JOURNAL OF BACTERIOLOGY, Nov. 1972, p. 950-958

Vol. 112, No. 2
Printed in U.S.A.

Cell Wall Peptidoglycan Mutants of *Escherichia coli* K-12: Existence of Two Clusters of Genes, *mra* and *mrb*, for Cell Wall Peptidoglycan Biosynthesis

TOKICHI MIYAKAWA, HIROSHI MATSUZAWA,[1] MICHIO MATSUHASHI, AND YOSHINOBU SUGINO[2]

Institute of Applied Microbiology, University of Tokyo, Bunkyo-ku, Tokyo, Japan, and Department of Biology, Faculty of Science, Osaka University, Osaka, Japan

Received for publication 24 August 1972

Temperature-sensitive mutants of *Escherichia coli* K-12 which cannot form cell wall peptidoglycan at 42 C were isolated. The thermosensitive steps were characterized biochemically, and the genes coding the enzymes of peptidoglycan synthesis were mapped. These genes were in two clusters: one group, located at about 1.5 min between *leu* and *azi*, was designated as *mra* (murein a); the second group, located at about 77.5 min close to *argH* and *metB*, was designated as *mrb* (murein b, with the order *metB-argH-mrb*). No simple relations were found between the gene location and the order or localization of enzymes involved in the sequence of reactions of cell wall peptidoglycan biosynthesis.

The material forming the basal structure in *Escherichia coli* cell wall is peptidoglycan, also called "murein" by several investigators (23). It consists of repeating units of *N*-acetylglucosaminyl (GlcNAc)-*N*-acetylmuramyl (MurNAc)-tetrapeptide(L-Ala-D-Glu-*meso*-diaminopimelic acid [Dpm]-D-Ala) about 50% cross-linking between the carboxy terminal of D-alanine and the D-amino terminal of *meso*-Dpm (23). There have been many studies on the biosynthetic pathway of peptidoglycan (18), and only a few steps are still unknown. The biosynthetic pathway can be divided in three sequences of reactions according to the nature of the enzymes involved: (i) formation of uridine diphosphate (UDP)-MurNAc-pentapeptide (L-Ala-D-Glu-*meso*-Dpm-D-Ala-D-Ala) from UDP-GlcNAc by soluble cytoplasmic enzymes; (ii) lipid cycle reactions involving the formation of lipid-linked intermediates, polymerization of repeating units, and regeneration of carrier lipid by enzymes bound to the cytoplasmic membrane; and (iii) formation of cross-linking by transpeptidation and formation of cell wall saccules by enzymes located outside the cytoplasmic membrane (18). The enzymes involved are listed in Table 1.

The present genetic studies on the biosynthesis of peptidoglycan in *E. coli* strain K-12 were to determine the position on the chromosome of genes specifying the enzymes involved in sequences of the biosynthetic reactions described above. At present we have identified two clusters of genes *mra* at ca. 1.5 min and *mrb* at ca. 77.5 min and characterized several mutants biochemically. A preliminary report of this work, including a rough map of these two clusters of genes, has appeared (17). A similar study has been carried out in *Bacillus subtilis* 168 (T. Miyakawa and M. Matsuhashi, *manuscript in preparation*).

MATERIALS AND METHODS

Bacterial strains. The properties of bacterial strains used are summarized in Table 2. The transducing phage P1kc was obtained from A. Matsushiro.

Media. Nutrient broth (Difco) was used for most experiments involving the isolation of temperature-sensitive (ts) mutants and mating experiments. Difco antibiotic medium 3 (Penassay Broth) was used for preparation of cells at 30 C for in vitro enzyme assay. L-broth (10) was used for transduction experiments. Davis minimal medium (19) was used for determination of auxotrophic markers and for selection of auxotrophic mutants. EM medium (19) and EMB

[1] Present address: Department of Agricultural Chemistry, University of Tokyo, Bunkyo-ku, Tokyo, Japan.

[2] On leave of absence at the National Institute of Arthritis and Metabolic Diseases, Bethesda, Md.

TABLE 1. *List of enzymes involved in the biosynthesis of the cell wall peptidoglycan in Escherichia coli*

Enzymes
Soluble, cytoplasmic enzymes
Enzymes of UDP-MurNAc synthesis:
Phosphoenolpyruvate-UDP-GlcNAc transferase
UDP-GlcNAc-3-enolpyruvate reductase
Enzymes of peptide formation:
L-Alanine-adding enzyme [EC 6.3.2.8]
D-Glutamate-adding enzyme [EC 6.3.2.9]
meso-Diaminopimelate-adding enzyme
D-Alanyl-D-alanine synthetase [EC 6.3.2.4]
D-Alanyl-D-alanine-adding enzyme [EC 6.3.2.10]
Enzymes of D-amino acid formation:
Unidentified D-glutamate-synthesizing enzyme
Alanine racemase [EC 5.1.1.1]
Membrane-bound enzymes related to lipid cycle reactions
Enzymes of lipid cycle reactions:
Phospho-*N*-acetylmuramyl-pentapeptide translocase
N-acetylglucosamine translocase
Peptidoglycan polymerase
Undecaprenol-pyrophosphatase
Enzymes of undecaprenol-phosphate synthesis:
Undecaprenol phosphokinase
Unidentified undecaprenol synthetase (possibly soluble enzymes)
Membrane-bound or periplasmic enzymes involved in formation of peptidoglycan saccules
Synthetic enzymes:
Peptidoglycan transpeptidase
D-Alanine carboxypeptidase
Unidentified enzyme metabolizing released D-alanine
Degradative enzymes:
Peptidoglycan lytic enzymes

medium (19) were used for determination and selection of defective sugar fermentation. Peptone yeast extract medium (22), containing 3.4 mM sodium azide, was used for determination of azide sensitivity. For solidifying media 1.5% agar (Wako Pure Chemical Co., Osaka, Japan) was added.

Isolation of ts mutants. Mutants were isolated from *E. coli* strain JE1011 (derived from *E. coli* K-12). Mutation of bacteria in the logarithmic phase of growth at 30 C (titer 3×10^8 cells per ml) was induced with 100 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) per ml, essentially by the method of Adelberg et al. (1). After treatment, cells were washed once with saline, transferred to 7 ml of nutrient broth, and incubated at 30 C for 3 hr with shaking. Then 0.1-ml samples were spread on plates of nutrient agar at a cell concentration giving several hundred colonies per plate (master plates). Plates were incubated at 30 C, and colonies were replicated (10) on nutrient agar plate. Colonies which did not grow at 42 C on the replica plates were collected from the master plates and purified by single-colony isolation. The thermosensitivity, requirement for 0.5 M sucrose at 42 C (17), and genotype of mutants were examined on appropriate plates.

Mating procedure. The methods used were essentially those of Taylor and Thoman (21) and were as described previously (19) except that the temperature was kept at 30 C throughout the mating procedure.

Transduction techniques. The procedure used for transduction was based on that described by Lennox (11).

P1kc lysates were prepared by making confluent plate lysates in 2.5 ml of 0.7% agar poured onto L-agar plates containing 2.5 mM $CaCl_2$. After overnight incubation at appropriate temperatures, the soft agar layer was scraped off, mixed with an equal volume of L-broth, and centrifuged to remove debris.

For transduction, recipient cells were grown in 7 ml of L-broth containing 2.5 mM $CaCl_2$ to a titer of 2×10^8 cells per ml, and P1kc lysate was added at a multiplicity of infection of 0.3. After incubation for 30 min to allow adsorption, cells were washed, suspended in saline, and plated on selective media (30 C). The unselected markers described in the text were scored by replica plating.

Radioactive compounds, chemicals, and enzymes. ^{14}C-L-alanine, specific activity 125 mCi per mmole, and ^{14}C-D-glucosamine, specific activity 50 mCi per mmole, were purchased from New England Nuclear Corp. ^{14}C-DL-alanine was prepared by autoclaving an aqueous solution of ^{14}C-L-alanine for 2 hr at 200 C. (We are obliged to H. Wakamatsu of

TABLE 2. *Strains used*[a]

Strain	Sex	Genotype	Origin
JE1011	F⁻	*thr, leu, trp, his, thy, thi, lac, gal, xyl, mtl, strA, azi*	M. Ishibashi
JE1031	HfrH	*met, thi*	
W2252	HfrC	*met*	
AB1450	F⁻	*his, ilvD, metB, argH, thi, gal, lac, mal, xyl, tsx, strA*	J. Pittard
AB1206	F	Prototroph (carries F-14)	J. Pittard
AT997	Hfr KL16	*dapC*	A. I. Bukhari

[a] Gene symbols: *thr*, threonine; *leu*, leucine; *trp*, tryptophan; *his*, histidine; *met*, methionine; *arg*, arginine; *ilv*, isoleucine-valine; *dap*, diaminopimelate; *thi*, thiamine; *thy*, thymine; *lac*, lactose; *gal*, galactose; *xyl*, xylose; *mtl*, mannitol; *mal*, maltose; *str*, streptomycin; *tsx*, resistance to phage T6; *azi*, azide. The time scale of reference markers is that of Taylor (20).

Ajinomoto Co., Japan for informing us of this technique.) ^{14}C-D-Ala-^{14}C-D-Ala was prepared enzymatically from ^{14}C-DL-alanine (8). ^{14}C-labeled UDP-MurNAc-pentapeptide(^{14}C-D-Ala-^{14}C-D-Ala), specific activity 120 mCi per mmole, was prepared enzymatically from UDP-MurNAc-tripeptide(L-Ala-D-Glu-*meso*-Dpm) and ^{14}C-D-Ala-^{14}C-D-Ala (8). ^{14}C-D-GlcNAc-labeled UDP-GlcNAc, specific activity 12 mCi per mmole, was prepared by the method of Endo et al. (7). UDP-MurNAc, UDP-MurNAc-L-Ala, and UDP-MurNAc-L-Ala-D-Glu were isolated from *Staphylococcus aureus* and UDP-MurNAc-L-Ala-D-Glu-*meso*-Dpm from *Bacillus cereus* by methods described previously (8, 9).

^{14}C-L-alanine or ^{14}C-D-glucosamine incorporation in vivo. Cells in 150-ml cultures in the logarithmic phase of growth in nutrient broth at 30 C were harvested by centrifugation. Cells were washed with cold 0.05 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride buffer, *p*H 7.5 containing 0.01 M $MgCl_2$. Then a 2% suspensions of cells (wet weight per volume) were prepared in the incubation mixture (final volume 2.5 to 5.0 ml). The incubation mixture consisted of 0.04 M Tris-hydrochloride buffer (*p*H 7.5), 0.008 M $MgCl_2$, 0.01 M glucose, 0.1 μCi of ^{14}C-L-alanine (specific activity 125 mCi per mmole), and 0.2 mg of chloramphenicol per ml; or 0.04 M Tris-hydrochloride buffer (*p*H 7.5), 0.008 M $MgCl_2$, and 0.04 μCi of ^{14}C-D-glucosamine (specific activity, 50 mCi per mmole). After incubation for 60 min at either 30 C or 42 C, cells were collected by centrifugation, washed once with cold water, and suspended in 3 ml of water. The cells were then heated for 3 min in a boiling water bath, and the cells and insoluble materials were precipitated by centrifugation and washed once with water. The pellet was digested with trypsin and subjected to paper chromatography with isobutyric acid-1 M ammonia (5:3, v/v) as solvent. The radioactivity remaining at the origin (peptidoglycan) was counted in a liquid scintillation counter.

The supernatants obtained from the boiled cell suspensions contained radioactive nucleotides. The solutions were adjusted to *p*H 2 with cold 50% trichloroacetic acid, and the resulting precipitate was removed by centrifugation. Then 50 mg of activated charcoal (Darco KB) was added to the supernatant and, after stirring for 20 min at room temperature, was collected and washed with centrifugation. The labeled nucleotides were eluted with three 1-ml volumes of 0.03 M NH_4OH in 50% ethanol. The eluates were combined and evaporated to dryness in vacuo, and the radioactive materials were dissolved in 150 μliters of water and subjected to paper chromatography with isobutyric acid-1 M ammonia (5:3, v/v) as solvent. The radioactive nucleotides were located by autoradiography and identified from their mobilities. Occasionally two-dimensional paper chromatography (second solvent: 96% ethanol-1 M ammonium acetate, *p*H 7.2 [5:2, v/v]) was used for their identification.

Assay of UDP-GlcNAc-3-enolpyruvate reductase. The reaction mixture in a final volume of 50 μliters contained Tris-hydrochloride buffer (*p*H 7.8), 2.5 μmoles; KCl, 250 nmoles; $MgCl_2$, 250 nmoles; the cyclohexyl ammonium salt of phosphoenolpyruvate, 20 nmoles; reduced nicotinamide adenine dinucleotide phosphate (NADPH), 20 nmoles; 2-mercaptoethanol, 50 nmoles; ^{14}C-UDP-GlcNAc (specific activity, 12 mCi per mmole), 0.005 μCi; and cell extracts of the parent strain (98 μg of protein) or mutant ST5 (110 μg of protein) which had been treated with Sephadex G25. Mixtures were incubated at 42 C for 1 hr. The reaction was stopped by adding 10 μliters of isobutyric acid-1 M ammonia (5:3, v/v), and the mixture was subjected to paper chromatography in the latter solvent. The area corresponding to UDP-GlcNAc (R_F 0.30) and UDP-MurNAc (R_F 0.35) was cut out, and the radioactive materials were eluted with water and separated by paper electrophoresis in 0.1 M ammonium formate-formic acid, *p*H 5.2. Electrophoresis was carried out at 14 v/cm for 2.5 hr under cooling with carbon-tetrachloride. The paper was scanned with a 4π gas-flow strip scanner (Packard).

Cell extracts were prepared by sonic disintegration of cells harvested in the logarithmic phase of growth (8). Protein was determined by the method of Lowry et al. (12).

Assay of L-alanine-adding enzyme. Reaction mixture in a final volume of 50 μliters contained Tris-hydrochloride buffer (*p*H 8.0), 2 μmoles; $MnCl_2$, 100 nmoles; adenosine triphosphate (ATP), 200 nmoles; UDP-MurNAc, 10 nmoles; D-cycloserine, 80 nmoles; ^{14}C-L-alanine (specific activity, 125 mCi per mmole), 0.08 μCi; and cell extracts of the parent strain (98 μg of protein) or mutant ST222 (105 μg of protein) which had been treated with Sephadex G25. Mixtures were incubated at 30 or 42 C for 1 hr. The reaction was stopped by boiling the mixture for 3 min in a water bath. The mixtures were subjected to paper chromatography in isobutyric acid-1 M ammonia (5:3, v/v). Radioactivity corresponding to UDP-Mur-NAc-L-Ala was counted.

Assays of the D-Ala-D-Ala-adding enzyme and D-Ala-D-Ala synthetase. The reaction mixture in a final volume of 50 μliters contained Tris hydrochloride buffer (*p*H 7.8), 2 μmoles; $MnCl_2$, 100 nmoles; ATP, 200 nmoles; UDP-Mur-NAc-L-Ala-D-Glu-*meso*-Dpm, 10 nmoles; ^{14}C-L-alanine (specific activity, 125 mCi per mmole); ^{14}C-DL-alanine (specific activity, 125 mCi per mmole) or ^{14}C-D-Ala-^{14}C-D-Ala (specific activity, ca. 40 mCi per mmole), 0.02 μCi; and an extract of the parent strain (250 μg of protein) or mutant ST640 (226 μg of protein) after treatment with Sephadex G25. Mixtures were incubated at 30 or 42 C for 1 hr. The reaction was stopped by boiling the mixture for 3 min in a water bath. The mixtures were then subjected to paper chromatography in isobutyric acid-1 M ammonia (5:3, v/v). Radioactivity corresponding to UDP-MurNAc-pentapeptide or D-Ala-D-Ala was counted.

Assays of cell membrane-bound peptidoglycan synthetase, peptidoglycan transpeptidase, and D-alanine carboxypeptidase. The methods used were as described previously (2, 9) with UDP-MurNAc-L-Ala-D-Glu-*meso*-Dpm-^{14}C-D-Ala-^{14}C-D-Ala (specific activity, 120 mCi per mmole) as substrate.

RESULTS

Isolation of ts mutants with mutations

causing defects in synthesis of cell wall peptidoglycan. The defects in the biosynthesis of cell wall peptidoglycans are usually lethal, and most mutant cells require isotonic conditions for growth resulting in formation of spheroplasts. Such cells cannot be stored, and they are unsuitable for genetic analysis by sexual mating and phage transduction. Therefore, ts mutants were isolated and investigated.

More than 600 ts mutants, which grew at 30 C but not at 42 C, were isolated by the replica plating technique. Among those cells that lysed at 42 C in the hypotonic medium, only seven mutant strains (ST35, 59, 222, 353, 408, 454, and 640) could be grown by addition of 20% sucrose to the medium (17). Mutant ST5 which also lysed at 42 C could be grown by addition of 20% sucrose and 10 mM $MgCl_2$ to the medium. Therefore, in further experiments isolation of cells was carried out as follows. Cells were treated with NTG, washed with saline, and transferred to 7 ml of nutrient broth supplemented with 0.5 M sucrose. After incubation for 7 hr at 42 C with shaking, cells were collected by centrifugation and suspended in 0.5 ml of the same medium. The cell suspension was layered on 10 ml of nutrient broth containing 0.6 M sucrose and centrifuged at 1,000 $\times$ *g* for 30 min at room temperature. Three milliliters from the top of the supernatant was incubated overnight at 30 C with shaking. ts mutants requiring sucrose were isolated efficiently from the overnight culture by plating on nutrient agar after sequential dilution with nutrient broth containing 0.5 M sucrose. Forty mutants were isolated, and those with low enough reverting frequency to allow genetic analysis were selected. They were designated strains ST728, 755, 772, 775, and 778.

Accumulation of UDP-bound precursor compounds in mutant cells at the higher temperature. The ability of the mutants listed in Table 3 to incorporate ^{14}C-L-alanine into peptidoglycan was 1/20 to 1/2 less at the elevated temperature (42 C) than at 30 C (Table 3). At 42 C inhibition of peptidoglycan formation was accompanied by accumulation of UDP-linked acetamidosugar (-peptide) compounds, precursors of peptidoglycan. The accumulation of these compounds was measured using appropriate radioactive compounds, such as ^{14}C-uridine, ^{14}C-D-glucosamine, or ^{14}C-L-alanine. The labeled compounds accumulated were identified by paper chromatography, and the results are shown in Table 4. The thermosensitive enzymes could be approximately deduced from the compounds accumulated. Strain ST5, which accumulated ^{14}C-UDP-

TABLE 3. *Inhibition of peptidoglycan synthesis at 42 C in vivo*

Strain	Incorporation of ^{14}C-L-Ala into peptidoglycan[a]	
	30 C	42 C
Expt 1 JE1011	190	250
ST5	520	10
ST35	430	140
ST59	560	180
ST222	250	50
ST353	360	30
ST408	460	90
ST454	360	30
ST486	290	10
ST640	200	80
Expt 2 JE1011	506	1,030
ST728	460	111
ST755	617	214
ST772	381	200
ST775	358	166
ST778	422	158

[a] Counts per minute per milligram (wet weight) of cells.

TABLE 4. *Accumulation of labeled compounds at 42 C*[a]

Strain	Labeled compounds accumulated
JE1011 (parent)	None[b]
ST5	UDP-GlcNAc-enolpyruvate[c]
ST222	UDP-GlcNAc, UDP-MurNAc
ST408	UDP-MurNAc-L-Ala-D-Glu
ST454	UDP-MurNAc-L-Ala-D-Glu
ST640	UDP-MurNAc-L-Ala-D-Glu-*meso*-Dpm
ST728	UDP-MurNAc-L-Ala-D-Glu-*meso*-Dpm
ST755	UDP-MurNAc-L-Ala-D-Glu-*meso*-Dpm
ST35	UDP-MurNAc-L-Ala-D-Glu-*meso*-Dpm-D-Ala-D-Ala
ST772	UDP-MurNAc-L-Ala-D-Glu-*meso*-Dpm-D-Ala-D-Ala
ST775	UDP-MurNAc-L-Ala-D-Glu-*meso*-Dpm-D-Ala-D-Ala
ST778	UDP-MurNAc-L-Ala-D-Glu-*meso*-Dpm-D-Ala-D-Ala

[a] ^{14}C-uridine was used as labeled substrate for ST5, ^{14}C-glucosamine for ST222, and ^{14}C-L-alanine for all other strains.

[b] A trace amount of UDP-MurNAc pentapeptide usually appeared.

[c] For details of identification of this compound, see another paper (Y. Sugino, *manuscript in preparation*).

GlcNAc-3-enolpyruvate at 42 C, presumably has a thermosensitive reductase which reduces this compound to form UDP-GlcNAc-3-lactate, i.e., UDP-MurNAc. Similarly strain ST222, which accumulated ^{14}C-UDP-GlcNAc and ^{14}C-UDP-MurNAc at 42 C, presumably has a thermosensitive L-alanine-adding enzyme. Strains ST408 and ST454, which accumulated UDP-MurNAc-L-Ala-D-Glu, were deduced to be Dap$^-$ mutants (4) from their requirement for diaminopimelic acid (50 μg of a mixture of the LL, DD, and DL compounds per ml). Strains ST640, ST728, and ST755, which accumulated UDP-MurNAc-L-Ala-D-Glu-*meso*-Dpm, may have thermosensitive enzymes required for formation of D-alanine or D-Ala-D-Ala, or for addition of the latter compound to UDP-MurNAc-tripeptide to form UDP-MurNAc-pentapeptide. Finally strains ST35, ST772, ST775, and ST778 which accumulated UDP-MurNAc-L-Ala-D-Glu-*meso*-Dpm-D-Ala-D-Ala at 42 C may have thermosensitive steps in the cell membrane-bound enzyme systems involved after formation of this precursor.

Assay of soluble thermosensitive enzymes in strains ST5, ST222, and ST640 in vitro. The presence of a thermosensitive UDP-GlcNAc-3-enolpyruvate reductase in strain ST5 was demonstrated by the results shown in Fig. 1. In this experiment cell extracts of the parent strain JE1011 and mutant ST5 were incubated at 42 C with ^{14}C-UDP-GlcNAc, phosphoenolpyruvate, and NADPH. The radioactive materials formed during incubation were separated by paper electrophoresis. With the extract of the parent strain, the radioactivity appeared in the position of UDP-MurNAc (upper line). With the extract of mutant strain, the radioactive product had higher mobility than UDP-MurNAc (lower line).

The product from the mutant was eluted from the paper. On hydrolysis with weak acid (*p*H 2) at 100 C for 20 min, it gave GlcNAc, but on enzymatic hydrolysis with venom phosphodiesterase (Worthington) and *E. coli* alkaline phosphomonoesterase (Nutritional Biochemical Corp.) it gave a compound which had the same mobility as MurNAc at *p*H 5.2. Furthermore, the unhydrolyzed product was completely converted to UDP-MurNAc in the presence of NADPH by an enzyme from *E. coli* JE1011 as shown in Table 5.

The activities of the L-alanine-adding enzyme in the parent and in mutant ST222 were as shown in Table 6. In the mutant strain no L-alanine-adding enzyme activity was detected at 42 C, and its activity was low at 30 C. It is generally known that thermosensitive enzymes are often labile in a cell-free state even at lower temperatures.

There are several possible explanations of why UDP-MurNAc-tripeptide accumulated in strains ST640, ST728, and ST755. Previously we reported that in strain ST640 alanine racemase might be thermosensitive, as this mutant became thermostable if the nutrient agar was fortified with 1 mg of D-alanine per ml (Table 7) (17). Wijsman (Ph.D. thesis, Amsterdam Univ., Holland, 1970) suggested that this concentration of D-alanine is too high to be required to overcome thermosensitive alanine racemase deficiency of cells, and he suspected that the thermosensitive enzyme might be D-Ala-D-Ala-adding enzyme since deficiency

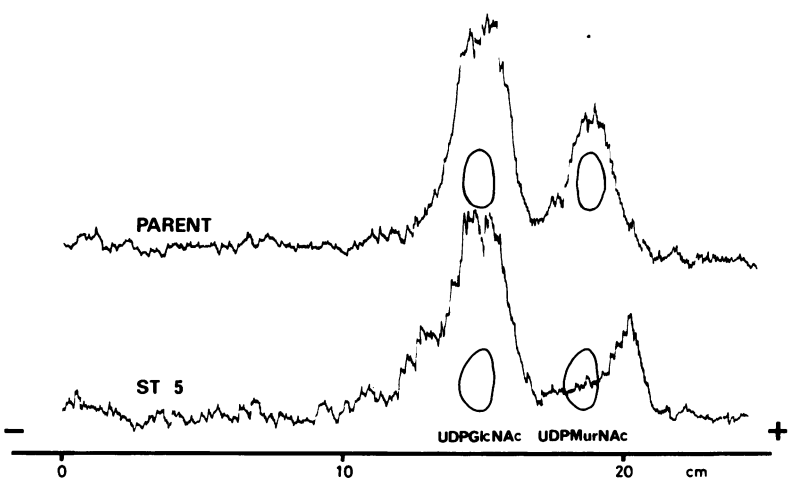

FIG. 1. *Accumulation of UDP-GlcNAc-3-enolpyruvate in a cell free extract of mutant ST5. Abscissa: distance of migration from the origin.*

TABLE 5. *Identification of ^{14}C-UDP-GlcNAc-3-enolpyruvate*[a]

Condition	UDP-GlcNAc-3-enolpyruvate (counts/min)	UDP-MurNAc (counts/min)
Complete, 0 min	362	26
Complete, 60 min	9	379
−NADPH, 60 min	282	63

[a] Experimental conditions were as described in Materials and Methods except that ^{14}C-UDP-GlcNAc-3-enolpyruvate formed in the experiment shown in Fig. 1 was used as substrate. An enzyme preparation from the parent strain was used.

TABLE 6. *L-Alanine-adding enzyme activity in the parent strain and in mutant ST222*

Strain	Temp of incubation (C)	^{14}C-L-alanine incorporation into UDP-MurNAc-L-Ala (counts/min)
Parent	30	4,712
	42	2,111
ST222	30	101
	42	0

TABLE 7. *Recovery of growth at 42 C by addition of D-alanine or DL-Ala-DL-Ala*

Supplement (μg/ml)		JE1011	ST640	ST728	ST755
D-Alanine	0	+	−	−	−
	50	+	−	−	+
	200	+	−	+	+
	800	+	+	+	+
DL-Ala-DL-Ala					
	200	+	−	−	+

of the adding enzyme could be overcome by a high concentration of D-alanine. Our results in Table 8 show that at 42 C cell extracts of mutant ST640 could not utilize ^{14}C-L-alanine or ^{14}C-D-alanine to form UDP-MurNAc-pentapeptide, although they could utilize ^{14}C-D-Ala-^{14}C-D-Ala. Here also the enzyme utilizing D-alanine seemed to be unstable even at 30 C. These results indicate that D-Ala-D-Ala synthetase in mutant ST640 is thermosensitive.

It was more difficult to identify the thermosensitive sites of the other two strains, ST728 and ST755, accumulating UDP-MurNAc-tripeptide. Cell extracts of these mutant strains utilized all three substrates, ^{14}C-D-Ala-^{14}C-D-Ala, ^{14}C-D-alanine, and ^{14}C-L-alanine at 42 C about as well as extracts of the parent strain (Table 8). However, at 42 C these mutant cells could be rescued by addition of 200 μg of D-alanine per ml (Table 7). Surprisingly, strain ST755 could also be rescued at 42 C by addition of 200 μg of DL-Ala-DL-Ala per ml (corresponding to 50 μg of D-Ala-D-Ala per ml, Table 7), but strains ST728 and ST640 could not. The reasons for these phenomena are unknown.

Survey of thermosensitive steps by assay of peptidoglycan synthesis in particulate preparations of enzymes. Peptidoglycan synthesis from UDP-MurNAc-pentapeptide(^{14}C-D-Ala-^{14}C-D-Ala) and UDP-GlcNAc was measured at 30 and 42 C by using enzymes prepared from mutant strains accumulating UDP-MurNAc-pentapeptide (Table 4) at 42 C. The rates of peptidoglycan synthesis, lipid intermediate formation, and penicillin-sensitive D-alanine release in preparations from these mutants were about the same at 30 and 42 C as the preparations of the parent strain. However, preparations from strains ST778 and ST772 showed low peptidoglycan synthesis at 42 C. The preparation from strain ST778 showed a characteristic change in formation of lipid intermediates on elevation of the temperature from 30 to 42 C (Fig. 2, bottom). In preparations from the parent and other mutants, formation of lipid intermediates was similar at the two temperatures (Fig. 2, top). Formation of these intermediates was complete within the first 20 min of incubation and then remained unchanged, so carrier lipid somehow becomes unavailable after the low initial level of lipid intermediates has been reached (Fig. 2).

In strain ST772 both the rates of peptidoglycan formation and alanine release decreased at 42 C, whereas that of formation of the lipid intermediate did not (Table 9). Peptidoglycan formation was very low at 42 C so it was impossible to determine whether the peptidoglycan transpeptidase was thermosensitive.

TABLE 8. *D-Ala-D-Ala-adding enzyme and D-Ala-D-Ala synthetase activities in the parent strain and mutant ST640*

^{14}C-substrate	Temp of incubation (C)	UDP-MurNAc-L-Ala-D-Glu-*meso*-Dpm-^{14}C-D-Ala-^{14}C-D-Ala formed (counts/min)	
		Parent	ST640
L-Alanine	30	1,269	73
	42	637	6
DL-Alanine	30	2,977	125
	42	1,544	16
D-Ala-D-Ala	30	8,361	7,172
	42	6,613	4,826

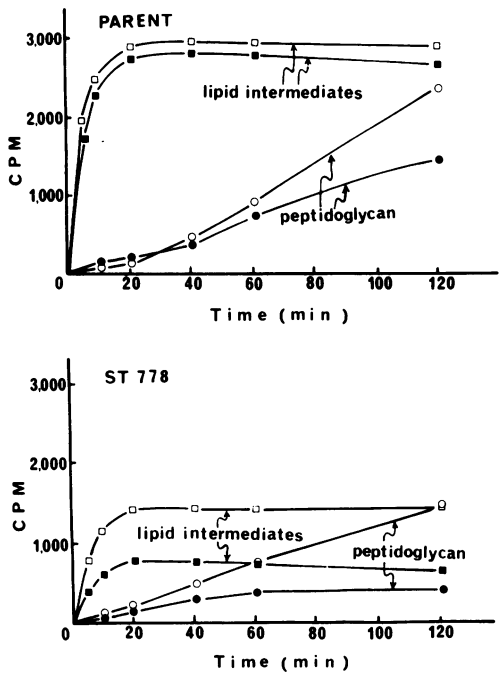

FIG. 2. *Kinetics of formation of peptidoglycan and lipid intermediates by particulate enzymes from the parent strain and mutant ST778. Incorporation of MurNAc-L-Ala-D-Glu-meso-Dpm-^{14}C-D-Ala-^{14}C-D-Ala from its UDP-bound precursor into lipid intermediates at 30 C (□) and 42 C (■) and peptidoglycan at 30 C (○) and 42 C (●). Enzyme samples contained 55 μg of protein (parent) and 49 μg of protein (mutant ST778).*

TABLE 9. *Formation of peptidoglycan and lipid intermediates and release of alanine in the parent and mutant ST772*[a]

Strain	Temp of incubation (C)	Counts/min		
		Peptidoglycan	Lipid intermediates	Alanine
Parent	30	850	4,890	1,985
	42	625	4,764	2,219
ST772	30	1,080	4,649	2,409
	42	606	4,255	1,441

[a] A 55-μg amount of protein (parent) and 58 μg of protein (mutant ST772) were used in reaction mixtures of 50-μliter final volume.

However, D-alanine carboxypeptidase, which is not supposed to be responsible for the cross-linking reaction of the peptidoglycans, was assayed using ^{14}C-UDP-MurNAc-pentapeptide (^{14}C-D-Ala-^{14}C-D-Ala) as substrate (omitting UDP-GlcNAc). Table 10 shows that the D-alanine carboxypeptidase activity of the particulate enzyme preparation from strain ST772 was low at 42 C. This table also shows that the activity of this enzyme in the supernatant of cell-free extracts of strain ST772 was not thermolabile.

TABLE 10. *D-Alanine carboxypeptidase activity in the parent and mutant ST772*[a]

Strain	Temp of incubation (C)	^{14}C-D-alanine released (counts/min)	
		Particulate enzyme	Soluble enzyme
Parent	30	1,186	577
	42	1,129	520
ST772	30	1,416	422
	42	640	392

[a] ^{14}C-labeled UDP-MurNAc-L-Ala-D-Glu-*meso*-Dpm-^{14}C-D-Ala-^{14}C-D-Ala was used as substrate, and cold UDP-GlcNAc was omitted from the reaction mixture. Particulate enzyme preparations contained 55 μg of protein (parent) and 58 μg of protein (mutant ST772), and soluble enzymes contained 47 μg of protein (parent) and 41 μg of protein (mutant ST772).

Mapping of the genes coding enzymes for peptidoglycan synthesis. The ts loci of the isolated mutants were roughly mapped by sexual conjugation with Hfr H, Hfr C and an F′ strain carrying the F-14 genote. The mutants can be divided in two groups. In the first group, *mra*, the ts genes could be transferred either by Hfr H or Hfr C and the loci of the ts genes were roughly located between *ara* and *lac* (ST222, 640, 728, and 772). In the second group, *mrb*, the ts genes could be transferred by Hfr C but not by Hfr H in the first 2 hr of mating at 30 C (ST5, 35, 755, 775, and 778). The genes could also be transferred by F-14, an F genote carrying the chromosome fragment located between *argH* and *ilv* (ST5 and 35 among the mutants examined so far).

The two gene clusters, *mra* and *mrb*, were mapped precisely by transduction with phage P1kc. The *mra* genes were transduced with *leu*, *azi*, and *dapC*, and the *mrb* genes were transducible with *argH* and *metB*. Three-factor crosses were performed with respect to the markers, and the results are shown in Tables 11 and 12 and are summarized in Fig. 3. Table 11 clearly shows that orientation of four ts mutations (four *mra* mutations) with respect to *leu* and *azi* is *leu-mra-azi*. The orientation of five *mrb* mutations with respect to *metB* and *argH* is seen from Table 12 to be *metB-argH-mrb*. The markers of the donor strains and of the

TABLE 11. *Analysis of three-factor transductions involving the leu, mra, and azi markers*[a]

Selected marker *leu*	Unselected markers		No. of transductants			
	ts	*azi*	ST222	ST640	ST728	ST772
1	1	1	13	31	50	49
1	1	0	28	33	17	13
1	0	1	0	0	0	0
1	0	0	60	37	34	39

[a] Donor: AT997 (*leu*$^+$ *ts*$^+$ *azi*$^+$), recipient: ST222, ST640, ST728, ST772 (*leu ts azi*). 1 and 0 represent markers from donor and recipient strains, respectively.

TABLE 12. *Analysis of three-factor transduction involving the metB, argH, and mrb markers*[a]

Selected marker *met*	Unselected markers		No. of transductants				
	arg	*ts*	ST5	ST35	ST755	ST775	ST778
1	1	1	6	10	5	12	2
1	1	0	18	21	22	17	12
1	0	1	0	1	1	1	1
1	0	0	64	67	73	71	20

[a] Donor: ST5, ST35, ST755, ST775, ST778 (*met*$^+$, *arg*$^+$, *ts*); recipient: AB1450 (*met, arg, ts*$^+$). 1 and 0 represent markers from donor and recipient strains, respectively.

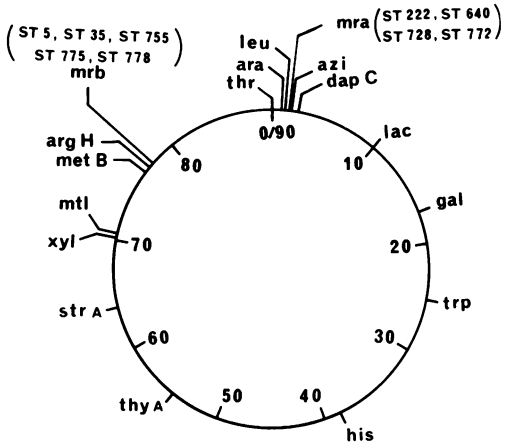

FIG. 3. *Linkage map of E. coli JE1011 showing two clusters of genes for peptidoglycan biosynthesis.*

recipient strains are given in the tables. Figure 3 shows a chromosome map with the *mra* and *mrb* genes.

DISCUSSION

There seems to be no clear relation between the order of enzymes in the biosynthetic pathway of peptidoglycan and the position of genes specifying these enzymes. Thus, *mra* is a cluster of genes coding for the L-alanine-adding enzyme (ST222), D-Ala-D-Ala synthetase (ST640), an unknown enzyme related to the requirement for D-alanine (ST728), and membrane-bound D-alanine carboxypeptidase (ST772). The *mrb* cluster contains the genes coding for UDP-GlcNAc-3-enolpyruvate reductase (ST5), another unknown enzyme related to the requirement for D-alanine (ST755), an unidentified enzyme involved in the formation of lipid intermediates (ST778), and also unidentified enzymes which are probably involved in membrane-bound biosynthetic reactions (ST35 and ST775).

The requirement of ST728 and ST755 for D-alanine at 42 C could be due to the thermosensitivity of some enzymes related to D-alanine metabolism, or to membrane transport or leakage of D-alanine or D-Ala-D-Ala. The relation of these mutations to the D-cycloserine-resistance genes (*cyc*) reported by Curtiss (6) is under investigation. Recently a gene for biotin retention (*bir*) has been mapped at 77 min (5). A similar mechanism might be considered for D-alanine metabolism. Strain ST772 was a mutant with thermosensitive membrane-bound D-alanine carboxypeptidase activity. Recently, Strominger et al. showed that the inhibition by penicillin of membrane-bound D-alanine carboxypeptidase in *B. subtilis* is not lethal for the cells (3), but it is still uncertain whether D-alanine carboxypeptidase and peptidoglycan transpeptidase are the same enzyme or not. In general, mutations in a membrane-bound multienzyme system are difficult to identify, as pleiotropic effects must be taken into account in a system which requires supramolecular, structural organization for expression of full activity.

The clusters of genes, *mra* and *mrb*, might be responsible for a variety of enzymes, not only those directly involved in the sequence of biochemically established reactions, but also those responsible for organization of the physiologically active state for cell wall synthesis, for instance retention of the intermediates or assembly of synthetic enzymes, etc. In this respect, we designate the two groups of genes responsible for cell-wall peptidoglycan synthesis as *mra* and *mrb* by analogy to the *rfa* and *rfb* clusters in *S. typhimurium* given to groups of enzymes involved in the biosynthesis of lipopolysaccharides (16). Wijsman (Ph.D. thesis,

Amsterdam Univ., Holland, 1970) recently mapped the gene position of the adding enzymes of L-alanine, *meso*-Dpm, and D-Ala-D-Ala at 1 to 1.5 min, and this gene may be included in our *mra* cluster. Among the genes investigated so far, only that of alanine racemase located at 17 min (Wijsman, Ph.D. thesis, Amsterdam Univ., Holland, 1970) seems to be separated from these two clusters. More recently Lugtenberg et al. performed the isolation and identification of *E. coli* ts mutants deficient in several amino acid and D-Ala-D-Ala-adding enzymes (13-15).

LITERATURE CITED

1. Adelberg, E. A., M. Mandel, and G. C. C. Chen. 1965. Optimal conditions for mutagenesis by N-methyl-N'-nitro-N-nitrosoguanidine in *Escherichia coli* K-12. Biochem. Biophys. Res. Commun. **18**:788-795.
2. Anderson, J. S., M. Matsuhashi, M. A. Haskin, and J. L. Strominger. 1967. Biosynthesis of the peptidoglycan of bacterial cell walls. II. Phospholipid carriers in the reaction sequence. J. Biol. Chem. **242**:3180-3190.
3. Blumberg, P. M. and J. L. Strominger. 1971. Inactivation of D-alanine carboxypeptidase by penicillins and cephalosporins is not lethal in *Bacillus subtilis*. Proc. Nat. Acad. Sci. U.S.A. **68**:2814-2817.
4. Bukhari, A. I., and A. L. Taylor. 1971. Genetic analysis of diaminopimelic acid- and lysine- requiring mutants of *Escherichia coli*. J. Bacteriol. **105**:844-854.
5. Campbell, A., A. del Campillo-Campbell, and R. Chung. 1972. A mutant of *Escherichia coli* that requires high concentrations of biotin. Proc. Nat. Acad. Sci. U.S.A. **69**:676-680.
6. Curtiss, R. III., L. J. Charamella, C. M. Berg, and P. E. Harris. 1965. Kinetic and genetic analysis of D-cycloserine inhibition and resistance in *Escherichia coli*. J. Bacteriol. **90**:1238-1250.
7. Endo, A., K. Kakiki, M. Hori, H. Abe, and T. Misato. 1970. A simple procedure for the preparation of UDP-N-Acetylglucosamine-^{14}C. Biochem. Biophys. Res. Commun. **39**:718-722.
8. Ito, E., S. G. Nathenson, D. N. Dietzler, J. S. Anderson, and J. L. Strominger. 1966. Formation of UDP-acetymuramyl peptides, p. 324-337. *In* E. F. Neufeld and V. Ginsberg (ed.), Methods in enzymology, vol. 8. Academic Press Inc., New York.
9. Izaki, K., M. Matsuhashi, and J. L. Strominger. 1968. Biosynthesis of the peptidoglycan of bacterial cell walls. XIII. Peptidoglycan transpeptidase and D-alanine carboxypeptidase: penicillin sensitive enzymatic reactions in the strains of *Escherichia coli*. J. Biol. Chem. **243**:3180-3192.
10. Lederberg, J., and E. M. Lederberg. 1952. Replica plating and indirect selection of bacterial mutants. J. Bacteriol. **63**:399-406.
11. Lennox, E. S. 1955. Transduction of linked genetic characters of the host by bacteriophage P1. Virology **1**:190-206.
12. 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.
13. Lugtenberg, E. J. J., L. de Hass-Menger, and W. H. Ruyters. 1971. Murein synthesis and identification of cell wall precursors of temperature-sensitive lysis mutants of *Escherichia coli*. J. Bacteriol. **109**:326-335.
14. Lugtenberg, E. J. J., and A. van Schijndel-van Dam. 1972. Temperature-sensitive mutants of *Escherichia coli* K-12 with low activities of the L-alanine adding enzyme and the D-alanyl-D-alanine adding enzyme. J. Bacteriol. **110**:35-40.
15. Lugtenberg, E. J. J., and A. van Schijndel-van Dam. 1972. Temperature-sensitive mutants of *Escherichia coli* K-12 with low activity of the diaminopimelic acid adding enzyme. J. Bacteriol. **110**:41-46.
16. Mäkelä, P. H., and B. A. D. Stocker. 1969. Genetics of polysaccharide biosynthesis. Annu. Rev. Genet. **3**:291-322.
17. Matsuzawa, H., M. Matsuhashi, A. Oka, and Y. Sugino. 1969. Genetic and biochemical studies on cell wall peptidoglycan synthesis in *Escherichia coli* K-12. Biochem. Biophys. Res. Commun. **36**:682-689.
18. Strominger, J. L., K. Izaki, M. Matsuhashi, and D. J. Tipper. 1967. Peptidoglycan transpeptidase and D-alanine carboxypeptidase: penicillin-sensitive enzymatic reactions. Fed. Proc. **26**:9-22.
19. Tamaki, S., T. Sato, and M. Matsuhashi. 1971. Role of lipopolysaccharide in antibiotic resistance and bacteriophage adsorption of *Escherichia coli* K-12. J. Bacteriol. **105**:968-975.
20. Taylor, A. L. 1970. Current linkage map of *Escherichia coli*. Bacteriol. Rev. **34**:155-175.
21. Taylor, A. L., and M. S. Thoman. 1964. The genetic map of *Escherichia coli*. Genetics **50**:659-677.
22. Yura, T., and C. Wada. 1968. Phenetyl alcohol resistance in *Escherichia coli*. I. Resistance of strain C600 and its relation to azide resistance. Genetics **59**:177-190.
23. Weidel, W., and H. Pelzer. 1964. Bagshaped macromolecules—a new outlook on bacterial cell walls. Advan. Enzymol. **26**:193-232.