Regulation of the Biosynthesis of Aminoacyl-Transfer Ribonucleic Acid Synthetases and of Transfer Ribonucleic Acid in *Escherichia coli*

V. Mutants with Increased Levels of Valyl-Transfer Ribonucleic Acid Synthetase†

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Spontaneous revertants of a temperature-sensitive *Escherichia coli* strain harboring a thermolabile valyl-transfer ribonucleic acid (tRNA) synthetase were selected for growth at 40°C. Of these, a large number still contain the thermolabile valyl-tRNA synthetase. Three of these revertants contained an increased level of the thermolabile enzyme. The genetic locus, valK, responsible for the enzyme overproduction, is adjacent to the structural gene, valS, of valyl-tRNA synthetase. Determination (by radioimmunoassay) of the turnover rates of valyl-tRNA synthetase showed that the increased level of valyl-tRNA synthetase is due to new enzyme synthesis rather than decreased rates of protein degradation.

Aminoacyl-tRNA synthetases are enzymes of central importance for protein synthesis in cells. These enzymes are responsible for the correct attachment of each amino acid to its cognate tRNA (33). In addition, through their catalysis of aminoacyl-tRNA esterification, the synthetases in bacteria are involved in the regulation of various biosynthetic operons (4) and of amino acid transport (32), as well as in operation of the pleiotropic stringent response (5).

Because these enzymes are involved in so many aspects of cellular metabolism (15), the manner in which their levels are regulated is of great interest. In the past 10 years much work has been done in attempts to unravel the mechanisms by which the levels of aminoacyl-tRNA synthetases are controlled (23, 26). Synthetase levels in bacterial cells have been found to vary in response to a variety of environmental factors, including carbon source in the growth medium (29), growth temperature (17), and restriction of cognate amino acids (26).

Genetic alterations affecting the regulation of biosynthesis of several synthetases have been discovered. Mutants with increased levels of seryl-tRNA synthetase (8, 30, 35), leucyl-tRNA synthetase (16, 35), threonyl-tRNA synthetase (24, 27), and methionyl-tRNA synthetase (6) have been found.

In this paper we describe the isolation and characterization of *Escherichia coli* K-12 strains with genetic alterations leading to increased levels of valyl-tRNA synthetase. These strains were found among temperature-resistant revertants of a temperature-sensitive strain harboring a thermolabile valyl-tRNA synthetase. This type of selection procedure has been previously used successfully for obtaining *E. coli* mutants with increased levels of other aminoacyl-tRNA synthetases (23).

**MATERIALS AND METHODS**

Uniformly labeled [14C]valine (235 mCi/mmol) was obtained commercially. All other radioactive amino acids used had specific activities of 200 to 325 mCi/mmol. Pure *E. coli* valyl-tRNA synthetase was prepared by Jeremy Knowles. Bolton-Hunter reagent of specific activity 500 to 4,000 Ci per mmol was obtained commercially. This was used to prepare [125I]-labeled pure valyl-tRNA synthetase by the method of Bolton and Hunter (3). Sheep anti-rabbit antiserum was a gift of R. Rosenstein.

**Bacterial strains.** The bacterial strains used are described in Table 1.

**Media.** A modified Luria and Burrous (LB) medium and L agar were prepared as described previously (19). For various growth curves and selection of transductants, minimal medium 56 was used (1) supplemented with any necessary growth factors such as thiamine (0.1 mg/ml), amino acids (each 100 μg/ml), purines and pyrimidines (each 40 μg/ml), and glucose (0.2%).

**Revertant strains.** Spontaneous temperature-resistant derivatives of strain MB1 were selected on LB agar at 40°C. Portions (0.1 ml) were removed from a fresh 5-ml LB culture grown at 30°C and plated on

LB agar. The colonies which appeared after incubation at 40°C were picked, and markers were retested by replica plating (18). The highest temperature at which a strain is able to grow is designated the growth limit of the strain. These were determined by replica plating and subsequent incubation at 25, 30, 37, 40, 42, and 45°C for 12 to 16 h.

Genetic manipulations. Bacterial crosses and transductions were performed as described previously (18, 19).

Preparation of cell extracts. Cells were grown and cell extracts were prepared as described previously (35), except that after centrifugation the resulting supernatant fluid was dialyzed against buffer containing 30% polyethylene glycol, thus reducing the volume of the extract. Glycerol was added to a final concentration of 50%, and the mixture was stored at −20°C. The extracts normally contained about 5 mg of protein per ml as determined by the method of Groves et al. (11) or Lowry et al. (20) with crystalline bovine plasma albumin as standard.

Preparation of tRNA. This was done as described previously (22).

Aminoacylation reaction. Aminoacyl-tRNA synthetases were assayed at 25 or 30°C by the aminoacylation reaction as described earlier (32). Enzyme activity was determined as picomoles of aminoacyl-tRNA formed per microgram of S-100 protein per minute.

Heat inactivation studies. Cell extracts, adjusted to a protein concentration of 1 mg/ml with bovine serum albumin in 10 mM sodium cacodylate (pH 7.2) -10% (vol/vol) glycerol, were incubated at 30 or 37°C. Aliquots were removed at various time intervals and assayed at 25°C for residual enzyme activity.

Antiserum preparation. Pure E. coli valyl-tRNA synthetase was used to prepare antibodies in a white New Zealand rabbit. Control antiserum was prepared from the blood of the rabbit before it had received injections of foreign proteins.

Antiserum titration by assay of residual activity. Cell extracts (each containing the same amount of protein of the strains to be compared) were mixed with a specified amount of wild-type cell extract diluted in a solution of 10 mM sodium cacodylate (pH 7.2), 1 mg of bovine serum albumin per ml, and 1% (vol/vol) glycerol. Increasing amounts of antiserum, diluted in the same buffer, were added to separate samples, and precipitation was allowed to proceed for 30 min at 25°C. After this time, the remaining enzyme activity in the samples was assayed at 25°C in the aminoacylation reaction. No correction was made for enzyme inactivation during the incubation period. The amount of valyl-tRNA synthetase contained in the various strains is derived from (and proportional to) the amount of antiserum necessary for inactivation of one-half of the enzyme activity.

Radioimmunoassays. For the radioimmunoassays a modification of the procedure of Aakensen and Leonard (2) was used. Antibody to valyl-tRNA synthetase was linked to a solid plastic support (clear plastic Falcon test tubes [2 by 7.5 cm) or the walls of plastic Linbro microtiter plates).

The amount of valyl-tRNA synthetase in an S-100 extract was measured by two different radioimmunoassays.

### Table 1. E. coli strains

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
<th>Source or derivation</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB3441</td>
<td>F88(gal+)/galK2 thi-1 metC56 xyl ara-1 lacY1 tr-5 tsx-5 supE44</td>
<td>E. A. Adelberg collection</td>
</tr>
<tr>
<td>AB3584</td>
<td>As for AB3441 but valS7</td>
<td>E. A. Adelberg collection, EMS mutagenesis of AB3441</td>
</tr>
<tr>
<td>200YS</td>
<td>thi pyrB31 thr leu pro his rpsL lac gal mal xyl</td>
<td>AB3584 × 200YS Thr+ Leu+ (Strt) (Iv Pro Met Arg only in liquid)</td>
</tr>
<tr>
<td>MB1</td>
<td>thi valS7 his pyrB31 ara gal lac rpsL ilvB</td>
<td>P1(AB3441) × MB1 → 45°C survival Spontaneous TR revertant of MB1</td>
</tr>
<tr>
<td>MB2</td>
<td>As for MB1 but valS7* and pyrB31</td>
<td>Spontaneous TR revertant of MB1</td>
</tr>
<tr>
<td>MB1381</td>
<td>As for MB1 but partially temperature-resistant valX12</td>
<td>Spontaneous TR revertant of MB1</td>
</tr>
<tr>
<td>MB1951</td>
<td>As for MB1 but partially temperature-resistant valX13</td>
<td>Spontaneous TR revertant of MB1</td>
</tr>
<tr>
<td>MB2101</td>
<td>As for MB1 but partially temperature-resistant valX14</td>
<td>Spontaneous TR revertant of MB1</td>
</tr>
<tr>
<td>MB3121</td>
<td>As for MB1 but partially temperature-resistant val-16</td>
<td>Spontaneous TR revertant of MB1</td>
</tr>
<tr>
<td>MB1382</td>
<td>As for MB1381 but pyrB*</td>
<td>Spontaneous PyrB+ revertant of MB1381</td>
</tr>
<tr>
<td>MB3</td>
<td>As for MB1 but pyrB*</td>
<td>Spontaneous PyrB+ revertant of MB1</td>
</tr>
<tr>
<td>MB4</td>
<td>As for MB2 but pyrB*</td>
<td>Spontaneous PyrB+ revertant of MB2</td>
</tr>
<tr>
<td>NF269</td>
<td>valS20 pyrB45 leu-6 his-1 argG6 metB1 rpsE rpsL104 lacY1 or 24 gal-6 xyl-7 mil-2 maI1 sup-S5 tonA2 λ</td>
<td>N. Filil via J. Parker</td>
</tr>
<tr>
<td>MB5</td>
<td>As for NF269 but valS7 pyrB+</td>
<td>P1(MB3) × MB7 → PyrB+</td>
</tr>
<tr>
<td>MB6</td>
<td>As for NF269 but valS7 pyrB+</td>
<td>P1(MB4) × NF269 → PyrB+</td>
</tr>
<tr>
<td>MB7</td>
<td>As for NF269 but valS*</td>
<td>P1(MB4) × NF269 → 45°C survival</td>
</tr>
<tr>
<td>MB1383</td>
<td>As for NF269 but partially temperature-resistant valX12 valS7 pyrB*</td>
<td>P1(MB1382) × NF269 → PyrB+</td>
</tr>
</tbody>
</table>

J. Bacteriol. 

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(i) Radioimmunoassay I. Radioimmunoassay I determines the relative amounts of valyl-tRNA synthetase in a series of S-100 extracts. The resultant curves demonstrated the antigenic similarity of the enzyme in the various extracts. In this procedure various concentrations of nonradioactive S-100 protein diluted with a solution of 0.01 M sodium phosphate (pH 7.5) - 0.9% NaCl - 0.1% bovine serum albumin (BSA) - 0.01% sodium azide (final volume, 40 μl) were added to antibody-coated tubes. After incubation at room temperature for 1 h, 125I-labeled valyl-tRNA synthetase (ca. 31,000 cpm in 20 μl) was added. Incubation was continued at room temperature for 1 h. Then excess liquid was removed and the tubes were washed with a solution of 0.01 M sodium phosphate (pH 7.4) - 0.9% NaCl. The radioactivity adhering to the tubes was measured in a gamma counter. The radioactivity of antigen adhering to tubes coated only with 0.1% BSA solution served as the control. Data were plotted as percentage of radioactive valyl-tRNA synthetase bound in the tubes. The radioactivity in the tube with labeled antigen only, i.e., without nonradioactive S-100 protein, marked the 100% point. The amount of S-100 protein required to inhibit the binding of 50% of the maximum amount of radioactive valyl-tRNA synthetase bound was taken as a measure of the amount of the enzyme present in a strain; the less S-100 protein required for this, the higher the amount of enzyme in the strain.

(ii) Radioimmunoassay II. Radioimmunoassay II determines the amount of valyl-tRNA synthetase present per microgram of S-100 protein. In this procedure a standard curve was generated by incubating various concentrations of pure unlabeled synthetase in 0.01 M sodium phosphate (pH 7.5) - 0.9% NaCl - 0.1% BSA - 0.01% sodium azide (50 μl) for 4 h at 4°C in the antibody-coated well of a microtiter plate. Buffer without enzyme was added to control wells. Then 125I-labeled valyl-tRNA synthetase (50,000 cpm in 20 μl) was added to each well, including wells precoated only with BSA buffer (blank control) and wells precoated with antibody but incubated only with BSA buffer (100% control). After incubation for 16 to 20 h at 4°C, the wells were emptied and rinsed with 0.01 M sodium phosphate (pH 7.4) - 0.9% NaCl and tap water. The radioactivity adhering to the wells was measured in a gamma counter. The 100% control wells contained ca. 6,000 cpm. Results were plotted as the percentage of radioactive valyl-tRNA bound versus the number of milligrams of unlabeled pure enzyme added.

Assays of the amount of enzyme in any particular S-100 preparation were done in a similar fashion. It was determined that 50 μl of a twofold dilution of S-100 protein (ca. 0.1 mg/ml) from a 5-ml cell sample (2 x 10^8 cells per ml) would inhibit the binding of 30 to 50% of the 125I-labeled antigen in the radioimmunoassay. Therefore, 50 μl of a twofold dilution of each 5-ml S-100 cell sample to be assayed was added to the precoated wells of the microtiter plate as nonradioactive competitor antigen, and the procedure described above was carried out. From the percentage of 125I-labeled valyl-tRNA synthetase still binding to a well in the presence of nonradioactive S-100 protein as compared to the 125I radioactivity in wells with no nonradioactive S-100 protein, the concentration of the enzyme in an S-100 extract was obtained.

Preparation of labeled material for the monitoring of valyl-tRNA synthetase degradation in vivo. Bacterial cultures (30 to 40 ml) were grown at 28°C with aeration in minimal medium 56 supplemented with glucose (0.33%, wt/vol), B1, and required amino acids. Upon reaching a cell density of 2 x 10^8 cells per ml, 0.1 mCi of [3H]lysine (specific activity, 50 to 60 Ci per mmol) was added per 10 ml of cell culture. After 5 min, 5 ml of the cell cultures were removed for the zero time readings and 22 μmol of unlabeled lysine (a 2,600-fold excess) was immediately added as a chase. Samples (5 ml) of the cell cultures were removed thereafter at various times. Immediately upon its removal each sample was chilled. The cells were lysed in a French press, and S-100 extracts were prepared as described above.

Immunoprecipitation for the monitoring of valyl-tRNA synthetase degradation in vivo. Valyl-tRNA synthetase was isolated from the 3H-labeled S-100 preparation by immunoprecipitation. The procedure used is similar to that described by Hunter (13).

The amount of the enzyme in each S-100 extract was first assayed by radioimmunooassay II. For each set of S-100 extracts (made from cell samples taken from the same culture), care was taken to add equal amounts of the enzyme to form the immunoprecipitate. 3H-labeled S-100 extract (50 to 100 μl) and rabbit anti-valyl-tRNA synthetase antiserum diluted in 0.01 M sodium phosphate (pH 7.5) - 0.9% NaCl - 0.1% BSA - 0.01% sodium azide to make the total volume of 150 μl were gently mixed together and allowed to sit for 1 h at room temperature. Then sheep anti-rabbit antiserum (150 μl) was added to each mixture. After 16 to 20 h at 4°C, the precipitates were collected by centrifugation, washed with 0.01 M sodium phosphate (pH 7.4) - 0.9% NaCl, and then dissolved in 0.05 M NaOH (0.5 ml). The 3H radioactivity was determined by liquid scintillation counting. Blank controls containing an equal amount of rabbit serum from an unimmunized rabbit in place of the anti-valyl-tRNA synthetase antiserum were always processed along with the experimental assay mixtures.

A competition assay was run to prove that the synthetase was indeed the protein precipitated in this assay was carried out by using the above-described procedure except that before addition of the 3H-labeled S-100 extract various dilutions of pure unlabeled enzyme were incubated for 1 h at room temperature with the anti-valyl-tRNA synthetase antiserum.

All assays were done in duplicate. The error in separate determinations was approximately 10%. The blank was generally 35 to 40% of the total radioactivity precipitated. This was always subtracted from the total radioactivity to give the net radioactivity precipitated, and these net values were used to calculate the results.

Rate of protein synthesis. Cell cultures were grown in supplemented minimal medium at 28 or 33°C to a cell density of 2 x 10^8 cells per ml. [3H]lysine (15 mCi/mmol) was then added to the cultures (final concentration, 20 μg/ml). Aliquots of 0.1 ml were removed every 15 min and spotted on filter paper. The
rate of protein synthesis was determined by the amount of acid precipitable radioactivity per cell number.

In vivo levels of valyl-tRNA. These were determined as described previously (34).

Measurement of the amount of valyl-tRNA synthetase produced in various strains grown in different media. Cultures were grown at 25°C in LB medium or in minimal medium 56 containing vitamin B1 and required amino acids supplemented either with 0.33% glucose or with 1.0% disodium succinate. Upon reaching a cell density of 2 × 10^8 cells per ml, a 5-ml portion of cell culture was removed and immediately chilled, and the cells were harvested. After the cells were washed with 10 mM MgCl₂-10 mM NH₄Cl-10 mM Tris (pH 7.5), they were frozen at −78°C. S-100 preparations of these cells were prepared in the above buffer. The amount of valyl-tRNA synthetase was determined by radioimmunoassay II.

RESULTS

Selection of temperature resistant phenotypic revertants. Spontaneous temperature-resistant derivatives of strain MB1 (carrying a thermolabile valyl-tRNA synthetase) were isolated at 40°C on LB agar plates as described in Materials and Methods. Temperature-resistant colonies appeared at a frequency of 10⁻⁶. These “revertants” were classified on the basis of their differing growth limits on LB and minimal agar plates. Strains which were able to grow at 45°C on LB and minimal agar plates (15 of 68 tested) were not studied further because these were thought to be true revertants. Those strains showing temperature sensitivity at 42 and 45°C were selected for further study, since they still harbored some temperature sensitive lesions.

Thermosensitivity of valyl-tRNA synthetase in revertant strains. To determine whether these revertant strains still possessed the thermolabile enzyme of the parent strain MB1, standard heat inactivation studies of S-100 cell extracts were performed. Of 12 strains tested, 4 still contained a labile valyl-tRNA synthetase similar to that from parental strain MB1 (Fig. 1). The valS⁺ strain MB2 is shown for purposes of comparison. The synthetases from the other eight strains also were found to be thermolabile, but less so than the parental (MB1) enzyme. This was, therefore, presumed to be due to second site mutations in the valS gene; these strains were not studied further.

Valyl-tRNA synthetase levels in revertant strains. The valyl-tRNA synthetase content of the S-100 extracts of these four revertants was determined by antiserum titration. Three strains, MB1381, MB1951, and MB2101, were found to have synthetase levels two to four times as high as the parent strain MB1 (Table 2). A representative experiment is shown in Fig. 2.

At 30°C all strains have similar growth rates. At 37°C, however, the synthetase-overproducing strains and strain MB2 grew approximately twice as fast in LB medium as strain MB1 grew at 30°C. Because of this growth rate difference, one might expect increased amounts of valyl-tRNA synthetase in the revertant strains compared to the temperature-sensitive parent (25).

However, no increased levels of the synthetase from cultures grown at 37°C, as compared to 30°C, were found in these strains (Table 2). This is in accord with the work of LeMaux et al. (17). In fact, the amounts of valyl-tRNA synthetase in the revertant strains as compared to that in the parent strain MB1, although still greater than in MB1, were less so than they had been when all strains were grown at the same temperature (Table 2). This is probably due to increased degradation of the thermolabile enzyme in the revertants grown at 37°C as will be discussed below.

To rule out the possibility that the increased levels of synthetase observed by antiserum titration were caused by differences in antigenic determinants of the enzyme from various strains, their enzyme content was determined by radioimmunoassay. Comparison of the shapes of the radioimmunoassay curves obtained would be a measure of similarity of the antigenic de-
TABLE 2. Valyl-tRNA synthetase levels in various strains*

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Aminoacylation activity (U/μg of protein)</th>
<th>Amt of valyl-tRNA synthetase as determined by antiserum titration (μlitters of antiserum/μg of S-100)*</th>
<th>Amt of valyl-tRNA synthetase as determined by radioimmunoassay (1/μg of S-100)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB2</td>
<td>valS+</td>
<td>12.2</td>
<td>1.1 (0.8)</td>
<td>1.0</td>
</tr>
<tr>
<td>MB1</td>
<td>valS7</td>
<td>0.7</td>
<td>0.6 (0.5)*</td>
<td>0.77</td>
</tr>
<tr>
<td>MB1381</td>
<td>valS7</td>
<td>6.2</td>
<td>2.3 (1.2)</td>
<td>3.3</td>
</tr>
<tr>
<td>MB1951</td>
<td>valS7</td>
<td>3.9</td>
<td>1.9 (0.7)</td>
<td>2.0</td>
</tr>
<tr>
<td>MB2101</td>
<td>valS7</td>
<td>2.3</td>
<td>1.8 (0.7)</td>
<td>3.3</td>
</tr>
<tr>
<td>MB3121</td>
<td>valS7</td>
<td>1.0</td>
<td>0.5 (ND)</td>
<td>0.67</td>
</tr>
<tr>
<td></td>
<td>valS7</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>valX12</td>
<td></td>
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<td></td>
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<td></td>
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<td></td>
<td>valX14</td>
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<td></td>
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<tr>
<td></td>
<td>valX16</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Strains were grown at 30 and at 37°C (values in parentheses).

† Determined at 25°C.

‡ Antiserum was identically diluted in assays or results were normalized to identical dilution values. Results represent the amount of antiserum required for 50% inactivation of valyl-tRNA synthetase activity.

§ Results represent the reciprocal of the amount of S-100 protein required to inhibit the binding of 50% of 125I-labeled valyl-tRNA synthetase.

* Strain MB1 was regrown at 29.5°C while the other strains were grown at 37°C. The different values obtained for two identically treated cultures illustrate the experimental variation in separate determinations.

ND, Not determined.

FIG. 2. Antiserum titration of valyl-tRNA synthetase by assay of residual activity. Various amounts of anti-valyl-tRNA synthetase antiserum were incubated with fixed amounts of S-100 cell extracts of strains grown at 30°C. After immunoprecipitation, the mixtures were assayed for their residual valyl-tRNA synthetase activity. An S-100 extract from strain MB2 (valS+) was assayed alone and was also added as a carrier in all assays of S-100 extracts from either the temperature-sensitive or revertant strains. ●, MB2; ○, MB3121 + MB2; ×, MB1 + MB2; ---, MB1381 + MB2.

Aminoacylation activity was determined at 30°C. The radioimmunoassay curves (representative ones are shown in Fig. 3) showed similar shapes; thus the enzymes from all of the strains tested were antigenically similar. This experiment also confirmed the increased enzyme levels in three revertant strains and the lack of overproduction in strain MB3121 (Table 2).

Due to the thermostability of the valyl-tRNA synthetase in the parent and revertant strains, the determination of valylation activity is not an accurate measure of the levels of this enzyme. However, the observed (Table 2) synthetase activity in these strains supports the antiserum titration data.

tRNA levels in the revertant strains. Next, we wanted to determine whether elevated enzyme levels in some revertant strains would cause an increase in tRNAVal and also whether the revertant strain with normal levels is stabilized at the higher temperature by an increased tRNAVal concentration. Such a tRNA increase was observed in two temperature-resistant revertants of glutaminyl-tRNA synthetase (7, 22). When the four revertant strains were assayed for the amounts of tRNAVal, no increased level...
was found relative to strains MB1 and MB2 (data not shown).

Levels of other aminoacyl-tRNA synthetases in the revertant strains. Are the levels of other aminoacyl-tRNA synthetases also elevated? When the activities of four other aminoacyl-tRNA synthetases in the aminoaacylation reaction were determined, no significant increase of the enzymes for leucine, isoleucine, serine, and arginine was seen (data not shown). Thus the revertants appear to be selectively overproducing valyl-tRNA synthetase. Interestingly, neither leucyl- nor isoleucyl-tRNA synthetase is elevated, although evidence for the existence of a common control element in the regulation of production of branched-chain aminoacyl-tRNA synthetases has been presented (14).

Genetic characterization of revertants. Before mapping the revertant loci the stability of the revertant strains was checked. Since among approximately 1,000 colonies of each strain grown at 30°C no back revertant to 40°C temperature sensitivity was found, the mutations appeared to be stable.

Mutations linked to the structural genes of aminoacyl-tRNA synthetases and resulting in overproduction of these enzymes have been previously found (8, 16, 30). Therefore, transductions were performed in an attempt to determine whether or not the loci causing temperature resistance in the revertant strains were located close to valS and pyrB. P1 was grown on strain AB3584 (valS7, pyrB+), and transduced into strain MB2 (pyrB31, valS+) and into revertant strains MB1381, MB1951, MB2101, and MB3121. Selection was for uracil prototrophy on minimal agar plates (Table 3). The revertant locus in each case was closely linked to pyrB+ and the valS structural gene, since the P1 grown on strain AB3584 was able to eliminate the revertant phenotype by bringing in pyrB+. However, the revertant loci are not necessarily closely adjacent to the structural gene for valyl-tRNA synthetase, for there is some difference between the percentage of PyrB+ transductants of the temperature-resistant MB2 strain which acquire the temperature-sensitive phenotype, and the percentage of PyrB+ transductants of the revertants MB1381, MB2101, MB1951, and MB3121 which become temperature sensitive (Table 3).

Examination of strains MB1 and MB2 and the revertants revealed that this set of isogenic strains harbors a cryptic auxotrophic mutation which could be overcome by the addition of isoleucine, valine, proline, arginine, and methionine to minimal medium. We therefore transduced one of the loci, valX12, causing overproduction of a temperature-sensitive valyl-tRNA synthetase into a genetically well-defined background. First, spontaneous uracil prototrophs were selected from the revertant strain MB1381 (valX12) from the parent strain MB1 (valS7) and from MB2 (valS+). P1 grown on one of these strains was transduced into strain NF269 (pyrB41, valS20), a strain nonviable at 40°C, because of a thermolabile valyl-tRNA synthetase of intermediate thermolability in heat-inactivation assays. (The mutation in this strain, valS20, is a different allele from that carried by strain MB1.) These transductions yielded Pyr+ strains MB5 (valS7), MB6 (valS+), and MB1383
VOL. 139, AB3441

PYRBY approximately synthetase than strain found was synthetase tRNA AB3584 strains three in strain (pyrB+ AB3584 AB3584 (pyrB+ thermostable valyl-tRNA synthetase effects of levels of synthesis of rates of alterations strain ts in vivo The incorporation (valS7). The (valS7 MB1383 Revertant of MB1 MB1383 Revertant MB3121 Revertant MB1 via- antimetabolite (21, 25, 29). When an E. coli strain is grown in different media containing various carbon sources, both its growth rate and its aminocyl-tRNA synthetase levels increase with the richness of the growth medium. In particular, valyl-tRNA synthetase levels have been shown to increase 2.5-fold over a 7-fold increase.

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### Table 3. Transductions to test the linkage of revertant loci to valS

<table>
<thead>
<tr>
<th>P1 donor strain</th>
<th>Relevant* phenotype</th>
<th>Recipient strain</th>
<th>Relevant* phenotype</th>
<th>Selected marker</th>
<th>Total transductants</th>
<th>Unselected* marker scored</th>
<th>Percent cotransduction selected and unselected markers</th>
</tr>
</thead>
<tbody>
<tr>
<td>AB3584 (pyrB+ valS7)</td>
<td>Valyl-tRNA synthetase ts nonviable at 37°C, PyrB+</td>
<td>MB2 (pyrB31 valS7)</td>
<td>Valyl-tRNA synthetase wild type viable at 45°C, PyrB+</td>
<td>PyrB+</td>
<td>175</td>
<td>Nonviable at 37°C</td>
<td>69</td>
</tr>
<tr>
<td>AB3441 (pyrB+ valS7*)</td>
<td>Valyl-tRNA synthetase wild type viable at 45°C, PyrB+</td>
<td>MB1 (pyrB31 valS7)</td>
<td>Valyl-tRNA synthetase ts nonviable at 37°C, PyrB+</td>
<td>PyrB+</td>
<td>46</td>
<td>Viable at 45°C</td>
<td>72</td>
</tr>
<tr>
<td>AB3584 (pyrB+ valS7)</td>
<td>Valyl-tRNA synthetase ts nonviable at 37°C, PyrB+</td>
<td>MB3181 (pyrB31 valS7 valX12)</td>
<td>Revertant of MB1 overproduces valyl-tRNA synthetase ts viable at 40°C, PyrB+</td>
<td>PyrB+</td>
<td>355</td>
<td>Nonviable at 37°C</td>
<td>48*</td>
</tr>
<tr>
<td>AB3584 (pyrB+ valS7)</td>
<td>Valyl-tRNA synthetase ts nonviable at 37°C, PyrB+</td>
<td>MB2101 (pyrB31 valS7 valX14)</td>
<td>Revertant of MB1 overproduces valyl-tRNA synthetase ts viable at 40°C, PyrB+</td>
<td>PyrB+</td>
<td>272</td>
<td>Nonviable at 37°C</td>
<td>57*</td>
</tr>
<tr>
<td>AB3584 (pyrB+ valS7)</td>
<td>Valyl-tRNA synthetase ts nonviable at 37°C, PyrB+</td>
<td>MB1951 (pyrB31 valS7 valX13)</td>
<td>Revertant of MB1 overproduces valyl-tRNA synthetase ts viable at 40°C, PyrB+</td>
<td>PyrB+</td>
<td>103</td>
<td>Nonviable at 37°C</td>
<td>60*</td>
</tr>
<tr>
<td>AB3584 (pyrB+ valS7)</td>
<td>Valyl-tRNA synthetase ts nonviable at 37°C, PyrB+</td>
<td>MB3121 (pyrB31 valS7 val-16)</td>
<td>Revertant of MB1 viable at 40°C, PyrB+</td>
<td>PyrB+</td>
<td>290</td>
<td>Nonviable at 37°C</td>
<td>83*</td>
</tr>
</tbody>
</table>

* Temperature limits were observed on Luria agar.

### Table 4. Valyl-tRNA synthetase levels in strains created by transduction into strain NF269

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Aminoacylation activity* (U/µg of protein)</th>
<th>Amt of valyl-tRNA synthetase as determined by antisera titration (µl of antisera/µg of S-100)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>MB5</td>
<td>valS7</td>
<td>1.4</td>
<td>0.55</td>
</tr>
<tr>
<td>MB6</td>
<td>valS7*</td>
<td>23.2</td>
<td>1.08</td>
</tr>
<tr>
<td>MB1383</td>
<td>valS7</td>
<td>2.7</td>
<td>1.87</td>
</tr>
<tr>
<td>MB1383</td>
<td>valX12</td>
<td>0.5</td>
<td>0.66</td>
</tr>
</tbody>
</table>

* Determined at 25°C.

+ Antiserum was identically diluted in assays or results were normalized to identical dilution values. Results represent the amount of antisera required for 50% inactivation of valyl-tRNA synthetase activity.
in growth rate (28). Therefore we wanted to determine whether strain MB1383, a strain with altered valyl-tRNA synthetase levels, strain MB5, a strain with an altered valyl-tRNA synthetase enzyme, and wild-type strain MB6 would exhibit metabolic regulation of this particular synthetase.

The three strains were grown in LB, glucose minimal, and succinate minimal media at 28°C. Their growth rates were monitored, and the levels of the synthetase were measured by radioimmunoassay II. Only a 2.4-fold growth rate difference was achieved (Table 6) by growing the strains in these media, whereas Parker et al. (28) had obtained a 6-fold growth rate difference by using the same carbon sources. This may be due to lower growth temperature (28°C) or possibly to the nature of the strains themselves (acetate medium could not be used to extend the growth rate difference, as our strains showed no sign of growth in this medium after 4 days of incubation). However, the relative production of the synthetase among the three strains was nearly the same regardless of the growth medium. Strain MB1383 always appears to have an increased level of enzyme relative to strain MB5. There is generally a lower level of synthetase production in all three strains with growth in media poorer than LB. However, only the wild-type strain MB6 exhibits a parallel decrease in valyl-tRNA synthetase production with decrease in growth rate. Neither strain MB5 (valIS7) nor strain MB1383 (valIS7 valIX12) do so; they do not follow the pattern of metabolic control described by Neidhardt et al. (25). This may be due to the mutant nature of the enzyme and raises the question of whether synthetases are involved in their own control.

Degradation of valyl-tRNA synthetase in strains MB5, MB6, and MB1383. Mutant proteins are degraded much more rapidly than normal E. coli proteins (10, 31, 36). Thus, it was possible that the increased level of valyl-tRNA synthetase in strain MB1383 was due not to increased formation but rather to an enhanced stability of the enzyme in vivo as compared to the enzyme in strain MB5. Therefore the in vivo turnover rate of the synthetase was determined. The three strains were pulse-labeled with [3H]lysine and then subjected to a 60- to 110-min chase period with the unlabeled amino acid. During this time samples were removed and the decrease of radioactive valyl-tRNA synthetase in S-100 extracts was assayed by immunoprecipitation of the enzyme (see Materials and Methods). Valyl-tRNA synthetase precipitation in this assay was ascertained by inhibiting the binding of [3H]valyl-tRNA synthetase with different concentrations of unlabeled pure enzyme (Fig. 4). The results (Fig. 5) show the enzyme from wild-type strain MB6 to be stable after 60 min while the thermolabile enzymes of strains MB5 and MB1383 are equally unstable and significantly degraded after 110 min. The zero point in these experiments, taken just before addition of the unlabeled L-lysine chase, was consistently lower than the 5- or 10-min postchase points. This point is lower for strains MB1383 and MB6 than it is for strain MB5. This may be due to different times required for the unlabeled L-lysine to enter the cells or to the growth rate difference between the strains.

**DISCUSSION**

Selection of temperature-resistant revertants from a strain harboring a thermolabile valyl-tRNA synthetase has led to the isolation of three mutant strains which produce increased amounts of the enzyme. This was demonstrated by radioimmunoassays and antiserum titrations of enzyme activity. The radioimmunoassays have also confirmed the antigenic identity of the enzymes in the various strains; these enzymes react in an identical manner with the anti-valyl-tRNA synthetase antibody population.

In each of these strains only valyl-tRNA synthetase levels are elevated. No evidence for a generalized control mechanism affecting the pro-
Fig. 4. Specific precipitation of valyl-tRNA synthetase by rabbit anti-valyl-tRNA synthetase antiserum + sheep anti-rabbit antiserum. A fixed amount of $^3$H-labeled valyl-tRNA synthetase from a representative S-100 cell extract was mixed with increasing amounts of unlabeled pure valyl-tRNA synthetase and fixed amounts of rabbit anti-valyl-tRNA synthetase antiserum and sheep anti-rabbit antiserum. The remaining radioactivity in the precipitates was measured. A 100% value represents approximately $5,500$ net cpm.

Fig. 5. Breakdown of valyl-tRNA synthetase in vivo in strains grown at $28^\circ$C. $\times$, MB5; $\bullet$, MB6; $\circ$, MB1383. For description see the text. A 100% value represents ca. $15,000$ net cpm above background $(9,000$ cpm). The zero time point was taken immediately before addition of an excess of unlabeled lysine. The experimental points represent the average of four determinations.

Production of all the synthetases or even of just the branched-chain aminoacyl-tRNA synthetases has been observed. This is in accord with previous findings in E. coli that each regulatory mutation affects the production of only one synthetase (7, 8, 16, 35). In addition, no link between synthetase production and production of the cognate tRNA has been found here.

The mutations leading to elevated levels of the synthetase in these three strains are closely linked to the $valS$ gene. As such they are similar to $serO$ (8) and $leuX$ (16) in their relationship to their respective synthetase structural genes. However, further work must be done to determine whether the mutations are actually of the operator/promoter type as are $serO$ and $leuX$.

The relationship between the various types of synthetase control mechanisms which have been found to give rise to increased levels of valyl-tRNA synthetase and the control mechanism which is genetically altered in these strains remains obscure. Experiments in which strains were grown in various media showed that the increased production of valyl-tRNA synthetase seen in a representative strain occurs independently of the growth medium. The increase does not seem to be a response of the mutant strain to a particular metabolite as was seen by Hirshfield and Zamecnik (12) in studies on lysyl-tRNA synthetase production.

A crucial and long-standing question in all studies on the regulation of aminoacyl-tRNA synthetase synthesis concerns the rates of protein turnover and new biosynthesis. By using...
pure labeled valyl-tRNA synthetase and antiserum made against this protein, we have shown that the rates of valyl-tRNA synthetase degradation in the parent and in a representative temperature resistant revertant strain are similar. Thus the increased level of this enzyme found in the revertant strain is due to new synthesis. These experiments have also shown that the thermolabile valyl-tRNA synthetase isolated from the temperature-sensitive strain is more rapidly degraded than wild-type enzyme.

ACKNOWLEDGMENTS

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