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Temperature-Sensitive Mutant of *Escherichia coli* K-12 with an Impaired D-Alanine: D-Alanine Ligase

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The temperature-sensitive *Escherichia coli* mutant strain ST-640 lyses at the restrictive temperature except when an osmotic stabilizer or a high concentration of D-alanine is present. The presence of DL-alanyl-DL-alanine does not prevent lysis. The rate of murein synthesis, followed in a wall medium, is decreased at both 30 and 42 C. D-Alanyl-D-alanine and uridine diphosphate-*N*-acetyl-muramyl (UDP-MurNAc)-pentapeptide are synthesized in decreased amounts, accompanied by accumulation of UDP-MurNAc-tripeptide at 42 C but not at 30 C. Uridine nucleotide precursors leak into the medium, especially out of the mutant cells. This leakage is prevented when NaCl is present. The D-alanine: D-alanine ligase (ADP) (EC 6.3.2.4) of the mutant strain, assayed in crude extracts, is temperature sensitive. The impaired ligase is relatively resistant to D-cycloserine and other inhibitors of the enzyme. Combined genetic and enzymatic results show that the low ligase activity is due to a mutation in the *ddl* gene, the structural gene for D-alanine: D-alanine ligase.

Matsuzawa et al. (7) found that one of their temperature-sensitive cell wall mutants of *Escherichia coli* K-12 (strain ST-640) accumulated uridine-diphosphate-*N*-acetyl-muramyl-tripeptide (UDP-MurNAc-tripeptide) at the restrictive temperature. A high concentration of D-alanine (1 mg/ml) restored the growth at 42 C. The authors therefore assumed that this mutant contains a temperature-sensitive alanine racemase (EC 5.1.1.1). The gene was localized on the *E. coli* linkage map (7) at about min 3, a region where a number of other genes involved in murein synthesis are located (H. J. W. Wijsman, Ph.D. thesis, Amsterdam, 1970; and references 2, 5, and 6). Recently, it was found that a temperature-sensitive lysis mutant of *E. coli* K-12, strain TKL-10, contains an impaired L-alanine: D-alanine racemase (2; H. J. W. Wijsman, Ph.D. thesis, Amsterdam, 1970). It accumulated UDP-MurNAc-tripeptide under restrictive conditions (4). This mutation was mapped left of the *thr* marker (H. J. W. Wijsman, *personal communication*). This mutant was able to grow at 42 C in the presence of a low concentration of D-alanine (20 μg/ml).

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On the basis of the different location of these two mutants on the *E. coli* map, as well as quantitative difference in D-alanine requirement between strains TKL-10 and ST-640, we decided to study strain ST-640 more extensively. The results show that in strain ST-640 the D-alanine: D-alanine ligase is impaired and that alanine racemase is not changed by the mutation.

MATERIALS AND METHODS

Media. The compositions of yeast broth (3) and of the minimal medium A (4) have been described previously. Medium C, a low osmotic medium, contained per liter: Na_2HPO_4, 1.5 g; KH_2PO_4, 0.75 g; NH_4Cl, 0.5 g; $MgSO_4$, 0.05 g; glucose, 0.2%; and the auxotrophic requirements in small amounts (5-100 mg). The final *p*H was 7.2. Media B and D were identical to media A and C, respectively, except that they were supplemented with Casamino Acids (0.1%). The media were sometimes solidified with 1.25% Davis agar. The composition of the cell wall synthesis medium CWSM-IV was the same as that of CWSM-I (2), except that the salts of medium C were substituted for the salts of medium A.

Bacterial strains. Only *E. coli* K-12 strains were used. The properties of the strains are summarized in Table 1. Strains JE-1011 and ST-640 were obtained from M. Matsuhashi, University of Tokyo, Tokyo, Japan. The origins of the donor strains H and R4 are

TABLE 1. *Properties of Escherichia coli K-12 strains*

Strain	PC no.[a]	Mating type	Relevant genes[b]
KMBL-146	0417	F⁻	*thr leu arg ilvA his thyA pyrF thi*
TKL-10	1243	F⁻	*thr leu arg ilvA his thyA pyrF thi trp asp alr*
JE-1011	1356	F⁻	*thr leu his thi trp strA*
ST-640	1358	F⁻	*thr leu his thi trp ddl strA*
Hfr H	0008	Hfr	*thi*
Hfr R4	0031	Hfr	Prototroph

[a] Phabagen Collection number.

[b] Gene symbols are according to Taylor (9), except the symbols *alr* and *ddl*, that have been proposed for the genes for L-alanine:D-alanine racemase and D-alanine:D-alanine ligase, respectively (E. J. J. Lugtenberg, Ph.D. thesis, Utrecht, 1971).

located at 87 and 9.5 min, respectively, on the *E. coli* map (9). Strain R4 injects its chromosome counterclockwise, whereas strain H injects clockwise. Spontaneous temperature-resistant revertants of strain ST-640 were obtained by inoculation of 50 ml of medium D with 3.10^8 cells derived from a temperature-sensitive colony, followed by incubation at 42 C for 16 hr. One revertant of each batch was purified twice on solid medium. Revertants were called Rev, followed by the batch number.

Recombinants of strain ST-640 were obtained as follows. Donor strains, as well as strain ST-640, were diluted 10-fold in yeast broth and incubated at 37 and 30 C, respectively, for 90 and 120 min, respectively. One volume of a culture of the donor strain was mixed with nine volumes of a culture of strain ST-640. After 15 min at 30 C, the suspension was carefully diluted 1:100. After 60 min at 30 C, the mixture was violently shaken, and samples of 0.1 ml of the undiluted, as well as the 1:10 diluted, suspension were plated and incubated at 30 C. Auxotrophic recombinants were selected on medium C by omitting one growth factor for the acceptor strain. The donor strain was killed with streptomycin (100 μg/ml). Recombinants were purified twice on the same medium without streptomycin and tested for temperature sensitivity on medium C at 42 C.

Chemicals and radiochemicals. Streptomycin was obtained from KNG & SF, Delft, The Netherlands. DL-alanine-T (G) (specific activity 2.4 mCi/μmole), obtained from the Radiochemical Centre, Amersham, England, was used to prepare tritiated D-alanyl-D-alanine by the procedure described previously for the preparation of ^{14}C-D-alanyl-^{14}C-D-alanine (4). The origins of other chemicals and ^{14}C-labeled radiochemicals have been published previously (2–4).

Synthesis of murein and of its alanine-containing precursors. Cells growing exponentially in yeast broth were washed with and resuspended in CWSM-IV to a final concentration of 0.24 mg (dry weight) per ml. After incubation for 15 min at the desired temperature, ^{14}C-L-alanine was added at a final concentration of 6.25×10^{-5} M (32 μCi/μmole). Lysis of the temperature-sensitive mutants at the restrictive temperature is prevented because this medium contains chloramphenicol and lacks a number of amino acids that are necessary for protein synthesis and because the strains used are multi-amino acid auxotrophs (4). Samples of 0.1 ml, taken at various intervals, were either added to cold 3% perchloric acid to determine the acid-precipitable radioactivity, or heat inactivated and chromatographed (3). The radioactive components were observed by autoradiography. Cell-bound precursors were isolated from washed, disintegrated cells by charcoal adsorption and were identified by chromatography (4).

Permeability for L-alanine and D-alanyl-D-alanine. Cells growing exponentially in yeast broth at 30 C were harvested and washed twice with a cold solution of the salts of medium A, supplemented with chloramphenicol (200 μg/ml). They were resuspended in one-fifth of the original volume (1.2 mg [dry wt]/ml), stored at 4 C, and used within 1 hr. To assay the permeability, four volumes of a solution of the same salts, supplemented with chloramphenicol and glucose (0.2%), at 25 C were added to one volume of the suspension. After incubation at 25 C for 5 min, ^{14}C-L-alanine (specific activity, 152 μCi/μmole) or ^{3}H-D-alanyl-^{3}H-D-alanine (specific activity 4.8 mCi/μmole) were added in final concentrations of 10 and 20 μCi/ml, respectively. Samples of 0.2 ml, taken at intervals of 60 sec for 5 min, were filtered through an Oxoid filter (pore size, 0.45 μm), and the filter was immediately washed twice with 5 ml of the salt solution, mentioned above, supplemented with chloramphenicol. The radioactivity of the air-dried filter was determined as described in an earlier paper (3).

Enzyme assays. The method for the preparation of crude cell extracts was described earlier (2). Activities of adding enzymes were assayed by incubation of the appropriate uridine nucleotide precursor with the corresponding ^{14}C-labeled amino acid or dipeptide. The radioactivity of the nucleotide-product was used as a measure for enzyme activity (2). The activity of D-alanine:D-alanine ligase was assayed with ^{14}C-D-alanine as the labeled substrate. As the ligase is inhibited by its own product (2) and as the activity of the UDP-MurNAc-L-alanyl-D-glutamate-*m*-diaminopimelate:D-alanyl-D-alanine ligase (ADP) (D-alanyl-D-alanine adding enzyme) in wild-type extracts is considerably higher than that of the ligase, UDP-MurNAc-tripeptide was included in the incubation, and the activity of the resulting UDP-MurNAc-pentapeptide was taken as a measure for ligase activity. As the L-alanine:D-alanine racemase activity is much lower than that of the ligase, the former enzyme was assayed as the latter one, except that ^{14}C-L-alanine was the labeled substrate (2). Specific activities are expressed relative to the specific activities of the parent strain JE-1011. When the ligase of ST-640 was assayed, chromatography was carried out for 40 hr to ascertain that UDP-MurNAc-pentapeptide was separated from other spots (*see* Results).

RESULTS

Growth properties. Initially, it was difficult to show that strain ST-640 was temperature sensitive. At 42 C it grew on both yeast broth and media A and B. As the strain is osmotic pressure dependent (7), we used medium C or D, containing decreased amounts of salts. In contrast to the parent strain, strain ST-640 was unable to form colonies at 42 C when plated on these low osmotic media. When sucrose (20%) or NaCl (0.1–0.4 M) was added to medium D, strain ST-640 also formed colonies at 42 C. The concentrations of D-alanine and DL-alanine required for growth on solid medium D of strain ST-640 and of the alanine racemase mutant TKL-10 were compared. Strain ST-640 required at least 1 mg of D-alanine per ml, whereas strain TKL-10 could grow at 42 C in the presence of only 10 μg/ml. Whereas strain TKL-10 grew in the presence of 20 μg of DL-alanyl-DL-alanine per ml, strain ST-640 did not grow even when 80 μg of DL-alanyl-DL-alanine was added. In liquid medium D, supplemented with D-alanine or DL-alanyl-DL-alanine, the growth response of strain ST-640 was similar to that observed on solid medium (Fig. 1): DL-alanyl-DL-alanine (100 μg/ml) had no influence on the growth curve, whereas 100 μg of D-alanine per ml delayed the moment of lysis, and 1 mg of D-alanine per ml prevented the lysis completely.

Murein synthesis. Cells were incubated in a cell wall synthesis medium supplemented with ^{14}C-L-alanine. Samples of the complete suspension were chromatographed, and radioactive compounds were detected by autoradiography. To determine the distribution of radioactivity among the precursors, the acid-soluble charcoal-adsorbable material of disintegrated cells was isolated and chromatographed (4).

When the wall medium CWSM-I (3) was used, no extreme difference was found at 42 C between the strains JE-1011 and ST-640 with respect to the rate of murein synthesis. After separation of the precursors, we found that UDP-MurNAc-tripeptide contained 20% of the total radioactivity of the precursors of strain ST-640 compared with 3% in the parent strain JE-1011. Much higher percentages of this precursor have been found in strains with impaired alanine racemase (4) or D-alanyl-D-alanine adding enzyme (5). As strain ST-640 was able to grow at 42 C in medium B containing the minimal medium salts of CWSM-I, but failed to grow in the low osmotic medium D, the minimal medium salts of CWSM-I were substituted by low osmotic medium salts. When

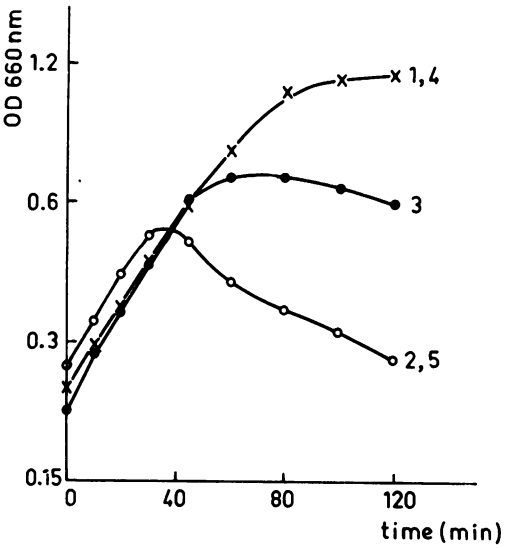

FIG. 1. *Influence of* D-*alanine and* DL-*alanyl-*DL-*alanine on the growth of strain ST-640. Strains JE-1011 and ST-640 growing exponentially at 30 C in medium D were diluted in the same medium, supplemented as indicated below. The optical density at 660 nm was measured at various intervals. (1) Strain JE-1011, no additions. (2) Strain ST-640, no additions. (3) Strain ST-640 + 100 μg of* D-*alanine per ml. When* D-*alanine was present in a concentration of 10 μg per ml, the measured curve was intermediate between curves 2 and 3. (4) Strain ST-640 + 1 mg of* D-*alanine per ml. (5) Strain ST-640 + 100 μg of* DL-*alanyl-*DL-*alanine per ml. Because curves 1 and 4, as well as 2 and 5, did not differ significantly, only one curve is presented for each type.*

murein synthesis was followed in the resulting CWSM-IV medium, much larger differences between parent and mutant strains were obtained. The radioactivities in D-alanyl-D-alanine, the combined precursor spots, and murein were plotted against time (Fig. 2). As described previously (3, 4), the activity of D-alanyl-D-alanine is almost completely found in the medium, while its total amount increases with the incubation temperature. Strain JE-1011 clearly shows these effects. The amount of the dipeptide that is present in the suspension of the mutant strain ST-640 at 30 C is decreased (Fig. 2A). After incubation at 42 C for 2 hr, the situation is more dramatic: the culture of strain ST-640 contains only 13% of the amount of D-alanyl-D-alanine that is present in the parent strain. The low amount of D-alanyl-D-alanine suggests that an enzyme involved in the synthesis of this dipeptide is impaired in the mutant strain.

Because the uridine nucleotide precursors

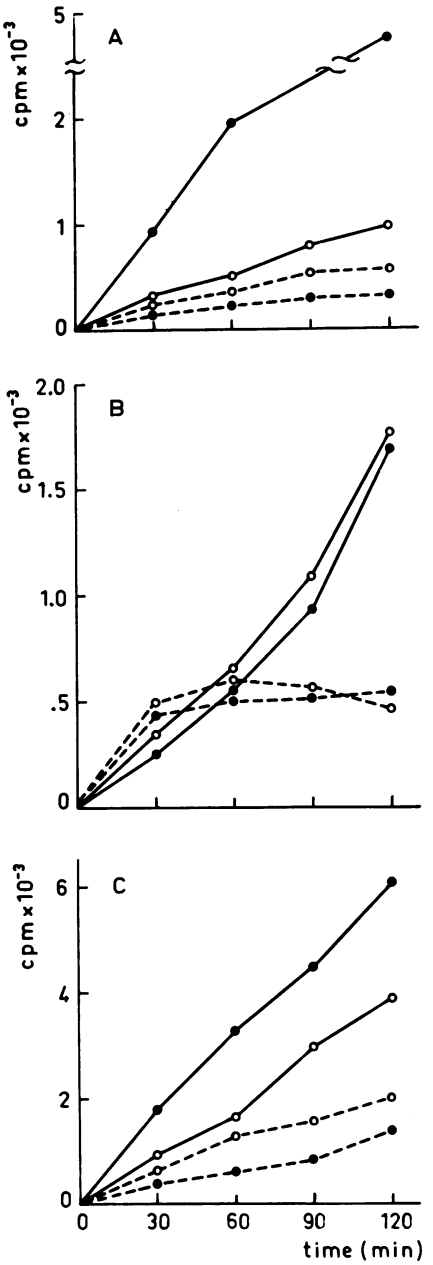

FIG. 2. *Synthesis of murein and its alanine-containing precursors. Strains JE-1011 (solid lines) and ST-640 (broken lines) were grown in yeast broth, washed, and incubated in CWSM-IV medium supplemented with ^{14}C-L-alanine at 30 C (open circles) or 42 C (closed circles). Samples (0.1 ml) were heat inactivated and chromatographed. The radioactive compounds were observed by autoradiography. Details of the procedure are given in the text and in references 2 and 3. The radioactivities in D-alanyl-D-alanine (A), combined uridine nucleotide precursors (B), and murein (C), are plotted against time.*

could not be separated completely on the chromatogram without losing other components, they were combined. Their radioactivity, plotted in Fig. 2B, is a measure of the amount of precursors present in the cells as well as in the supernatant. In the beginning, this activity is higher for strain ST-640. The situation becomes different after about 30 min, when a level is reached for the mutant strain, and the radioactivity of the precursors of parent strain increases. In a previous paper (3), it was shown that the total precursor activity of *E. coli* K-12 strain KMBL-146 reached a constant level within a few minutes when incubated under the same conditions, except that CWSM-I, containing a higher salt concentration, was used. The explanation probably is that part of the precursors of *E. coli* leak into the medium when the low osmotic medium CWSM-IV is used, in contrast to the situation in CWSM-I where all precursor activity is recovered in the cells (3). In a separate experiment, it was shown that after 60 min of incubation in CWSM-IV 20% and 56% of the radioactivities of the precursors of strains JE-1011 and ST-640, respectively, were present in the medium. However, when the medium was supplemented with 0.3 M NaCl, essentially all radioactivity of the precursors of both strains was found in the cells.

The total radioactivity of the precursors, present in the cells after 60 min of incubation, is presented in Table 2. The data of Fig. 2 and Table 2 are from the same experiment. Com-

TABLE 2. *Distribution of radioactivity among the murein precursors*[a]

Strain	Temperature (C)	Total precursor activity[b] (counts/min)	Distribution of radioactivity among the precursors[c] (counts/min)			
			X-1	X-2	X-3	X-5
JE-1011	30	17,000	320	1,700	320	14,600
JE-1011	42	25,100	750	3,250	500	20,600
ST-640	30	6,500	780	980	390	4,350
ST-640	42	5,700	340	400	3,140	1,820

[a] The experiment was carried out as described in the legend of Fig. 2. After incubation in the presence of ^{14}C-L-alanine for 60 min at the indicated temperature, samples of 2 ml were harvested and washed. The charcoal-adsorbable material was isolated and identified as described in an earlier paper (4). All radioactivity was recovered in murein precursors. The optical density of both strains decreased during incubation for 120 min: 8% at 30 C and 20% at 42 C.

[b] Calculated for cells of 2 ml of CWSM-IV suspension.

[c] X = UDP-MurNAc. The number represents the number of amino acids attached to it.

parison of Table 2 with Fig. 2B shows that the percentage of radioactive precursors present in the cells of parent and mutant strain is much higher for the parent strain, indicating that a considerable fraction of the precursors leaks into the medium, especially out of the mutant cells.

Table 2 also shows the distribution of radioactivity among the cell-bound precursors. At 30 C the majority of the radioactivity of the precursors of both strains is found in UDP-MurNAc-pentapeptide, although the absolute amount of this precursor is lower in the case of strain ST-640, causing a reduced rate of murein synthesis (Fig. 2C). Accumulation of UDP-MurNAc-tripeptide does not occur in strain ST-640 at 30 C, but the amount of UDP-MurNAc-L-Ala is significantly higher than in the parent strain. The latter result probably is not due to a second mutation because a relatively high amount of UDP-MurNAc-L-Ala has also been found at 30 C in a mutant with a temperature-sensitive D-alanyl-D-alanine adding enzyme (5). The distribution of radioactivity among the precursors at 42 C is simple to explain. Compared with the parent strain, UDP-MurNAc-tripeptide is accumulated sixfold in the mutant, whereas the amount of UDP-MurNAc-pentapeptide is largely reduced (Table 2).

Summarizing, the impaired ligase causes the following effects, especially at 42 C: (i) a reduced amount of D-alanyl-D-alanine is synthesized, resulting in decreased rates of synthesis of UDP-MurNAc-pentapeptide and murein; (ii) overproduction of UDP-MurNAc-tripeptide occurs; and (iii) the cell wall of the mutant, even when grown at 30 C, is so weak that it cannot withstand the pressure of the cytoplasmic membrane, resulting in considerable leakage of murein precursors (and probably also of other components) into the medium.

Enzyme assays. Crude extracts of strains ST-640 and JE-1011 were tested at 30 C for activities of the following enzymes: L-alanine:D-alanine racemase; D-alanine:D-alanine ligase; UDP-MurNAc:L-alanine ligase (ADP) (EC 6.3.2.8) (L-alanine adding enzyme); UDP-MurNAc-L-alanine:D-glutamate ligase (ADP) (EC 6.3.2.9) (D-glutamate adding enzyme); UDP-MurNAc-L-alanyl-D-glutamate:*m*-diaminopimelate ligase (ADP) (*m*-diaminopimelate [Dpm] adding enzyme); and D-alanyl-D-alanine adding enzyme. The results presented in Table 3 show that low activities of both the racemase and the ligase were measured in extracts of strain ST-640. As this racemase assay requires an active ligase (2), we assumed that the racemase activity might be normal. When the extracts of the two strains were mixed before assay, the specific activity of the racemase in the mixture was the same as that of an extract of strain JE-1011, whereas that of the ligase was half that of the parental strain (Table 3). These results show that the racemase of strain ST-640 is normal and that only the ligase activity of strain ST-640 is impaired. It can also be concluded that the low enzyme activity is not due to the (over)production of a possible enzyme inhibitor, but that it is the result of a defect in the enzyme itself.

Properties of the impaired ligase in crude extracts. Because the in vitro activity of the D-alanine:D-alanine ligase of strain ST-640 is very low, the protein concentration in extracts of the mutants was made higher than in those of the parent strain to obtain an amount of product that could be measured sufficiently accurately. During the assay of the ligase of strain ST-640, two radioactive components were synthesized with R_F-values of about 0.25.

We concluded that the two new components had nothing to do with the ligase from the following observations. (i) When the extract was preincubated for 10 min at 42 C, followed by assay of the ligase at 30 C, the radioactivity of each of the unknown components was reduced to less than 15%, whereas the activity of the ligase was not affected. (ii) The addition of concentrations of D-alanyl-D-alanine, D-cycloserine, glycine, or glycyl-glycine, that severely inhibited the ligase activity, did not influence the radioactivity of the two unknown components.

The impaired ligase is temperature sensitive

TABLE 3. *Specific activities of enzymes of strain ST-640 in crude extracts*[a]

Enzyme	Specific activity (%) in extracts of strains	
	ST-640	ST-640 + JE-1011 (mixed 1+1)
L-Alanine adding enzyme	86	
D-Glu adding enzyme	99	
m-Dpm adding enzyme	109	
D-Ala-D-Ala adding enzyme	100	
L-Ala:D-Ala racemase	9	91
D-Ala:D-Ala ligase	4	49

[a] Enzyme assays were carried out as described in Materials and Methods. UDP-MurNAc-tripeptide was present in the assay mixtures for the racemase and ligase. Specific activities are expressed relative to the specific activities of strain JE-1011 (= 100%).

in vitro (Fig. 3). As discussed in a previous paper (5), it is unlikely that the in vitro activity of the impaired enzyme at any temperature is an accurate measure of its in vivo activity at the same temperature.

D-Alanine:D-alanine ligase activity of *E. coli* K-12 is inhibited by D-cycloserine, glycine, glycyl-glycine, and D-alanyl-D-alanine (2). The impaired ligase exhibited an increased resistance to all of these inhibitors. The concentrations of D-alanyl-D-alanine, D-cycloserine, glycine, and glycyl-glycine that were required to inhibit 50% of the enzyme activity were increased by factors of 25, 9, 2, and 4, respectively (Table 4; Fig. 4).

Enzyme activities in revertants and recombinants. The specific activities of reference enzymes of the two spontaneous revertant strains Rev-1 and Rev-2, with wild-type growth rate and morphology at 42 C, were significantly altered. The relative specific activities of their ligases assayed at 30 C were 63 and 22%, respectively, whereas 6% was found for the mutant strain. The temperature dependence of the specific activity of the ligase of strain Rev-1 differed from the parent as well as from the mutant strain, whereas no significant difference was found between the strains Rev-2 and ST-640 (Fig. 3). These changed properties of the ligase of strain Rev-1 suggest that the reversion

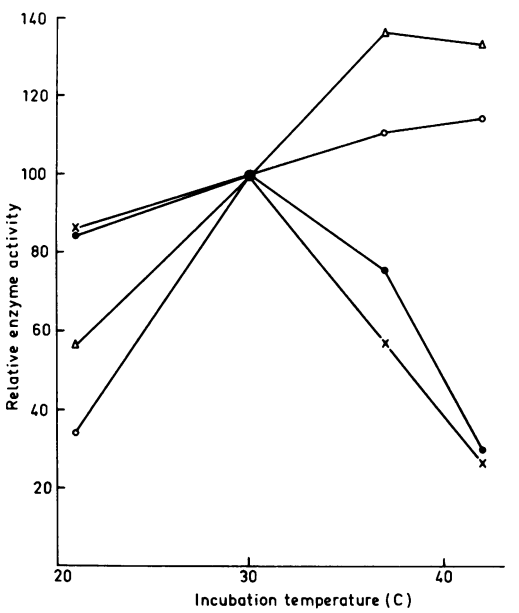

FIG. 3. *Influence of incubation temperature on the relative specific activities of D-alanine:D-alanine ligase of strains JE-1011* (○), *ST-640* (●), *Rev-1* (△), *and Rev-2* (×). *Incubations were carried out for 60 min at the indicated temperature. The radioactivity of the products of all assays was nearly completely found in UDP-MurNAc-pentapeptide. The specific activities for each strain are given relative to the enzyme activity of that strain at 30 C. The relative specific activities at 30 C are given in the text.*

TABLE 4. *Influence of inhibitors on the activity of D-alanine:D-alanine ligase*[a]

Inhibitor	Final concn (mM)	Remaining activity in strain	
		JE-1011	ST-640
D-Alanyl-D-alanine	0.001	77	93
	0.010	43	81
	0.100	12	52
D-Cycloserine	0.002	85	97
	0.02	33	72
	0.2	6	28
Glycine	1	76	83
	5	47	57
	25	17	27
Glycyl-glycine	5	81	87
	25	47	64
	125	15	37

[a] Enzyme assays were carried out at 30 C as described in Materials and Methods. The given activities were relative to those of samples without inhibitor (100%).

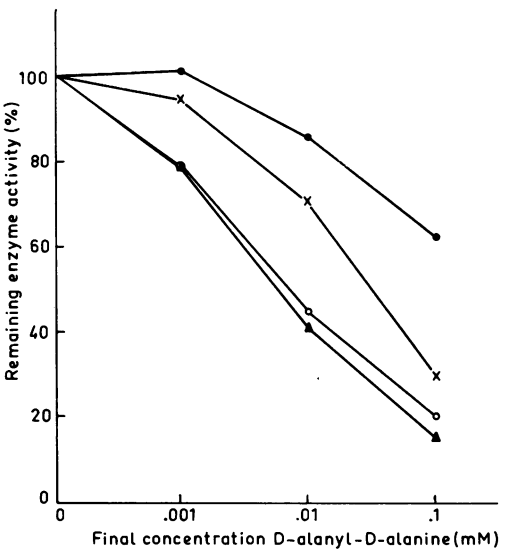

FIG. 4. *Inhibitory effect of D-alanyl-D-alanine on the ligases of strains JE-1011* (○), *ST-640* (●), *Rev-1* (△), *and Rev-2* (×). *Enzyme assays were carried out at 30 C as described in the text. The specific activities for each strain are plotted relative to the specific activity of its ligase assayed in the absence of D-alanyl-D-alanine (100%).*

is due to a second change in the structural gene of the ligase.

When extracts of strains JE-1011, ST-640, Rev-1, and Rev-2 were mixed in pairs, the resulting activities had the mean activity of those of the individual extracts. This result shows that the revertant phenotype is not due to inactivation of a possible inhibitor of the ligase.

The possibility that the reversion in strain Rev-2 was due to the production of an increased number of copies of the impaired (mutant) ligase was excluded by the finding that the ligase of strain Rev-2 was far more sensitive for D-alanyl-D-alanine (Fig. 4) than that of strain ST-640, suggesting that the reversion in strain Rev-2 is also due to a second mutation, different from that in strain Rev-1, in the structural gene of the ligase.

Temperature-sensitive and temperature-resistant recombinants contained temperature-sensitive and wild-type ligase activities, respectively.

The results show that the lysis of strain ST-640 at 42 C is due to a temperature-sensitive D-alanine:D-alanine ligase.

Permeability for alanyl-alanine. For reasons described in a previous paper (4) we believed that an *E. coli* K-12 mutant with an impaired D-alanine:D-alanine ligase would be able to grow in the presence of DL-alanyl-DL-alanine (80 μg/ml), and we were surprized to find that strain ST-640 was unable to grow under these conditions. We tested the possibility that this strain was unable to utilize a sufficient amount of the dipeptide for murein synthesis, in contrast to strain KMBL-146 (4). Both strains were incubated in the wall medium CWSM-I supplemented with ^{14}C-D-alanyl-^{14}C-D-alanine (2 μCi/ml; 81.6 μCi/μmole), while unlabeled L-alanine was omitted. The rate of murein synthesis was followed by determination of the acid-precipitable radioactivity. The acid-precipitable activities of 0.1-ml samples of strains KMBL-146 and ST-640, taken after incubation for 90 min at 42 C, were 4,520 and 500 counts/min, respectively. The acid-precipitable radioactive material of strain KMBL-146 was lysozyme-degradable (3) to a much larger extent (77%) than that of strain ST-640 (42%). These results show that exogenous D-alanyl-D-alanine is poorly incorporated into the murein of strain ST-640. As a control, ^{14}C-L-alanine was used instead of ^{14}C-D-alanyl-^{14}C-D-alanine. In this case the strains showed about the same rate of incorporation. The low activity derived from exogenous D-alanyl-D-alanine in the murein of strain ST-640 therefore was probably due to a lower permeability for the dipeptide. This possibility was tested. ^{3}H-D-alanyl-^{3}H-D-alanine of a high specific activity had to be used to achieve a measurable incorporation. The permease for ^{14}C-L-alanine was determined as a control. The results are summarized in Table 5 and show that strain ST-640, as well as its parental strain, had greatly reduced permeability for D-alanyl-D-alanine when compared with strain KMBL-146. The L-alanine permeases of the three strains were about equally active. These experiments show that the permeability of strain JE-1011 and its mutant strain ST-640 for D-alanyl-D-alanine is extremely low and that this is the reason for the inability of strain ST-640 to grow in the presence of DL-alanyl-DL-alanine at 42 C.

Sensitivity for D-cycloserine. The antibiotic D-cycloserine, an analogue of D-alanine (8), inhibits the ligase (4). Therefore, we tested whether strain ST-640, in which this enzyme is changed by mutation, had a changed sensitivity for this antibiotic. Mutant and parent strain were plated on medium D, supplemented with D-cycloserine concentrations increasing with steps of a factor three. At both 30 and 42 C (medium supplemented with 0.3 M NaCl), the mutant showed the same sensitivity as the parent strain at a D-cycloserine concentration that was three times lower. At 42 C, a threefold higher D-cycloserine concentration was necessary to prevent colony formation for both strains.

DISCUSSION

The assumption of Matsuzawa et al. (7) that strain ST-640 lyses at 42 C because its alanine racemase would be impaired was based on their observation that the strain was able to grow on D-alanine. Our experiments show that the L-alanine:D-alanine racemase activity is as in the parent strain, but that the D-alanine:

TABLE 5. *Permeability for L-alanine and D-alanyl-D-alanine*[a]

Strain	Radioactivity in the cells (counts/min)	
	L-Alanine	D-Alanyl-D-alanine
KMBL-146	7,000	9,850
JE-1011	7,350	300
ST-640	8,150	950

[a] Experimental details are given in the text. The radioactivity of samples, taken after incubation for 4 min, is given.

D-alanine ligase is temperature sensitive.

Strain ST-640 is able to grow at 42 C on both yeast agar and medium B, but does not form colonies on the low osmotic medium D. This means that this mutant would not have been scored as a temperature-sensitive strain on media that we normally use. This suggests that it is useful to use a low salt medium for the isolation of temperature-sensitive lysis mutants. However, the mutant strain TKL-15 (6) is temperature sensitive when plated on medium A, but it does not grow on the low osmotic medium C (H. J. W. Wijsman, Ph.D. thesis, Amsterdam, 1970), showing that a number of potential mutants would also be missed when a low osmotic selection medium is used.

In the low osmotic wall medium CWSM-IV, uridine nucleotide precursors leak into the medium, especially out of the mutant cells. The percentage of leaked precursors is not greatly dependent on the incubation temperature (cf. Fig. 2B and Table 2) but is mainly determined by the properties of the cells when suspended in the wall medium. We believe that the degree of leakage is determined by two factors: (i) the rigidity of the murein of the cells, and (ii) the osmotic value of the medium. The murein of the mutant cells grown at 30 C is probably less rigid than that of the parent cells because stained preparations show that the mutant culture contains many rounded cells. Moreover, when murein synthesis of the parent is inhibited by D-cycloserine, UDP-MurNAc-tripeptide can be recovered from the cells in concentrations that are 10- to 50-fold of that of the control. Accumulation of this precursor in mutants under restrictive conditions is only three- to sevenfold. The weak murein of the mutants probably causes the inner membrane to swell to such an extent that murein precursors and probably also other components can leak into the medium. The observation that 0.3 M NaCl prevents this leakage supports this idea.

The influence of the salt concentration on murein synthesis has been studied in the *m*-Dpm adding enzyme mutant strain TKL-15 incubated in CWSM-I at 42 C (6). Compared with the wild-type strain, its rate of murein synthesis was 30%, and the radioactivity in the precursors was 50%. The latter activity was mainly found in UDP-MurNAc-dipeptide (92%), whereas this percentage was only 28% in the wild type. In the presence of 0.3 M NaCl, the rate of murein synthesis of the mutant was increased by 30%, and the radioactivity in the cellular precursors was decreased to 60%. In the parent strain, no influence of NaCl was found. The distribution of the radioactivity among the precursor was not significantly different from the wild type. It was stated that the stimulation of murein synthesis by NaCl was apparently caused by stimulation of the synthesis of UDP-MurNAc-pentapeptide, although the salt had no measurable effect on the activity of the impaired enzyme in vitro (6). The observation, described in the present paper, that an increasing salt concentration apparently decreases leakage of uridine nucleotides, suggests that it may also decrease leakage of other low-molecular-weight compounds that are necessary for synthesis of murein precursors, e.g., adenosine triphosphate and divalent cations. When these effects occur, especially for the mutants, the addition of salt would stimulate the synthesis of UDP-MurNAc-pentapeptide and therefore of murein, especially in the mutant.

Because the murein of the mutant, even at the permissive temperature, seems to be weaker than that of the parent strain, it would be interesting to analyze the murein structure of both mutant and parent strains.

The rate of murein synthesis for the mutants is, because of the leakage, not a good measure for the activity of the impaired enzyme in vivo. When the leakage is prevented by salt, the rate of murein synthesis increases, suggesting that the impaired enzyme of the mutants is only slightly less active than that of the parent strain. On the other hand, the rate of synthesis of UDP-MurNAc-pentapeptide is inhibited probably by feedback (4), especially in the parent strain, because this strain contains the highest level of UDP-MurNAc-pentapeptide (6). When these observations are considered, it is nearly impossible to conclude anything about the potential activity of the impaired enzyme in vivo.

At the moment, many *E. coli* mutants in enzymes concerned with the synthesis of UDP-MurNAc-pentapeptide are known (5–7, 10). Many of the genes have been mapped between *leu* and *azi*: *murC* and *murE* and *murF* (H. J. W. Wijsman, Genet. Res., Cambridge, *in press*). No *murD* mutant is known at the moment. The gene for D-Ala:D-Ala ligase, *ddl*, is also contransducile with *leu* (H. J. W. Wijsman, *personal communication*). Strain ST-5 is probably a *murB* mutant, because it accumulates UDP-*N*-acetylglucosamine-enolpyruvate. The gene is cotransducible with *argH* at about 77 min on the chromosome map (7). A mutant in the first enzyme in the pathway, the transferase, has recently been described (10) as a mutant resistant to the antibiotic phosphonomycin (1). The gene *murA* also maps near *argH* (10). The gene for L-alanine:D-alanine

racemase is neither cotransducible with *leu* nor with *argH* (H. J. W. Wijsman, *personal communication*). These genetic data show that two genes involved in the synthesis of UDP-MurNAc-pentapeptide map near *argH* and that four genes map near *leu*. The mapping may indicate that each of the two groups forms or is part of an operon. This question is currently under investigation in cooperation with H. J. W. Wijsman.

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