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Overproduction of Tyrosyl-tRNA Synthetase Is Toxic to *Escherichia coli*: a Genetic Analysis

HUGUES BEDOUELLE,* VALÉRIE GUEZ, ANNE VIDAL-CROS, AND MONIQUE HERMANN†

Unité de Biochimie Cellulaire (Centre National de la Recherche Scientifique URA D1129), Institut Pasteur, 28 rue du Docteur Roux, 75724 Paris Cedex 15, France

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The *tyrS* genes from *Escherichia coli* and *Bacillus stearothermophilus* were toxic to *E. coli* when they were carried by plasmids with very high copy numbers (pEMBL8 and pEMBL9). We quantified this effect by comparing the efficiencies of plating of *E. coli* derivatives harboring recombinant plasmids in various experimental conditions. The toxicity was apparent at both 30 and 37°C. It increased with the growth temperature, the strength of the *tyrS* promoter, and the copy number of the plasmidic vector. Two- to threefold enhancement of *tyrS* expression raised the toxicity 300-fold. Point mutations in *tyrS* that prevent interaction between its product, tyrosyl-tRNA synthetase, and $tRNA^{Tyr}$ but do not alter the rate of formation of tyrosyl-adenylate abolished the toxicity. Thus, the toxic effect was due to high cellular levels of synthetase activity. At 30°C, the cellular concentration of tyrosyl-tRNA synthetase reached 55% of that of soluble proteins and led to decreased β-galactosidase stability. We discuss possible causes of this toxic effect and describe its applications to the study of the recognition and interaction between the synthetase and $tRNA^{Tyr}$.

Some DNA fragments are difficult to clone into multicopy plasmids of *Escherichia coli* because an encoded product or function or merely their nucleotide sequence interferes with replication of the vector or with cellular metabolism. For example, basic proteins can inhibit transcription or translation by binding to the nucleic acids (8, 15), exported proteins can jam the cellular export machinery (13, 19), and strong promoters (17) or long palindromes (9) can prevent plasmid replication. Although such cloning problems are common, they are rarely analyzed in detail. We performed such an analysis for the gene of an aminoacyl-tRNA synthetase.

The *tyrS* genes of *E. coli* ($tyrS_{Ec}$) and *Bacillus stearothermophilus* ($tyrS_{Bs}$) code for tyrosyl-tRNA synthetase (TyrTS), an enzyme which catalyzes the aminoacylation of $tRNA^{Tyr}$ with tyrosine in a two-step reaction. The tyrosine is first activated with ATP to form tyrosyl-adenylate (Tyr-AMP) and PP_i and then Tyr-AMP is attacked by the 3′-terminal ribose of the tRNA to form tyrosyl-$tRNA^{Tyr}$ and AMP.

The $tyrS_{Ec}$ and $tyrS_{Bs}$ genes were originally cloned as *Sau*3aI fragments into the *Bam*HI site of plasmid pBR322 (1). $tyrS_{Bs}$ was subsequently subcloned into an M13-derived phage after trimming of unnecessary sequences. It is strongly expressed from recombinant bacteriophage M13-BY(M24) under the control of its own promoter. Mutations in the untranslated 5′ region of $tyrS_{Bs}$ were constructed in vitro. One such mutation, $tyrS_{Bs}$(M24.89), results in two- to threefold enhancement of *tyrS* expression (21). Numerous mutations have been constructed in this overexpressed gene (4, 7, 11).

In this report, we show that when subcloned into a very high-copy-number plasmid, the *tyrS* gene was toxic to the host cell. We report a genetic analysis of this phenomenon and describe its potential uses for the study of the interaction between TyrTS and $tRNA^{Tyr}$.

* Corresponding author.
† Permanent address: Institut Français du Pétrole, 92506 Rueil-Malmaison Cedex, France.

MATERIALS AND METHODS

Media and strains. The media used, LB, M63, and MacConkey, have already been described (14). M63 buffer is M63 medium without a carbon source. Ampicillin was added at 100 μg/ml, and kanamycin was added at 50 μg/ml. The plasmids and bacteriophages used are listed in Table 1. Strain TG2 of *E. coli* K-12 [Δ(*lac-pro*) *supE thi hsdD5 recA56 srl*::Tn*10*(F′ *traD36 proA*$^+$*B*$^+$ *lacI*^{q} *lacZ*Δ*M15*)] was a gift from T. Gibson. HB2201 is a female derivative of TG2; HB2202 is identical to TG2 except that it carries a *lac*$^+$ episome, F′ *proA*$^+$*B*$^+$ *lacI*^{q1} *lacZ*$^+$*Y*$^+$ (a gift from J. Miller).

Plasmid constructions. Plasmids pEMBL8$^+$ and pEMBL9$^+$ have their cloning sites in opposite orientations (6). Plasmids pEMBL8$^+$K and pEMBL9$^+$K were constructed by inserting the kanamycin resistance cartridge of plasmid pUC-4K into the *Pst*I sites of plasmids pEMBL8$^+$ and pEMBL9$^+$, respectively. A double cut of pEMBL9$^+$K with *Hin*dIII and *Eco*RI or of pEMBL8$^+$K with *Sal*I and *Hin*dIII releases a fragment which corresponds to the cartridge and can easily be visualized by electrophoresis in an agarose gel. Insertion of a fragment in place of the cartridge can be monitored by loss of kanamycin resistance (10a).

In our first subcloning experiments, we purified all of the DNA fragments before ligation, as previously described (3). Subsequently, for subcloning of derivatives of the $tyrS_{Bs}$ gene from M13 recombinant phages into plasmids pEMBL9$^+$K and pBR322, the phage and plasmid DNAs were digested with *Hin*dIII and *Eco*RI and a fixed amount of the phage DNA was mixed with decreasing amounts of the plasmid DNA in separate ligation reactions. After transformation of the ligated DNAs into HB2201, a female strain that does not allow M13 to propagate, we analyzed the colonies coming from the ligation with the highest possible dilution of the plasmid. The transformants of HB2201 or HB2202 were selected as follows: the mixture of competent cells and DNA was heat shocked, incubated for 2 h at 30°C, spread on LB plates containing ampicillin, and grown at 30°C.

Plating experiments. The strains were grown on LB plates containing ampicillin for 2 days at 30°C (master plates). An isolated colony showing no trace of papillae was suspended

TABLE 1. Phages and plasmids

Phage or plasmid	Relevant characteristics[a]	Source or reference
M13 phages		
M13-BY(M24)	*B. stearothermophilus tyrSp*$^+$ *tyrS*$^{+b}$	21
M13-BY(M24.89)	Like M13-BY(M24) but with *tyrSp* deleted of residues −44 to −98; overproduces TyrTS	21
M13-Ptac-BY	Like M13-BY(M24) but with the nucleotides upstream of −78 replaced by *Ptac*	A. Vidal-Cros
M13-BY(Tr)	Like M13-BY(M24.89) but codes for a mutant TyrTS deleted of amino acid residues 318–417	22
M13-BY(K410N)	Like M13-BY(M24.89) but codes for a mutant TyrTS (Lys-410→Asn)	4
M13-BY(K411N)	Like M13-BY(M24.89) but codes for a mutant TyrTS (Lys-411→Asn)	4
Plasmids		
pUC-4K	*bla*$^+$; carries a kanamycin resistance cartridge	20
pEMBL8$^+$, pEMBL9$^+$	*bla*$^+$; carries the intergenic region of phage f1	6
pEMBL8$^+$K, pEMBL9$^+$K	Like pEMBL8$^+$ and pEMBL9$^+$ but with the kanamycin resistance cartridge of pUC-4K inserted into the *Pst* site	This work
pBR322-EY	*E. coli tyrSp*$^+$ *tyrS*$^+$; *bla*$^+$	1
pEMBL9-BY(M24)	*B. stearothermophilus tyrSp*$^+$ *tyrS*$^+$ inserted between the *Hin*dIII and *Eco*RI sites of pEMBL9$^+$	This work
pEMBL9-BY(M24.89)	Like pEMBL9-BY(M24) but *tyrSp* is deleted of nucleotides −44 to −98	This work
pEMBL9-BY(Ptac)	Like pEMBL9-BY(M24) but with the nucleotides upstream of −78 replaced by *Ptac*	This work
pBR322-BY(M24)	*B. stearothermophilus tyrSp*$^+$ *tyrS*$^+$ inserted between the *Hin*dIII and *Eco*RI sites of pBR322	This work
pEMBL9-BY(K410N)	Like pEMBL9-BY(M24.89) but codes for a mutant TyrTS (Lys-410→Asn)	This work
pEMBL9-BY(K411N)	Like pEMBL9-BY(M24.89) but codes for a mutant TyrTS (Lys-411→Asn)	This work
pEMBL9-BY(Tr)	Like pEMBL9-BY(M24.89) but codes for a mutant TyrTS deleted of amino acid residues 318–417	This work
pEMBL9-BY(Δ*Bss*HII)	Like pEMBL9-BY(M24.89) but *tyrS* is deleted of nucleotides +294 to +1107	This work
pEMBL8-EY	*E. coli tyrSp*$^+$ *tyrS*$^+$ inserted between the *Sal*I and *Hin*dIII sites of pEMBL8$^+$	This work

[a] The *tyrS* gene has the same orientation as *lacZ* in the phages and the same as *bla* in the plasmids.
[b] The *B. stearothermophilus* fragment extends from −325 to +1368, taking the first nucleotide residue of the initiation codon for the *tyrS* gene as +1. $TyrTS_{Bs}$ has 419 amino acid residues (24).

from a fresh master plate into 1 ml of M63 buffer; 200-μl samples of serial dilutions in the same buffer were spread on plates that had been preincubated at the appropriate temperatures. Alternatively, an isolated colony from a master plate was used to inoculate 4 ml of LB broth containing ampicillin. The culture was grown overnight at 30°C, and dilutions were plated as described above.

Concentration of TyrTS in crude extracts. Overnight cultures grown at 30°C in LB broth containing ampicillin were diluted to a starting A_{600} of 0.070 cm^{-1} and grown in the same conditions to an A_{600} of 1.0 cm^{-1} to 1.2 cm^{-1}. HB2202[pEMBL9-BY(M24.89)] grew to an A_{600} of 0.2 cm^{-1} and then remained stationary. The cellular extracts were prepared at 0 to 4°C. The cells were washed with half of the culture volume of 50 mM Tris chloride–1 mM EDTA (pH 7.5) and suspended in 1/10 of a volume of standard buffer (144 mM Tris chloride [pH 7.78], 10 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride), and samples were stored frozen at −20°C. After thawing, the cell suspensions were sonicated in a cup horn and the debris was removed by centrifugation. Active-site titration of TyrTS in the soluble extracts was performed as already described (23). Sodium PP_i was added to the reaction mixture to displace unlabeled Tyr-AMP from TyrTS and then hydrolyzed with pyrophosphatase to initiate formation of ^{14}C-labeled Tyr-AMP. The concentrations of protein in the extracts were measured with the Bio-Rad reagent with bovine serum albumin as the standard.

Inactivation of β-galactosidase. Several isolated colonies without papillae were suspended from a fresh master plate containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) into 200 μl of M63 buffer, and samples were stored frozen at −20°C. The soluble extract was prepared as described above and then diluted about 20 times in M63 buffer. Samples (100 μl) were incubated for various times at either 56.0 or 57.0°C (±0.05°C). One sample was removed every 5 min for 30 min and immediately cooled on ice. After centrifugation of the protein precipitate, the supernatant was assayed for β-galactosidase activity (14).

RESULTS

Cloning of $tyrS_{Bs}$ and $tyrS_{Ec}$ into pEMBL. In phage M13-BY(M24), the *tyrS* gene and its promoter belong to a *Hin*dIII-*Eco*RI fragment. We inserted this fragment between the *Hin*dIII and *Eco*RI cloning sites of plasmids pEMBL9$^+$K and pBR322. Similarly, we subcloned several mutant alleles of $tyrS_{Bs}$ from M13-BY(M24) derivatives into pEMBL9$^+$K. We also inserted the *Sal*I-*Hin*dIII fragment of pBR322-EY that contains $tyrS_{Ec}$ and its promoter between the *Sal*I and *Hin*dIII cloning sites of pEMBL8$^+$K (Fig. 1; Table 1).

In preliminary experiments, we grew the transformed bacteria at 37°C. However, when we tried to insert the $tyrS_{Bs}$ (M24.89) allele into pEMBL9$^+$K, only rare transformants appeared on the plates of selective medium at 37°C. Moreover, when they were streaked on plates or inoculated in broth of the same medium, these transformants either did not grow further at 37°C or contained plasmids which did not have the correct structure. Use of pEMBL8$^+$K, instead of pEMBL9$^+$K, as a vector or of various strains as bacterial hosts did not help. We subsequently grew the transformed cells at 30°C and obtained all of the plasmids described above at this last temperature. These experiments suggested to us that recombinant plasmid pEMBL9-BY(M24.89) was toxic to *E. coli* at 37°C.

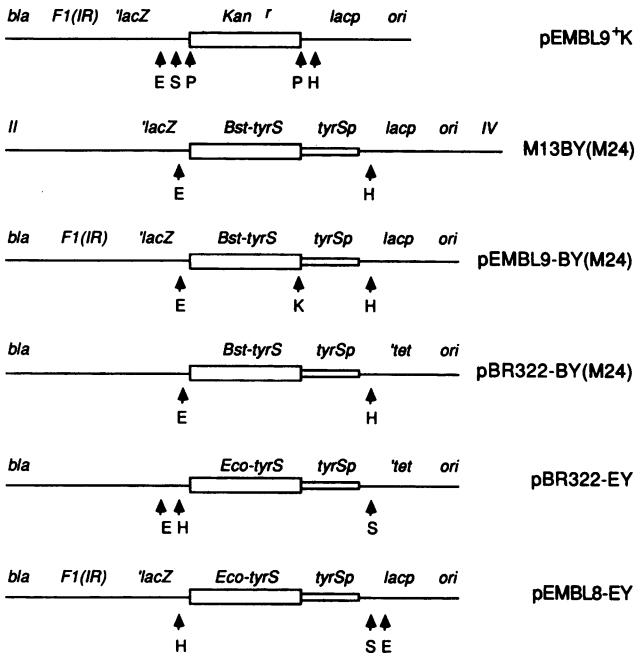

FIG. 1. Structure of the parental and recombinant plasmids and phages used. E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; S, *Sal*I. In pEMBL9-BY(Ptac), the *Hin*dIII-*Kpn*I fragment contains promoter *Ptac* in place of $tyrSp_{Bs}$.

Toxicity of pEMBL9-BY(M24.89) at 37°C. To quantify the toxicity of plasmid pEMBL9-BY(M24.89) for *E. coli*, we compared the plating efficiencies of two recombinant strains, HB2202(pEMBL9$^+$) and HB2202[pEMBL9-BY(M24.89)], in different growth conditions. Table 2 (top) gives the ratios of these plating efficiencies. For HB2202(pEMBL9$^+$), the four ratios (30°C without ampicillin/37°C without ampicillin, 30°C with ampicillin/37°C with ampicillin, 30°C without ampicillin/30°C with ampicillin, and 37°C without ampicillin/37°C with ampicillin) were close to 1. In contrast, there was a large effect of temperature on the plating efficiencies of HB2202[pEMBL9-BY(M24.89)], 800-fold without ampicillin and 2,000-fold with ampicillin. The 30°C without ampicillin/30°C with ampicillin ratio was close to 1, which showed that the plasmid was present in all of the cells at the time of plating. The 37°C without ampicillin/37°C with ampicillin ratio showed that only one of five cells was able to replicate the plasmid or express a sufficient amount of β-lactamase to become resistant to ampicillin at 37°C.

Genetic analysis of toxicity. To understand the molecular basis of the toxicity of pEMBL9-BY(M24.89), we performed a genetic analysis of the phenomenon. We varied the following parameters: (i) copy number of the plasmidic vector, (ii) strength of the promoter, (iii) activity, and (iv) origin of the synthetase (Table 1). For parameter i, we inserted the *tyrS* gene into plasmid pBR322 and into pEMBL8$^+$ or pEMBL9$^+$, which have around 10 times more copies than pBR322 in saturated cultures (6; unpublished data). For parameter ii, we put the $tyrS_{Bs}$ gene under control of its wild-type promoter which is present in $tyrS_{Bs}$(M24), a mutant promoter which is present in $tyrS_{Bs}$(M24.89) and is two to three times more efficient than the wild-type promoter (21), or promoter *Ptac*, which is normally repressed and can be induced with IPTG (5). For parameter iii, we introduced the following four mutations into $tyrS_{Bs}$: two point mutations which change Lys-410 or Lys-411 of $TyrTS_{Bs}$ to Asn and result in a 100-fold increase of the K_m for $tRNA^{Tyr}$; a deletion of the 3′ end of the gene which removes the C-terminal domain of the synthetase (residues 318 to 417) and prevents binding and charging of $tRNA^{Tyr}$; and an internal deletion between the two *Bss*HII sites which removes residues 98 to 370 of $TyrTS_{Bs}$, introduces a frameshift, and abolishes all activity. The four mutations were carried by the overexpressed gene $tyrS_{Bs}$(M24.89); the first three mutations do not affect formation of tyrosyl-adenylate

TABLE 2. Comparison of plating efficiencies

Plasmid	Ratio of results obtained at[a]:			
	30° − amp/37° − amp	30° + amp/37° + amp	30° − amp/30° + amp	37° − amp/37° + amp
Suspended colonies[b]				
pEMBL9$^+$	1.07	1.15	0.91	0.97
pEMBL9-BY(M24.89)	807	1,939	1.10	4.30
pEMBL9-BY(M24)	11.0	5.95	1.05	0.58
pEMBL9-BY(Ptac)	1.14	1.23	0.96	1.04
pBR322-BY(M24)	1.11	1.00	1.04	0.95
pEMBL9-BY(K410N)	1.86	1.97	0.96	1.03
pEMBL9-BY(K411N)	2.08	2.18	1.05	1.11
pEMBL9-BY(Tr)	1.10	1.09	0.98	0.98
pEMBL9-BY(Δ*Bss*HII)	1.18	1.38	0.93	1.09
pEMBL8-EY	18.9	11.2	0.80	0.58
pBR322-EY	1.01	1.12	0.93	1.02
Saturated cultures[c]				
pEMBL9$^+$	1.08	1.03	0.96	0.92
pEMBL9-BY(M24.89)	1.15	539	3.70	2,668

[a] Abbreviations: 30° − amp, 30°C without ampicillin; 30° + amp, 30°C with ampicillin. Average values from three independent experiments are shown.

[b] Suspended colonies. An isolated colony of HB2202 containing one of the plasmids listed was suspended in buffer, and dilutions of the suspension were spread on LB plates (see Materials and Methods). The plates either did or did not contain ampicillin, and they were incubated at either 30 or 37°C. The colonies were counted after 48 h of growth. The ratios of the colony counts in the different conditions are shown. For strain HB2202[pEMBL9-BY(Ptac)], the plates contained 1 mM IPTG.

[c] Saturated cultures. Isolated colonies of the HB2202 derivatives were used to inoculate 4 ml of LB medium containing ampicillin. The bacteria were grown overnight at 30°C, and dilutions of the saturated cultures were spread on LB plates as described in footnote *b*. The efficiencies of plating of the saturated cultures on ML medium at 30°C per A_{600} unit were 0.19×10^8 for HB2202[pEMBL9-BY(M24.89)] if the plates contained ampicillin and 0.94×10^8 if they did not. For HB2202(pEMBL9$^+$), the plating efficiency was 5.6×10^8 in both cases.

by the synthetase (4, 22). For parameter iv, we tested the *tyrS* genes from *E. coli* and *B. stearothermophilus*.

Plating experiments similar to those described above were performed with these derivatives of $tyrS_{Bs}$ and $tyrS_{Ec}$ cloned into pBR322, pEMBL8$^+$, or pEMBL9$^+$. The derivatives for which the four resulting ratios were close to 1 (Table 2, top) had efficiencies of plating similar to that of HB2202 (pEMBL9$^+$). The main results were as follows. The $tyrS_{Bs}$ gene was toxic to *E. coli* at 37°C when carried by pEMBL9$^+$ (Table 2, lines 2 and 3). The mutant genes whose products were still able to catalyze formation of Tyr-AMP but not to bind $tRNA^{Tyr}$ were not toxic to cells (Table 2, lines 6 to 8). Toxicity increased with the strength of the promoter (Table 2, lines 2 and 3). $tyrS_{Bs}$ was not toxic when expressed from promoter *Ptac*, which was induced with IPTG (Table 2, line 4). Both $tyrS_{Bs}$ and $tyrS_{Ec}$ were toxic (Table 2, lines 2, 3, and 10). The toxicity increased with the copy number of the plasmidic vector (Table 2, lines 3, 5, 10, and 11).

In summary, an excess of TyrTS activity was strongly toxic to *E. coli* at 37°C. Mutations that prevent interaction between TyrTS and $tRNA^{Tyr}$ abolished the toxicity. Using a promoter that is two- to threefold more efficient increased the toxicity by a factor of 300.

Growth in broth. The plating experiments described above were done with suspended colonies. In preliminary experiments, we plated saturated cultures grown in liquid medium, but the results were more difficult to interpret (Table 2, bottom). For example, there was a strong effect of temperature when the bacteria were plated on a medium with ampicillin but no effect when they were spread on plates without ampicillin. At least 1 of 5 cells still harbored the plasmid at the time of plating, but only 1 in 2,700 expressed resistance to ampicillin at 37°C (Table 2, line 13). Thus, the genetic or physiological state of the cells was modified during growth in broth. We determined the efficiencies of plating on LB medium at 30°C per A_{600} unit of the cultures grown in broth (Table 2, footnote *c*). The plating of the strain overexpressing $tyrS_{Bs}$ was 6 to 30 times less efficient than that of the control strain. Inspection of the cell cultures under a light microscope did not show filamentation. These results suggested that cells of HB2202[pEMBL9-BY(M24.89)] were unable to restart growth or that a large proportion of them were dead.

Cellular concentrations of TyrTS at 30°C. To correlate the cellular level of TyrTS with its toxicity in the plating experiments, we determined its concentration in crude extracts of HB2202-derived strains. We had to grow the strains at 30°C to avoid the problems of strong toxicity encountered at 37°C. We diluted saturated cultures of the HB2202 derivatives into fresh medium and grew them for four generations, until the late exponential phase, except for HB2202 [pEMBL9-BY(M24.89)], which stopped growing after 1.5 generations. Thus, even at 30°C, plasmid pEMBL9-BY (M24.89) inhibited cell growth. The concentration of TyrTS was determined by active-site titration, an assay which measures the number of molecules that are active in forming Tyr-AMP.

TyrTS was overproduced to up to 360 times its endogenous level in *E. coli* cells and constituted up to 55% of the soluble proteins (Table 3, lines 1 and 2). Its concentration was strongly dependent on the strength of the *tyrS* promoter (Table 3, lines 2 to 5). The weak expression under control of *Ptac* can explain the lack of toxicity of plasmid pEMBL9-BY(Ptac) in the plating experiments (Table 2). The two point mutants were produced at levels that were intermediate between those obtained for wild-type $TyrTS_{Bs}$ under the control of promoters M24 and M24.89 (Table 3, lines 2, 3, 7, and 8). Changing the plasmid vector from pBR322 to pEMBL had no effect on production of $TyrTS_{Bs}$ but resulted in a 1.5-fold increase in production of $TyrTS_{Ec}$ (Table 3, lines 3, 6, 11, and 12).

TABLE 3. Toxicity and cellular concentration of TyrTS at 30°C

Plasmid[a]	% of soluble proteins[b]	Diam of colonies (mm)[c]
pEMBL9$^+$	0.15	1.50
pEMBL9-BY(M24.89)	55.11	0.00
pEMBL9-BY(M24)	19.04	0.25
pEMBL9-BY(Ptac) with IPTG	3.58	ND
pEMBL9-BY(Ptac) without IPTG	0.20	1.50
pBR322-BY(M24)	19.92	1.50
pEMBL9-BY(K410N)	27.70	1.00
pEMBL9-BY(K411N)	29.72	1.00
pEMBL9-BY(Tr)	6.93	1.00
pEMBL9-BY(Δ*Bss*HII)	0.11	0.50
pEMBL8-EY	7.42	0.25
pBR322-EY	4.83	1.50

[a] Recombinant plasmids harbored by the derivatives of strain HB2202.

[b] The HB2202 derivatives were grown in LB broth at 30°C for four generations, until an A_{600} of 1.0 cm^{-1} to 1.2 cm^{-1} was reached. The culture of HB2202[pEMBL9-BY(M24.89)] grew for only 1.5 generations and then remained stationary at an A_{600} of 0.2 cm^{-1}. The concentrations of TyrTS in the soluble extracts determined by active-site titration (23) varied between 1.14×10^{-8} and 293×10^{-8} M. The total concentrations of protein varied between 0.90 and 1.43 mg/ml, except for the soluble extract of HB2202[pEMBL9-BY(M24.89)], which contained only 0.13 mg of protein per ml. The molecular weights of $TyrTS_{Bs}$, $TyrTS_{Bs}$(Tr), and $TyrTS_{Ec}$ were taken as 94,632, 72,648 and 94,806, respectively (2, 22, 24). The concentrations of TyrTS were normalized with respect to the total concentrations of protein in the soluble extracts and are expressed as percentages.

[c] The HB2202 derivatives were streaked on minimal M63B1 medium containing glucose as the carbon source, proline, and ampicillin. After 48 h of growth at 30°C, the diameters of the colonies were recorded. ND, Not determined; 0.00, no colony.

Thus, although the titration and plating experiments were done in different conditions, they showed a clear correlation between the cellular concentration of active TyrTS during growth at 30°C and its toxicity at 37°C.

Toxicity at 30°C. The results obtained with cultures in LB broth had suggested to us that plasmid pEMBL9-BY (M24.89) inhibited the growth of its bacterial host at 30°C (see above). To further substantiate this observation, we streaked strain HB2202 and its derivatives on minimal glucose and on lactose or galactose indicator plates containing ampicillin and incubated them at 30°C for 48 h. On minimal medium, the diameters of the colonies (Table 3) correlated with the cellular concentrations of active TyrTS at 30°C and with its toxicity at 37°C (Tables 2 and 3). In particular, bacteria containing pEMBL9-BY(M24.89) did not grow at all, whereas those containing pEMBL9$^+$ gave large colonies. Thus, overexpression of the *tyrS* gene prevented cell growth on minimal medium at 30°C. On MacConkey plates, all of the HB2202 derivatives were strongly Lac$^+$ and Gal$^+$, except HB2202[pEMBL9-BY(M24.89)], which was only weakly Lac$^+$ and Gal$^+$ (pink). This suggested to us that the activity or stability of β-galactosidase and essential proteins might be altered in a strain overexpressing *tyrS*.

Thermal stability of β-galactosidase. To determine whether overexpression of *tyrS* alters the structural properties of cellular proteins, we suspended colonies of HB2202 derivatives in buffer, prepared their soluble extracts, and determined the kinetics of inactivation of β-galactosidase, taken

TABLE 4. β-Galactosidase inactivation half-times[a]

Plasmid	Inactivation half-time (min) at:	
	56.0°C	57.0°C
pEMBL9$^+$	28.5	10.1
pEMBL9-BY(M24.89)	15.5	6.1
pEMBL9-BY(K410N)	27.0	11.5

[a] Soluble extracts were prepared from suspended colonies. Each number is the average of the values obtained in two independent experiments.

as a test protein, at 56 and 57°C (Table 4). When β-galactosidase was synthesized by HB2202[pEMBL9-BY (M24.89)], its inactivation half-time was about half of the normal value, i.e., the value obtained with an extract of the control strain, HB2202(pEMBL9$^+$). This decrease in stability was abolished by point mutation K410N. These results showed that it was overproduction of active TyrTS during growth at 30°C that decreased the stability of β-galactosidase.

DISCUSSION

We found that overproduction of active TyrTS was toxic to *E. coli*. This toxicity was apparent at both 30 and 37°C, but its effect was stronger at 37°C. Overproduction of TyrTS decreased the stability of β-galactosidase.

The results showed a clear correlation between the toxicity of the plasmids carrying the *tyrS* gene and the cellular concentration of active TyrTS, although it was not possible to measure them in identical conditions. Indeed, the toxicity in the plating experiments at 37°C, the inhibition of growth in broth or on minimal medium at 30°C, and the concentration of TyrTS in cells growing at 30°C increased together with the strength of the *tyrS* promoter and, to a lesser extent, with the copy number of the plasmidic vector.

In cells growing at 30°C, the concentration of active $TyrTS_{Bs}$ increased threefold when we replaced the wild-type promoter *tyrSp*(M24) with mutant promoter *tyrSp*(M24.89), which is two to three times stronger (21), and it amounted to 55% of the soluble proteins. Replacement of the wild-type promoter by the mutant promoter resulted in strong inhibition of growth at 30°C and in a 300-fold increase of toxicity at 37°C. Thus, toxicity occurred at high concentrations of TyrTS and at these cellular levels, a weak increase in TyrTS expression resulted in a strong enhancement of toxicity.

Inhibition of growth at 30°C and toxicity at 37°C were abolished by two point mutations of TyrTS which strongly affect $tRNA^{Tyr}$ binding and aminoacylation but not Tyr-AMP formation. At 30°C, the cellular concentrations of these point mutant proteins were high, intermediate between those obtained for wild-type TyrTS under control of promoters M24 and M24.89. Therefore, if the relative concentrations of wild-type and mutant TyrTS remained constant under the various experimental conditions that we used, these results imply that the toxicity was due to interaction of TyrTS with tRNAs and not to formation of Tyr-AMP.

Toxicity was not due to defective folding of overproduced TyrTS (16). Indeed, our titrations of active sites in crude extracts showed that TyrTS could be produced at very high levels, up to 55% of the soluble proteins, and still remain active and, thus, correctly folded. Moreover, point mutations in TyrTS abolished the toxicity.

The toxicity of TyrTS was abolished by two different point mutations which changed Lys-410 or Lys-411 to Asn and by a deletion, *tyrS*(Δ*Bss*HII), which removed codons 93 to 370 but left unchanged the 3′-terminal end of *tyrS*, where the two point mutations lie. These results eliminate the nucleotide sequence of *tyrS*, at either the DNA or the mRNA level, as the cause of the toxicity, in particular through formation of palindromes, synthesis of antisense RNA, or transcription of adjacent regions of the plasmid (see the introduction for references). It is remarkable that the strong promoter $tyrSp_{Bs}$(M24.89) did not destabilize pEMBL9$^+$. Similarly, $tyrSp_{Bs}$(M24) and $tyrSp_{Ec}$ did not destabilize pBR322. Thus, insertion of a strong terminator at the 3′ end of cloned genes is not a general requirement.

The half-time of β-galactosidase inactivation at 56 and 57°C was reduced when the enzyme was synthesized by a strain that overproduced TyrTS compared with when it was synthesized by strains that did not overproduce it or overproduced a partially inactive synthetase, TyrTS(K410N). The effect was small (about twofold) but reproducible (Table 4). On the basis of the results, we can conclude that it was overexpression of TyrTS activity (more precisely, its interaction with tRNAs but not formation of Tyr-AMP) that decreased the stability of β-galactosidase. Swanson et al. (18) have shown that if *E. coli* glutaminyl-tRNA synthetase is overproduced in vivo, it incorrectly acylates $tRNA^{Tyr}$ with Gln. A similar mechanism could explain the decreased stability of β-galactosidase in our experiments: high cellular levels of TyrTS could result in mischarging of noncognate tRNAs and thus in misincorporation of tyrosine in β-galactosidase. Hall and Gallant have shown that amino acid starvation results in thermolabile β-galactosidase (10). High levels of TyrTS could drain the tRNA pool and starve the cell for $Tyr\text{-}tRNA^{Tyr}$, resulting in mistranslated proteins. A low level of mistranslation in β-lactamase could explain the fourfold difference between the efficiencies of plating of strain HB2202[pEMBL9-BY(M24.89)] at 37°C on medium with or without ampicillin (Table 2, line 2).

How can we explain the effect of temperature on TyrTS toxicity? The average coefficient of temperature for enzymatic reactions is $Q_{10} = 2$ (12); therefore, decreasing the temperature from 37 to 30°C should decrease the activity of TyrTS about 1.4 times. Such a decrease could have an important effect on toxicity, since our results showed that a 2- to 3-fold decrease in the strength of the *tyrS* promoter resulted in a 325-fold increase in cell viability. We found that overproduction of TyrTS decreased the stability of β-galactosidase. If this effect is general, essential proteins might be active at 30°C and become partially inactive at 37°C, which would affect cell growth or viability.

The correlations between our different experiments were not absolute. We see two possible causes. The plasmid copy numbers are strongly dependent on the physiological state of the host cells and on their growth rate (17). The toxicity of wild-type TyrTS could contribute to its own overproduction relative to the other cellular proteins by slowing down or precociously stopping bacterial growth.

The toxicity of TyrTS should be useful for the study of the recognition of $tRNA^{Tyr}$ by TyrTS. Indeed, an increase of its production by a factor of only 2 to 3 resulted in a toxic effect multiplied 300-fold. Thus, by cloning mutant *tyrS* genes into pEMBL9$^+$ and by testing the toxicity of the recombinant plasmids, we shall be able to strongly amplify little differences in the activity or specificity of charging of their product, which, by in vitro kinetic experiments, would be near the limit of detection. By mutagenizing the *tyrS* gene under the control of promoter *Ptac*, we shall be able to screen for conditionally lethal mutants with increased TyrTS

activity or decreased specificity in the recognition of $tRNA^{Tyr}$. By selecting bacteria that contain pEMBL9-BY(M24.89) and are able to grow on minimal medium, we could isolate mutants with decreased activity or increased specificity.

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