JOURNAL OF BACTERIOLOGY, Dec. 1970, p. 1145-1150
Vol. 104, No. 3

Printed in U.S.A.

Growth Inhibition in *Thiobacillus neapolitanus* by Histidine, Methionine, Phenylalanine, and Threonine[1]

CORINNE L. JOHNSON AND WOLF VISHNIAC
Department of Biology, University of Rochester, Rochester, New York 14627

Received for publication 29 June 1970

Thiobacillus neapolitanus, a strict chemoautotroph, is sensitive to the addition of 10^{-4} M methionine, histidine, threonine, or phenylalanine to the thiosulfate medium on which it grows. When histidine, threonine, or phenylalanine are added at the time of inoculation, spontaneous mutants tolerant to the three amino acids are selected. These mutants appear to result from a single genetic change; of 18 independently isolated histidine-tolerant mutants, all are also tolerant to phenylalanine and threonine. The uptake of ^{14}C-phenylalanine into exponentially growing cells of one such mutant is negligible in contrast with the uptake observed in the phenylalanine-sensitive parent. The addition of methionine to the medium slows growth, but spontaneous mutants are not selected. Inhibition of growth by these amino acids is observed only under conditions of amino acid imbalance; the addition of an equimolar mixture of 16 amino acids, in which each component is present at a concentration of 10^{-3} M, causes no inhibition. Histidine and threonine inhibition may be released by equimolar amounts of any one of seven amino acids: serine, alanine, glycine, leucine, valine, tryptophan, or tyrosine; histidine inhibition is also released by isoleucine, and threonine inhibition by methionine. None of the inhibiting amino acids inhibits oxidation of thiosulfate in cell suspensions. A group of hexoses, pentoses, and Krebs cycle intermediates were tested for inhibition of growth or release of inhibition by histidine, phenylalanine, or threonine, but no effects, either inhibition or relief of inhibition, were found.

The metabolism of autotrophic bacteria is under study in two areas: (i) description of oxidative pathways of inorganic substrates and (ii) exploration of intermediary metabolism and biosynthesis, with the aim of defining the unique features of autotrophy.

This paper reports growth inhibition by four amino acids in a strict autotroph, and relationships with other amino acids in the release of inhibition. These observations are similar to ones made in heterotrophs, for which elegant explanations have been proposed, involving the regulation of amino acid biosynthesis. The evidence suggests that these amino acid inhibitions may be related to those in heterotrophs, and also poses the question whether other amino acid inhibitions in autotrophs could be due to similar causes, or require other explanations.

[1] The data in this paper are taken from a thesis submitted by C.L.J. to the University of Rochester in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

MATERIALS AND METHODS

Maintenance of the organism. *Thiobacillus neapolitanus* (*Thiobacillus* X) was grown at 30 C in the medium described by Vishniac and Santer (13). Mutants were maintained in the presence of selecting amino acids at concentrations of 10^{-3} M to suppress the growth of revertants. Cultures were routinely checked for purity from heterotrophic contaminants by streaking on tryptic soy agar, and were purified by subculturing single colonies from thiosulfate agar when necessary.

Growth curves. Growth curves were recorded from 10-ml portions of culture taken from 300 ml of medium which had been inoculated with 1 to 3 ml of a fresh stock culture. They were grown in 300-ml Nephelo culture flasks (Bellco Glass Co., Vineland, N.J.), and optical density at 660 nm was measured by using a Spectronic 20 Colorimeter (Bausch & Lomb, Rochester, N.Y.). Small amounts of concentrated stock solutions of amino acids and other metabolites which had been sterilized by passage through membrane filters (pore size 0.45 μm; Millipore Corp., Bedford, Mass.) were added as indicated; each cul-

ture was set up in duplicate. Flasks were aerated by shaking on a rotary shaker at 300 rev/min.

The concentration of potassium phosphate buffer in the medium was raised to 0.132 M. Raising the phosphate concentration to this level in unsupplemented medium does not affect the length of the lag period or the early stages of exponential growth, but increases the yield of cells by allowing 80 to 90% of the thiosulfate in the medium to be oxidized before *p*H limitation ends the growth cycle.

Batch cultures. Batch cultures were grown either in 2-liter round-bottom flasks or in 1-liter gas washing cylinders. They were inoculated by diluting a stock culture 1:100, and were incubated initially for 4 to 5 hr without aeration. Forced aeration was then begun slowly (<1 culture volume per minute) and, as the culture became turbid with cells, was gradually increased to a maximum rate of 4 to 10 culture volumes per minute during late exponential growth. This procedure avoids the difficulty encountered with tetrathionate formation in cultures with small cell populations (14). Batch cultures were neutralized intermittently by adding sterile Na_2CO_3.

Measurements of oxygen uptake. Oxygen uptake by cell suspensions was measured at 30 C by manometry.

Measurements of radioactivity. Samples of ^{14}C-labeled cells and culture filtrates were counted in a liquid scintillation counter (model 722, Nuclear-Chicago Corp., Des Plaines, Ill.), and counting efficiency was assessed directly by the channels ratio method. Cells were separated from suspending media by filtering through membrane filters (pore size 0.45 μm). Filters were washed with 0.9% NaCl and distilled water, and dried at 80 C for 30 min; solvent and fluors were then added. Filtered cells were counted in 20 ml of toluene and 0.8 ml of a toluene solution of 100 g of PPO (2,5-diphenyloxazole) per liter and 1.25 g of POPOP [2-*p*-phenylene-bis(5-phenyl) oxazole] per liter. Aqueous culture filtrate samples were counted in 17 ml of toluene and 3 ml of methanol, with 0.8 ml of fluor solution.

Chemicals. Chemicals were of reagent grade and were obtained from commercial sources. L-Amino acids were used. Phenylalanine, uniformly labeled with ^{14}C, was obtained from New England Nuclear Corp., Boston, Mass.

RESULTS

Low concentrations of histidine, methionine, phenylalanine, and threonine, added at the time of inoculation, inhibit the growth of *T. neapolitanus* (Table 1). After 24 hr, when normal cultures reach the late exponential or early stationary phase of growth, inhibited cultures are not turbid and the *p*H of the medium is unchanged. Concentration dependence of histidine, phenylalanine, and threonine inhibitions is shown in Fig. 1. The inhibiting concentration of methionine is a function of the concentration of the potassium phosphate buffer in the medium. With 0.132 M buffer, 10^{-4} M methionine inhibits growth completely, but inhibition is less marked in the presence of 0.054 M buffer. None of these four amino acids inhibits growth when present at otherwise inhibitory concentrations as components of a balanced amino acid mixture; inhibition is much less marked when the inhibiting amino acids are added to exponentially growing cultures (Fig. 2).

In cultures which have been inhibited by histidine, phenylalanine, or threonine, the growth of stable variants, which appear to be mutants, occurs after several days. After this long lag period, populations, virtually every member of which is capable of growth in the presence of the inhibitory amino acid without delay, are selected. Many transfers through unsupplemented medium do not result in reestablishing a long lag period when cells are transferred again into medium with the inhibitory amino acids, and nearly the entire tolerant population is able to form colonies on plates with the inhibiting amino acids (Table 2). Since tolerance persists in the absence of the inhibiting amino acids and is a stable character, it has been considered due to spontaneous mutation. Commonly the mutants observed under these conditions are tolerant not only to the amino acid on which they are selected, but also to the other two of these three amino acids.

The examination of 18 mutants which arose independently in unsupplemented medium, and were then selected on plates for histidine tolerance, illustrates this point (Table 3) and suggests that a single genetic change may be responsible for the acquisition of tolerance to these three amino acids. These tolerant mutants may well be permeability mutants; examination of one selected on phenylalanine (and also tolerant to threonine and histidine) showed it to fail to take up or incorporate ^{14}C-phenylalanine significantly, compared with the sensitive parent, during two generations of exponential growth (Fig. 3).

Examination of suspensions of the parent organism for the effect of inhibiting amino acids on the rate of thiosulfate oxidation did not show significant inhibition.

A survey of amino acids which are able to release inhibition by histidine, phenylalanine, or threonine at equimolar concentrations showed phenylalanine inhibition to be released only by tyrosine, but histidine and threonine inhibition to be released by any one of seven amino acids (Table 4). Other metabolites, none of which causes growth inhibition, were also tested for ability to release histidine, phenylalanine, and threonine inhibitions, but none was active. Lactate, pyruvate, glucose, fructose, ribose, citrate, acetate, succinate, malate, glycerol, propionate, and glycolate were tested against histidine, phenylalanine, and threonine at concen-

trations of 3×10^{-4} M; the non-amino acid metabolites were tested for their ability to release amino acid inhibition at concentrations of 3×10^{-4} M and 3×10^{-3} M.

DISCUSSION

We have shown that the growth of *T. neapolitanus* is inhibited by several amino acids and that inhibition is not observed when these amino acids are present in a balanced mixture. This pattern of growth inhibition was first described for leucine, isoleucine, and valine in *Bacillus anthracis* by Gladstone (4), who suggested that the relationships between inhibiting amino acids not required for growth involved control of biosynthesis. The extensive literature on the control of amino acid biosynthesis needs no discussion here; it was recently reviewed by Umbarger (12).

It is easy, however, to overlook relationships between amino acids in experimenting with an autotroph, since no organic metabolite is required to be added to the growth medium. Rittenberg (11), in a review of carbon metabolism in autotrophs, suggested that amino acid imbalance might account for amino acid growth inhibitions, and Kelly (7–9) elucidated the roles of a group I amino acid permease and the enzyme DAHP synthetase (phospho-2-keto-3-deoxy-heptonate aldolase; EC 4.1.2.15) in phenylalanine inhibition of *T. neapolitanus* strain C. It is not known whether amino acid growth inhibitions in the nitrifying autotrophs *Nitrosomonas europaea* (2, 5) and *Nitrobacter agilis* (3) are due to amino acid imbalance.

Our data should be compared with those of

TABLE 1. *Amino acids tested for growth inhibition in T. neapolitanus*[a]

Addition to thiosulfate medium[b]	Growth at 24 hr
None	+
Alanine	+
Arginine	+
Aspartate	+
Cysteine	+
Glutamate	+
Glycine	+
Histidine	0
Isoleucine	+
Leucine	+
Lysine	+
Methionine[c]	0
Phenylalanine	0
Proline	+
Serine	+
Threonine	0
Tryptophan	+
Tyrosine	+
Valine	+
Complete mixture[d]	+

[a] Criteria for growth inhibition: no increase in turbidity, measured as optical density at 660 nm, and no change in *p*H of the medium detected by bromocresol purple indicator. Cultures scored + were turbid and had *p*H values less than 6.0.

[b] Amino acid concentrations were 3×10^{-4} M.

[c] In 0.132 M phosphate, methionine inhibits completely at 24 hr, but in 0.054 M phosphate, growth is observed before this time.

[d] Complete mixture contains individual components at concentrations of 3×10^{-4} M, except cysteine and tyrosine, which are at concentrations of 3×10^{-5} M.

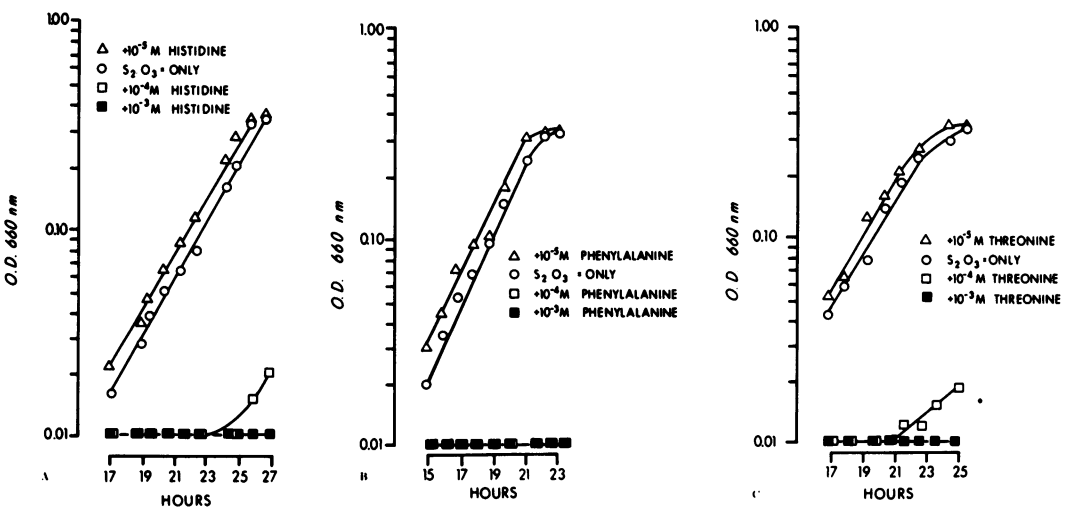

FIG. 1. *Growth inhibition by histidine, phenylalanine, and threonine. At time 0, a newly inoculated culture was divided into 10-ml portions, and each was delivered into a sterile Nephelo flask, to which the indicated concentrations of amino acids were added. Ordinate is a logarithmic scale of optical density.*

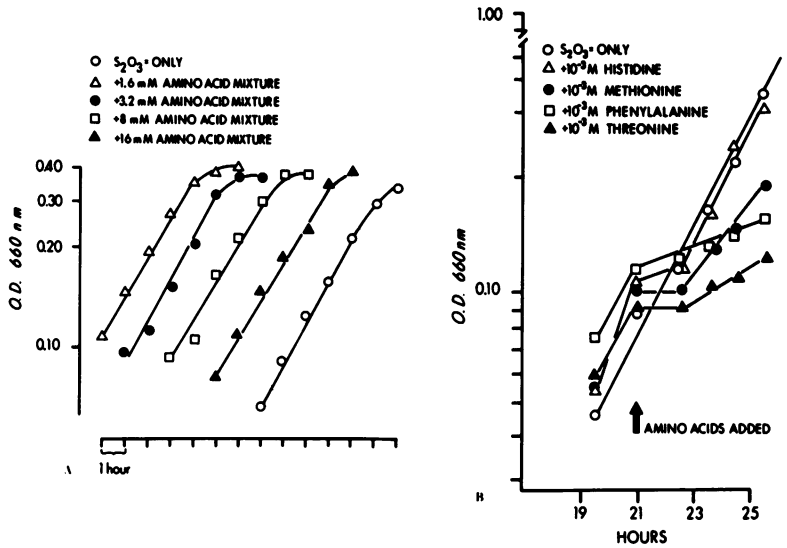

FIG. 2. *A, Growth in the presence of an equimolar mixture of 16 amino acids. At time 0, newly inoculated cultures received the amino acid mixtures in the concentrations indicated. All curves have the first point at 19 hrs; each curve after the one farthest left is displaced 1 hr from the preceding one. B, Growth, in the presence of inhibiting amino acids added to exponential-phase cultures.*

TABLE 2. *Ability of T. neapolitanus and mutants tolerant to histidine, threonine, or phenylalanine to grow on $S_2O_3^=$ with these amino acids*

Strain	Colonies per plate on[a]			
	$S_2O_3^=$	$S_2O_3^=$ + histidine	$S_2O_3^=$ + phenylalanine	$S_2O_3^=$ + threonine
Parent	264 (SD[e] 18)	18[b]	8[c]	160[d]
Histidine-tolerant	332 (SD 24)	287 (SD 34)	213 (SD 24)	213 (SD 14)
Phenylalanine-tolerant	465 (SD 38)	483 (SD 36)	436 (SD 43)	454 (SD 28)
Threonine-tolerant	389 (SD 56)	375 (SD 11)	357 (SD 30)	425 (SD 34)

[a] Cultures were plated as 10^5 dilution except where noted. Plates contained 3×10^{-4} M amino acids. Cultures were plated after three to nine sequential transfers through unsupplemented medium with inocula diluted 1:100 at each transfer.

[b] Plated as 10^3 dilution (frequency 6.8×10^{-5}).

[c] Plated as 10^3 dilution (frequency 3.0×10^{-5}).

[d] Plated as 10^3 dilution (frequency 6.1×10^{-3}).

[e] SD = standard deviation of count.

Kelly. We did not observe release of phenylalanine inhibition by tryptophan or by other group I amino acids, but the phenylalanine concentration was higher and amino acids were added to cultures with small inocula, which went through lag phase before growth. These conditions select tolerant mutants and are more drastic than the addition of amino acids to growing cultures. Our data on the uptake of ^{14}C-phenylalanine suggest that the tolerant mutant which we described may possess an altered permease for group I amino acids, and be resistant to phenylalanine, histidine, and threonine by simple exclusion from the cell.

This permease could account for the similarity of compounds releasing histidine and threonine inhibition, which with histidine, threonine, methionine, and phenylalanine comprise the group I amino acids. If release of inhibition acts by competition for the common permease, the intracellular sites for histidine and threonine inhibition could either be separate or the same. The latter would presumably involve cross-pathway control in amino acid biosynthesis, which has been

TABLE 3. *Independent histidine-tolerant mutants*[a]

Culture	Frequency of histidine-tolerant colonies	No. and character of colonies tested[b]			
		HPT	HT	HP	H
1		—[c]	—	—	—
2	1.57×10^{-6}	3	—	—	—
3	1.46×10^{-7}	1	—	—	—
4	8.63×10^{-7}	3	—	—	—
5	2.10×10^{-6}	2	1	—	—
6	4.70×10^{-6}	3	—	—	—
7	3.40×10^{-7}	2	—	—	—
8	5.93×10^{-6}	3	—	—	—
9	1.52×10^{-6}	3	—	—	—
10	2.12×10^{-6}	3	—	—	—
11	1.58×10^{-6}	3	—	—	—
12	1.55×10^{-6}	3	—	—	—
13	1.19×10^{-6}	3	—	—	—
14	1.10×10^{-6}	3	—	—	—
15	2.24×10^{-6}	2	—	—	—
16		—	—	—	—
17	1.02×10^{-6}	3	—	—	—
18	6.30×10^{-6}	1	1	—	1
19	1.76×10^{-6}	3	—	—	—
20	3.49×10^{-6}	3	—	—	—

[a] Inocula for cultures contained 10^8 cells. Frequency of histidine tolerance in original culture used for inocula was 1.1×10^{-6}. Amino acid concentrations in replica plates were 3×10^{-3} M.

[b] If possible, three colonies from each culture were tested for tolerance. HPT: Tolerant to histidine, phenylalanine, and threonine; HT: tolerant to histidine and threonine; HP: tolerant to histidine and phenylalanine; H: tolerant to histidine only.

[c] No colonies observed.

TABLE 4. *Amino acids affecting growth of T. neapolitanus*

Growth inhibited by	Inhibition released by
Histidine	Alanine Glycine Isoleucine Leucine Serine Tryptophan Tyrosine Valine
Threonine	Alanine Glycine Leucine Methionine Serine Tryptophan Tyrosine Valine
Phenylalanine	Tyrosine

demonstrated in *B. subtilis* (1, 10; Kane and Jensen, and Rebello and Jensen, Bacteriol. Proc., p. 123, 1969); it is of interest that Kelly observed release of phenylalanine inhibition of DAHP synthetase by histidine.

We think that the role of the inhibiting amino acids in *T. neapolitanus* is likely to be confined to biosynthesis, since several amino acids taken up during exponential growth were found incorporated into protein metabolically unaltered, or in structures of other amino acids for which they are precursors in synthetic pathways established in other organisms (6). Since we were unable to

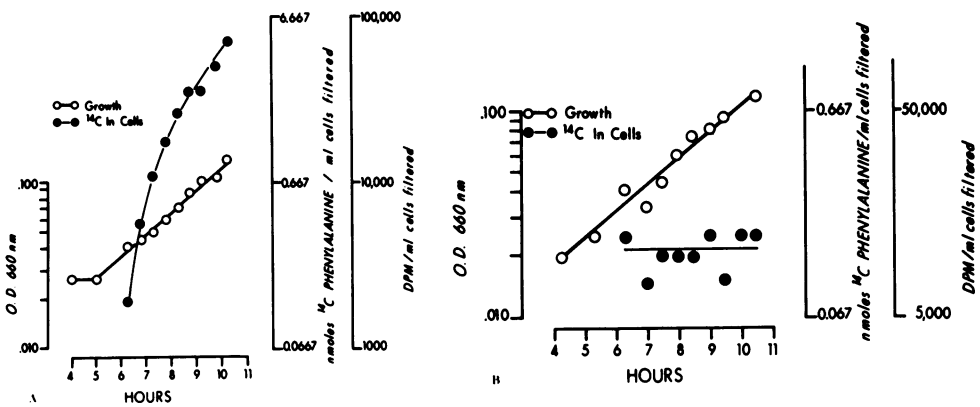

FIG. 3. *Uptake of L-phenylalanine-UL-^{14}C by exponentially growing wild-type T. neapolitanus (A) and by a histidine-phenylalanine-threonine-tolerant mutant (B). Cultures (15 ml) in Nephelo flasks received 2×10^{-5} M phenylalanine uniformly labeled with 2 μCi of ^{14}C (A) or 10 μCi of ^{14}C (B) at 6.25 and 6.5 hr, respectively. Growth was measured turbidimetrically; ^{14}C in filtered cells by liquid scintillation counting.*

observe inhibition of thiosulfate oxidation by whole cells in the presence of the inhibitory amino acids, we have no evidence which suggests oxidative metabolism as a direct site of inhibition. The ability to release amino acid growth inhibition was restricted to certain other amino acids, and did not extend to other metabolites tested.

Our experiments indicate a similarity between autotrophic and heterotrophic physiological regulation which has hitherto not received much attention. These observations reinforce the notion that the distinct features of autotrophic physiology lie in electron transport and energy metabolism, on the one hand, and CO_2 fixation on the other, and many other major features of metabolism are shared with heterotrophs.

ACKNOWLEDGMENTS

This investigation was supported by NSF grants GB-2758 and GB-8217 and by NASA grant NSG 209-62, supplements 6 and 7. Corinne Johnson was the recipient of a Public Health Service graduate traineeship.

LITERATURE CITED

1. Chapman, L. F., and E. W. Nester. 1968. Common element in the repression control of enzymes of histidine and aromatic amino acid biosynthesis in *Bacillus subtilis*, J. Bacteriol. **96**:1658–1663.
2. Clark, C., and E. L. Schmidt. 1967. Growth response of *Nitrosomonas europaea* to amino acids. J. Bacteriol. **93**: 1302–1308.
3. Delwiche, C. C., and M. S. Finstein. 1965. Carbon and energy sources for the nitrifying autotroph *Nitrobacter*. J. Bacteriol. **90**:102–107.
4. Gladstone, G. P. 1939. Interrelationships between amino acids in the nutrition of *B. anthracis*. Brit. J. Exp. Pathol. **20**:189–200.
5. Gundersen, K. 1955. Effects of B-vitamins and amino acids on nitrification. Physiol. Plant. **8**:136–141.
6. Kelly, D. P. 1967. Influence of amino acids and organic antimetabolites on growth and biosynthesis of the chemoautotroph *Thiobacillus neapolitanus* strain C. Arch. Mikrobiol. **56**:91–105.
7. Kelly, D. P. 1969. Regulation of chemoautotrophic metabolism. I. Toxicity of phenylalanine to *Thiobacilli*. Arch. Mikrobiol. **69**:330–342.
8. Kelly, D. P. 1969. Regulation of chemoautotrophic metabolism. II. Competition between amino acids for incorporation into *Thiobacillus*. Arch. Mikrobiol. **69**:343–359.
9. Kelly, D. P. 1969. Regulation of chemoautotrophic metabolism. III. DAHP synthetase in *Thiobacillus neapolitanus*. Arch. Mikrobiol. **69**:360–369.
10. Nester, E. W. 1968. Cross pathway regulation: effect of histidine on the synthesis and activity of enzymes of aromatic amino acid biosynthesis in *Bacillus subtilis*. J. Bacteriol. **96**:1649–1657.
11. Rittenberg, S. C. 1969. The roles of exogenous organic matter in the physiology of chemolithotropic bacteria. Adv. Microbial. Physiol. **3**:159–196.
12. Umbarger, H. E. 1969. Regulation of amino acid metabolism. Annu. Rev. Biochem. **38**:323–378.
13. Vishniac, W., and M. Santer. 1957. The thiobacilli. Bacteriol. Rev. **21**:195–213.
14. Vishniac, W., and P. A. Trudinger. 1962. Symposium on autotrophy. V. Carbon dioxide fixation and substrate oxidation in the chemosynthetic sulfur and hydrogen bacteria. Bacteriol. Rev. **26**:168–175.