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Kinetic and Genetic Analyses of D-Cycloserine Inhibition and Resistance in *Escherichia coli*

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ABSTRACT

CURTISS, ROY, III (Oak Ridge National Laboratory, Oak Ridge, Tenn.), LEIGH J. CHARAMELLA, CLAIRE M. BERG, AND PAULA E. HARRIS. Kinetic and genetic analyses of D-cycloserine inhibition and resistance in *Escherichia coli*. J. Bacteriol. **90**:1238-1250.1965.—Wild-type cells of *Escherichia coli* growing at 37 C in mineral salts-glucose medium with vigorous aeration were lysed at maximal exponential rates by 10^{-4} to 10^{-2}M D-cycloserine. At concentrations above 2×10^{-2}M, D-cycloserine was bacteriostatic. Low levels of D-cycloserine (10^{-5} M) and pencillin G (10 units per ml) interacted synergistically to cause a rapid exponential rate of lysis. Spontaneous mutations to D-cycloserine resistance occurred in discrete steps at frequencies of 10^{-6} to 10^{-7} for each step. First-, second-, and third-step D-cycloserine-resistant mutants were lysed at maximal exponential rates by D-cycloserine concentrations of 10^{-3}, 3×10^{-3}, and 5×10^{-3} M, respectively. D-Alanine, L-alanine, and DL-alanyl-DL-alanine reversed D-cycloserine-induced lysis, in that order of effectiveness. On the basis of these observations, a D-cycloserine-enrichment cycling technique was developed for isolation of auxotrophic mutants. D-Cycloserine at 2×10^{-3} M was as efficient as penicillin G (1,000 units per ml) for mutant enrichment in *E. coli* and should be useful for isolation of mutants in penicillin-resistant microorganisms. Bacterial conjugation experiments indicated that all three mutations conferring D-cycloserine resistance were linked to the met_1 locus. Transduction experiments showed that the mutation conferring first-step resistance was at least 0.5 min away from the mutations conferring second- and third-step resistance. The latter two mutations possibly occurred in the same gene, since they were sometimes carried in the same transducing phage. Studies on expression of D-cycloserine resistance indicated that these mutations were neither dominant nor recessive to each other nor to the D-cycloserine-sensitivity allele. Each allelic state exerted its influence on the phenotype independently of the others. These results are discussed in terms of the known inhibition of alanine racemase and D-alanyl-D-alanine synthetase by D-cycloserine.

D-Cycloserine (D-4-amino-3-isoxazolidone) is a broad-spectrum antibiotic showing activity against many bacterial genera (Welch, Putnam, and Randall, 1955). Following the suggestion by Reilly (1956) that D-cycloserine interferes with amino acid metabolism, Bondi, Kornblum, and Forte (1957) showed that the action of D-cycloserine is prevented by DL-alanine. Shockman (1959) with *Streptococcus faecalis*, Zygmunt (1962) with *Escherichia coli* W, Moulder, Novosel, and Officer (1963) with members of the psittacosis group of microorganisms, and Zygmunt (1963) with *Mycobacterium smegmatis* and *M. phlei* have shown that this antagonism is competitive and is most efficiently brought about by D-alanine, the structural analogue of D-cycloserine. After the observation that D-cycloserine causes lysis of *E. coli* B in synthetic medium (Ciak and Hahn, 1959), Strominger, Ito, and Threnn (1960) were able to show that D-cycloserine is a competitive inhibitor of alanine recemase and D-alanyl-D-alanine synthetase in *Staphylococcus aureus*. Neuhaus and Lynch (1962) confirmed this finding in *Streptococcus faecalis*. The full activity of these two enzymes is necessary for synthesis of the cell-wall mucopeptide, which explains why D-cycloserine causes the accumulation of a uridine nucleotide-mucopeptide deficient in D-alanine (Strominger, Threnn, and Scott, 1959). D-Cycloserine does not interfere with alanine transport into the cell (Shockman, 1959; Strominger et al., 1960), nor does it interfere with the D-alanine activating enzyme described by Baddiley and Neuhaus (1960) (Neuhaus and Lynch, 1962) or with the incorporation of D-alanyl-D-alanine into the mucopeptide (Stominger et al., 1960). At very high concentration, however, D-cycloserine does interfere with several transaminases that require pyridoxal phosphate as coenzyme (Barbieri et al., 1960; Braunstein, Azarkh, and Seng, 1961).

TABLE 1. *Escherichia coli K-12 strains*

Strain no.	Mating type	D-Cycloserine response[a]	Genetic markers[b]												P1kc lysogeny	Derivation[c]
			thr	*ara*	*leu*	*T1*	*pro*$_1$	*pro*$_2$	*lac*	*T6*	*str*	*xyl*	*mel*$_1$	*thi*		
χ57	Hfr H	s	+	+	+	s	+	+	+	s	s	+	+	−	−	3000
χ235	Hfr Cav	s	+	+	+	s	+	+	+	s	s	+	−1	+	−	χ11
χ269	F⁻	r1														
χ270	F⁻	r1	+	−2	−	r	−1	+	−2	r	r	−2	+	−	−	χ131
χ271	F⁻	r1														
χ289	F⁻	s	+	+	+	s	+	+	+	s	s	+	+	+	−	χ15
χ316	F⁻	r1	+	+	+	s	+	+	+	s	s	+	+	+	−	χ289
χ323	F⁻	r1	+	+	+	s	+	+	+	s	s	−3	+	+	−	χ316
χ426	Hfr AB313	s	−	+	−	s	+	+	+	s	r	+	+	−	−	AB313
χ444	F⁻	r2	+	+	+	s	+	+	+	s	s	+	+	+	−	χ316
χ447	F⁻	r1	+	+	+	s	+	+	+	s	s	+	+	+	−	χ289
χ449	F⁻	r3	+	−3	−	r	−[d]	−[d]	−y	r	r	+	+	−	−	χ134
χ453	F⁻	r3	+	+	+	s	+	+	+	s	s	+	+	+	−	χ444
χ471	F⁻	s	+	+	+	s	+	+	+	s	s	+	+	+	+	χ289

[a] The *cyc*^{s}, *cyc*^{r1}, *cyc*^{r2}, and *cyc*^{r3} terms are phenotypic designations indicating sensitivity to and low, medium, and high resistance to D-cycloserine. All *cyc*^{r3} strains have three mutations to D-cycloserine resistance, *cyc*^{r2} strains have two such mutations, and *cyc*^{r1} strains only one mutation to D-cycloserine resistance. Mutants to D-cycloserine resistance were independently isolated unless a filial relationship is indicated under derivation.

[b] The markers are arranged in the order in which they occur on the chromosome. The following abbreviations are used: *thr*, threonine; *ara*, arabinose; *leu*, leucine; *T1* and *T6*, bacteriophages T1 and T6 (*T1*^{r} mutation also confers resistance to bacteriophage T5); *pro*, proline; *lac*, lactose; *ade*, adenine; *str*, streptomycin; *xyl*, xylose; *mtl*, mannitol; *ile*, isoleucine; *met*, methionine; *arg*, arginine; *thi*, thiamine; +, ability to synthesize or utilize; −, inability to synthesize or utilize; s, sensitive; and r, resistant. The subscript numbers used after some gene symbols (e.g., *pro*$_1$, *ade*$_3$, etc.) are cistron designations. Numbers used in the body of the table refer to independent mutant alleles. All strains except χ426 were nonlysogenic for bacteriophage λ.

[c] For derivation of strains χ11, χ15, χ131, and χ134, see Curtiss (1964); for 3000, see Curtiss (1965); and, for AB313, see Taylor and Adelberg (1960).

[d] χ449 contains a *pro*$_1$-*pro*$_2$ deletion mutation which also confers resistance to bacteriophages T3, T7, λ, and P1kc (Curtiss, 1965).

We became interested in D-cycloserine inhibition and resistance in *E. coli* for three reasons. First, we wanted a resistance mutation which would be useful as a genetic marker in conjugation experiments. Second, we wished to use mutations to D-cycloserine resistance to be able to reisolate selectively a genetically marked *E. coli* strain from mice with a nonselected microbial flora and from mice with a defined flora (Curtiss et al., *in preparation*). Third, if possible, we wanted to use D-cycloserine to enrich for auxotrophic mutants. This would provide a useful alternative to the penicillin method, especially when the organism is relatively penicillin-resistant.

In this communication, we report the isolation of several types of D-cycloserine-resistant mutants from *E. coli* K-12, rates of D-cycloserine-induced lysis in sensitive and in resistant mutants, and prevention of D-cycloserine-induced lysis by antagonists of D-cycloserine. We also have mapped the loci controlling D-cycloserine resistance by conjugation techniques and have done transduction experiments to analyze phenotypic expression of D-cycloserine resistance. A D-cycloserine-cycling enrichment procedure of broad applicability has been developed for isolation of auxotrophic mutants. A preliminary report has been given (Curtiss, Berg, and Charamella, Bacteriol. Proc., p. 17, 1965). Information on use of D-cycloserine-resistant mutants for studies on the intestinal flora of mice will be presented separately (Curtiss et al., *in preparation*).

MATERIALS AND METHODS

Bacteriophages. The methods for propagation and storage of the seven T phages, λ, and R17 have been given (Curtiss, 1965). Methods for P1kc bacteriophage are described under *Transduction methods.*

Bacterial strains. Derivatives of *E. coli* K-12 used are listed in Table 1. Cultures were maintained on Pennassay agar slants at 4C and were transferred at 3-month intervals.

Media and reagents. The synthetic media employed were: ML (liquid) and MA (agar) (Curtiss, 1965), Davis (Davis and Mingioli, 1950), and Medium E (Vogel and Bonner, *unpublished data*). These were supplemented with amino acids and vitamins at optimal concentrations. Carbohydrates were added to 0.5% and streptomycin sulfate to 200 μg/ml final concentrations. EMB agar (Curtiss, 1965), L broth and L agar (Lennox, 1955), and Penassay broth and agar (Difco) were employed as complete media. L soft agar had the same composition as L agar except that the agar concentration was at 0.65%.

D-Cycloserine (molecular weight, 102) was the gift of Eli Lilly & Co., Indianapolis, Ind. (The biologically produced D-isomer can be purchased inexpensively from several drug and biochemical supply firms.) It was prepared as a 0.2 M solution in sterile 0.1 M phosphate buffer (*p*H 8.0). A fresh solution was prepared for each experiment, and all plates containing D-cycloserine were poured 1 day before the experiment. Using inhibition of strain χ289 as the indicator, we found that D-cycloserine had a half-life of 4 to 5 days in MA (minimal agar) and about 2 days when dissolved in distilled water.

Sodium penicillin G was purchased from E. R. Squibb & Sons, New York, N.Y., and was prepared fresh for each experiment. The stock solution was made in sterile distilled water at 4×10^4 units per ml.

D-Alanine and L-alanine were purchased from Calbiochem and DL-alanyl-DL-alanine from Nutritional Biochemicals Corp., Cleveland, Ohio. They were prepared fresh as 0.2 M solutions in sterile distilled water.

Determination of rate of lysis. Strain χ289 and D-cycloserine-resistant mutants isolated from it were grown overnight with aeration at 37 C in ML (minimal liquid) with 0.5% glucose. This culture, still in log phase, was then diluted 1:200 into 20 ml of prewarmed ML. Six to eight of these subcultures were then aerated at 37 C for about 3 hr. When these reached 10^7 cells per milliliter, the experiment commenced. D-Cycloserine, with or without various antagonizing agents, was added at 4-min intervals. Dilutions were made in ML, followed by plating 0.05-ml amounts on MA containing 0.5% glucose. Plates were counted after 24-hr incubation at 37 C. To prevent D-cycloserine inhibition of colony formation, 0.05-ml samples were plated from dilutions of 1:50 or greater. It should be noted that data from these experiments are plotted in terms of cells surviving as a function of time of treatment. The slope of these survival curves is directly related to the rate of lysis.

Mating procedure. Conjugation methods have been described (Curtiss, 1965). The genotypes of recombinants were determined after purification on the medium used to select the recombinant class. Unselected nutritional, fermentation, and antibiotic-resistance markers were scored by replica plating (Lederberg and Lederberg, 1952).

Transduction methods. P1kc-transducing lysates were prepared by the confluent-plate lysis technique. L agar, L broth, and L soft agar contained 2.5×10^{-3} M $CaCl_2$ for all work with P1kc. Adsorption of P1kc was for 20 to 30 min at 37 C in L broth with log-phase cells at a density of 2×10^8 to 3×10^8 per milliliter. One-day-old L agar plates were inoculated by the soft-agar layer technique with 6×10^5 to 8×10^5 P1kc plaque-forming units. The phage was harvested from the plates after 6 hr of incubation at 37 C. P1kc lysates prepared in this manner had titers exceeding 10^{11} plaque-forming units per ml. They were stored over $CHCl_3$ in the refrigerator.

Strain χ471, a P1kc lysogenic derivative of χ289, was used as recipient for transduction of D-cycloserine resistance. Strain χ471 was grown in ML containing 1.25×10^{-3} M $CaCl_2$, and, when a titer of 2×10^8 cells per milliliter had been reached, P1kc grown on the *cyc*^{r3} strain χ453 was added at various multiplicities of infection. Aeration was continued at 37 C for 40 min, after which the mixture was diluted into prewarmed ML lacking $CaCl_2$. Dilutions and platings on MA containing various D-cycloserine concentrations were performed at intervals. Control experiments using χ471 with no P1kc and χ471 with P1kc grown on the homologous *cyc*^{s} strain χ289 were always performed. Plate counts were done after 48-hr incubation at 37 C.

RESULTS

D-Cycloserine inhibition of colony-forming ability on minimal agar. Figure 1 illustrates the concentrations of D-cycloserine necessary to prevent colony formation by *cyc*^{s}, *cyc*^{r1}, *cyc*^{r2}, and *cyc*^{r3} strains. Note that each strain mutated to higher levels of D-cycloserine resistance and that these mutants occurred at frequencies of 10^{-6} to 10^{-7}. No attempt was made to isolate *cyc*^{r4} mutants. All strains plated on MA containing noninhibitory concentrations of D-cycloserine formed normal-appearing colonies. As concentrations of D-cycloserine were increased, colonies became mucoid. Confluent mucoid growth was observed when very high cell densities were plated on an inhibitory concentration of D-cycloserine, whereas a 10-fold lower dilution gave discrete nonmucoid *cyc*^{r} mutant colonies. This was probably due to liberation of cell contents by lysed cells, which could then partially prevent the D-cycloserine inhibition of unlysed cells.

Response of cyc^r *mutants to bacteriophages and drugs.* Various *cyc*^{r1}, *cyc*^{r2}, and *cyc*^{r3} strains were no different from *cyc*^{s} strains in sensitivity to the seven T phages, λ, R17, and P1kc, or in sensitivity to 22 antibiotics. Antibiotic sensitivities were tested by placing Unidisks (Difco) over soft-agar layers containing log-phase cells on Penassay agar plates. Changes in sensitivity to penicillin, strep-

tomycin, and chlortetracycline were checked thoroughly, without any discernible differences noted between *cyc*^{s} and *cyc*^{r} strains.

D-Cycloserine-induced lysis in liquid medium. To determine concentrations of D-cycloserine needed for the mutant enrichment procedure described later in this paper, it was necessary to measure rates of lysis induced by D-cycloserine. At the beginning of these studies, it became apparent that lytic rates were dependent on the "age" of the D-cycloserine solution, on the growth rate of the strain being tested, and on the initial cell density of the culture. As pointed out in Materials and Methods, it was necessary to use freshly prepared D-cycloserine in 0.1 M phosphate buffer (*p*H 8.0) to eliminate the first variable. A comparison of the slopes of the χ289 control growth curves (*b* in the formula $N = e^{bt}$) and the slopes of the survival curves in 10^{-4} M D-cycloserine ($-b$ in the formula $N = e^{-bt}$) for 13 determinations indicated that slow growth rates were associated with suboptimal rates of lysis. To minimize this effect, we used vigorously aerated log-phase cultures.

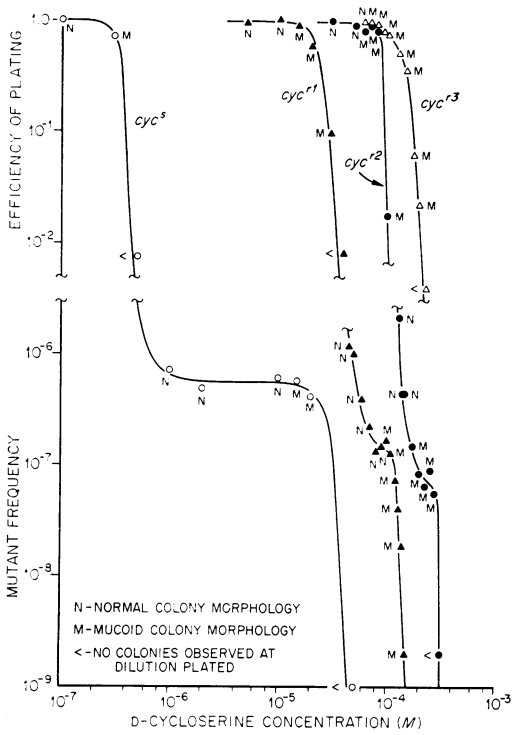

FIG. 1. *Inhibition of colony formation on minimal agar by D-cycloserine. χ289 (cyc^s), χ316 (cyc^{r1}), χ444 (cyc^{r2}), and χ453 (cyc^{r3}) grown at 37 C with aeration to log phase in ML with glucose were used for these experiments. Plate counts were made after 48 hr and again after 72 hr of incubation at 37 C. There were few if any changes in colony counts. Mucoid colony types varied and consisted of different sizes with some opaque and some transparent. χ316 was isolated from the experiment with χ289; χ444 from the experiment with χ316; χ453 from the experiment with χ444.*

We observed that in experiments with initial cell densities above 5×10^7 cells per milliliter there was marked decrease in the rate of lysis after several hours. Cells surviving several hours of treatment were not *cyc*^{r} mutants but presumably had been supplied, by the cells which had lysed, with compounds which could prevent D-cycloserine-induced lysis. It was also noted that with high initial cell densities there was considerable clumping. To eliminate these difficulties, we used cultures with initial cell densities of about 10^7 cells per milliliter.

Figure 2A and B shows results of two experiments with the *cyc*^{s} strain χ289. It can be noted that rates of lysis increased over the range of D-cycloserine concentrations from 5×10^{-6} to 5×10^{-5} M, remained constant from 5×10^{-5} M up to 1.2×10^{-2} M, and then declined at higher concentrations. D-Cycloserine at a concentration of 3×10^{-2} M was bacteriostatic (Fig. 2B).

Figure 3 shows a plot which summarizes all the lytic rate determinations done for χ289 at various D-cycloserine concentrations.

It has been reported that D-cycloserine acts synergistically with penicillin and several other antibiotics in complex media (Harris et al., 1955) and in mice (Cuckler et al., 1955). To verify this and to compare rates of lysis induced by penicillin G with those induced by D-cycloserine, several experiments like those shown in Fig. 2C were performed. The synergistic effect was most striking when suboptimal concentrations of penicillin (10 units per ml) and D-cycloserine (10^{-5} M) were combined. Penicillin at 1,000 units per ml (Fig. 2C) caused lysis at rates comparable to those obtained with optimal concentrations of D-cycloserine (Fig. 2A and B).

Since *cyc*^{r1} mutants occurred at frequencies of 10^{-6} to 10^{-7} (Fig. 1), it was necessary to determine D-cycloserine concentrations needed to lyse these mutants. Otherwise, the use of suboptimal D-cycloserine concentrations in the auxotroph enrichment procedure would enrich for *cyc*^{r1} mutants. Results of these experiments with *cyc*^{r1}, *cyc*^{r2}, and *cyc*^{r3} mutants are presented in Fig. 4. Note that D-cycloserine will lyse all mutant types at a concentration of 2×10^{-3} to 3×10^{-3} M.

Prevention of D-cycloserine-induced lysis by D-alanine. Figure 5 presents data on prevention of D-cycloserine-induced lysis of a *cyc*^{s} strain by D-alanine. Note that 50% antagonism of 10^{-4} M D-cycloserine was obtained with about one-

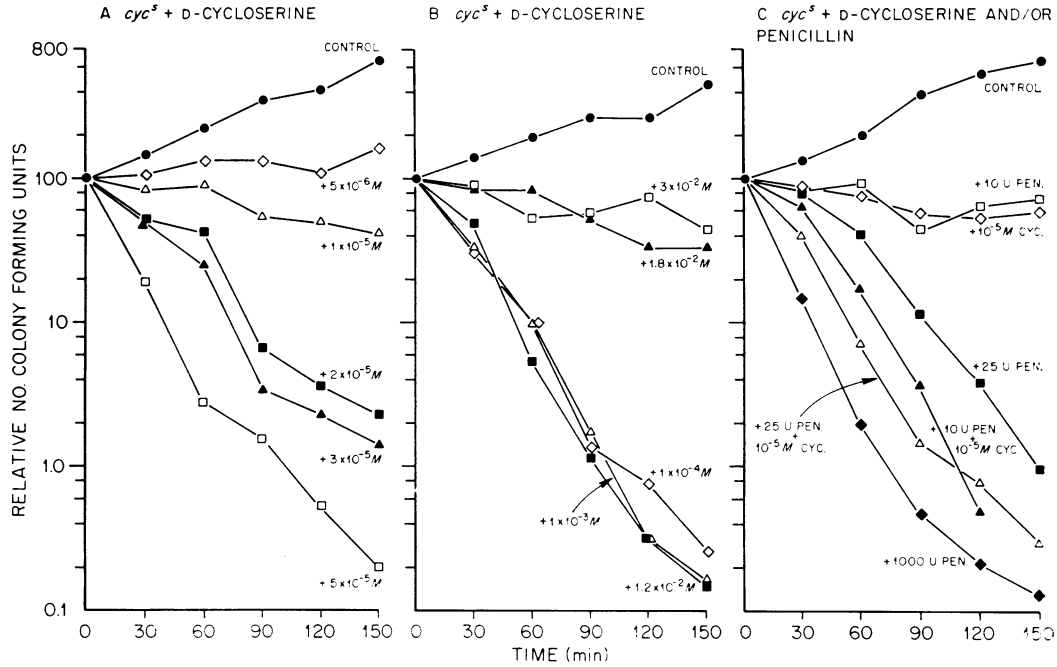

FIG. 2. *D-Cycloserine-induced lysis in the cyc⁵ strain χ289. A and B: χ289 tested with varying D-cycloserine concentrations. C: Synergistic interaction between penicillin G and D-cycloserine.*

twentieth of this concentration of D-alanine (Fig. 5A). As the D-cycloserine concentration was increased (Fig. 5B and C), the antagonism of D-cycloserine by D-alanine became less efficient. A quantitative analysis and comparison of the data on antagonism of D-cycloserine-induced lysis will be presented in the Discussion.

Figure 6 presents data on D-alanine antagonism of D-cycloserine-induced lysis in *cyc*^{r1}, *cyc*^{r2}, and *cyc*^{r3} cultures. With the *cyc*^{r1} strain χ316, 2×10^{-3} M D-alanine was required to give about 50% antagonism of lysis by 10^{-3} M D-cycloserine (Fig. 6A). In contrast, D-alanine was efficient in preventing D-cycloserine-induced lysis of the *cyc*^{r2} and *cyc*^{r3} strains χ444 and χ453 (Fig. 6B and C). Recall that the mutation to D-cycloserine resistance in χ316 is also present in χ444 and χ453. Since the results with χ316 (Fig. 6A) were out of line with those shown in Fig. 5 and Fig. 6 (B and C), we repeated the experiment four times over a period of 5 months. Similar results were obtained in each experiment. We then repeated the experiment with χ447, another *cyc*^{r1} mutant independently isolated from χ289. The results obtained with this strain were almost identical to those shown in Fig. 6A for χ316. Thus, the low efficiency of antagonism of D-cycloserine-induced lysis by D-alanine in *cyc*^{r1} strains is probably characteristic of first-step mutants and is a property which disappears upon introduction of a second mutation to give the *cyc*^{r2} phenotype.

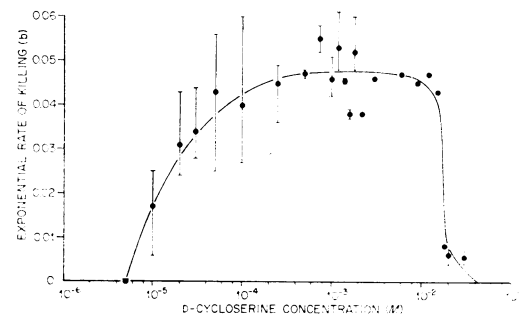

FIG. 3. *Summary of exponential rates of lysis induced by various concentrations of D-cycloserine in χ289. The exponential rate of killing is expressed in units of b in the equation* $N = e^{-bt}$ *in which N = per cent survival and t = time in minutes. The slopes b were calculated from data like those presented in Fig. 2A and B by the method of least squares. Seventy determinations are included, and for those concentrations of D-cycloserine which were tested two or more times the ranges are given.*

Prevention of D-cycloserine-induced lysis by L-alanine and by DL-alanyl-DL-alanine. Figure 7A shows the results of a representative experiment on L-alanine antagonism of D-cycloserine-induced lysis in the *cyc*^{s} strain χ289. Note that L-alanine is

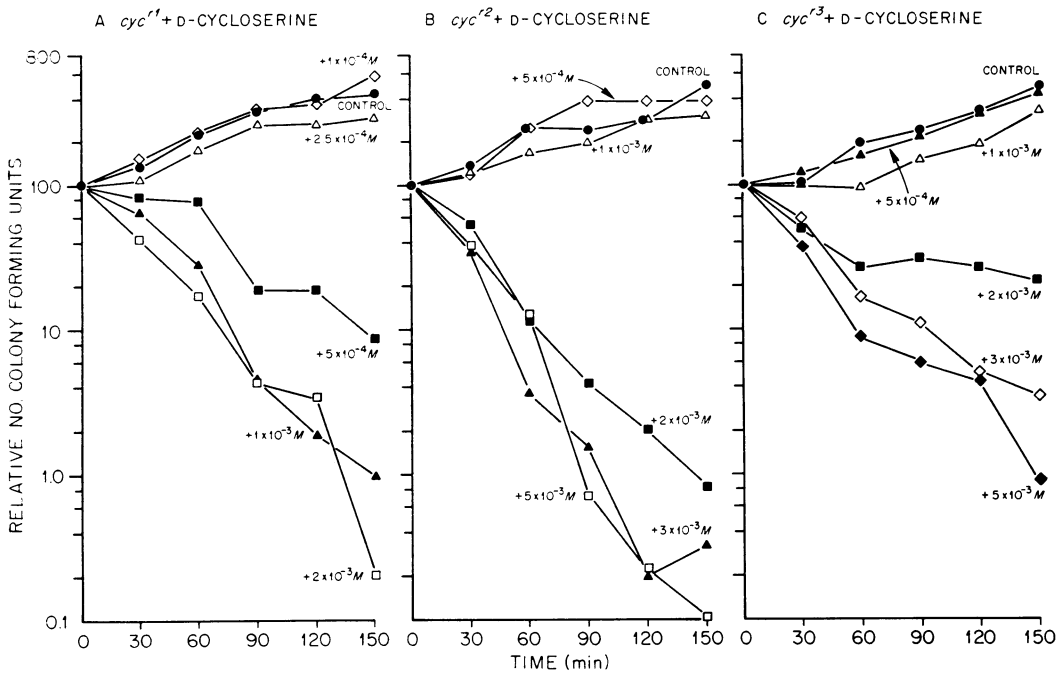

FIG. 4. *D-cycloserine-induced lysis in cyc^{r1}(A), cyc^{r2}(B), and cyc^{r3}(C) strains. χ316, χ444, and χ453 were the bacterial strains used.*

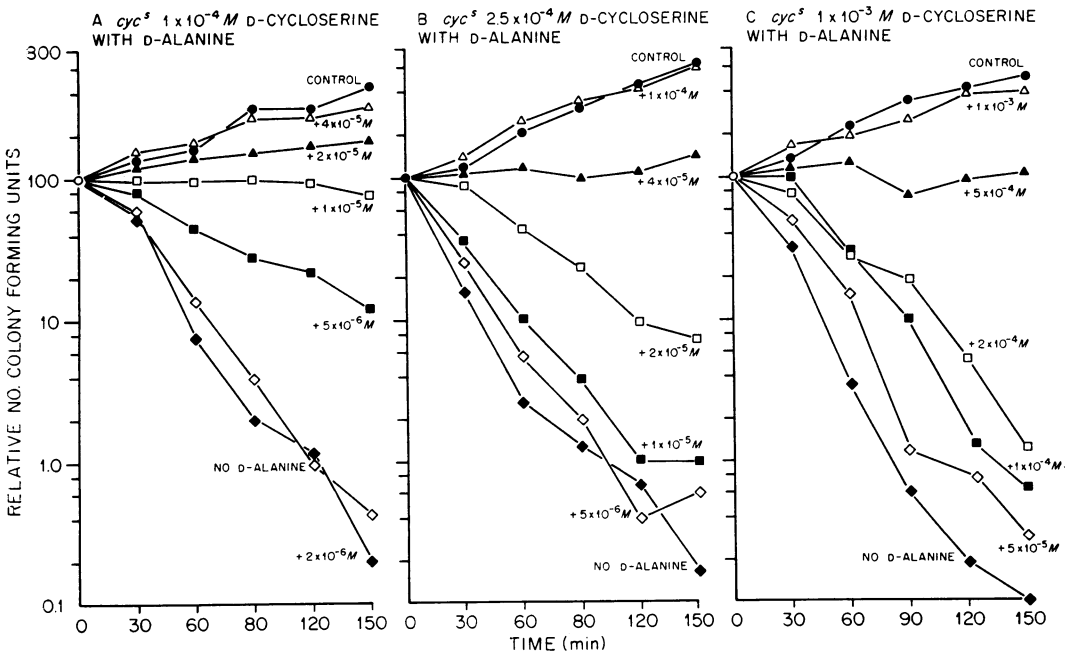

FIG. 5. *D-Alanine antagonism of lysis induced by 10^{-4}M (A), 2.5 × 10^{-4}M (B), and 10^{-3}M (C) D-cycloserine in the cyc^{s} strain χ289.*

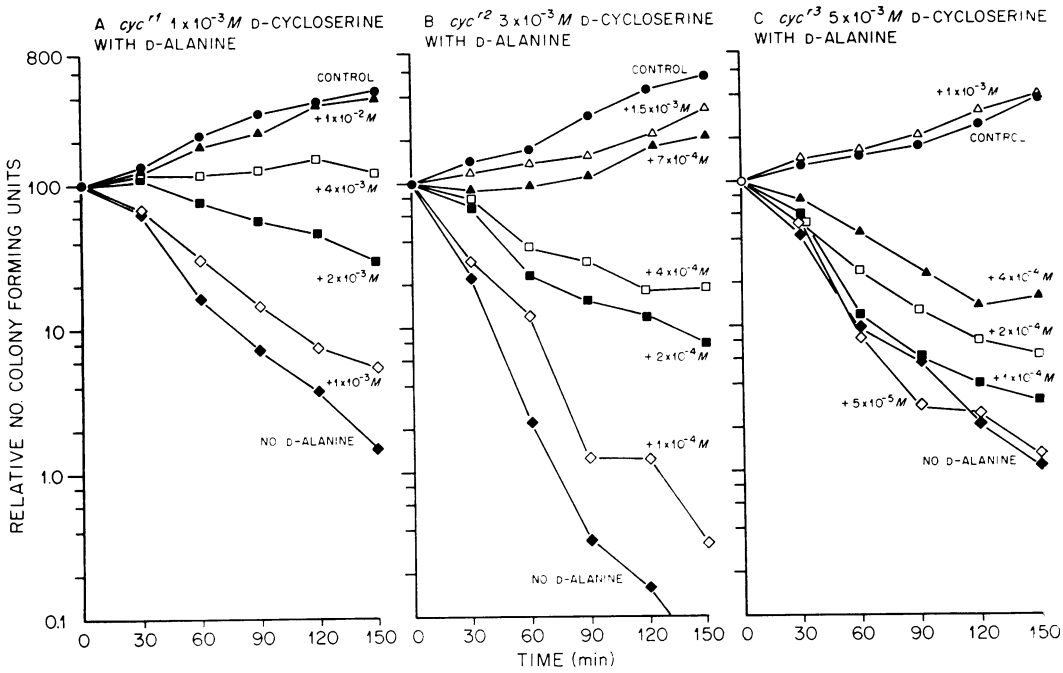

FIG. 6. *D-Alanine antagonism of lysis induced by 10^{-3}M D-cycloserine in χ316 (A), by 3×10^{-3}M D-cycloserine in χ444 (B), and by 5×10^{-3}M in χ453 (C).*

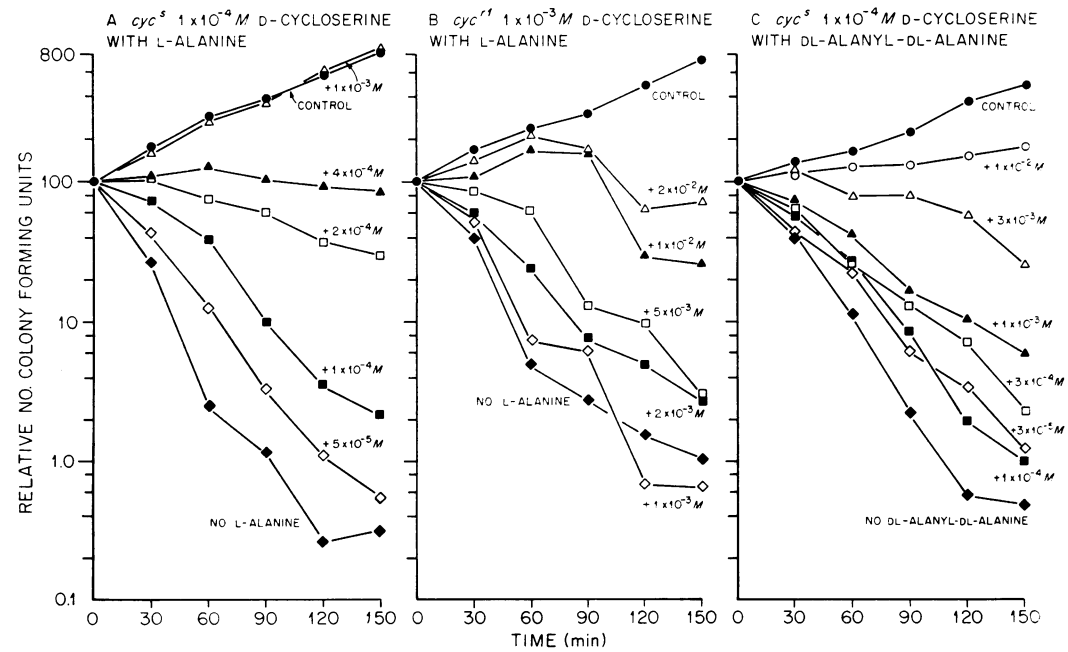

FIG. 7. *L-Alanine antagonism of lysis induced by 10^{-4}M D-cycloserine in χ289 (A) and by 10^{-3}M D-cycloserine in χ316 (B), and antagonism of 10^{-4}M D-cycloserine-induced lysis by DL-alanyl-DL-alanine in χ289 (C).*

much less efficient than D-alanine in preventing lysis by D-cycloserine (compare Fig. 5A and 7A). Similarly, L-alanine is less efficient than D-alanine in antagonizing D-cycloserine-induced lysis in the *cyc*^{r1} strain χ316 (compare Fig. 6A and 7B). We also did an experiment with the *cyc*^{r2} strain χ444, which showed that antagonism of D-cycloserine-induced lysis by L-alanine was more efficient than in the *cyc*^{r1} strain χ316. Thus, cells with the *cyc*^{r1} phenotype respond less well to the D-cycloserine-antagonizing action of D- and L-alanine than cells with *cyc*^{s} and *cyc*^{r2} phenotypes.

Since the sequence of reactions for synthesizing D-alanyl-D-alanine is inhibited by D-cycloserine, it would be expected that this dipeptide would prevent D-cycloserine inhibition. Although DL-alanyl-DL-alanine does this, it is obvious that it is the least efficient D-cycloserine antagonist used (Fig. 7C).

Development of a mutant-enrichment cycling technique using D-cycloserine and penicillin. The general method outlined below was based on the observations of Lederberg (1950), Gorini and Kaufman (1960), and Lubin (1962). The actual specifications were based on the foregoing results and upon direct experimentation during mutant isolation. The steps of the D-cycloserine cycling technique were as follows. (i) Treat bacterial suspension with mutagen. (ii) Grow treated suspension with aeration for at least four to five generations in minimal medium supplemented to permit growth of desired mutant type(s). (iii) Wash cells by centrifugation or by collection on a membrane filter (0.45-μ pore diameter) with medium which will support growth only of nonmutants. Resuspend in this medium at a density of 5×10^6 cells per milliliter. (iv) Aerate for 1 to 3 hr to starve mutant cells and to allow nonmutants to begin growing. (v) Freshly prepare D-cycloserine in 0.1 M phosphate buffer (*p*H 8.0) and add to culture (10^7 to 2×10^7 cells per milliliter) to 2×10^{-3} M. Continue aerating for 2 to 3 hr (depending on growth rate). (vi) Collect unlysed cells by filtration on a membrane filter, and wash. Resuspend cells by aerating filter in minimal medium supplemented for desired mutant type(s). (vii) Repeat steps ii to vi or dilute (if necessary), plate on supplemented minimal medium, and then replica-plate.

The above method has been used to obtain auxotrophic mutants which occurred spontaneously or which were induced by nitrogen mustard, X rays, and ultraviolet (UV). We have isolated mutants unable to synthesize proline, methionine, leucine, tyrosine, isoleucine, isoleucine and valine, serine, histidine, ornithine, arginine, lysine (from diaminopimelic acid), phenylalanine, adenine, uracil, pantothenic acid, and pyridoxal. One or several mutant types can be enriched for simultaneously. We have also successfully employed general enrichment for any type of auxotroph by using broth as the complete medium.

Success in isolation of mutants was not dependent on the medium used, and the minimal media of Davis and Mingioli (1950), Vogel and Bonner (*unpublished data*), and Curtiss (1965) were equally satisfactory. In allowing for segregation and expression after mutagen treatment (step ii), care should be taken to prevent the culture from reaching stationary phase before proceeding to step iii. As the culture becomes denser, the supplement required by the desired mutant type may become exhausted, which would permit the nonmutant cells to overgrow the culture. We have circumvented this possible problem by giving mutagen treatment in late afternoon and then inoculating supplemented minimal medium with 10^3 to 10^4 surviving cells per milliliter. Such a culture will reach 10^7 to 10^8 cells per milliliter by the next morning and will be ready for step iii.

We have used the microfilter apparatus manufactured by Carl Schleicher & Schuell Co., Keene, N. H., with 25-mm diameter membrane filters for steps iii and vi. In this way, washing could be accomplished very rapidly. When the filters were kept moist during filtration, cells came off readily during aeration of the suspending medium. To keep manipulations to a minimum, we collected only enough cells on the filter in step iii to yield a titer of 5×10^6 cells per milliliter when the entire filter was placed in the suspending minimal medium. The filter was then left in the medium until refiltration during step vi.

It is important to keep the titer below 2×10^7 cells per milliliter upon addition of D-cycloserine (step v). This is necessary both to prevent initiation of growth of mutants by cellular contents from lysed cells and to prevent antagonism of D-cycloserine-induced lysis by liberated D- and L-alanine.

Penicillin G at a concentration of 1,000 to 2,000 units per ml was as satisfactory for mutant enrichment by the procedure outlined above as was D-cycloserine. A combination of D-cycloserine and penicillin G should be very efficient in view of their synergistic interaction (Fig. 2C), although we have not tried this.

Mapping of cyc^{r} *loci by conjugation.* One of our reasons for isolating *cyc*^{r} mutants was to seek a resistance mutation which would be unlinked to the *str* locus. During bacterial conjugation, a given Hfr strain (donor) transfers its chromosome in a linear, oriented manner to an F^- strain (recipient). For another study, we had been interested in the chromosome region between the *xyl* and *his* loci, which is the chromosome region

transferred at high frequency by Hfr strains like Hfr AB313 (Taylor and Adelberg, 1960). Unfortunately, the *str* locus is transferred soon after *xyl*$^+$ by donors like Hfr AB313 (Fig. 8), thus preventing the use of the F$^-$ *str*^{r} marker to select against the Hfr.

In our first experiment, χ323, a *xyl*$^-$ *cyc*^{r1} F$^-$ strain, was mated with Hfr AB313 (*thr*$^-$ *leu*$^-$). After 60 min, we plated the diluted mixture on minimal agar lacking threonine and leucine and containing xylose as sole carbon source. There were no *cyc*^{s} recombinants found among 200 *xyl*$^+$ *thr*$^+$ *leu*$^+$ recombinants tested. Therefore, the *cyc*^{r} mutation was not in the chromosome region transferred at high frequency at Hfr AB313. Our next matings were between Hfr H and three F$^-$ *leu*$^-$ *str*^{r} strains with independently isolated *cyc*^{r1} mutations (χ269, χ270, χ271). All *leu*$^+$ *str*^{r} recombinants tested (100 from each mating) remained *cyc*^{r1} in phenotype. Since the results with Hfr H and Hfr AB313 suggested that the *cyc*^{r1} locus was between their respective origins of transfer (Fig. 8), it was decided to do matings with Hfr Cav (*leu*$^+$ *met*$_1$$^-$ *cyc*^{s} *str*^{s}). The same three F$^-$ strains used in mating with Hfr H were employed, and *leu*$^+$ *str*^{r} recombinants were selected for in the presence of methionine. The Hfr Cav *cyc*^{s} marker was inherited with the *met*$_1$$^-$ marker 89, 95, and 94% of the time in matings with χ269, χ270, and χ271, respectively. The *cyc*^{s} marker was also inherited in association with the *leu*$^+$ marker more often than was the *met*$_1$$^-$ marker. Thus, it is our conclusion that the *cyc*^{r1} locus is between the origin of Hfr H and the *met*$_1$ locus (Fig. 8).

To determine whether the three mutations resulting in the *cyc*^{r3} phenotype were linked, several crosses were done with χ449 (F$^-$ *cyc*^{r3} *leu*$^-$ *met*$_1$$^+$ *str*^{r}). All *leu*$^+$ *str*^{r} recombinants obtained in crosses with Hfr H remained as resistant to D-cycloserine as the parent F$^-$, χ449. In a cross between χ449 and Hfr Cav, the number of *leu*$^+$ *str*^{r} recombinants was low, but, of 40 analyzed, 17 were *cyc*^{s} *met*$_1$$^-$ and two were *cyc*^{s} *met*$_1$$^+$. There were no *cyc*^{r} *met*$_1$$^-$ recombinants, and no recombinants with D-cycloserine resistance intermediate between the two parents were observed. Thus, it would appear that all three mutants resulting in the *cyc*^{r3} phenotype are linked.

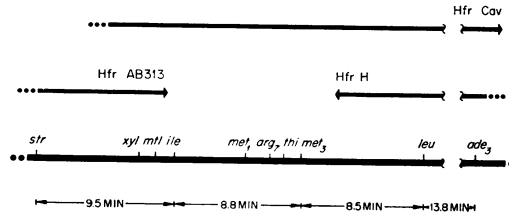

FIG. 8. *Partial chromosome map of Escherichia coli K-12 showing the origins of transfer for the donor strains Hfr H, Hfr AB313, and Hfr Cav. Each Hfr strain transfers its chromosome from a unique starting point (origin) and at a constant rate at 37 C. The time at which a given Hfr marker is first transferred to an F$^-$ recipient is the time of entry for that marker. Thus, distances between genetic loci are measured as the differences in their times of entry.*

Transduction of D-cycloserine resistance. P1kc grown on the *cyc*^{r3} strain χ453 was used for trans-

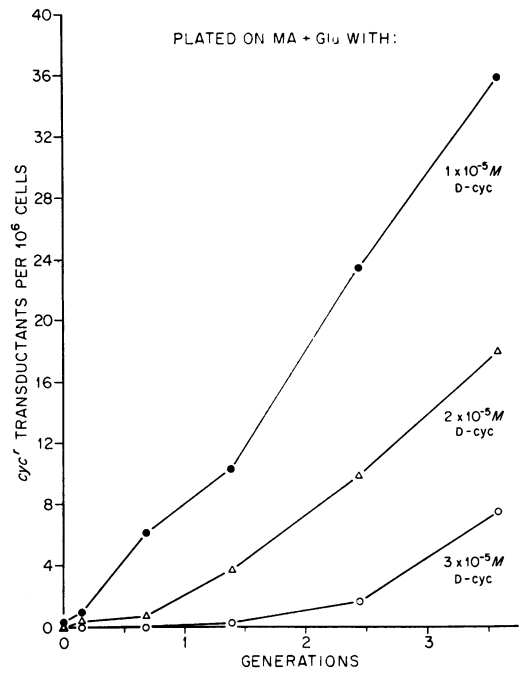

FIG. 9. *Transduction of the cyc*^{s} *strain χ471 with P1kc grown on the cyc*^{r3} *strain χ453. χ471 was at an initial density of 3.0 × 10*^{8} *cells per milliliter upon addition of the P1kc to a final titer of 1.5 × 10*^{10} *phage per milliliter. (The high multiplicity of infection was necessary because of poor adsorption in ML.) The phage-bacterial mixture was diluted 1:2 at 40 min and again at 140 min after commencement of the experiment. χ471 cultures with no added phage and with P1kc grown on χ289 were used as controls, with the same results. The mean cyc*^{r1} *mutant frequencies obtained for each time and on each type of medium were subtracted from the values obtained when P1kc had been grown on χ453. The corrected values are plotted. The mean mutation frequencies for platings at 20, 40, 70, 100, 140, and 180 min were 1.53 × 10*$^{-6}$ *(10*$^{-5}$ *MD-cycloserine), 0.93 × 10*$^{-6}$ *(2 × 10*$^{-5}$ *MD-cycloserine), and 0.47 × 10*$^{-6}$ *(3 × 10*$^{-5}$ *MD-cycloserine). The 20-min plating was used as the zero generation point.*

duction experiments with the *cyc*^{s} P1kc lysogenic strain χ471 as recipient. These experiments were initiated to determine whether all three mutations resulting in the *cyc*^{r3} phenotype were contransducible, and to gain some information on phenotypic expression of D-cycloserine resistance.

The results of such an experiment are presented in Fig. 9. No transductants capable of colony formation on minimal agar with 5×10^{-5} M D-cycloserine were obtained. Thus, there was no cotransduction of even the two mutations resulting in a *cyc*^{r2} phenotype, since *cyc*^{r2} cells can form colonies at 5×10^{-5} M D-cycloserine (Fig. 1).

It is more difficult to arrive at the conclusions on expression of D-cycloserine resistance. When plating was on MA with 10^{-5} M D-cycloserine, there was a linear increase in the number of transductants (Fig. 9). If the mutation resultine in the *cyc*^{r1} phenotype was dominant, then there should have been an immediate increase in the number of transductants with the rapid attainment of a plateau number, presuming, of course, that expression at the genic level would be immediate whether the trait was recessive or dominant. This assumption is based on the belief that D-cycloserine resistance is manifested at the enzyme level (see below) and on the principles set forth by Pardee, Jacob, and Monod (1959) for the expression of the enzyme β-galactosidase. If this mutation were recessive, then there should have been a delay in the apperance of transductants proportional to the time necessary to segregate, from multinucleate cells, daughters no longer having any genes conferring sensitivity to D-cycloserine. Since neither of these situations was observed (Fig. 9), it is concluded that the alleles responsible for the *cyc*^{s} and *cyc*^{r1} phenotypes act independently. With independent action, alleles present in the same cell would each contribute to the phenotype. Based on studies with the independently acting alleles *azi*^{r} and *azi*^{s} and *val*$_1^{r}$ and *val*$_1^{s}$ in both haploid and stable partial diploid strains of *E. coli* (Curtiss, 1962; 1964; *unpublished data*), it would be predicted that the number of transductants expressing the *cyc*^{r1} phenotype would depend on the concentration of D-cycloserine in the selecting medium. Thus, if low D-cycloserine concentrations had been used (10^{-6} M), the curve for the expression of *cyc*^{r1} should have simulated the result expected of a dominant mutation. On the other hand, with the use of higher concentrations of D-cycloserine (3×10^{-5} M), one should theoretically obtain the results predicted for a recessive mutation—a result confirmed by experiment (Fig. 9).

Three *cyc*^{r1} transductants obtained from the above-described experiment were used for further transduction experiments with P1kc grown on the *cyc*^{r3} strain χ453. In these transduction experiments, MA plates with from 5×10^{-5} to 2×10^{-4} M D-cycloserine were used, and platings were made after 0 (40 min after adding phage), 1.8, and 3.3 generations of growth. (The recipient cultures only grew 0.25 generation during the 40-min phage-adsorption period.) In all three of these experiments, transductants were obtained which grew on MA up to 1.5×10^{-4} M D-cycloserine. Since the recipient cultures, without added transducing phage, contained no mutants able to form colonies on MA with over 8×10^{-5} M D-cycloserine, it is concluded that there was cotransduction of the two mutations, which would result in the *cyc*^{r3} phenotype. From a comparison of the frequencies of transductants forming colonies on MA with 1.4×10^{-4} to 1.5×10^{-4} M D-cycloserine and on MA with 7×10^{-5} M D-cycloserine, one can estimate that the two mutations resulting in the *cyc*^{r3} phenotype were cotransduced 5 to 10% of the time.

From the results obtained on expression of the *cyc*^{r2} and *cyc*^{r3} phenotypes, it is concluded that the mutations conferring these properties are also independently acting. Over 25% of the maximal number of transductants capable of growth on MA with 5×10^{-5} to 7×10^{-5} M D-cycloserine formed colonies when plating was done at zero generations. On the other hand, transductants capable of colony formation on MA with 1.2×10^{-4} M D-cycloserine were not detected until plating after 1.8 generations of growth.

DISCUSSION

It can be noted by a comparison of Fig. 1 with Fig. 2 and 4 that D-cycloserine is more efficient in inhibiting colony formation than in causing lysis. Table 2 gives a comparison of D-cycloserine concentrations that cause various levels of inhibition on solid and in liquid media. The maximal concentrations for no inhibition of growth in ML are four to six times higher than the maximal noninhibitory concentrations tolerated on solid medium. Similarly, the D-cycloserine concentrations which permit stasis in liquid medium are 5 to 10 times higher than the minimal concentrations needed to inhibit colony formation. Our experiments suggest no explanation for the greater "sensitivity" of *cyc*^{s}, *cyc*^{r1}, *cyc*^{r2}, and *cyc*^{r3} cells on solid medium. Possibly the difference is due to the increased length of time allowed for D-cycloserine to interact with cells on solid medium.

The bacteriostatic effect of D-cycloserine at concentrations of 2×10^{-2} M and above (Fig. 2B and 3) is most likely due to inhibition of transaminases (Barbieri et al., 1960; Braunstein et al., 1961). The stoppage of transaminase activity would cause protein synthesis to cease, which

TABLE 2. *Comparison of D-cycloserine concentrations to inhibit under different growth conditions**

Strain genotype	Solid medium (MA)		Liquid medium (ML)		
	Maximal concn with no inhibition	Minimal concn for complete inhibition	Maximal concn with no inhibition	Concn for stasis	Minimal concn for maximal lysis
	M	*M*	*M*	*M*	*M*
cyc^s	3×10^{-7}	6×10^{-7}	—	5×10^{-6}	5×10^{-5}
cyc^{r1}	2×10^{-5}	4×10^{-5}	1×10^{-4}	3.5×10^{-4}	1×10^{-3}
cyc^{r2}	8.5×10^{-5}	1.2×10^{-4}	5×10^{-4}	1.2×10^{-3}	3×10^{-3}
cyc^{r3}	1.2×10^{-4}	3×10^{-4}	5×10^{-4}	1.5×10^{-3}	5×10^{-3}

* Strains used were χ289 (*cyc*^{s}), χ316 (*cyc*^{r1}), χ444 (*cyc*^{r2}), and χ453 (*cyc*^{r3}). Values are based on analysis of experiments like those presented in Fig. 1, 2, and 4.

would in turn prevent further growth. Nongrowing cells are not lysed by D-cycloserine (Ciak and Hahn, 1959).

The lack of increase in the resistance of *cyc*^{r} mutants to chlortetracycline is notable, since Moulder, Novosel, and Tribby (1965) found that a chlortetracycline-resistant strain of mouse pneumonitis bacteria was also resistant to D-cycloserine. As discussed by Park (1958), the A ring of tetracyclines is structurally similar to D-glutamic acid, which is a component of the cell-wall mucopeptide (*see* Salton, 1960). Thus, if chlortetracycline inhibits by interfering with D-glutamic-acid synthesis or incorporation into the mucopeptide, or both, it is possible that some chlortetracycline-resistant mutants would be able to perform this (these) step(s) without interference by chlortetracycline. It is known that D-cycloserine inhibits alanine racemase and D-analyl-D-alanine synthetase (Strominger et al., 1960), and we believe that mutations to D-cycloserine resistance affect these two enzymes (see below). These explanations do not, however, offer any reason for the joint resistance to D-cycloserine and chlortetracycline found by Moulder et al. (1965). One possible explanation would be that the structural genes for alanine racemase and glutamic acid racemase are contiguous and that mutations can occur which eliminate both functions. Strains having such mutations would be resistant to both D-cycloserine and chlortetracycline, and would require D-alanine and D-glutamic acid for growth. Our method of selecting *cyc*^{r} mutants on minimal agar precluded the discovery of such auxotrophic mutants. Lending some credence to the idea of clustering of genes responsible for mucopeptide synthesis or polymerization is the fact that the *cyc*^{r} loci are in the same region of the chromosome as the gene for ampicillin resistance (Eriksson-Greenberg et al., 1965).

Table 3 summarizes the data on prevention of D-cycloserine-induced lysis for strains with different genotypes. D-alanine is the most efficient antagonist to D-cycloserine. In the *cyc*^{s} strain

TABLE 3. *Prevention of D-cycloserine-induced lysis**

Strain genotype	D-Cycloserine concn	50% antagonism of lysis		Ratio of D-cycloserine (M) to antagonist (M)
		Antagonizing compound	Concn	
	M		*M*	
cyc^s	1×10^{-4}	D-Alanine	4.5×10^{-6}	22.2
	2.5×10^{-4}	D-Alanine	1.8×10^{-5}	13.9
	1×10^{-3}	D-Alanine	2.3×10^{-4}	4.3
cyc^{r1}	1×10^{-3}	D-Alanine	1.4×10^{-3}	0.71
cyc^{r2}	3×10^{-3}	D-Alanine	1.5×10^{-4}	20.0
cyc^{r3}	5×10^{-3}	D-Alanine	3.6×10^{-4}	13.9
cyc^s	1×10^{-4}	L-Alanine	1.2×10^{-4}	0.83
cyc^{r1}	1×10^{-3}	L-Alanine	7.6×10^{-3}	0.13
cyc^{r2}	3×10^{-3}	L-Alanine	9.2×10^{-3}	0.33
cyc^s	1×10^{-4}	DL-Alanyl-DL-alanine	1.0×10^{-3}	0.10

* Data calculated from experiments shown in Fig. 5, 6, and 7, and from an experiment on prevention of D-cycloserine inhibition by L-alanine in χ444. The concentrations of antagonizing compounds needed to cause 50% antagonism of lysis were determined by probit analysis (Finney, 1952) after calculating the survival-curve slopes (see Fig. 3 legend).

χ289, it becomes less efficient as the concentration D-cycloserine is increased. In the *cyc*^{r1} strains χ316 and χ447, D-alanine is a very inefficient antagonist. Although the mutation in χ316 is also present in the *cyc*^{r2} strain χ444, D-alanine is very effective in preventing the action of D-cycloserine in this strain. L-alanine is a much poorer antagonist than D-alanine, although the relative efficiencies of antagonism by L-alanine compared with D-alanine for *cyc*^{s}, *cyc*^{r1}, and *cyc*^{r2} strains are similar. Whereas D-alanyl-D-alanine should be a very efficient antagonist, the isomeric mixture containing it is not (Table 3). All of the

above observations are most likely explicable on the basis of permeability to, and active transport of, the various D-cycloserine antagonists.

The results of the conjugation experiments indicated that all the mutations resulting in the *cyc*^{r3} phenotype were linked. Transduction experiments indicated that the mutation conferring first-step resistance was not cotransducible with mutations that confer second- and third-step resistance. The latter two mutations were cotransducible. Thus, it would seem that the second and third mutations in χ453 were in the same (or neighboring) gene(s), whereas the first mutation occurred in a gene at least 0.5 min away on the bacterial chromosome. From the results on expression, it was concluded that the various mutations to D-cycloserine resistance were neither dominant nor recessive to each other or to D-cycloserine sensitivity but were independently acting. This conclusion suggests that the mutations to D-cycloserine resistance are expressed phenotypically at the eznyme level. If the phenotypic expression of D-cycloserine resistance required alteration of some structural component of the cell, then the appearance of D-cycloserine-resistant transductants would have been delayed much longer than was observed, and it would have been impossible to obtain fully expressed *cyc*^{r2} transductants by plating immediately after P1kc adsorption to the *cyc*^{r1} recipient strain. Thus, the genetic data are in complete accord with the fact that D-cycloserine inhibits the enzymes alanine racemase and D-alanyl-D-alanine synthetase (Strominger et al., 1960). We suggest that the mutation giving rise to the *cyc*^{r1} phenotype causes an alteration in one of these enzymes, whereas the second and third mutations, combining (with the first) to yield the *cyc*^{r3} phenotype, cause alterations in the other enzyme. Neuhaus and Lynch (1962) showed that there are two sites on the D-alanyl-D-alanine synthetase enzyme which can interact with D-cycloserine. Thus, it may be that the mutation in the *cyc*^{r1} strains affects alanine racemase, wherease both the second and third mutations found in the *cyc*^{r3} strain affect D-alanyl-D-alanine synthetase.

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