Title

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Publication Date

2016-11-01

DOI

10.1016/j.mrfmmm.2016.10.003

Peer reviewed

Mutation Research 793–794 (2016) 22–31

Contents lists available at ScienceDirect

Mutation Research/Genome instability and disease

journal homepage: www.elsevier.com/locate/molmut
Community address: www.elsevier.com/locate/mutres

Transposon-mediated activation of the *Escherichia coli glpFK* operon is inhibited by specific DNA-binding proteins: Implications for stress-induced transposition events

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ARTICLE INFO

Article history:
Received 5 July 2016
Received in revised form 18 August 2016
Accepted 22 October 2016
Available online 27 October 2016

Keywords:
Stress-induced mutagenesis
Transposon
Glycerol utilization
Nutrient starvation
Cyclic AMP-Crp
cAMP

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 activator of the glycerol utilization operon, *glpFK*. We have previously shown that a transposon, Insertion Sequence 5 (IS*5*), can insert into the upstream regulatory region of the operon to activate the *glpFK* promoter and enable glycerol utilization. GlpR, which represses *glpFK* transcription, binds to the *glpFK* upstream region near the site of IS*5* insertion and inhibits insertion. By adding cAMP to the culture medium in Δ*cyaA* cells, we here show that the cAMP-Crp complex, which also binds to the *glpFK* upstream regulatory region, inhibits IS*5* hopping into the activating site. Control experiments showed that the frequencies of mutations in response to cAMP were independent of parental cell growth rate and the selection procedure. These findings led to the prediction that *glpFK*-activating IS5 insertions can also occur in wild-type (Crp^+) cells under conditions that limit cAMP production. Accordingly, we found that IS5 insertion into the activating site in wild-type cells is elevated in the presence of glycerol and a non-metabolizable sugar analogue that lowers cytoplasmic cAMP concentrations. The resultant IS5 insertion mutants arising in this minimal medium become dominant constituents of the population after prolonged periods of growth. The results show that DNA binding transcription factors can reversibly mask a favored transposon target site, rendering a hot spot for insertion less favored. Such mechanisms could have evolved by natural selection to overcome environmental adversity.

1. Introduction

Wild type *E. coli* cells can grow on glycerol as a sole carbon source, but cells lacking the cAMP receptor protein (Crp) cannot [1–3]. In a previous communication [4], 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*) [5]. 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 [6]. The *glpFK*-activating insertional event occurred at high frequency in the presence of glycerol, but not in the presence of glucose or another carbon source. Glycerol increased insertion of IS*5* at this specific site but not in other operons [4,7]. Glycerol-promoted IS*5* insertion into the *glpFK*-activating site proved to be regulated by binding of the glycerol repressor, GlpR, to its 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 [4]. Moreover, insertion could be shown to occur independently of the selection procedure [4]. Thus, the inhibition 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 [7]. Finally, we demonstrated that IS5 can precisely excise, showing that its insertion can be considered to be fully reversible [8].

In this communication, we first report that in Δ*cyaA* Crp^+ cells, which lack the cyclic AMP biosynthetic enzyme, adenylate cyclase, Cya [9], IS5-mediated *glpFK* activation occurs in a manner strictly analogous to that observed in Δ*crp* cells. We further show that addition of cAMP to the growth medium, known to increase the cytoplasmic cAMP concentration [10], greatly suppresses IS*5* insertion specifically at this site. This effect occurred independently of

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http://dx.doi.org/10.1016/j.mrfmmm.2016.10.003
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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 [4,11]. 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 lowers 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 [12]. These sugar analogues include 2-deoxy-D-glucose (2DG) and methyl-α-D-glucoside (αMG) [10]. 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. Our results are consistent with a scenario in which environment-sensitive transcription factors such as GlpR and Crp reversibly mask transposition target sites so as to suppress or promote IS5 insertional activation of genes, depending on conditions. We discuss these results in the context of the current understanding of mutagenic mechanisms that are proposed to be active in the absence of appreciable growth.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Strains and DNA oligonucleotides used in this study are described in Supplementary Tables S1 and S2, respectively. The *cyaA* deletion mutant was generated from the parental strain (*E. coli* K-12 strain BW25113) using the method of [13]. 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 S2), 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 °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 [4] 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 [4], was used. In this plasmid, the *cat* gene is located upstream of a FRT-flanking *km* gene [13]. The *cat* structural gene with its own ribosome binding site (RBS), together with the downstream *km* gene, was amplified from pKD13-*cat* using primers glpFKcat1-P1 and glpFKcat2-P2 (Supplementary Table S2). 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 8th nucleotide and the 94th 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*/*glp* 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 37 °C or 30 °C. When appropriate, kanamycin (Km; 25 μg/ml), ampicillin (Ap; 100 μg/ml), or chloramphenicol (Cm; 20–60 μg/ml) was added to the media.

2.2. 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* [4], 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 $PglpFK_{CrpI\&II}$-F and $PglpFK_{CrpI\&II}$-R (Supplementary Table S2), both Crp operators (CrpI and 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_{Crp}, as template, the region containing the *km* gene and P*glpFK_*$O_{CrpI\&II}$ was PCR amplified using the primers $PglpFK_{CrpI\&II}$-P1 and $PglpFK_{CrpI\&II}$-P2 (Supplementary Table S2). The PCR products were integrated into the Δ*cyaA_cat* and Δ*cyaA* Δ*glpR_cat* mutant chromosome to replace the wild type P*glpFK*. The nucleotide substitutions in both CrpI and CrpII operators were confirmed by sequencing. The *km* gene was removed, and the resultant strains were named Δ*cyaA* O_{Crp}*_cat* and Δ*cyaA* Δ*glpR* O_{crp}*_cat*, respectively (Supplementary Table S1).

2.3. Glp^+ mutation assay using a Δ*cyaA* 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 [4]. 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 °C incubator and were examined daily for the appearance of Glp^+ colonies with each colony representing an independently arising 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 [14]. 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 S1) 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 20 μg/ml. Preliminary experiments showed that all Δ*cyaA_cat* cells resistant to Cm at 20 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 °C water bath shaker. After 15 h, the cells were washed 1 x (to remove residual cAMP) with carbon source-free M9 salts, serially diluted, and applied onto LB + glucose agar plates and LB + glucose + Cm agar plates. The plates were incubated at 37 °C for 15 to 18 h. Total populations and Glp^+ populations were determined based on numbers of colonies on LB + glucose plates and on LB + glucose + Cm plates,

respectively. The frequencies of Glp$^+$ mutation were determined by the ratios of Cm^r populations to total populations.

To see if there is any difference in growth rate between *cyaA* Glp$^-$ and *cyaA* Glp$^+$ cells, these two strains (*ΔcyaA*:*km_cat* and *ΔcyaA_cat* Glp$^+$) were cultured in LB without or with cAMP (0 to 1 mM). Briefly, overnight LB cultures from single colonies were diluted into fresh 5 ml of LB ± cAMP (final OD_{600} = 0.005). The tubes were shaken (250 rpm) at 30 °C. Samples were collected every 45 min to 1 h, and their optical cell densities (OD_{600}) were measured using a Bio-Rad SmartSpect 3000 machine.

To compare their competitive abilities, the Glp$^-$ strain, *ΔcyaA*:*km_cat* [in which a Km^r gene subsitutes for *cyaA*, so that it is resistant to kanamycin (Km^r) but sensitive to chloramphenicol (Cm^s)] and the Glp$^+$ strain, *ΔcyaA_cat* Glp$^+$ (that is Cm^r but Km^s), were mixed in a 1:1 ratio in the same tubes. The tubes were agitated (250 rpm) at 30 °C. Samples were taken after 5, 10 and 15 h of growth and serially diluted. The dilutions were applied onto LB + Km plates for determination of Glp$^-$ cells and LB + Cm plates for determination of Glp$^+$ cells.

To examine if the presence of *glpFK* has an effect on IS insertions, the *cat* gene was substituted for the *glpFK* operon. In the resultant strain, the *cat* gene is directly under the control of the *glpFK* promoter [4]. This construct was moved to a *ΔcyaA ΔglpR* background by P1 transduction, yielding strain *ΔcyaA ΔglpR_cat2* (see Table S1). The same methods as above were used to determine cAMP effects on IS5 insertion.

To determine if cAMP affects IS5 insertion into another chromosomal site, we chose to analyze mutants resistant to Furazolidone (FZD) using *ΔcyaA_cat* cells. First, step I mutants were isolated by spreading the cells onto nutrient broth (NB) agar plates with a low concentration (1 μg/ml) of FZD. The cells of a step I mutant were then applied onto the agar plates with higher concentrations (5–7.5 μg/ml) of FZD. The plates were incubated at 30 °C for 36 h or more, and colonies obtained were examined for the presence of IS elements in the *nfsB* gene by PCR using oligos nfsB-ver-F and nfsB-ver-R (Supplementary Table S2). Among those mutants carrying IS elements (such as IS1, IS2 and IS5), IS5 insertional mutants were determined by two rounds of PCR, using oligos IS5-ver-F/nfsB-ver-R and nfsB-ver-F/IS5-ver-F, respectively. The ratio of IS5 mutants to total IS insertion mutants was calculated by dividing total IS mutant numbers by IS5 mutant numbers.

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

To determine the effect of *glpR* overexpression on the appearance of Glp$^+$ mutations, the *glpR* structural gene was amplified from the wild type genomic DNA using primers glpR-KpnI and glpR-BamHI (Supplementary Table S2). The PCR products were digested with *Kpn*I and *BamH*I, gel purified, and then ligated to the same sites of pZA31 [15], 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 [16]. To repress P*tet* activity, the constitutively expressed *tetR* cassette, located at the *attB* site, was transferred to *ΔcyaA_cat* carrying pZA31-*glpR* from BW-RI [16] by P1 transduction. Therefore, the expression of *glpR* could be induced using a tetracycline analogue, chlorotetracycline (cTc). The resultant strain containing the *tetR* repressor, and pZA31-*glpR* was tested for Glp$^+$ mutations in LB ± 0.1 mM 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 short-term experiment by transferring cultures to new media at various intervals. To do this, an LB culture (10 μl) of *ΔcyaA_cat* was used to inoculate M9 + glycerol ± cAMP (0 to 1 mM) media. Before the first transfer, when all cells were in the original test tube, samples were removed and analyzed about every 12 h. 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 about 2 or 2.5 days, the cultures were 1000 x diluted into new tubes with the same media. Then at one-day intervals, the cultures were 1000 x diluted into fresh media. For each transfer, the total cells and the Glp$^+$ mutant populations were determined as stated above.

2.4. IS5 insertional mutation assay using a wild type genetic background

2-deoxyglucose (2DG) is a non-metabolizable glucose analogue that reduces the level of cytoplasmic cAMP level when added to the media [17]. 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 S1) were tested for IS5 insertion on M9 + glycerol (0.2%) + 2DG (0.12%) ± Cm (60 μg/ml) agar plates. The plates were incubated at 30 °C, and the colonies were examined for the presence of IS5 in the upstream *glpFK* operon control region by PCR followed by gel electrophoresis.

2.5. 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 (0.12%) ± Cm (60 μg/ml) in 30 ml glass tubes. The tubes were incubated with shaking (250 rpm) in a 30 °C water bath shaker. After 2.5 days of incubation, the cultures (i.e., mutant cells resistant to 2DG and Cm) were 1000 x diluted into new tubes with the same media. Every two days the mutants were 1000 x diluted into new media of the same composition. For analysis of the cell populations after 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 °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. This experiment was also conducted without Cm to show that the beneficial consequences of IS5 insertion were not related to the *cat* gene fusion.

2.6. Chromosomal lacZ fusions and β-galactosidase assays

Using pKD13-P*glpFK* [4] and pKD13-P*glpFK_*O_{Crp} (Supplementary Table S1) as templates, P*glpFK* (-204 to +66 relative to the transcriptional start site) and P*glpFK_*O_{Crp} plus their upstream FRT-flanked *km^r* gene were amplified using oligos P*glpFK*z-P1 and P*glpFK*z-P2 (Supplementary Table S2). Using the method of [13], the promoters plus the upstream *km^r* gene (*km^r*:P*glpFK* or *km^r*:P*glpFK_*O_{Crp}) 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* [18]. This chromosomal replace-

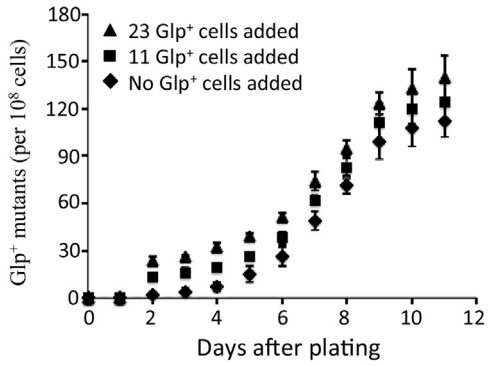

Fig. 1. Glp$^+$ mutations in Δ*cyaA* cells on M9 + glycerol agar plates. Δ*cyaA* cells (~10^8) from a fresh LB culture were spread on M9 + glycerol (0.2%) agar plates. The plates were incubated at 30 °C and examined for the appearance of Glp$^+$ colonies (each colony represents an IS5 insertional mutation) at 24 h intervals. The mutation frequencies were determined as in Zhang and Saier [4]. ♦ = no Glp$^+$ cells added before initially plating; ■ and ← = 11 and 23 Glp$^+$ (IS5 insertional mutant) cells were included before plating.

ment 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$_{Crp}$*. 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 S1).

For β-galactosidase assays, strains were cultured in liquid LB media ± 1 mM cAMP at 30 °C. When cultures entered the exponential phase, samples were collected for measurement of β-galactosidase activities as described by Miller, [19]).

3. Results

3.1. IS5 insertional activation of the glpFK operon occurs in Δ*cyaA* 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 [4]. 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 appeared (Fig. 1). These mutants were IS5 insertional mutants carrying IS5 in the same position and orientation upstream of the *glpFK* promoter as those isolated previously from Δ*crp* cells. The time course (Fig. 1) for their appearance and the properties of these double mutants were indistinguishable from those isolated previously [4]. 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 mutants 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 (Fig. 1).

3.2. IS5 activation of the glpFK operon in Δ*cyaA* cells is suppressed by exogenous cAMP

Exogenous cAMP can enter cells to increase the cytoplasmic concentration of this nucleotide [10]. Fig. 2A shows the effect of increasing concentrations of external cAMP on the *glpFK*-specific

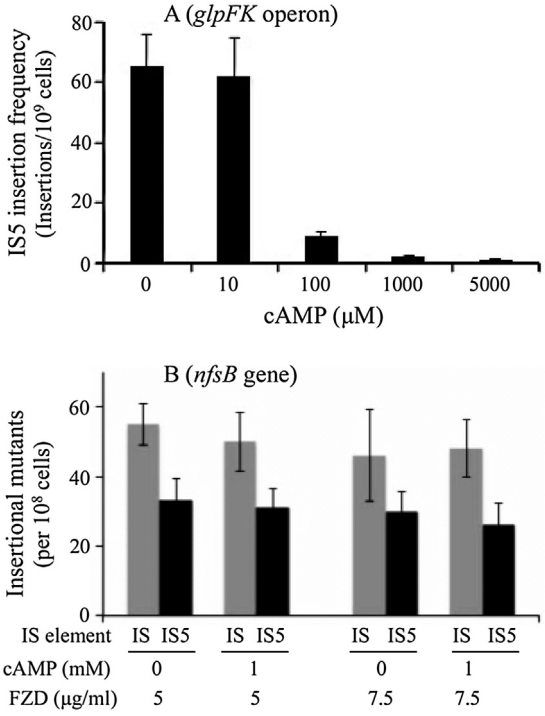

Fig. 2. Effects of cAMP on (A) IS5 insertion upstream of the *glpFK* regulatory region in Δ*cyaA* cells, and (B) in the *nfsB* gene as a control. In A, a fresh LB culture from a single Δ*cyaA-cat* (see Supplementary Table S1) colony was diluted 1000 x into 5 ml of LB ± cAMP (0 to 5 mM) in 30 ml glass tubes (2.5 cm x 20 cm). The tubes were shaken at 250 rpm in a 30 °C water bath shaker. After ~15 h, the cells were washed 1 x (to remove residual cAMP) with a carbon source-free M9 salts solution, serially diluted, and applied onto LB + glucose agar plates (for total population determination) and LB + glucose + Cm (20 μg/ml) agar plates (for IS5 insertional mutant population determination). In B, the cells (~2×10^8) 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 FZD (5 or 7.5 μg/ml) ± cAMP (1 mM). The plates were incubated at 30 °C for 36 h before being examined for the appearance of FZD^r mutants. Among these FZD^r mutants, IS5 and other IS insertional mutants in the *nfsB* gene were determined and quantitated by PCR (see Materials and methods). In all cases, the proportions of IS5 mutants (~60%) was the same.

IS*5* insertional frequency in Δ*cyaA_cat* cells (see *Materials and Methods* and Supplementary Table S1). 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 (Fig. 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 IS5 insertion into the *nfsB* gene in Δ*cyaA* cells. Mutational inactivation of *nfsB* confers resistance to furazolidone (FZD) [20] and a substantial fraction of inactivating mutations (20%) are due to IS insertions. Of these, 60% are due to IS5. As shown in Fig. 2B, cAMP did not influence the frequency of either total insertional events (grey bars) or of IS5 insertional events (black bars) among FZD-resistant mutants within experimental error.

3.3. Control experiments that eliminate preferential growth and selection as causes of increased IS5 insertion

We have conducted control experiments to eliminate the possibility that the changes in IS5 insertion observed in response to cAMP were due to differentiated growth or to the selection procedure used. The results are presented in Fig. 3 and Fig. S1. We grew the *cyaA* Glp$^-$ and *cyaA* Glp$^+$ (IS5 insertion) cells separately (Fig. 3A) or together in a 1:1 ratio (Fig. 3B). It can be seen that the two strains grew in LB medium at essentially the same rate when the exogenous cAMP concentration was 0, 0.1 or 1.0 mM (Fig. 3A).

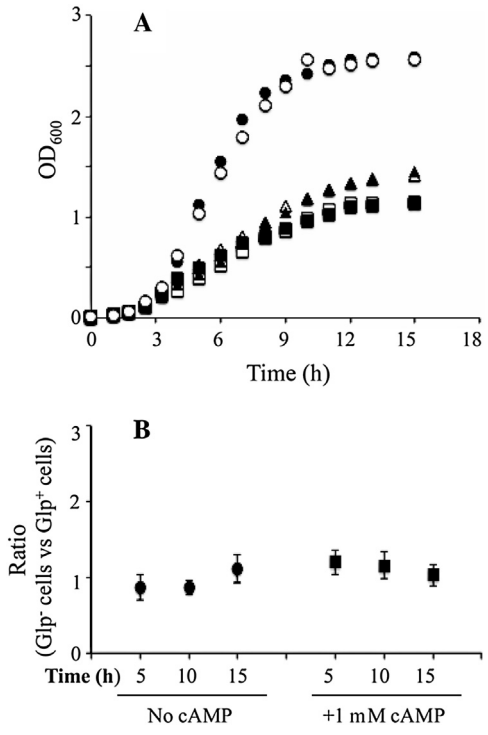

Fig. 3. Growth rates and competition assays for *ΔcyaA* Glp⁻ and *ΔcyaA* Glp⁺ cells. Strains *ΔcyaA:km_cat* (Glp⁻, Km^r and Cm^s) and *ΔcyaA_cat* Glp⁺ (Glp⁺, Km^s and Cm^r) (see Table S1) and LB ± cAMP (0 to 1 mM) were used for these assays. In A, these two strains were cultured in separate tubes. The tubes were shaken at 250 rpm at 30 °C. Samples were taken every 45 min to 1 h, and their optical cell densities (OD_{600}) were measured. ■, ▲ and ● = *ΔcyaA:km_cat*. □, △ and ○ = *ΔcyaA_cat* Glp⁺. In B, the two strains were mixed at a 1:1 ratio in the same tube. After 5, 10 and 15 h of growth with shaking at 30 °C, samples were taken to determine the populations of each strain. The cell samples were serially diluted and applied onto LB + Km plates for *ΔcyaA:km_cat* Glp⁻ population determination and LB + Cm plates for *ΔcyaA_cat* Glp⁺ population determination. ■, no cAMP; ▲, 0.1 mM cAMP; ●, 1.0 mM cAMP.

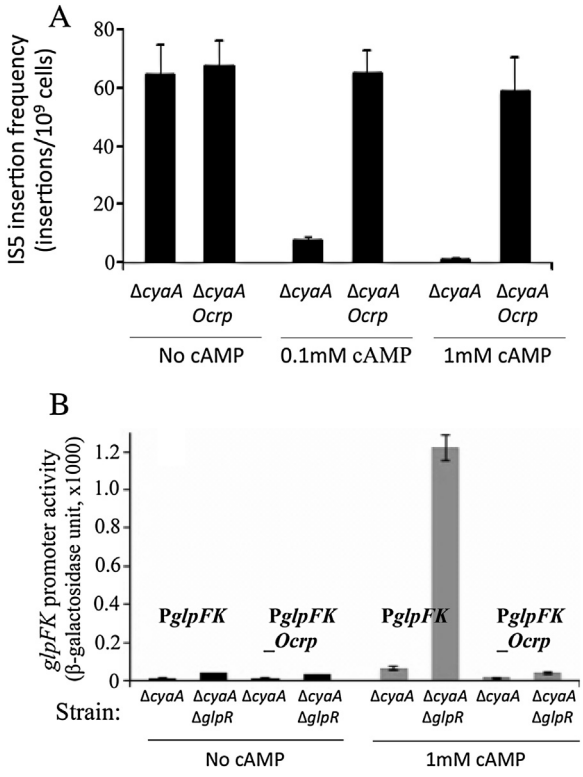

Fig. 4. Effect of Crp binding site mutations on (A) IS5 insertion upstream of the *glpFK* promoter region and (B) the promoter activity in *ΔcyaA* and *ΔcyaA ΔglpR* cells. In both A and B, cells were grown in LB medium ± cAMP. The concentrations of cAMP are indicated below the x-axes. O_{crp} indicates the mutated Crp binding sites (CrpI and CrpII) in the upstream regulatory region of the *glpFK* operon to prevent Crp binding. In A, strains used carry the *cat* gene fused to and downstream of *glpFK*. In B, the activities of the *glpFK* promoter (P*glpFK*) and the same promoter (P*glpFK*_O_{crp}) mutated in CrpI and CrpII were measured using the LacZ reporter in both *ΔcyaA* cells and *ΔcyaA ΔglpR* cells. See Supplementary Table S1 for the detailed strain information.

The duration of the experiment was 15 h (11 generations), the duration of the experiments reported in Fig. 2 and Figs. 4–6 (see below). Similarly, when cells were first mixed in a 1:1 ratio and then grown for the same period of time, the ratio remained constant within 10%, the error of the measurements (Fig. 3B). This was true regardless of whether cAMP was absent or present at strongly inhibitory concentrations (see Fig. 2).

In a separate experiment, we used an isogeneic strain in which the *glpFK* promoter was fused to a *cat* (chloramphenicol acetyl transferase) gene with both the *glpF* and *glpK* genes deleted. The experiment (Fig. S1) was conducted without and with 0.1 mM cAMP in a *ΔcyaA ΔglpR* double mutant. There can not be selection on glycerol with such strains because glycerol utilization is absolutely dependent on GlpK. It can be seen that 0.1 mM cAMP strongly inhibited the frequency of IS5 insertion. Thus, these experiments separate mutation rate from any possible effect of glycerol utilization, growth, or selection. These results confirm our earlier report [4] demonstrating that IS5 insertion into the *glpFK* promoter activating site was dependent on GlpR in a *crp* genetic background under conditions where the *glpFK* promoter was fused directly to the *cat* gene in the absence of GlpF and GlpK function.

3.4. The effect of cAMP on the IS5 insertion frequency requires the Crp binding sites in the glpFK promoter region

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 (CrpI and CrpII), present in the *glpFK* promoter region, we analyzed the consequences of point mutations within these binding sites. These mutations essentially abolished the inhibitory effect of Crp on IS*5* insertion (Fig. 4A). 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 (Fig. 4B). 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.

3.5. 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 *ΔcyaA* background, yielding a *ΔcyaA ΔglpR* double mutant (Supplementary Table S1). Higher IS*5* insertional frequencies were observed in the *ΔcyaA ΔglpR* cells than in the *ΔcyaA* cells when grown in LB (no glycerol added) (Fig. 5A). 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 (compare column 2 and column 4 in Fig. 5A). 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.

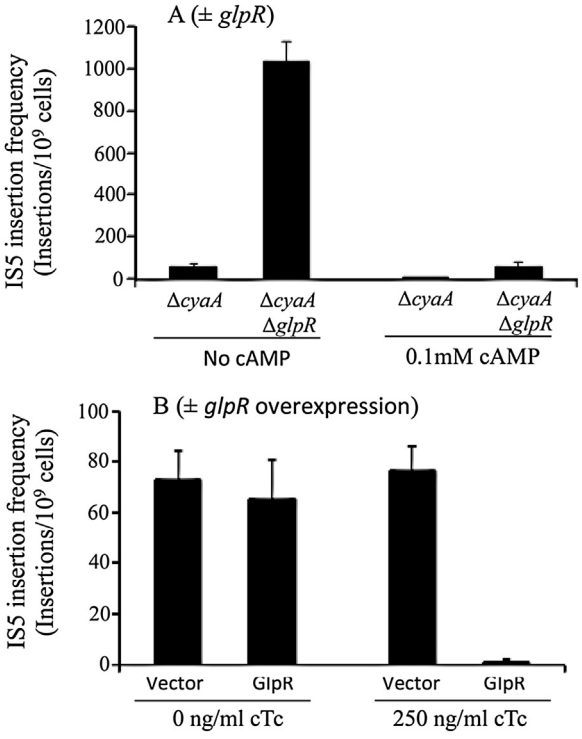

Fig. 5. Effects of *glpR* deletion (A) and *glpR* overexpression (B) on IS*5* insertion in the control region of the *glpFK* operon in Δ*cyaA* cells grown in LB ± cAMP. In A, the exogenous cAMP concentration was either 0 (left) or 0.1 mM (right). In B, vector: no *glpR* present (control); GlpR indicates that the *glpR* gene is present downstream of the *tet* promoter, P*tet*, in an expression vector. cTc = chloro-tetracycline (an inducer of the *tet* promoter) at a concentration of 0 (left) or 250 ng/ml (right). *glpR* expression is thus under the control of the cTc induced promoter, P*tet*, in the expression vector. Δ*cyaA* cells that constitutively produce TetR (repressing P*tet*) were used in these experiments. Note the scale difference for Fig. 4A and B. All strains carry the *cat* gene fused to and downstream of the *glpFK* genes.

When GlpR was over-produced in the absence of cAMP, GlpR still exerted its strong inhibitory effect (Fig. 5B). 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 Fig. 5B). The same experiments were conducted in the presence of cAMP (0.1 mM). 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 GlpR and the cAMP-Crp complex can bind to the *glpFK* control region simultaneously.

3.6. IS5 insertional activation of the glpFK operon occurs in a wild type background in the presence of 2-deoxyglucose

Since a reduction in the cytoplasmic cAMP concentration promotes IS5 insertion in the *glpFK* activating site, we sought to determine whether environmental conditions that lead to a reduction in cAMP concentrations elevate IS*5* insertion into the *glpFK* promoter. Non-metabolizable glucose analogues, such as 2-deoxyglucose (2DG) and α-methylglucoside (αMG) [21–24,25] are known to lower cytoplasmic cAMP levels by inhibiting adenylate cyclase activity [26–29]. 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

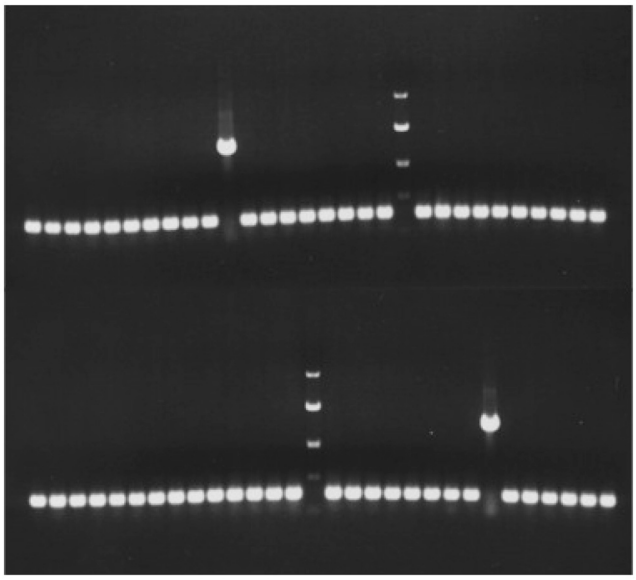

Fig. 6. IS*5* insertion upstream of the *glpFK* control region in wild type cells. Wild type cells carrying the *glpFK_cat* (chloroamphenicol acetyl transferase) fusion (i.e., BW_*cat*, see Methods section) were plated onto M9 + glycerol (0.2%) + 2DG (0.12%) + chloramphenicol (Cm) (60 μg/ml) agar plates. The plates were incubated at 30 °C. After 5 days of incubation, colonies were examined for the presence of IS5 upstream of the *glpFK* control region by PCR. In both the upper and the lower panels, only one type of IS5 insertion was obtained. A DNA marker showing four bands with known sizes (4 kb, 2 kb, 1 kb and 0.5 kb from above) is indicated in each panel.

kinase [30–33]. 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 (strain, BW_ *cat*) was used to measure IS*5* insertion (see Supplementary Table S1 and *Materials and Methods*). Regardless of the inhibitory glucose analogue used, analogue-resistant mutants (which were also Glp^+) could be isolated in a wild type *E. coli* genetic background [34–36]. As shown in Fig. 6, 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 (cra)* gene [which appears to regulate *crp* gene expression [37]]. 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 when high cytoplasmic concentrations of the cAMP-Crp complex are available [37].

To demonstrate that the Glp^+ phenotype of IS5 mutants results solely from the IS5 insertional event, we carried out P1 transduction experiments to determine if the phenotypes of $2DG^r$, Cm^r and Glp^+, could be transferred together into another *E. coli* genetic background. In these experiments, we used two "wild type" strains, BW25113 and BW_ *cat* (Supplementary Table S1) (with or without the *glpFK_cat* fusion) as recipients. For both recipient strains, transductants were obtained using the IS*5* insertional mutant (expressing the fused *glpFK_cat* operon) as donor, when plated on M9 + glycerol + 2DG ± Cm agar plates. The regulatory regions of 23

Table 1
Growth of a wild type strain, 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 salts 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 °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 colony size while + indicates decreased size.

independently isolated transductants were amplified by PCR, and all were found to carry the IS*5* element. DNA sequencing showed that IS5 was located in the same position and in the same orientation as described previously [4,6]. These results showed that in these mutants, IS*5* insertion is necessary and sufficient to give rise to the $2DG^r$ Glp^+ phenotype.

3.7. Short-term and long-term evolution experiments to evaluate if IS5-mediated activation of glpFK operon expression could have evolved in wild type cells

Initially, we conducted short-term evolutionary experiments using *ΔcyaA_cat* cells in which a chloramphenicol-resistance gene was transcriptionally fused to the *glpFK* operon such that *glpFK* activation by IS5 insertion led simultaneously to a Glp^+ and a Cm^r phenotype (see *Materials and Methods*). These short-term experiments (several transfers) were conducted by incubating Δ*cyaA* cells in the presence of glycerol alone (Fig. 7A), glycerol plus 0.1 mM cAMP (Fig. 7B), and glycerol plus 1.0 mM cAMP (Fig. 7C). In the absence of cAMP, IS*5* insertional mutants appeared as the only species after 37 h of incubation with shaking. After several subsequent transfers, virtually 100% of the cells contained IS*5* in the *glpFK*-activating site (Fig. 7A). When cAMP was added at 0.1 mM, IS*5* insertional mutants again appeared as the only species after a 61 h incubation with shaking. After the first transfer, virtually all cells contained IS*5* (Fig. 7B). When exogenous cAMP was added at 1 mM, the appearance of these mutants was strongly inhibited. As a result, after the 6th transfer, only about 20% of the population were IS*5* insertional mutants (Fig. 7C). These observations are consistent with the conclusion that the cAMP-Crp complex inhibits IS*5* insertion upstream of the *glpFK* operon.

In order to conduct long-term evolutionary experiments in a wild type genetic background, we initially used *glpFK_cat* cells (i.e., BW_*cat*) in which a chloramphenicol-resistance (*cat*) gene was transcriptionally fused to the *glpFK* operon such that *glpFK* activation by IS*5* insertion led simultaneously to a Glp^+ and Cm^r phenotype (see *Materials and Methods*). Prolonged incubation of *glpFK_cat* wild type cells under conditions analogous to those described above for the short-term Δ*cyaA* experiments revealed that IS*5* mutants became an appreciable fraction of the population after several generations in minimal glycerol medium with 2-deoxyglucose, with (Fig. 8A) or without (Fig. 8B) chloramphenicol. When chloramphenicol was present, IS*5* insertional mutants first appeared after six transfers, and then continued to accumulate during the remainder of the experiment (17 transfers), after which about 25% of the cells bore the IS5 insertion (Fig. 8A). When chloramphenicol was absent, it took a little longer; insertional mutants became appreciable following the eighth transfer, and accounted for over 20% of the cells after 20 transfers (Fig. 8B). In both experiments, the percentages were still increasing when the time course

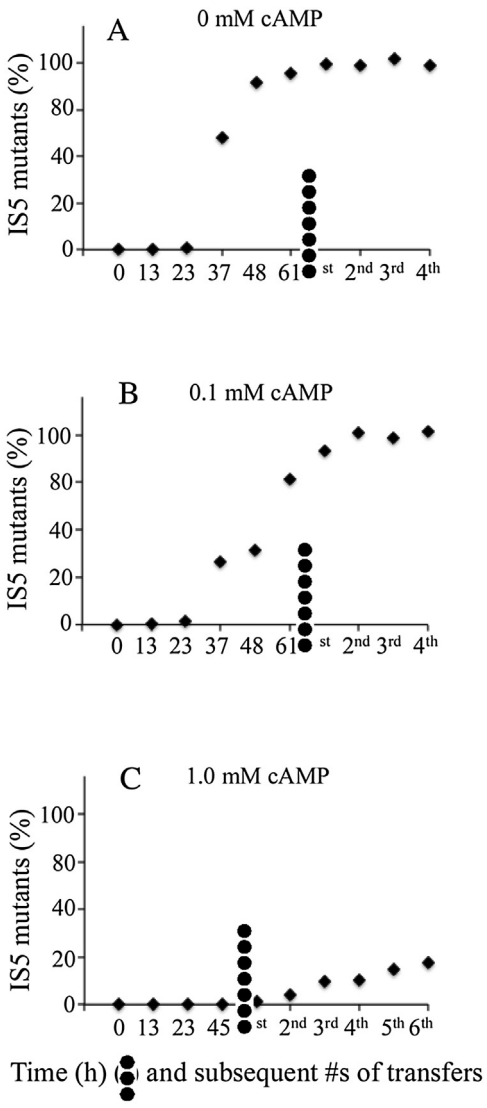

Fig. 7. Percentages of IS*5* mutant populations vs the total populations during growth in M9 + glycerol ± cAMP over time using strain Δ*cyaA_cat*. For the first period [up to 61 h (A and B) or 45 h (C)], time is expressed in hours. Thereafter (vertical dotted line), time is expressed in numbers of transfers where each transfer occurred about once per day. Figures A-C show the effects of increasing cAMP concentrations: A, 0; B, 0.1 mM; C, 1 mM.

was terminated. These results show that *glpFK* activation by IS*5* is advantageous when wild type cells are exposed to glycerol in the presence of a sugar analogue inhibitor of adenylate cyclase such as 2-deoxyglucose [38]. The process could, therefore, have evolved by natural selection.

4. Discussion

4.1. Primary features of the IS5-glpFK experimental system

In wild type *E. coli* cells, glycerol utilization requires expression of genes that are poorly expressed in the presence of glucose or a glucose analogue. The first operon in the glycerol utilization pathway, *glpFK*, codes for the glycerol uptake facilitator (GlpF) and glycerol kinase (GlpK) that phosphorylates glycerol to glycerol-3-phosphate (G3P). G3P, the inducer of the *glp* regulon, is then oxidized by GlpD to dihydroxyacetone phosphate, an intermediate of glycolysis. The *glpFK* operon is repressed by the binding of the GlpR repressor to its four binding sites (operators) in the promoter

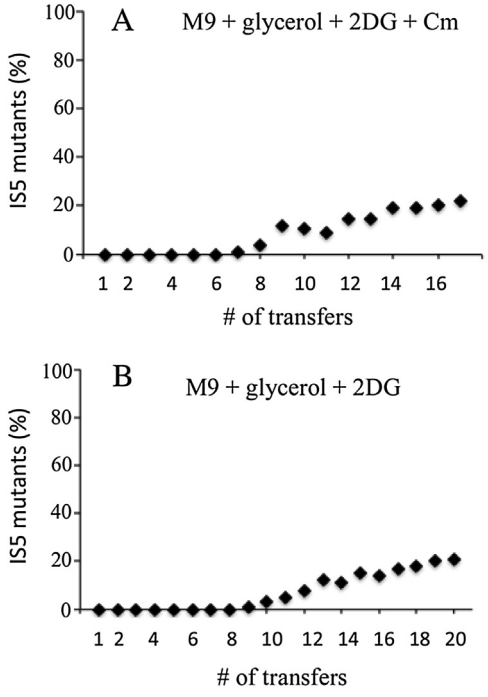

Fig. 8. Percentages of IS5 insertional mutant populations versus total mutant populations over time using the "wild type" strain carrying the chromosomal *glpFK-cat* fusion. Cells were incubated with shaking (250 rpm) at 30 °C in 5 ml of M9 + glycerol (0.2%) + 2DG (0.12%) medium plus (A) or minus (B) chloramphenicol (Cm) (60 μg/ml) in glass tubes (20 mm x 200 mm). After 2.5 days of incubation, 5 μl culture samples were transferred to 5 ml of the same media (1st transfer) and incubated with shaking at 30 °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 °C, 100 colonies were examined by PCR for the presence of IS*5* in the *glpFK* control region (See Fig. 6).

region, and is activated by the binding of the cAMP-Crp complex to its two binding sites that partially overlap the GlpR binding sites [4,11]. G3P binds to and inactivates GlpR, which, however, is insufficient to activate the weak native *glpFK* promoter, the activation of which requires the binding of the cAMP-Crp complex to the two binding sites (CrpI and CrpII) in the promoter. Thus, cells lacking the *crp* (Crp) or *cyaA* (biosynthesis of cAMP) gene are Glp^- because the *glpFK* operon cannot be activated.

Prolonged incubation of *Δcrp* (Glp^-) cells or *ΔcyaA* (Glp^-) cells on glycerol minimal plates results in the continuous generation of Glp^+ colonies. Sequence analysis of several hundred independent Glp^+ colonies revealed that in each and every case, IS*5* is found to be inserted upstream of the *glpFK* promoter at the same specific location and in the same orientation. Subsequent work established several key features of this *glpFK*-activating insertional mutation as reviewed elsewhere [39–41], and we have interpreted the results to mean that the IS*5*-*glpFK* system represents an example of a novel mutagenic mechanism.

4.2. Formation and selection of mutations under stressful conditions

Whether specialized mutagenic mechanisms are activated in response to environmental conditions has been the subject of active investigation for nearly three decades. Much of the work addressing this question is based on the so-called Foster-Cairns *E. coli* experimental system [recent review: [42]]. This system is based on the *E. coli* strain FC40/F'*lac* which cannot utilize lactose because a frameshift mutation prevents expression of the gene (*lacZ*) for β-galactosidase, an enzyme required for lactose utilization. However, when FC40 cells are subjected to prolonged incubation on minimal lactose plates, on which Lac^- cells cannot grow, Lac^+ colonies appear, starting on day 3, and accumulate linearly over several subsequent days. Foster and coworkers provided evidence that these "adaptive" or "stress-induced" mutations are dependent on DinB (DNA Polymerase IV) and homologous recombination functions, and are due to error-prone DNA synthesis associated with the processing of recombination intermediates [42]. Rosenberg and coworkers proposed an alternative scenario, suggesting that adaptive mutations arose in a hypermutable subset of cells in which starvation stress activated a specialized error-prone double-stranded DNA break repair pathway [43,44].

Without disputing the experimental observations made by proponents of adaptive mutagenesis, Roth and coworkers proposed that the Lac^+ colonies in the Foster-Carins system arose solely from mutation-capture by selection, a mechanism to be contrasted with induction of a novel mutagenic mechanism [for an updated summary of this view, see: [45]]. In this "capture" model, all Lac^+ revertants arise from a pre-existing subset of cells in which the F plasmid is replicated without concomitant cell division. However, Stumpf et al. [46] have provided concrete evidence that gene amplification cannot account for adaptive mutation in the *E. coli lac* system. Both the "adaptive mutagenesis" and "selection-capture" hypotheses, although originally proposed to explain mutations in the Foster-Carins system, are extendable to other experimental systems with minor modifications.

The IS*5*-*glpFK* experimental system, when Glp^- cells are subjected to prolonged incubation on minimal glycerol medium on which they cannot grow, Glp^+ colonies appear after day 3. However, because the mutation is due to transposon (IS*5*) insertion at a single specific location and orientation, there is no need for the participation of either cellular DNA polymerases or homologous recombination. Not surprisingly, loss of the *recA* gene has little effect on IS*5*-*glpFK* mutation [4].

4.3. "Directed transposition" vs. "Selected transposition" models

In our "directed transposition" model, we propose that the DNA sequence upstream of the *glpFK* promoter is a conditional hotspot (Z. Humayun, Z. Zhang & M. Saier, unpublished results) for IS*5* insertion that is somehow masked by the vicinal binding of one or more DNA binding proteins. In *Δcrp* cells, GlpR is known to bind near the IS*5* target sequence to suppress IS*5* insertion [4] and in this work, Crp has now been shown to also suppress IS5 insertion. In this model, passive diffusion of glycerol, or entry via GlpF [47], followed by its phosphorylation to glycerol-3-phosphate (G3P) by leaky expression of GlpK leads to unmasking of the IS*5* target site.

Under the plausible "mutation-capture" hypothesis [45], a subset of the plated Glp^- cells amplify the *glpFK* region and are selectively subjected to the "positive feedback loop" in which availability of small amounts of energy from leaky expression of GlpFK leads to further amplification of the *glpFK* region. Either the accumulation of G3P, which inactivates GlpR, or simple titration of the limited amount of the GlpR protein by the amplified excess of *glpFK* DNA, or both, may lead to the unmasking of the IS5 insertion site. It is important to note, that regardless of the mechanism(s) by which binding of GlpR and the cyclic AMP-Crp complex inhibits IS5 insertion, the system appears to exhibit the characteristics defined as "directed mutation" [48].

4.4. IS5-glpFK mutation as a "last resort" option for survival

Regardless of the mechanism considered above, the discovery that GlpR occupation of O_1 [4] and cAMP-Crp occupation of CrpI and CrpII (this communication) inhibit IS*5* insertion suggests that the IS*5* insertion target is unmasked in wild-type cells only under

a specific set of environmental circumstances. Wild type cells preferentially use glucose and repress the pathways required for the utilization of other carbon sources as long as glucose continues to be available in the medium. This repression (confusingly named "catabolite repression") is accomplished by blocking cAMP synthesis, and therefore, the formation of the cAMP-Crp complex. Since the cAMP-Crp complex is a required positive transcriptional activator for genes necessary for utilizing alternative sugars under normal conditions of glucose depletion, the cAMP-Crp complex is expected to bind to and activate most operons subject to catabolite repression, including the *glpFK* operon. Thus, insertional activation in the IS*5*-*glpFK* system can be seen as a "last resort" survival mechanism that is activated only when glycerol is present, but utilization is blocked due to an environmental condition that interferes with the production of the cAMP-Crp complex, such as occurs in the presence of a non-utilizable glucose analogue, as shown here. We note that numerous toxic and non-metabolizable sugar analogues are synthesized by microorganisms, plants, fungi and man. They include deoxy sugars such as 2-deoxyglucose, methylated sugars such as 3- and 6-O methylglucose, fluoro-sugars, and a variety of α- and β-glycosides such as methyl α-glucoside [21–24,49,50].

4.5. Other IS-mediated stress-induced mutations

In addition to the IS*5*-*glpFK* system, a number of reports describe beneficial transposition events that appear to relieve a variety of environmental stress conditions. Target operons include the *flhDC* operon encoding the flagellar master regulator, FlhDC [51], zinc inducible zinc resistance [52], β-glucoside utilization [53], growth on propandiol involving the *fuc* regulon [8], and resistance to toxic nitro-substituted compounds [20]. In an early review article, Hall summarized several situations where transposon insertions activate cryptic operons apparently at elevated frequencies under conditions of starvation [54].

The example of IS5 insertion that occurs at higher frequencies when beneficial, giving rise to activation of the *E. coli* flagellar master regulator operon, *flhDC*, than when neutral or detrimental [51], seems particularly relevant to the proposal of directed mutagenesis. Insertional activation of *flhDC* substantially enhances bacterial migration through semisolid agar media, and this phenotype is beneficial under adverse conditions such as nutrient depletion or a need to escape a toxic substance. Under conditions where swarming is not beneficial or permitted (i.e., in liquid or on solid media, respectively), insertional activation of the *flhDC* operon occurs at greatly reduced frequencies. 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 and extended the observations of [51] in our laboratory [38]; Kukita et al., manuscript submitted for publication). Few mechanistic molecular details are available for these experimental systems, but they have the potential of testing the generality of hypotheses that seek to explain the relationships between stress and transposon-mediated mutations that can relieve such stresses. Further work will be required to define the details of these processes.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

We thank Fengyi Tang, Joshua Asiaban, Sabrina Phan, Anne Chu and Yongxin Hu for assistance with manuscript preparation, and Chika Kukita and Robert Palido for technical assistance with some of the experiments. Professor Zafri Humayan provided valuable assistance with manuscript presentation and clarification. This work was supported by NIH grants GM109895 and GM077402.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.mrfmmm.2016.10.003.

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