Unusual Valyl-Transfer Ribonucleic Acid Synthetase Mutant of Escherichia coli

JAMES J. ANDERSON and FREDERICK C. NEIDHARDT

Department of Biological Sciences, Purdue University, Lafayette, Indiana 47907

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Escherichia coli strain NP2907 was isolated as a spontaneous mutant of strain NP29, which possesses a thermostabile valyl-transfer ribonucleic acid (tRNA) synthetase. The valyl-tRNA synthetase of the new mutant, unlike that of its immediate parent, retains enzymatic activity in vitro but differs from the wild-type enzyme in stability and apparent $K_m$ for adenosine triphosphate. The new mutant locus, $valS^{-102}$, cotransduces with $pyrB$ at the same frequency as does the parental locus, $valS^{-1}$. Cultures of strain NP29 cease growth immediately in any medium when shifted from 30 to 40 C. The new mutant grows normally at 30 C, and upon a shift to 40 C growth quickly accelerates exactly as for normal cells. Exponential growth, however, cannot be sustained at 40 C. At a point characteristic for each medium, growth becomes linear with time. This transition occurs almost immediately in rich media and after 1.5 generations in glucose minimal medium. Net synthesis of valyl-tRNA synthetase ceases in the new mutant as soon as the temperature is raised to 40 C, irrespective of the growth medium. We conclude that it is the amount of valyl-tRNA synthetase activity that limits the rate of growth in the linear phase at 40 C. This property of the mutant makes it possible to evaluate the in vivo efficiency of this enzyme at different growth rates and thereby to determine the concentration that is necessary for a given rate of protein synthesis. The results of our measurements indicate that cells of E. coli growing in minimal medium normally possess a functional excess of valyl-tRNA synthetase with respect to protein synthesis and to repression of threonine deaminase.

Escherichia coli strain NP29 (formerly I-9) was isolated by Eidlic and Neidhardt (5) and shown to possess a thermostabile valyl-transfer ribonucleic acid (tRNA) synthetase (VRS) which failed to charge tRNA at restrictive temperatures (2). Consequently, growth in any medium halted immediately at 40 C. Enzyme activity, however, could not be detected in vitro in extracts made from cells grown at any temperature. VRS mutants with similar properties have been independently isolated elsewhere by others (11, 23). Partial reversion to temperature resistance yields a spectrum of altered valyl-tRNA synthetases, many of which fail to regain full wild-type activity in vitro (23; Eidlic, Anderson, and Neidhardt, unpublished data). The phenotype of the above mutants, and that of most other aminoacyl-tRNA synthetase mutants, was readily understandable in terms of the particular enzyme alteration each had incurred. For example, amino acid analogue-resistant mutants were found to have synthetases with altered $K_m$ values for the analogues (7, 10), and certain pseudopseudoauxotrophs requiring high external concentrations of a particular amino acid were found to have a synthetase with an increased $K_m$ for the natural amino acid (3, 15). All of these mutants have been useful in assessing the role aminoacyl-tRNA synthetases play in protein synthesis and especially in the regulation of macromolecule synthesis (16).

Our accidental discovery of a new VRS mutant, E. coli strain NP2907, in which the level of this enzyme can be manipulated by temperature, enabled us to extend the usefulness of mutant analysis to a unique determination of the minimum concentration of VRS which can

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1 The studies reported here are taken from a thesis submitted by James J. Anderson to Purdue University in partial fulfillment of the requirements for the Ph.D. degree. A preliminary report of some of these findings has appeared (17).

2 Present address: Department of Biological Sciences, Dartmouth College, Hanover, N. H. 03755.

3 Present address: Department of Microbiology, The University of Michigan, Ann Arbor, Mich. 48104.
sustain a particular rate of protein synthesis in vivo. Our findings indicate that under all but the most favorable conditions of growth there is a considerable excess of VRS for the requirements of protein synthesis and for the maintenance of repression of the valine-controlled biosynthetic enzymes.

In this paper we present this analysis together with an account of the origin of the new mutant and a description of its phenotype. An accompanying paper (1) concerns the molecular basis of the mutant phenotype.

**MATERIALS AND METHODS**

**Organisms.** *E. coli* strain NP2 is a prototrophic wild-type strain derived from R. Benzer. Strain NP29, a temperature-sensitive mutant derived from NP2, has a thermolabile VRS (5). Strain NP2907 is a spontaneous mutant of strain NP29 and is described below. Bacteriophage P1kc was obtained from Barbara Maling.

*E. coli* strain PA200-Y (SM" leu", thr", pyrB", F") was supplied by D. Fraenkel of Harvard Medical School, Boston, Mass.

**Media and methods of cultivation.** Bacterial cultures were grown aerobically on a rotary-action shaker at the desired temperatures. Minimal medium contained solution P of Fraenkel and Neidhardt (8) supplemented with 0.2% (NH4)2SO4, 0.4% glucose, and other supplements as noted. TGYE medium consisted of 1% tryptone, 0.5% yeast extract, and 0.1% glucose. L-broth had the same composition as TGYE, supplemented with 0.5% NaCl and 2.5 x 10^-2 M CaCl2.

Temperature shifts were performed in two ways. Where medium composition remained the same, a low-temperature sample was diluted at least fivefold into prewarmed fresh medium at the higher temperature. Shifts involving a change in medium composition required a centrifugation and washing step at 5 C before dilution into prewarmed medium. Growth was monitored by optical density at 420 nm with a Beckman model DB spectrophotometer. Unless specifically noted, all growth rate measurements were made under conditions of cell concentration and medium composition which would permit balanced growth. When it was necessary to monitor growth over many generations, intermittent dilutions of cultures into fresh medium were performed.

The specific growth rate constant, k, is defined as: k (hr^-1) = ln 2/mass doubling time (expressed in hours).

**Aminoaacyl-tRNA synthetase assays.** The aminoaacylation of tRNA was assayed by minor modifications of the procedure of Fangman and Neidhardt (7). Reaction mixtures (0.5 ml) contained: tRNA (0.5 mg) prepared from *E. coli* K-12 by General Biochemicals Corp.; reduced glutathione, pH 6.6 (1 μmole); diatomaceous adenosine triphosphate (ATP), pH 6.6 (1 μmole); KCl (5 μmoles); tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.3 (50 μmoles); MgCl2 (5 μmoles); bovine serum albumin (100 μg, Pentex); a limited amount of enzyme; and either L-[^14]C]valine (0.05 μmole, 5 μCi/μmole) or other L[^14]C]amino acids (0.01 μmole, 20 μCi/μmole). Mixtures were incubated at 30 C, and the reactions were terminated at 10 min by the addition of 5% trichloroacetic acid (3 ml) containing D,L-amino acids at 1 mg/ml. Precipitates were collected on cellulose ace rate filters (Schleicher and Schuell, B4) and washed with two rinses of 5% trichloroacetic acid (5 ml apiece), and one rinse of 67% ethanol (5 ml) at -10 C. The air-dried filters were counted in a Nuclear-Chicago thin end window gas flow counter. Kinetic experiments employed the following modifications. The specific activity of the isotope was increased to 20 μCi/μmole, and variable concentrations of ATP or L-valine were used. The precipitates were counted in a toluene-based scintillation fluid by a Packard liquid scintillation counter.

**Preparation of extracts.** Rapidly chilled culture samples were centrifuged at 9,000 x g for 10 min at 0 C, and the cells were washed twice with one culture volume sample of cold 0.1 M Tris-hydrochloride (pH 7.3) containing 2-mercaptoethanol (0.006 M) and L-valine (0.001 M). The final pellet was resuspended in this buffer (approximately 3 mg of protein/ml of buffer) and sonically disrupted for 3 min at 3 on a Branson sonifier, model S-75. The mixture was then centrifuged at 20,000 x g for 15 min to remove whole cells and insoluble debris. Extracts were stored at 0 C and assayed within 24 hr. Dialysis did not significantly affect enzyme activity and was not routinely performed.

Protein content of extracts was determined by the method of Lowry (13), using bovine serum albumin (Pentex) as a standard.

**Threonine deaminase assay.** Cell extracts were prepared as above, except that the buffer contained potassium phosphate (0.05 M, pH 7.2), ethylenediaminetetraacetic acid (EDTA, 0.001 M), and L-isoleucine (20 μg/ml). The method of Umbarger and Brown (20) was used. The reaction mixture (0.5 ml) contained: L-threonine (40 μmoles); NH4Cl (50 μmoles); pyridoxalphosphate (10 μg); Tris-hydrochloride (50 μmoles, pH 8.0); and limiting enzyme. Incubation at 37 C was halted after 10 min by chilling. A small portion (0.1 ml) was removed and added to 3 ml of 2.4-dinitrophenylhydrazine (0.2% in 0.5 N HCl). After 15 min, 40% KOH (0.5 ml) was added. Absorbance at 540 nm was read on a Zeiss (model PMQ II) spectrophotometer, and values were compared with those from a standard curve of known α-ketobutyrate concentrations.

**Transduction.** Media and techniques were essentially those of Lennox (12). High titer strains of bacteriophage P1kc were prepared by serially passing the phage four times on the donor strain NP2907. The recipient, *E. coli* strain PA200-Y, was infected in L broth at a multiplicity of infection of 1.0. Recombinants possessing the wild-type pyrB allele were selected by ability to grow on plates lacking uridine and screened for the unselected valS character by replica plating to TGYE agar plates at 40 C. The valS-102 lesion permitted only a fine lawn of growth after 24 hr of incubation and was readily distinguished both from the wild type and the parent mutant. All transductants were screened for the un-
selected amino acid markers, and mutational reversion to pyrB+ was monitored.

RESULTS

Isolation of strain NP2907. A culture of strain NP29 which had been serially cultured four times at 30 C on 1.8% agar slants (TGYE) over a period of several months was found to have accumulated a secondary mutant. The selective agent was not obvious, but it was noted that lengthy storage of strain NP29 at 5 C on slants resulted in a prolonged lag in growth upon inoculation into fresh medium, liquid or solid, at 30 C. Neither the parent strain, NP2, nor the new mutant exhibited this lag. The possibility exists that the VRS of strain NP29 is unstable upon long-term storage of cultures and that a partial revertant of the mutant strain is selected. The new mutant was cloned and designated NP2907.

Growth of strain NP2907. When cultures of strain NP29 are shifted from 30 to 40 C, growth ceases immediately in any medium (5). Figure 1 contrasts the growth of the wild type, NP2, with that of strain NP2907 in TGYE medium, minimal medium plus 18 amino acids, and minimal medium plus L-valine and L-isoleucine. In each, growth was comparable to the wild type at 30 C and was initially so at 40 C. Growth lapsed into a linear mode immediately in TGYE medium, at approximately 0.5 generation in the minimal medium plus 18 amino acids, and at 1.5 generations in minimal medium plus L-valine and L-isoleucine. When L-valine was not included in the medium (isoleucine was routinely added with L-valine because the strain is valine-sensitive), the persistence of log growth was highly variable. In all subsequent experiments, the minimal medium was supplemented with the two amino acids. This supplementation did not change the growth rate of either mutant or wild strain at 30 C.

When growth of a bacterial culture proceeds linearly with time, it usually signifies either dilution of a catalytic component of the cells necessary for growth or some environmental limitation such as a linear rate of gas exchange or production of an inhibitor. In the present case it was easily determined that an altered VRS of strain NP2907 was responsible for the peculiar growth pattern, as will now be shown.

Altered valyl-tRNA synthetase of strain NP2907. Strain NP2907 possessed in vitro VRS activity by the attachment assay, in contrast to extracts of the parental strain which lack such activity. Figure 2 shows that the activity from strain NP2907 is unstable in the absence of L-valine and at low ionic strength at 0 C, differing from the stable wild-type enzyme of strain NP2. Yaniv and Gros (23) have also reported substrate stabilization of thermolabile VRS's. When the molarity of the buffer cation is raised to 0.1 M (Na+, K+, or NH4+) can substitute for Tris+) and 0.001 M L-valine is added, the VRS activity is fully stable for several days.

When extracted under stabilizing conditions, the specific activity of strain NP2907 was identical in extracts from cells grown in different media (Table 1). The differences in the levels in the wild-type strain grown in different media were reproducible, and dialysis of the crude extracts had no effect upon these levels. The arginyl-tRNA synthetase level was constant in both strains, in all media.
FIG. 2. Stability of valyl-tRNA synthetase in crude extracts of strains NP2 and NP2907 at 0 C. Valyl-tRNA synthetase activity was measured by the tRNA attachment assay. Extracts of strain NP2907 were prepared in 0.006 M potassium phosphate buffer, pH 7.3, containing 0.006 M 2-mercaptoethanol only (A) or 0.001 M L-valine in addition (O), or in 0.1 M Tris-hydrochloride (pH 7.3) containing 0.006 M 2-mercaptoethanol and 0.001 M L-valine (C). Strain NP2 was prepared in 0.006 M potassium phosphate buffer, pH 7.3, containing 0.006 M 2-mercaptoethanol (O). Data are plotted as per cent initial activity (within one hour of preparation of the extract) versus time of incubation at 0 C.

Figure 3 is an Eadie (4) plot of kinetic data obtained from crude extracts of strain NP2 and strain NP2907, measuring the apparent Michaelis constants of the VRS for L-valine and ATP. There is no significant difference in apparent K_m for L-valine, but the K_m for ATP is increased fourfold in the mutant strain. Yaniv and Gros (23) have reported K_m-type mutants among their collection of VRS mutants; all carried increased K_m values for both L-valine and ATP.

Behavior of the altered valyl-tRNA synthetase in vivo at restricting temperatures. Figure 4 depicts the accumulation of aminoacyl-tRNA synthetase as a function of growth when strain NP2907 was shifted from 30 to 40 C in rich (TGYE) and in poor (minimal) medium. The temperature shift had no effect upon the differential rate of accumulation of the arginyl-tRNA and phenylalanyl-tRNA synthetases, but accumulation of VRS was halted in both media. The measurable enzyme activity per milliliter of culture remained constant in both media for over an eightfold increase in cell mass; the concentration (enzyme units/milligram of protein) of this enzyme progressively declined at a rate which matched the accumulation of new cellular protein.

Either of two circumstances could produce this effect. Synthesis of the enzyme might be halted at 40 C, and preexisting, stable enzyme would be diluted during cell growth; or there might be an exact matching of rates of synthesis of the enzyme and its inactivation. This question is dealt with in our accompanying paper (1), in which it is shown that it is the stability of the enzyme and not its synthesis that is abnormal at 40 C.

The effect of reduced concentration of VRS upon the growth rate of the mutant at 40 C is illustrated in Fig. 5. In rich medium, all but the initial concentration of the enzyme are growth-limiting, resulting in lower values of k as the concentration falls. This occurs when the concentration is lowered gradually by growth or when the enzyme level is reduced by incubating nongrowing cells in the absence of L-valine (1). In contrast, cells whose k is low for nutritional reasons (minimal medium) can sustain a threefold reduction in VRS concentration before further reduction in k. These

<table>
<thead>
<tr>
<th>Organism</th>
<th>Medium*</th>
<th>Valyl-tRNA synthetase</th>
<th>Arginyl-tRNA synthetase</th>
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<tr>
<td>NP2</td>
<td>Minimal</td>
<td>0.27</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>Minimal + 18 amino acids</td>
<td>0.36</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>TGYE</td>
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</tr>
<tr>
<td>NP2907</td>
<td>Minimal</td>
<td>0.11</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>Minimal + 18 amino acids</td>
<td>0.11</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>TGYE</td>
<td>0.13</td>
<td>0.12</td>
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</table>

* The various media are described in the text.
* Specific activities are expressed as micromoles of aminoacyl-tRNA formed per milligram of crude protein per hour.
cells must possess a threefold excess of VRS under these conditions, at least with respect to overt growth requirements.

When growth becomes limiting at 40 °C, it is most probably at the expense of valyl-tRNA. A comparison can be made between the calculated requirement for valyl-tRNA and the actual measure of the activity of the enzyme producing it, when it is known to be limiting. A culture of the mutant containing 1 mg of protein and growing at a K of 1.0 is producing protein at the rate of 1 mg/hr. From the molar proportion of L-valine residues in E. coli protein, 5.5% (18), it is seen that this amount of protein requires the production of 0.5 μmole of valyl-tRNA, or the functioning of 0.5 unit of VRS. From Fig. 5 it can be seen that at a k of 1.0, 0.12 unit of VRS is rate-limiting, when enzyme activity is measured in vitro at 30 °C. Allowing for a twofold increase in enzyme activity at 40 °C, it is evident that the mutant enzyme must be twice as active in vivo as in vitro to account for the observed growth.

Repression of threonine deaminase. Eidlic and Neidhardt (5) demonstrated a requirement for an active VRS in maintaining repression of the valine-controlled biosynthetic enzymes of the branched-chain amino acid pathway. Freundlich (9) and Williams and Freundlich (21) have shown that only those valine analogues which the VRS can transfer to tRNA are effective in repression. Figure 6 depicts the ability of strain NP2907 to maintain repression of threonine deaminase, in the presence of the four multivalent repressors of that enzyme, while the VRS concentration is declining during growth at 40 °C. Both rapidly growing and slowly growing cultures exhibit a fivefold derepression of threonine deaminase after the temperature shift, but the onset of derepression is delayed one mass doubling time in the more slowly growing cells. This finding shows that there is an excess of VRS in the latter case not only for growth but also for repression.

The extent of derepression of threonine deaminase in TGYE-grown cultures of strain NP2907 adapted to temperatures other than 40 °C is shown in Fig. 7, along with the temperature dependence of the VRS level of the mutant during balanced growth. The steady state values of k at these different temperatures are given in Table 2. It is evident that there is a correlation between the amount of VRS a cell can maintain and the temperature of growth, even below 30 °C. Surprisingly, at temperatures at which there is a severe growth limitation, such as 34 °C, at which the mutant k is half that of the wild type, there is only 50% of the derepression of threonine deaminase that occurs at 40 °C. A similar phenomenon has been reported for another aminocyl-tRNA synthetase mutant (14); well adapted cultures of E. coli growing under a histidyl-tRNA limitation which severely restricted growth yielded only an intermediate level of derepression of the histidine biosynthetic enzymes.

Analysis of the mutant by transduction. Tingle and Neidhardt (19) showed that the original VRS locus, valS-1, was cotransducible with pyrB at a frequency of 85% and probably maps on the side of pyrB opposite the fdp
locus. Accordingly, the transducing phage Plkc was grown on strain NP2907 and used to infect E. coli strain PA200-Y (pyrB, valS+). The pyr+ colonies were selected and tested for the NP2907 character by temperature challenge as described above. By this criterion, 85% of the 100 pyr+ colonies were NP2907-like. Twelve of the latter were also tested for in vitro valine-mediated stability, and all were found to possess the NP2907 character. All of the temperature-resistant colonies possessed a wild-type VRS. It is therefore highly likely that the lesions in both strain NP29 and strain NP2907 are alleles; we have given the new mutant the designation valS-102. The frequency of reversion of strain NP2907 to wild-type growth on plates at 40 C is low, approximately 10^-8.

DISCUSSION

The work presented here has demonstrated that, under ordinary nutritional restriction of growth rate, there is no apparent adjustment of the cellular concentration of VRS to match closely the reduced demand for valyl-tRNA. This conclusion is based upon the unique opportunity afforded by strain NP2907 to vary the internal concentration of VRS and to assess its in vivo efficiency in protein synthesis and in repression of the L-valine-controlled biosynthetic enzymes. By means of an alteration in the VRS protein, the concentration of this enzyme can be gradually lowered by growth at 40 C while the effect of the reduction is monitored by observations of growth rate and state of repression of threonine deaminase. Despite the probably different milieu within cells growing at widely different growth rates, it is clear that in slowly growing cells there is a true excess of this enzyme, since its concentration could be lowered at least two-

![Figure 4](http://jb.asm.org/)  
**Fig. 4.** Differential rate of aminoacyl-tRNA synthetase accumulation in strain NP2907 after a temperature shift. Two cultures of strain NP2907 growing in balanced growth at 30 C were shifted to 40 C, and samples were taken periodically to determine aminoacyl-tRNA synthetase activities. The culture growing in TGYE medium (described in the text) was measured for phenylalanyl-tRNA synthetase activity (A), arginyl-tRNA synthetase activity (■), and valyl-tRNA synthetase activity (○). The other culture, growing in minimal medium plus 150 μg of L-valine and 50 μg of L-isoleucine per ml, was measured for valyl-tRNA synthetase only (○). Enzymes units (micromoles of aminoacyl-tRNA formed per hour per milliliter of culture) of the three synthetases have been-normalized at their 30 C levels for the sake of comparison. Data are plotted as relative enzyme units per milliliter of culture versus mass per milliliter of culture, where mass was measured by optical density at 420 nm.

![Figure 5](http://jb.asm.org/)  
**Fig. 5.** Specific growth rate (k) dependence upon concentration of valyl-tRNA synthetase in Escherichia coli strain NP2907 at 40 C. The data are combined results of experiments similar to those illustrated in Fig. 1 and 4. Where k was continuously changing (as in Fig. 1a), it was estimated as the tangent to the curve at the point at which the cell sample was withdrawn for measurement of enzyme activity. Growth at 40 C in TGYE medium (○) and in minimal medium plus L-valine and L-isoleucine (×) was followed spectrophotometrically by optical density at 420 nm. A separate culture of TGYE-grown cells was resuspended in 0.7% NaCl at 40 C, and at various times portions were removed for enzyme assays and reconstitution of the original TGYE medium at 40 C. In this case, k was estimated as the initial rate of growth at 40 C after reconstitution (○).
fold before any physiological stress could be detected. This behavior occurs in a mutant strain that has an altered enzyme but presumably a normal regulatory system for that enzyme (1).

This conclusion is relevant to the studies of Nass and Neidhardt (15) and of Williams and Neidhardt (22) who found that at least some and possibly all aminoacyl-tRNA synthetases are regulated by a repression-like mechanism. Manipulation of the amino acid supply to growing cells can result in an alteration in the differential rate of formation of the cognate synthetases; 30-fold variations in rate have been measured. Nevertheless, most synthetases are maintained at the same level during slow growth in minimal medium as during fast growth in rich medium, and even in the exceptional case of VRS in strain NP2 (Table 1) a threefold reduction in growth rate from rich to minimal medium is accompanied by only a 35% reduction in synthetase level.

One possible explanation for this apparent failure of the regulatory device to match synthetase level with growth rate is that these enzymes are inefficient in cells growing in minimal medium, and therefore the level being

![Graph](image)

**Fig. 6.** Differential rate of synthesis of threonine deaminase in strain NP2907 after a temperature shift. Cultures of strain NP2907 growing in TGYE medium (○) and in minimal medium plus 150 μg of L-valine per ml, 50 μg of L-isoleucine and L-leucine per ml, and 10 μg of calcium pantothenate per ml (▲) were shifted from 30 to 40°C, and samples were removed periodically for measurement of threonine deaminase activity (1 enzyme unit = 1 μmole of α-ketobutyrate formed per hr). The two experiments have been normalized to adjust for different cell concentrations. Data are plotted as the amount of threonine deaminase per milliliter of culture versus mass per milliliter of culture, where mass was monitored spectrophotometrically at 420 nm.

maintained is actually the minimum appropriate level. One could envision, for example, that the amino acid concentration in cells growing in minimal medium is subsaturating for the enzyme. Such an argument is made difficult, however, by the fact that supplementation of minimal medium with individual amino acids does not repress the cognate synthetases (15, 22; Anderson and Neidhardt, unpublished data). Furthermore, in strain NP2907, if the results reported here have been correctly interpreted, the level of VRS in cells growing in minimal medium is in excess by two- to threefold of what these cells need.

If it is generally true that the regulation of aminoacyl-tRNA synthetases in normal cells is

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**Table 2.** Growth rates (k) of strain NP2907 during balanced growth in TGYE medium at different temperatures

<table>
<thead>
<tr>
<th>Temp (°C)</th>
<th>Growth rate (k)</th>
<th>Temp (°C)</th>
<th>Growth rate (k)</th>
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<tr>
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* Cultures were adapted to the indicated temperatures, as described in the legend to Fig. 7, before k(h⁻¹) was determined.
* This figure is an overestimate; balanced growth could not be confidently demonstrated.
not designed to match their level to immediate functional demand, then one should entertain the possibility that novel biochemical signals are employed in the regulatory process.

ACKNOWLEDGMENTS
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LITERATURE CITED