Co-transcription of *Rhizobium meliloti* lysyl-tRNA synthetase and glutamyl-tRNA synthetase genes

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Abstract An open reading frame encoding a putative polypeptide very similar to several lysyl-tRNA synthetases was found 10 nucleotides downstream of *Rhizobium meliloti gltX* encoding glutamyl-tRNA synthetase. Expression of this gene complemented a mutation in *lysS* of *Escherichia coli* and led to the overexpression of a polypeptide of the expected mass (62 kDa), thus confirming that it encodes *R. meliloti* lysyl-tRNA synthetase. Reverse transcription/polymerase chain reaction was used to demonstrate that this *lysS* gene is co-transcribed with *gltX* in *R. meliloti*. This is the first reported case of two immediately adjacent and co-transcribed genes encoding aminoacyl-tRNA synthetases.

Key words: Co-transcription; *Rhizobium meliloti gltX*; *Rhizobium meliloti lysS*; Glutamyl-tRNA synthetase; Lysyl-tRNA synthetase

1. Introduction

Aminoacyl-tRNA synthetases (aaRSs) constitute a family of enzymes, each of which catalyzes the formation of an ester linkage between a specific amino acid and the 3′-end ribose of its cognate tRNA(s) [1]. Each aaRS falls into one of two evolutionarily distinct classes according to their structures and catalytic properties [1–3]. The only exception to this rule is lysyl-tRNA synthetase (LysRS). LysRS of most organisms is a class II enzyme whereas that of several euryarchaea and *Borrelia burgdorferi* contains the amino acid sequence motifs characteristic of class I enzymes [4,5].

In bacterial genomes, there have been only three known cases of co-transcribed aaRS genes described to date. Two of them are the *gltX-cysE-cysS* operons of *Bacillus subtilis* and *Staphylococcus xylosus*, in which the genes encoding two aaRSs specific for glutamate and cysteine, respectively, are separated by a gene encoding the first enzyme of cysteine biosynthesis [6,7]. The third case is the *thrS-infC-rpmL-rplT-pheS-pheT-himA* cluster of *Escherichia coli* in which the genes encoding the aaRSs specific for threonine (*thrS*) and phenylalanine (*pheS-pheT*), respectively, are separated by three genes [8–11]. Open reading frames (ORFs) apparently encoding the histidyl- and the aspartyl-tRNA synthetase are adjacent on the *B. subtilis* genome [12], but there has been no report about their co-transcription.

Two aaRS genes of *Rhizobium meliloti* have been cloned to date: *alaS*, which is located downstream of *recA* [13], and *gltX* [14]. We report here that a gene encoding a LysRS in *R. meliloti* starts 10 nucleotides downstream of the UAA stop codon of *gltX*, and that it is co-transcribed with *gltX*. This is the first known operon containing immediately adjacent aaRS genes.

2. Materials and methods

2.1. Chemicals, enzymes and oligonucleotides

Restriction endonucleases and modification enzymes were purchased from Bethesda Research Laboratory Inc., Pharmacia LKB Biotechnology, Boehringer Mannheim, Sigma Chemical Co., New England Biolabs or International Biotechnologies, Inc. and were used according to the manufacturers' instructions. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) were obtained from IBI and BRL, respectively. The labeled nucleotides [α-^{32}P]dCTP (800 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from DuPont Canada Inc. Double-stranded and single-stranded DNA were sequenced by the dideoxyribonucleotide chain termination technique [15], using the T7 sequencing kit from Pharmacia. The following oligonucleotides were synthesized: RHLYSS (5′-GGTCGAGCTTCTGGG-3′), RHLYS1 (5′-GACCGACAAGACACAGG-3′) and RHGLTX (5′-CCATGGCGGGCAGTG-3′).

2.2. Bacterial cell growth and transformation

E. coli DH5α (F$^-$ *recA1 endA1 gyrA96 thi*-1 *hsdR17* (R_K^-, M_K^+) *supE44 relA*1 λ$^-$) [16] was used to maintain and propagate plasmids. *E. coli* strains XA103 (F$^-$ Δ(*lac-pro*) *gyrA*96 *rpoB metB argE(Am) ara supF*) [17] and PAL3103KΔRS (XA103; Δ*lysS::kan, srl*-300::Tn*10 recA56*) [18] were used in complementation assays. *E. coli* and *R. meliloti* A2 were grown respectively on LB medium [19] and YMB medium [20,21]. Ampicillin was added to a final concentration of 100 μg/ml. *E. coli* cells were transformed according to Chung et al. [22]. Plasmid DNA was purified according to Brun et al. [23].

2.3. Plasmid construction

Chromosomal DNA was isolated from *R. meliloti* A2 as previously described [14]. Vector pTrc99b (Pharmacia) was used to express the putative *lysS* gene. This vector contains the IPTG-inducible *trc* promoter and the *lacI*^{q} gene, which increases the amount of *lac* repressor in the cell [24]. A homologous radiolabeled *Eco*RV-*Sal*I DNA fragment was used as a Southern hybridization probe [19] to identify a 2.5 kb *Bam*HI fragment containing the putative *lysS* gene (Fig. 1), which was inserted into the unique *Bam*HI site of pTrc99b. The recombinant plasmids were mapped to determine the fragment orientation relative to the *trc* promoter. In pYGM305, the *trc* promoter and *lysS* are oriented in the same direction; this plasmid was digested to delete the upstream *Bam*HI-*Stu*I region of the fragment which is not included in *lysS* (Fig. 1), to produce pYGM306. pYGM307 contains *lysS* inserted in the opposite orientation with respect to the *trc* promoter, and was used as control. Expression of proteins encoded by recombinant plasmids in *E. coli* was monitored as described by Amann et al. [24].

2.4. RT-PCR amplification of lysS mRNA

RNA was extracted from *R. meliloti* A2 according to Gray et al. [25]. mRNA sequences were amplified by PCR according to Ausubel et al. [19]. *R. meliloti* A2 total RNA (10 μg) was mixed with 4 pmol of

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RHLYSS (a 15-mer oligonucleotide starting 70 nt after the beginning of *lysS*) (Fig. 1) in 20 μl of reverse transcription buffer (50 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 80 mM KCl). The mixture was heated for 5 min at 65°C and cooled on ice for 10 min. Reverse transcription buffer (20 μl) containing 2 mM dNTP, 10 mM DTT and 14 U of AMV reverse transcriptase was added and the extension reaction mixture was incubated for 60 min at 45°C before stopping the reaction by the addition of 450 μl of TE buffer pH 8.0. Aliquots (10 μl) of this reaction mixture were used as a template in a polymerase chain reaction using $Vent_R$ (exo-) DNA polymerase and the appropriate oligonucleotides. Samples were analyzed by electrophoresis in a 1.5% agarose gel, stained with ethidium bromide, transferred onto a nylon membrane (Amersham Hybond-N) and covalently linked by UV irradiation. Hybridization was performed according to Ausubel et al. [19] using 5′-radiolabeled RHLYS1 as a probe.

2.5. GenBank accession number and computer analysis

The sequence reported in this study has been deposited in the GenBank data base with the accession number M27211, which initially contained only *R. meliloti gltX* but was recently updated to include the sequence of the 4119 nucleotides of the *Sal*I-*Bam*HI fragment shown in Fig. 1. The programs of the Genetics Computer Group [26] were used for nucleotide and amino acid sequence analysis. In particular, Wordsearch and Tfasta programs were used to scan the GenBank (version 8.5) database, and PileUp was used to align multiple amino acid sequences.

3. Results

3.1. Nucleotide sequence of an ORF immediately downstream of R. meliloti gltX

We previously reported the cloning and sequencing of the *R. meliloti gltX* gene encoding glutamyl-tRNA synthetase [14]. We sequenced the region downstream of *gltX*, and identified an incomplete ORF of at least 469 codons, which revealed a high degree of sequence similarity with the *lysS* and *lysU* genes encoding the lysyl-tRNA synthetases of *E. coli*. In order to clone the entire ORF, we identified by Southern hybridization (data not shown) a 2.5 kb *Bam*HI fragment that contains the 3′-end region of the putative *lysS* gene (see Fig. 1) and cloned it into the unique *Bam*HI site of pTrc99b. Hybridization of this purified, cloned fragment to Southern transfers of *R. meliloti* chromosomal DNA digested with various restriction endonucleases (results not shown) indicated that no rearrangements occurred during the cloning procedures.

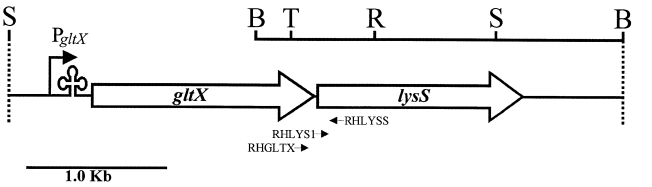

Fig. 1. Physical map of the *R. meliloti gltX-lysS* genes with their flanking region. The cloned *R. meliloti* 2.5 kb *Bam*HI DNA fragment containing the *lysS* gene is presented together with the positions of *gltX* and *lysS*. The homologous radiolabeled *Eco*RV-*Sal*I DNA fragment was used as the hybridization probe to clone the entire *lysS* gene. A cloverleaf-like secondary structure was identified between the putative promoter for *gltX* (P_{gltX}; see Fig. 2) and *gltX*. Oligonucleotides used for RT-PCR are indicated by arrows. Recognition sites for restriction endonucleases are identified as follows: B, *Bam*HI; R, *Eco*RV; S, *Sal*I; T, *Stu*I.

The complete nucleotide sequence of the ORF downstream of *gltX* revealed that it encodes a polypeptide of 499 amino acid residues with a calculated molecular mass of 62 907 Da. The putative AUG initiator codon of this gene is located 10 nucleotides downstream of the UAA stop codon of *gltX*, suggesting that the two genes are co-transcribed (Fig. 2A). Analysis of the nucleotide sequence upstream of the ORF, including *gltX*, did not reveal the presence of a sequence similar to the consensus for *E. coli* −35/−10 promoters. However, one such sequence was found upstream of *gltX* (Fig. 2A). The sequence between this putative *gltX* promoter and the *gltX* initiation codon was predicted to form a putative clover-

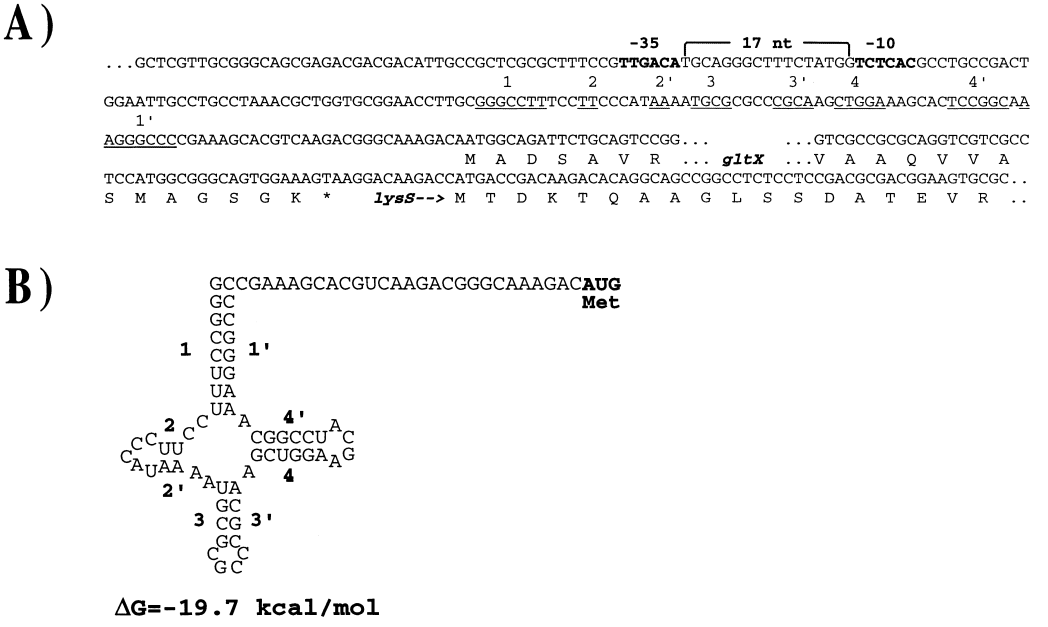

Fig. 2. Intergenic sequence between *R. meliloti gltX* and *lysS*, and putative secondary structure of the 5′ leader region of the *gltX-lysS* mRNA. A: The amino acid sequence deduced from the nucleotide sequence for the beginning of *gltX* and *lysS* is given in single-letter code. The putative promoter of *gltX* is shown in bold. The underlined nucleotides correspond to the stems, numbered from 1 to 4, of the putative cloverleaf-like secondary structure (B) found upstream of and near *gltX*.

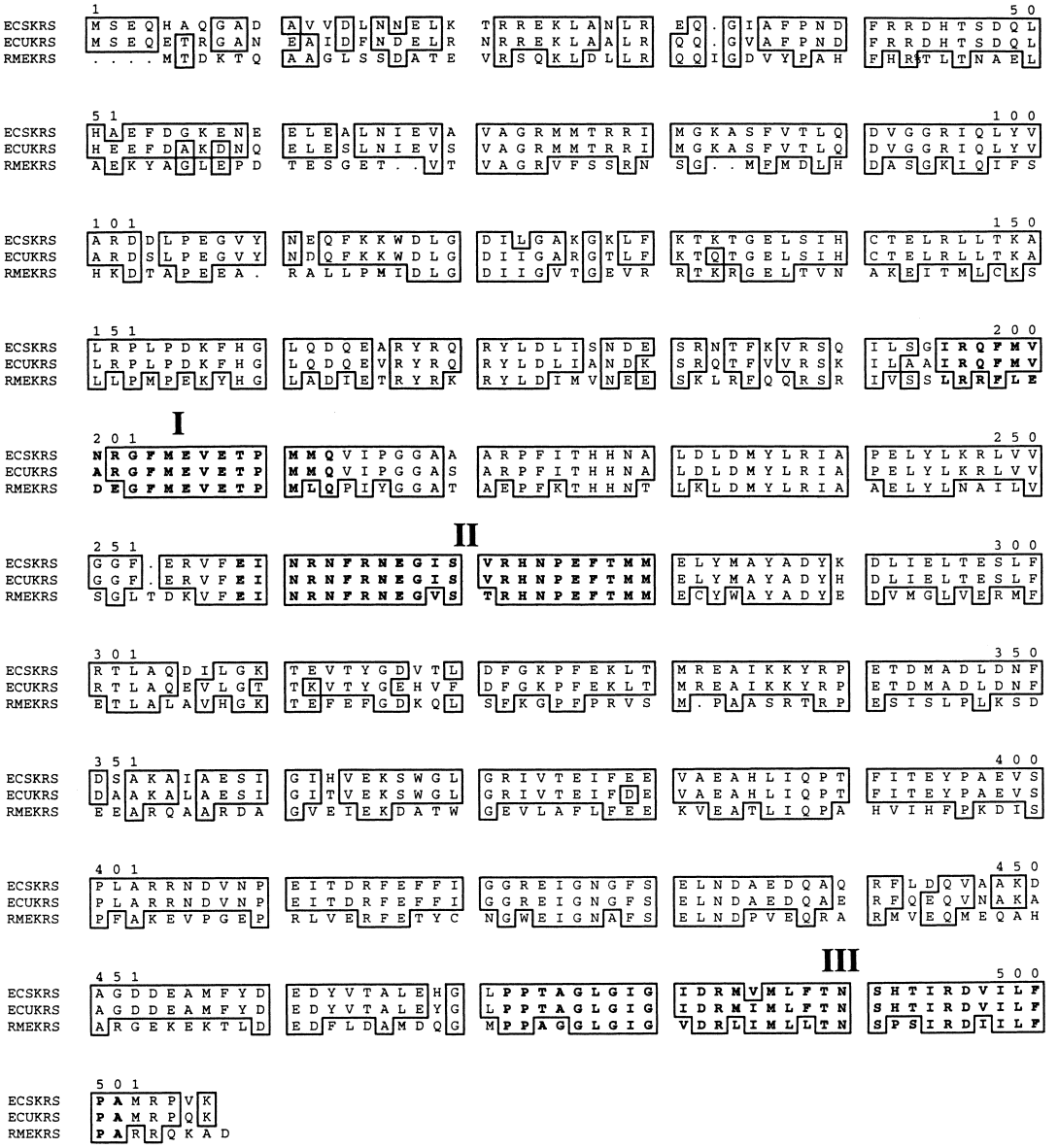

Fig. 3. Alignment of the deduced amino acid sequences of *R. meliloti* LysRS and those of the two *E. coli* LysRSs. The three conserved motifs (I, II and III) of the class II aaRSs are shown in bold. The sequences are identified as follows: *E. coli lysS*: ECSKRS [41]; *E. coli lysU*: ECUKRS [42]; *R. meliloti lysS*: RMEKRS.

leaf-like secondary structure ($\Delta G = -19.7$ kcal/mol) [27] (Fig. 2B).

Computer analysis of the deduced amino acid sequence of the protein encoded by the *R. meliloti* ORF showed higher similarities with the two *E. coli* LysRSs than with any other aaRS (Fig. 3). Moreover, the predicted sequence of this protein is about 47% identical to that of the two *E. coli* LysRSs and less than 30% identical to those of AspRS and AsnRS from *E. coli*, which together with LysRS constitute a subclass of class II aaRSs [3,28]. The three conserved motifs unique to class II aaRSs are present in the *R. meliloti* ORF. Although these motifs are known to be involved in the catalytic activity [2], their respective sequences are not highly conserved between aaRSs of different specificities [3]. However, the respective sequences of these motifs are highly conserved in aaRSs of the same specificity, enabling their use in identifying unknown class II aaRSs. The fact that the sequences of the three motifs found in the protein encoded by the *R. meliloti* ORF immediately downstream of *gltX* show the highest identity (~70%) with those of LysRSs suggests that this ORF encodes a LysRS.

3.2. Expression of the putative *R. meliloti lysS* gene in *E. coli* and complementation of a *lysS* mutation

To demonstrate that the ORF downstream of *R. meliloti gltX* encodes a functional LysRS, it was expressed in *E. coli* and its ability to complement a mutant of the *lysS* gene was verified. Since no promoter was found in front of the putative *R. meliloti lysS*, this gene was expressed in *E. coli* using the inducible *trc* promoter. Two plasmids, pYGM306 and pYGM307, were constructed (see Section 2) and introduced into *E. coli*. The protein content of strain PAL3103KΔRS bearing different plasmids was examined by Coomassie blue-stained SDS polyacrylamide gels (Fig. 4). A band correspond-

ing to a polypeptide of apparent molecular mass 62 kDa was detected in IPTG-induced cells containing pYGM306, which contains the insert in the right orientation (Fig. 4, lane 1). This value is consistent with the molecular mass calculated from the deduced amino acid sequence of LysRS (62 907 Da). The same band was not detected in cells containing pYGM307, in which *lysS* is inserted in the wrong orientation (Fig. 4, lanes 3 and 4). Finally, the band was also detected in cells containing pYGM306 even when they were not induced with IPTG (Fig. 4, lane 2), suggesting that the control of the *trc* promoter was leaky.

The function of the putative *R. meliloti lysS* gene was tested by complementation assays using *E. coli* strain PAL3103KΔRS. This strain has a cold-sensitive phenotype resulting from the disruption of *lysS* and grows very slowly at 25°C [18]. The slow growth of this strain was complemented efficiently by the recombinant plasmid pYGM306 in the presence of 1 mM IPTG, and only partially in its absence. In contrast, this phenotype was not complemented by pYMG307. These results show that the ORF immediately downstream of *gltX* in *R. meliloti* encodes a functional LysRS.

3.3. Co-transcription of gltX and lysS in R. meliloti

The initiation codon (AUG) of *lysS* is situated 10 nt downstream of the termination codon (UAA) of *gltX*, suggesting that these two genes are co-transcribed. To verify this co-regulation, the RNA extracted from *R. meliloti* A2 was reverse-transcribed using RHLYSS, an oligonucleotide situated 70 nt downstream of the beginning of *lysS* (Fig. 1). Two segments of the corresponding cDNA were amplified by the polymerase chain reaction, using RHLYSS and either an oligonucleotide situated at the beginning of *lysS* (RHLYS1) or an oligonucleotide situated near the end of *gltX* (RHGLTX) (Fig. 5A). The positions of the sequences corresponding to these oligonucleotides are indicated in Fig. 1. The identity of these amplicons was confirmed by their hybridization with radiolabeled RHLYS1 (Fig. 5B). The resulting amplicons of the *gltX-lysS* and *lysS* regions were of the expected size of 113 nt and 68 nt, respectively (Fig. 5, lanes 1 and 3). These amplicons were not observed when the reverse transcription reaction was omitted (Fig. 5, lanes 2 and 4), indicating that they did not result from the amplification of contaminating chromosomal DNA in the purified RNA preparation. These results show that *gltX* and *lysS* are co-transcribed in vivo. For unknown reasons, in vivo transcripts of *gltX* and/or *lysS* could not be detected by Northern hybridization or primer extension using several different primers.

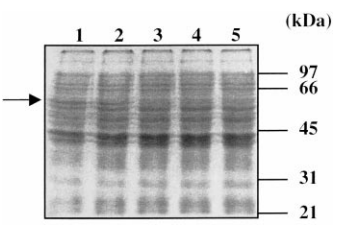

Fig. 4. Expression of the *R. meliloti lysS* gene in *E. coli*. Total protein extracts of *E. coli* PAL3103KΔRS harboring the indicated plasmids were separated by SDS-PAGE on a 10% gel. Lanes 1 and 2: PAL3103KΔRS bearing plasmid pYGM306. Lanes 3 and 4: PAL3103KΔRS bearing plasmid pYGM307. The extracts analyzed in lanes 1 and 3 were from cells induced with 1 mM IPTG. Lane 5: PAL3103KΔRS without plasmid. The arrow indicates the position of a 62 kDa polypeptide.

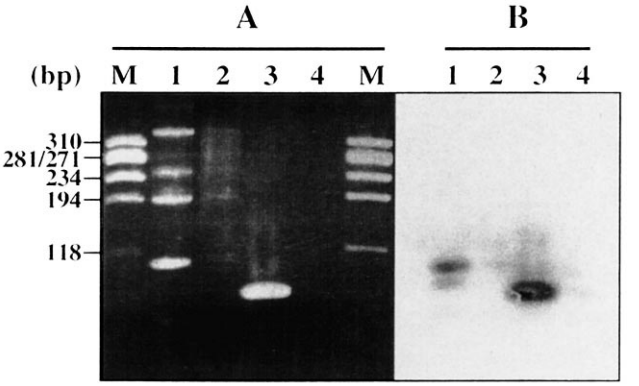

Fig. 5. Detection by RT-PCR of a transcript overlapping *R. meliloti gltX* and *lysS*. A: cDNA obtained by reverse transcription of total *R. meliloti* A2 RNA was amplified using an oligonucleotide situated 70 nt after the beginning of *lysS* (RHLYSS) and another one situated either at the end of *gltX* (RHGLTX; lane 1) or at the beginning of *lysS* (RHLYS1; lane 3) (see Fig. 1). Controls were performed using the same oligonucleotides on RNA prior to reverse transcription (lanes 2 and 4). B: Samples were transferred onto a nylon membrane and hybridized with radiolabeled RHLYS1 to confirm their identity. M, *Hae*III-digested ϕX174 DNA.

4. Discussion

We report the identification, cloning, and the complete nucleotide sequence of the *R. meliloti lysS* gene encoding the lysyl-tRNA synthetase and its co-transcription with the *gltX* gene encoding the glutamyl-tRNA synthetase. The deduced amino acid sequence of the *R. meliloti* LysRS contains the three motifs conserved among the class II aaRSs and shows strong similarity to the two LysRSs of *E. coli*. The overexpression of the *R. meliloti lysS* gene in *E. coli* functionally complemented a mutation in the latter's *lysS* and was correlated with the presence of a polypeptide of 62 kDa.

The 5′-flanking region of *R. meliloti gltX* contains two hexamers, TTGACA-15 nt-GG-TCTCAC-G (Fig. 2A), which have a total of nine identities with the consensus sequence of the −35/−10 promoters of *E. coli* (TTGACA-17 nt-TATAAT) [29]. The two hexamers also have several identities with the two characterized vegetative promoters of *R. meliloti* (TTGACC-15 nt-GG-GAGAAT-G) [30]. This putative promoter of the *gltX-lysS* operon would specify the transcription of a 5′ leader of about 125 nt, most of which can be folded in a putative cloverleaf-like secondary structure ($\Delta G = -19.7$ kcal/mol) (Fig. 2B). It is known that mRNA secondary structure influences the expression of *E. coli thrS* and of many Gram-positive aaRSs genes [8,31]. In *E. coli*, the 5′ leader of *gltX* mRNA can adopt a stable secondary structure (close to the ribosome binding site) that is similar to the anticodon and part of the dihydroU stem and loop of $tRNA^{Glu}$, and could be implicated in mRNA processing upstream of the *E. coli gltX* gene [32]. The function of the putative secondary structure upstream of the *R. meliloti gltX-lysS* bicistronic mRNA has yet to be determined; it could be involved in its stabilization, as shown for secondary structures at the 5′ end of other mRNAs [33], or it could be a recognition signal for specific RNases.

Several bacterial aaRS genes are adjacent to and, at least in some cases, probably co-regulated with other protein biosynthesis genes [11]; for instance, in several organisms, the promoter upstream of *gltX* also drives the transcription of another protein biosynthesis gene. In *B. subtilis* and *S. xylosus*, the *gltX* gene is co-transcribed with *cysE* and *cysS* encoding the serine acetyltransferase and the cysteinyl-tRNA synthetase respectively [6,7]. However, the expression of *cysE* and *cysS* is partly uncoupled from that of *gltX* by the presence of a $tRNA^{Cys}$-dependent transcriptional attenuator between *gltX* and *cysE* and by a specific cleavage of the long *gltX-cysE-cysS* transcript in the same region [34]. In higher eukaryotes, the largest polypeptide of the multisynthetase complex is a multifunctional aminoacyl-tRNA synthetase endowed with glutamyl-tRNA synthetase and prolyl-tRNA synthetase activities in its N-terminal and C-terminal halves, respectively [35]. A low molecular weight form of prolyl-tRNA synthetase has also been found in *Drosophila* and in rat salivary glands. Messenger RNAs encoding the full length glutamyl-prolyl-tRNA synthetase and just the prolyl-tRNA synthetase are controlled by two independent promoters in *Drosophila* [36,37]. It was recently observed that the initiation codon of *Azospirillum brasilense cysS* gene is situated 34 nt from the termination codon of *gltX* [38], but the possibility that they are co-transcribed was not tested. Furthermore, *gltX* in many organisms is adjacent to a tRNA operon [32,39,40]. These gene arrangements may have been selected to optimize protein biosynthesis.

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