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Selection and Characterization of Aspartokinase Feedback-Insensitive Mutants of *Azotobacter vinelandii*

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Aspartokinase feedback-insensitive mutants of *Azotobacter vinelandii* were selected as resistant to L-threonine, β-hydroxynorvaline, or *S*-(2-aminoethyl)-L-cysteine. L-Threonine-resistant strains were classified into three groups based on their ability to transport L-threonine and their growth response to *O*-methylthreonine and β-hydroxynorvaline. Most of the mutants were transport defective; however, some were desensitized to feedback regulation.

The aspartate family of amino acids includes L-threonine, L-methionine, L-lysine, and L-isoleucine, which are synthesized from the common precursor aspartate. The first reaction of the pathway is the phosphorylation of aspartate to produce β-aspartyl phosphate. In *Escherichia coli* and *Salmonella typhimurium* this reaction is catalyzed by three isofunctional aspartokinases (ATP:L-aspartate-4-phosphotransferase [EC 2.7.2.4]), the inhibition and repression of which respond to different end products (25). Two of these enzymes are bifunctional, having both aspartokinase and homoserine dehydrogenase activities (i.e., AKI-HDHI and AKII-HDHII). The third isofunctional enzyme, AKIII, has only aspartokinase activity. AKI-HDHI is encoded by the *thrA* gene, AKII-HDHII is encoded by the *metL* gene, and AKIII is encoded by the *lysC* gene. The structural genes of the three aspartokinases and of the two homoserine dehydrogenases of *E. coli* have been mapped, cloned, and sequenced, and details of their regulation have been resolved (2, 14, 25, 29).

In this report we show that *Azotobacter vinelandii* was inhibited by exogenous threonine and that mutants selected as resistant to threonine and the analogs *O*-methylthreonine (OMT), β-hydroxynorvaline (HNV), and *S*-(2-aminoethyl)-L-cysteine (AEC) were defective for threonine transport or processed a feedback-insensitive aspartokinase.

Wild-type *A. vinelandii* grew in Burk's sucrose nitrogen-free medium (24); however, growth of this azotroph was inhibited in medium containing concentrations of threonine as low as 0.5 mM (Fig. 1). Maximum inhibition of growth was observed at concentrations of L-threonine between 2 and 10 mM. Neither exogenous methionine nor lysine inhibited the growth of *A. vinelandii*. However, the addition of 0.2 mM methionine to growth medium containing threonine reversed threonine growth inhibition by as much as 80% (Fig. 1). Threonine growth inhibition was also reversed in the presence of 10 mM homoserine, cystathionine, and to some extent D-threonine. These results suggest that threonine limits methionine biosynthesis, which contributes to the growth inhibition of *A. vinelandii*. Exogenous threonine added to medium A does not inhibit growth of *E. coli* because of the independent end product control of its three isofunctional aspartokinases (10, 24).

Spontaneous threonine-resistant *A. vinelandii* mutants were isolated from Burk's sucrose plates containing 10 mM L-threonine and were characterized by their ability to transport threonine and to grow in the presence of the analogs OMT and HNV. In *E. coli*, OMT inhibits a number of metabolic functions and is an isosteric analog of isoleucine, a threonine analog with respect to transport, and also an inhibitor of threonine deaminase (21, 22). HNV is an isomer of OMT known to inhibit aspartokinase activity (22). Growth of strain AMOP was sensitive to concentrations of OMT and HNV as low as 1 μM (Table 1). Spontaneous threonine-resistant mutants were divided into three distinct classes. Class I includes strains AM4, AM03, AM08, AM9, AM11, AM15, AM016, AM20, and AM024, which were resistant to OMT and HNV. One possible explanation for the resistance to threonine in these mutants is their inability to transport exogenous threonine. In order to investigate this possibility, [^{14}C]threonine (specific activity of 50 μCi/mmol) uptake studies were performed as previously described by McKenny and Melton (9). All mutants in class I failed to transport threonine (Table 1). Strains AM188 and AM189, which were selected as spontaneously resistant to HNV, demonstrated cross-resistance to threonine and OMT (Table 1). However, strain AM188 was positive for transport of threonine and therefore was placed in class II. The third class of mutant, strain AM21, was resistant to threonine and positive for threonine transport but remained sensitive to OMT and HNV, as shown in Table 2.

Spontaneous AEC-resistant mutants of *A. vinelandii* were also selected. AEC, an antagonistic thioester analog of lysine, has been used to select mutants exhibiting aspartokinases desensitized to feedback regulation and capable of excreting lysine (8, 15, 18–20, 27, 28). Feedback-insensitive mutants have been isolated from a number of bacterial species: *E. coli*, *Bacillus subtilis*, *Bacillus licheniformis*, *Cornynebacterium glutamicum*, and a methylotrophic *Bacillus* sp. Aspartokinase activity in *A. vinelandii* AMOP was measured according to the procedure of Stadtman et al. (23) as modified by Robert-Gero et al. (17). The wild-type strain AMOP exhibited a level of aspartokinase of about 15 U/mg of protein which was inhibited by both threonine and lysine (Fig. 2). Aspartokinase activity in strain AMOP was more sensitive to lysine than to threonine. Greater than 85% inhibition of aspartokinase activity occurred in the presence of 10 mM lysine, compared to only 30% inhibition by the same concentration of threonine. Lysine at 20 mM inhibited aspartokinase activity more than 90%, while at the same concentration threonine inhibited the activity of the enzyme only about 60%. However, low concentrations of both L-lysine and L-threonine simultaneously elicited very strong concerted inhibition of aspartokinase (Fig. 2).

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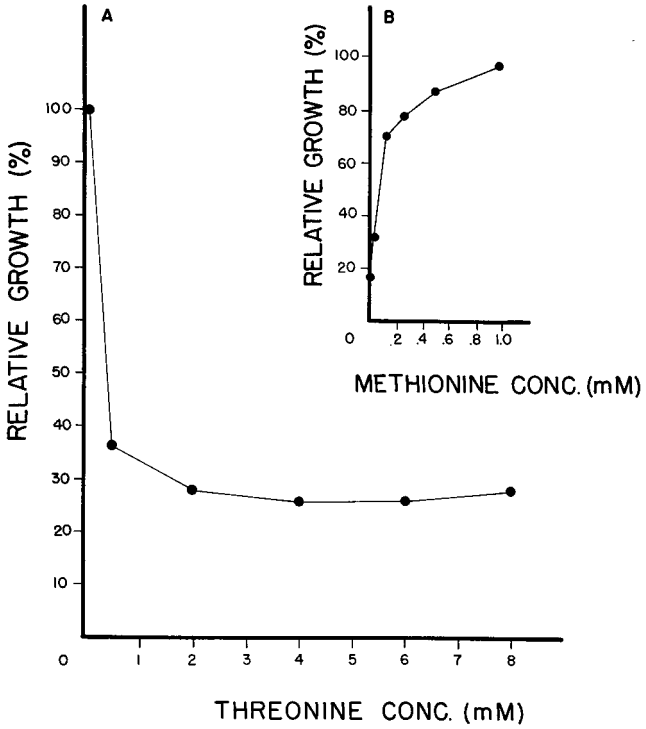

FIG. 1. Effects of various concentrations of threonine and methionine on growth inhibition and recovery of strain AMOP. Cells were cultured in 20 ml of Burk's sucrose medium overnight. The overnight culture was centrifuged, and the cell pellet was resuspended in 1 ml of Burk's buffer. Then 20 μl of these cells was used to inoculate tubes containing 10 ml of Burk's sucrose medium with various concentrations of threonine (A) or 2 mM threonine and various concentrations of methionine (B).

All of the AEC-resistant mutants were also resistant to threonine, OMT, and HNV (Table 2). The *A. vinelandii* AEC-resistant mutants were screened for aspartokinase activity (Table 2). Wild-type *A. vinelandii* exhibited an aspartokinase activity of 15.5 U/mg of protein, while aspartokinase activity in the AEC-resistant strains ranged from 12.9 to 20.4 U/mg of protein. All of the *A. vinelandii* AEC-resistant strains possessed aspartokinase activity which was desensitized to feedback inhibition by both threonine and lysine, as shown in Table 2. Aspartokinase activity of AEC-resistant mutants ranged

TABLE 1. Classes of *A. vinelandii* threonine-resistant mutants

Mutant class	Strain(s)	Threonine transport[a]	Growth on[b]:		
			Thr	OMT	HNV
	AMOP	+	−	−	−
I	AM4, AM03, AM08, AM9, AM11, AM189, AM15, AM016, AM20, AM024	−	+	+	+
II	AM06, AM010, AM188	+	+	+	+
III	AM21	+	+	−	−

[a] Strains were grown and transport of [^{14}C]threonine was measured as described in text. Symbols: +, positive for uptake; −, negative for uptake.

[b] Strains were pregrown in 5 ml of Burk's sucrose medium at 28°C overnight. Cells were streaked onto Burk's sucrose plates containing either 10 mM L-threonine, 1 mM HNV, or 1 mM OMT. Plates were incubated for 2 days at 28°C and scored for growth (+) or no growth (−).

TABLE 2. Inhibition of aspartokinase activity of different *Azotobacter* mutants by threonine and lysine

Strain	Aspartokinase activity (U/mg)[a]			% Inhibition	
	No addition	+Thr	+Lys	+Thr	+Lys
AMOP	15.5	3.4	3.1	78	80
AEC2	14.4	10.1	12.0	30	17
AEC3	20.4	16.7	10.2	18	50
AEC4	12.9	6.1	2.0	53	84
AEC8	16.5	7.8	12.6	53	24
AEC20	11.2	14.3	15.8	0	0
AEC18	13.5	5.0	9.5	63	30

[a] Cultures were grown and aspartokinase was assayed as described in Materials and Methods. Aspartokinase was assayed in the presence of 0.5 mM L-threonine or 2.5 mM L-lysine. One unit is defined as the amount of aspartokinase causing the formation of 1 nmol of β-aspartylhydroxymate per min.

from two- to about fivefold higher than that of the wild type in the presence of threonine. Similar results were observed for aspartokinase activity of AEC-resistant strains measured in the presence of lysine (Table 2). AEC-resistant strain AEC20 exhibited aspartokinase activity which was four to five times higher than that of the wild-type strain in the presence of either threonine or lysine. The aspartokinase activity of strain AEC4 was relatively resistant to inhibition by threonine but remained as sensitive as the wild-type with respect to lysine inhibition. These represent the first aspartokinase mutants of *A. vinelandii* to be selected and characterized. Petricek et al. (15) recently mapped two mutations, *aecA* and *aecB*, which cause resistance to AEC in *B. subtilis*. The *aecA* and *aecB* loci map at 250 and 290 degrees, respectively. The *aecB* locus has been suggested as the structural gene for aspartokinase in *B.*

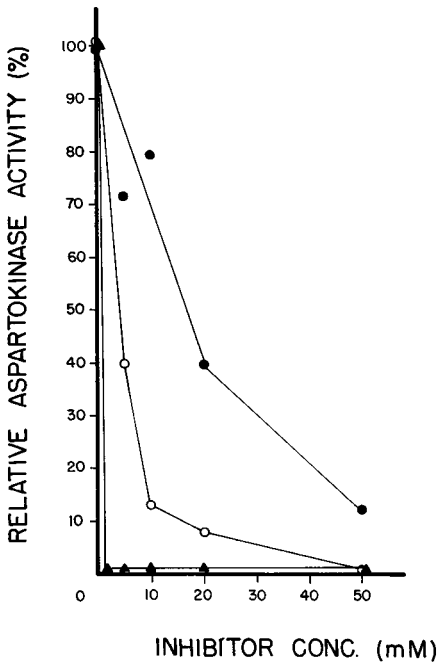

FIG. 2. Inhibition of aspartokinase activity by L-threonine and L-lysine. Strain AMOP was grown and crude extracts were prepared as described by McKenny and Melton (9). Aspartokinase was assayed as described in the text in the presence of L-threonine (●), L-lysine (○), or L-lysine plus L-threonine (▲) at the appropriate concentrations.

subtilis. The AEC resistance gene of *C. glutamicum* has recently been cloned to a 1.2-kb chromosomal fragment (26). The aspartokinase activity encoded by the cloned AEC resistance gene was resistant to inhibition by mixtures of lysine and threonine or of AEC and threonine.

Among the various genera of bacteria, it appears that aspartokinase activity is regulated by different mechanisms (25). Bacterial isofunctional aspartokinases are usually regulated by an independent end product control mechanism (1, 3, 4, 12, 23), while in those bacteria processing a single aspartokinase, the corynebacteria and brevibacteria (8, 21, 26), the enzyme is regulated by a concerted feedback inhibition mechanism (6, 13). The sensitivity of the single aspartokinase to the allosteric effectors threonine and lysine varies within different bacterial genera (4, 5, 7, 11, 16, 21). In 1971, Robert-Gero et al. examined the patterns of regulation of aspartokinase in five *Azotobacter* species, one of which was *A. vinelandii*, and proposed that the genus *Azotobacter* has a single aspartokinase that is sensitive to feedback inhibition by threonine (17). Our results suggest that aspartokinase of *A. vinelandii* is subjected to feedback inhibition by both lysine and threonine and that aspartokinase activity of *A. vinelandii* AMOP is more sensitive to lysine than to threonine. Since in these studies threonine did not limit the biosynthesis of lysine in *A. vinelandii*, our results do not exclude the possibility that this bacterium contains two aspartokinases.

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