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Temperature sensitive mutants of Escherichia coli for tRNA synthesis

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ABSTRACT An efficient method was devised to isolate temperature sensitive mutants of E. coli defective in tRNA biosynthesis. Mutants were selected for their inability to express suppressor activity after su3$^+$-transducing phage infection. In virtually all the mutants tested, temperature sensitive synthesis of tRNA^{Tyr} was demonstrated. Electrophoretic fractionation of ^{32}P labeled RNA synthesized at high temperature showed in some mutants changes in mobility of the main tRNA band and the appearance of slow migrating new species of RNA. Temperature sensitive function of mutant cells was also evident in tRNA synthesis directed by virulent phage T4 and BF23. We conclude that although the mutants show individual differences, many are temperature sensitive in tRNA maturation functions.

In spite of much information on the structure and function of transfer RNA (tRNA), our knowledge concerning the biosynthesis of tRNA is relatively poor. It is generally assumed that complete tRNA molecules are made via a series of processing steps from the original transcription products of tRNA genes which are presumably unmodified and longer than mature tRNA molecules. In the case of tyrosine suppressor tRNA of su3$^+$, an unmodified precursor RNA carrying additional residues at the 3' and 5' ends has been isolated (1,2), and an endonuclease cleaving at the 5' side of this precursor has been identified in E. coli (3). In the case of T4 encoded tRNA, a large precursor molecule for several tRNA's has been reported (4). Some enzymes that catalyze the modifications have also been described (5). However, the over-all picture

and the precise mechanisms of tRNA maturation are as yet largely unkown.

For study of tRNA biosynthesis in E. coli, a genetic approach may prove useful, as has been the case in other biosynthetic pathways. In order to obtain mutants blocked in any of the intermediary steps of tRNA synthesis, we have developed an efficient selection system that enriches these mutants. Since any mutational block in tRNA biosynthesis might well be lethal, we looked for conditional lethal mutants in which the defect in tRNA synthesis occurs only at high temperature. In this selection system, the su3 gene carried by a temperate phage was newly introduced into cells(su^-) and those cells incapable of synthesizing $su3^+$ tRNA at high temperature were selected. Such mutants were easily enriched by using conditions in which cells expressing suppressor activity were killed by two virulent phages. In this communication, we report the method for isolation of mutants and some characterization of tRNA synthesis in these mutants. Recently, Schedl and Primakoff (6) have independently isolated thermosensitive mutants of E. coli defective in tRNA synthesis which may or may not be different types from ours.

MATERIALS AND METHODS

Phages and Bacteria. $\lambda psu3^+$ was constructed from $\phi 80psu3^+$ (Cambridge strain(7), generous gift of Dr. J. Abelson) by replacing both sides of the $su3^+$ transducing segment with λ genome. Unless otherwise noted, $\phi 80psu3^+$ used in our experiments was a Kyoto strain described by Andoh and Ozeki (8). Phage BF23 has been described by Nishioka and Ozeki (9). Phages T4D and φ80plac were supplied by Drs. T. Minagawa and Y. Oshima, respectively. From E. coli CA85lac^-_{am} (8), strain 4273 su^- lac^-_{am} $T6^r_{am}$ $BF23^r_{am}$ was isolated and used as a basic strain.

Media and Buffers. λ broth and φ80 adsorption buffer have been described elsewhere (10). M9-casamino-glycerol is M9 medium enriched with 0.2% casamino acid (Difco) and 0.2% glycerol as a carbon source. Selection medium used was λ broth supplemented with 40 μg/ml L-tryptophan, 0.1 M NaCl, 1 mM $CaCl_2$. Low

phosphate medium used for ^{32}P labeling has been described by Landy et al. (11).

Mutagenesis. For mutagenesis, cells were grown to a density of 2 x 10^8 cells/m in λ broth, washed twice with Tris-malate buffer (pH 6.0), and treated with 200 μg/ml of N-methyl-N'-nitro-N-nitrosoguanidine (NG) in Tris-malate buffer at 30°C for 30 min. The NG treated cells were washed once with λ broth and subsequently grown overnight at 30°C.

β-Galactosidase Assay. IPTG(isopropyl-β-D-thiogalactoside)-induced synthesis of β-galactosidase was assayed by essentially the same method as described by Pardee et al. (12).

Polyacrylamide Gel Electrophoresis. Gel slab preparation (20 x 40 x 0.3 cm) and electrophoresis were described by DeWachter and Fiers (13). Polyacrylamide gel contains 10% acrylamide, 0.25% bisacrylamide in 0.1 M Tris-acetate at pH 8.3. Electrophoresis was carried out in 0.1 M Tris-acetate (pH 8.3) at 60 mA for 22 hr. Details of two-dimensional (10%-20%) polyacrylamide gel electrophoresis have been described elsewhere (14). The conditions used are as follows first dimension (10%), 420 volts/17 cm, 3 hr; second dimension (20%), 450 volts /17 cm, 15 hr. Electrophoresis was performed at 15°C in half the concentration c Peacock's Tris-borate-EDTA buffer at pH 8.3 (15).

RESULTS

Isolation of Mutants. For the primary selection of mutants, we isolated a strain, 4273(lac^-_{am}, $T6^r_{am}$, $BF23^r_{am}$, su^-), which carries amber mutations in the lactose gene and in the genes specifying the receptor sites for two virulent phages, T6 and BF23. Accordingly when these amber mutations are suppressed, the cells become susceptible to these phages. For the selection, mutagenized 4273(λCI857) was infected with λpsu3$^+$ and subsequently incubated with T6 and BF23 at 42°C for 3 hr. Those cells that could make functional su3$^+$ tRNA were thus eliminated. We used λpsu3$^+$ instead of φ80psu3$^+$ to introduce the suppressor tRNA gene into the cells, because at high temperature adsorption of φ80psu3$^+$

was rather poor and in addition, the cells were somehow killed by ϕ80psu3$^+$ itself if infected at high multiplicity. The heat-inducible λCI857 lysogen was employed to exclude cells escaped from λpsu3$^+$ infection. The introduction of CI$^+$ gene into the cells accompanied with λpsu3$^+$ infection is essential to rescue them from the thermal induction of λCI857. In this way, the uninfected cells that would behave like the mutants we looked for were eliminated together with those cells capable of synthesizing the suppressor tRNA, thereby enriching the mutants (Fig. 1).

Among the survivors after the phage selection, those colonies showing temperature sensitive growth were picked, because mutants unable to synthesize tRNA at high temperature were expected to be, in all likelihood, temperature sensitive (ts) for growth. As most of the survivors were still lysogenic for λCI857, the growth test was performed after superinfecting with λCI$^+$ on agar plates to antagonize thermal induction of prophage λCI857. About one fourth of the survivors were unable to grow at 42°C.

In order to screen the ts mutants for defective tRNA function, we measured the amount of β-galactosidase synthesized by these mutants at 42°C after ϕ80psu3$^+$ infection. Since the ts mutants carry lac^-_{am} mutation, the synthesis of β-galactosidase in the cells after ϕ80psu3$^+$ infection depends on the synthesis of su3$^+$ tRNA at 42°C. On the other hand, when the cells are infected with ϕ80plac, β-galactosidase might be synthesized for some period even after the cells are shifted to 42°C, as tRNA molecules made at low temperature may still function. Accordingly the mutants we looked for should be those that synthesize β-galactosidase at 42°C after ϕ80plac infection but not after ϕ80psu3$^+$ infection. Of 2000 mutants which were temperature sensitive for growth, about one tenth proved to satisfy these criteria. For further experiments, cured strains missing λCI857 were selected from each of these mutants by examining the survivors after a heat-pulse treatment.

Fig. 2 shows the kinetics of β-galactosidase synthesis in one of the mutants,

TS241, after ϕ80plac or ϕ80psu3$^+$ infection. When infected with ϕ80psu3$^+$, this mutant hardly synthesized β-galactosidase at 42°C. At 30°C, however, amounts of the enzyme produced were nearly comparable to that of wild type. On the other hand, if infected with ϕ80plac, the mutant produced appreciable amounts of β-galactosidase even at 42°C. When this mutant was previously lysogenized with ϕ80psu3$^+$, thus allowing synthesis of su3$^+$ tRNA, β-galactosidase was synthesized even at 42°C (data not shown). Therefore, the temperature sensitive synthesis of β-galactosidase of TS241 after ϕ80psu3$^+$ infection is not due to a ts mutation in the lactose gene. Essentially the same kinetic pattern of β-galactosidase synthesis was observed with other ts mutants. These results strongly suggest that the synthesis of su3$^+$ tRNA is temperature sensitive in the mutants.

Polyacrylamide Gel Electrophoresis. In order to analyze tRNA molecules produced in these mutants, ϕ80psu3$^+$ infected cells were labeled with ^{32}P inorganic phosphate. RNA was extracted by the direct phenol method (1), precipitated repeatedly with ethanol, and fractionated by electrophoresis on 10% polyacrylamide gel slab. Fig. 3 shows a radioautogram of the gel separation of TS241 RNA. As shown in the figure, mutant RNA labeled at 42°C shows slightly, but reproducibly altered migration of "4S tRNA" and contains additional bands of RNA (1, 2, 3, 4 in Figure 3) compared to wild type RNA or mutant RNA labeled at 30°C. Other mutant RNA's have also been analyzed in this way. In some the synthesis of 4S RNA at 42°C was greatly decreased relative to 16S rRNA, and in others both 4S and 5S RNA synthesis were depressed (Figure 4). We conclude that in many of the mutants studied the tRNA which accumulates at non-permissive temperature is abnormal. Detailed analysis of the separated RNA species, some of which may represent tRNA precursors, is in progress.

In order to measure the synthesis of tRNA^{Tyr} and possibly other tRNA's at permissive and non-permissive temperature, RNA from ϕ80psu3$^+$ infected cells

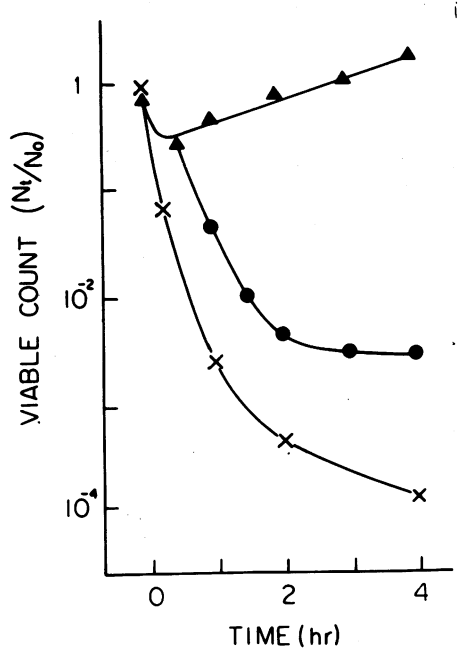
VIABLE COUNT (N_t/N_0)
1
10^{-2}
10^{-4}
0
2
4
TIME (hr)

Fig. 1

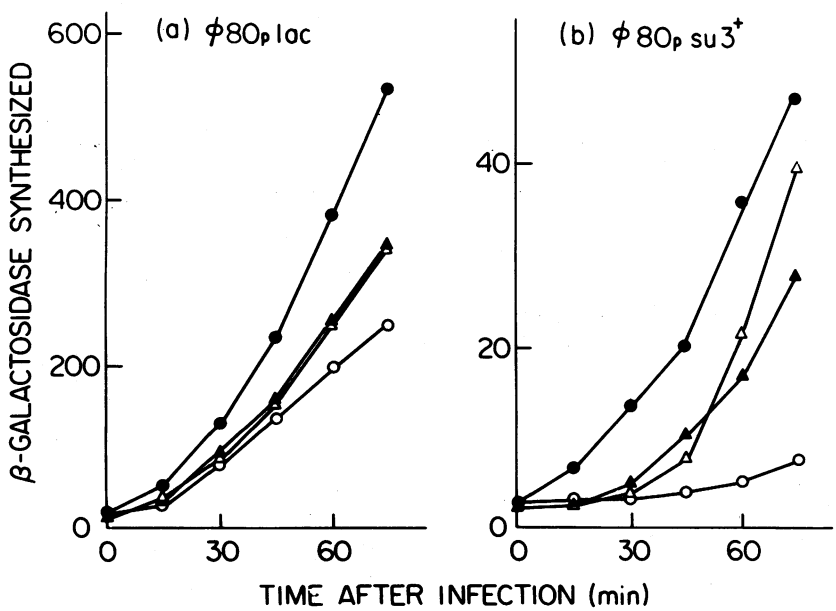
(a) φ80p lac
(b) φ80p su3+
β-GALACTOSIDASE SYNTHESIZED
600
400
200
0
40
20
0
30
60
TIME AFTER INFECTION (min)

Fig. 2

FIGURE LEGENDS

Fig.1. Killing of cells by T6 and BF23 after λpsu3$^+$ infection. NG treated cells were grown to 2x10^8 cells/ml, washed once with 10mM $MgSO_4$, and suspended in the same solution to give a density of 2x10^9 cells/ml. The cells were infected with λpsu3$^+$ at a multiplicity of 10 at 42°C. 10 min after infection, the cells were diluted with selection medium (prewarmed at 45°C) to 2x10^8 cells (0 min) and incubated at 42°C with shaking. At 30 min, T6 and BF23 were added a multiplicity of 20 for each phage. At the time indicated, viable counts (Nt) were assayed on λ-plates by plating the culture after adequate dilutions. The viable counts are expressed relative to the number of cells before λpsu3$^+$ infection (N_0). λpsu3$^+$ infected, —▲—▲— ; λpsu3$^+$, T6, and BF23 infected, —●—●— ; uninfected, —✕—✕—

Fig.2. β-Galactosidase synthesis after ϕ80plac or ϕ80psu3$^+$ infection. Cells grown to 2x10^8 cells/ml at 30°C in M9-casamino-glycerol, were concentrated in ϕ80 adsorption buffer, and infected with ϕ80plac or ϕ80psu3$^+$ at a multiplicity of 10. The infected cells were diluted with M9-casamino-glycerol to 2x10^8 cells/ml (0 min) and incubated at 30°C or 42°C. At 5 min, IPTG (0.5 mM) was added. At the time indicated, 0.5 ml samples were removed for assay of β-galactosidase. 4273, 42°C; —●—●— : 4273, 30°C; —▲—▲— : TS241, 42°C; —○—○— : TS241, 30°C; —△—△— .

$$\text{Enzyme activity} = \frac{A_{420} - 1.65 \times A_{550}}{\text{Reaction time (Min)}} \times 10^3$$

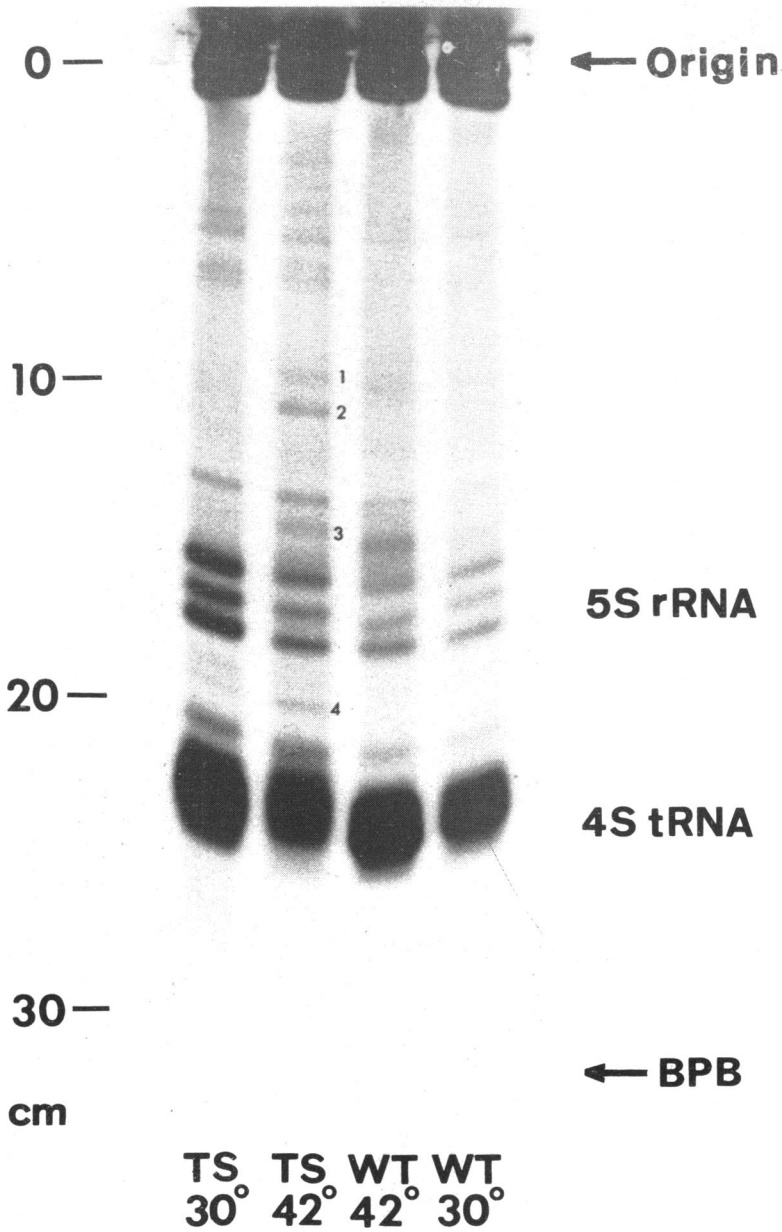
0
10
20
30
cm
Origin
1
2
3
4
5S rRNA
4S tRNA
BPB
TS 30°
TS 42°
WT 42°
WT 30°

Fig. 3

Fig. 3. Fractionation of ^{32}P-labeled RNA from ϕ80psu3^{+} infected cells by polyacrylamide slab gel electrophoresis. Cells, grown at 30°C in low phosphate medium to 3x10^{8} cells/ml, were concentrated in ϕ80-adsorption buffer, and infected with ϕ80psu3^{+} at a multiplicity of 10 at 30°C. At 15 min after infection, the cells were diluted with low phosphate medium to 3x10^{8} cells/ml and incubated at 30°C for 10 min to allow phage DNA replication to start. The culture was then divided into two 2ml portions, transferring one to 42°C, and another to 30°C. After 10 min, 0.5 mCi of carrier free (^{32}P)-orthophosphate was added to each culture (0 min). At 60 min, labeling was stopped by addition of an equal volume of water-saturated phenol (prewarmed at 45°C) and RNA was extracted as described by Altman (1). The RNA samples were washed 6 times by ethanol precipitation and finally disolved in 0.1 M Tris-acetate (pH 8.3), containing 20% sucrose and a trace amount of bromophenol blue (BPB). Electrophoresis and autoradiography were performed as described in Materials and Methods. TS and WT in the figure represent TS241 and strain 4273, respectively.

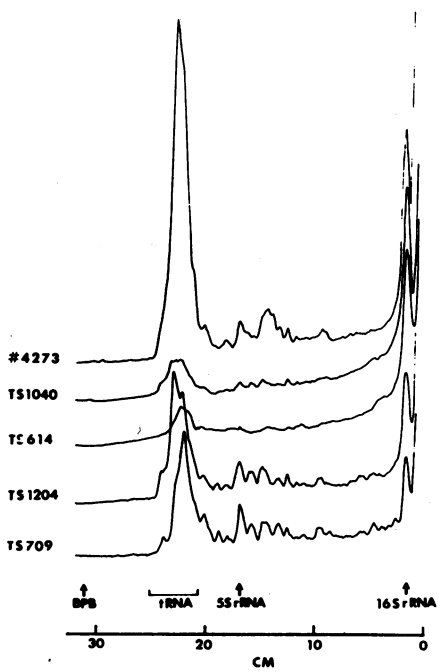

Fig. 4. Densitometer tracings of the autoradiograms of ^{32}P-labeled RNA. ϕ80psu3^{+} infected cells were labeled at 42°C for 30 min. RNA was prepared and electrophoresed as described in Fig. 3. The autoradiograms were traced with a Joyce-Loebl recording microdensitometer. Origin is at 0 cm in the figure.

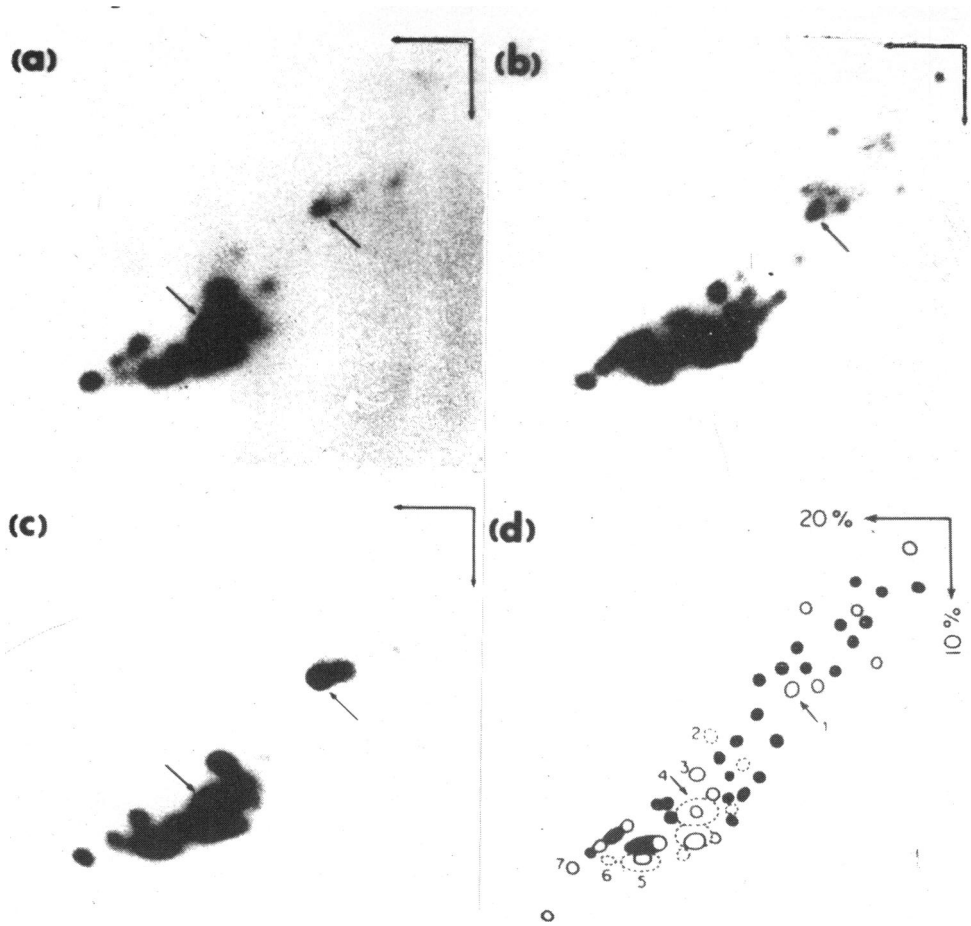

Fig. 5. Two dimensional separation of ^{32}P-labeled RNA on 10% - 20% polyacrylamide gel. ^{32}P-labeled RNA was prepared as described in Fig. 3. Electrophoresis was carried out as described in Materials and Methods. (a) ø80psu3^{+} infected 4273, labeled at 42°C. (b) ø80psu3^{+} infected TS241, labeled at 42°C. (c) ø80psu3^{+} infected TS241, labeled at 30°C. (d) Diagrammatic representation of the gel pattern of (b): Filled circles; new spots appearing in TS241 at 42°C; Open circles; spots present in 4273; Broken circles, spots absent in TS241 at 42°C. On the basis of the results of Ikemura and Dahlberg(14,16), the numbered spots are tentatively assigned: 1, 5S rRNA; 2, 4.5S RNA; 3, $tRNA_{I}^{Leu}$; 4, $tRNA^{Tyr}$; 5, $tRNA_{III}^{Gly}$; 6, $tRNA_{f}^{Met}$; 7, $tRNA_{II}^{Glu}$.

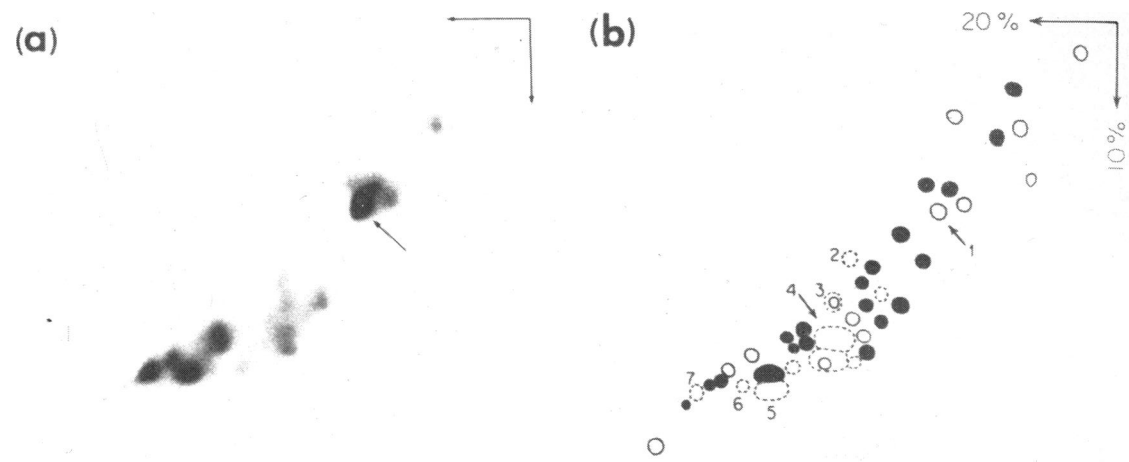

Fig. 6. Two dimensional separation of TS709 RNA. ^{32}P-labeled RNA was prepared as described in Fig. 3. Electrophoresis was carried out as described in Materials and Methods. (a) ø80psu3^{+} infected TS709, labeled at 42°C. (b) Diagrammatic representation of the gel pattern of (a): Filled circles, new spots appearing in TS709 at 42°C; Open circles, spots present in 4273; Broken circles, spots absent in TS709 at 42°C. The spots in (b) are numbered as in Fig. 5(d).

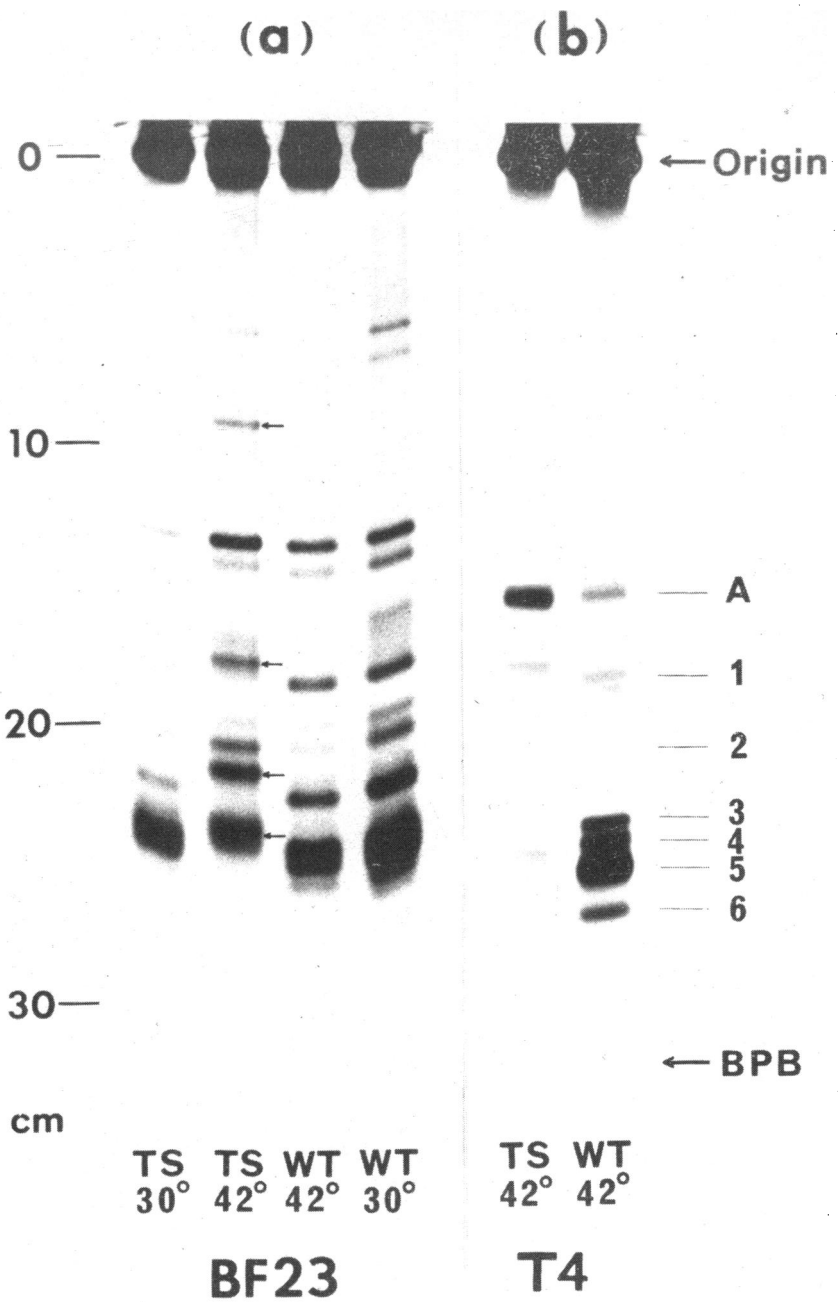
(a)
(b)
0
10
20
30
cm
Origin
A
1
2
3
4
5
6
BPB
TS 30°
TS 42°
WT 42°
WT 30°
TS 42°
WT 42°
BF23
T4

Fig. 7

Fig. 7. (a) Fractionation of ^{32}P-labeled RNA from BF23 infected cells by polyacrylamide slab gel electrophoresis. The cells were grown at 30°C in low phosphate medium containing $CaCl_2$ to a density of $2x10^8$ cells/ml, and infected with BF23 at a multiplicity of 10. At 5 min after infection, 2 ml portions of the culture were transferred to 42°C or 30°C, and incubated for 5 min. 0.4 mCi of carrier-free ^{32}P-orthophosphate was then added and the cells were labeled for 60 min. (b) Fractionation of ^{32}P-labeled RNA from T4 infected cells. Cells were grown at 30°C to a density of $2x10^8$ cells/ml, concentrated in glucose-free low phosphate medium, and infected with T4D at a multiplicity of 10 at 30°C. At 15 min after infection, the cells were diluted with the same medium supplemented with glucose to give a density of $3x10^8$ cells/ml, and incubated at 42°C for 5 min. The cells were superinfected at the same multiplicity to cause lysis inhibition. Subsequently (^{32}P)-orthophosphate was added to give a final concentration of 0.3 mCi/ml. The cells were labeled at 42°C for 60 min. RNA was prepared and electrophoresed as described in Fig. 3. The bands are numbered according to Wilson and Abelson (20). The following abbreviations are used: TS, TS241; WT, strain 4273; BPB, bromophenol blue.

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was fractionated by two-dimensional polyacrylamide gel electrophoresis developed by Ikemura and Dahlberg (14). By this method, about 15 tRNA species of E. coli can be fractionated in a pure form and 11 of those have been assigned by fingerprint analysis (16). In ϕ80psu3$^+$ infected cells, tyrosine tRNA is synthesized up to almost half the total tRNA as a result of the amplification of the su3 gene during phage growth (Fig. 5a,c). In contrast, the spot corresponding to $tRNA^{Tyr}$ is missing in TS241 RNA labeled at 42°C (Fig. 5b). Although the two-dimensional tRNA patterns are complex, it appears that the synthesis of particular species of tRNA other than $tRNA^{Tyr}$ is also altered in mutant cells, and that the pattern of tRNA's synthesized varies in the different mutants (Fig. 6). Further analysis of specific tRNA species isolated by

two-dimensional electrophoresis is in progress.

Virulent Phage Directed tRNA Produced in TS241. Virulent phages T4 and T5 are known to direct the synthesis of phage specific tRNA's upon infection (17). It has been recently found in our laboratory that BF23, a virulent phage closely related to T5, also codes for its specific tRNA (18). As with T4, several nonsense suppressor mutations have been isolated from double-amber mutants in BF23 (Okada et al.; unpublished). It is interesting to see if the phage encoded tRNA's are synthesized using the maturation machinery of the host. TS241(ϕ80psu3$^+$) a lysogen made sensitive to BF23, was infected with BF23 and subsequently labeled with ^{32}P. RNA was isolated and subjected to electrophoresis on 10% polyacrylamide gel. Since host RNA synthesis is halted after BF23 infection, synthesis of ^{32}P-labeled RNA is thought to be directed by the phage. As shown in Fig. 7a, the electrophoretic gel pattern of BF23 RNA made in the mutant at 42°C differs from that of the same RNA made in wild type. There are RNA molecules that probably correspond to phage tRNA of wild type but move more slowly. In addition, at least three new bands appear in the mutant as indicated by arrows. Again, if the phage encoded RNA was made in the same mutant at 30°C, the gel pattern is more like that from wild type, although smaller amounts of the new RNA species found at 42°C are present, suggesting there is some defect even at 30°C.

Similarly, the synthesis of T4 encoded tRNA was analyzed in TS241. From the electrophoretic gel pattern (Fig.7b), it is clear that T4 tRNA molecules (bands 3, 4, 5, and 6) were not seen if the infected mutant was grown at 42°C. In addition, there was a sharp increase of an RNA band that electrophoresed in the vicinity of band A, which may correspond to the dimer precursor of tRNA^{Pro} tRNA^{Ser} (19). From these results, we conclude that host functions take part in the synthesis of these phage encoded tRNA's.

DISCUSSION

We have developed a selection procedure to isolate mutants of tRNA biosynthesis in E. coli. In this selection, the cells that make the suppressor tRNA at high temperature are killed by two virulent phages. Obviously, the rationale of this procedure is that the enzymes involved in the processing steps of suppressor $tRNA^{Tyr}$ must be common to those used for cellular synthesis of many tRNA species, if not all. By this method, we have isolated many mutants for $tRNA^{Tyr}$ biosynthesis and, in fact, some of them are shown to be defective in the synthesis of various tRNA's, including cellular as well as phage encoded tRNA's.

Although characterization of these mutants is still incomplete, it appears that the mutants may be grossly classified into three groups from electrophoretic gel patterns of RNA formed at high temperature; i.e., (1) those that hardly synthesize or accumulate 4S and 5S RNA, but seem to make a considerable amount of rRNA, (2) those that fail to form some tRNA species and accumulate abnormal 4S and larger RNA's including tRNA precursors, and (3) those that produce almost normal amount of $tRNA^{Tyr}$, although its suppressor activity is not expressed at 42°C, as judged by the suppressed synthesis of β-galactosidase. Each group, however, may still contain various types of mutants. For instance, TS241 and TS709 are apparently distinguishable from their patterns of "4S RNA's" synthesized at high temperature (Fig.5b and Fig.6). In addition, genetic analysis of the mutants should also help in classification. A preliminary experiment indicates that the mutation of TS709 is covered by F'141 (carrying the 60'-66' region of the E. coli chromosome), whereas that of TS241 is not.

Although our collection of mutants seem to be of various types, the kinds of mutants obtainable in the present procedure may be limited as far as a suppressor gene for $tRNA^{Tyr}$ is used for mutant selection. In this connection, it would be possible get a different set of mutants if another suppressor tRNA gene is used. In this way it may be possible to work out the biosynthetic pathways of tRNA through the analysis of these mutants.

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