Supplementary Information

AUA codon decoding by preferential use of $tRNA^{Ile}(UAU)$ in *Lactobacillus casei*

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Supplementary Methods

Purification of *E. coli* TsaB, TsaD, and TsaE

The expression plasmids (pET28a_Ec_tsaB (yaeZ), pET21b_Ec_tsaD (ygjD), and pET28a_Ec_tsaE (yjeE)) were kindly provided by Dr. Tsutomu Suzuki (The University of Tokyo). These proteins were purified as described previously (1).

TsaB and TsaE were expressed in *E. coli* BL21(DE3) at 37°C by induction with 1 mM isopropyl-β-D-1-thiogalactopyranoside (IPTG) and cultured for 4 h. TsaD was expressed in *E. coli* BL21(DE3) at 20°C by induction with 1 mM IPTG and 0.5 μM $ZnCl_2$, and cultured for 16 h. Cells were harvested and stored at −80°C. TsaB was purified as described for *L. casei* TsaC in the main text.

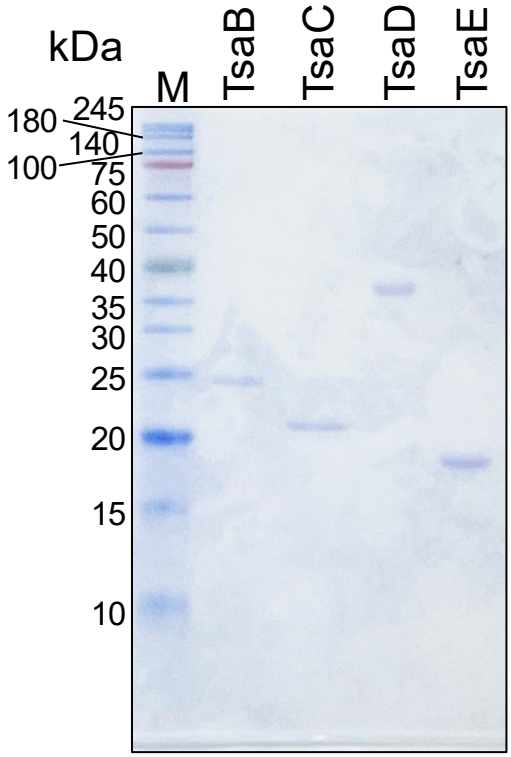
Cell pellets of *E. coli* TsaD were resuspended in buffer A (100 mM Tris–HCl, pH 8.1, 300 mM KCl, 2 mM β-mercaptoethanol, 20 mM imidazole, and 10% glycerol) and lysed by sonication. The lysate was centrifuged at 9,000 × g for 15 min at 4 ºC. The supernatant was applied to a Ni Sepharose High Performance column (Cytiva) equilibrated with buffer A. After washing with buffer A, bound proteins were eluted with a linear gradient of buffer B (100 mM Tris–HCl, pH 8.1, 300 mM KCl, 2 mM β-mercaptoethanol, and 10% glycerol) containing 30–250 mM imidazole. The purified sample was concentrated using a Vivaspin-15 centrifugal filter unit (MWCO 10 kDa; Sartorius Stedim Biotech).

Cell pellets of *E. coli* TsaE were resuspended in buffer C (25 mM Tris–HCl, pH 7.5, 200 mM NaCl, 6 mM β-mercaptoethanol, and 1 mM phenylmethanesulfonyl fluoride) and lysed by sonication. The lysate was centrifuged at 9,000 × g for 15 min at 4ºC. The supernatant was applied to a Ni Sepharose High Performance column equilibrated with buffer C, and bound proteins were eluted stepwise with buffer C containing 20, 50, 100, 200, or 400 mM imidazole. Fractions eluting at 100, 200, and 400 mM imidazole were pooled and dialyzed against buffer D (25 mM Tris–HCl, pH 7.5, 50 mM NaCl, and 6 mM β-mercaptoethanol). The dialyzed sample was reapplied to a Q-Sepharose column equilibrated with buffer D. After washing, bound proteins were eluted with buffer D containing 150, 250, 350, and 500 mM NaCl. Fractions eluting at 250, 350, and 500 mM NaCl were pooled, concentrated, and exchanged into buffer D using a Vivaspin-15 centrifugal filter unit.

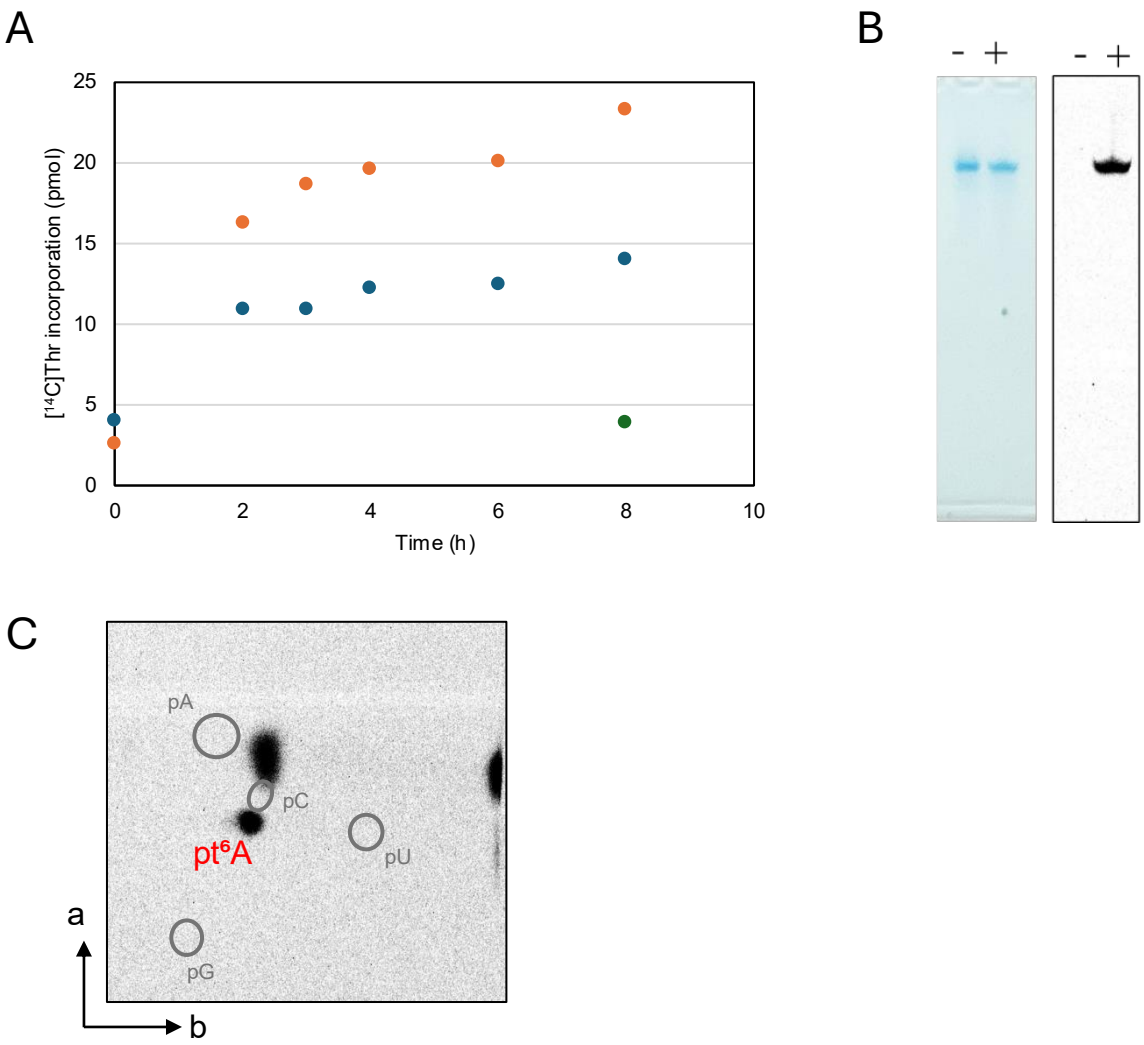
The purified *proteins* were concentrated and stored at −30°C supplemented with 50% (v/v) glycerol. Protein concentration was determined using a Bradford assay (Bio-Rad), and purity was assessed by 15% SDS–PAGE.

REFERENCE

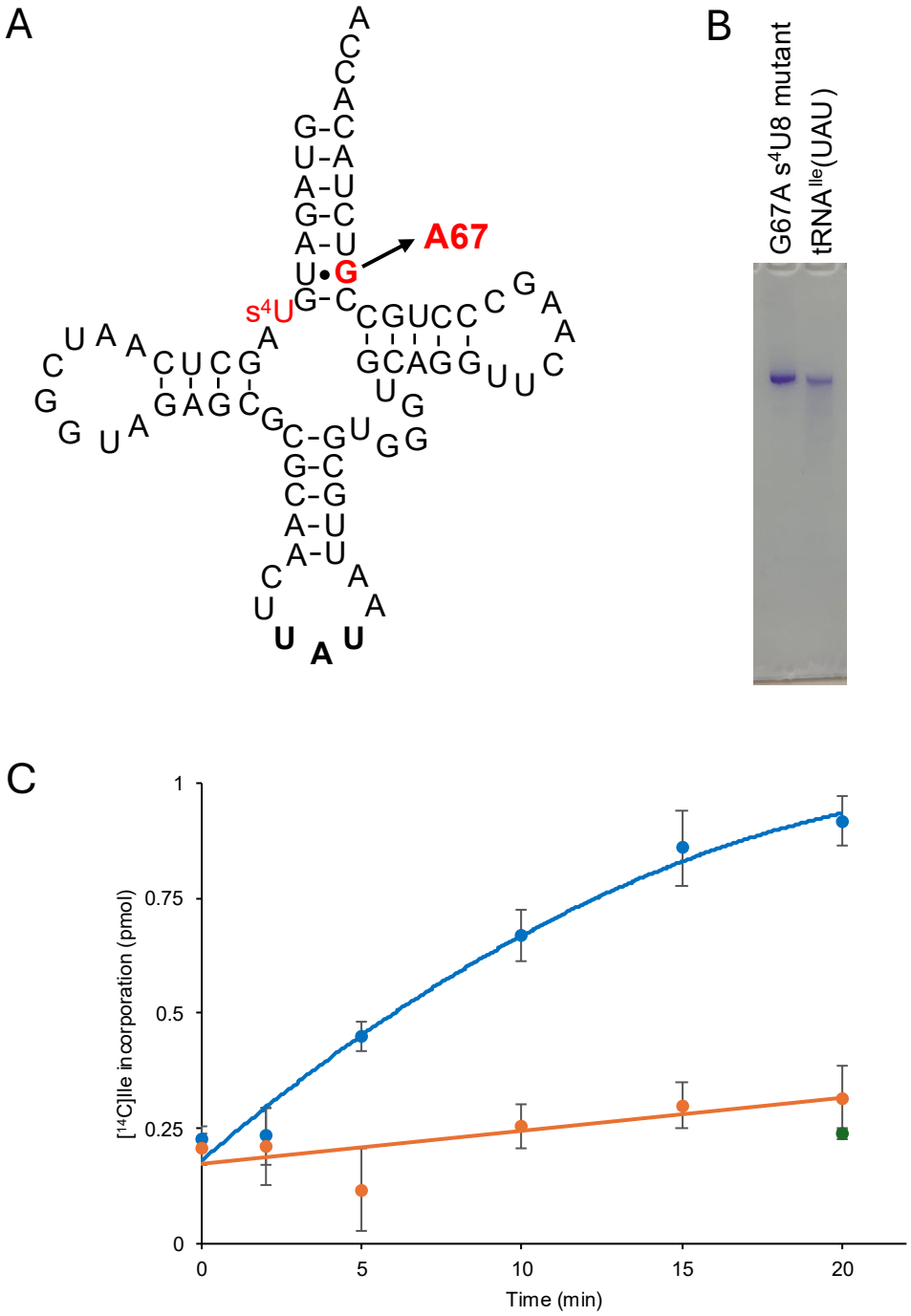
1. Deutsch,C., Yacoubi,B.E., Crécy-Lagard,V. de and Iwata-Reuyl,D. (2012) Biosynthesis of Threonylcarbamoyl Adenosine (t^6A), a Universal tRNA Nucleoside. *Journal of Biological Chemistry*, **287**, 13666–13673.



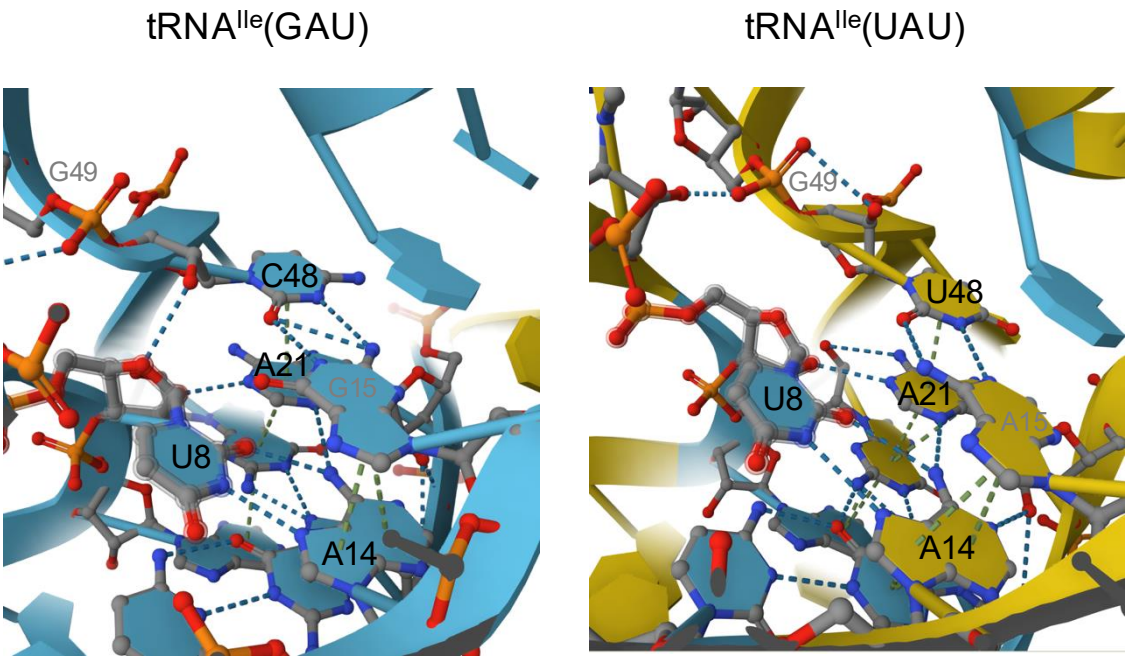
Supplementary Figure S1. Purified t^6A37 biosynthesis enzymes: *E. coli* TsaB, *L. casei* TsaC, *E. coli* TsaD, and *E. coli* TsaE. Protein purity was assessed by 15% SDS—PAGE.



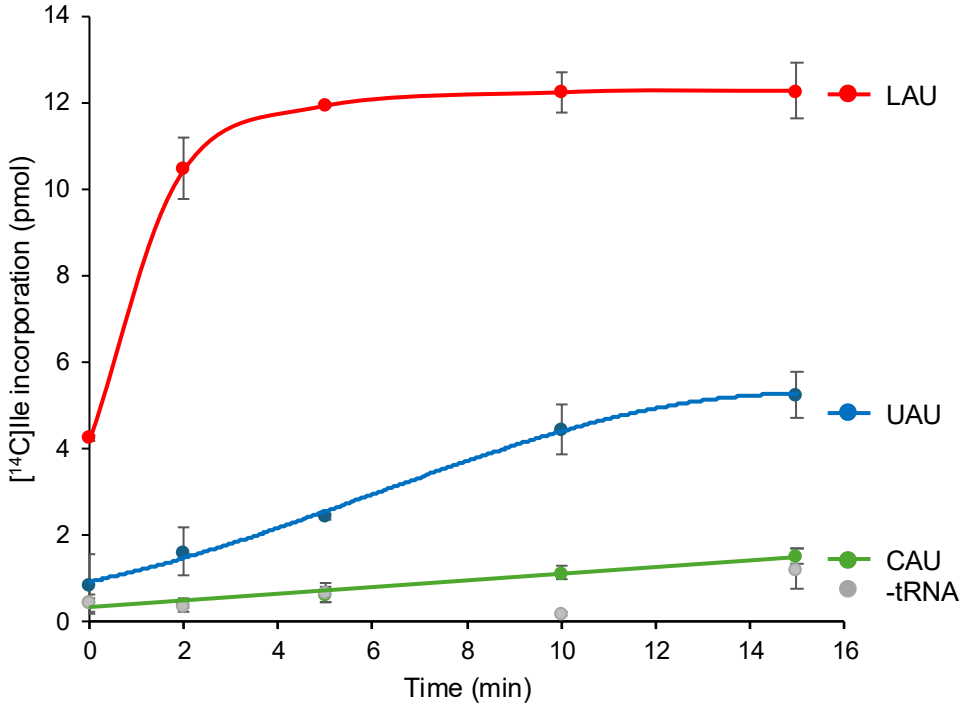
Supplementary Figure S2. Detection of t^{6}A formation by purified t^{6}A biosynthesis enzymes. tRNA^{Ile}(UAU) transcript was incubated with t^{6}A-modifying enzymes TsaC, TsaB, TsaD, and TsaE, and the activity was analyzed. (A) Filter-binding assay to detect [^{14}C]Thr incorporation into tRNA^{Ile}(GAU) and tRNA^{Ile}(UAU) transcripts from [^{14}C]threonine. Orange and blue dots indicate tRNA^{Ile}(GAU) and tRNA^{Ile}(UAU), respectively. The sample without tRNA is shown as a green dot. (B) Gel assay to detect [^{14}C]Thr incorporated into tRNA^{Ile}(UAU) with (+) or without (-) purified t^{6}A biosynthesis enzymes. The left panel shows toluidine blue staining of a 10% denaturing gel (7 M urea), and the right panel shows the corresponding autoradiogram of the same gel. Incorporation of [^{14}C]Thr into tRNA^{Ile}(UAU) was detected only in the presence of the enzymes. (C) Detection of t^{6}A formation by two-dimensional thin-layer chromatography (2D TLC). tRNA^{Ile}(UAU) transcript was incubated with t^{6}A biosynthesis enzymes and [^{14}C]Thr, digested to nucleotides with nuclease P1, and analyzed by 2D TLC. The solvent systems were as follows: (a) isobutyric acid, concentrated ammonia, and water (66:1:33, v/v/v) and (b) isopropanol, HCl, and water (75:15:15, v/v/v). The positions of nucleotide standards (pA, pG, pC, and pU) are indicated by gray circles. A signal corresponding to t^{6}A was detected.



Supplementary Figure S3. Isoleucine acceptance activity of the tRNA^{Ile}(UAU) G67A mutant containing s^{4}U8 modification. (A) Cloverleaf structure of the G67A mutant containing s^{4}U8. (B) Purity of the tRNA transcripts (10% PAGE containing 7 M urea, toluidine blue staining). (C) IleRS activity toward the wild-type tRNA^{Ile}(UAU) transcript and the G67A mutant containing s^{4}U8 is shown as blue and orange dots, respectively. The sample without tRNA is shown in green.



Supplementary Figure S4. Predicted structure of the region around uridine at position 8 in tRNA^{Ile}(GAU) and tRNA^{Ile}(UAU). U8 interactions with surrounding residues are shown. In tRNA^{Ile}(GAU), U8 is predicted to interact with the ribose at position 48, whereas this interaction is not conserved in tRNA^{Ile}(UAU). Structures predicted by AlphaFold are shown in their default color scheme.



Supplementary Figure S5. Isoleucine acceptance activity of the tRNA^{Ile} transcripts. *L. casei* IleRS activity toward the tRNA^{Ile}(CAU) with or without L34 (lysidine introduced by TilS) and tRNA^{Ile}(UAU) is shown as red, green, and blue dots, respectively. The sample without tRNA is shown in gray.

Supplementary Table S1. Oligonucleotide sequences of primers and probes

Name	Sequence	Use
Lc $tRNA^{Ile}$(GAU) 71-47 3' biotin	GGCCTAGATGGACTTGAACCATCGA	Isolation of $tRNA^{Ile}$ (GAU)
Lc $tRNA^{Ile}$(LAU) 71-48 3' biotin	GACCTTGTAGGGCTCGAACCTACG	Isolation of $tRNA^{Ile}$ (LAU)
Lc $tRNA^{Ile}$(UAU) 61-38 3' biotin	GGCTTGAACCTGCACCCACGCAAT	Isolation of $tRNA^{Ile}$ (UAU)
Lc $tRNA^{Ile}$(UAU) F	GGGGCTGCAGTAATACGACTCACTATAGTAGAT GTAGCTCAATCGGTAGAGCGCGCAACTTATA	Template for tRNA transcription
Lc $tRNA^{Ile}$(UAU) R	TGGTGTAGACGGCAGGGCTTGAACCTGCACCCA CGCAATTATAAGTTGCGCGCTCTACC	Template for tRNA transcription
Lc $tRNA^{Ile}$(GAU) F	GGGGCTGCAGTAATACGACTCACTATAGGGCCT ATAGCTCAGTTGGTtAGAGCGCACGCTTGATAA	Template for tRNA transcription
Lc $tRNA^{Ile}$(GAU) R	TGGTGGGCCTAGATGGACTTGAACCATCGaCCTC ACGCTTATCAAGCGTGCGCTCTAAC	Template for tRNA transcription
Lc $tRNA^{Ile}$(CAU) F	GGGGCTGCAGTAATACGACTCACTATAGGACCT TTAGCTCAGTTGGTTAGAGCAGACGGCTCAT	Template for tRNA transcription
Lc $tRNA^{Ile}$(CAU) R	TGGTGGACCTTGTAGGGCTCGAACCTACGACCG GACGGTTATGAGCCGTCTGCTCTAACC	Template for tRNA transcription
Lp $tRNA^{Asp}$(GUC) F	GGGGCTGCAGTAATACGACTCACTATAGGTCCG TTGGTCTAGTTGGTTTAGGACGCCTGCCTGTC	Template for tRNA transcription
Lp $tRNA^{Asp}$(GUC) R	TGGCGGTCCGTACGAGACTCGAACTCGTGATCT CCTGCGTGACAGGCAGGCGTCCTAAA	Template for tRNA transcription
Lc $tRNA^{Ile}$(UAU) U6-A67 mutant R	TGGTGTAGATGGCAGGGCTTGAACCTGCACCCA CGCAATTATAAGTTGCGCGCTCTACC	Template for tRNA transcription
$tRNA^{Ile}$(UAU) containing s4U8 1-21	GUAGAUGs^4UAGCUCAAUCGGUA	Template for tRNA transcription
Lc $tRNA^{Ile}$(UAU) 22-CCA	GGGGCTGCAGTAATACGACTCACTATAGAGCGC GCAACTTATAATTGCGTG	Template for tRNA transcription
Splint DNA for LctRNA^{Ile}(UAU) 39-4	ATTATAAGTTGCGCGCTCTACCGATTGAGCTAC ATC	Template for tRNA transcription

Lc $tRNA^{Ile}$(UAU) 61-38	CTTGAACCTGCACCCACGCAATTA	Hybridization probe
Lc $tRNA^{Ile}$(LAU) 32-11	GCCGTCTGCTCTAACCAACTGAG	Hybridization probe
Lc $tRNA^{Ile}$(GAU) 34-13	CAAGCGTGCGCTCTAACCAACTG	Hybridization probe
Lp $tRNA^{Ile}$(LAU) 33-14	AGCCGTCTGCTCTGACCAACT	Hybridization probe
mRNA T7 SD 5'	GCTAATACGACTCACTATAGGGTTAACTTAAGA AAGGAGGTTATACT	Template for mRNA transcription
mRNA AspMetAUG3'	TTTTTTCTGCAGTTACATGTCAGTATAACCTCCT TT CTTAAGTT	Template for mRNA transcription
mRNA AspIleAUA3'	TTTTTTCTGCAGTTATATGTCAGTATAACCTCCT TT CTTAAGTT	Template for mRNA transcription
mRNA AspProCCA3'	TTTTTTCTGCAGTTATGGGTCAGTATAACCTCCT TT CTTAAGTT	Template for mRNA transcription
pET22b hybrid His NdeI Lcasei TsaC F	C GTC CTC TTC CAG GGT CCT ATGTCAAAAC GTTATCACGA ATC	Recombinant of Lc TsaC
pET22b hybrid casei TsaC R	TGTTAGCAGCCGGATCTCAGTT ATCCTTGAAA ATGCATATCG C	Recombinant of Lc TsaC
pET22b hybrid LcEF-Tu F	CTTTAAGAAGGAGATATACATATGGCTGAAAAA GAACACTAT	Recombinant of Lc EF-Tu
pET22b hybrid LcEF-Tu HRV His R	GTGGTGGTGGTGCTCGAGAGGACCCTGGAAGAG TTCTAGGACGTCAAGGATTTCGGAAACAAC	Recombinant of Lc EF-Tu