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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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**Abstract**

*Escherichia coli* cells deleted for the cyclic AMP (cAMP) receptor protein (Crp) gene (Δcrp) 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 (IS5), 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 IS5 insertion and inhibits insertion. By adding cAMP to the culture medium in ΔcyaA cells, we here show that the CAMP-Crp complex, which also binds to the glpFK upstream regulatory region, inhibits IS5 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.

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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 Δcrp strain could mutate to rapid glycerol utilization due to insertion of the small transposon, Insertion Sequence 5 (IS5) [5]. To cause activation, IS5 hops into a single site, in a single orientation, upstream of the glpFK operon promoter. The presence of IS5 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 IS5 at this specific site but not in other operons [4,7]. Glycerol-promoted IS5 insertion into the glpFK-activating site proved to be regulated by binding of the glycerol repressor, GlpR, to its four adja-
GlpR, but it depended exclusively on Crp and the two adjacent Crp binding sites (Crp1 and Crp2) 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 predispense the glpFK operon to activation by IS5 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 (mMG) [10]. Here we show that incubation of wild type E. coli cells in glycerol media together with 2 DG or mMG 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 cyaΔ 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 ~20bp region at the 3′ end that is complementary to the FRT-flanking km gene sequence, and a ~50bp region at the 5′ end that is homologous to cyaA. The PCR products were gel purified, treated with DpnI, and then electroporated into BW25113 cells expressing the lambda-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 resistant cells 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 chaperonin-glucocerebroside 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 glpFKcat-P1 and glpFKcat-P2 (Supplementary Table S2). The PCR products were electroporated into wild type, ΔcyaA and ΔcyaA ΔglpR 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+ colonies were verified for the substitution of the 85 bp glpk/glpk intergenic region by PCR and subsequent DNA sequencing. In the resultant strains (named BW_cat, ΔcyaA_cat and ΔcyaA ΔglpR_cat, respectively), glpF, glpK and cat form a single operon with its expression solely under the control of the glpFK promoter (PglpFK).

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 chromosonal Crp operators

To modify the chromosomal Crp binding sites in the control region of the glpFK operon, the previously made plasmid pKD13-PglpFK [4], was used. In this plasmid, PglpFK and the FRT-flanking km gene were oriented in opposite directions. Using the quick-change site-directed mutagenesis kit (Agilent) and oligos PglpFK_Crp1aceous-F and PglpFK_Crp2aceous-R (Supplementary Table S2), both Crp operators (Crp1 and Crp2) in the glpFK control region, contained within pKD13-PglpFK, were mutated by changing tat-gacgccacacacatattaag (-69 to -44 relative to +1 of PglpFK) to gacgcgagctgattgcattataac (substitutions are underlined). The substitutions were confirmed by sequencing. Using the resultant plasmid, pKD13-PglpFK_O1, as template, the region containing the km gene and PglpFK_O1 was PCR amplified using the primers PglpFK_Crp1aceous-P1 and PglpFK_Crp2aceous-P2 (Supplementary Table S2). The PCR products were integrated into the ΔcyaA_cat and ΔcyaA ΔglpK_cat mutant chromosome to replace the wild type PglpFK. The nucleotide substitutions in both Crp1 and Crp2 operators were confirmed by sequencing. The km gene was removed, and the resultant strains were named ΔcyaA_O1_cat and ΔcyaA ΔglpR_O1_cat, respectively (Supplementary Table S1).

2.3. Glp* mutation assay using a ΔcyaA mutant strain

Using the ΔcyaA 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 (~105 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 ΔcyaA cells, together with small numbers of ΔcyaA 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 ΔcyaA_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 (ΔcyaA Glp* cat) is resistant to Cm at 20 μg/ml. Preliminary experiments showed that all ΔcyaA cat cells resistant to Cm at 20 μg/ml were due to IS5 insertion in front of PglpFK. To determine the effect of cAMP on IS5 insertion, an 8-h old culture from a single ΔcyaA_cat colony was diluted 1000x 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⁺ populations to total populations.

To see if there is any difference in growth rate between cyaA Glp⁻ and cyoA Glp⁺ cells, these two strains (ΔcyaA::km cat and ΔcyoA::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 OD₆₀₀ = 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 (OD₆₀₀) were measured using a Bio-Rad SmartSpec 3000 machine.

To compare their competitive abilities, the Glp⁻ strain, ΔcyaA::km cat [in which a Km gene substitutes for cyaA, so that it is resistant to kanamycin (Km⁺) but sensitive to chloramphenicol (Cm⁺)] and the Glp⁺ strain, ΔcyoA::cat Glp⁺ (that is Cm⁺ but Km⁻), 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 Ocr, cat cells with mutations in operators, Crp1 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-Kpnl and glpR-BamHI (Supplementary Table S2). The PCR products were digested with Kpnl and BamHI, 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 (Ptet). The same plasmid carrying a random fragment (RF), pZA31-RF, served as a control [16]. To repress Ptet activity, the constitutively expressed tetR cassette, located at the attB site, was transferred to ΔcyaA::cat carrying pZA31-glpR from BW-R1 [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 + glucose ± 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 PglpFK 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 inoculated 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 10⁵-fold and 10⁶-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 function.

2.6. Chromosomal lacZ fusions and β-galactosidase assays

Using pKD13-PglpFK [4] and pKD13-PglpFK_Ocr (Supplementary Table S1) as templates, PglpFK (~204 to +66 relative to the transcriptional start site) and PglpFK_Ocr plus their upstream FRT-flanked km⁻ gene were amplified using oligos PglpFK-P1 and PglpFKz-P2 (Supplementary Table S2). Using the method of [13], the promoters plus the upstream km⁻ gene (km⁻:PglpFK or km⁻:PglpFK_Ocr) 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-
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 PglpFK or PglpFKΔcrp. Both constructs were transferred into ΔcyAΔ and ΔcyAΔglpK strains by P1 transduction, yielding ΔcyAΔPglpFK-lacZ, ΔcyAΔPglpFKΔcrp-lacZ, ΔcyAΔΔglpK-PglpFK-lacZ, and ΔcyAΔΔglpK-PglpFKΔcrp-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. ISS insertional activation of the glpFK operon occurs in ΔcyAΔ cells

We previously demonstrated that Δcrp mutant cells of E. coli could regain the ability to utilize glycerol by ISS-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 ΔcyAΔ mutant cells lacking the cAMP biosynthetic enzyme, adenylate cyclase (Cya). Like the Δcrp mutant cells, the ΔcyAΔ mutant cells cannot utilize glycerol as the sole carbon source for growth. However, after a prolonged incubation on M9+glycerol agar plates, Glp+ cells appeared (Fig. 1). These mutants were ISS insertional mutants carrying ISS in the same position and orientation upstream of the glpFK promoter as those isolated previously from Δcrp 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 ΔcyAΔ Glp+ insertional mutants were added to the ΔcyAΔ cells prior to plating, they gave rise to colonies within a shorter time period (Fig. 1).

3.2. ISS activation of the glpFK operon in ΔcyAΔ 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 ISS insertional frequency in ΔcyAΔ 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 ISS insertion, but at 100 μM, cAMP inhibited over 90%, whereas at 1 mM, cAMP essentially abolished ISS insertion (Fig. 2A).

To determine whether the decrease in ISS insertion frequency due to the presence of cAMP was specific to the glpFK promoter, we analyzed ISS insertion into the nfsB gene in ΔcyAΔ cells. Mutational inactivation of nfsB confers resistance to furazolidone (FZD) [20] and a substantial fraction of activating 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 insertion events (grey bars) or of ISS 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 ISS insertion

We have conducted control experiments to eliminate the possibility that the changes in ISS 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 cyaAΔ Glp− and cyaAΔ Glp+ (ISS 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, 0.1 or 1.0 mM (Fig. 3A).
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 isogenic 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 ΔcytA ΔglpK 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 ISS 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 ISS 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 ISS insertion frequency requires the Crp binding sites in the glpFK promoter region

To determine whether the inhibition of ISS 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 (Crl 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 cAMP on ISS 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 cytA deletion background, and the promoter activity was still under the control of GlpK (Fig. 4B). Thus, it can be concluded that inhibition by cAMP-Crp of ISS 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 The ISS insertion frequency in the glpFK promoter

To determine if GlpR plays a role in the inhibitory effect of Crp on ISS insertion, we deleted the glpR gene in the ΔcytA background, yielding a ΔcytA ΔglpR double mutant (Supplementary Table S1). Higher ISS insertional frequencies were observed in the ΔcytA ΔglpR cells than in the ΔcytA 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 ISS 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 (Fig. 5B). In the left panel, the glpR gene was expressed at an extremely low level, and GlpR exerted only a minimal effect because chlorotetraacycline (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 IS5 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 IS5 insertion frequency decreased, while overexpression of GlpR further inhibited IS5 insertion (data not shown). These results suggest that GlpR and the cAMP-Crp complex exert their effects on IS5 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 IS5 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 kinase [30–33]. We therefore asked if we could isolate IS5 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 IS5 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-IS5 mutants" here), and (ii) a minority (<10%; "IS5 mutants") that had IS5 inserted into the glpFK promoter-activating site.

Sequencing revealed that the non-IS5 insertion mutants did not have genetic alterations in the glpFK operon, or in the fraR (cra) gene [which appears to regulate cpr gene expression [37]]. Unlike IS5 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 sucrose (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+, Cm+ 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 IS5 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 sources1, 2.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Glycerol</th>
<th>Sorbitol</th>
<th>Succinate</th>
<th>Xylose</th>
<th>Mannitol</th>
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<td>++</td>
<td>*</td>
<td>++</td>
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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 IS5 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, IS5 insertion is necessary and sufficient to give rise to the 2DG2 Glp2 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 \( \Delta \text{cyaA-cat} \) 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 Cmr phenotype (see Materials and Methods). These short-term experiments (several transfers) were conducted by incubating \( \Delta \text{cyaA} \) 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, IS5 insertion mutants appeared as the only species after 37 h of incubation with shaking. After several subsequent transfers, virtually 100% of the cells contained IS5 in the glpFK-activating site (Fig. 7A). When cAMP was added at 0.1 mM, IS5 insertion mutants again appeared as the only species after a 61 h incubation with shaking. After the first transfer, virtually all cells contained IS5 (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 IS5 insertion mutants (Fig. 7C). These observations are consistent with the conclusion that the cAMP-Crp complex inhibits IS5 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 IS5 insertion led simultaneously to a Glp+ and Cmr phenotype (see Materials and Methods). Prolonged incubation of glpFK_cat wild type cells under conditions analogous to those described above for the short-term \( \Delta \text{cyaA} \) experiments revealed that IS5 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, IS5 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 was terminated. These results show that glpFK activation by IS5 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
ever, 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-Cairns 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-Cairns system, are extendable to other experimental systems with minor modifications.

The IS5-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 (IS5) 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 IS5-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 IS5 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 IS5 target sequence to suppress IS5 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 IS5 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 O1 [4] and cAMP-Crp occupation of CrpI and CrpIl (this communication) inhibit IS5 insertion suggests that the IS5 insertion target is unmasked in wild-type cells only under

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 CrpIl) in the promoter. Thus, cells lacking the crp (Crp) or cytA (biosynthesis of cAMP) gene are Glp− because the glpFK operon cannot be activated.

Prolonged incubation of Δcrp (Glp−) cells or ΔcytA (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, IS5 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 insertion mutation as reviewed elsewhere [39–41], and we have interpreted the results to mean that the IS5-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. How-
Conflict in stressful conditions such as nutrient deprivation or starvation [54]. The example of ISS insertion that occurs at higher frequencies when beneficial, giving rise to activation of the E. coli flagellar master regulator operon, fliDC, than when neutral or detrimental [51], seems particularly relevant to the proposal of directed mutagenesis. Insertional activation of fliDC 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 swimming is not beneficial or permitted (i.e., in liquid or on solid media, respectively), insertional activation of the fliDC operon occurs at greatly reduced frequencies. Thus, insertion of ISS upstream of the fliDC 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.

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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.

References


