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The Directed Mutation Controversy in an Evolutionary Context

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ABSTRACT: Neo-Darwinists have long held that random mutations produce genetic differences among individuals, and selection increases the frequency of advantageous alleles.[1] In 1988, Cairns *et al.* claimed that an environmental pressure can cause advantageous mutations to occur in specific genes to alleviate that particular pressure. Directed mutation, as proposed by Cairns, has been all but eradicated from evolutionary thinking. However, more than a decade of research spurred by the Cairns *et al*. paper has cast doubt on three neo-Darwinian principles: (1) mutations occur independently of the environment, (2) mutations are due to replication errors, and (3) mutation rates are constant. This mini-review explores the history of the controversy and the decade of research that followed so as to place it in an evolutionary context. Several of the cellular mechanisms and models that explain the increased genetic diversity in populations experiencing adverse environmental pressure are described. In most cases it is clear that the increased genetic diversity is due to breakdowns of cellular machinery or alleles evolved for a purpose other than increasing genetic diversity, rather than to cellular systems that have been evolutionarily selected to increase the genetic diversity in times of stress.

I. INTRODUCTION

An organism's ability to change into a superorganism when advantageous has been the subject of comic strips and science fiction for centuries. Like in George Lucas' *Star Wars*, the ideas of science fiction have spilled over into science proper. In 1982, Walter Fitch commented " . . . the organism might be better off if it could vary its mutation rate upward in stressful times and downward in favorable times . . . If the organism needs to change only a few of its genes, one would prefer to increase the mutation rate in those genes specifically".[2] Although an intriguing idea, there was no evidence that organisms can direct their evolution.

A. Neo-Darwinism

Neo-Darwinism has held that mutations occur at random,[1] and the novel allele is subsequently selected for or against.[3,4] That is, heritable mutations are independent of the environment, occurring only during replication and at a constant rate. This replication-dependent mutation rate is determined by replication fidelity, which is governed by the inherent imperfection of cellular machinery and the amount of energy allocated to repair. Mutation rates were assumed to be constant per generation because organisms were thought to have evolved to allow the lowest number of mutations per unit energy allocated to repair.[5]

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B. The Cairnsian Revolution

In 1988, John Cairns and his collaborators published the controversial paper: *The Origin of Mutants*,[6] arguing that bacteria may be able to sense environmental pressures and subsequently mutate specific genes to relieve the selective disadvantage. The predicted mechanism involves an environmental cue to cause the gene under pressure to produce a highly variable set of mRNA, the most advantageous of which is reverse transcribed and recombined into the genome. Cairns believed that the mutations he observed were not replication dependent because the cells they occurred in were in stationary phase. Cairns' refutations of the three previously described neo-Darwinian postulates, (1) nondirected mutations, (2) replication-dependent errors, and (3) constant mutation rate, hereafter will be referred to as Cairnsian for simplicity in contrasting with neo-Darwinism.

II. DIRECTED MUTATION

Results from Luria and Delbrück's fluctuation test established that mutations were not directed to cope with selection pressures in bacteria.[7] In the fluctuation test, an *Escherichia coli* strain with a mutation in the bacteriophage T1-resistance allele is plated in the presence of bacteriophage T1 to distinguish mutations arising as a response to a selection pressure from those that arise randomly and are then selected. If mutations arose as a response to the selection pressure, each independent replicate would harbor a small number of mutant colonies, approximating a Poisson distribution (variance equal to the mean). According to this hypothesis, the number of mutant colonies is the product of the mutation rate during selection and the density of cells plated. If the mutations arose randomly prior to exposure to selection, some cultures experience jackpots, causing a high variance to mean ratio. A jackpot occurs when a mutation that is preadapted for the selective conditions occurs early in a lineage while growing in permissive conditions. Luria and Delbrück found a high variance to mean ratio across cultures, indicating jackpots, and supporting an important tenet of neo-Darwinism, that mutations are independent of the environment.

Cairns believed that the fluctuation test was poorly designed for finding directed mutations because of the lethal pressure on the gene under selection. Lethal selection pressures such as bacteriophage necessitate the prior evolution of resistance; bacteria without a preexisting resistance gene will die on exposure to the selection pressure. In addition, phage resistance mutations are atypical in that they are not expressed for several generations, again requiring a resistance allele upon exposure to selection.[6]

Cairns *et al.* used a *lacZ* amber mutation when they repeated the fluctuation test to rectify these mistakes. When *E. coli* was plated on lactose minimal media plates, a jackpot distribution occurred in the first 2 days, as in the Luria and Delbrück fluctuation test. After day 2, a Poisson distribution of colonies formed.[6] The reversion mutations in these colonies occurred while in stationary phase. There was assumed to be no DNA replication in bacteria in stationary phase, leading Cairns *et al.* to postulate that these mutations occurred by mechanisms other than replication error and were directed to allow the use of lactose as an energy source.

A. Neo-Darwinists Take Aim

Supporters of neo-Darwinism found three flaws in *The Origin of Mutants*: the use of the F' sex plasmid, the lack of an appropriate control, and the failure to validate assump-

tions. In many cases, general evolutionary conclusions should not be made from F' data because genes on plasmids, especially sex plasmids, are peculiar compared with chromosomal genes. For instance, *lacZ* amber mutations on the chromosome do not revert on lactose minimal plates,[8] where the same mutation on the F' plasmid readily reverts.[9] In addition, RecA and RecG, proteins involved in recombination, are not needed for reversion of the *lacI33* allele when it is on the chromosome, but are essential when it is on a plasmid.[8] Cairnsians imagined that similar mechanisms may be used for stationary phase mutation in both plasmid- and chromosomally borne alleles, supporting the general applicability of the plasmid system.[10,11] For example, *recD* and *recG* mutants have elevated mutation rates at unselected loci on the chromosome as well as on the F' plasmid in Lac^+ revertant strains.[12] Because of the atypical behavior of plasmids and plasmid-borne genes, most evolutionary biologists now support the idea that projects using genes on sex plasmids reflects more about plasmid biology than adaptive mutagenesis.[13]

Another criticism was the use of the chromosomally located *val*^{R} allele as the negative control. Lac^+ mutant colonies did not also accumulate Val^R mutations when starved for lactose, prompting the conclusion that mutations were directed specifically to the allele under selection.[6] The control used had two errors: the *val* allele is chromosomally located and valine resistance is assumed to be neutral in the absence of valine.[14] If Val^R mutations are deleterious, strains with the mutation would have lower fitness and a decreased population size. Lac^+ revertants would be less likely to occur in the Val^R subpopulations because there are fewer cells. In addition, the Cairns team only screened the Lac^+ revertant colonies for the Val^R mutations. In a random mutation model, it is unlikely that there will be multiple mutations in the same clone. Had Lac^- strains been screened, Val^R may have been more prevalent even without the selection pressure of valine.

Cairns *et al.* assumed that Lac^+ revertants and Lac^- cells had equal fitness when grown in minimal media without lactose. Cairns found that when lactose was added to the minimal media 1 to 3 days post-plating, colonies formed 1 to 3 days later than those plated directly onto lactose minimal media plates. The density of colonies were not elevated after the addition of lactose, as would be expected if revertants were spontaneously accumulating in the absence of lactose. However, Lac^+ mutants may be at a selective disadvantage when no lactose is present,[15,16] reducing the number of their progeny, and thus reducing jackpots.[16] Cairns found significantly fewer viable bacteria on the plates when lactose was added 3 days after plating than he found in cultures plated directly onto lactose containing plates, indicating significant death on the former plates.[14,17] Death of revertants prior to the addition of lactose leads to a negative correlation between time spent on minimal media and the number of colonies formed when lactose is added.[14] This is, in fact, what the data show, contrary to the interpretation by Cairns and his colleagues. The Lac^+ revertants are not selectively neutral on minimal media without lactose, biasing the results toward a Poisson distribution and an incorrect directed mutation conclusion.

III. THE CURRENT DEBATE

Although Cairns retracted the reverse information flow hypothesis,[18] his work stimulated other scientists to question three neo-Darwinian principles: (1) mutations occur independently of the environment, (2) mutations are due solely to replication errors, and (3) mutation rates are constant. The most parsimonious explanation of the

data accumulated that was stimulated by *The Origin of Mutants* is that mutation rates can depend on the environmental conditions, mutations do occur in the absence of division and mutation rates are not constant. The question that remains is have bacteria evolved to manipulate mutation rates in specific circumstances or are these mutations simply the consequence of aberrant cellular activities that are a response to stress?

IV. QUESTIONS OF INTEREST

A. Is There A Better Method?

The temporal experimental design used in most directed mutation studies assumes that revertants appearing after 2 days mutated while in the stationary phase. This assumption could be invalid if bacteria with different mutations have different growth rates,[15,19] or if transcription of mutant alleles is delayed.[6] An alternative method using conditional lethal alleles to spatially distinguish stationary phase mutations from mutations arising during growth avoids these problems. In the spatial design, revertants at a particular locus die under restrictive conditions, while nonrevertants continue to grow logarithmically. The bacteria experience the selective environment when the culture is moved to permissive conditions. For example, into a kanamycin-resistant strain of *E. coli,* a temperature-sensitive *kan*^{R} element was spliced into the *lacZ* allele, allowing the bacteria to be kanamycin-resistant at 42°C (the restrictive condition), but leaving the cell unable to utilize lactose.[20] Lac$^+$ revertants are kanamycin sensitive at high temperatures because the temperature-sensitive *kan*^{R} element needs to be excised to produce active LacZ. Lac$^+$ revertants expressing functional LacZ die when plated at a restrictive 42°C on lactose minimal media (the selective environment) with kanamycin. Lac$^-$ cells remain viable at 42°C on lactose minimal media, but enter stationary phase due to the lack of a usable energy source. Moving these cultures to 37°C, the tolerant condition, allows the growth of Lac$^+$ revertants in the presence of kanamycin. This method assures that all revertant colonies incurred mutations post-plating on the lactose minimal media.[20] This protocol makes fewer assumptions and is a more powerful method than the temporal design and should be employed in future studies when applicable.

B. How Much DNA Synthesis Occurs in Stationary Phase?

Given that stationary phase mutations occur suggests either DNA synthesis in static cells or a nonreplication-dependent mutation generation mechanism. Early reports indicated that *E. coli*, which divide approximately every half hour when growing logarithmically, replicate between 0.005 to 0.05 genomes per day in stationary phase.[21] More advanced methods increased that estimate to nearly 0.25 genomes,[22] but contend that this too is likely an underestimate.[23] At this rate of DNA synthesis, 10% of the stationary phase mutations can be accounted for by a replication-dependent mutation model.[24] The occurrence of DNA replication in static cells allows for the possibility that mutations are replication dependent, as claimed in neo-Darwinism. However, mutation rates should not be measured per generation, as not all replication leads to reproduction.

V. HOW ARE MUTATIONS GENERATED IN STATIONARY PHASE?

Prior to *The Origin of Mutants,* supporters of neo-Darwinism had accepted that ex-

ternal agents such as UV light, cigarette smoke, and car exhaust can cause DNA damage. The damage was considered transient because repair machinery would replace the damaged bases, thus leading to very few heritable mutations in natural environments. However, in stationary phase cells, DNA damage-causing agents, such as alkylating and oxidative molecules, are generated more rapidly and remain at higher concentrations than in growing cells, increasing the mutation rate. These agents alter the chemical structure of the nucleotides, allowing mispairing in the double-stranded complex. Oxidatively damaged guanine preferentially pairs with adenine,[25] causing 100 times more G to T transversions in static than growing cells.[24] Therefore, nonreplication-dependent mutations may have an evolutionarily significant influence on the mutation rate in stationary phase cells, as suggested by Cairnsians.

Repair polymerases III[26,27] and IV,[28] which are implicated in most of the DNA synthesis in static cells, are inherently error prone. Polymerase III is constitutively expressed, but polymerase IV is only expressed when DNA damage is extensive. Polymerase IV accounts for up to 50% of reversion mutants in the lac system in wild-type polymerase cells,[28] and increases the mutation rate 800-fold when overexpressed.[29] Repair polymerases generate mutations due to the evolved ability to recognize damaged bases, leading to decreased fidelity in damaged areas.[30] Repair polymerases cause replication-dependent mutations, supporting neo-Darwinism, but at an elevated rate in times of severe damage, endorsing the Cairnsians.

A. Hypermutable Subpopulations

The Hypermutable Subpopulation hypothesis states that small subsets of the cells in stationary phase have an increased genome-wide mutation rate.[31] Strains that randomly acquire a beneficial mutation subsequently exit the hypermutable state and resume growth, while other hypermutable cells suffer deleterious mutations and die. This hypothesis was welcomed by most neo-Darwinists because it reconciled the directed mutation data of Cairns *et al.* with neo-Darwinism. It was later reported that only 10% of all stationary phase mutations arise in a hypermutable subpopulation,[24] leaving 90% to be accounted for by other mechanisms. However, Lac^+ revertants had an increased mutation rate at several other loci as well, indicating they may be derived from hypermutable cells.[10] Lac^+ revertants bearing second unselected mutations increased in late-arising colonies, suggesting that cells can accumulate incorrect mutations without imminent death.[24] These unselected mutants are 10 to 100 times more prevalent in revertant colonies than in nonrevertant or unstressed controls.[10] Coincidentally, the rate at which unselected mutations arose was found to be equal to the advantageous mutation rate.[10] The subpopulations mutation rate, combined with the estimate of replication in stationary phase cells,[23] accounts for all of the revertant colonies in Cairns' study by a random mutation model.

Hypermutable subpopulations can arise either from a transient mutator phenotype or a heritable element. Transient mutator phenotypes, which are the more prevalent of the two, can develop due to a down-regulation of the error correction pathway or an up-regulation of error producers. Although mutations are created at random, the emergent properties of the transient hypermutable subpopulations may be maintained by selection. If the emergent properties are advantageous, the ability of cells to be transiently hypermutable may be a system that has evolved for the purpose of creating genetic variability during times of stress.

Heritable mutator phenotypes do not account for many of the cases of stationary phase mutation, although there is evidence that high mutation rates can be selected for.[24,32] One-quarter of the 12 *E. coli* populations evolved on glucose-limited media for 10,000 generations showed an increase in mutation rates several orders of magnitude above the ancestral population.[33] The ancestral mutation rate was restored in all of the derived populations when wild-type methyl-directed mismatch repair (MMR) alleles were transformed into cells of the derived population. Mutators are selected for in rapidly changing environments because novel alleles are often the factor limiting evolution.[34] In static environments, mutators are probably deleterious, due to the deleterious mutations they create, but the mutator genes can fix in a population if they hitchhike along with an advantageous allele.[33]

B. What Is The Role Of Mismatch Repair In Stationary Phase Mutation?

Methyl-directed mismatch repair proteins inefficiently correct mismatches in stationary phase cells, allowing mutations to accumulate and become fixed. Of the three MMR proteins investigated in static cells, there is a 10-fold decrease in MutS concentration and a 3-fold drop in MutH, while MutL remains at a level equivalent to growing cells.[35] Surprisingly, neither MutS nor MutH affect the mutation rate when experimentally depressed or overexpressed. However, concomitant defects in MutS and MutL increase the reversion rate in some systems 100-fold, indicating a synergistic effect.[13] Conversely, MutL overexpression depresses the stationary phase mutation rate, but not the growth-dependent mutation rate.[35,36] It was proposed that MutL might be transiently depressed during stationary phase,[10] or is not functional under the physiological conditions of stationary phase cells.[36,37] Decreases in the concentration or function of MMR during stationary phase appear to be a result of a mechanistic breakdown, not a directed or evolved process designed to operate during times of stress. A fluctuating mutation rate caused by the environment contradicts a tenet held by neo-Darwinism. Evolutionary models need to incorporate this fluctuation in order to gain a clear picture of microbial evolution.

C. Are Stationary Phase Mutations Different?

Neo-Darwinists claim that stationary phase mutation can be explained by conventional replication-dependent mutation and subsequent selection, while Cairnsians claim that there is "some process" predominating in the stationary phase that facilitates the generation and/or fixation of adaptive mutations.[38] A project attempting to demonstrate the inherent difference of mutational mechanisms in stationary- and growth-phase involves the recombination pathway.[4,39] A mutation in the *recABCD* pathway dramatically reduces the stationary-phase mutation rate, but not the mutation rate in growing cells, indicating that recombination is necessary for stationary phase mutations but not mutations occurring in growing cells.[4,39] In the F' plasmid system, nearly all of the adaptive reversions are –1 frameshift deletions in a four-cytosine mononucleotide repeat region, while mutations conferring a Lac^+ phenotype in growing cells are heterologous (*e.g.* -1, +2, -5). However, the –1 frameshift mutations in stationary phase cells are characteristic of DNA polymerase errors that accumulate in the absence of post-synthesis mismatch repair,[10] denoting a breakdown of MMR machinery. The decrease in the stationary phase mutations observed when the

recombination pathway is mutated may be due to a peculiarity of the plasmid used in these experiments.

Both the stationary-phase and growth-dependent spontaneous mutation spectra are dominated by insertion elements.[40] Experiments in *Saccharomyces cerevisiae* support neo-Darwinism, demonstrating identical single base deletions in monotonic runs in stationary phase and in growing cells. These reversion events seemed to cluster near the 3' end, similar to those found in growing cells with deficient proofreading machinery.[41] There has yet to be conclusive evidence that mutational events in stationary phase occur by mechanisms that are absent in growing cells. If mutational mechanisms were found to operate solely in the stationary phase, it may suggest that these mechanisms had evolved to create genetic diversity when it is needed. The lack of such findings signifies that the increased mutation rates in stationary phase are the result of a breakdown of normal cellular processes.

VI. MUTATIONAL MECHANISMS IN STATIONARY PHASE CELLS

Many scientists modeled stationary phase mutation mechanisms, increased mutation rates in static cells, and the seeming directionality of these mutations. Although these mechanisms are modeled for stationary phase mutations, all require some DNA synthesis, suggesting all may be active in logarithmically growing cells as well. The models are a combination of classic neo-Darwinian thinking and revolutionary Cairnsian concepts.

A. Recombination

The recombination-dependent mutation model requires the RecABCD pathway and an inherent predisposition to form double-stranded breaks, as found on plasmids.[4] Double-stranded breaks can be produced when a replication fork crosses a single-stranded nick, causing a replication fork collapse.[28] When a double-stranded break is formed, the exonuclease RecBCD complex loads onto the DNA, degrading the ends until RecD falls off. RecBC continues as a helicase, denaturing the DNA that is subsequently lined with RecA, catalyzing strand exchange.[4] The 3' end invades the double-stranded complex of the other fork, resuming replication and creating a four-stranded intermediate (Holliday junction). Resolution of the Holliday junction by RecG moves the junction away from the fork, keeping newly synthesized DNA with a methylated strand. Resolution by RuvAB moves the strand toward the replication fork, putting two newly synthesized strands together. Because neither strand is methylated, MMR proteins will replace either base in a mismatched pair, fixing mutations half of the time. This model suggests that mutations will be most prevalent near sites that are prone to nicking or double-strand breaks, such as the *oriT* site on plasmids.[28] In addition, recombination events are often accompanied by tracts of error-prone DNA synthesis.[39,42-44] If DNA synthesis associated with recombination is prone to mutations, then recombination can increase genetic variation by both rearranging and creating alleles.[39]

B. SOS

The SOS system is a cell cycle checkpoint consisting of more than 20 genes[29] that evolved to handle DNA damage that blocks synthesis.[26,45] The SOS system is initiated when RecA is activated (RecA*) by double-strand breaks.[29] By cleaving the LexA repressor, RecA* permits the production of SOS proteins.[4] This system is down-regu-

lated by PsiB, which functions to bring the cell out of SOS by deactivating RecA*. The SOS system is not necessary for the production of stationary phase mutations, but is known to elevate mutation rates.[24,29,46] It has been suggested that the SOS system is regulated to produce the highest number of revertant colonies because the greatest number of colonies form in populations that have a RecA* concentration within an "optimal" range.[29] However, very high levels of RecA* may lead to an increase in mortality, leading to reduced numbers of revertant colonies formed, not a decrease in reversion mutations. The SOS system may be tightly regulated, but more convincing data on the fate of cells with increased levels of RecA* are needed to conclude it has evolved to increase genetic variability. It is more parsimonious that increased genetic variability is a byproduct of the inadequacy of the SOS system to accurately repair heavily damaged DNA.

In addition to the presence of RecA*, the deactivation of CRP (cAMP receptor protein) by high concentrations of cAMP is essential to activate the SOS system. This additional level of control allows the induction of SOS solely in cells that have high levels of cAMP, such as starving cells.[47] Artificially lowered cAMP concentration in auxotrophic *trp* mutant cells with an abundant carbon source decreases the number of Trp^+ revertants to a level similar to SOS-deficient cells. This added level of control indicates that this system has evolved to increase genetic variability in times of starvation. If this is true, the mutation-generation systems may have evolved to respond to stressful conditions.[47]

There is still considerable debate as to whether the SOS and MMR systems have evolved to increase the mutation rate, if they were preadapted and are maintained for this purpose, or if this is merely a byproduct of their true function. Most of the evidence points to the latter for both systems, although support is slowly accumulating for the preadaptation explanation in SOS.

CONCLUSION

The Origin of Mutants led to many experiments concluding that mutation rates depend on the environment in which the organism lives, and those rates fluctuate over evolutionary time. In addition, mutations occur in nondividing cells, indicating mutation rates should be measured per nucleotide turnover, not per generation. All mutational mechanisms conceived thus far involve some DNA synthesis occurring during the stationary phase. The reaction in the scientific community to Cairns *et al.* (1988) has led to a softening of some neo-Darwinian tenets in evolutionary thinking. Most now believe that the environment affects mutation rates, although not in a directed manner, and many different replication-dependent mutational mechanisms contribute to genetic variation.

Are these adaptive mutations a result of an adaptive strategy to increase mutation rates during times of stress (Genetic Engineer), or a byproduct of other cellular mechanisms that function suboptimally or abnormally due to the stress placed on the cell (Sick Watchmaker)[32]? Cairnsians champion the Genetic Engineer, concluding that bacteria have evolved the ability to "shuffle their genetic deck" when needed.[4] Neo-Darwinism is most akin to the Sick Watchmaker, claiming that DNA damage accumulates in stationary phase cells and that fixed mutations are simply the best the cell can do in stressful periods.[45] The Sick Watchmaker resembles a Spandrel of San Marco:[48] are these mutation generating systems really evolved to produce genetic variation when it is needed, or is that merely a byproduct of their original function.[30] Example systems strengthening the Sick Watchmaker hypoth-

esis include polymerase IV,[30] resolvase,[49] and MMR[34] It is also possible that the cell may not have the resources to repair DNA that is damaged or accurately replicate DNA when it is under stress, permitting mutations to accumulate[34] The breakdown of cellular mechanisms resulting in a fortuitous adaptive mutation does not indicate that the system has evolved to fail under stressful conditions. Because of myriad mutation generating mechanisms and the differential response at various loci,[39] it is unlikely that they evolved for the purpose of producing adaptive mutations. Although there is some evidence that systems such as SOS and heritable mutator alleles have evolved to increase genetic variability, all of the mechanisms may truly be Spandrels of San Marco, leaving the Cairnsian explanation resembling those of Dr. Pangloss.

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