

Temperature-Sensitive Mutants of *Escherichia coli* K-12 with Low Activities of the L-Alanine Adding Enzyme and the D-Alanyl-D-Alanine Adding Enzyme¹

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A number of properties of temperature-sensitive mutants in murein synthesis are described. The mutants grow at 30 C but lyse at 42 C. One mutant possesses a temperature-sensitive D-alanyl-D-alanine adding enzyme, has an impaired rate of murein synthesis *in vivo* at both 30 and 42 C, and contains elevated levels of uridine diphosphate-*N*-acetyl-muramyl-tripeptide (UDP-MurNAc-L-Ala-D-Glu-*m*-diaminopimelic acid) at 42 C. The other mutant possesses an L-alanine adding enzyme with a very low *in vitro* activity at both 30 and 42 C. Its *in vivo* rate of murein synthesis is almost normal at 30 C but is much less at 42 C. When the murein precursors were isolated after incubation of the cells in the presence of ¹⁴C-L-alanine, they contained only a fraction of the radioactivity that could be obtained from a wild-type strain. A genetic nomenclature for genes concerned with murein synthesis is proposed.

This paper describes part of a research project on the biochemical and genetic characterization of *Escherichia coli* K-12 mutants which grow at 30 C but lyse at 42 C. A number of these mutants certainly have defects in murein synthesis, because diaminopimelic acid (Dpm) or D-alanine could prevent lysis of a number of mutants while (under these conditions) the growth pattern at 42 C was identical to that of the wild-type strain (5). The first results of the biochemical (5) and genetic (H. J. W. Wijsman, Thesis, Amsterdam, 1970) work have been published recently. In the former paper we described a number of mutants with a decreased rate of ¹⁴C-L-alanine incorporation from a "wall medium" into murein (5). Mutants were found with an unusual distribution of radioactivity among the murein precursors (5). One of these mutants (strain H-1119) had nearly no radioactivity in its precursor fraction. Two mutants with an unusually high content of uridine diphosphate-*N*-acetyl-muramyl-tripeptide (UDP-MurNAc-tripeptide) were found. One of them (strain TKL-10) grew in the presence of D-alanine or DL-alanyl-DL-

alanine, while the other one (strain TKL-46) did not. The former mutant possessed an impaired alanine racemase (3; Wijsman, Thesis, Amsterdam, 1970). These results suggested that the mutants possessed an impaired enzyme in the synthesis of UDP-MurNAc-L-Ala-D-Glu-*m*-Dpm-D-Ala-D-Ala. Therefore we decided to screen these mutants for their levels of the four adding enzymes, L-alanine:D-alanine racemase, and D-alanine:D-alanine ligase. The results obtained with strains H-1119 and TKL-46 are presented in this report.

MATERIALS AND METHODS

Strains. Only *E. coli* K-12 and strains derived therefrom were used, with the possible exception of strain H-1119 and its revertant. Strain H-1119 and all other mutants isolated in the same experiment from a culture that was thought to be inoculated with strain H-669 possessed only a *pur* marker, in contrast to strain H-669, which possessed many other markers. Because the *pur* strains that are present in our laboratory stock are only K-12 strains, we assume that the culture was inoculated by accident with a K-12 purine auxotroph instead of with strain H-669. Strains KMBL-146 and TKL-46 were obtained from A. Rörsch. Strain H-1119 and spontaneous temperature-resistant revertants of strains H-1119 and TKL-46 were isolated in our laboratory. The relevant markers of these strains have been described in a previous paper (5).

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Growth of bacteria. Cells were grown in yeast-broth (4). The optical density at 660 nm was followed in a Unicam SP-600 spectrophotometer. Yeast-agar, sometimes supplemented with sucrose or NaCl, was used as the solid medium.

Chemicals and radiochemicals. The origin of these compounds was reported earlier (3). Radiochemicals were labeled with carbon-14.

Uridine nucleotides. The methods of accumulation and purification of the uridine nucleotides were published earlier (3, 5, 6).

Rate of murein synthesis. Exponentially growing cells were washed and incubated in the "wall medium" CWSM-I (4), supplemented with ^{14}C -L-alanine. CWSM-I medium consists of minimal medium salts in phosphate buffer, supplemented with uracil, chloramphenicol, and murein amino acids. Protein synthesis is almost completely inhibited under these conditions because of the presence of chloramphenicol and the absence from the CWSM-I "wall medium" of a number of amino acids that would be required by the mutiauxotrophic strains. Murein synthesis was determined by measuring the activity of the perchloric acid-precipitable material (4).

Other methods. The isolation, identification, and determination of radioactive precursors, synthesized in CWSM-I supplemented with ^{14}C -L-alanine, have been described earlier (5). The isolation of crude soluble enzyme and the enzyme assays were described in a previous paper (3).

RESULTS

Growth experiments. Growth curves of wild-type and mutant strains in yeast-broth were obtained at 30 and 42 C. No differences were observed at the permissive temperature. At the restrictive temperature, the optical density increase of the mutants was for a short period identical to that of the wild-type strain KMBL-146. Thereafter, the mutants lysed (Fig. 1). Strain H-1119 stopped growing after about 50 min, followed by a rapid decrease in optical density. The decrease in optical density of strain TKL-46 started later and was less dramatic.

None of the mutants formed colonies on yeast-agar at 42 C. When the medium was supplemented with 20% sucrose, strain H-1119 could form colonies at 42 C, but strain TKL-46 could not. Both mutants grew at 30 C in the presence of sucrose. NaCl, tested in concentrations between 0.2 and 0.4 M, had no stimulatory effect on colony formation. The parental strain grew under all conditions mentioned.

Rate of murein synthesis. The rate of murein synthesis was measured as ^{14}C -L-alanine incorporation from CWSM-I in acid-precipitable material (4). Earlier experiments had shown that the rate of incorporation varied from culture to culture (4). Therefore, the vials in which the rates of murein synthesis at 30

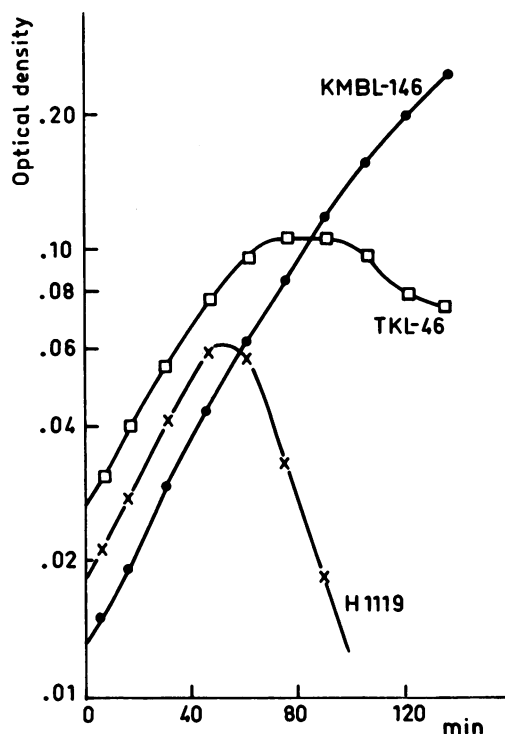


FIG. 1. Growth curves at 42 C of *Escherichia coli* strain KMBL-146 and of two temperature-sensitive lysis mutants, H-1119 and TKL-46. The optical density was measured at 660 nm.

and 42 C were compared were inoculated from the same batch of exponentially growing cells. The rate of murein synthesis in strain KMBL-146 was the same at 30 and 42 C (Fig. 2). However, the activities measured after 2 hr of incubation in a number of experiments varied between 10,000 and 20,000 counts/min. At 30 C, the lysis mutant H-1119 showed a rather low rate of incorporation, but this rate was equal to the low rate of incorporation that was sometimes measured in strain KMBL-146. The rate of murein synthesis of strain TKL-46 was considerably lower at 30 C. At 42 C both mutants showed a low rate of incorporation, especially during the second hour (Fig. 2), indicating that murein synthesis was impaired in both mutants.

Distribution of radioactivity among the murein precursors. The distribution of radioactivity among the alanine-containing murein precursors was determined after incubation in CWSM-I, supplemented with ^{14}C -L-alanine, at 30 and 42 C. In some experiments, the 42 C suspension was incubated at 30 C before isolation of the precursors, to study whether the effects observed at the restrictive

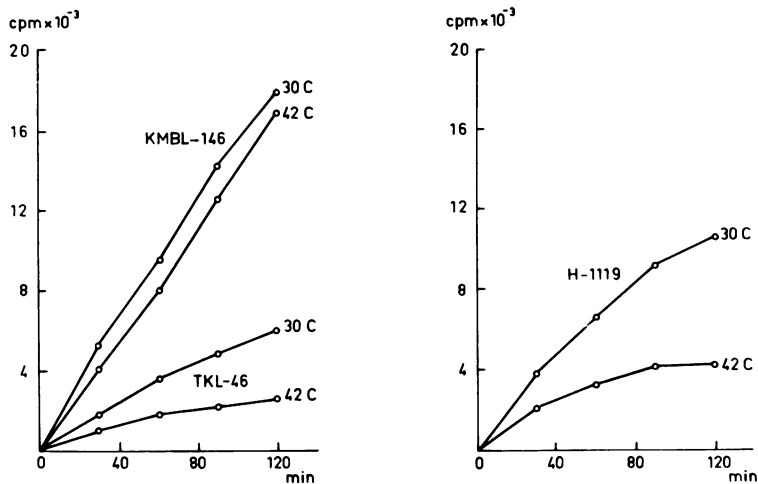


FIG. 2. Rate of murein synthesis of wild-type strain KMBL-146 and of the temperature-sensitive lysis mutants H-1119 and TKL-46. Bacteria growing exponentially in yeast-broth were washed with and resuspended in "wall medium" CWSM-I. After 15 min of incubation at 30 and 42 C, ¹⁴C-L-alanine was added in a final concentration of 1 μ Ci/ml. The perchloric acid-precipitable activity of 0.2-ml samples was determined.

temperature were reversible. The results are summarized in Table 1. The total radioactivity of the precursors of the wild-type strain KMBL-146 was rather constant, as was the distribution of the radioactivity among the precursors. High activities were found in UDP-MurNAc-pentapeptide (59-72%) and UDP-MurNAc-dipeptide (20-30%).

Strain TKL-46 contained less activity in the total precursor fraction under all conditions (Table 1). At 30 C the absolute amount of UDP-MurNAc-pentapeptide was lower than the corresponding amount in the strain KMBL-146. This decrease was not accompanied by an increase in the amounts of the other three alanine-containing precursors. This result suggests that the synthesis of UDP-MurNAc-L-Ala is impaired. The situation at 42 C is different. A small decrease in the total precursor activity was observed. The absolute amount of UDP-MurNAc-tripeptide increased considerably, and the amounts of the other three precursors decreased. This result suggests that the D-alanyl-D-alanine adding enzyme is impaired at 42 C. The possibility that the synthesis of D-alanyl-D-alanine is impaired was excluded in an earlier experiment, in which it was found that DL-alanyl-DL-alanine could not prevent lysis of this strain (5). The ratio of precursor activity over murein activity was considerably increased at 42 C. This ratio decreased when the cells were subsequently incubated at 30 C. After 1 hr this ratio and the distribution of the precursors were almost equal to those observed after incubation at 30

C only. This result indicates that the enzyme which is responsible for the decrease in the rate of murein synthesis is temperature-sensitive and that the temperature effect is reversible.

Strain H-1119 also contained a low amount of alanine-containing precursors at 30 C (Table 1). At 42 C a further decrease in the total precursor activity was observed. At this temperature the highest activity was observed in a spot where UDP-MurNAc-L-alanine is usually found. The total activity in this spot was about equal to the activity found in the corresponding spot from precursors of the wild-type strain. This means that strain H-1119 does not accumulate any of the alanine-containing precursors, suggesting that the mutant is impaired in the synthesis of UDP-MurNAc-L-alanine. If this is true, the activity in the "UDP-MurNAc-L-alanine" spot could be due to accumulated UDP-N-acetyl-glycosamyl-enolpyruvate or UDP-MurNAc. The chromatographic mobilities of these two components are identical to that of UDP-MurNAc-L-alanine; the radioactivity then could be derived from the small amount of pyruvate, synthesized from L-alanine under the experimental conditions (4).

Enzyme activities of the mutants. The enzyme assays have been described in detail in a previous paper (3). Strain TKL-46 was expected to possess a temperature-sensitive D-alanyl-D-alanine adding enzyme. Enzyme assays showed a low D-alanyl-D-alanine adding enzyme activity, while the other enzyme activ-

TABLE 1. Murein synthesis and precursor composition of the non-temperature-sensitive strain KMBL-146 and the temperature-sensitive lysis mutants TKL-46 and H-1119^a

Strain	(Subsequent) incubation(s) in CWSM-I + ¹⁴ C-L-alanine	Total precursors (counts/min) ^b	Murein (counts/min) ^c	Precursors/ murein × 10 ²	Distribution among precursors ^d (% of total counts/min)			
					X-1 ^e	X-2	X-3	X-5
KMBL-146	60 min 30 C	1,320	10,400	12.5	4	30	3	63
	60 min 42 C	1,830	9,950	18.5	9	30	2	59
	60 min 42 C	1,260	8,760	14.5	6	20	2	72
	60 min 42 C, 60 min 30 C	1,300	16,600	7.8	7	29	3	61
TKL-46	60 min 30 C	537	4,300	12	8	39	5	48
	60 min 42 C	365	870	42	4	12	69	15
	60 min 42 C, 10 min 30 C	336	1,530	22	6	14	59	21
	60 min 42 C, 30 min 30 C	497	2,540	19.5	5	22	30	43
	60 min 42 C, 60 min 30 C	772	4,090	19	8	38	12	42
H-1119	60 min 30 C	223	8,140	2.7	39	19	0	42
	60 min 42 C	33	1,370	2.4	78	22	0	0
	60 min 42 C, 10 min 30 C	47	1,710	2.7	49	25	0	26
	60 min 42 C, 30 min 30 C	71	2,350	3.0	87	8	0	5
	60 min 42 C, 60 min 30 C	83	3,190	2.6	28	22	0	47

^a Cells growing exponentially at 30 C in yeast-broth were washed with and incubated in CWSM-I at the indicated temperature. After 15 min, ¹⁴C-L-alanine was added in a final concentration of 2 μCi/ml. After additional incubation(s), the perchloric acid-precipitable activity in 0.1 ml was determined. The remaining suspension (1.9 ml) was used to isolate the precursors. A sample of this fraction was mixed with reference compounds and chromatographed. Precursors were detected under ultraviolet light and by autoradiography. The radioactivity of the spots was counted.

^b Total precursor activity of 0.1 ml of suspension is given.

^c Total murein activity of 0.1 ml of suspension is given.

^d X = UDP-MurNAc, where the numbers represent the amino acids in the peptide chain; 1 = L-Ala; 2 = L-Ala-D-Glu; 3 = L-Ala-D-Glu-m-Dpm; 5 = L-Ala-D-Glu-m-Dpm-D-Ala-D-Ala.

^e This activity could be due to UDP-GlcNAc-enolpyruvate, X, or X-1, which could not be distinguished from one another in the separation system used.

ities were similar to those found in its parental strain KMBL-146 (Table 2). Incubations at temperatures between 19 and 42 C showed that the D-Ala-D-Ala adding enzyme was temperature-sensitive (Fig. 3). Compared to strain KMBL-146, its activity was 25% and 3.5% at 19 and 42 C, respectively. When the extract was preincubated at 42 C for 10 min, followed by assay at 30 C, the D-alanyl-D-alanine adding enzymes of strains TKL-46 and KMBL-146 lost 75.5% and 5%, respectively, of their initial activities.

When extracts of strains KMBL-146 and TKL-46 were mixed in the appropriate dilutions, followed by assay of the D-alanyl-D-alanine adding enzyme, the enzyme activity found was equal to the sum of the activities of the individual extracts from these strains, indicating that the low activity in strain TKL-46 is not due to the (over)production of an inhibitor of the enzyme.

One temperature-resistant revertant of strain TKL-46 was studied with respect to growth and enzyme activities. No significant

TABLE 2. Relative levels of enzyme activities in extracts of temperature-sensitive lysis mutants

Strain	Relative activity ^a					
	L-Ala adding enzyme	D-Glu adding enzyme	m-Dpm adding enzyme	D-Ala-D-Ala adding enzyme	D-Ala : D-Ala ligase ^b	L-Ala : D-Ala race-mase ^b
TKL-46	99	123	81	20	65 ^c	98 ^c
H-1119	5	104	54	104	63	119

^a Enzyme assays were carried out at 30 C for 1 hr. The activities are presented as percentages relative to the corresponding activities of strain KMBL-146, determined under the same conditions. The values are corrected for zero-time controls.

^b Determined in the presence of UDP-MurNAc-tripeptide. The activity in UDP-MurNAc-pentapeptide was counted. The activity in D-alanyl-D-alanine was very low except when extracts of strain TKL-46 were used.

^c This value was obtained by addition of the activities of UDP-MurNAc-pentapeptide and D-alanyl-D-alanine. The latter activity was high because of the impaired D-alanyl-D-alanine adding enzyme.

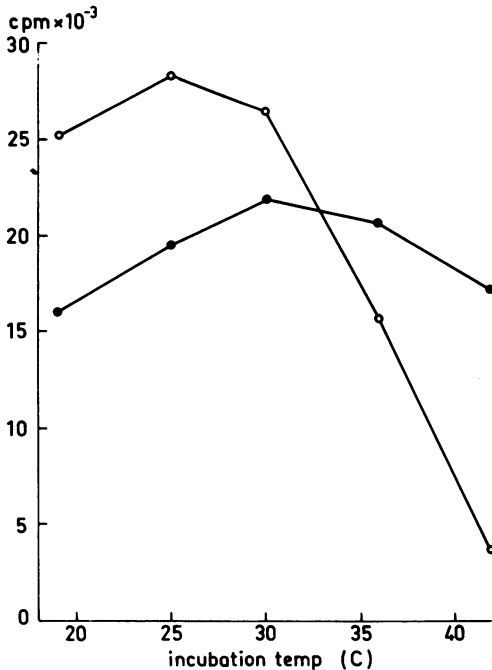


FIG. 3. The temperature-sensitive character of the *D*-alanyl-*D*-alanine adding enzyme of strain TKL-46. The *D*-alanyl-*D*-alanine adding enzymes of strains KMBL-146 and TKL-46 were assayed at different temperatures. Protein concentrations in the assay mixtures were 1.3 and 8.1 mg/ml for strain KMBL-146 and strain TKL-46, respectively. Closed circles: strain KMBL-146; open circles: strain TKL-46.

differences from strain KMBL-146 were found. The activities of the adding enzymes for L-alanine, *m*-Dpm and *D*-alanyl-*D*-alanine differed by less than 25% from those of the parental strain. These results show that the lysis of strain TKL-46 is due to its temperature-sensitive *D*-alanyl-*D*-alanine adding enzyme.

Enzyme assays with extracts of strain H-1119 showed that the L-alanine adding enzyme activity was extremely low whereas the activities of the other enzymes were comparable with the activities in extracts of strain KMBL-146 (Table 2). It was impossible to compare the enzyme levels of strain H-1119 with those of its parental strain, because the origin of strain H-1119 is unknown (5). A temperature-resistant revertant of strain H-1119 was isolated and this strain had a growth rate similar to that of strain KMBL-146 at 42 C. The activities of its adding enzymes for L-alanine and *m*-Dpm, compared with those of strain KMBL-146, were 113% and 104%, respectively.

Assay of the enzymes of strains H-1119 and KMBL-146 at different temperatures sug-

gested that the L-alanine adding enzyme of H-1119 was temperature-sensitive. At 22, 30, and 42 C, the relative activities of this enzyme of strain H-1119 were 4.6, 5.0, and 0%, respectively. We are not completely convinced of its temperature-sensitive character, as the initial activity of the enzyme is already very low at 22 C.

Assays of the L-alanine adding enzyme activity in mixed extracts of strains H-1119 and KMBL-146 suggested that the low L-alanine adding enzyme activity of strain H-1119 was not due to (over)production of an inhibitor by the mutant. These results strongly suggest that the L-alanine adding enzyme of strain H-1119 is impaired. The results of the enzyme assays make it very likely that the activity in the "UDP-MurNAc-L-Ala" spot from strain H-1119 is due to UDP-MurNAc, labeled in its lactic acid residue.

DISCUSSION

The rate of murein synthesis of the strains H-1119 and TKL-46 is certainly impaired at 42 C; at 30 C it is slightly decreased for strain H-1119 and impaired for strain TKL-46 (Fig. 2). The level and distribution of the alanine-containing murein precursors in both mutants differ from strain KMBL-146 at both temperatures (Table 1). The low activities of the impaired enzymes limit the rate of murein synthesis even at 30 C, although the activities are sufficient to prevent lysis (Fig. 2). The *D*-alanyl-*D*-alanine adding enzyme of strain TKL-46 has a low activity *in vitro*, and it is temperature-sensitive (Fig. 3). The *in vitro* activity of the L-alanine adding enzyme of strain H-1119 at 30 C is about 5% of that of the wild type. We suppose that this activity is higher *in vivo*, because it is unlikely that *E. coli* K-12 wild type contains an activity of this enzyme that is 20 times higher than required. Low *in vitro* enzyme activities of temperature-sensitive mutants have been described for L-alanine:*D*-alanine racemase (6; H. J. W. Wijsman, Thesis, Amsterdam, 1970), valyl-sRNA synthetase (2, 8), phenylalanyl-sRNA synthetase (2), and fructose-1,6-diphosphate aldolase (1). Therefore one can assume that the L-alanine adding enzyme of strain H-1119 is altered in such a way that its *in vitro* activity at any temperature is not an accurate measure of its *in vivo* activity at that temperature. Matsuzawa et al. (7) reported that their temperature-sensitive lysis mutant, ST-222, accumulated an unknown precursor. We have received this mutant from M. Matsushashi. Its behavior was similar to that of strain H-1119 with respect to

rate of murein synthesis and to level and distribution of radioactivity among the precursors. In addition, we have found a similar low level of the L-alanine adding enzyme. No evidence for an inhibitor was found.

The mutation that causes the temperature sensitivity of strain TKL-46 has been shown to be on the *E. coli* chromosome between the leucine and azide markers (H. J. W. Wijsman, Thesis, Amsterdam, 1970). The mutation of strain H-1119 was found in the same region (H. J. W. Wijsman, *personal communication*). The temperature-sensitive mutation of strain ST-222 has been roughly mapped in a conjugation experiment about 3 min at the right side of threonine (7), which is less than 2 min from the site of the mutation of strain H-1119. Because the localization in strain ST-222 was inaccurate and because the impaired enzyme in strains H-1119 and ST-222 is identical, it seems likely that the mutated genes are identical.

Because the proteins corresponding to a number of genes concerned with murein synthesis have been identified, we think that it is necessary to indicate the loci according to a systematic three-letter code. Wijsman and we propose *mur* for genes that are directly concerned with murein synthesis. We do not consider genes involved in the synthesis of either D-alanyl-D-alanine or amino acids required for murein synthesis as *mur* genes. The first two enzymes specific for murein synthesis are then the pyruvate:UDP-*N*-acetylglucosamine transferase and the UDP-*N*-acetylglucosamine-enolpyruvate reductase; *murA* and *murB*, respectively, are the proposed names for the corresponding genes. Genes corresponding to the adding enzymes for L-alanine, D-glutamic acid, *m*-Dpm, and D-Ala-D-Ala are then called

murC, *D*, *E*, and *F*, respectively. According to this proposal the strains H-1119 and TKL-46 are *murC* and *murF* mutants, respectively.

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