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Transposon-mediated directed mutation controlled by DNA binding proteins in *Escherichia coli*

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**INTRODUCTION**

It is a basic principle of genetics that the likelihood of a particular mutation occurring is independent of its phenotypic consequences. The concept of directed mutation, defined as genetic change that occurs is independent of its phenotypic consequences, was specifically induced by the stress conditions that the mutation relieves (Cairns et al., 1988), challenges this principle (Foster, 1999; Rosenberg, 2001; Wright, 2004). The topic of directed mutation is controversial, and its existence, even its potential existence, has been altogether questioned (Roth et al., 2006).

Part of the justifiable skepticism concerning directed mutation resulted from experiments that were purported to demonstrate this phenomenon, but were subsequently shown to be explainable by classical genetics (Roth et al., 2006). Mutation rates vary with environmental conditions (e.g., growth state) and genetic background (e.g., mutator genes), a phenomenon known as “adaptive” mutation (Wright, 2004; Foster, 2005), but this does not render the mutation “directed.”

To establish the principle of directed mutation, it is necessary to show that the adaptive mutation is “directed” to a specific site, characterize the mechanism responsible, identify the proteins involved, and provide the evolutionary basis for its appearance.

One frequently encountered type of mutation results from the hopping of transposable genetic elements, transposons, which can activate or inactivate critical genes (Mahillon and Chandler, 1998; Chandler and Mahillon, 2002). For example, activation of the normally cryptic β-glucoside (bgl) catabolic operon in *E. coli* can be accomplished by insertion of either of two insertion sequences, IS1 or IS5, upstream of the *bgl* promoter (Schnetz and Rak, 1992).

The *E. coli* glycerol (*glp*) regulon consists of five operons, two of which (*glpFK* and *glpD*) are required for aerobic growth on glycerol (Lin, 1976). Both operons are subject to negative control by the DNA-binding *glp* regulon repressor, GlpR (Zeng et al., 1996), which also binds glycerol-3-phosphate, the inducer of the *glp* regulon. The *glpFK* operon is additionally subject to positive regulation by the cyclic AMP receptor protein (CRP) complexed with cyclic adenosine monophosphate (cAMP; Freedberg and Lin, 1973; Campos et al., 2013), although *glpD* is not appreciably subject to regulation by this mediator of catabolite repression (Weissenborn et al., 1992). The *glpFK* regulatory region contains four GlpR binding sites, O1–O4, and two CRP binding sites, Crp1 and CrpII, which overlap O2 and O3, respectively, (Figure 1A). The strong CRP dependency of *glpFK* transcription is reflected by the fact that *crp* and *cyo* (adenylate cyclase) mutant cells are unable to utilize glycerol (Zhang and Saier, 2009a). We have found that binding of GlpR and the cAMP-CRP complex to the *glpFK* upstream control region negatively influences IS5 hopping specifically into the single site that strongly activates *glpFK* promoter activity (Zhang and Saier, 2009a, unpublished observations).

### Glp+ Mutations in a *crp* Genetic Background

When *crp* cells were incubated on solid glycerol minimal medium, Glp+ colonies appeared. We tested the growth of a *crp* Glp+ strain on glycerol in defined liquid medium. The growth rate was greater than that of wild type (*wt*) *E. coli* (Zhang and Saier, 2009b).

The relative rates of Glp+ mutation were determined in minimal and complex media. On glycerol plates, colonies first appeared after about 3 days (Figure 1B) although wild type (*wt*) and *crp* Glp+ *E. coli* cells formed visible colonies in <2 days (Figure 1C). When the same cells were plated as before, but small numbers of *crp* Glp+ mutant cells were included with the *crp* cells before plating, colonies appeared from the *crp* Glp+ cells within 2 days, and new Glp+ mutants arose at the same rate as before (Figure 1C). This experiment showed that the mutants that arose on the plates had not existed in the cell population when initially plated. Thus, the Glp+ mutants arising from *crp* cells on these plates arose during incubation on the plate, and no growth inhibitor was present. The rate of mutation on glycerol plates proved to be 10 times higher than in minimal sorbitol or complex LB medium, and it was over 100 times higher than in...
glucose-containing medium (Zhang and Saier, 2009a).

Mutation proved to be due to IS5 hopping to a discrete site, between base pairs 126 and 127 upstream of the transcriptional start site, and always in the same orientation. The increase in mutation rate was specific to the glpFK operon and did not occur in three other operons examined (Zhang and Saier, 2009a). Only the downstream 177 bp region of IS5 was required for activation of the glpFK operon, and this proved to be due to the presence of a permanent bend and an overlapping IHF binding site, each of which was responsible for half of the activation (Zhang and Saier, 2009b). This mechanism of activation (but not the actual transposon hopping event), presumably involving DNA looping, could also be demonstrated for the lac operon in a crp genetic background of E. coli (Zhang and Saier, 2009b).

**DEPENDENCY OF THE Glp+ MUTATION RATE ON GlpR**

Glycerol is phosphorylated by glycerol kinase (GlpK) to glycerol-3-phosphate which binds to and releases GlpR from its operators (Lin, 1976). When GlpR dissociates from its operators, due to glycerol-3-phosphate binding, a conformational change could be transmitted through the DNA, promoting insertion of IS5 at the target CTAA site upstream of the glpFK promoter. In other words, GlpR binding might have two functions: repression of gene expression and suppression of IS5 transposition to the upstream activating site.

To test this possibility, the glpR gene was deleted, and the rates of appearance of Glp+ mutations in the crp glpR double mutant background were measured in the absence and presence of glycerol. The numbers of Glp+ cells arising was 10-fold higher in the crp glpR double mutant than in the crp mutant when glycerol was absent. In the presence of glycerol, the loss of GlpR was without effect. Thus, deletion of glpR is equivalent to the inclusion of excess glycerol in the growth medium. High level overexpression of glpR decreased mutation rate to background levels (Zhang and Saier, 2009a). Clearly, GlpR regulates the increased rate of IS5-mediated insertion activation of the glpFK promoter by glycerol.

**GlpR OPERATORS DIFFERENTIALLY CONTROL glpFK EXPRESSION AND Glp+ MUTATION RATE**

There are four GlpR binding sites, O1–O4, in the upstream glpFK operon regulatory region (see Figure 1A), identified by DNA footprinting (Freedberg and Lin, 1973; Zeng et al., 1996). We mutated the far upstream site (O1) and the far downstream site (O4) so they no longer could...
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bind GlpR, and compared the effects on glpFK expression using a lacZ reporter gene fusion construct vs. mutation rate to Glp+ during growth in LB medium. Mutation of O4 increased glpFK operon expression about 5-fold although mutation of O1 was almost without effect (Zhang and Saier, 2009a). By contrast, loss of O1 yielded a 7-fold increase in mutation rate although loss of O4 had only a 2-fold effect on mutation rate. We confirmed that IS5 was always in the same position and orientation (Zhang and Saier, 2009a). Thus, O1 primarily controls the rate of IS5 insertion into the activating site, while O4 primarily controls glpFK expression.

CONTROL OF IS5-MEDIATED glpFK OPERON ACTIVATION BY THE cAMP-CRP COMPLEX

As noted above, IS5-mediated activation of the glpFK promoter was observed in a crp genetic background. Initial attempts in our laboratory and elsewhere to isolate such mutants in a wild type genetic background proved unsuccessful (Ibarra et al., 2002; Honisch et al., 2004; Fong et al., 2005; Zhang and Saier, 2011, unpublished observations; Cheng et al., 2014). Since crp mutants are not found in nature, this brought into question the suggestion that our discovery of directed mutation in a crp mutant of E. coli were relevant to the wild type situation, and hence whether it had actually evolved under the pressures of natural selection.

We consequently undertook a systematic analysis of the cAMP dependency of IS5-mediated glpFK activation to understand why crp mutants but not wt cells gave rise to IS5-activated mutants. The cya gene, encoding the cAMP biosynthetic enzyme, adenylate cyclase (Cya), was deleted, and as expected, IS5-mediated glpFK activation was observed at high frequency. When sub-mM concentrations of cAMP were added to the growth medium, the frequency of these mutations was drastically reduced; 1 mM exogenous cAMP essentially prevented the appearance of these mutants. When glpR was deleted in

![Schematic diagram illustrating dual GlpR-mediated/cAMP-CRP-mediated control of (right) the level of glpFK transcription and (left) the rate of IS5 hopping into the activating site upstream of the glpFK promoter (directed mutation).](image-url)
the cya genetic background, the frequencies of IS5 insertion increased by about 20-fold (Zhang and Saier, unpublished observations).

These observations led us to experiment with wt E. coli cells. Glycerol utilization in these cells is strongly inhibited by the presence of the non-metabolizable glucose analogs, 2-deoxyglucose and α-methyl glucoside, which also lower cytoplasmic cAMP levels by inhibiting adenylate cyclase activity (Saier and Feucht, 1975; Castro et al., 1976; Saier et al., 1976; Feucht and Saier, 1980; Saier, 1989). Wild type cells were therefore plated on minimal salts medium containing 0.2% glycerol and 0.1% 2-deoxyglucose or 0.5% α-methyl glucoside. Not surprisingly, IS5 insertional directed mutants could be isolated under these conditions (Zhang and Saier, unpublished results). Abolition of the CRP binding sites in the glpFK upstream regulatory region greatly enhanced the frequency of these mutants in an otherwise wt genetic background, even in the absence of a non-metabolizable glucose analog, showing that binding of the CAMP-CRP complex to the glpFK control region negatively regulates IS5 insertion. These experiments showed that directed mutation is negatively regulated by binding of both the glycerol repressor, GlpR, and the CAMP-CRP complex to the glpFK control region. This explains why IS5 insertion into the activating site of the glpFK regulatory region in wild type cells depends on both high glycerol and low cyclic AMP concentrations.

**DIRECTED MUTATIONS PROMOTING EXPRESSION OF OTHER OPERONS IN E. COLI**

Following the reports of Zhang and Saier (2009a,b) cited above, Wang and Wood (2011) demonstrated directed mutation of the operon (flhDC) encoding the flagellar transcriptional master switch in E. coli, FlhDC. IS5 insertion into the upstream control region of the flhDC operon was responsible. Although the mechanism was not established, the frequency of IS5 insertion clearly increased under swarming conditions in soft agar compared to growth in liquid medium or on solid agar plates where swarming does not occur (Wang and Wood, 2011). We have confirmed and extended their results (Zhang et al., 2013). Moreover, preliminary results suggest that the E. coli fuc (fucose; propanediol) operon may also be subject to IS5-mediated directed mutation (Zhang and Saier, 2011; Zhang et al., 2013). It seems that transposon-mediated directed mutation will prove to be important to microbial evolution, and possibly to that in other organisms, partly accounting for the prevalence of these genetic elements in virtually all living organisms.

**CONCLUSIONS AND PERSPECTIVE**

Directed mutation has been defined as genetic change that is specifically induced by the stress conditions that the mutation relieves, but until recently, in no case had such a mechanism been established. We have demonstrated that mutations in the glpFK control region, allowing growth of E. coli crp or cya mutants on glycerol plates, or wt cells on glycerol plus 2-deoxyglucose or α-methyl glucoside plates, are specifically induced by the presence of these compounds. The glycerol regulon repressor, GlpR, which binds to its four operators (O1–O4) in front of the glpFK operon (Figure 1A) and is displaced from these sites when α-glycerol phosphate binds allosterically to GlpR (Zeng et al., 1996), not only controls gene expression, but also controls mutation rate. In this case, GlpR binding negatively influences both glpFK operon expression and operon activation by IS5. Our results established that binding of GlpR to O4, which overlaps the —10 promoter region, primarily controls gene expression, while binding to O1 primarily controls IS5 hopping into the specific CTAA site, between 127 and 122 base pairs upstream of the glpFK transcriptional start site, that activates the glpFK promoter. Binding of the CAMP-CRP complex to its two binding sites overlapping O2 and O3 also blocks IS5 hopping to the activating site. This dual mechanism may involve changes in DNA conformation or supercoiling. The results serve to dissociate two functions of both GlpR and CRP.

The mechanism of IS5-mediated glpFK promoter activation in wild type cells provides relief from starvation when glycerol is present and a CAMP-depressing toxic substance, such as 2-deoxyglucose, is simultaneously present. Such non-metabolizable sugar derivatives are synthesized by many organisms and therefore are present in nature. This example of directed mutation could therefore have been selected for during evolution. It appears to be a genuine example of directed mutation, with mutations arising at a greater rate under conditions that allow benefit to the organism. The fact that mutation rate is influenced by the presence of glycerol in a process mediated by the glycerol repressor, and by cAMP in a process mediated by CRP, provides mechanistic explanations for IS5-mediated directed mutational control. This mechanism, illustrated in Figure 2, allows rationalization of the presence of four GlpR binding sites and two CRP binding sites in the control region of the glpFK operon. Our studies also provide the rationale for the evolution of this elaborate mechanism of gene activation.

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