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Resistance to D-Cycloserine in the Tubercle Bacilli: Mutation Rate and Transport of Alanine in Parental Cells and Drug-Resistant Mutants

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A single transport system was found to accumulate L- and D-alanine, glycine and D-serine in *Mycobacterium tuberculosis*. The results of inhibition experiments suggested that the antibiotics D-cycloserine and *O*-carbamyl-D-serine were also transported by the alanine-glycine-D-serine system. A D-cycloserine-resistant permease-competent (D-CS^r/perm$^+$) mutant and a D-cycloserine-resistant permease-defective (D-CS^r/perm$^-$) mutant were isolated. The D-CS^r/perm$^-$ mutant was not found to be more resistant to the drug than was the D-CS^r/perm$^+$ mutant. The data were consistent with the conclusion that resistance to D-cycloserine in the tubercle bacilli is primarily due to mutations in the gene(s) controlling the enzyme D-alanyl-D-alanine synthetase. The mutation rate was calculated to be about 10^{-10} mutations per bacterium per generation.

Genetic investigations in *Escherichia coli* indicated that resistance to D-cycloserine (D-CS) was the result of spontaneous mutations and that mutants with increasing levels of resistance occurred at frequencies of 10^{-6} to 10^{-7} (1). Biochemical investigations by Reitz and collaborators revealed that the mutations in *Streptococcus faecalis* would result in an increase in levels of some of the enzymes participating in the utilization of alanine in the pathway leading to the synthesis of the cell wall peptidoglycan or in an impaired transport system for alanine (6). Wargel et al. (9) observed in *E. coli* that D-CS was transported into the cell by a D-alanine-glycine system, whereas L-alanine was transported by a separate system. They noted that the study of the D-alanine-glycine transport is important in the understanding of the mechanism of action of D-CS and the acquisition of D-CS resistance (D-CS^r). Following the suggestion that alanine-permease mutants (ala-perm$^-$ mutants) would be resistant to D-CS because of their inability to accumulate the drug, the investigations described in this report were started. In this communication we report the mutation rate to D-CS resistance and the isolation of two types of D-CS^r mutants [D-cycloserine-resistant with a competent alanine-permease system (D-CS^r/perm$^+$) and D-cycloserine-resistant with a defective alanine transport system (D-CS^r/perm$^-$)], and we describe some properties of the alanine-permease system of the tubercle bacilli.

MATERIALS AND METHODS

Bacterial strain. The strain $H_{37}Rv$ of the *Mycobacterium tuberculosis* was used in all experiments. The strain was maintained in Middlebrook and Cohn 7H9 medium (Difco) containing 0.05% Tween 80. The D-CS^r mutants were also maintained by successive transfers in the same medium.

Isolation of mutants. The D-CS^r mutants were isolated by applying the Luria and Delbrück fluctuation test (5). Colonies that grew on Middlebrook 7H-10 medium (Difco) containing 20 μg of D-CS per ml were transferred to 7H-9 liquid medium and were then subjected to five cycles of direct selection in liquid medium containing 10 μg of the drug per ml. After the various cycles of selection were completed, the mutants were maintained in liquid medium without the drug.

The phenotypes of the parental cells (D-CS^s) and the mutants [wild-type parental strain (D-CS^s/perm$^+$)] were analyzed by testing in 7H-9 medium containing increasing concentrations of D-CS (1.25, 2.5, 5.0, 10.0, and 20.0 μg/ml), 100.0 μg of *O*-carbamyl-D-serine (*O*-CS) per ml, and combinations of the two drugs. The effect exhibited by the combination of the two drugs has been described (2, 7).

The fluctuation test was performed, and the data were analyzed as described previously (3).

Transport of alanine. Two- or three-week-old cultures in 100-ml volumes of 7H-9 medium were used in all experiments. The cells were harvested by centrifu-

gation and washed twice in cold 0.01 M phosphate buffer, *p*H 7.0. To the washed cells resuspended in 10 ml of the same buffer, L- or D-alanine-*1*-^{14}C was added to the desired concentration, and the mixture was then preincubated for 15 min in ice. Samples (1.0 ml) were transferred to small test tubes that were incubated in a temperature block (Scientific Products, Evanston, Ill.) set at 30 C. At the desired time, the entire contents of a test tube were filtered by suction by using 0.8-μm membrane filters (Millipore Corp., Bedford, Mass.), and the collected cells were washed two times with 10-ml volumes of distilled water maintained at 25 C. The membrane filters were transferred to counting vials and were dried by warming the open vials over a slide-warmer (Cleveland Electric Co., Inc., Chicago) set at 65 to 70 C for 1 hr or more. A 15-ml amount of scintillation fluid was added, the membranes were dissolved by vigorous shaking, and the amount of radioactivity was established in a scintillation spectrometer model 6850 "Unilux I" (Nulcear-Chicago Corp., Des Plains, Illinois). The weight of cells in each experiment was estimated by subtracting the weight of the filter from the weight after the cells were collected. The amount of accumulated alanine is expressed as nanomoles per milligram (wet weight) of cells.

Chemicals and reagents. D-CS was donated by Eli Lilly & Co., Indianapolis, Ind., and *O*-CS was donated by Commercial Solvents Corp., Terre Haute, Ind. D-Alanine was purchased from Mann Research Laboratories, New York, and L-alanine from Nutritional Biochemicals Corp., Cleveland, Ohio. L-Alanine-*1*-^{14}*C* (specific activity, 13.2 mCi/mmole) and D-alaine-*1*-^{14}*C* (specific activity, 17.9 mCi/mmole) were purchased from New England Nuclear Corp., Boston, Mass. 2,5-Diphenyloxazole and 1,4-bis-2-(5-phenyloxazolyl)-benzene were purchased from Amersham Searle Co., Des Plains, Ill., and dioxane and toluene (scintillation grade) were purchased from Fisher Scientific Co., Atlanta, Ga.

RESULTS

Transport of alanine. The uptake of alanine in the tubercle bacilli is essentially completed in 5 hr (Fig. 1). It was also verified that the rate of uptake was increased when the cells were previously exposed to L-alanine. This observation is in agreement with previous investigations indicat-

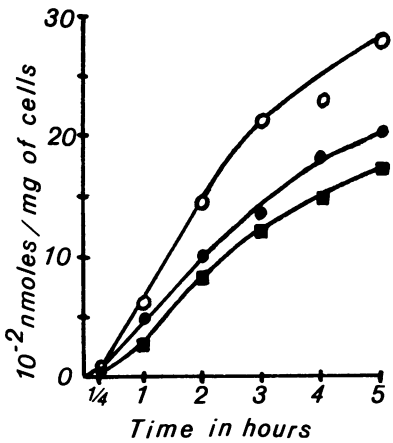

FIG. 1. *Time course of alanine uptake. The reaction mixture contained 10.0 ml of the cell suspension in 0.01 M phosphate buffer, pH 7.0, and either L-alanine-1-^{14}C (3.4 × 10^{-5} M) or D-alanine-1-^{14}C (3.1 × 10^{-5} M). At the indicated intervals, a 1.0-ml sample was taken and the cells were collected in membrane filters (Millipore Corp.) as described in the text. Symbols: ○, uptake of L-alanine in cells previously exposed to L-alanine; ●, uptake of L-alanine; ■, uptake of D-alanine.*

TABLE 1. *Effect of D-cycloserine (D-CS) and of O-carbamyl-D-serine (O-CS) on the induction of an alanine transport system*[a]

Inhibitor	Inducer	Uptake of L-alanine-*1*-^{14}*C*	
		Amt (10^{-2} nmoles/mg of cells)	Test/control
None	None	39.0	1.0
None	1 mM L-Alanine	68.0	1.7
1 mM D-CS (4-hr pulse)	1 mM L-Alanine	87.4	2.2
1 mM *O*-CS (4-hr pulse)	1 mM L-Alanine	73.6	1.8
None	10 mM L-Alanine	333.0	8.3
10 mM D-CS	10 mM L-Alanine	100.0	2.5
10 mM *O*-CS	10 mM L-Alanine	184.0	4.6

[a] In one type of experiment, the cells suspended in 7H-9 medium were exposed to 1 mM of either D-CS or *O*-CS for 4 hr; the cells were then washed in medium containing 1 mM L-alanine and incubated overnight for induction. In the second type of experiment, the cells were exposed in the medium to 10 mM L-alanine and to 10 mM of either D-CS or *O*-CS; then the cell suspensions were incubated overnight for induction. The transport of alanine was then determined as indicated in the Materials and Methods section. The concentration of L-alanine-*1*-^{14}*C* was 0.6 × 10^{-5} M. The amount of radioactivity was established after 5 hr of incubation at 30 C.

that the alanine-permease system is inducible in these bacteria (2) and is in agreement with Yabu's investigations in *M. smegmatis* (10). Pulse experiments indicated that neither drug interferes with the subsequent induction of the permease system by L-alanine (Table 1); it was also observed that exposing the cells to the drugs during the whole period of induction did not interfere with the induction process. The lower accumulation of alanine under these conditions was probably due to the competitive inhibition of the transport system by D-CS and *O*-CS.

Inhibition experiments (Table 2) indicated that L-alanine was a very effective inhibitor of D-alanine uptake and, conversely, D-alanine was a very effective inhibitor of L-alanine uptake. Glycine, D-serine, D-CS, and *O*-CS were also effective inhibitors of the transport of both alanine diasterioisomers. However, the antibiotics were found to be less effective than the amino acids. The study of the relationship of the D-alanine uptake to the concentration of D-CS and *O*-CS (Fig. 2) revealed that D-CS was an effective inhibitor at lower concentrations than *O*-CS.

The previous observations indicate that L- and D-alanine are transported in *M. tuberculosis* by a single transport system. Similar observations were made in *M. smegmatis* (10). The data are also consistent with the conclusion that the same system transports glycine, D-serine, D-CS, and *O*-CS.

Isolation of resistant mutants. Mutants resistant to D-CS were identified by performing a fluctuation test as indicated in Table 3. From the data at the concentration of 20 μg of D-CS per ml, a mutation rate 10^{-10} mutations per bacterium per generation was calculated, and the highest proportion of mutants was about 10^{-8}. After five cycles of selection in 7H-9 medium containing 10

TABLE 2. *Inhibition of alanine uptake*[a]

Inhibitor (10 mM)	Uptake of L-alanine		Uptake of D-alanine	
	Amt (10^{-2} nmoles/mg)	Per cent inhibition	Amt (10^{-2} nmoles/mg)	Per cent inhibition
None	74.5		146.2	
L-Alanine			8.4	99.4
D-Alanine	7.6	89.8		
Glycine	15.4	79.4	14.7	90.0
D-Serine	37.3	50.0	15.7	89.3
D-Cycloserine	54.8	26.5	48.7	68.1
O-carbamyl-D-serine	44.3	40.6	100.6	31.2

[a] Reaction mixture contained 1.0 ml of cell suspension in 0.01 M phosphate buffer (*p*H 7.0) and 0.7×10^{-5} nmoles of L-alanine-*1*-^{14}C or 0.6×10^{-5} nmoles of D-alanine-*1*-^{14}C. The amount of radioactivity was established after 5 hr of incubation at 30 C.

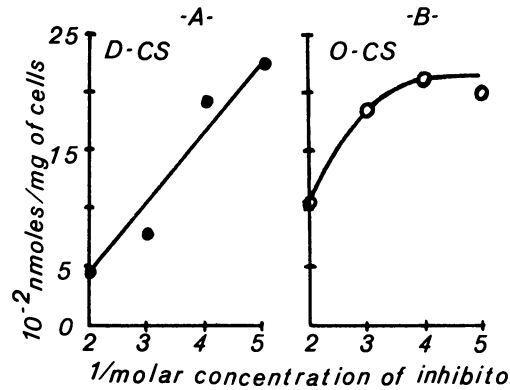

FIG. 2. *Uptake of D-alanine-1-^{14}C as a function of the concentration of D-CS (A) or O-CS (B). The concentrations of D-CS and O-CS were 10^{-2}, 10^{-3}, 10^{-4}, and 10^{-5} M; the abscissa in the figures is, therefore, the logarithm of the reciprocal of the molar concentration of the inhibitors. The reactive mixtures contained 1.0 ml of cell suspension in 0.01 M phosphate buffer (pH 7.0), 0.6×10^{-5} M D-alanine-1-^{14}C, and the desired concentration of the inhibitor. The amount of radioactivity was established after 5 hr of incubation at 30 C.*

TABLE 3. *Results of a fluctuation test*[a]

No. of mutants	Frequency of mutants at various concn of D-cycloserine[b]				
	2.5	5.0	10.0	20.0	≥30.0
0	0	0	2	12	18
1	0	0	1	2	0
2	0	1	1	1	0
3–4	0	0	0	0	0
5–8	0	1	1	2	0
9–16	1	0	1	0	0
17–32	0	0	0	0	0
33–64	0	0	1	1	0
>64	17	16	11	0	0

[a] Initial inoculum in the small, identical cultures contained about seven cells. Twenty cultures were initiated but growth was obtained in only 18; the time of incubation required to obtain full growth was 4 weeks. The average number of bacteria was 3.5×10^9 cells per ml, and the number of generations that elapsed in the 18 small cultures was 30.8. Average number of mutants at 20 μg of D-CS per ml: 9.5. Highest proportion of mutants: 10^{-8}; mutation-rate: 0.9×10^{-10}.

[b] Volume of cultures: 5.0 ml; volume of samples, 0.1 ml.

TABLE 4. *Characteristics of the parental cells and the D-cycloserine-resistant mutants*

Phenotype[a]	Ratio of cell density in cultures of various concn[b]											Control	Uptake of alanine	
	D-CS (μg per ml)					O-CS (100 μg per ml)	D-CS plus 100 μg per ml of O-CS						L-Alanine	D-Alanine
	1.25	2.5	5.0	10.0	20.0		1.25	2.5	5.0	10.0	20.0			
Parental (D-CS^s/perm$^+$)	0.86	0.75	0	0	0	0.62	0.65	0.51	0	0	0	1.0	24.1	30.4
D-CS^r/perm$^+$	1.0	0.90	0.76	0.57	0	0.52	0.80	0.80	0.66	0	0	1.0	25.5	29.0
D-CS^r/perm$^-$	0.95	0.95	0.95	0.90	0	0.95	0.74	0.75	0.75	0.74	0	1.0	13.4	3.7

[a] Abbreviations: D-CS, D-cycloserine; O-CS, O-carbamyl-D-serine; D-CS^s/perm$^+$, wild-type parental strain; D-CS^r/perm$^+$, D-CS-resistant mutant with a competent alanine-permease system; D-CS^r/perm$^-$, D-CS resistant mutant with a defective alanine transport system.

[b] Susceptibility of the strains is expressed as the ratio of the cell density in the test culture to the cell density in the controls. The cell density was established after 10 days of incubation by using a Coleman Jr. spectrophotometer, and the readings were done at 650 nm. The uptake of alanine is expressed as $10^{-2} \times$ nmoles per mg of cells (wet weight).

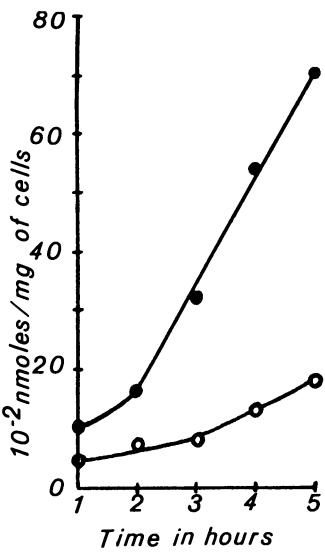

FIG. 3. *Time course of alanine uptake by the parental cells (●) and the D-CS^r/perm$^-$ mutant (○). The experiment was performed as indicated in Fig. 1.*

μg of D-CS per ml, two distinct phenotypes were identified (Table 4). The phenotypes were as follows: the D-CS^s/perm$^+$ wild-type parental strain was inhibited by 5.0 μg of D-CS per ml, the cell density in the presence of 100 μg of O-CS was 38% less than the control, and the strain was alanine permease-competent; a D-CS^r/perm$^+$ mutant was inhibited by 20 μg of D-CS per ml, the cell density in the presence of 100 μg of O-CS was 48% less than the control, and the mutant was alanine permease-competent; and, finally, a D-CS^r/perm$^-$ mutant was inhibited by 20 μg of D-CS per ml, the cell density in the presence of 100 μg per ml of O-CS was not significantly different from the control, and this mutant was alanine permease-defective.

The time course of the accumulation of L-alanine by the D-CS^r/perm$^-$ mutant (Fig. 3) confirmed that this mutant had an impaired transport system.

DISCUSSION

The genetics of D-CS resistance in the tubercle bacilli was investigated before by Tsumakura and collaborators (8). These authors studied the changes in the proportion of mutants after successive cycles of direct selection. They observed that the proportion of mutants in the wild-type parental cells was about 6×10^{-8} and observed two distinct discontinuities in the proportion of mutants during the various selective cycles. On this basis, they suggested that two separate genes control the sensitivity to D-CS.

According to our investigations, resistance to D-CS is the result of spontaneous and random mutations that occur at a rate of 10^{-10} mutations per bacterium per generation; the highest proportion of mutants to be expected in unselected populations of the tubercle bacilli was found to be 10^{-8}.

The two D-CS^r mutants that we isolated were equally resistant to D-CS, and, because they exhibited a low degree of resistance, they were probably first-step mutants. One mutant was alanine permease-competent and was as resistant to O-CS as the parental strain; the second mutant was alanine permease-deficient and was more resistant to O-CS than the parental strain. These observations suggest that the tubercle bacilli have

a normally high level of D-alanine racemase and that the inhibition of this enzyme by D-CS must play a minor role in the mechanism of action of the drug. As suggested before (4), the level of D-alanyl-D-alanine synthetase must be relatively low. This notion is in agreement with our present observations because a mutation(s) resulting in an alanine permease-deficient mutant was not accompanied by an increase in the degree of resistance to D-CS. On the other hand this indicates that enough of the drug accumulates by simple diffusion to inhibit the enzyme and bacterial growth. It is possible, however, that D-CS may also be transported by a separate system in the tubercle bacilli. Attempts to isolate a mutant that is both alanine permease-defective and sensitive to D-CS are now in progress.

We conclude that resistance to D-CS in the tubercle bacilli is due primarily to mutations in the gene(s) controlling D-alanyl-D-alanine synthetase; these mutations occur at a rate of 10^{-10} per bacterium per generation.

We have suggested previously that the application of the fluctuation test may give information that is useful in the performance and the interpretation of drug sensitivity tests in tuberculosis (3). In the present report, we showed that the fluctuation test can also be used to find the critical concentration of a drug; the critical concentration of D-CS in the 7H-10 medium was found to be 20 μg of the drug per ml (Table 3). Because a plasmid carrying a marker to streptomycin resistance was recently demonstrated in *M. smegmatis* (Jones and David, *manuscript in preparation*), we should emphasize that in applying the fluctuation test one is analyzing the resistance that is due to mutations in chromosomal genes. The physiological basis of resistance caused by plasmids may not necessarily be the same.

LITERATURE CITED

1. Curtiss, R., L. J. Charamella, C. Berg, and P. E. Harris. 1965. Kinetic and genetic analysis of D-cycloserine inhibition and resistance in *Escherichia coli*. J. Bacteriol. **90**:1238–1250.
2. David, H. L. 1970. Effect of *O*-carbamyl-D-serine on the growth of *Mycobacterium tuberculosis*. Amer. Rev. Resp. Dis. **102**:68–74.
3. David, H. L. 1970. Probability distribution of drug-resistant mutants in unselected populations of the tubercle bacilli. Appl. Microbiol. **20**:810–814.
4. David, H. L., K. Takayama, and D. S. Goldman. 1969. Susceptibility of mycobacterial D-alanyl-D-alanine synthetase to D-cycloserine. Amer. Rev. Resp. Dis. **100**:579–581.
5. Luria, S. E., and M. Delbrück. 1943. Mutations of bacteria from virus sensitivity to virus resistance. Genetics **28**:491–511.
6. Reitz, R. H., H. D. Slade, and F. C. Neuhaus. 1967. The biochemical mechanisms of resistance by streptococci to the antibiotics D-cycloserine and *O*-carbamyl-D-serine. Biochemistry **6**:2561–2570.
7. Tanaka, N., and H. Umezawa. 1964. Synergism of D-4-amino-3-isoxazolidone and *O*-carbamyl-D-serine. J. Antibiot.[A] (Tokyo) **17**:8–10.
8. Tsumakura, M., Y. Noda, M. Hayashi, and M. Yamamoto. 1959. A genetic study on the cycloserine-resistance of *Mycobacterium tuberculosis* var. *hominis*. Jap. J. Microbiol. **3**:1–8.
9. Wargel, R. J., C. A. Shadur, and F. C. Neuhaus. 1970. Mechanism of D-cycloserine action: transport systems for D-alanine, D-cycloserine, L-alanine, and glycine. J. Bacteriol. **103**:778–788.
10. Yabu, K. 1970. Amino acid transport in *Mycobacterium smegmatis*. J. Bacteriol. **102**:6–13.