Co-Expression of Yeast Amber Suppressor tRNA^{Tyr} and Tyrosyl-tRNA Synthetase in *Escherichia coli*: Possibility to Expand the Genetic Code[1]

Satoshi Ohno,* Takashi Yokogawa,* Isao Fujii,* Haruichi Asahara,* Hachiro Inokuchi,[†] and Kazuya Nishikawa*,[2]

*Department of Biomolecular Science, Faculty of Engineering, Gifu University, Yanagido 1-1, Gifu 501-1193; and
†Department of Biophysics, Graduate School of Science, Kyoto University, Sakyo-ku, Kyoto 606-8502

An efficient system was developed for the co-expression of a yeast tRNA^{Tyr}/tyrosyl-tRNA synthetase (TyrRS) pair in *Escherichia coli*. Analysis of suppression patterns using several sets of *E. coli* and λ phage mutants indicated that the expressed yeast suppressor tRNA^{Tyr} was aminoacylated only with tyrosine by its cognate yeast TyrRS and not by *E. coli* TyrRS or other aminoacyl-tRNA synthetases. This extra tRNA/TyrRS pair is expected to be a key bridgehead for developing an *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 *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 or the results of trials to establish such suppressor tRNA/aminoacyl-tRNA synthetase pairs that are functional *in vivo* (*6–8*).

We considered that a yeast tRNA^{Tyr}/TyrRS pair, when introduced into *Escherichia coli* cells, would be an ideal candidate for this purpose for the following reasons. (i) Yeast tRNA^{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 *E. coli* tRNAs except for tRNA^{Pro} (*9*). (ii) The size of the variable arm in yeast tRNA^{Tyr} is completely different from that in *E. coli* tRNA^{Tyr}, and therefore *E. coli* TyrRS will not aminoacylate yeast tRNA^{Tyr}. (iii) Yeast tRNA^{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 *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^{Tyr} and wild-type TyrRS in *E. coli*.

Series of plasmids for the expression of yeast tRNAs and/or yeast TyrRS in *E. coli* were derived from pMW118 (Nippon Gene). Their expression is under the control of the *lac* promoter. pMWTyr contains a tRNA gene sequence derived from *Saccharomyces cerevisiae* tRNA^{Tyr} between *Xba*I and *Hind*III sites in the cloning site of pMW118 (Fig. 1a). The intervening sequence was deleted and a CCA sequence was added to the 3′ end of the gene to facilitate direct expression of a mature tRNA^{Tyr} coding sequence in *E. coli*. pMWSup has the same sequence as pMWTyr except that the first letter of the anticodon has been changed from G to C to allow the decoding of the amber codon, UAG (Fig. 1b). pMWYRS has a ribosome binding site (rbs) derived from *E. coli* expression vector pET21a (+) (Novagen), and the gene of *Saccharomyces cerevisiae* TyrRS (*9*) between *Xba*I and *Sph*I sites (Fig. 1c). For co-expression of yeast tRNA^{Tyr} and TyrRS, pMWTyrYRS and pMWSupYRS were constructed in such a way that the

[2] To whom correspondence should be addressed. E-mail: zuy@ap-chem.gifu-u.ac.jp
Abbreviations: TyrRS, tyrosyl-tRNA synthetase; IPTG, isopropyl-thio-β-D-galactoside; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

tRNA gene (wild type or amber suppressor), ribosome binding site and TyrRS gene were inserted in this order between *Xba*I and *Sph*I sites (Fig. 1d).

E. coli CA274 (HfrC lac_{am125} trp_{am}) 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 I). 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
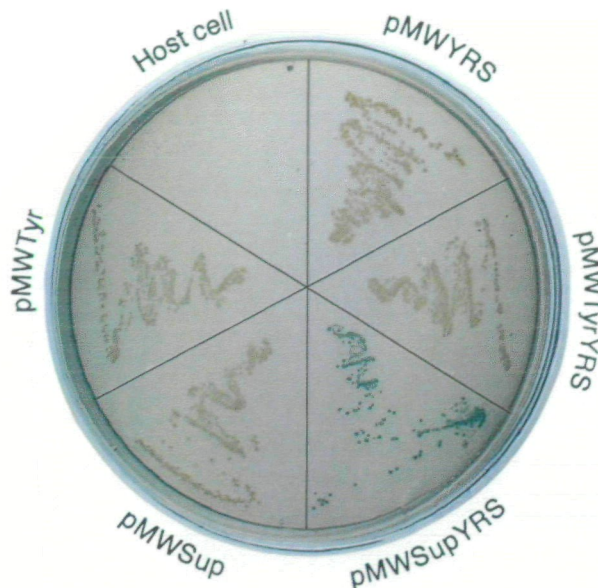

Fig. 2. **Suppression of** lac_{am}**125 in** *E. coli* **CA274 on co-expression of yeast amber suppressor tRNA^{Tyr} and TyrRS.** Plasmids carrying yeast tRNA^{Tyr} and/or TyrRS were transformed into the host cells, *E. coli* CA274, on a Luria-Bertani plate containing 50 μg/ml ampicillin, 0.5 mM IPTG, and 0.004% (w/v) X-Gal. pMWTyr carries the gene of yeast tRNA^{Tyr}, and pMWSup carries that of amber suppressor tRNA^{Tyr}. pMWYRS is the plasmid for the expression of yeast TyrRS. pMWTyrYRS is that for the co-expression of yeast tRNA^{Tyr} and TyrRS. Similarly, pMWSupYRS is that for the co-expression of amber suppressor tRNA^{Tyr} and TyrRS. The plate was incubated at 37°C overnight.
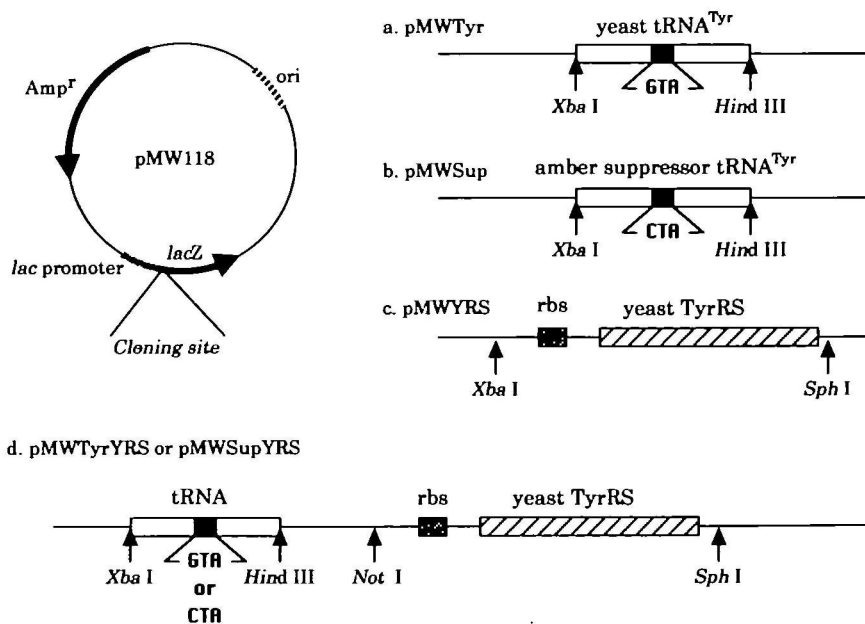

Fig. 1. **Construction of plasmids carrying the yeast tRNA^{Tyr} and/or TyrRS.** Plasmids pMWTyr, pMWSup, pMWYRS, pMWTyrYRS, and pMWSupYRS were constructed by inserting the corresponding DNA fragments containing the gene(s) for yeast tRNA^{Tyr} (wild type or amber suppressor) and/or TyrRS into the indicated restriction sites within the cloning site of pMW118 (upper left). Amp^r, ampicillin resistance gene; ori, replication origin; *lac*Z, β-galactosidase gene; rbs, ribosome binding site.

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 $A_{600} \doteqdot 4$, 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^{-3} unit) of β-galactosidase divided by the amount (mg) of total protein in the cell extract.

Host cells and transformants	Genes expressed			Specific activity (milli unit/mg)	Relative
	tRNA^{Tyr}	Suppressor tRNA^{Tyr}	TyrRS		
CA274	−	−	−	0.9	1.00
CA274 [pMWYRS]	−	−	+	1.6	1.78
CA274 [pMWTyr]	+	−	−	0.9	1.00
CA274 [pMWTyrYRS]	+	−	+	0.5	0.56
CA274 [pMWSup]	−	+	−	0.7	0.78
CA274 [pMWSupYRS]	−	+	+	130	144

TABLE II. **Suppression patterns of E. coli amber mutants.** The suppression of lac_{am125} (E. coli CA274) and lac_{am1000} (E. coli 1000BT) was determined as the growth of mutant bacteria as blue colonies, which were transformed with plasmids carrying the yeast tRNA^{Tyr} and/or TyrRS genes. + indicates growth as blue colonies (suppression +); −, growth as white colonies (suppression −). The suppression of met_{am3} was determined as the growth of the mutant bacteria (E. coli BT3) on minimal plates, which were transformed similarly. + indicates normal growth; −, no growth. The plates were incubated at 32°C for 3 days.

Plasmids transformed	Amber mutant		
	lac_{am125}	lac_{am1000}	met_{am3}
None	−	−	−
pMWYRS	−	−	−
pMWTyr	−	−	−
pMWTyrYRS	−	−	−
pMWSup	−	−	−
pMWSupYRS	+	−	+

TABLE III. **Suppression patterns of λ phage amber mutants.** λ phage amber mutants were examined as to the appearance of phage plaques on E. coli CA274 (Su$^-$) or the transformants of several yeast genes, and E. coli BT22 (Su$^+$2) or BT32 (Su$^+$3) as a control. + indicates the appearance of phage plaques; − no phage plaques; N.D., not done.

Host cells and transformants	λ phage amber mutants	
	P_{am3}	S_{am7}
CA274	−	−
CA274 [pMWYRS]	−	−
CA274 [pMWTyr]	−	−
CA274 [pMWTyrYRS]	−	−
CA274 [pMWSup]	−	−
CA274 [pMWSupYRS]	−	+
BT22	+	N.D.
BT32	N.D.	+

β-galactosidase of E. coli 1000BT (F$^-$ lac_{am1000} trp_{am} str^- su^-) is active only when it is suppressed by Gln or Trp (12). E. coli BT3 (F$^-$ lac_{am1000} trp_{am} str^- su^- met_{am3}) 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 (12).

As shown in Table II (lac_{am1000} 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 pMWSup-YRS could grow on minimal media plates (Table II; met_{am3} 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, P_{am3} (λcI857 b2 P_{am3}) did not form plaques on E. coli CA274 (Su$^-$) or its transformants, but did so on the control cells, E. coli BT22 (Su$^+$2), and S_{am7} (λcI857 S_{am7}) formed plaques on E. coli CA274 transformed with pMWSupYRS, similarly to the control cells, BT32 (Su$^+$3). Since it is known that P_{am3} is unable to form plaques when its amber codon is suppressed by Su$^+$3 (tyrosine), and that S_{am7} forms plaques only when its amber

codon is suppressed by Su$^+$3 (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^{Tyr} was efficiently charged by yeast TyrRS (their unpublished results), we think their

result is questionable. We could not aminoacylate *E. coli* tRNA^{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^{Tyr} but not in *E. coli* tRNA^{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^{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 *Not*I and *Sph*I, 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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