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A simple turbidimetric method for monitoring the inhibition of tRNA-dependent
amidotransferase GatCAB

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ABSTRACT

In eubacteria that lack glutamyl-tRNA synthetase (GlnRS), a tRNA-dependent amidotransferase (GatCAB) recognizes mischarged Glu-tRNA^{Gln} and converts it into Gln-tRNA^{Gln}. An inhibitor specific for GatCAB could therefore act as an antibiotic with a novel mode of action against multidrug-resistant bacteria such as *Staphylococcus* strains. However, there is no rapid, simple and efficient screening method for specifically monitoring the inhibition of GatCAB activity. We have focused on developing a simple system for monitoring the inhibition of GatCAB activity using *Escherichia coli* Top10 co-expressing the ndGluRS and GatCAB genes from *Staphylococcus aureus* Mu50. First, growth repression was confirmed by introducing *ndgluRS* from *S. aureus* Mu50 into *E. coli*. Then, we verified that co-expression of the *gatCAB* operon alleviated growth repression in the host *E. coli*. The screening system consisted of these two transformants and non-expressing *E. coli* Top10. The transformant harbors both ndGluRS gene and GatCAB operon could be co-expressed in the presence and in the absence of chemical compound of interest. Since there is no inhibitor that inactivates GatCAB activity, we expressed two inactive GatCAB deletion variants, GatCAB^{Δ10} and GatCAB^{ΔCHD} together with ndGluRS in *E. coli* Top10. The expressed *E. coli* showed repressed growth as well as ndGluRS expressed. These results indicate that if GatCAB activity is inhibited in this co-expressed *E. coli*, the inhibition can be monitored by the decrease in O.D. of the co-expressed *E. coli*.

Keywords

Escherichia coli

inhibitor

non-discriminating glutamyl-tRNA

tRNA-dependent amidotransferase GatCAB

turbidimetric assay

1. Introduction

A tRNA-dependent amidotransferase (GatCAB) plays an important role in eubacteria that lack glutamyl-tRNA synthetase (GlnRS) (Wilcox and Nirenberg, 1968, Schön *et al.*, 1988, Ibba *et al.*, 1999). In these bacteria, tRNA^{Gln} is first misaminoacylated with glutamate by a non-discriminating glutamyl-tRNA synthetase (ndGluRS), which, in addition to generating Glu-tRNA^{Glu}, also forms Glu-tRNA^{Gln}. The resulting mischarged tRNA is then recognized by

GatCAB and converted into Gln-tRNA^{Gln} (Wilcox, M., 1968, Curnow *et al.*, 1997, Ibba *et al.*, 2000). Therefore, the inhibition of GatCAB might result in growth repression or lethality in these bacteria, in which case a GatCAB inhibitor could be used as a drug that has a novel target and mode of action.

New targeted antimicrobial agents are urgently required to combat pathogens such as *Staphylococcus aureus* that have acquired multidrug resistance. Whole-cell bioassays involving *S. aureus* cannot effectively screen for inhibitors specific for GatCAB as various compounds affect the growth of bacteria in a variety of ways. An in vitro assay would require the preparation of a large amount of Glu-tRNA^{Gln}, the substrate for GatCAB, which would be both time-consuming and costly. To screen a new GatCAB inhibitor from thousands of chemical compounds or natural products, it is important to construct a simple method to evaluate the inhibition of this enzyme. Moreover, a recent study showed that a non-discriminating aspartyl-tRNA synthetase (ndAspRS), tRNA^{Asn}, and a tRNA-dependent amidotransferase, assemble into a specific ribonucleoprotein complex, the transamidosome, that remains stable during the overall catalytic process (Bailly *et al.*, 2007). It remains unclear whether or not ndGluRS, Glu-tRNA^{Gln} and GatCAB also assemble into a similar complex. Regardless, a bioassay monitoring the growth repression of living cells due to inhibition of the whole transamidosome is both more practical and specific than an in vitro assay for GatCAB activity.

In *Bacillus subtilis*, a single ndGluRS is responsible for the aminoacylation of both tRNA^{Gln} and tRNA^{Glu} with glutamate. This enzyme can also mischarge *Escherichia coli* tRNA₁^{Gln} with glutamate, but not *E. coli* tRNA₂^{Gln} and tRNA^{Glu} in vitro (Lapointe *et al.*, 1986). Early attempts to clone the *B. subtilis* ndGluRS gene in *E. coli* were unsuccessful. Over-expression of an intact *B. subtilis* ndGluRS was found to be lethal in *E. coli* (Pelchat *et al.*, 1998). A controlled vector system using *B. subtilis* ndGluRS expression in *E. coli* verified that reduced numbers of colony formation units in the *E. coli* was due to the mischarging of tRNA^{Gln} with glutamate (Baick *et al.*, 2004).

In this work, we describe a new system for monitoring GatCAB inhibition in *E. coli* Top10 in which both the ndGluRS and GatCAB genes from *Staphylococcus aureus* Mu50 are expressed in the presence and in the absence of chemical compound or natural product of interest, and compared the growth of these bacteria. The design and strategy of the system is shown in Fig.1. We first confirmed that growth repression was induced by the expression of *ndgluRS* from *S. aureus* Mu50 in *E. coli*. Then, we verified that co-expression of the *gatCAB* operon alleviated growth repression by ndGluRS in the host *E. coli*. In addition, two GatCAB deletion variants, GatCAB^{Δ10} and GatCAB^{ΔCHD}, containing the deletion of the entire helical bundle domain and the deletion of 64 residues from the C terminus of GatB

respectively, were expressed in *E. coli* together with ndGluRS. It was already known that these deletion variants are incapable of binding tRNA, as shown by an in vitro gel-shift assay (Nakamura *et al.*, 2006). We assumed that the two expressed GatCAB deletion variants were also inactive, and could not alleviate growth repression in *E. coli*. We therefore expressed inactive GatCABs in *E. coli*, since no chemical inhibitor presently exists. The growth of the transformants was measured and compared to that of the control, ndGluRS and GatCAB co-expressed *E. coli*.

2. Materials and Methods

2.1. Bacterial strains

The bacterial strains, *Escherichia coli* XL10Gold (Stratagene, La Jolla, Calif, USA) and *E. coli* Top10 (Invitrogen Corp., Carsbad, CA, USA), were used for general manipulation and propagation of plasmids. *E. coli* Top10 was used as a host strain for the expression of the ndGluRS gene and the *gatCAB* operon from *Staphylococcus aureus* Mu50.

2.2. DNA and vector construction

The plasmids used or constructed in this study are shown in Fig. 2, and the primer sequences are listed in Table 1. The entire ndGluRS gene from *Staphylococcus aureus* Mu50 was polymerase chain reaction (PCR) amplified using the primer pair of ndGluRS-f-NcoI and ndGluRS-r-XbaI. The gene was then cloned into the NcoI/XbaI site of pBAD/ Myc-His B vector (Invitrogen) to construct pBADndgluRS. The *gatCAB* operon from pET-28/*gatCAB* (Nakamura *et al.*, 2006) was PCR amplified using the primer pair GatC-f-NcoI and GatB-lumio-r-XhoI. The operon was then cloned first into the NcoI/XhoI site of pCOLADuet-1 (Novagen®, Darmstadt, Germany), with the ColA origin, fused with a linker and the lumio tag (Cys-Cys-Pro-Gly-Cys-Cys) at the C-terminus of GatB. The lumio tag fused *gatCAB* operon was PCR amplified using the primer pair gat C-f-NcoI and lumio-r-KpnI, and cloned into the NcoI/KpnI site of pTrcHis2A (Invitrogen) to obtain a *trc* promoter element. Then, together with the *trc* promoter region and terminator, the *gatCAB* operon with the lumio tag was PCR amplified using the primer pair pTrc-f-BamHI and rrnB-r-XhoI, and cloned back into the BamHI/XhoI site of pCOLADuet-1 to form pCTgatCAB. Because of the different origin of replication, plasmids derived from pCOLADuet-1 are compatible with the pBAD/Myc-His B derivative. Each insertion of plasmid was sequenced to confirm the construct.

2.3. DNA manipulation and transformation

The standard procedures were as described in Molecular Cloning (Sambrook and Russell, 2001). All plasmids were extracted using GE Healthcare illustra plasmid prep Mini Spin kit (GE Healthcare, USA). Restriction enzymes were purchased from Takara Bio Ohtsu, Japan. Dephosphorylation was performed with Takara alkaline phosphatase (Takara Bio, Ohtsu, Japan). A T4 DNA ligation kit ver.2 (Takara Bio) was used for ligation. Transformation was achieved by electroporation using a Gene pulser II electroporation system (Bio-Rad, California, USA).

2.4. PCR and sequencing

For the amplification of genes and DNA fragments, KOD DNA polymerase-plus ver.2 (Toyobo, Osaka, Japan) was used. All sequencing reactions were conducted using a BigDye Terminator v1.1 cycle sequencing kit, and the products were analyzed by an ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA)

2.5. Co-expression of ndGluRS and GatCAB in *E. coli* Top10

Transformant *E. coli* Top10/pBADndgluRS/pCTgatCAB cells were grown at 30°C in LB medium containing 50 µg/mL each kanamycin and carbenicillin. The expression of ndGluRS was induced by initially adding 0.2% L-arabinose under constitutively co-expressed GatCAB. After running the cell lysate on an SDS polyacrylamide gel, the expression of ndGluRS was detected by western blotting using anti-His Antibody (Invitrogen), as described in the manufacturer's protocol.

The expression of GatCAB in *E. coli* Top10 was confirmed by detecting the fluorescent fusion protein using a Lumio™ Green Detection Kit (Invitrogen) as described in the protocols.

2.6. Growth and viability of the bacteria.

Bacterial cell growth during incubation was monitored by optical density (O.D.) at 600 nm or 650 nm, and by counting colony formation units (CFU) in triplicate. Damaged and living cells were discriminated by LIVE/DEAD BacLight Bacterial Viability Kits (Invitrogen) under a fluorescence microscope (OLYMPUS BX50, Olympus, Japan). Survival rate was calculated

from the average number of living cells and the average number of damaged cells, by counting 20 fields of fluorescence overlay images from non-induced, ndGluRS-expressed and ndGluRS, GatCAB co-expressed *E. coli*.

2.7. Activities of GatCAB deletion variants in *E. coli* Top10

The heterotrimeric GatCAB deletion variant contains GatB deletion mutant. The GatCAB Δ^{CHD} and GatCAB Δ^{10} genes, containing a deletion of the entire helical bundle domain (Δ CHD) or 64 residues from the C terminus of the GatB gene (Δ 10) (Nakamura *et al.*, 2006), respectively, were cloned first into the *NcoI/XhoI* site of pCOLADuet-1 fused with a linker and the lumio tag at the C-terminus of GatB. Then, pCTgatCAB Δ^{CHD} and pCTgatCAB Δ^{10} were constructed as described in method 2.2. They were expressed in *E. coli* Top10 together with pBADndgluRS by incubating with 0.2% L-arabinose at 30 °C, and the O.D. was monitored at 650 nm every 15 min for 12 hrs using a plate reader (SpectraMax M2, Molecular Devices Corp. USA). The O.D. readings were compared with those of ndGluRS, GatCAB co-expressed and ndGluRS expressed *E.coli* Top10.

3. Results

3.1. Growth inhibition by the expression of pBADndgluRS and its alleviation by co-expression of GatCAB in *E. coli* TOP10

We constructed two plasmids, pBADndgluRS and pCTgatCAB, which harbor the ndGluRS gene and the GatCAB gene operon, respectively. *E. coli* RNA polymerase can transcribe both genes. The pBADndgluRS, which strictly regulates the expression of ndGluRS under the araBAD promoter (Guzman *et al.*, 1995), was transformed into *E. coli* Top10 together with pCTgatCAB. When *E. coli* Top10 /pBADndgluRS/pCOLADuet-1 was incubated in the presence of 0.2% L-arabinose (without pCTgatCAB), ndGluRS was expressed after 6 h incubation, but expression decreased at 9h (Fig. 3, B). The growth of the bacteria was repressed throughout the incubation period (Fig. 4). In contrast, the expression of both ndGluRS and GatCAB in *E. coli* Top10 /pBADndgluRS/pCTgatCAB with 0.2% L-arabinose was confirmed after 3 hrs incubation (Fig. 3, A, B). The total growth and the viable cell counts of the strain were higher than that of ndGluRS alone expressed bacteria. Presumably constitutively expressed GatCAB alleviated the lethality of ndGluRS products (Fig. 3, A, B, and Fig.4). GatCAB was stably expressed under the *trc* promoter, probably without *lacI^q* expression and without IPTG induction. Moreover, ndGluRS expression was

also increased by the addition of L-arabinose with GatCAB co-expression (Fig.3, A, B). Damaged and living microbial cells after 6h incubation were discriminated by fluorescence microscopy using two fluorescence dyes. The bacteria with intact cell membranes stained fluorescent green with SYTO9, whereas bacteria with damaged membranes stained fluorescent orange with propidium iodide (Fig. 5, A). The survival rate of cells co-expressed with GatCAB, as well as non-induced cells, was about 10 times higher than that of GluRS expressed cells alone (Fig. 5, B). These results demonstrated that ndGluRS from *Staphylococcus aureus* Mu50 produced Glu-tRNA₁^{Gln} in *E. coli* Top10, and co-expressed GatCAB from *Staphylococcus aureus* Mu50 also alleviated the lethal effect of Glu-tRNA₁^{Gln} in *E. coli* Top10.

The O.D. values in the three types *E. coli* Top10 (i.e., non-induced, ndGluRS expressed, and both ndGluRS and gatCAB expressed), after 6h incubation were highly reproducible. Based on the difference in O.D. values between ndGluRS expressed *E. coli*, and ndGluRS and gatCAB co-expressed *E. coli*, we constructed a screening system for monitoring inhibition specific for GatCAB activity. In this system, two types of transformants, *E. coli* Top10 /pBADndgluRS/pCOLADuet-1 and *E. coli* Top10 /pBADndgluRS/pCTgatCAB are incubated in the presence and in the absence of chemical compounds or natural products in LB medium with 50 µg/mL each kanamycin and carbenicillin, and with/without 0.2% L-arabinose. After 6 h incubation, the O.D. of each *E. coli* culture is measured and compared. If the O.D. of co-expressed *E. coli* in the presence of a certain compound is lower, as well as the O.D. of the ndGluRS expressed *E. coli*, whereas the O.D. of the non-induced *E. coli* is unchanged, this would suggest that the chemical compound inhibits GatCAB activity. Therefore, Glu-tRNA₁^{Gln} produced by ndGluRS would not be converted to Gln-tRNA₁^{Gln}, thereby repressing growth.

3.2. Activities of GatCAB deletion variants in *E. coli* Top10

In the constructed system, we expressed inactive gatCABs in *E. coli*, since adding an inhibitor was not possible, as none presently exist. The time course of total growth of the transformants was measured and compared to that of controls. Although GatCAB^{ΔCHD} and GatCAB^{Δ10} were co-expressed as proteins together with ndGluRS (Fig. 6, A and B), the transformants harboring GatB deletion mutants (10 and CHD in Fig. 6, C) showed a lower growth rate than ndGluRS and GatCAB co-expressed *E. coli* (Co in Fig. 6, C) as well as ndGluRS expressed *E. coli* (RS in Fig. 6, C). These results demonstrate that the Glu-tRNA₁^{Gln} produced by ndGluRS could not be converted to Gln-tRNA₁^{Gln} by GatCAB^{ΔCHD} and GatCAB^{Δ10} in this system. Therefore, if a compound inhibited GatCAB activity in

ndGluRS and GatCAB co-expressed *E. coli* Top10, the co-expressed *E. coli* could no longer grow as quickly as the control, resulting in decreased growth rate and lower O.D.

4. Discussions

We have developed a simple turbidimetric method for monitoring the inhibition of *S. aureus* GatCAB expressed in *E. coli* Top10. It is supposed that in *E. coli* cells, the introduced *S. aureus*-ndGluRS produces lethal Glu-tRNA^{Gln} from *Escherichia-coli* tRNA^{Gln} charged with glutamate, thereby reducing cell growth. However, the introduction of *S. aureus*-GatCAB eliminated the lethal component and restored the growth of *E. coli* Top10. If a mutation in *S. aureus*-GatCAB inhibited GatCAB activity, or if an inhibitor specific for *S. aureus*-GatCAB could be developed, protein synthesis would be impaired and cellular growth would be repressed. Thus, by detecting the repressed growth in *S. aureus*-ndGluRS, *S. aureus*-GatCAB co-expressed *E. coli* in the presence of compound, we thought that we could monitor the inhibiting effect of compound.

GatCAB is a heterotrimeric protein composed of A, B, and C subunits. GatCAB catalyzes three reactions: GatA acts as a glutaminase, and GatB acts as both a kinase and transamidase. These reactions are tightly coupled, and the binding of Glu-tRNA^{Gln} regulates the glutaminase reaction (Nakamura, *et al.*, 2006). In order to screen for inhibitors specific for this coupling reaction, we established a screening system using *E. coli* Top10 /pBADndgluRS /pCTgatCAB, *E. coli* Top10 /pBADndgluRS /pCOLADuet-1 and non-inductive *E. coli* Top10. A compound that can decrease the growth of co-expressed *E. coli* Top10 /pBADndgluRS /pCTgatCAB, but not that of the non-inducer, could be an inhibitor specific for GatCAB. Such screening is now in progress in our laboratory using culture supernatants from various isolates, as well as synthesized chemical compounds. Because *E. coli* cells produce enzymes, *S. aureus*-nuGluRS and *S. aureus*-GatCAB and substrate for GatCAB in this system, it could be important for a specific inhibitor to be transported through the membrane.

Table 1. Primers used in this study and their sequences

Name	Sequence (5'-3')	Reference
ndGluRS-f-NcoI	CATGCCATGGGCGATCGTATAAGAG	this study
ndGluRS-r-XbaI	GCTCTAGACCCTTATATTGTTTTAAACGGTTTAGC	this study
GatC-f-NcoI	CTAGCCATGGCAAAAGTAACACGTG	Nakamura <i>et al.</i>
GatB-r-XhoI	CCGCTCGAGTCTTTTATCTAATTCTTGTTTTAATAGTTG	Nakamura <i>et al.</i>
GatB-lumio-r-XhoI	CCGCTCGAGTTAACAGCAGCCCGGACAGCAGCTGCCGC TGCCTCTTTTATCTAATTCTTGTTTTAATAG	this study
lumio-r-KpnI	GGGGTACCTTAACAGCAGCCCGGACA	this study
pTrc-f-BamHI	CGGGATCCCTGTTGACAATTAATCATCCGG	this study
rrnB-r-XhoI	CCGCTCGAGAAAGGCCAGTCTTTCGAC	this study

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Legends

Fig. 1. Strategy and design for the screening system. Total growth of three types of *Escherichia coli* Top10 is compared. RS: *ndgluRS* from *Staphylococcus aureus* Mu50 is expressed, Co: *ndgluRS* and *gatCAB* operons from *S. aureus* Mu50 are co-expressed, No: both *ndgluRS* and *gatCAB* operons are not expressed. In *E. coli* Top10 type Co, Glu-tRNA₁^{Gln} is converted to Gln-tRNA₁^{Gln} and the resulting reduction in cellular growth is alleviated by the expression of GatCAB. When *E. coli* type Co is expressed in the presence and in the absence of chemical compound of interest and the type No is incubated in the presence of the same compound, specific inhibitor for GatCAB is identified by the ability to repress total growth of type Co but not type No.

Fig. 2. Construction of plasmids harboring the *ndgluRS* and *gatCAB* operons

A: pBAD*ndgluRS*, B: pCT*gatCAB*

Amp: ampicillin resistance, Kan: kanamycin resistance.

araBAD: *araBAD* promoter, T7: T7 promoter, *trc*: *trc* promoter

Source or references for each plasmid are described in the manuscript.

Primers and their sequences are listed in Table 1.

Fig. 3. Time course of expression in three transformants of *Escherichia coli* Top10, A: GatB detected by the fluorescence of the lumio tag, B: ndGluRS detected by western blotting using anti-His antibody.

No: no-induction (*E. coli* Top10 /pBAD*ndgluRS*/pCOLADuet-1 without L-arabinose),

RS: ndGluRS expression (*E. coli* Top10 /pBAD*ndgluRS*/pCOLADuet-1 with 0.2% L-arabinose)

Co: co-expression (*E. coli* Top10 /pBAD*ndgluRS*/pCT*gatCAB* with 0.2% L-arabinose)

M: molecular size standard markers, lumio tagged in A, His tagged in B.

Fig. 4. Plots showing optical density at 600 nm and viable cell count at 0, 3, 6, 9, 12, and 24 h incubation time. Incubation broth was removed at each time point and the optical density and number of colony forming units (CFU) were measured. CFU were measured after spreading the broth aliquot on a LB plate containing 50 µg/mL each kanamycin and carbenicillin and incubating overnight at 37 °C. The CFU reflect the number of cells that can proliferate at that incubation time, which is equivalent to the number of viable cells present at that time point.

Fig. 5. Fluorescence overlay images and survival rate of *E. coli* determined using a LIVE/DEAD BacLight Bacterial Viability Kit. A: fluorescence microscope overlay images of *Escherichia coli* Top10 cells after 6 h incubation. No: no induction, RS: ndGluRS expression and Co: co-expression of ndGluRs and GatCAB. B: Survival rate was calculated from the average number of living cells and the average number of all cells. Cells were counted in 20 fields of fluorescence overlay images, where living cells are stained fluorescent green and dead cells are stained fluorescent orange. Results of two independent experiments are shown as mean $\pm$ SEM.

Fig. 6. Growth repression in *E. coli* expressing GatCAB deletion variant.

A: GatB and its deletion mutants were detected by the fluorescence of the lumio tag after 6 h incubation at 30 °C. B: ndGluRS detected by western blotting using anti-His antibody at the same incubation time. C: Plot showing time course of optical density at 650 nm.

M: molecular size standard markers, lumio tagged in A, His tagged in B.

Co: co-expression in *E. coli* Top10 /pBADndgluRS/pCTgatCAB with 0.2% L-arabinose

RS: ndGluRS expression in *E. coli* Top10 /pBADndgluRS/pCOLADuet-1 with 0.2% L-arabinose.

10: ndGluRS and GatCAB Δ^{10} deletion variant co-expression in *E. coli* Top10 /pBADndgluRS/ pCTgatCAB Δ^{10} with 0.2% L-arabinose. CHD: ndGluRS and GatCAB Δ^{CHD} co-expression in *E. coli* Top10 /pBADndgluRS/ pCTgatCAB Δ^{CHD} with 0.2% L-arabinose. See materials and methods for details regarding the deletion mutants.

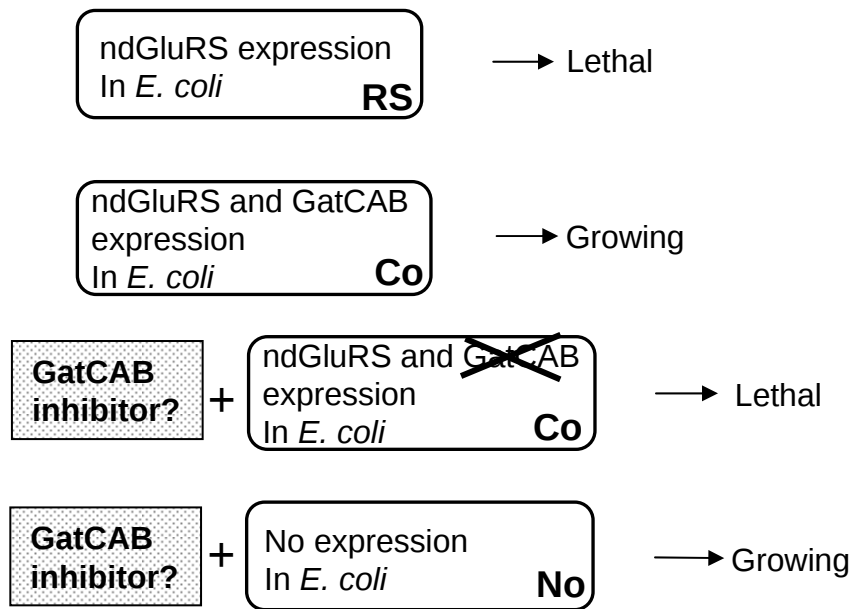


Fig. 1

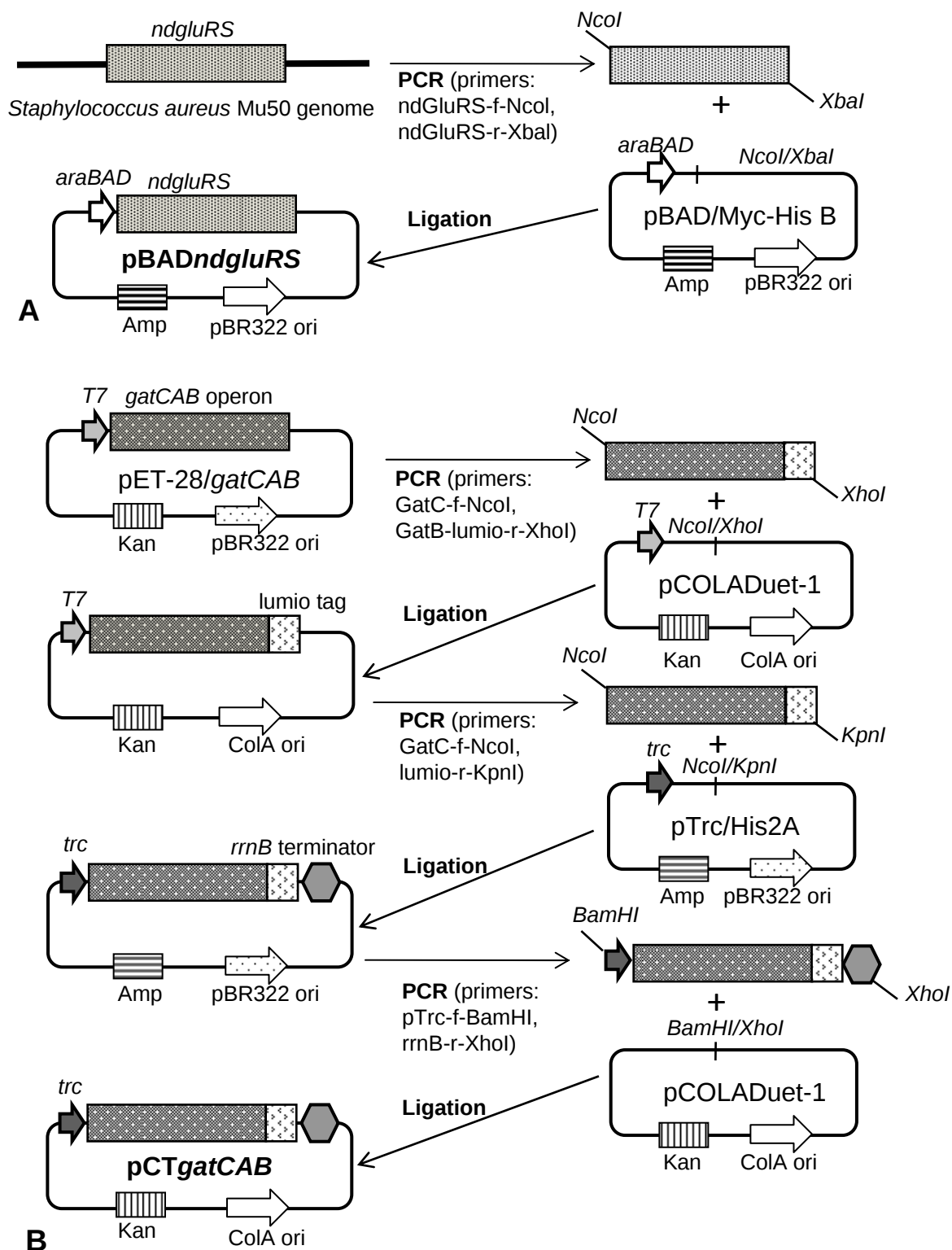


Fig. 2

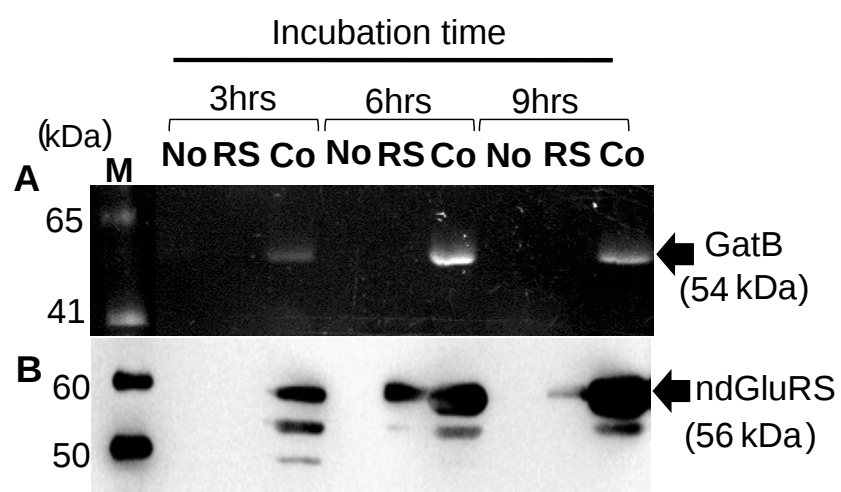


Fig. 3

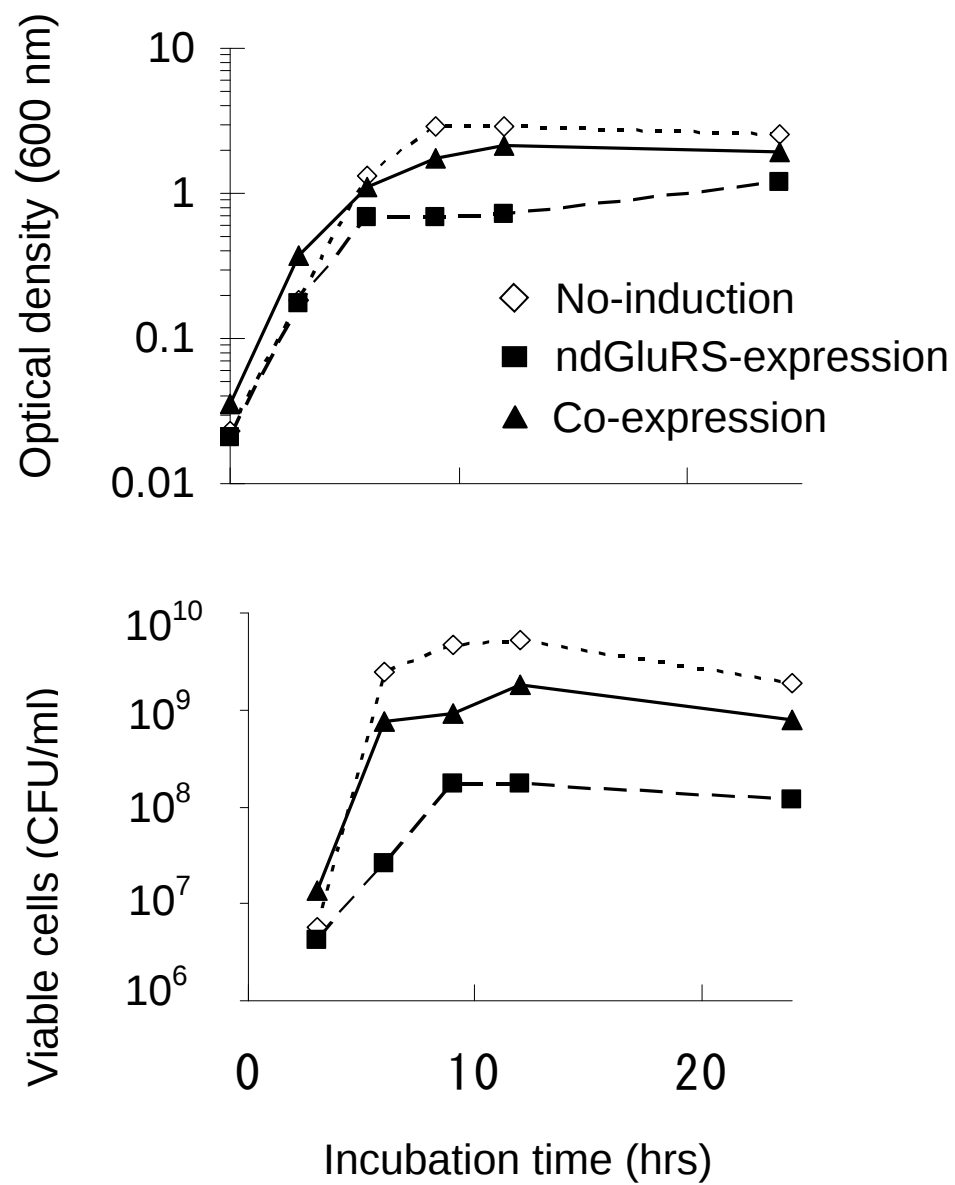


Fig. 4

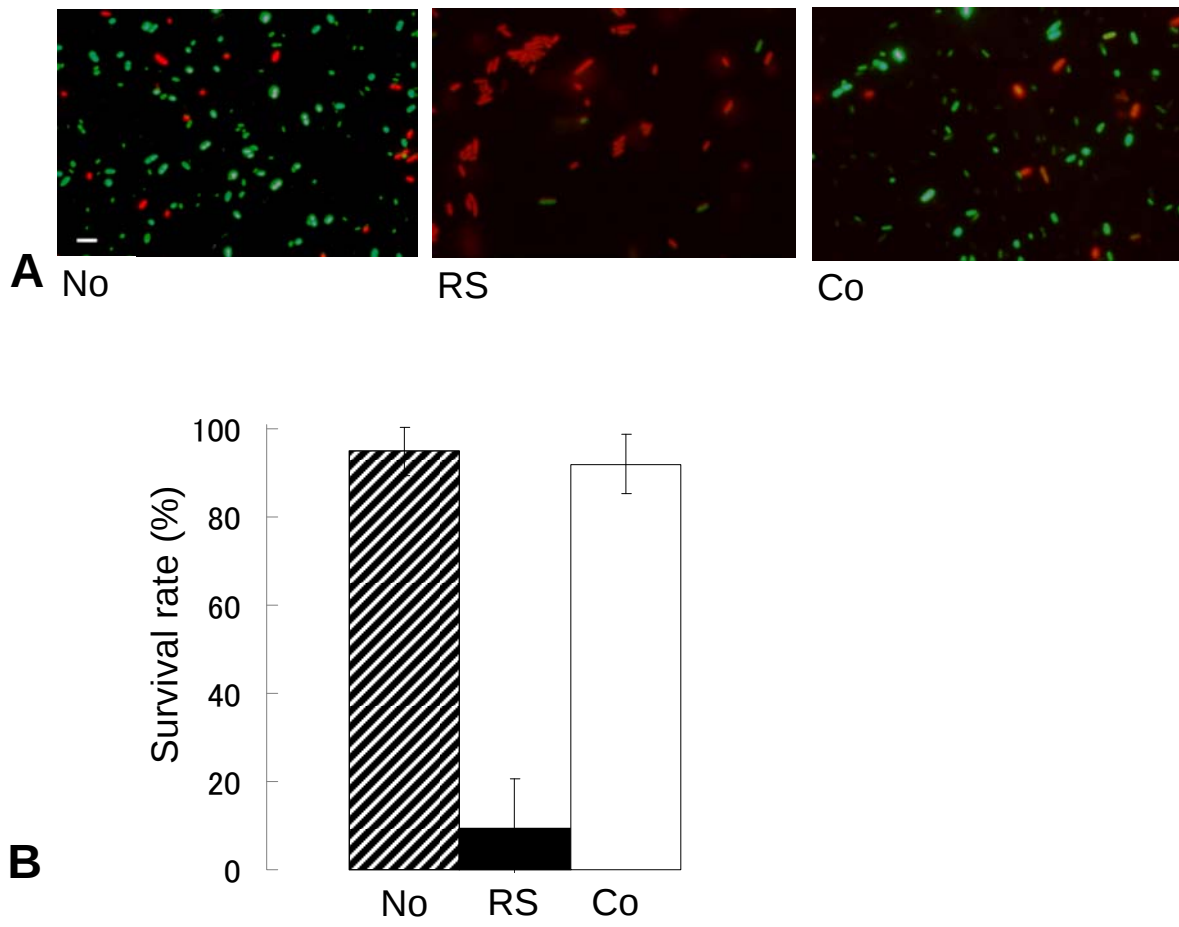


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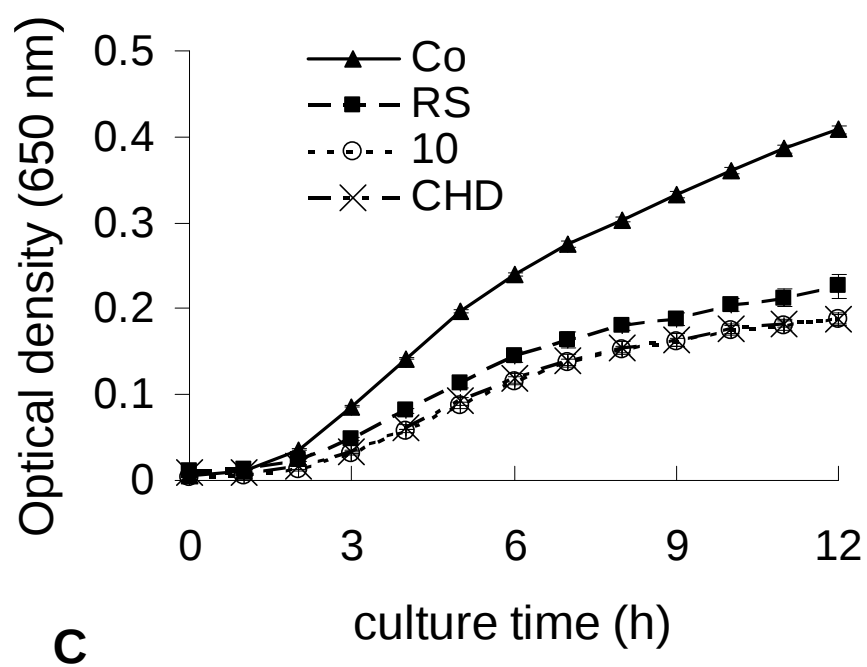
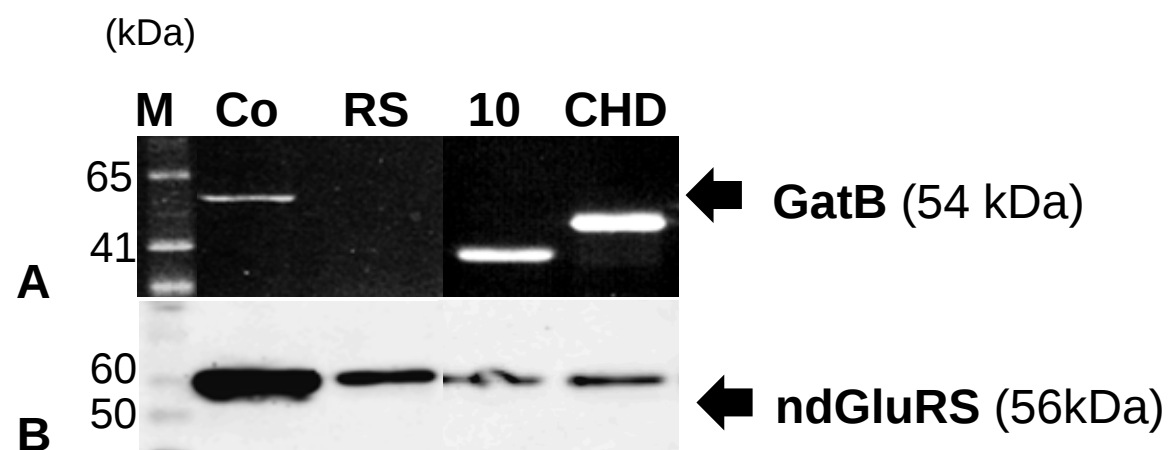


Fig. 6