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Transport Systems for Alanine, Serine, and Glycine in *Escherichia coli* K-12

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At least two transport systems serve for the entry of alanine, glycine, and serine into *Escherichia coli*. One of these systems serves mainly for glycine, D-alanine, and D-serine and to some extent for L-alanine, whereas the second serves for L-alanine and perhaps L-serine. These two transport systems have been characterized by kinetic studies and by inhibition analysis. Reciprocal plots for L-alanine entry are distinctly biphasic, giving rise to K_m values of about 2 and 27 μM. The major route of glycine entry can be described by a single K_m value of about 4 μM. A higher K_m value for glycine of around 70 to 100 μM shows that other routes of entry may serve at high concentrations of amino acid. The glycine, D-serine and D-alanine transport system is defective in a D-serine-resistant mutant, strain EM1302. The mutation, *dagA*, is recessive in *dagA*/*dagA*$^+$ merodiploids and is 7 to 12% linked by phage P1 transduction to the *pyrB* locus of *E. coli*. *E. coli* with the *dagA* mutation are unable to utilize D-alanine as a carbon source, providing an additional basis for selecting such mutants. The remaining L-alanine uptake in *dagA* mutants is subject to inhibition by L-serine, L-threonine, and L-leucine. It is also sensitive to osmotic shock treatment and repressed by growth of the cells on L-leucine. It appears from a comparison of the properties of the second L-alanine system with those of the leucine, isoleucine, and valine system (LIV system) that the LIV system also serves for the transport of L-alanine and L-threonine and perhaps L-serine.

We previously reported that the amino acids alanine, serine, and glycine appeared to share a common transport system in *Escherichia coli* K-12 (17). An examination of the quantitative nature of the mutual inhibition among these amino acids in various strains of *E. coli* has revealed heterogeneity in the transport systems, particularly for the uptake of L-alanine (J. R. Piperno, Ph.D. thesis, Univ. of Mich., 1966; 1, 5, 7, 9, 13, 14, 20, 21). The use of transport mutants has greatly facilitated the identification of transport families. The toxic effects of D-serine on *E. coli* W (5) and of the antibiotic D-cycloserine on *E. coli* W (7) and *E. coli* K-12 (3) have provided the basis for mutant selections. Wargel et al. (20, 21) showed that D-cycloserine, D-alanine, and glycine are transported into *E. coli* K-12 by a common system separate from that serving for L-alanine uptake. Kessel and Lubin (7) isolated similar mutants in *E. coli* W and obtained similar results. Cosloy (1) has isolated and described a D-serine-resistant mutant (EM1302) in a D-serine deaminaseless strain (EM1301) of *E. coli* K-12, which is defective in the transport of D-serine, D-alanine, and glycine. The transport of L-alanine is partially impaired in this mutant. Levine and Simmonds (8) also described an *E. coli* K-12 mutant apparently defective in glycine, but not L-serine, transport.

The present report examines in greater detail the specificity and degree of overlap of the two transport systems for glycine and the isomers of alanine and serine.

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MATERIALS AND METHODS

Materials. Chemicals were purchased from normal commercial sources. Membrane filters (0.45-μm pore size) were bought from the Millipore Corp.

Strains and media. All bacterial strains used in this study (Table 1) are derivatives of *E. coli* K-12. Strains EM1301, 1302, and 1304 were gifts of E. M. McFall. Strain EM1301 was used in this study because it lacks D-serine deaminase activity and is the parental strain for EM1302 and EM1304. We have

TABLE 1. *Description of E. coli K-12 strains used*

Strain	Parental strain	Relevant genetic markers (19)
EM1301	W3828	*dsdC3 dsdA*[a, b]
EM1302	EM1301	*dsdC3 dsdA, dagA*
EM1304	EM1302	*dsdC3 dsdA, dagA, metB*
909		*pyrB*
910		*pyrB*
4255	3000	F'117[c]
4258	J4	F'111[c]
4260	Ra-2	F'112[c]
4265	Kl-25	F'133[c]

[a] Abbreviations: *dsdC3 dsdA*, constitutive, but inactive D-serine deaminase. *dagA*, Glycine, D-serine, DL-alanine permease A, apparently the same as *cycA*, D-cycloserine resistance (18, 20, 21).

[b] Cosloy and McFall have reported (2) that strain EM1301 also contains an additional mutation, making it more resistant than other W3828-derived strains to the transient L-serine inhibition of growth which they report for *E. coli* K-12 strains in general.

[c] See Fig. 5.

obtained essentially similar data for *E. coli* K-12 (ATCC 14948 [17]). D-Serine is toxic in strain EM1301 and has been used to select resistant transport mutants. A substantial fraction of D-serine-resistant mutants isolated in this laboratory from strain EM1301 are transport mutants. (Of 100 D-serine-resistant mutants, 27 failed to grow on D-alanine as a carbon source. All of these 27 were defective in D-alanine transport.) Strain EM1302, which is an example of this mutant type (*dagA*), was studied in most detail. It is also resistant to 100 μM D-cycloserine. Strains 909 (CGSC-4517) and 910 (AT2535) were given to us by G. R. Greenberg. The F'-containing strains 4255, 4258, 4260, and 4265 came from the Coli Genetic Stock Center at Yale University. Vogel-Bonner minimal medium was used in most experiments, with glycerol as the carbon source unless otherwise indicated. Frequently, cells were grown overnight in minimal medium with limiting (0.01–0.02%) glycerol. In the morning the cells could be started into log growth with only a short lag time by addition of excess (0.20%) glycerol. Amino acids used as carbon sources were 0.1%.

Methods. The transport assay is similar to that previously described (17). Exponentially growing cells (red filter Klett reading of 80–100, about 1.3–1.8 mg of cells [wet wt] per ml) were harvested by centrifugation or filtration, washed, and suspended in ice-cold phosphate buffer (10^{-2} M potassium phosphate, 10^{-4} M magnesium sulfate, pH 6.9). The cells could be stored on ice for 1 h. For uptake measurements, the cell suspension was incubated for 10 to 15 min at 37 C, and samples were added to radioactive amino acids in the same warm buffer. At the desired times, 1.0-ml portions of the uptake mixture (generally 0.5–1.5 mg [wet wt] of cells) were membrane filtered, and the cells were washed with 5 ml of buffer at 37 C. After the filters were dried, the radioactivity was determined by liquid scintillation counting, and amino acid uptake was calculated from comparison with suitable standards. Uptake values were generally linear with time for 30 s (see Fig. 3), so that 20-s uptake values could be taken to approximate initial rates.

Mutagenesis, penicillin selection, mating, and transduction were done by standard techniques.

RESULTS

Kinetics of uptake of L-alanine and glycine into strain EM1301. The initial rates of entry for glycine and L-alanine into strain EM1301 were measured over the concentration range of 10^{-6} to 10^{-4} M. A reciprocal plot of the initial rates for these amino acids is presented in Fig. 1. The reciprocal plot of L-alanine entry is distinctly biphasic over the concentration range tested, while the plot for glycine is essentially linear. The kinetic constants for L-alanine were calculated by applying methods previously indicated by Winter and Christensen (22) and Neal (11) for obtaining two sets of K_m and V_{max} values from biphasic reciprocal plots. When concentrations of glycine above 10^{-4} M were used, the reciprocal plots deviated from linearity, suggesting additional entry routes with relatively high K_m values of 70 to 100 μM. The kinetic constants obtained from reciprocal plots of the rates of entry for several amino acids are presented in Table 2.

Inhibition analysis in strain EM1301. The inhibitory action of several amino acids on the initial rates of entry of L-alanine and glycine into strain EM1301 was measured (Fig. 2A and B). Glycine uptake is strongly inhibited by

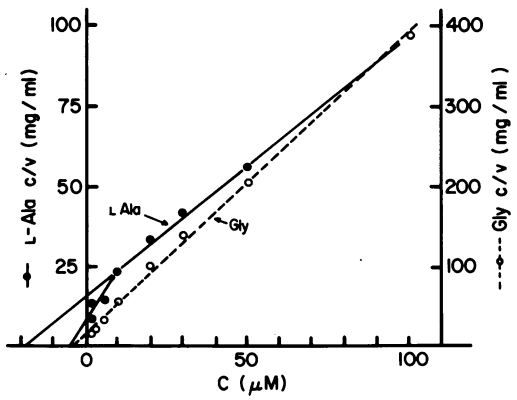

FIG. 1. *Reciprocal plot of glycine and L-alanine transport by strain EM1301. Uptake velocities (v, nanomoles per milligram of cells per 20 s) were measured as described in Materials and Methods over the indicated range of concentrations of L-alanine or glycine.*

TABLE 2. *Kinetic constants of uptake*[a]

E. coli strains	K_m (μM)			V_{max} (nmol/mg/20 s)		
	L-Alanine	D-Alanine	Glycine	L-Alanine	D-Alanine	Glycine
EM1301	2 27	2	2.8–4.8	0.4 0.8	0.11	0.4–0.6
EM1302	1 34	2	1.5–3.5	0.3 0.3	0.002	<0.004

[a] Values for D-alanine and glycine were obtained directly from $1/v$ versus $1/c$ and/or c/v versus c graphs. Values for L-alanine were calculated by the method of Neal (11) to separate the biphasic kinetics of one experiment into contributions of the two transport systems. Several other experiments gave similar results for L-alanine.

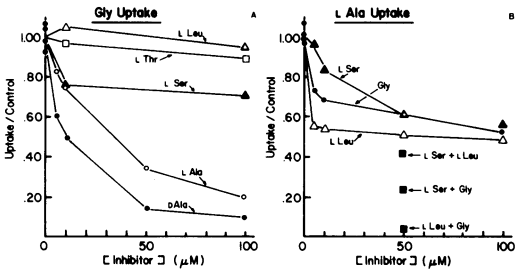

FIG. 2. *Relative inhibition of glycine and L-alanine transport in strain EM1301. The glycine concentration was 1.7 μM (A); L-alanine, 1.6 μM (B). The abscissa indicates final concentrations of the inhibitory amino acids shown in the figure. The four values (filled circles) on the ordinate indicate the experimental variation observed in controls.*

L-alanine as well as by D-alanine (Fig. 2A) and D-serine (not shown). Glycine, D-serine, and D-alanine always gave similar uptake and inhibition results in our studies, so that in most experiments only one of them was used to represent the group. L-Leucine did not produce significant inhibition of glycine uptake in strain EM1301. Figure 2B shows that glycine, L-serine, and L-leucine all produce partial inhibition of L-alanine entry, while the combination of L-leucine and glycine produces essentially complete inhibition. In experiments that are not shown, competitive inhibition of the low-K_m L-alanine uptake component by L-leucine (K_I = 0.2 μM) and of the high-K_m component by glycine (K_I = 4 μM) were demonstrated with the mutant strain EM1302 and the leucine-repressed parental strain EM1301, which (see below) lack, respectively, the high- and low-K_m L-alanine components. The K_I values of these inhibitors are similar to their K_m values for transport (Table 2 and reference 17). In neither case were V_{max} values of L-alanine transport significantly changed.

Transport activity of the D-serine-resistant mutant strain EM1302. The time course of entry of glycine and L-alanine into strain EM1302 is compared to that of the parental strain EM1301 in Fig. 3A and B. The figure shows that glycine uptake is seriously impaired in the mutant while L-alanine uptake is slightly impaired. The uptakes of D-serine and D-alanine (not shown) are also defective in strain EM1302. Table 2 shows that the V_{max} values for glycine, D-alanine, and high-K_m L-alanine uptakes are reduced in strain EM1302 without significant changes in K_m. This mutant is unable to utilize D-alanine as a carbon source. The uptakes of the L-isomers of leucine, isoleucine, valine, proline, and phenylalanine are not altered in the mutant strain, and the accumulation of α-methyl-D-glucoside is not defective. These results support interpretations that the *dagA* mutation results in a specific transport defect in a single system responsible for the uptake of glycine, D-serine, and D-alanine.

Inhibition of residual L-alanine transport in strain EM1302. The specificity of the component of L-alanine uptake not shared by glycine can easily be examined using the D-serine-resistant mutant, strain EM1302, which lacks the glycine-shared component. The inhibitory action of various amino acids is shown in Fig. 4. Essentially all of the L-alanine uptake by strain EM1302 is subject to inhibition by L-leucine, L-serine, and L-threonine. Glycine and other members of the glycine transport system (data not shown for D-alanine and D-serine) do not produce significant inhibition of L-alanine uptake. The inhibition of the second component of

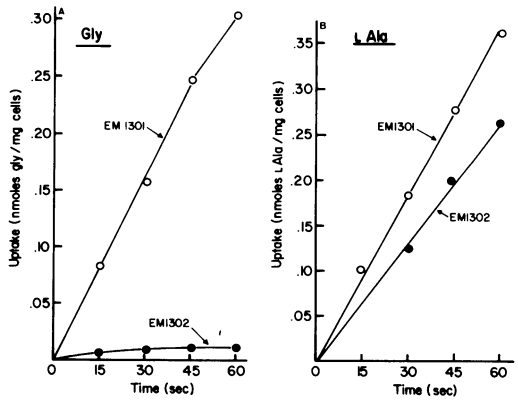

FIG. 3. *Time courses of glycine and L-alanine uptake by strains EM1301 and EM1302. Glycine (4 μM) (A) and L-alanine (2 μM) (B) uptakes were measured as described in Materials and Methods.*

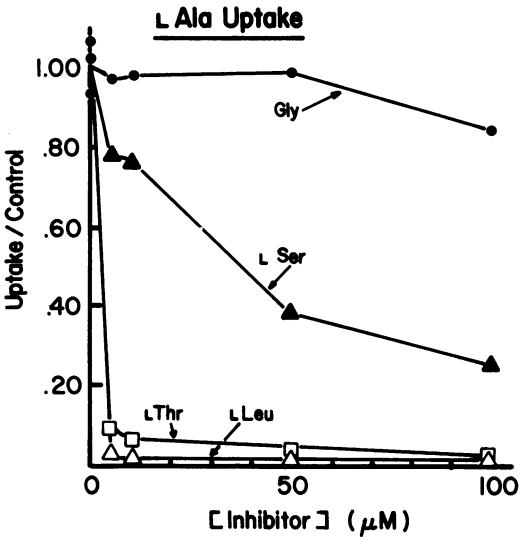

FIG. 4. *Inhibition of* L-*alanine (1.6 μM) transport in strain EM1302. Performed as indicated in legend to Fig. 2.*

L-alanine entry by the branched-chain amino acids suggests that the previously described leucine, isoleucine, and valine transport system (17) may also serve for L-alanine entry. To test this possibility further, we examined the effects of osmotic shock treatment and growth of the cells in the presence of L-leucine on the uptake of L-alanine. Cells were subjected to osmotic shock treatment by the procedure of Neu and Heppel (12). We found (data not shown) that the L-alanine uptake into shock-treated EM1302 cells was sharply decreased, while L-alanine uptake into shock-treated EM1301 cells was only slightly decreased when compared to effects on L-proline uptake in these same cells. L-Proline uptake in *E. coli* has been shown to be insensitive to osmotic shock treatment (16). The sensitivity to osmotic shock treatment of L-alanine entry into EM1302 cells is consistent with the hypothesis that the leucine, isoleucine, and valine (LIV) system also serves for L-alanine uptake.

The effect of the presence of 25 μg of L-leucine per ml in the growth medium on the subsequent uptake of L-alanine in strains EM1301 and EM1302 was examined (Table 3). Growth in the presence of L-leucine severely represses the L-alanine transport activity in strain EM1302 but has no effect on the L-alanine transport activity in strain EM1301. L-Leucine in the growth medium causes a significant stimulation of the uptake of glycine in strain EM1301 (Table 3), although inhibition studies (Fig. 2A) indicate that L-leucine is not a substrate of the glycine system. The stimulatory effect on the glycine system, which is under further investigation, may balance the repressive effect on the other L-alanine system, thus accounting for the failure to observe repression of L-alanine uptake in strain EM1301. Since the LIV transport system and the LIV-binding protein are highly repressed under these same conditions (15), we can tentatively conclude that L-alanine also enters *E. coli* by the LIV-system.

Mapping of dagA mutation in strain EM1302. The *dagA* mutation in strain EM1302 renders the strain resistant to D-serine and to D-cycloserine. Resistance to these analogues has been used by Cosloy (1) to show that the *dagA* mutation is located on the *E. coli* chromosome to the right of *malB* (Fig. 5). A recent report shows that D-cycloserine resistance (*cycA*) of strains obtained by Curtiss et al. (3) is near *pyrB* on the *E. coli* chromosome (18). We found the inability of *dagA* strains to utilize D-alanine as a carbon source to be a more useful phenotype in some genetic experiments than antibiotic resistance because of a high background of spontaneous resistance. Strain EM1304 (*dagA metB*), kindly furnished to us by S. Cosloy, was used for transduction experiments (not shown) to demonstrate that the *dagA* mutation was not

TABLE 3. *L-Leucine repression and stimulation of transport*[a]

Uptake of	Additions to minimal medium	Strain	
		EM1301	EM1302
L-Alanine	None	0.236	0.175
	L-Leucine	0.240	0.004
Glycine	None	0.165	
	L-Leucine	0.310	

[a] Uptake of L-alanine (2 μM) and glycine (1 μM) (nanomoles per milligram per 20 s) were measured (see Methods) with cells grown in glycerol minimal medium with and without addition of 25 mg of L-leucine per liter.

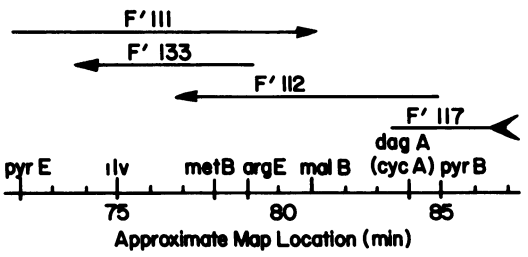

FIG. 5. *F' factors used. Description according to Low (10).*

linked to *metB*, the reported location of certain D-cycloserine resistance mutations studied by Wargel et al. (21). The rough mapping was accomplished by introducing various episomes containing genes around the *ilv*, *metB*, and *pyrB* regions. A diagram of this region of the *E. coli* chromosome is presented in Fig. 5 (19). As indicated in the diagram, *dagA*/*dagA*$^+$ merodiploids could be formed by the transfer of F'117 into strain EM1302 or EM1304. These merodiploids could grow on D-alanine, showing that F'117 carried the *dagA*$^+$ gene and, furthermore, that the *dagA* mutation was recessive. More accurate mapping was accomplished by phage P1 transduction with phage lysates prepared from strains EM1302 and EM1304 with two *pyrB* strains as recipients. The *pyrB*$^+$ transductants were examined for loss of the ability to grow on D-alanine. The *dagA* mutation was 7 to 12% linked to the *pyrB* locus, similar to the reported location of *cycA* (18) and consistent with the approximate position reported for *dagA* (1) (Table 4). The uptakes of L-alanine, D-alanine, and L-proline were examined in *pyrB*$^+$ transductants. The transductants that had lost the ability to grow on D-alanine showed the EM1302 transport phenotype. We also demonstrated that strain EM1302 could be transduced to give the EM1301 transport phenotype by using a direct selection for D-alanine utilization. The results reported above make it clear that the transport defect in strain EM1302 and the inability to grow on D-alanine are cotransducible with each other and with *pyrB*.

DISCUSSION

Early studies on *E. coli* W, the growth of which is strongly inhibited by D-serine, led to the isolation of D-serine-resistant mutants that had an impaired ability to accumulate D-serine, glycine, and L-alanine (6, 7). Although the earlier studies were often interpreted in terms of a common transport system, a more careful examination of the transport kinetics and competitive actions of these amino acids in both *E. coli* W and K-12 clearly showed that more than one system served for their transport.

TABLE 4. *Linkage of dagA to pyrB*[a]

Phage	Strain transduced	
	909	910
$P1_{1302}$	4/56 = 7.1%	3/38 = 7.9%
$P1_{1304}$	15/199 = 7.5%	23/200 = 11.5%

[a] Phage grown on strains EM1302 and EM1304 were used to transduce the *pyrB* strains *909* and *910* to *pyrB*$^+$. These uracil-independent transductants were then tested for ability to grow on D-alanine as a sole carbon source. The fraction of transductants that failed to grow on D-alanine is shown.

The heterogeneity in the transport activity for L-alanine is apparent when the kinetics of uptake into wild-type *E. coli* K-12 cells are examined. Figure 1 shows that at least two K_m values are necessary to describe the entry of L-alanine in the concentration range of 10^{-4} to 10^{-6} M. The entry of glycine in this same concentration range can be described by a single K_m value. Measurements of the uptake at higher concentrations (10^{-3} to 10^{-4} M) of both of these amino acids reveal partially saturable components of entry which are difficult to quantitate. Lineweaver-Burk plots of the entry of glycine at these higher levels are nonlinear and can be analyzed in terms of biphasic plots. Such biphasic plots have also been reported for glycine and D-alanine by Wargel et al. (20). The additional entry at the higher levels may result, in part, from an overlap with other amino acid transport systems since the specificity for entry at the higher levels is considerably broader.

From the results of the inhibition analysis presented in Fig. 2 and similar studies (not shown), it is clear that one component of L-alanine entry—the high-K_m component—is shared by glycine and D-alanine (with higher affinities than L-alanine if the K_m values are indicating affinity), and by D-serine, but an additional significant route of entry is not shared by these amino acids. This second component is inhibited by L-serine, L-threonine, and the branched-chain amino acids. The inhibitory actions of glycine and L-leucine on L-alanine entry are additive and together produce a complete inhibition of L-alanine uptake. Glycine entry is subject to essentially complete inhibition by L-alanine, D-alanine, and D-serine, but it is not greatly affected by L-serine, L-threonine, or L-leucine.

The isolation of D-cycloserine- and D-serine-resistant mutants of *E. coli* W and K-12 has provided additional strong support for the existence of two transport systems for these amino acids (1, 3, 5, 20, 21). In particular, Cosloy (1) has studied the glycine, D-alanine, D-serine, transport system of *E. coli* K-12 by comparison of the mutant strain EM1302 with a strain related to the parental strain EM1301. These mutants show an almost complete loss of transport of glycine, D-alanine, D-serine, and D-cycloserine (not all transports are reported for each mutant) but only a partial loss of L-alanine transport. We have confirmed (Fig. 3) this earlier work and have studied the remaining

L-alanine uptake in such a mutant (see below).

A partially completed mapping of the EM1302 transport defect (*dagA*) has been reported (1), with the mutation said to lie to the right (Fig. 5) of *malB*. The D-cycloserine resistance mutation obtained by Curtiss (3) (*cycA*) had been mapped near *pyrB* (min 85) (18). Since these two mutants are phenotypically similar, we attempted to transduce the transport mutation from strain EM1302 into *E. coli pyrB* strains (909 and 910) using the inability to grow on D-alanine as a phenotype to identify the *dagA* genotype. We found that the linkage to *pyrB* was 7 to 12%, which is similar to that found for the linkage of *cycA* to *pyrB* (18) and consistent with the partial mapping of *dagA* (1). This mutation appears to be recessive in *dagA*/*dagA*$^+$ merodiploids.

D-Cycloserine resistance develops from multistep mutations, some of which are genetically separable (3). Cosloy (1) reports that the transport mutation of strain EM1302 appears to be multistep, but we found the determinant or determinants of D-serine- and D-cycloserine resistance, D-alanine growth, and glycine, DL-alanine, and D-serine transport to be transduced together, and to lie at or near the site reported by Russell (18) for *cycA*.

The characteristics of the second L-alanine component of entry have been examined in strain EM1302, which lacks the glycine transport system. L-Alanine uptake in this mutant is strongly inhibited by L-leucine, L-serine, and L-threonine, while glycine is no longer an effective inhibitor (Fig. 4). L-Alanine entry in strain EM1302 has properties very similar to those of the previously identified (17) branched-chain amino acid transport system (LIV system) on the basis of the following findings. (i) L-Leucine and L-isoleucine are competitive inhibitors. (ii) Growth of the cells in the presence of L-leucine strongly represses the transport activity of L-alanine in strain EM1302 (Table 3). (iii) L-Alanine transport activity in strain EM1302 is very sensitive to osmotic shock treatment, in contrast to the behavior observed for strain EM1301, which retains the shock-resistant glycine system. (iv) Leucine-binding activity of the LIV-binding protein is inhibited by L-alanine (Rahmanian et al., submitted for publication). From the above lines of evidence, it appears that the LIV transport system also serves for L-alanine uptake. The inhibition of L-alanine uptake by L-serine (Fig. 4) indicates that L-serine may share this second L-alanine transport system, although we did not find a strong inhibition of L-serine uptake by L-alanine (not shown). Initial rates of L-serine uptake, however, are very difficult to measure with precision because of a rapid metabolic degradation. It seems likely that at least part of the L-serine uptake is by the LIV transport system, but it is also possible that L-serine inhibits the activity without itself being transported by this system. The findings of broader specificity of the LIV transport system than previously described (17) are discussed more fully in a second paper (Rahmanian et al., submitted for publication).

We are at the present time examining mutants of strain EM1302 that have been selected for the inability to utilize L-alanine as a carbon source to determine if transport mutants can be obtained for the second L-alanine component of entry in *E. coli*.

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