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Effect of Alanine, Leucine, and Fructose on Lysyl-Transfer Ribonucleic Acid Ligase Activity in a Mutant of *Escherichia coli* K-12

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A mutant of *Escherichia coli* K-12 was examined which has growth medium-dependent lysyl-transfer ribonucleic acid (tRNA) ligase activity. In minimal medium or 0.5% yeast extract, the activity of the enzyme in the mutant strain was 5 to 10% of wild type. However, when the mutant was grown in a highly enriched medium, such as AC broth (Difco), the activity of the mutant ligase increased 10- to 20-fold. We found that the supplementation of 0.5% yeast extract by L-alanine plus D-fructose replaces the need for the highly enriched medium. Fructose plus L-leucine and fructose plus L-α-amino-*n*-butyric acid were also stimulatory, but not as effective as fructose and alanine. With minimal medium, a combination of carbohydrate (fructose or glucose) plus alanine and leucine was required to replace the enriched medium. The most effective combination was fructose, glucose, alanine, and leucine. Lysyl-tRNA ligase was stimulated 1.5- to 2-fold in the wild-type strain or Hfr H (Hayes) by fructose plus alanine when these strains were cultured in 0.5% yeast extract. Experiments employing the combined technique of density labeling with D_2O and isopycnic centrifugation in cesium chloride indicated that the increased activity of lysyl-tRNA ligase observed in AC broth or in the presence of fructose, glucose, alanine, and leucine is due to the synthesis of new enzyme.

The mutant of *Escherichia coli* K-12 (IH 2001) with growth medium-dependent lysyl-transfer ribonucleic acid (tRNA) ligase (L-lysine:tRNA ligase [adenosine monophosphate], EC 6.1.1.6) activity examined in this report was isolated by its resistance to the lysine analogue thiosine (9). When this mutant was grown in minimal-glucose medium or 0.5% yeast extract, the lysyl-tRNA ligase activity was 5 to 10% that of the wild-type strain. However, upon growing the strain in a highly enriched medium such as 5% yeast extract or Difco AC broth, the mutant ligase activity approximated that of the wild-type enzyme (9). Studies on the lysyl-tRNA ligase from the mutant have revealed that the enzyme is altered, and is more resistant to thermal and urea denaturation than the wild-type ligase (8).

Earlier work (9) showed that alanine but not lysine can stimulate lysyl-tRNA ligase activity five- to eightfold in mutant IH 2001 when it is grown in minimal-glucose or 0.5% yeast extract medium. The influence of alanine on lysyl-tRNA ligase in the mutant strain is peculiar, since other studies have shown that the activity of an aminoacyl-tRNA ligase is usually regulated by its cognate amino acid (1, 15, 22).

Previous studies with mutant IH 2001 have suggested that at least one other factor in addition to alanine is required to restore the activity of lysyl-tRNA ligase in the strain to the wild-type level. One approach which might be used to find the putative missing factor(s) is to look for biological parallels. One biological system in which alanine alone or in combination with other compounds is important is the initiation of spore germination in *Bacillus* (5, 11, 20). We therefore decided to use combinations of compounds effective in initiating spore germination in *Bacillus* as a guide in an attempt to find the other factor(s) needed in addition to alanine to restore lysyl-tRNA ligase activity in mutant IH 2001 to the wild-type level. By using this strategy, we have found that the addition of the combination of alanine and fructose to 0.5% yeast extract restores the

lysyl-tRNA ligase activity in the mutant to the wild-type levels. In minimal medium, the restoration of mutant lysyl-tRNA ligase activity to the wild-type level is best accomplished by the combination of glucose, fructose, alanine, and leucine.

MATERIALS AND METHODS

Strains. Strains IH 2000 (wild-type) and IH 2001 (mutant) have been described previously. Strain Hfr Hayes (Hfr H) is Thi^- and Str^s. Strain Hfr Cavalli (Hfr C) is prototrophic and Str^s.

Media. Cells were grown in minimal medium (3) or yeast extract medium, supplemented as described in the text. Unless stated otherwise, the carbon source used with minimal medium was 0.4% glucose.

Yeast extract was stored as a 25% solution and diluted in minimal medium to a concentration of 0.5% for the experiments. Stock solutions of 25% yeast extract over 3 weeks old were discarded. AC broth (Difco) was also made up in minimal medium.

All experiments were conducted under aerobic conditions at 37 C. Growth of cells in minimal medium was monitored at 490 nm, and growth in 0.5% yeast extract or AC broth was monitored at 580 nm.

Materials. Chemical compounds were of highest quality from commercial institutions. Uniformly labeled L-[^{14}C]amino acids were obtained from New England Nuclear Corp., Boston, Mass. or Schwarz/Mann, Orangeburg, N.Y. Cesium chloride was purchased from Fisher Scientific Co., Boston, Mass. Ultrafiltration membranes and apparatus were purchased from Amicon Corp., Lexington, Mass.

Aminoacyl-tRNA ligase assays. The activity of lysyl-, glutamyl-, arginyl-, and methionyl-tRNA ligase was measured by the ability of each to charge tRNA with the appropriate L-[^{14}C]amino acid. The assay conditions and standard incubation mixture for lysyl-, arginyl-, and glutamyl-tRNA ligase have been previously described (9). However, in these experiments, 10 mM mercaptoethanol was substituted for glutathione, and the specific activity of L-[^{14}C] glutamic acid was 10 μCi/μmole.

The methionyl-tRNA ligase activity was assayed under the same conditions as the other ligases, but for only 3 min. In these assays, 0.02 μmole of methionine (10 μCi/μmole) was used. All assays were conducted at *p*H 7.25 unless noted otherwise in the text. One unit of enzyme activity is defined as that amount of enzyme which catalyzes the incorporation of 1 μmole of amino acid into tRNA per min at 37 C. Specific activity is defined as the number of units per milligram of total protein.

Preparation of crude enzyme extracts. In all experiments, except those involving isopycnic centrifugation in cesium chloride, cells were harvested by centrifugation and washed once in 0.01 M tris(hydroxymethyl)aminomethane (*p*H 7.3)-0.01 M $MgCl_2$. The samples in the above buffer were treated ultrasonically for 20 to 30 sec at setting 5 on a Branson sonic cell disruptor and were then centrifuged at 12,000 $\times$ *g* for 15 min on a Sorvall refrigerated centrifuge at 0 to 4 C to sediment unbroken cells and cell debris. The supernatant fluid served as the crude extract. In experiments involving isopycnic centrifugation in cesium chloride, the cells were washed and sonically treated in 0.006 M potassium phosphate buffer, *p*H 7.4.

Protein determination. Protein was determined by the method of Lowry et al. (14), with crystalline bovine serum albumin (Sigma Chemical Co.) as a standard.

Density labeling and isopycnic centrifugation in cesium chloride. The mutant strain IH 2001 was grown in minimal medium plus lysine (100 μg/ml) with 0.4% glucose as a carbon source. The strain was then adapted to growth in minimal-lysine-glucose medium containing 20, 40, and 80% D_2O by volume. The strain was grown for at least eight generations at each step. It might be noted that growth in the presence of deuterium slows the rate of growth of IH 2001 from a generation time of 83 min in minimal-lysine-glucose medium to 150 to 180 min when 80% D_2O is present. After growth in 80% D_2O, the culture was centrifuged in a table-model centrifuge at top speed for 10 min at room temperature to pellet the cells. The cells were suspended in minimal medium (no carbon source present) and recentrifuged. The pellet was then resuspended in a small volume of AC broth filtrate and subsequently added to a flask containing the broth filtrate to an absorbance of ~0.1 at 580 nm. A sample was taken at this time and after one generation of growth in the broth filtrate for analysis by isopycnic centrifugation in cesium chloride.

The AC broth filtrate was prepared as follows. The mutant strain IH 2001 was grown in AC broth to an absorbance of 0.65 to 0.75 at 580 nm. The culture was then centrifuged at 12,000 $\times$ *g* for 15 min at 0 to 4 C in a Sorvall refrigerated centrifuge. The supernatant fluid was filtered (65 μm pore size; Millipore Corp.) to remove any contaminating microorganisms. The supernatant fluid was then subjected to Diaflow ultrafiltration through a UM-10 and UM-2 membrane, the latter having a molecular weight cut-off point of 1,000. After each step, the filtrate was passed through a 65-μm filter to remove microbial contamination. The UM-2 filtrate was the one used in the experiment.

In a second experiment, cells were grown in minimal-lysine-glucose medium and adapted to grow in the presence of 80% D_2O as described above. The cells were also centrifuged and washed as above and then resuspended in a small volume of minimal medium (no D_2O) containing fructose (20 mM), glucose (20 mM), L-alanine (10 mM), L-leucine (10 mM), and L-lysine (100 μg/ml). The cells were then added to a flask of this medium to an absorbance of 0.1 at 490 nm. A sample was saved at this time and after one generation of growth for analysis by isopycnic centrifugation in cesium chloride.

The cesium chloride isopycnic centrifugation was run according to the procedure of Williams and Neidhardt (22). In the experiments reported here, mercaptoethanol was omitted from the buffer (potassium phosphate, *p*H 7.4, 0.006 M). From 0.28 to 1.0

mg of protein was applied to the gradient. Upon completion of centrifugation, the tubes were pierced at the bottom, and 30 four-drop or 40 three-drop fractions were collected. Lysyl-tRNA ligase activity was assayed at pH 7.8 for 10 minutes by use of 5 to 10 μliters of each fraction. The refractive index was determined with a Zeiss refractometer and converted into density by use of standard tables.

RESULTS

Effect of alanine and its analogues on lysyl-tRNA ligase activity. As shown in Table 1, L-α-amino-n-butyric acid (ABA) was the only compound other than alanine which enhanced lysyl-tRNA ligase activity greater than twofold. However, ABA was not as effective as alanine at a 10 mM concentration. ABA is also regarded as an analogue of valine, since it can be activated by the valyl-tRNA ligase in bacteria (21) and yeast (4), but in this study valine had no effect.

Effect of leucine on lysyl-tRNA ligase activity. A combination of L-alanine (10 mM) and inosine or adenosine (0.015 to 0.15 mM) did not enhance the activity of lysyl-tRNA ligase more than alanine alone (data not shown). As indicated in Table 2, leucine clearly had an effect on lysyl-tRNA ligase activity. Although leucine analogues were not extensively tested, the enhancement of lysyl-tRNA ligase activity by leucine was not found with isoleucine or the keto analogue of leucine, α-ketoisocaproic acid.

Leucine was also effective in stimulating lysyl-tRNA ligase in combination with alanine, and the effect of alanine and leucine was additive. Moreover, the combination of alanine plus glycine, or serine, did not increase lysyl-tRNA ligase activity any more than alanine alone, indicating that the combination of alanine and leucine is specific, and does not produce its effect nonspecifically by increasing the ionic strength of the medium.

This is further emphasized by the stimulation of lysyl-tRNA ligase activity by the combination of L-alanine and ABA. In this case, the stimulation by the combination was even less than that by L-alanine alone (data not shown), suggesting that L-alanine and ABA compete for a common site.

Effect of carbohydrate and amino acid combinations on lysyl-tRNA ligase activity. The combination of alanine and leucine routinely increased lysyl-tRNA ligase activity 10- to 12-fold in the mutant strain grown in 0.5% yeast extract. However, shifting strain IH 2001 from 0.5% yeast extract to 5% yeast extract or AC broth usually resulted in a 15- to 20-fold increase in the activity of the enzyme. This greater effect could be reproduced if D-fructose was added in combination with alanine or alanine plus leucine to 0.5% yeast extract medium as shown in Table 3. The combination of fructose and alanine was synergistic in stim-

TABLE 1. *Effect of alanine and its analogues on lysyl-tRNA ligase activity in strain IH 2001 grown in 0.5% yeast extract medium*[a]

Addition to medium[b]	Specific activity[c] (units/mg)
None	0.6×10^{-4}
L-Alanine	3.60×10^{-4}
L-α-Amino-n-butyric acid	2.3×10^{-4}
D L-β-Aminobutyric acid, 20 mM	1.05×10^{-4}
1-Aminoethylphosphoric acid	0.90×10^{-4}
β-Chloroalanine	0.70×10^{-4}
2-Methylalanine	0.75×10^{-4}
D-Alanine	0.55×10^{-4}
β-Alanine	0.60×10^{-4}
Pyruvate	0.70×10^{-4}
L-γ-Aminobutyric acid	0.60×10^{-4}
Glycine	0.50×10^{-4}
L-Serine	0.40×10^{-4}
L-Cysteine	0.40×10^{-4}

[a] Mutant strain IH 2001 was grown in 0.5% yeast extract medium from an A_{680} of 0.02 to an A_{680} of 0.3 to 0.35. All samples were tested at least in duplicate in this experiment and those presented in Tables 2–4.

[b] The concentration of the compounds was 10 mM except where noted.

[c] The basal specific activity of lysyl-tRNA ligase in IH 2001 in 0.5% yeast extract varied from 0.4×10^{-4} to 0.8×10^{-4} units/mg, and in Tables 1–4 is normalized to 0.6×10^{-4} units/mg.

TABLE 2. *Stimulation of lysyl-tRNA ligase activity in strain IH 2001 by leucine*[a]

Addition to medium[b]	Specific activity (units/mg)	Fold increase over control[c]
None	0.6×10^{-4}	
L-Leucine	2.6×10^{-4}	4.3
L-Isoleucine	0.5×10^{-4}	
α-Ketoisocaproic acid	0.6×10^{-4}	
L-α-Amino-n-butyric acid (L-ABA)	2.3×10^{-4}	3.6
L-Alanine	3.5×10^{-4}	5.9
L-Serine	0.5×10^{-4}	
L-Glycine	0.5×10^{-4}	
L-Alanine + L-leucine	6.9×10^{-4}	11.5
L-Alanine + L-serine	3.3×10^{-4}	5.7
L-Alanine + glycine	3.4×10^{-4}	5.7
L-Leucine + L-ABA	6.0×10^{-4}	10.0

[a] Mutant strain IH 2001 was grown in 0.5% yeast extract medium from an A_{680} of 0.02 to an A_{680} of 0.3 to 0.35.

[b] The concentration of all compounds was 10 mM.

[c] Control sample was 0.5% yeast extract alone.

TABLE 3. *Stimulation of lysyl-tRNA ligase activity by a combination of carbohydrates and amino acids in strain IH 2001 grown in 0.5% yeast extract*[a]

Addition of medium[b]	Specific activity (units/mg)	Fold increase over control[c]
None	0.6×10^{-4}	
L-Alanine	3.5×10^{-4}	5.9
L-Leucine	2.5×10^{-4}	4.2
L-α-Amino-n-butyric acid (L-ABA)	1.8×10^{-4}	3.0
D-Fructose	1.4×10^{-4}	2.3
D-Glucose	0.6×10^{-4}	
L-Alanine + D-glucose	3.3×10^{-4}	5.5
L-Leucine + D-glucose	2.8×10^{-4}	4.7
L-Alanine + D-fructose	11.3×10^{-4}	18.8
L-Leucine + D-fructose	5.3×10^{-4}	8.8
L-ABA + D-fructose	4.6×10^{-4}	7.7
L-Alanine + L-leucine + D-fructose	13.3×10^{-4}	22.2
L-Alanine + glycerol	3.5×10^{-4}	5.9
L-Alanine + succinate	4.0×10^{-4}	6.7
L-Alanine + D-sorbitol	4.1×10^{-4}	6.8
L-Alanine + L-xylose	3.3×10^{-4}	5.5
L-Alanine + D-mannose	3.9×10^{-4}	6.5
L-Alanine + L-arabinose	5.0×10^{-4}	8.3

[a] Mutant strain IH 2001 was grown in 0.5% yeast extract medium from an A_{580} of 0.02 to an A_{580} of 0.3 to 0.35.

[b] Concentration of amino acids was 10 mM and carbohydrates 0.4% (~20 mM) except where indicated. Glycerol and succinate were also used at 20 mM.

[c] Control was 0.5% yeast extract alone.

ulating lysyl-tRNA ligase activity in the mutant, in that the two together were considerably more effective in enhancing the ligase activity than would be expected from their individual effects. The stimulation of lysyl-tRNA ligase activity by fructose and alanine in IH 2001 grown in 0.5% yeast extract was nearly 20-fold. Thus, the combination of these two compounds can completely replace the need for a highly enriched medium in elevating lysyl-tRNA ligase activity in the mutant strain to the wild-type level when the mutant is grown in 0.5% yeast extract medium. In these experiments, the mutant was grown for four generations to allow maximal stimulation of lysyl-tRNA ligase to be expressed.

Addition of such other carbon sources as D-glucose, D-mannose, L-arabinose, L-xylose, D-sorbitol, glycerol, or succinate to 0.5% yeast extract medium along with alanine was not more effective than alanine alone in enhancing lysyl-tRNA ligase activity in the mutant. Figure 1 presents a concentration curve for the effectiveness of D-fructose, and Fig. 2, for L-

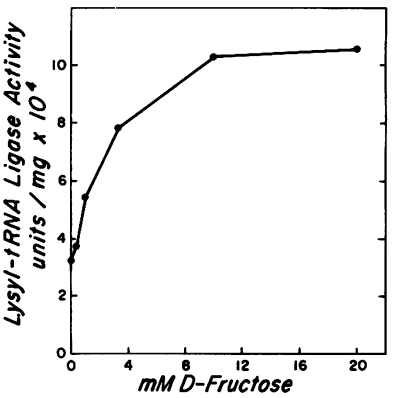

FIG. 1. *Effect of increasing the concentration of D-fructose on the stimulation of lysyl-tRNA ligase activity in strain IH 2001. The strain was grown in 0.5% yeast extract medium for four generations with each concentration of D-fructose. The concentration of D-fructose was varied from 0.4 to 20 mM in these experiments, and alanine (10 mM) was present in all cultures. Lysyl-tRNA ligase activity was measured as described in Materials and Methods.*

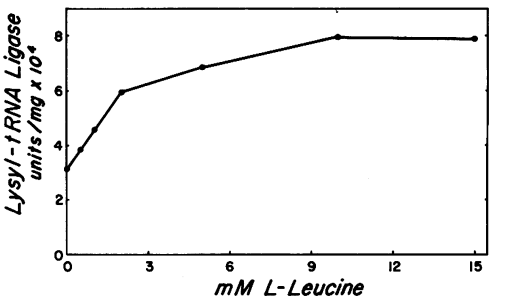

FIG. 2. *Effect of concentration of L-leucine on the stimulation of lysyl-tRNA ligase activity in strain IH 2001. The strain was grown in 0.5% yeast extract medium for four generations with each concentration of L-leucine. The concentration of L-leucine was varied from 0.5 to 15 mM, and alanine (10 mM) was present in all cultures. Lysyl-tRNA ligase activity was measured as described in Materials and Methods.*

leucine, in elevating lysyl-tRNA ligase activity in the mutant in 0.5% yeast extract. The optimal effect for fructose was achieved at 10 to 20 mM and for leucine at 10 to 15 mM. These experiments were run in the presence of 10 mM alanine, which has previously been shown to be the optimal concentration in 0.5% yeast extract (9). It might be added that alanine had only a slight effect on the ligase activity at 1 mM, but the effect gradually increased with concentration to an optimum at 10 mM. In Fig. 3 are presented kinetic data on the increase in lysyl-tRNA ligase activity in mutant IH 2001 grown in 0.5% yeast extract with fructose and alanine.

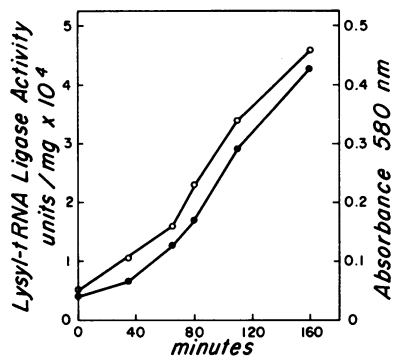

FIG. 3. *Kinetics of lysyl-tRNA ligase activity in strain IH 2001 grown in 0.5% yeast extract plus fructose and alanine. The strain was grown at 37 C for three generations in this experiment. The concentration of* D-*fructose was 20 mM and* L-*alanine was 10 mM. Lysyl-tRNA ligase activity was assayed as described in Materials and Methods.* (○) *Absorbance at 580 nm;* (●) *specific activity of lysyl-tRNA ligase.*

The increase was gradual over three generations. The greatest increase in lysyl-tRNA ligase activity in one generation that we have observed in this medium is fourfold (Hirshfield and Buklad, *unpublished data*).

We then asked whether fructose and alanine would have the same stimulatory effect on lysyl-tRNA ligase activity if the mutant strain was grown in minimal medium instead of 0.5% yeast extract. We found that the requirements for stimulating lysyl-tRNA ligase in minimal medium were different (Table 4). Either glucose or fructose in combination with alanine or leucine, or both, was able to stimulate lysyl-tRNA ligase activity when IH 2001 was grown in minimal medium. Moreover, leucine plus glucose or fructose enhanced lysyl-tRNA ligase activity more effectively than alanine plus the carbohydrates. No data are presented for alanine or leucine alone, because at 10 mM these amino acids could not serve as carbon sources; however, the combination of the two was able to support the growth of the culture. The addition of other carbon sources such as glycerol and succinate to alanine plus leucine resulted in a higher lysyl-tRNA ligase activity in the mutant than that attained with alanine plus leucine. Glycerol and succinate were not as effective as glucose and fructose. Like glucose, glycerol and succinate had little effect in 0.5% yeast extract medium (see Table 3). The best stimulation of lysyl-tRNA ligase activity was achieved by the combination of fructose, glucose, alanine, and leucine. The stimulation was approximately 15-fold, and amounts to full restitution of lysyl-tRNA ligase activity, as the wild-type

TABLE 4. *Stimulation of lysyl-tRNA ligase activity by a combination of carbohydrates and amino acids in strain IH 2001 grown in minimal medium*[a]

Addition to medium[b]	Specific activity (units/mg)
D-Glucose	0.52×10^{-4}
D-Fructose	0.57×10^{-4}
Glycerol	0.50×10^{-4}
Succinate	0.65×10^{-4}
D-Glucose + L-alanine	2.70×10^{-4}
D-Fructose + L-alanine	2.75×10^{-4}
D-Glucose + L-leucine	3.30×10^{-4}
D-Fructose + L-leucine	4.30×10^{-4}
L-Alanine + L-leucine	2.00×10^{-4}
D-Glucose + L-alanine + L-leucine	5.30×10^{-4}
D-Fructose + L-alanine + L-leucine	5.80×10^{-4}
Glycerol + L-alanine + L-leucine	3.60×10^{-4}
Succinate + L-alanine + L-leucine	2.90×10^{-4}
L-Xylose + L-alanine + L-leucine	1.50×10^{-4}
D-Glucose + D-fructose + L-alanine + L-leucine	7.20×10^{-4}

[a] Cells were grown from an A_{490} of 0.01 to an A_{490} of 0.16 to 0.20 in minimal medium.

[b] The concentration of amino acids was 10 mM; that of carbohydrates, glycerol, and succinate was 20 mM.

level in minimal medium is 7×10^{-4} to 8×10^{-4} units/mg. It should be noted that in these experiments, as in 0.5% yeast extract medium, the cells were grown for four generations to allow for maximal stimulation of lysyl-tRNA ligase activity.

Effect of glucose, fructose, alanine, and leucine on the wild-type lysyl-tRNA ligase. It was of importance to know whether the effects of the carbohydrates and amino acids were peculiar to the mutant strain, or whether this combination would also stimulate lysyl-tRNA ligase activity in the wild-type strain. A positive result in the wild-type strain would suggest that the effect of the carbohydrate-amino acid combination on the lysyl-tRNA ligase of the mutant was an amplification of normal events. Moreover, it was of interest to know whether only lysyl-tRNA ligase activity would be stimulated by the carbohydrate-amino acid combination. The data given in Table 5 show that with 0.5% yeast extract medium not only lysyl- but also methionyl-, arginyl-, and glutamyl-tRNA ligase activity is increased 1.5 to 2-fold by fructose plus alanine in IH 2000 (wild-type) and another K-12 strain, Hfr H. This is the range of stimulation of wild-type lysyl-tRNA ligase when IH 2000 is grown in AC broth (9). However, in minimal medium, only lysyl-tRNA ligase activity was

TABLE 5. *Effect of fructose, glucose, alanine, and leucine on the activity of lysyl-, arginyl-, glutamyl-, and methionyl-tRNA ligase in wild-type strains*

Growth medium[a]	Strain	Addition to medium[b]	Aminoacyl-tRNA ligase	Specific activity (unis/mg)
Yeast extract	IH 2000	—	Lysine	11.3×10^{-4}
	IH 2000	Alanine + fructose	Lysine	19.4×10^{-4}
	Hfr H	—	Lysine	11.0×10^{-4}
	Hfr H	Alanine + fructose	Lysine	16.6×10^{-4}
	IH 2000	—	Arginine	35.1×10^{-4}
	IH 2000	Alanine + fructose	Arginine	55.7×10^{-4}
	Hfr H	—	Arginine	46.1×10^{-4}
	Hfr H	Alanine + fructose	Arginine	82.7×10^{-4}
	IH 2000	—	Glutamic acid	4.3×10^{-4}
	IH 2000	Alanine + fructose	Glutamic acid	6.7×10^{-4}
	Hfr H	—	Glutamic acid	8.0×10^{-4}
	Hfr H	Alanine + fructose	Glutamic acid	13.1×10^{-4}
	IH 2000	—	Methionine	11.5×10^{-4}
	IH 2000	Alanine + fructose	Methionine	17.8×10^{-4}
	Hfr H	—	Methionine	19.3×10^{-4}
	Hfr H	Alanine + fructose	Methionine	27.9×10^{-4}
Minimal	IH 2000	Glucose	Lysine	7.7×10^{-4}
	IH 2000	Glucose + fructose + alanine + leucine	Lysine	13.9×10^{-4}
	IH 2000	Glucose	Arginine	29.4×10^{-4}
	IH 2000	Glucose + fructose + alanine + leucine	Arginine	27.8×10^{-4}
	IH 2000	Glucose	Glutamic acid	5.6×10^{-4}
	IH 2000	Glucose + fructose + alanine + leucine	Glutamic acid	6.3×10^{-4}
	IH 2000	Glucose	Methionine	13.5×10^{-4}
	IH 2000	Glucose + fructose + alanine + leucine	Methionine	13.1×10^{-4}

[a] Cells were grown in 0.5% yeast extract medium from an A_{580} of 0.02 to an A_{580} of 0.3 to 0.35. In minimal medium, cells were grown from an A_{490} of 0.01 to an A_{490} of 0.25.

[b] The concentration of amino acids was 10 mM; that of carbohydrates was 20 mM.

substantially stimulated in IH 2000 by glucose, fructose, alanine, and leucine.

Density labeling and isopycnic centrifugation experiments. A critical point is whether the increase in lysyl-tRNA ligase activity observed when the mutant strain is grown in AC broth (9) or AC broth filtrate (9), or in the presence of glucose, fructose, alanine, and leucine, is due to the activation of preexisting but inactive enzyme or to the formation of new enzyme. Previous work from this laboratory suggested that the increase in lysyl-tRNA ligase activity in the mutant strain was due to the formation of new protein (9). This conclusion was based on indirect experiments with chloramphenicol and rifampin, and here direct evidence is presented which substantiates the earlier interpretation.

The experiments presented here utilize the technique of density labeling with D_2O, and then separating heavy and light protein in a cesium chloride gradient (10, 22). In one experiment, mutant IH 2001 was shifted from D_2O-minimal medium to AC broth filtrate (no deuterium) for one generation of growth. Under these conditions, as much as a 10- to 15-fold stimulation of lysyl-tRNA ligase activity can be found in one doubling. If the increased activity is due to activation of preexisting but inactive enzyme, then one would expect lysyl-tRNA ligase activity to be almost exclusively associated with the heavy (deuterium) fraction on the gradient. However, if the increased activity of the ligase is due to the synthesis of new enzyme, then the activity should be associated with the light peak (water medium), and this is what was found (see Fig. 5). Figure 4 gives standard curves depicting the density of lysyl-tRNA ligase when IH 2001 was grown in minimal-glucose medium (80% D_2O) or in AC broth (water medium). In different experiments, the density (ρ) of the heavy peak varied in 80% D_2O minimal medium from 1.313 to 1.323; in medium with water, the density of the light peak varied from 1.291 to 1.297 (Fig. 4 and 5). The density of the heavy peak is somewhat lighter than reported in the work of Williams and Neidhardt (22), presumably because strain IH 2001, having several amino acid requirements, incorporated these compounds directly from

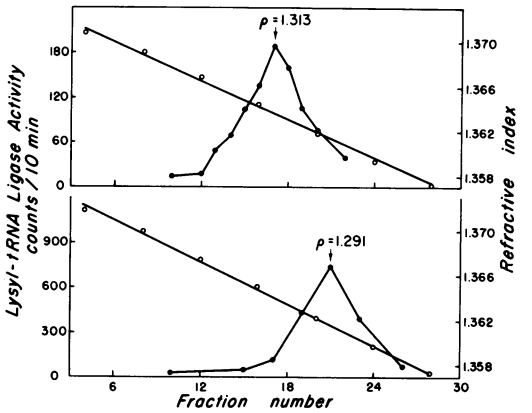

FIG. 4. *Isopycnic centrifugation of extracts from IH 2001 grown in 80% D_2O minimal medium or AC broth. In the top graph, strain IH 2001 was grown in 80% minimal medium with glucose (20 mM) as a carbon source. A cell extract was prepared by sonic treatment, and 280 μg of protein was centrifuged in a cesium chloride gradient at 39,000 rev/min for 65 hr. After the centrifugation was completed, 30 four-drop fractions were collected, and the refractive index of every fourth fraction was measured. Lysyl-tRNA ligase activity was assayed at pH 7.8, 37 C, for 10 min. Refractive index (○); lysyl-tRNA ligase activity (●). In the lower graph, IH 2001 was grown in AC broth (water medium) to an A_{580} of 0.7. A cell extract was prepared by sonic treatment, and 340 μg of protein was subjected to cesium chloride centrifugation at 39,000 rev/min for 65 hr. Fractions were collected and examined as above. Refractive index (○); lysyl-tRNA ligase (●).*

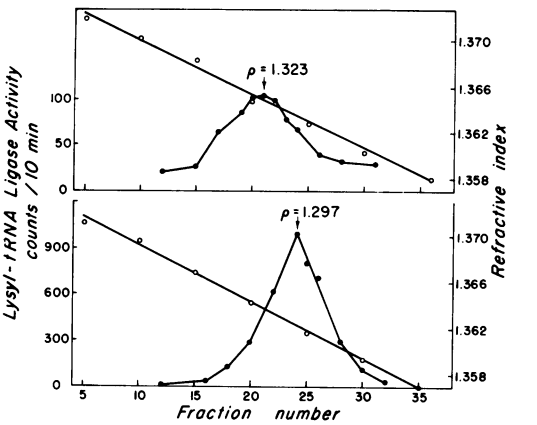

FIG. 5. *Effect of shifting from D_2O minimal medium to AC broth filtrate on the density distribution of lysyl-tRNA ligase from strain IH 2001 in a cesium chloride gradient. Strain IH 2001 was pregrown in 80% D_2O minimal medium with glucose (20 mM) as a carbon source. The culture was centrifuged sterilely to remove the medium, and the cells were added to AC broth filtrate to an initial A_{580} of 0.1. The cells were grown for one generation in this medium. Samples were taken from the initial inoculum (A_{580}, 0.1) and after one generation of growth for analysis by cesium chloride centrifugation. The cesium chloride centrifugation was run for 65 hr at 39,000 rev/min. In these experiments, 40 three-drop fractions were collected, and the refractive index of every fifth tube was measured. Lysyl-tRNA ligase activity was assayed as in Fig. 4. The top graph shows the results of the cesium chloride centrifugation of the initial inoculum (A_{580}, 0.1); 1.0 mg of protein was used in this experiment. The lower graph shows the results with the sample after one generation of growth; 600 μg of protein was used in this experiment. Refractive index (○); lysyl-tRNA ligase (●).*

the D_2O medium with less net deuterium therefore being present in any newly synthesized protein. As a confirmation of this statement, Hfr C, which is prototrophic, was grown in 80% D_2O-minimal medium, and an extract of it was run on a cesium chloride gradient. The density of the protein peak was 1.33 (figure not shown).

Table 6 shows that, when assayed at pH 7.25 or 7.8, the increase in lysyl-tRNA ligase activity after one doubling in the AC broth filtrate was about 10-fold. Control experiments have shown that (i) growth in D_2O does not inactivate the enzyme, and (ii) growth in D_2O does not diminish the response to stimulating agents (Hirshfield and Buklad, *unpublished data*).

In a second experiment, IH 2001 was grown in 80% D_2O minimal-glucose medium and shifted to minimal medium plus glucose, fructose, alanine, and leucine for one generation of growth. Under these conditions, a four- to fivefold increase in lysyl-tRNA ligase activity was obtained in one generation (Table 7). As with AC broth filtrate, the increase in lysyl-tRNA ligase activity was associated with the light fraction (Fig. 6). In this experiment, the light fraction was skewed toward the deuterium peak (see Fig. 6, lower graph). This was due to the fact that the heavy and light peaks overlapped somewhat, and because in this particular experiment after one generation of growth a significant fraction of the total activity was due to the deuterated protein that remained in the extract. The activity of the deuterated fraction accounts for approximately 12 to 14% of the total activity shown in the lower graph in Fig. 6.

DISCUSSION

The results reported in this manuscript are a continuation of the analysis of mutants with growth medium-dependent lysyl-tRNA ligase activity previously published (8, 9). These mutants have only 5 to 10% of the wild-type

TABLE 6. *Increase in lysyl-tRNA ligase activity in strain IH 2001 after one generation of growth in AC broth filtrate*[a]

Absorbance at 580 nm	Specific activity (units/mg)	pH[b]
0.1	0.39×10^{-4}	7.25
0.2	4.10×10^{-4}	7.25
0.1	1.24×10^{-4}	7.8
0.2	12.10×10^{-4}	7.8

[a] Strain IH 2001 was grown in 80% D_2O minimal medium and transferred to AC broth filtrate for one generation of growth. Lysyl-tRNA ligase was assayed from the initial sample in AC broth (A_{580}, 0.1) and after one generation of growth.

[b] The enzyme incubation was conducted for 5 min at pH 7.25, and for 3 min at pH 7.8 at 37 C. All assays were conducted in duplicate.

TABLE 7. *Increase in lysyl-tRNA ligase activity in strain IH 2001 upon shifting from 80% D_2O minimal-glucose medium to minimal medium + glucose, fructose, alanine, and leucine for one generation of growth*[a]

Absorbance at 490 nm	Specific activity[b] (units/mg)
0.1	0.7×10^{-4}
0.2	3.6×10^{-4}

[a] Mutant IH 2001 was grown in 80% D_2O-minimal medium + glucose (20 mM) and shifted to minimal medium + glucose (20 mM), fructose (20 mM), alanine (10 mM), and leucine (10 mM) for one generation of growth. Lysyl-tRNA ligase was measured from the initial inoculum (A_{490}, 0.1) and after one generation of growth.

[b] The assay was conducted at pH 7.25 for 5 min at 37 C in duplicate.

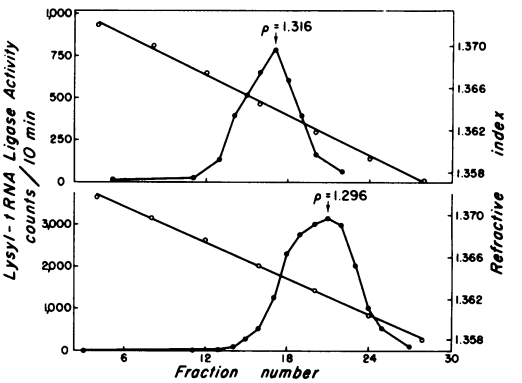

FIG. 6. *Effect of shifting from D_2O minimal medium to minimal medium containing glucose, fructose, alanine, and leucine on the density distribution of lysyl-tRNA ligase from strain IH 2001 in a cesium chloride gradient. Cells were pregrown in 80% D_2O minimal medium with glucose (20 mM) as a carbon source. The culture was centrifuged under sterile conditions to remove the medium, and the cells were added to a minimal medium culture supplemented with glucose and fructose (each 20 mM) and with leucine and alanine (each 10 mM) to an optical density of 0.1 at 490 nm. This culture was grown for one generation, and samples were taken from the initial inoculum (A_{490}, 0.1) and after one generation of growth for analysis by cesium chloride density centrifugation. The cesium chloride centrifugation was run at 39,000 rev/min for 65 hr. Upon completion of centrifugation, 30 four-drop fractions were collected, and in every fourth fraction the refractive index was measured. Lysyl-tRNA ligase activity was assayed as in Fig. 4. The top graph shows the density pattern of the initial inoculum (A_{490}, 0.1), and the lower graph shows the density distribution after one generation of growth. In each experiment, 800 μg of protein was used. Refractive index (○); lysyl-tRNA ligase activity (●).*

lysyl-tRNA ligase activity when grown in 0.5% yeast extract medium or minimal medium, respectively, but have normal ligase activity when grown in highly enriched media such as Difco AC broth or 5% yeast extract. In this work, analysis was confined to the mutant strain IH 2001. Of significance in the work reported here is the finding that a combination of carbohydrates and amino acids can enhance lysyl-tRNA ligase activity to the same degree as the highly enriched media, and can thus replace them. In 0.5% yeast extract medium, the combination of fructose and alanine sufficed, although the combination of fructose, alanine, and leucine was slightly more effective. In minimal medium, the maximal stimulation of lysyl-tRNA ligase activity was found with the combination of fructose, glucose, alanine, and leucine.

Whether alanine, leucine, fructose, and glucose are the factors in the highly enriched media which stimulate lysyl-tRNA ligase activity is not known. The presence of alanine and leucine in yeast extract filtrates has been demonstrated by thin-layer chromatography (Buklad, *unpublished data*), but the amounts have not yet been quantitated.

Another set of key experiments reported here show that the increase in lysyl-tRNA ligase activity seen upon shifting mutant IH 2001 from minimal medium to AC broth, or to minimal medium plus glucose, fructose, alanine, and leucine, is due to the formation of new enzyme, and not activation of preexisting but inactive enzyme. However, what cellular process is responsible for the fluctuation in the lysyl-tRNA ligase level in the mutant strain

remains to be discovered. When the mutant strain is shifted from 5 to 0.5% yeast extract medium, the activity of lysyl-tRNA ligase decreases by approximately one-half per generation (9). Such a result can be interpreted in several ways. For instance, the regulation of lysyl-tRNA ligase may be altered in such a fashion that the messenger RNA (mRNA) for the enzyme is transcribed at a normal rate only when sufficient alanine and fructose are present in the medium. A second interpretation is that the mutant ligase is susceptible to degradation, and in 0.5% yeast extract medium the rate of synthesis is equal to the rate of degradation. According to this view, fructose and alanine control some aspect of the degradative process and prevent the degradation. Alternatively, if the mRNA for lysyl-tRNA ligase were rapidly inactivated in 0.5% yeast extract, then no new protein would be made, and the ligase activity would drop by one-half per generation. This hypothesis suggests an mRNA stabilizing role for fructose and alanine or some derivative of these compounds. Lastly, it is conceivable that the mRNA for lysyl-tRNA ligase is synthesized but not translated unless alanine, leucine, or fructose is present. The enhancement of lysyl-tRNA ligase activity in wild-type strains by fructose, glucose, alanine, and leucine suggests that whatever process is involved occurs normally in the cell even if only to a limited extent, and that, in the strains with growth medium-dependent lysyl-tRNA ligase activity, this process has been amplified by mutation.

As stated in the introduction, we have used the initiation of spore germination in *Bacillus* as a guide to find compounds which in combination with alanine would restore lysyl-tRNA ligase activity in the mutant strain to the wild-type level. Of particular value to us were the studies of Foerster and Foster (5), Hyatt and Levinson (11), and Wax et al. (19, 20). In the comprehensive study by Foerster and Foster (5), for example, it was observed that a class of strains termed "reluctant" would not germinate in the presence of glucose, alanine, and inosine, but germinated if 1.0 mM L-leucine was supplied. Hyatt and Levinson (11) have shown that carbohydrates, particularly hexoses, can initiate spore germination in *B. megaterium*, and furthermore demonstrated that the germination of heat-treated spores of *B. megaterium* is stimulated synergistically by the combination of alanine and fructose.

Wax et al. (19) reported experiments on mutants of the transformable Marburg strain of *B. subtilis* which require alanine, glucose, and potassium ions for maximal germination. The wild-type strain only needs alanine and K^+. Alanine could be replaced by ABA. Subsequent work has shown that alanine can also be replaced in the mutant by asparagine (20).

The finding that glucose, fructose, alanine, and leucine stimulate lysyl-tRNA ligase activity in the mutant strain raises the question of whether these compounds act without change or by conversion to other metabolites. The *Bacillus* system we have followed most attentively involves work on the Marburg strain of *B. subtilis*, which requires fructose, glucose, and alanine or asparagine for maximal germination (19, 20). Recently, a publication by Prasad et al. (16) has appeared which analyzes these mutants in greater depth. These authors have concluded that fructose-6-phosphate, an amino acid (as amino donor), and reducing power by means of reduced nicotinamide adenine dinucleotide (NADH) or reduced nicotinamide adenine dinucleotide phosphate are required for germination. Glucose acts as a source of NADH via glucose dehydrogenase rather than as a carbon source. Prasad et al. (16) suggested that fructose-6-phosphate reacts with asparagine or alanine to form an unknown compound which enhances the response of the spores to NADH. In the instance of spore germination initiation by leucine, leucine dehydrogenase may play a role and generate NADH (7).

It is not clear at the present time whether similar metabolic changes must take place in *E. coli* in order for glucose, fructose, alanine, and leucine to stimulate lysyl-tRNA ligase activity. *E. coli* does not appear to possess glucose dehydrogenase (S. R. Kupor, Ph.D. Thesis, Harvard Univ., Cambridge, Mass., 1971), alanine dehydrogenase (12), or, as far as we know, leucine dehydrogenase. If reducing power is required, it must be generated by a different pathway in *E. coli*, perhaps through glucose-6-phosphate dehydrogenase. A requirement for reducing power could possibly explain why glucose is needed for maximal stimulation of lysyl-tRNA ligase in minimal medium, but not in 0.5% yeast extract medium. It may be that enough glucose is already present in the latter, or that reducing power is provided through other components of the yeast extract. Fructose metabolism is apparently the same in both *E. coli* and *B. subtilis*, with fructose being converted primarily to fructose-1-phosphate (6, 16). Hence, it is conceivable that the same putative compound is formed in both organisms from fructose-6-phosphate and alanine. Of course, the alternative should be considered that alanine and leucine act without change, and that the carbohydrates act via a cyclic

nucleotide (17).

At whatever site(s) alanine is exerting its influence, our data, compared with those of Lambert and Neuhaus (12), suggest that alanine does not act on the alanine racemase system. For example, Lambert and Neuhaus (12) found that D-alanine, as well as L-alanine, increases the specific activity of alanine racemase, whereas only L-alanine stimulates lysyl-tRNA ligase activity. Moreover, they reported that in minimal medium alanine and glycerol are considerably more effective in enhancing alanine racemase activity than alanine and glucose, but with lysyl-tRNA ligase alanine and glucose form the more effective combination.

The amino acids alanine and leucine appear to have unique biological effects in other systems as well as in spore germination initiation in *Bacillus* and the *E. coli* lysyl-tRNA ligase mutant. L-Alanine is by far the most effective initiator of spore germination with untreated spores of *Clostridium bifermentans* (18). With *E. coli*, it has been reported that the addition of 125 μM alanine or leucine, but no other amino acids, to succinate-minimal medium abets the effect of adenosine 3',5'-cyclic monophosphoric acid (3',5'-cyclic adenosine monophosphate) in overcoming glucose repression of β-galactosidase synthesis (2). Lee and Kenney (13) have found that 5 mM leucine can stimulate tyrosine-α-ketoglutarate transaminase activity 8- to 10-fold in cultured Reuber (H-35) hepatoma cells. Twelve other amino acids which were tested had no effect (alanine was not tested). In their study, leucine was shown to increase the amount of the enzyme by both decreasing the rate of degradation and increasing the rate of synthesis. Whether there is any common mode by which alanine and leucine act in these various systems remains to be seen.

Lastly, it should be stressed that it is not our intention to imply either that lysyl-tRNA ligase is involved in the mechanism of spore germination, or that changes in the amount of aminoacyl-tRNA ligases are important in the mechanism of spore germination. What we find intriguing is that certain combinations of metabolites can both initiate spore germination in certain strains of *Bacillus* and markedly enhance lysyl-tRNA ligase activity in certain mutants of *E. coli*. The only connection we envision at the present time is that a common control mechanism might be involved.

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