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Daniel Kim 4/15/2015

# Mutation of the *gltT* gene decreases the efficiency of growth of *Neisseria gonorrhoeae*

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An abstract of
a thesis submitted to the Faculty of Emory College of Arts and Sciences
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**ABSTRACT**

Mutation of the *gltT* gene decreases the efficiency of growth of *Neisseria gonorrhoeae*

By Daniel Kim

The *gltT* gene encodes for an L-glutamate ABC transporter. In this work, I studied the effects of a *gltT*-negative mutation in streptomycin resistant *Neisseria gonorrhoeae* FA19 (FA19St$^R$). A *gltT*-negative mutant, FA19St$^R$*gltT*::*kan*, was shown to be deficient for growth in GC broth in comparison with its parental strain FA19. In the related pathogen *N. meningitidis*, acquisition of L-glutamate through GltT promotes evasion of polymorphonuclear leukocyte (PMN)-generated reactive oxygen species. Accordingly, oxidative stress due to hydrogen peroxide of FA19St$^R$*gltT*::*kan* was tested, although the mutant FA19St$^R$*gltT*::*kan* always appeared more sensitive to $H_2O_2$ than its isogenic parental strain FA19St$^R$, the difference in sensitivity was not statistically significant. This work reveals genetic pathways that could potentially weaken gonococcal survival in PMN. Lastly, attempts to complement the *gltT*-negative mutant using the pGCC4 vector were unsuccessful. Sequence of the construct used for complementation (pGCC4*gltT*) revealed the presence of a new mutation in the coding region of the *gltT* gene that could result in the production of a non-functional protein.

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**List of Tables and Figures:**

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## INTRODUCTION

### *Neisseria gonorrhoeae*

*Neisseria gonorrhoeae* (gonococci) is a Gram-negative diplococcus bacterium that is the causative agent of the sexually transmitted disease gonorrhea. The disease is also known as “the clap,” where this term was first introduced in 1378 and likely to have originated from an old Parisian district that housed prostitutes – “Les Clapiers” (1). This disease also has ancient references to the Old Testament (Leviticus 15:1-3). The genus *Neisseria* was named after the German bacteriologist Albert Neisser, who discovered in 1879 the first example of the genus, *Neisseria gonorrhoeae* (2). Records of treatment against this organism have been around since the mid 1850s, where botanicals such as balsam of copaiba were used because of its ability to cease discharge (3). This organism has caused much public health concern worldwide and has been the focus of research to develop antimicrobials against the bacteria. But because of the growing resistance to antibiotics, much more extensive research on genetic pathways of *Neisseria gonorrhoeae* is being done. In 2003, the first complete genome of *Neisseria gonorrhoeae* was sequenced through shotgun sequencing (4). The genome, in one contiguous sequence, is approximately 2.1 Mb in size.

### *Burden of gonorrhea*

#### *Symptoms and Statistics*

Gonococcal infections generally present as urethritis in men and cervicitis in women. Women are frequently asymptomatic and untreated infections can result

in severe complications such as pelvic inflammatory disease, which can lead to infertility (5, 6). A minority of men (<10%) and a larger proportion of women (>50%) have asymptomatic urogenital infections. Most commonly, rectal and pharyngeal gonorrhea are asymptomatic. If these urogenital infections are left untreated, it may ascend to the upper genital tract and result in reproductive complications. Failure in curbing the transmission of gonorrhea also increases transmission of other sexually transmitted infections (STI), such as HIV infection (7).

In 2013, there were 333,004 reported cases of gonorrhea (128.6 cases per 100,000 population) in the United States with increasing number of cases throughout 2009-2013 (8). In the world, an estimate of 106 million cases is known to occur every year (7). The WHO Western Pacific Region was estimated to have the highest estimate of 42 million cases, with WHO South-East Asia Region (25.4 million cases) and WHO Africa Region (21.1 million cases) having the next highest estimated number of cases. The number of reported cases is substantially smaller than the actual number of cases because of poor diagnostic methods, incomplete reporting and epidemiological surveillance.

*Antibiotic Resistance*

Gonorrhea has been successfully treated through the use of antimicrobials such as penicillin for the last 70 to 80 years. However, there is now a high prevalence of antibiotic resistant *N. gonorrhoeae* internationally. Many *N.*

*gonorrhoeae* strains are becoming more resistant to antimicrobials previously widely available for treatment, such as penicillins, sulfonamides, tetracyclines and earlier cephalosporins. Recently, extended-spectrum cephalosporins (ESCs) cefixime and ceftriaxone have failed to treat gonorrhea (9). While ceftriaxone remains the last option for empirical first-line antimicrobial therapy, there is fear that gonorrhea will soon be untreatable using this method. As a response, dual-antimicrobial regimens are implemented where ceftriaxone and azithromycin are used. Ceftriaxone is a β-lactam antibiotic, which has a β-lactam ring fused to a thizolidine ring (penicillin), or a dihydrothiazine ring (cephalosporins).

β-lactam antibiotics target the penicillin-binding proteins or PBPs - a group of enzymes found anchored in the cell membrane, which are involved in the cross-linking of the bacterial cell wall (10)**.** Resistance to β-lactam drugs occurs due to the production of β-lactamases, which cleave the amide bond of the β-lactam molecule (10) or mutations of the PBPs. Eventually the cell ruptures as the bacterium loses its ability to maintain integrity of the cell wall.

Azithromycin is a macrolide antibiotic and the general chemical structure consists of a macrolide ring, which is a large macrocyclic lactone ring (11)**.** Azithromycin and other macrolide antibiotics bind to the 50S subunit of the bacterial ribosome and inhibit the translation of mRNA (12). Resistance occurs due to methylation of a 23S rRNA nucleotide (13). These types of methylations sterically hinder antibiotic activity on these ribosomes (14).

As susceptibility of gonococci to these antimicrobials is decreasing, these dual-regimen therapies of azithromycin and ceftriaxone could become ineffective in the long term. Therefore, the lack of a vaccine and widespread antibiotic resistance expressed by geographically disperse strains complicate the control of infection. It is important to attain a better understanding of how *N. gonorrhoeae* survives *in vivo* and what mechanisms make the bacterium virulent. A more advanced understanding of its genetic regulation pathways may provide an impetus for infection control of gonococci and development of better drugs for targeting *N. gonorrhoeae.*

***Regulation of glutamate ABC-transporter***

In meningococci, Tala *et al.* (15) showed that the acquisition of L-glutamate through the GltT transporter promoted evasion of polymorphonuclear neutrophil leukocyte (PMN)-generated reactive oxygen species (ROS) produced by the oxidative burst. Because *Neisseria meningitidis* and *Neisseria gonorrhoeae* are highly related species, gonococci might exhibit similar regulatory pathways as meningococci. Both are obligate human pathogens, with more than 80% DNA homology (16) with majority of genes conserved (17, 18).

***Relationship of glutamate to metabolism and oxidative stress***

Glutamate is an important molecule for all living organisms that plays various roles in metabolic processes. It is an amino acid involved in protein synthesis and other essential processes such as glycolysis and the Krebs cycle (19). Glutamate dehydrogenase, composed of 400-500 amino acids, exists in all living organisms

and catalyzes the reversible oxidation deamination of L-glutamate to α-ketoglutarate (20).

L-glutamate is also used in the production of glutathione in most organisms. Glutathione is a tripeptide composed of amino acids L-cysteine, L-glycine and L-glutamate. The tripeptide exists in high intracellular concentrations (1-10mM) and is the most abundant low molecular weight thiol of bacterial, plant and animal cells (21). Glutathione is also a key antioxidant for maintaining redox potential and protects many organisms from reactive oxygen species such as hydrogen peroxide by existing as a cofactor for the glutathione peroxidase (GSH peroxidase) and S-transferase enzyme families (22, 23). When GSH peroxidase converts hydrogen peroxide ($H_2O_2$) into water, the thiol groups in two molecules of GSH, the reduced form of glutathione, donate a reducing equivalent to unstable oxygen species (24). Upon this reaction, which is catalyzed by glutathione reductase, oxidized glutathione (GSSG) is formed through a disulfide linkage between two GSH molecules.

*N. gonorrhoeae* is exposed to reactive oxygen species when it inhabits the host mucosal surfaces such as the urethra in males and cervix in females. Exposure to ROS can occur in various ways. It may first come from byproducts created during the bacterium's aerobic respiration process (24). *N. gonorrhoeae* is also routinely exposed to oxidative killing by the host due to the innate immune response (24). One hallmark of gonococcal infection is the high level of PMNs found at the site of infection (25). Gonococci can be seen inside macrophages

and PMNs in an anaerobic environment. Most commonly discussed ROS in biological systems are $H_2O_2$ and superoxide ($O_2^{\cdot -}$) (26). Exposure to these ROS results in damage to DNA, lipids and proteins (27). As bacterial response to oxidative stress is being recognized as an important factor for virulence, understanding the microbial oxidative stress response may help develop future treatment and prevent infections caused by bacteria.

## OBJECTIVES

1. Determine if a *gltT*-negative mutant, that does not produce a GltT glutamate transporter, will show a difference in growth efficiency compared to its parental wild-type strain in GC broth.

2. Investigate if a *gltT* mutation will affect gonococcal sensitivity to hydrogen peroxide using a disc diffusion inhibition test.

3. Complementation of the *gltT*-negative mutant and rescue experiments to determine if phenotypes will be restored.

## METHODS

### Bacterial Strains and Culture Conditions

Bacterial strains are as described in Table 1. Streptomycin-resistant ($St^R$) derivatives of wild-type *N. gonorrhoeae* strain FA19 and its isogenic *gltT* mutant strain were used in this study. This mutant strain was previously constructed in the Shafer laboratory (personal communications).

### Growth curves

Strains were harvested from GC agar plates after 18 hours of incubation at 37°C and inoculated into 20 mL of GCB broth (Difco Laboratories, Detroit, MI) supplemented with glucose supplements, iron and 0.043% (v/v) $NaHCO_3$ to a starting absorbance value $A_{600}$ = 0.2. Cultures were incubated at 37°C in a shaking incubator as previously described (28). OD measurements were made every hour. The $A_{600}$ value at each time point is an average of values from three independent experiments. Standard deviation for each average was calculated. The *p* value was also calculated through a one-tail t-test for the average $A_{600}$ values at the fourth hour.

### $H_2O_2$ sensitivity analysis using a disc diffusion assay test

Overnight plate cultures of $FA19St^R$ and $FA19St^R$*gltT*::*kan* were resuspended in GC broth. Each bacterial suspension was set to $A_{600}$=0.2. 40 mL of each bacterial suspension were plated onto GC plates supplemented with 1mM $NaNO_2$. Paper disks were placed on the center of each plate, and 0.12 µg of

$H_2O_2$ were added to the center of each disk. The plates were placed at 37°C for 48 hours in both aerobic and anaerobic conditions. Inhibition diameters were measured after 48 hours at 37°C in both aerobic and anaerobic conditions. Three independent experiments were performed and the inhibition diameters were averaged. Standard deviation for each average was calculated. The *p* value was also calculated through a one-tail t-test for each average measurement.

**Complementation**

The complementation of FA19St$^R$*gltT*::*kan* was done by inserting a wild-type copy of the *gltT* gene at the *lctP* locus of FA19St$^R$*gltT*::*kan* using the pGCC4 vector (30). The pGCC4 vector plasmid was digested by Pac1 and Pme1 (Figure 3A). The *gltT* gene was amplified by PCR using primers pac12117b (5'-ATGTT CTCCGGCTCGCCGCAG-3') and pme12117b (5'-GATCGTTTAAACCGC GTTTCGTCTTCAGACAG-3'). The resulting PCR product was subsequently digested by PmeI and PacI. The pGCC4 vector and *gltT* insert fragment were then ligated (Figure 3B). The ligation was transformed into DH5α *E. coli*, and transformants were selected on GC plates supplemented with 50 mg/mL of kanamycin. Transformants were confirmed by PCR using pac12117b and pme12117b primers (Table 2). Qiaprep of pGCC4*gltT* was performed in order to transform the pGCC4*gltT* construct into FA19St$^R$*gltT*::*kan*. Transformants were selected on GC agar plates supplemented with erythromycin (1mg/mL). Transformants were checked by colony PCR using primers pac12117b, pme12117b and kanF (Table 2, Figure 3F).

**Structural Model Prediction of GltT and mutant GltT**

The Phyre program was used to predict the three-dimensional structure formed by the amino acid sequences of the wild type and mutant *gltT* gene (31).

## RESULTS

### Growth Curve Experiments of FA19St$^R$ versus FA19St$^R$*gltT*::*kan*

Mutation in the *gltT* gene slows the growth rate in meningococci (29). When cultured in GC broth under standard conditions, FA19St$^R$*gltT*::*kan* grew slower in comparison to its wild type parental strain FA19St$^R$ (Figure 1). The mutant strain took a longer time to reach the late logarithmic phase. The average $A_{600}$ value at the fourth hour FA19St$^R$ reached $A_{600}$=0.924 (STD=0.05) while FA19St$^R$*gltT*::*kan* reached $A_{600}$=0.555 (STD=0.06). By the fourth hour, the difference in the average $A_{600}$ between the wild type and the mutant was statistically significant ($p$=0.00089).

### Determination of gonococcal susceptibility to $H_2O_2$

As described above, gonococcal susceptibility to $H_2O_2$ was assessed using a disc diffusion assay. The zone of inhibition was determined by taking an average of three independent experiments. Under aerobic conditions, the average zone of inhibition for FA19St$^R$ was 2.13cm (STD=0.15), while for FA19St$^R$*gltT*::*kan* the average was 2.33cm (STD= 0.15) (Figure 2A). Under anaerobic conditions, the average zone of inhibition for FA19St$^R$ was 2.37cm (STD=0.21), and the average for FA19St$^R$*gltT*::*kan* was 2.63cm (STD=0.15) (Figure 2B). The difference in the average value in FA19St$^R$ and FA19St$^R$*gltT*::*kan* in both aerobic and anaerobic conditions was calculated to be statistically insignificant, with $p$ values all exceeding 0.05.

**Complementation of FA19St$^{R}$*gltT*::*kan***

Based on PCR results, the genetic complementation of the *gltT* mutant was successful (Figure 3). When gene products are amplified using the pacI2117b and pmeI2117b primers, FA19St$^{R}$*gltT*::*kan* shows one band with a product size of 1769 bp in lane 1 (Figure 3F). This product corresponds to the *gltT* gene with the kanamycin insert in the genome of FA19St$^{R}$*gltT*::*kan*. FA19St$^{R}$*gltT*::*kan*C' shows two bands at lane 2, one faint band at 1769 bp and the second band at 990 bp (Figure 3F). The band at 990 bp represents the wild type FA19 *gltT* gene that was recombined in the bacterial genome for complementation. When kanF and pacI2117b primers were used to amplify gene products from FA19St$^{R}$*gltT*::*kan* and FA19St$^{R}$*gltT*::*kan*C', one band at 905 bp appeared for both FA19St$^{R}$*gltT*::*kan* in lane 3 and FA19St$^{R}$*gltT*::*kan*C' in lane 4. After this confirmation, FA19St$^{R}$, FA19St$^{R}$*gltT*::*kan,* and FA19St$^{R}$*gltT*::*kan*C' strains were cultured separately in GC broth with supplements as described in materials and methods. FA19St$^{R}$ grew to $A_{600}$=0.841 by the fourth hour, while FA19St$^{R}$*gltT*::*kan*C' reached 0.358 and FA19St$^{R}$*gltT*::*kan* reached 0.476 (Figure 4A). In that the complemented strain did not show a wild-type pattern of growth, it was suspected that the cloned gene might have a mutation. Indeed, sequencing of the FA19 *gltT* gene recombined into FA19St$^{R}$*gltT*::*kan* showed a thymidine to cytosine point mutation 84 base pairs downstream of the start codon, when compared to the wild type *gltT* gene (Figure 4B and 4C). This mutation resulted in a leucine to serine substitution in the 29$^{th}$ position of the amino acid sequence (Figure 4D), which is in the first

transmembrane domain of the GltT protein (Figure 4E). Phyre prediction of the protein structures shows that the mutant GltT protein has a truncated α-helix and shortened loops connecting the helices (Figure 4F).

## DISCUSSION

As antibiotic resistance increases over time in *Neisseria gonorrhoeae,* a better understanding of these microorganisms must be attained in order to find new ways to fight resistant gonococcal strains. In this study, the phenotype of a *gltT*-negative mutant (FA19St$^R$*gltT*::*kan*), that lacks the GltT glutamate ABC-transporter, was evaluated through growth curve analysis and $H_2O_2$ diffusion inhibition tests. Subsequently, complementation of FA19St$^R$*gltT*::*kan* was attempted to rescue the phenotypic difference observed in the mutant strain.

The growth curve experiments suggested that the *gltT* mutation impaired the growth of FA19St$^R$*gltT*::*kan*. The glutamate ABC transporter is embedded in the inner membrane of *Neisseria gonorrhoeae*. One explanation of the growth deficiency of FA19St$^R$*gltT*::*kan* may be that the absence of the GltT protein in the membrane of the mutant resulted in a destabilized membrane so that the mutant strain lysed more easily than its parental strain FA19St$^R$. An indication that the membrane of FA19St$^R$*gltT*::*kan* was perturbed by the absence of this transporter was that mutant bacteria grown on GC agar plates were extremely difficult to harvest. Their resuspension into GC broth was also very challenging in comparison with FA19St$^R$.

In regards to metabolism, low glutamate uptake levels could also have altered the conversion of glutamate to α-ketoglutarate. The oxidative deamination of L-glutamate to α-ketoglutarate is catalyzed by glutamate dehydrogenase (20). If a major component of the Krebs cycle like α-ketoglutarate is affected due to

low glutamate levels, it is plausible the affected Krebs cycle resulted in lower energy production and hence, inhibited growth of the mutant strain.

The absence of the GltT glutamate transporter also seemed to affect the response of FA19St$^R$*gltT*::*kan* to oxidative stress. The larger average zone of inhibition value of FA19St$^R$*gltT*::*kan* in both aerobic and anaerobic conditions suggested that the mutant strain was more sensitive to $H_2O_2$ than the wild type strain under the same conditions, although shown to be statistically insignificant based on the three experiments. The potential difference in sensitivity to $H_2O_2$ between FA19St$^R$ and FA19St$^R$*gltT*::*kan* is worth discussing in terms of the role of glutathione in protecting cells from oxidative stress. GSH, the reduced form of glutathione converts $H_2O_2$ into $H_2O$ by donating a reducing equivalent to unstable ROS like superoxide ($O_2^{\cdot -}$). While the glutathione levels in the FA19St$^R$ and FA19St$^R$*gltT*::*kan* were not measured for this study, as glutamate is one of the amino acids that compose the tripeptide glutathione, there could be effects on the ability of FA19St$^R$*gltT*::*kan* to process $H_2O_2$ into $H_2O$ because glutathione production is insufficient due to low glutamate levels in FA19St$^R$*gltT*::*kan*.

The diffusion inhibition test was also done in anaerobic conditions in order to replicate the environment that gonococci are often exposed to *in vivo*. Because gonococci have been seen intracellularly in PMNs, exposing FA19St$^R$ and FA19St$^R$*gltT*::*kan* to $H_2O_2$ emulate gonococcal exposure to PMN-generated ROS.

In order to show that the change in phenotype of FA19St$^R$*gltT*::*kan* was due only to the *gltT* mutation, FA19St$^R$*gltT*::*kan* was complemented by integrating

the wild type FA19 *gltT* gene at the *lctP* locus. PCR results showed genetically that the complementation was successful (Figure 3F).

One trial of vitro growth kinetic analysis done with FA19St$^R$, FA19St$^R$*gltT*::*kan* and FA19St$^R$*gltT*::*kan*C' (Figure 4A) shows that the wild type phenotype growth efficiency was not rescued in FA19St$^R$*gltT*::*kan*C'. This result can be explained by the sequencing results of the wild type FA19 *gltT* gene that was integrated into the complemented strain.

The amino acid substitution due to the point mutation (Figure 4D) likely disrupts the structure of the GltT protein (Figure 4E and 4F). Serine is a hydrophilic amino acid with a hydroxyl group in its side chain, while leucine is a hydrophobic amino acid with only hydrocarbons in its side chain. This change in polarity may have effects on the overall structure of the GltT protein, altering its normal function. Because the lipid bilayer is a hydrophobic environment, the substitution of a hydrophobic to a hydrophilic amino acid within the transmembrane domain could greatly disrupt the integrity of the protein in the membrane. The predicted protein models of the wild type and mutant GltT proteins further show that a change in the structure of the protein may have occurred due to the mutation. The mutant GltT protein has a truncated α-helix and shortened loops that connect the helices. This change in protein structure further explain why the phenotype was not restored in FA19St$^R$*gltT*::*kan*C'.

## FUTURE RESEARCH

Currently minimal bactericidal concentration (MBC) assays are being performed in order to test more accurately the sensitivity of FA19St$^R$ and FA19St$^R$*gltT*::*kan* to reactive oxygen species. Two compounds, $H_2O_2$ and tert-Butyl hydroperoxide are being used for the current assay. The intracellular survival capacity of FA19St$^R$ and FA19St$^R$*gltT*::*kan* could also be evaluated using cultured phagocyte cell lines. Lastly, if significant difference in MBC and intracellular survival capacity is displayed between FA19St$^R$ and FA19St$^R$*gltT*::*kan*, their glutathione levels could be studied to test whether the loss of glutamate uptake has a potential effect on glutathione synthesis. If the two strains FA19St$^R$ and FA19St$^R$*gltT*::*kan* exhibit these differences, FA19St$^R$*gltT*::*kan*C' also needs to be reconstructed and tested in these experiments in order to evaluate whether any difference in phenotype can be rescued.

## TABLES AND FIGURES

**Table 1. Bacterial Strains**

| Strain | Relevant Characteristics | Reference |
|---|---|---|
| FA19St$^R$ | Streptomycin-resistant strain of FA19 | Shafer laboratory; strain FA19 was obtained from P.F. Sparling's laboratory in 1982 |
| FA19St$^R$*gltT*::*kan* | *gltT*-negative mutant of strain FA19St$^R$ | Shafer laboratory |
| FA19St$^R$*gltT*::*kan*C' | *gltT* complemented of FA19St$^R$*gltT*::*kan* | Present study |

**Table 2. Oligonucleotide Primers Used in This Study**

| Primer | Sequence (5' to 3') |
|---|---|
| pmeI2117b | GATCGTTTAAACCGCGTTTCGTCTTCAGACAG |
| pacI2117b | ATGTTCTCC GGCTCGCCGCAG |
| kanF | GATTATCGAGCTGTATGCGGA |

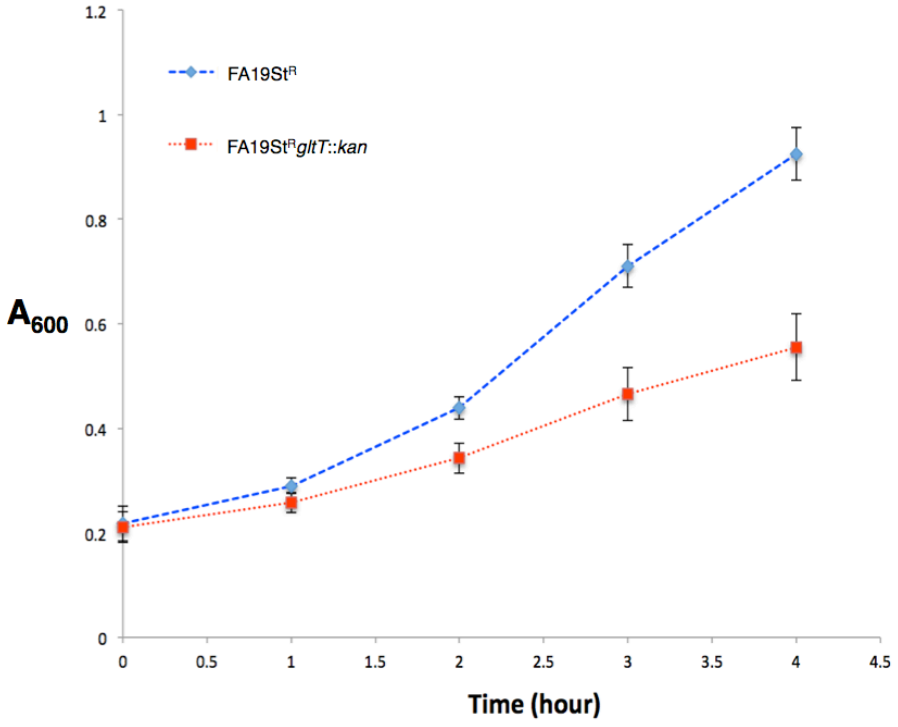


Figure 1. In vitro growth kinetics of FA19St$^R$ and FA19St$^R$*gltT*::*kan* in GC broth supplemented with glucose supplements, iron and 0.043% (v/v) $NaHCO_3$. $A_{600}$ level for each time point was averaged from three individual experiments.

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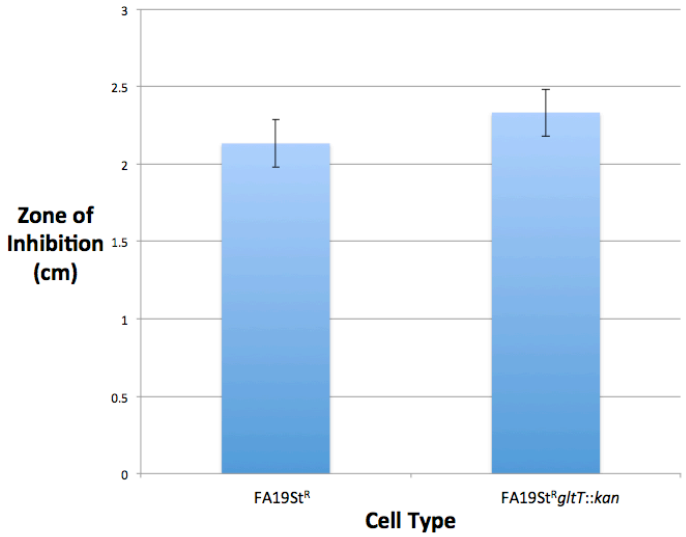


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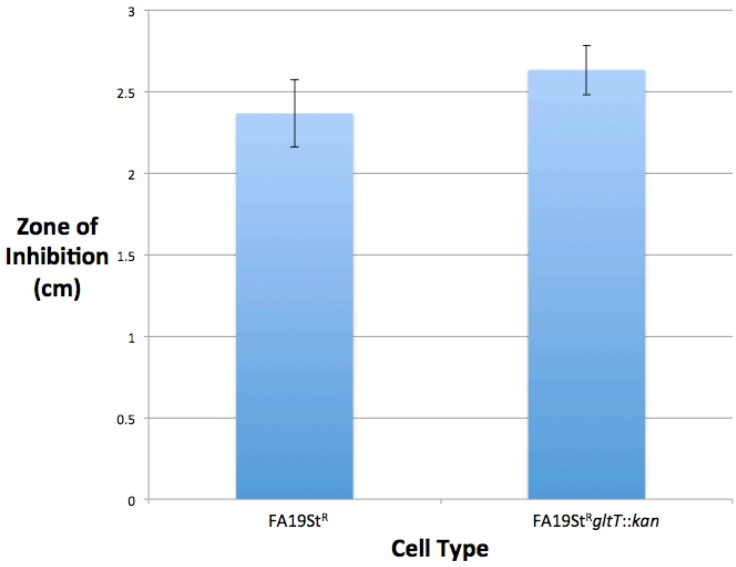
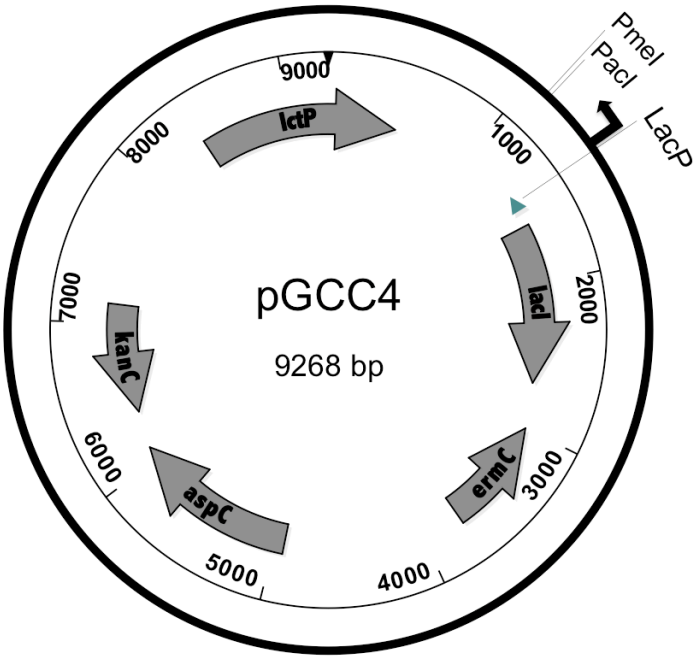



Figure 2. $H_2O_2$ Diffusion inhibition test on FA19St$^R$*gltT* and FA19St$^R$*gltT*::*kan* under aerobic (A) and anaerobic (B) conditions. Values are average of three independent experiments.

A.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LacP
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pGCC4
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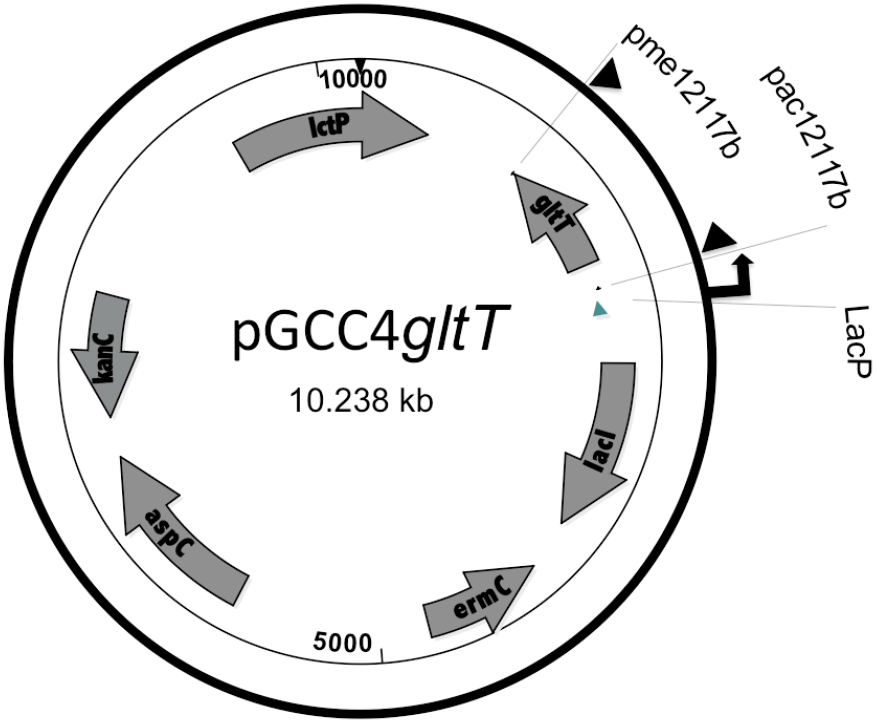
B.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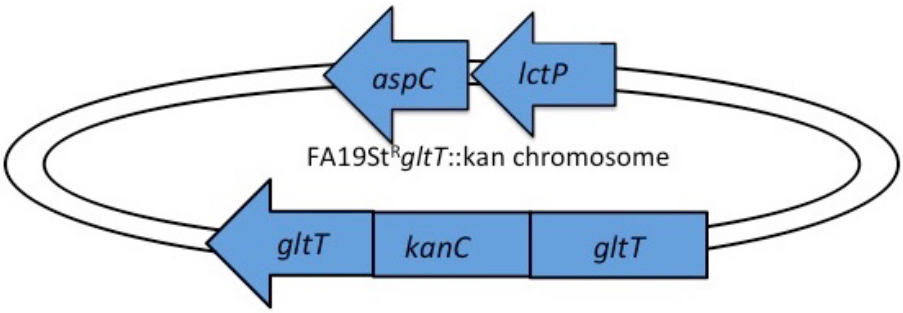
C.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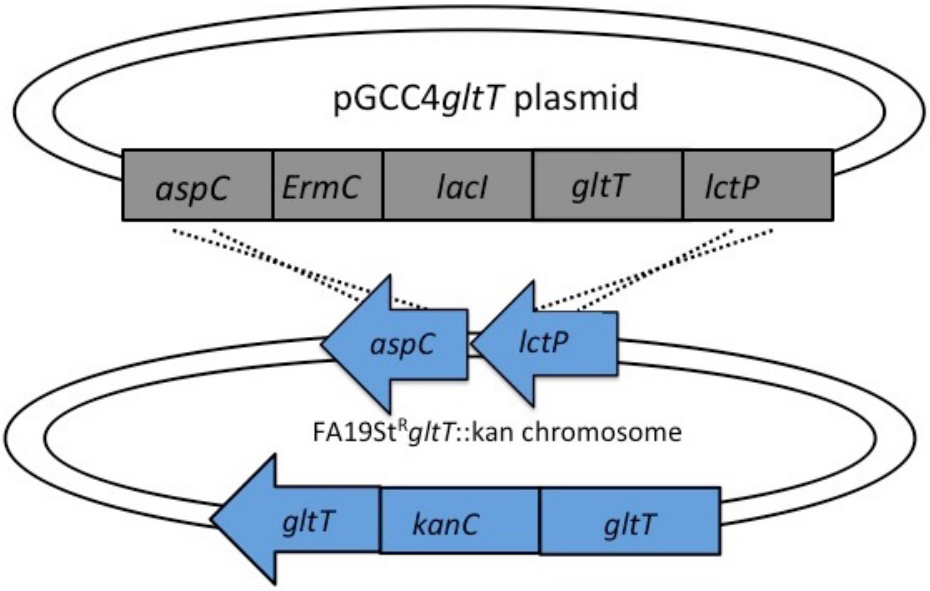
FA19St^R gltT::kan chromosome
gltT
kanC
gltT

D.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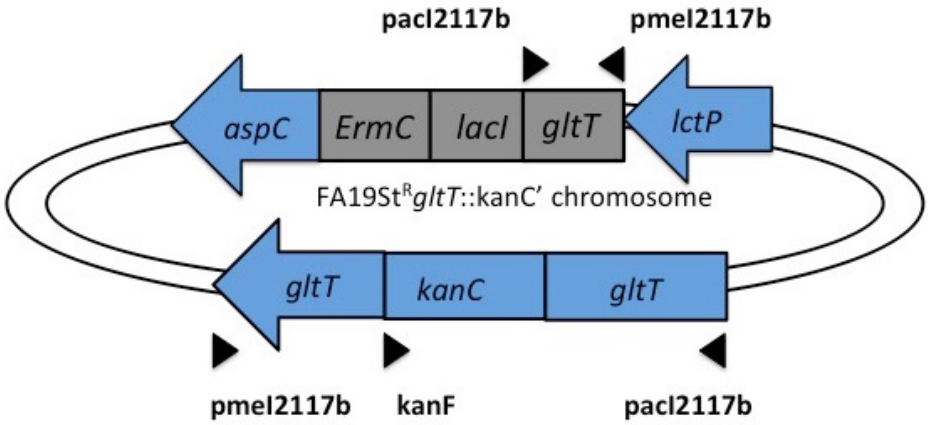
lctP
FA19St^R gltT::kan chromosome
gltT
kanC
gltT

E.


pacI2117b
pmeI2117b
aspC
ErmC
lacI
gltT
lctP
FA19St^R gltT::kanC' chromosome
gltT
kanC
gltT
pmeI2117b
kanF
pacI2117b

F.

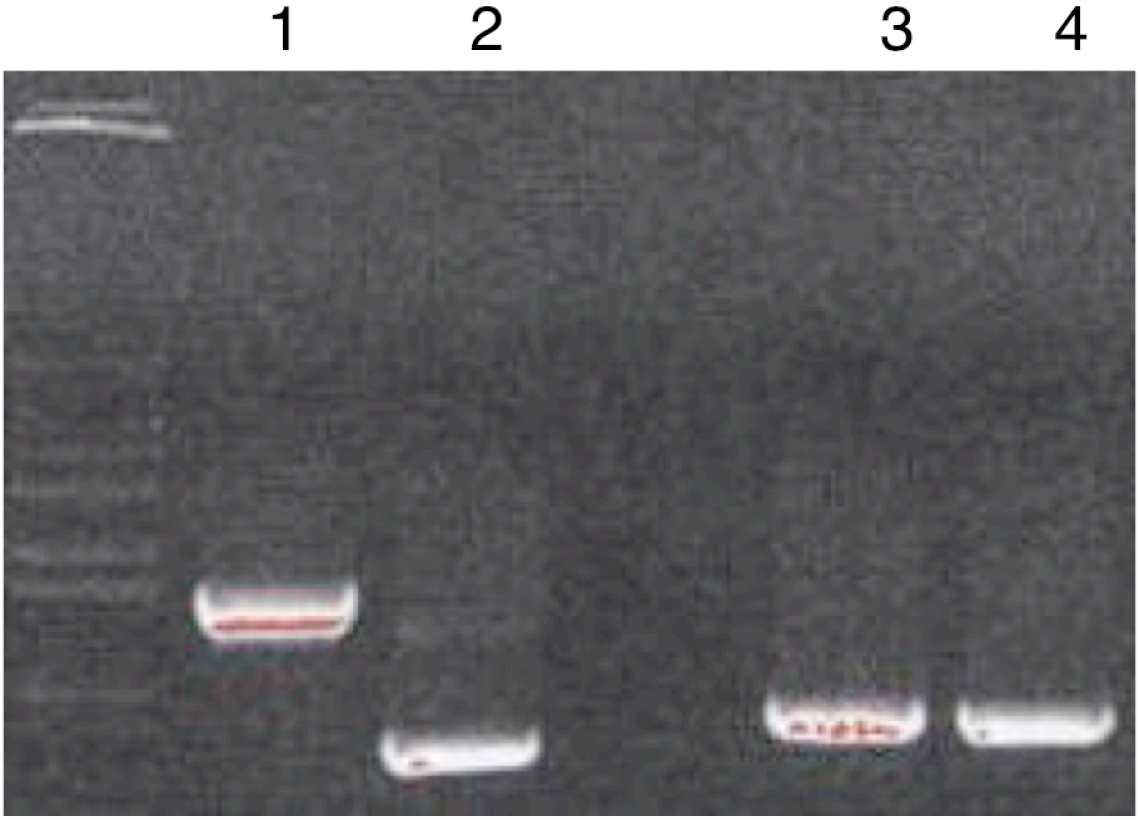
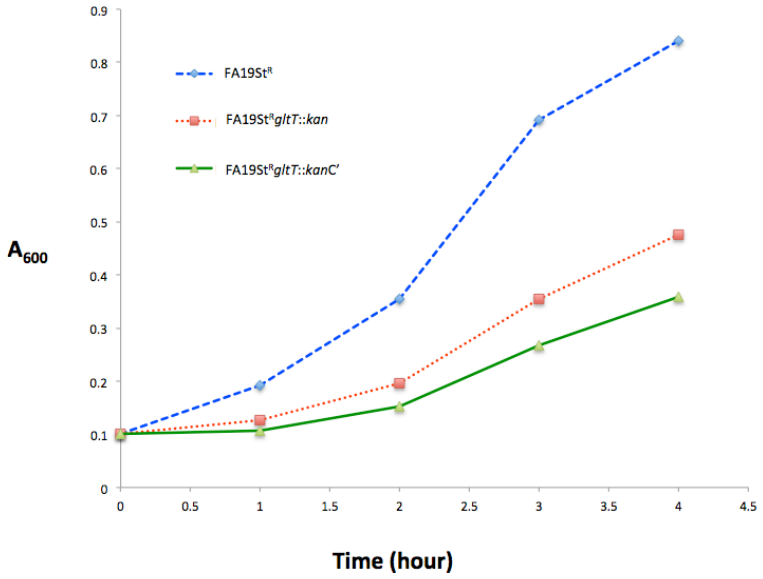
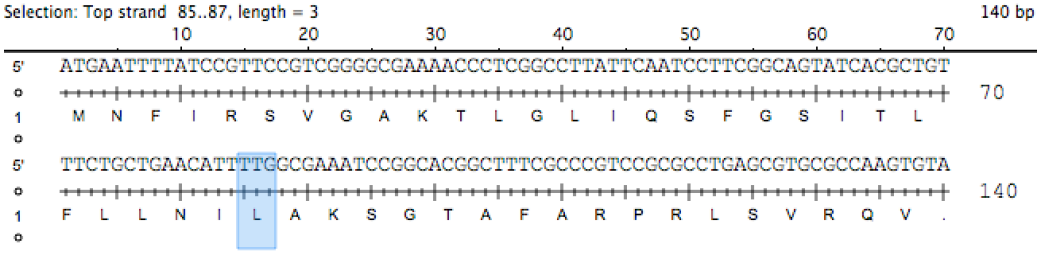



Figure 3. Genetic complementation of the FA19St$^R$*gltT*::*kan* mutant.
A. Map of the pGCC4 vector. B. Cloning of the *gltT* gene into pGCC4 resulting in the pGCC4*gltT* construct. Primers are represented by black triangles.
C. Schematic representation of the bacterial chromosome of FA19St$^R$*gltT*::*kan*.
D. Homologous recombination of the pGCC4*gltT* construct at the *lctP* locus.
E. Schematic representation of FA19St$^R$ *gltT*::*kan*C'.
F. PCR products. pmeI2117b/pacI2117b on FA19St$^R$ *gltT*::*kan* (lane 1), pmeI2117b/pacI2117b on FA19St$^R$ *gltT*::*kan*C'(lane 2), and kanF/pacI2117b on FA19St$^R$ *gltT*::*kan* (lane 3), kanF/pacI2117b on FA19St$^R$ *gltT*::*kan*C'(lane 4).

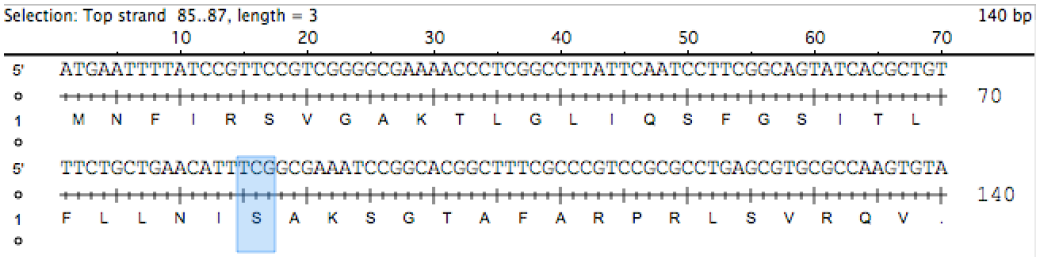
A)




B)



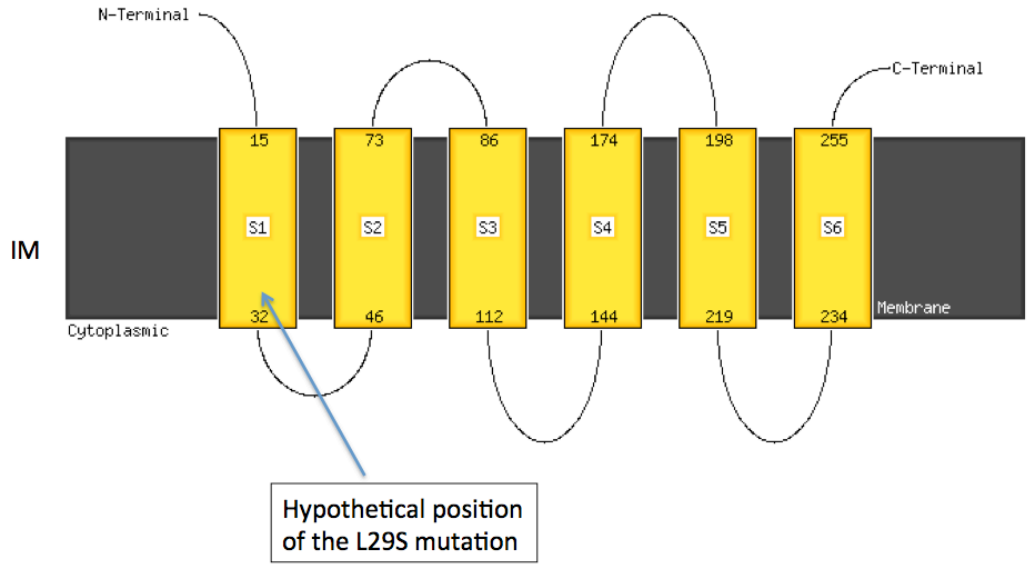

C)



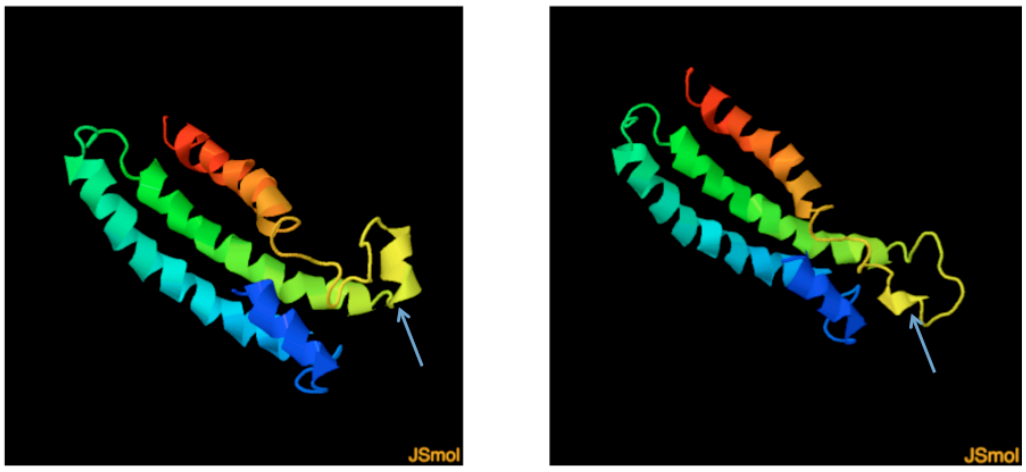

D)

```
WT              MNFIRSVGAKTLGLIQSFGSITLFLLNILAKSGTAFARPRLSVRQVYFAGVLSVLIVAVS 60
mut             MNFIRSVGAKTLGLIQSFGSITLFLLNISAKSGTAFARPRLSVRQVYFAGVLSVLIVAVS 60
                ***************************** ********************************
```

E)




F)




WT FA19 GltT transporter

L29S FA19 GltT transporter

Figure 4. FA19St$^{R}$ *gltT*::*kan*C'. (A) In vitro growth kinetics of FA19St$^{R}$, FA19St$^{R}$*gltT*::*kan* and FA19St$^{R}$*gltT*::*kan*C'in GC broth with supplements. (B) Sequence of the FA19 *gltT* gene (C) Sequence of the *gltT* gene integrated at the *lctP* locus in FA19St$^{R}$*gltT*::*kan*C' (D) Clustal 2.1 amino acid sequence alignment of the wild type and mutant *gltT* gene (E) Hypothetical domains of the GltT protein (F) Hypothetical structural model of the wild type GltT protein (left) and mutant GltT protein with leucine to serine substitution at the 29$^{th}$ position of amino acid sequence (right)

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