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Growth Inhibition of *Escherichia coli* during Heterologous Expression of *Bacillus subtilis* Glutamyl-tRNA Synthetase that Catalyzes the Formation of Mischarged Glutamyl-$tRNA_1^{Gln}$

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It is known that *Bacillus subtilis* glutamyl-tRNA synthetase (GluRS) mischarges *E. coli* $tRNA_1^{Gln}$ with glutamate *in vitro*. It has also been established that the expression of *B. subtilis* GluRS in *Escherichia coli* results in the death of the host cell. To ascertain whether *E. coli* growth inhibition caused by *B. subtilis* GluRS synthesis is a consequence of Glu-$tRNA_1^{Gln}$ formation, we constructed an *in vivo* test system, in which *B. subtilis* GluRS gene expression is controlled by IPTG. Such a system permits the investigation of factors affecting *E. coli* growth. Expression of *E. coli* glutaminyl-tRNA synthetase (GlnRS) also ameliorated growth inhibition, presumably by competitively preventing $tRNA_1^{Gln}$ misacylation. However, when amounts of up to 10 mM L-glutamine, the cognate amino acid for acylation of $tRNA_1^{Gln}$, were added to the growth medium, cell growth was unaffected. Overexpression of the *B. subtilis gatCAB* gene encoding Glu-$tRNA^{Gln}$ amidotransferase (Glu-AdT) rescued cells from toxic effects caused by the formation of the mischarging GluRS. This result indicates that *B. subtilis* Glu-AdT recognizes the mischarged *E. coli* Glu-$tRNA_1^{Gln}$, and converts it to the cognate Gln-$tRNA_1^{Gln}$ species. *B. subtilis* GluRS-dependent Glu-$tRNA_1^{Gln}$ formation may cause growth inhibition in the transformed *E. coli* strain, possibly due to abnormal protein synthesis.

***Key words*:** misaminoacylation, $tRNA_1^{Gln}$, glutamyl-tRNA synthetase, Glu-$tRNA^{Gln}$ amidotransferase

Protein biosynthesis is a key process in the metabolism of all living organisms. In bacterial protein synthesis, aminoacyl-tRNA synthetases (aaRSs) initially catalyze the covalent attachment of the correct amino acid to the cognate tRNA. The elongation factor EF-Tu then carries aminoacyl-tRNA to the mRNA-programmed ribosome (Zuurmond *et al.*, 2000). Therefore, the fidelity of protein biosynthesis depends on the degree of correct aminoacylation of the tRNAs by respective aaRS. It is generally accepted that 20 kinds of aminoacyl-tRNA synthetase exist, for 20 standard amino acids (Meinnel *et al.*, 1995). The process of aminoacyl-tRNA formation, in which aaRS attaches the amino acid to the cognate tRNA, is known as a direct aminoacylation pathway (Fig. 1A). However, in most gram-positive bacteria, some gram-negative bacteria, archaea, and organelles in the eukaryotes, the glutaminyl-$tRNA^{Gln}$ (Gln-$tRNA^{Gln}$) is formed by the indirect aminoacylation pathway (Fig. 1B) via mischarged Glu-$tRNA^{Gln}$ (Wilcox and Nirenberg 1968; Schön *et al.*, 1988a; Schön *et al.*, 1988b; Curnow *et al.*, 1996; Gagnon *et al.*, 1996). In this process, the $tRNA^{Gln}$ is first misaminoacylated with glutamate by the nondiscriminating GluRS, resulting in the formation of Glu-$tRNA^{Gln}$. The glutamate on the mischarged Glu-$tRNA^{Gln}$ is then converted to glutamine by Glu-$tRNA^{Gln}$ amidotransferase (Glu-AdT) (Curnow *et al.*, 1997). Such an indirect aminoacylation pathway (transamidation) is known to be involved not only in the synthesis of glutaminyl-tRNA, but also in the synthesis of asparaginyl-tRNA in some bacteria and archaea (Becker *et al.*, 2000; Raczniak *et al.*, 2001). In this pathway, the mischarged amino acid on the tRNA is converted to the amino acid which corresponds to the tRNA anticodon. Following this conversion, the corrected aminoacyl-tRNA interacts with the EF-Tu for the correct decoding of the mRNA codon. In *Bacillus subtilis*, a single nondiscriminating GluRS is responsible for the aminoacylation of both $tRNA^{Gln}$ and $tRNA^{Glu}$ with glutamate. This enzyme also can mischarge *Escherichia*

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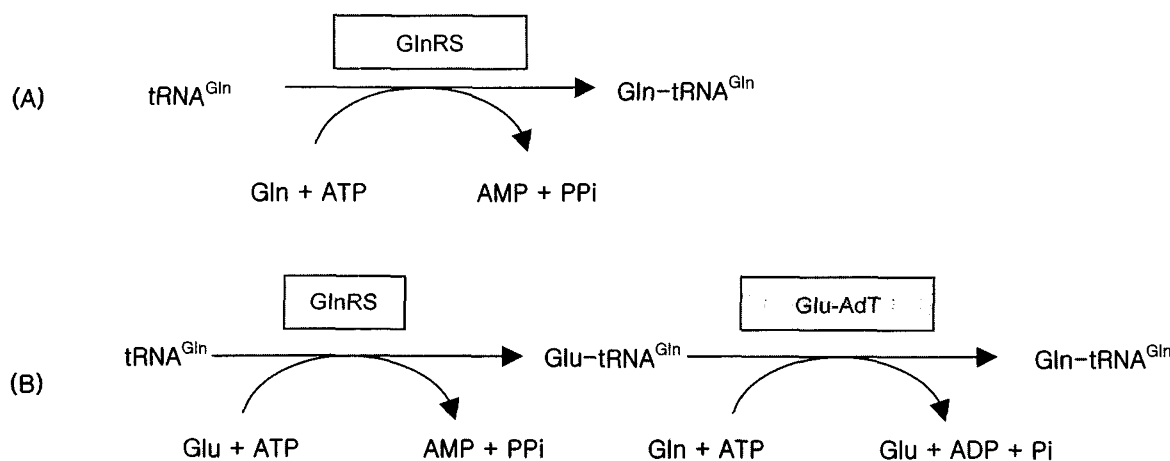

Fig. 1. Direct and indirect aminoacylation pathways of Gln-$tRNA^{Gln}$ formation. (A) Direct aminoacylation pathway: cytoplasm of eukaryotes, some gram (-) bacteria including *E. coli*. (B) Indirect aminoacylation pathway (transamidation, tRNA-dependent amino acid transformation): organelles in eukaryotes, archea, most gram (–) and gram (+) bacteria including *B. subtilis*.

Table 1. Plasmids used in this study

Plasmids	Characteristics	References
pKS-fAP	pBluescript II KS-*gatCAB* (*B. subtilis*)	Curnow *et al.*, 1997.
pTrc*gatCAB*	pTrc99a-*gatCAB* (*S. aureus*)	Namgoong *et al.*, 2001.
pKS-*glnS*	pBluescript II KS-*glnS* (*E. coli*)	Hong *et al.*, 1998.
pEBER	pET11a-*gltX* (*B. subtilis*)	Oh *et al.*, 2002.
pYBER	pACYC184-$_{T7}$*gltX* (*B. subtilis*)	in this study
pEBGF	pET22b-*gatCAB* (*B. subtilis*)	in this study

coli $tRNA_1^{Gln}$ with glutamate, but not *E. coli* $tRNA_2^{Gln}$ and $tRNA^{Glu}$ *in vitro* (Lapointe *et al.*, 1986). Early attempts to clone the *B. subtilis* GluRS gene (*gltX*) in *E. coli* were unsuccessful (Pelchat *et al.*, 1998). Overexpression of an intact *B. subtilis* GluRS was found to be lethal in *E. coli*. Therefore, it was assumed that, this toxicity was due to the formation of mischarged Glu-$tRNA^{Gln}$, which can be directly used in protein biosynthesis (Pelchat *et al.*, 1998).

In this study, we constructed a system which allows the control of *B. subtilis gltX* expression in *E. coli*. This verified that the reduced *E. coli* growth was due to the mischarging of $tRNA^{Gln}$ with glutamate, when *B. subtilis* GluRS was overexpressed in *E. coli*.

Materials and Methods

Bacterial strains and plasmids

E. coli DH5a [F^- 80d *lacZΔM15 recA1 endl gyrA96 thil hsdR17* (m_k^- r_k^-) *supE44 relA1 deoR Δ(lacZYA-argF)* U169] was used for recombinant plasmid construction. NovaBlue (DE3) [*endA1 hsdR17* (m_k^- r_k^+) *supE44 thil recA1 gyrA96 relA1 lac* (F' *proA⁺B⁺ lacI^qZΔM15*::Tn10)] was used as a host strain for the expression of the *B. subtilis gltX* gene under the control of the T7-inducible promoter. The *B. subtilis* 168 strain served as a source for genomic DNA.

The plasmids used in this study are listed in Table 1. Plasmid pKS-$glnS_{Ec}$ and pKS-$gatCAB_{Bs}$ (pKS-fAP) are derivatives of pBluescript II KS (Curnow *et al.*, 1997). Plasmid pKS-$glnS_{Ec}$ contains the entire *E. coli glnS* gene, and plasmid pKS-$gatCAB_{Bs}$ (pKS-fAP) contains the *B. subtilis gatCAB* gene. Plasmid pET11a-$gltX_{Bs}$ (pEBER) is a derivative of expression vector pET11a (Oh *et al.*, 2002), bearing a 1.5 kb insert in the *Nde*I and *Bam*HI site containing the *B. subtilis gltX* gene. Plasmid pTrc-$gatCAB_{Sa}$ is a derivative of pTrc99a (Namgoong *et al.*, 2001), containing *Staphylococcus aureus gatCAB* gene, thus resulting in an overexpression of Glu-AdT.

Expression of B. subtilis *GluRS and Glu-AdT in* E. coli

E. coli NovaBlue (DE3) cells harboring a recombinant plasmid were grown at 37°C in 10 ml of antibiotic-enhanced Luria-Bertani (LB) medium. Gene expression was induced by the addition of IPTG; when the A_{600} of the culture reached a level of 1.0, properly diluted transformant cells, different concentrations of IPTG, and 0.8% top agar were mixed, and poured into a LB plate containing antibiotics. The culture was then incubated at 37°C for 48 h.

RNA isolation

Total RNAs were isolated from *E. coli* NovaBlue (DE3) and its transformants, using a SV Total RNA Isolation kit (Promega, UK), according to the manufacturer's instruc-

tions. IPTG-treated cells (10^7-10^8) were disrupted by incubation with lysozyme (0.4 mg/ml) for 5 min, followed by addition of lysis buffer and 95% ethanol. RNA in this solution was bound to the silica surface of the glass fibers in the Spin Column. Contaminating DNA was completely removed using RNase-Free DNase according to the manufacturer's instructions. The silica was washed twice with Wash Solution and the RNA was eluted using 100 ul of Nuclease-Free Water, followed by centrifugation at 12000 ×g for 1 min. The purified RNA was stored at -70°C until use.

Reverse transcriptase-polymerase chain reaction

Primer sequences utilized were *gltX* 5'-TGTGGATGTCGGAGGAGAGTA-3' (forward) and 5'-ACAATCAGCGTCATGTGTCCG-3' (reverse); and *gatCAB* 5'-AGCACGTTGCGCACCTTGCAA-3' (forward) and 5'-ATC-ATCAACCGCTTGGATGCG-3' (reverse). Reverse transcription PCR was carried out using reagents and protocols included in the One Step RNA PCR kit (Takara, Japan). DNase-treated RNA (ca. 100 ng) was amplified with 5 U of AMV RTase XL and AMV-Optimized *Taq*, 1×buffer, 20 uM each of forward and reverse primers, 1 mM dNTP, and 5 mM $MgCl_2$. This mixture was volumized to 50 ul with RNase-free distilled water. The RT-PCR samples were amplified in a two-step thermal program: cDNA was synthesized at 50°C for 30 min, and then at 94°C for 2 min, in order to inactivate reverse transcriptase. The cDNA was then amplified for 30 cycles of 94°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec. The RT-PCR samples (5 ul) were electrophoresed on a 1.2% agarose gel in 1×TAE buffer.

Results and Discussion

Construction of an inducible expression system for* B. subtilis *GluRS and Glu-AdT

It has been postulated that the overexpression of nondiscriminating *B. subtilis* GluRS is toxic to *E. coli* due to the $tRNA_1^{Gln}$ mischarging with glutamate (Pelchat *et al.*, 1998). Therefore, it can be assumed that *E. coli* may overcome these toxic effects if mischarged Glu-$tRNA_1^{Gln}$ can be converted to correctly charged Gln-$tRNA_1^{Gln}$ by Glu-AdT. To ascertain whether this toxic effect is indeed caused by mischarged Glu-$tRNA^{Gln}$, an inducible coexpression system for *B. subtilis* GluRS and Glu-AdT in *E. coli* was constructed. pEBER is a high copy-number plasmid containing a *B. subtilis gltX* gene under the control of the T7 promoter (Oh *et al.*, 2002). The *gltX* gene from the pEBER plasmid was subcloned in pACYC184, a low copy-number plasmid which harbors the chloramphenicol resistance gene, in order to reduce the expression level of cytotoxic GluRS. The resulting 5.2 kb portion of the pACYC184-$gltX_{Bs}$ plasmid was dubbed pYBER. Also, to control the expression of *B. subtilis* Glu-AdT through the T7 promoter, the *gatCAB* gene in the pKS-$gatCAB_{Bs}$ (pKS-fAP) plasmid was subcloned into a pET22b vector which contained the ampicillin resistance gene. The subcloning process yielded 8.9 kb of pET22b-$gatCAB_{Bs}$ (pEBGF) plasmid (Fig. 2).

Determination of IPTG concentration for expression of B. subtilis GluRS

To determine the optimal concentration of IPTG for the induction of *B. subtilis gltX*, properly diluted NovaBlue

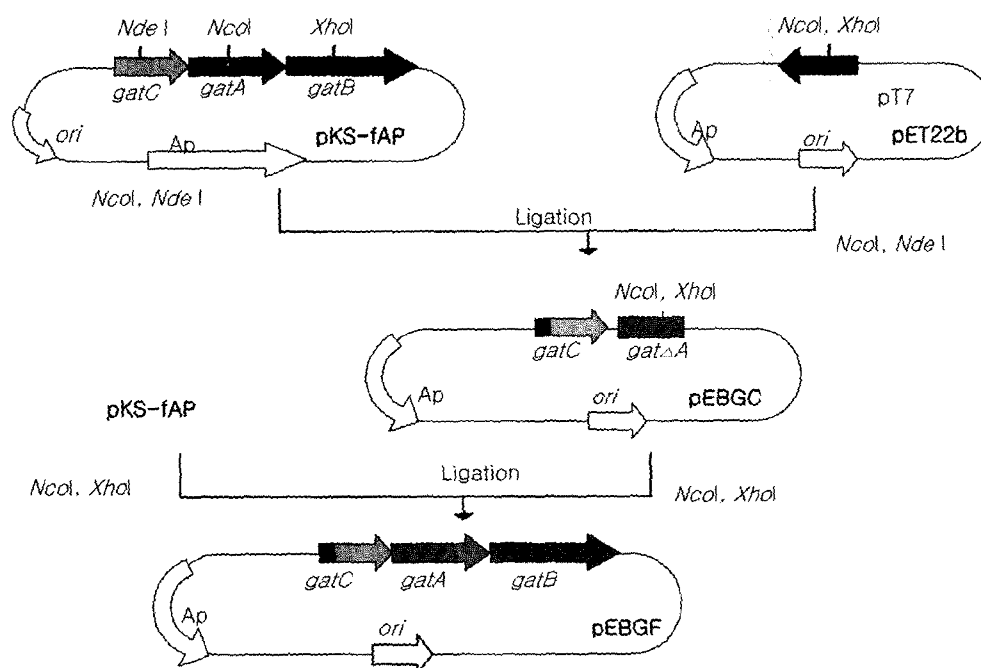

Fig. 2. Construction of pET22b-$gatCAB_{Bs}$ (pEBGF) plasmid containing *B. subtilis* Glu-AdT gene with T7 promoter.

(DE3)/pYBER transformant cells, different concentrations of IPTG (0, 0.125, 0.25, and 0.5 mM), and 0.8% top agar were mixed, and then poured onto an LB plate which also contained chloramphenicol. The plate was then incubated at 37°C for 48 h. When IPTG was not added to the medium, the transformants grew normally; but when 0.125 mM IPTG was added, growth rates underwent a slight reduction (Fig. 3A). When IPTG concentrations increased above 0.25 mM, cell growth was completely inhibited. These results suggest that *B. subtilis* GluRS expression can be controlled by IPTG concentration, and that the growth inhibition of *E. coli* was proportional to the IPTG concentration. An IPTG concentration of 0.25 mM was selected for our studies of the effects of factors that might lessen the growth inhibition phenotype caused by GluRS expression.

There are several reports that elongation factor EF-Tu can discriminate between mischarged aminoacyl-tRNAs and correctly charged aminoacyl-tRNAs (Stanzel *et al.*, 1994; Becker and Kern, 1998; LaRiviere *et al.*, 2001); however, such discrimination by EF-Tu seems to have limitations in *in vivo* situations (Min *et al.*, 2003). *E. coli* can tolerate mischarged tRNA to some extent, but it tends to undergo abnormal protein synthesis when an excessive amount of mischarged tRNA is recognized by elongation factors. This abnormal protein synthesis is uniformly fatal for the host cell.

Toxic effects of B. subtilis *GluRS in* E. coli *are reduced by the overexpression of Glu-AdT and GlnRS, but not by glutamine*

To investigate whether *B. subtilis* Glu-AdT can convert mischarged Glu-tRNA^{Gln} into correctly-charged Gln-tRNA^{Gln} in *E. coli*, *B. subtilis* GluRS and Glu-AdT were coexpressed in an *E. coli* NovaBlue (DE3) strain. In the presence of 0.25 mM IPTG in the growth medium, transformants possessing both GluRS and Glu-AdT genes evidenced only a slight growth inhibition (Fig. 3B), while NovaBlue (DE3)/pYBER (GluRS) transformants could not grow at all (Fig. 3A). The constitutive or inducible overexpression of *B. subtilis* Glu-AdT in *E. coli* had little influence on the growth of *E. coli* (Curnow *et al.*, 1997). These results suggest that *B. subtilis* Glu-AdT, expressed in *E. coli,* could recognize mischarged Glu-tRNA$_1^{Gln}$ and convert it into correctly-charged Glu-tRNA$_1^{Gln}$. This consequently implies that the presence of mischarged Glu-tRNA$_1^{Gln}$ in the cell is the main cause of growth inhibition of host cells.

Considering the fact that toxicity seems to be due to the mischarging of *E. coli* tRNA$_1^{Gln}$ with glutamate *in vivo*, *E. coli* GlnRS and *B. subtilis* GluRS may compete to use the substrate tRNA$_1^{Gln}$. If the *E. coli* GlnRS gene is overexpressed in a condition such that the amount of tRNA$_1^{Gln}$ in cells is limited, it is expected that the ratio of mischarged tRNA$_1^{Gln}$ to correctly charged tRNA would be decreased, hence ameliorating the toxic effects of *B. subtilis* GluRS. In service of this hypothesis, we obtained transformants containing both *B. subtilis* GluRS and *E. coli* GlnRS genes, and compared their growth under the same conditions. Upon addition of 0.25 mM of IPTG, the transformants could grow, although their growth was slightly less vigorous than when Glu-AdT was overexpressed (Fig. 3C). Considering that its own promoter was used for *glnS* gene expression while Glu-AdT gene expression was driven by the strong T7 promoter, the transcription level difference between Glu-AdT and *E. coli* GlnRS may be a factor in the suppression of growth inhibition. At any rate, it seems that *E. coli* GlnRS, when overexpressed in cells, tends to produce a higher ratio of correctly charged tRNA$_1^{Gln}$ with glutamine to mischarged tRNA$_1^{Gln}$, therefore reducing the toxic effects caused by mischarged tRNA$_1^{Gln}$ *in vivo*. These results indicate that the formation of such misacylated tRNA is a major cause of growth inhibition in *E. coli.*

E. coli tRNA$_1^{Gln}$ can be charged with glutamine by *E. coli* GlnRS, or, at the same time, mischarged with glutamate by *B. subtilis* GluRS. It is known that the glutamic acid pool within cells is generally larger in gram-positive bacteria, which synthesize Gln-tRNA^{Gln} via transamidation pathways, than in gram-negative bacteria (Hong *et al.*, 1998). It has also been demonstrated that *E. coli* GlnRS can charge tRNA$_1^{Gln}$ with glutamine more efficiently than can *B. subtilis* GluRS *in vitro* (Lapointe *et al.*, 1986). It is possible that an excessive quantity of glutamine, when added into an *E. coli* growth medium, may induce the correct aminoacylation of tRNA$_1^{Gln}$ with glutamine, therefore reducing toxicity caused by the

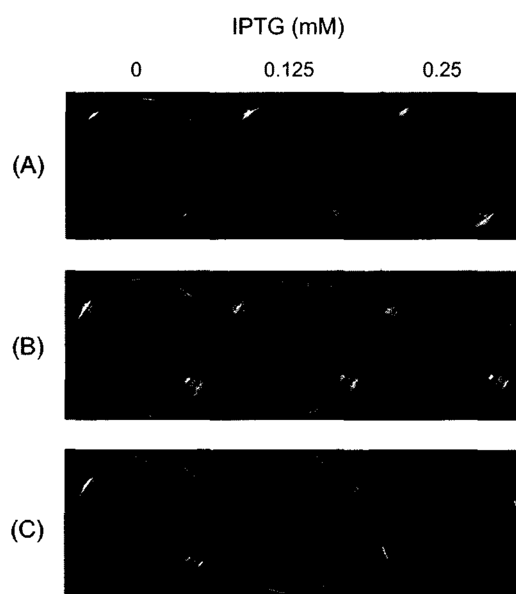

Fig. 3. Effect of expression of *B. subtilis* Glu-AdT and *E. coli* GlnRS on the growth inhibition by *B. subtilis* GluRS in *E. coli*. Cells were grown on LBCm (A) and LBCmAp (B and C) plates containing different concentrations of IPTG at 37°C for 48 h. A: *E. coli* NovaBlue (DE3)/pYBER; B: NovaBlue (DE3)/pYBER+pEBGF; C: NovaBlue (DE3)/pYBER+pKS-fAP.

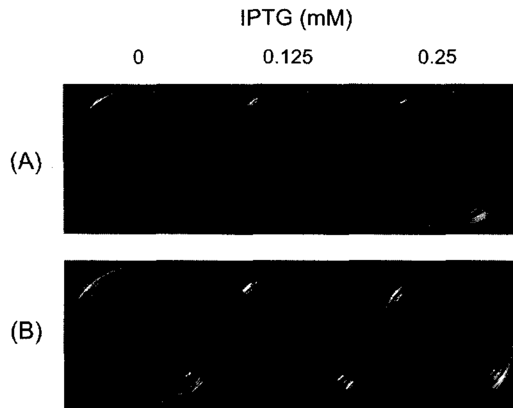

Fig. 4. Addition of L-glutamine to the growth medium. Cells were grown on LBCm plates containing different concentrations of IPTG at 37°C for 48 h. A: *E. coli* NovaBlue (DE3)/pYBER; B: NovaBlue (DE3)/pYBER+1 mM L-glutamine.

expression of *B. subtilis* GluRS. We added an excessive quantity of glutamine into the growth medium and induced the expression of nondiscriminating GluRS, using IPTG. We then examined the growth of the NovaBlue (DE3)/pYBER transformant. But the addition of 1 mM glutamine into the medium did not lessen *B. subtilis* GluRS-related toxicity (Fig. 4), and even the addition of 10 mM glutamine had no attenuating effects on growth inhibition (data not shown).

These results show that, although mischarged Glu-tRNA-^{Gln} may be tolerated to some extent in *E. coli* without a transamidation pathway for the formation of Gln-tRNA^{Gln}, if the amount of mischarged tRNA is excessive, it results in abnormal protein synthesis and, ultimately, cell death.

Detection of B. subtilis *gltX and* gatCAB *genes expression by RT-PCR*

Previous results showed that overexpression of the *B. subtilis gatCAB* gene rescued *E. coli* cells from toxic effects caused by the formation of mischarging *B. subtilis* GluRS. Measurement of the activities of both enzymes is required in order to identify the coexpression of *B. subtilis gltX* and *gatCAB* genes in *E. coli* transformants. In the case of *B. subtilis* GluRS, however, its activity cannot be independently measured, due to interference from *E. coli* GluRS. Therefore, we examined the presence of mRNAs from *B. subtilis gltX* and *gatCAB* genes, using RT-PCR in *E. coli* cells, after 0.5 mM IPTG induction for 3 h (Fig. 5). The controls on this gel (lanes 1 and 2) indicated that RT-PCR had amplified the 512 and 392 bp fragments from *gltX* and *gatCAB* target mRNAs, respectively, in *B. subtilis*. However, both mRNAs were undetectable in the *E. coli* host, as expected (lanes 3 and 4). The 512 and 392 bp products which were amplified from the mRNA of the *E. coli* transformant harboring *B. subtilis gltX* and *gatCAB* genes showed that both genes were expressed simultaneously in *E. coli* (lanes 7 and 8).

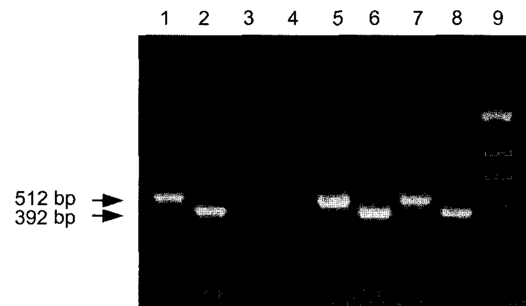

Fig. 5. RT-PCR detection of *B. subtilis gltX* (512 bp) and *gatCAB* (392 bp) mRNA from *E. coli* transformants induced by IPTG treatment for 3 h. Lane 1 and 2, *B. subtilis* genomic DNA was amplified with *gltX* and *gatCAB* specific primers, respectively; lane 3 and 4, *E. coli* NovaBlue (DE3) genomic DNA was amplified with *gltX* and *gatCAB* specific primers, respectively; lane 5, Total RNA from *E. coli* NovaBlue (DE3)/pYBER was amplified with *gltX* specific primers ; lane 6, Total RNA from *E. coli* NovaBlue (DE3) /pEBGF was amplified with *gatCAB* specific primers; lane 7 and 8, Total RNA from *E. coli* NovaBlue (DE3)/pYBER+pEBGF was amplified with *gltX* and *gatCAB* specific primers, respectively; lane 9, molecular size standards (3.6, 1.3, 0.8, and 0.45 kb).

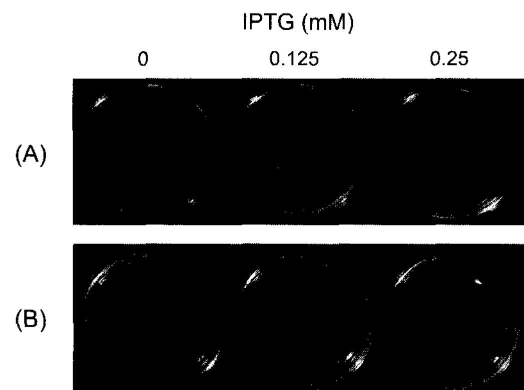

Fig. 6. Effect of expression of *S. aureus* Glu-AdT on the growth inhibition by *B. subtilis* GluRS in *E. coli*. Cells were grown on LBCm (A) and LBCmAp (B) plates containing different concentrations of IPTG at 37°C for 48 h. A: *E. coli* NovaBlue (DE3)/pYBER; B: NovaBlue (DE3)/pYBER+ pTrc*gatCAB*.

S. aureus *Glu-AdT also reduces the toxic effect of* B. subtilis *GluRS in* E. coli

When Glu-AdT of the pathogenic gram-positive bacterium *S. aureus* was used instead of that of *B. subtilis* in the complementation assay, a similar degree of suppression was observed (Fig. 6). Therefore, this complementation system could be utilized to measure the *in vivo* activity of Glu-AdT genes from different organisms. The activity of Glu-AdT, which is involved in Gln-tRNA formation in a large number of bacteria, is essential for cell viability (Curnow *et al.*, 1997), but this enzyme and pathway do not exist in the cytoplasm of mammalian cells. Therefore, we propose that Glu-AdT is a target candidate in the development of new antibiotics, and that this com-

plementation system can be utilized for the *in vivo* screening of compounds that may be developed into viable antibiotics.

Acknowledgement

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