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Formation of D-Tyrosyl-tRNA^{Tyr} Accounts for the Toxicity of D-Tyrosine toward *Escherichia coli**

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D-Tyr-tRNA^{Tyr} deacylase cleaves the ester bond between a tRNA molecule and a D-amino acid. In *Escherichia coli*, inactivation of the gene (*dtd*) encoding this deacylase increases the toxicity of several D-amino acids including D-tyrosine, D-tryptophan, and D-aspartic acid. Here, we demonstrate that, in a Δ*dtd* cell grown in the presence of 2.4 mM D-tyrosine, ~40% of the total tRNA^{Tyr} pool is converted into D-Tyr-tRNA^{Tyr}. No D-Tyr-tRNA^{Tyr} is observed in *dtd*$^+$ cells. In addition, we observe that overproduction of tRNA^{Tyr}, tRNA^{Trp}, or tRNA^{Asp} protects a Δ*dtd* mutant strain against the toxic effect of D-tyrosine, D-tryptophan, or D-aspartic acid, respectively. In the case of D-tyrosine, we show that the protection is accounted for by an increase in the concentration of L-Tyr-tRNA^{Tyr} proportional to that of overproduced tRNA^{Tyr}. Altogether, these results indicate that, by accumulating *in vivo*, high amounts of D-Tyr-tRNA^{Tyr} cause a starvation for L-Tyr-tRNA^{Tyr}. The deacylase prevents the starvation by hydrolyzing D-Tyr-tRNA^{Tyr}. Overproduction of tRNA^{Tyr} also relieves the starvation by increasing the amount of cellular L-Tyr-tRNA^{Tyr} available for translation.

D-amino acids found in the living world contribute to various functions (reviewed in Refs. 1 and 2). On the other hand, the selectivity of ribosomal protein synthesis prevents their incorporation into polypeptides. Aminoacyl-tRNA synthetases ensure the first step of exclusion of D-amino acids. However, the stereospecificity of these enzymes is not absolute. Thus, it was observed early on that *Escherichia coli* and *Bacillus subtilis* tyrosyl-tRNA synthetases catalyze the formation of D-Tyr-tRNA^{Tyr} *in vitro* (3, 4). More recently, *Saccharomyces cerevisiae* tyrosyl-tRNA synthetase (5), as well as *E. coli* tryptophanyl- and aspartyl-tRNA synthetases (6), were also shown to catalyze the transfer of the D-isomer of their cognate amino acid to the corresponding tRNA species *in vitro*. In all these cases, the catalytic efficiency with the D-enantiomer is measured only 15–2000-fold lower than that with the L-enantiomer.

Soon after the discovery of the D-tyrosyl-tRNA synthesis by tyrosyl-tRNA synthetases, it was shown that extracts of *E. coli* and some other organisms contain an activity capable of hydrolyzing D-tyrosyl-tRNA into D-tyrosine and free tRNA (7). This deacylase can also hydrolyze D-Trp-tRNA^{Trp} and D-Asp-tRNA^{Asp} (6) but not L-aminoacyl-tRNAs (6, 7). D-Tyr-tRNA^{Tyr} deacylase is encoded by the *dtd* gene in *E. coli* (8) and by the *DTD1* gene in *S. cerevisiae* (5). Homologs of *dtd*/*DTD1* genes occur in many bacterial and eukaryotic cells. Upon inactivation of *E. coli dtd* or *S. cerevisiae DTD1* gene, cell growth becomes more sensitive to the presence of various D-amino acids in the culture medium: D-Tyr, D-Trp, D-Asp, D-Gln, and D-Ser in *E. coli* and D-Tyr and D-Leu in *S. cerevisiae* (6). This behavior suggests that the toxicity of various D-amino acids can be at least partially contributed for by the formation of D-aminoacyl-tRNAs at the expense of the corresponding L-aminoacyl-tRNAs and that D-Tyr-tRNA^{Tyr} deacylase prevents this toxicity by recycling D-aminoacyl-tRNAs into free tRNA molecules.

To establish the involvement of tRNA in the toxicity of D-amino acids *in vivo*, we analyzed the nature of the amino acid linked to tRNA^{Tyr} when bacteria were grown in the presence of D-tyrosine. In a strain lacking D-Tyr-tRNA^{Tyr} deacylase, about 40% of the cellular tRNA^{Tyr} was found esterified with D-tyrosine. In the parental *dtd*$^+$ strain, no D-Tyr-tRNA^{Tyr} could be detected. These results indicate that D-Tyr-tRNA^{Tyr} formation can starve the cell for L-Tyr-tRNA^{Tyr} and that the deacylase prevents the starvation by hydrolyzing D-Tyr-tRNA^{Tyr}. We also studied the effect of tRNA overproduction on the toxicity of D-Tyr, D-Trp, or D-Asp for Δ*dtd E. coli* cells. A protection was observed when the overproduced tRNA corresponded to the D-amino acid added to the growth medium. Upon a ~3-fold tRNA^{Tyr} overproduction, the concentration of L-Tyr-tRNA^{Tyr} increases proportionally and, in a Δ*dtd* cell, such an increase in the L-Tyr-tRNA^{Tyr} concentration accounts for the relief of the D-tyrosine toxicity.

MATERIALS AND METHODS

D-[*methylene*-^{3}H]Tyrosine (211 GBq/mmol) was custom-prepared by Amersham Biosciences. L-[^{14}C]Tyrosine (18.3 GBq/mmol), L-[5-^{3}H]tryptophan (740 GBq/mmol), D-[2,3-^{3}H]aspartic acid (666 GBq/mmol), and L-[2,3-^{3}H]aspartic acid (703 GBq/mmol) were from PerkinElmer Life Sciences. Non-radioactive D-amino acids and Brij 58 (polyoxyethylene 20 cetyl ether) were from Sigma.

E. coli D-Tyr-tRNA^{Tyr} deacylase and tyrosyl-, aspartyl-, and tryptophanyl-tRNA synthetases were purified as described previously (6, 8). The bacterial strains and plasmids used in this study are listed in Table I. Strain K37ΔrecA was obtained from strain K37 by P1 transduction of the *recA938*::*cat* allele of strain GW5552 (9).

Determination of D-Amino Acid Toxicity—To study the effect of 2.4 mM D-tyrosine or 5 mM D-tryptophan on the generation times of different *E. coli* strains, cells were grown at 37 °C in M9-glucose minimal medium containing 100 μg of ampicillin/ml, in the presence or in the absence of the D-amino acid. Cultures were started in M9-glucose without D-amino acid. After ~8 h of growth, bacteria were diluted at a final OD_{650} of 0.0007–0.001 in medium containing or not the D-amino acid. When the OD_{650} of the culture reached 0.2–0.3, bacteria were diluted again into the medium under study to obtain a final OD_{650} of 0.005. These cultures were used to measure the generation times.

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TABLE I
Strains and plasmids used in this study

	Description	Reference
Strains		
K37	*galK rpsL*	Ref. 31
K37ΔTyrH	K37 Δ*dtd*::*kan*	Ref. 8
K37ΔrecA	K37 Δ*recA938*::*cat*	This work
K37ΔTyrHΔrecA	K37 Δ*dtd*::*kan* Δ*recA938*::*cat*	Ref. 8
Plasmids		
pBR322	Ap^r Tc^r	Ref. 32
pBRtyrT	Ap^r *tyrT* derivative of pBR322	This work
pBRtrpT	Ap^r *trpT* derivative of pBR322	This work
pBRaspT	Ap^r *aspT* derivative of pBR322	This work

The toxicity indices of D-aspartate were determined as described earlier for the toxicity indices of D-cysteine (10). Briefly, cells were pregrown to mid-exponential phase (0.2–0.3 OD_{650}) at 37 °C in M9-glucose minimal medium. Cells were then inoculated in liquid M9-glucose medium containing different concentrations of D-aspartate to give an OD_{650} of 0.005. The toxicity index is defined as the D-aspartate concentration causing a 2-fold reduction in the optical density of the culture after an 8-h incubation at 37 °C, as compared with the optical density of a culture without D-aspartate.

The D-tryptophan toxicity was also assayed on solid medium. Cells were grown at 37 °C for 40–48 h on M9-glucose minimal medium agar plates supplemented with ampicillin (100 μg/ml) and different concentrations of D-tryptophan (0.5, 1, 2.5, and 5 mM).

Overproduction of $tRNA^{Tyr}$, $tRNA^{Asp}$, and $tRNA^{Trp}$—Construction of pBSTNAV derivatives harboring *tyrT* (8), *trpT*, or *aspT* (6) tRNA genes was described previously. Possibly because of a high overproduction of tRNA, introduction of these plasmids into strain K37ΔrecA or K37ΔTyrHΔrecA resulted in the formation of colonies of variable size when plated on M9-glucose minimal medium supplemented with ampicillin. To obtain a lower overproduction of tRNA, the BamHI-XhoI fragments of the various pBSTNAV derivatives were introduced into the BamHI and SalI sites of pBR322. These fragments carried both the tRNA gene and the P_{lpp} promoter of pBSTNAV. The resulting plasmids (pBRtyrT, pBRtrpT, and pBRaspT) were used to transform *E. coli* strains K37ΔrecA and K37ΔTyrHΔrecA. Growth of the transformed cells on minimal medium was homogeneous and reproducible.

Amino Acid Acceptance of Crude tRNA Extracts—Strains harboring the pBRtyrT, pBRtrpT, or pBRaspT plasmids were grown overnight in 40 ml of 2× TY medium (1.6% bacto-tryptone, 1% bacto-yeast extract, 0.5% NaCl) containing 100 μg of ampicillin/ml. After the preparation of crude tRNA extracts (11), L-tyrosine, L-tryptophan, and L-aspartate acceptances were measured as described earlier (6). The reaction mixtures contained 20 mM Tris-HCl (pH 7.8), 2 mM ATP, 7 mM $MgCl_2$, 0.1 mM EDTA, 50 μg/ml bovine serum albumin, 2.5 mM 2-mercaptoethanol, 30 μM radioactive L-amino acid (L-[^{14}C]tyrosine, L-[^{3}H]tryptophan, or L-[^{3}H]aspartic acid, 500 Ci/mol), 0.3 A_{260} units of tRNA, and 1.5 μM the corresponding aminoacyl-tRNA synthetase. Prior to L-tryptophan acceptance measurements, tRNA was renatured at 60 °C in the presence of $MgCl_2$, as described previously (6, 12, 13).

Isolation of Aminoacyl-tRNAs—Aminoacyl-tRNAs were isolated under acidic conditions, essentially as described by Menninger (14). Cells were grown at 37 °C in 60 ml of M9-glucose minimal medium. When the optical density of the culture reached 0.8 at 650 nm, 6.6 ml of ice-cold 50% (w/v) trichloroacetic acid and 0.66 ml of 1% (w/v) Brij 58 were added. After incubation in an ice bath for 10 min, cells were collected by centrifugation (20 min, 4 °C, 12,000 × *g*) and suspended in 1 ml of 0.25 M sodium acetate (pH 5.0) containing 1 mM EDTA and 0.05% Brij 58. An equal volume of phenol equilibrated with 20 mM sodium acetate (pH 5.0) was added, and the mixture was sonicated for a total of 2 min (three 40-s strokes, with 1-min intervals, on ice). After centrifugation at 15,000 × *g* for 20 min at 4 °C, the aqueous layer was collected and precipitated with ethanol.

Aminoacylation Level Measurements—To evaluate the amounts of L-Tyr-$tRNA^{Tyr}$, D-Tyr-$tRNA^{Tyr}$, and peptidyl-$tRNA^{Tyr}$ in the aminoacyl-tRNA preparation, four aliquots were treated in parallel. (i) tRNA in the first aliquot was fully deacylated by an incubation at 37 °C for 1.5 h in the presence of 2 M Tris-HCl (pH 8.0). (ii) In the second aliquot, D-aminoacyl-tRNAs were specifically deacylated by a treatment with D-Tyr-$tRNA^{Tyr}$ deacylase. For this purpose, the sample was suspended in 20 mM MES-KOH[1] (pH 6.0) containing 5 mM $MgCl_2$, and pure *E. coli* D-Tyr-$tRNA^{Tyr}$ deacylase was added at a final concentration of 1 μM. We verified that this concentration of deacylase was sufficient to fully deacylate D-[^{3}H]Tyr-$tRNA^{Tyr}$ substrate at a concentration close to that of total $tRNA^{Tyr}$ in the test (data not shown). The mixture was incubated at 28 °C for 5 min. L-aminoacyl-tRNAs, which are resistant to the action of the deacylase, were then acetylated to avoid deacylation during the subsequent incubation in the presence of tyrosyl-tRNA synthetase. Therefore, the sample was precipitated with ethanol and resuspended in 5 mM sodium acetate (pH 5.0). Acetylation of the aminoacyl-tRNAs remaining in the sample was performed as described earlier (15). (iii) The third aliquot was processed exactly as the second one except that D-Tyr-$tRNA^{Tyr}$ deacylase was omitted from the reaction mixture. (iv) Finally, the fourth aliquot was incubated in the presence of $CuSO_4$ to hydrolyze aminoacyl but not *N*-acyl-aminoacyl tRNA ester linkages (16, 17). The sample was suspended in 0.5 ml of 0.3 M sodium acetate (pH 5) containing 10 mM $CuSO_4$. After a 30-min incubation at 37 °C, the reaction was terminated by the addition of 15 mM EDTA. The tRNA species were precipitated with ethanol. The pellet was washed with a solution containing 1 mM EDTA, 1 mM $MgSO_4$, and 75% ethanol and then with a solution containing 2 mM sodium acetate (pH 5), 1 mM $MgSO_4$, and 75% ethanol. To remove remaining copper ions, the sample was dissolved in 500 μl of water and applied on a Micro Bio-Spin chromatography column (from Bio-Rad) filled with 500 μl of Chelex 100 resin (from Bio-Rad). The column was eluted with the same volume of water by gravity flow.

The four aliquots were precipitated with ethanol. Pellets were washed with 70% ethanol and dissolved in 60 μl of water. To control the yield of nucleic acid recovery, the absorbances of the samples were measured at 260 nm. The values obtained for each of the four aliquots were found equal within ±5% error. L-[^{14}C]Tyrosine acceptance of tRNA in each aliquot was measured using *E. coli* tyrosyl-tRNA synthetase (28 °C, 10 min), as described earlier (6). The reaction mixture contained 20 mM Tris-HCl (pH 7.8), 2 mM ATP, 7 mM $MgCl_2$, 0.1 mM EDTA, 50 μg/ml bovine serum albumin, 2.5 mM 2-mercaptoethanol, 7 μM L-[^{14}C]tyrosine (500 Ci/mol), 1 A_{260} unit of tRNA, and 0.03 μM tyrosyl-tRNA synthetase.

From these measurements, the amount of non-esterified $tRNA^{Tyr}$ was deduced from the L-tyrosine acceptance of the third aliquot. The difference between the acceptances of the second and third aliquots reflected the amount of D-tyrosyl-$tRNA^{Tyr}$, the difference between the fourth and second aliquots corresponded to L-tyrosyl-$tRNA^{Tyr}$, and the difference between the first and fourth aliquots corresponded to peptidyl-$tRNA^{Tyr}$. Intracellular concentration of $tRNA^{Tyr}$ was calculated using the tyrosine acceptance of the sample treated with 2 M Tris-HCl and the OD_{650} of the culture used for sample preparation. We assumed that the extraction yield of tRNA was 100% and that, in the cell culture, 1 OD_{650} corresponded to 0.4 μl of intracellular volume (18).

RESULTS

Measurement of D-Tyr-$tRNA^{Tyr}$—Exogeneous D-tyrosine slows down the growth rate of *E. coli* mutants lacking D-Tyr-$tRNA^{Tyr}$ deacylase (8). One likely reason for the toxicity of D-tyrosine is the immobilization as D-Tyr-$tRNA^{Tyr}$ of part of the $tRNA^{Tyr}$ pool. As a consequence, the growth rate would be decreased because of a starvation in L-Tyr-$tRNA^{Tyr}$. To test this idea, aminoacyl-tRNAs were extracted from cells grown in the presence or absence of D-tyrosine, and the nature of the amino acid attached to $tRNA^{Tyr}$ was analyzed. Part of the sample was treated by D-Tyr-$tRNA^{Tyr}$ deacylase, whereas another part was incubated in the same conditions without deacylase. Then, the L-tyrosine acceptances of the two samples were compared. If the aminoacyl-tRNA preparation contained D-Tyr-$tRNA^{Tyr}$, the L-tyrosine acceptance of the deacylase-treated sample should be higher than that of the non-treated sample. The deacylase treatment did not change the L-tyrosine acceptance of the tRNA samples prepared from the *dtd*$^+$ strain grown in the presence or absence of D-tyrosine or from the Δ*dtd* strain grown in the absence of D-tyrosine. On the other hand, the incubation with deacylase increased about 4-fold the tyrosine acceptance of the sample from the Δ*dtd* strain grown in the presence of 2.4 mM

[1] The abbreviations used are: MES, 4-morpholineethanesulfonic acid; EF, elongation factor.

TABLE II
Level of tRNA^{Tyr} aminoacylation in vivo

Strain	Growth conditions[a]	Total tRNA^{Tyr}[b]	D-Tyr-tRNA^{Tyr}[c]	L-Tyr-tRNA^{Tyr}[c]
		μM	%	%
K37ΔTyrH	No D-Tyr	9 ± 1	1.5 ± 4	87 ± 3
K37	No D-Tyr	8 ± 3	2.5 ± 3	82 ± 3
K37ΔTyrH	2.4 mM D-Tyr	11 ± 6	36 ± 5	56 ± 3.5
K37	2.4 mM D-Tyr	12 ± 4	0.2 ± 4	88 ± 4
K37ΔTyrHΔrecA(pBRtyrT)	2.4 mM D-Tyr	30 ± 4	29 ± 3	60 ± 6
K37ΔTyrHΔrecA(pBR322)	2.4 mM D-Tyr	7 ± 1	44 ± 1	45 ± 3
K37ΔTyrHΔrecA(pBRtyrT)	no D-Tyr	24 ± 3	3 ± 2	88 ± 4

[a] Cells were grown at 37 °C in M9-glucose minimal medium supplemented or not with 2.4 mM D-tyrosine. Then, tRNA was isolated from the cultures and used to estimate the quantities of total tRNA^{Tyr}, L-Tyr-tRNA^{Tyr}, and D-Tyr-tRNA^{Tyr}, as described under "Materials and Methods." Measurements were performed at least twice.
[b] Total cellular concentration of tRNA^{Tyr} was calculated with an OD_{650} of 1.0 corresponding to 0.4 μl of intracellular volume (18).
[c] Indicated in the table are the calculated quantities of D- or L-Tyr-tRNA^{Tyr} as percentages of total tRNA^{Tyr}.

TABLE III
Effect of D-tyrosine on the growth of various E. coli strains

Strain	Generation time[a]	
	Control	D-Tyrosine
	min	
K37ΔrecA(pBR322)	69	71
K37ΔTyrHΔrecA(pBR322)	70	95
K37ΔrecA(pBRtyrT)	67	69
K37ΔTyrHΔrecA(pBRtyrT)	67	78
K37ΔTyrHΔrecA(pBRaspT)	69	96

[a] Cells were grown at 37 °C in M9-glucose minimal medium containing or not 2.4 mM D-tyrosine. The medium also contained 100 μg of ampicillin/ml. Cultures were inoculated with cells pregrown to mid-exponential phase (0.2–0.3 OD_{650}) in the medium under study. Shown values are within ± 5%.

D-tyrosine (Table II). These results indicate that D-tyrosylated tRNA^{Tyr} can be formed *in vivo* and that D-Tyr-tRNA^{Tyr} deacylase is involved in the deacylation of this misaminoacylated tRNA species. About 40% of the cellular tRNA^{Tyr} was esterified with D-tyrosine in the Δ*dtd* context.

Measurement of Peptidyl-tRNA^{Tyr}—We also considered the possible formation of D-peptidyl-tRNA^{Tyr}, *i.e.* tRNA^{Tyr} species with a peptide esterified to the terminal adenosine via D-tyrosine. During protein biosynthesis, peptidyl-tRNAs dissociate sometimes prematurely from ribosomes. Such peptidyl-tRNAs are recycled by peptidyl-tRNA hydrolase, a ubiquitous enzyme found in bacterial, archaeal, and eukaryotic cells (14, 15, 19–21). This enzyme hydrolyzes the ester linkage between a peptide and the terminal adenosine of tRNA. If D-aminoacyl-tRNAs participate in protein synthesis, D-peptidyl-tRNA should be released from the ribosome. Moreover, accumulation of such molecules could be favored by the fact that D-peptidyl-tRNAs are poorer substrates of peptidyl-tRNA hydrolase than L-peptidyl-tRNAs (8, 22).

To investigate the presence of D-peptidyl-tRNA^{Tyr}, an aliquot of the tRNA preparation was incubated in the presence of $CuSO_4$ to hydrolyze any aminoacyl-tRNAs while preserving all peptidyl-tRNAs. Another aliquot was fully deacylated by an incubation in the presence of 2 M Tris. Finally, the L-tyrosine acceptances of the two samples were compared. Whatever the strain (Δ*dtd* or *dtd*$^+$) or the presence or absence of 2.4 mM D-tyrosine in the growth medium, the same levels of tyrosylation were obtained within ±5% uncertainty. Therefore, we concluded that neither L- nor D-peptidyl-tRNA^{Tyr} species were present in significant amounts. This conclusion is in agreement with a previous measurement showing that L-peptidyl-tRNA concentrations remain low in exponentially growing *E. coli* cells (14).

Overproduction of tRNA^{Tyr} Reduces the Toxicity of D-Tyrosine—To confirm the idea that accumulation of D-aminoacyl-tRNA at the expense of metabolically active L-aminoacyl-tRNA may be involved in the origin of the toxicity of D-amino acids, we studied the effect of tRNA^{Tyr} overproduction on the toxicity of D-tyrosine. Indeed, overproduction of this tRNA is expected to cause an increase in the L-Tyr-tRNA^{Tyr} concentration available for translation.

TABLE IV
Toxicity indices of D-aspartate for the growth of various E. coli strains

Cells were pregrown to mid-exponential phase (0.2–0.3 OD_{650}) in M9-glucose minimal medium containing 100 μg of ampicillin/ml without D-amino acid. Then, they were inoculated at an optical density of 0.005 at 650 nm in liquid M9-glucose minimal medium supplemented with 100 μg of ampicillin/ml and containing or not different D-aspartate concentrations. After an 8-h incubation at 37 °C, the optical densities of the cultures were measured at 650 nm. Under such conditions, the optical densities of the control cultures without added aspartate reached values between 0.4 and 0.5. D-aspartate concentrations were varied as follows: 0.8, 1.6, 3.2, 6.4, 12.8, and 25.6 mM. Indicated in the table are the D-aspartate concentrations, designated as toxicity indices, causing a 2-fold reduction in the optical density at the end of the incubation time, as compared with the optical density of a control culture without aspartate. These indices were calculated by interpolation between the optical density values measured for different aspartate concentrations. Shown values are within ±10%.

Strain	Toxicity index of D-aspartate
	mM
K37ΔrecA(pBR322)	>25
K37ΔrecA(pBRaspT)	>25
K37ΔTyrHΔrecA(pBR322)	4 ± 1
K37ΔTyrHΔrecA(pBRaspT)	13 ± 1
K37ΔTyrHΔrecA(pBRtrpT)	3 ± 1
K37ΔTyrHΔrecA(pBRtyrT)	6 ± 1

A plasmid overexpressing tRNA^{Tyr} (pBRtyrT) was constructed and introduced into *E. coli* strains K37ΔrecA (*dtd*$^+$) and K37ΔTyrHΔrecA (Δ*dtd*). The presence of this plasmid increased by a factor of ~3 the total cellular amount of tRNA^{Tyr}, as compared with the tRNA^{Tyr} amount in the same strains transformed with the control plasmid pBR322. The presence of the plasmid pBRtyrT significantly diminished the toxicity of D-tyrosine for the Δ*dtd* strain. Thus, the generation time of strain K37ΔTyrHΔrecA(pBRtyrT) was equal to 78 min when bacteria were grown in M9-glucose minimal medium containing 2.4 mM D-tyrosine. Under the same growth conditions, the generation time of strain K37ΔTyrHΔrecA(pBR322) was equal to 95 min (Table III). In the absence of D-tyrosine, the generation times of these two strains were similar (67 and 70 min, respectively). They were also similar to the generation times of the *dtd*$^+$ strains K37ΔrecA(pBRtyrT) and K37ΔrecA(pBR322) grown in the presence or in the absence of D-tyrosine (Table III).

tRNA^{Asp} or tRNA^{Trp} Overproduction Protects against the Toxicity of D-Aspartate or D-Tryptophan, Respectively—D-Tyr-

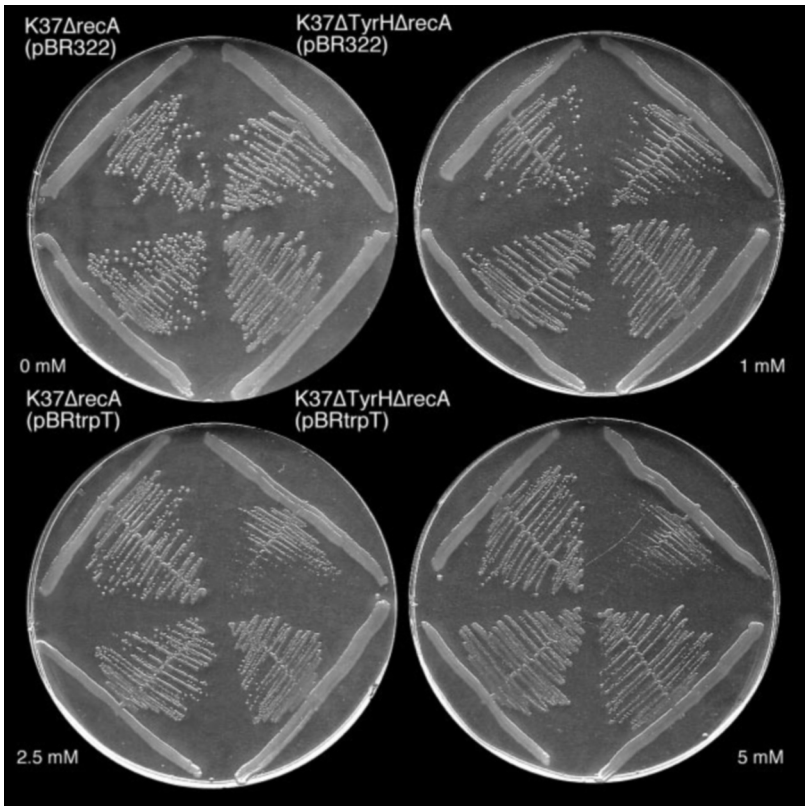

FIG. 1. **Effect of D-tryptophan on the growth of *E. coli* strains K37ΔrecA (*dtd*$^+$) and K37ΔTyrHΔrecA (Δ*dtd*) carrying plasmid pBRtrpT or control plasmid pBR322.** Cells were left to grow for 42 h at 37 °C on M9-glucose minimal medium agar plates supplemented with the indicated concentration of D-tryptophan (0, 1, 2.5, or 5 mM).

tRNA^{Tyr} deacylase protects *E. coli* against D-tyrosine but also against a few other D-amino acids including D-Asp and D-Trp (6). Therefore, we determined whether, in the case of these two D-amino acids also, an overproduction of tRNA affected the behavior of the Δ*dtd* strain. For this purpose, plasmids overproducing tRNA^{Asp} (pBRaspT) or tRNA^{Trp} (pBRtrpT) were introduced in the strains K37ΔTyrHΔrecA and K37ΔTyrH. The level of tRNA^{Asp} in the strains transformed by pBRaspT was ~2.7-fold higher than in control strains transformed with the plasmid pBR322, whereas the plasmid pBRtrpT increased ~3.7-fold the level of tRNA^{Trp}.

The presence of the plasmid pBRaspT conferred to the Δ*dtd* strain (K37ΔTyrHΔrecA) a significant protection against the toxic effect of D-aspartate. Indeed, the D-asparate toxicity index (10) was found to be three times greater in the presence of the pBRaspT plasmid than in the presence of the control plasmid (13 mM *versus* 4 mM). In the *dtd*$^+$ context, the toxicity index of D-aspartate was greater than 25 mM, whatever the plasmid harbored (pBRaspT or pBR322) (Table IV).

To estimate the toxicity of D-tryptophan, *dtd*$^+$ or Δ*dtd* strains overproducing or not tRNA^{Trp} were grown on solid M9-glucose minimal medium containing different concentrations of D-tryptophan. As shown in Fig. 1, the presence of the plasmid pBRtrpT significantly reduced the toxic effect of D-tryptophan on the Δ*dtd* strain K37ΔTyrHΔrecA. At 5 mM D-tryptophan, the growth of cells transformed by the control plasmid pBR322 was clearly more affected than that of cells harboring the plasmid pBRtrpT. The same behavior was observed in liquid M9-glucose minimal medium containing 5 mM D-tryptophan (Table V). Introduction of the plasmid pBRtrpT inside the Δ*dtd* strain significantly improved the growth rate in the presence of the D-amino acid.

The above results show that overproduction of a given tRNA species helps D-Tyr-tRNA^{Tyr} deacylase-deficient bacteria overcome the toxic effect of the corresponding D-amino acid. Nevertheless, it should be noted that each of the Δ*dtd* strains overproducing one given tRNA species remained slightly more sensitive to the corresponding D-amino acid than the parental *dtd*$^+$ strain.

TABLE V
Effect of D-tryptophan on the growth of various E. coli strains

Strain	Generation time[a]	
	Control	D-Tryptophan
	min	
K37ΔrecA(pBR322)	71	95
K37ΔTyrHΔrecA(pBR322)	70	130
K37ΔrecA(pBRtrpT)	73	110
K37ΔTyrHΔrecA(pBRtrpT)	72	113

[a] Cells were grown at 37 °C in M9-glucose minimal medium containing or not 5 mM D-tryptophan. The medium also contained 100 μg of ampicillin/ml. Cultures were inoculated with cells pregrown to mid-exponential phase (0.2–0.3 OD_{650}) in the medium under study. Shown values are within ± 5%.

Next, we determined whether the protective effect of a given tRNA was specific of a given amino acid. In liquid medium, the plasmid pBRaspT did not change the generation time of the Δ*dtd* strain in the presence of D-tyrosine (Table III). Similarly, plasmids overproducing tRNA^{Tyr} or tRNA^{Trp} did not enhance the resistance to D-aspartate (Table IV). Finally, plasmids overproducing tRNA^{Tyr} or tRNA^{Asp} were shown on solid medium not to interfere with the resistance of the Δ*dtd* strain to D-tryptophan (data not shown).

Overproduction of tRNA^{Tyr} Increases the Cellular Concentrations of D- and L-Tyr-tRNA^{Tyr}—As shown above, overproduction of tRNA^{Tyr} increases the resistance to D-tyrosine. To understand more precisely the cause of this enhanced resistance, we measured the amounts of D- and L-Tyr-tRNA^{Tyr} under overproduction conditions. When 2.4 mM D-tyrosine was added to growth medium, the relative concentrations of L- and D-Tyr-tRNA^{Tyr} in the extract of the strain K37ΔTyrHΔrecA(pBRtyrT) were equal to 60 ± 6% and 29 ± 3% of total tRNA^{Tyr}, respectively (Table II). These proportions are similar to those measured in the non-overproducing Δ*dtd* strains K37ΔTyrH and K37ΔTyrHΔrecA(pBR322) (Ta-

ble II). Since $tRNA^{Tyr}$ concentration has been increased by a factor of ~3 in the strain carrying the pBRTyrT plasmid, we can deduce that, upon overproduction of the tRNA, the intracellular concentration of L-Tyr-$tRNA^{Tyr}$ has become 2–3 times higher than that in the wild-type strain grown in the absence of D-tyrosine. Such an increase in the L-Tyr-$tRNA^{Tyr}$ concentration available for translation explains how $tRNA^{Tyr}$ overproduction can relieve the toxicity of D-tyrosine in a Δ*dtd* context.

DISCUSSION

Our results establish that D-tyrosyl-tRNAs can be formed *in vivo*. Thus, in a deacylase-deficient strain (Δ*dtd*), more than one-third of the total $tRNA^{Tyr}$ pool can become aminoacylated with the D-isomer of tyrosine when bacteria are grown in the presence of 2.4 mM D-tyrosine. In strains expressing an active D-Tyr-$tRNA^{Tyr}$ deacylase, accumulation of D-Tyr-$tRNA^{Tyr}$ is no longer detected, probably because these molecules are rapidly recycled by the deacylase.

The catalytic efficiency of *E. coli* tyrosyl-tRNA synthetase with the D-enantiomer of tyrosine is 15-fold lower than with the L-enantiomer (7). Therefore, enzymatic production of D-Tyr-$tRNA^{Tyr}$ must be small if compared with that of L-Tyr-$tRNA^{Tyr}$. The observation that one-third of total $tRNA^{Tyr}$ becomes aminoacylated with D-tyrosine in a Δ*dtd* context suggests that, being metabolically inactive, D-tyrosyl-tRNA molecules accumulate in the cell.

As shown above, overproduction of $tRNA^{Tyr}$, $tRNA^{Trp}$, or $tRNA^{Asp}$ significantly diminishes the toxicity of D-tyrosine, D-tryptophan, or D-aspartate, respectively. This result is in agreement with the idea that accumulation of D-aminoacyl-tRNA reduces the concentration of L-aminoacyl-tRNAs available for translation. Upon ~3-fold tRNA overproduction, D-Tyr and L-Tyr-$tRNA^{Tyr}$ concentrations are each increased by a factor of 2–3. We assume that the increase in the concentration of the only L-Tyr-$tRNA^{Tyr}$ species is enough to reverse the inhibition of protein synthesis.

The protective effect against D-tyrosine resulting from tRNA overproduction is not 100%, however. A deacylase-deficient strain overproducing one given tRNA species still exhibits more sensitivity to the corresponding D-amino acid than the wild-type strain. Therefore, although the L-Tyr-$tRNA^{Tyr}$ concentration has become 2–3 times higher than that found in the wild-type strain, the presence of D-Tyr-$tRNA^{Tyr}$ continues to slow down the growth of a deacylase-deficient strain. Such a residual toxicity of D-tyrosine can originate from at least two non-exclusive mechanisms. Firstly, D-aminoacyl-tRNA might be a ligand of the elongation factor Tu (EF-Tu). Indeed, weak binding of D-Tyr-$tRNA^{Tyr}$ to the EF-Tu·GTP complex has been reported (23). Competition between EF-Tu-complexed D-aminoacyl- and L-aminoacyl-tRNAs may then occur for the binding to the cognate codon in the A site. Moreover, non-enzymatic binding of D-aminoacyl-tRNA (without the help of EF-Tu) might hinder the A site (24, 25). The second mechanism that possibly accounts for the residual toxicity of a D-amino acid deals with the formation of non-functional proteins through full participation of D-aminoacyl-tRNAs to translation. Possible incorporation of D-amino acids into proteins has already been questioned. Stereospecificity of the translational machinery appears to favor L-amino acids (23). Thus, the affinity of L-Tyr-$tRNA^{Tyr}$ for the EF-Tu·GTP complex is 25-fold greater than that of D-Tyr-$tRNA^{Tyr}$. Similarly, the formation of the ribosome·aminoacyl-tRNA complex from the ternary complex EF-Tu·GTP·aminoacyl-tRNA occurs about 10-fold more efficiently when the amino acid is in L-conformation. Finally, a factor of 5 in favor of the L-amino acid arises from the more frequent detachment of D-aminoacyl-tRNA from the ribosome than that of L-aminoacyl-tRNA, before the reaction of transpeptidation. The combined effects of these three steps would provide a total discrimination factor of ~1200 in favor of L-tyrosine. Such a factor suggests that D-tyrosine is excluded from incorporation into peptides under conditions in which L-tyrosine is also present. In agreement with this view, several attempts to incorporate D-amino acids into proteins using chemically misacylated tRNAs in cell-free protein synthesizing systems have been unsuccessful (26–28).

However, Calendar and Berg (7) have reported that D-tyrosine from D-tyrosyl-tRNA was incorporated into peptide linkage in an *in vitro* prokaryotic protein-synthesizing system. The D-tyrosine incorporation was about 6-fold smaller than that of L-tyrosine incorporation from L-tyrosyl-tRNA. Labeled tyrosine incorporated from D-tyrosyl-tRNA was converted to the keto-acid with D-amino acid oxidase but not with L-amino acid oxidase, suggesting that incorporation of D-tyrosine into a polypeptide product occurred without inversion of the amino acid configuration. Champney and Jensen (29) have reported an *in vivo* incorporation of D-tyrosine into the cellular proteins of a *B. subtilis* strain that efficiently imports this D-amino acid. The radioactivity found in ribosomes and soluble proteins suggested the substitution of about 1% of the L-tyrosine molecules by the D-isomer. In favor of the incorporation of D-tyrosine without pretranslational change in the configuration of the amino acid, no labeled *p*-hydroxyphenylpyruvate was detected when acid hydrolysates of labeled proteins were treated with L-amino acid oxidase. However, treatment with D-amino acid oxidase was also ineffective in liberating *p*-hydroxyphenylpyruvate. An inhibition of D-amino acid oxidase by aromatic compounds in the hydrolysate was proposed to be at the origin of this contradictory observation. Finally, very recently, efficient incorporation of D-phenylalanine and D-methionine could be obtained *in vitro* provided that mutant ribosomes were used (30). With wild-type ribosomes, the incorporation was lower but still detectable.

In the present study, accumulation in the cell of D-peptidyl-tRNA could not be evidenced. At first glance, the lack of detection of this tRNA species argues against that idea that D-tyrosine could be incorporated into proteins. However, before we can reach a conclusion, the slight activity of peptidyl-tRNA hydrolase against D-peptidyl-tRNA molecules has to be taken into consideration. Indeed, the *in vitro* activity of the hydrolase toward *N*-blocked-D-Tyr-$tRNA^{Tyr}$ is only 150-fold smaller than that measured with *N*-blocked-L-Tyr-$tRNA^{Tyr}$ (8). Therefore, one cannot exclude that, if D-peptidyl-tRNAs were eventually produced *in vivo*, these molecules would be destroyed by the hydrolase. Clearly, more studies are necessary to unambiguously decide whether some D-amino acid misincorporation into proteins can occur *in vivo*.

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