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Characterization of a *B. subtilis* Minor Isoleucine tRNA Deduced from tDNA Having a Methionine Anticodon CAT[1]

Jitsuhiro Matsugi,[2] Katsutoshi Murao, and Hisayuki Ishikura

Laboratory of Chemistry, Jichi Medical School, 3311-1 Minamikawachi-machi, Tochigi 329-04

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***Bacillus subtilis*, which belongs to Gram-positive eubacteria, has been predicted to have a minor isoleucine tRNA transcribed from the gene possessing the CAT anticodon, which corresponds to methionine. We isolated this tRNA and determined its sequence including modified nucleotides. Modified nucleotide analyses using TLC, UV, and FAB mass spectroscopy revealed that the first letter of the anticodon is modified to lysidine [4-amino-2-(N^6-lysino)-1-β-D-ribofuranosyl pyrimidine]. As a result, this tRNA agrees with the minor one predicted from the DNA sequence and is thought to decode the isoleucine codon AUA.**

Key words: *Bacillus subtilis*, FAB mass spectroscopy, lysidine, modified nucleotide, tRNA.

In the codon table, isoleucine is assigned to three codons, AUU, AUC, and AUA, but theoretically two kinds of anticodon (GAU and UAU) are sufficient to decode these three codons. To decode the AUA codon, some organisms utilize a minor species of $tRNA^{Ile}$ whose first letter of the anticodon is a unique or an unknown modified nucleotide. For example, in *Escherichia coli*, a Gram-negative eubacteria, and *Mycoplasma capricolum*, a derivative of a Gram-positive eubacteria, it has been reported that their minor $tRNA^{Ile}$s have lysidine [4-amino-2-(N^6-lysino)-1-β-D-ribofuranosyl pyrimidine; referred to as L in this paper], which is posttranscriptionally converted from cytidine (*1, 2*). Some plant mitochondria and chloroplasts, and phage T4 have an unknown modified nucleotide possibly converted from cytidine (*3-5*).

Bacillus subtilis is a Gram-positive eubacteria, and its modification patterns used in the codon-anticodon relationship show some differences from those of Gram-negative bacteria such as *E. coli*. In addition, *B. subtilis* is known to have characteristic tRNA gene clusters. Green and Vold have reported that *trnB*, one of the tRNA gene clusters, encodes 21 tRNAs (*6*). Analysis of their sequences revealed that 2 of them are isoleucine tDNAs. One is a $tDNA^{Ile}$ with the GAT anticodon and the other is one with the CAT anticodon [referred to tDNA(GAT) and tDNA(CAT) respectively]. Comparison with other known $tRNA^{Ile}$s suggested that the tDNA(CAT) accepts isoleucine, though its anticodon sequence corresponds to methionine. Thus, if there is no change in its anticodon, competition with the methionine codon by $tRNA^{Met}$ and $tRNA^{Ile}$ will occur and lead to mistranslation. Therefore, a mechanism such as posttranscriptional modification targeting the anticodon, which is also one of the strong identity sites, must be required to change the codon recognition. In fact, in *E. coli*, lysidine in the wobble position can form a base pair only with adenosine, not with guanosine, and consequently the minor $tRNA^{Ile}$ can read only the AUA codon. At the same time, lysidine contributes to a change in charging specificity from methionine to isoleucine (*7*).

In *B. subtilis*, as described above, the structure of the tRNA transcribed from tDNA(CAT) remained unknown. Since we were studying the tRNA structure and function of *B. subtilis* (*8, 9*), this prompted us to attempt to determine the RNA sequence including its modification pattern. In this paper, we report the structure of the $tRNA^{Ile}$ transcribed from tDNA(CAT).

MATERIALS AND METHODS

Isolation and Purification of the Minor Isoleucine tRNA—B. subtilis W168 unfractionated tRNA was prepared as described previously (*10*). The bulk tRNA (134,500 A_{260} unit) was purified successively by DEAE Sepharose CL-6B, Sepharose 4B, and BD cellulose column chromatographies. For the fractionation, measurement of [^{14}C]isoleucine acceptor activity using *E. coli* aminoacyl-tRNA synthetase (*11*) and the dot blot hybridization methods (see below) were employed. The final purification was done by semipreparative HPLC (hydroxyapatite column, 21.4× 100 mm, TAPS, TONEN, buffer A; 5 mM sodium phosphate, pH 8.0, buffer B; 300 mM sodium phosphate, pH 8.0) (*12*) and 360 A_{260} of the tRNA was obtained.

Dot Blot Hybridization—A DNA probe for the dot blot hybridization was prepared using the phosphoramidite method (*13*). [γ-^{32}P]ATP (3,000 Ci/mmol) was purchased from Amersham. The hybridization was done by the procedure of Yokogawa *et al.* with several modifications (*14*). The tRNA solution (2 μl) was directly spotted onto a nylon membrane (Hybond-N, Amersham) and dried. The membrane was irradiated by UV light at 254 nm for 5 min and preincubated in 5×Denhardt's solution, 5×SSPE (20× SSPE contains 3 M NaCl, 0.2 M sodium phosphate, pH 7.4, and 20 mM Na_2EDTA) and 0.5% SDS at 60°C for 1 h. The

[1] The nucleotide sequence data of a *B. subtilis* minor $tRNA^{Ile}$ reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases with the accession number D26446.

[2] To whom correspondence should be addressed. Phone: +81-285-44-2111 (Ext. 3366), Fax: +81-285-44-2858

membrane was then incubated in new hybridization buffer (same composition as the prehybridization solution) containing 5′-[^{32}P]-labeled probe (obtained from the kination of 100 pmol oligonucleotide with [γ-^{32}P]ATP) at 37°C overnight. After the hybridization, the membrane was washed five times with 3×SSC (450 mM NaCl, 45 mM sodium citrate pH 7.0) at room temperature. The membrane was autoradiographed using X-ray film with an intensifying screen for 6 h at −70°C. Spots in the membrane were cut out and the radioactivity was measured with a liquid scintillation counter.

Sequencing of the tRNA—Two-dimensional (2D) TLC (Funacel SF plate, Funakoshi) for nucleotide analysis was developed with two systems. System A: 1st. isobutyric acid : 0.5 M NH_4OH = 5 : 3 (v/v), 2nd. 2-propanol : c.HCl : H_2O = 70 : 15 : 15 (v/v); system B: 1st. isobutyric acid : 0.5 M NH_4OH = 5 : 3, 2nd. 0.1 M Na_2HPO_4 (pH 6.8) 100 ml, $(NH_4)_2SO_4$ 60 g, propanol 2 ml (*11, 15*). A post-labeling method was primarily used for the confirmation of tRNA species and the search for modified nucleotides. For further confirmation of the modified nucleotides, the purified tRNA (150 A_{260}) was digested with RNase T1 and the digest was applied to urea column chromatography (Sephadex A-25, 0.6 cm×100 cm) (*16*). The oligomer containing the anticodon sequence was digested completely with nuclease P1 and analyzed by 2D TLC using system A. Mononucleotides were examined by UV spectroscopy, and the nucleotide mixture located in the same position as adenosine 5′-monophosphate (pA) was further subjected to a paper electrophoresis [pH 7.5 20 mM TEAB (triethylammonium bicarbonate) buffer]. As a result, 0.08 A_{260} (0.28 A_{220}) of unidentified nucleotide (pN) was obtained. pN was then analyzed by FAB mass spectroscopy. The FAB mass spectrum was recorded utilizing JEOL SX102 spectrometer with 10 kV accelerating voltage, FAB priming energy of 6 kV using xenon, and diethanolamine as a matrix. Positive ions were collected and analyzed by DA6000 data system. For the identification of 7-methylguanosine and other modified nucleotides, Sanger's method (2D electrophoresis) was used in a preparative scale together with UV spectroscopy (*17, 18*).

Aminoacylation by B. subtilis S30 Fraction—tRNA (1 μg) was aminoacylated in the following buffer (total volume 100 μl): 100 mM HEPES (pH 7.2), 10 mM $Mg(OAc)_2$, 10 mM KCl, 2 mM ATP, [^{14}C]isoleucine (specific activity 387 mCi/mmol, NEN) or [^{14}C]methionine (225 mCi/mmol, NEN), 20 μl of S30 fraction (*19*). After the reaction, radioactivity was measured by the procedure used for *E. coli* (*11*).

RESULTS

In *B. subtilis*, four isoleucine tRNA genes have been identified so far in *rrnA*, *rrnO* (*20*), and *rrnB* (*6*). The two tDNAs with the GAT anticodon, described as tDNA(GAT), have only one different base pair in the T-loop (Fig. 1A). However, the tDNA with the CAT anticodon, tDNA(CAT), differs from the tDNA(GAT) in 16 positions (Fig. 1B). We attempted to distinguish the tRNAs corresponding to the two kinds of anticodons by dot blot hybridization. A pentadecadeoxyribonucleotide corresponding to the region from 38 to 52 of the tDNA(CAT) was used as a probe because 5 of the 16 different nucleotides were concentrated around this region. The 5′-terminus of the probe was labeled for the hybridization by [γ-^{32}P]ATP and polynucleotide kinase.

By large-scale cultivation of *B. subtilis*, we obtained 134,500 A_{260} of the unfractionated tRNA. To isolate the $tRNA^{Ile}$ from the bulk tRNA, several column chromatographies were employed. As an example of one of the steps, the elution profile of DEAE Sepharose CL-6B column chromatography is shown in Fig. 2. We assayed the eluted fraction by two methods: dot blot hybridization using 5′-[^{32}P]-labeled probe, and acceptor activity of [^{14}C]isoleucine. For the dot blotting, eluted tRNA was directly immobilized on a nylon membrane by UV irradiation and then hybridized with the probe. The profile reveals that the

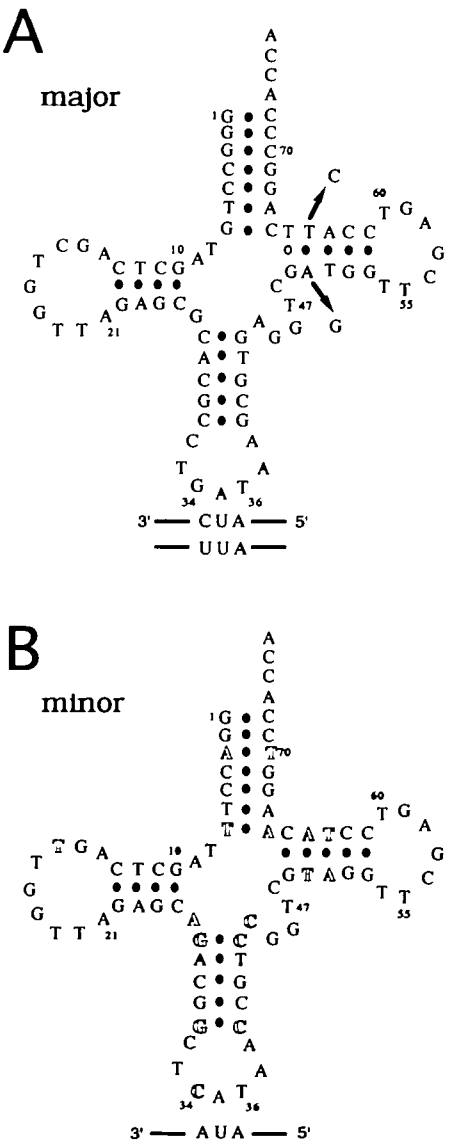

Fig. 1. **Secondary structures of *B. subtilis* isoleucine tDNAs deduced from the genes.** A: Major tDNAs and arrows mean different base-pairs between two major tDNAs coded in *rrnA*, *rrnO*, and *rrnB*. The major tDNA coded in *rrnB* has an AT pair in the T-stem. *rrnA* and *rrnO* code identical major tDNA, which has a GC pair in the T-stem. B: Minor tDNA and outlined letters represent different nucleotides from the major one.

peak of the dot blot preceded that of the major accepting activity. Thus, by the combination of assays, we confirmed that the desired tRNA was eluted in the front portion of the isoleucine-accepting fractions. In addition, the comparison of peak areas showed that the amount of the tRNA corresponding to tDNA(CAT) is considerably less than that of the one from tDNA(GAT), and thus we ranked this tRNA as a minor species. After this column chromatography, Sepharose 4B and BD cellulose were used. For the final purification, hydroxyapatite HPLC was employed and it gave satisfactory separation (see "MATERIALS AND METHODS"). The obtained tRNA was pure enough to determine the RNA sequence.

Sequencing was carried out by a postlabeling method (*21*). The tRNA was partially hydrolyzed under alkaline conditions to yield one scission per molecule. Then the 5′-ends of 3′-half molecules were labeled by [γ-^{32}P]ATP uniformly. The mixture of the 5′-labeled fragments was separated by 15% PAGE under denaturing conditions. Each band was extracted and digested completely to 5′-nucleotides with nuclease P1. Analyses of the 5′-labeled nucleotides on 2D TLC (system A) revealed the existence of several modified nucleotides, pΨ (55), pT (54), pm^{6}A (37), pD (16, 20, 20a). In regard to the first letter of the anticodon, the location of the corresponding spot on the 2D TLC was almost coincident with that of pA (Fig. 3A). However, in 15% PAGE for the separation of the 5′-labeled oligomers, a relatively big gap was observed between the bands corresponding to the first and second letters of the anticodon. This suggested the presence of a modified nucleotide having a bulky and/or charged group. To clarify whether this modified nucleotide showed the same mobility as pA or was carried over from the second letter of the anticodon, developing system B was tested. As shown in Fig. 3B, a new spot appeared between pA and pC. This revealed that this 5′-nucleotide (referred to as pN) differed from pA, pG, pC, pU, and other modified nucleotides mentioned above. Andachi *et al.* have reported about the behavior of lysidine on 2D TLC in system A and B, and it seemed to be consistent with the data of our pN (*2*). For further confirmation of the structure, the oligomer containing pN was prepared in a large scale by the combination of RNase T1 digestion and column chromatography with urea. Each oligomer was subjected to nuclease P1 digestion and identified by the content of nucleotides. As the fragment containing the anticodon sequence has m^{6}A, its use as a marker nucleotide allowed us to find the RNA fragment easily. The nuclease P1 hydrolysate from the fragment was separated by 2D TLC (system A) and the position corresponding to pA was scraped out. The reason why system A was used is that system B contained a large amount of salt that might interfere with the following analyses. From the

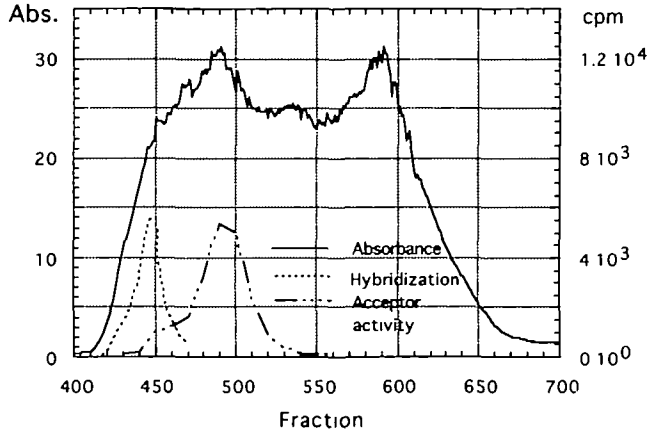

Fig. 2. **Elution profile of DEAE Sepharose CL-6B column chromatography.** The pattern of UV absorption, amino acid acceptor activity and dot blot hybridization are shown. The scale on the left ordinate axis gives the absorbance at 260 nm and the right one gives radioactivity in cpm.

TABLE I. **R_m values on paper electrophoresis.**

Compound	R_m value[a]
5′-XMP[b]	1.00
5′-VMP[c]	1.44
5′-NMP[d]	0.61

[a]Values were measured in 20 mM TEAB buffer at pH 7.5. [b]5′-XMP means the mixture of 5′-AMP, GMP, CMP, and UMP. [c]5′-VMP means uridine-5-oxyacetic acid 5′-monophosphate. [d]5′-NMP means the unidentified 5′-nucleotide studied in this paper.

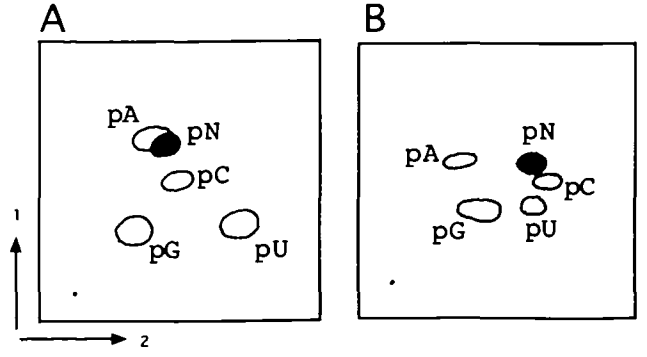

Fig. 3. **Two-dimensional TLC of the first letter of the minor tRNA^{Ile} anticodon.** A: TLC was developed with system A. B: TLC was developed with system B (see "MATERIALS AND METHODS"). pN is represented by a black spot and standard 5′-nucleotides (pA, pG, pC, and pU) are represented by outlined spots.

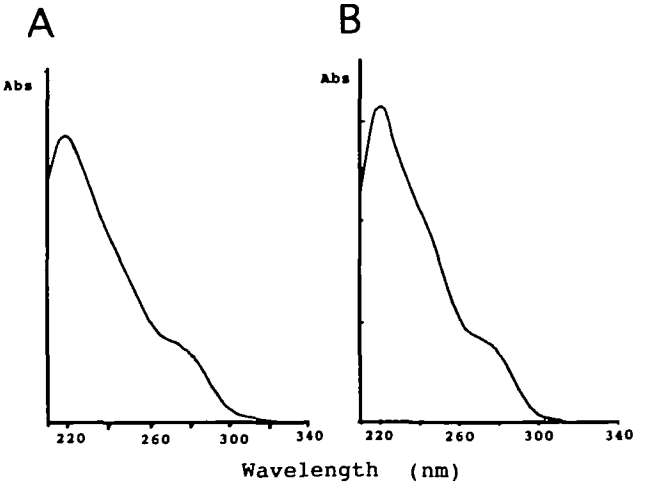

Fig. 4. **UV absorption spectra.** A: Spectrum of pN. B: Spectrum of pL. Both spectra were taken in H_2O.

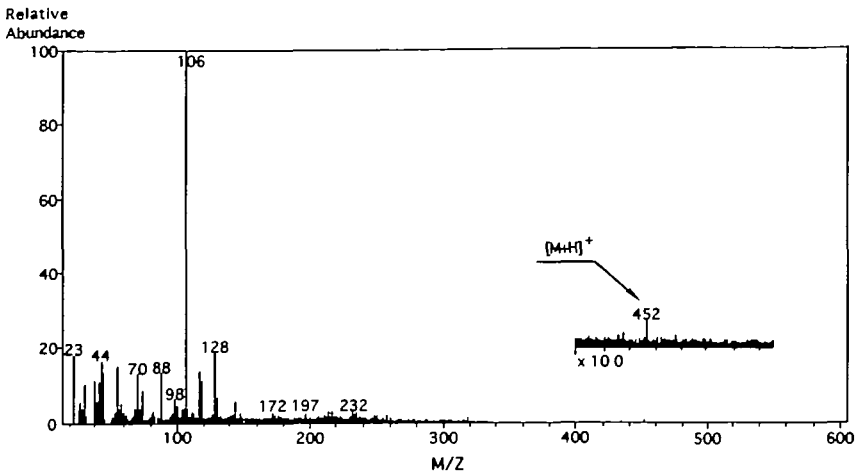

Fig. 5. **Positive FAB mass spectrum of pN.** Inset spectrum is expanded tenfold. The signal at m/z 452 is $[M+H]^+$ peak of lysidine monophosphate.

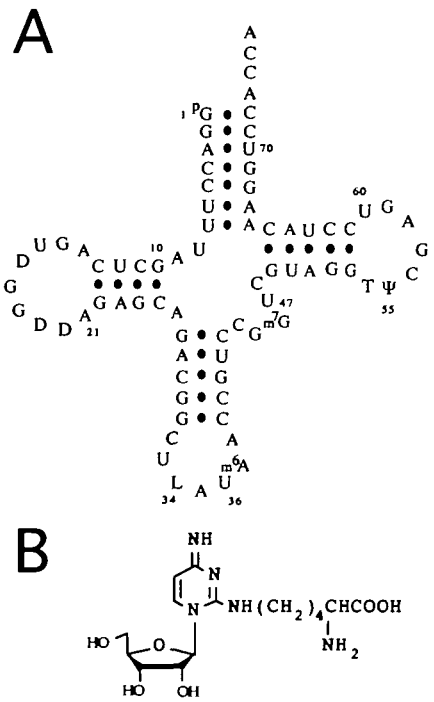

Fig. 6. **A: Secondary structure of the *B. subtilis* minor tRNA^{Ile}. B: Structure of lysidine.** This tRNA sequence will appear in the DDBJ with accession number D26446.

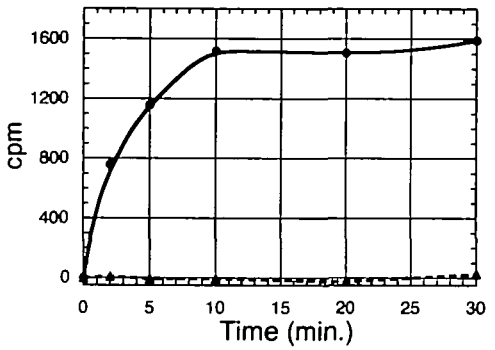

Fig. 7. **Time course of aminoacylation using *B. subtilis* S30 fraction and amino acid, [^{3}H]Ile (●) and [^{3}H]Met (▲).** One microgram of purified minor tRNA^{Ile} was used.

mixture of pA and pN, we separated pN by paper electrophoresis (pH 7.5) using 5′-[^{32}P]pN as a marker. The nucleotide pN showed two thirds of the mobility of the standard 5′-nucleotide and a half of that of uridine-5-oxyacetic acid 5′-monophosphate (pV) (Table I). In combination with the result of 15% PAGE, this result again indicated the presence of a considerably bulky residue and/or an extra positive charge in pN. The UV spectrum of pN showed characteristic absorption at 212 and 270 nm (Fig. 4A) and a resemblance to that of lysidine (Fig. 4B) (*1*). To obtain direct information about its structure, we used a FAB (fast atom bombardment) mass spectrometry. This technique is suitable for analysis of nucleotides and oligonucleotides because of its mild fragmentation and, in addition, it affords a molecular ion peak without the need for chemical derivatization such as trimethylsilylation. Figure 5 shows the result of the positive FAB mass spectrometry using diethanolamine as a matrix reagent. $[M+H]^+$ peak appeared at m/z 452, and this value agreed with the molecular mass of lysidine monophosphate (451). Taking account of all data, we concluded that in *B. subtilis* minor tRNA^{Ile}, the first letter of the anticodon is posttranscriptionally modified to lysidine and the sequence of the tRNA obtained in this study agreed with the one predicted from the DNA sequence. Figure 6 shows the determined tRNA structure including modified nucleotides.

This tRNA carries the anticodon sequence originating from the methionine anticodon and, in fact, two of three nucleotides of the anticodon still coincident with that of methionine after the lysidylation. But it showed no acceptor activity of methionine using *B. subtilis* S30 fraction as shown in Fig. 7.

DISCUSSION

To date, nine tRNA gene clusters have been reported in *B. subtilis* (*6, 20, 22-28*), of which *trnB* codes two isoleucine tRNAs having 5′GAU3′ (major) and 5′NAU3′ (minor) anticodons (*6*). In the minor one, as we revealed in this paper, cytidine in the first letter of the anticodon is posttranscriptionally modified to lysidine to decode the

AUA codon.

In the case of *E. coli*, the same type of the modification has been reported to switch the codon recognition and the tRNA identity simultaneously (*1, 7*). As for the codon recognition, the anticodon LAU can recognize only the isoleucine AUA codon, not methionine AUG, after the base modification. In regard to the tRNA identity, MetRS, which recognizes the anticodon CAU as a strong identity site, can no longer recognize the minor tRNA^{Ile} having the LAU anticodon. On the recognition by IleRS, it has been reported that identity sites of the *E. coli* tRNA^{Ile} are widely distributed over its entire structure. Of these, the critical identity sites are the nucleotides around the anticodon loop (G34, A35, U36, t^{6}A37, and A38). As IleRS must recognize the major and minor tRNA^{Ile}s, it is thought that *E. coli* IleRS recognizes a guanidino group as a signature site common to guanosine and lysidine (G for the major tRNA^{Ile} and L for the minor) (*29*). On the other hand, the sequence of *B. subtilis* IleRS is not known. However, judging from its substrate tRNA, its recognition pattern may be the same as the *E. coli* enzyme to some extent, because 82% sequence homology exists between *B. subtilis* and *E. coli* major tRNA^{Ile}s. In particular, the nucleotides recognized as the major identity sets in *E. coli* are retained in *B. subtilis*, except for the modification of A37. In *E. coli*, all isoacceptor tRNA^{Ile}s have t^{6}A37, which modification has been shown to play an important role in IleRS recognition (*29*). Interestingly, in *B. subtilis*, the major tRNA^{Ile}s have t^{6}A, and the minor one has m^{6}A. Thus, the enzyme recognition pattern for the modification at A37 might be different in the two species.

The *B. subtilis* minor tRNA^{Ile} shows considerably high homology (76%) to its elongator tRNA^{Met} and, in addition, both tRNAs have m^{6}A37. Because of these similarities, there might be a relationship between the two tRNAs concerned with the codon evolution. This is consistent with the hypothesis that AUA codon can be assigned to methionine in the early code (*30*). Without the lysidylation, misacylation by MetRS will occur and the system of protein synthesis in *B. subtilis* may not work properly. Thus, we speculate that the modification to lysidine has two significances for *B. subtilis*. (i) The modification affords the guanidino group as the recognition site for the first letter of the anticodon. This provides a new recognition site for the minor tRNA^{Ile} and, simultaneously, results in the avoidance of methionylation by the MetRS. (ii) In regard to the codon recognition, lysidine can form a base pair with adenosine, but not with guanosine. Thus, although this tRNA is transcribed from the tDNA having a CAT sequence in its anticodon, it recognizes AUA as an isoleucine codon after the lysidylation.

Why is such a special modification needed? Is not a modified uridine like xm^{5}s^{2}U (5-substituted 2-thiouridine derivative) enough to recognize the AUA codon as isoleucine? It is well-known that xm^{5}s^{2}U prefers adenosine to guanosine (*31, 32*). However, the recognition frequency for guanosine is not null, and even if the frequency is very low in this case, the formation of such a base pair would lead to the critical mistranslation of the AUG codon as isoleucine, because methionine is assigned to the sole AUG codon. On the other hand, lysidine shows the restricted recognition pattern only for adenosine, and at the same time, it has the ability to change its identity. In that sense, it seems that the lysidylation is a restricted and effective mechanism to decode the AUA codon.

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