Mechanism of D-Cycloserine Action: Transport Mutants for D-Alanine, D-Cycloserine, and Glycine[1]

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The accumulation of D-alanine and the accumulation of glycine in *Escherichia coli* are related and appear to be separate from the transport of L-alanine. The analysis of four D-cycloserine-resistant mutants provides additional support for this conclusion. The first-step mutant from *E. coli* K-12 that is resistant to D-cycloserine was characterized by the loss of the high-affinity line segment of the D-alanine-glycine transport system in the Lineweaver-Burk plot. This mutation, which is linked to the met_1 locus, also resulted in the loss of the ability to transport D-cycloserine. The second-step mutation that is located 0.5 min from the first-step mutation resulted in the loss of the low-affinity line segment for the D-alanine-glycine transport system. The transport of L-alanine was decreased only 20 to 30% in each of these mutants. A multistep mutant from *E. coli* W that is 80-fold resistant to D-cycloserine lost >90% of the transport activity for D-alanine and glycine, whereas 75% of the transport activity for L-alanine was retained. *E. coli* W could utilize either D- or L-alanine as a carbon source, whereas the multistep mutant could only utilize L-alanine. Thus, a functioning transport system for D-alanine and glycine is required for both D-cycloserine action and growth on D-alanine.

The antibacterial activity of D-cycloserine (D-4-amino-3-isoxazolidone) has been correlated with the inhibition of enzymes necessary for peptidoglycan synthesis (for a review, see reference 6). In the case of *Streptococcus faecalis*, it has been proposed that the primary site of D-cycloserine action at low concentrations is at the donor site of D-alanine: D-alanine ligase (ADP) and that the acceptor site of this enzyme and that of alanine racemase are secondary sites of action (7). In an analysis of D-cycloserine-resistant mutants from *Streptococcus* strain Challis, the transport system(s) for alanine was implicated as an integral aspect of both D-cycloserine action and the acquisition of resistance to this antibiotic (9). Thus, a single mutation that results in a reduction of D-cycloserine transport will "protect" the mul-

tiple inhibition sites associated with D-alanine: D-alanine ligase (ADP) and alanine racemase.

In *Escherichia coli*, the transport system for alanine has also been implicated in the accumulation of D-cycloserine (3, 11). In an analysis of alanine transport in *E. coli*, Wargel et al. (11) proposed that the transport systems for D-alanine and glycine are related and separate from that involved in the accumulation of L-alanine and that the D-alanine-glycine system is responsible for the transport of D-cycloserine.

As a result of the importance of the alanine transport system(s) to D-cycloserine action and the acquisition of resistance to this antibiotic, studies were undertaken of D-cycloserine-resistant mutants in an attempt to dissect the alanine transport system in *E. coli*. The results described in this paper provide additional evidence for a D-alanine-glycine transport system that is separate from the system for L-alanine. The primary mutation responsible for D-cycloserine resistance is characterized by the loss of the high-affinity line segment of the D-alanine-glycine transport system in the Lineweaver-Burk plot. This mutation is linked to the met_1 locus of the *E. coli* chromosome. In addition, an estimate of the amount of overlap between the D-alanine-glycine system and

the L-alanine system is presented.

MATERIALS AND METHODS

Materials. We are indebted to Roger Harned of Commercial Solvents Corp., Terre Haute, Ind., for generous samples of D-cycloserine and D-[^{14}C]cycloserine. The specific activity of the labeled antibiotic was 0.03 μCi/μmole and was used without dilution of the specific activity. The sources of labeled amino acids and other materials have been previously described (11).

Strains. Stock cultures were maintained in vacuo at -20 C. The strain of *E. coli* W (ATCC 9637) was a gift from Wallace Brockman. The D-cycloserine-resistant mutant (D-5) of *E. coli* W was isolated by serial plating on 1% agar containing minimal media (11) with 0.4% glucose and stepwise increasing concentrations of D-cycloserine. Since D-cycloserine slowly loses its bactericidal activity in agar plates (2), the plates were prepared immediately before use. Colonies from plates with the highest concentrations of D-cycloserine were picked and transferred to minimal media. The cycle was repeated until no additional increase in resistance could be developed (eight cycles). The mutants cyc^{r1}, cyc^{r2}, and cyc^{r3} were derived from *E. coli* K-12 and are the kind gift of Roy Curtiss III. They are designated as χ^{289}, cyc^s; χ^{316}, cyc^{r1}; χ^{444}, cyc^{r2}; and χ^{453}, cyc^{r3} (2).

The minimal inhibitory concentration (MIC) is defined as that concentration of D-cycloserine which limits the specific growth rate to 70% of the corresponding rate for the control culture. The MIC values of the strains are summarized in Table 1. For comparison, the MIC values for L-cycloserine are also presented.

The first-step mutant, cyc^{r1}, is approximately 25-fold more resistant to D-cycloserine when compared with the parent cyc^s. The mutant D-5 derived from *E. coli* W is approximately 80-fold more resistant than the parent. The MIC values summarized in Table 1 indicate that the first mutation, cyc^{r1}, is a primary mutation responsible for the acquisition of D-cycloserine resistance.

Methods. The growth and preparation of cells for these experiments have been previously described (11).

The procedure for measuring the accumulation of labeled amino acid was similar to the method described by Kessel and Lubin (3). A portion of a cell suspension (5 $\times$ 10^9 cells) was added to 0.4 ml of minimal growth medium that contained chloramphenicol (200 μg/ml), and the mixture was maintained at 25 C for 5 min before the addition of the labeled amino acid (0.05 ml). Minimal growth medium contained: 0.1% NH$_4$Cl, 0.68% NaH$_2$PO$_4 \cdot$H$_2$O, 0.30% K$_2$HPO$_4$, 0.02% MgSO$_4$, 0.05% NaCl, and 0.4% glucose. Samples (0.2 ml) were removed at the desired time, and the cells were collected on a membrane filter (0.45-μm pore size; Millipore Corp.). The cells were washed with minimal media minus glucose at either 4 or 25 C. The wash temperature is reported for each experiment.

Measurements of radioactivity were made in polyethylene vials by using the liquid scintillation spectrometer (Packard Tri-Carb model 314-EX2). The *damp* membrane filter and cells were dissolved by vigorous shaking in 15 ml of scintillation fluid, and the amount of radioactivity was established. The scintillation fluid was

TABLE 1. *Minimal inhibitory concentration (MIC) of the parent and mutant strains of E. coli K-12 and E. coli W*

Strain	MIC			
	D-Cycloserine			L-Cycloserine (10^{-5} M)^d
	10^{-5} M^a	10^{-5} M^b	10^{-3} M^c	
E. coli K-12 (cyc^s) .	0.3	0.1	0.1	1.4
cyc^{r1}	8.5	4.0	1.0	2.3
cyc^{r2}	15.0	12.0	3.0	2.6
cyc^{r3}	17.0	30.0	5.0	2.4
E. coli W	0.5			0.7
D-5	42.0			0.9

^{a} MIC is defined as that concentration of D-cycloserine which limits the rate of growth during the linear exponential phase to 70% of the rate of the control culture during exponential growth.

^{b} Minimum concentration of D-cycloserine needed for complete inhibition of growth on solid minimal medium. Data is from Curtiss et al. (2).

^{c} Minimum concentration of D-cycloserine needed for maximal lysis of cells growing in liquid minimal medium. Data is from Curtiss et al. (2).

^{d} MIC for L-cycloserine is defined in the same manner as that described for D-cycloserine (column 1).

Triton X-100-toluene (1:2, v/v) as described by Patterson and Green (8) and evaluated by Benson (1). The amount of accumulated amino acid is expressed as micromoles per milligram (dry weight) of cells. The data presented in this paper refer to the azide-sensitive transport of D-alanine, glycine, L-alanine, and D-cycloserine. The azide-insensitive component is minor (11).

RESULTS

Accumulation of L-alanine, D-alanine, and glycine by E. coli K-12, E. coli W, and the D-cycloserine-resistant mutants. The accumulations of L-alanine, D-alanine, and glycine in the strains derived from *E. coli* K-12 are shown in Fig. 1A and B. The amounts accumulated at 30 sec are estimates of the initial velocities and are tabulated in Table 2. In the first-step mutant, cyc^{r1}, the initial velocities of D-alanine and glycine uptake decreased to 33 and 42% of the parent value, respectively. In contrast, the initial velocity of L-alanine uptake in cyc^{r1} was 87% of that observed in the parent. Thus, a mutation markedly affecting the transport of D-alanine and glycine decreased the transport of L-alanine by only 13%.

In the second-step mutant, cyc^{r2}, the ability to accumulate D-alanine and glycine was further reduced to approximately 50% of the value obtained for cyc^{r1}, whereas the ability to accumulate L-alanine was not greatly affected (Fig. 1A). The third-step mutation caused a 25% reduction in the initial velocity of transport of L-alanine, D-
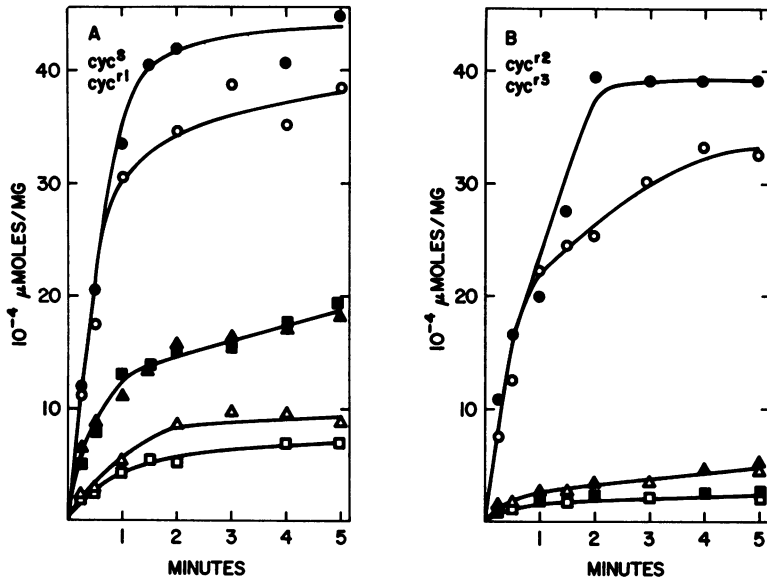
FIG. 1. *Accumulation of L-alanine, D-alanine, and glycine by* cyc^s (●, ■, ▲) *and* cyc^{r1} (○, □, △), *A, and by* cyc^{r2} (●, ■, ▲) *and* cyc^{r3} (○, □, △), *B. The concentrations are:* L-[^{14}C]alanine (●, ○), 2.6 × 10^{-5} M; D-[^{14}C]-alanine (■, □), 2.0 × 10^{-5} M; [^{14}C]glycine (▲, △), 2.5 × 10^{-5} M. *The accumulation of these amino acids was measured with a wash procedure at 25 C described in the Materials and Methods.*

TABLE 2. *Initial rates of accumulation of* L-, D-[^{14}C]-*alanine and* [^{14}C]glycine^a

Strain	Amino acid accumulated		
	L-Alanine	D-Alanine	Glycine
cys^s	20.1^b	7.8	8.6
cys^{r1}	17.4	2.6	3.6
cyc^{r2}	16.6	1.2	1.8
cyc^{r3}	12.6	0.9	1.4
E. coli W	13.5	7.8	10.1
D-5	10.1	0.6	0.6

^{a} Initial rates of accumulation are established from the data presented in Fig. 1A and B and Fig. 2.

^{b} Amount = 10^{-1} nmoles per mg (dry weight) of cells per 30 sec.

alanine, and glycine when compared with cyc^{r2}. The ability of D-5 (Fig. 2) to accumulate D-alanine and glycine decreased to 8 and 6%, respectively, of that observed for the parent. In contrast, the ability to accumulate L-alanine decreased to only 75%. Thus, the most resistant mutants, cyc^{r3} and D-5, lost a significant fraction of their ability to transport D-alanine and glycine, whereas they retained a large part of their ability to transport L-alanine.

Kinetic analysis of resistant mutants. In an analysis of the alanine transport systems in *E. coli* K-12 and *E. coli* W, a linear Lineweaver-Burk plot is observed for the azide-sensitive
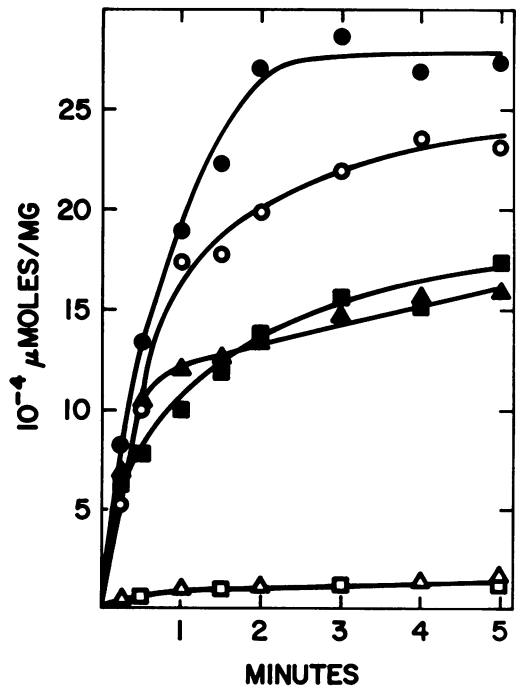

FIG. 2. *Accumulation of L-alanine, D-alanine, and glycine by E. coli W and D-5. The uptake of labeled amino acid by E. coli W* (●, ■, ▲) *and D-5* (○, □, △) *was measured as described in Fig. 1.*
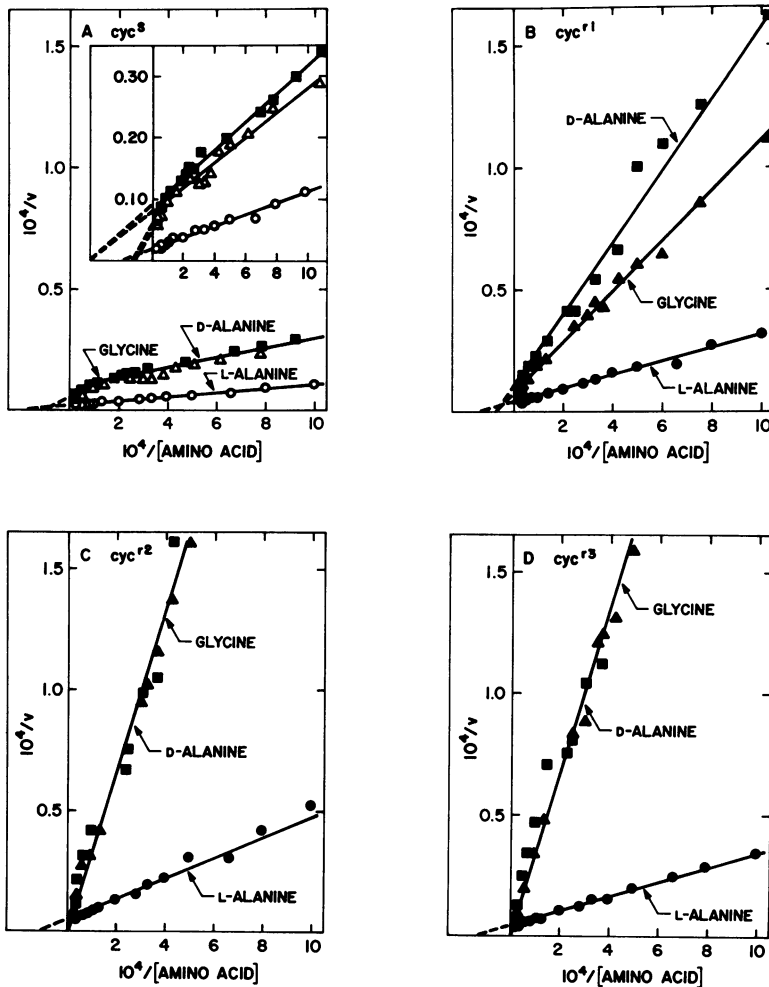
FIG. 3. *Lineweaver-Burk plots of the uptake of* L-[^{14}C]*alanine,* D-[^{14}C]*alanine, and* [^{14}C]*glycine in cyc^s (A), cyc^{r1} (B), cyc^{r2} (C), and cyc^{r3} (D). The cells were assayed as described in Fig. 1. All velocities have been corrected for the uptake of the corresponding amino acid by azide-treated cells. The velocity (v) is expressed as micromoles per milligram per 30 sec.*

transport of L-alanine. In the case of D-alanine and glycine, the double reciprocal plots are characterized by two intersecting line segments (11). These lines are referred to as the "high"- and "low"-affinity segments. This designation is not intended to imply the presence of two components with a particular affinity but serves only to characterize the transport system.

Lineweaver-Burk plots of the accumulation of L-alanine, D-alanine, and glycine by cyc^s, cyc^{r1}, cyc^{r2}, and cyc^{r3} are shown in Fig. 3A, B, C, and D. The double reciprocal plots for each organism are plotted on the same scale for ease of comparison. The insert in Fig. 3A emphasizes on an enlarged scale the two line segments for D-alanine

and glycine. The K_m values are listed in Table 3. The accumulation of D-alanine and glycine by cyc^{r1} is characterized by a single line segment with a Michaelis-Menten constant of 1.4×10^{-4} M. This value of K_m is similar to the value of the low-affinity segment mediating D-alanine ($K_m = 8.2 \times 10^{-5}$ M) and glycine ($K_m = 9.1 \times 10^{-5}$ M) uptake by cyc^s. Thus, the first-step mutation is characterized by the loss of the high-affinity D-alanine-glycine segment in the Lineweaver-Burk plot (Fig. 3B).

The second-step mutation is more difficult to characterize. Our observations indicate that this mutation has resulted in the loss of the saturable low-affinity line segment for D-alanine and gly-

TABLE 3. *Summary of values of $K_m{}^a$*

Strain	K_m (M)		
	L-Alanine	D-Alanine	Glycine
cyc^s	5.7×10^{-5}	2.5×10^{-5} 8.2×10^{-5}	2.5×10^{-5} 9.1×10^{-5}
cyc^{r1}	7.1×10^{-5}	— 14.0×10^{-5}	— 14.0×10^{-5}
cyc^{r2}	7.1×10^{-5}	— —	— —
cyc^{r3}	7.1×10^{-5}	— —	— —
E. coli W	9.1×10^{-5}	2.6×10^{-5} 8.7×10^{-5}	1.7×10^{-5} 9.9×10^{-5}
D-5	9.1×10^{-5}	— —	— —

^{a} Values for K_m for the accumulation of L- and D-alanine and glycine were established from the Lineweaver-Burk plots in Fig. 3A, B, C, D.

cine. The lack of a saturable component is based on an extrapolation that yields an infinite V_{max} and K_m, whereas the presence of a saturable component is based on a finite V_{max}. In Fig. 4, the sequential loss of the high and low-affinity line segments of D-alanine transport is illustrated. The Michaelis-Menten constant for L-alanine ($K_m = 7.1 \times 10^{-5}$ M) is the same for each mutant and similar to the value obtained for the parent (5.7×10^{-5} M). The V_{max} for L-alanine is 2.4, 2.1, and 2.5 nmoles per mg per 30 sec for cyc^{r1}, cyc^{r2},
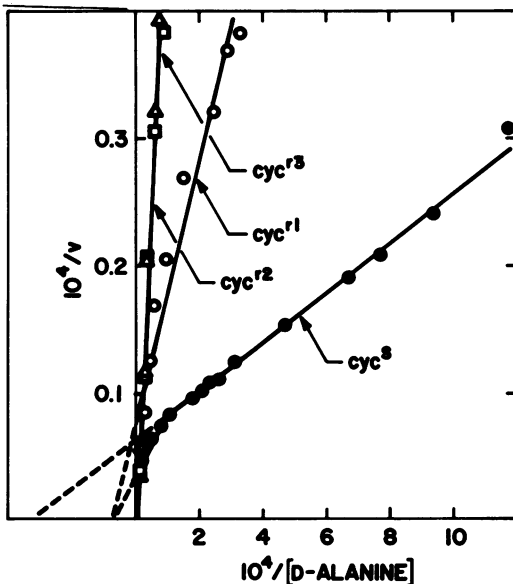

FIG. 4. *Comparison of Lineweaver-Burk plots of D-alanine uptake in cyc^s, cyc^{r1}, cyc^{r2}, and cyc^{r3}. The data are taken from a separate set of experiments than those presented in Fig. 3.*

and cyc^{r3}, respectively. These values are lower than the V_{max} (5.2 nmoles/mg) for L-alanine uptake by the parent.

The double reciprocal plots for the rate of accumulation of D-alanine by E. coli W and D-5 are shown in Fig. 5. These results indicate that D-5 is similar to cyc^{r2} and cyc^{r3}. This mutant is characterized by the loss of the high- and low-affinity line segments for D-alanine and glycine accumulation. The Michaelis-Menten constant (9.1×10^{-5} M) for L-alanine uptake is identical for the mutant and the parent.

In an effort to correlate the resistance to D-cycloserine with a defective transport system for the antibiotic, the ability of the D-cycloserine-resistant mutants cyc^{r1} and D-5 to accumulate D-cycloserine was established. As illustrated in Fig. 6 (A, B), the ability of these mutants to accumulate D-cycloserine was impaired. In the case of cyc^{r1}, it can be concluded that a significant portion of the ability to transport D-cycloserine was lost as a consequence of the mutation that results in the loss of the high-affinity line segment characteristic of the D-alanine-glycine transport system.

Growth of E. coli W and mutant (D-5) on L- and D-alanine. The conclusions drawn from the kinetic data may be tested in growth experiments by using either D- or L-alanine as the carbon source. As illustrated in Fig. 7A, E. coli W grew equally well on either L- or D-alanine. In contrast, mutant D-5 did not grow on D-alanine but grew on L-alanine at approximately the same rate as
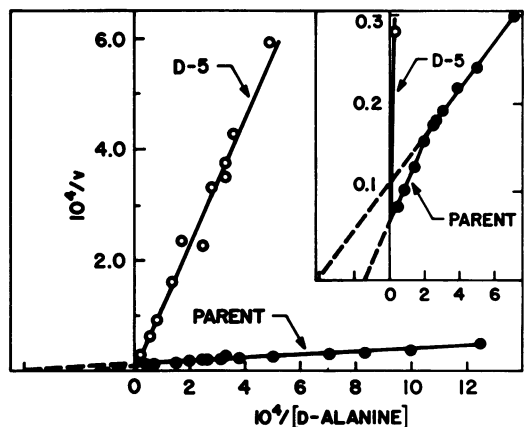

FIG. 5. *Lineweaver-Burk plots of the uptake of D-[^{14}C]alanine by E. coli W and D-5. The accumulation of labeled D-alanine was measured with the assay described in Fig. 1 by using a buffer wash at 4 C. As shown previously (11), the temperature of the buffer wash affects V_{max} but does not affect K_m. All velocities have been corrected for the uptake of D-[^{14}C]alanine by azide-treated cells.*
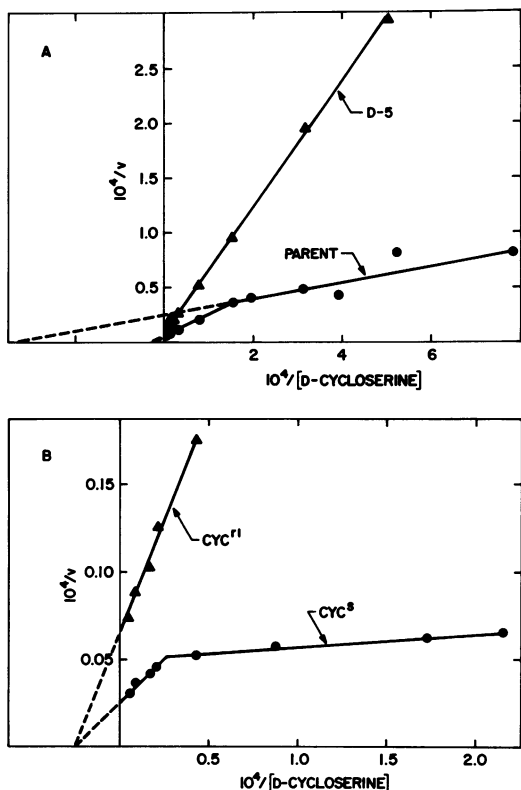
FIG. 6. *Lineweaver-Burk plots of the uptake of D-[14C]cycloserine in E. coli W and D-5 (A) and cyc* and cyc^{r1} (B). The rate of accumulation was measured by the procedure described in Materials and Methods. The velocities established for A were performed with a wash at 25 C and those in B were performed with a wash at 4 C (see legend to Fig. 5).*

FIG. 7. *Growth of E. coli W (A) and D-5 (B) on either glucose, L-alanine, or D-alanine. The growth medium contained the components described in the Materials and Methods with either 0.1 % glucose (●), 0.1% D-alanine (▲), or 0.1% L-alanine (■). Growth was monitored by measuring the turbidity at 650 nm at 37 C.*

the parent (Fig. 7B). Thus, the growth experiments with D-5 also suggest that the transport system for D-alanine is defective and that the ability to transport L-alanine is essentially intact. Since glycine cannot substitute as the sole carbon source, an analogous experiment with this amino acid was not possible.

Alanine racemase and D-alanine: D-alanine ligase (ADP). Acquisition of resistance to D-cycloserine may also result from elevated levels of the sensitive target enzymes alanine racemase (EC 5.1.1.1) and D-alanine: D-alanine ligase (ADP) (EC 6.3.2.4; reference 9). As shown in Table 4, the specific activities of these enzymes were essentially unchanged for each mutant. Thus, the absence of increased concentrations of the target enzymes in these mutants eliminated this mechanism for the acquisition of resistance to D-cycloserine. Modification of the target enzyme, another possible mechanism of D-cycloserine resistance, has not been eliminated as one of the mechanisms
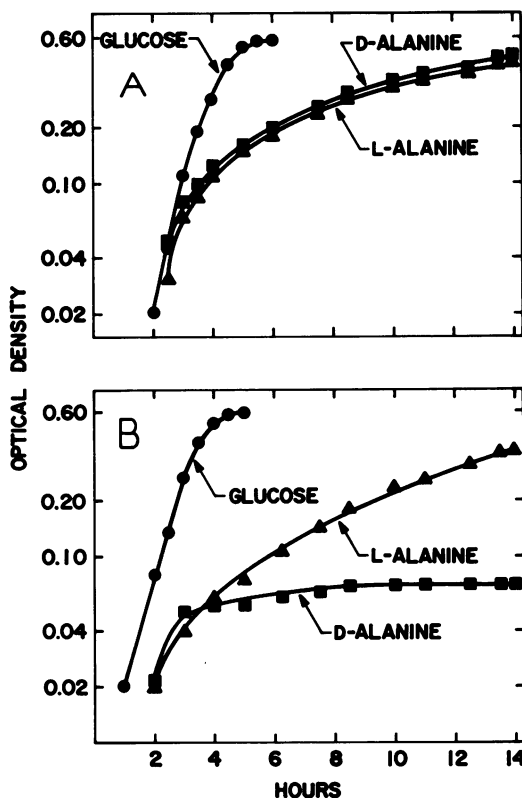
of D-cycloserine resistance in these mutants. In the case of the mutants of *Streptococcus* strain Challis that are resistant and have elevated enzyme levels, no differences in the target enzymes could be demonstrated (9).

DISCUSSION

Wargel et al. (11) suggested that the accumulation of D-alanine and the accumulation glycine in *E. coli* are related and appear to be separate from the transport of L-alanine. The data presented in this paper provide additional and more conclusive evidence in support of this suggestion and establish the amount of overlap between the two systems. A single mutation (cyc^{r1}) as well as the multiple mutations (cyc^{r2}, cyc^{r3}, and D-5) markedly affect the transport of D-alanine and glycine, whereas the transport of L-alanine is decreased to only a small extent.

In the first-step mutant, cyc^{r1}, the high-affinity line segment for D-alanine and glycine transport

TABLE 4. Specific activities of D-alanine: D-alanine ligase (ADP) and alanine racemase in extracts of cyc[s], cyc[r1], cyc[r2], and cyc[r3] [a]

Strain	D-Alanine: D-alanine ligase (ADP)	
cyc[s]	309[b]	57
cyc[r1]	318	51
cyc[r2]	309	50
cyc[r3]	364	48

[a] One-liter cultures of these strains were grown to an optical density of 0.40 in minimal medium containing 0.4% glucose. The cells were harvested and washed two times with 0.02 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.8. The cells (1.4 g) suspended in 10 ml of 0.02 M Tris-hydrochloride, pH 7.8, containing two drops of antifoam were disrupted with glass beads (9 g) in a mechanical cell homogenizer (Braun model MSK) at 4,000 cycles/min for 5 min. The suspension was centrifuged at 100,000 × g for 1 hr. Samples of the supernatant fraction were assayed for alanine racemase (4) and D-alanine: D-alanine ligase (ADP) (9).

[b] Values expressed as nanomoles per hour per milligram of protein.

has been lost. The ability to accumulate D-alanine and glycine has been further reduced by the second mutation. Lineweaver-Burk plots of D-alanine and glycine uptake by cyc[r2] and cyc[r3] indicate that the low-affinity line segment has been lost as a result of the second-step mutation. The locus for cyc[r1] is between the origin of Hfr H and the met[1] locus (2). Transduction experiments showed that the mutations conferring second- and third-step resistance are at least 0.5 min away from the first-step mutation. Thus, the loss of D-alanine and glycine transport activity occurs in at least two distinct genetic sites. In a genetic analysis of a serine auxotroph, Richter (Ph.D. Thesis, Univ. of Wisconsin, Madison, 1959) located a glycine transport gene near the met[1] region.

The mutant (D-5) isolated in this laboratory represents the highest level of D-cycloserine resistance that we have attained with E. coli. The mutant (D-5) is 80-fold resistant when compared with E. coli W. A Lineweaver-Burk analysis indicates that the high- and low-affinity segments of the D-alanine-glycine transport systems are missing. The results summarized in Table 2 indicate that >90% of the transport activity for D-alanine and glycine has been lost, whereas 75% of the transport activity for L-alanine is retained. A consequence of the impaired transport for D-alanine in mutant D-5 was readily observed in growth experiments. E. coli strain W can utilize either D- or L-alanine as a carbon source. In contrast, mutant D-5 is unable to grow on D-alanine, whereas it grows on L-alanine. Thus, a functioning transport system for D-alanine is required for growth on this isomer. The slower rate of

growth on L-alanine as the sole carbon source may be due to a partial loss of the total L-alanine transport activity.

The decreased transport of L-alanine in these mutants may reflect the amount of overlap between the systems, i.e., the amount of L-alanine that is transported over the D-alanine-glycine system. The results with mutants D-5 and cyc[r3] indicate that 25 and 35% of the L-alanine transport activity is lost in these mutants, respectively. In previous studies (11), it was observed that D-alanine, glycine, and D-cycloserine inhibit from 25 to 35% of the L-alanine accumulation by cyc[s]. The inhibition of L-alanine uptake by D-alanine, glycine, and D-cycloserine may represent the amount of L-alanine transported by the D-alanine-glycine systems in cyc[s]. Thus, the results with the D-cycloserine-resistant mutants and the inhibitor studies (11) suggest that 25 to 35% of the L-alanine accumulated at a concentration of 2.5 × 10^{-5} M is transported by the D-alanine-glycine system(s).

Schwartz et al. (10) observed that a D-serine-resistant mutant was defective for transport of D-serine, glycine, and L-alanine. From their results, it was concluded that these amino acids are on a common transport system. However, examination of the data reveals that the ability to transport glycine was reduced 93%, whereas the ability to transport L-alanine was only reduced by 65%. Kessel and Lubin (3) examined a D-serine-resistant mutant Trgly[-] for the ability to transport L-alanine, D-alanine, and glycine. In this strain, the uptake of glycine and D-alanine was depressed to about one-twentieth of the normal rate, whereas the uptake of L-alanine was depressed to only one-half of the normal rate. Moreover, Kessel and Lubin (3) isolated six additional mutants for resistance to D-cycloserine or D-serine. These mutants showed the same profile of uptake as Trgly[-]. Thus, the mutants examined by these workers, the mutants isolated by Curtiss et al. (2), and the one isolated in this laboratory all possess defective mechanisms for the transport of D-alanine and glycine. The decrease in the accumulation of L-alanine probably results from the overlap in L-alanine transport over the D-alanine-glycine transport system.

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