Co-Expression of Yeast Amber Suppressor tRNA\textsuperscript{\text{Tyr}} and Tyrosyl-tRNA Synthetase in \textit{Escherichia coli}: Possibility to Expand the Genetic Code\textsuperscript{1}

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An efficient system was developed for the co-expression of a yeast tRNA\textsuperscript{\text{Tyr}}/tyrosyl-tRNA synthetase (TyrRS) pair in \textit{Escherichia coli}. Analysis of suppression patterns using several sets of \textit{E. coli} and \textit{\lambda} phage mutants indicated that the expressed yeast suppressor tRNA\textsuperscript{\text{Tyr}} was aminoacylated only with tyrosine by its cognate yeast TyrRS and not by \textit{E. coli} TyrRS or other aminoacyl-tRNA synthetases. This extra tRNA/TyrRS pair is expected to be a key bridgehead for developing an \textit{in vivo} system for the site-directed incorporation of unnatural amino acids into proteins.

Key words: amber mutation, co-expression, non-canonical amino acid, suppressor tRNA, tyrosyl-tRNA synthetase.

Recently, it became possible to incorporate non-canonical (i.e., not specified in the genetic code) amino acids into proteins site-specifically in a cell-free translation system (1). This method involves chemically aminoacylated suppressor tRNAs, which incorporate the non-canonical amino acid into the programmed “stop” codons. Although a large number of novel amino acids have already been successfully introduced into “alloproteins” (2–5), the usefulness of this system seems to be rather limited because it requires many steps of laborious biochemical treatment and the final yields of the alloproteins are typically not enough for sample-intensive methods such as NMR spectroscopy. Therefore, the development of an \textit{in vivo} approach for the site-specific incorporation of non-canonical amino acids into proteins could potentially enhance the value of this technique. Such a system would require at least two translational apparatus components: (i) a suppressor tRNA that is charged with the novel amino acid but is not a substrate for any other endogenous aminoacyl-tRNA synthetases, and (ii) an aminoacyl-tRNA synthetase with substrate specificity for the suppressor tRNA and for the novel amino acid. Very recently, several groups described a strategy for the in vivo approach for the site-specific incorporation of non-canonical amino acids into proteins could potentially enhance the value of this technique. Such a system would require at least two translational apparatus components: (i) a suppressor tRNA that is charged with the novel amino acid but is not a substrate for any other endogenous aminoacyl-tRNA synthetases, and (ii) an aminoacyl-tRNA synthetase with substrate specificity for the suppressor tRNA and for the novel amino acid. Very recently, several groups described a strategy for the results of trials to establish such suppressor tRNA/aminoacyl-tRNA synthetase pairs that are functional in vitro or in vivo (6–8).

We considered that a yeast tRNA\textsuperscript{\text{Tyr}}/TyrRS pair, when introduced into \textit{Escherichia coli} cells, would be an ideal candidate for this purpose for the following reasons. (i) Yeast tRNA\textsuperscript{\text{Tyr}} has a unique identity element, i.e., a C1-G72 base pair at the end of the acceptor stem, which is not found in \textit{E. coli} tRNAs except for tRNAPrO (9). (ii) The size of the variable arm in yeast tRNA\textsuperscript{\text{Tyr}} is completely different from that in \textit{E. coli} tRNA\textsuperscript{\text{Tyr}}, and therefore \textit{E. coli} TyrRS will not aminoacylate yeast tRNA\textsuperscript{\text{Tyr}}. (iii) Yeast tRNA\textsuperscript{\text{Tyr}} can be converted to amber or ochre suppressor tRNAs that are functional in vitro or in vivo (10, 11). (iv) We have already obtained, by means of genetic engineering, several yeast TyrRS mutants whose amino acid specificity has been significantly changed, at least in vitro (Ohno et al., manuscript in preparation). As the first step to construct an efficient \textit{in vivo} system for synthesizing proteins containing non-canonical amino acids at desired sites, we report here the co-expression of yeast amber suppressor tRNA\textsuperscript{\text{Tyr}} and wild-type TyrRS in \textit{E. coli}.

Series of plasmids for the expression of yeast tRNAs and/or yeast TyrRS in \textit{E. coli} were derived from pMW118 (Nippon Gene). Their expression is under the control of the \textit{lac} promoter. pMWYTyrs contains a tRNA gene sequence derived from \textit{Saccharomyces cerevisiae} tRNA\textsuperscript{\text{Tyr}} between XbaI and SphI sites (Fig. 1c). For co-expression of yeast tRNA\textsuperscript{\text{Tyr}} and TyrRS, pMWYTYR and pMWSupYRS were constructed in such a way that the
tRNA gene (wild type or amber suppressor), ribosome binding site and TyrRS gene were inserted in this order between XbaI and SphI sites (Fig. 1d).

E. coli CA274 (HfrC lac<sub>am</sub>125 trp<sub>am</sub>) has an amber mutation in the β-galactosidase gene. It is known that the β-galactosidase activity is restored when this amber codon is suppressed by any of the following amino acids so far studied, i.e., Ser, Gln, Tyr, Lys, Leu, or Trp (12). Figure 2 shows the suppression patterns when the series of plasmids were transformed into E. coli CA274 on a Luria-Bertani plate containing 50 μg/ml ampicillin, 0.5 mM IPTG and 0.004% (w/v) X-Gal (LB-amp-X-Gal plate). As all the transformants grew on the plate, the introduction of plasmids itself has little or no effect on cell growth. Blue colonies were observed only when pMWSupYRS was introduced into E. coli CA274. This result clearly indicates that the transformant with pMWSupYRS co-expresses the functional suppressor tRNA and cognate TyrRS. On the other hand, the finding that the transformant with pMWSup grew as a white colony indicates that the suppressor tRNA alone does not function efficiently in E. coli. This is in conflict with the results of Rossi et al. (11), who showed that yeast ochre suppressor tRNA alone suppresses amber and ochre mutations in E. coli. Since the only difference between their ochre and our amber suppressor tRNAs is the first letter of the anticodon, this may be explained by assuming that some E. coli aminoacyl-tRNA synthetase(s) that strongly recognize uridine at position 34 in tRNA might have allowed their ochre suppressor tRNA to function in vivo.

To determine the extent of suppression quantitatively, the β-galactosidase activity of each transformant or the host cells, E. coli CA274, was measured spectrophotometrically using a Beta-Galactosidase Enzyme Assay System (Promega) (Table 1). All transformants except for that with pMWSupYRS gave very low levels of β-galactosidase expression, almost background levels similar to that of the host cells. Only the activity of the transformant with pMWSupYRS was clearly detected; it was at least 144-fold higher than the background level. This result also indicates that only the co-expressed amber suppressor tRNA and cognate TyrRS pair could suppress the amber mutation efficiently.

To determine whether or not an amber codon is suppressed by the insertion of tyrosine, several amber mutants that show amino acid dependence were used as markers. The
TABLE I. Functional testing of yeast tRNA and/or TyrRS by means of β-galactosidase measurements. β-Galactosidase measurements were carried out according to the protocol recommended by the manufacturer of the assay kit (Promega). The host cells, E. coli CA274, or transformants were grown to A600 = 4, 1.5 ml aliquots were harvested, and the supernatant (13,000 rpm × 2 min) of the lysate obtained using the lysis buffer (Promega) was used for the enzyme assay and protein determination. One unit of β-galactosidase hydrolyzes 1 μmol of o-nitrophenyl-β-D-galactopyranoside to o-nitrophenol and galactose in 1 min at 37°C. Specific activity was defined as the activity (× 10−4 unit) of β-galactosidase divided by the amount (mg) of total protein in the cell extract.

<table>
<thead>
<tr>
<th>Host cells and transformants</th>
<th>tRNA&lt;sup&gt;Tyr&lt;/sup&gt;</th>
<th>Suppressor tRNA&lt;sup&gt;Tyr&lt;/sup&gt;</th>
<th>TyrRS</th>
<th>Specific activity (milli unit/mg)</th>
<th>Relative</th>
</tr>
</thead>
<tbody>
<tr>
<td>CA274</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.9</td>
<td>1.00</td>
</tr>
<tr>
<td>CA274 [pMWSupYRS]</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.6</td>
<td>1.78</td>
</tr>
<tr>
<td>CA274 [pMWTryr]</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>0.9</td>
<td>1.00</td>
</tr>
<tr>
<td>CA274 [pMWTryrYRS]</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>0.5</td>
<td>0.56</td>
</tr>
<tr>
<td>CA274 [pMWSup]</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>0.7</td>
<td>0.78</td>
</tr>
<tr>
<td>CA274 [pMWSupYRS]</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>130</td>
<td>144</td>
</tr>
</tbody>
</table>

β-galactosidase of E. coli 1000BT (F<sup>lac</sup>am1000 trp<sub>am</sub> str<sub>am</sub> su<sup>am</sup>) is active only when it is suppressed by Gln or Trp (12). E. coli BT3 (F<sup>lac</sup>am1000 trp<sub>am</sub> str<sub>am</sub> su<sup>am</sup>) has an amber mutation in the methionine synthase gene. It can grow at 32°C in minimal media when its amber codon is suppressed by Tyr, Gln, or Trp, or Trp (12).

As shown in Table II (lac<sub>am</sub>1000 column), no transformant derived from 1000BT grew as blue colonies on LB-amp’ X-Gal plates. This means that the suppression of an amber codon is not due to the incorporation of Gln or Trp. Moreover, only the BT3 cells transformed with pMWSupYRS could grow on minimal media plates (Table II; met<sub>am</sub> column). This indicates that Tyr, Gln, or Trp was incorporated at the amber codon. These results together strongly suggest that the amber suppression in these cases is due to the incorporation of tyrosine, and that the amber suppressor tRNA and TyrRS from yeast can function as an extra pair in E. coli cells.

To further confirm that the amino acid introduced by the yeast amber suppressor tRNA/TyrRS pair is really limited to only tyrosine, the suppression patterns of λ phage amber mutants were tested on E. coli mutants and their transformants. As shown in Table III, F<sup>anm</sup> (λc857 h2 F<sup>anm</sup>) did not form plaques on E. coli CA274 (Su<sup>−</sup>) or its transformants, but did so on the control cells, E. coli BT22 (Su<sup>−</sup>), and S<sup>anm</sup> (λc857 S<sup>anm</sup>) formed plaques on E. coli CA274 transformed with pMWSupYRS, similarly to the control cells, BT32 (Su<sup>−</sup>). Since it is known that F<sup>anm</sup> is unable to form plaques when its amber codon is suppressed by Su<sup>+</sup> (tyrosine), and that S<sup>anm</sup> forms plaques only when its amber codon is suppressed by Su<sup>3</sup> (12), these results unambiguously indicate that the co-expressed amber suppressor tRNA and TyrRS pair suppressed the amber codon by inserting tyrosine. In other words, the amber UAG codon in this situation is assigned to an “extra” tyrosine specifically decoded by the extra amber suppressor tRNA/TyrRS pair transplanted from yeast into E. coli cells. This means that once a TyrRS mutant with specificity to non-canonical amino acids is obtained, this co-expression system will be applicable to the site-directed incorporation of non-canonical amino acids into proteins in vivo. We have already obtained several TyrRS mutants with altered amino acid specificities by genetic engineering (Ohno et al., manuscript in preparation), and construction of the in vivo system is underway.

As already mentioned, several research groups recently described a strategy for or the results of trials to establish such tRNA/aminoacyl-tRNA synthetase pairs that are functional in vivo (6-8). We think our system has some advantages compared to them. First, the strictness of the decoding specificity of our system is excellent since neither the suppressor tRNA nor TyrRS expressed alone seems to affect the endogenous protein synthesis in E. coli (Fig. 2 and Table II). As the host cells transformed with pMWYRS could grow without any apparent problem (Fig. 2), the expressed yeast TyrRS seems to be unable to recognize endogenous E. coli tRNAs. In addition, the suppressor tRNA alone does not seem to be charged with any amino acids in E. coli cells (Table I, CA274 [pMWSup] row; Table II, suppressor tRNA row). Although Perret et al. (13) reported that E. coli tRNA<sup>Tyr</sup> was efficiently charged by yeast TyrRS (their unpublished results), we think their
result is questionable. We could not aminoacylate E. coli tRNA\textsuperscript{TYr} with yeast TyrRS in our in vitro experiments, and Chow and RajBhandary (9) have reported that yeast TyrRS strongly recognizes the C1-G72 base pair which is present in yeast tRNA\textsuperscript{TYr} but not in E. coli tRNA\textsuperscript{TYr}. There remains a possibility that a low level of proline was actually incorporated by the yeast suppressor tRNA/TyrRS system since the C1-G72 base pair was found to be a part of the identity determinant for E. coli prolyl-tRNA synthetase (14, 15). However, we could not detect any charging of proline to yeast tRNA\textsuperscript{TYr} by E. coli prolyl-tRNA synthetase, at least under the in vitro reaction conditions so far tested (data not shown). Taking all the above into consideration, the amber UAG codon in our system will have very low chance of suffering from “double-meaning” as to amino acid assignment as it does in other systems (6-8). This will help increase the homogeneity of amber-mutated target proteins. Second, the coding sequence of yeast TyrRS is readily changeable in our system. Since the TyrRS gene is inserted into the site between NotI and SphI, and these restriction sites exist only once in pMWSupYRS (Fig. 1d), the coding sequence of a mutant TyrRS with the desired amino acid specificity can be easily substituted for that of wild-type TyrRS, like a cassette. Third, since the replication origin of pMWSupYRS is different from that of usual expression vectors commercially available, one more plasmid can be co-transfected simultaneously to supply the mRNA for the synthesis of the target protein. We believe that our system will be one of the best systems currently available for the site-directed incorporation of non-canonical amino acids into proteins in vivo.

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REFERENCES