JOURNAL OF BACTERIOLOGY, June 1975, p. 1310-1321
Vol. 122, No. 3

Printed in U.S.A.

D-Alanine-Requiring Cell Wall Mutant of *Escherichia coli*

KENNETH OLDEN,* S. ITO, AND T. HASTINGS WILSON

Departments of Physiology and Anatomy, Harvard Medical School, Boston, Massachusetts 02115*

Received for publication 26 February 1975

A mutant of *Escherichia coli* is described whose cells show a spherical or irregular morphology, associated with leakage of β-galactosidase and other intracellular proteins. The expression of the morphologic abnormality is most marked when the mutant is grown in rich media and is suppressed by D-alanine, D-serine, D-glutamate, or glycine supplementation. D-Alanine is the most effective amino acid supplement, half maximally supressing this anomalous property at a concentration of 75 μg/ml, as measured by the reduction in β-galactosidase released from the cells. The mutant is more sensitive to penicillin G, D-methionine, and D-valine and it is relatively resistant to lysozyme. These phenotypic abnormalities are likewise corrected by the above supplementations. The relative rates of peptidoglycan synthesis in mutant and parent, grown under restrictive conditions, were measured both in vivo and in vitro by rates of incorporation of L-[^{14}C]alanine and uridine-5'-diphosphate-*N*-acetyl-D-[1-^{14}C]-glucosamine, respectively. There is no metabolic block in the biosynthesis of uridine-5'-diphosphate-*N*-acetyl-muramyl-pentapeptide as shown by enzymic analysis and the lack of accumulation of uridine-5'-diphosphate-*N*-acetylmuramyl-peptide precursors. These preliminary studies suggest that the mutant possesses a defect in the biosynthesis of peptidoglycan although the exact lesion has not yet been established.

Our knowledge of the chemistry and biosynthesis of the polyfunctional cell envelope of bacteria is considerable although much of the work has been carried out with only a few organisms such as *Staphylococcus* and *Bacillus*. Still largely unanswered are the biological questions of the nature of the extracellular assembly process, the control mechanisms involved, and the physiological relationship of the various molecular constituents. Since elements of the cell wall provide the characteristic shape of microorganisms, one approach has been the isolation and investigation of mutants showing morphological abnormalities. Such mutants have previously been reported in *Escherichia coli* (1, 14, 20, 28, 29, 31), *Bacillus subtilis* and *B. licheniformis* (4, 10, 12, 36-39, 45), *Staphylococcus aureus* (8, 13), and *Agrobacterium tumefaciens* (11). However, the structural or enzymatic lesions were established only in *Staphylococcus*.

In a previous communication (32), we reported the isolation and properties of several mutants of *E. coli* which leaked β-galactosidase into the external medium. One of these, X71-415, was studied in greater detail by morphological and biochemical methods. When this cell was grown in rich medium it developed large irregular shaped morphology, leaked several intracellular proteins, and showed abnormal synthesis of peptidoglycan. All of these abnormalities were returned toward normal by the addition of D-alanine to the growth medium. Evidence is presented to support the view that this cell possesses a defect in either the peptidoglycan layer or in the interaction of this macromolecule with other components of the cell envelope.

MATERIALS AND METHODS

Bacterial strains. The parental strain used in this study was X71 ($i^-z^+y^+a^-$, $ProC^-$, try^-, B_1^-, Sm^R, F^-), a transacetylase-negative strain derived from X5072, (43) a K-12 strain obtained from Jonathan R. Beckwith (Harvard Medical School). From this strain (X71) a conditional rod mutant (X71-415) was isolated after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (32). *Bacillus cereus* T was obtained from Jack L. Strominger (Harvard University).

Growth of bacteria. Bacteria were grown with continuous shaking on a gyrotatory shaker at about 200 rpm at 37 C in mineral medium 63 (9) which consisted of KH_2PO_4 (13.6 g), $(NH_4)_2SO_4$ (2.0 g), $MgSO_4 \cdot 7H_2O$ (0.2 g), and $FeSO_4 \cdot 7H_2O$ (5 mg) added to 1 liter of distilled water and adjusted to pH 7.0 with KOH. The medium was supplemented with NaCl (0.29%), L-tryptophan (10 μg/ml), L-proline (100 μg/ml), and thiamine (0.5 μg/ml). The minimal media carbon sources were 10 mM glucose, or 20 mM succinate, lactate, or glycerol. The rich media carbon sources were 1% tryptone (Difco) peptone, proteose

peptone, yeast extract, nutrient broth, or Trypticase soy broth (Fisher). The individual amino acid supplements were used at a concentration of 1 mg/ml.

The bacteria were usually grown in 30 ml of medium in 300-ml side arm flasks, or in 1 liter of medium in a 2-liter Erlenmeyer flask. For large scale preparations the bacteria were grown in 14 liters of medium in a fermenter (Microferm, New Brunswick Scientific Co.). Growth was monitored by the change in absorbance of the culture with a Klett-Summerson colorimeter (filter no. 97).

Chemicals. Sodium dodecyl sulfate, glass beads (acid washed, type III, 150 to 200 μm), *N*-acetyl-D-glucosamine (GlcNAc), *N*-acetylmuramic acid (MurNAc), D-glucosamine, muramic acid, DL-alanyl-DL-alanine, glycylglycine, ribonuclease, deoxyribonuclease, penicillin G, chloramphenicol, egg white lysozyme (*N*-acetylmuramidase, grade I), pronase, D-cycloserine (D-4-amino-3-isoxazolidone) (DCS), pyridoxal phosphate, and unlabeled amino acids were obtained from Sigma Chemical Co., St. Louis. D-Alanyl-D-alanine was obtained from Cyclo Chemical Co., Los Angeles. D-Amino acid oxidase was obtained from Worthington Biochemical Co., N.J. Bio Gel P-2 (200 to 400 mesh) and Dowex-1-chloride (2% crosslinked) were obtained from Pharmacia Co., N.J. Vancomycin was the generous gift of Jack L. Strominger. D-[^{14}C]alanyl-D-[^{14}C]alanine was prepared from D-[U-^{14}C]alanine as described by Lugtenberg et al. (26). Other radiochemicals were obtained from New England Nuclear Corporation, Boston.

Isolation of uridine-5′-diphosphate (UDP)-N-acetylmuramyl-peptides. Two-liter cultures of logarithmic cells were harvested, washed, and extracted with 5% (wt/vol) trichloroacetic acid. The nucleotides were separated by chromatography as described by Lugtenberg et al. (25) and Good and Tipper (13). The pooled, concentrated fractions were desalted and further fractioned by gel filtration on a Bio Gel P-2 (200 to 400 mesh) column (2.5 by 90 cm) eluted with deionized water. Further purification was achieved by descending paper chromatography on Whatman no. 3 filter paper in isobutyric acid-0.5 M NH_4OH (5:3). UDP-MurNAc-tripeptide (UDP-MurNAc-L-Ala-D-Glu-*m*-Dap) and UDP-MurNAc-pentapeptide (UDP-MurNAc-L-Ala-D-Glu-*m*-Dap-D-Ala-D-Ala) were isolated from *Bacillus cereus* T after their accumulation in the presence of DCS (50 mg/ml) and vancomycin (12.5 μg/ml), respectively, as described by Lugtenberg et al. (25, 26). Reference nucleotides were the generous gift of Jack L. Strominger.

Peptidoglycan synthesis. The rate of peptidoglycan (or its alanine containing precursors) synthesis in vivo was determined by incubating the washed cells in cell wall synthesis medium I supplemented with L-[U-^{14}C]alanine (15.6 Ci/M) as described by Lugtenberg and deHaan (24). Peptidoglycan synthesis in vitro (particulate preparation) was carried out by labeling with UDP-[1-^{14}C]GlcNAc (5.65 Ci/M) in the presence of UDP-*N*-acetylmuramly-pentapeptide as described by Lugtenberg et al. (25).

Preparation of enzyme extracts. Bacteria were grown in 2 liters of tryptone medium to mid- or late logarithmic phase. The cells were harvested by centrifugation at 10,000 $\times$ *g* for 10 min and washed once with 0.01 M potassium phosphate (pH 6.8). The remainder of the procedures were the same as that described by Neuhaus (30). The ammonium sulfate fraction served as the source of enzyme for L-alanine-D-alanine racemase, D-alanyl-D-alanine ligase, D-alanyl-D-alanine adding enzyme, and D-alanine-D-glutamate transaminase. The ammonium sulfate fraction was further purified by heat treatment, protamine sulfate, and acetone fractionation according to the procedure described by Neuhaus (30). The enzyme preparations were stored in 2-ml aliquots at −60 C.

Enzyme assays. L-Alanine:D-alanine racemase was determined by the rate at which L-[U-^{14}C]alanine (156 Ci/M) was coupled to UDP-MurNAc-tripeptide (2 mM) after its conversion to D-[^{14}C]alanyl-D-[^{14}C]alanine, to form UDP-[^{14}C]MurNAc-pentapeptide according to the procedure described by Lugtenberg (23). This enzyme was also assayed by the D-amino acid oxidase procedure described by Julius et al. (17). D-Alanyl-D-alanine ligase was likewise assayed by two procedures. Firstly, by the rate of D-alanyl-D-alanine formation using D-alanine or D-[U-^{14}C]alanine (179 Ci/mol) as substrate with both the ammonium sulfate and acetone fractions according to the procedure described by Neuhaus (30). Secondly, the enzyme was assayed by the rate of UDP-MurNAc-pentapeptide formation with D-[U-^{14}C]alanine and UDP-MurNAc-tripeptide as substrates as described by Lugtenberg (23). D-Alanyl-D-alanine adding enzyme was assayed by the rate at which D-[^{14}C]alanyl-D-[^{14}C]alanine and UDP-MurNAc-tripeptide were converted to UDP-[^{14}C]MurNAc-peptapeptide as described by Lugtenberg (23). D-Alanine-D-glutamate aminotransferase was assayed colorimetrically by the rate of formation of pyruvate with D-alanine and α-ketoglutarate as substrates as described by Martinez-Carrion and Jenkins (27). The thiodigalactoside-resistant *o*-nitrophenyl-β-D-galactopyranoside hydrolysis of washed cells was taken as a measure of β-galactosidase within the periplasmic space (32).

Isolation of peptidoglycan. The cell wall fraction was prepared by suspending 25 g (wet weight) of cells in 120 ml of distilled water containing 2.5 mg of deoxyribonuclease. The suspension was transferred to a Waring blender, and 100 g of acid-washed glass beads was added gradually while stirring at low speed. After 15 min the beads were allowed to settle out and the disrupted cell suspension was decanted. An additional 120 ml of distilled water was added to the glass beads and the residual broken cells were extracted by a 10-min homogenization at low speed. The beads were again allowed to settle out and the supernatant was decanted and combined with the first supernatant fluid. Potassium-ethylenediaminetetraacetic acid (0.1 M, pH 7.4) was added to a final concentration of 10 mM, and the suspension was centrifuged at 9,000 $\times$ *g* for 1 h. The cell wall sediment was washed three times with distilled water. The above manipulations were carried out at 4 C.

The peptidoglycan was isolated from the cell wall preparation by precipitating the peptidoglycan-lipoprotein complex with boiling 4% sodium dodecyl sulfate followed by repeated washings with water, and

cleavage of the lipoprotein from the peptidoglycan with trypsin according to the procedure described by Braun and Rehn (5).

Uptake of L-alanine and D-alanine. For uptake experiments, a washed cell suspension (0.9 mg [dry weight]/ml) was mixed with L-[U-^{14}C]alanine or D-[U-^{14}C]alanine (0.42 μCi/ml) and incubated at 24 C. Portions (0.2 ml) were removed at various time intervals, filtered through a presoaked membrane filter (0.45-μm pore size; Millipore Corp.), and washed with 10 ml of minimal medium. The filters containing the cells were placed in vials, 15 ml of Brays (6) liquid scintillation fluid was added, vials were capped and shaken vigorously, and radioactivity was determined by liquid scintillation spectrophotometry. Calculation of intracellular concentrations was based on an earlier report (44) that 1 ml of cells with absorbance of 100 Klett units (filter no. 42) contained 0.6 μl of intracellular water.

Analytical procedures. Protein determinations were made according to the procedure described by Lowry et al. (21), with bovine serum albumin as standard. Amino sugar was determined by the procedure described by Reissig et al. (35). Amino acids and dipeptides were identified by descending paper chromatography on Whatman no. 3MM filter paper in 0.5 M isobutyric acid-1 M ammonium hydroxide (5:3), or butanol-acetic acid-water (4:1:5); they were also identified by chromatography on the amino acid analyzer (Beckman model 120B). Radioactivity on the paper chromatograms was determined by strip scanning or by cutting out 1 cm^2 strips and counting by liquid scintillation spectrophotometry as described above. The perchloric acid precipitates and peptides were hydrolyzed for 18 h at 105 C in 6 N HCl for amino acid analysis. The peptidoglycan was hydrolyzed for 8 h at 100 C in 3 N HCl for amino sugar analysis. The peptides were cleaved from UDP with 0.01 N HCl by heating in a boiling water bath for 15 min.

Morphological studies. For electron microscopy evaluation, the control strain (X71) and the mutant strain (X71-415) were grown in glycerol minimal medium or tryptone plus or minus D-alanine. Centrifuged microorganisms in the logarithmic growth phase were fixed by resuspending them in diluted Karnovsky fixative (18) containing 1% glutaraldehyde and 1.25% formaldehyde made up in 0.25 M phosphate buffer, pH 7.4, for 1 h at 4 C. The cells were centrifuged, washed with phosphate buffer, and treated with 0.5% uranyl acetate in maleate buffer at pH 5.2 for 1 h. Dehydration was carried out through a graded series of cold ethanol solutions and the microorganisms were embedded in diepoxy octane which is a very low viscosity epoxy embedding medium (22). Sections were cut with diamond knives, stained with uranyl acetate in 50% acetone followed by lead citrate (42), and examined without a supporting film with a Jeol 100 B electron microscope.

Specimens for freeze-cleave replicas of *E. coli* were grown, fixed as for the thin section, but the fixation period was reduced to 15 min at 4 C, washed, and placed in 20% glycerol for 1 to 2 h at 25 C. Small drops of a thick suspension of microorganisms were placed on cardboard disks and frozen in Freon 22 (chlorodiflouromethane) cooled with liquid nitrogen and stored in liquid nitrogen. Replicas were made with a Balzers apparatus, cleaned in a 4 to 6% hypochlorite solution, collected on bare grids after several distilled water washes, and examined in the electron microscope.

RESULTS

Growth properties and morphology. The growth rate of the mutant as judged by increase in turbidity was almost the same as that of the parent in both rich and minimal media, and it was not dependent on the presence of an osmotic stabilizing agent. The morphology, however, was markedly different when cells were grown in various media. When grown in tryptone or other rich media the mutant cells were several times larger than normal *E. coli* and had a spherical or heteromorphic shape (Fig. 1a and b). Mutant cells grown in tryptone supplemented with D-alanine (Fig. 1c) showed normal rod morphology. The morphology of mutant cells grown in glycerol or other minimal media was not as abnormal as that seen in rich media, but they have a more elongated and irregular appearance than normal (Fig. 1d). When mutant cells were transferred from rich media to minimal media, or vice versa, they underwent the morphological transition in one to two generations. Cells grown in minimal media leaked less β-galactosidase and showed reduced sensitivity to deoxycholate (32) compared with similar cells grown in rich media. When mutant cells, grown in either rich or minimal media, were diluted and plated on nutrient agar, there were fewer colonies per unit of optical density than observed for the parent cells grown under similar conditions consistent with increased cellular mass.

Electron microscopy examination of ultrathin sections of the mutant cells showed no obvious defect in the structure and organization of the cell envelope. The characteristic trilaminar structure with inner and outer discrete electron dense areas separated by a thicker clear zone can be seen (Fig. 2a and b). Likewise, no dissimilarities between mutant and parent cell membranes were observed by freeze-fracture electron microscopy (Fig. 3a and b).

Suppression of abnormalities by amino acid supplementation. Since the shape and rigidity of bacterial cells is attributed to the peptidoglycan component of the cell (33), it seemed plausible that the mutant was deficient in the biosynthesis or assembly of this macromolecule. Previous studies (32) have shown that the alteration is not strictly growth rate dependent since the abnormalities are expressed when cells were grown at 23 C or in

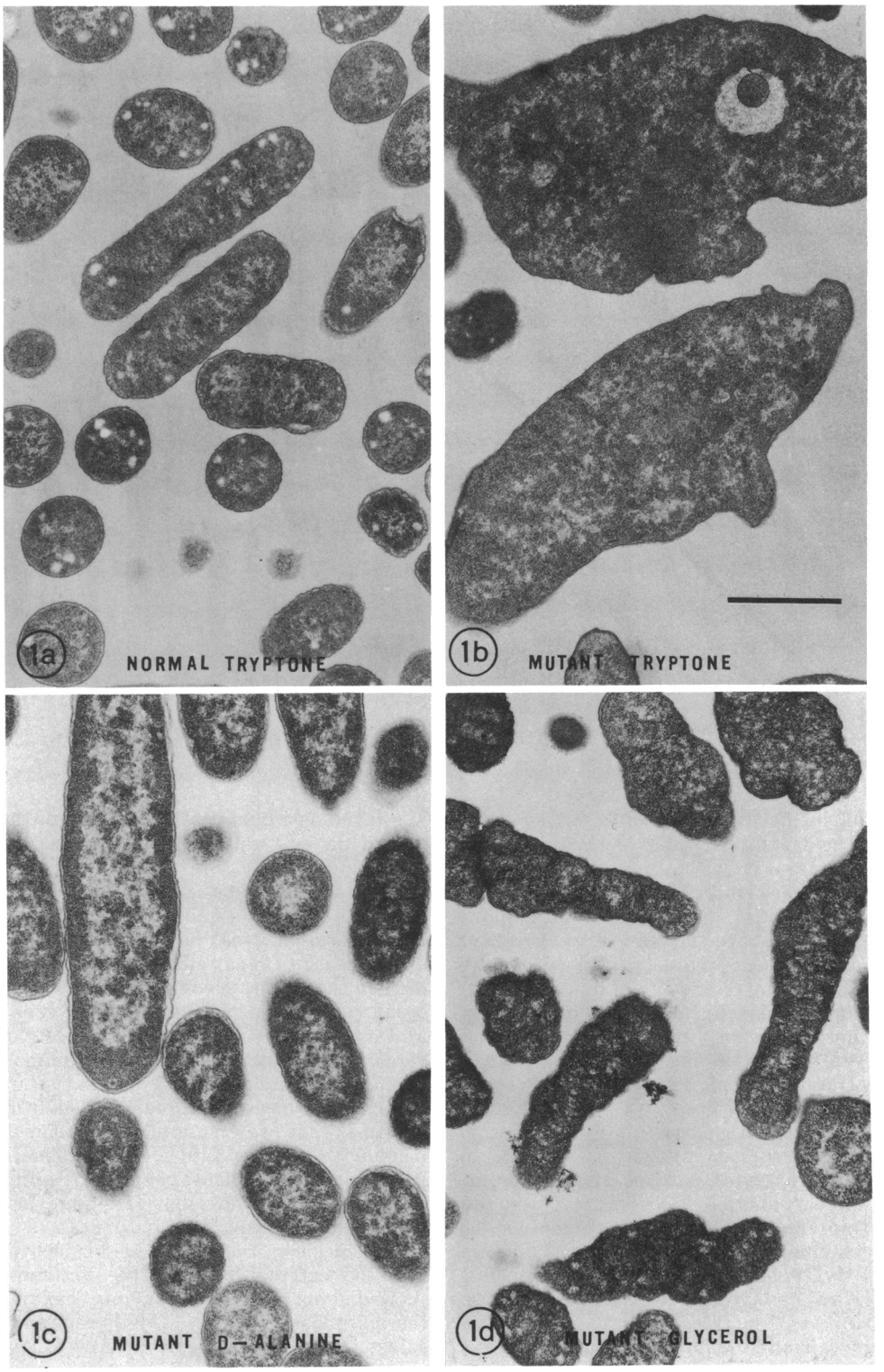

FIG. 1. *Electron micrographs of parental and mutant cells. (a) Parental strain (X71) grown in tryptone medium. (b) Mutant (X71-415) grown in tryptone medium. (c) The mutant grown in tryptone medium supplemented with* D*-alanine (1 mg/ml). (d) The mutant grown in glycerol medium. Bar, 1 μm.*

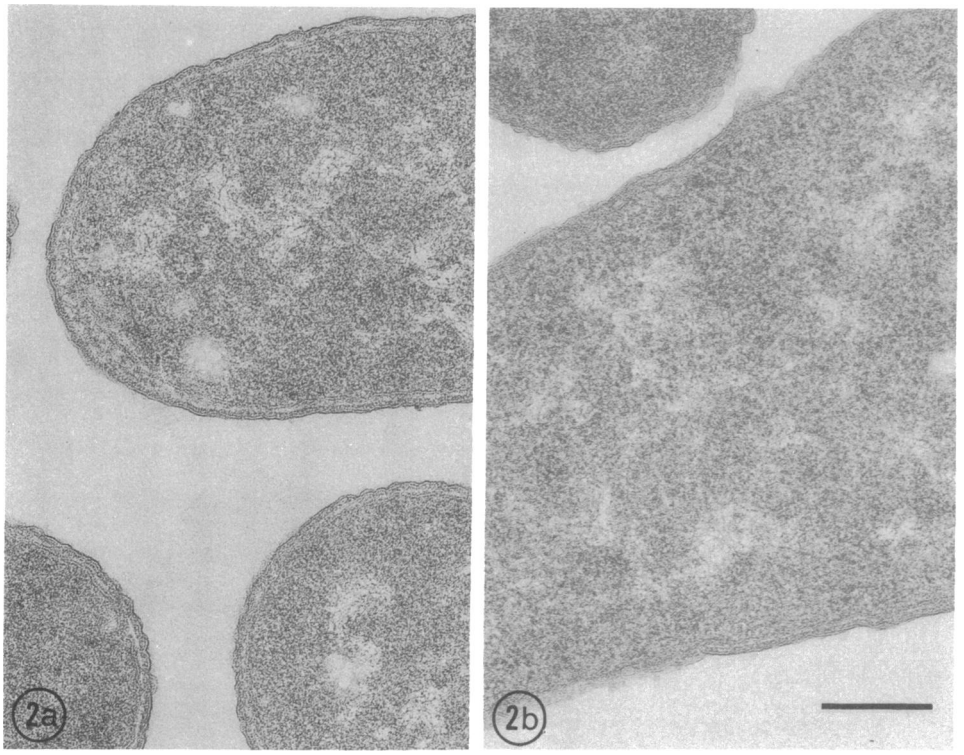

FIG. 2. *High power electron micrographs of parental and mutant cells. Both cells grown in tryptone medium. (a) Parental cell; (b) mutant cell. Bar, 0.2 μm.*

minimal media containing glucose, succinate, glycerol or lactate. It was possible that one of the precursors was synthesized at a limiting rate during rapid growth in rich media and was less restrictive during slower growth in minimal media. This was investigated by supplementing tryptone with the individual peptidoglycan constituents. D-Alanine and D-glutamate were the only constituents effective in suppressing the abnormal morphology and leakage of β-galactosidase. (The following amino acids, at a concentration of 1 mg/ml, did not repress the morphologic abnormality: L-serine, L-glutamate, L-lysine, L-aspartate, L-methionine, L-leucine, L-isoleucine, L-valine, L-threonine, L-proline, L-hydroxyproline, L-tryptophan, L-tyrosine, L-phenylalanine, D-histidine, D-arginine, D-methionine, D-leucine, D-threonine, D-phenylalanine, D-valine, D-isoleucine, D-lysine, and D-tryptophan. In addition, supplementations with carbon sources, such as glucose, succinate, and lactate, did not suppress the morphologic abnormality.

A quantitative comparison was made between several amino acids for their capacity to alleviate the abnormality in the mutant strain. In these experiments leakage of β-galactosidase was taken as a quantitative measure of the extent of the cell wall defect. Although this is a rather indirect measure of the membrane defect, it proved to be more quantitative than other mutant phenotypic alterations. In a previous publication it has been demonstrated that the presence of β-galactosidase and other proteins in the culture medium was due to leakage by all cells rather than lysis of a few cells (32). Mutant cells were grown in tryptone containing various concentrations of the test amino acid and the appearance of β-galactosidase in the medium was measured. It can be seen in Fig. 4 that D-alanine was more effective than either D-serine or glycine, with a half maximal suppression at a concentration of 75 μg/ml; D-glutamate and D-aminobutyrate were less effective (not shown).

In addition to the release of β-galactosidase into the external medium the mutant cells showed abnormally large amounts of enzyme in the periplasmic space. This periplasmic β-galactosidase was markedly reduced by supplementing the growth medium with D-alanine. Alkaline phosphatase was released into the medium by X71-415 growing in tryptone but this release was inhibited by addition of D-ala-

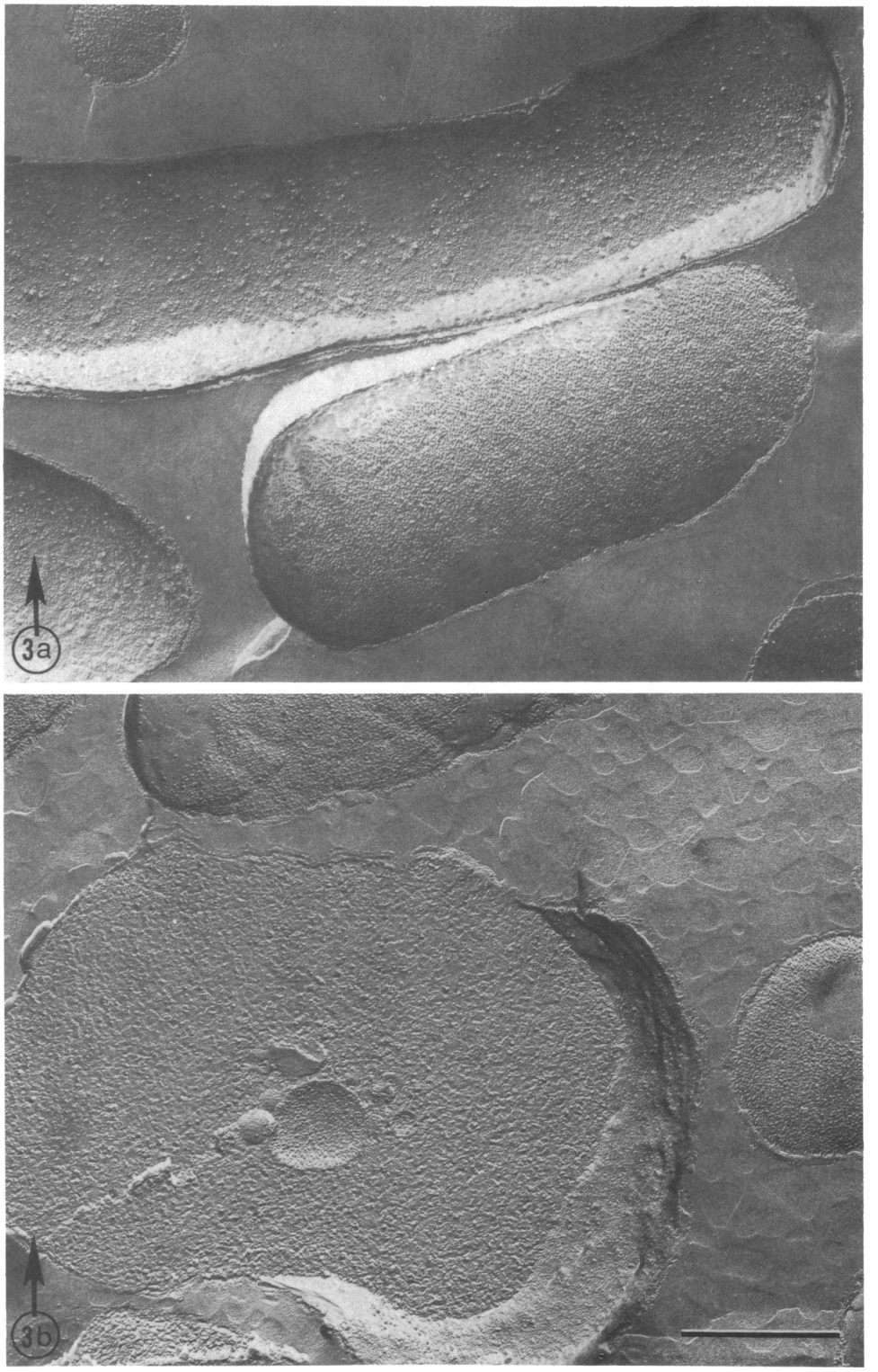

FIG. 3. *Freeze-cleaved specimens of parental and mutant cells. The convex A-face is studded with numerous particles and the concave B-face has only a few large particles. Both cells were grown in tryptone medium. (a) Parental cell; (b) mutant cell. Bar, 0.5 μm.*

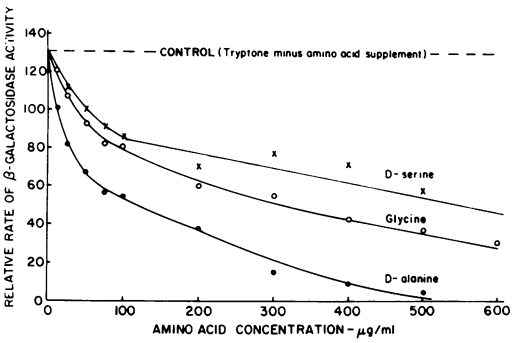

FIG. 4. *The correlation between β-galactosidase release and the concentration of* D-*alanine,* D-*serine, or glycine added to the growth medium. The cells were grown in tryptone supplemented with varying concentrations of these amino acids and separated from the growth medium by filtration at early stationary phase, and the filtrate was assayed for β-galactosidase activity by the rate of o-nitrophenyl-β-*D-*galactopyranoside (2mM) hydrolysis.*

nine to the growth medium. The increased sensitivity of the mutant to deoxycholate was likewise reduced by D-alanine.

Other experiments employed to test for structural modification in the cell envelope involved the use of penicillin G and lysozyme. The mutant was lysed more readily than the parent by penicillin when growing logarithmically in tryptone (Fig. 5a). For example, 100 U of penicillin per ml caused immediate lysis of mutant cells, whereas the parental strain continued to grow logarithmically for more than 1 h. Similarly, growth of the mutant in tryptone was more strongly inhibited by D-methionine and D-valine than the parent. Normal lysis by these agents was restored by D-alanine supplementation during growth. In addition, the mutant was found to be much more resistant to lysozyme than its parent. Figure b5 demonstrates that during a 35-min period the parental cell showed almost complete lysis whereas the turbidity of the mutant cell remained unchanged. Similarly, isolated cell envelopes of the mutant were more resistant to lysozyme than those of the parent. The addition of potassium-ethylenediaminetetraacetic acid (10 mM) did not render mutant cells susceptible to lysozyme. However, when the mutant strain was grown in tryptone supplemented with D-alanine it was as sensitive to lysozyme as the parental strain. To summarize, D-alanine supplementation restores normal morphology, permeability, and lysis by penicillin, deoxycholate, and lysozyme.

Enzyme activities. Since D-alanine was the most effective amino acid in correcting the phenotypic abnormality, a defect in the biosynthesis or utilization of this precursor in peptidoglycan synthesis was possible. This was examined by studying the kinetics and other properties of partially purified L-alanine-D-alanine racemase, D-alanyl-D-alanine ligase, D-alanyl-D-alanine adding enzyme from both mutant and

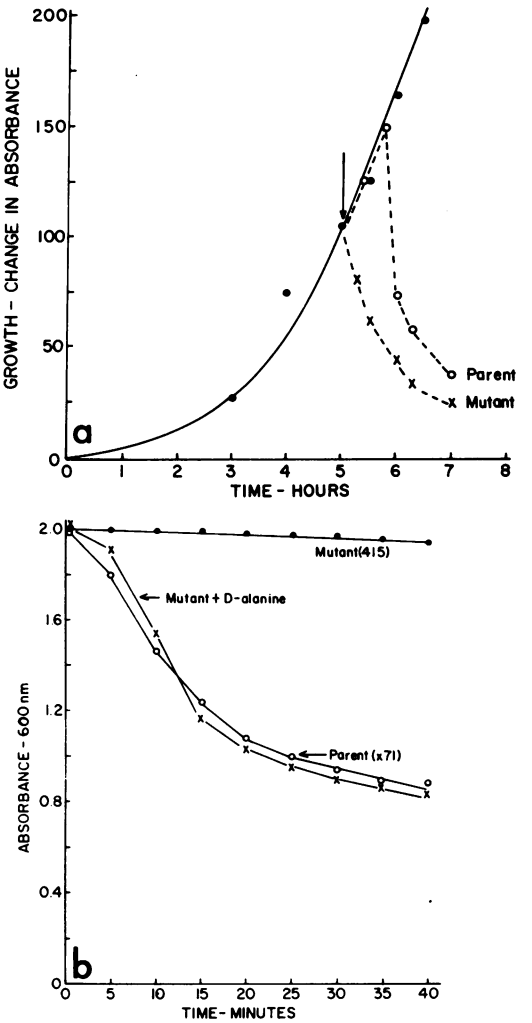

FIG. 5. (a) *Penicillin sensitivity. Penicillin (100 U per ml final concentration) was added to logarithmic cultures at the time designated by the arrow and incubation was continued as before. The rate of cell lysis was monitored by the decrease in absorbance of the culture.* (b) *Lysozyme sensitivity. Late logarithmic-phase cells were harvested by centrifugation and washed once in 10 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.0). The cells were resuspended in 30 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0). Lysozyme was added to a final concentration of 20 μg/ml and the suspension was incubated at 24 C. The change in absorbance at 600 nm was recorded continuously.*

parent cells grown in tryptone. A Lineweaver-Burk plot illustrating the kinetics of alanine racemase is shown in Fig. 6. There was no difference in the affinity (K_m 2.7 mM) of the racemase for L-alanine compared with the parent. Although the V_{max} of the mutant enzyme was somewhat greater than that of the parent in this particular experiment, smaller differences were observed in three additional experiments. No differences were found in the K_m (19 mM) and V_{max} of D-alanyl-D-alanine ligase, or the V_{max} of D-alanyl-D-alanine adding enzyme between parent and mutant strains.

Both alanine racemase and D-alanyl-D-alanine ligase from parent and mutant were equally sensitive to DCS, glycine, glycyl-glycine, and heat inactivation. D-Alanyl-D-alanine ligase from both sources was absolutely specific for amino acids of the D configuration or glycine, and the capacity of this enzyme from both cells to form mixed dipeptides with glycine, D-serine, or D-aminobutyrate was similar.

It is plausible that the observed differences in the ability of D-alanine and D-glutamate to correct the abnormal morphology might result from differences in their uptake or the efficiency in their utilization when provided exogenously, in which case D-glutamate might be the metabolically deficient substrate since L-glutamate may be synthesized from D-alanine by the action of D-alanine-D-glutamate aminotransferase. The activity of this enzyme in the ammonium sulfate fraction was detectable, however, and showed similar activity in both parent and mutant with a K_m of 10 mM for D-alanine. Whether this is the physiological mechanism of D-glutamate formation was not determined. The uptake of D-glutamate by washed cells was similar in both parent and mutant strains.

Intracellular levels of nucleotide-bound hexosamine. If one of the initial steps in alanine incorporation into peptidoglycan were blocked then the nucleotide-acceptor substrate for L-alanine (UDP-MurNAc) or for D-alanine (UDP-MurNAc-tripeptide) would be expected to accumulate abnormally in mutant cells grown in rich media. This was examined by extraction of the nucleotides with trichloroacetic acid (5%) from a heat-treated cell suspension. The concentrated extracts were quantitatively applied to a Dowex-1-chloride column and eluted with a NaCl linear gradient. This resulted in the partial separation of nucleotides and exclusion of amino acids.

The elution patterns of parent and mutant extracts are shown in Fig. 7a. The nucleotide peak I was ninhydrin positive and contained acetylamino sugars whereas peaks II and III were negative for amino acids and sugars by the

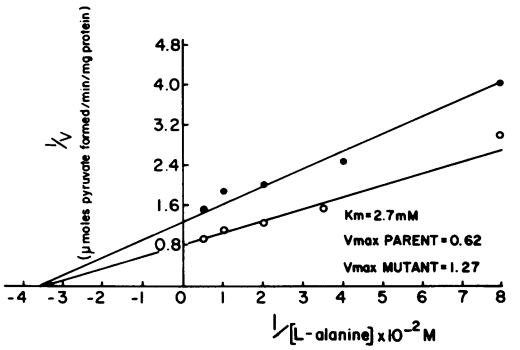

FIG. 6. *Relation between velocity of L-alanine-D-alanine racemase activity and L-alanine concentration. The reaction was performed in 0.1 M tris(hydroxymethyl)aminomethane-hydrochloride buffer (pH 8.9) containing 5 × 10^{-4} M pyridoxal phosphate purified as the flavin nucleotide complex. The reaction was terminated by heating at 85 C for 5 min. The D-alanine formed in 10 min at 37 C was quantitatively converted to pyruvate by incubation with D-amino acid oxidase (0.045 U/mg of protein) for 15 min at 37 C. Pyruvate was determined by incubation with 2,4-dinitrophenylhydrazine (0.25 mg/ml) for 5 min at 24 C. NaOH (1.5 M) was added and the absorbance was read at 520 nm. The concentration of D-alanine was determined by comparison to the absorbance of standards.*

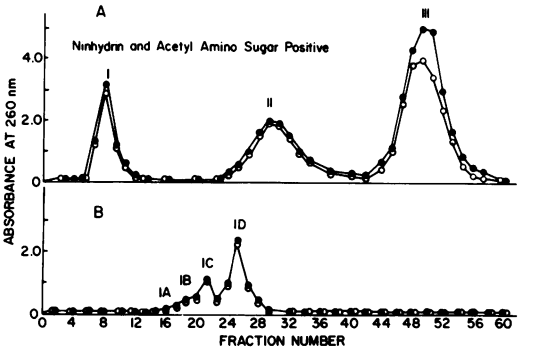

FIG. 7. *(A) Dowex I-X8 (chloride) ion exchange chromatography of trichloroacetic acid-soluble nucleotides. The column was equilibrated with 0.01 N HCl, and 5 ml of the trichloroacetic acid-soluble extract was applied to the column and eluted with a sodium chloride linear gradient (0 to 0.3 M) in 0.01 N at 4 C. The flow rate was 25 ml per h, and 10-ml fractions were collected. Peak I was positive for ninhydrin and acetyl amino sugar. The data represents the mean values for seven experiments. (B) Gel filtration on Bio Gel P-2. Five milliliters of concentrated fraction I was applied to the column and eluted with deionized water at 4 C at a flow of 20 ml per h, and 10-ml fractions were collected. The open circles represent the parent, and the closed circles the mutant.*

same criteria. The ultraviolet absorption spectra of peaks II and III indicated that they did not contain uridine. These experiments indicate that the parent and the mutant contain similar concentrations of precursors. Mutant cells grown in the presence of D-alanine do not show abnormal concentrations of these precursors.

Peak I was concentrated and desalted by quantitatively applying to Bio Gel P-2, and four subfractions were eluted with deionized water (Fig. 7B). Although fractions IA and IB were not very prominent here, we have demonstrated that they are distinct components by other chromatographic procedures. Identification of these fractions was facilitated by the use of DCS which has been shown to block peptidoglycan synthesis by specifically inhibiting alanine racemase (23, 41). Fraction IC accumulated when the growth medium was supplemented with DCS (50 μg/ml) (Fig. 8B), indicating that this peak corresponds to UDP-MurNAc-tripeptide (23). Further identification of these fractions was carried out by the elution patterns of L-[U-^{14}C]alanine-labeled nucleotides (Fig. 8A and B) (1), paper co-chromatography with reference nucleotides (31), and amino acid analyses (14). The major fraction (ID) is composed of UDP-GlcNAc and UDP-MurNAc, fraction IC is UDP-MurNAc-tripeptide, fraction IB is UDP-MurNAc-pentapeptide, and fraction IA was not identified.

Peptidoglycan synthesis. Although the results already presented indicate that there is no metabolic block in the biosynthesis of UDP-MurNAc-pentapeptide, it is clear that there is an alteration in the cell envelope which is corrected by glycine and by several amino acids having the D configuration. The possibility remained that the mutant might be defective in the utilization of the soluble precursors (UDP-GlcNAc and UDP-MurNAc-pentapeptide).

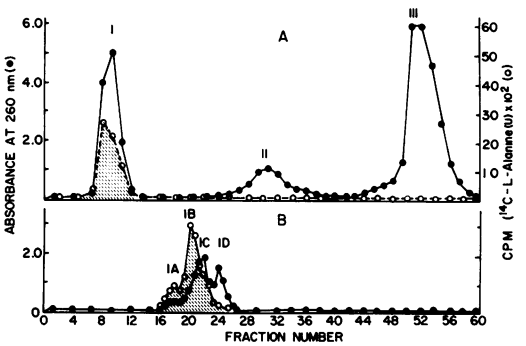

FIG. 8. *Dowex I-X8 chromatography of DCS-inhibited cells. Chloramphenicol (100 μg/ml), DCS (80 μg/ml), and* L-[U-^{14}C]*alanine were added to a logarithmic culture of parental cells, and incubation was continued as before for an additional 60 min. The nucleotides were extracted as described in the text and the concentrated extract was applied to (A) Dowex I-X8 (chloride) column (B) peak I from the first column rechromatographed on Bio Gel P-2. Elution was as described in Fig. 7. The closed circles represent the absorption at 260 nm, and the open circles represent* L-[^{14}C]*alanine incorporation.*

Since L-alanine is the first amino acid added to UDP-MurNAc in the sequence leading to UDP-MurNAc-pentapeptide synthesis, the incorporation of this radioactive amino acid, in the absence of protein synthesis, provides a method for studying the kinetics of the synthesis of peptidoglycan or its alanine-containing nucleotide precursors with intact cells (26). The average of five experiments (shown in Fig. 9) indicate that the level of L-[U-^{14}C]alanine incorporation into the peptidoglycan of the mutant is only about one-half that found in the parent after 2.5 h. The incorporation is specifically inhibited by DCS, a well characterized inhibitor of peptidoglycan synthesis. In this experiment protein synthesis was blocked due to the presence of chloramphenicol (100 μg/ml) and the absence of the amino acids proline and tryptophan for which the cells are auxotrophic. This was confirmed by the low level of incorporation of L-[^{14}C]arginine. The reduction in level of alanine incorporation in the mutant is not due to cell lysis as the viable count and optical density of the cell suspension remained constant during the course of the experiment. More than 95% of the label in the perchloric acid precipitate chromatographed as alanine after acid hydrolysis.

When the rate of peptidoglycan synthesis was determined with the membrane preparations using UDP-[1-^{14}C]GlcNAc and UDP-MurNAc-pentapeptide as substrate in buffer B (25), there was no observable difference between the parent and mutant (Fig. 10), suggesting that there is no biochemical lesion associated with the polymerization of these substrates and the subsequent insertion of the GlcNAc-MurNAc-pentapeptide subunit into the peptidoglycan.

Peptidoglycan amino sugar composition. The amino sugar composition of the isolated peptidoglycan from mutant and parent strains was determined. The mutant had the same quantitative amino sugar content as the parent (0.35 μmol/mg of cell envelope), suggesting that there was no reduction in the glycan component.

DISCUSSION

A defect in cell wall biosynthesis is clearly evident in the mutant 415. When growing rap-

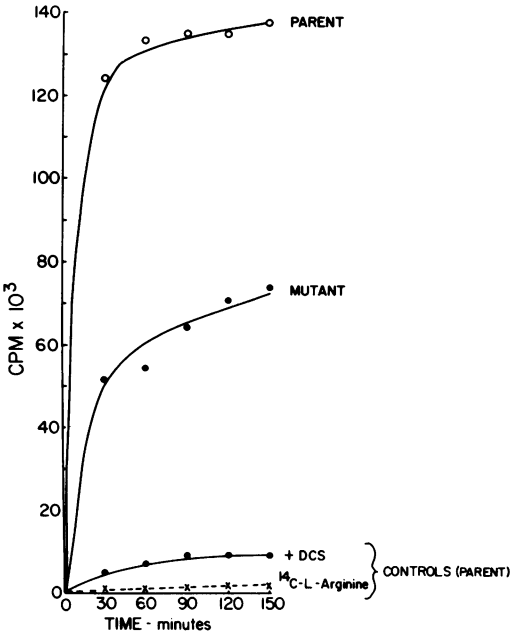

FIG. 9. *Incorporation of L-[^{14}C]alanine into perchloric acid-precipitable compounds. The cells were grown in tryptone and harvested when the cell density was 0.24 mg (dry weight) per ml. The cells were washed once with 1 volume of CWSM-I and allowed to equilibrate at 37 C for 15 min with vigorous aeration. Then 0.2 ml of L-[U-^{14}C]alanine (10 μg/ml) was added to 1.8 ml of the equilibrated suspension, which made the concentration of L-alanine 6.25 × 10^{-5} M, and incubation was continued. Two-tenth-milliliter aliquots were removed at zero time and subsequently at 30-min intervals for 2.5 h and were added to 5 ml of ice-cold 3% perchloric acid with 1 drop of bovine serum albumin (1 mg/ml) as carrier. After 30 min at 24 C, the precipitate was collected by membrane filtration (HA filters, pore size 0.45 μm; Millipore Corp.), and washed five times with 3% perchloric acid. The precipitates were dissolved in 15 ml of Brays scintillation fluid and counted by liquid scintillation spectrophotometry. DCS was added to a final concentration of 20 μg/ml. L-[^{14}C]arginine (1 μg/ml) replaced L-[^{14}C]alanine in control experiments to estimate protein synthesis.*

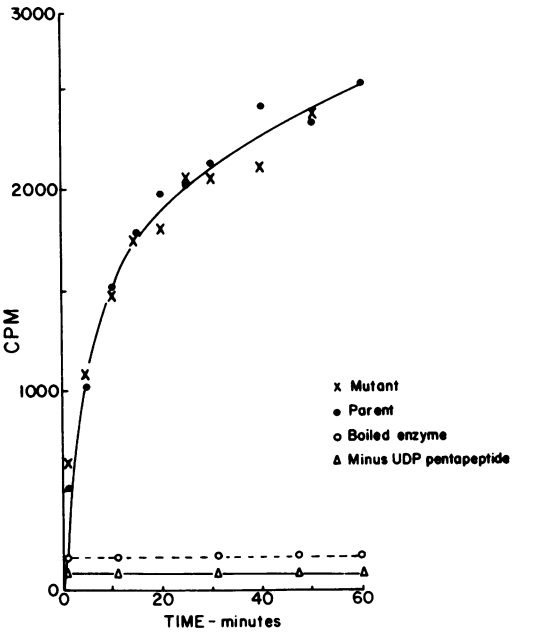

FIG. 10. *Incorporation of UDP-[1^{14}C]GlcNAc into perchloric acid-precipitable compounds. The particulate fraction was prepared from a logarithmic culture by disruption with glass beads. The membrane fraction was washed three times with buffer A (which contained 5 × 10^{-2} M tris(hydroxymethyl)aminomethane-hydrochloride and 10^{-2} M $MgCl_2$ (pH 7.8) (22) and was resuspended in the same buffer. The assay mixture contained 0.1 ml of UDP-[1^{14}C]GlcNAc (2.5) μCi/ml, 1 μmol/ml), 0.2 ml of UDP-MurNAc-pentapeptide (4 μmol/ml), 0.2 ml of buffer B which contained 1.5 M tris(hydroxymethyl)aminomethane-hydrochloride and 0.4 ml of particulate enzyme preparation (2 mg of protein per ml final concentration). The perchloric acid-precipitable counts were determined as described in Fig. 6.*

idly in rich media it becomes spherical in shape, osmotically fragile, leaks intracellular protein, and is lysed readily by penicillin, D-methionine, D-valine, and deoxycholate. D-Alanine, a constituent of the peptidoglycan layer, was able to largely repair these abnormalities when this amino acid was added to the growth medium. This view that the structural layer was defective was strengthened by the findings that the mutant was more resistant to lysozyme than the parent. This enzyme is an endoacetylmuramidase which is specific for β-1,4-glycosidic linkages between MurNAc and GlcNAc. An abnormality in the peptide could interfere with the enzymatic hydrolysis by lysozyme. Lysozyme resistance has previously been accounted for by the occurrence of *N*-nonsubstituted glucosamine residues (14, 31, 33), the interaction of the peptidoglycan with other components of the cell envelope (19), the number of free amino groups in the peptide moiety (34), the degree of cross-linking (40), or *O*-acetylation of the peptidoglycan (7).

Although the specific biochemical lesion has not been elucidated a partial analysis has been made of several stages of peptidoglycan biosynthesis. The results suggest that the mutant defect is not due to a deficiency in the biosynthesis or utilization of D-alanine in the formation of UDP-MurNAc-pentapeptide since nucleotide-bound hexosamine precursors did not accumulate abnormally. Furthermore, the ac-

tivities of alanine racemase, D-ala-D-ala ligase, and D-ala-D-ala adding enzyme are not reduced under the assay conditions used.

It is significant that D-alanine and glycine found to correct the morphologic abnormalities, restore normal lysozyme sensitivity, and other anomalous properties of mutant cells are among those shown to reverse the transpeptidation reaction in vitro (15). This reaction, catalyzed by D-alanine transpeptidase, involves the release of the carboxy terminal D-alanine from GlcNAc-MurNAc-pentapeptide after its incorporation into peptidoglycan, and it results in the formation of peptide cross-linking. Although the mutant appears to possess some defect in the peptidoglycan, we have insufficient data at present to speculate further on the specific abnormality.

Similar conditional or nonconditional rod mutants have previously been described. The abnormal morphology of several of these mutants is suppressed by growth in media with a high salt content (8, 12, 14, 36, 37) and others have a similar requirement for D-alanine (20, 29). Unlike the mutant described here, the D-alanine requiring temperature-sensitive mutants described by Miyakawa et al. (29) accumulated UDP-MurNAc-tri-peptide and failed to incorporate significant amounts of L-[^{14}C]alanine into peptidoglycan even though the activities of the soluble and particulate enzyme known to be involved in peptidoglycan synthesis were normal in disrupted cell preparation (29). Although the conditional rod mutant described by Lazdunski and Shapiro (20) was repaired by D-alanine supplementation, the cell showed normal morphology when grown in rich media and was not resistant to lysozyme. Thus, none of the reported mutants show identical properties to the one described in this report.

Comment should be made concerning the leakage of β-galactosidase, a property which permitted the isolation of the mutant. A defect in the peptidoglycan layer would lead to partial loss of rigidity of the wall and osmotic swelling of the cell. Such swelling would lead to stretching of the plasma membrane with a resulting leakage of a variety of molecules including proteins. This hypothesis is supported by the observation that the leakage of β-galactosidase is greatly reduced by growing the cells in the presence of 0.4 M sucrose as an osmotic stabilizer (32).

ACKNOWLEDGMENTS

We would like to express our appreciation to J. L. Strominger and Paul Linnett for their many helpful suggestions and stimulating discussions.

This work was supported by a Public Health Service grant (AM-05736) from the National Institute of Arthritis and Metabolic Diseases. One of us (K.O.) was supported by a Public Health Service Fellowship (5-FO2 GM46608-02) from the National Institutes of General Medical Sciences and by a Macy Faculty Fellowship awarded by The Josiah Macy, Jr., Foundation.

LITERATURE CITED

1. Adler, H. I., C. E. Terry, and A. A. Hartigree. 1968. Giant cells of *Escherichia coli*. J. Bacteriol. **95**:139–142.
2. Araki, Y., T. Nakatani, H. Hayashi, and E. Ito. 1971. Occurrence of non-N-substituted glucosamine residues in lysozyme-resistant peptidoglycan from *Bacillus cereus* cell walls. Biochem. Biophys. Res. Commun. **42**:691–697.
3. Araki, Y., T. Nakatani, K. Nakayama, and E. Ito. 1972. Occurrence of N-nonsubstituted glucosamine residues in peptidoglycan of lysozyme-resistant cell walls from *Bacillus cereus*. J. Biol. Chem. **247**:6312–6322.
4. Boylan, R. J., and N. H. Mendelson. 1969. Initial characterization of a temperature-sensitive rod mutant of *Bacillus subtilis*. J. Bacteriol. **100**:1316–1321.
5. Braun, V., and K. Rehn. 1969. Chemical characterization, spatial distribution and function of a lipoprotein (murein-lipoprotein) of the *Escherichia coli* cell wall. Eur. J. Biochem. **10**:426–438.
6. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. **1**:279–285.
7. Brumfitt, W., A. C. Wardlow, and J. T. Park. 1958. Development of lysozyme-resistance in *Micrococcus lysodiekticus*. Nature (London) **181**: 1783–1784.
8. Chatterjee, A. N., and F. E. Young. 1972. Regulation of bacterial cell wall: isolation and characterization of peptidoglycan mutants of *Staphylococcus aureus*. J. Bacteriol. **111**:220–230.
9. Cohen, G. N., and H. V. Rickenberg. 1956. Concentration specifique reversible des amino acids chez *Escherichia coli*. Ann. Inst. Pasteur Paris **91**:693–720.
10. Cole, R. M., J. T. Popkin, R. J. Boylan, and N. H. Mendelson. 1970. Ultrastructure of a temperature sensitive rod-mutant of *Bacillus subtilis*. J. Bacteriol. **103**:793–810.
11. Fujimore, T., and S. Fukui. 1972. Isolation of morphological mutants of *Agrobacterium tumefaciens*. J. Bacteriol. **110**:743–746.
12. Gilpin, R. W., F. E. Young, and A. N. Chatterjee. 1973. Characterization of a stable L-form of *Bacillus subtilis* 168. J. Bacteriol. **113**:486–499.
13. Good, C. M., and D. J. Tipper. 1972. Conditional mutants of *Staphylococcus aureus* defective in cell wall precursor synthesis. J. Bacteriol. **111**:231–241.
14. Henning, U., K. Rehn, V. Braun, and B. Höhn. 1972. Cell envelope and shape of *Escherichia coli* K12. Eur. J. Biochem. **26**:570–586.
15. Izaki, K., M. Matsuhashi, and J. L. Strominger. 1968. Biosynthesis of peptidoglycan of bacterial cell walls. XIII. Peptidoglycan transpeptidase and D-alanine carboxypeptidase. J. Biol. Chem. **243**:3180–3192.
16. Izaki, K., and J. L. Strominger. Biosynthesis of the peptidoglycan of bacterial cell walls. XIV. Purification and properties of two D-alanine carboxypeptidases from *E. coli*. J. Biol. Chem. **243**:3193–3201.
17. Julius, M., C. A. Free, and G. T. Barry. 1970. Alanine racemase, p. 171–176. *In* L. Grossman and K. Maldave (ed.), Methods in enzymology, vol. 17A. Academic Press Inc., New York.
18. Karnovsky, M. J. 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J. Cell Biol. **27**:137A.

19. Krause, R. M., and M. McCarty. 1961. Studies on the chemical structure of the streptococcal cell wall. J. Exp. Med. **114**:127-140.
20. Lazdunski, C., and B. M. Shapiro. 1972. Relationship between permeability cell division, and murein metabolism in a mutant of *Escherichia coli*. J. Bacteriol. **111**:499-509.
21. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265-275.
22. Luft, J. H. 1973. Embedding media—old and new, p. 1-34. *In* J. K. Koehler (ed.), Advanced techniques in biological electron microscopy. Springer-Verlag, New York.
23. Lugtenberg, E. J. J. 1972. Studies on *Escherichia coli* enzymes involved in the synthesis of uridine diphosphate-*N*-acetyl-muramyl-pentapeptide. J. Bacteriol. **110**:26-34.
24. Lugtenberg, E. J. J., and P. G. deHaan. 1971. A simple method for following the fate of alanine containing components in murein synthesis in *Escherichia coli*. Antonie van Leeuwenhoek J. Microbiol. Serol. **37**:537-552.
25. Lugtenberg, E. J. J., A. van Schijndel-Van Dam, and T. H. M. van Bellegem. 1971. In vivo and in vitro action of new antibiotics interfering with the utilization of *N*-acetyl-glucosamine. *N*-acetyl-muramyl-pentapeptide. J. Bacteriol. **108**:20-29.
26. Lugtenberg, E. J. J., L. de Haas-Menger, and W. H. M. Ruytgers. 1972. Murein synthesis and identification of cell wall precursors of temperature-sensitive lysis mutants of *Escherichia coli*. J. Bacteriol. **109**:326-335.
27. Martinez-Carrion, M., and W. T. Jenkins. 1965. D-alanine-D-glutamate transaminase. J. Biol. Chem. **240**:3538-3546.
28. Matsuzama, H., K. Hayakawa, T. Sato, and K. Imahori. 1973. Characterization and genetic analysis of a mutant of *Escherichia coli* K-12. J. Bacteriol. **115**:436-442.
29. Miyakama, T., H. Matsuzama, M. Matsuhoshi, and Y. Sugino. 1972. Cell wall peptidoglycan mutants of *Escherichia coli* K-12: existence of two clusters of genes, Mra and Mrb, for cell wall peptidoglycan biosynthesis. J. Bacteriol. **112**:950-958.
30. Neuhaus, F. C. 1962. The enzymatic synthesis of D-alanyl-D-alanine. J. Biol. Chem. **237**:778-786.
31. Normark, S. 1969. Mutation in *Escherichia coli* K-12 mediating spherelike envelopes and changed tolerance to ultraviolet irradiation and some antibiotics. J. Bacteriol. **98**:1274-1277.
32. Olden, K., and T. H. Wilson. 1972. Isolation and characterization of mutants of *Escherichia coli* K12 which leak β-galactosidase. Biochim. Biophys. Acta **288**:54-64.
33. Osborn, M. J. 1969. Structure and biosynthesis of the bacterial cell wall. Annu. Rev. Biochem. **38**:501-538.
34. Perkins, H. R. 1965. The action of hot formamide on bacterial cell walls. Biochem. J. **95**:876-882.
35. Reissig, J. L., J. L. Strominger, and L. F. Lehoir. 1955. A modified colorimetric method for the estimation of N-acetylamine sugars. J. Biol. Chem. **217**:959-966.
36. Rogers, H. J., and M. McConnell. 1970. The role of L-glutamine in the phenotypic change of a rod mutant derived from *Bacillus subtilis* 168. J. Gen. Microbiol. **61**:173-181.
37. Rogers, H. J., M. McConnell, and I. D. J. Burdett. 1968. Cell wall or membrane mutants of *Bacillus subtilis* and *Bacillus licheniformis* with grossly deformed morphology. Nature (London) **219**:285-288.
38. Rogers, H. J., M. McConnell, and I. D. J. Burdett. 1970. The isolation and characterization of mutants of *Bacillus subtilis* and *Bacillus lichenformis* with disturbed morphology and cell division. J. Gen. Microbiol. **61**:155-171.
39. Rogers, H. J., M. McConnell, and J. R. C. Hughes. 1971. The chemistry of the cell walls of rod mutants of *Bacillus subtilis*. J. Gen. Microbiol. **66**:297-308.
40. Strominger, J. L., and J. M. Ghuysen. 1967. Mechanisms of enzymatic bacteriolysis. Science **156**:213-221.
41. Strominger, J. L., R. H. Threnn, and S. S. Scott. 1959. Oxamycin, a competitive antagonist of the incorporation of D-alanine into a uridine nucleotide in *Staphylococcus aureus*. J. Am. Chem. Soc. **81**:3803-3804.
42. Venable, J. H., and R. Cogyeshall. 1965. A simplified lead citrate stain for use in electron microscopy. J. Cell. Biol. **25**:407-408.
43. Wilson, T. H., and E. R. Kashket. 1969. Isolation and properties of a thiogalactoside transacetylase-negative mutants of *Escherichia coli*. Biochim. Biophys. Acta **173**:501-508.
44. Winkler, H. H., and T. H. Wilson. 1966. The role of energy coupling in the transport of β-galactosides by *Escherichia coli*. J. Biol. Chem. **241**:2200-2211.
45. Young, F. E., P. Haywood, and M. Pollack. 1970. Isolation of L-forms of *Bacillus subtlis* which grow in liquid medium. J. Bacteriol. **102**:867-870.