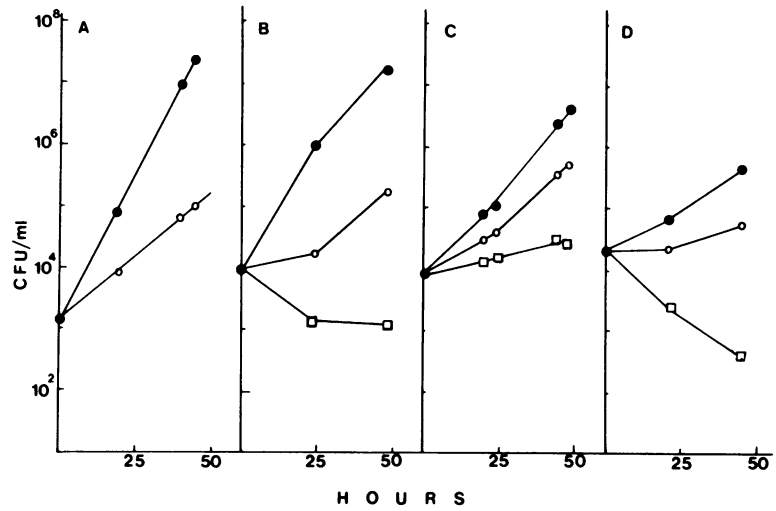

FIG. 6. *Inhibition of growth of amino acid auxotrophs by individual amino acids. Amino acid auxotrophs were grown at threshold concentrations of the requirement and at a 10- to 20-fold excess of other amino acids, applied individually. The general inhibitory amino acids (see text) were not included. (A) SNL grown at 200 μM leucine in the presence of* (○) *2 mM isoleucine, valine, alanine, glycine, or tryptophan or* (●) *2 mM of any other amino acid. (B) SNT grown at 10 μM tryptophan in the presence of* (○) *100 μM leucine, isoleucine, valine, or glycine,* (□) *2 mM alanine, or* (●) *2 mM of any other amino acid. (C) SNP grown at 120 μM proline in the presence of* (□) *1.2 mM valine,* (○) *1.2 mM glutamate, or* (●) *1.2 mM of any other amino acid. (D) SNH grown at 70 μM histidine in the presence of* (□) *1 mM leucine, isoleucine, valine, tryptophan, alanine, or glycine,* (○) *1 mM arginine or proline,* (●) *1 mM of any other amino acid.*

DISCUSSION

It appeared that physiological, rather than genetic, peculiarities of obligate chemo- or photolithotrophs were the limiting factors in the isolation of auxotrophs from these bacteria. It was shown here that spontaneous forward mutation or reversion frequencies and frequencies of mutations induced by 1-methyl-3-nitro-1-nitrosoguanidine were not in any way unusual, as shown previously for *A. quadruplicatum* (18).

The limited information on amino acid synthesis in chemo- or photolithotrophs suggests that these pathways are indistinguishable from those of organotrophic heterotrophs, e.g., phenylalanine synthesis in *T. neapolitanus* (22, 23, 26), tryptophan synthesis in *A. quadruplicatum* (18), and leucine synthesis in *T. thioparus* (this work). Regulation of biosynthesis at the enzyme level through feedback inhibition of the first specific reaction also seems to be similar to that of heterotrophs. However, it should not be concluded that chemolithotrophs such as *T. thioparus* are entirely comparable to other bacteria in terms of metabolic regulation patterns; unusual responses by these organisms to certain organic compounds are well-documented (19, 23, 36, 44, 51, 56). The response of *T. thioparus* is dependent on the growth rate of auxotrophs at limiting concentrations of their requirement. The same phenomenon is known in heterotrophs but at amino acid requirement concentrations several-hundred-fold below those encountered here. A similar response occurs in a tryptophan auxotroph of *A. quadruplicatum* (18) and in amino acid auxotrophs of heterotrophic bacteria which have lost their high-affinity transport system (33). Accordingly, the absence of high-affinity transport system for amino acids in *T. thioparus* is suggested.

Amino acids are taken up by *T. thioparus* via a mechanism(s) manifesting the properties of active transport: uptake is dependent on the presence of an energy source, greatly reduced at low temperature, and inhibited by respiration, coupling, and ATPase inhibitors. Amino acids accumulate against a concentration gradient and are not metabolized during transport in the absence of protein synthesis. Based on the present data, it is impossible to conclude whether ATP was actually involved in amino acid transport. The data suggest that leucine and tryptophan transport may depend on ATP. Proline transport stimulation by DCCD may be explained by two possible mechanisms. (i) The situation may be similar to that occurring in ATPase mutants of *E. coli* (5, 47), where it was suggested that ATPase plays a structural rather than a catalytic role in amino acid transport; DCCD may block the leak of protons and thus enhance membrane

TABLE 4. *Competitive amino acid uptake in T. thioparus*

Competitor amino acid	Relative uptake rate (%)				
	[^{14}C]Leu[a] (SN[f])	[^{14}C]Leu[b] (SNL[f])	[^{14}C]Trp[c] (SNT[f])	[^{14}C]Pro[d] (SNP[f])	[^{14}C]His[e] (SNH[f])
(−)	100	100	100	100	100
Ala	40	5	20	93	7
Arg	86	70	66	87	55
Asp	82	75	86	132	100
Glu	100	60	100	118	100
Gly	47	6	30	90	4
His	80	62	55	82	10
Ile	45	5	15	70	4
Leu	45	4	29	84	5
Lys	100	75	100	112	100
Pro	100	70	100	43	100
Trp	60	9	20	87	19
Tyr	100	60	64	88	86
Val	47	5	15	63	9
Cys	5	5	NT[g]	NT	NT
Met	NT	9	NT	97	12
Phe	NT	6	NT	87	5
Ser	NT	6	NT	100	10
Thr	NT	8	NT	82	13

[a] Reaction mixture contained: 780 μg of total SN cell protein per ml, 30 nmol of [^{14}C]leucine per ml, and 340 nmol of any other amino acid per ml.

[b] Reaction mixture contained: 570 μg of total SNL cell protein per ml, 1.5 nmol of [^{14}C]leucine per ml, and 7,700 nmol of any other amino acid per ml.

[c] Reaction mixture contained: 900 μg of total SNT cell protein per ml, 40 nmol of [^{14}C]tryptophan per ml, and 400 nmol of any other amino acid per ml.

[d] Reaction mixture contained 430 μg of total SNP cell protein per ml, 10 nmol of [^{3}H]proline per ml, and 2,000 nmol of any other amino acid per ml.

[e] Uptake assay mixture contained 260 μg of total SNH cell protein per ml, 63 nmol of [^{14}C]histidine per ml, and 630 nmol of any other amino acid per ml. SNH was grown at 50 μg of [^{14}C]histidine per ml and suspended as described in the text.

[f] Strain.

[g] NT, Not tested.

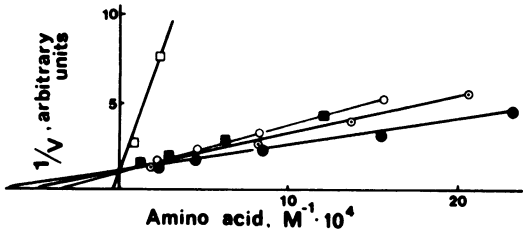

FIG. 7. *Competitive inhibition of leucine transport in SNL. Mixtures contained 350 μg of cell protein per ml, 1.7 μM [^{14}C]leucine, and 4 to 80 μM of competitor amino acids. Velocity is defined as the percentage of inhibition of [^{14}C]leucine uptake by competitor amino acids, where uptake with no competitor is 100%.* (●) *Leucine, apparent K_t = 20 μM,* (⊙) *valine, K'_t = 26 μM;* (○) *isoleucine, K'_t = 32 μM;* (■) *alanine, K'_t = 32 μM; and* (□) *glycine, K'_t = 330 μM.*

potential. (ii) DCCD could block a pathway (ATP synthesis) that competes with proline transport. We have no ready explanation for the resistance of histidine transport to DCCD. With regard to energetics, amino acid transport in *T. thioparus* is similar to that in membrane vesicles of *T. neapolitanus* (37) or in many heterotrophic bacteria.

The existence of a limited number of amino acid transport systems with broad specificities observed here is common among obligate chemolithotrophs (19, 26). The idea expressed by Matin (36), that the low specificity of transport systems in obligate chemolithotrophs may be an adaption to their unusual carbon metabolism and that the purpose of such transport is to minimize organic imbalances to which they are usually prone, is compatible with our findings.

The kinetic constants of amino acid transport observed in *T. thioparus* are different from those of heterotrophs. It is suggested here that, compared with heterotrophs, amino acid transport in *T. thioparus* is a low-affinity process manifested by high K_t values for transport and high K_i values for competition (which reflect high K_t values for the competing amino acids). V_{max} values were also lower than those measured in heterotrophs. Such low-affinity transport

seemed to be a property of wild-type *T. thioparus*, since four independently isolated auxotrophs manifested the same phenomenon both in growth and transport experiments.

Low-affinity amino acid transport is not rare among heterotrophs, such as *E. coli, Pseudomonas aeruginosa, P. putida, Salmonella typhimurium, Staphylococcus aureus*, and *Mycobacterium phlei* (13, 14, 16, 20, 29, 40, 50). However, in almost every case, these systems are accompanied by a battery of very low- and high-affinity systems (2–4, 7–9, 11, 13, 15, 20, 29, 31, 38, 40–43, 45, 46, 48, 54, 55). K_t values of transport in *T. thioparus* are 10-fold higher than the values observed in *T. neapolitanus* (37) but V_{max} values are comparable and are in the range of picomoles per minute per milligram of protein. It seems that, similarly to the phenomenon of broad specificity of amino acid transport, low-affinity transport systems for organic matter are more common among obligate chemo- or photolithotrophs than among heterotrophs. What is more, every auxotrophic mutant isolated so far from the former group of organisms manifests the properties of transport-deficient mutants of heterotrophs which have lost the high-affinity systems but retained the low-affinity ones (27, 33). Growth rates of four auxotrophs and the tryptophan-requiring mutant of *A. quadruplicatum* (17, 18) depend on exogenous concentration. The question whether low-affinity transport is a cause or a consequence of obligate chemolithotrophy cannot be answered yet, but the existence of such systems may contribute to the explanation of the poor response of these organisms to organic matter.

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