JOURNAL OF BACTERIOLOGY, May 1973, p. 499-506

Vol. 114, No. 2
Printed in U.S.A.

Properties of a D-Glutamic Acid-Requiring Mutant of *Escherichia coli*

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Received for publication 22 January 1973

Some properties of a D-glutamic acid auxotroph of *Escherichia coli* B were studied. The mutant cells lysed in the absence of D-glutamic acid. Murein synthesis was impaired, accompanied by accumulation of uridine-5'-diphosphate-*N*-acetyl-muramyl-L-alanine (UDP-MurNAc-L-Ala), as was shown by incubation of the mutant cells in a cell wall medium containing L-[^{14}C]alanine. After incubation of the parental strain in a cell wall medium containing L-[^{14}C]glutamic acid, the acid-precipitable radioactivity was lysozyme degradable to a large extent. Radioactive UDP-MurNAc-pentapeptide was isolated from the L-[^{14}C]glutamic acid-labeled parental cells. After hydrolysis, the label was exclusively present in glutamic acid, the majority of which had the stereoisomeric D-configuration. Compared to the parent the mutant incorporated less L-[^{14}C]glutamic acid from the wall medium into acid-precipitable material. Lysozyme degraded a smaller percentage of the acid-precipitable material of the mutant than of that of the parent. No radioactive uridine nucleotide precursors could be isolated from the mutant under these conditions. Attempts to identify the enzymatic defect in this mutant were not successful. The activity of UDP-MurNAc-L-Ala:D-glutamic acid ligase (ADP; EC 6.3.2.9) (D-glutamic acid adding enzyme) is not affected by the mutation. Possible pathways for D-glutamic acid biosynthesis in *E. coli* B are discussed.

In recent years many mutants impaired in the biosynthesis of uridine-5'-diphosphate-*N*-acetyl-muramyl-L-Ala-D-Glu-meso-diaminopimelic acid-D-Ala-D-Ala (UDP-MurNAc-pentapeptide) have been identified (8). In the collection of temperature-sensitive lysis mutants under study in our laboratories, no mutants were found that required D-glutamic acid for growth (5). Recently W. Messer isolated a D-glutamic acid auxotroph of *Escherichia coli* B (2). Some properties of this mutant are described in the present paper. Unfortunately we did not succeed in identifying the enzymatic defect in this mutant.

MATERIALS AND METHODS

Bacterial strains. *E. coli* B/r, F$^-$ (relevant markers including *leu*, *pro*, *trp*, *his*, *arg*, *thy*, *met*, *lac*, *gal*, *strA*, *hsrK*, and *hsmK*) and its D-glutamate-requiring mutant were obtained from W. Messer, Max-Planck-Institut für Molekulare Genetik, Berlin, Germany (2).

Growth conditions. The cells were grown at 37 C in CGPY medium (4) containing salts, 0.5% glucose, peptone, and yeast extract. D-Glutamic acid (50 μg/ml) was supplemented if necessary. The optical density of bacterial cultures, determined at various times, was measured in a Unicam-SP600 spectrophotometer at 660-nm wave length. Yeast agar (4) was used as the solid medium.

Murein synthesis. Exponentially growing cells were harvested and then washed with and suspended in the cell wall synthesis medium CWSM-I, which contains glucose, minimal medium salts, chloramphenicol, uracil, and cell wall amino acids. After incubation for 15 min at 37 C, L-[*U*-^{14}*C*]alanine was added to a final concentration of 6.25×10^{-5} M (1 or 2 μCi/ml). The suspension was incubated at 37 C. Murein synthesis was followed by measuring the acid-precipitable radioactivity of 0.1- or 0.2-ml samples. Under these conditions L-alanine is nearly completely incorporated into murein (4). Protein synthesis does not occur under these conditions because chloramphenicol is present and a number of amino acids, for which the strains are auxotrophic, are not present in CWSM-I medium. Details of the method have been described previously (4). This method was also used to test whether L-[*U*-^{14}*C*]glutamic acid (1 or 2 μCi/ml) can be incorporated into murein. To obtain a reasonable incorporation, it

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was necessary to omit unlabeled L-glutamic acid (usually 120 μg per ml) from CWSM-I. The concentration of L-alanine was 50 mg/liter. Incorporation of the radioactivity in murein was tested by measuring the lysozyme-degradable fraction of the acid-precipitable material.

Degradation with lysozyme. After incubation in CWSM-I, 2.0 ml of the suspension was cooled and washed twice with and suspended in one volume of 10^{-4} M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, *p*H 7.0. After ultrasonic disintegration (4), the suspension was heated for five min at 100 C, cooled, and incubated with and without lysozyme (100 μg/ml) for one hour at 37 C. The acid-precipitable radioactivity of 0.2 ml of the washed cell suspension before and after lysozyme treatment was determined. Under these conditions between 20 and 40% of the murein was found to be resistant to lysozyme treatment (4).

Uridine nucleotides. Accumulation and purification of reference uridine nucleotides was carried out as described previously (5, 9), except the accumulation of UDP-MurNAc-pentapeptide. This nucleotide was accumulated in *Bacillus cereus* T growing under vigorous aeration at 37 C in CGPY medium. When the optical density was 0.7 (0.37 mg [dry wt] per ml suspension), chloramphenicol was added to a final concentration of 100 μg/ml. Fifteen minutes later, vancomycin (10 μg/ml) was added. After further incubation for 40 min, the cells were cooled and harvested (9). With this procedure, the yield of crude uridine nucleotides was about 300 μmol/8 liters of culture, which is many times higher than that obtained with the method described in a previous paper (9).

Radioactive uridine nucleotide precursors were isolated and identified as described previously (5). The conditions for growth and labeling were the same as described for murein synthesis. L-[^{14}C]Alanine or L-[^{14}C]glutamic acid were present in final concentrations of 2 μCi/ml. After incubation for 40 or 60 min at 37 C, the cells of a 2.0-ml suspension were harvested, suspended in distilled water, and heated for 15 min at 100 C to liberate the precursors into the supernatant fluid. The charcoal-adsorbable material was isolated (5).

Chromatography. Samples were applied to Whatman 1 chromatography paper and chromatographed in isobutyric acid-1 M ammonia (5:3; vol/vol). Hydrolysates were chromatographed for 12 to 16 h, and charcoal-adsorbable material was chromatographed for 90 h. Radioactive areas were detected by autoradiography (4). R_f values were: glutamate, 0.42; 2-ketoglutarate, 0.35; UDP-MurNAc-L-Ala, 0.28; UDP-MurNAc-dipeptide, 0.2; UDP-MurNAc-tripeptide, 0.05; UDP-MurNAc-pentapeptide, 0.1.

Determination of D-glutamate. After incubation of the parent strain in the CWSM-I medium used for incorporation of L-[^{14}C]glutamate (2 μCi/ml), UDP-MurNAc-pentapeptide was isolated by charcoal adsorption and preparative paper chromatography (5). After hydrolysis in 6 N HCl for 16 h at 105 C, samples were neutralized and incubated in an assay mixture for the D-glutamic acid adding enzyme for 16 h at 30 C as described previously (3) except that excess enzyme was used. As a control on the stereospecificity of the enzyme, commercial samples of DL- and L-[^{14}C]glutamic acid were also tested as the radioactive substrate.

Enzyme assays. Procedures for the assay of UDP-MurNAc:L-alanine ligase (ADP) (EC 6.3.2.8) (L-alanine adding enzyme), D-glytamic acid adding enzyme); UDP-MurNAc-L-Ala-D-Glu:meso-diaminopimelic acid (m-Dpm) ligase (ADP) (m-Dpm adding enzyme, and UDP-MurNAc-L-Ala-D-Glu-m-Dpm:D-Alanyl-D-alanine ligase (ADP) (D-alanyl-D-alanine adding enzyme) have been described previously (Fig. 1) (3). The radioactivity of the ^{14}C-labeled amino acid or dipeptide that was bound to the appropriate uridine nucleotide precursor was the measure for enzyme activity. D-[^{14}C]Alanine was the substrate for D-alanine:D-alanine ligase (ADP; EC 6.3.2.4) (D-alanyl-D-alanine synthetase). The enzyme was assayed in the presence of UDP-MurNAc-tripeptide. The product of D-alanine:D-alanine ligase was immediately coupled to UDP-MurNAc-tripeptide because the specific activity of the D-alanyl-D-alanine adding enzyme is about eightfold higher than that of D-alanine:D-alanine ligase. This procedure prevents inhibition of D-alanine:D-alanine ligase by its product (3). L-Alanine:D-alanine racemase (EC 5.1.1.1) (alanine racemase) was assayed essentially as D-alanine:D-alanine ligase, except that L-[^{14}C]alanine was the labeled substrate. This procedure could be used because the racemase activity is about 20 times lower than that of D-alanine:D-alanine ligase (3). All substrates and products were separated by chromatography, detected by autoradiography, and counted. Details of the assay procedures were described previously (3).

For the assay of D-alanine:2-oxo-glutarate aminotransferase (EC 2.6.1.6; D-alanine aminotransferase) two methods were tested. Method A was the colorimetric determination of pyruvate with salicylaldehyde in alkali, as described by Martinez-Carrion and Jenkins (10). Either sonically disrupted cells or soluble protein after ammonium sulfate precipitation (3) was used as the enzyme source in a final protein concentration of 2 mg/ml. Ketoglutarate was omitted from the control. The mixtures were incubated for 1 h at 37 C. Method B was a microassay, based on the conversion of radioactive 2-ketoglutarate to glutamate. The assay mixture contained (i) [2-^{14}*C*]ketoglutarate (2 μCi/ml); (ii) D-alanine (50 mM); (iii) pyridoxal-5'-phosphate (0.4 mM); (iv) Tris-hydrochloride (100 mM), final pH of 7, 8, or 9; (v) $MnCl_2$ (2 mM); (vi) adenosine-5'-triphosphate (4 mM); (vii) UDP-MurNAc-L-Ala (0.4 mM); and (viii) enzyme as used in method A. The final volume was 50 μliters. The mixtures were incubated for 1, 2, 4, and 16 h at 37 C. D-Glutamate, if synthesized by the transaminase, would be added to UDP-MurNAc-L-Ala by the D-glutamic acid adding enzyme (3). Incubations without UDP-MurNAc-L-Ala were used as controls. Substrates and products were separated by chromatography (3) and detected by autoradiography (4). The D-glutamic acid adding enzyme was sufficiently active under these conditions as was shown when

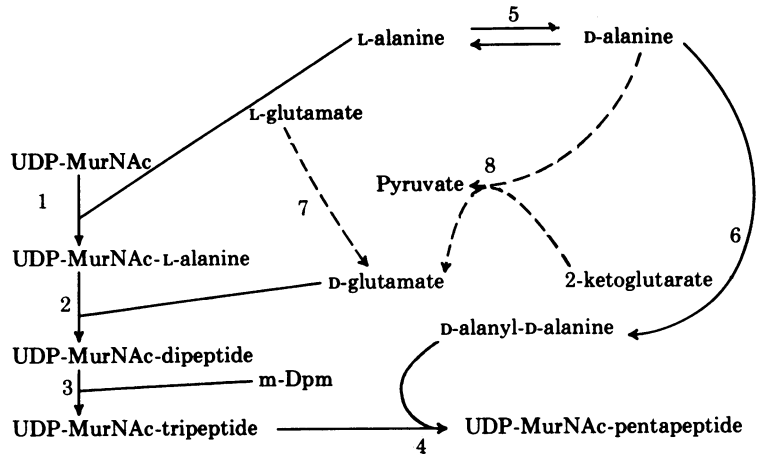

FIG. 1. *Relevant reactions for the enzyme assays in this paper. Reactions indicated by dotted lines represent activities that could not be demonstrated by the authors of the present paper. The numbers correspond with the following enzymes: 1, L-alanine adding enzyme; 2, D-glutamic acid adding enzyme; 3, meso-diaminopimelic acid adding enzyme; 4, D-alanyl-D-alanine adding enzyme; 5, L-alanine: D-alanine racemase; 6, D-alanine: D-alanine ligase; 7, L-glutamate: D-glutamate racemase; 8, D-alanine specific D-alanine: 2-ketoglutarate transaminase.*

[2-^{14}C]ketoglutarate was replaced by DL-[^{14}C]glutamate. When only 0.02 μCi of DL-[^{14}C]glutamate was substituted for the substrate, labeled UDP-MurNAc-dipeptide could still be detected. It therefore seems reasonable to assume that 1 nmol of D-glutamate product could still be detected.

L-Glutamate: D-glutamate racemase (EC 5.1.1.3) was tested in a mixture containing 10 μliters of each of the following solutions: (i) L-[^{14}C]glutamate, 20 μCi/ml; (ii) pyridoxal-5'-phosphate, 10 mM; (iii) pyruvate, 2 mM; (iv) universal buffer adjusted to the appropriate pH (containing per liter: citric acid-water (1:1), 6 g; KH_2PO_4, 3.89 g; H_3BO_3, 1.77 g; diethyl-barbituric acid, 5.27 g), and (v) one of each enzyme preparation that are described above for the transaminase assays. The assay was carried out at pH values varying from 5.0 to 11.0 for 2 h at 30 C. After chromatography and autoradiography, glutamate was eluted overnight with distilled water. The amount of the D-stereoisomer was determined by incubation of the eluted material in an assay mixture for the D-glutamic acid adding enzyme for 16 h at 30 C as described previously (3) except that excess of enzyme was used. When, as a positive control, various amounts of DL-[^{14}C]glutamate were substituted for L-[^{14}C]glutamate, the radioactive product UDP-MurNAc-dipeptide could still be detected when as little as 0.002 μCi of DL-glutamic acid (corresponding with 0.1 nmol) was substituted for the substrate L-[^{14}C]glutamic acid. About 3% of the radioactivity of the substrate L-glutamic acid, corresponding with 0.01 nmol, was added to UDP-MurNAc-L-Ala, even when no enzyme was present. The same value was obtained when the remaining glutamic acid was eluted and used as a substrate for the D-glutamic acid adding enzyme. It is not known whether this result is due to spontaneous racemization or to enzymatic addition of L-glutamic acid by the enzyme.

Pyruvate was included in the assay mixture to prevent a possible transamination reaction between pyridoxal-5'-phosphate and ornithine (17), possibly present in the extract. When pyruvate was omitted, the results were essentially the same.

Chemicals. D-Glutamic acid and D-cycloserine were obtained from Fluka, Buch, Switzerland, and vancomycin was from Eli Lilly & Co., Indianapolis, Ind.

Radiochemicals. L-[*U*-^{14}C]Alanine (specific activity 270 mCi/mmol), L-[*U*-^{14}C]glutamic acid (specific activity 270 mCi/mmol), DL-[*1*-^{14}C]glutamic acid (specific activity 19.8 mCi/mmol), and sodium [5-^{14}C]2-ketoglutarate (specific activity 17.5 mCi/mmol) were obtained from the Radiochemical Center, Amersham, England.

RESULTS

Growth characteristics. Parent and mutant cells, growing exponentially in CGPY medium supplemented with D-glutamic acid, were washed with the same medium without D-glutamic acid, resuspended, and incubated at 37 C in CGPY medium with or without D-glutamic acid. The optical density was followed (Fig. 2). The addition of D-glutamic acid had no influence on the growth of the parent strain. The mutant cells lysed in the absence of D-glutamic acid, whereas in the presence of this amino acid no differences between parent and mutant strains were observed with respect to growth rate and morphology.

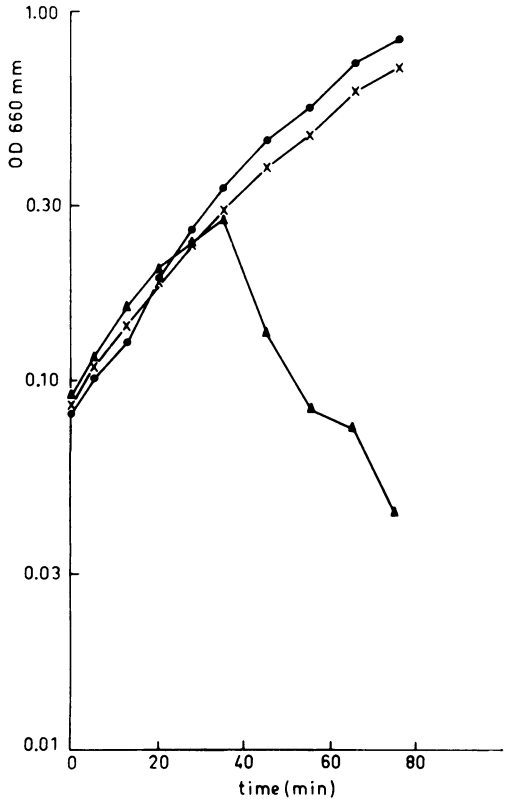

FIG. 2. *Effect of* D-*glutamic acid starvation on the growth. Cells, growing exponentially in CGPY medium supplemented with* D-*glutamic acid (50 μg/ml), were washed with CGPY medium and suspended in four volumes of CGPY medium without or supplemented with* D-*glutamic acid. The suspensions were incubated at 37 C. The optical density was measured at various intervals. The curves obtained for the parent strain under both conditions (●), the mutant strain without (▲) and in the presence (×) of* D-*glutamic acid are presented.*

Lysis of mutant cells under restrictive conditions has been observed with mutants impaired in the biosynthesis of murein (5–8, 11), unsaturated fatty acids, and phospholipids (J. H. F. F. Broekman and J. F. Steenbakkers, personal communications). Since D-glutamic acid is an amino acid that is found in the murein sacculus (14), the lysis observed under restrictive conditions is probably the result of an impaired synthesis of murein.

Murein synthesis. When temperature-sensitive mutants impaired in murein synthesis were incubated in CWSM-I medium, no significant lysis was observed (5). However, the D-glutamate-requiring strain, unlike its parent strain, showed considerable lysis under these conditions. Compared with its parent strain, the mutant seemed to be defective in the synthesis of murein (Table 1). However, the optical density of the mutant culture was decreased by 50% in the course of the experiment (60 min). The decreased incorporation of L-[^{14}C]alanine can therefore at least partly be explained by the extensive decrease in optical density. Definite proof for an impaired murein synthesis came from the distribution of the radioactivity among the murein precursors (Table 1). Whereas the majority of the radioactivity of the precursors of the parent strain was found in UDP-MurNAc-pentapeptide and UDP-MurNAc-dipeptide, virtually all radioactivity of the precursors of the mutant was found in UDP-MurNAc-L-Ala. This result shows that the lysis of the mutant strain in the absence of D-glutamic acid is caused by a defect in murein synthesis. Accumulation of UDP-MurNAc-L-Ala can be explained either by an altered D-glutamic acid adding enzyme with increased K_m for D-glutamic acid or by a defective enzyme in the biosynthesis of D-glutamic acid. No significant differences between parent and mutant strains were found in the activities of the adding enzymes for L-alanine, D-glutamic acid, meso-Dpm, or D-alanyl-D-alanine (data not presented). The mutant strain is therefore defective in the synthesis of D-glutamic acid. It seemed to offer a unique opportunity for study-

TABLE 1. *Distribution of radioactivity among the alanine-containing murein precursors*[a]

Strain	Murein (counts/min)	Total precursors (counts/min)[b]	Radioactivity among murein precursors[c]			
			X-1	X-2	X-3	X-5
Parent	8,760	1,270	6	20	2	72
Mutant	2,170	320	96	1.5	1	1.5

[a] Cells growing exponentially at 37 C in CGPY medium supplemented with D-glutamic acid were washed with and incubated in CWSM-I medium. After incubation for 15 min at 37 C, L-[^{14}C]alanine was added to 3.0 ml of the suspension in a final concentration of 2 μCi/ml. After additional incubation for 60 min, the perchloric acid-precipitable activity of duplicate samples of 0.1 ml was determined. At the same time, 2.0 ml of suspension was used to isolate the precursors. Reference cultures without radioactivity were incubated under identical conditions and used to determine the optical density, which was constant for the parent strain and decreased by 50% for the mutant strain.

[b] Corresponding with 0.1 ml of suspension.

[c] X = UDP-MurNAc. The number represents the number of amino acids in the peptide chain. Radioactivity was measured as the percentage of total counts per minute.

ing the pathway of the biosynthesis of D-glutamate.

Attempts to show the presence of an enzyme in the pathway for the biosynthesis of D-glutamic acid. Cota-Robles and Duncan suggested that the immediate precursors of D-glutamic acid were or could be D-alanine and 2-ketoglutaric acid. The transaminase involved in the reaction was found to be specific for D-alanine (1). We tried to assay the D-alanine-specific transaminase (1) by the two methods described previously. Crude extract or sonically disrupted cells (3) of the parent strain were used as the enzyme. No activity could be detected. These results are not in agreement with those of Cota-Robles and Duncan, who showed that extracts of *E. coli* B were able to synthesize D-glutamate and pyruvate from D-alanine and 2-ketoglutarate (1). As these authors have not described their assay method, it is difficult to ascertain the reason for this discrepancy. Because earlier results from this laboratory (5) strongly suggest that D-alanine is not required for the synthesis of D-glutamate (see below), we decided to test the possibility that D-glutamate, like D-alanine (5, 16), is synthesized by racemization. The tests were performed at pH values between 5 and 11. In vitro conversion of L-glutamate to D-glutamate could not be demonstrated. We therefore concluded that *E. coli* either does not contain the tested enzyme activities or that the conditions used for isolation or assay of these enzymes were far from optimal.

In vivo incorporation of exogenous L-glutamate into murein. Because we were unable to demonstrate the in vitro activity of an enzyme that could synthesize D-glutamate from either 2-ketoglutarate or L-glutamate, we found it necessary to check whether the label from exogenous L-glutamate could be detected in murein and, if so, whether it was present as D-glutamate. Although a positive result would not distinguish racemization from transamination, failure to label murein with L-glutamate would strongly suggest the operation of other biosynthetic pathways.

The cells of the parent strain were incubated in CWSM-I medium, as described previously, to prevent protein synthesis and therefore to increase the possibility of detecting labeled murein in acid-precipitable material. When unlabeled L-glutamate was omitted from CWSM-I, a good incorporation of exogenous L-[^{14}C]glutamate into acid-precipitable material was observed. After hydrolysis of this material followed by chromatography in isobutyric acid-1 M ammonia (5:3; vol/vol), more than 98% of the label was found in glutamate. The effect of the cell wall antibiotic vancomycin (300 μg/ml) on the incorporation of L[^{14}C]glutamate and, as a control, of L-[^{14}C]-alanine was studied. After incubation for 2 h at 37 C, the incorporation of L-alanine was inhibited by 41% and that of L-glutamic acid by 26%, suggesting that part, but not all, of the L-glutamate taken up was incorporated into murein.

The idea that label from exogenous L-glutamate was present in murein was checked by testing the sensitivity of acid-precipitable material to lysozyme. Both parent and mutant strains were used. As a control, lysozyme-sensitivity of L-[^{14}C]alanine incorporated into the cells of the parental strain was tested (Table 2). In the controls without lysozyme, about 17% of the activity was degraded. Degradation occurred during ultrasonic disintegration and is probably due to degradation by autolytic enzymes that are not completely inactivated by the heat treatment (4). Normally 20 to 40% of

TABLE 2. *Lysozyme sensitivity of labeled acid-precipitable material*[a]

Strain	Labeled amino acid	Acid-precipitable radioactivity		
		Before washing (counts/min)	After incubation[b]	
			+ Lysozyme	− Lysozyme
Parent	L-Ala	1,150	39	83
Parent	L-Glu	8,810	45	83
Mutant	L-Glu	4,590	70	82

[a] Parent and mutant strains were incubated in the wall medium and supplemented with the labeled amino acid (2 μCi/ml) as described previously. After incubation for 2 h at 37 C, the perchloric acid-precipitable radioactivity in a sample of 0.2 ml was determined. Another sample (2.0 ml) was centrifuged, washed twice with and resuspended into one volume of 10^{-4} M Tris (*p*H 7.0), disintegrated by sonic treatment, and heat inactivated as described previously. Samples were incubated with and without lysozyme. The acid-precipitable activity of samples of 0.2 ml was determined to measure the fraction of lysozyme-sensitive radioactive material. The sample was taken before sonic treatment and was considered to contain 100% activity. Reference cultures without radioactivity were incubated under identical conditions to measure the optical density, which was found to be equal and constant during the incubation in the wall medium for both strains.

[b] Expressed as percentage of acid-precipitable radioactivity relative to the values measured before ultrasonic disintegration of washed cells.

the murein is resistant to lysozyme under these conditions. In this experiment the percentage of lysozyme-resistant activity was quite high but was about the same for L-alanine- and L-glutamate-labeled parent cells, indicating that L-glutamate was incorporated into murein of the parent strain for at least 38% but probably much more. The mutant incorporated less radioactivity into the acid-precipitable material. Both the absolute and relative radioactivity of lysozyme-degradable material was decreased compared with the parent strain, which is in agreement with the proposed defect in murein synthesis.

Analysis of the charcoal-adsorbable material of the parent strain showed that the label of exogenous L-glutamate is found in UDP-MurNAc-dipeptide and UDP-MurNAc-pentapeptide. These components had about the same radioactivity which after hydrolysis was only found in glutamate. The addition of D-cycloserine (80 μg/ml) under these conditions resulted in accumulation of radioactive UDP-MurNAc-tripeptide. No radioactive precursors could be isolated from the mutant. This result is in agreement with the observed accumulation of UDP-MurNAc-L-Ala in this strain (Table 1). The results indicate that exogenous L-glutamate is incorporated into murein via uridine nucleotide murein precursors. The label is still present as glutamate. The steroisomeric configuration of this amino acid remained to be investigated. We therefore decided to isolate L-glutamate-labeled UDP-MurNAc-pentapeptide for further analysis.

Investigation of the stereoisomeric form of labeled glutamate in UDP-MurNAc-pentapeptide. The parent strain was incubated in 6 ml of wall medium supplemented with L-[^{14}C]glutamate (2 μCi/ml) and vancomycin (300 μg/ml) for 40 min at 37 C. In the presence of vancomycin, the radioactivity of the precursors was twice as high as in its absence. UDP-MurNAc-pentapeptide was isolated by charcoal adsorption and purified by paper chromatography using the solvents isobutyric acid-1 M ammonia (5:3; vol/vol) and ethanol-1 M ammonium acetate, pH 7.2 (5:2; vol/vol). The purified material was hydrolyzed with 6 N HCl. The acid was removed and the material was dissolved in a small volume of distilled water. All radioactivity was present as glutamate as was shown by chromatography and autoradiography. The stereoisomeric form was investigated, as previously described, with the help of the D-glutamic acid adding enzyme. The stereospecificity of this enzyme was tested with commercial samples of L- and DL-glutamate. Under the experimental conditions, 36% of the label of pure DL-glutamate was added to UDP-MurNAc-L-Ala, while about 3% of L-glutamate was added to this uridine nucleotide (Table 3). In both cases UDP-MurNAc-dipeptide was the only product. We concluded that the enzyme is sufficiently stereospecific to differentiate between L- and D-glutamate. The stereospecificity of the D-glutamic acid adding

TABLE 3. *Determination of the stereoisomeric configuration of glutamic acid from UDP-MurNAc-pentapeptide*[a]

Substrate	Radioactivity (counts/min)		
	X-2[b]	X-5	Glutamate
DL-Glutamate	35,300 (36)[c]		63,600 (64)
L-Glutamate	2,520 (3)		83,400 (97)
Hydrolyzed sample	7,400 (30)	12,100 (50)	4,900 (20)
Hydrolyzed sample	4,500 (34)	5,200 (39)	3,500 (27)
Hydrolyzed sample	2,400 (32.5)	2,600 (35)	2,400 (32.5)
Hydrolyzed sample	1,280 (30)	1,420 (34)	1,500 (36)
Hydrolyzed sample without enzyme	20 (0)	0 (0)	10,200 (100)

[a] Hydrolyzed samples of UDP-MurNAc-pentapeptide, isolated from the parent strain after incubation in the wall medium supplemented with L-[^{14}C]glutamate (2 μCi/ml), were prepared as described previously. As the specific activity of ^{14}C-glutamic acid in the hydrolysates is unknown, various amounts of the hydrolysate were tested. As a control on the stereospecificity of the enzyme, commercial samples of DL- and L-[^{14}C]glutamic acid were also tested as the radioactive substrate. The substrates were incubated for 16 h at 30 C in the mixture used for assay of the D-glutamic acid adding enzyme (3) except that the amount of enzyme was doubled.

[b] X = UDP-MurNAc. The number represents the number of amino acids in the peptide chain.

[c] The percentage of the total radioactivity is given in parentheses.

enzyme of *Staphylococcus aureus* has been shown by Nathenson et al. (12).

As the specific activity of glutamic acid in the hydrolysate was unknown, various amounts were given as a substrate (Table 3). We found radioactivity in both UDP-MurNAc-dipeptide and UDP-MurNAc-pentapeptide. This result was expected because all enzymes and substrates necessary to convert UDP-MurNAc-dipeptide to UDP-MurNAc-pentapeptide are present in the assay mixture. Depending on the dilution of the hydrolyzed sample, 64.5 to 80% of the input radioactivity is converted by the D-glutamic acid adding enzyme. As not all of the stereoisomeric D-form of the reference DL-glutamate is converted under the experimental conditions (in earlier experiments over 50% of its radioactivity could be recovered in the product), it is likely that the labeled glutamic acid of in vivo synthesized UDP-MurNAc-pentapeptide is (nearly) completely present as the D-stereoisomeric form.

Preliminary genetic location of the mutation. Because revertants are easily obtained, the phenotype of the mutant is the result of only one mutation. Judging from the number of prototrophic recombinants in relation to several reference markers in crosses with a number of Hfr strains, the mutation responsible for the D-glutamate requirement is situated on the *E. coli* genome in the segment between the *str* marker and the origin of the Hfr H strain (data not presented). Of known murein genes, *murA* (13) and *murB* (11), involved in UDP-MurNAc synthesis, as well as *alr* (16), for alanine racemase, have been located in the same region, in contrast to genes coding for the adding enzymes which are located close to the *leu* marker (15).

DISCUSSION

The present paper describes a number of properties of a D-glutamic acid auxotroph of *Escherichia coli*. Under restrictive conditions, the cells show the lysis pattern (Fig. 2) that has been observed for mutants impaired in murein synthesis (5, 6). Experiments in a cell wall medium (4) showed that less ^{14}C-alanine is incorporated into the murein of the mutant (Table 1). The mutant accumulates UDP-MurNAc-L-alanine, indicating that D-glutamic acid either cannot be added or cannot be synthesized. As the D-glutamic acid adding enzyme of mutant and parent were equally active, the latter possibility is more likely. Unfortunately we were unable to show in vitro activity of a D-glutamic acid-synthesizing enzyme in extracts of the parent strain.

We were able to show that exogenous L-[^{14}C]glutamate is partly incorporated into the murein of *E. coli* (Table 2) where it is present as the D-isomer (Table 3). This result indicates that D-glutamic acid can be synthesized from L-glutamic acid. The mutant that is impaired in the synthesis of D-glutamic acid incorporated considerably less exogenous L-glutamate into its murein, indicating that the enzyme (or one of the enzymes) that is involved in the biosynthesis of D-glutamic acid via L-glutamic acid, is defective in the mutant strain. The residual murein synthesis in the absence of D-glutamic acid (Tables 1 and 2) indicates either that the defective enzyme is leaky or that its product can also be synthesized in small amounts by another enzyme. The enzyme(s) responsible for D-glutamate biosynthesis remain(s) unknown. If D-alanine is required for D-glutamate biosynthesis, as was assumed by Cota-Robles and Duncan (1), D-glutamate synthesis should be blocked in a L-alanine:D-alanine racemase mutant under restrictive conditions, and accumulation of UDP-MurNAc-L-alanine should then be expected (Fig. 1). However, Lugtenberg et al. (5) have found that such a mutant accumulates UDP-MurNAc-tripeptide. The possibility that this tripeptide contains L-glutamate instead of D-glutamate is very unlikely because the L-glutamate can hardly be added to UDP-MurNAc-L-alanine (Table 3). The possibility that D-glutamate is replaced by another amino acid is unlikely as this then should also occur in the D-glutamate requiring mutant, in which accumulation of UDP-MurNAc-L-alanine was found.

ACKNOWLEDGMENTS

We thank Arna van Schijndel-van Dam for excellent technical assistance in part of the experiments, H. C. Wu for important criticisms and suggestions, and W. Messer for supplying us with the bacterial strains.

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