วัตถุประสงค์

1. To construct mutants of the non-discriminating aspartyl-tRNA synthetase (ND-AspRS) from *Helicobacter pylori* for the investigation of tRNA specificity.
2. To construct the ND-AspRS chimeras by swapping anticodon binding domain of *H. pylori* ND-AspRS with that of *E. coli* discriminating AspRS (D-AspRS) and asparaginyl-tRNA synthetase (AsnRS).
3 To evaluate a correlation between tRNA specificity and heterologous toxicity of these mutants and chimeras *in vivo*.
4. To determine the kinetic parameters of these mutants and chimeras.

วิธีทดลอง

Materials. All reagents and chemicals were purchased from Sigma Aldrich or Acros Organics (Thermo Fisher Scientific). Aspartic acid [2,3-^{3}H] was purchased from American Radiolabeled Chemicals Inc. (ARC). Oligonucleotides were purchased from Pacific Science. Plasmids were purified using plasmid purification kit from Qiagen. The chemical (calcium chloride) competent cells (DH5α) were purchased from Invitrogen. DNA sequencing was performed by MACROGEN (Macrogen Inc., Seoul, Korea). The entire reading frame was fully sequenced for every construct.

Isolation of Genomic DNA (E. coli K12). An overnight culture of *E. coli* K12 (DH5α) was used to inoculate 20 mL of LB broth. The culture was rigorously shaken at 37 $^{\circ}$C until the OD_{600} reached 0.4. The cells were harvested by centrifugation at 5,000 rpm for 15 minutes. The cell pellets were then resuspended in 0.75 mL 1X TES buffer (10 mM Tris-HCl, 10 mM EDTA pH 8.0, 0.2% SDS) and incubated at 75 $^{\circ}$C for 5 minutes. The cells were then extracted by phenol:chloroform (3:1 V/V) twice. After centrifugation at 3,000 rpm for 10 minutes, a clear aqueous layer was transferred to a new eppendorf tube and the genomic DNA was then precipitated by adding 1/10 of the volume of 3 M sodium acetate followed by 3X of the volume of ice-cold ethanol and the mixture was left overnight at -20 $^{\circ}$C. The genomic DNA was then recovered after centrifugation at 14,000 rpm for 30 minutes followed by a wash using 70% ice-cold ethanol. The agarose gel electrophoresis showed plenty amount of *Ec.* genomic DNA. The resulting genomic DNA was used as a template for gene amplification without any further purification.

Construction of A105G mutant. The A105G mutation (C464G in *aspS* gene) was introduced into plasmid pPTC001 (A plasmid containing *aspS* gene from *H. pylori*) using QuickChange Site-Directed Mutagenesis (Stratagene) according to the manufacturer's instruction. The resulting plasmid (pPC001) was generated using Pt#005 and Pt#006 (See supporting information for primer sequences). The correct introduction of C464G point mutation was confirmed by DNA sequencing of the entire open reading frame.

Construction of Chimera-D and Chimera-N of H. pylori ND-AspRS. The genes encoding the N-terminal domains of the discriminating AspRS and the AsnRS from *E. coli* were amplified from the *E. coli* (DH5α) genomic DNA. The *E. coli* genomic DNA sample was obtained according to the general protocol. The gene encoding the N-terminal domains of the *E. coli* AspRS was generated using primers Pt#007 and Pt#008 (See supporting information for primer sequences). These primers introduced flanking *BamHI* and *KpnI* sites onto 5' and 3' ends of the gene. The primers Pt#009 and Pt#010 were used in the amplification of the gene encoding the N-terminal domain of *E. coli* AsnRS. These primers also introduced flanking *BamHI* and *KpnI* restriction sites in order to facilitate the subsequent cloning into pPC001 plasmid. The PCR product generated from primers Pt#007 and Pt#008 was then cloned into the shuttle vector pGEM-T Easy according to the manufacturer's protocol (Promega) to generate pPC005, a plasmid containing the N-terminal anticodon binding domain AspRS gene from *E. coli*. Similarly, the PCR product generated from primers Pt#009 and Pt#010 was also cloned into pGEM-T Easy shuttle vector to generate pPC006, a plasmid containing the N-terminal anticodon binding domain AsnRS gene from *E. coli*.

The gene encoding the N-terminal anticodon binding domain of *E. coli* AspRS was obtained from the *BamHI* and *KpnI* digestion reaction of pPC005. The gene was then inserted into pPC001 to generate pPC007, a plasmid encoding *H. pylori* ND-AspRS Chimera-D. Correspondingly, the plasmid encoding chimera-N of *H. pylori* ND-AspRS, pPC008, was generated by insertion of the gene from *BamHI* and *KpnI* digestion reaction of pPC006 into pPC001. The identity of every construct was confirmed by DNA sequencing of the entire open reading frame.

Expression and purification of the wild type H. pylori ND-AspRS, Chimera-D, and Chimera-N. The wild type *Hp* ND-AspRS, Chimera-D, and Chimera-N were expressed and purified according to the protocol previously reported. Briefly, *E. coli* (DH5α) calcium chloride chemical competent cells were transformed with an appropriate

plasmid (pPTC001 for wild type ND-AspRS, pPC007 for Chimera-D, and pPC008 for Chimera-N) and selected on Luria-Broth (LB) agar plates containing 100 μg/mL ampicillin. The ND-AspRS, Chimera-D, and Chimera-N were expressed for 30 minutes in order to avoid toxicity effects. The crude lysate was purified by Ni-NTA affinity chromatography as described previously. The purity of expressed proteins was confirmed to be >95% homogeneity by means of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The purified proteins were kept at -20 °C in protein storage buffer containing 33 mM phosphate buffer (pH 7.4), 3 mM Tris-Cl, 1.5 mM β-mercaptoethanol (BME), 0.5 mM phenylmethanesulfonyl fluoride (PMSF), and 50% glycerol. Final protein concentrations were determined in triplicate by the Bradford Protein Assay (Bio-Rad) according to the manufacturer's instruction.

In vivo transcription and purification of Hp tRNA^{Asp} and tRNA^{Asn}. Hp tRNA^{Asp} and tRNA^{Asn} were each transcribed *in vivo* according to the protocol previously reported. Total tRNA was isolated by Nucleobond Kit (CloneTech) according to the manufacturer's instruction. This total tRNA contains a mixture of *E. coli* tRNAs enriched with overtranscribed *Hp* tRNA. Since the post-transcriptional modification of tRNA^{Asp} is crucial for tRNA recognition of AspRS, this approach seems to be a reasonable method of choice for obtaining *Hp* tRNA^{Asp} and tRNA^{Asn} as the post-transcriptional modification of tRNA is conserved in different bacteria. The amount of tRNA^{Asp} and tRNA^{Asn}, obtained from the *in vivo* overtranscription mentioned above, were quantified in triplicate using the plateau aminoacylation assays previously described.

Aminoacylation Assays. Aminoacylation assays were performed in 100 mM Hepes (pH 7.5), 2 mM ATP, 4 mM $MgCl_2$, 10 μM aspartic acid and 80 μCi/mL L-[2,3-^{3}H] aspartic acid. The ND-AspRS, Chimera-D, and Chimera-N were each added to a final concentration of 0.2 μM. Each tRNA was denatured at 75 °C for 5 minutes and then refolded by addition of 8 mM $MgCl_2$ at 65 °C and cool to room temperature. Each tRNA was assayed at the concentration of 0.2 μM.

Native polyacrylamide gel electrophoresis (Native-PAGE). Each protein was analyzed by Native-PAGE in order to estimate its ability to form dimer under native and slightly denatured conditions. The native-PAGE was run using 7% polyacrylamide gel with running buffer (without SDS in every component). The slightly denatured condition