Pattern of Valine Transfer Ribonucleic Acid of *Bacillus subtilis* under Different Growth Conditions*

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SUMMARY

The relative amounts of two valyl-tRNA species from *Bacillus subtilis* W23 were compared by methylated albumin Kieselguhr column chromatography and by diethylaminoethyl cellulose column chromatography after T1 ribonuclease digestion. The ratio of the two valyl-tRNAs changes significantly during sporulation, during step-up and step-down growth transitions, during the stationary phase of an asporogenous mutant, and during sporulation of *B. subtilis* 168 Marburg. The change in ratio could not be induced *in vitro* by treating the tRNA preparations with several chemicals and was not the result of differential hydrolysis of the two valyl-tRNA species during the experimental procedures. The change in ratio was due to the increased valine-accepting ability of one of the two valine tRNAs.

These results suggest that the valyl-tRNA ratio changes may be a function of the growth condition and that a regulatory mechanism exists which controls the amino acid accepting capacity of the two valine accepting tRNAs.

An analysis of *Bacillus subtilis* tRNA by methylated albumin Kieselguhr column chromatography revealed that the pattern of valyl-tRNA obtained from cells in early sporulation stages was significantly different from that obtained from log phase cells (1). The altered valyl-tRNA profile of early sporulation cells returned to the log phase cell pattern during the late sporulation stages preceding endospore formation. The primary pattern change during early sporulation was in the relative amounts of valyl-tRNA fractions that eluted as two peaks from the MeAK column. This process is reversible, since addition of glucose to a culture in the early stages of sporulation causes resumption of growth of the culture and the return of the valyl-tRNA pattern to that found in growing log phase cells (2). In related experiments Lazzarini (3) has found that spores of *B. subtilis* contained a second lysyl-tRNA fraction, which was not present in vegetative cells. The occurrence of this altered lysyl-tRNA was strongly dependent on the growth medium on which the cells were sporulated and was not correlated directly with sporulation per se (4). Since tRNAs play a vital role in protein synthesis, it is of interest to determine the mechanism and function of these changes. To decide whether tRNAs may have some role in the regulation of protein synthesis, studies have been initiated to determine what growth conditions lead to altered valyl-tRNA patterns and to see whether there is any direct correlation between tRNA changes and sporulation.

The results illustrate that the ratio of two valyl-tRNA species changes during sporulation, during drastic alterations in growth conditions, and during the stationary phase of an asporogenous mutant blocked at an early stage of sporulation. It appears that the alteration of the valine tRNA fraction is correlated with either some factor in the growth medium or in the growth rate; however, it is not possible to decide as yet whether any direct relationship exists between sporulation and tRNA changes.

METHODS

Organism and Media—*B. subtilis* W23 was grown at 37° and used as the source of tRNA and enzymes. *B. subtilis* W23 (PR-BH-SP-), which is protease-less, antibiotic-less, and asporogenous, was kindly provided by Dr. John Spizizen. The SCM medium used for the sporulation experiments contained 0.055 M NH₄Cl, 0.170 M NaCl, 0.054 M KCl, 6 mM MgSO₄, 2.5 mM PO₄³⁻, 0.050 M Tris buffer (pH 7.2), 0.1 mM MnCl₂, 0.2 mM CaCl₂, 3.7 × 10⁻³ M FeCl₃·6H₂O, 5.5 mM glucose, and 0.3% casein hydrolysate (Calbiochem). For the step-down experiments, cells grown in Penassay (Difco) medium were transferred during the log phase of growth to a modified SCM medium. For the modified SCM medium, the glucose concentration was increased to 16.5 mM and the casein hydrolysate was replaced by 5

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mm monosodium L-glutamate and 3.4 mm sodium citrate. During the step-down transition 1 mm MgCl₂ and 10% sucrose were added to the modified SCM medium to prevent cell lysis. For the step-up experiment, cells growing in modified SCM medium were transferred during the log phase to Penassay medium. Since log phase cells tended to lyse during harvesting, the growth medium was made 1 mm MgCl₂ immediately before harvesting and kept at 4° during centrifugation.

Preparation of tRNA—The tRNA was extracted from cells by the method of von Ehrenstein and Lipmann (5). The bound amino acids were removed by treatment of the tRNA with 0.5 M Tris-HCl buffer, pH 7.3, for 60 min at 35°, which was followed by ethanol precipitation and dialysis against water. The tRNA was purified from ribosomal RNA by passing the RNA preparation through a Sephadex G-100 column (1.7 x 80 cm). The column was eluted with a solution containing 0.05 M NaCl and 1 M Tris-Cl buffer, pH 8.8, for 60 min at 35°, which was followed by treatment of the tRNA with 0.5 M NaCl and 10% sucrose. During the step-down transition 1 mm MgCl₂ and 10% sucrose were added to the reaction mixture in an ice bucket, which was then shaken. Two volumes of 100% ethanol were added, and after centrifugation the precipitate was resuspended with 0.1 M acetic buffer, pH 5.5.

Preparation of Methylated Albumin Kieselguhr Columns—The MEAK column was made as described by Sueoka and Yamane (8). The only modification was the use of a shallower linear elution gradient; 160 ml each of 0.3 M and 1.1 M NaCl in 0.05 M phosphate buffer, pH 6.8, were used; 2-ml fractions were collected, and each fraction was analyzed for its optical density at 260 mμ and its radioactivity as described previously (9).

T₁ Ribonuclease Digestion of Valyl-tRNA—The method of Ishikia and Miura (10) was used for T₁ RNase digestion and DEAE-cellulose column chromatography of the digest. The valyl-tRNA was incubated in 2 ml of 0.1 M HCl buffer, pH 5.5, containing 2 x 10⁻⁴ M EDTA in the presence of 500 units of T₁ RNase for 4 hours at 37°. These conditions were sufficient for the complete digestion of tRNA. After the end of this incubation period the digest was diluted to 15 ml with cold water and absorbed to a DEAE-cellulose column (0.8 x 5.0 cm), which had been equilibrated previously with 0.01 M ammonium formate, pH 6.7. After the free amino acids had been eluted with 100 ml of 0.01 M ammonium formate, pH 6.7, a linear gradient from 0.01 M ammonium formate, pH 6.7, to 1.0 M ammonium formate, pH 7.1, was applied with 250 ml of each buffer. The 2-ml fractions were analyzed for optical density at 260 mμ, dried on plastic planchets, and counted with a Packard scintillation counter.

Treatment of tRNA and Valyl-tRNA—The treatment of tRNA and aminoacyl-tRNA with EDTA, Mg++, and high temperatures has been described (11, 12). The oxidation with iodine and reduction with thiourea were performed by the method of Carbon, Hung, and Jones (13).

RESULTS

Comparison of Valyl-tRNA Obtained from Log Phase and Sporulating Cells of B. subtilis—Cells were harvested during the log phase (Fig. 1, Point 1) and sporulation phase (Fig. 1, Point 3) of B. subtilis growing in SCM medium. The tRNA preparations isolated from these cells were stripped of amino acids by alkaline treatment, differentially labeled with valine-¹⁴C and valine-²H, and cochromatographed through an MEAK column (see "Methods"). Two peaks of valyl-tRNA eluted from the MEAK column (Fig. 2). The ratio of the two valyl-tRNA fractions varied markedly, depending on the growth phase of the cell. The first valyl-tRNA peak was much larger than the second peak in the log phase cells, whereas the opposite was true for the valyl-tRNA fractions from sporulating cells.

Several experimental conditions were employed to determine whether the valyl-tRNA patterns could be altered in vitro. 1. Incubation of Isolated tRNA with 0.5 M Tris-HCl Buffer, pH 8.8, at 37° for up to 8 Hours Prior to Aminoacylation—Longer periods of alkali treatment for removal of bound amino acids were used to determine whether the rates of deacylation were similar.
and complete for the two valine-specific tRNAs. No significant increase in total valyl-tRNA formation was observed after treatment of the tRNA preparation for 1 hour at the alkaline pH. The valyl-tRNA formed from the tRNA preparation treated for 3 hours at pH 8.8 still had an elution profile from an MeAK column similar to that shown in Fig. 2. These results indicate that the difference in the relative amounts of the two valyl-tRNA peaks and the altered pattern found in tRNA from sporulating cells are not the result of incomplete deacylation and partial charging of the two valine tRNAs.

2. Treatment of Isolated tRNA with EDTA and Mg++—It has been shown that the MeAK column elution profile of tryptophanyl-tRNA from Escherichia coli can be altered by the presence or absence of bound Mg++. The Mg++ can be removed from the tRNA during the phenol purification procedure by exposure to EDTA or by incubation at low pH in the absence of Mg++. Mg++ can be reinserted into the tRNA structure by incubating the tRNA in the presence of Mg-acetate and phenol or 0.1 M sodium acetate buffer, pH 3.5. Furthermore, Mg++ associated with leucyl-tRNA of yeast could be reversibly removed by incubation at 60° (12). The difference in the valyl-tRNA patterns could be a function of the presence or absence of bound Mg++. However, when the tRNA preparations were treated as discussed above to restore Mg++ either before or after charging with valine, the same MeAK column elution profiles were obtained as in Fig. 2. Therefore the difference in elution patterns is not a function of the presence or absence of Mg++ in the valine tRNA.

3. Treatment of tRNA with Iodine and Thiosulfate—The relative stabilities of the two valyl-tRNAs were tested by incubating part of each peak (indicated by the arrows in Fig. 3) at 37° at pH 6.5 and testing for the decrease in acid-precipitable 14C-valyl-

![Fig. 2.](http://www.jbc.org/issue/10/1968/issue.jpg) MeAK column elution profile of aminoacyl-tRNA can be modified by prior treatment with iodine oxidation or thiosulfate reduction (13, 14), which affect the sulfur-containing bases in tRNA. It has been shown that iodine oxidation of thioacyl-containing tRNA species prior to aminoacylation could prevent the tRNA from accepting amino acids. This treatment can alter the MeAK column pattern of several aminoacyl-tRNAs, which contain more than one tRNA species for a specific amino acid, since the inactivation of only one or part of the tRNA species will result in the absence of these species from the elution pattern. The iodine inactivation can be reversed by thiosulfate reduction, resulting in the reappearance of the sensitive species. When B. subtilis tRNA was treated with either iodine or thiosulfate prior to aminoacylation and then charged with valine, no alteration of the valyl-tRNA pattern was observed. Also, the treatment of valyl-tRNA had no effect. These results indicate that the elution patterns are not the result of partial inactivation of the valine-specific tRNAs and suggest that the valine tRNAs do not contain thiobases.

4. Relative Stabilities of the Two Valyl-tRNAs—The relative stabilities of the aminoacyl linkage of the two valyl-tRNA peaks were determined. The two peaks were separated by passing a large quantity of valine-labeled tRNA through an MeAK column with twice the usual capacity (Fig. 3). Peak 1 has a prominent shoulder on the trailing edge (Fractions 36 to 45). The size of this shoulder varied with different tRNA preparations. An analysis of the valyl-tRNA in this region by T1 RNase digestion followed by DEAE-cellulose chromatography (see "Methods") indicated that it represented a portion of Peak 2 that was eluting earlier than the major part of Peak 2. These inconsistencies of the MeAK column, which resulted in variable peak ratios, prompted us to analyze valyl-oligonucleotides. These analyses will be discussed fully in the following sections.

The stabilities of the two valyl-tRNAs were tested by incubating part of each peak (indicated by the arrows in Fig. 3) at 37° at pH 6.5 and testing for the decrease in acid-precipitable 14C-valyl-

![Fig. 3.](http://www.jbc.org/issue/10/1968/issue.jpg)
FIG. 4. A comparison of the hydrolysis rate of valyl-tRNA from Peaks 1 and 2 from an MeAK column. The two fractions illustrated by arrows in Fig. 3 were incubated at 37°C at pH 6.5. At the indicated times, 0.1 ml was removed from each fraction and precipitated with 10% trichloroacetic acid at 0°C, filtered, washed, and assayed for acid-precipitable counts. The ordinate represents the percentage of radioactivity remaining relative to 0 hour of incubation. The open and closed circles represent Peaks 1 and 2, respectively.

FIG. 5. DEAE-cellulose chromatography of T1 ribonuclease digest of 3H-valyl tRNA and 14C-valyl tRNA. The 3H-valyl-tRNA and 14C-valyl-tRNA were obtained from Peaks 1 and 2, respectively, of two valyl-tRNA preparations fractionated by MeAK columns, as illustrated in Fig. 3. The peak tubes were treated with T1 RNase, as described in “Methods.” The DEAE-cellulose column was eluted with a linear elution gradient of 0.01 M ammonium formate, pH 6.7, and 1.0 M ammonium formate, pH 7.1. Fractions (2 ml) were collected, and the optical density at 260 mp and the radioactivity were measured. The whole fraction was dried on clear plastic planchets and counted with a Packard scintillation counter. The closed and open circles represent 14C and 3H, respectively.

tRNA counts with increasing time. The results in Fig. 4 show that both valyl-tRNA fractions are degraded at the same rate. Therefore the difference in the amount of each peak is not a function of differential hydrolysis rates of the two valyl-tRNA fractions during the experimental procedures.

Analysis of Valyl-tRNA after Digestion with T1 Ribonuclease—It was shown previously that two valyl-tRNA species with different nucleotide sequences at the 3’ hydroxyl end could be detected after hydrolysis with T1 RNase and DEAE-cellulose chromatography (10, 15). Since this method would indicate more clearly whether the two valyl-tRNA peaks from the MeAK column were indeed two species with two different nucleotide sequences, B. subtilis tRNA was analyzed by labeling one fraction with valine-14C and one with valine-1H. Each preparation was chromatographed separately through an MeAK column as shown in Fig. 3. Then 1H-valyl-tRNA from Peak 1 and 14C-valyl-tRNA from Peak 2 were mixed and digested with T1 RNase to obtain the differentially labeled valyl-oligonucleotides from each peak. When this hydrolysate was passed through the DEAE-cellulose column, two peaks of valyl-oligonucleotides were obtained (Fig. 5). Peak 1 and Peak 2 from the MeAK column eluted as Peak 2 and Peak 1, respectively, from the DEAE-cellulose column. The recovery of radioactivity applied to the DEAE-cellulose column was 95 to 99% in this and subsequent experiments. The percentage of recovered radioactivity in valyl-oligonucleotides, free valine, and other labeled fragments was 80 to 90%, 10 to 20%, and 1 to 3%, respectively. The presence of a small amount of 14C in Peak 2 (Fig. 5) represents the contamination of Peak 2 of the MeAK column by Peak 1 of the MeAK column. Better separation of the two valyl-tRNA species was obtained compared with the MeAK column. These results allowed a more quantitative evaluation of the two peaks and gave strong evidence for the presence of two distinct valyl-tRNA species with different nucleotide sequences at the 3’-hydroxyl end.

The valyl-tRNA of log phase and sporulating cells was analyzed subsequently by this procedure. The results with tRNA from log phase cells (see Fig. 1, Point 1) illustrate that the ratio of Peak 2 to Peak 1 is 2.11 (Fig. 6), whereas the ratio of the two peaks for tRNA from sporulating cells (see Fig. 1, Point 2) is 1.02 (Fig. 7). The results in Figs. 6 and 7 show that the first valyl-oligonucleotide peak is smaller than the second peak in tRNA from log phase cells but is approximately equal to the second peak in tRNA from early sporulation cells. A slight variation in the ratios was observed when tRNAs were prepared from different growth cultures; however, the pattern of the changes was always consistent when sporulating cells were ob-
**TABLE I**

<table>
<thead>
<tr>
<th>Growth Phase</th>
<th>Ratio of Two Valyl-tRNA Species</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Peaks 1/2</td>
</tr>
<tr>
<td>1 (Mid-log)</td>
<td>3.1</td>
</tr>
<tr>
<td>2 (Late log)</td>
<td>2.9</td>
</tr>
<tr>
<td>3 (Early sporulation)</td>
<td>1.3</td>
</tr>
<tr>
<td>4 (Late sporulation)</td>
<td>1.8</td>
</tr>
</tbody>
</table>

**Valyl-tRNA Pattern of an Asporogenous Mutant**—It was of interest to determine whether the tRNA pattern change was correlated with sporulation. Transfer RNA was isolated from an asporogenous mutant of B. subtilis W23 (PR-BH-SP−), which is deficient in both protease and antibiotic production and is blocked in early sporulation. The cells were harvested in log phase and at early and late stationary phases, as indicated in Fig. 8. Some lysis was evident in the late stationary phase culture. The valyl-tRNA from these cells was analyzed by DEAE-cellulose chromatography after T1 RNase digestion. The ratios of the valyl-oligonucleotides peaks were determined as before, and the results are summarized in the inset of Fig. 8. The ratios of the two valyl-tRNAs from the log phase and stationary phase mutant cells were similar to those seen in Figs. 6 and 7 for the wild type strain. However, one difference was noted in that the ratio of Peak 2 to Peak 1 remained low and did not rise in the mutant at late stationary phase. In the wild type the ratio rose during late sporulation (see Table I, line 4).

**Valyl-tRNA Pattern of Cells Subjected to Different Growth Phases**

**TABLE II**

<table>
<thead>
<tr>
<th>Growth Phase</th>
<th>Peak 1</th>
<th>Peak 2</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log phase</td>
<td>181</td>
<td>342</td>
<td>524</td>
</tr>
<tr>
<td>Stationary phase</td>
<td>406</td>
<td>462</td>
<td>868</td>
</tr>
</tbody>
</table>

The valyl-tRNA preparations were analyzed by DEAE-cellulose column chromatography after T1 RNase hydrolysis. See "Methods" for the procedures.

![Fig. 7. DEAE-cellulose column chromatography of T1 ribonuclease digest of 14C-valyl-tRNA from early sporulation phase cells of B. subtilis. See "Methods" for procedures. The open and closed circles represent optical density at 260 nm and radioactivity, respectively.](image)

![Fig. 8. Growth curve of B. subtilis W23 (PR-BH-SP−) in SCM medium at 37°. The inset shows the ratio of Peaks 1 and 2 of the valyl-tRNA isolated from the three growth phases, as indicated by the arrows. The tRNA preparations isolated from these cells were treated with T1 RNase and fractionated by DEAE-cellulose column chromatography, as described in the "Methods." The closed circles represent optical density at 600 nm.](image)
Conditions—Since sporulation occurs at a time when the culture medium becomes unfavorable for growth, B. subtilis cells were subjected to sudden changes in growth conditions to determine whether these changes had any effect on their tRNA fractions. The tRNA pattern was examined during transition of cells from a rich medium to a poor medium (step-down) and from a slow to a fast rate of growth (step-up). For the step-down experiments, cells growing at a generation time of 25 min were centrifuged out of the rich medium and transferred to a prewarmed synthetic medium in which the generation time was 50 min. The cells were harvested 30 min after transfer into the poorer medium. The ratio of the valyl-tRNA peaks was determined by DEAE-cellulose column chromatography after T1 RNase digestion. The ratio of Peak 2 to Peak 1 was found to decrease from 2.85 to 2.67, which approaches the ratio of 2.39 found in cells growing exponentially in the poorer medium. The growth curve for the culture under these conditions is shown in Fig. 9. In the reciprocal experiments the cells were transferred from the poor synthetic medium to the rich Penassay medium. The ratio of the two valyl-tRNAs rose from 2.39 to 2.57 after the cells had been transferred to the rich medium for 30 min. These results are summarized in Table III. By use of large cultures and identical experimental conditions, the changes in ratios were found consistently. Duplicate experiments gave ratios that varied by ±4%.

Analysis of B. subtilis Marburg 168—This analysis was extended partially to B. subtilis 168, which is closely related to B. subtilis W23. Homologous tRNA and synthetase preparations were used for synthesizing valyl-tRNA from this species. The relative amounts of valyl-oligonucleotides were determined after T1 RNase hydrolysis and DEAE-cellulose column chromatography. The results are presented in Table IV. For B. subtilis 168 the relative amounts of Peak 2 to Peak 1 in log phase cells was 7.2, which is considerably higher than that found in B. subtilis W23. As in the case with B. subtilis W23, the relative amount of Peak 1 was larger in preparations of tRNA from sporulating cells, since the ratio was only 4.7. Thus, in the case of these two closely related strains, a similar change in the ratio of the two tRNA fractions is observed.

DISCUSSION

Changes in tRNA patterns have been demonstrated after phage (16–18) and virus (19, 20) infections, in tumor and normal mammalian cells (21), in organs from the same organism (21), in cells grown in enriched and semisynthetic media (22), and in sporulating bacteria and spores (1–4). The changes have been examined primarily by comparing elution profiles from an MEA column. The profile changes could be the result of the appearance of a new tRNA species or the alteration of an existing species, which allows it to elute in a different position. The results of valyl-oligonucleotide fractionation by DEAE-cellulose column chromatography provide convincing evidence that two or more distinct species of these tRNAs are involved in the case of sporulating B. subtilis cells. Furthermore, these results demonstrate that the cell has a mechanism for controlling the functional quantity of specific tRNA species. This regulatory mechanism is flexible, since the ratio of the two valyl-tRNA species can be raised or lowered, depending on the metabolic requirements of the cell. The alteration in ratios was most evident during sporulation, when the growth medium is depleted, and during step-up and step-down growth transitions.

The alteration of the functional concentration of the two valyl-tRNAs could occur either by differential synthesis of the two species or by differential modification of the amino acid accepting ability. None of the common nonenzymatic procedures used to alter accepting function was effective in modifying the ratio of the two valyl-tRNAs. If any modification occurred in the cell, an enzyme would be actively involved. The time that
The ratio is related to induction of sporulation. One difference was follows. (a) The change is related to the initiation of sporulation, during step-up and step-down transitions. Experiments are in progress to determine this point. The fact that the valyl-tRNA pattern changes during step-down transition in a manner similar to that in sporulating cells suggests that the alteration of the pattern during sporulation may be related to a deficiency in the growth medium. This regulation may be particularly important when the growth medium of the cells has been altered drastically. The reversible nature of this mechanism was revealed by the reciprocal changes in ratios during step-up and step-down transitions.

The change in ratio of the two valyl-tRNAs during the stationary phase of the asporogenous mutant can be interpreted as follows. (a) The change is related to the initiation of sporulation, and the mutation affected is a function subsequent, to this event. Or (b) the change is related to a deficiency of some component of the growth medium and may or may not be related directly to sporulation. The evidence obtained from analysis of the step-down and step-up cultures favors the second view; it is not possible to decide from the available data whether the change in ratio is related to induction of sporulation. One difference was noted in the patterns between the mutant and the wild type strains. In the mutant, the valyl-tRNA ratio of cells in late stationary phase did not begin to approach that of log phase cells but remained low. This was in contrast to the wild type, in which the ratio began to rise during the late sporulation stages. The significance of this difference for sporulation will be investigated.

Since tRNA is so intimately associated with protein synthesis, these changes in valyl-tRNA patterns may reflect either a cause or an effect of enzyme synthesis during metabolic shifts. The elucidation of the control mechanism of tRNA functional capacity should give some insight into the regulation of protein synthesis (23, 24). Evidence has been presented previously concerning the possible role of aminoacyl-tRNA in cellular regulatory functions (25, 26). Experiments are in progress to determine the mechanism by which functional concentrations of the two valyl-tRNA species are altered and the role of transfer RNA in the regulation of protein synthesis.

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