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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 (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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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,