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Screening of *Escherichia coli* Temperature-sensitive Mutants by Pretreatment with Glucose Starvation

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A system for screening *Escherichia coli* temperature-sensitive mutants is described. The system involves glucose starvation and minimizes ambiguities introduced by the interdependencies of macromolecular synthesis during balanced growth. The system permits the quick recognition of protein synthesis mutants and their classification into two general catagories. Complete protein synthesis mutants are unable to make any polypeptide material, whereas partial protein synthesis mutants are able to produce inactive proteins. The phenotypes of several mutants are described.

Auxotrophic mutants have been a valuable aid in the elucidation of many biochemical pathways (1, 3, 6, 31). The analysis of such mutants as a biochemical approach to intermediary metabolism has been extended to control systems; for example, control of deoxyribonucleic acid (DNA), synthesis (21), ribonucleic acid (RNA) synthesis (28), and protein synthesis (17). At present, attempts are being made to use mutants to study the steps required to synthesize a biologically active RNA (14) or DNA molecule (7), and to look at *individual* steps in protein synthesis (8). However, no investigation reported has analyzed a large number of bacterial mutants to outline the number and nature of the steps required to synthesize a protein molecule.

Mutations affecting the synthesis of all cellular proteins would normally be lethal; however, certain mutants can be obtained where the mutation is fully expressed only under certain conditions, e.g., at high temperatures. Such conditionally lethal mutants were first used by Edgar and coworkers (9). They have since been employed by many investigators in the study of viruses (4, 5, 7, 9, 10, 12, 14, 19, 24, 30), bacteria (11, 16, 20, 22, 25), and other organisms (13, 15, 27). We have surveyed several temperature-sensitive (T^s) mutants of *Escherichia coli* in an attempt to screen specifically for protein-synthesis mutants.

Two conditions must be fulfilled if one is to categorize T^s mutants with confidence. First, the screening system must be able to distinguish mutants that (i) do not affect the synthesis of proteins, (ii) affect protein synthesis secondarily, and (iii) affect protein synthesis directly. Simply shifting a growing culture of bacteria from a low to a high temperature and following the synthesis of protein with time is not sufficient. We do not know the length of time at the high temperature required to inactivate any given temperature-sensitive function; hence, we cannot distinguish between primary and secondary effects on protein synthesis. For this reason, it is preferable to inactivate the temperature-sensitive function in the absence of metabolic activity.

Second, the data must be expressed in a way that allows one to assign a specific metabolic lesion for each mutant unequivocally. The usual way of expressing screening data for T^s mutants is by means of the ratio of biosynthetic capacity per cell at 42 C to biosynthetic capacity per cell at 30 C.

However, it must be remembered that, unlike most auxotrophs, T^s mutants usually reflect a rate-constant change in a given cellular reaction, not the total absence of that reaction. Moreover, the rate constant is usually different from that of the parent strain *at both temperatures*. For these reasons, we express our results as the ratio (reported hereafter as the index) of biosynthetic capacity per mutant cell at 42 C to biosynthetic capacity per parent cell at 42 C.

We describe here the ability of several T^s mutants to make virus, to synthesize the enzyme β-galactosidase, and to incorporate ^{14}C-leucine, ^{3}H-thymidine, and ^{3}H-uridine at 42 C. From these data, we have been able to specify particular lesions for some mutants. Results from the starvation procedure are compared with data obtained from the usual procedure for screening temperature-sensitive mutants, i.e., simply shifting a logarithmically growing culture from 30 to 42 C; the advantages of the former procedure are indicated.

MATERIALS AND METHODS

Bacteria and media. The bacterium used in this study was *E. coli* A232 and was obtained from S. E. Luria (this is strain AB1152 of E. Adelberg's collection). The strain is an F^- and requires arginine, tryptophan, histidine, and vitamin B_1. It is streptomycin-sensitive, λ-sensitive, T6-resistant, and is able to ferment lactose.

The minimal medium (SC) contained 2.5×10^{-2} M tris(hydroxymethyl)aminomethane (Tris), *p*H 7.3; 5×10^{-3} M PO_4, *p*H 7.0; 4×10^{-1} M NaCl; 5×10^{-4} M KCl; 1.5×10^{-4} M $MgCl_2$; 2×10^{-4} M Na_2SO_4; 10^{-6} M $FeCl_3$; and 2 mg of glucose per ml. Minimal $PATHB_1$ medium contained, in addition, proline, arginine, tryptophan, and histidine at 20 μg/ml, and vitamin B_1 at 1 μg/ml. The complete medium was either Difco Nutrient Broth or L broth (0.8 g of glucose, 8 g of Difco tryptone, 4 g of Difco yeast extract, 4 g of NaCl, and 1.8 ml of 1 N NaOH in 800 ml of distilled water). Penassay medium was Difco Antibiotic Medium No. 3; 10× broth was Nutrient Broth at 10 times the concentration specified by the manufacturer. Solid media, except Nutrient Agar, contained 2% minimal Difco agar in addition to the above components. Nutrient Agar was 2.3% Difco Nutrient Agar.

Selection of mutants. About 5×10^7 cells from a mid-logarithmic phase culture of *E. coli* A232 grown at 25 C in Nutrient Broth were spread on Nutrient Agar plates and irradiated at 35 cm for about 60 sec with a Sylvania G15T8 lamp, to give 100 to 200 survivors. The plates were incubated at 30 C for 3 days, and then replicated onto two fresh sets of nutrient plates. One set was incubated at 30 C and the other at 43 C. Colonies too small to replica-plate were picked and analyzed for growth on agar at 30 and 43 C. Single colony isolates from mutants able to grow at 30 C, but not at 43 C, were tested for their temperature-sensitive phenotype on several media. T^s mutants were finally tested on Nutrient Agar for their ability to recover from a 3-day exposure at 43 C.

Glucose starvation. Cells were grown at 25 C in Nutrient Broth to late logarithmic phase, harvested, washed once with SC medium lacking glucose, and resuspended in SC medium lacking glucose at an optical density (660 mμ) of 0.7 to 0.9. The culture was starved with gentle shaking for 17 to 20 hr at 30 C, then for 2 hr at 42 C. At this point (time-zero), 0.1 volume of 10× Nutrient Broth was added to the culture, and incubation was continued at 42 C with vigorous shaking.

Ability of T^s mutants to support virus production at 43 C. Mutants were made lysogenic for λC_1857 at 30 C by selecting and purifying survivors from an infected culture. This strain of λ is induced at 42 C (29). Lysogenic T^s cells were starved for glucose as described above and preincubated at 43 C.

The virus was induced during the preincubation, but the production of metabolites requiring energy for their synthesis did not begin until broth was added back to the culture. After the addition of broth, the optical density at 660 mμ of the culture was followed. If the culture lysed, plaque-forming units (free phage plus infective centers) were assayed at the end of the lytic cycle by the soft-agar overlay technique. A 0.1-ml amount of a dilution of the induced recovering culture was mixed with 0.1 ml of logarithmically growing sensitive cells in melted soft (0.5%) L agar, and the mixture was poured over 2% L agar. The plates were incubated at 30 C for 1 hr, and then at 43 C overnight. If the culture did not lyse, the same assay was performed 120 min after the addition of broth. In addition, the number of cells and the number of plaque-forming units were measured before the 42 C preincubation.

Ability of T^s mutants to synthesize β-galactosidase at 43 C. Mutants were starved for glucose as described above. Isopropylthiogalactoside (IPTG; Mann Biochemical, New York, N.Y.) at a final concentration of 2×10^{-4} M was added to the culture with the 10× nutrient broth. At 15-min intervals, 0.5-ml samples were removed from the culture and placed in 2 to 3 drops of toluene at 4 C. The toluene-treated samples were shaken for 20 min at room temperature, and 0.1 ml was assayed for β-galactosidase activity by the assay of Pardee, Jacob, and Monod (26) with slight modifications. The assay was carried out at 40 C, and the *rate* of appearance of *o*-nitrophenol from *o*-nitrophenyl galactoside (Calbiochem, Los Angeles, Calif.) was followed by observing the absorbancy at 420 mμ with a Klett-Summerson colorimeter. One unit of enzyme is equal to the number of Klett units per minute of assay per milliliter of culture divided by the optical density at 660 mμ of the culture at the time of sampling. β-Galactosidase indices for T^s mutants are expressed as: (enzyme units per minute of recovery for T^s mutant at 42 C)/(enzyme units per minute of recovery for A232 at 42 C).

The mutants were always identified before and after the experiment by nutritional tests on $PATHB_1$, Nutrient Agar, and L agar.

Ability of T^s mutants to incorporate ^{14}C-leucine, ^{3}H-thymidine, and ^{3}H-uridine at 42 C. Mutants were starved for glucose as described above. ^{14}C-leucine at 0.1 μc/ml and ^{3}H-thymidine or ^{3}H-uridine at 1 μc/ml were added to the culture with the 10× broth. The source of radioactive isotopes is presented in the accompanying communication (18). At 20-min intervals, 0.5-ml samples were withdrawn and placed in 0.5 ml of cold 2% trichloroacetic acid. The samples were assayed for trichloroacetic acid-precipitable radioactivity. No difference was found between ^{14}C radioactivity in hot (heated to 80 C for 20 min) and cold trichloroacetic acid precipitates, so cold trichloroacetic acid precipitation was routinely used. The trichloroacetic acid assay used was the crude radioactivity assay described by Jacobson and Gillespie (18). The results were confirmed, where so specified in the text, by purified radioactivity assays (18). The latter assay gives a more dependable measure of the incorporation of thymidine into DNA. One unit of incorporation equals the number of counts per minute incorporated per 0.5 ml of culture divided by the optical density at 660 mμ of the culture at the time of sampling. Again indices were expressed as: (incorporation units per minute of recovery for T^s mutant at 42 C)/(incorporation units per minute of recovery for A232 at 42 C). Mutants were always identified nutritionally before and after the experiment.

The chemical procedures for assaying DNA, RNA, and protein, and the method for analyzing the cellular ribosome content, have been described (18). Where indicated, ribosome content of mutants was determined at 60 min of recovery. Both untreated and ribonuclease-treated extracts were examined in all cases. The rough map positions reported here were obtained from conjugation experiments in which Hfr strains with different origins were used. The procedural details will be reported when the map positions have been determined more accurately.

RESULTS

Nutritional responses of T^s mutants. All of the mutants described here were induced by intense ultraviolet (UV) irradiation (6 decades of killing) and were selected on Difco nutrient medium without allowing phenotypic segregation. Of 13,000 UV survivors tested, about 0.7% were T^s on Nutrient Agar. When these mutants were tested for their temperature-sensitive phenotype on several different media, many nutritional patterns were obtained (Table 1). For the sake of simplicity, we have divided the response into three categories with respect to growth on minimal $PATHB_1$ medium.

Class 1 is temperature-sensitive on $PATHB_1$ and nutrient medium. This class can be subdivided with respect to growth on other media, but we see no operational basis for doing this yet.

Class 2 mutants do not grow on $PATHB_1$ medium at any temperature and are T^s on nutrient medium. Some of these are double mutants (as judged from the nutritional responses of revertants), but many appear to be pleitropic single mutants. Most Class 2 mutants are temperature-independent (T^i) on L agar.

Class 3 mutants grow on $PATHB_1$ at both temperatures and are T^s on nutrient medium. About half of our mutants fell into this last class. Many of these appear to be mutants that do well if allowed to produce their own intermediates, but are unable to grow when nutrients are supplied exogenously at the concentrations found in Nutrient Broth (so-called nutrient-sensitive mutants). Other class 3 mutants are extremely sensitive to *p*H changes or require relatively high concentrations of phosphate, sulfate, magnesium, or other minimal medium components.

Virus production at 43 C by T^s mutants. Column 2 of Table 2 presents the data obtained from the virus assays. In general, three phenotypes were found—normal virus production (80 to 100% of the parent strain), low virus production, and no virus production. So far, we have not been able to make the virus production assay in the parent strain reproducible enough to justify expressing the results with mutants as an absolute ratio (mutant/parent). Low virus production is on the order of one plaque-forming virus particle per cell, and may represent the expression of infective centers at 30 C during the soft-agar assay. No virus production reflects the absence of free (active) virus and the destruction of infective centers.

Column 3 of Table 2 indicates the strains in which virus-induced lysis occurred.

Growth and β-galactosidase synthesis at 43 C by T^s mutants. Column 4 of Table 2 presents the data obtained from β-galactosidase induction experiments. This assay is highly reproducible with the parent strain, with respect to the amount of enzyme synthesized and the time of the initiation of enzyme synthesis. With reference to the latter phenomenon, the extent of glucose starvation (i.e., the extent of ribosome depletion) is critical. Incomplete starvation reduces the lag before enzyme appearance, but does not alter the rate or amount of enzyme made.

Four general phenotypes were obtained—hyper enzyme synthesis, normal enzyme synthesis (index = 1.0 to 1.2), half normal enzyme synthesis (index = 0.4 to 0.5), and low enzyme synthesis (index = 0 to 0.2). In most mutants and in the parent strain, the enzyme increased linearly for at least 75 min, but in a few mutants enzyme synthesis ceased abruptly after 15 to 30 min of production (Fig. 1, Table 2). The latter mode of synthesis represents a delayed (secondary) effect on protein synthesis.

Column 5 of Table 2 shows the type of growth at 42 C exhibited by T^s mutants during recovery from glucose starvation. The parent strain typically demonstrates a biphasic growth pattern (Fig. 2, 3, 7).

Incorporation of ^{14}C-leucine, ^{3}H-thymidine, and ^{3}H-uridine at 43 C by T^s mutants. The data obtained from radioactive precursor incorporation experiments are presented in Table 3. Graphs are presented for those mutants whose incorporation curves were of a different shape than those of the parent strain (Fig. 3–14). Mutants cannot be as easily categorized with this test as they can with the virus and β-galactosidase assays, but the test is the most informative of all when interpreted in connection with the other assays. For example, mutants that make little or no virus or enzyme may (T^s-41, 53, 58, 63, 67, 73, 83, 86, and 128) or may not (T^s-68) incorporate ^{14}C-leucine. We are calling the former class partial protein synthesis mutants and the latter complete protein synthesis mutants. Other examples are presented in connection with specific mutants.

Categorization of specific mutants. T^s-40 is unable to make DNA (low thymidine incorporation, no detectable DNA increase by the diphenylamine

TABLE 1. *Nutritional responses of T^s mutants*

T^s no.	PATHB$_1$		Nutrient		Lagar		Penassay		PATHB$_1$ + yeast extract		Nutrient + yeast extract		Requirement at 30 C on
	30 C	43 C	30 C	43 C	30 C	43 C	30 C	43 C	30 C	43 C	30 C	43 C	PATHB$_1$
Class 1													
40	+++	−	+++	−	+++	−	+++	−	+++	−	+++	−	
41	+++	+	+++	−	+++	+	+++	±	+++	+	+++	−	
42	+++	−	+++	+	+++	+	+++	+	+++	+	+++	+	
45	+++	−	+++	−	+++	+++	+++	+++	+++	+	+++	+	
53	+++	+	+++	+	+++	+++	+++	+++	+++	++	+++	+	
58	+++	−	+++	−	+++	+	+++	+++			+++	++	
67	+++	−	+++	+	+++	+	+++	+	+++	+	+++	+	
68	+++	−	+++	−	+++	+	+++	++	+++	++	+++	−	
80	+++	−	+++	−	+++	+++	+++	+++			+++	+++	
84	+++	−	+++	±	+++	+	+++	+	+++	+	+++	−	
92	+++	−	+++	−			+++	+	+++	−	+++	−	
103	+++	−	+++	−	+++	(−)			+++	++	+++	−	
114	+++	−	+++	−			+++	−	+++	−	+++	−	
115	+++	+	+++	+			+++	+++	+++	+++	+++	+	
119	+++	+	+++	+			+++	+	+++	++	+++	+	
131	+++	−	+++	−	+++	−	+++	−					
Class 2													
38	−	−	+++	+	+++	+++	+++	+++	+++	+++	+++	++	Adenine or guanine
51	−	−	+++	−	+++	++	+++	++	+++	++	+++	++	Adenine or guanine
52	−	−	+++	++	+++	+++	+++	+++	+++	+++	+++	++	Casamino Acids and any nucleoside
63	−	−	+++	−	+++	++	+++	++	+++	+++	+++	+	Pyridoxine
64	−	−	+	−	+++	+	+++	−			+	−	Casamino Acids
72	−	−	+++	+	+++	+++	+++	+++	+++	+++	+++	+++	Vitamins and Casamino Acids
73	−	−	+	−	+++	+++	+++	+++			+++	−	N.S.
76	−	−	+++	+	+++	+++	+++	+++	+++	+++	+++	++	N.S.
83	−	−	+++	+	+++	+++	+++	+++	+++	+++	+++	+++	N.S.
86	−	−	+++	−	+++	+++	+++	+++	+++	+++	+++	+++	Methionine and lysine
87	−	−	+++	+	?	−	+++	−	+++	−	+++	++	N.S.
101	−	−	+++	−	+++	+++	+++	+++	+++	+++	+++	+++	N.S.
102	−	−	+++	+	+++	+++	+++	+++	+++	+++	+++	+++	Casamino Acids and adenine or guanine
116	−	−	+++	−	+++	++							
122	+	−	+++	+			+++	+++	+++	++	+++	++	Casamino Acids
127	−	−	+++	±			+++	+++	+++	+++	+++	++	Adenine or Guanine
128	+	++	+++	+			+++	+++	+++	+++	+++	+++	Glycine
Class 3													
43	+++	+++	+++	+	+++	+	+++	++	+++	++	+++	+	
71	+++	+++	+++	−	+++	+	+++	+	+++	+++	+++	−	
99	+++	+++	+++	+		−		−	+++	+++	+++	++	
113	+++	+++	+++	+			+++	+++	+++	+++	+++	++	
125[a]	+++	+++	+++	−			+++	+++	+++	+++	+++	+++	
126	+++	++	++	−			+++	+++	+++	+++	+++	+++	

[a] Mutants 37, 39, 55, 60, 62, 66, 69, 79, 81, 85, 88, 89, 90, 91, 93, 95, 97, 98, 100, 104, 106, 107, 108, 118, 120, 121, 123, 124, 129 are all class 3—like T^s-125. Unlisted mutants 34–131 proved leaky or not T^s upon retesting. Nutrient Broth cultures of mutants were diluted to 10^7 cells/ml; 0.01-ml samples were spotted on agar medium of the specified composition, and incubated for 36 hr at 30 or 43 C. Symbols: +++ = normal growth; − = no growth; and + and ++ are relative intermediate growth levels. A232 is +++ on all media at either temperature. N.S. = not satisfied by a combination of Casamino Acids, nucleosides, and vitamins.

assay), but can make RNA, protein, and ribosomes. ^{14}C-leucine incorporation is normal, but β-galactosidase is only half normal. T^s-40 does not recover from an exposure to 42 C.

If this mutant is cultured in Nutrient Broth at 30 C, then shifted directly to 42 C without intervening glucose starvation (direct shift), the incorporation of ^{3}H-thymidine is half normal for 20 min, then stops completely. Incorporation of ^{14}C-leucine and ^{3}H-uridine is normal for at least 2 hr after the temperature shift. Without information from the glucose starvation experiments, it would not be apparent whether the residual thymidine incorporation during the first 20 min represented the time required to inactivate a temperature-sensitive protein directly involved in DNA synthe-

TABLE 2. *Virus and enzyme production and growth at 43 C of T^s mutants*

T^s no.	Virus production	Lysis	β-Galactosidase index	Growth at 42 C
Class 1				
40	Low	No	0.5	Normal
41	0	No	**0**	Very slow, linear
42	Normal	Yes	—	—
45	Low	Yes	1.0, slow after 30 min	Normal
53	Low	Yes	**0.2,** increase after 100 min	Normal
58	0	No	**0.2,** lags	No increase
67	0	Yes	**0.2,** lags	Very slow, linear
68	—	—	**0**	Slow, linear
80	0	Yes (slow)	**0.3,** lags	1st phase slow, 2nd phase absent
84	Low	No	**0**	Very slow, linear
92	—	—	1.1, stops in 30 min	Slow, linear
103	—	—	**0.2**	Very slow
114	Normal	Yes (slow)	**0.2**	Normal
115	Normal	Yes (slow)	1.0, stops in 60 min	Normal
119	Normal	Yes	1.0	Normal
131	Normal	Yes	3.8, stops in 20 min	1st phase slow
Class 2				
38	Normal	? (incomplete)	1.0	Normal
51	Normal	Yes	0.4	Normal
52	½	Yes (slow)	—	—
63	Low	No	**0**	Very slow, linear
64	Normal	Yes	1.2	2nd phase slow
72	Normal	Yes	**0**	Slow, linear
73	0	No	**0.1**	Very slow, linear
76	Low	Yes (sharp)	1.0, stops in 45 min	Normal
83	0	No	**0**	1st phase short
86	0	No	0.4–1.0, biphasic	Biphasic
87	Low	Yes (slow)	0.4	Slow
101	?	Yes (from T = 0)	**0.1,** lags	Lyses
102	Low	Yes (slow)	1.0	Normal
116	Normal	Yes (slow)	**0.2**	Slow, linear
122	Low	Yes	0.4, slows after 45 min	Normal
127	Normal	Yes	1.0	Normal
128	Low	Yes	**0.2**	2nd phase slow
Class 3				
43	Normal	Yes	1.5, after 30 min slows to 1.0	Normal
71	Low	Yes (slow)	0.5	Slow
99	Normal	Yes (slow)	0.5	Slow
113	Low	Yes	0.3, lags 30 min	Normal
125	Low	Yes	0.7	Normal
126	½	Yes (slow)	0.3, stops in 60 min	Slow

sis, or represented a secondary effect on DNA synthesis. The glucose starvation data support the former interpretation.

So far, we have not been able to demonstrate temperature sensitivity of the DNA polymerase of this mutant in vitro.

This mutant is very stable genetically, but will occasionally revert to T^i. The mutation maps at about ten o'clock on the *E. coli* chromosome.

T^s-103 may be an RNA synthesis mutant. It makes very low amounts of RNA and β-galactosidase during recovery from glucose starvation (Fig. 3, 4). The incorporation of ^{14}C-leucine is initially very low, but eventually reaches the level of the parent strain. The early incorporation of ^{3}H-thymidine is slightly suppressed, but this incorporation continues for 100 min, finally reaching a level twice that of the parent strain. The incorporated thymidine can be recovered in DNA.

In a direct-shift experiment of the type described for T^s-40, the incorporation of ^{3}H-uridine, ^{14}C-leucine, and ^{3}H-thymidine at 42 C occurs at 50, 60, and 35% of the 30 C rates, respectively, for at least 60 min. These figures correspond to

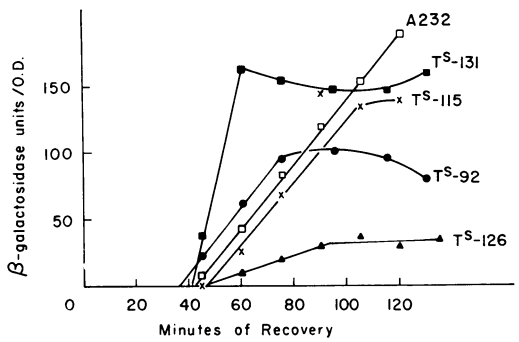

FIG. 1. *Short-term β-galactosidase synthesis at 42 C in Escherichia coli T^s mutants; 20 ml of cells were starved for glucose and preincubated at 42 C. Nutrient Broth and IPTG were added to the starved culture at T = 0, and 0.1-ml samples were assayed for β-galactosidase activity at 15-min intervals. The optical density at 660 mμ of the culture was also determined at 15-min intervals.*

25, 30, and 17.5% of the parent strain rates at 43 C. The results of the direct-shift experiment are not interpretable in themselves, but the glucose starvation experiments indicate that the mutation directly affects RNA production. The mutation maps roughly at eight to nine o'clock, near mannitol.

T^s-87 gives low values with all of the screening assays. The small amount of thymidine incorporated can be recovered in DNA. This mutant is able to make about half the normal amount of ribosomes during recovery from glucose starvation. T^s-87 is a single mutant (reverts in a single step to the parent strain phenotype), but requires an additional factor for growth at 30 C. This requirement cannot be satisfied by Casamino Acids, the nucleosides, or the common vitamins, singly or in combination. It is, however, satisfied fully by ribose and partially by glycerol, but only in the absence of glucose and only at 30 C. The 30 C auxotrophy (and probably, therefore, the temperature-sensitive mutation) map at seven to eight o'clock, below streptomycin. This mutant may be blocked early in nucleic acid synthesis or, alternatively, may be very sensitive to catabolite repression.

T^s-84 behaves like T^s-87, except that T^s-84 has no additional auxotrophy at 30 C. The mutation maps near streptomycin.

T^s-68 is a complete protein synthesis mutant; that is, it cannot make active enzyme or inactive polypeptides. The incorporation of ^{3}H-thymidine is partially impaired (Fig. 5), and none of the incorporated thymidine can be recovered in completed DNA (18). The incorporation of ^{3}H-uridine is unaffected. This mutant makes some new 30*S* ribosomes during recovery, as well as other ribosome precursor particles. T^s-68 is impaired at 30 C and is a "mucoid" at this temperature (makes capsular material), although nonmucoid derivatives behave identically. It recovers very slowly from an exposure to 42 C.

In a direct shift experiment of the type described for T^s-40, the ability of T^s-68 to incorporate ^{14}C-leucine at 42 C is 30% of the 30 C rate, and continues for at least 60 min after the temperature shift. Incorporation of ^{3}H-thymidine is unaffected by the temperature shift, whereas the incorporation of ^{3}H-uridine is initially stimulated.

T^s-41 is a partial protein-synthesis mutant; it cannot make biologically active β-galactosidase, but it can incorporate leucine into polypeptide material. The incorporation of ^{3}H-thymidine is secondarily affected and, as in the case of T^s-68, the thymidine does not enter completed DNA molecules. Synthesis of RNA, as measured by ^{3}H-uridine incorporation is unaffected.

This mutant makes no detectable ribosomes during recovery, and all of the ^{3}H-uridine radioactivity is tightly associated with the membrane. This radioactivity cannot be released from the membrane with ribonuclease (18). T^s-41 recovers from an exposure at 43 C. This mutation maps roughly at eight o'clock, and is linked to the mannitol and streptomycin loci.

In a direct shift experiment of the type described for T^s-40, the capacity of T^s-41 to incorporate ^{3}H-uridine or ^{14}C-leucine decreases logarithmically with time after the shift to 43 C. The half-life in both instances is about 10 min. T^s-41 is completely unable to make β-galactosidase after the temperature shift when induced with IPTG at the time of the shift. The direct shift data are more difficult to interpret than the glucose starvation data, since there is a secondary effect on uridine incorporation in the former case.

T^s-83 is also a partial protein-synthesis mutant. During recovery at 42 C, it can make no virus, but

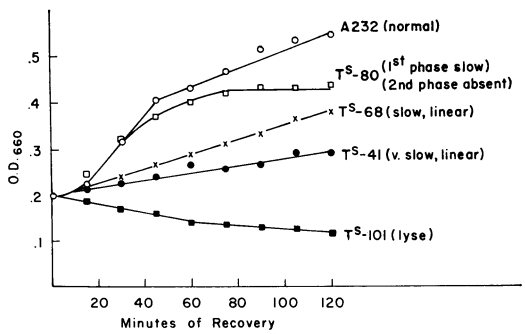

FIG. 2. *Characteristic growth responses at 42 C of T^s mutants during recovery from glucose starvation.*

TABLE 3. *Incorporation of radioactive precursors at 43 C by T^s mutants*

T^s no.	^{14}C-leucine index	^{3}H-thymidine index	^{3}H-uridine index	Special characteristics
Class 1				
40	0.8	**0.2**	0.7	Thymidine, leucine lag 20 min
41	**0.5**	0.5	0.8	
42	—	—	—	
45	1.0	0.8	0.7	Thymidine lags
53	0.5	0.7	—	
58	1.0	1.0	1.0	
67	0.3–0.7	2.0	0.6	Thymidine lags 45 min, leucine biphasic
68	**0.05**	0.4	1.0	Thymidine linear for 100 min
80	**0.4**	0.5	1.0	
84	**0.2**	**0.1**	**0.1**	
92	0.8	1.7	0.9	Leucine slows at 60 min
103	**0.2**	1.9	**0.1**	Leucine increases logarithmically Thymidine continues for 100 min
114	0.7	0.8	—	Thymidine lags
115	0.6	0.4	—	
119	—	—	—	
131	1.1	0.8	—	
Class 2				
38	—	—	—	
51	0.7	0.4	—	Leucine lags 15 min
52	—	—	—	
63	**0.3**	0.5	1.0	Leucine stops in 50 min
64	—	—	—	
72	**0.3**	0.4–0.8	0.6	Thymidine increases at 75 min
73	**0.4**	0.5	1.0	Leucine, thymidine lag
76	1.4	1.6	—	Total thymine 3×
83	**0.2**	0.6	0.6	
86	0.1–1.0	0.2–1.0	—	Biphasic
87	**0.3**	**0.1**	**0.2**	Leucine lags 15 min, uridine lags 10 min
101	0.6	0.3	0.3	Lyses
102	1.0	1.0	0.7	
116	0.8	0.6	—	Thymidine lags 25 min
122	0.7	0.6	—	
127	—	—	—	Leucine lags 20 min
128	1.0	1.0	—	
Class 3				
43	—	—	—	
71	0.7	0.6	—	Thymidine reaches normal level
99	0.4	0.8	—	Thymidine, leucine lag
113	0.5	0.2	—	
125	1.5	1.2	—	
126	0.7	0.9	—	

the original infective centers are destroyed and the mutant is lysed. No β-galactosidase can be detected during recovery, and the incorporation of ^{14}C-leucine is low. This mutant has an additional growth requirement at 30 C that is not satisfied by the usual concentrations of amino acids, nucleosides, or the common vitamins. The 30 C auxotrophy maps roughly at three o'clock. It is not temperature-sensitive on L agar and recovers from an exposure to 43 C in Nutrient Agar. The leucine incorporation is sensitive to chloramphenicol and streptomycin, and so appears to be ribosome (at least 30*S*) directed. The mutant makes few detectable ribosomes during recovery from glucose starvation; the few it makes are 30 and 50*S*, and again the ^{3}H-uridine radioactivity is found tightly associated with the cell membrane. As in the case of T^s-41, this radioactivity cannot be released by ribonuclease.

In a direct-shift experiment of the type described for T^s-40, T^s-83 can incorporate no ^{14}C-leucine nor synthesize β-galactosidase at any time after the shift to 42 C. Its ability to incorporate ^{3}H-uridine decreases logarithmically at 42 C, with a half-life of 20 min.

T^s-128 is a partial protein synthesis mutant

and has a requirement at 30 C for glycine. If the rate constant of an enzyme in this strain involved with the availability of glycine for protein synthesis is low at 30 C, then this deficiency might be overcome by adding an excess of substrate to the medium. The fact that T^s-128 is less able to incorporate glycine than leucine into protein (Fig. 9) supports the idea that the mutation selectively prevents the insertion of glycine into proteins.

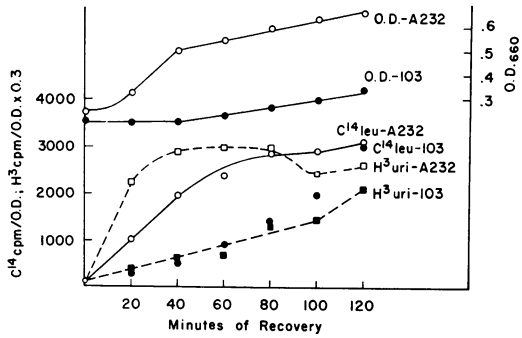

FIG. 3. *Growth and uridine and leucine incorporation by T^s-103 at 42 C. Note that uridine incorporation is linear, while leucine incorporation is logarithmic.*

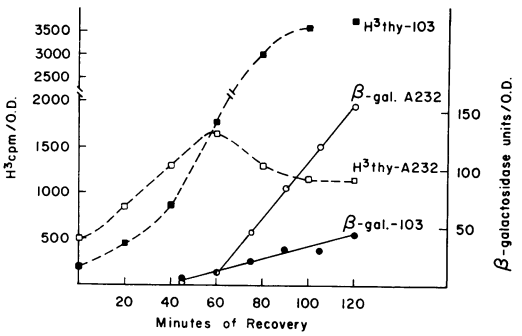

FIG. 4. *Thymidine incorporation and β-galactosidase synthesis by T^s-103 at 42 C.*

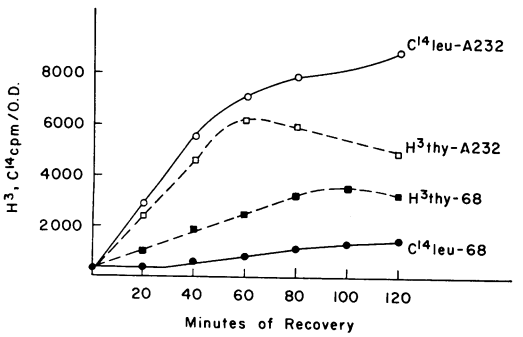

FIG. 5. *Thymidine and leucine incorporation by T^s-68 at 42 C.*

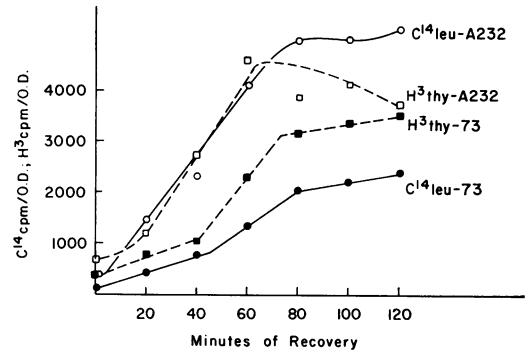

FIG. 6. *Thymidine and leucine incorporation by T^s-73 at 42 C.*

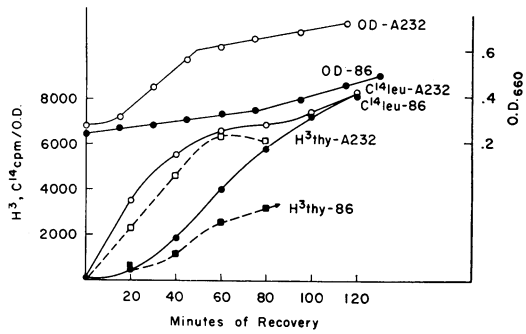

FIG. 7. *Growth and thymidine and leucine incorporation by T^s-86 at 42 C.*

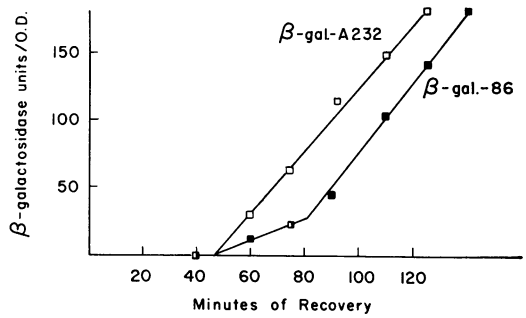

FIG. 8. *Uridine incorporation and β-galactosidase synthesis by T^s-86 at 42 C.*

T^s-53, 58, 73, 86, and 122 all appear to be partial protein synthesis mutants.

T^s-101 begins to lyse immediately upon the addition of broth to the medium. Though this mutant does not lyse during the starvation procedure, starvation at 30 C is bactericidal (in terms of colony-forming ability). T^s-101 is an unstable single mutant but has an additional growth requirement at 30 C and is satisfied by none of the supplements we tested, including cell wall precursors (D-amino acids, glucosamine,

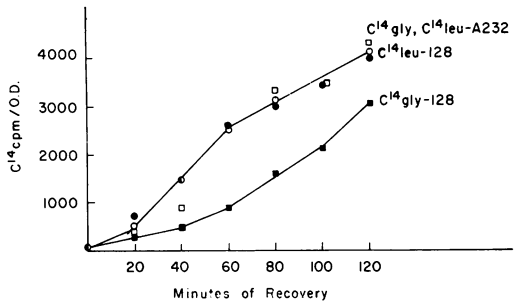

FIG. 9. *Leucine and glycine incorporation by T^s-128 at 42 C. Symbols: ○, ^{14}C-leucine by A232; ●, ^{14}C-leucine by T^s-128; □, ^{14}C-glycine by A232; and ■ ^{14}C-glycine by T^s-128.*

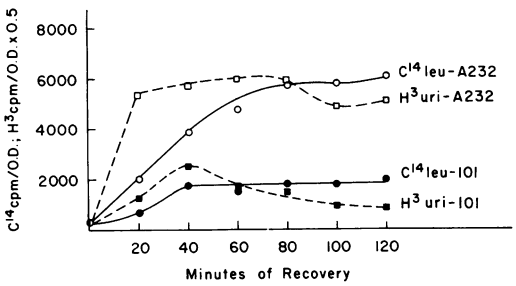

FIG. 10. *Uridine and leucine incorporation by T^s-101 at 42 C.*

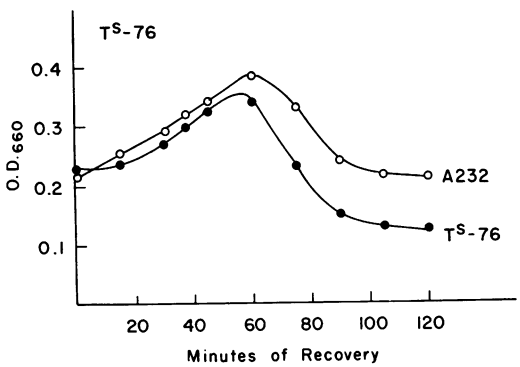

FIG. 11. *Virus-induced lysis in T^s-76 at 42 C; 20 ml of lysogenic cells were starved for glucose and preincubated at 42 C. A sample of the culture was diluted to 1,000 cells/ml, and broth was added to the diluted and undiluted cultures. The optical density at 660 mμ of the undiluted cultures was followed, and at the end of the lytic cycle (at 90 min) 0.1 ml of the diluted culture was assayed for plaque-forming units (PFU). A232 gave 20 PFU per viable cell, while T^s-76 gave 2 PFU per viable cell.*

and muramic acid). The mutation affects a protein involved with the metabolism of the cell envelope.

T^s-76 is also a cell envelope mutant. In contrast to T^s-101, however, T^s-76 is able to do everything but make virus at least as well as the parent strain. We think it is a cell envelope mutant because of the rather sharp λ-induced lysis (Fig. 11). Although this effect is small, it has been reproducible in several experiments. T^s-76 has an additional growth requirement at 30 C, and is like T^s-101 in that the requirement is satisfied by none of the nutrients we tested.

In addition to the above mutants which can be categorized more or less specifically, there are

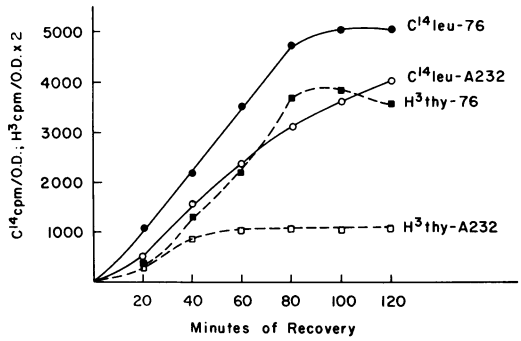

FIG. 12. *Thymidine and leucine incorporation by T^s-76 at 42 C. The thymidine scale has been depressed to allow visualization of the T^s-76 thymidine incorporation.*

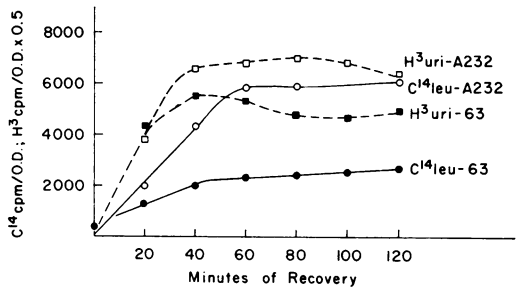

FIG. 13. *Uridine and leucine incorporation by T^s-63 at 42 C.*

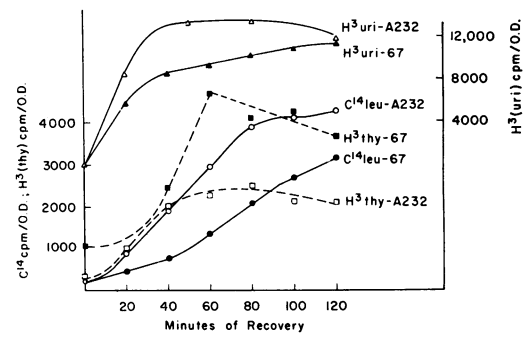

FIG. 14. *Thymide, uridine, and leucine incorporation by T^s-67 at 42 C.*

two classes of interest. One class (T^s-72, 114, and 116) makes viruses normally, but cannot make substantial amounts of enzyme. In these cases, the incorporation of radioactive precursors does not give us much of a clue concerning the condition required for enzyme synthesis that is not required for virus production. A second class of mutants also can be found with the reverse phenotype. These mutants make enzyme at the normal initial rate, but cannot make any active viruses (T^s-45, 76, and 102). The β-galactosidase assays revealed that two of these strains carry mutations that exert a delayed secondary effect on enzyme production (Table 2).

T^s-38, 42, 52, 64, 115, 119, 127, and 131 all behave like the parent strain in the screening tests employed.

DISCUSSION

It is preferable, when doing screening assays, to inactivate the temperature-sensitive function in the absence of metabolic activity. We originally chose to inactivate this function in glucose-starved cells for this reason only, but the starvation system proved to have other desirable features. We have characterized the events occurring during starvation and recovery (18). During starvation, DNA and protein remain unchanged, while two-thirds of the RNA is lost. At least 95% of the detectable ribosomes disappear, and they are degraded to functional ribosomal protein and RNA fragments and nucleotides. Upon the addition of broth, RNA synthesis begins immediately and is relaxed. It is followed in 2 to 5 min by protein synthesis and in 20 min by DNA synthesis. ^{3}H-thymidine incorporation begins in 5 to 10 min, but does not appear in DNA for 20 min. β-Galactosidase does not appear for 45 min. These syntheses have been measured both by chemical means and by the incorporation of radioactive precursors into trichloroacetic acid-insoluble material. The important feature, with respect to the screening of T^s protein-synthesis mutants, is that after glucose starvation the cells must begin protein synthesis by rebuilding the protein synthesizing machinery, but that new protein synthesis may not be required for the formation of ribosomes.

The three principal assays we have used to screen temperature-sensitive mutants are the abilities of the mutants to: (i) make biologically active viruses, (ii) synthesize the inducible enzyme β-galactosidase, and (iii) incorporate radioactive precursors into DNA, RNA, and protein. All assays were performed at 42 C after pretreatment by glucose starvation.

The production of active viruses requires the synthesis of DNA, RNA, and protein, and the participation of other elements. In addition to measuring the production of active virus particles, the virus assay can also be used to measure virus-induced lysis (presumably requiring the synthesis of endolysin) and virus-induced lethality. The synthesis of β-galactosidase requires the synthesis of RNA and protein. In the glucose starvation system, full enzyme production requires DNA synthesis as well; mutants unable to make DNA should make β-galactosidase at about half the normal rate, since DNA duplication normally occurs before the onset of β-galactosidase induction (18). Mutants unable to make virus or β-galactosidase, or both, are assayed for their ability to incorporate radioactive precursors into macromolecules.

Mutants that are low in thymidine incorporation, only, are classified as DNA-synthesis mutants. Since not all of the thymidine incorporated can be recovered in DNA, the extent of DNA synthesis in these mutants during recovery from glucose starvation is verified with purified radioactivity or diphenylamine determinations. Mutants low in uridine and leucine incorporation are classified as RNA-synthesis mutants, and those low in leucine and thymidine incorporation, as complete protein-synthesis mutants. Synthesis of a new protein during recovery is required for DNA synthesis (18), so protein-synthesis mutants will be unable to make DNA. Similarly, RNA-synthesis mutants that can make *no* new proteins during recovery will be unable to make DNA, so those mutants unable to incorporate any precursor into macromolecules are tentatively classified as either RNA-synthesis or energy-generating mutants. Classifications must be consistent with the virus and β-galactosidase assays.

The screening system we have described has allowed us to categorize a large proportion of randomly selected temperature-sensitive mutants. We have compared the results with data obtained from the usual procedure for screening temperature-sensitive mutants, i.e., simply shifting a logarithmically growing culture from 30 to 42 C. In no case were the results of the two types of screening procedures inconsistent, but the results derived from glucose starvation experiments were, in most cases, easier to interpret.

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LITERATURE CITED

1. AMES, R., R. GOLDBERGER, R. HARTMAN, R.

MARTIN, AND J. ROTH. 1967. The histidine operon, p. 272. *In* V. V. Konongsberger and L. Bosch [ed.], Regulation of nucleic acid and protein biosynthesis. Elsevier Publishing Co., Amsterdam.

2. BACK, K. J. C., AND E. G. WESTAWAY. 1962. Studies on a mutant strain of *Escherichia coli* which requires both methionine and leucine for growth. J. Gen. Microbiol **27**:41–56.
3. BEADLE, G. W., AND E. L. TATUM. 1941. Genetic control of biochemical reactions in *Neurospora*. Proc. Natl. Acad. Sci. U.S. **27**:499–506.
4. BURGE, B. W., AND E. R. PFEFFERKORN. 1966. Complementation between temperature-sensitive mutants of Sindbis virus. Virology **30**:214–223.
5. COOPER, P. D., R. T. JOHNSON, AND D. J. GARVES. 1966. Physiological characterization of heat-defective (temperature-sensitive) poliovirus mutants: preliminary classification. Virology **30**:638–649.
6. DEMEREC, M. 1958. Genetic structure of the *Salmonella* chromosome. Intern. Congr. Genet., 10th **1**:55–62.
7. DOWELL, C. E., AND R. L. SINSHEIMER. 1966. The process of infection with bacteriophage ϕX174 IX. Studies on the physiology of three ϕX174 temperature-sensitive mutants. J. Mol. Biol. **16**:374–386.
8. EIDLIC, L., AND F. C. NEIDHARDT. 1965. Protein and nucleic acid synthesis in two mutants of *Escherichia coli* with temperature-sensitive amino acyl ribonucleic acid synthetases. J. Bacteriol. **89**:706–711.
9. EPSTEIN, R. H., A. BOLLE, C. M. STEINBERG, E. KELLENBERGER, E. BOY DE LA TOUR, R. CHEVALLEY, R. S. EDGAR, M. SUSMAN, G. H. DENHARDT, AND A. LIELANSIS. 1963. Physiological studies of conditional lethal mutants of bacteriophage T4D. Cole Spring Harbor Symp. Quant. Biol. **28**:375–394.
10. FATTIG, W. D., AND F. LANNI. 1965. Mapping of temperature-sensitive mutants in bacteriophage T5. Genetics **51**:157–166.
11. FORCHHAMMER, J., AND N. KJELDGAARD. 1967. Decay of messenger RNA *in vivo* in a mutant of *E. coli* 15. J. Mol. Biol. **24**:459–470.
12. FRIED, M. 1965. Cell transforming ability of a temperature-sensitive mutant of polyoma virus. Proc. Natl. Acad. Sci. U.S. **53**:486–491.
13. HARTWELL, L. H. 1967. Macromolecule synthesis in temperature-sensitive mutants of yeast. J. Bacteriol. **93**:1662–1670.
14. HORIUCHI, K., H. F. LODISH, AND N. D. ZINDER. 1966. Mutants of bacteriophage f2. VI. Homology of temperature-sensitive and host dependent mutants. Virology **28**:438–447.
15. IGARASHI, S. 1966. Temperature-sensitive mutation in *Paramecium aurelia*. I. Induction and inheritance. Mutation Res. **3**:13–24.
16. IRANORICS, G. 1963. Unbalanced growth induced by a temperature shift in a mutant of *Bacillus anthracis*. Biochem. Biophys. Res. Commun. **11**:343–345.
17. JACOB, F., AND J. MONOD. 1961. Genetic regulatory mechanisms in the synthesis of proteins. J. Mol. Biol. **3**:318–356.
18. JACOBSON, A., AND D. GILLESPIE. 1968. Metabolic events occurring during recovery from prolonged glucose starvation in *Escherichia coli*. J. Bacteriol. **95**:1030–1039.
19. KAHAN, E. 1966. A genetic study of temperature-sensitive mutants of the subtilis phage SP82. Virology **30**:650–660.
20. KOHIYAMA, M., D. COUSIN, A. RYTER, AND F. JACOB. 1966. Mutants thermosensibles d' *Escherichia coli* K12. I. Isolement et characterisation. Ann. Inst. Pasteur **110**:465–486.
21. MAALØE, O., AND P. C. HANAWALT. 1961. Thymine deficiency and the normal DNA replication cycle. J. Mol. Biol. **3**:144–155.
22. MACDONALD, R., G. TURNOCK, AND J. FORCHHAMMER. 1967. The synthesis and function of ribosomes in a new mutant of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S. **57**:141.
23. NIRENBERG, M., P. LEDER, M. BERNFIELD, R. BRIMACOMBE, J. TRUPIN, F. ROTTMAN, AND C. O'NEAL. 1965. RNA codewords and protein synthesis. VII. On the general nature of the RNA code. Proc. Natl. Acad. Sci. U.S. **53**:1161–1168.
24. NISHIHARA, M., AND W. R. ROMIG. 1964. Temperature-sensitive mutants of *Bacillus subtilis* bacteriophage SP3. II. In vivo complementation studies. J. Bacteriol. **88**:1230–1239.
25. NOZAWA, R., T. HORIUCHI, AND D. I. MIZANO. 1967. Degradation of ribosomal RNA in a temperature-sensitive *Escherichia coli*. Arch Biochem. Biophys. **118**:402–409.
26. PARDEE, A. B., F. JACOB, AND J. MONOD. 1959. The genetic control and cytoplasmic expression of "inducibility" in the synthesis of β-galactosidase by *E. coli*. J. Mol. Biol. **1**:165–178.
27. PRINTZ, D. B., AND S. R. GROSS. 1967. An apparent relationship between mistranslation and an altered leucyl tRNA synthetase in a conditional lethal mutant of *Neurospora crassa*. Genetics **55**:451–467.
28. STENT, G. S., AND S. BRENNER. 1961. A genetic locus for the regulation of ribonucleic acid synthesis. Proc. Natl. Acad. Sci. U.S. **47**:2005–2014.
29. SUSSMAN, R., AND F. JACOB. 1962. Sur un système de répression thermosensible chez le bacteriophage λ d'*Escherichia coli*. Compt. Rend. **254**:1517–1519.
30. WITTMAN-LIEBOLD, B., B. J. JAUREGUE-ADELL, AND H. G. WITTMAN. 1965. The primary protein structure of temperature-sensitive mutants of tobacco mosaic virus. II. Chemically induced mutations. Z. Naturforsch. **20b**:1235–1249.
31. YANOFSKY, C., D. R. HELINSKI, AND B. D. MALING. 1961. The effects of mutation on the composition and properties of the A protein of *E. coli* tryptophan synthetase. Cold Spring Harbor Symp. Quant. Biol. **26**:11–24.