# Control of Transposon-mediated Activation of the *glpFK* Operon of *Escherichia coli* by two DNA binding Proteins

Zhongge Zhang[†] and Milton H. Saier, Jr.[* †]

[†]Department of Molecular Biology, Division of Biological Sciences, University of California at San Diego, La Jolla, CA 92093-0116.

# DNA-binding Protein Directed Transposon Insertion

*Corresponding Author: Tel +1 858 534 4084
Fax: +1 858 534 7108
E-mail: msaier@ucsd.edu
Mail: 9500 Gilman Drive, La Jolla, CA

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## Abstract

*Escherichia coli* cells deleted for the cyclic AMP (cAMP) receptor protein (Crp) gene (Δ*crp*) cannot utilize glycerol because cAMP-Crp is a required positive activator of glycerol utilization operon *glpFK*. We have previously shown that a transposon, Insertion Sequence 5 (IS*5*), can reversibly insert into the upstream regulatory region of the operon so as to activate *glpFK* and enable glycerol utilization. GlpR, which represses *glpFK* transcription, binds to the *glpFK* upstream region near the site of IS*5* insertion, and prevents insertion. We here show that the cAMP-Crp complex, which also binds to the *glpFK* upstream regulatory region, also inhibits IS*5* hopping into the activating site. This finding allowed us to identify conditions under which wild type cells can acquire *glpFK*-activating IS5 insertions. Maximal rates of IS*5* insertion into the activating site require the presence of glycerol as well as a non-metabolizable sugar analogue that lowers cytoplasmic cAMP concentrations. Under these conditions, IS5 insertional mutants accumulate and outcompete the wild type cells. Because of the widespread distribution of glucose analogues in nature, this mechanism of gene activation could have evolved by natural selection.

## Introduction

Wild type *E. coli* cells can grow on glycerol as a sole carbon source, but cells lacking the cAMP receptor protein (Crp) cannot (Lin, 1976, Won et al., 2009, Fic et al., 2009). In a previous communication (Zhang and Saier, 2009a), we showed that a Δ*crp* strain could mutate to rapid glycerol utilization due to insertion of the small transposon, Insertion Sequence *5* (IS*5*) (Sousa et al., 2013). To cause activation, IS*5* hops into a single site, in a single orientation, upstream of the *glpFK* operon promoter. The presence of IS*5* at this site activates the *glpFK* promoter so that it becomes stronger than that in wild type cells (Zhang and Saier, 2009a). Interestingly, a gene-activating IS*5* insertion is capable of undergoing subsequent precise excision (Zhang et al., 2010), showing that IS5-mediated operon activation can be a fully reversible process. The *glpFK*-activating insertional event occurred at high frequency in the presence of glycerol, but not in the presence of glucose or other carbon sources. Glycerol increased insertion of IS*5* at this specific site, but not in other operons (Zhang and Saier, 2009a, Zhang and Saier, 2011). Glycerol-promoted IS*5* insertion into the *glpFK*-activating site proved to be regulated by binding of the glycerol repressor, GlpR, when bound to the four adjacent *glpFK* operators, *O1*, *O2*, *O3* and *O4* in the *glpFK* control region. However, it became clear that the effect of GlpR-binding on IS*5* insertion was not mediated by increased expression of *glpFK*, or by increased growth, since binding to *O1* primarily controlled IS5 insertion without a significant impact on transcription, while binding to *O4* primarily controlled transcription (Zhang and Saier, 2009a). Thus, the negative control of IS*5* insertion into the upstream activating site is a newly recognized function of GlpR that is distinct from the previously recognized function of repressing *glpFK* transcription (Zhang and Saier, 2011).

In a previous study, Hall has summarized situations where transposon insertion to activate cryptic operons is elevated under conditions of starvation (Hall, 1999). More recently, Wang and Wood (Wang and Wood, 2011) described another example of IS5 insertion that occurs at a higher frequency when beneficial, giving rise to activation of the *E. coli* flagellar master switch operon, *flhDC*. Insertional activation of *flhDC* substantially enhances bacterial swarming in semisolid agar media, and the swarming phenotype is beneficial under adverse conditions such as nutrient depletion. Under conditions where swarming is not permitted (liquid or on solid agar media), insertional activation of the *flhDC* operon is not beneficial, and the frequency of insertion is greatly reduced. Thus, insertion of IS*5* upstream of the *flhDC* operon is another example of a mutation whose frequency is elevated under conditions where the mutation is advantageous. We have now confirmed the observations of Wang and Wood, 2011, in our laboratory (Zhang et al., 2013).

Since *de novo* IS*5* insertion at a single site in the *glpFK* operon in Δ*crp* cells is promoted by starvation in the presence of glycerol, and the insertion event relieves the starvation by enabling glycerol utilization, the question arises whether this constitutes an evolved mechanism. However, in wild type (*crp*$^+$) cells, under the tested laboratory conditions, no such IS*5* insertion mutants were obtained (Zhang and Saier, 2009a, Herring et al., 2006, Cheng et al., 2014). Our earlier studies had been conducted in a Δ*crp* deletion strain which grows poorly and consequently is not present under normal environmental conditions.

In this communication, we first report that in Δ*cyaA crp*$^+$ cells, IS5-mediated *glpFK* activation occurs in a manner strictly analogous to that observed in Δ*crp* mutant cells. The *cyaA* gene codes for the cyclic AMP biosynthetic enzyme, adenylate cyclase, Cya (Gancedo, 2013). We further show that addition of cAMP to the growth medium, known to increase the

cytoplasmic cAMP concentration (Saier et al., 1982), greatly suppressed IS*5* insertion specifically to this site. This effect occurred independently of GlpR, but it depended exclusively on Crp and the two adjacent Crp binding sites (CRPI and CRPII) that overlap the two GlpR binding sites *O2* and *O3*, in the *glpFK* control region (Zhang and Saier, 2009a, Weissenborn et al., 1992). It thus became clear that the conditions that predispose the *glpFK* operon to activation by IS*5* in wild type cells were (i) the presence of glycerol, and (ii) the presence of an environmental agent that could lower cytoplasmic cAMP levels.

Non-metabolizable glucose analogues and other sugar substrates of the phosphoenolpyruvate (PEP):sugar phosphotransferase system (PTS) are among the compounds known to lower cellular cAMP concentrations by inhibiting adenylate cyclase (Feucht and Saier, 1980). These include 2-deoxy-D-glucose (2DG) and methyl-α-D-glucoside (αMG) (Saier et al., 1982). Here we show that incubation of wild type *E. coli* cells in glycerol media together with 2DG or αMG promotes *glpFK*-activating IS5 insertional events. To determine whether under these conditions IS*5* mutants can accumulate and take over the population, we carried out long-term growth experiments. Our results are consistent with a scenario in which IS5 insertional activation of *glpFK* evolved in wild type cells as a mechanism to overcome adverse environmental conditions.

## Materials and Methods

### *Bacterial Strains and Growth Conditions*

Strains and DNA oligonucleotides used in this study are described in Supplementary Tables 1 and 2, respectively. The *cyaA* deletion mutant was generated from the parental strain (*E. coli* K-12 strain BW25113) using the method of Datsenko and Wanner (Datsenko and Wanner, 2000b).

Briefly, a kanamycin resistance gene (*km*), flanked by the FLP recognition site (FRT) was amplified from the template plasmid pKD4 using mutation oligos cyaA1-P1 and cyaA2-P2 (Supplementary Table 2), each of which is composed of a ~20 bp region at the 3' end that is complementary to the FRT-flanking *km* sequence, and a ~50 bp region at the 5' end that is homologous to *cyaA*. The PCR products were gel purified, treated with *Dpn*I, and then electroporated into BW25113 cells expressing the lamada-Red proteins encoded by plasmid pKD46. The pKD46 plasmid, which carries a temperature-sensitive origin of replication, was removed by growing the mutant cells overnight at 40 $^{o}$C. The Km$^{r}$ mutants were verified for the replacement of the target gene by the FRT-flanking *km* gene by PCR. The *km* gene was subsequently eliminated (leaving an 85-bp FRT sequence) using plasmid pCP20 that bears the FLP recombinase. The *cyaA glpR* double mutant was constructed by transferring a *km* insertional mutation of the *cyaA* gene into the *glpR* deletion mutant background (Zhang and Saier, 2009a) using P1 transduction.

To fuse the chloramphenicol-resistance gene (*cat*) with the *glpFK* operon, downstream of *glpK* in the chromosome, the plasmid pKD13-*cat* made previously (Zhang and Saier, 2009a), was used. In this plasmid, the *cat* gene is located upstream of a FRT-flanking *km* gene (Datsenko and Wanner, 2000b). The *cat* structural gene with its own ribosome binding site (RBS), together with its downstream *km* gene, was amplified from pKD13-*cat* using primers *glpFK*cat1-P1 and *glpFK*cat2-P2 (Supplementary Table 2). The PCR products were electroporated into wild type, Δ*cyaA* and Δ*cyaA* Δ*glpR* cells to replace the 85-bp downstream region between the 8$^{th}$ nucleotide and the 94$^{th}$ nucleotide relative to the *glpK* stop codon in the chromosome. After electroporation, the cells were selected on LB + Km agar plates. The Km$^{r}$ colonies were verified for the substitution of the 85 bp *glpK*/*glpA* intergenic region by PCR and subsequent DNA sequencing.

In the resultant strains (named BW_Cat, Δ*cyaA*_Cat and Δ*cyaA* Δ*glpR*_Cat, respectively), *glpF*, *glpK* and *cat* form a single operon with its expression solely under the control of the *glpFK* promoter (P*glpFK)*.

Strains were cultured in LB, NB or minimal M9 media with various carbon sources at 37ºC or 30ºC. When appropriate, kanamycin (Km; 25 μg/ml), ampicillin (Ap; 100 μg/ml), or chloramphenicol (Cm; 16-60 μg/ml) was added to the media.

***Mutations of Chromosomal Crp Operators***

To modify the chromosomal Crp binding sites in the control region of the *glpFK* operon, the previously made plasmid pKD13-P*glpFK* (Zhang and Saier, 2009a), was used. In this plasmid, P*glpFK* and the FRT-flanking *km* gene were oriented in opposite directions. Using the quick-change site-directed mutagenesis kit (Agilent) and oligos P*glpFK*$_{CrpI\&II}$-F and P*glpFK*$_{CrpI\&II}$-R (Supplementary Table 2), both Crp operators ($O_{CrpI}$ and $O_{CrpII}$) in the *glpFK* control region, contained within pKD13-P*glpFK*, were mutated by changing tatgacgaggcacacacattttaagt (-69 to –44 relative to +1 of P*glpFK*) to gacagcgaggcatctgcattttaatc (substitutions are underlined). The substitutions were confirmed by sequencing. Using the resultant plasmid, pKD13-P*glpFK*_$O_{CrpI\&II}$, as template, the region containing the *km* gene and P*glpFK*_$O_{CrpI\&II}$ was PCR amplified using the primers P*glpFK*$_{CrpI\&II}$-P1 and P*glpFK*$_{CrpI\&II}$-P2 (Supplementary Table 2). The PCR products were integrated into the Δ*cyaA* mutant chromosome to replace the wild type P*glpFK*. The nucleotide substitutions in both $O_{CrpI}$ and $O_{CrpII}$ operators were confirmed by sequencing. The *km* gene was removed, and the resultant strains were named Δ*cyaA* $O_{CrpI\&II}$ (Supplementary Table 1).

***Glp$^+$ Mutation Assay Using a ΔcyaA Mutant Strain***

Using the Δ*cyaA* deletion mutant, mutation to $Glp^+$ was first measured on minimal M9 + 0.2% glycerol agar plates as described previously (Zhang and Saier, 2009a). Briefly, cells from an overnight LB culture were washed and inoculated onto plates (~$10^8$ cells/plate). The plates were then incubated in a 30 ºC incubator and were examined daily for the appearance of $Glp^+$ colonies with each colony representing a $Glp^+$ mutation. On these glycerol minimal agar plates, any colonies appearing by day 2 were considered to be from $Glp^+$ cells initially present when applied to the plates. They were therefore subtracted from the subsequent measurements. The total numbers of $Glp^-$ cells were determined as described by Cairns and Foster (Cairns and Foster, 1991). The $Glp^+$ mutations were determined by counting the $Glp^+$ colonies that appeared on the original agar plates. The frequencies of $Glp^+$ mutations on glycerol M9 plates were determined by dividing the numbers of $Glp^+$ colonies by the total $Glp^-$ populations. To determine if any of the $Glp^+$ colonies arose from $Glp^+$ cells initially plated, the Δ*cyaA* cells, together with small numbers of Δ*cyaA* $Glp^+$ cells, were plated onto the same M9 + 0.2% glycerol plates. The plates were incubated and examined as above.

To determine the effect of cAMP on the frequency of IS5 insertion into the *glpFK* activating site, strain Δ*cyaA*_Cat (in which *glpF*, *glpK* and *cat* are fused in a single operon, see Supplementary Table 1) was used. This strain is sensitive to Cm at 8 μg/ml while the same strain with the IS5 insertion (Δ*cyaA* $Glp^+$_Cat) is resistant to Cm at 16 μg/ml. Preliminary experiments showed that all Δ*cyaA*_Cat cells resistant to Cm at 16 μg/ml were due to IS5 insertion in front of P*glpFK*. To determine the effect of cAMP on IS5 insertion, an 8-h old culture from a single Δ*cyaA*_Cat colony was diluted 1000 x into 5 ml LB ± cAMP (0 to 5 mM) contained in 30 ml glass tubes (2.5 cm x 20 cm). The tubes were shaken at 250 rpm in a 30 $^oC$ water bath shaker. After 15 h, the cells were washed 1x (to remove residual cAMP) with carbon source-free M9

191 salts, serially diluted, and applied onto LB + glucose agar plates and LB + glucose + Cm agar
192 plates. The plates were incubated at 37 $^{o}$C for 15 to 18 h. Total populations and Glp$^{+}$ populations
193 were determined based on numbers of colonies on LB + glucose plates and on LB + glucose +
194 Cm plates, respectively. The frequencies of Glp$^{+}$ mutation were determined by the ratios of Cm$^{r}$
195 populations to total populations.

196 To determine if cAMP affects IS5 insertion into other chromosomal sites, we chose to
197 analyze mutants resistant to Furazolidone (FZD) using Δ*cyaA*_Cat cells. First, step I mutants
198 were isolated by spreading the cells onto nutrient broth (NB) agar plates with a low
199 concentration (1 μg/ml) of FZD. The cells of a step I mutant were then applied onto the agar
200 plates with higher concentrations (5-7.5 μg/ml) of FZD. The plates were incubated at 30 $^{o}$C for
201 36 h or above, and colonies obtained were examined for the presence of IS elements in the *nfsB*
202 gene by PCR using oligos nfsB-ver-F and nfsB-ver-R (Supplementary Table 2). Among those
203 mutants carrying IS elements, IS5 insertional mutants were determined by two rounds of PCR,
204 using oligos IS5-ver-F / nfsB-ver-R and nfsB-ver-F / IS5-ver-F, respectively. The ratio of IS5
205 mutants was calculated by dividing total IS mutant numbers by IS5 mutant numbers.

206 To establish the effects of mutations in the Crp operators in the *glpFK* control region on
207 the appearance of Glp$^{+}$ IS5 insertional mutations, the Δ*cyaA* $O_{CrpI\&II}$_Cat cells with mutations in
208 operators, $O_{CrpI}$ and $O_{CrpII}$, were examined for the appearance of Glp$^{+}$ mutations in LB media
209 with or without cAMP as described above. To determine the effect of loss of *glpR* on Glp$^{+}$
210 mutation frequency, the Δ*cyaA* Δ*glpR* double mutant was examined for Glp$^{+}$ mutations in liquid
211 LB ± cAMP (0.1 mM) as compared to the single Δ*cyaA* mutant as described above.

To determine the effect of *glpR* overexpression on the Glp$^+$ mutations, the *glpR* structural gene was amplified from the wild type genomic DNA using primers glpR-KpnI and glpR-BamHI (Supplementary Table 2). The PCR products were digested with *Kpn*I and *BamH*I, gel purified, and then ligated to the same sites of pZA31 (Lutz and Bujard, 1997), yielding pZA31-*glpR*, in which *glpR* is driven by a synthetic *tet* promoter (P*tet*). The same plasmid carrying a random fragment (RF), pZA31-RF, served as a control (Levine et al., 2007a). To repress P*tet* activity, the constitutively expressed *tetR* cassette, located at the *attB* site, was transferred to Δ*cyaA*_Cat carrying pZA31-*glpR* from BW-RI (Levine et al., 2007b) by P1 transduction. Therefore, the expression of *glpR* could be induced by a tetracycline analog, chlorotetracycline (cTc). The resultant strain containing the *tetR* source and pZA31-*glpR* was tested for Glp$^+$ mutations in LB ± 0.1mM cAMP as described above. To induce expression of *glpR* in pZA31-*glpR*, cTc (250 ng/ml) was added to the medium.

To further demonstrate cAMP inhibitory effects on the appearance of IS5 insertional mutants, and the competitive abilities of the mutants under low cAMP conditions, we performed a long-term experiment by transferring cultures to new media at various intervals. To do this, an LB culture (10 µl) of Δ*cyaA*_Cat was used to inoculate M9 + glycerol ± cAMP (0 to 1 mM) media. Before the first transfer, every 12 h or so, the cultures were serially diluted onto LB + glucose plates for total population determination and onto LB + glucose + Cm plates for Glp$^+$ (IS5 insertion) mutant population determination. After 2 or 2.5 days, the cultures were 1000x diluted into new tubes with the same media. Then at one-day intervals, the cultures were 1000x diluted into fresh media. For each transfer, the total cells and the Glp$^+$ mutant populations were determined.

***IS5 Insertional Mutation Assay Using A Wild Type Background***

2-deoxyglucose (2DG) is a non-metabolizable glucose analog that reduces the level of cytoplasmic cAMP level when adding to the media (Saier, 1989). Preliminary experiments showed that *E. coli* cells are sensitive to this compound at 0.1%. To determine if IS5 insertion upstream of P*glpFK* occurred in a wild type background, BW_Cat cells (Supplementary Table 1) were tested for IS5 insertion on M9 + glycerol (0.2%) + 2DG (0.13%) ± Cm (60 μg/ml) agar plates. The plates were incubated at 30 $^{o}$C, and the colonies were examined for the presence of IS5 in the upstream *glpFK* operon control region by PCR followed by gel electrophoresis.

***Long Term Evolutionary Experiments.***

At least two types of mutations arose when BW_Cat cells were incubated on M9 + glycerol + 2DG ± Cm agar plates, the IS5 insertional mutation and a non-IS5 mutation of unknown nature. To determine if the IS5 insertional mutants are more competitive than the non-IS5 insertional mutants, 10 μl of a fresh LB culture of BW_CAT was used to inoculate 5 ml of M9 + glycerol (0.2%) + 2DG (0.13%) ± Cm (60 μg/ml) in 30 ml glass tubes. The tubes were incubated with shaking (250 rpm) in a 30 $^{o}$C water bath shaker. After 2.5 days of incubation, the cultures (i.e., mutant cells resistant to 2DG and Cm) were 1000x diluted into new tubes with the same media. On every other day, the mutants were 1000x diluted into new tubes. For each transfer, the mutant cultures were serially diluted using carbon source-free M9 salts, and the $10^{5}$-fold and $10^{6}$-fold dilutions were applied onto M9 + glycerol + 2DG ± Cm agar plates before incubation at 37 $^{o}$C. After 2 days, 100 colonies from each transfer were subjected to PCR and subsequent gel electrophoresis analyses to determine the percentages of IS5 insertion mutants to the total mutants. Note that the parental cells do not grow under these conditions.

***Chromosomal lacZ Fusions and β-Galactosidase Assays***

Using pKD13-P*glpFK* (Zhang and Saier, 2009a) and pKD13-P*glpFK_O*$_{CrpI\&II}$ (Supplementary Table 1) as templates, P*glpFK* (-204 to +66 relative to the transcriptional start site) and P*glpFK_O*$_{CrpI\&II}$ plus their upstream FRT-flanked *km*$^{r}$ gene were amplified using oligos P*glpFK*z-P1 and P*glpFK*z-P2 (Supplementary Table 2). Using the method of Datsenko and Wanner (Datsenko and Wanner, 2000a), the promoters plus the upstream *km*$^{r}$ gene (*km*$^{r}$:P*glpFK* or *km*$^{r}$:P*glpFK_O*$_{CrpI\&II}$) were integrated into the chromosome to replace the *lacI* gene and the native *lac* promoter (including the 5' UTR of *lacZ*) of MG1655 deleted for *lacY* (Klumpp et al., 2009). This chromosomal replacement was confirmed by PCR and subsequent DNA sequencing analysis. The resultant strains are deleted for both *lacI* and *lacY*, but they carry the *lacZ* gene that is expressed under the control of P*glpFK* or P*glpFK_O*$_{CrpI\&II}$. Both constructs were transferred into Δ*cyaA* and Δ*cyaA* Δ*glpR* strains by P1 transduction, yielding Δ*cyaA*_P*glpFK-lacZ*, Δ*cyaA*_P*glpFK_O*$_{Crp}$*-lacZ*, Δ*cyaA*Δ*glpR* _P*glpFK-lacZ*, and Δ*cyaA*Δ*glpR* _P*glpFK_O*$_{Crp}$*_lacZ* (Supplementary Table 1).

For β-galactosidase assays, strains were cultured in liquid LB media ± 1 mM cAMP at 30 $^{o}$C. When cultures entered the exponential phase, samples were collected for measurement of β-galactosidase activities as described by Miller (Miller, 1972).

## Results

### *IS5 insertional activation of the glpFK operon occurs in ΔcyaA mutant cells*

We previously demonstrated that Δ*crp* mutant cells of *E. coli* could regain the ability to utilize glycerol by IS5-mediated insertional mutations that specifically occurred at a single site, upstream of the *glpFK* promoter, preferentially in the presence of glycerol (Zhang and Saier,

2009a). In this study, we first used Δ*cyaA* mutant cells lacking the cAMP biosynthetic enzyme, adenylate cyclase, Cya. Like the Δ*crp* mutant cells, the Δ*cyaA* mutant cells cannot utilize glycerol as the sole carbon source for growth. However, after a prolonged incubation on M9 + glycerol agar plates, $Glp^+$ mutants could be observed (Figure 1). These mutants were IS*5* insertional mutants carrying IS*5* in the same position and orientation upstream of the *glpFK* promoter as those isolated previously from Δ*crp* cells. The time course (Figure 1) for their appearance and the properties of these double mutants were indistinguishable from those isolated previously (Zhang and Saier, 2009a). Among at least 100 independent $Glp^+$ mutants analyzed, no other types of mutants arose under the conditions used. There was an approximately two-day delay before mutant colonies appeared, and these clearly arose during incubation on the plates, since when small numbers (e.g. 11 and 23) of identical Δ*cya* $Glp^+$ insertional mutants were added to the Δ*cyaA* cells prior to plating, they gave rise to colonies within a shorter time period (Figure 1).

***IS5 activation of the glpFK operon in ΔcyaA cells is suppressed by exogenous cAMP***

Exogenous cAMP can enter cells to increase the cytoplasmic concentration of this nucleotide (Saier et al., 1982). Figure 2A shows the effect of increasing concentrations of external cAMP on the *glpFK*-specific IS*5* insertional frequency in Δ*cyaA glpFK_cat* cells (see *Materials and Methods* and Supplementary Table 1). At a concentration of 10 μM, exogenous cAMP had only a slight inhibitory effect on IS*5* insertion, but at 100 μM, cAMP inhibited over 90%, whereas at 1 mM, cAMP essentially abolished IS*5* insertion (Figure 2A).

To determine whether the decrease in IS*5* insertion frequency due to the presence of cAMP was specific to the *glpFK* promoter, we analyzed IS*5* insertion at the *nfsB* gene in Δ*cyaA* cells. Mutational inactivation of *nfsB* confers resistance to furazolidone (FZD) (Whiteway et al.,

1998), and a significant fraction of inactivating mutations are due to IS*5* insertion (see *Materials and Methods*). As shown in Figure 2 B, cAMP did not influence the frequency of total insertional events (grey bars) or of IS5 insertional events (black bars) among FZD-resistant mutants within experimental error.

***The effect of cAMP on IS5 insertion frequency requires* Crp-*binding sites in the glpFK promoter***

To determine whether the inhibition of IS*5* insertion upstream of the *glpFK* promoter by cAMP is due to the binding of the cAMP-Crp complex to the two adjacent Crp binding sites ($O_{CrpI}$ and $O_{CrpII}$), present in the *glpFK* promoter, we analyzed the consequences of point mutations within these binding sites. These mutations essentially abolished the inhibitory effect of Crp on IS*5* insertion (Figure 3A). Although these Crp operator mutations eliminated binding of the cAMP-Crp complex, they did not change the *glpFK* promoter strength in the *cyaA* deletion background, and the promoter activity was still under the control of GlpR (Figure 3B). Thus, it can be concluded that inhibition by cAMP-Crp of IS*5* insertion into the *glpFK* activating site is due to the binding of the cAMP-Crp complex solely to these two operators present in the *glpFK* promoter region.

**Crp *and* GlpR *independently affect IS5 insertion upstream of the glpFK promoter***

To determine if GlpR plays a role in the inhibitory effect of Crp on IS*5* insertion, we deleted the *glpR* gene in the Δ*cyaA* background, yielding a Δ*cyaA* Δ*glpR* double mutant (Supplementary Table 1). Higher IS*5* insertional frequencies were observed in the Δ*cyaA* Δ*glpR* cells than in the Δ*cyaA* cells when grown in LB (no glycerol added) (Figure 4A). However, in the absence of GlpR, cAMP still exerted its inhibiting effect, presumably by binding to Crp,

which then bound to its two *glpFK* operon binding sites, $O_{CrpI}$ and $O_{CrpII}$ (compare column 2 and column 4 in Figure 4A). Comparable inhibition was observed regardless of the presence of glycerol or GlpR. It was therefore concluded that regulation of IS*5* insertion by the cAMP-Crp complex occurs independently of glycerol and GlpR.

When GlpR was over-produced in the absence of cAMP, GlpR still exerted its strong inhibitory effect (Figure 4B). In the left panel, the *glpR* gene was expressed at an extremely low level, and GlpR exerted only a minimal effect because chlorotetracycline (cTc), the inducer, was not present. When the cTc concentration was high (250 ng/ml), *glpR* was expressed at a high level, and the rate of IS*5* insertion into the *glpFK* upstream site was greatly reduced (see right panel of Figure 4b). The same experiments were conducted in the presence of cAMP (0.1mM). The IS*5* insertion frequency decreased, while overexpression of GlpR further inhibited IS*5* insertion (data not shown). These results suggest that GlpR and the cAMP-Crp complex exert their effects on IS*5* insertion independently of each other. These experiments also provide evidence that that GlpR and the cAMP-Crp complex can bind to the *glpFK* control region simultaneously.

**IS*5* *insertional activation of the glpFK operon occurs in a wild type background in the presence of 2-deoxyglucose***

Since a reduction in cAMP promotes IS5 insertion in the *glpFK* activating site, we sought to determine whether environmental conditions that could lead to a reduction in cAMP concentrations could elevate IS*5* insertion into the *glpFK* promoter. Non-metabolizable glucose analogues, such as 2-deoxyglucose (2DG) and α-methylglucoside (αMG), (He and Liu, 2002, Holst and Williamson, 2004, Kumar et al., 2013, Moller, 2010, Tantanarat et al., 2012, Saier and Ballou, 1968, Xi et al., 2014) are known to lower cytoplasmic cAMP levels by inhibiting

adenylate cyclase activity (Gabor et al., 2011, Gershanovich, 2003, Saier et al., 1996, Vastermark and Saier, 2014). These analogues also strongly inhibit growth on glycerol, at least in part due to inhibition of both cytoplasmic cAMP production by adenylate cyclase, and of cytoplasmic glycerol-3-phosphate (substrate/inducer ) production by glycerol kinase (Kuroda et al., 2001, Peterkofsky et al., 2001, Schlegel et al., 2002, Saier and Reizer, 1994). We therefore asked if we could isolate IS*5* insertional mutants in a wild-type background on minimal M9 agar plates containing glycerol, inhibitory concentrations of 2DG or αMG and chloramphenicol. The results for αMG proved to be same as for 2DG, and consequently, only those obtained with 2DG are presented here.

In these experiments, a *glpFK-cat* (chloramphenicol acetyl transferase) fusion was used to measure IS*5* insertion (see Supplementary Table 1 and *Materials and Methods*). Regardless of the inhibitory glucose analogue used, 2DG/αMG, resistant ($2DG^r$/ $\alpha MG^r$) mutants (which were also $Glp^+$) could be isolated in a wild type *E. coli* genetic background (Jones-Mortimer and Kornberg, 1980, Kornberg et al., 2000, Rephaeli and Saier, 1980). As shown in Figure 5, PCR analyses of colonies that appeared after a five-day incubation at 30°C revealed two types of $Glp^+$ mutants: (i) a majority that did not have an insertion in the *glpFK* promoter (dubbed "non-IS*5* mutants" here), and (ii) a minority (<10%; "IS*5* mutants") that had IS*5* inserted into the *glpFK* promoter-activating site.

Sequencing revealed that the non-IS*5* insertion mutants did not have genetic alterations in the *glpFK* operon, or in the *fruR* gene [which appears to regulate *crp* gene expression (Zhang et al., 2014)]. Unlike IS*5* insertional mutants, the non-IS5 mutants showed a pleiotropic phenotype in addition to their increased growth in a glycerol + 2DG medium, such as poor utilization of sorbitol (D-glucitol) and succinate (Table 1) which are utilized efficiently only

370 when high cytoplasmic concentrations of the cAMP-Crp complex are available (Zhang et al.,
371 2014).

372 To demonstrate that the $Glp^+$ phenotype of IS5 mutants results solely from the IS5
373 insertional event, we carried out P1 transduction experiments to determine if the phenotypes of
374 $2DG^r$, $Cm^r$ and $Glp^+$, could be transferred together into another *E. coli* genetic background. In
375 these experiments, we used two "wild type" strains, BW25113 and BW_Cat (Supplementary
376 Table 1) (with or without the *glpFK-cat* fusion), as recipients. For both recipient strains,
377 transductants were obtained using the IS*5* insertional mutant (expressing the fused *glpFK_cat*
378 operon) as donor, when plated on M9 + glycerol + 2DG ± Cm agar plates. The regulatory
379 regions of 23 independently isolated transductants were amplified by PCR, and all were found to
380 carry the IS*5* element. DNA sequencing showed that IS5 was located in the same position and in
381 the same orientation as described previously (Zhang and Saier, 2009a, Zhang and Saier, 2009c)).
382 These results showed that in these mutants, IS*5* insertion is necessary and sufficient to give rise
383 to the $2DG^r$ $Glp^+$ phenotype.

384 ***Short-term and long-term evolution experiments to evaluate if* IS*5*-*mediated activation***
385 ***of glpFK operon expression could have evolved in wild type cells***

386 Initially, we conducted short term evolutionary experiments (several transfers) using the
387 Δ*cyaA* strain in the presence of glycerol as sole carbon source (Figure 6A), glycerol plus 0.1 mM
388 cAMP (Figure 6B), and glycerol plus 1.0 mM cAMP (Figure 6C). In the absence of cAMP, IS*5*
389 insertional mutants appeared as the only species after 37 h of incubation with shaking. After
390 several transfers, virtually 100% of the cells contained IS*5* in the *glpFK*-activating site (Figure
391 6A). When cAMP was added at 0.1 mM, IS*5* insertional mutants again appeared as the only
392 species after a 61 h incubation with shaking. After the first transfer, virtually all cells contained

393 IS*5* (Figure 6B). When exogenous cAMP was added at 1 mM, the appearance of these mutants
394 was strongly inhibited. As a result, after the 6th transfer, only about 20% of the population were
395 IS*5* insertional mutants (Figure 6C). These observations are consistent with the conclusion that
396 cAMP-Crp complex inhibits IS*5* insertion upstream of the *glpFK* operon.

397 In order to conduct long-term evolutionary experiments in a wild type background, we
398 used *glpFK_cat* cells in which a chloramphenicol-resistance gene was transcriptionally fused to
399 the *glpFK* operon in wild type cells such that *glpFK* activation by IS*5* insertion led
400 simultaneously to a $Glp^+$ as well as a $Cm^r$ phenotype (see *Materials and Methods*). Prolonged
401 incubation of *glpFK_cat* wild type cells under conditions analogous to those described above for
402 the short-term Δ*cyaA* experiments revealed that, IS*5* mutants became an appreciable fraction of
403 the population after several generations in minimal glycerol medium with 2-deoxyglucose, with
404 (Figure 7A) or without (Figure 7B) chloramphenicol. When chloramphenicol was present, IS*5*
405 insertional mutants first appeared after six transfers, and then continued to accumulate during the
406 remainder of the experiment (17 transfers) at the end of which about 25% of the cells bore the
407 IS*5* insertion (Figure 7A). When chloramphenicol was absent, it took a little longer; insertional
408 mutants became appreciable following the eighth transfer, and accounted for over 20% of the
409 cells after 20 transfers (Figure 7B). These experiments show that *glpFK* activation by IS*5* is
410 advantageous when wild type cells are exposed to glycerol in the presence of a sugar analogue
411 inhibitor of adenylate cyclase such as 2-deoxyglucose (Novotny et al., 1985). Thus, the process
412 we had previously described in Δ*crp* and Δ*cyaA* mutants could have evolved in wild type cells in
413 response to specific environmental conditions.

414

415

## Discussion

Transposons (transposable elements; jumping genes) were first described by Barbara McClintock (McClintock, 1950), recipient of the 1983 Nobel Prize (Kenez, 1984, van de Putte, 1983). Initially identified in corn, transposable elements occur in virtually all living organisms, where they cause mutations that can inactivate genes, activate genes, and mediate chromosomal rearrangements (Zhang and Saier, 2011). Different types of transposons (especially retrotransposons) comprise about 30% of the human genome (Cordaux and Batzer, 2009). Why are they ubiquitous, and what are their primary functions? What is responsible for their broad occurrence in genomes of all types of living organisms? The answers to these questions are not yet in hand, but it is clear that transposons contribute to genomic instability and genetic innovation (Huang et al., 2012).

Long-terminal-repeat (LTR) retrotransposons are known to jump around within eukaryotic genomes (Curcio et al., 2015). To avoid damaging resident genes, they have been selected to integrate away from protein-coding sequences. For instance, the fission yeast LTR retrotransposon, Tf1, inserts at nucleosome-free regions in gene promoters. Jacobs et al. (Jacobs et al., 2015) recently showed that Tf1 is directed to these insertion sites by specific DNA binding proteins such as Sap1, a situation that parallels in some respects the observations reported here. Thus, regulation of transposon insertion by DNA-binding proteins may prove to be common in Nature, perhaps even ubiquitous.

Adaptive mutation, the genome-wide acceleration of mutation rate under stress conditions, is well-documented, and in some cases, the mechanisms involved are understood (Shee et al., 2011). An understanding of the basis for mutations that increase fitness, give rise to drug resistance, and enable the emergence of pathological traits in microorganisms, are also

439 relevant to human, animal and plant health, at both the individual and the population levels
440 (Gordo et al., 2011).

441 Over the last several decades, a number of mechanisms that accelerate mutagenesis under
442 stressful conditions have been described. Among these have been those that activate cyptic
443 operons by transposon insertion under starvation conditions where such activation is beneficial to
444 the cell (Hall, 1999). Another example is provided by enhanced IS insertional mutations causing
445 metal resistance in bacterial cells subjected to toxic levels of zinc (Vandecraen et al., 2016). The
446 activation of the *glpFK* operon by IS*5* insertion represents an adaptive mutagenic mechanism
447 where some, but not all, molecular mechanisms leading to the activation of the operon are
448 understood (Zhang and Saier, 2009b, Zhang and Saier, 2009d). In this paper we advance the
449 state of knowledge of this system by showing that transposition into the upstream promoter
450 region of *glpFK* is controlled independently by two defined DNA binding proteins (GlpR and the
451 Crp-cAMP-Crp complex). We show that IS*5* insertion into the *glpFK* promoter can be regulated
452 by exposure to environmental toxic sugar analogues, and propose that such exposure may
453 constitute a selective environment that favored the evolution of a gene activation mechanism that
454 requires a specific transposition event.

455 In our experiments with wild type *E. coli* cells, we used 2-deoxyglucose (2DG) as
456 described in this report or methyl α-glucoside (αMG) (data not presented) to lower cytoplasmic
457 cAMP levels and to inhibit glycerol utilization (Saier, 1998, Saier, 1989, Saier, 2001, Saier et al.,
458 1995). But are comparable non-metabolizable glucose analogues found in Nature? In fact,
459 numerous toxic and non-metabolizable sugar analogues are synthesized by microorganisms,
460 plants, fungi and man. They include deoxy sugars such as 2-deoxyglucose, methylated sugars,
461 such as 3- and 6-0 methyl glucose, fluoro sugars and a variety of α- and β-glycosides such as

462 methyl α-glucoside (He and Liu, 2002, Holst and Williamson, 2004, Kumar et al., 2013, Moller,
463 2010, Tantanarat et al., 2012, Saier and Ballou, 1968, Xi et al., 2014). Thus, the conditions that
464 promote IS*5* hopping into the *glpFK*-activating site could have evolved under the pressures of
465 natural selection. Supporting a possible evolutionary role for transposition under starvation
466 conditions, it was recently reported that prolonged starvation leads to significantly elevated
467 expression of transposases in *E. coli*, including the *insA* gene, encoding the IS*5* transposase
468 (Arunasri et al., 2014).

469

470

471 **Concluding Remarks**

472 We have shown that the IS*5*-mediated *glpFK*-activating insertional site is a conditional
473 transposition hotspot. It is an insertional hotspot in low cyclic AMP and high glycerol, but
474 insertion at this site is essentially undetectable in the presence of other carbon sources, or high
475 levels of cyclic AMP in the absence of glycerol. The experimentation described in this
476 report explains the effect of cAMP for the first time, and provides plausible conditions under
477 which such a mutational mechanism could have evolved in a wild type genetic background.
478 Thus, our results demonstrate that the activated cAMP-Crp complex strongly inhibits IS5 hoping
479 into the *glpFK* activating site when bound to its DNA-binding sites in the upstream *glpFK*-
480 promoter region. Our results also showed definitively that in the lab, one can isolate these
481 activating mutants in a wild type genetic background in the presence of glycerol and inhibitory
482 concentrations of 2DG or α-MG that represent adverse environmental conditions.

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## Conflict of Interest

The authors declare no conflict of interest.

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## Figure legends

**Figure 1. Glp$^+$ mutations in Δ*cyaA* cells on M9 + glycerol agar plates.** Δ*cyaA* cells (~$10^8$) from a fresh LB culture were spread on M9 + glycerol (0.2%) agar plates. The plates were incubated at 30$^o$C and examined for the appearance of Glp$^+$ colonies (each colony represents an IS5 insertional mutation) at 24-h intervals. The mutation frequencies were determined as in Zhang and Saier (Zhang and Saier, 2009a). ♦ = no Glp$^+$ cells added before initially plating; ■ and ▲ = 11 and 23 Glp$^+$ cells were included before plating.

**Figure 2. Effects of cAMP on (A) IS5 insertion upstream of the *glpFK* regulatory region in Δ*cyaA* cells, and (B) in the *nfsB* gene as a control.** In A, a fresh LB culture from a single Δ*cyaA*-Cat (see Supplementary Table 1) colony was diluted 1000 x into 5 ml of LB ± cAMP (0 to 5 mM) in 30 ml glass tubes (2.5 cm x 20 cm). The tubes were shaken at 250 rpm in a 30 $^o$C water bath shaker. After ~15 h, the cells were washed 1x (to remove residual cAMP) with carbon source-free M9 salts, serially diluted, and applied onto LB + glucose agar plates (for total population determination) and LB + glucose + Cm (16 μg/ml) agar plates (for IS5 insertional mutant population determination). In B, the cells (~2x $10^8$) of a step I furazolidone (FZD) resistant (FZD$^r$) mutant strain isolated from a Δ*cyaA*-Cat strain, were applied to nutrient broth (NB) agar plates, with furazolidone (5 or 7.5 μg/ml) ± cAMP (1mM). The plates were incubated at 30 $^o$C for 36 h before being examined for the appearance of FZD$^r$ mutants. Among these FZD$^r$ mutants, IS5 or other IS insertional mutants in the *nfsB* gene were determined by PCR (see Materials and Methods). In all cases, the proportion of IS5 mutants (~60%) was the same.

**Figure 3. Effect of Crp binding site mutations on IS5 insertion upstream of the *glpFK* promoter region (A) and the promoter activity in Δ*cyaA* cells grown in LB medium ±**

686 **cAMP (B).** The concentrations of cAMP are indicated below the x-axis. $O_{crp}$= the mutated Crp
687 binding sites ($O_{crpI}$ and $O_{crpII}$) in the upstream regulatory region of the *glpFK* operon to prevent
688 Crp binding. In b, the activities of the *glpFK* promoter (P*glpFK*) and the same promoter
689 (P*glpFK*_$O_{crp}$) mutated in $O_{crpI}$ and $O_{crpII}$ were measured using the LacZ reporter in both Δ*cyaA*
690 cells and Δ*cyaA* Δ*glpR* cells. See Supplementary Table 1 for the detailed strain information.

691 **Figure 4. Effects of *glpR* deletion (A) and *glpR* overexpression (B) on IS*5* insertion in the**
692 **control region of the *glpFK* operon in Δ*cyaA* cells grown in LB ± cAMP.** In A, the exogenous
693 cAMP concentration was either 0 (left) or 0.1 mM (right). In B, vector: no *glpR* expression
694 (control); GlpR indicates *glpR* expression; cTc = chloro-tetracycline (an inducer of the *tet*
695 promoter) at the concentrations of 0 (left) or 250 ng/ml (right). *glpR* expression is under the
696 control of the cTc induced promoter, P*tet*, in an expression vector. The Δ*cyaA* cells that
697 constitutively produce TetR (repressing P*tet*) were used in these experiments. Note scale
698 difference for Figure 4A and 4B.

699 **Figure 5. IS*5* insertion upstream of the *glpFK* control region in wild type cells.** Wild type
700 cells carrying the *glpFK-cat* (chloroamphenicol acetyl transferase) fusion (See Methods section)
701 were plated onto M9 + glycerol (0.2%) + 2DG (0.12%) + chloramphenicol (Cm) (55 μg/ml) agar
702 plates. The plates were incubated at 30 $^{o}$C. After 5 days of incubation, colonies were examined
703 for the presence of IS5 upstream of the *glpFK* control region by PCR. In both the upper and the
704 lower panels, a single IS5 insertion was obtained. A DNA marker showing four bands with
705 known sizes (4 kb, 2 kb, 1 kb and 0.5 kb from above) is indicated in each panel.

706 **Figure 6. Percentages of IS*5* mutant populations vs the total populations during growth in**
707 **M9 + glycerol ± cAMP over time using strain Δ*cyaA*_Cat.** For the first period [up to 61 h (A
708 and B) or 45 h (C)], time is expressed in hours. Thereafter (vertical dotted line), time is

expressed in numbers of transfers where each transfer took about a day. Figures A-C show the effects of increasing cAMP concentrations: A, 0; B, 0.1 mM; C, 1 mM.

**Figure 7. Percentages of IS5 insertional mutant populations versus total mutant populations over time using the "wild type" strain carrying the chromosomal *glpFK-cat* fusion.** Cells were incubated with shaking at 30 $^{o}$C in 5 ml of M9 + glycerol (0.2%) + 2DG (0.13%) plus (A) or minus (B) chloramphenicol (Cm) (60 μg/ml) in glass tubes (20 mm x 200 mm). After 2.5 days of incubation, 5 μl cultures were transferred to 5ml of the same media (1$^{st}$ transfer) and incubated with shaking at 30 $^{o}$C. Later, every two days, 5 μl cultures were transferred to new media and grown under the same conditions. For every transfer, the cultures were diluted with carbon source-free M9 salt solution and plated onto M9 + glycerol + 2DG + Cm agar plates. After 2 days of incubation at 30 $^{o}$C, 100 colonies were examined by PCR for the presence of IS*5* in the *glpFK* control region (See Figure 5).

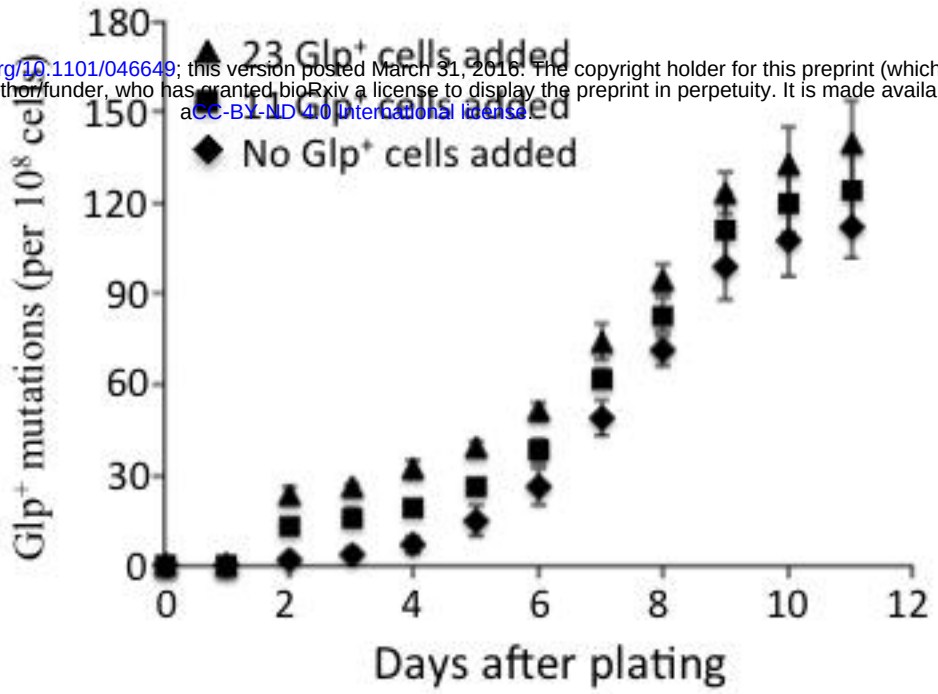
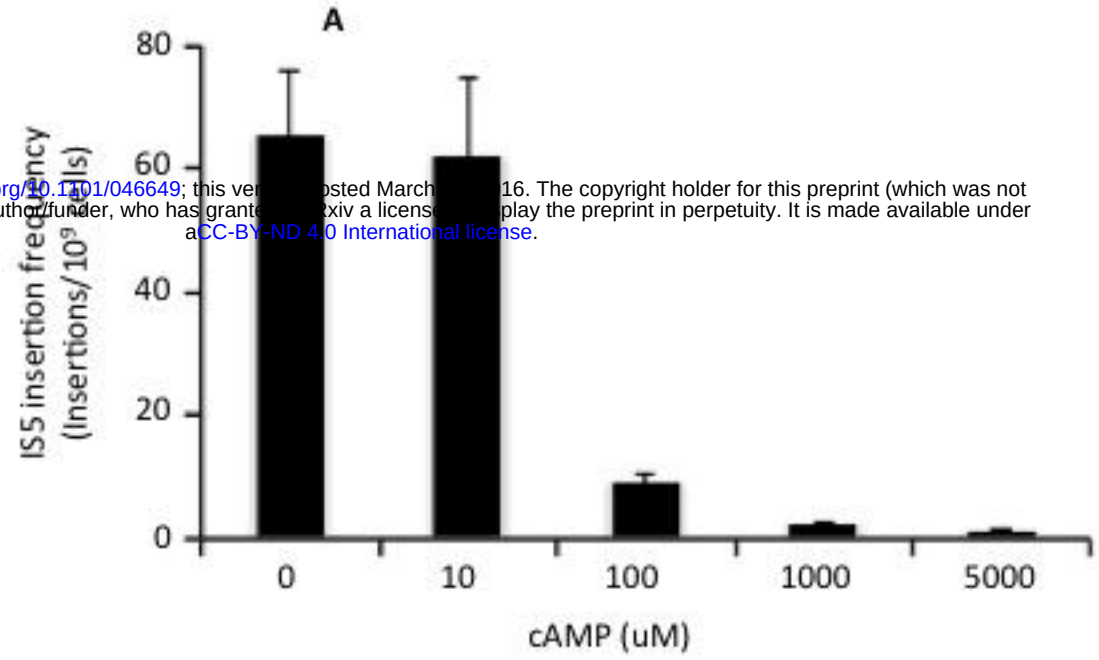



Figure 1



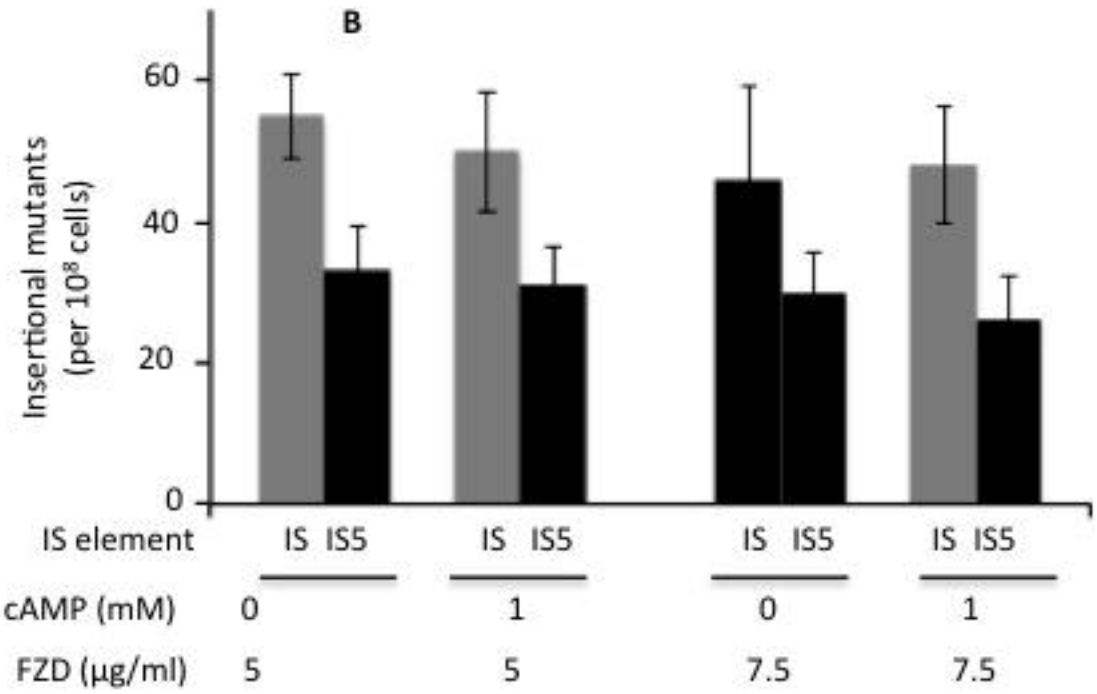




Figure 2

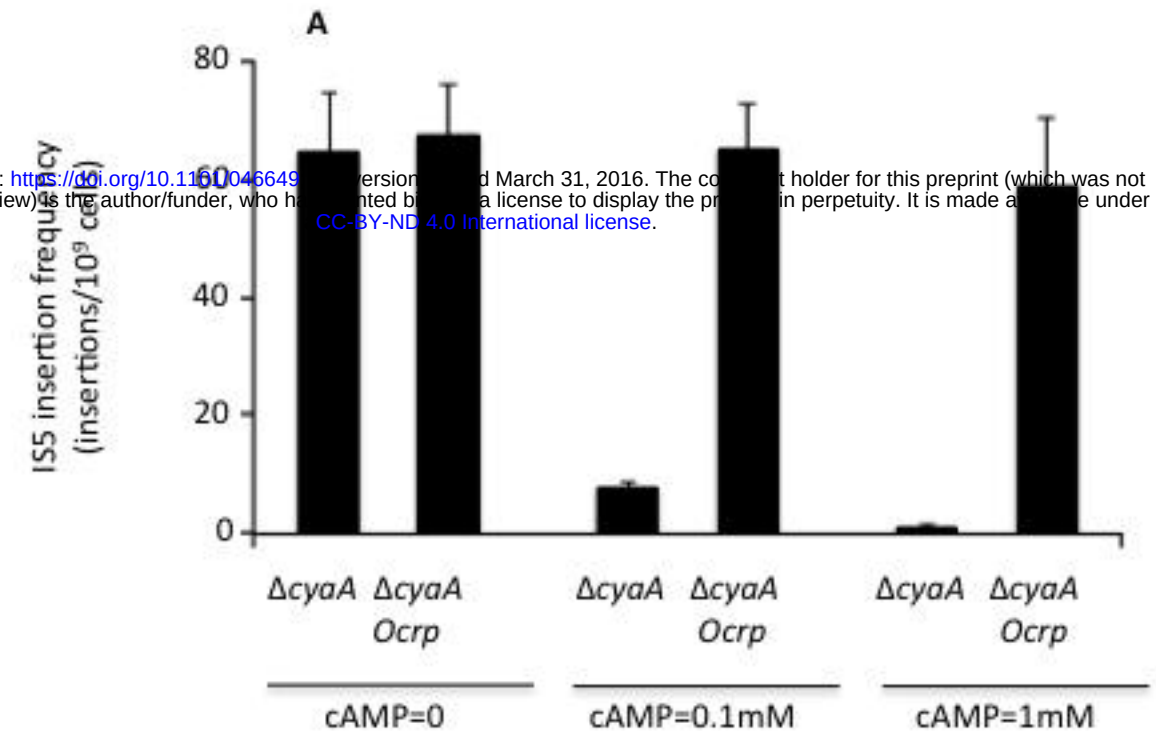
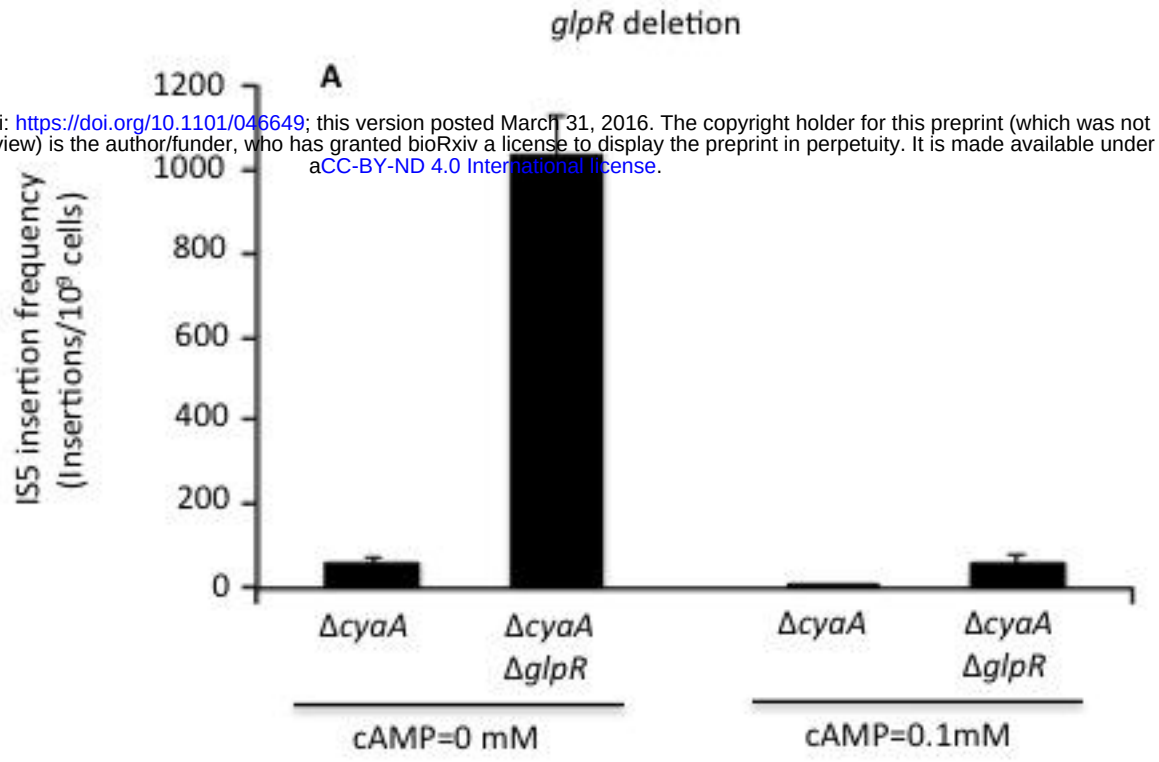
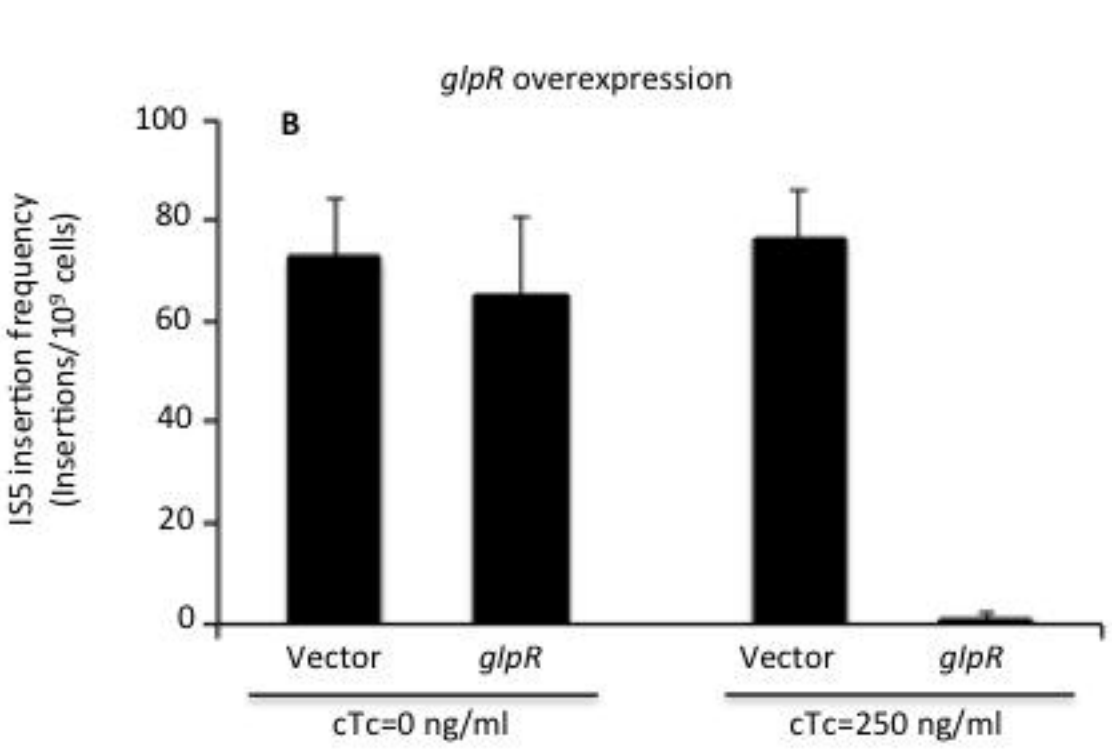



Figure 3

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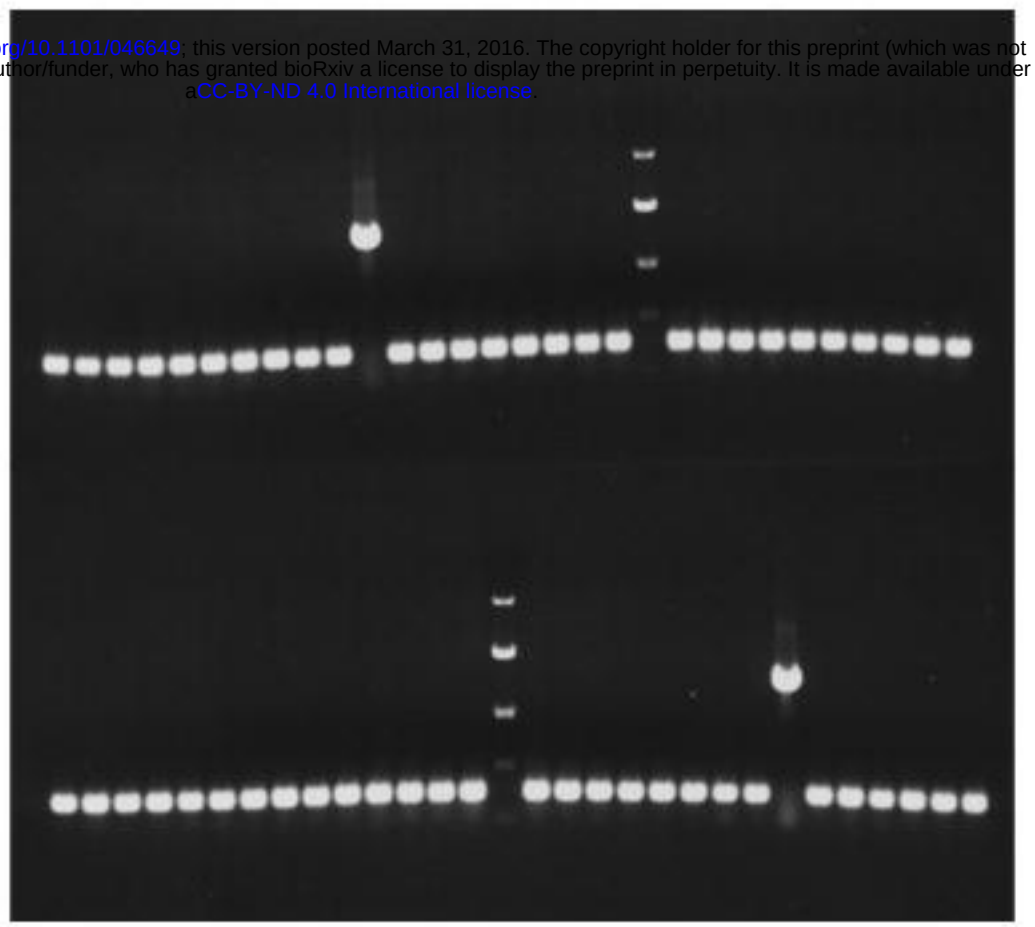
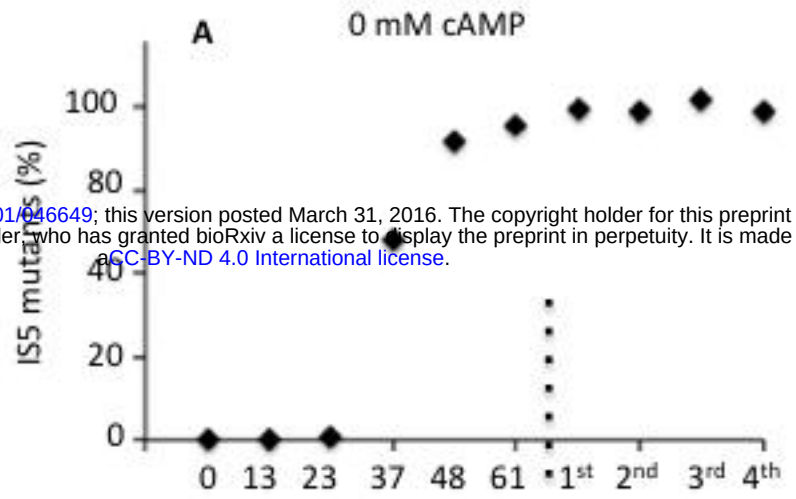
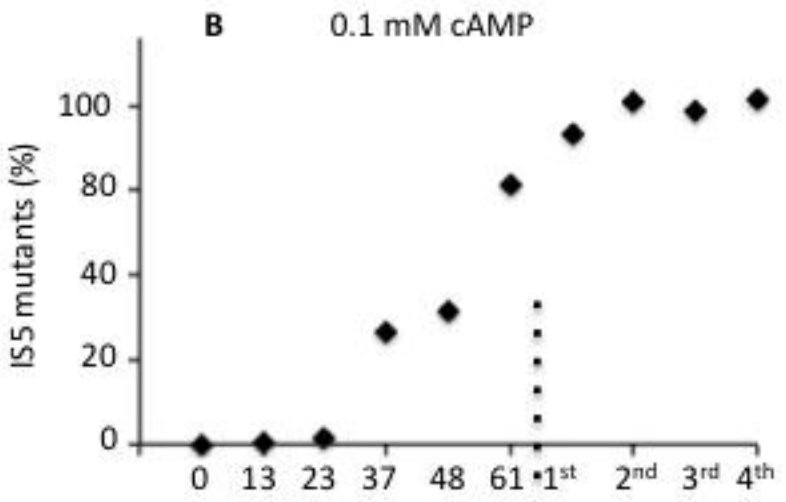


Figure 5



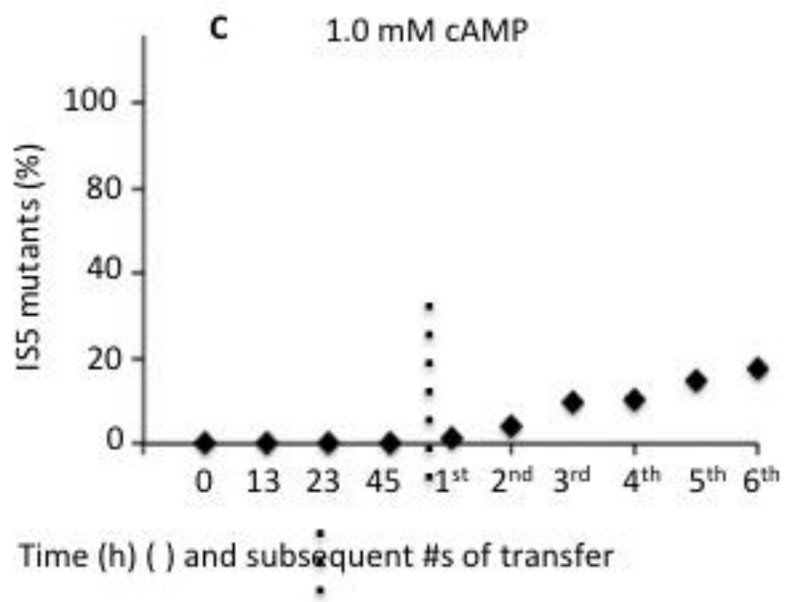
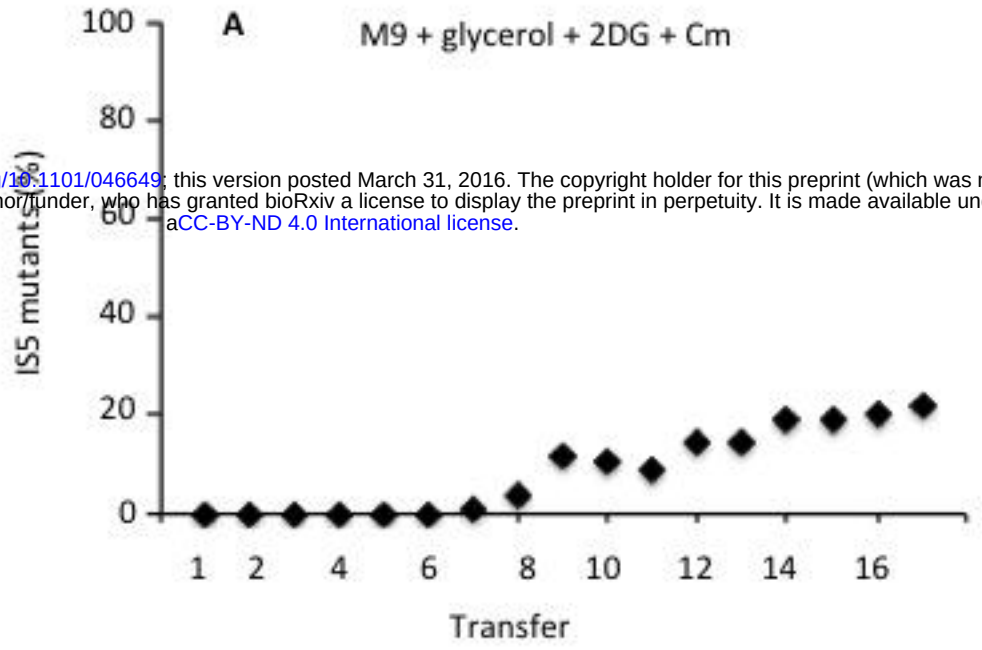




Figure 6



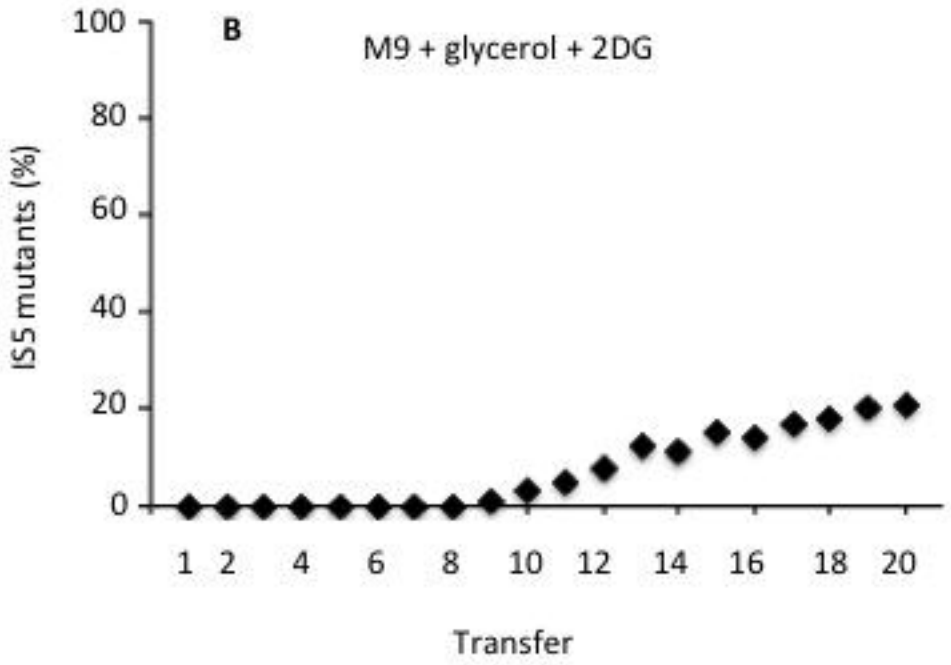




Figure 7

Table 1. Growth of wild type, an IS5 insertional mutant and a non-IS5 mutant on minimal M9 agar plates supplemented with various carbon sources[1,2].

| Strain | Glycerol | Sorbitol | Succinate | Xylose | Mannitol |
|---|---|---|---|---|---|
| Wild type | ++ | ++ | ++ | ++ | ++ |
| IS5 mutant | +++ | ++ | ++ | ++ | ++ |
| Non-IS5 mutant | ++ | + | + | ++ | ++ |

[1]Overnight LB cultures from single colonies were washed once with a carbon source-free M9 salt solution. The pellets were resuspended in the same M9 solution, and cells were inoculated onto the agar plates by streaking using a sterile transfer loop. The plates were incubated at 30 $^{o}$C for 36 h before being examined for colony sizes.

[2]+, ++ and +++ denote the relative sizes of the colonies. All values are relative to growth of the wild type strain (++) on agar plates containing a single carbon source as indicated. Thus, +++ indicates increased size while + indicates decreased colony size.