The Biochemical Mechanisms of Resistance by Streptococci to the Antibiotics D-Cycloserine and *O*-Carbamyl-D-serine*

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ABSTRACT: The antibacterial activity of D-cycloserine (D-4-amino-3-isoxazolidone) (D-CS) and *O*-carbamyl-D-serine (O-CS) has been correlated with the inhibition of enzymes necessary for peptidoglycan biosynthesis. D-Cycloserine inhibits alanine racemase (EC 5.1.1.1) and D-alanine:D-alanine ligase (ADP) (EC 6.3.2.4) (Strominger, J. L., Ito, E., and Threnn, R. H. (1960), *J. Am. Chem. Soc. 82*, 998). *O*-Carbamyl-D-serine inhibits only alanine racemase (Lynch, J. L., and Neuhaus, F. C. (1966), *J. Bacteriol. 91*, 449). Mutants of *Streptococcus* strain Challis selected for resistance to *O*-carbamyl-D-serine have increased levels of alanine racemase. Enzymes that are not sensitive to *O*-carbamyl-D-serine (D-alanine:D-alanine ligase (ADP), UDP-NAc-muramyl-L-Ala-D-isoGlu-L-Lys:D-Ala-D-Ala ligase (ADP) (EC 6.3.2.10); UDP-NAc-muramyl-L-Ala-D-isoGlu:L-lysine ligase (ADP) (EC 6.3.2.7)) are not elevated in mutant (O-CS). Resistance to *O*-carbamyl-D-serine develops in steps, and each increase in resistance is accompanied by an increase in the specific activity of alanine racemase. In the most resistant mutant, alanine racemase is increased eightfold. Two types of D-cycloserine-resistant mutants have been isolated and examined. The first type, mutant (D-CS$_a$), has elevated levels of D-alanine:D-alanine ligase (ADP) (five times) and alanine racemase (eight times) and does not differ from the parent strain in its ability to concentrate alanine. Alanine racemase and D-alanine:D-alanine ligase (ADP) in mutant (D-CS$_a$) are similar to the enzymes in the parent strain with respect to Michaelis–Menten constants, susceptibility to inhibition by D-cycloserine, and heat stability. The second type of D-cycloserine resistant mutant, mutant (D-CS$_b$), produces normal levels of the drug-sensitive enzymes and differs from the parent strain in its ability to concentrate alanine. Since D-cycloserine inhibits the accumulation of alanine by the parent strain and since mutant (D-CS$_b$) possesses a defective transport system for alanine, it would appear that this mutant is unable to concentrate D-cycloserine. Transformation experiments with deoxyribonucleic acid prepared from mutant (D-CS$_a$) suggest the existence of two genetic markers associated with resistance to D-cycloserine in this strain.

One marker is associated with increased levels of alanine racemase whereas the other marker is associated with increased levels of D-alanine:D-alanine ligase (ADP).

D-Cycloserine (D-4-amino-3-isoxazolidone) and *O*-carbamyl-D-serine, analogs of D-alanine, are inhibitors of peptidoglycan biosynthesis (Park, 1958, 1960; Ciak and Hahn, 1959; Tanaka *et al.*, 1963). Cultures of bacteria that have been grown in the presence of these antibiotics have defective walls and are susceptible to lysis (Shockman, 1959; Ciak and Hahn, 1959). With each antibiotic there is an accumulation of the incomplete cell wall precursor, UDP[1]-NAc-muramyl-L-Ala-D-isoGlu-L-Lys, which lacks the terminal D-Ala-D-Ala moiety (Strominger *et al.*, 1959; Lynch and Neuhaus, 1966). The addition of D-alanine to the medium overcomes the inhibition of growth by D-cycloserine and *O*-carbamyl-D-serine and decreases the accumulation of UDP-NAc-muramyl-L-Ala-D-isoGlu-L-Lys (Strominger *et al.*, 1959; Lynch and Neuhaus, 1966). These results have been correlated with the effects of the analogs on enzymes which catalyze the incorporation of D-alanine from L-alanine into the wall precursor. D-Cycloserine is an effective inhibitor of D-alanine:D-alanine ligase (EC 6.3.2.4)

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[1] Abbreviations used: UDP, uridine diphosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; FUDP, 5-fluorouridine 5′diphosphate; D-CS, D-4-amino-3-isoxazolidone; O-CS, *O*-carbamyl-D-serine.

(ADP) (D-Ala-D-Ala synthetase) (Strominger *et al.*, 1960; Neuhaus and Lynch, 1964) and alanine racemase (EC 5.1.1.1) (Strominger *et al.*, 1960). In contrast, *O*-carbamyl-D-serine inhibits only alanine racemase (Lynch and Neuhaus, 1966). D-Cycloserine and *O*-carbamyl-D-serine do not inhibit the enzyme that adds D-Ala-D-Ala to UDP-NAc-muramyl-L-Ala-D-isoGlu-L-Lys (Strominger, 1962; Lynch and Neuhaus, 1966).

The emergence of resistance to D-cycloserine has been observed in many clinical situations (for a review, see Neuhaus, 1967). In addition, D-cycloserine-resistant mutants have been isolated in the laboratory (Howe *et al.*, 1964; Chambers *et al.*, 1963; Curtiss *et al.*, 1965). Curtiss *et al.* (1965) studied the kinetic and genetic aspects of D-cycloserine resistance in *Escherichia coli*.

It is the purpose of this paper to establish the biochemical mechanism(s) of resistance to D-cycloserine and *O*-carbamyl-D-serine in *Streptococcus* (serological group H) strain Challis.

Experimental Section

Materials

D-[1-^{14}C]Alanine, L-[1-^{14}C]alanine, and L-[U-^{14}C]-lysine were purchased from California Corp. for Biochemical Research. The plastic beads (styrenedivinylbenzyne copolymer, 20–50 mesh, 8% cross-linked), Dowex 50-X8 (100–200 mesh), and Dowex 1 were gifts of the Dow Chemical Co. We are indebted to Dr. O. K. Behrens of the Eli Lilly Co., and Drs. M. C. Bachman and P. H. Hidy of Commercial Solvents Corp. for D-cycloserine and *O*-carbamyl-D-serine, respectively. Todd–Hewitt broth, blood agar base, proteose peptone, and yeast extract were obtained from Difco Co. Defibrinated sheep's blood was supplied by Dr. Robert Galvin (Beecher, Ill.). D-Amino acid oxidase (EC 1.4.3.3) (4.5 units/mg, electrophoretically purified) and catalase (CTR) were purchased from Worthington Biochemical Corp. For the D-Ala-D-Ala adding enzyme, a 55–70% ammonium sulfate fraction (0.12 unit/mg) was used (Neuhaus and Struve, 1965).

D-[^{14}C]Ala-D-[^{14}C]Ala and UDP-NAc-muramyl-L-Ala-D-isoGlu-L-Lys were prepared according to the procedures described by Neuhaus and Struve (1965) and Stickgold and Neuhaus (1967). UDP-NAc-muramyl-L-Ala-D-isoGlu was prepared with *Staphylococcus aureus* Copenhagen in a manner similar to that described by Strominger and Threnn (1959) (see Reitz, 1966). The isolation of the nucleotide from the extract is identical with that described by Stickgold and Neuhaus (1967) for FUDP-NAc-muramyl-pentapeptide. Cell walls were prepared according to the method described by Struve *et al.* (1966).

Mutant Isolation. Streptococcus strain Challis (10^7–10^8 cells) from an 18-hr culture (Todd–Hewitt broth) was spread on a blood agar plate containing 4% blood agar base, 4% defibrinated sheep's blood, and either D-cycloserine or *O*-carbamyl-D-serine (*e.g.*, see Table VI). Since D-cycloserine slowly loses its bactericidal activity in agar plates (Curtiss *et al.*, 1965), the plates were prepared immediately before use. In every case, D-cycloserine and *O*-carbamyl-D-serine were sterilized by passage through a Millipore filter (0.2 μ). Colonies from the plates with the highest concentration of antibiotic were transferred to Todd–Hewitt broth. This cycle was repeated until a high degree of resistance was obtained. The antibiotic sensitivity was quantitated in a medium which contained 1% peptone T, 1% yeast extract, 0.5% K_2HPO_4, and 0.5% glucose. The minimal inhibitory concentration is defined as the lowest concentration of antibiotic that limits the culture to a turbidity of 0.9 under conditions in which the control tube reaches 1.3. Turbidimetric measurements were performed in a Bausch and Lomb Spectronic 20 in 18 × 150 mm test tubes at 650 mμ.

The mutant strains resemble the parent strain in cell morphology, Gram stain, colonial appearance, α-hemolysis on blood agar, and group H typing reaction (Swift *et al.*, 1943). The strains were maintained as lyophilized cultures *in vacuo* at −20°. The mutants that have been studied in detail are summarized in Table I. Mutant (D-CS$_a$) has been in culture 5 years and upon retesting has demonstrated a stable mutation for D-cycloserine resistance.

TABLE I: Summary of Mutants Used.

Streptococcus strain Challis	Minimal Inhibitory Concentration (moles/l.)	
	D-Cycloserine	*O*-Carbamyl-D-serine
Parent	5×10^{-4}	2×10^{-3}
Mutant (D-CS$_a$)	100×10^{-4}	30×10^{-3}
Mutant (D-CS$_b$)	40×10^{-4}	*b*
Mutant (O-CS)[a]	20×10^{-4}	20×10^{-3}

[a] See Table VI for summary of isolation. [b] Not determined.

Preparation of Cell-Free Extracts. The bacteria were grown at 37° in 500 ml of a medium which contained 1% peptone T, 1% yeast extract, 0.5% K_2HPO_4, and 0.5% glucose. When the turbidity had reached 0.7, the cells were chilled and harvested by centrifugation at 6000*g* for 20 min. The bacteria (1.6–2.2 g wet wt) were washed once in cold 0.01 M Tris-HCl buffer (pH 7.8) and then resuspended in 20 ml of the same buffer containing 17.5 g of plastic beads and 2 drops of antifoam. The bacteria were disrupted in a Bronwill mechanical cell homogenizer (Braun Model MSK) at 4000 cycles/min for 5 min. During the disruption the temperature was maintained between 2 and 8° with liquid carbon dioxide. After removing the beads by filtration through a 110-mesh nylon cloth, the unbroken cells and cell walls were removed by centrifugation at 14,000*g* for 10 min. The extracts were

frozen in 0.6-ml polyethylene capsules and stored in liquid nitrogen until used. In order to establish meaningful enzyme profiles, each capsule was thawed only once.

Assays. The unit used in each assay is defined as 1 μmole of product formed/hr. The D-Ala-D-Ala adding enzyme was assayed according to the method described by Neuhaus and Struve (1965). Alanine racemase was assayed in the "L-alanine to D-alanine assay" described by Lynch and Neuhaus (1966).

L-Lysine Adding Assay. The L-lysine adding enzyme was assayed by the addition of L-[^{14}C]lysine to UDP-NAc-muramyl-L-Ala-D-isoGlu with the formation of UDP-NAc-muramyl-L-Ala-D-isoGlu-L-[^{14}C]Lys. The standard assay mixture contained 0.4 μmole of ATP neutralized with NaOH, 0.4 μmole of $MgCl_2$, 10 μmoles of Tris-HCl buffer (pH 7.8), 32 nmoles of UDP-NAc-muramyl-L-Ala-D-isoGlu, 0.1 μmole of L-[^{14}C]lysine (6×10^4 cpm), and enzyme preparation in a total volume of 0.10 ml. The composition of the mixture is modified from that described by Ito and Strominger (1962b). The sample of extract was added to the assay mixture at 37° and incubated for 10 min at this temperature. The reaction was terminated by the addition of 0.5 ml of 0.2 N sodium citrate buffer (pH 2.2). The separation of UDP-NAc-muramyl-L-Ala-D-isoGlu-L-[^{14}C]Lys from L-[^{14}C]lysine is identical with that described for the D-Ala-D-Ala Adding Assay (Neuhaus and Struve, 1965). With these assay conditions, the amount of product is proportional to the enzyme concentration.

D-Ala-D-Ala Synthetase Assay. Previously, the routine assay for D-Ala-D-Ala synthetase in extracts involved the separation of D-[^{14}C]alanine from D-[^{14}C]Ala-D-[^{14}C]Ala by paper chromatography (Neuhaus, 1962). The P_i Assay and ADP Assay that were described are not satisfactory for the assay of this enzyme in extracts. In the present work this enzyme was assayed by adding the product of the enzyme reaction, D-[^{14}C]Ala-D-[^{14}C]Ala, to UDP-NAc-muramyl-L-Ala-D-isoGlu-L-Lys and separating the product from the remaining D-[^{14}C]alanine in the incubation.

The reaction mixture contained 5 μmoles of Tris-HCl buffer (pH 7.8), 2.5 μmoles of KCl, 0.5 μmole of $MgCl_2$, 0.5 μmole of ATP neutralized with NaOH, 2 μmoles of D-[^{14}C]alanine (25,000 cpm/μmole), and enzyme preparation in a total volume of 0.05 ml. The enzyme was added to the reaction mixture at 37° and incubated for 10 min at this temperature. The reaction was terminated by placing the tube in a boiling water bath for 2 min.

In the second stage, a solution (0.05 ml) containing 0.25 μmole of UDP-NAc-muramyl-L-Ala-D-isoGlu-L-Lys, 0.20 μmole of ATP neutralized with NaOH, 2 μmoles of $MgCl_2$, and 500 μg of D-Ala-D-Ala adding enzyme was added, and the mixture was incubated for 20 min at 37°. The reaction was terminated by the addition of 0.5 ml of 0.2 N sodium citrate buffer (pH 2.2). The UDP-NAc-muramyl-L-Ala-D-isoGlu-L-Lys-D-[^{14}C]Ala-D-[^{14}C]Ala was separated from D-[^{14}C]alanine by the procedure described for the D-Ala-D-Ala adding enzyme (Neuhaus and Struve, 1965). Under the conditions of the second stage, 0.2 μmole of D-[^{14}C]Ala-D-[^{14}C]Ala was quantitatively transferred to the nucleotide tripeptide. The amount of UDP-NAc-muramyl-L-Ala-D-isoGlu-L-Lys-D-[^{14}C]Ala-D-[^{14}C]Ala was proportional to the concentration of D-Ala-D-Ala synthetase added.

Alanine Accumulation Assay. The amount of [^{14}C]-alanine concentrated by cells of *Streptococcus* strain Challis (or mutants) was established in the following procedure. The bacteria were grown in 500 ml of medium that contained 1% peptone T, 1% yeast extract, 0.5% K_2HPO_4, and 0.5% glucose. When the turbidity reached 0.8, the cultures were chilled (4°) and harvested by centrifugation at 6000*g* for 20 min. The cells (2 g) were resuspended in 100 ml of 0.05 M potassium phosphate buffer (pH 8.0) and recentrifuged. The pellet of cells was suspended in 20 ml of the same buffer and equilibrated at 25°.

The cells were preloaded with alanine in a procedure similar to that described by Winkler and Wilson (1966). The preloading was accomplished in the following incubation mixture: 180 mg of cells (wet weight), 20 mg of glucose, 2 μmoles of D- or L-alanine, and 90 μmoles of potassium phosphate buffer (pH 8.0) in a volume of 2 ml. The cells were incubated for 5 min at 25°. After preloading, 50 nmoles of [^{14}C]-alanine (5×10^5 cpm) was added and the cells were allowed to accumulate the [^{14}C]amino acid for the desired length of time. The uptake was terminated by adding 4.0 ml of 0.05 M potassium phosphate buffer (pH 8.0) (0°) and immediately immersing each tube in an ice-water bath. The tubes were immediately centrifuged at 27,000*g* for 5 min at 0–4°. The cells were then washed with 5 ml of 0.05 M phosphate buffer (0–4°) (pH 8.0). After centrifuging, the pellet was resuspended in 1 ml of distilled water and transferred to a vial containing 10 ml of toluene–Triton X-100 scintillation fluid (Patterson and Greene, 1965).

Transformation of D-Cycloserine Resistance. For the transformation of D-cycloserine resistance, DNA was prepared from mutant (D-CS_8) by the method of Perry and Slade (1962). Transformation was performed according to the procedure described by Perry and Slade (1966). After 15 min DNase (10 μg) was added to terminate the transformation. The cells were then allowed to incubate an additional 2 hr at 37° before plating samples on blood agar plates containing 300 μg of D-cycloserine/ml. After 48 hr at 37°, the plates were examined for transformants. About 1% of the viable cells exposed to DNA was able to form colonies on the plates containing 300 μg/ml of D-cycloserine. When a control culture of the *Streptococcus* strain Challis (DNase added before the transforming DNA) was carried through this procedure and plated (10^5 cells/plate), no D-cycloserine-resistant colonies were observed.

Analytical Procedures. Protein was determined by the method of Lowry *et al.* (1951). For the analysis of amino acids, the walls (1.7 mg wet wt) were hydrolyzed in 5.7 N HCl for 12 hr and the amino acids were

determined on the Beckman Model 120 amino acid analyzer according to the methods described by Spackman *et al.* (1958). Measurements of radioactivity were made in polyethylene vials using the Packard Tri-Carb liquid scintillation spectrometer (Model 314-EX). The scintillation fluid (15 ml) was described by Patterson and Greene (1965) and evaluated by Benson (1966).

Results

Resistance to D-*Cycloserine.* Cultures of *Streptococcus* strain Challis, *Streptococcus* strain Challis (D-CS_a), and *Streptococcus* strain Challis (D-CS_b) were grown under identical conditions to a turbidity of 0.7 and harvested. Cell-free extracts were prepared from these cultures and assayed for the following enzymes: (1) alanine racemase, (2) D-Ala-D-Ala synthetase, (3) D-Ala-D-Ala adding enzyme, and (4) L-lysine adding enzyme. The specific activities of these enzymes are summarized in Table II. In the case of mutant (D-CS_a), an eight- and fivefold increase was observed for alanine racemase and D-Ala-D-Ala synthetase, respectively. The specific activities of the L-lysine adding enzyme and the D-Ala-D-Ala adding enzyme are not significantly different from that observed for the parent strain. With mutant (D-CS_b) no significant increase in the specific activities of either alanine racemase or D-Ala-D-Ala synthetase was detected.

TABLE II: Enzyme Profiles of *Streptococcus* Strain Challis, Mutant D-CS_a, and Mutant D-CS_b.[a]

Enzyme	Specific Activity (units/mg of protein)		
	Parent	D-CS_a	D-CS_b
Alanine racemase	1.2 ± 0.1	9.6 ± 1.0	1.7
D-Ala-D-Ala synthetase	0.033 ± 0.003	0.16 ± 0.02	0.043
D-Ala-D-Ala adding	0.35 ± 0.15	0.29 ± 0.15	—
L-Lysine adding	0.12 ± 0.01	0.17 ± 0.01	—

[a] The results for the parent and mutant (D-CS_a) are the average of five separate experiments. Blank spaces not determined.

Since the specific activities of the enzymes may be a function of the growth phase (*e.g.*, see Ito and Strominger, 1962a), extracts were prepared from cultures of the parent strain and mutant (D-CS_a) at early, middle, and late log phase, and the specific activities of the enzymes were established at each phase. From the results in Table III, it is apparent that the increases in specific activities of alanine racemase and D-Ala-D-Ala synthetase are independent of the growth phase.

TABLE III: Specific Activities as a Function of the Growth Phase in *Streptococcus* Strain Challis and Mutant D-CS_a.[a]

Enzyme	Specific Activity (units/mg of protein)		
	Turbidity		
	0.4	0.7	1.0
Parent			
Alanine racemase	1.3	1.2	1.1
D-Ala-D-Ala synthetase	0.030	0.033	0.031
D-Ala-D-Ala adding	0.26	0.42	0.46
Mutant D-CS_a			
Alanine racemase	10.7	9.6	8.3
D-Ala-D-Ala synthetase	0.16	0.16	0.17
D-Ala-D-Ala adding	0.30	0.28	0.35

[a] The assays and growth conditions are described in the Experimental Section.

The increase in specific activities observed with mutant (D-CS_a) could be either the result of an endogenous inhibitor in the parent strain or the result of a limitation in an essential cofactor. If either of these were correct, one would observe an apparent stimulation or inhibition of the activity in one extract by the addition of the other extract. The results in Table IV illustrate that the sum of the activities in the extracts assayed separately equals the activities when the extracts are assayed simultaneously. Thus, the increased specific activities do not appear to be the result of an endoge-

TABLE IV: Summation of Activities in Extracts from the Parent and Mutant (D-CS_a).

Extract	Activity (nmoles/10 min)	
	Alanine Racemase	D-Ala-D-Ala Synthetase
1. *Streptococcus* strain Challis	5.5 (40)[a]	6.3 (40)
2. Mutant (D-CS_a)	5.6 (5)	9.1 (10)
Sum	11.1	15.4
3. Combined[b]	11.2	14.3

[a] The number in parentheses is micrograms of extract protein added. [b] In 3 the Alanine Racemase Assay contains 40 μg of the parent extract and 5 μg of the mutant extract and the D-Ala-D-Ala Synthetase Assay contains 40 μg of the parent extract and 10 μg of the mutant extract.

nous inhibitor or the result of a limitation in an essential cofactor (*e.g.*, pyridoxal phosphate) in the parent strain.

In several cases resistance to an antibiotic may be associated with the production of modified enzymes. For example, the elevated dihydrofolate reductase isolated from an amethopterin-resistant *Diplococcus pneumoniae* (ame^r-3) has a heat stability that differs from that for the enzyme prepared from the parent strain (Sirotnak *et al.*, 1964). In Figure 1 the heat inactivation of alanine racemase and D-Ala-D-Ala synthetase is shown. In the presence or absence of ATP, no difference could be detected between the synthetase from the parent strain and that from the mutant strain (D-CS$_a$). Moreover, the rate constants for the first-order decay of alanine racemase were identical for both strains.

In addition to the heat stability, the Michaelis–Menten constants for the synthetase and racemase have been established for the parent and mutant (D-CS$_a$) strain (Figure 2A,B). Identical intercepts on the abscissa ($-1/K_m$) indicate that the Michaelis constants are not different. The inhibition of the synthetase and racemase by D-cycloserine was examined in an attempt to distinguish between the enzymes from mutant (D-CS$_a$) and the parent. No difference in the values of K_i could be detected (Figure 3A,B). Thus, this suggests that alanine racemase and D-Ala-D-Ala synthetase of the mutant strain are identical with those of the parent strain. In addition, these results also indicate that formation of an altered enzyme with decreased sensitivity to inhibition by the antibiotic is not a mechanism of resistance in this strain.

A mutant strain might be resistant to D-cycloserine if it were capable of forming an altered cell wall in which D-alanine was not an essential component. Cell walls were isolated from the parent and mutant strain (D-CS$_a$) and analyzed for amino acids. The results in Figure 4 are presented as molar ratios of amino acid to glutamic acid. These analyses indicate that the walls of the parent and mutant strain (D-CS$_a$) contain almost identical ratios of alanine to glutamic acid.

Many of the D-cycloserine-resistant mutants that were isolated did not show increased specific activities of alanine racemase and D-Ala-D-Ala synthetase. For example, mutant (D-CS$_b$) is eightfold resistant to the action of D-cycloserine (Table II), and yet the levels of the D-cycloserine-sensitive enzymes are not significantly different from those in the parent strain. It seemed likely that the antibiotic resistance of this strain might be the result of an alteration in the permeability of the cells to D-cycloserine.

Mora and Snell (1963) reported that *Streptococcus faecalis* R 8043 contains a transport system for glycine, L-alanine, and D-alanine which requires an energy source, possesses a high temperature coefficient, and is saturated at relatively low external concentrations of alanine. In addition, they reported that D-cycloserine (1×10^{-4} M) inhibited the accumulation of [^{14}C]-alanine.

Cells of the parent, mutant (D-CS$_a$), and mutant (D-CS$_b$) were examined for their ability to concentrate D-[^{14}C]alanine and L-[^{14}C]alanine. The results with L-[^{14}C]alanine are illustrated in Figure 5A. Mutant (D-CS$_a$) and the parent strain concentrate L-[^{14}C]-alanine at almost identical rates. However, mutant (D-CS$_b$) lacks the ability to concentrate L-[^{14}C]alanine (Figure 5A) and D-[^{14}C]alanine (Table V). A preloading procedure similar to that described by Winkler and Wilson (1966) was found to increase the initial rate of uptake of [^{14}C]alanine. The amount accumulated is proportional to time for at least 5 min (Figure 5B). If glucose is omitted or if sodium azide and iodoacetate are added, the uptake is decreased to 10% or less (Figure 5B). After 5 min of accumulation, Mora and Snell (1963) observed that all of the alanine concentrated in *S. faecalis* R 8043 could be extracted as free alanine. With *Streptococcus* strain Challis that was not preloaded with alanine, a portion of the radioactivity extracted after 10 min was found in a nucleotide which

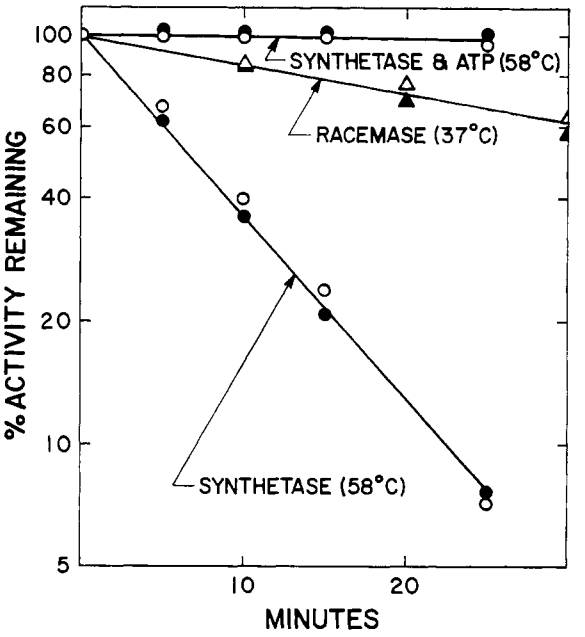

FIGURE 1: Heat denaturation of alanine racemase and D-Ala-D-Ala synthetase in cell-free extracts of *Streptococcus* strain Challis and mutant (D-CS$_a$). Samples of the extracts were incubated in the following mixtures: (1) "Synthetase + ATP (58°)," 0.01 M $MgCl_2$; 0.01 M ATP neutralized with NaOH; 2 mg of bovine serum albumin/ml; 0.02 M potassium phosphate buffer (pH 8.0); and 400 μg of extract protein/ml. (2) "Racemase (37°)," all components except ATP and $MgCl_2$. (3) "Synthetase (58°)," all components except ATP and $MgCl_2$. The samples were incubated at the indicated temperature and aliquots (50 μl) were removed at the indicated time and assayed for enzyme activity according to the procedures described in the Experimental Section. The open symbols represent the parent extract and the closed symbols represent the extract from mutant (D-CS$_a$).

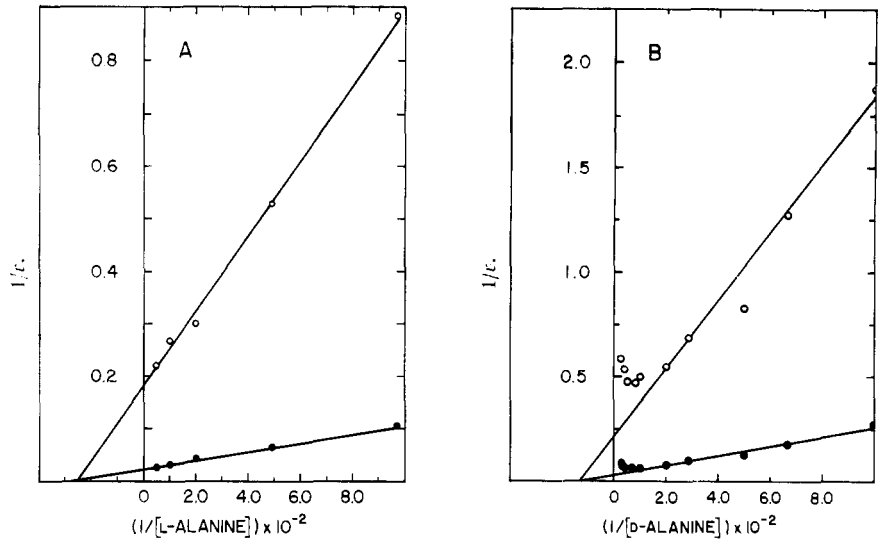

FIGURE 2: Lineweaver–Burk plots of alanine racemase (A) and D-Ala-D-Ala synthetase (B) from *Streptococcus* strain Challis (O—O) and mutant (D-CS_a) (●—●).

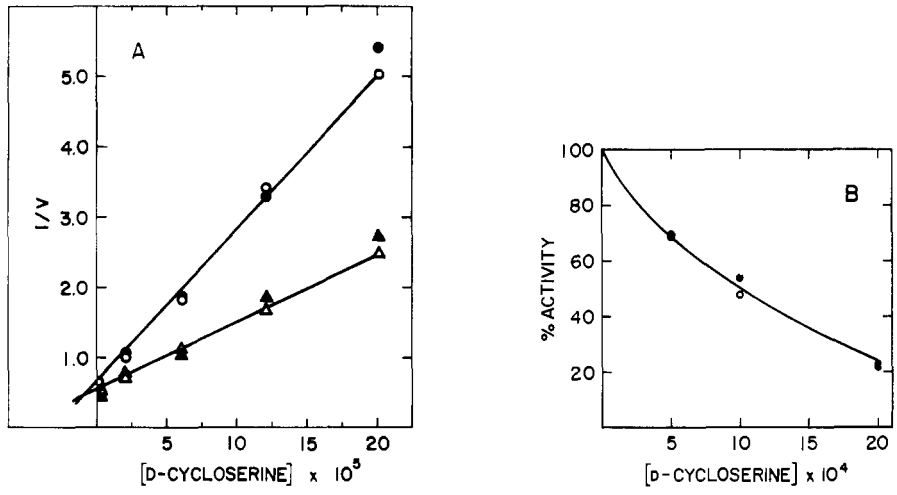

FIGURE 3: Inhibition of D-Ala-D-Ala synthetase (A) and alanine racemase (B) by D-cycloserine. For A the concentrations of D-alanine are (O, ●) 0.005 M, (△, ▲) 0.01 M. For A and B the open symbols and closed symbols are enzymes from the parent and mutant, respectively.

had an R_F equal to that of UDP-NAc-muramyl-pentapeptide in two solvent systems (Figure 6). The remainder was extracted as free alanine. Thus, in order to minimize secondary reactions, initial rates of accumulation were established during short time intervals (2 min) in preloaded cells (see above).

In agreement with the results of Mora and Snell (1963), D-alanine (1 mM) reduces the uptake of L-[^{14}C]-alanine (1 mM), and L-alanine (1 mM) reduces the uptake of D-[^{14}C]alanine (1 mM) (Table V). This indicates that D-alanine and L-alanine enter *Streptococcus* strain Challis by the same transport system. In a similar manner, D-cycloserine reduces the uptake of both D-[^{14}C]alanine and L-[^{14}C]alanine (Table V).

Resistance to O-Carbamyl-D-serine. Mutants resistant to *O*-carbamyl-D-serine were isolated by the procedure outlined in Table VI. Cell-free extracts were prepared and assayed for alanine racemase, D-Ala-D-Ala synthetase, L-lysine adding enzyme, and D-Ala-D-Ala adding enzyme. As shown in Table VII, an increase in the specific activity of alanine racemase is observed at each step with no significant changes in the specific activities of the other enzymes. Further, a correlation is observed between the minimal inhibitory concentration and the relative specific activity of alanine racemase.

Transformation of D-Cycloserine Resistance. The enzyme profiles of five transformants were examined

TABLE V: Initial Rates of Accumulation of L-[^{14}C]-Alanine and D-[^{14}C]Alanine in *Streptococcus* Strain Challis, Mutant (D-CS$_a$), and Mutant (D-CS$_b$).[a]

Additions	[^{14}C]Alanine Accumulated (pmoles/min mg^{-1})		
	Parent	D-CS$_a$	D-CS$_b$
D-[^{14}C]Alanine			
1. Complete system	620	810	74
2. +Sodium azide +Iodoacetate	48	—	—
3. +D-Cycloserine	220	—	—
4. +L-Alanine	170	—	—
L-[^{14}C]Alanine			
1. Complete system	570	720	63
2. +Sodium azide +Iodoacetate	54	—	—
3. +D-Cycloserine	361	—	—
4. +D-Alanine	274	—	—

[a] The Alanine Accumulation Assay was used with 1×10^{-3} M L- or D-[^{14}C]alanine, and 180 mg of bacteria (wet weight). The additions were (2) 0.03 M sodium azide and 1×10^{-3} M iodoacetate; (3) 1×10^{-3} M D-cycloserine; and (4) 1×10^{-3} M alanine. Blank spaces not determined.

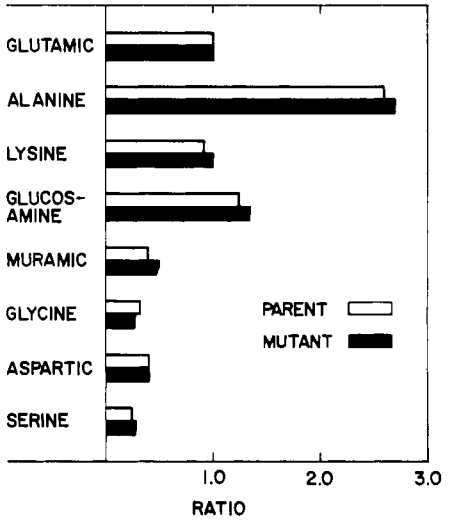

FIGURE 4: Composition of the cell walls from *Streptococcus* strain Challis and mutant (D-CS$_a$). The bars represent the molar ratio of the compounds to glutamic acid. The amino acid composition was determined by methods described in the Experimental Section. The preparation of walls was described by Struve *et al.* (1966).

(Table VIII). These transformants were isolated as described in the Experimental Section from competent cells exposed to DNA isolated from mutant (D-CS$_a$). In contrast to mutant (D-CS$_a$), none of the transformants showed an elevation in both enzymes. However, in every case either D-Ala-D-Ala synthetase or alanine racemase was elevated. The two classes of transformants may be further distinguished by their sensitivity to *O*-carbamyl-D-serine. One class (increased alanine racemase) is tenfold resistant to the action of this antibiotic whereas the other class (increased D-Ala-D-Ala synthetase) is almost as sensitive to this agent as the parent strain (Table VIII).

Discussion

Mutant (D-CS$_a$) has elevated levels of the antibiotic-sensitive enzymes, alanine racemase and D-Ala-D-Ala synthetase. It is not possible with the present results to distinguish between an elevated level (concentration) of enzyme and a modified rate-limiting catalytic constant. In other respects, however, the enzymes from the parent and mutant (D-CS$_a$) have identical Michaelis–Menten constants, inhibitor constants, and heat stabilities.

With regard to mutant (D-CS$_a$), four alternative mechanisms have been eliminated as possibilities for the acquisition of D-cycloserine resistance. For example, mutant (D-CS$_a$) has an alanine-concentrating system

TABLE VI: Isolation of *O*-Carbamyl-D-Serine Resistant Mutants.[a]

O-Carbamyl-D-serine[b] (moles/l. $\times 10^3$)	Step[e]			Minimal Inhibitory Concn (moles/l. $\times 10^3$)
	1	2	3	
0	>300			
2	6			
3	0			
4	0			
5	2[c]	>300		5
6		>300		
7		>300		
8		10		
9		3		
10		1[c]	>300	12
13			>300	
16			>300	
20			2[d]	20

[a] Approximately 10^7 cells were spread on each plate. The colonies growing on the plate with the highest concentration of antibiotic were grown in Todd–Hewitt broth (see mutant isolation) and 10^7 cells were transferred to the next series. [b] Concentration of *O*-carbamyl-D-serine in the blood agar plate. [c] Mutant grown up for transfer to the next series. [d] Mutant referred to as mutant (O-CS). [e] Colonies per plate.

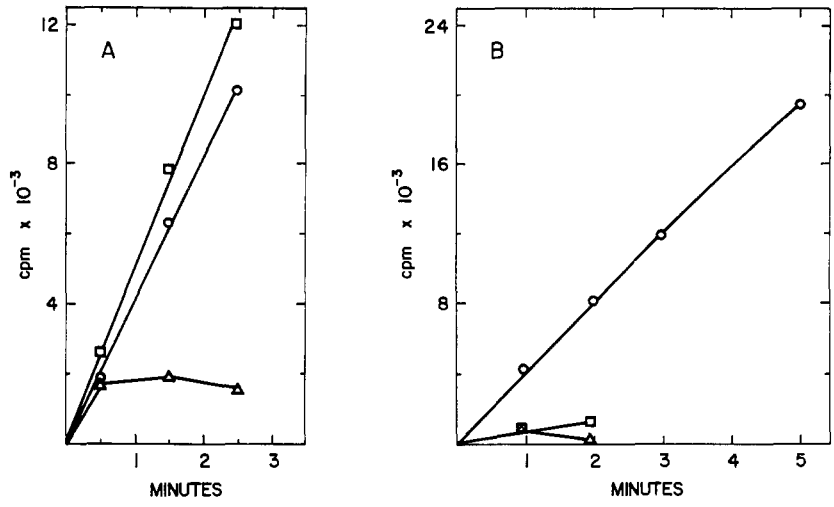

FIGURE 5: Accumulation of alanine by *Streptococcus* strain Challis, mutant (D-CS$_a$), and mutant (D-CS$_b$). The cells were preloaded and assayed as described in the Alanine Accumulation Assay. In A the following strains were used: (O—O) parent, (□—□) mutant (D-CS$_a$), and (△—△) mutant (D-CS$_b$). In B the additions are as follows (O—O) complete system, (□—□) minus glucose, (△—△) 0.03 M sodium azide and 1×10^{-3} M iodoacetate.

TABLE VII: Enzyme Profiles in *O*-Carbamyl-D-serine-Resistant Mutants.[a]

Enzyme	Specific Activity (units/mg of protein)			
	Parent	1	2	3[b]
Alanine racemase	0.89	3.1	6.6	7.1
D-Ala-D-Ala synthetase	0.029	0.030	0.043	0.023
D-Ala-D-Ala adding	0.45	0.33	0.42	0.40
L-Lysine adding	0.14	0.17	0.15	0.12
Minimal inhibitory concentration $\times 10^3$	1.5	5.0	12	20

[a] The mutant isolation is described in Table VI.
[b] Mutant (O-CS) described in other experiments is the third-step mutant.

which is similar to that of the parent.[2] In addition, it does not have the ability to degrade D-cycloserine (unpublished observations). Since the cell walls from the parent and mutant have the same molar ratio of alanine to glutamic acid, there does not appear to be a decreased requirement for alanine. Moreover, the inhibitor constants for alanine racemase and D-Ala-D-Ala synthetase are identical for the enzymes from the parent and mutant (D-CS$_a$).

In the mutants selected for resistance to *O*-carbamyl-D-serine, only alanine racemase is increased. The production of D-Ala-D-Ala synthetase, D-Ala-D-Ala adding enzyme, and L-lysine adding enzyme is not significantly different from the parent strain. In addition, at each step there is a correlation between the minimal inhibitory concentration and the specific activity of alanine racemase. Therefore, it is concluded that increased production of the drug-sensitive enzymes is a primary mechanism of antibiotic resistance in mutant (D-CS$_a$) and mutant (O-CS).

Increased production of the drug-sensitive enzymes has been implicated as a mechanism of resistance in other situations. For example, mutants selected for resistance to 5-methyltryptophan produce elevated levels of the enzymes in the tryptophan pathway (Cohen and Jacob, 1959). Similarly, strains of bacteria resistant to arginine analogs, *e.g.*, canavanine, produce elevated levels of the enzymes of the arginine pathway (Maas, 1961).

The elevated levels of alanine racemase and D-Ala-D-Ala synthetase in mutant (D-CS$_a$) and mutant (O-CS) may be the result of a breakdown in the regulatory mechanism governing the rate of their synthesis. The stepwise development of resistance in the selection of the *O*-carbamyl-D-serine-resistant mutants with the associated increase in enzyme levels may be the cumulative result of mutations which alter the structure of either the repressor or operator (Jacob and Monod, 1961).[3] The end result of the selection procedures

[2] Since D-cycloserine inhibits the accumulation of alanine in *S. faecalis* (Mora and Snell, 1963) and *E. coli* W (Kessel and Lubin, 1965), it has been suggested that the concentrating system for alanine also accumulates D-cycloserine.

[3] An alternative explanation for these results would involve changes in the control of constitutive enzyme synthesis.

TABLE VIII: Enzyme Profiles in Transformants Resistant to D-Cycloserine.[a]

Enzyme	Specific Activity (units/mg of protein)				
	Parent	T11	T12	T1A	T2B
Alanine racemase	1.2	6.7	8.9	1.8	1.5
D-Ala-D-Ala synthetase	0.035	0.033	0.036	0.22	0.21
D-Ala-D-Ala adding	0.35	0.37	0.22	0.60	0.45
L-Lysine adding	0.12	0.13	0.09	0.08	*b*
	Minimal Inhibitory Concentration				
[D-Cycloserine] $\times 10^4$	5	75	100	50	50
[*O*-Carbamyl-D-serine] $\times 10^3$	2	20	*b*	5	5

[a] The transformants, T11, T12 were isolated in one series whereas, T1A and T2B were isolated in another series.
[b] Not determined.

with *O*-carbamyl-D-serine could be a total absence of control in the rate of synthesis of the antibiotic-sensitive enzyme. This is consistent with the numerous isolations of *O*-carbamyl-D-serine-resistant mutants in which alanine racemase is increased to the same extent (eightfold). In addition, mutant (D-CS_a) also possesses an eightfold increase in alanine racemase.

In many cases, when the enzymes of a biosynthetic pathway are subject to regulation, all of the enzymes are either coordinately repressed or derepressed. For example, Ames and Garry (1959) observed that the enzymes involved in the biosynthesis of histidine remained in constant proportion to each other over a wide range of repressed and derepressed levels. In contrast, the four enzymes studied in this paper involved in the biosynthesis of UDP-NAc-muramyl-L-Ala-D-isoGlu-L-Lys-D-Ala-D-Ala appear to be under independent control. This conclusion is based on the following observations. (1) In mutant (D-CS_a) alanine racemase and D-Ala-D-Ala synthetase are increased eight- and fivefold, respectively. In contrast, the D-Ala-D-Ala adding enzyme and L-lysine adding enzyme are not increased. (2) In mutant (O-CS), alanine racemase is increased eightfold, whereas the specific activities of the other enzymes are identical with those of the parent. (3) Transformation studies show that it is possible to transfer independently the genetic determinants in mutant (D-CS_a) (increased alanine racemase or increased D-Ala-D-Ala synthetase) to a recipient strain. No transformants were detected with increased levels of both enzymes.

Mutant (D-CS_b) produces normal levels of the drug-sensitive enzymes, alanine racemase and D-Ala-D-Ala synthetase, as well as normal levels of D-Ala-D-Ala adding enzyme and L-lysine adding enzyme. In contrast to mutant (D-CS_a), this mutant differs in its ability to concentrate alanine. On the basis of the experiments presented in this paper and those presented by Mora and Snell (1963) and Kessel and Lubin (1965), we propose that D-cycloserine is also concentrated by the alanine-transport system. Since mutant (D-CS_b) possesses a defective transport system for alanine, it would appear that it is also unable to concentrate D-cycloserine.

A mutation which results in a decreased permeability

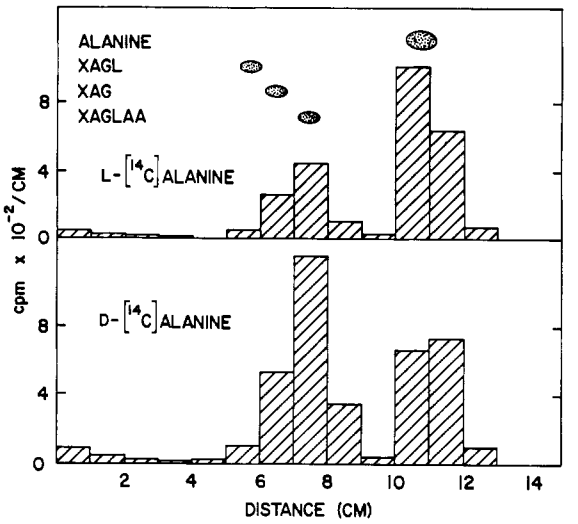

FIGURE 6: Chromatography of ^{14}C-labeled compounds extracted from cells incubated with D-[^{14}C]alanine and L-[^{14}C]alanine. The hot-water extract was prepared from cells (20 mg dry wt) of *Streptococcus* strain Challis which had been incubated for 10 min at 25° in a medium that contained 20 mg of glucose, 2 μmoles of [^{14}C]alanine (10^6 cpm), and 50 μmoles of potassium phosphate buffer (pH 8.0) in a volume of 2 ml. The hot-water extract of these cells was chromatographed on Whatman 3MM with 1-butanol–acetic acid–water (200:30:75, v/v) in a descending system. In contrast to the Alanine Accumulation Assay, these cells were not preloaded with alanine. The abbreviations are XAGL, UDP-NAc-muramyl-L-Ala-D-isoGlu-L-Lys; XAG, UDP-NAc-muramyl-L-Ala-D-isoGlu; XAGLAA, UDP-NAc-muramyl-L-Ala-D-isoGlu-L-Lys-D-Ala-D-Ala.

to the antibiotic is a common mechanism of drug resistance (Moyed, 1964). For example, Schwartz (1959) isolated two mutants of *E. coli* that are unable to concentrate D-serine and canavanine, respectively. As a result, they are resistant to the action of these compounds. Recently Unowsky and Rachmeler (1966) observed that *E. coli* which had acquired antibiotic resistance through the resistance-transfer factor had an altered permeability to several [^{14}C]antibiotics.

Curtiss *et al.* (1965) isolated three cycloserine-resistant mutants (cyc^{r1}, cyc^{r2}, and cyc^{r3}). On the basis of bacterial conjugation experiments, it was concluded that all three mutations were linked to the met_1 locus. The mutations in cyc^{r2} and cyc^{r3} appear to be in the same gene. They proposed that the mutation in the cyc^{r1} strains affects alanine racemase, whereas both the second and third mutations found in cyc^{r3} strain affect D-Ala-D-Ala synthetase. It would be very interesting to apply the methods described in this paper to the mutants isolated by Curtiss *et al.* (1965).

When antibiotics with more than one site of action (*e.g.*, D-cycloserine) are utilized, alterations in permeability may be more important than in those cases where the antibiotic has a single site of action (*e.g.*, *O*-carbamyl-D-serine). Decreased uptake of D-cycloserine would "protect" both enzymes, whereas a mutation which caused increased production of one of the sensitive enzymes would still find the biosynthetic sequence restricted by inhibition of the other sensitive enzyme. Except for mutant (D-CS_a), the other D-cycloserine-resistant mutants that were isolated resembled mutant (D-CS_b). On the other hand, all of the mutants which were selected for resistance to *O*-carbamyl-D-serine contained increased levels of alanine racemase.

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