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Transposable Elements Mediate Adaptive Debilitation of Flagella in Experimental *Escherichia coli* Populations

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Abstract

Although insertion sequence (IS) elements are generally considered genomic parasites, they can mediate adaptive genetic changes in bacterial genomes. We discovered that among 12 laboratory-evolved *Escherichia coli* populations, three had experienced at least six different IS *I*-mediated deletions of flagellar genes. These deletions all involved the master flagellar regulator *flhDC*, and as such completely incapacitate motility. Two lines of evidence strongly suggest that these deletions were adaptive in our evolution experiment: (1) parallel evolution in three independent populations is highly unlikely just by chance, and (2) one of these deletion mutations swept to fixation within ~1000 generations, which is over two million times faster than expected if this deletion was instead selectively neutral and thus evolving by genetic drift. Because flagella are energetically expensive to synthesize and operate, we suspect that debilitating their construction conferred a fitness advantage in our well-stirred evolution experiment. These findings underscore the important role that IS elements can play in mediating adaptive loss-of-function mutations in bacteria.

Keywords

adaptive evolution; parallel evolution; natural selection; insertion sequence element; loss-of-function mutation; experimental evolution

Introduction

Insertion sequences (IS) are common transposable elements in bacterial genomes (Siguier et al. 2014). IS elements have long been considered genetic parasites (Doolittle and Sapienza 1980; Orgel and Crick 1980), and genomic analyses across the bacterial domain support this contention (Plague 2010; Wagner 2006). Despite their generally deleterious fitness effects,

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IS elements can mediate beneficial mutations (Casacuberta and González 2013), including in bacterial laboratory evolution experiments (Chou et al. 2009; Chou and Marx 2012; Cooper et al. 2001; de Visser et al. 2004; Edwards et al. 2002; Gaffé et al. 2011; Philippe et al. 2009; Raeside et al. 2014; Stoebel et al. 2009; Treves et al. 1998; Zhong et al. 2004).

Several years ago, we conducted a long-term laboratory evolution experiment with *Escherichia coli* to test whether relaxed natural selection could be responsible for the high IS loads often observed in intracellular bacterial pathogens (Plague et al. 2011). We did not find support for our hypothesis, and instead found that IS elements were generally quiescent during our 4000 generation experiment. However, one of the seven IS *I* element copies in the genome (locus b1893/1894) did exhibit activity in three of our 12 populations: in one population, a 274 bp deletion immediately upstream of the IS *I* element had apparently become fixed, and two other populations contained clones with unidentified genetic variation at this locus (specifically, PCRs using primers surrounding this element failed, which could be indicative of chromosomal inversions or deletions at this locus) (Plague et al. 2011). IS *I* elements exhibit replicative transposition (Turlan and Chandler 1995) (often referred to as ‘copy-and-paste’ transposition, wherein an IS is copied and inserted into a new location while the parental copy remains intact), and may also be capable of conservative transposition (Chandler and Mahillon 2002) (often referred to as ‘cut-and-paste’ transposition, wherein an IS leaves one location and inserts into another). Interestingly, the particular IS *I* element that was active in our experiment is just upstream of 15 flagellar genes (Blattner et al. 1997), and its presence increases motility by enhancing the expression of the downstream *flhDC* operon (Barker et al. 2004).

The flagellum is an ancient and widely distributed locomotory organelle in Bacteria (Liu and Ochman 2007). In *E. coli*, >50 genes are involved in flagellar biosynthesis and function (Macnab 1996). Although they help orient bacteria toward beneficial environments and away from deleterious conditions, flagella are costly to synthesize and operate: ~2% and ~0.1% of total energy expended under normal growth conditions, respectively (Macnab 1996). As such, we hypothesized that the genetic variation we uncovered at IS *I* locus b1893/1894 in our experimental *E. coli* populations (Plague et al. 2011) may have debilitated flagella biosynthesis and thus may have been selectively advantageous, since flagella would have been essentially useless in the constant 120 rpm shaking of our experiment. We assessed this hypothesis by characterizing the genetic diversity and evolution of this IS *I* locus across all 12 experimental populations.

Results and Discussion

We found that large deletions were relatively common across our experimental populations (Table 1) in the IS *I* locus b1893/1894 chromosomal neighborhood. Specifically, we identified six unique deletion genotypes among the 300 isolates we analyzed from generation 4000 (N=25 isolates per population) (Fig. 1). These deletions, which ranged from 274 – 15,979 bp, arose in three of the 12 populations (Fig. 1), all three of which were maintained at a high effective population size ($N_e \approx 1.2 \times 10^9$) (Table 1). Most of these deletion genotypes were relatively rare within their respective populations (four of the six genotypes had frequencies < 0.08; Fig. 1), although collectively they comprised a substantial

proportion of the genotypes in their resident populations (20%, 100%, and 72% of populations 2, 3, and 9, respectively). These deletions affect various numbers of flagellar-associated genes (Fig. 1): the shortest deletion (274 bp) does not disrupt any open reading frames, but does eliminate the promoter of the FlhDC transcription factor (Fig. 2), while the longest deletion excises these regulatory genes, as well as genes involved in the flagellar motor (*motA*, *motB*), chemotaxis (*cheA*, *cheW*, *tar*, *tap*, *cheR*, *cheB*, *cheY*, *cheZ*), flagellar protein export (*flhB*, *flhA*), and an incompletely characterized flagellar protein (*flhE*) (Keseler et al. 2013).

The concordant architecture of these deletions, with the IS *I* element located immediately downstream of every one (Fig. 1), suggests that one of two possible molecular events was responsible for their origin. The first possibility is a two-step process, wherein an IS *I* element initially transposes to a new site within this chromosomal neighborhood, inserting in the same orientation as the resident IS *I* at locus b1893/1894. Then, homologous recombination between the new and resident elements leads to deletion of everything between them, as well as one of the elements (Andersson and Kurland 1998). Cooper et al. (2001) proposed this scenario to explain the repeated deletion of ribose catabolism genes by IS *I50* elements in their long-term laboratory evolution experiment with *E. coli*, which were structurally identical to the flagellar deletions we observed (Fig. 1). The second possibility is a one-step process that results from IS *I*'s replicative (copy-and-paste) transposition. When replicative transposition occurs to the same strand (i.e., same orientation) of the same DNA molecule as the parental IS element, everything between the parental and daughter ISs is deleted, and these nucleotides along with one of the ISs form a circular, extrachromosomal molecule (He et al. 2015; Shapiro 1979). This extrachromosomal molecule will not survive unless it contains an origin of replication (He et al. 2015). Therefore, replicative transposition of IS *I* locus b1893/1894 into this flagellar neighborhood and onto the same strand would also lead to chromosomal deletions identical to the ones we observed (Fig. 1).

So which of these two molecular events was likely responsible for these deletions? If it was the former (two-step) mechanism, then tell-tale signs of the initial IS *I* transposition into this flagellar neighborhood would be evident. Specifically, if the new IS *I* arrived via conservative (cut-and-paste) transposition, then we would have detected a deletion of one of the six other native IS *I* elements (Blattner et al. 1997) when we screened for IS activity (Plague et al. 2011). We did not detect any such deletions. If the new IS *I* arrived via replicative transposition, then transposition would have to be from a parental IS *I* on the opposite DNA strand (since replicative transposition to the same strand ultimately leads to deletion of the IS; see above). This causes a chromosomal inversion between the parental and daughter elements (He et al. 2015; Shapiro 1979), which we similarly did not observe (Plague et al. 2011). Also noteworthy is that our experimental *E. coli* K-12 strain does not contain a plasmid, so intermolecular IS *I* transposition to this region was not possible. Therefore, since we did not see tell-tale signs of an initial IS *I* transposition into this chromosomal neighborhood, the one-step process of replicative deletion by IS *I* locus b1893/1894 is the likely molecular mechanism responsible for these deletions.

Each of these deletions (Fig. 1) of course originated in one bacterium in their respective populations, so the initial frequency of each was 1/N. Since these populations were

bottlenecked daily to 2.5×10^8 CFU and then grew ~100-fold in size (Plague et al. 2011), the frequency of each deletion would have been no greater than 4.0×10^{-9} at the time they originated. By generation 4000, all had reached a frequency 0.04 (Fig. 1), so another outstanding question is: which evolutionary mechanism, genetic drift or natural selection, was likely responsible for these genotypes reaching relatively high frequencies in their respective populations? Two lines of evidence strongly suggest that these deletions are selectively advantageous under our experimental conditions, and as such evolved by natural selection. First, parallel genetic evolution in independently evolving populations is often strong evidence for adaptive evolution (Stern 2013). All of these deletions disrupt or remove at least some flagellar genes. Even the shortest 274 bp deletion eliminates the *flhDC* promoter (Fig. 2), which is the master regulator of flagellum biosynthesis and motility; i.e., *flhDC* is the first flagellar operon to be expressed, and it positively regulates all other flagellar genes (Kutsukake et al. 1990). Simply put, turning off *flhDC* turns off the construction and operation of flagella. Importantly, we found that deleting the *flhDC* promoter does disable motility in this clone (Fig. 3). As such, we suspect that the primary fitness advantage of all these deletions (Fig. 1) was to inactivate *flhDC*; not surprisingly, these deletion clones are all non-motile (Fig. 3).

The second line of evidence supporting the adaptive benefit of these deletions (Fig. 1) comes from tracking the evolution of the 274 bp deletion in population 3. This deletion was fixed in population 3 by generation 4000 (Fig. 1), so analyzing archived glycerol stocks provided us a unique opportunity to investigate the origin and evolution of this mutation. We first uncovered the deletion in generation 303, when it comprised 2% of the population, and it became fixed by generation 1305 (Fig. 4) (it is worth noting that although we did not find the deletion genotype in generation 204 or the wildtype genotype in generation 1305, both may have been present at frequencies too low to detect). This progressive and relatively rapid march to fixation (Fig. 4) strongly suggests that this 274 bp deletion confers a selective advantage over the non-deleted wildtype. Indeed, if this mutation was instead selectively neutral, then it would have taken on average $2N_e$ (or $\sim 2.4 \times 10^9$) generations to drift to fixation (Kimura and Ohta 1969), which is over two million times longer than it actually took. Furthermore, we can use the change of genotype frequencies over time (Fig. 4) to calculate the relative fitness of the two genotypes (using equation 6.3 in Hartl and Clark 1997). From generation 303 to 1200, the wildtype's fitness was 0.991 relative to the mutant, so this mutation conferred ~1% fitness advantage over the wildtype.

In our long-term evolution experiment (Plague et al. 2011), all *E. coli* populations were propagated under constant 120 rpm shaking. Consequently, we suspect that the fitness advantage conferred by deletions (Fig. 1) that disable motility (Fig. 3) comes simply from relieving these genotypes of the relatively high expense (Macnab 1996) of constructing a useless motor. Not surprisingly, flagellar genes are common targets for IS-mediated mutations in laboratory evolution experiments with well-stirred *E. coli* populations (Edwards et al. 2002; Zhong et al. 2004). IS-mediated flagellar knockouts have also been documented in several pathogenic bacteria (Parkhill et al. 2003; Parkhill et al. 2001; Song et al. 2010), so this phenomenon is not restricted to lab-reared populations. Indeed, since IS element transposition rates (Sousa et al. 2013) are several orders of magnitude greater than point

mutation rates (Lee et al. 2012), IS elements can clearly play an important role in selectively advantageous loss-of-function mutations in bacteria (Hottes et al. 2013).

Materials and Methods

Laboratory evolution experiment

Our 12 experimental populations (Plague et al. 2011) were all initiated with *E. coli* K-12 MG1655 strain FB21284 (obtained from Dr. Frederick Blattner, Univ. Wisconsin) (Table 1). This strain has a kanamycin-resistance gene inserted into an IS150 transposase gene (locus b3558) (Kang et al. 2004). We propagated six large ($N_e \approx 1.2 \times 10^9$) and six small populations ($N_e \approx 2.0 \times 10^2$) by differentially bottlenecking them every 24 or 48 hrs, respectively. Half of the large and half of the small populations were maintained in MOPS Rich medium, and the other half of each were maintained in MOPS Minimal medium (Neidhardt et al. 1974), all supplemented with 0.2% glucose and 50 µg/mL kanamycin. All populations were reared for 4000 generations, and 15% glycerol stocks of each population were stored at -80°C at 100-generation intervals.

IS1 activity screening

We screened all 12 populations for IS1 locus b1893/1894 activity (i.e., transposition out of or into this locus, or recombination-mediated deletion) by performing diagnostic PCRs on 300 clones isolated at generation 4000 ($N=25$ clones from each population), using primers in the *uspC* and *flhD* genes flanking the IS element (EcIS1F6: CAATAAACGTCTTGTCACGGG, EcIS1R6: AACTGAGTAATCGTCTGGTGGC). For any clones that failed to amplify, and thus that did not exhibit the ancestral genotype, we used additional PCR primers in this chromosomal region (EctarR1: TCCCAGTTTGGATCTTGTTTCAG, EcycTR1: ACCATGACATTTTCAGCCATCA) in combination with the EcIS1F6 primer to amplify and ultimately sequence this locus.

Motility screening

We qualitatively assessed the motility, and thus flagellar function, of flagellar gene deletion mutants by growing stab cultures in Motility Test Medium (BBL, Franklin Lakes, NJ) supplemented with 0.005% tetrazolium. For these cultures, we revived frozen glycerol stocks of the deletion clones, as well as the *E. coli* K-12 generation 0 ancestor, overnight in LB broth supplemented with 50 µg/mL kanamycin. We then used a sterile inoculating needle to inoculate each culture tube, and incubated them for 24 hrs at 37°C.

Population genetic analysis of the 274 bp deletion

We investigated the origin and evolution of the fixed 274 bp deletion in population 3 by reviving and isolating individual clones from frozen glycerol stocks stored at 100-generation intervals, and genotyping them with diagnostic PCRs using primers EcIS1F6 and EcIS1R6 (see above). Once we identified the earliest generation to harbor this deletion genotype, we went back 100 generations and genotyped 100 individuals, confirming that all were wildtype at this locus (of course, the deletion mutant may have been present, but was <1% of the population). We then estimated the deletion and wildtype frequencies every 100 generations by genotyping 50 individuals, until the 100-generation interval in which the deletion became

fixed within the population (as confirmed by genotyping an additional 50 individuals, so that the deletion mutant was >99% of the population). We also analyzed 100 individuals from generation 1200, even though the deletion was not fixed, because we uncovered one wildtype individual in our analysis of 50 additional individuals (after the first 50 individuals we genotyped were deletion mutants).

We estimated the fitness of the wildtype genotype relative to the deletion genotype using equation 6.3 in Hartl and Clark (1997):

$$\log(p_t/q_t) = \log(p_0/q_0) + t \times \log(w)$$

In this equation, p_t and q_t are the frequencies of the wildtype and mutant genotypes at generation 1200 (0.01 and 0.99, respectively), p_0 and q_0 are the frequencies of the wildtype and mutant genotypes at generation 303 (0.98 and 0.02, respectively), t is the number of elapsed generations (897), and w is the relative fitness, which is what we solved for.

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Fig. 1. IS *I*-mediated deletions of flagellar genes in evolving *E. coli* populations. The map of this chromosomal region (loci b1878 – b1893/1894) is shown above, with arrows indicating gene orientation and colors indicating gene class (blue: flagellar, red: *IS1*). The six deletions are represented as horizontal lines, with the length of the line representing the extent of the deletion. The frequency of each deletion in its respective population at generation 4000 is provided.

Fig. 2. Intergenic region between *flhD* and *IS1* in *E. coli* K-12 MG1655, showing the 274 bp deletion fixed in population 3 (deleted nucleotides are shaded in the gray box). Nucleotides are numbered from the *E. coli* K-12 MG1655 genome sequence (GenBank accession no. NC_000913.3). The 5' and 3' ends of *flhD* and *IS1*, respectively, are depicted in the open boxes (arrows show the orientation of each gene, and dotted lines signify the continuation of additional nucleotides of the gene), the -10 and -35 regions of the promoter are depicted by horizontal lines, and the transcription start site is depicted by a bent arrow (Soutourina et al. 1999). Note that the entire promoter sequence was eliminated by this deletion.

Fig. 3. Stab cultures of the *E. coli* wildtype (generation 0 ancestor) and flagellar deletion genotypes in Motility Test Medium. Each deletion genotype is identified by the length of its deletion (see Fig. 1 for details of each). All stabs were grown for 24 hrs at 37°C. Note that the flagellated wildtype is growing throughout the agar, while the growth of each deletion genotype is limited to the immediate stab area.

Fig. 4.

Frequencies of the wildtype and 274 bp flagellar deletion genotypes in *E. coli* population 3 from generation 204 to 1305.

Table 1

Culture conditions of our 12 *E. coli* K-12 MG1655 experimental populations (Plague et al. 2011).

Population number	Culture medium ^a	Population size ^b
1–3	MOPS Minimal	large
4–6	MOPS Minimal	small
7–9	MOPS Rich	large
10–12	MOPS Rich	small

^a all media was supplemented with 0.2% glucose and 50 µg/mL kanamycin; MOPS Rich contains four nucleotides, five vitamins, and 20 amino acids that are lacking in MOPS Minimal

^b effective population size (N_e); large: $\sim 1.2 \times 10^9$ CFU/mL, small: $\sim 2.0 \times 10^2$ CFU/mL