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Regulation of Dipeptide Transport in *Saccharomyces cerevisiae* by Micromolar Amino Acid Concentrations

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Prototrophic *Saccharomyces cerevisiae* X2180, when grown on unsupplemented minimal medium, displayed little sensitivity to ethionine- and *m*-fluorophenylalanine-containing toxic dipeptides. We examined the influence of the 20 naturally occurring amino acids on sensitivity to toxic dipeptides. A number of these amino acids, at concentrations as low as 1 μM (leucine and tryptophan), produced large increases in sensitivity to leucyl-ethionine, alanyl-ethionine, and leucyl-*m*-fluorophenylalanine. Sensitivity to ethionine and *m*-fluorophenylalanine remained high under either set of conditions. The addition of 0.15 mM tryptophan to a growing culture resulted in the induction of dipeptide transport, as indicated by a 25-fold increase in the initial rate of L-leucyl-L-[^{3}H]leucine accumulation. This increase, which was prevented by the addition of cycloheximide, began within 30 min and peaked approximately 240 min after a shift to medium containing tryptophan. Comparable increases in peptidase activity were not apparent in crude cell extracts from tryptophan-induced cultures. We concluded that *S. cerevisiae* possesses a specific mechanism for the induction of dipeptide transport that can respond to very low concentrations of amino acids.

The yeast *Saccharomyces cerevisiae* takes up small oligopeptides from a growth medium. Results of studies have shown that this uptake is carrier mediated, energy dependent, and of broad substrate specificity (4). Similar systems have been characterized in bacteria (8, 18) and other fungi (6, 10, 21). The regulation of these systems is of particular interest because a range of peptides and peptide derivatives are antimicrobial agents (19). Small peptides can also play an important role in satisfying the nutritional requirements of some microorganisms (18).

Significant progress has been made in studies of the regulation of peptide uptake in bacterial systems. Jamieson and Higgins (9) have shown that the *opp*-encoded peptide transport system in *Salmonella typhimurium* is constitutive, while the *tppB*-encoded system is induced by leucine or anaerobiosis. In *Escherichia coli*, however, it is the *opp*-encoded system that is regulated. Andrews and co-workers (1, 2) have reported that not only leucine and anaerobiosis but also alanine and leucine- or alanine-containing oligopeptides induce expression of the *E. coli opp* operon.

In a number of reports the regulation of peptide transport in fungi has been addressed, but significant details are not yet available. In one study, Wolfinbarger (22) concluded that peptide transport in *Neurospora crassa* is constitutive because neither derepression as a result of carbon or nitrogen limitation nor induction was observed. However, we found (3) that the initial rate of trimethionine uptake in *S. cerevisiae* is much higher in cells that are grown on a poor nitrogen source, such as proline, than in cells that are grown on glutamate or ammonium sulfate. These results were confirmed by Nisbet and Payne (16) for other peptide substrates and more recently by Moneton et al. (13). Thus, nitrogen repression appears to play a role in the regulation of peptide transport in this organism. The dimorphic fungus *Candida albicans* also adjusts peptide transport activity to environmental conditions. Yadan et al. (23) found that the apparent K_m for uptake of trimethionine is much lower in rich medium than in minimal medium. In addition, a mutant that is resistant to the peptidyl nucleoside Nikkomycin Z and that is subsequently deficient in the uptake of dimethionine had a greatly increased capacity for the transport of trimethionine.

Three peptide transport-deficient mutants of *S. cerevisiae*, which were selected for their resistance to ethionyl-alanine (11), bacilysin (17), or Nikkomycin Z (14), did not display such complexity, however. Each strain lost the ability to transport both di- and tripeptides. One interpretation is that a single system mediates the entry of di- and tripeptides in *S. cerevisiae*. Alternatively, these mutants may represent only one class of peptide transport-deficient strains. During the isolation and characterization of additional mutants, we noted an important regulatory phenomenon associated with this system. Supplements that are required for the growth of some auxotrophic strains resulted in changes in the level of peptide uptake, the specificity of peptide uptake, or both. We undertook this study with radiolabeled and toxic dipeptides and found that dipeptide transport in *S. cerevisiae* is subject to induction by micromolar concentrations of numerous amino acids.

MATERIALS AND METHODS

Strains, media, and growth conditions. *S. cerevisiae* X2180-1A and X2180-1B, which were obtained from the Yeast Genetic Stock Center, Berkley, Calif., were routinely maintained on 1% yeast extract–2% Bacto-Peptone (Difco Laboratories, Detroit, Mich.)–2% glucose medium. All experiments were conducted with these strains in minimal medium (MM) containing yeast nitrogen base (Difco) without amino acids and without ammonium sufate, 1 mg of allantoin per ml, 2% glucose, and individual amino acid supplements if indicated (Table 1). The concentration of an amino acid was 0.15 mM unless stated otherwise. Solid media contained 2% Noble agar. Shifts from MM to MM plus tryptophan (MM-Trp) were performed as follows. A total of 300 ml of an exponentially growing yeast culture in MM was split, and separate 100-ml fractions were filtered onto filters (pore size, 0.65 μm; type DA; Millipore Corp.,

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TABLE 1. Effect of Amino acid supplements on sensitivity to toxic peptides[a]

Supplement	Diameter (cm) of zone of inhibition of plates containing[b]:				
	Eth	F-Phe	Leu-Eth	Ala-Eth	Leu-F-Phe
None	4.6	3.7	None	1.5	None
Leucine	5.0	3.4	4.1	4.5	4.2
Tryptophan	4.5	2.4	4.3	4.4	3.6
Lysine	5.7	2.7	4.8	4.0	None
Methionine	None	3.2	None	None	3.7[c]
Isoleucine	4.9	3.4	3.9	3.5	4.0[d]
Threonine	4.5	2.6	3.9	3.3	3.0[d]
Phenylalanine	3.7	None	3.6	3.5[c]	None
Valine	4.4	3.1	3.4	2.8[c]	None
Histidine	4.0	3.8	3.2	2.6	None
Alanine	4.4	3.2	3.0	2.5[c]	None
Serine	4.5	3.5	2.9	2.3[c]	None
Tyrosine	3.8	3.4	2.4	2.3[c]	None
Asparagine	4.2	3.0	2.1[c]	Trace	None
Cysteine	4.7	3.8	None	1.8	None
Glycine	4.5	3.6	Trace	1.5[c]	None
Aspartate	4.6	3.9	None	1.3[c]	None
Proline	4.5	4.0	None	1.1[c]	None
Glutamate	4.6	3.5	None	Trace	None
Glutamine	4.7	3.5	None	None	None
Arginine	4.6	2.8	None	None	None

[a] Strain X2180-1B; plates contained MM with 0.15 mM amino acid supplements.
[b] None, no inhibition; trace, very slight decrease in growth next to disk.
[c] Heavy background growth, especially at the outer edges of the zone.
[d] Only an outline of the zone was visible.

Bedford, Mass.); the filters were washed with 100 ml of either MM or MM-Trp (30°C) and were suspended in an equal volume of the same medium (30°C). Each culture was incubated at 30°C and 140 rpm during the experiment. Where indicated, cycloheximide was added at 10 μg/ml.

Transport assays. Uptake of L-leucyl-L-[^{3}H]leucine over the course of induction was determined as follows. At various times before or after a shift to fresh medium (MM or MM-Trp), 1-ml portions of yeast cultures were harvested by centrifugation for 1 min at 12,000 × *g*. The pellet was washed two times by suspending it in an equal volume of cold, sterile water and centrifuged as described above. The washed pellet was finally suspended to 0.5 ml in cold 2% glucose and incubated at 30°C for 10 min. The cells (0.25 ml) were then added to an equal volume of reaction mixture (30°C) and incubated at 30°C. At 0.12, 1, 2, 3, and 4 min, 0.08-ml portions were removed, filtered over filters (pore size, 0.45 μm; GN-6; Gelman Sciences, Inc., Ann Arbor, Mich.) and washed 2 times with 5 ml of cold water. The filtered cells were placed in scintillation vials with 5 ml of Bray solution and counted (liquid scintillation counter; LS-7000; Beckman Instruments, Inc., Fullerton, Calif.). The final concentration of components in the uptake assay was 1% glucose, 40 mM sodium citrate–potassium phosphate buffer (pH 5.5), and 1.6×10^{-4} M L-leucyl-L-[^{3}H]leucine (specific activity, 9.4 mCi/mmol). Uptake is expressed as nanomoles per minute per milliliter of cells per optical density at 660 nm unit. One unit at an optical density at 660 nm corresponds to approximately 2.5×10^7 cells per ml.

Disk assays. Sensitivity to toxic peptides was measured by using disk assays. Cells were grown overnight on MM and suspended in sterile water at 5.0×10^7 cells per ml. Fractions (0.1 ml) were added to 0.8% Noble agar and plated on MM containing single amino acid supplements, as indicated in Table 1. A total of 0.38 μmol of L-ethionine (Eth), D,L-*m*-fluorophenylalanine (F-Phe), L-leucyl-L-ethionine (Leu-Eth), L-alanyl-L-ethionine (Ala-Eth), or L-leucyl-D,L-*m*-fluorophenylalanine (Leu-F-Phe) was added to a disk made of Whatman 3MM filter paper. Disks containing each compound were placed on the suface of the plates. After 48 h of incubation, zones of inhibition were measured for all plates except those containing lysine, which required an additional 12 to 24 h of incubation.

Cell extracts and peptidase assays. Cell extracts were prepared from yeast cultures 180 min after a shift to fresh MM and MM-Trp. Approximately 95 ml of each culture was harvested by centrifugation (10 min at 2,000 × *g*) and washed 2 times with cold water. Cell extracts were prepared by vortexing with glass beads (diameter, 0.45 μm) essentially as described previously (20). The concentration of protein was determined by using protein assay (Bio-Rad Laboratories, Richmond, Calif.), with bovine serum albumin used as the standard. Peptidase activity in crude cell extracts was measured by the cleavage of pertinent dipeptide substrates (Table 2) to constituent amino acids. The appearance of specific amino acids was quantitated from standard curves by using the coupled L-amino acid oxidase–peroxidase assay described by Fujita et al. (7). Cleavage of leucine *p*-nitroanilide was monitored by the appearance of *p*-nitroaniline (405 nm). Rates are expressed as nanomoles cleaved per milligram of protein per minute.

Synthesis of L-leucyl-L-[^{3}H]leucine trifluoroacetic acid and toxic dipeptides. L-leucyl-L-[^{3}H]leucine trifluoroacetic acid (TFA) and the toxic dipeptides Leu-Eth, Ala-Eth, and Leu-F-Phe were synthesized by standard solution phase techniques (15). L-leucyl-L-[^{3}H]leucine TFA comigrated with an authentic sample of Leu-Leu TFA on a C_{18} column (K', 1.71; methanol-water-TFA; 300:700:0.25 [vol/vol/vol]). The toxic dipeptides were homogenous on silica thin layers (butanol-acetic acid-water; 4:1:5 [vol/vol/vol]) and had the expected ^{1}H nuclear magnetic resonance spectra.

RESULTS

Increase in sensitivity to toxic dipeptides by amino acids. When *S. cerevisiae* was grown on MM with allantoin as the sole nitrogen source, it was not sensitive to Leu-Eth or Leu-F-Phe and was only slightly sensitive to Ala-Eth (Table 1 and Fig. 1). In contrast, the presence of any one of a number of amino acids greatly increased the sensitivity to one or all of the toxic dipeptides. In general, neutral amino acids were most effective as inducers of this sensitivity.

Not all amino acids produced the same response to the three toxic dipeptides. Only leucine and tryptophan resulted in clear sensitivity to Leu-F-Phe, and these amino acids also resulted in a high degree of sensitivity to Leu-Eth and

TABLE 2. Peptidase activity in cell extracts from tryptophan-induced and uninduced cultures[a]

Peptide substrate	Sp act (nmol cleaved/min per mg of protein) of[b]:		Ratio of induced/uninduced
	Induced	Uninduced	
Leu-Eth	1,620 ± 240	1,210 ± 60	1.3
Ala-Eth	5,120 ± 40	3,770 ± 90	1.4
Leu-F-Phe	1,950 ± 30	1,460 ± 40	1.3
Leu-Leu	1,760 ± 20	1,360 ± 80	1.3
Leu-p-Na[c]	11 ± 0.3	8 ± 0.1	1.4

[a] Cell extracts were prepared 180 min after a shift to MM (uninduced) or MM-0.15 mM Trp (induced).
[b] Values are the average of two experiments.
[c] Leu-p-Na, Leucine *p*-nitroanilide.

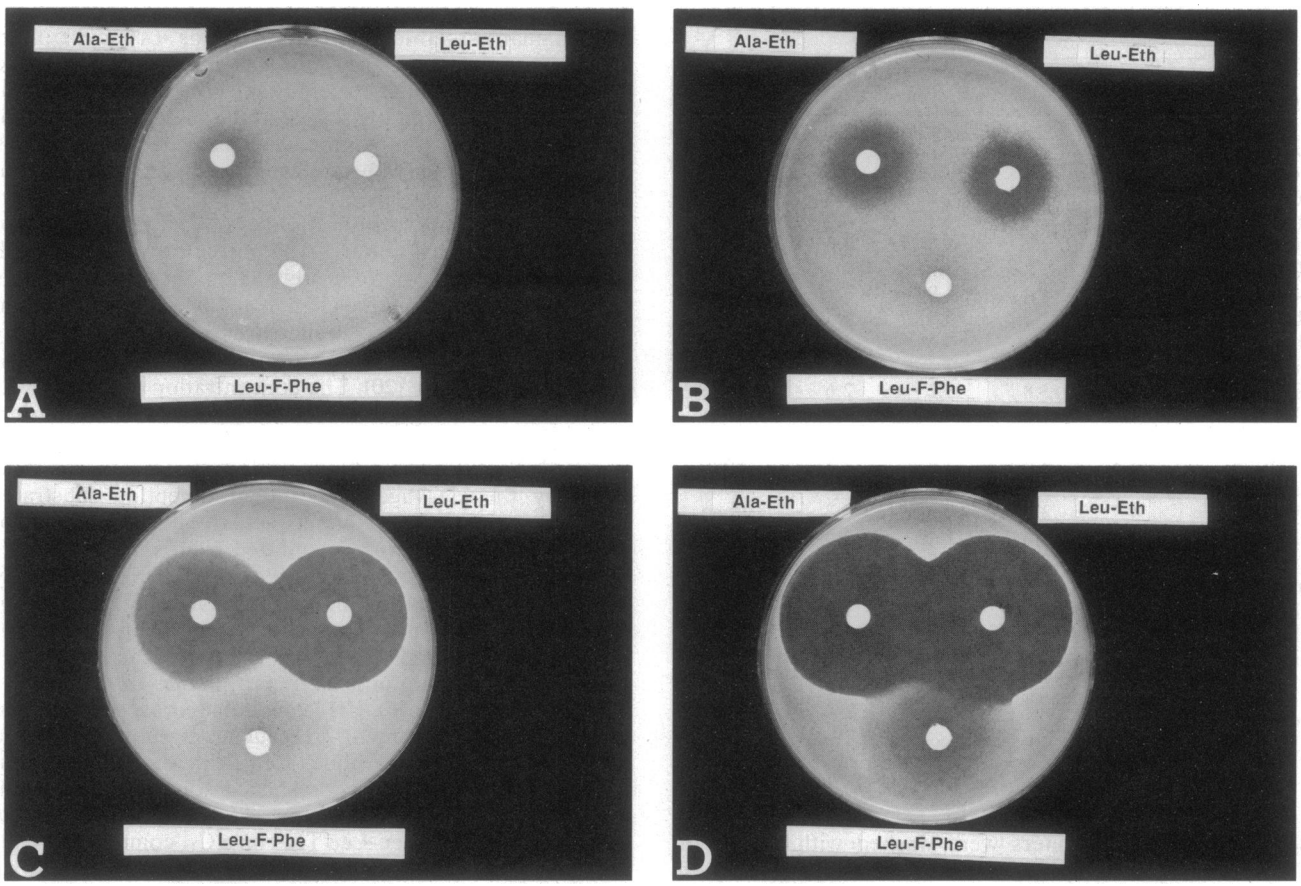

FIG. 1. Induction of toxic peptide sensitivity by increasing concentrations of leucine. A suspension of *S. cerevisiae* X2180-1B in 0.8% top agar was plated onto yeast nitrogen base containing allantoin as the nitrogen source (see text). The medium contained leucine at 0 μM (A), 1 μM (B), 10 μM (C), or 100 μM (D). Disks contained 0.38 μmol of the indicated toxic dipeptide.

Ala-Eth. Only very slight sensitivity to Leu-F-Phe was seen in the presence of isoleucine, methionine, and threonine due to heavy background growth. In general, those amino acids that had a significant effect caused Leu-Eth sensitivity greater than or equal to that for Ala-Eth; this was apparent in the diameter of and the amount of background growth within zones of inhibition (Table 1 and Fig. 1). However, unsupplemented MM or MM containing cysteine, proline, aspartate, or glycine resulted in slight sensitivity to Ala-Eth in the absence of sensitivity to other dipeptides.

The sensitivity to each toxic peptide on media containing different amino acid supplements could be influenced at either of two levels: (i) changes in sensitivity to the toxic amino acid analogs or (ii) changes in the transport or hydrolysis of toxic peptides. The latter case included differences in the ability to cleave toxic peptides with various compositions or in the regulation of the activity or characteristics of peptide transport systems. Although phenylalanine and methionine prevented sensitivity to their toxic structural analogs, the addition of other amino acid supplements caused only small changes in sensitivity to Eth and F-Phe, and these changes did not correlate with changes in toxic peptide sensitivity. These results do not support the argument that increased sensitivity to toxic peptides is solely due to increased sensitivity to the pertinent amino acid analog. Therefore, increases in sensitivity to dipeptides containing these toxic moieties are probably attributable to changes in transport, hydrolysis of the peptide, or both. Experiments (see below) in which the uptake of a radiolabeled dipeptide was measured support this interpretation.

Sufficiency of micromolar concentrations of leucine to increase sensitivity to toxic dipeptides. Leucine and tryptophan induced high levels of sensitivity to the three dipeptides that were examined. Leucine was used subsequently in the titration of the minimal concentration of amino acid required to induce dipeptide transport. Leucine induced Leu-Eth and Ala-Eth sensitivity at a concentration as low as 1 μM (Fig. 1); zone diameter and clarity increased with increasing concentrations of leucine. Sensitivity to Leu-F-Phe, however, was first detected in the presence of 10 to 100 μM leucine; at 100 μM leucine, concentric zones of inhibition were observed for Leu-F-Phe. An increase in the concentration of leucine had relatively little effect on sensitivity to Eth, but sensitivity to F-Phe decreased as the concentration of leucine increased (data not shown). Similar results were obtained by using tryptophan as the inducer (data not shown).

Increase in uptake of L-leucyl-L-[^{3}H]leucine by tryptophan. We synthesized a radiolabeled dipeptide (see above) to analyze the induction process and to verify that the initial rate of dipeptide transport, in fact, increased after growth in the presence of certain amino acids. When an exponentially growing yeast culture was split and a portion was shifted to fresh MM-Trp, we observed over a 25-fold increase in the initial rate of tritiated leucyl-leucine uptake (Fig. 2). The rate began to increase within 30 min and peaked approximately

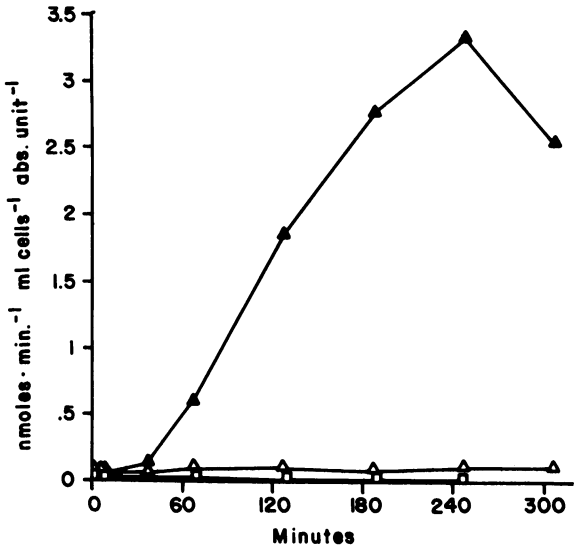

FIG. 2. Initial rate of L-leucyl-L-[^{3}H]leucine uptake at various times after a shift from MM to MM (△), MM-0.15 mM Trp (▲), MM-0.15 mM Trp and 10 μg of cycloheximide per ml (□).

240 min after the shift to MM-Trp. Transport in the control culture, which was shifted to fresh MM, increased less than twofold. Thus, an increase in the initial rate of dipeptide uptake was a possible explanation for the increased toxic peptide sensitivity (Table 1). The time period that was required for induction suggests that synthesis of new protein occurs under these conditions. Indeed, a shift to medium containing cycloheximide in addition to tryptophan prevented the increase in the initial rate of L-leucyl-L-[^{3}H]leucine accumulation (Fig. 2).

No increase in cellular peptidase activity by tryptophan. We questioned whether increases in dipeptide uptake were associated with similar increases in peptidase activity. The induction of peptide transport could be part of a more general response which includes increases in peptidohydrolytic activity. In addition, changes in the amount or activity of cellular peptidases could contribute to the increases in dipeptide transport and in the sensitivity to toxic peptides that we observed.

Three hours after induction with tryptophan, when L-leucyl-L-[^{3}H]leucine uptake was increased approximately 20-fold, comparable increases in peptidase activity could not be detected in crude cell extracts (Table 2). A uniform 29 to 38% increase in the rate of hydrolysis of each peptide substrate was observed; however, the total protein contained in cell extracts from induced cultures (corrected for culture density) was only 65 to 70% of that in uninduced cultures. Thus, the increase in specific activity may reflect a change more in cell protein content than in peptidase activity. In addition to decreases in total protein, tryptophan-induced increases in dipeptide transport were accompanied by a 20% decrease in growth rate during the experiment.

DISCUSSION

The transport of small peptides into *S. cerevisiae* is subject to repression by nitrogen sources that support a high growth rate (3, 13, 16). We found that an additional, sensitive regulatory control acts on this system. In the presence of allantoin, which is one of the least repressive nitrogen sources in yeast (5), micromolar concentrations of any one of a number of amino acid supplements induce large increases in dipeptide uptake. In the case of induction by tryptophan, which was examined closely, a 25-fold increase in the initial rate of L-leucyl-L-[^{3}H]leucine uptake was observed. Induction did not occur in the presence of cycloheximide.

Unlike the *tppB*-encoded system of *Salmonella typhimurium* and the *opp*-encoded system of *E. coli*, in which only leucine or leucine and alanine, respectively, induce peptide uptake, a range of amino acids affects dipeptide transport in *S. cerevisiae*. Three general categories are apparent. Several amino acids (cysteine, glycine, aspartate, and proline) promote only slight sensitivity to Ala-Eth and therefore approximate the derepressed state that is seen on MM lacking amino acid supplements. Some amino acids that are good nitrogen sources in *S. cerevisiae* (glutamate, glutamine, and arginine) in turn repress the sensitivity to Ala-Eth that is seen on unsupplemented medium. The remaining amino acids (Table 1) are inducers of sensitivity to one or more of the toxic dipeptides tested. This interpretation is consistent with the previously noted repression-derepression of peptide uptake and identifies induction as an additional phenomenon associated with dipeptide transport; other oligopeptides have not been tested.

Most importantly, we detected induction at micromolar concentrations of amino acids. Our results place into perspective the observation of Moneton et al. (13) that a high concentration of leucine (34 mM), when used as the sole nitrogen source, influences both sensitivity to toxic peptides and the kinetic parameters of peptide transport in *S. cerevisiae*. Moreover, we suggest that, rather than a general influence of the nitrogen source, the induction of dipeptide transport is mediated by a very sensitive, specific metabolic sensor that triggers the rapid synthesis of an additional permease, permease component, or catalytic activity that is capable of modifying existing peptide transporters. It is not yet clear which molecule(s) is the actual inducer, nor is it clear what role the overall nutritional state plays in the induction process. Although both protein content and growth rate, which are sensitive indicators of nutritional state, decrease when tryptophan is added to induce dipeptide transport, peptones also induce dipeptide uptake (data not shown). The doubling time is significantly reduced in the presence of peptone, and nutritional deprivation is absent.

Multiple peptide transport systems have been documented in bacteria (8, 18) and in *C. albicans* (12). The *Salmonella typhimurium* system includes both a component that is induced by leucine and a component that is constitutive. Our data raise the possibility of multiple peptide permeases in *S. cerevisiae*. A single dipeptide permease, whether induced or uninduced, would be expected to display a consistent affinity for each toxic peptide. However, our results show that at least two cases can exist. On unsupplemented MM and on medium containing the amino acids in the first category presented above, sensitivity to Ala-Eth is seen in the absence of sensitivity to the other toxic dipeptides. In contrast, those amino acids that induce sensitivity to toxic dipeptides tend to produce larger, cleaner zones of inhibition to Leu-Eth and not Ala-Eth. Induction of an additional dipeptide permease, with different affinities for these two peptides, is a possible explanation and is consistent with the observation that cycloheximide prevents induction. Definitive evidence for multiple transport systems and further insights into the mechanism of peptide transport regulation must await the characterization of additional mutants.

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