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Aspartyl-tRNA Synthetase from *Acidithiobacillus ferrooxidans* Aspartylates Both $tRNA^{Asp}$ and $tRNA^{Asn}$

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Aspartyl-tRNA synthetase (AspRS) exists in two different forms with respect to tRNA recognition. The discriminating enzyme (D-AspRS) recognizes only $tRNA^{Asp}$, while the non-discriminating one (ND-AspRS) also recognizes $tRNA^{Asn}$ and therefore forms both Asp-$tRNA^{Asn}$ and Asp-$tRNA^{Asp}$. Plus primary sequence distinguishes two general groups of AspRS. There is a predominantly bacterial-type, larger AspRS (about 580 aa) in addition to a shorter archaeal/eukaryotic type (about 430 aa). *In vitro* data made clear that discriminating and non-discriminating enzymes exist in both groups. The determinants in the protein sequence responsible for tRNA discrimination are not known. The AspRS from *Acidithiobacillus ferrooxidans* might be suggested ND-AspRS for missing of AsnRS in genomic sequencing data. Therefore, we analyzed the AspRS from *A. ferrooxidans* with in vitro aminoacylation assay with *E. coli* unfractionated tRNA, *in vivo* missense suppression assay with trpA34 mutant and Northern hybridization with probes which were specific with $tRNA^{Asp}$ or $tRNA^{Asn}$. The AspRS from *A. ferrooxidans* produced more Asp-tRNA than that from *E. coli*. Only *aspS* gene from *A. ferrooxidans* suppressed trpA34 strain in minimal media without tryptophan. Only AspRS from *A. ferrooxidans* showed mischarged Asp-$tRNA^{Asn}$ band. Therefore, AspRS from *A. ferrooxidans* is definitely ND-AspRS.

Key Words: Nondiscriminating aspartyl-tRNA synthetase, Discriminating aspartyl-tRNA synthetase, Mischarging, Aminoacylation, missense suppression

INTRODUCTION

Aminoacyl-tRNA synthetase (AARS) recognizes a specific amino acid and transfers amino acid to tRNA which has the anticodon corresponding to the amino acid. There are at least 20 AARSs and tRNAs relating to 20 amino acids in protein biosynthesis. But there is identified a lack of some aminoacyl-tRNA synthetases from the result of the full genome sequencing projects (Tumbula et al., 1999). Especially, it is revealed that a lot of eubacterium including most pathogenic bacterium are lacked an asparaginyl-tRNA synthetase (AsnRS) and asparagine synthetase. But they have a tRNA-dependent amidotransferase (AdT) instead of asparagine synthetase and AsnRS (Min et al., 2002). Plus the two different types of aspartyl-tRNA synthetases (AspRS) are existed in nature. The one of two AspRSs is a discriminating type (D-AspRS) which aspartylates the only $tRNA^{Asp}$. The other is a nondiscriminating type (ND-AspRS) which aspartylates not only $tRNA^{Asp}$ but also $tRNA^{Asn}$. An asparaginyl-tRNA is synthesized through two different pathways with these enzymes (Becker et al., 1997; Curnow et al., 1998; Becker et al., 2000; Min et al., 2002). One is "direct pathway" which asparaginylates the $tRNA^{Asn}$ by AsnRS. The other is "indirect pathway" which is misacylated the $tRNA^{Asn}$ with aspartate by ND-AspRS, and then corrected to asparaginyl-$tRNA^{Asn}$ by AdT. Both AspRSs consist of N-terminal domain recognizing the anticodon of tRNA and the catalytic domain and insertion domain. The primary sequence distinguishes two general groups of AspRS. There is a predominantly bacterial type of AspRS that is about 580 amino acids, in addition to a shorter archaeal-eukaryotic type of about 430 amino acids. *In vitro* and *in vivo* data have made clear that discriminating and

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nondiscriminating enzymes exist in both groups. The determinants in the protein sequence responsible for tRNA discrimination are not known (Raczniak et al., 2001; Tumbula-Hansen et al., 2002; Min et al., 2003). Therefore, *in vitro* enzyme assay and *in vivo* missense suppression analysis can differentiate between the D-AspRS and the ND-AspRS.

Genomic sequencing of *Acidithiobacillus ferrooxidans* was finished at 2006. However, it was not published yet. According to genomic data, AsnRS is missing but Asp/Glu-tRNA dependent amidotransferase (Asp/Glu-AdT) is coded in genome. It means that AspRS of *A. ferrooxidans* may be ND-type. So far, no specific sequences or domains can give the clue for nondiscriminating properties of AspRSs. Therefore, we want to determine the nondiscriminating properties of *A. ferrooxidans* AspRS.

MATERIALS AND METHODS

1. Oligonucleotides, DNA Sequencing and Radiochemicals

Oligonucleotides were synthesized by Bioneer (Daejeon, Korea) and DNAs were sequenced by Solgent (Daejeon, Korea). [^{3}H]Asp (37 Ci/mmol) was purchased from Amersham Biosciences (Piscataway, NJ).

2. Plasmids and strains

AspRS genes were cloned into pCR2.1-TOPO (Invitrogen: Carlsbad, CA), while aspS complementation studies were carried out with pCBS1 (Ibba et al., 1999) and over-expression of AspRSs were performed with pCYB1 (NEB: Ipswich, MA). *E. coli* DH5α was used for most of the cloning experiments. *E. coli* trpA34 strains carrying a D60N mutation in *trpA* were used in missense suppression tests (Min et al., 2003).

3. Preparation of *E. coli* AspRS and *A. ferrooxidans* AspRS

DH5α strains which contained plasmid pCYB1-ECaspS or pCYB1-AFaspS were cultured in LB broth for overnight at 37℃. And then these were inoculated the fresh LB broth. After 2~3 hours, 0.5 mM of IPTG was added for induction of AspRSs. Overexpressed AspRSs were purified by one-step purification method using chitin bead resin from NEB. The purified AspRSs were dialyzed with storage buffer containing 50% glycerol and then stored at -70℃.

4. Preparation of *E. coli* unfractionated tRNA

Unfractionated *E. coli* tRNA was prepared as described (Curnow et al., 1998). *E. coli* DH5α cells were harvested during logarithmic phase growth. The cells were suspended in buffer A (20 mM Tris-HCl, pH 7.4, 20 mM $MgCl_2$, 10 mM 2-mercaptoethanol). And then unfractionated *E. coli* tRNA was prepared. The purified total tRNA was also stored at -70℃.

5. Aminoacylation of *E. coli* unfractionated tRNA

Aminoacylation were performed as described previously (Tumbula-Hansen et al., 2002). Reaction were performed at 37℃ and contained 100mM HEPES (pH 7.2), 50 mM KCl, 5 mM DTT, 6 μM [^{3}H] aspartate, 2.5 mM unlabeled aspartate, 2 mM ATP, and 100 nM each AspRSs. Aliquits of the reaction were removed at various time intervals and spotted onto 3 MM filter disk that was immersed in 10% TCA. The filters were rinsed in ethanol, dried and counted in 3 ml of scintillation mixture fluid.

6. *In vivo* complementation

The trpA34 strain was transformed with pCBS1 plasmid containing *aspS* gene from *E. coli* or *A. ferrooxidans*. Ampicillin-resistant colonies were streaked onto M9 minimal agar plates supplemented with 19 amino acids (20 μg/ml) in the presence or absence of tryptophan (20 μg/ml), incubated at 37℃ for 3 days, and scored daily.

7. Northern hybridization analysis

Total *E. coli* tRNA were purified, aminoacylated for 60 min and analyzed as described previously (Feng et al., 2003).

RESULTS

1. Aminoacylation of *E. coli* AspRS and *A. ferrooxidans* AspRS

To determine the nondiscriminating property of *A. ferrooxidans* AspRS, aminoacylation assay was carried out with aspartate and *E. coli* unfractionated tRNA (1,800 pmole). The activity of *E. coli* AspRS was also determined as a control (Fig. 1). *E. coli* AspRS (EC) aminoacylated 2.4% of unfractionated tRNA from *E. coli*. However, *A.*

ferrooxidans AspRS aminoacylated 4.1% of this tRNA (AF). Even though *E. coli* unfractionated tRNA was used for this assay, the activity of *A. ferrooxidans* AspRS was higher than that of *E. coli* AspRS. It means that *A. ferrooxidans* AspRS might misaminoacylated to another tRNA with aspartate. Probably, it might be a $tRNA^{Asn}$. Therefore, we carried out two different experiments to prove these mischarging. These were *in vivo* missense suppression and Northern hybridization.

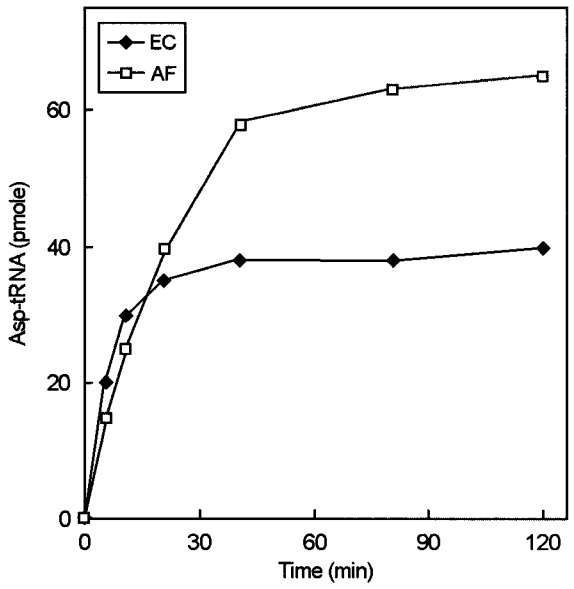

Fig. 1. Aspartylation of *E. coli* unfractionated tRNA (1,600 pmole) by *E. coli* AspRS (EC) and A. *ferrooxidans* AspRS (AF).

2. Missense suppression of *E. coli* trpA34 mutant strain

The *E. coli* trpA34 mutation is a GAT-->AAT change in codon 60^{th} of the *trpA* gene (Shirvanee et al., 1990). This mutation changed the 60^{th} amino acid, aspartate (D) to asparagines (N). D-->N mutation causes loss of the catalytically essential D60 residues in α subunit of tryptophan synthetase and leads to enzyme inactivation. As a consequence, the *E. coli* trpA34 mutant strain is a Trp auxotroph (Shirvanee et al., 1990). *In vivo* missense suppression system with this trpA34 strain was already established (Min et al., 2003). Fig. 2 shows the missense suppression system with trpA34 strain. The presence in *E. coli* of mischarged Asp-$tRNA^{Asn}$ should lead to reinsertion of D at the AAU codon (specifying N) and enable synthesis of wild-type tryptophan synthetase and restoration of prototrophic growth. The missense suppression was performed with trpA34 strains transformed the plasmid vector pCBS1 containing *E. coli aspS* or *A. ferrooxidans aspS* genes. Each single colony was transferred to M9 minimal plate with or without tryptophan. And then these were incubated for 3 to 5 days at 37℃ (Fig. 3). Both grew well in M9 plate with tryptophan. However, the trpA34 strain transformed the plasmid pCBS1 containing *A. ferrooxidans aspS* gene could grow well in M9 plate without tryptophan, while the trpA34 strain transformed the plasmid pCBS1 containing *E. coli aspS* gene could not. This means that the trpA34 strain transfor-

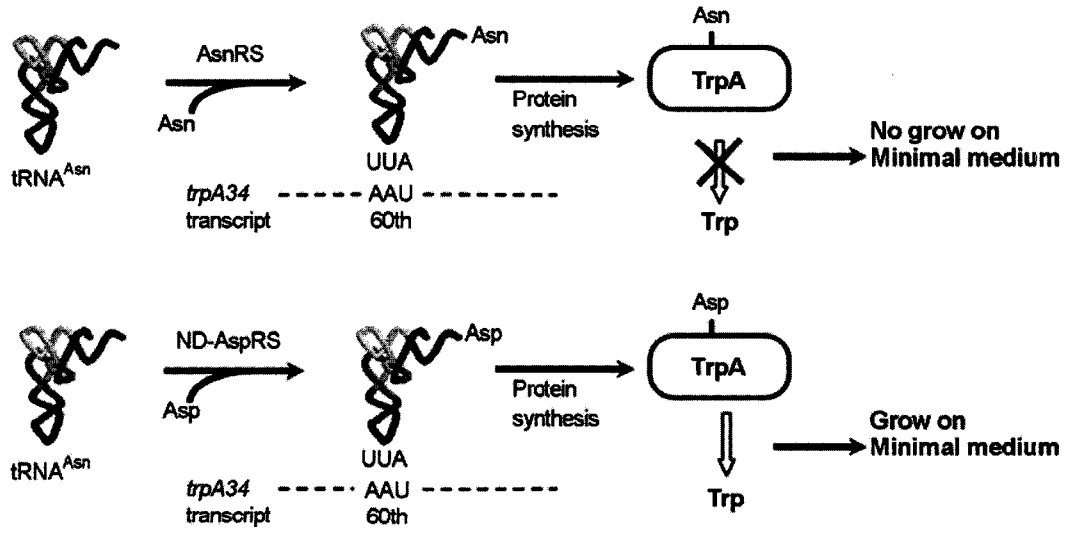

Fig. 2. Diagrams for missense suppression using trpA34 mutant strain.

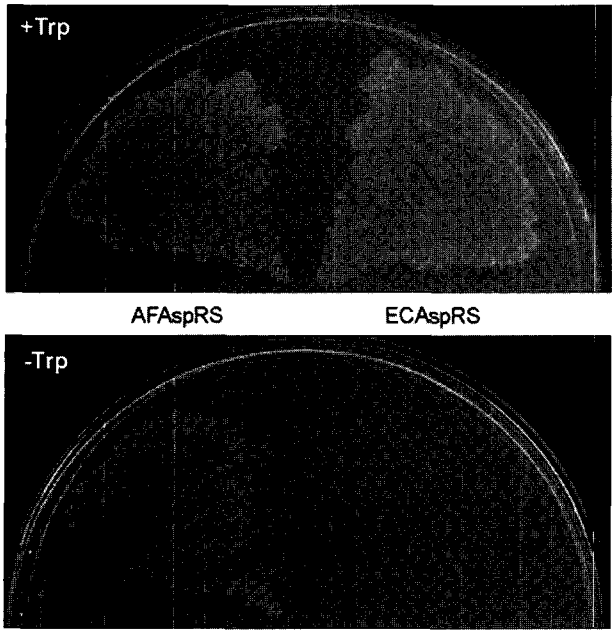

Fig. 3. *In vivo* missense suppression using trpA34 strain on M9 minimal agar plates with 19 amino acids in the absence (-Trp) or presence (+Trp) of tryptophan. These strains cultured for 3 to 5 days at 37℃. These pictures were taken after 5 days.

med the plasmid pCBS1 containing *A. ferrooxidans aspS* gene could synthesize the mischarged Asp-tRNA^{Asn}.

3. Direct determination of mischarged Asp-tRNA^{Asn} using Northern hybridization analysis

The results from *in vitro* aminoacylation assay and *in vivo* missense suppression experiments strongly supported the presence of mischarged Asp-tRNA^{Asn}. However, these results did not show a direct evidence for the presence of mischarged Asp-tRNA^{Asn}. Therefore, we did Northern hybridization analysis to show the mischarged Asp-tRNA^{Asn}.

The unfractionated *E. coli* tRNA was aminoacylated with aspartate by AspRSs from *E. coli* or *A. ferrooxidans*. The half of each sample was transferred to another tubes and then deacylated. The aminoacylated or deacylated samples were separated by 6% acid urea PAGE for 30~40 hours at 4℃, transferred to nitrocellulose membrane using electroblotting and hybridized with probes for tRNA^{Asp} or tRNA^{Asn}, respectively (Fig. 4). Both AspRSs from *E. coli* or *A. ferrooxidans* aminoacylated the tRNA^{Asp} with aspartate. However, only *A. ferrooxidans* AspRS could charge the tRNA^{Asn} with aspartate. In case of aspartylation of AspRS, all signal of tRNA^{Asp} have two bands, even the signal of Asp-tRNA^{Asp}. It might be caused some modification. These

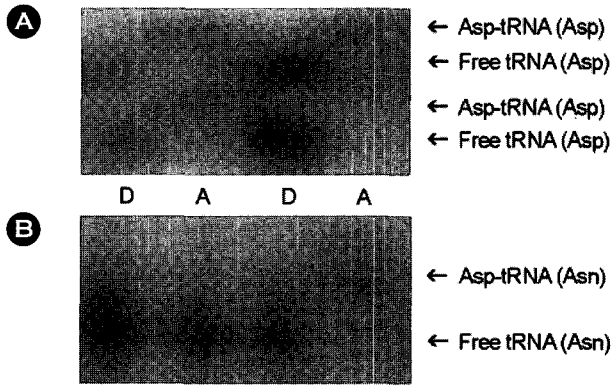

Fig. 4. Northern hybridization analysis for mischarging. Acylated tRNA (A) and deacylated tRNA (D) were separated using 6% acid urea PAGE (pH 4.5, 50% urea). Panel **A)** shows tRNA^{Asp} and panel **B)** shows tRNA^{Asn}. Lane 1, 2: ECAspRS, Lane 3, 4: AFAspRS

phenomenons were showed several previous reports (Skouloubris et al., 2003; Chuawong and Hendrickson, 2006). And both bands shift in all of acylated lanes. Thus both tRNA^{Asp} are acylated by *E. coli* or *A. ferrooxidans* AspRSs. The band of correctly charged Asn-tRNA^{Asn} located much higher than mischarged Asp-tRNA^{Asn} (data not shown). This result directly showed the mischarged Asp-tRNA^{Asn} band. This mischarged Asp-tRNA^{Asn} was produced by AspRS from *A. ferrooxidans*. Therefore, AspRS from *A. ferrooxidans* is a nondiscriminating AspRS. It is consistent with the suggestion from genomic sequence data.

DISCUSSION

Twenty amino acids and its cognate twenty tRNAs need the cognate twenty AARS to charge correctly. Accurate aminoacyl-tRNA synthesis is essential for faithful translation of the genetic code and consequently has been intensively studies for about forty years.

Recently, so much data were spouted out from genome projects. These genomic sequencing results have given so many clues for presence of new enzymes and pathways. Investigations with archaea and eubacteria have already led to the discoveries of novel pathways and enzymes for the synthesis of numerous aminoacyl-tRNAs. The most surprising discoveries have been a transamidation pathway for the synthesis of asparaginyl-tRNA (Curnow et al., 1996; Curnow et al., 1998; Min et al., 2002) and glutaminyl-tRNA (Curnow et al., 1997), a novel lysyl-tRNA (Ibba et al., 1997), the 22nd pyrrolysyl-tRNA (Hao et al., 2002; Srinivasan et

al., 2002; Polycarpo et al., 2003) and a novel indirect synthetic pathway for cysteinyl-tRNA formation (Sauerwald et al., 2005). These results have revealed completely unexpected levels of complexity and diversity.

The transamidation pathway for the synthesis of asparaginyl-tRNA is related with ND-AspRS and GatCAB, and is indirect pathway. However, this ND-AspRS does not have any specific residues or motifs differentiated from D-AspRS, so far. *A. ferrooxidans* have only one AspRS and doesn't have AsnRS. Fig. 1, 3 and 4 prove that AspRS from *A. ferrooxidans* aminoacylated both $tRNA^{Asp}$ and $tRNA^{Asn}$ with aspartate. However, discriminating *E. coli* AspRS aminoacylated only $tRNA^{Asp}$. Therefore, AspRS from *A. ferrooxidans* is nondiscriminating type. These new finding enzymes and pathways strongly suggest much more possibility for presence of new enzymes and pathways. New techniques, like a missense suppression assay which was used in this experiment, accelerate to find new enzymes and pathways. And these techniques will be useful for characterization of new enzymes and pathways, too.

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