Formation of d-Tyrosyl-tRNA\textsubscript{Tyr} Accounts for the Toxicity of d-Tyrosine toward \textit{Escherichia coli}*

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D-Tyr-tRNA\textsubscript{Tyr} deacylase cleaves the ester bond between a tRNA molecule and a d-amino acid. In \textit{Escherichia coli}, inactivation of the gene (\textit{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 \textit{Δdtd} cell in vivo, the presence of 2.4 mM d-tyrosine, \textit{Δdtd} cells in which no d-Tyr-tRNA\textsubscript{Tyr} is observed in \textit{dtd} cells. In addition, we observe that overproduction of tRNA\textsubscript{Tyr}, tRNA\textsubscript{Asp}, or tRNA\textsubscript{Asp} prevents a \textit{Δdtd} 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\textsubscript{Tyr} proportional to that of overproduced tRNA\textsubscript{Tyr}. Overproduction of tRNA\textsubscript{Tyr} also relieves the starvation by increasing the amount of cellular L-Tyr-tRNA\textsubscript{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 \textit{Escherichia coli} and \textit{Bacillus subtilis} tyrosyl-tRNA synthetases catalyze the formation of d-Tyr-tRNA\textsubscript{Tyr} in \textit{vivo} (3, 4). More recently, \textit{Saccharomyces cerevisiae} tyrosyl-tRNA synthetase (5), as well as \textit{E. coli} tryptophanyl- and aspartyl-tRNA synthetases (6), were also shown to catalyze the transfer of the d-amino acid to the corresponding tRNA species in \textit{vivo}. In all these cases, the catalytic efficiency with the d-enantiomer is measured only 15–2000-fold lower than with the l-enantiomer.

Soon after the discovery of the tRNATyr synthesis by tyrosyl-tRNA synthetases, it was shown that extracts of \textit{E. coli} are able to hydrolyze d-Tyr-tRNA\textsubscript{Tyr} and free tRNA (7). This deacylase can also hydrolyze d-Trp-tRNA\textsubscript{Trp} and d-Asp-tRNA\textsubscript{Asp} (6) but not l-aminocarboxyl-tRNAs (6, 7). D-Tyr-tRNA\textsubscript{Tyr} deacylase is encoded by the \textit{dtd} gene in \textit{E. coli} (8) and by the \textit{DTD1} gene in \textit{S. cerevisiae} (5). Homologs of \textit{dtd/DTD1} genes occur in many bacterial and eukaryotic cells. Upon inactivation of \textit{E. coli} \textit{dtd} or \textit{S. cerevisiae DTD1} genes, 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 \textit{E. coli} and d-Tyr and d-Leu in \textit{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-aminocarboxyl-tRNAs at the expense of the corresponding L-aminocarboxyl-tRNAs and that d-Tyr-tRNA\textsubscript{Tyr} deacylase prevents this toxicity by recycling d-aminocarboxyl-tRNAs into free tRNA molecules.

To establish the involvement of tRNA in the toxicity of d-amino acids in \textit{vivo}, we analyzed the nature of the amino acid linked to tRNA\textsubscript{Tyr} when bacteria were grown in the presence of d-tyrosine. In a strain lacking d-Tyr-tRNA\textsubscript{Tyr} deacylase, about 40% of the cellular tRNA\textsubscript{Tyr} was found esterified with d-tyrosine. In the parental \textit{dtd} strain, no d-Tyr-tRNA\textsubscript{Tyr} could be detected. These results indicate that d-Tyr-tRNA\textsubscript{Tyr} formation can starve the cell for L-Tyr-tRNA\textsubscript{Tyr} and that the deacylase prevents the starvation by hydrolyzing d-Tyr-tRNA\textsubscript{Tyr}. We also studied the effect of tRNA overproduction on the toxicity of d-Tyr, d-Trp, or d-Asp for \textit{Δdtd} \textit{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\textsubscript{Tyr} overproduction, the concentration of L-Tyr-tRNA\textsubscript{Tyr} increases proportionally and, in a \textit{Δdtd} cell, such an increase in the L-Tyr-tRNA\textsubscript{Tyr} concentration accounts for the relief of the d-tyrosine toxicity.

**MATERIALS AND METHODS**

\textit{d-methylen}e\textsubscript{3}H\textsubscript{2}Tyrosine (211 GBq/mmol) was custom-prepared by Amersham Biosciences. \textit{L-[1\textsuperscript{3}C]}Tyrosine (18.3 GBq/mmol), \textit{L-[5\textsuperscript{3}H]}tryptophan (740 GBq/mmol), \textit{L-[2\textsuperscript{3}H]}H\textsubscript{2}aspartic acid (866 GBq/mmol), and \textit{L-[2\textsuperscript{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.

\textit{E. coli} tRNA\textsubscript{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 K37\textsubscript{ΔrecA} was obtained from strain K37 by P1 transduction of the rec\textsubscript{A}::cat allele of strain JW5532 (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 \textit{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\textsubscript{650} of 0.0007–0.001 in medium containing or not d-amino acid. When the OD\textsubscript{650} of the culture reached 0.2–0.3, bacteria were diluted again into the medium under study to obtain a final OD\textsubscript{650} of 0.005. These cultures were used to measure the generation times.

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The toxicity indices of d-asp-tRNA 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 OD600) at 37 °C in M9-glucose minimal medium. Cells were then inoculated in liquid M9-glucose medium containing different concentrations of d-asp-tRNA to give an OD600 of 0.065. The toxicity index is defined as the d-asp-tRNA concentration causing a 50% 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-asp-tRNA.

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\textsuperscript{\textalpha}y—tRNA\textsuperscript{\textalpha}y, and tRNA\textsuperscript{\textalpha}y—Construction of pBSTNAV derivatives harboring tyrT\textsuperscript{\textalpha}—Construction of pBSTNAV derivatives harboring tyrT (8), tyrT, or aspT (6) tRNA genes was described previously. Possibly because of a high overproduction of tRNA, introduction of these plasmids into strain K37/H9004 or K37/H9262 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 Sall sites of pBR322. These fragments carried both the tRNA gene and the P\textalpha\textbeta promoter of pBR322. The resulting plasmids (pBRtyrT, pBRtrypT, and pBRaspT) were used to transform E. coli strains K37/H9004 and K37/H9262. Growth of the transformed cells on minimal medium was homogeneous and reproducible.

Amino Acid Acceptance of Crude tRNA Extracts—Strains harboring the pBRtyrT, pBRtrypT, or pBRaspT plasmids were grown overnight in 40 ml of 2x 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), t-tyrosine, t-tryptophan, and t-asp-tRNA acceptances were measured as described earlier (6). The reaction mixtures contained 20 μM Tris-HCl (pH 7.8), 2 mM ATP, 7 mM MgCl\textalpha, 0.1 mM EDTA, 50 μg/ml bovine serum albumin, 2.5 mM 2-mercaptoethanol, 7 μM \textalpha\textbeta-tyrolysine (500 Ci/mM), 1 A\textalpha\textbeta\textepsilon unit of tRNA, and 0.03 μM tyrosyl-tRNA synthetase. From these measurements, the amount of non-esterified tRNA\textsuperscript{\textalpha}y was deduced from the tyrosine acceptance of the third aliquot. The difference between the acceptances of the second and third aliquots reflected the amount of d-tryosyl-tRNA\textsuperscript{\textalpha}y. If t-tyrosyl-tRNA\textsuperscript{\textalpha}y, the difference between the fourth and second aliquots corresponded to t-tryosyl-tRNA\textsuperscript{\textalpha}y, and the difference between the first and fourth aliquots corresponded to peptide-tRNA\textsuperscript{\textalpha}y. Intracellular concentration of tRNA\textsuperscript{\textalpha}y was calculated using the tyrosine acceptance of the sample treated with 2 μM Tris-HCl and the OD\textalpha\textbeta 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\textalpha\textbeta corresponded to 0.4 μl of intracellular volume (18).

RESULTS

Measurement of d-Tyr-tRNA\textsuperscript{\textalpha}y—Exogeneous d-tyrosine slows down the growth rate of E. coli mutants lacking d-Tyr-tRNA\textsuperscript{\textalpha}y decylase (8). One likely reason for the toxicity of d-tyrosine is the immobilization as d-Tyr-tRNA\textsuperscript{\textalpha}y of part of the tRNA\textsuperscript{\textalpha}y pool. As a consequence, the growth rate would be decreased because of a starviation in t-Tyr-tRNA\textsuperscript{\textalpha}y. 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\textsuperscript{\textalpha}y was analyzed. Part of the sample was treated with d-Tyr-tRNA\textsuperscript{\textalpha}y decylase, whereas another part was incubated in the same conditions without decylase. Then, the t-tyrosine acceptances of the two samples were compared. If the aminoacyl-tRNA preparation contained d-Tyr-tRNA\textsuperscript{\textalpha}y, the t-tyrosine acceptance of the decylase-treated sample should be higher than that of the non-treated sample. The decylase treatment did not change the t-tyrosine acceptance of the tRNA samples prepared from the ddl\textsuperscript{\textalpha} strain grown in the presence or absence of d-tyrosine or from theddl strain grown in the absence of d-tyrosine. On the other hand, the incubation with decylase increased about 4-fold the tyrosine acceptance of the sample from theddl strain grown in the presence of 2.4 mM

\textsuperscript{1} The abbreviations used are: MES, 4-morpholinêthanesulfonic acid; EF, elongation factor.

| Table 1 | Strains and plasmids used in this study |
|---|---|
| **Strains** | Description | Reference |
| K37 | gaiK rpsL | Ref. 31 |
| K37/TyrH | K37Δtdr::kan | Ref. 8 |
| K37/TdrAec | K37Δtdr::Aec938:cot | This work |
| K37/TyrH/ArecA | K37Δtdr::kan Aec938::cot | Ref. 8 |
| **Plasmids** | | |
| pBR322 | Ap\textbeta Ty\textalpha derivative of tRNA\textalpha | This work |
| pBRtryT | Ap\textbeta tyrT derivative of tRNA\textalpha | This work |
| pBRaspT | Ap\textbeta aspT derivative of tRNA\textalpha | This work |


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*The abbreviations used are: MES, 4-morpholinêthanesulfonic acid; EF, elongation factor.*

In 20 mM MES-KOH (pH 6.0) containing 5 mM MgCl\textalpha, and pure E. coli v-Tyr-tRNA\textsuperscript{\textalpha}y decylase was added at a final concentration of 1 μl. We verified that this concentration of decylase was sufficient to fully deacylate n-[\textalpha\textbeta]H[Tyr-tRNA\textsuperscript{\textalpha}y] substrate at a concentration close to that of total tRNA\textsuperscript{\textalpha}y in the test (data not shown). The mixture was incubated at 28 °C for 5 min. t-aminocyl-tRNAs, which are resistant to the action of the decylase, 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). Acylation 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\textsuperscript{\textalpha}y decylase was omitted from the reaction mixture. (iv) Finally, the fourth aliquot was incubated in the presence of CuSO\textalpha 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\textalpha. 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\textalpha, and 75% ethanol and then with a solution containing 2 mM sodium acetate (pH 5), 1 mM MgSO\textalpha, 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 Chelox 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. \textalpha\textbeta-tyrolysine 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\textalpha, 0.1 mM EDTA, 50 μg/ml bovine serum albumin, 2.5 mM 2-mercaptoethanol, 7 μM \textalpha\textbeta-tyrolysine (500 Ci/mM), 1 A\textalpha\textbeta\textepsilon unit of tRNA, and 0.03 μM tyrosyl-tRNA synthetase.
tRNA species. About 40% of the cellular tRNATyr was esterified by an enzyme that hydrolyzes the ester linkage between a peptide and the terminal adenosine. This enzyme is involved in the deacylation of this misaminoacylated tRNA.

To investigate the presence of D-peptidyl-tRNATyr, an aliquot of such molecules could be favored by the fact that D-peptidyl-tRNAs should be released from the ribosome. Moreover, accumulation of such molecules could be favored by the fact that D-peptidyl-tRNAs participate in protein synthesis, D-peptidyl-tRNA TyrH, as compared with the tRNA Tyr amount in the same strain. Thus, the generation time of strain K37TyrH 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 times of these two strains were similar (67 and 78 min, respectively). They were also similar to the generation times of the dtd strains K37TyrHarecA(pBR322) and K37TyrHarecA(pBRaspt) grown in the presence or in the absence of D-tyrosine (Table III).

Overproduction of tRNATyr 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 tRNATyr 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.

A plasmid overexpressing tRNATyr (pBRtyrT) was constructed and introduced into E. coli strains K37TyrH and K37TyrHarecA (dtd^+). The presence of this plasmid increased the level of intracellular volume (18). The presence of this plasmid in E. coli strains K37TyrH and K37TyrHarecA (dtd^+). The presence of this plasmid increased the level of intracellular volume (18). The presence of this plasmid increased the level of intracellular volume (18).

To investigate the presence of D-peptidyl-tRNATyr^, 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 (dtd or dtd^+), the presence of D-tyrosine was detected within ±5% uncertainty. Therefore, we concluded that neither L- nor D-peptidyl-tRNATyr^ 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 tRNATyr^ 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 tRNATyr 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.

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tRNA\textsuperscript{Tyr} deacylase protects \textit{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 \textit{ddtd} strain. For this purpose, plasmids overproducing tRNA\textsuperscript{Asp} (pBRaspT) or tRNA\textsuperscript{Tyr} (pBRtrpT) were introduced in the strains \textit{K37}\textit{TyrH}\textit{ΔrecA} and \textit{K37}\textit{TyrH}. The level of tRNA\textsuperscript{Asp} in the strains transformed by pBRaspT was \textasciitilde 2.7-fold higher than in control strains transformed with the plasmid pBR322, whereas the plasmid pBRtrpT increased \textasciitilde 3.7-fold the level of tRNA\textsuperscript{Tyr}.

The presence of the plasmid pBRaspT conferred to the \textit{ddtd} strain (\textit{K37}\textit{TyrH}\textit{ΔrecA}) a significant protection against the toxic effect of d-aspartate. Indeed, the d-aspartate 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 \textit{versus} 4 mM). In the \textit{ddtd} context, the toxicity index of d-aspartate was greater than 25 µM, whatever the plasmid harbored (pBRaspT or pBR322) (Table IV).

To estimate the toxicity of d-tryptophan, \textit{ddtd} or \textit{ddtd} strains overproducing or not tRNA\textsuperscript{Tyr} 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 \textit{ddtd} strain \textit{K37}\textit{TyrH}\textit{ΔrecA}. 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 \textit{ddtd} strain (\textit{K37}\textit{TyrH}\textit{ΔrecA}(pBRtrpT)) 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\textsuperscript{Tyr} deacylase-deficient bacteria overcome the toxic effect of the corresponding d-amino acid. Nevertheless, it should be noted that each of the \textit{ddtd} strains overproducing one given tRNA species remained slightly more sensitive to the corresponding d-amino acid than the parental \textit{ddtd} strain.

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 \textit{ddtd} strain in the presence of d-tyrosine (Table III). Similarly, plasmids overproducing tRNA\textsuperscript{Tyr} or tRNA\textsuperscript{Asp} did not enhance the resistance to d-aspartate (Table IV). Finally, plasmids overproducing tRNA\textsuperscript{Tyr} or tRNA\textsuperscript{Asp} were shown on solid medium not to interfere with the resistance of the \textit{ddtd} strain to d-tryptophan (data not shown).

\textbf{Overproduction of tRNA\textsuperscript{Tyr} Increases the Cellular Concentrations of d- and L-Tyr-tRNA\textsuperscript{Tyr}}—As shown above, overproduction of tRNA\textsuperscript{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\textsuperscript{Tyr} under overproduction conditions. When 2.4 mM d-tyrosine was added to growth medium, the relative concentrations of L- and d-Tyr-tRNA\textsuperscript{Tyr} in the extract of the strain \textit{K37}\textit{TyrH}\textit{ΔrecA}(pBRtrpT) were equal to 60 \textpm 6% and 29 \textpm 3% of total tRNA\textsuperscript{Tyr}, respectively (Table II). These proportions are similar to those measured in the non-overproducing \textit{ddtd} strains \textit{K37}\textit{TyrH} and \textit{K37}\textit{TyrH}\textit{ΔrecA}(pBR322) (Ta-
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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 Δtdt context.

**DISCUSSION**

Our results establish that D-tyrosyl-tRNAs can be formed in vivo. Thus, in a deacylase-deficient strain (∆tdt), 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 deacetylase, accumulation of D-Tyr-tRNA^Tyr is no longer detected, probably because these molecules are rapidly recycled by the deacetylase.

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 Δtdt 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^Met 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-D-aminoacyl-tRNA complex from the ternary complex EF-Tu-GTP-aminocacyl-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 the 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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**REFERENCES**

1. Friedman, M. (1999) *J. Agric. Food Chem.* 47, 3457–3479
2. Yang, H., Zheng, G., Peng, X., Qiang, B., and Yuan, J. (2003) *FEBS Lett.* 552, 95–98
3. Calendar, R., and Berg, P. (1966) *Biochemistry* 5, 1681–1690
4. Calendar, R., and Berg, P. (1966) *Biochemistry* 5, 1690–1695
5. Soutourina, J., Blanquet, S., and Plateau, P. (2000) *J. Biol. Chem.* 275, 11626–11630
6. Soutourina, J., Blanquet, S., and Plateau, P. (2000) *J. Biol. Chem.* 275, 32535–32542
7. Calendar, R., and Berg, P. (1967) *J. Mol. Biol.* 26, 39–54
8. Soutourina, J., Plateau, P., Delort, F., Peirotes, A., and Blanquet, S. (1999) *J. Biol. Chem.* 274, 19109–19114
9. Marsh, L., and Walker, G. C. (1987) *J. Bacteriol.* 169, 1818–1823
10. Soutourina, J., Blanquet, S., and Plateau, P. (2001) *J. Biol. Chem.* 276, 40864–40872
11. Meinnel, T., Mechulam, Y., and Fayat, G. (1988) *Nucleic Acids Res.* 16, 8095–8096
12. Garlant, W. J., and Sueoka, N. (1966) *Proc. Natl. Acad. Sci. U. S. A.* 55,
13. Lindahl, T., Adams, A., and Fresco, J. R. (1966) Proc. Natl. Acad. Sci. U. S. A. 55, 941–948
14. Menninger, J. R. (1976) J. Biol. Chem. 251, 3392–3398
15. Schmitt, E., Mechulam, Y., Fromant, M., Plateau, P., and Blanquet, S. (1997) EMBO J. 16, 4760–4769
16. Schofield, P., and Zamecnik, P. C. (1968) Biochim. Biophys. Acta 155, 410–416
17. Guillon, J. M., Mechulam, Y., Schmitter, J. M., Blanquet, S., and Fayat, G. (1992) J. Bacteriol. 174, 4294–4301
18. Coste, H., Brevet, A., Plateau, P., and Blanquet, S. (1987) J. Biol. Chem. 262, 12096–12103
19. Cuzin, F., Kretchmer, N., Greenberg, R. E., Hurwitz, R., and Chapeville, F. (1987) Proc. Natl. Acad. Sci. U. S. A. 84, 2275–2277
20. Rosas-Sandoval, G., Ambrogelly, A., Rinehart, J., Wei, D., Cruz-Vera, L. R., Graham, D. R., Stetter, K. O., Guarneros, G., and Soll, D. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 16707–16712
21. Fromant, M., Ferri-Fioni, M. L., Plateau, P., and Blanquet, S. (2003) Nucleic Acids Res. 31, 3227–3235
22. Kissel, H., and RajBhandary, U. L. (1968) J. Mol. Biol. 35, 539–560
23. Yamane, T., Miller, D. L., and Hopfield, J. J. (1981) Biochemistry 20, 7059–7064
24. Kemkhadze, K. S., Odintsov, V. B., Semenkov, Y. P., and Kirillov, S. V. (1981) FEBS Lett. 125, 10–14
25. Rheinberger, H. J., and Nierhaus, K. H. (1986) J. Biol. Chem. 261, 9133–9139
26. Heckler, T. G., Roesser, J. R., Xu, C., Chang, P. I., and Hecht, S. M. (1988) Biochemistry 27, 7254–7262
27. Roesser, J. R., Xu, C., Payne, R. C., Surratt, C. K., and Hecht, S. M. (1989) Biochemistry 28, 5185–5195
28. Bain, J. D., Dalia, E. S., Glabe, C. G., Wacker, D. A., Lyttle, M. H., Dix, T. A., and Chamberlin, A. R. (1991) Biochemistry 30, 5411–5421
29. Champney, W. S., and Jensen, R. A. (1970) J. Bacteriol. 104, 170–176
30. Dedkova, L. M., Fahmi, N. E., Golovin, S. Y., and Hecht, S. M. (2003) J. Am. Chem. Soc. 125, 6616–6617
31. Miller, H. I., and Friedman, D. I. (1980) Cell 20, 711–719
32. Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heyneker, H. L., and Boyer, H. W. (1977) Gene (Amst.) 2, 95–113

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