[Frontiers In Bioscience, Landmark, 22, 1458-1468, March 1, 2017]

Transposon-mediated directed mutation in bacteria and eukaryotes

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1. ABSTRACT

Transposon-mediated "directed" mutations occur at higher frequencies when beneficial than when detrimental and relieve the stress that causes them. The first and best-studied example involves regulation of Insertion Sequence-5 (IS5) insertion into a specific activating site upstream of the glycerol utilization operon in *Escherichia coli*, *glpFK*. This event promotes high level expression of the *glpFK* operon, allowing glycerol utilization in wild type cells under inhibitory conditions. The phosphoenolpyruvate-dependent, sugar transporting, phosphotransferase system (PTS) influences this process by regulating cytoplasmic glycerol-3-phosphate and cyclic AMP concentrations. Insertion frequencies are determined by IS5-specific tetranucleotide target sequences in stress-induced (DNA) duplex destabilization (SIDD) structures counteracted by two DNA binding proteins, GlpR and Crp which directly inhibit insertion, responding to cytoplasmic glycerol-3-phosphate and cyclic AMP, respectively. Expression of the *E. coli* master regulator of flagellar gene control, *flhDC*, is subject to activation by IS elements by a directed mechanism, and zinc-induced transposon-mediated zinc resistance has been demonstrated in *Cupriavidus metallidurans*. The use of DNA conformation and DNA binding proteins to control transposon hopping also occurs in eukaryotes.

2. INTRODUCTION

2.1. Darwin and Lamarck

Charles Darwin has been considered to be the greatest Biologist of all time because he proposed and provided evidence that all living organisms on Earth arose in an evolutionary process, accounting for their similarities, differences and relatedness (the Third Law of Biology) (1). However, long before Darwin, the French naturalist, Jean Baptiste Pierre Antoine de Monet Chevalier de la Marck (Lamarck) had proposed that living organisms evolved according to natural laws. He developed the first "theory of inheritance of acquired characteristics" which became known as "Lamarckism." As for Darwin, he did not know how this might occur, but he suggested the existence of a "complexifying force" driven by physiological need and the use (or disuse) of phenotypic characteristics (2, 3).

Figure 1. Caricature of Lamarck, showing his face replacing that of a giraffe. Lamarck considered that because the giraffe repeatedly tried to stretch its neck to reach the leaves of a tree, its neck, and those of its offspring, became permanently longer. Such inheritance, termed "Lamarckian Inheritance", implies that evolutionary change is directed by need. We and others consider recent finding in epigenetics and directed mutation to occur in a pseudo-Lamarckian way (DeLisi and Vaughn, 2015, Skinner, 2015, Wang and Wood, 2011). This Figure was reproduced with the permission of Chris Madden.

Thus, he thought a giraffe might acquire its long neck and an elephant its long nose because they stretched and used these organs for benefit. This proposal, was reasonable at the time, and perhaps at some level even today (4, 5). But people have ridiculed Lamarck for his ideas as illustrated in Figure 1. Today, however, there is a rebirth of molecular concepts more akin to the proposals of Lamarck (2, 4, 5) .

2.2. The proposal of directed mutation

In the late 1980s and early '90s, John Cairns, Barry Hall and others introduced the concept of directed mutation (6-8). They considered that organisms could respond to stresses by altering their genes purposefully. Thus, such mutations might occur with increased frequencies if they relieved the stress that caused them. Any population, repeatedly subjected to an environmental shift, might respond by increasing the probability of mutations that relieve a stress in response to the specific stressful environmental condition. Although reasonable and easily explainable by Darwinian natural selection, the demonstration of directed point mutations has not been convincing (9-11).

Recently, strong support for directed mutation has emerged, not for point mutations as independently proposed by Cairns, Hall and their collaborators, but for transposon-mediated mutations (12, 13). If accepted by the scientific community, this concept could advance (or revise) our perception of evolution, allowing increased rates of mutational change in times of need. But this concept goes against the current dogma that states that mutations occur randomly, and only the beneficial ones are selected for (14, 15). The concept of directed mutation, if established, would require the reversal of a long accepted precept.

2.3. Transposons

Transposons were discovered over 65 years ago by Barbara McClintock while studying pigment variegation in corn seed kernals (16, 17). They are "jumping genes", DNA elements that move independently of other genetic elements, to other locations in the host DNA (18). These "hopping" or transposition events give rise to mutations that can occur at high frequencies, relative to normal mutation rates. These jumping genes have been found in virtually all living organisms from bacteria to humans.

Bacterial Insertion Sequence (IS) elements are small transposons that encode the transposase that catalyzes the hopping event(19, 20). These elements are common in most prokaryotes, and other

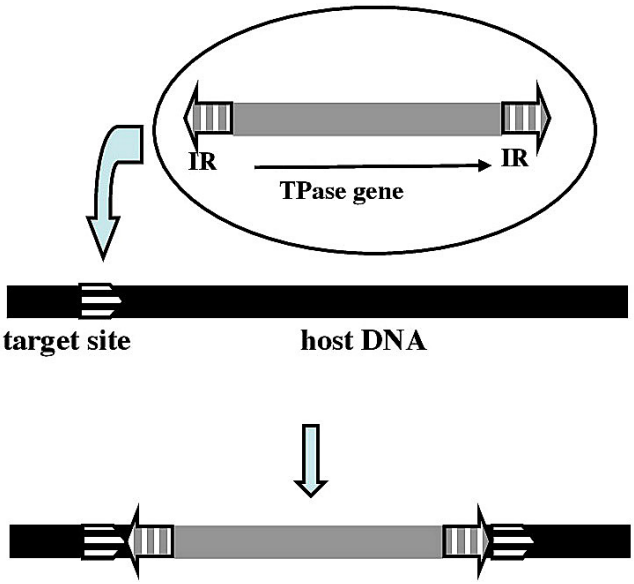

Figure 2. Depiction of a small transposon, a prokaryotic insertion sequence (IS) element. It identifies a target site in the chromosome (indicated by the horizontal white bars) and inserts into that site while duplicating the flanking target site. IR, inverted repeat, represented by the vertical bars; TPase, the transposase that catalyzes IS element transposition.

types of transposons, such as retrotransposons, are common in eukaryotes. Some of the bacterial and eukaryotic transposons and viruses may be related (21). Moreover, in mammals, over 30% of the total chromosomal DNA was derived through evolutionary time from retroviruses and (retro)transposons (18).

Transposons usually recognize specific target sites in the host chromosome (Figure 2). Hopping can inactivate a gene if the transposon hops into it, but can activate expression if it hops into the upstream regulatory region. Thus, they can be hugely detrimental, but eukaryotes have devised ways to prevent their destructive actions. The means that have been demonstrated involve DNA binding proteins. In eukaryotes, DNA binding proteins prevent detrimental transposon insertions into structural genes while promoting potentially beneficial insertions in regulatory regions upstream of structural genes (22). Interestingly, in bacteria, the same may be true. For example, loss of the nucleoid protein, H-NS, drastically reduces transposon hopping in E. coli (23). Moreover, in E. coli, DNA binding proteins, such as the nucleoid protein, IHF, and chromosomal structural elements, such as permanent DNA bends, can promote the gene activation process. Such is the case for IS5-mediated activation of the glpFK operon (13).

2.4. The glpFK Operon of *E. coli*

The glpFK operon is essential for aerobic growth of E. coli cells on glycerol as a sole carbon source (24). It encodes the glycerol facilitator (GlpF), which facilitates glycerol uptake from the medium, and glycerol kinase (GlpK), which phosphorylates cytoplasmic glycerol using ATP as the phosphoryl donor to give glycerol-3-phosphate, the inducer of the glp regulon (25, 26). The glpD gene encodes the aerobic glycerol-3-phosphate dehydrogenase (GlpD), completing glycerol-specific catabolism, and yielding dihydroxyacetone-phosphate that can feed directly into glycolysis to provide the cell with carbon and energy (27). Glycerol-3-phosphate is also a primary precursor of phospholipid biosynthesis. The glycerol repressor, GlpR, controls expression of both glpFK and glpD, but the cyclic AMP receptor protein, Crp, controls transcription only of the glpFK operon, not glpD (28, 29). Thus, the glpFK operon, but not glpD, is subject to cyclic AMP control.

The upstream transcriptional control region of the glpFK operon includes four binding sites (operators) for GlpR (O1-O4) and two binding sites for Crp, (Crp I and Crp II) (Figure 3) (12, 13). The -35 hexanucleotide component of the promoter overlaps O3 and Crp II, while the -10 hexanucleotide component overlaps O4. GlpR, without glycerol-3-phosphate bound to it, binds to O1-O4 to repress glpFK operon expression, while Crp, in the cyclic AMP-bound form, binds Crp I and Crp II to activate glpFK operon expression. Thus, the free form of GlpR negatively regulates (represses) glpFK expression, while Crp, with cyclic AMP bound, positively regulates (activates) expression (30, 31). The ligand-bound form of GlpR does not bind to its operators and therefore does not repress transcription. This mechanism is similar, in essence, to that of the lactose (lac) operon of E. coli in which a repressor, LacI, controls expression in response to cytoplasmic

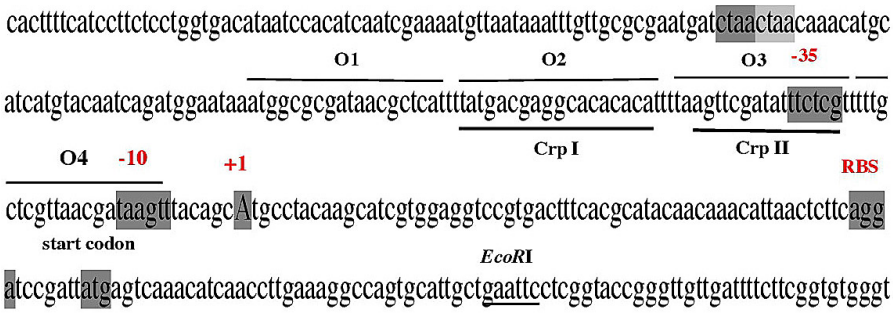

Figure 3. The *E. coli glpFK* promoter region showing (1) the ctaa insertion site (duplicated following IS5 insertion as shown), (2) the four adjacent GlpR operators (binding sites), O1-O4, (3) the two adjacent Crp binding sites, CrpI and CrpII, (4) the -35 and -10 hexanucleotide regions of the promoter, (5) the transcriptional start site (+1) and (6) the ribosome binding site (RBS, agga) for initiation of translation of the first structural gene, the *glpF* gene, within the *glpFK* operon. The start codon of the *glpF* gene (atg) and a downstream *EcoRI* restriction site are also shown. Modified from Zhang and Saier, 2009a with permission.

β-galactoside availability while Crp controls its expression in response to carbon availability (32).

Because of the dependency of glpFK operon expression on the cyclic AMP-Crp complex, mutants lacking Crp or Cya (adenylate cyclase) cannot utilize glycerol. However, the wild-type strain has Crp and can make cyclic AMP, and can therefore grow on glycerol. It cannot grow in the presence of a nonmetabolizable glucose analogue such as 2-deoxyglucose (2DG) or α-methylglucoside (αMG) which inhibits glycerol kinase and adenylate cyclase activities (33, 34). The mutational event involving IS5 activation of glpFK expression, described below, allows the cell to overcome the inhibitory effect of a non-metabolizable sugar analogue, enabling glycerol utilization in its presence (35).

2.5. Evidence for IS5-mediated mutations activating the glpFK Operon

When crp or cya mutant cells were plated on minimal glycerol agar plates on which they could not grow, or when wild type cells were plated on the same plates containing the growth inhibitor, 2DG or αMG, IS5 insertion mutations arose after a lag period during which the cells were responding to starvation stress (12, 13, 35). Starvation in the presence of glycerol proved to activate the hopping of IS5 to the glpFK activating site, and insertion of this transposon relieved this stress by allowing rapid glycerol utilization. Activation proved to be specific for this one insertional site upstream of the glpFK promoter (12).

By eliminating the glpR gene, encoding the glycerol repressor, it was demonstrated that this non-liganded transcription factor bound to the DNA in the absence of glycerol-3-phosphate and inhibited the activational insertion of IS5 when glycerol was not present in the medium. However, because the presence of glycerol in the medium of wild type cells allowed the generation of the inducer, cytoplasmic glycerol-3-phosphate, an increase in IS5-mediated mutation rate, mediated by the dissociation of GlpR from the DNA, was observed. In other words, the bound GlpR protein greatly depressed the IS5-mediated glp^+mutation rate when glycerol was absent, but not when it was present. Thus, GlpR mediates the activating effect of glycerol on the insertion of IS5 into the glpFK-activating site (12). This raises the question of whether this is an example of an evolved mechanism that can sense a type of stress and promote mutational events tailored to relieve that specific stress.

2.6. GlpR regulates transcription and IS5-mediated mutation independently

Cytoplasmic GlpR, without glycerol-3-phosphate bound, represses transcription of the glpFK operon and blocks IS5 insertion into the glpFK activating site. It was possible that one of these two events was a consequence of the other, so this possibility needed to be tested. As noted above, GlpR binds to four operators, O1-O4, overlapping the glpFK promoter, but it was important to know which of these binding sites was/were most important for the two regulatory events, induction of transcription and regulation of IS5 insertion into the activating site. When O1 and O4 (Figure 3) were separately mutated so each one could not bind GlpR, O4, but not O1, proved to strongly control transcription while O1 but not O4 strongly controlled IS5-mediated insertion (12). It was therefore apparent that the IS5-insertion rate was controlled by GlpR independently of glpFK expression. Consequently, one is not the result of the other. They are independently controlled by the same DNA binding protein.

2.7. Crp regulates is5-mediated activation of the glpFK Operon

IS5 insertion into the activating site, upstream of the glpFK promoter, proved to be suppressed when the cyclic AMP-Crp complex bound to the two Crp

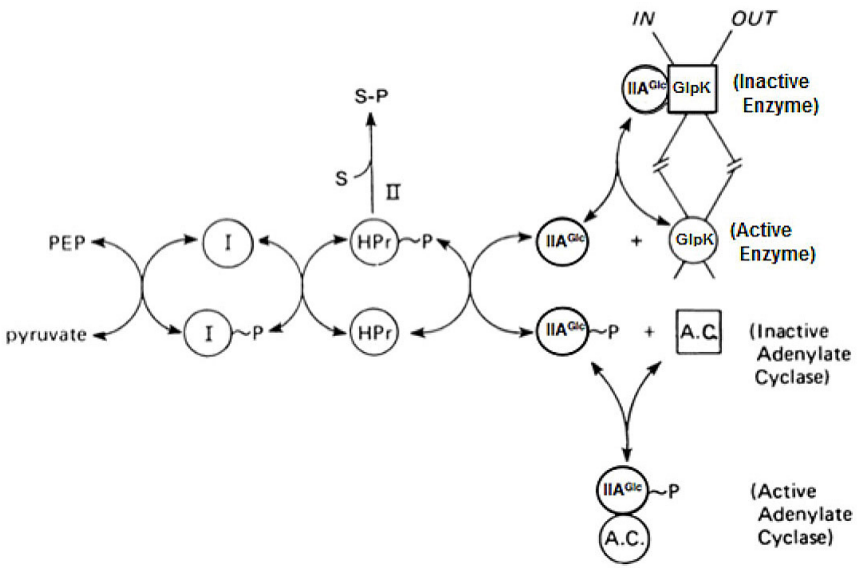

Figure 4. Proposed mechanism for the regulation of glycerol kinase (GlpK) and adenylate cyclase (A.C.) by the phosphoenolpyruvate (PEP)-dependent sugar phosphorylating phosphotransferase system (PTS) in *E. coli*. Enzyme IIA^{Glc} (IIA^{Glc}) is the central regulatory protein that is reversably phosphorylated by the two general energy-coupling proteins of the PTS, Enzyme I (I) and HPr, which are sequentially phosphorylated using PEP as the phosphoryl donor. IIA^{Glc} interacts directly with target enzymes, GlpK and A.C. Because all of the phospho-proteins of the PTS are high energy, their phosphorylation is reversible. Only the free form of IIA^{Glc} inhibits GlpK, and only the phosphorylated form of IIA^{Glc} activates A.C. II is a sugar transporting enzyme II specific for a particular sugar (S). *Modified from Saier, 1989, Microbiol. Rev. 53:109-120 with permission.*

binding sites in the glpFK control region, CrpI and CrpII (Figure 3). Since wild type cells have Crp that can prevent IS5 insertion, is this mutational mechanism relevant to wild type E. coli? Possibly it evolved to allow glycerol utilization when growth is inhibited by the presence of a bacteriostatic compound such 2-deoxyglucose. This glucose analogue, a substrate of the PTS (see below), is known to inhibit both adenylate cyclase and glycerol kinase activities (see Figure 4) (33, 36, 37). This could be important since non-metabolizable sugar analogues are common in nature (36, 38-42). Moreover, the process of IS5 hopping is fully reversible (43), leading to the possibility that this process could have evolved by natural selection. Maybe the upstream GlpR binding site (O1) and the tetranucleotide IS5 target site, upstream of the glpFK promoter, evolved to allow glycerol utilization under adverse conditions such as those used in the described experiments (35).

2.8. The E. coli phosphotransferase system (PTS)

The phosphoenolpyruvate-dependent sugar-transporting phosphotransferase system (PTS), which couples transport of sugars to their phosphorylation, regulates numerous physiological processes (34). One of these in E. coli is known as “inducer exclusion”, and another is referred to as “catabolite repression”. Early genetic and physiological evidence supported a mechanism whereby the non-phosphorylated form of a PTS protein, the enzyme IIA specific for glucose (IIA^{Glc}), allosterically inhibits the activities of a number of permeases and catabolic enzymes, one of which is glycerol kinase, giving rise to inducer exclusion (Figure 4, top) (33, 44). Extensive biochemical evidence as well as high resolution protein structural data now supports this model. The PTS also mediates regulation of cyclic AMP synthesis by adenylate cyclase, giving rise to catabolite repression (Figure 4, bottom) (45). Allosteric activation of adenylate cyclase by phospho-IIA^{Glc} (IIA^{Glc}~P) occurs in the absence of a PTS sugar substrate (Figure 4). Since PTS-catalyzed IIA^{Glc} phosphorylation controls both glycerol kinase and adenylate cyclase, and since GlpR and the cyclic AMP-Crp complex control IS5 insertion into the glpFK activating site, it follows that this unified mechanism regulates transposon-mediated activation of the glpFK operon. Thus, the PTS can influence mutation rate; it serves as a sensor of extracellular carbon availability.

2.9. Evolutionary significance

If the phenomena summarized above arose by natural selection, then the IS5 insertional mutants should eventually take over a bacterial culture under selective conditions. This proved to be the case (Figure 5). As noted above, binding of either GlpR or the activated Crp complex to the glpFK control region inhibits IS5 insertion into the upstream site, although GlpR represses while Crp activates transcription (Figure 6 and Table 1). Since the IIA^{Glc} protein of the PTS directly regulates glycerol kinase (GlpK), which makes glycerol-3-phosphate, and adenylate cyclase (Cya), which makes cyclic AMP (Figure 4), it seems likely that

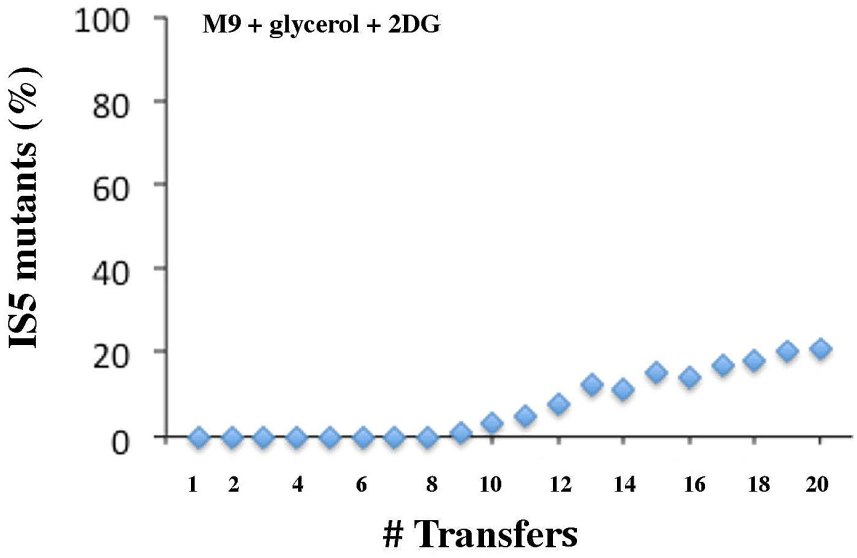

Figure 5. Slow accumulation of IS5 insertional mutants over time when wild type bacteria are incubated in minimal medium M9 in the presence of glycerol (0.5.%) and 2-deoxyglucose (2DG; 0.1.%). A single transfer is equivalent to about 8 generations. IS5 mutants are expressed as percent of the total population.

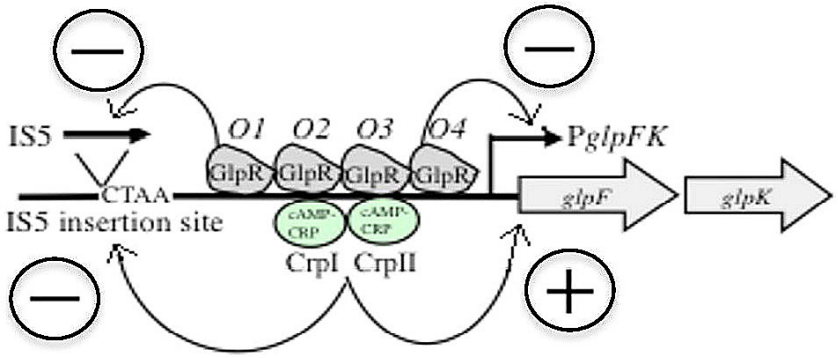

Figure 6. Schematic depiction of the proposed regulation of *glpFK* operon transcription (indicated by the arrow in front of the *glpF* gene) and operon activation by IS5 insertion, upstream of the promoter, by GlpR and the cyclic AMP (cAMP)-Crp complex when they bind to their binding sites, O1-O4 for GlpR, and CrpI and CrpII for Crp. The possible outcomes, depending on conditions, are presented in Table 1. The "+" means activation and the "–"means inhibition or repression.

the PTS indirectly controls IS5 insertion into the glpFK upstream activating site as depicted schematically in Figures 4 and 6. Possibly, transposon-mediated directed mutation of the glpFK operon evolved initially by the INTRODUCTION into the upstream control region of an IS5 tetranucleotide target site as well as the O1 GlpR operator. Such events would increase the probability of a beneficial mutation under conditions of inhibited glycerol utilization (35). Since such mutations are of obvious benefit to the organism, it is reasonable to suggest that this mechanism evolved via natural selection.

An important condition for the evolution of this proposed mechanism is that the bacteria must have repeatedly faced a situation in the past in which glycerol utilization was blocked by the presence of non-metabolizable sugar analogues. This is reasonable since both glycerol and sugar analogues are commonly found in nature (36, 38-42). This mechanism, promoting phenotypic plasticity, allows an E. coli population to make this switch as soon as the environment changes for the worst, creating starvation conditions.

2.10. Hotspots for IS insertion?

It is known that Insertion Sequence elements insert into intergenic regions of bacterial chromosomes at higher frequencies than in intragenic

Table 1. Effects of exogenous glycerol and cytoplasmic cyclic AMP on glpFK transcription and activating IS5 insertion

Glycerol	cyclic AMP	Transcription	IS5 Insertion
-	-	-	±
-	+	±	-
+	-	-	+
+	+	+	-

The results revealed that while both GlpR and the cyclic AMP-Crp complex both negatively influence IS5 hopping to the glpFK activating site, the cyclic AMP-Crp complex plays a quantitatively more important role

regions, even though coding regions cover about 10-fold more of the chromosome than non-coding regions (46, 47). Moreover, while all IS elements have short target sites, some of these are used frequently while others are never used. This is true even though the used and non-used sites are identical in sequence. Recently, the molecular explanation has come to light (M.Z. Humayun, Z. Zhang and M. H. Saier, manuscript in preparation). It was found that "hot spots" for IS insertion in E. coli correspond to regions of supercoiling stress-induced DNA duplex destabilization (SIDDs) which occur much more frequently in intergenic regions than in intragenic regions in bacteria. Moreover, by destabilizing a SIDD, so it opens up, yielding single stranded DNA with increased facility, the frequency of insertion increases. The reciprocal also proved to be true (Humayun *et al.,* unpublished results). It was therefore clear that DNA secondary structures such as SIDDs can influence the ease of IS element insertion.

2.11. Directed mutation of the operon encoding the flagellar master switch, flhDC

In 2011, Wang and Wood reported apparent directed mutation by IS5 of the operon encoding the flagellar master switch of E. coli, flhDC (48). These mutations arise only when environmental conditions allow swarming, or when cells form communal biofilms. We have confirmed their results, showing that when cells are grown in liquid medium or on solid agar (1.5.%) plates, IS insertional mutations do not arise at an appreciable rate. By contrast, in semi-solid agar (0.2. - 0.3.%) media, the swarming cells, in the near-stationary growth phase, inserted IS5 as well as IS1 and IS3 in positions upstream of the promoter that activated expression of the flhDC operon. These mutations arise at rates >100-fold above the background rate (C. Kukita, Z. Zhang and M.H. Saier, 2016, Micrbiology, in press). Moreover, preliminary evidence suggests that one of the two fucose (fuc) catabolic operons of E. coli, fucAO, is subject to IS5-mediated mutation under well defined stress conditions (43 and unpublished results). We suggest that this phenomenon may be much more prevalent than currently recognized. For other potential examples, see (49-52).

2.12. Zinc-induced Zinc Resistance in Cupriavidus metallidurans

Recently, a report of potential directed mutation in the β-proteobacterium, Cupriavidus (formerly Ralsonia) metallidurans, has appeared (53). These researchers discovered that zinc induces transposition of several different IS elements (as for flhDC operon activation in E. coli), and the insertion events give rise to zinc resistance. Thus, the agent of induction, toxic levels of zinc, adaptively overcomes the heavy metal toxicity that promoted IS insertion. Interestingly, in this case, some of the transposon insertions affect a regulatory locus, cnrYX, that causes derepression of a gene encoding an RNA polymerase sigma factor, CnrH, which enhances expression of the cnrCBA operon that encodes a heavy metal RND-type efflux pump (TC# 2.A.6.1.1.; 54). In this case, transposase expression appeared to be zinc-responsive, leading to the possibility that this mechanism of zinc resistance was not truly "directed" because expression of other loci might also be promoted. However, in a cnrH deletion mutant, zinc-induced transposon-mediated adaptation still occurred in a process dependent on outwardly directed IS element promoters, driving cnrCBAT transcription. It was also reported that IS reshuffling enhanced adaptation to subsequent environmental challenges. Evidently, IS elements can be induced to hop by various stresses to promote adaptation to the very stresses that cause appearance of the adaptive mutations. The observations reported, together with previously published work, indicate that multiple mechanisms of transposon-mediated adaptive and directed mutation are likely to emerge.

2.13. Transposon-mediated mutation in eukaryotes?

If transposon-mediated mutations are of benefit to E. coli, possibly they are similarily important in the evolution of other bacteria, archaea and eukaryotes. Recent research suggests that this is the case. For example, Jacobs *et al* (2015) have reported that DNA binding proteins can guide retrotransposons to preferred target sites. The locations of targeting and the efficiency of the translocation events are determined by the DNA-binding activities of the guiding proteins (22).

Additionally, Elbarbary *et al.* have reviewed the evidence that retrotransposons can function as regulators of gene expression by several different mechanisms (55).

The consequential and mechanistic similarities of the transposon-mediated regulation in eukaryotes to the regulation of IS5-mediated activation of E. coli operons are worthy of note. As Jacques Monod once said, "What is true for E. coli is also true for the elephant." Or more generally and precisely "Everything comes from experience accumulated by the entire ancestry of the species in the course of its evolution." And after all, bacteria were the evolutionary precursors of the eukaryotic cell!

2.14. A parallel between uninformed evolution and cognitive learning?: Back to Lamarck

In a paper entitled "How Can Evolution Learn?", Richard Watson and Eors Szathmary interrelate "uninformed" evolutionary theory with cognitive learning theory, and suggest that the principles may be the same, particularly the evolution of evolvability (56). In fact, they noted that the capacity for the evolutionary process to "learn" can explain apparent intelligent designs. After all, the genetic basis for any biological process including learning must have evolved according to Darwinian principles, involving mutation, adaptation and selection. Facilitation of adaptation must follow a course analogous to that by which a learning system can exploit knowledge obtained from past experiences. Thus, evolution progresses from past selection as do learning systems when they acquire knowledge based on past experiences. Both anticipate actions that will confer future benefits, even though neither can actually "see" the future; they generalize and extrapolate to the future based on the past. In fact, learning theory involves the study of processes that change over time in accordance with past experience, just as does evolution (56).

Interestingly, consideration of the concept of transposon-mediated directed mutation allows a synthesis of ideas attributed to Lamarck and Darwin. Thus, the proposed molecular mechanisms are consistent with the phenotypic Lamarckian postulates, but they could have evolved as the result of straightforward Darwinian selection. Others have suggested that recent advances in epigenetic molecular mechanisms similarily warrant a reconsideration of Lamarckism (2, 4, 5). Let the past be the guide to the future, in evolution as in all of life.

3. ACKNOWLEDGEMENTS

The authors declare no conflict of interest. Work from the Saier Laboratory included in this review was supported by NIH grants GM077402 and GM109895. Some Figures were modified from our previous publications with permission. We thank Sabrina Phan for assistance with manuscript preparation.

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Key Words: Adaptive Mutation, Directed Mutation, Transposons, Insertion Sequence Elements, DNA Binding Proteins, Evolution, Review

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