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Identification of a Temperature-Sensitive Asparaginyl-Transfer Ribonucleic Acid Synthetase Mutant of *Escherichia coli*†

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A temperature-sensitive mutant of *Escherichia coli* K-12 isolated previously (H. Ohsawa and B. Maruo, J. Bacteriol. 127:1157-1166, 1976) was found to have an alteration in asparaginyl-transfer ribonucleic acid synthetase. This alteration can account for the temperature-sensitive phenotype of the mutant. No evidence was obtained to support the previous suggestion that ribosomal protein S1 is altered in this mutant. Combined with the previous genetic studies, we conclude that the newly defined genetic locus, *asnS*, for the asparaginyl-transfer ribonucleic acid synthetase maps near *pyrD* at 21 min on the *E. coli* chromosome.

Recently, Ohsawa and Maruo isolated a mutant of *Escherichia coli* K-12 defective in protein synthesis at high temperatures and mapped the gene responsible for its temperature sensitivity at 21 min on the *E. coli* chromosome (7). Using a cell-free protein synthesis system, they looked for factors in the wild-type cell extract that could stimulate activity of the mutant extracts. Such stimulatory activity was found associated with a ribosomal-wash fraction and, furthermore, with a purified ribosomal protein S1 obtained from a wild-type strain. Although no direct evidence was presented, it was suggested that ribosomal protein S1 was altered in the mutant (7). Since S1 has been implicated as having an important function in protein synthesis (3, 8, 9), we attempted further characterization of this mutation.

Yamamoto and Nomura first isolated λ-transducing phages that can suppress the temperature sensitivity of the mutant (unpublished data). Using ultraviolet-irradiated cells, proteins were examined whose synthesis was stimulated by infection with the transducing phages. None of them, however, comigrated with reference ribosomal protein S1 in sodium dodecyl sulfate-polyacrylamide gel electrophoresis, suggesting that the structural gene for ribosomal protein S1 is not located on the segment of the bacterial chromosome carried by these transducing phages (M. Yamamoto and M. Nomura, unpublished data). This observation led us to consider other possibilities to explain the mutational defects in this mutant.

It was previously noted that this temperature-sensitive mutation not only caused immediate inhibition of protein synthesis but also that of ribonucleic acid (RNA) synthesis in a *relA*$^+$ strain upon temperature shift from 30 to 42°C. However, when the same mutation was introduced into an isogenic *relA* strain, RNA synthesis continued for more than 1 h at the higher temperature (7). Such behavior is similar to that of several known temperature-sensitive aminoacyl-transfer RNA (tRNA) synthetase mutants, such as valyl-tRNA synthetase temperature-sensitive mutants. Therefore, we have examined aminoacyl-tRNA synthetase activities of the mutant and found that asparaginyl-tRNA synthetase (AsnRS; abbreviated according to Neidhardt et al. [6]) was the altered component responsible for temperature sensitivity of the mutant. In this communication, we report evidence supporting this conclusion.

MATERIALS AND METHODS

Bacterial strains. Two strains of *E. coli* K-12 were used in this study. HO201 is F *thi rpsL*(*str*) *relA*. HO202 carried the same genetic markers and, in addition, the temperature-sensitive mutation (*tss*) mentioned in the introduction. These strains were described previously (7).

Media. LB medium contained per liter: 10 g of tryptone, 5 g of yeast extract, and 5 g of NaCl. ABG minimal medium contained per liter: 2 g of $(NH_4)_2SO_4$, 6 g of $Na_2HPO_4 \cdot 2H_2O$, 3 g of KH_2PO_4, 2 g

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of NaCl, $MgCl_2$ to 2 mM, $CaCl_2$ to 120 μM, $FeCl_3$ to 3.6 μM, 2 g of glucose, and 1 mg of thiamine.

Chemicals. Sources of the chemicals used in this study were as follows: nonradioactive amino acids, Calbiochem and Sigma; ^{14}C-labeled amino acids, Amersham/Searle; ^{3}H-labeled amino acids, New England Nuclear Corp.; deoxyribonuclease (ribonuclease-free), Worthington Biochemicals Corp.; *E. coli* asparaginase (500 IU/mg), Calbiochem; and *E. coli* B tRNA (uncharged), GIBCO.

Growth studies. The overnight culture of the parent strain (HO201) or the mutant (HO202), grown at 30°C in ABG medium, was diluted about 50-fold into 10 ml of fresh ABG medium. Appropriate amino acids were supplemented to a final concentration of 1 mg/ml. Incubation was then started either at 30 or 40°C with aeration by shaking. The growth rates of each strain at 30 or 40°C, in the presence or absence of certain amino acids, were measured by following the increases of turbidity with a Klett-Summerson photoelectric colorimeter.

Preparation of cell extracts for aminoacyl-tRNA synthetase assays. Strain HO201 or HO202 was grown in 1 liter of LB meidum at 30°C until the culture's absorbance at 550 nm (A_{550}) reached 1.0. Cells were harvested by low-speed centrifugation at 4°C and washed once with 250 ml of a solution containing 10 mM tris(hydroxymethyl)aminomethane-hydrochloride, 10 mM $MgCl_2$, 30 mM NH_4Cl, and 1 mM dithiothreitol at pH 7.4 (TMAI). About 1 g (wet weight) of a bacterial pellet was obtained for each strain. All of the following procedures were carried out at 0 to 4°C. Cells were ground with 2 g of alumina and extracted with 10 ml of TMAI. Centrifugation was done at 15,000 rpm for 15 min, and then at 50,000 rpm for 4 h. The supernatant (S100 fraction) was applied to a diethylaminoethyl-cellulose column (0.8 by 8 cm) equilibrated with TMAI. Proteins adsorbed to the column were then eluted with TMAI containing 0.25 M NH_4Cl. Fractions of about 1 ml each were collected. The absorbance at 280 nm (A_{280}) of each fraction was measured, and the peak fractions containing most of the eluted proteins were combined. The preparations obtained in this way are called ARS fractions in this paper. They were frozen and kept at −70°C until use.

Assay of aminoacyl-tRNA synthetase activities. The assay for amino acid-charging activity was carried out according to Muench and Berg (4). One batch of reaction mixture had 50 μl of total volume and contained 5 μmol of sodium cacodylate buffer, pH 6.9, 0.05 μmol of adenosine 5′-triphosphate, 0.5 μmol of $MgCl_2$, 0.5 μmol of KCl, 0.2 μmol of reduced glutathione, 10 μg of bovine serum albumin, 200 μg of *E. coli* B tRNA, 1.5×10^{-3} A_{280} unit ARS fractions, and an appropriate amount of radioactive amino acids. For the assay of AsnRS, 0.2 μmol of nonradioactive aspartic acid was included in the reaction mixture (see below). After incubation at 30 or 42°C for certain lengths of time, ice-cold 5% trichloroacetic acid was added. Precipitated materials containing radioactive aminoacyl-tRNA's were collected on nitrocellulose membrane filters, and the radioactivity was determined.

When AsnRS activity was to be assayed, nonradioactive aspartic acid was added to the reaction mixture. Addition of nonradioactive aspartic acid decreased the apparent asparagine-charging activity of enzyme fractions (Table 1). Our interpretation of this observation is that the ARS fractions probably contain asparaginase, produce radioactive aspartic acid from radioactive asparagine, and charge the radioactive aspartic acid to tRNA, and that the presence of excess nonradioactive aspartic acid reduces such "false" charging reactions. The effects of pretreatment of radioactive asparagine with asparaginase (Table 1) are consistent with this interpretation; with asparaginase pretreatment, the apparent charging activity was much higher than that obtained without the treatment, but this apparent charging activity was almost completely abolished when nonradioactive aspartic acid was added to the reaction mixture. Without asparaginase treatment, only a partial reduction of the apparent activity was observed in the presence of nonradioactive aspartic acid. We conclude that the charging activity, which was not affected by excess nonradioactive aspartic acid but was abolished by pretreatment with asparaginase, reflects real asparagine-charging activity. Therefore, we always added nonradioactive aspartic acid to the assay mixture (0.2 μmol/50 μl of the reaction mixture) when AsnRS activity was assayed.

RESULTS

Partial recovery of the growth of the mutant at high temperatures by addition of asparagine to medium. It is known that growth defects in several temperature-sensitive mutants of aminoacyl-tRNA synthetases, for instance, a temperature-sensitive mutant of histidyl-tRNA synthetase of *E. coli* (5), are partially suppressible by the presence of corresponding amino acids in growth medium (6). Because we suspected a defect in some aminoacyl-tRNA synthetases in the mutant HO202 (see above), we examined the effects of amino acids on the

TABLE 1. *Charging of tRNA with [^{14}C]asparagine*[a]

Addition to the assay mixture	Pretreatment with asparaginase[b]	
	− (cpm)	+ (cpm)
None	1,290	2,666
Aspartic acid[c]	964	36

[a] 1.5×10^{-3} A_{280} units of aminoacyl-tRNA synthetase fraction obtained from the parent strain (HO201) and 0.1 μCi of [^{14}C]asparagine (specific activity, 133 mCi/mmol) were used for each assay. Incubation was done at 30°C for 15 min.

[b] [^{14}C]asparagine (0.1 μCi) was treated with *E. coli* asparaginase (0.1 IU) at 37°C for 30 min in 10 mM tris(hydroxymethyl)aminomethane buffer (pH 7.4) before the assay.

[c] A total of 0.2 μmol of nonradioactive aspartic acid was added to the assay mixture.

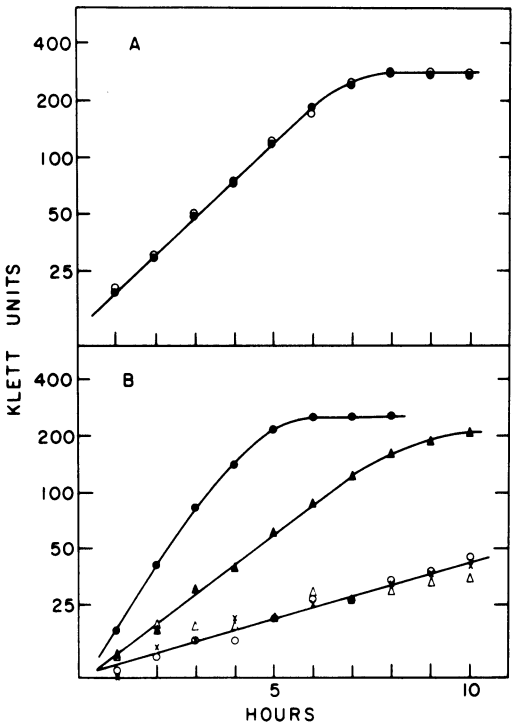

FIG. 1. *Growth of the parent (HO201) and the mutant (HO202) at 30°C (A) and at 40°C (B). The plots are: HO201 in minimal medium* (●), *HO202 in minimal medium* (○), *and HO202 in minimal medium supplemented with 1 mg of asparagine per ml* (▲), *aspartic acid* (△), *or glutamine* (×).

growth of this mutant. At 42°C, the mutant did not grow in several media tested. However, at 40°C, the mutant strains grew very slowly in minimal medium and considerably faster when it was supplemented with 20 mg of Casamino Acids per ml. Among 20 common amino acids tested (saturating amount for tyrosine, 1 mg/ml for others), only asparagine showed a stimulating effect on the growth of the mutant at 40°C. The doubling time was shortened from about 5 to 2 h by addition of asparagine (Fig. 1). Other amino acids such as aspartic acid or glutamine showed no stimulatory effect (Fig. 1). At 42°C, the same amount of asparagine did not restore the growth of HO202 (data not shown). These observations suggested a possibility that AsnRS is the altered component in the mutant HO202.

AsnRS and aspartyl-, glycyl-, and phenylalanyl-tRNA synthetase activities of extracts from mutant and parent strains. ARS fractions of HO201 and HO202 were analyzed for AsnRS and aspartyl- and glycyl-, and phenylalanyl-tRNA synthetase activities in vitro at 30 and 42°C. ARS fractions containing equal amounts of proteins were used for the enzyme assay (Fig. 2).

As shown in Fig. 2A through D, the ARS fraction derived from HO202 completely lacked AsnRS activity at 42°C, and showed only very weak activity at 30°C. (It was noted that the charging of tRNA with asparagine at 42°C by AsnRS of the parent strain, HO201, did not proceed linearly. It is possible that even the wild-type enzyme is slightly thermolabile under our assay conditions and undergoes gradual inactivation at 42°C as the incubation proceeds. Alternatively, the apparent decrease of activity could be a result of a decrease in [^{14}C]asparagine due to action of asparaginase in the sample at 42°C [cf. Materials and Methods].) In contrast to the large difference between HO201 and HO202 with respect to AsnRS activity, no significant difference was observed in glycyl-tRNA synthetase activity at either temperature. Glycyl-tRNA synthetase activity was assayed using [^{3}H]glycine in the same reaction mixtures simultaneously with the assay of AsnRS activity, which was done using [^{14}C]-asparagine. Two other synthetases, namely aspartyl- and phenylalanyl-tRNA synthetases, were also assayed in a similar way. Again, no significant difference was observed between HO201 and HO202 (Fig. 2E through H). It is unlikely that the ARS fraction derived from the mutant HO202 contained some substance(s) that inhibits an asparagine-charging reaction, since addition of the mutant sample to the sample from the parent strain did not cause any inhibition of the reaction by the latter sample (data not shown). We conclude that AsnRS is the mutationally altered component in HO202. (However, we cannot rigorously exclude the possibility that the mutation affects the synthesis, rather than the structure, of this enzyme [see below].)

The above experiments were done in a laboratory in Madison, Wis. Similar experiments were done in a laboratory in Tokyo using ribosome-free S100 extracts without the diethylaminoethyl-cellulose column fractionation step. The same conclusion was obtained.

It should be noted that the AsnRS of HO202 was completely inactive in vitro at 42°C, the temperature at which that strain cannot grow, but that the enzyme showed only a very weak activity even at 30°C in vitro, although HO202 can grow as fast as the parental strain, HO201, at this temperature (Fig. 1A). A similar kind of instability of aminoacyl-tRNA synthetases in vitro at permissive temperatures was reported with temperature-sensitive mutants of phenylalanyl- and valyl-tRNA synthetases (2). It is possible that weak activities of these enzymes

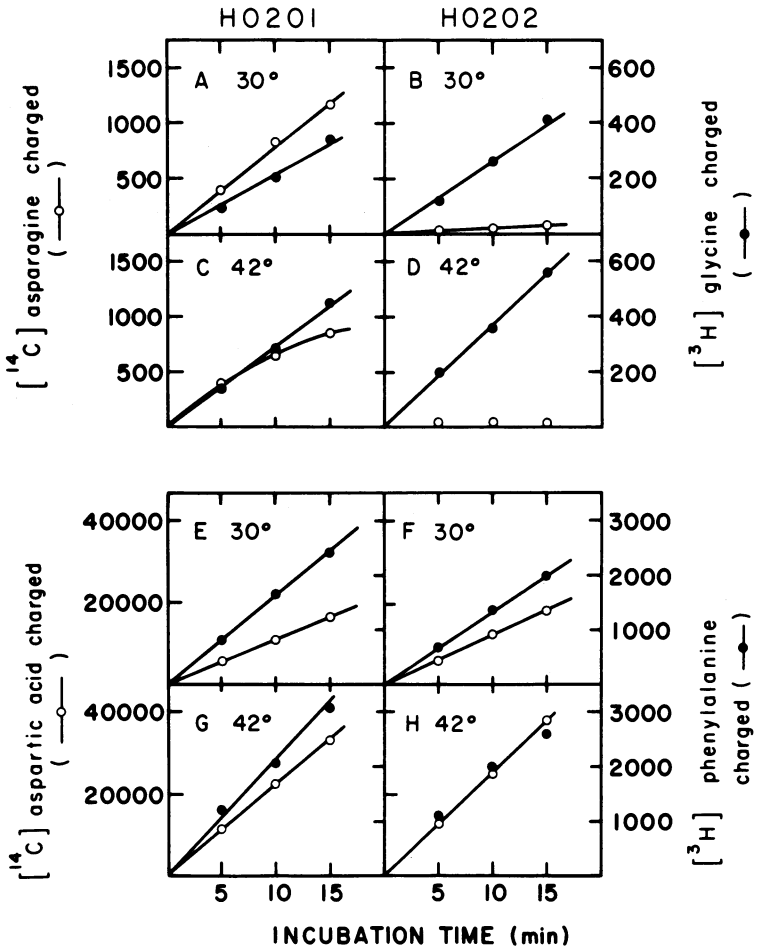

FIG. 2. *Charging of tRNA with asparagine, glycine, aspartic acid, and phenylalanine by aminoacyl-tRNA synthetase fractions from HO201 and HO202. The assay procedure is described in the text. Each assay sample for one time point (50 μl) contained 0.1 μCi of [^{14}C]asparagine and 1 μCi of [^{3}H]glycine (A through D) or 0.1 μCi of [^{14}C]aspartic acid and 1 μCi of [^{3}H]phenylalanine (E through H). The sources of the enzyme fraction and the temperatures for the assay were: HO201, 30°C (A and E); HO202, 30°C (B and F); HO201, 42°C (C and G); HO202, 42°C (D and H). For the assay at 42°C, the reaction mixtures without radioactive amino acids were preincubated at 42°C for 3 min, and the reactions were started by the addition of radioactive amino acids. The specific activities of the amino acids used were as follows: [^{14}C]asparagine, 133 mCi/mmol; [^{14}C]aspartic acid, 220 mCi/mmol; [^{3}H]glycine, 11.4 Ci/mmol; and [^{3}H]phenylalanine, 40 Ci/mmol.*

may be enough to support the normal cell growth. Alternatively, the enzymes may be stable at 30°C in vivo, and the instability may manifest itself only after disruption of cellular structure. The latter explanation is, perhaps, more likely.

DISCUSSION

We have demonstrated that the temperature-sensitive mutant, HO202, has an alteration in AsnRS. The mutant enzyme seems to be very unstable, especially at 42°C. The altered AsnRS can account for the temperature-sensitive growth of the strain, since a high concentration (1 mg/ml) of asparagine shows specific stimulation of growth at quasirestrictive temperatures. In addition, Ohsawa and Maruo previously showed that the temperature shift-up causes so-called stringent response in RNA synthesis in this mutant strain (7). Our conclusion that the mutant has a temperature-sensitive AsnRS explains this phenomenon. The phenomenon is similar to those observed with other temperature-sensitive aminoacyl-tRNA synthetase mutants (2); that is, at 42°C, the mutant probably fails to charge $tRNA^{Asn}$ with asparagine, accumulates guanosine tetraphosphate, and stops the accumulation of stable RNAs.

In the previous studies, Ohsawa and Maruo showed that ribosomal protein S1 fractions prepared from a wild-type strain could stimulate the activity of the mutant extracts in a cell-free protein-synthesizing system, and they inferred that the mutation in HO202 affected the function of S1. Subsequent experiments showed that the ribosomal-wash fraction and the ribosomal-protein S1 preparation used in the previous work had AsnRS activity (H. Ohsawa and B. Maruo, unpublished data). Furthermore, a highly purified S1 preparation supplied by T. Yokota and K. Arai failed to rescue the defects in the mutant extracts; this S1 preparation was active in stimulating polyuridylic acid-dependent polyphenylalanine synthesis (T. Yokota, K. Arai, and Y. Kaziro, personal communication) and did not show any AsnRS activity (Ohsawa and Maruo, unpublished data). Therefore, the stimulation observed by the S1 preparation in the previous work could be explained by the activity of AsnRS present in the S1 preparation as a contaminant. We conclude that the temperature-sensitive mutation in HO202 affects asparaginyl-tRNA synthesis but not ribosomal protein S1.

Mutations affecting AsnRS have not been reported before. Although we have not proven rigorously that the mutation affects the structural gene of the enzyme, the observed apparent instability of the mutant enzyme suggests that the mutation is in a structural gene. In addition, in preliminary experiments, the AsnRS activity of some of the temperature-resistant "revertants" isolated from HO202 was found to have temperature sensitivity different from that of AsnRS from the parent (Ohsawa and Maruo, unpublished data). This observation also indicates that the mutation is in the structural gene of AsnRS. We, therefore, suggest *asnS* as the name for the gene affected by the mutation studied in this paper. From the genetic experiments reported previously (7), we conclude that *asnS* is located near *pyrD* at 21 min on the revised *E. coli* genetic map (1).

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