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THIOSINE-RESISTANT MUTANTS OF *ESCHERICHIA COLI* K-12 WITH GROWTH-MEDIUM-DEPENDENT LYSYL-tRNA SYNTHETASE ACTIVITY

I. ISOLATION AND PHYSIOLOGICAL CHARACTERIZATION*

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SUMMARY

1. Thiosine-resistant mutants of *Escherichia coli* K-12 have been isolated which have decreased lysyl-tRNA synthetase (L-lysine: tRNA ligase (AMP), EC 6.1.1.6) activity.

2. These mutants have three unusual characteristics.

A. The activity of lysyl-tRNA synthetase is growth-medium-dependent, and in some strains is 15–20 times higher in an enriched medium than in minimal medium.

B. The activity of the lysyl-tRNA synthetase from the wild-type strain declines in stationary phase in AC broth, but the activity of the enzyme from mutant strains does not.

C. The lysyl-tRNA synthetase from the mutant strains is thermostable in comparison to the wild-type enzyme.

3. Alanine can stimulate the lysyl-tRNA synthetase activity in minimal medium. and thus can partially replace the requirement for an enriched growth medium.

INTRODUCTION

The aminoacyl-tRNA synthetases are a class of enzymes indispensable to the cell because of their role in amino acid activation, and in insuring the fidelity of translation in protein synthesis by interacting selectively with the proper tRNA species[1]. There have been a number of publications on the possible role of aminoacyl-tRNA synthetase in the regulation of amino acid biosynthesis (*e.g.* refs.[2–7]), but only a few reports have appeared on the regulation of the synthesis and activity of the synthetases themselves[8–12]. It was initially thought that the synthesis of these enzymes is constitutive[8], but the work of NASS AND NEIDHARDT[8] demonstrated that the formation of the phenylalanyl- and isoleucyl-tRNA synthetases is subject to control by a repression-like mechanism. By use of the more sophisticated technique of density labeling, WILLIAMS AND NEIDHARDT[9] extended the above experiments to other aminoacyl-tRNA synthetases. The major conclusion from their experiments is

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that the rate of synthesis of aminoacyl-tRNA synthetases can vary considerably (10–50 fold), and that this rate can be modulated by a repression-like mechanism.

A number of mutant aminoacyl-tRNA synthetases have been isolated by various techniques (*e.g.* refs. 13–19), but they all appear to be structural gene mutants. To the authors' knowledge no mutant strain has been reported in which the amount of an aminoacyl-tRNA synthetase is depressed or elevated, although at least one attempt has been made to identify such a mutant[16]. We have isolated a number of thiosine-resistant strains of *Escherichia coli* K-12 which have reduced lysyl-tRNA synthetase (L-lysine: tRNA ligase (AMP), EC 6.1.1.6) activity. These mutants are most unusual in that the activity of the lysyl-tRNA synthetase is growth-medium-dependent, and in some strains the specific activity of the enzyme is 15–20 times higher in an enriched medium than in minimal medium. Another singular aspect of these strains is that the lysyl-tRNA synthetase is more heat stable than the wild-type enzyme. In this report we describe the isolation and physiological studies on these strains. The enzymological studies are presented in the accompanying paper[20].

MATERIALS AND METHODS

Strains, media, and growth conditions

The strains of *E. coli* K-12 used in the experiments described in this report are listed in Table I.

Cells were grown at 37° with aeration in minimal medium[21], minimal medium supplemented with amino acids, vitamins, purines and pyrimidines[22] (supplemented minimal medium), 1 % bactopeptone–0.5 % yeast extract, 1 % bactopeptone–5 % yeast extract or Difco AC broth. Growth of the bacterial cultures was determined by measuring the absorbance at 490 nm for minimal medium and 580 nm for the other media.

When the lysine bradytrophs were grown in the supplemented minimal medium, arginine and histidine were omitted, and with the arginine bradytrophs, lysine and histidine were omitted[5]. This precaution was taken because the basic amino acids inhibit the growth of the bradytrophs. Amino acids were used at a final concentration of 100 μg/ml unless noted otherwise. In experiments in which the cells were shifted from supplemented minimal medium to the enriched medium, the cells were not washed.

Mutagenesis

(*A*) *Spontaneous.* Individual cultures of the parent strain were grown in AC broth to about $1 \cdot 10^9$ cells per ml. After washing twice with minimal medium and concentrating the cells about 10 fold, 0.1 ml was plated on 2 % agar (Difco) containing supplemented minimal medium *plus* 100 μg/ml thiosine. No lysine was present in this medium. These plates were incubated at 37° for 48 h to permit thiosine-resistant mutants to grow.

(*B*) *Ethyl methane sulfonate.* The procedure was that of LOVELESS AND HOWARTH[23] using the modification of COOPER *et al.*[24]. Cells were incubated with ethyl methanesulfonate at 30° for 90 min or at 37° for 15 min. Samples of the mutagenized cells were grown overnight in AC broth, and then plated on agar as described above.

Enzyme assay

The activities of lysyl-, arginyl-, and glutamyl-tRNA synthetases were measured by the ability of each to charge tRNA with the appropriate L-[^{14}C]amino acid. The standard incubation mixture for the assay of lysyl-tRNA synthetase consisted of the following in a volume of 0.1 ml: 10 μmoles Tris–maleate buffer, pH 7.25; 0.1 μmole ATP; 1.0 μmole $MgCl_2$; 1.0 μmole KCl; 0.001 μmole of L-[^{14}C]lysine (80 μC/μmole); and *E. coli* K-12 tRNA, 0.45 mg. The pH chosen (7.25) was below the optimum in order to increase the chance of finding a mutant enzyme, for it had previously been found that the activity of mutant arginyl-tRNA synthetases decreased more rapidly than the activity of the wild-type enzyme as the pH was lowered from the optimum of 8.0—5.5[15]. In the assays for arginyl- and glutamyl-tRNA synthetase activity, 0.4 μmole of glutathione was also included. In these latter assays 0.02 μmole of either arginine (20 μC/μmole) or glutamic acid (7 μC/μmole) was used.

The assays were performed at 37° for 5 min unless otherwise noted. The rate of labeling of tRNA was constant during this time interval. After the incubations were completed the samples were prepared for counting by the membrane filter technique[25] and the radioactivity determined on a gas-flow counter. 1 unit of enzyme activity represents the amount of enzyme that catalyzes the incorporation of 1 nmole of lysine into tRNA in 10 min at 37°, pH 7.25. Specific activity is defined as the number of units per mg of total protein.

Preparation of crude enzyme extracts

Crude extracts were prepared by sonic disruption as previously described[5]. In the synthetase assays 3–5 μg of crude extract protein was routinely used.

Protein determination

Protein was determined by the method of LOWRY *et al.* [26] using crystalline bovine serum albumin as a standard.

Chemicals

The amino acids and vitamins used in preparing media were highest quality products from either the Mann Research Laboratories or Sigma Chemical Company. L-Thiosine (*S*-(β-aminoethyl)-L-cysteine) was obtained from Cyclo Chemicals, Los Angeles, California. All ^{14}C-labeled amino acids were obtained from New England Nuclear. Transfer RNA was purchased from either General Biochemicals or Schwarz Bioresearch. Crystalline bovine serum albumin was obtained from Pentex, Kankakee, Ill. AC broth, bactopeptone and yeast extract were obtained from Difco Laboratories, Detroit, Mich. Ethyl methanesulfonate was purchased from Eastman Organic Chemicals. Chloramphenicol was obtained from Parke, Davis and Company, and lastly rifampicin was a gift of Dr. K. Brooks Low of Yale University.

RESULTS

Isolation of thiosine-resistant mutants with reduced lysyl-tRNA synthetase activity

L-Thiosine (*S*-(β-aminoethyl) L-cysteine) is an analog of lysine and can replace it in the pyrophosphate exchange reaction, and in the aminoacylation of

tRNA in the presence of lysyl-tRNA synthetase[27]. It has also been shown by STERN AND MEHLER[27] that L-[^{35}S]thiosine can be incorporated into the protein of *E. coli* although thiosine would not support the growth of a lysine auxotroph. The growth of *E. coli* K-12 is inhibited by thiosine[27]. The above properties of thiosine made it a likely device for selecting lysyl-tRNA synthetase mutants, and such mutants can be selected on the basis of their resistance to the growth-inhibitory property of the analog.

In the experiments reported here the parent strain is IH2000, which is a lysine 7 bradytroph (conversion of diaminopimelic acid to lysine) derived from strain MA50, a lysine 7 auxotroph (see Table I for other properties of IH2000). A bradytrophic (leaky) strain was used for the following reasons: It is necessary to have an endogenous supply of lysine when selecting thiosine-resistant mutants, for lysine added to medium containing thiosine will interfere with the growth-inhibitory action of the latter. Lysine is, however, a better substrate for lysyl-tRNA synthetase than is thiosine[27]. By using a bradytroph, the amount of lysine synthesized is reduced, but is sufficient to support growth. Moreover, the reduced amount of lysine enables thiosine to compete more effectively as a substrate for lysyl-tRNA synthetase.

TABLE I

DESCRIPTION OF STRAINS

Strain	*Nutritional requirements*						*Response to*			*Genotype of Arg and Lys loci*			*Mating type*
	Arg	*Lys*	*Cys*	*Thr*	*Leu*	*Thi*	*Can*	*Thio*	*Str*	*Arg B*	*Arg S*	*Lys 7*	
IH 2000 (parent)	+	br	−	−	−	−	S	S	S	+	+	−	F⁻
IH 2001	+	br	−	−	−	−	S	R	S	+	+	−	F⁻
IH 2002	+	br	−	−	−	−	S	R	S	+	+	−	F⁻
MA 5000 (parent)	br	+	+	+	+	+	S	S	S	−	+	+	F⁺
MA 5003	br	+	+	+	+	+	R	S	S	−	−	+	F⁺
MA 5005	br	+	+	+	+	+	R	S	S	−	−	+	F⁺

Abbreviations used: Arg, arginine; Lys, lysine; Cys, cysteine; Thr, threonine; Leu, leucine; Thi, thiamine; Can, canavanine; Thio, thiosine; Str, streptomycin; br, bradytrophic; S, sensitive; R, resistant, +; no requirement; −, complete or partial requirement.

Spontaneously arising or ethyl methanesulfonate-induced thiosine-resistant mutants were selected on 2 % agar plates containing supplemented minimal medium with 100 μg/ml thiosine. No lysine was present in the medium. Only slow growing, thiosine-resistant colonies were chosen for further study, as it was reasoned that such strains would be more likely to contain a defective lysyl-tRNA synthetase. The colonies chosen for further study were purified by restreaking on agar plates containing thiosine, and were then grown in liquid culture (supplemented minimal medium+lysine) to prepare crude extracts. Assays for lysyl-tRNA synthetase were performed with sonic extracts by measuring the incorporation of L-[^{14}C]lysine into tRNA. Approx. 400 strains were tested, and about 50 of these had reduced lysyl-tRNA synthetase activity, which ranged from 5–50 % of the parent strain. In Table II a representative sample of mutants is listed. The largest percentage of this kind of mutant resulted from treatment with ethyl methanesulfonate at 30°. In the

TABLE II

THIOSINE-RESISTANT STRAINS WITH REDUCED LYSYL-tRNA SYNTHETASE ACTIVITY

Lysyl-tRNA synthetase was assayed according to the procedure described under MATERIALS AND METHODS. The figures listed under % activity are average values. The range of activities observed is given in parentheses

Strain	*Mutagenesis*	*Temp.*	*% Parental lysyl-tRNA synthetase activity*
IH 2001	EMS	30°	5 (3.5–6.5)
IH 2002	EMS	30°	5 (3.5–6.5)
IH 2003	Spontaneous		48 (43–53)
IH 2004	EMS	30°	11 (9–13)
IH 2005	EMS	30°	7 (5–9)
IH 2006	EMS	30°	37 (33–41)
IH 2007	EMS	30°	14 (12–16)
IH 2008	EMS	30°	50 (45–55)
IH 2009	EMS	30°	12 (10.5–14.5)
IH 2010	EMS	30°	24 (21–27)
IH 2011	EMS	37°	5 (3.5–6.5)
IH 2012	EMS	37°	26 (23–29)

remainder of this presentation, discussion will be confined primarily to the wild-type strain, IH2000, and the mutant strain, IH2001. The generation times of these strains in various media are listed in Table III.

TABLE III

GENERATION TIME OF THE PARENT AND THIOSINE-RESISTANT STRAINS IN VARIOUS GROWTH MEDIA

Growth was determined as described under MATERIALS AND METHODS. Each figure quoted represents the average of at least three experiments.

Strain	*Generation time (min)*		
	Supplemented minimal medium	*Supplemented minimal medium plus lysine*	*AC broth*
IH 2000 (parent)	65	35	28
IH 2001	93	42	31
IH 2002	61	51	30

Growth-medium dependent lysyl-tRNA synthetase activity

It was found that growing the mutant strains in AC broth, which is a complex enriched medium instead of supplemented minimal medium, resulted in a significant increase in lysyl-tRNA synthetase activity. This phenomenon is depicted in Fig. 1. In these experiments the strains were first grown in supplemented minimal medium *plus* lysine, and then shifted to the AC broth. The specific activity of the lysyl-tRNA synthetase from mutant strain IH2001 typically increases 15–20 fold in such an experiment whereas the enzyme from the wild-type strain characteristically shows a 1.5–2-fold increase. Addition of lysine (100 μg/ml) to the AC broth had no effect on the observed increase in lysyl-tRNA synthetase activity. A number of mutant

strains have been tested in AC broth, and in every instance the activity of lysyl-tRNA synthetase increased. The percentage increase in AC broth depends on the intitial activity of the enzyme in supplemented minimal medium; the lower the activity in this medium, the larger the percentage increase when the strain is grown in AC broth.

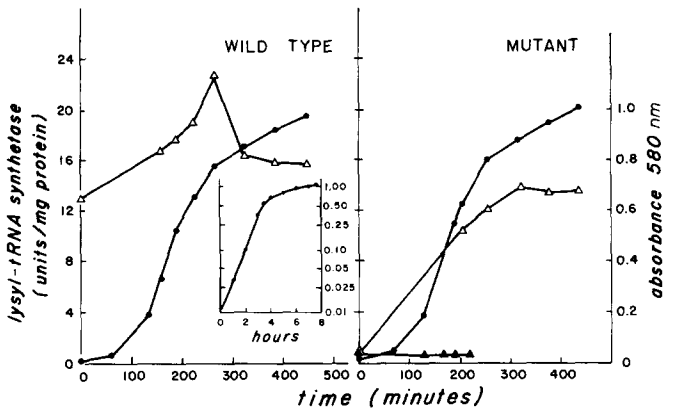

Fig. 1. Activity of lysyl-tRNA synthetase from IH 2000 and IH 2001 upon shifting them from supplemented minimal medium *plus* lysine to AC broth. Samples were taken at various points upon growth in the AC broth, sonic extracts were prepared and lysyl-tRNA synthetase activity measured by incorporation of [^{14}C]lysine into tRNA at 37°, pH 7.25 for 5 min. The cultures were grown aerobically at 37°. The insert shows growth on a log absorbance *vs.* time plots for the wild type strain. The mutant's growth curve is superimposable on the wild-type curve. ●–●, absobance; △–△, lysyl-tRNA synthetase activity in AC broth; ▲–▲, lysyl-tRNA synthetase activity in 1 % bactopeptone–0.5 % yeast extract medium.

The activity of the lysyl-tRNA synthetase from the mutant strains grown in AC broth usually approximates the activity of the wild-type enzyme when that strain is grown in supplemented minimal medium. However, the activity of the mutant strain synthetase never reaches the level of the wild-type activity in AC broth.

Another interesting feature of Fig. 1 is the fate of enzyme activity in stationary phase. The activity of the wild-type enzyme increases during early stationary phase, but declines as the cells continue into the stationary phase of growth. This is consistent with the studies of SHORTMAN AND LEHMAN[28] and HEINONEN[10] on other aminoacyl-tRNA synthetases. Several mutant strains (IH2001 is depicted here) which have been examined differ from the wild-type pattern. The activity of these enzymes also increases in early stationary phase, but does not decline thereafter.

Mutant strain IH2001 has been tested in other media, and it has been found that in minimal medium or 1 % bactopeptone–0.5 % yeast extract medium, the lysyl-tRNA synthetase activity is equivalent to that in supplemented minimal medium. With the wild-type strain the lysyl-tRNA synthetase activity is equivalent in supplemented minimal medium and 1 % bactopeptone–0.5 % yeast extract, but the enzyme's activity is lower when wild type is grown in minimal medium (see Tables VII and VIII). With either strain the increase in lysyl-tRNA synthetase activity is observed if a 1 % bactopeptone–5 % yeast extract medium replaces AC broth.

The increase in specific activity of the lysyl-tRNA synthetase in AC broth is not related merely to the density of the culture, for if the mutant strain IH2001 is grown in 1 % bactopeptone–0.5 % yeast medium to an $A_{580\,nm}$ of 0.65–0.70, the specific activity of the synthetase remains low, whereas in AC broth at the same density the activity has increased 8–10 fold (see Fig. 1).

Specificity

It was of interest to determine whether (A) other aminoacyl–tRNA synthetases in the mutant strain had reduced activity or (B) showed a large increase in specific activity when the strains were shifted from supplemented minimal medium *plus* lysine to AC broth. Glutamyl- and arginyl-tRNA synthetase were tested in the mutant and wild-type strains for this purpose (Table IV). The results show that the specific activity of these enzymes in the wild-type and mutant strains is quite similar, and only a small increment in specific activity is observed with these synthetases when either strain is grown in AC broth.

TABLE IV

ACTIVITY OF GLUTAMYL- AND ARGINYL-tRNA SYNTHETASES FROM PARENT AND A THIOSINE-RESISTANT STRAIN GROWN IN SUPPLEMENTED MINIMAL MEDIUM *plus* LYSINE OR AC BROTH

Cells were grown in the supplemented minimal medium to late log phase. Samples were taken at various points upon growth in AC broth and growth was concluded at $A_{580\,nm}$ 0.75. In (A) the values expressed are the specific activities found in the supplemented minimal medium. In (B) the figures represent the maximum ratio of the specific activity found in AC broth to that in supplemented minimal medium. Assays on the synthetases were run as described under MATERIALS AND METHODS

Strain	*(A) Supplemented minimal medium plus lysine*		*(B) Increase in specific activity in AC broth*	
	Glutamyl-tRNA synthetase (units/mg)	*Arginyl-tRNA synthetase (units/mg)*	*Glutamyl-tRNA synthetase*	*Arginyl-tRNA synthetase*
IH 2000	3.7	32.7	1.3	1.3
IH 2001	3.3	30.1	1.4	1.5

Next it was asked whether there was anything unique about the increase in lysyl-tRNA synthetase activity which was observed when the mutant strains were grown in AC broth. Perhaps any mutant aminoacyl–tRNA synthetase would exhibit the same response. To test this point we used strains MA5003 and MA5005, which are strains with a defective arginyl-tRNA synthetase, and their parent strain MA5000. The arginyl-tRNA synthetase from MA5003 has been shown to have an increased K_m for arginine whereas the corresponding synthetase from MA5005 has an increased K_m for ATP[15]. When these strains were shifted from growth in supplemented minimal medium *plus* arginine (100 μg/ml) to AC broth, less than a 2-fold increase was observed in the specific activity of arginyl-tRNA synthetase. The increases found were MA5000, 1.5; MA5003, 1.3; and MA5005. 1.6. These results suggest that the mutation in IH2001 and related strains possibly affects only the lysyl-tRNA synthetase, and that the increase in activity observed in AC broth is not due to a non-specific effect which would be displayed by any mutant aminoacyl-tRNA synthetase.

Is cell density in AC broth critical for obtaining an increase in lysyl-tRNA synthetase activity?

The next major question posed was whether the number of generations of growth or the cell density obtained in AC broth was the more critical factor in determining whether lysyl-tRNA synthetase activity would increase. The experiment described in Fig. 2 examines this question. The mutant strain IH2001 was grown for varying numbers of generations in AC broth, and as the results show, the increase in activity was independent of this variable but apparently related to the density of the culture. This is most obvious for the culture which was started at an $A_{580\,nm}$ of 0.00035. This culture grew eight generations in order to reach an $A_{580\,nm}$ of 0.1, but as the results show, there is virtually no increase in the lysyl-tRNA synthetase activity at this point. In all three cases, the specific activity of lysyl-tRNA synthetase increases most rapidly after an $A_{580\,nm}$ of 0.3 is reached. This experiment suggests that some factor(s) accumulates in the medium and is responsible for the stimulation of lysyl-tRNA synthetase activity.

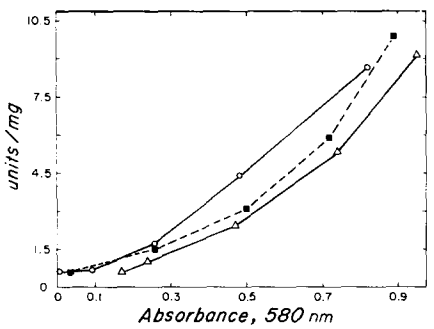

Fig. 2. Lack of correlation between number of generations of growth and the increase in lysyl-tRNA synthetase activity in mutant strain IH 2001. Cells were pregrown in supplemented minimal medium *plus* lysine and then grown in AC broth for varying numbers of generations. Culture conditions and lysyl-tRNA synthetase assay were the same as previously stated. ○–○, initial absorbance in AC broth, 0.00035; ■- - -■, initial absorbance in AC broth, 0.035; △–△, initial absorbance in AC broth, 0.17.

The above results prompted the next experiment in which mutant IH2001 was grown in AC broth or 1 % bactopeptone–5 % yeast extract to late log or early stationary phase (a point at which lysyl-tRNA synthetase activity is high) and then the medium was filtered through a millipore filter. To the filtrate was added fresh cells of IH2001 grown in suppplemented minimal medium *plus* lysine. These cells were incubated for 2 h in the filtrate. Table V shows that a marked stimulation of lysyl-tRNA synthetase activity in IH2001 was achieved by this procedure, and it was not necessary for the cells to reach a high density in the filtrate in order to observe the stimulation. Moreover, it is obvious by the slow rate of growth of IH2001 in the AC broth filtrate that rapid growth of the cells is not a critical determinant for stimulation of lysyl-tRNA synthetase activity. In an analogous experiment the filtrate from a wild-type AC broth culture stimulated lysyl-tRNA synthetase activity about 2-fold in cells of the same strain pregrown in supplemented minimal medium. This rules out the possibility that the factor(s) produced in the mutant culture is due to an aberration in the mutant's metabolism.

TABLE V

EFFECT OF INCUBATING MUTANT STRAIN IH 2001 IN FILTRATE FROM AN AC BROTH CULTURE

Mutant strain IH 2001 was pregrown in supplemented minimal medium and lysine and then transferred to AC broth. The mutant was grown in the AC broth to early stationary phase, and then the culture was filtered through a sterile millipore. Cells of IH 2001 which had been growing in supplemented minimal medium were added to the filtrate to give a $A_{580\ nm}$ of 0.1. These cells were then incubated in the AC broth filtrate for 2 h. All cultures were grown at 37°, and lysyl-tRNA synthetase was prepared and assayed as described under MATERIALS AND METHODS.

Growth medium	*Time of incubation (h)*	$A_{580\ nm}$	*Lysyl-tRNA synthetase (units/mg protein)*
Supplemented minimal medium *plus* lysine	-	0.35	0.48
AC broth	-	0.75	9.8
AC filtrate	0	0.1	0.48
AC filtrate	1	0.165	4.6
AC filtrate	2	0.24	8.1
AC filtrate + rifampicin (100 μg/ml)	0	0.11	0.48
AC filtrate + rifampicin	2	0.12	0.46
AC filtrate + chloramphenicol (100 μg/ml)	0	0.14	0.48
AC filtrate + chloramphenicol	2	0.18	0.52

Is the increase in lysyl-tRNA synthetase activity due to enzyme activation or to a change in the amount of enzyme?

If the RNA synthesis inhibitor, rifampicin[29,30], or the protein synthesis inhibitor, chloramphenicol[31,32], was added to the AC broth filtrate and IH2001 incubated as above, no increase in lysyl-tRNA synthetase activity was observed (see Table V). These results suggested that the AC filtrate factor(s) does not directly activate the synthetase. The requirement for protein synthesis could be a direct one, *i.e.* more lysyl-tRNA synthetase is synthesized), or an indirect one (*i.e.* the filtrate factor(s) can only act by being converted *via* metabolism to an active form).

The question of enzyme activation *versus* enzyme synthesis was probed in another manner. In the following experiment (Table VI), the mutant IH2001 was grown in 1 % bactopeptone–5 % yeast extract medium to late log phase, and then an aliquot of cells was transferred to a 1 % bactopeptone–0.5 % yeast extract medium, a medium in which the lysyl-tRNA synthetase activity in the mutant strain is low. The cells were allowed to grow for two generations in the latter medium, and it was our intent to observe whether the activity of the enzyme decreased rapidly or slowly following the shift. A rapid decline would suggest that the increase in specific activity of lysyl-tRNA synthetase observed in the 5 % yeast extract medium was due to an activator which was diluted out in the 0.5 % yeast extract medium. A slow decline in the synthetase's activity would suggest a turnoff of synthesis of the enzyme. As the results clearly indicate (Table VI), the decline in lysyl-tRNA synthetase activity is slow, the activity being diluted out by approximately 1/2 per generation.

What is the nature of the stimulating factor(s)?

There are at least two possible approaches to this question. One is to grow the strains in AC broth or 1 % bactopeptone–5 % yeast extract and then fractionate the

TABLE VI

EFFECT OF SHIFTING MUTANT IH 2001 FROM 1 % BACTOPEPTONE–5 % YEAST EXTRACT TO 1 % BACTOPEPTONE–0.5 % YEAST EXTRACT MEDIUM ON LYSYL-tRNA SYNTHETASE ACTIVITY

The mutant strain was pregrown in supplemented minimal medium *plus* lysine, and then shifted to a 1 % bactopeptone–5 % yeast extract medium (rich). The cells were grown to an $A_{580\ nm}$ of 0.7 in this medium. An aliquot of cells was removed, centrifuged, and then washed in a 1 % bactopeptone–0.5 % yeast extract medium. The washed cells were added to the latter medium to an $A_{580\ nm}$ of 0.1 and then grown for two generations. All cultures were grown at 37°, and lysyl-tRNA synthetase assayed as before.

Growth medium	$A_{580\ nm}$	*Lysyl-tRNA synthetase (units/mg protein)*
Supplemented minimal medium + lysine	0.35	0.53
1 % bactopeptone–5 % yeast extract	0.7	10.6
1 % bactopeptone–0.5 % yeast extract	0.1	10.6
1 % bactopeptone–0.5 % yeast extract	0.2	5.5
1 % bactopeptone–0.5 % yeast extract	0.4	3.5

filtrate which contains the active factor(s) in order to identify it (them). The second approach would be to search for compounds which, when added to minimal or 1 % bactopepton–0.5 % yeast extract medium, would bring the activity of lysyl-tRNA synthetase to the level found in AC broth. Due to a chance observation, we have at this time utilized the second approach. That observation was that when a concentrated solution of amino acids and purines and pyrimidines was added to 1 % bactopeptone–0.5 % yeast extract culture of the mutant IH2001, there was a 5–8-fold stimulation of lysyl-tRNA synthetase activity upon growth. All of the stimulatory activity of the concentrated solution was determined experimentally to be accounted for by L-alanine. The optimal concentration for alanine in the 1 % bactopeptone–0.5 % yeast extract medium is 0.01 M, and it is not replaced at an equivalent concentration by lysine, glycine, serine, threonine, methionine, β-alanine, pyruvate or acetate (see Table VII). It may be mentioned that alanine was able to stimulate lysyl-tRNA synthetase activity in mutant strains other than IH2001. The kinetics of stimulation of lysyl-tRNA synthetase activity by alanine is shown in Fig. 3.

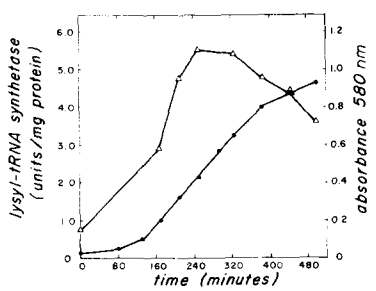

Fig. 3. Kinetics of lysyl-tRNA synthetase activity in strain IH 2001 grown in 1 %bactopeptone–0.5 % yeast extract medium containing 0.01 M alanine. The strain was pregrown in supplemented minimal medium *plus* lysine. The cultures were grown aerobically at 37°, and lysyl-tRNA synthetase activity measured as previously described. ●–●, absorbance; △–△, lysyl-tRNA synthetase activity.

TABLE VII

STIMULATION OF LYSYL-tRNA SYNTHETASE ACTIVITY BY ALANINE IN IH 2001 GROWN IN 1 % BACTOPEPTONE–0.5 % YEAST EXTRACT MEDIUM

Cells were grown in 1 % bactopeptone–0.5 % yeast extract medium to $A_{580\ nm}$ of 0.5. Lysyl-tRNA synthetase was then prepared and assayed as before.

Additions to medium (0.01 *M*)	*Lysyl-tRNA synthetase* (*units/mg protein*)
—	0.60
Alanine	3.7
Lysine	0.73
Glycine	0.58
Serine	0.70
Threonine	0.61
Methionine	0.65
Pyruvate	0.63
Acetate	0.68
β-Alanine	0.66

It was of interest to know whether alanine would stimulate lysyl-tRNA synthetase activity in a simple, defined medium such as minimal medium, and also if alanine would have any effect on the wild-type lysyl-tRNA synthetase activity. The answers to both of these questions were positive (Table VIII). The optimal concentration of alanine in the minimal medium system is 0.01–0.02 M and with mutant strain IH2001 a 5–7-fold increase in lysyl-tRNA synthetase activity was routinely seen.

TABLE VIII

STIMULATION OF LYSYL-tRNA SYNTHETASE ACTIVITY BY ALANINE IN STRAIN IH 2000 AND IH 2001 IN MINIMAL MEDIUM

Cells were grown in minimal medium to a $A_{490\ nm}$ of 0.3, and in supplemented minimal medium to $A_{580\ nm}$ of 0.3. In 1 % bactopeptone–0.5 yeast extract IH 2000 was grown to $A_{580\ nm}$ of 0.5. Lysyl-tRNA synthetase was then prepared and assayed as described under MATERIALS AND METHODS.

Strain	*Growth medium*	*Lysyl-tRNA synthetase* (*units/mg protein*)
IH 2001	Minimal	0.60
IH 2001	Minimal *plus* 0.02 M alanine	4.10
IH 2000	Minimal	7.6
IH 2000	Minimal *plus* 0.02 M alanine	11.1
IH 2000	Supplemented minimal medium *plus* lysine	11.0
IH 2000	1 % Bactopeptone–0.5 % yeast extract	11.4
IH 2000	1 % Bactopeptone–0.5 % yeast extract *plus* 0.01 M alanine	14.2

Although alanine does stimulate the activity of the wild-type enzyme, the effect is considerably lower than that observed in the mutant strain. As mentioned previously, the activity of the wild-type enzyme is lower in minimal medium than in 1 % bactopeptone–0.5 % yeast extract or supplemented minimal medium. Lastly, as might be expected, the ability of alanine to stimulate lysyl-tRNA synthetase activity in either strain was prohibited by rifampicin or chloramphenicol.

DISCUSSION

In this report we have described studies on the isolation and physiological characterization of a novel class of aminoacyl-tRNA synthetase mutants. The most striking feature of these mutants is their growth-medium-dependent lysyl-tRNA synthetase activity. Although an exhaustive survey of other aminoacyl-tRNA synthetases was not taken, the glutamyl- and arginyl-tRNA synthetases had the same activity in the mutant and wild-type strains in all media used. Moreover, the activity of independently isolated arginyl-tRNA synthetase mutants[5] showed only a slight variation with changes in growth medium. These results suggest that only the lysyl-tRNA synthetase may be affected in the mutant strains and that the large variation in activity with the growth medium is a peculiar characteristic of this mutant class. Another characteristic of these strains, which will be described in greater depth in the accompanying paper[20], is the increased thermostability of the lysyl-tRNA synthetase from the mutant strains when compared to the wild type.

At this stage of our work the increase in lysyl-tRNA synthetase specific activity cannot be definitely ascribed to any particular mechanism. However, the results based on the studies with the inhibitors, rifampicin and chloramphenicol as well as those described in Table VI, suggest that the fluctuation in the activity of lysyl-tRNA synthetase is not the result of an activation–inactivation phenomenon, but due to changes in the level of the enzyme. At least four hypotheses could account for the variation in the enzyme's level. (A) There are two distinct lysyl-tRNA synthetases, but one is synthesized only under certain growth conditions, such as in AC broth. (B) What we have been observing is the suppression of a missense mutation by some factor in the AC broth. According to this hypothesis, only the apparent level of the enzyme would increase in the AC broth, as the suppression would result in a more active form of the enzyme. (C) A mutation has affected the control of the enzyme's synthesis, and there is a factor(s) in the AC broth which restores the rate of enzyme synthesis to nearly normal. (D) A mutation has occurred in the structural gene for lysyl-tRNA synthetase which makes the enzyme susceptible to selective degradation under certain conditions of growth. According to this view, when the lysyl-tRNA synthetase activity is high, its degradation is prevented; and when the enzyme's activity is low, its degradation is proceeding at approximately the same rate as its synthesis. There is a precedent for the occurrence of such an event in bacteria, for recently GOLDSCHMIDT[33] has shown that β-galactosidase nonsence fragments are rapidly degraded in *E. coli*, whereas PLATT *et al.*[34] have reported that a mutant lac repressor is selectively degraded in the same organism. In the following paper[20] evidence is presented which appears to eliminate the first two hypotheses, but cannot unequivocally distinguish between the latter two.

Although alanine does stimulate lysyl-tRNA synthetase activity, it does not appear to account for the entire increase in activity that occurs when either the mutant or wild-type strains are grown in AC broth. Moreover, it remains to be seen whether alanine is a factor present in the broth filtrate. It is somewhat disturbing that such a high concentration of alanine is required to produce its maximum effect. If alanine is actually a stimulating factor, it may be that it acts cooperatively with another factor, and under such circumstances lower concentrations of alanine would be required. That this might actually happen is suggested by the finding that

lysyl-tRNA synthetase activity in the wild-type strain is approximately the same whether the strain is grown in supplemented minimal medium or in minimal medium *plus* alanine (see Table VIII). In the former medium the alanine concentration is 0.002 M, whereas in the latter it is 0.02 M. An alanine concentration of 0.002 M has no effect on lysyl-tRNA synthetase activity in minimal medium.

It is interesting that the activity of the wild-type lysyl-tRNA synthetase is lowest in minimal medium, which is the poorest growth medium, and highest in AC broth, which is the richest medium. This suggests that the factor(s) which can influence the activity of lysyl-tRNA synthetase in the mutant strain may normally have some role in regulating the activity of the enzyme in the wild-type strain. This thought is reinforced by the fact that alanine stimulates the activity of the enzyme in both strains. Both SUSSMAN AND GILVARG[35], and GOLDBERG[36] have presented evidence which indicates that an inverse relationship exists between net RNA synthesis and protein degradation. That is, under circumstances where RNA synthesis is high, protein degradation is low. The fact that the wild-type lysyl-tRNA synthetase level is lowest in minimal medium and highest in AC broth is consistent with the idea that some degradation of the enzyme may occur in poorer growth media even during exponential growth, and that the events in the mutant strain are an amplification of this process.

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REFERENCES

1 P. C. ZAMECNIK, *Harvey Lect.*, 54 (1960) 256.
2 L. EIDLIC AND F. C. NEIDHARDT, *Proc. Natl. Acad. Sci. U.S.*, 53 (1965) 539.
3 R. J. ROTH AND B. N. AMES, *J. Mol. Biol.*, 22 (1966) 325.
4 F. C. NEIDHARDT, *Bacteriol. Rev.*, 30 (1966) 701.
5 I. N. HIRSHFIELD, R. DE DEKEN, P. C. HORN, D. A. HOPWOOD AND W. K. MAAS, *J. Mol. Biol.*, 35 (1968) 83.
6 W. F. DOOLITTLE AND C. YANOFSKY, *J. Bacteriol.*, 95 (1968) 1283.
7 A. SZENTERMAI, M. SZENTERMAI AND H. E. UMBARGER, *J. Bacteriol.*, 95 (1968) 1672.
8 G. NASS AND F. C. NEIDHARDT, *Biochim. Biophys. Acta*, 134 (1967) 347.
9 L. S. WILLIAMS AND F. C. NEIDHARDT, *J. Mol. Biol.*, 43 (1969) 529.
10 J. HEINONEN, *Acta Chem. Scand.*, 22 (1968) 883.
11 J. HEINONEN, *FEBS Lett.*, 9 (1970) 252.
12 M. BRENNER, F. DE LORENZO AND B. N. AMES, *J. Biol. Chem.*, 245 (1970) 450.
13 W. L. FANGMAN AND F. C. NEIDHARDT, *J. Biol. Chem.*, 239 (1964) 1839.
14 A. BOCK, L. E. FAIMAN AND F. C. NEIDHARDT, *J. Bacteriol.*, 92 (1966) 1076.
15 I. N. HIRSHFIELD AND H. P. J. BLOEMERS, *J. Biol. Chem.*, 244 (1969) 2911.
16 M. YANIV AND F. GROS, *J. Mol. Biol.*, 44 (1969) 31.
17 S. KAPLAN AND D. ANDERSON, *J. Bacteriol.*, 95 (1968) 991.
18 E. J. MURGOLA AND E. A. ADELBERG, *J. Bacteriol.*, 103 (1970) 178.
19 W. STEINBERG AND C. ANAGNOSTOPOULOS, *J. Bacteriol.*, 105 (1971) 6.
20 I. N. HIRSFIELD, J. W. TOMFORD AND P. C. ZAMECNIK, *Biochim. Biophys. Acta*, 259 (1972) 344.
21 B. D. DAVIS AND E. S. MINGIOLI, *J. Bacteriol.*, 60 (1950) 17.

22 R. P. NOVICK AND W. K. MAAS, *J. Bacteriol.*, 81 (1961) 236.
23 A. LOVELESS AND S. HOWARTH, *Nature*, 184 (1959) 1780.
24 P. H. COOPER, I. N. HIRSHFIELD AND W. K. MAAS, *Mol. Gen. Genet.*, 104 (1969) 383.
25 J. F. SCOTT, in L. GROSSMAN AND K. MOLDAVE, *Methods in Enzymology*, Vol. 12, Academic Press, New York, 1968, p. 173.
26 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
27 R. STERN AND A. H. MEHLER, *Biochem. Z.*, 342 (1965) 400.
28 K. SHORTMAN AND I. R. LEHMAN, *J. Biol. Chem.*, 239 (1964) 2964.
29 G. P. TOCHINI-VALENTINI, P. MARINO AND A. J. COVILL, *Nature*, 220 (1968) 275.
30 E. DI MAURO, L. SNYDER, P. MARINO, A. LAMBERTI, A. COPPO AND G. P. TOCCHINI-VALENTINI, *Nature*, 222 (1969) 533.
31 E. F. GALE AND L. P. FOLKES, *Biochem. J.*, 53 (1953) 493.
32 C. L. WISSEMAN, J. E. SMADEL, F. E. HAHN AND H. E. HOPP, *J. Bacteriol.*, 67 (1954) 662.
33 R. GOLDSCHMIDT, *Nature*, 228 (1970) 1151.
34 T. PLATT, J. H. MILLER AND K. WEBER, *Nature*, 228 (1970) 1154.
35 A. J. SUSSMAN AND C. GILVARG, *J. Biol. Chem.*, 244 (1969) 6304.
36 A. L. GOLDBERG, *Proc. Natl. Acad. Sci. U.S.*, 68 (1971) 362.