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Inhibition of Growth of *Mycobacterium smegmatis* and of Cell Wall Synthesis by D-Serine

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D-Serine inhibited the growth of *Mycobacterium smegmatis* and induced the morphological alteration of the bacilli. The growth inhibitory action of D-serine was partially reduced by an equimolecular concentration of D-alanine. The combination of glycine with D-alanine reversed the growth inhibition produced by D-serine more than did D-alanine alone. In cells cultured in the presence of D-serine, the amounts of alanine, diaminopimelic acid, and glycine inserted into the cell wall mucopeptide were reduced, and serine was increased. The intracellular accumulation of a precursor of cell wall mucopeptide was increased by D-serine, and this accumulation was reduced by D-alanine. D-Serine competed with glycine for incorporation into the cell wall mucopeptide. The incorporation of L-aspartic acid into diaminopimelic acid residues in the cell wall mucopeptide was markedly inhibited by D-serine. Three mutants resistant to D-serine were isolated by nitrosoguanidine treatment. In these mutants the effects of D-serine on the sites of cell wall mucopeptide synthesis were all reduced. Thus, D-serine inhibition of the growth is due to replacement of glycine residues of the cell wall mucopeptide with D-serine and inhibition of the cell wall synthesis by blocking the formation of D-alanine and diaminopimelic acid.

The inhibitory effects of D-serine on growth have been shown in a variety of bacteria (4, 5, 11, 20, 29, 35, 36, 39). It has also been reported that D-serine induces the morphological alteration and the inhibition of cell division of some bacteria (9, 10, 18). In *Micrococcus lysodeikticus*, the inhibition of growth (39) and cell division (10) caused by D-serine were reversed by the addition of D-alanine to the growth medium. This effect is similar to the effects of *O*-carbamyl-D-serine and D-cycloserine, which are inhibitors of the alanine racemase that is the enzyme essential for the synthesis of uridine diphosphate-*N*-acetylmuramylpentapeptide, a precursor of cell wall mucopeptide (19, 28, 34). Some studies concerning the alanine racemase have shown that D-serine inhibits the racemization of alanine (13, 19). Glycine also reversed the inhibitory action of D-serine on the growth of *M. lysodeikticus* (39) and *Pasteurella pestis* (29). Whitney and Grula (40) observed that *M. lysodeikticus* cells incorporate large amounts of D-serine into the cell wall mucopeptide with a concomitant decrease in levels of glycine when cultured in a defined medium containing D-serine. The peptide moiety of the cell wall mucopeptide of *M. lysodeikticus* is composed of a basal peptide subunit, L-alanyl-D-glutamyl($^{\alpha}$glycine)$^{\gamma}$L-lysyl-D-alanine (7, 37). It has been suggested that approximately 75 to 80% of the incorporated D-serine can substitute for glycine and be attached via its amino group to the α-carboxyl group of D-glutamic acid (40). Recently, Kotani et al. (16) found in *Mycobacterium tuberculosis* the existence of a new tetrapeptide, L-alanyl-D-glutamyl($^{\alpha}$glycine)$^{\gamma}$-diaminopimelic acid, besides a tripeptide (L-alanyl-D-glutamyl-diaminopimelic acid) and a tetrapeptide (L-alanyl-D-glutamyl-diaminopimelyl-D-alanine), as basal peptide subunits of the cell wall mucopeptide. The overall structure of the mycobacterial cell wall mucopeptide seems to be more complicated, since a peptide moiety of the mucopeptide contains several minor amino acids in addition to the usual three amino acids as the major constituents (21, 25, 41). Since D-serine inhibits the growth of mycobacterial cells, it was of interest to determine whether or not growth inhibition by D-serine reflects a mode of action seen in *M. lysodeikticus*.

The purpose of the present paper is to elucidate the mechanisms of D-serine on the growth

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and cell wall synthesis in *Mycobacterium smegmatis* and its mutants selected for resistance to D-serine.

MATERIALS AND METHODS

Microorganism and measurement of growth. The strain studied was *M. smegmatis* ATCC 607. The growth experiments were conducted in a modified Dubos liquid medium. The components of the basal medium were prepared as follows. Solution I contained in 900 ml: KH_2PO_4, 1.0 g; $Na_2HPO_4 \cdot 12H_2O$, 6.3 g; $MgSO_4 \cdot 7H_2O$, 0.01 g; $CaCl_2$, 0.5 mg; $ZnSO_4$, 0.1 mg; $CuSO_4$, 0.1 mg; sodium L-glutamate, 3.4 g; sodium citrate, 0.13 g; ferric ammonium citrate, 0.01 g; and Tween 80, 0.5 g (final pH 6.8). D-Serine and other test materials were added before autoclaving. Solution II contained in 100 ml: NaCl, 0.85 g; albumin fraction V, 5.0 g; and glucose, 7.5 g; it was sterilized by passage through membrane filters (Millipore Corp., Bedford, Mass.). The test tubes for growth measurements contained 4.5 ml of solution I with or without the test materials and 0.5 ml of solution II. The inoculum consisted of 0.2 ml of cells in the early log-phase of growth. The tubes were maintained at 37 C and shaken twice daily to suspend the cells. The growth was measured turbidimetrically by using a Hitachi model 101 spectrophotometer at a wavelength of 470 nm. Experiments were performed at least twice to insure reproducibility, and all measurements were run in duplicate. Means of duplicate measurements from one experiment are presented in Fig. 1.

Amino acid analysis of cell wall mucopeptide. The cells were grown for three days in the same medium as described for the growth measurement. To isolate the cell wall, the fractionation procedure of Kotani et al. (15) was employed, except that sonic disruption with a Kubota 10-kc sonic oscillator was shortened to 10 to 20 min. Lipid was removed from cell wall preparations at room temperature by repeated extraction with chloroform-methanol (2:1, vol/vol). The delipidated cell walls were hydrolyzed in sealed tubues with 6 N HCl at 100 C for 16 h and then dried in vacuo over concentrated H_2SO_4 and solid NaOH. The residues were dissolved in water, adsorbed on the column of Amberlite CG-120, and eluted. The desalted samples were subjected to descending two-dimensional paper chromatography on Whatman no. 1 filter paper using solvent A (*n*-butanol-pyridine-water-acetic acid; 60:40:30:3, vol/vol/vol/vol) in the first direction and solvent B (pyridine-water; 3:1, vol/vol) in the second direction. After drying, areas containing the individual amino acids were detected by spraying the paper with 0.2% ninhydrin in water-saturated *n*-butanol and subsequently drying at 70 C for 20 min. These areas were cut out so that all spots of a given amino acid and appropriate paper blanks were equivalent in size. Each area was eluted with 4 ml of methanol containing 0.5% cadmium acetate. The absorbance of the eluates was measured at 500 nm. The amounts of each amino acid present were estimated by comparison with the standards tested in the same manner.

Intracellular accumulation of N-acylamino sugar. The cells were grown in a Saunton medium which contained (per liter): K_2HPO_4,0.5 g; $MgSO_4 \cdot 7H_2O$ 0.5 g; L-asparagine, 4.0 g; citric acid, 2.0 g; ferric ammonium citrate, 0.05 g; and glycerol, 50.0 g (final pH 7.0). They were harvested at the log-phase on filter paper in a funnel, washed with water, and then suspended, by grinding in a mortar, in ice-cold water at a concentration of 23 to 41 mg (dry weight)/ml. Incubations were carried out in 50-ml Erlenmeyer flasks at 37 C with vigorous shaking for 6 h. We used a 10-ml amount of incubation medium containing (per milliliter): phosphate buffer (pH 7.0), 40 μmol; glucose, 40 μmol; sodium L-glutamate, 10 μmol; $MgSO_4$, 0.1 μmol; the test materials in varying amounts; and 23 to 41 mg of cells. The cells were collected by centrifugation and washed with cold water. The cell pellet was taken up in 1 ml of water and extracted in a boiling-water bath for 10 min. The *N*-acylamino sugar of the extract was measured by a modified method (30) of the Morgan-Elson reaction using *N*-acetylglucosamine as the standard.

Analyses of an accumulated uridine nucleotide were carried out as follows. The log-phase cells from the Sauton medium were suspended in the incubation medium containing 25 mM D-serine, incubated at 37 C with shaking for 6 h, and then collected by centrifugation. The cells were extracted with 5% trichloroacetic acid overnight. The nucleotides in the extract were adsorbed onto charcoal and recovered in the cold by elution with ammoniacal ethanol. The nucleotide sample was fractionated on a column of Dowex 1 (Cl^- form, 200 to 400 mesh) as described by Nakatani et al. (24). The *N*-acylhexosamine fraction was recovered from the eluates by charcoal adsorption and elution and then subjected to two-dimensional paper chromatography on Whatman no. 3 MM paper in isobutyric acid-0.5 N NH_4OH (5:3) and ethanol-1 M ammonium acetate (7.5:3). The ultraviolet-absorbing compound was eluted from the chromatogram with water. The base present was identified by its ultraviolet absorption spectrum in 0.1 N HCl and 0.1 N NaOH. Hydrolysis to release the amino sugar and glycolic acid was done in 2 N HCl at 110 C for 4 h. The glycolic acid was identified by paper chromatography as described by Adam et al. (1). The amino sugar was identified by the modified Morgan-Elson reaction (30). Examination of amino acids was carried out by hydrolyzing in 6 N HCl at 100 C for 6 h. After removal of HCl in vacuo, the sample was subjected to paper chromatography in solvent A. Optical configuration of alanine was determined by the D-amino acid oxidase (16). Inorganic phosphate liberated by heating the sample in 1 N HCl at 100 C for 20 min was assayed by the method of Fiske and Subbarow (6).

Measurement of incorporation of L-aspartic acid, diaminopimelic acid, glycine, and D-serine into cell wall mucopeptide. The cells grown in the Sauton medium were suspended at 3.5 to 5.0 mg (dry weight)/ml in the incubation medium which contained L-[^{14}C]aspartic acid (0.2 μCi/ml), [^{14}C]diaminopimelic acid (0.125 μCi/ml), [^{14}C]glycine (0.2 μCi/ml), or D-[^{14}C]serine (0.1 μCi/ml). The final concentration was 1.0 mM for L-aspartic acid, glycine, and D-serine, and 0.25 mM for diaminopimelic acid.

Incubations were done in the same conditions as described above. The cells were collected by centrifugation, washed with cold water, and fractionated to isolate the cell wall. The delipidated cell wall preparations were hydrolyzed, and the hydrolysates were adsorbed onto, and then eluted from, the columns of Amberlite CG-120 and CG-400, respectively. The desalted solutions were streaked onto sheets of Whatman no. 3 MM paper and subjected to chromatography in solvent A. In experiments for incorporation of L-aspartic acid and diaminopimelic acid, the band on the chromatograms corresponding to diaminopimelic acid was eluted with water. Suitable portions were taken for determination of the specific radioactivity of diaminopimelic acid. The radioactivity measurements were carried out with an Aloca gas-flow counter. Diaminopimelic acid was determined by the method of Gilvarg (8). In the experiment with [^{14}C]glycine or D-[^{14}C]serine, the radioactivity of the incorporated glycine or D-serine was detected after separation by two-dimensional paper chromatography in solvents A and B. Amino acid content was determined by the method of Moore and Stein (23).

Mutant isolation. The cells grown in the modified Dubos liquid medium overnight were suspended in 0.85% saline supplemented with 0.05% Tween 80. The suspensions were incubated at a concentration of 100 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml for 30 min. The mutagenized suspensions were washed with saline, incubated in the modified Dubos liquid medium overnight, and plated on a nutrient agar which contained (per liter) 900 ml of solution I, 100 ml of 5% glucose, and 10 mmol of D-serine. After incubation of the plates for 3 days, small colonies were picked, and their D-serine resistance was checked.

Measurement of D-serine uptake. The cells required for experimental procedures were grown for 20 h in the modified Dubos liquid medium. They were harvested, washed, and resuspended in 0.05% Tween 80 solution to give about 1 mg (dry weight) per ml of suspension. Uptake was measured at 37 C on a reciprocal shaking water bath in 50-ml Erlenmeyer flasks, each containing a total volume of 4 ml. A portion (1 ml, 5×10^7 cells) of the cell suspension was added to the incubation mixture. The final incubation mixture contained (per milliliter): phosphate buffer (pH 7.0), 40 μmol; glucose, 25 μmol; chloramphenicol, 100 μg; Tween 80, 0.25 mg; and D-[^{14}C]serine in varying amounts (12.5 to 100 μmol). Samples were removed at the desired time, and the cells were collected on a 0.45-μm membrane filter. The cells were washed with 15 ml of ice-cold 0.04 M phosphate buffer containing 0.025% Tween 80. Measurements of the radioactivity were made on a stainless-steel planchet by using the gas-flow counter. The initial velocity of uptake was calculated from samples taken at 5 min at which uptake was linear with time. The Lineweaver-Burk plot of the data exhibited a linear curve in all D-serine-resistant mutants and the parent strain.

Electron microscopy. The cells were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) for 2 h at 2 C. After washing with the same buffer, cells were postfixed for two days in 1% OsO_4 in 0.1 M phosphate buffer (pH 7.4) at 2 C. Stepwise dehydration in ethanol was carried out at room temperature, and embedding was performed in Epon 812. Sections were cut on a Porter-Blum ultramicrotome with glass knives and stained with lead citrate and uranyl acetate. Examination was carried out with a JEM 100B electron microscope.

Chemicals. L-[U-^{14}C]aspartic acid (227 mCi/mmol) and D-[3-^{14}C]serine (49 mCi/mmol) were purchased from Radiochemical Centre, Amersham, England; α,ϵ-[1-^{14}C]diaminopimelic acid (9.8 mCi/mmol) from Calatomic, Los Angeles, Calif.; and [2-^{14}C]glycine (21.2 mCi/mmol) from Daiichi Pure Chemical Co., Tokyo, Japan. Nonradioactive D- and L-alanine, L-aspartic acid, and D- and L-glutamic acid were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. DL-Alanyl-DL-alanine, α,ϵ-diaminopimelic acid, and D-serine were products of Sigma Chemical Co., St. Louis, Mo. All other chemicals used were of reagent grade.

RESULTS

Reversal of growth inhibitory effect of D-serine by amino acids. The results of the growth experiments are shown in Fig. 1. Approximately 90% of inhibition of the growth of *M. smegmatis* was obtained at 10 mM concentration of D-serine under the experimental conditions employed. This growth inhibition was reversed approximately 50% by the addition of 10 mM D-alanine to the growth medium. A similar effect was observed with 10 mM DL-alanyl-DL-alanine. Increasing the concentration of these compounds did not promote further reversal of the growth inhibitory action of D-serine. The addition of D-alanine and DL-alanyl-DL-alanine in combination produced no combined reversal of the growth inhibitory effect of D-serine. Glycine did not reverse the growth inhibition caused by D-serine, but the combination of glycine with D-alanine reversed more effectively than did D-alanine. The combination of glycine with DL-alanyl-DL-alanine resulted in more growth than that observed in the combination with D-alanine. L-Aspartic acid and diaminopimelic acid were found to possess no significant D-serine reversal activity.

Cultures grown in the modified Dubos liquid medium, with and without D-serine, were examined at varying intervals of time to observe any changes in morphology. Phase-contrast microscopy of the bacilli showed that in the control culture the cells were all rod-shaped, whereas in the presence of 10 mM D-serine they produced spheroplasts in 2 to 3 days in approximately 1 to 5% of the cell population, which was predominantly spheroidal. In electron microscopic studies of the spheroidal cells, detachment of the plasma membrane from the inner surface of cell walls, partial breakdown of cell walls of normal

thickness, and occurrence of plasmolysis could be observed. The spheroidal forms seemed to be the intermediate stage in the formation of round forms. The spheroplasts burst readily when subjected to vigorous mechanical shock, but the spheroidal cells were less fragile osmotically. This fact, together with spheroplast formation occurring in the medium without any osmotic stabilizer, suggests that structural alteration of the cell wall may be less profound in the D-serine spheroplasts and spheroidal cells compared with the spheroplasts produced by lysozyme and glycine (26).

With the addition of D-alanine or DL-alanyl-DL-alanine to the medium, no obvious change was observed in the morphological appearance of the bacilli. The results indicate that these compounds are capable of antagonizing the morphological alteration which is attributed to the cell wall degeneration induced by D-serine.

D-Serine inhibits the growth of *Escherichia coli* by affecting pantothenate biosynthesis, and this inhibition is competitively reversed by β-alanine and noncompetitively reversed by pantothenic acid (4, 20). The addition of β-alanine or pantothenic acid to the medium failed to reverse the growth inhibitory action of D-serine in *M. smegmatis*. It thus appears that D-serine has no significant effect on the pantothenate biosynthesis of the mycobacterial cells.

Alteration in amino acid composition of cell wall mucopeptide. Each 400-ml culture in the growth conditions, with and without 5 mM concentration of D-serine, was used for fractionation of the cell wall. Approximately 70% inhibition of the growth was obtained at 5 mM concentration of D-serine. The cell wall preparations were analyzed for amino acid composition (Table 1). By the analytical techniques employed, six amino acids were detected in both cell wall preparations. The cells grown in the presence of D-serine differed from the control in containing more amino acids per milligram of cell wall. Distinct differences with regard to molar ratios of four amino acids between these cell wall preparations were observed. The results of this analysis were similar in tendency to those obtained at 10 mM concentration of D-serine in the preliminary experiment. It is apparent that the addition of D-serine to the growth medium results in decreases in the

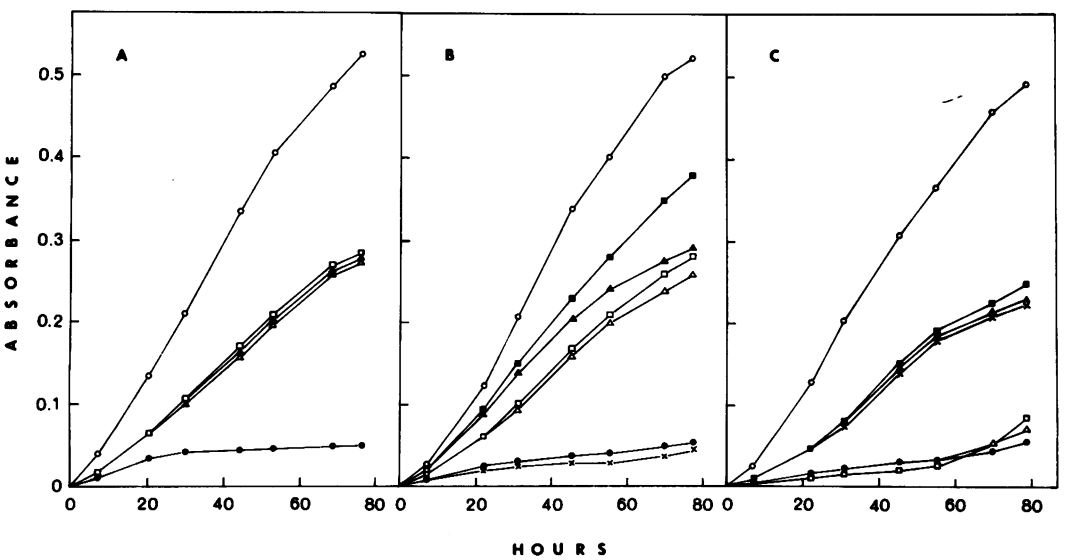

FIG. 1. *Effect of amino acids on the growth inhibition of M. smegmatis by D-serine. For details of the method, see text. The concentration of amino acids was 10 mM each. (A) Effect of D-alanine and DL-alanyl-DL-alanine singly and in combination. Symbols: ○, control; ●, D-serine; △, D-serine + D-alanine; ▲, D-serine + DL-alanyl-DL-alanine; □, D-serine + D-alanine + DL-alanyl-DL-alanine, (B) Effect of the combination of glycine with D-alanine and with DL-alanyl-DL-alanine. Symbols: ○, control; ●, D-serine; ×, D-serine + glycine; △, D-serine + D-alanine; □, D-serine + DL-alanyl-DL-alanine; ▲, D-serine + glycine + D-alanine; ■, D-serine + glycine + DL-ananyl-DL-alanine. (C) Effect of L-aspartic acid and diaminopimelic acid singly and in combination with D-alanine. Symbols: ○, control; ●, D-serine; △, D-serine + L-aspartic acid; □, D-serine + diaminopimelic acid; ×, D-serine + D-alanine; ▲, D-serine + D-alanine + L-aspartic acid; ■, D-serine + D-alanine + diaminopimelic acid.*

TABLE 1. *Amino acid composition of the cell wall mucopeptide from Mycobacterium smegmatis grown in the absence and presence of 5 mM D-serine*[a]

Medium additions	Amino acid (nmol/mg of dry cell wall)[b]					
	Aspartic acid	Alanine	Diamino-pimelic acid	Glutamic acid	Glycine	Serine
None	394 (1.65)	68 (0.28)	156 (0.65)	239 (1.00)	75 (0.31)	50 (0.21)
D-Serine	449 (1.54)	67 (0.23)	150 (0.52)	291 (1.00)	62 (0.21)	152 (0.52)

[a] Growth conditions were the same as in Fig. 1 except for D-serine concentration.
[b] Figures in parentheses represent molar ratio in which glutamic acid was set as 1 M amount.

molar ratios of alanine, diaminopimelic acid, and glycine and an increase in the amount of serine.

Intracellular accumulation of N-acylamino sugar and its reversal. Under the experimental conditions used, the cells of *M. smegmatis* accumulated *N*-acylamino sugar even in the absence of D-serine. This accumulation increased in the presence of D-serine. The net accumulation of *N*-acylamino sugar induced by the incubation in the presence of 10 mM D-serine was approximately 2 to 3 μmol/g of dry cells. This effect was markedly antagonized when DL-alanyl-DL-alanine was added at the concentration of 5 mM. D-Alanine also was capable of reducing the action of D-serine, whereas L-aspartic acid, diaminopimelic acid, and glycine were all ineffective (Table 2).

A 2-liter amount of culture at the log-phase growth was treated with 25 mM concentration of D-serine for 6 h. The cells were collected by centrifugation and extracted with 5% cold trichloroacetic acid. The *N*-acylamino sugar-containing nucleotide in the extract was recovered by charcoal adsorption and elution, separated on Dowex 1-chloride, and subjected to two-dimensional paper chromatography. An ultraviolet-absorbing area was observed on the chromatogram, and this area was eluted with water. The base present was identified by its ultraviolet absorption spectrum. Amino acid, phosphate, and acylamino sugar analyses were also carried out. By these techniques, the ultraviolet-absorbing compound was considered to contain uridine diphosphate-glycolylmuramylalanylglutamyldiaminopimelic acid. The amount of ultraviolet-absorbing compound present in the extract of a control culture was too small to analyze by these techniques.

Inhibition of incorporation of glycine into cell wall mucopeptide. Figures 2A and 2B show inhibition of the incorporation of [^{14}C]glycine and D-[^{14}C]serine into the cell wall preparations by D-serine and glycine, respectively. D-Serine inhibited the incorporation of glycine into the cell wall preparations, and, conversely, glycine inhibited the incorporation of D-serine into the cell wall preparations. The decrease in the incorporation with increasing concentrations of the inhibitor resembled each other. Radioactivity of the [^{14}C]glycine and D-[^{14}C]serine was found on only the glycine and serine, respectively, in the hydrolysates of cell wall preparations. Therefore, these findings indicate that D-serine competes with glycine for incorporation into the cell wall mucopeptide.

TABLE 2. *Effect of amino acids on the intracellular accumulation of N-acylamino sugar induced by 10 mM D-serine*

Amino acid additions	*N*-acylamino sugar accumulation[a] (μmol/g of dry cells)
None	3.30
D-Serine	5.50
+ D-Alanine, 5 mM	4.00 (68)
+ D-Alanine, 10 mM	3.98 (69)
+ DL-Alanyl-DL-alanine, 5 mM	3.48 (92)
+ DL-Alanyl-DL-alanine, 10 mM	3.60 (86)
+ L-Aspartic acid, 5 mM	5.70 (0)
+ L-Aspartic acid, 10 mM	5.70 (0)
+ Diaminopimelic acid, 5 mM	5.88 (0)
+ Diaminopimelic acid, 10 mM	6.38 (0)
+ Glycine, 5 mM	5.55 (0)
+ Glycine, 10 mM	5.55 (0)

[a] Figures in parentheses represent percent of reversal of accumulation. Cells were incubated at 37 C for 6 h in the presence of D-serine and the indicated compounds. The acylamino sugar was extracted from the cells and measured as described in the text.

Inhibition of incorporation of L-aspartic acid and diaminopimelic acid into cell wall mucopeptide. To investigate the effect of D-serine on the biosynthesis of diaminopimelic acid of the cell wall mucopeptide, the incorporation of L-[^{14}C]aspartic acid and [^{14}C]diaminopimelic acid into diaminopimelic acid residues in the cell wall mucopeptide was measured (Table 3). The incorporation of L-[^{14}C]aspartic acid

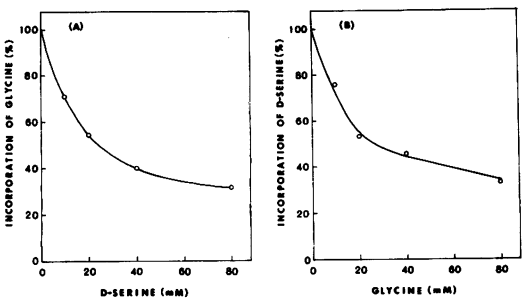

FIG. 2. *(A) Effect of D-serine on the incorporation of glycine into the cell wall preparations. Cells were incubated at 37 C for 6 h in the presence of [^{14}C]glycine (1 mM) and D-serine in varying amounts, as described in the text. The delipidated cell walls were fractionated and analyzed for radioactivity in unit weight. Results are plotted as percent of the control without D-serine. (B) Effect of glycine on the incorporation of D-serine into the cell wall preparations. Cells were incubated at 37 C for 6 h in the presence of D-[^{14}C]serine (1 mM) and glycine in varying amounts, as described in the text. The delipidated cell walls were fractionated and analyzed for radioactivity in unit weight. Results are plotted as percent of the control without glycine.*

TABLE 3. *Effect of D-serine on the incorporation of L-aspartic acid and diaminopimelic acid into diaminopimelic acid residues in the cell wall mucopeptide*

D-Serine concentration (mM)	Incorporation (counts per min per mg of mucopeptide diaminopimelic acid)[a]	
	L-Aspartic acid	Diaminopimelic acid
0	6,142	23,058
12.5	1,034 (83)	
25	760 (88)	21,731 (6)
50	605 (90)	21,238 (8)
100		18,470 (20)

[a] Figures in parentheses represent percent of inhibition. Cells were incubated at 37 C for 6 h in the presence of D-serine and either [^{14}C]aspartic acid or [^{14}C]diaminopimelic acid. The delipidated cell wall preparations were fractionated, hydrolyzed, and subjected to paper chromatography. The chromatograms were assayed for radioactivity in diaminopimelic acid.

was strikingly inhibited by D-serine. In contrast, a slight inhibition of the incorporation of [^{14}C]diaminopimelic acid was obtained with D-serine, even at concentrations from 25 to 100 mM at which the growth was completely inhibited. It is evident that D-serine inhibits synthesis of diaminopimelic acid from L-aspartic acid in *M. smegmatis*.

Effect of D-serine on three sites of cell wall mucopeptide synthesis in D-serine-resistant mutants. Three mutants resistant to the action of D-serine were isolated by nitrosoguanidine treatment. These mutants resembled the parent strain in cell morphology, acid-fast staining, and colonial appearance. The mutants that have been studied in detail are summarized in Table 4. These mutants were approximately four to six times more resistant to D-serine than the parent strain, but they showed no cross-resistance to D-cycloserine and *O*-carbamyl-D-serine. For the two mutants, DSR1 and DSR3, the Michaelis-Menten constant for D-serine transport was similar to the value obtained for the parent strain. The one mutant, DSR2, showed a little lowered affinity to D-serine, but this alteration seemed unlikely to be directly associated with resistance. Accordingly, the acquisition of D-serine resistance in these mutants is not concerned with the loss of the ability to transport D-serine.

The parent strain accumulated two times or greater the *N*-acylamino sugar in the presence of 10 and 20 mM concentrations of D-serine than in the absence of D-serine. With all the D-serine-resistant mutants the accumulation at a reduced rate was observed in the same concentrations of D-serine, although the basal level of *N*-acylamino sugar in the two mutants, DSR2 and DSR3, was higher than that of the parent strain (Table 5). In regard to the increased accumulation of *N*-acylamino sugar by the D-serine-resistant mutants in the absence of D-serine, the results are in accordance with the data obtained by Mora and Bojalil in cells of *M. acapulcensis* resistant to D-cycloserine (22). The incorporation of glycine into the cell wall mucopeptide in the parent strain was significantly inhibited by 10 and 20 mM D-serine, but that in the D-serine-resistant mutants, DSR1 and

TABLE 4. *Minimal inhibitory concentration and K_m[a] value for D-serine transport of the parent and mutant strains of M. smegmatis*

Strain	Minimal inhibitory concentration[b] (M)	K_m value[c] (M)
Parent	0.05	4.1×10^{-5}
Mutant DSR1	0.30	3.3×10^{-5}
Mutant DSR2	0.25	4.6×10^{-4}
Mutant DSR3	0.20	3.2×10^{-5}

[a] K_m, Michaelis constant.

[b] Minimal concentration of D-serine needed for complete inhibition of growth on agar medium.

[c] K_m value for D-serine transport was established from Lineweaver-Burk plots as described in Materials and Methods.

TABLE 5. *Effect of D-serine on the intracellular accumulation of N-acylamino sugar in the D-serine-resistant mutants and parent strain*[a]

D-Serine concentration (mM)	Accumulation (μmol/g of dry cells)[b]			
	Parent strain	Mutant DSR1	Mutant DSR2	Mutant DSR3
0	3.19 (100)	2.94 (100)	5.80 (100)	5.52 (100)
10	6.13 (192)	4.02 (137)	5.71 (98)	6.27 (112)
20	7.31 (230)	5.62 (191)	6.82 (118)	6.97 (125)

[a] Experimental conditions were as in Table 2.
[b] Figures in parentheses represent percent of value of the control.

DSR3, was affected to a small extent by the same concentrations of D-serine (Table 6). Inhibition of the incorporation of L-aspartic acid into diaminopimelic acid residues of the cell wall mucopeptide by 10 and 20 mM of D-serine was remarkable in the parent strain but not in the D-serine-resistant mutants (Table 7).

Thus, the mutation acquiring resistance to D-serine reduced the action of D-serine on the three sites of the cell wall mucopeptide synthesis. These data provide additional evidence in support of the suggestion that the growth inhibitory action of D-serine on *M. smegmatis* results from inhibition of at least three sites of the cell wall mucopeptide synthesis.

DISCUSSION

The growth inhibition of *M. smegmatis* produced by 10 mM D-serine could be decreased to approximately one-half of the control by the addition of an equimolecular concentration of D-alanine or DL-alanyl-DL-alanine to the growth medium. The effect of D-alanine and DL-alanyl-DL-alanine in combination was not additive in reversing the growth inhibitory action of D-serine. These findings indicate that the growth inhibition caused by D-serine is partially due to inhibition of the reaction in which D-alanine is synthesized. It has been reported that D-serine inhibits the racemization of alanine (13, 19). *O*-carbamyl-D-serine causes the accumulation of uridine diphosphate-*N*-acetylmuramyl-tripeptide, which occurs as a result of inhibition of the alanine racemase, and this effect is reversed by D-alanine (19, 34). In *Mycobacterium tuberculosis* D-cycloserine induces the accumulation of uridine diphosphate-*N*-glycolylmuramyltripeptide (33). D-Serine also induced the accumulation of this nucleotide in *M. smegmatis*, and this accumulation could be decreased by D-alanine and DL-alanyl-DL-alanine (Table 2). Therefore, the action of D-serine on the growth of *M. smegmatis* is partially inhibition of the alanine racemase which deprives the bacterium of the D-alanine necessary for synthesis of D-alanyl-D-alanine. This conclusion is compatible with the fact that the deletion of alanine of the cell wall mucopeptide occurred in the presence of D-serine.

Glycine in the normal cell wall mucopeptide is known to be attached via its amino group to the α-carboxyl group of glutamic acid (16). D-Serine competed with glycine for incorporation into the cell wall mucopeptide (Fig. 2). If D-serine is replacing glycine in the cell wall mucopeptide, one would expect such a result. Whitney and Grula (40) suggested that approximately 75 to 80% of the incorporated D-serine can substitute for glycine which is attached to the α-carboxyl group of glutamic acid in *M. lysodeikticus*. The amount of serine plus glycine in mucopeptide of the cell wall altered by 5 mM D-serine exceeded that of the normal cell wall, but did not raise the molar proportion of glutamic acid (Table 1). Presumably, some of the serine may occupy a position of free α-carboxyl group of glutamic acid.

Hydroxylysine can substitute for lysine in the cell wall of *Streptococcus faecalis* (38). D-Methionine, when present in the growth medium at high concentrations, is incorporated into the

TABLE 6. *Effect of D-serine on the incorporation of glycine into the cell wall mucopeptide in the D-serine-resistant mutants and parent strain*[a]

D-Serine concentration (mM)	Incorporation (counts per min per mg of dry cell wall)[b]			
	Parent strain	Mutant DSR1	Mutant DSR2	Mutant DSR3
0	3,746	3,861	4,594	4,280
10	2,667 (29)	3,445 (11)	3,318 (28)	3,577 (16)
20	2,042 (45)	2,587 (33)	2,834 (38)	3,327 (22)

[a] Experimental conditions were as in Fig. 2A.
[b] Figures in parentheses represent percent inhibition.

TABLE 7. *Effect of D-serine on the incorporation of L-aspartic acid into diaminopimelic acid residues in the cell wall mucopeptide in the D-serine-resistant mutants and parent strain*[a]

D-Serine concentration (mM)	Incorporation (counts per min per mg of diaminopimelic acid)[b]			
	Parent strain	Mutant DSR1	Mutant DSR2	Mutant DSR3
0	3,529	2,534	4,185	4,490
10	396 (89)	2,270 (10)	2,944 (30)	2,755 (39)
20	261 (92)	1,936 (24)	2,583 (38)	1,807 (60)

[a] Experimental conditions were as in Table 3.
[b] Figures in parentheses represent percent inhibition.

cell wall of *Alcaligenes faecalis*, presumably in place of some normal constituent (17). Also, glycine can be incorporated into the uridine nucleotide of *Staphylococcus aureus* in place of L-alanine (31). The incorporation of these amino acids into the cell wall materials induces inhibition of the growth, and this inhibition is reversed by the normal constituent amino acids. In the present study, glycine when combined with D-alanine was capable of reversing the growth inhibition caused by D-serine, but it alone was rather inhibitory at the concentration of 10 mM. The combination of glycine with DL-alanyl-DL-alanine resulted in more growth than that observed in the combination with D-alanine. This is explained by the fact that DL-alanyl-DL-alanine can reverse a growth inhibitory effect of glycine (42). Thus, glycine exerts its reversal activity on the growth inhibitory effect of D-serine, although the degree of reversal is less than that of D-alanine. The growth experiments further support the fact that the incorporation of D-serine into the cell wall mucopeptide in place of glycine is partially responsible for the growth inhibition caused by D-serine.

It was found that the decrease in diaminopimelic acid content of the cell wall mucopeptide was caused by D-serine. This is consistent with the finding that D-serine inhibited strikingly the incorporation of L-aspartic acid into diaminopimelic acid residues in the cell wall mucopeptide. Since L-aspartic acid is a precursor of four of the carbon atoms of diaminopimelic acid (8), it appears that D-serine inhibits the formation of diaminopimelic acid from L-aspartic acid. However, the addition of L-aspartic acid to the growth medium failed to reverse the growth inhibition caused by D-serine. The failure of added L-aspartic acid to reverse the growth inhibitory effect of D-serine in the culture medium indicates that the growth inhibition by D-serine involves factors other than L-aspartic acid concentration. The incorporation of diaminopimelic acid into the cell wall mucopeptide was only slightly inhibited even by high concentration of D-serine. It is probable that D-serine has no direct effect on the addition of diaminopimelic acid into the uridine nucleotide (12) and presumably the racemization of LL-diaminopimelic acid to the *meso*-isomer (2). Hence, it appears that D-serine may deprive *M. smegmatis* cells of diaminopimelic acid of the cell wall mucopeptide by interfering with a reaction associated with diaminopimelic acid synthesis.

On the basis of the present results, it may be concluded that D-serine acts on three sites of cell wall mucopeptide synthesis, i.e., the racemization of alanine, the incorporation of glycine, and the formation of diaminopimelic acid from L-aspartic acid. The effects of D-serine on the three sites of cell wall mucopeptide synthesis were all reduced in the D-serine-resistant mutants. This fact assumes that D-serine inhibits the growth of *M. smegmatis* by affecting the three sites of cell wall mucopeptide synthesis.

If the organism lacks the ability to concentrate D-serine or possesses the mechanism by which D-serine is transformed into nontoxic substance, resistance to the action of D-serine would be acquired. Schwartz et al. (27) isolated two mutants of *Escherichia coli* that were unable to concentrate D-serine and L-canavanine, respectively. As a result, they were resistant to the action of these amino acids. Kessel and Lubin (14) and Cosloy (3) selected mutants for resistance to D-serine or D-cycloserine. The mutants showed decreased uptake of glycine, D-alanine, and L-alanine. These amino acids and D-serine or D-cycloserine appear to be on the same transport system. We have not succeeded in isolating mutants defective for the transport system of D-serine. All the D-serine-resistant mutants isolated possessed a transport system for D-serine.

Since the three D-serine-resistant mutants showed no cross-resistance to *O*-carbamyl-D-serine and D-cycloserine, which are potent inhibitors of the alanine racemase, it would appear that the mutants are susceptible to inhibition of the alanine racemase by D-serine. Accordingly, it is not conceivable that the resistance acquired to the action of D-serine is due to the multiple mutations on the three sites in cell wall mucopeptide synthesis. A single mutation that results in elevated level of the enzyme which catalyses the transformation of D-serine into other compound, e.g., deaminase, transaminase, or racemase, could explain the phenotypic properties of the D-serine-resistant mutants. Such a mutation is considered to occur for the acquisition of resistance of D-serine in cells of *M. smegmatis*.

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