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Cloning of the Glu-tRNA^{Gln} Amidotransferase (*gatCAB*) Gene from *Staphylococcus aureus*

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Abstract In Gram(+) bacteria and organelles in higher eukaryotes, Gln-tRNA^{Gln} utilized for protein biosynthesis is formed by a tRNA-dependent amino acid transformation using mischarged Glu-tRNA^{Gln} as the intermediate. In this study, the *gatCAB* gene encoding Glu-tRNA^{Gln} amidotransferase (Glu-AdT) of *Staphylococcus aureus* was cloned and its nucleotide sequence was determined. The *S. aureus gatCAB* gene was organized in an operon structure consisting of three open reading frames (*gatC*, *gatA*, and *gatB*), similar to that of *Bacillus subtilis*. The gene sequences for the A and B subunits of Glu-tRNA^{Gln} amidotransferase showed significant homology (77 and 87% homology with amino acid sequence) with the *gatA* and *gatB* genes of *B. subtilis*, yet the C subunit (*gatC*) showed a relatively lower homology with the *B. subtilis gatC* gene and other orthologues. The cloned *S. aureus* Gln-tRNA^{Gln} amidotransferase gene was highly expressed in *Escherichia coli*, and the resulting crude enzyme could convert misacylated Glu-tRNA^{Gln} into Gln-tRNA^{Gln} *in vitro*.

Key words: tRNA, *Staphylococcus aureus*, Glu-AdT, Glu-tRNA^{Gln} amidotransferase, *gatCAB*

The formation of aminoacyl-tRNAs is one of the important steps in protein biosynthesis. It is generally accepted that 20 different aminoacyl-tRNA synthetases exist for the aminoacylation of 20 amino acids onto their cognate tRNAs [18]. However, in many Gram(+) bacteria and organelles in the higher organisms, the Gln-tRNA^{Gln} used in protein biosynthesis is formed by an indirect pathway via misacylated Glu-tRNA^{Gln} [9, 19, 22, 24, 28]. In this pathway (Fig. 1), the tRNA^{Gln} is first misaminoacylated with glutamic acid by nondiscriminable glutamyl-tRNA synthetase (GluRS) to form Glu-tRNA^{Gln}. In the second step, the Glu-tRNA^{Gln} is converted to Gln-tRNA^{Gln} by Glu-tRNA^{Gln} amidotransferase (Glu-AdT). The presence of Glu-AdT activity in *Bacillus sp.* was originally established in the 1960s [28] and further studies have revealed that this tRNA-dependent amino acid transformation is widespread in Gram-positive bacteria, chroloplasts in plants, and mitochondria in animals. [9, 24] However, the exact biochemical nature of this enzyme was still unknown until the gene encoding Glu-AdT (Glu-AdT) was identified in *B. subtilis* [4].

In *B. subtilis*, Glu-AdT exists as a heterotrimeric enzyme consisting of *gatC*, *gatA*, and *gatB* subunits. Remarkably, this enzyme does not show any homology with previously known aminoacyl-tRNA synthetases. The A subunit (*gatA*) has a typical amidase signature motif and an extensive homology with previously known amidases, indicating that this subunit acts as a catalytic subunit in a holoenzyme. The B subunit has a significant homology with the *Saccharomyces cerevisiae pet112* gene, which is involved in mitochondrial protein synthesis. This subunit is thus presumed to be involved in the binding of tRNA substrate. The role of the C subunit is still unknown. However, the coexpression of the A and C subunits in *E. coli* has been found to be essential for the activity of Glu-AdT [4]. The genes for the three subunits consist of an operon, and the disruption of the 5' portion of the operon in *B. subtilis* is lethal [4], thereby indicating that the formation of Gln-tRNA^{Gln} by Glu-AdT is the only pathway in this organism.

Another report concerning Glu-AdT is that of *Deinococcus radiodurans* [6]. The genomic *D. radiodurans* sequence data show that a conventional GlnRS gene is present as well as the Glu-AdT encoding genes. In this organism, the conventional GlnRS directly carried out glutaminylation, while Glu-AdT converted misacylated Asp-tRNA^{Asn} into Asn-tRNA^{Asn}. When considering that this organism does not have any conventional asparagine synthetase activity, the 'Glu-AdT' in this organism may be involved in tRNA-dependent

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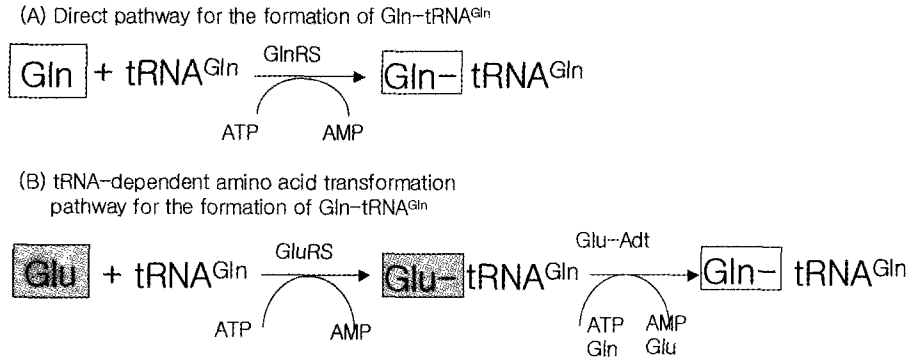

Fig. 1. Direct and indirect pathways for the generation of Gln-tRNA^{Gln}.
(A) Direct Gln-tRNA^{Gln} formation pathway by glutaminyl-tRNA synthetase used in some Gram (-) bacteria and the cytoplasm of eukaryotes.
(B) tRNA-dependent Gln-tRNA^{Gln} formation by Glu-AdT in Gram (+) bacteria, archea, and organelles of eukaryotes.

asparagine synthesis *in vivo*. This result suggests that the enzyme currently known as 'Glu-AdT' may play different physiological roles in various organisms. Therefore, to understand the role of this enzyme in protein biosynthesis, an investigation of Glu-AdT from a wide range of organisms is necessary.

Many human bacterial pathogens such as *Helicobacter pylori*, *Streptococcus pneumoniae*, and *Mycobacterium tuberculosis* rely on tRNA-dependent amino acid transformations for the formation of Gln-tRNA^{Gln}, while the cytoplasm of mammalian cells relies on direct aminoacylation for the formation of Gln-tRNA^{Gln} in protein biosynthesis. As such, this unique enzyme may be an ideal target for the development of antibiotics [22].

Accordingly, we cloned the Glu-AdT gene (*gatCAB*) from *Staphylococcus aureus*, a major human pathogen causing various infections [1], and performed some genetic and biochemical characterizations of the enzyme. Considering the fact that the rapid emergence of antibiotic resistant pathogenic bacteria in recent years [16, 22] is a major concern for human health, study on the unique translational machineries found in many pathogenic bacteria would provide valuable information for the development of novel antibiotics.

Material and Methods

Bacterial Strains and Plasmids

The *S. aureus* ATCC 25923 strain used as the source of chromosomal DNA was obtained from the American Type Culture Collection (ATCC). *E. coli* XL-1 Blue strains (Purchased from the Stratagene, La Jolla, U.S.A.) were employed for the routine molecular cloning. pBluescript II SK (+) phagemid (Stratagene) was used for the generation of a subgenomic library of *S. aureus* and pTrc99a vector (Amersham Pharmacia Biotech, Uppsala, Sweden) was used for the expression of the *S. aureus* Gln-tRNA^{Gln} amidotransferase gene in *E. coli*.

PCR Amplification of a Portion of *gatCAB* Operon

In order to amplify a portion of the *gatCAB* operon for use as the probe in the Southern hybridization, four degenerate primers (GatA1: 5'-AATATGGAYGARTTYGCNATGGG-3'; GatA2: 5'-GCWGGWTAYTAYGAYGCWTA-3'; GatB1: 5'-GGTTCWCCRAARTAW CKRTA-3'; GatB2: 5'-TCW-GGRTARAARTARTTYTT-3') corresponding to the most conserved amino acid sequence of the *gatA* or *gatB* gene in various organisms on the database were designed and chemically synthesized (Bioneer, Chungbuk, Korea). These primers were used for the PCR amplification using *S. aureus* genomic DNA as the template. PCR reactions were carried out using the combination of each primer (GatA1-GatB1, GatA2-GatB2, GatA2-GatB1, GatA1-GatB2). For the PCR reactions, 1 μM of each primer, 100 μM of dNTP, 2.5 unit of Extend-Long Template DNA polymerase (Roche Biochemical, Indianapolis, U.S.A.), and 100 ng of *S. aureus* genomic DNA were used with 30 cycles as follow: (60 s at 95°C, 60 s at 48°C, 90 s at 72°C per cycle). The resulting PCR products were cloned into a pGEM-Easy vector (Promega, Wisconsin, U.S.A.) and the nucleotide sequences were confirmed by DNA sequencing.

Southern Hybridization of *gatCAB* Gene and Generation of Subgenomic Library from *S. aureus*

The PCR products amplified using the degenerate primers were used as the probe in the Southern hybridization. The fragment produced by the PCR (0.5 kb) with the GatA2 and GatB2 primers (encompassing 3' of the *gatA* and 5' of the *gatB* gene) were labeled with biotin using the Bionick Labeling system (Life Technology, Rockville, U.S.A.) and then used as the probe for the Southern hybridization. *S. aureus* genomic DNA (10 μg) completely digested with various restriction enzymes were used for the Southern hybridization.

After the identification of the position of the hybridization signals, the corresponding region of digested *S. aureus* genomic DNA was isolated by an agarose gel elution using

Geneclean III kit (BIO101 Inc, Carlsbad, U.S.A.), ligated with pBluescript II SK (+) vector digested with *Sma*I or *Hin*dIII, and then transformed in *E. coli* XL-1 Blue. About 10^3 of independent clones were isolated using alpha-complementation.

Screening of *gatCAB* Clone from Subgenomic Library of *S. aureus*

A subgenomic library (about 10^3 independent clones) was screened using a PCR mediated method. About 1,000 independent clones were individually grown in LB Agar (containing 100 μg of ampicillin), pooled, and then plasmids were purified by groups of 30. The grouped recombinant plamids were diluted and used as the template for a PCR amplification with primers (GatA-2 and GatB-2). Groups containing the *gatCAB* gene were rescreened by PCR and restriction analysis.

Nucleotide Sequence Determination of Cloned *gatCAB* Gene

To generate a nested-deleted subclone to facilitate the sequence determination of the *gatCAB* gene, the Erase-a Base System (Promega, Madison, U.S.A.) was employed. The nucleotide sequence was determined using a BigDye™-terminator kit (Perkin-Elmer, U.S.A.) and analyzed with the ABI 310 genetic analyzer (Applied Biosystem Inc, U.S.A.). The nucleotide and deduced amino acid sequence homology with other *gatCAB* orthologues was analyzed with Blast2 algorithm using the Entrez database (National Center for Biotechnology Information, U.S.A.). A multiple amino acid sequence alignment was generated using the ClustalX program [12].

Expression of Glu-AdT in *E. coli*

To express Glu-AdT in *E. coli*, a pTrc99a vector (Phamarcia) was employed. In order to introduce a *Nco*I site in the region of the start codon of the *gatC* gene, primer GatC5 (5'-GG**CCATGG**CAAAAGTAACACGTG-3', bolded sequence indicates the *Nco*I Site) was designed and used for the PCR amplification with a T7 primer. The 2.8 kb PCR product corresponding to the 5' portion of the *gatCAB* operon was subcloned into a pGEM-T vector, yielding pGatCAB plasmid. The 2.2 kb *Nco*I and *Sal*I fragment containing the 5' portion of the *gatCAB* operon was then linked with the 1.4 kb *Sal*I and *Bam*HI fragments at the corresponding 3' region of operon, and inserted into a pTrc99a vector digested with *Nco*I and *Bam*HI, thereby yielding a pTrcgatCAB plasmid, where the whole *gatCAB* operon expression was directed by a strong trc promoter located in the 5' region of the operon.

E. coli XL-1 Blue cell harboring the pTrcgatCAB plasmid was cultivated in 100 ml of LB medium at 30°C, induced with the addition of 0.25 mM IPTG, and then further cultivated for an additional 12 h. The cells were harvested by centrifugation at 30,000 ×g. Harvested cells were resuspended in the buffer (25 mM of Hepes-KOH (pH 7.4), 25 mM of KCl, 10 mM of $MgCl_2$, and 1 mM of DTT), and disrupted by sonication (4 times with 30 s ultrasonic treatments). The cellular debris was then removed by centrifugation at 100,000 ×g for 1 h, and the S100 extract was obtained.

Assay of Glu-AdT and Glutaminase Activity in Crude Extract

A Glu-AdT assay was carried out as previously described by Curnow *et al.* [6] with a slight modification. The tRNA^{Gln} gene from *B. subtilis* was expressed in *E. coli* DH5α from the plasmid pGIF-tRNA^{Q} [4]. The expressed tRNA^{Gln} (*B. subtilis*) was purified using two-step ion-exchange chromatography as described by Curnow *et al.* [6], and then used as the substrate for the misacylation reaction with [C^{14}] labeled glutamate (250 μCi/mmol, Amersham Pharmacia biotech, Uppsala, Sweden). For the misacylation of tRNA^{Gln} with glutamate, partially purified *B. subtilis* GluRS was used. The misacylated Glu-tRNA^{Gln} was extracted with phenol: chloroform, ethanol precipitated, and then used for the Glu-AdT reaction. The reaction mixture (100 μl) containing 1 μM of Glu-tRNA^{Gln}, 1 mM of glutamine, 1 mM of ATP, 1.5 mM of $MgCl_2$ in 5 mM HEPES-KOH buffer (pH 7.0) was prepared, and 10 μg S100 extracts of *E. coli* XL-1 Blue/pTrc*gatCAB* or XL-1 Blue/pTrc99a were added, respectively. The reaction proceeded for 30 min at 37°C, then the amino acids attached to the tRNA were released by treatment with 0.025 N NaOH and concentrated using a vacuum concentrator. Amino acids released from the tRNA were resuspended in 2 μl of H_2O and separated on a cellulose-TLC plate (Merck, Germany) using the mobile phase of methanol:NH_4OH:chloroform:H_2O (4:1:1:1). The TLC plate was dried and analyzed using a Fuji BAS-25000 phosphorimager (Fuji Photo Film, Tokyo, Japan). The glutaminase activity of Glu-AdT was determined by measuring the glutamate formation using glutamate dehydrogenase and $NADP^+$, as previously described by Curthoys *et al.* [7].

RESULTS

Cloning of *S. aureus gatCAB* Gene

Considering the fact that *B. subtilis* and *S. aureus* are located in the same phylogenic group (low G+C content Gram (+) eubacteria), it was assumed the organization of the *gatCAB* gene in *S. aureus* may be similar to that in *B. subtilis*. For the PCR amplification of a fragment of the *gatCAB* operon from *S. aureus* chromosomal DNA, degenerated primers corresponding to the highly conserved regions of *gatA* and *gatB* amino acid sequences were designed (panel C in Fig. 2). The primer sets were designed to amplify 1.9, 1.2, 1.3, and 0.6 kb fragments encompassing portions of the *gatA* and *gatB* open reading

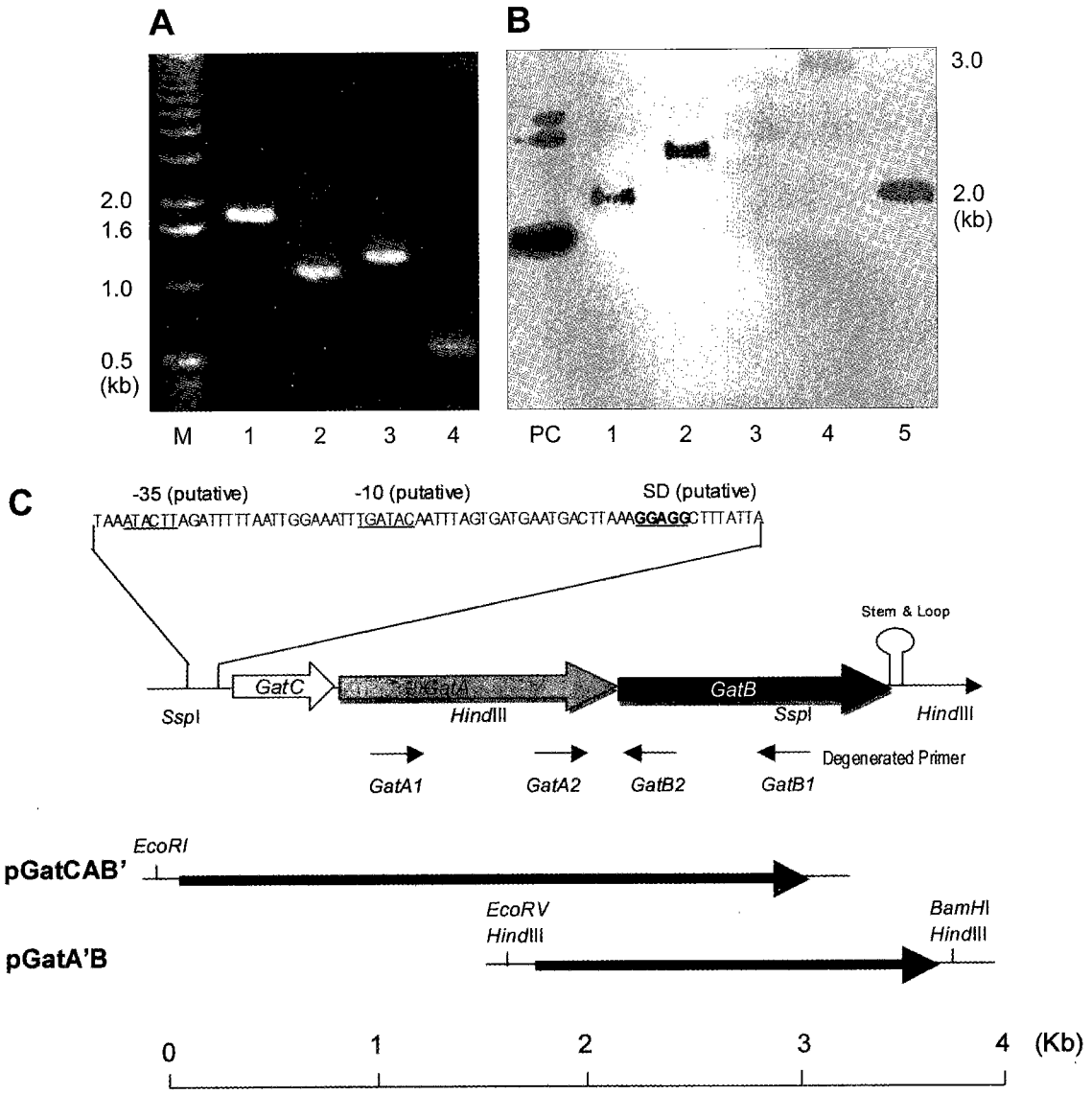

Fig. 2. Glu-tRNA^{Gln} amidotransferase (*gatCAB*) gene from *Staphylococcus aureus*.
(A) PCR amplification using degenerate primers based on a conserved sequence of the Glu-AdT amino acid sequence. M: 1 kb Ladder; Lanes 1,2,3,4: PCR product using GatA1/GatB1, GatA2/GatB1, GatA1/GatB2, and GatA2/GatB2 primers, respectively. (B) Southern hybridization of the *gatCAB* gene in *S. aureus* chromosome. PC: PCR product (GatA2/GatB2) cloned in pGEM-T Vector; Lanes 1, 2, 3, 4: *S. aureus* chromosomal DNA digested with *Eco*RI, *Hin*dIII, *Nde*I, *Sna*BI, and *Ssp*I, respectively. A cloned 0.5 kb region encompassing the *gatA* and *gatB* genes (GatA2/GatB2 PCR product) was used as the probe. (C) *S. aureus gatCAB* gene organization and location of degenerate primer and clones in *gatCAB* gene. The pGatCAB clone contained a 3.0 kb *Ssp*I fragment containing *gatC*, *gatA* and the 5' portion of *gatB* ORF. pGatAB clone contains a 2.3 kb *Hin*dIII fragment containing the 3' portion of *gatA* and whole *gatB* ORF.

frames when the *gatC*, *gatA*, and *gatB* genes in the *S. aureus* chromosome are located in a similar pattern to that in *B. subtilis*.

As shown in panel A of Fig. 2, each primer sets yielded the predicted size of PCR products. These PCR products were then cloned into pGEM-T vectors (Promega). The cloned PCR products were sequenced and confirmed as portions of the Glu-AdT gene (data not shown).

The 0.6 kb fragment of the 3' portion of the *gatA* gene and the 5' portion of the *gatB* gene was then used as probe for the Southern hybridization. As shown in panel B of Fig. 2, the probe was strongly hybridized with *S. aureus* genomic DNA completely digested with various restriction enzymes.

First, the 3.0 kb fragments of *Ssp*I-digested *S. aureus* genomic DNA (lane 5 in panel B) were used for the generation of subgenomic library, and one putative clone (pBS-*gatCAB'*) harboring on the region of the promoter and most of the portion of the *gatCAB* operon was found. However, it lacked the 3' terminal region of the *gatB* ORF. We also tried screening for *Hin*dIII fragments to find the remaining 3'

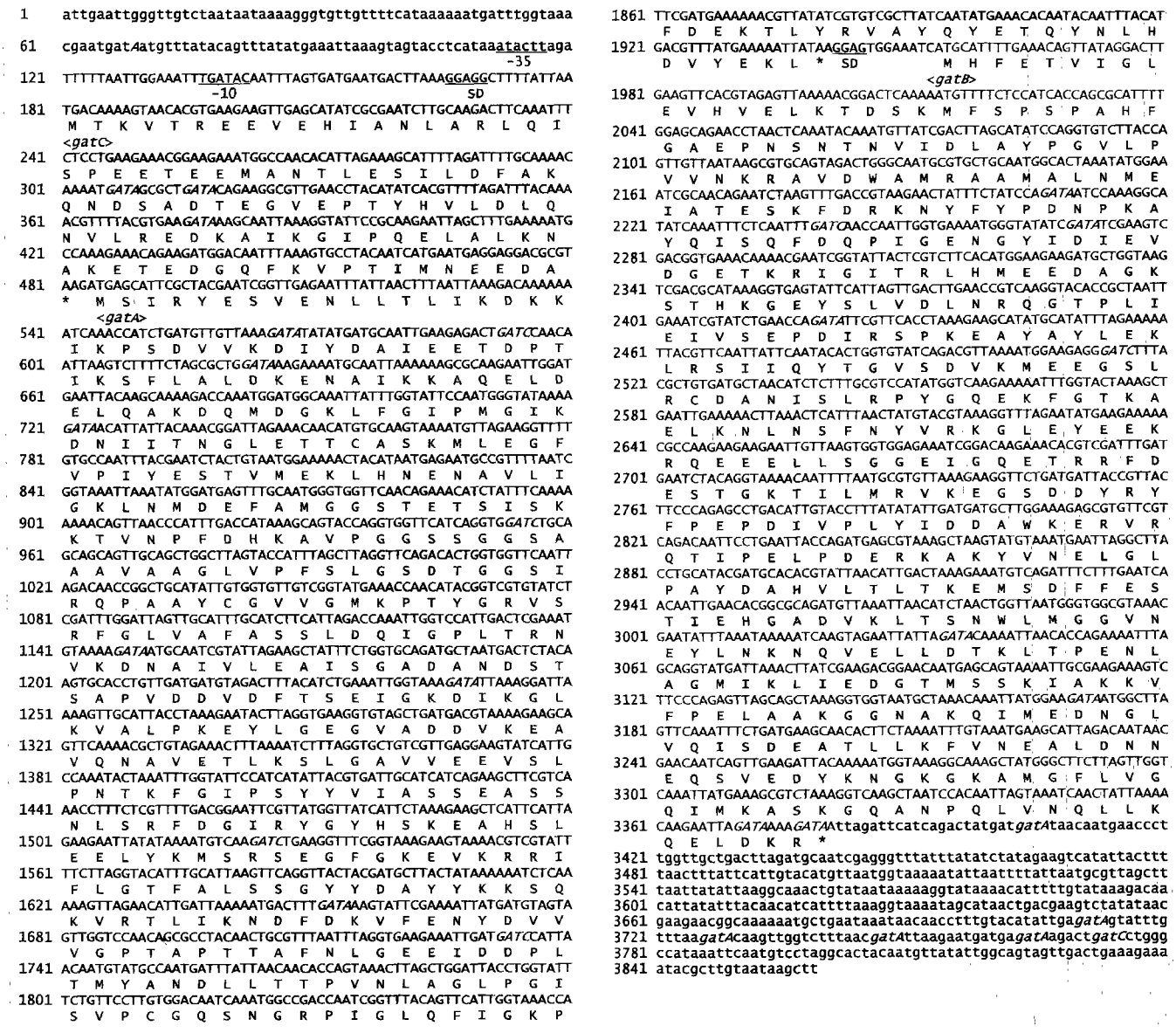

Fig. 3. Nucleotide sequence of the *S. aureus* Glu-AdT gene (*gatCAB*).
-35, -10, SD: putative Pribnow box (-35, -10) and Shine-Dalgano sequence. This sequence is registered in the GenBank (National Center of Biotechnology Information, U.S.A.) under accession number AF205033.

terminal region of the *gatCAB* operon. A clone containing a 2.4 kb *Hin*dIII fragment encompassing nearly half of the *gatA* ORF and the entire *gatB* ORF was found and designated as pBS-*gatA'B*.

Nucleotide Sequence of *gatCAB* Operon and Comparison with Orthologues of Other Organisms

The overall organization of the *gatCAB* operon of *S. aureus* is very similar to that of *B. subtilis* [4]. The nucleotide and deduced amino acid sequences of the full ORFs are shown in Fig. 3, where three ORFs corresponding to the C, A, and B subunits of *S. aureus* Glu-tRNA^{Gln} amidotransferase are organized in the order of C, A, and B. The amino acid homology of the cloned *S. aureus* Glu-AdT gene was analyzed. The amino acid sequences of the *gatA* and *gatB* subunits exhibited extensive homologies with those of *B. subtilis* (*gatA*: 77%, *gatB*: 87% positive, respectively). These high homologies between the two organisms may be due to their close phylogenic relationship. As shown in the phylogenic tree presented in Fig. 4, *gatA* and *gatB* are allocated to the same phylogenic group as *B. subtilis*, *Streptomyces coelicolor*, and *Synechocystis* sp.

However, in the case of the *gatC* subunit, a relatively low homology (45% homology with *B. subtilis gatC* protein) was observed. This sequence divergency of the *gatC* subunit

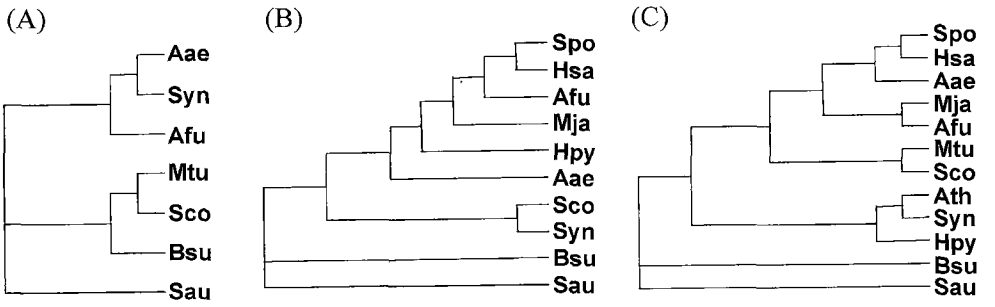

Fig. 4. Phylogenic tree of three Glu-AdT subunits based on the deduced amino acid sequences along with orthologues of other species. Panels A, B, C: *gatC*, *gatA*, and *gatB*, respectively. Sau: *Staphylococcus aureus*; Bsu: *Bacillus subtilis*; Syn: *Synechocystis* sp.; Sco: *Streptomyces coelicolor*; Hpy: *Helicobacter pylori*; Mtu: *Mycobacterium tuberculosis*; Aae: *Aquifex aeoquis*; Mja: *Methanococcus jannaschii*; Hsa: *Homo sapiences*; Spo: *Schizosaccharomyces pombe*, Afu: *Archaeoglobus fulgidus*.

suggests the possibility that the *gatC* subunit may not be involved in the catalytic reactions of the holoenzyme, but rather behave as some kind of species-specific function. Further biochemical and genetical characterization of this subunit is required to prove this possibility.

Another interesting result is that the upstream region of the *gatCAB* gene promoter was identical to that of the previously reported sequence of 3' region of *S. aureus* proline permease [27]. Whole genome sequencing of *B. subtilis* revealed that the proline permease gene was also located upstream of the *gatCAB* gene in the same configuration as *S. aureus* [3] These similarities in the genetic organization of the two organisms may represent a close phylogenetic relationship between *B. subtilis* and *S. aureus*.

Expression of Glu-AdT in *E. coli* and Its Activities

In order to confirm the biological activity of Glu-AdT *in vitro*, the expression of Glu-AdT in *E. coli* was attempted. The entire *gatCAB* operon was constructed to yield a single transcriptional unit under a strong *trc* promoter. As shown in panel A of Fig. 5, two protein bands corresponding to *gatA* and *gatB* appeared in the region around 55 kDa. It is interesting to note that the deduced molecular weights of each subunit were nearly similar (53 kDa), but the migrations in SDS-PAGE appeared to be slightly different. Subsequent experiments using expression plasmids containing the *gatA* or *gatB* gene alone showed that *gatB* was a faster migrating subunit (data not shown).

Next, the Glu-AdT and glutaminase activity of the S100 extracts obtained from *E. coli* XL-1 Blue containing the pTrc*gatC*AB plasmid and an empty vector were examined. Considering the fact that the nucleotide sequences of $tRNA^{Gln}$ between *S. aureus* and *B. subtilis* differ in only two bases (C29G, A45G) [10], the recombinant *B. subtilis* $tRNA^{Gln}$ expressed in *E. coli*, purified by two-step ion-exchange chromatography [4], was used as the substrate. The *B. subtilis* $tRNA^{Gln}$ was misacylated with partially purified *B. subtilis* GluRS and subjected to an amidotransferase reaction using the S100 extract of recombinant Glu-AdT.

As shown in Fig. 6, the S100 extracts prepared from the *E. coli* XL-1 Blue/pTrc*gatCAB* efficiently converted the mischarged Glu-$tRNA^{Gln}$ into Gln-$tRNA^{Gln}$. However, the S100 extract from the *E. coli* XL-1 Blue containing an empty expression vector did not convert the mischarged Glu-$tRNA^{Gln}$ into Gln-$tRNA^{Gln}$. These results indicate that the cloned *gatCAB* genes were expressed well in *E. coli* and yielded functional Glu-AdT proteins.

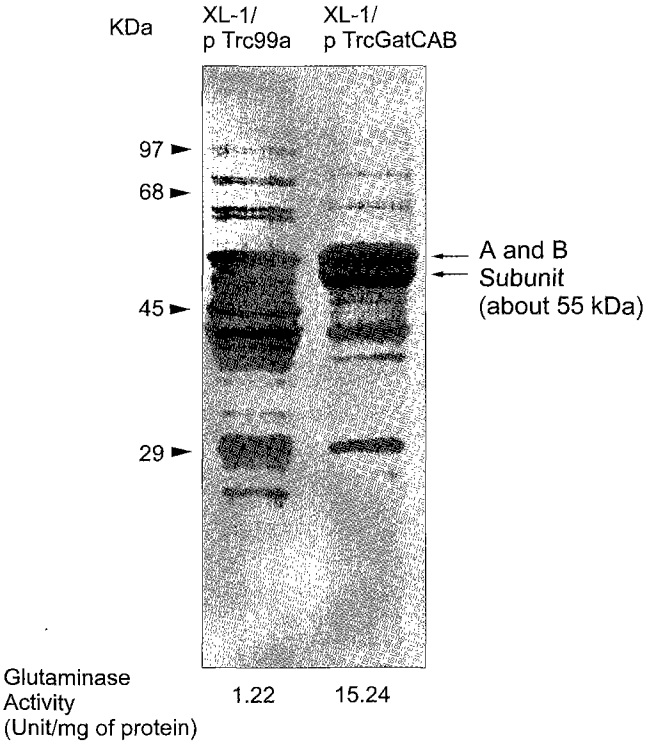

Fig. 5. Expression of *S. aureus* Glu-AdT in *E. coli*.
E. coli XL-1 Blue/pTrc99a (Left) and XL-1 Blue/pTrc*gatCAB* (Right) were incubated in 100 ml of LB medium at 30°C, and induced with 0.25 mM IPTG for 12 h. S100 extracts of each sample were applied on 10% SDS/PAGE gel and later stained with Coomassie blue G-250. (Top) Arrows indicate the bands corresponding to *gatA* and *gatB* subunits.

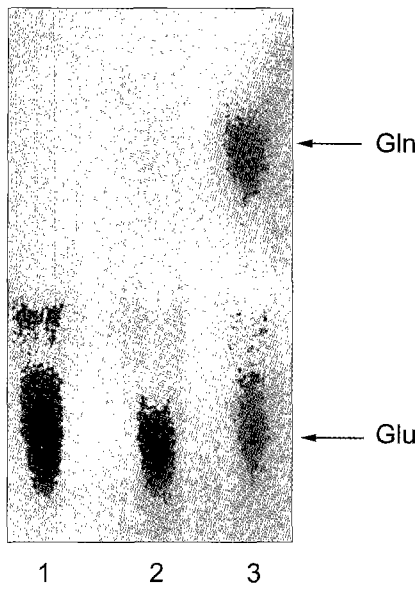

Fig. 6. Activity of *S. aureus* Glu-AdT.
Phosphorimage of thin-layer chromatography separation of ^{14}C-labeled glutamine and glutamic acid derived from deacylation of aminoacyl-tRNA recovered from the assay (see text). The Glu-AdT was from S100 extracts from *E. coli* XL-1 Blue harboring: Lane 1, pTrc99a; Lane 2; reaction mixture lacking S-100 extracts. Lane 3: pTrc*gatCAB*.

These results also show that *B. subtilis* tRNA^{Gln} could be used as a substrate for *S. aureus* Glu-AdT, and that the nucleotide sequence difference in tRNA^{Gln} between *B. subtilis* and *S. aureus* (C29G, A45G) has no affect on the amidotransferase reaction of Glu-AdT.

Since there has been a previous report that *B. subtilis* Glu-AdT also has glutaminase activity [4], an investigation on whether overexpressed *S. aureus* Glu-AdT has a glutaminase activity was performed. As displayed in Fig. 5 (bottom), the glutaminase activity in *E. coli* XL-1 Blue/pTrc*gatCAB* was increased about 10-fold compared to that of the control (*E. coli* XL-1 Blue/pTrc99a), thereby, indicating that *S. aureus* Glu-AdT also has glutaminase activity as in the case of *B. subtilis*. When considering the extensive homology of the GatA subunit with the previously characterized amidase family, this glutaminase activity may be dependent on the GatA subunit in the holoenzyme.

DISCUSSION

This study was undertaken to clone the Glu-AdT gene from *S. aureus* expressed in *E. coli* and confirm its activity *in vitro*. As in the case of *B. subtilis*, the Glu-AdT gene of *S. aureus* appeared to be organized as a single operon, in the order of *gatC*, *gatA*, and *gatB*. Organization of this gene is also seen in other Gram-positive eubacteria, such as *B. subtilis*, *Streptococcus pyogenes*, *Streptomyces coelicolor*, *B. stearothermophillus* (S. I. Kim, personal communication), and cynobacteria *Synechocystis* sp. PCC6803 [19]. However, in the case of certain eubacteria, such as *Neisseria gonorrhoeae*, *Mycoplasma genitalium*, *Rhodococcus* sp., *Helicobactor pylori*, *Deinococcus radiodurans*, and most archea, these genes are not present as an operon.

It is plausible that the operon organization seen in *S. aureus* may have some significant physiological role such as the regulation of its expression. Yet, possible regulatory sequences, such as the T-box, frequently found in most aminoacyl-tRNA synthetase genes of Gram-positive bacteria [11], were absent in the *S. aureus gatCAB* operon. However, it is still questionable that certain physiological conditions may exert some kind of regulation on Glu-AdT expression, because of its possible significance in protein synthesis and nitrogen metabolism.

The *gatA* subunit has a strong homology with the previously characterized amidase family, implying that this subunit may act as a catalytic subunit for transamidation in the holoenzyme. Curnow *et al.* [4] reported that the *gatA* subunit alone is sufficient to show glutaminase activity. However, the exact roles of the *gatB* and *gatC* subunits are still unknown. The function of the *gatC* subunit is more elusive, because it does not exhibit any significant homology with other known proteins. The only function of the *gatC* subunit is most likely that it may be involved in the formation of a functional holoenzyme, because the lack of the *gatC* gene in the *gatCAB* operon led to failure for the formation of active *B. subtilis* [4] and *S. aureus* (S. Namgoong, unpublished result). To elucidate the exact role of the GatC and GatB subunits in the holoenzyme for Glu-AdT in *E. coli*, their expression, purification, and a rigorous functional characterization are required.

Another interesting question concerning the natures of Glu-AdT is its substrate specificity. According to the results of Curnow *et al.* [4, 5], it specifically acts on mischarged Glu-tRNA^{Gln}, yet not on the Glu-tRNA^{Glu}. However, the *B. subtilis* Glu-AdT enzyme could efficiently convert mischarged Asp-tRNA^{Asn} formed by a nondiscriminating AspRS of *D. radiodurans* to Asp-tRNA^{Asn} [6]. Also, *D. radiodurans* Glu-AdT is able to transamidate Glu-tRNA^{Gln} and Asp-tRNA^{Asn} *in vitro*. These results imply that the tRNA binding specificity on the Glu-AdT is somewhat relaxed compared to those of conventional aminoacyl-tRNA synthetases. How can this enzyme discriminate misacylated tRNAs such as Glu-tRNA^{Gln} or Asp-tRNA^{Asn} from correctly acylated tRNAs such as Glu-tRNA^{Glu} or Asp-tRNA^{Asp}? To answer these questions, further extensive studies on the Glu-AdT from a wide range of organisms are required.

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