Overproduction of the *Bacillus subtilis* glutamyl-tRNA synthetase in its host and its toxicity to *Escherichia coli*

Martin Pelchat, Lucille Lacoste, Fu Yang, and Jacques Lapointe

Abstract: The *Bacillus subtilis* glutamyl-tRNA synthetase (GluRS), encoded by the *gltX* gene, aminoacylates its homologous $tRNA^{Glu}$ and $tRNA^{Gln}$ with glutamate. This gene was cloned with its σ^A promoter and a downstream region including a ρ-independent terminator in the shuttle vector pRB394 for *Escherichia coli* and *B. subtilis*. Transformation of *B. subtilis* with this recombinant plasmid (pMP411) led to a 30-fold increase of glutamyl-tRNA synthetase specific activity in crude extracts. Transformation of *E. coli* with this plasmid gave no recombinants, but transformation with plasmids bearing an altered *gltX* was successful. These results indicate that the presence of *B. subtilis* glutamyl-tRNA synthetase is lethal for *E. coli*, probably because this enzyme glutamylates $tRNA_1^{Gln}$ in vivo as it does in vitro.

Key words: glutamyl-tRNA synthetase overproduction, *Bacillus subtilis*, toxicity, *Escherichia coli*.

Résumé : La glutamyl-ARNt synthétase (GluRS) de *Bacillus subtilis*, codée par le gène *gltX*, aminoacyle l'$ARNt^{Glu}$ et l'$ARNt^{Gln}$ avec le glutamate. Ce gène a été cloné avec son promoteur σ^A et une région en amont incluant un terminateur ρ-indépendant dans le vecteur navette pRB394 pour *Escherichia coli* et *B. subtilis*. La transformation de *B. subtilis* avec ce plasmide recombinant (pMP411) mène à une augmentation de 30 fois de l'activité spécifique en glutamyl-ARNt synthétase dans l'extrait cellulaire. La transformation d'*E. coli* avec ce plasmide ne donne aucun recombinant, cependant sa transformation avec des plasmides incluant le gène *gltX* altéré permet la croissance. Ces résultats montrent que la présence de la glutamyl-ARNt synthétase de *B. subtilis* est létale pour *E. coli*, probablement à cause de la glutamylation de l'$ARNt_1^{Gln}$ in vivo, comme observé in vitro.

Mots clés : surproduction de la glutamyl-ARNt synthétase, *Bacillus subtilis*, toxicité, *Escherichia coli*.

Aminoacyl-tRNA synthetases (aaRS) play an important role in protein biosynthesis by catalyzing the accurate charging of tRNAs with amino acids. There are significant functional similarities among several aaRSs of *Bacillus* species and the corresponding ones in *E. coli*, as indicated by several examples of complementation by *Bacillus* synthetases of *E. coli* hosts whose corresponding synthetase is temperature-sensitive (Barker 1982; Brakhage et al. 1989; Brand and Fersht 1986; Gagnon et al. 1994; Jones et al. 1986). One of the most significant differences between aaRSs of Gram-positive bacteria and those of *E. coli* is the lack, in the former, of an enzyme capable of charging a tRNA with glutamine (Schön et al. 1988; Wilcox and Nirenberg 1968; Wilcox 1969*a*, 1969*b*). In those organisms $tRNA^{Gln}$ is first charged with glutamate, which is subsequently converted to glutamine by an amidotransferase while it is attached to tRNA. In *Bacillus subtilis*, a single glutamyl-tRNA synthetase (GluRS) is responsible for aminoacylating both $tRNA^{Glu}$ and $tRNA^{Gln}$ with glutamate (Lapointe et al. 1986). In vitro this enzyme can efficiently attach glutamate to *E. coli* $tRNA_1^{Gln}$ but not to *E. coli* $tRNA^{Glu}$ or $tRNA_2^{Gln}$.

As previous attempts at cloning the *B. subtilis gltX* gene (encoding GluRS) on a single DNA fragment in *E. coli* were unsuccessful (Breton 1990), it was cloned on two fragments (Breton et al. 1990). On the first (pLQB205), the $gltX^A$ promoter is immediately followed by the 5′-terminal part of *gltX*. On the second (pLQB206), the 3′-terminal part is followed by the genes *cysE* (encoding the serine acetyltransferase) and the beginning of *cysS* (encoding the cysteinyl-tRNA synthetase). Owing to low transformation frequencies using *B. subtilis* as the primary host, we conducted the initial cloning step with an *E. coli – B. subtilis* shuttle vector (pRB394; Brückner 1992) in *E. coli* and then transferred the recombinant plasmid for the last step to *B. subtilis*. We began by forming plasmids pMP205 and pMP206 by transferring the inserts of pLQB205 and pLQB206 in the vector pRB394 and pHM4, respectively. By combining fragments of pMP205 and pMP206, we constructed pMP211, which contains the *gltX* gene interrupted by the 1-kb *Sac*II segment shared by pMP206 and pMP205 (Fig. 1). Stable transformants of *E. coli* DH5α carrying pMP211 were obtained. By deleting this *Sac*II fragment, plasmid pMP411 containing the intact *gltX* gene, its σ^A promoter with a 77-bp region upstream from the –35 hexamer, and a 1.3-kbp downstream region, including a ρ-independent terminator, was constructed in vitro (Fig. 1) and was used to transform *B. subtilis* 168p.

SDS–PAGE analysis of the clear supernatant of a cell extract of *B. subtilis* 168p carrying pMP411 (about 100 copies/cell, as

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Fig. 1. Construction of pMP411. The *Hin*dIII fragment coming from pLQB205 (A), containing the beginning of *gltX* and its promoter, was cloned into pRB394 (Brückner 1992) to form pMP205 (B). The *Sal*I–*Eco*RI fragment of pLQB206 (A), containing the end of *gltX*, the intergenic region, *cysE*, and the beginning of *cysS*, was cloned into pHM4 to give pMP206 (B). By combining these two fragments to give pMP211 (B) and then deleting the repeated region, the plasmid pMP411 was formed (B). All plasmids were cloned in *E. coli*, except for pMP411, which was cloned in *B. subtilis*. These plasmids (except pMP206) contain both *E. coli* and *B. subtilis* replication origins. The *cat* gene is under the control of the *gltX* promoter. E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; L, *Sal*I; S, *Sac*II; V, *Eco*RV; *bla*, Ap^r; *cat*, Cm^r; *cysE*, SAT; *cysS*, CysRS; *erm*, Em^r; *gltX*, GluRS; *neo*, Km^r and Nm^r; T, terminator.

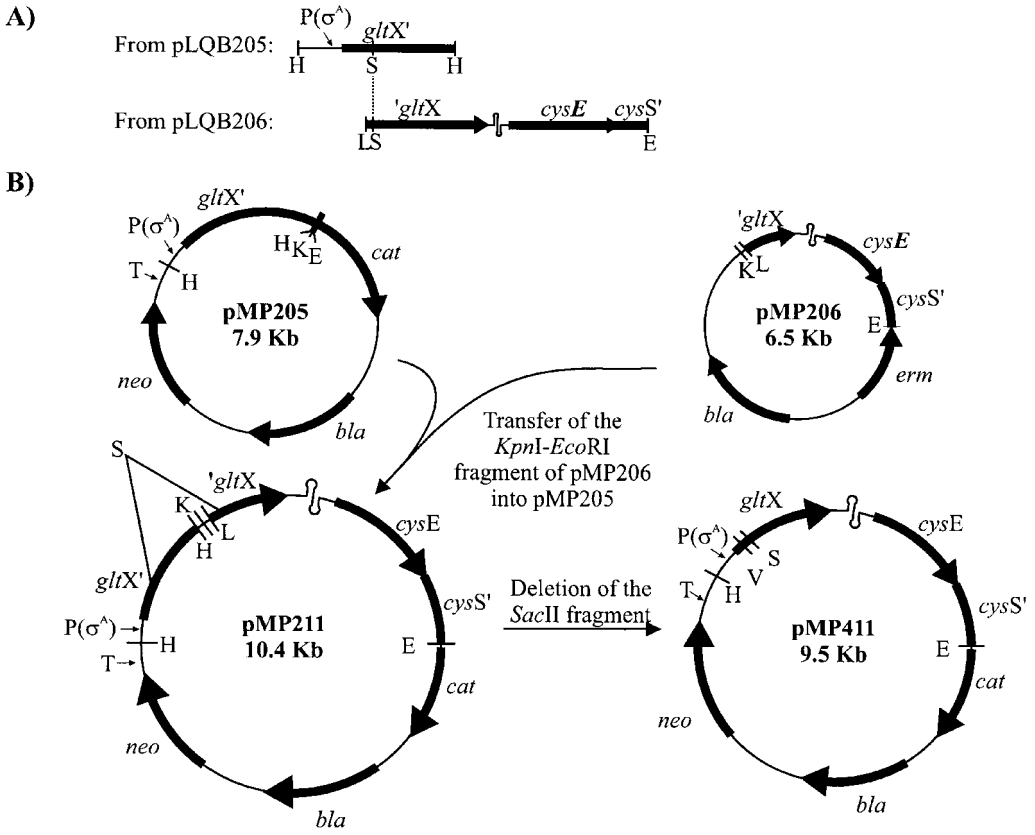


Fig. 2. Overproduction of *B. subtilis* GluRS by *B. subtilis* 168p(pMP411) revealed by SDS–PAGE. *Bacillus subtilis* 168p and *B. subtilis* 168p(pMP411) cells were harvested at 0.8 A_{600} during exponential growth in Luria–Bertani medium and lysed by incubation with lysozyme followed by sonication. Proteins were analyzed by SDS–PAGE. Lane A, Molecular mass markers; lane B, 6 µg *E. coli* GluRS (53.8 kDa); lane C, 30 µg protein of *B. subtilis* 168p cell extract; lane D, 90 µg protein of *B. subtilis* 168p cell extract; lane E, 30 µg protein of *B. subtilis* 168p(pMP411) cell extract; lane F, 90 µg protein of *B. subtilis* 168p(pMP411) cell extract.

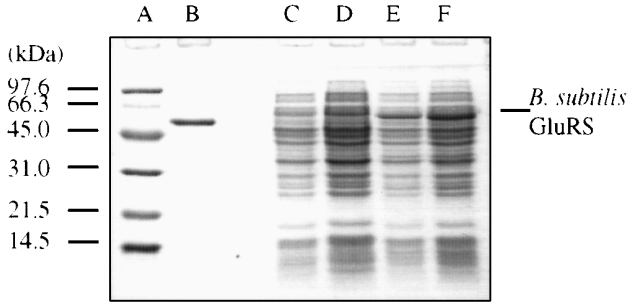
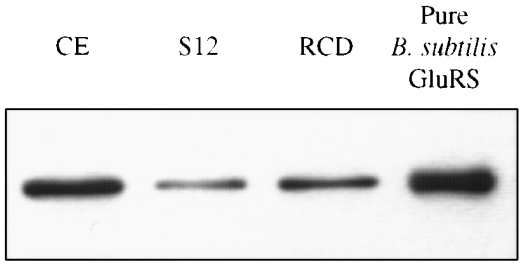


Fig. 3. Western Blot analysis of the GluRS content of cell debris and supernatant from *B. subtilis* 168p(pMP411). CE, crude extract; S12, soluble fraction at 12 000 × *g*; RCD, resuspended cell debris. Quantification of these signals by microdensitometry shows that about 30 and 70% of the GluRS protein present in CE are found in S12 and RCD, respectively.



measured by dot blot hybridization) reveals the overproduction of a polypeptide of about 55 kDa, which is not overproduced by the same cells in the absence of pMP411 (Fig. 2). To confirm that this polypeptide is *B. subtilis* GluRS, whose molecular mass is 55.7 kDa (Breton et al. 1990), we measured GluRS aminoacylation activity (Lin et al. 1992) of both extracts and found a 30-fold higher specific activity in cells car-

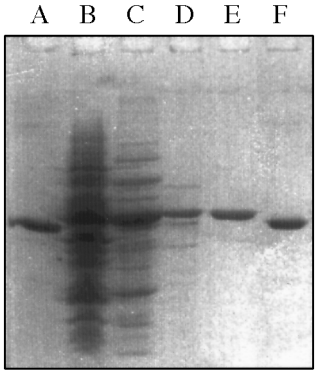
Table 1. Purification of the *B. subtilis* glutamyl-tRNA synthetase from an overproducing strain.

	Protein			Enzyme			
Fraction	Volume of fraction (mL)	Concn. (mg/mL)	Total amount (mg)	Specific activity (units/mg)	Total amount (units)[a]	Yield (%)	Purification factor (*n*-fold)
Cell extract	35	53	1 855	14	26 600	100	1.0
Two-phase system	39	15	585	12	7 300	27	0.9
Q-sepharose	20	2	40	153	6 100	23	10.9
Blue-sepharose	10	3	30	93	2 800	10.5	6.7
Hydroxyapatite	10	2	20	160	3 200	12	11.4

Note: The enzyme was purified from 11.6 g (wet weight) of *B. subtilis* 168p(pMP411) cells harvested during exponential growth in Luria–Bertani medium containing 5 μg chloramphenicol/mL.

[a]One unit of GluRS catalyzes the formation of 1 nmol of Glu-tRNA/min at 37°C under the conditions described by Lin et al. (1992).

Fig. 4. SDS–PAGE analysis of the fractions enriched for GluRS at various steps of its purification from *B. subtilis* 168p(pMP411). Lane A, 12 μg of *E. coli* GluRS; lane B, 400 μg of *B. subtilis* 168p(pMP411) cell extract; lane C, 35 μg of the Q-Sepharose fraction; lane D, 25 μg of the Blue-Sepharose fraction; lane E, 12 μg of the hydroxyapatite fraction; lane F, 12 μg of *E. coli* GluRS.



rying pMP411. As SDS–PAGE revealed the presence of a large amount of a polypetide of about 55 kDa in the pellet obtained by centrifugation of the crude extract of *B. subtilis* 168p(pMP411) but not in that from *B. subtilis* 168p (data not shown), we measured the amount of soluble and insoluble overproduced GluRS protein in the crude extract, the clear supernatant, and the resuspended cell debris by Western blot analysis and microdensitometry, using anti-*B. subtilis* GluRS antiserum. This analysis shows that the cell debris contained twice as much GluRS protein as the soluble supernatant (Fig. 3), but this protein has no aminoacylation activity and is not soluble in the absence of detergent, apparently because it is in inclusion bodies. We concluded that the total overproduction of GluRS is about 100-fold, as the cell debris in inclusion bodies contained about two-thirds of the total GluRS present in cell extracts. Attempts to lower the proportion of inclusions bodies by lowering or elevating the growth temperature were unsucessful. Furthermore, as pMP411 is present at about 100 copies/cell in *B. subtilis* 168p, we concluded that the observed GluRS overproduction reflects *gltX* gene dosage and suggests that *B. subtilis* GluRS does not regulate its own biosynthesis. We also found that the 30-fold increase in GluRS specific activity does not reduce the growth rate of *B. subtilis*, since these cells transformed with pM205 (which does not contain an intact *gltX*) grow at the same rate as those transformed with pMP411 (data not shown). We purified the overproduced GluRS as described by Proulx and Lapointe (1985) with an additional step, a chomatography on hydroxyapatite. Table 1 summarizes the purification of GluRS from *B. subtilis* 168p(pMP411). By this procedure, we obtained 15 mg of enzyme from 13 g of wet cells, with a 50-fold purification. This GluRS was pure to homogeneity as shown by SDS–PAGE (Fig. 4). It has a similar specific activity, in the aminoacylation reaction, as that of the enzyme purified from a wild-type strain (Proulx et al. 1983; Proulx and Lapointe 1985).

To determine if the previous failure at cloning the complete *gltX* gene of *B. subtilis* in *E. coli* was due to the toxicity of *B. subtilis* GluRS to *E. coli*, we deleted a part of the *gltX* gene carried by pMP411, without destroying its reading frame; the resulting plasmid, pMP400, lacks the 105-bp *Eco*RV–*Sac*II segment encoding the ^{91}I–^{125}R region of GluRS, which is a substantial part of the tRNA acceptor stem binding domain (For alignments of this region with *Thermus thermophilus* GluRS and *E. coli* GlnRS, whose three-dimensional structures are known (Nureki et al. 1995; Rould et al. 1989), see Liu et al. (1995).) By transforming *E. coli* DH5α with pMP400, pMP211, and pMP411, we found about 10^5 transformants/μg DNA with pMP400 and pMP211 (containing, respectively, a deletion or an insertion in the *gltX* gene), but none with pMP411 (containing an intact *gltX* gene), which proves the toxicity of this *B. subtilis* protein to *E. coli*. We used thermosensitive *E. coli* JP1449 (Russell and Pittard 1971; Lapointe and Delcuve 1975), whose altered *gltX* gene encoded a GluRS whose activity was undetectable in cell extracts, to test the activity of the product of the above-mentioned altered *gltX* gene. Extracts of JP1449 transformed with pMP400 or pMP211 had no detectable GluRS activity (results not shown). Furthermore, no *E. coli* transformants were obtained with plasmids carrying *B. subtilis gltX* under the control of the following promoters, in the absence of an inducer: (*i*) the *trc* promoter repressed by a high level of *lac* repressor owing to the presence of the *lacI*^{q} gene (Amann et al. 1988), (*ii*) the $\lambda p_R p_L$ promoters repressed by the temperature-sensitive repressor cI857, and (*iii*) a T7 RNA polymerase promoter repressed by the T7 lyzozyme (Studier et al. 1990).

The *B. subtilis* GluRS aminoacylates with glutamate both tRNA^{Glu} and tRNA^{Gln} in *B. subtilis* (Lapointe et al. 1986); the resulting mischarged Glu-tRNA^{Gln} is then transformed into the correct Gln-tRNA^{Gln} by a specific amidotransferase using

ATP and a donor of amino group such as glutamine or NH_3 (Wilcox and Nirenberg 1968). Because *E. coli* does not contain such an amidotransferase, Glu-tRNA^{Gln} may lead to the production of altered proteins. The observed lethality of the *B. subtilis* GluRS to *E. coli* is then probably due to the misacylation of tRNA$_1^{Gln}$ of *E. coli* with glutamate, as observed in vitro (Lapointe et al. 1986). Cells that synthesize Gln-tRNA^{Gln} via a mischarged Glu-tRNA^{Gln} intermediate could avoid the misincorporation of glutamate into proteins by a direct transfer of Glu-tRNA^{Gln} from GluRS to the specific amidotransferase or by having an elongation factor EF-Tu, which does not recognize the mischarged tRNA^{Gln}, as is the case for misacylated tRNA^{Selenocysteine} (reviewed by Baron and Böck 1995). The second strategy appears to have been chosen by chloroplasts of *Pisum sativum*, as its misacylated Glu-tRNA^{Gln} does not interact in vitro with its homologous EF-Tu (Stanzel et al. 1994). The fact that *B. subtilis* 168p(pMP411), which overproduces GluRS, grows normally is consistent with this model.

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