The Effect of Mobile Element IS10 on Experimental Regulatory Evolution in *Escherichia coli*

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Abstract

Mobile genetic elements are widespread in bacteria, where they cause several kinds of mutations. Although their effects are on the whole negative, rare beneficial mutations caused by insertion sequence elements are frequently selected in some experimental evolution systems. For example, in earlier work, we found that strains of *Escherichia coli* that lack the sigma factor RpoS adapt to a high-osmolarity environment by the insertion of element IS10 into the promoter of the *otsBA* operon, rewiring expression from RpoS dependent to RpoS independent. We wished to determine how the presence of IS10 in the genome of this strain shaped the evolutionary outcome. IS10 could influence the outcome by causing mutations that confer adaptive phenotypes that cannot be achieved by strains without the element. Alternatively, IS10 could influence evolution by increasing the rate of appearance of certain classes of beneficial mutations even if they are no better than those that could be achieved by a strain without the element. We found that populations evolved from an IS10-free strain did not upregulate *otsBA*. An *otsBA-lacZY* fusion facilitated the recovery of a number of mutations that upregulate *otsB* without involving IS10 and found that two caused greater fitness increases than IS10 insertion, implying that evolution could have upregulated *otsBA* in the IS10-free strain. Finally, we demonstrate that there is epistasis between the IS10 insertion into the *otsBA* promoter and the other adaptive mutations, implying that introduction of IS10 into the *otsBA* promoter may alter the trajectory of adaptive evolution. We conclude that IS10 exerts its effect not by creating adaptive phenotypes that could not otherwise occur but by increasing the rate of appearance of certain adaptive mutations.

Key words: *Escherichia coli*, mobile element, regulatory evolution, IS10, mutational neighborhood, mutation rate.

Introduction

Mobile genetic elements are usually considered to be genomic parasites that inflict a burden on their host. The detrimental effects of mobile elements have been inferred from population genetic and phylogenetic data (Charlesworth and Langley 1989; Touchon and Rocha 2007; Wagner 2009) and directly measured in laboratory experiments (Wilke and Adams 1992; Elena et al. 1998). Despite the potential cost of possessing these elements, mutations generated by mobile elements are sometimes beneficial (Chao et al. 1983; Wilke and Adams 1992). This has clearly been demonstrated by experimental evolution, where mobile elements can create adaptive deletions and duplications of regions of the genome (Schneider et al. 2000; Cooper et al. 2001; Zhong et al. 2004). Mobile elements have also been found to cause beneficial mutations that change patterns of gene expression (Treves et al. 1998; Chou et al. 2009; Stoebel et al. 2009).

In a previous experiment (Stoebel et al. 2009), the mobile element IS10 was involved in the evolution of stress responses in *Escherichia coli*. We experimentally evolved populations to a high-osmolarity environment and found that ten of ten lines adapted via the same mutation: IS10 transposition into the promoter of the *otsBA* operon, denoted P_{otsBA}. The *otsBA* operon codes for the enzymes required for the synthesis of trehalose, a carbohydrate that allows *E. coli* to maintain osmotic balance. These lines carried a deletion of the sigma factor RpoS, which is required for transcription of the wild-type (wt) *otsBA* promoter. The insertion of IS10 allows transcription in the absence of RpoS because this mobile element brings an RpoS-independent promoter with it.

Our finding that IS10 caused an important adaptive mutation suggests that strains that contain a mobile element like IS10 might, at least in some environments, evolve in different ways than strains without the same element. Differences in adaptation between strains with and without an IS insertion sequence ( ) element has the potential to be important in evolution in nature, as mobile elements are not distributed equally among strains. In a comprehensive survey of six IS elements in naturally occurring strains of *E. coli*, Sawyer et al. (1987) found that strains vary dramatically in the presence and copy number of IS elements, with even closely related strains of *E. coli* differing in their IS content. Their survey did not include IS10, but this element was surveyed by Matsutani (1991), who found that IS10 copy number varied from zero to nine copies in a set of *E. coli* and *Shigella* strains. This diversity of element carriage comes about because mobile elements do not persist in

bacterial lineages for long: they are either purged from the genome or drive the lineage extinct (Wagner 2006, 2009).

How does the carriage of an IS element alter the potential for adaptive evolution? At a trivial level, strains that differ in the carriage of an element must differ in their evolutionary potential because strains without the element cannot have IS-mediated mutations. The resulting differences in mutational spectrum might be evolutionarily important, for example, if they alter the rate at which adaptive mutations occur at some loci or if they change the identity of loci underlying adaptive evolution. To test the hypothesis that IS elements change the course of adaptive evolution, we experimentally evolved a strain that lacked IS10 but was otherwise isogenic to the strain used in our earlier experiments. We observed that the availability of IS10-mediated mutations does not alter the average increase in fitness of an evolving population, but it does change the locus of evolution: none of the strains free of IS10 evolved increased expression of otsBA, whereas all strains with IS10 evolved increased expression of this locus. Subsequent experiments demonstrated that mutations increasing expression of otsBA can occur in a strain free of IS10, but these mutations occur at a much lower rate than in a strain possessing IS10.

Materials and Methods

Strains, Plasmids, and Growth Media

All strains used are listed in supplementary table S1, Supplementary Material online. Long-term evolution and competition experiments were conducted in 3-(N-morpholino)propanesulphonic acid (MOPS) minimal medium (Neidhardt et al. 1974) with 0.2% glucose (hereafter MOPS MM) as a carbon source. In some treatments, an additional 0.3 M NaCl was added for osmotic stress. L medium was 0.5% yeast extract, 1% tryptone, and 0.5% NaCl. Antibiotics were used at 15 mg/l tetracycline, 50 mg/l kanamycin, 100 mg/l carbenicllin, and 20 mg/l chloramphenicol.

Strain Construction

Strain DMS2156 was the parental strain for all experiments with IS10-free strains. Construction of DMS2156 began with wt strain CF1684, an isolate of MG1655, from which DMS1688 is also derived. ΔpyrE::kan was transduced from JW3617-1 into CF1684 using phage P1 (Thomason et al. 2007). This strain was then transduced to pyrE+ rph+ by transduction from strain CF7968, creating strain DMS2144. lacIZY was replaced with the kan cassette from plasmid pKD4 in strain CF1684 using the method of Datsenko and Wanner (2000). ΔlacIZY::kan was transduced into DMS2144, and the kan cassette was removed by the plasmid pCP20, creating strain DMS2148. Finally, ΔrpoS::kan was introduced into DMS2148 by transduction from ZK1000, creating strain DMS2156. DNA sequencing confirmed that DMS2156 was rph+. In addition, we sequenced the spoT gene, as it is known to vary between different E. coli K-12 isolates (Spira et al. 2008). Both DMS2156 and DMS1688 contain the H255Y spoT mutation found in strain MC4100 (Spira et al. 2008) but neither contains the insertion

of Glutamine Aspartic acid (QD) between amino acids 82 and 83 of the SpoT protein.

To select mutants upregulating P_{otsBA} in a ΔrpoS background, otsB::lacZYcat was transduced from DMS2098 (Stoebel et al. 2009) into DMS2156. To examine epistasis between P_{otsBA}::IS10 and other adaptive mutations, the promoter insertion was cotransduced with araH::tetRA from DMS1945 (Stoebel et al. 2009).

To recombine mutations upregulating otsB::lacZY, we amplified the promoter along with the araH::tetRA element immediately upstream using primers araH verify3+ and araH verify3− (supplementary table S2, Supplementary Material online) and recombined the polymerase chain reaction (PCR) product into strain CF1684. DNA sequencing was used to confirm that the promoter mutations were recombined along with araH::tetRA, and the construct was transduced into DMS1688.

Long-term Experimental Evolution

Strain DMS2156 (the ancestral strain) was streaked onto L+kan plates, and ten colonies were inoculated into separate 2 ml MOPS MM cultures. These strains were grown overnight. The next day, 250 μl of culture was added to 25 ml of MOPS MM and grown for 2 h at 37 °C, shaken at 200 rpm. NaCl was then added to a final concentration of 0.3 M, and cultures were grown for another 22 h. The next day, these cultures were used to found the ten experimental evolution lines by inoculating 25 μl into 25 ml fresh medium. Strains were grown in 25 ml of MOPS MM + 0.3 M NaCl in 250-ml flasks, shaken at 200 rpm at 37 °C. Twenty-five microliters of culture was transferred to 25 ml of fresh media every 24 h. This 1:1,000 dilution results in $\log_2(1,000) = 9.96$ doublings per day. The long-term experiment was conducted for 25 days or approximately 250 generations. Cultures were frozen at −80 °C by the addition of glycerol to 20%.

Competition Experiments

Competition experiments were performed as described by Stoebel et al. (2009). Briefly, a pair of strains to be competed were each grown separately in MOPS MM + 0.3 NaCl for 24 h. The pair were then mixed and diluted 1:1,000 into fresh media, where they were grown together for 24 h. The density of the two strains was measured at the start and end of the experiment, and fitness was calculated as the ratio of the growth rates of the two strains.

Real-Time Quantitative Polymerase Chain Reaction

For all RNA work, strains were inoculated directly from −80 °C frozen culture into 2 ml of MOPS MM in a culture tube and grown overnight at 37 °C, shaken at 200 rpm. The next day, the culture was diluted 1:100 into MOPS MM and grown for 2 h at 37 °C, shaken at 200 rpm. NaCl was added to a final concentration of 0.3 M, and cultures were grown for another 22 h. The next day, the culture was diluted 1:100 into 25 ml of MOPS MM + 0.3 M NaCl and grown until the cells reached an Optical density at a light wavelength of 600 nanometers between 0.25 and 0.3. Growth

was stopped by the addition of 5 ml ice-cold phenol:ethanol (5:95 by volume), and the cells were left on ice for 30–60 min. Cells were pelleted by centrifugation, the supernatant removed, and frozen at −80 °C for up to 1 week. RNA was extracted using a Promega SV Total RNA Purification kit. Residual DNA contamination was removed by treatment with DNase I (Ambion DNA-free Kit). RNA was run on an agarose gel to confirm that it was not degraded.

Primer pair otsB QPCR+ and otsB QPCR− (supplementary table S2, Supplementary Material online) were used to amplify the *otsB* gene, and primer rho QPCR+ and rho QPCR− were used to amplify the control gene *rho*. One-step QPCR was performed with a QuantiTect SYBR Green Kit (Qiagen) on a RotorGene RG-3000 (Corbett Research). Three independent RNA samples were run for each strain and measured for both *otsB* and *rho*. In addition, a reaction without reverse transcriptase was run for each sample to ensure that there was no DNA contamination. The method of Pfaffl (2001) was used to quantify the level of *otsB* message. Statistical analysis was performed on log-transformed data.

Green Fluorescent Protein Reporter Fusions

Expression of wt P_{otsBA} was measured with a *gfp* fusion on plasmid pDMS123 (Stoebel et al. 2009). To measure expression, strains were cultured overnight in MOPS MM and then diluted 1:100 into 2 ml fresh MOPS MM and grown for 2 h. After 2 h, NaCl was added to a final concentration of 0.3 M. Cells were sampled by dilution into 4% formaldehyde in phosphate buffered saline and stored at 4 °C overnight. Fluorescence of 10,000 cells from each sample was measured by flow cytometery.

Selection of Mutants Upregulating *otsB::lacZY*

Mutants upregulating *otsB::lacZY* in DMS2191 were selected as described by Stoebel et al. (2009). Briefly, individual colonies of DMS2191 were grown to stationary phase in MOPS MM + 0.3 M NaCl and then plated on MOPS MM + 0.3 M NaCl, with lactose instead of glucose as the carbon source. A single colony was randomly selected off each plate and purified on the same media.

Measurement of Mutation Rate

Strains DMS2098 and DMS2191 were inoculated from frozen cultures onto L plates and grown overnight. One colony of each strain was inoculated into 2 ml MOPS MM and grown overnight. The next day, each strain was subcultured 1:100 into MOPS MM, NaCl was added to 0.3 M after 2 h of growth, and the strains were grown for a further 22 h. Each strain was then diluted 1:10,000 into twenty-four 2-ml cultures of MOPS MM + 0.3 M NaCl. Cultures were grown for 48 h, and then, the entire volume was plated on MOPS MM, with 0.5% lactose instead of glucose, 0.3 M NaCl, kanamycin, and 40 μg/ml X-gal. Plates were incubated at 37 °C for 3 days, after which colonies were counted. In addition, colony counts were determined at the start and

end of the experiment by plating appropriate dilutions on L plates. Mutation rate was estimated using the Ma-Sandri-Sarkar Maximum Likelihood Estimator method implemented in the program FALCOR (Hall et al. 2009).

β-Galactosidase Activity

Mutations in P_{otsBA} were transduced into a fresh *ΔrpoS* background (DMS1688) to ensure that strains did not have secondary mutations. For measurement of β-galactosidase activity, strains were inoculated from frozen cultures directly into 2 ml MOPS MM and grown overnight at 37 °C. The next day, strains were diluted 1:100 into 2 ml MOPS MM and grown for 2 h. NaCl was added to a final concentration of 0.3 M and grown for 2 h. Growth was stopped by placing cells on ice, and the activity of β-galactosidase was measured as described by Miller (1992).

Results

When IS10 was present in the genome of a *ΔrpoS* strain, we found (Stoebel et al. 2009) that ten of ten populations growing in high-osmolarity media evolved by insertion of IS10 into P_{otsBA}. How would evolution have proceeded if this particular mutation could not have occurred? The evolved strains might be different in terms of the level of fitness increase, the locus of evolution, or the phenotypic effect of the adaptive mutation. To explore this question, we performed long-term experimental evolution with a strain that lacked IS10 (DMS2156) but was otherwise isogenic to the *ΔrpoS* strain (DMS1688) used in previous long-term evolution experiments. Competition experiments confirmed that these two strains have equal fitness in the environment used for experimental evolution (*t*-test, $P > 0.1$).

We evolved ten lines of DMS2156 for 250 generations and selected one isolate from each line. These evolved isolates were competed against their ancestor, revealing that all lines increased fitness. Significant analysis of variance on fitness ($P = 0.002$) suggests that there is heterogeneity in the increase in fitness. A strain with IS10 in P_{otsBA} had an intermediate level of fitness that was not significantly different from any of the evolved strains (Tukey's Honestly Significant Difference [HSD] test, $P > 0.1$), indicating that strains that possess IS10 do not evolve with mutations of unusually large benefit.

Was the locus of evolution affected by the lack of IS10 in the genome? We sequenced P_{otsBA} in each of the ten lines and found that none of the strains had a mutation in the promoter, meaning that the presence of IS10 affects the locus of evolution. This might not have resulted in a difference in phenotypic evolution, however, if a mutation in *trans* upregulated *otsBA*, just as IS10 had by inserting into P_{otsBA}. To test for this, we used QPCR to measure the level of *otsB* messenger RNA (mRNA) in each of the ten evolved strains that lacked IS10, as well as their nonexpressing ancestor and two positive control strains (fig. 1). We found no evidence that any of the evolved strains without IS10 increased the level of *otsB* mRNA relative to their ancestor
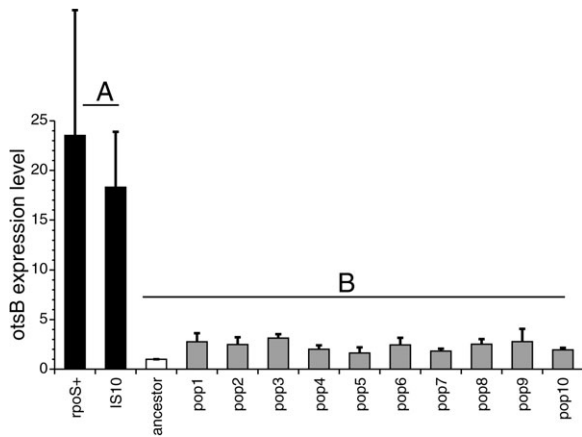
Fig. 1. Transcription of *otsB* from evolved lines that lacked IS*10* as measured by QPCR. *otsB* levels are not significantly higher in any of the evolved lines than in their ancestor (P > 0.2, Tukey's HSD on log-transformed data). As a control, note that *otsB* message levels are much higher in both the wt (*rpoS*+) and P$_{otsBA}$::IS*10* lines (P < 0.001, Tukey's HSD). *otsB* mRNA levels are normalized to *rho* mRNA and expressed as a fold difference relative to the *ΔrpoS* ancestor. mRNA levels were measured on three biological replicates.

(Tukey's HSD test, P > 0.2). To confirm that there was no mutation in *trans*, we introduced into each evolved line a plasmid (pDMS123) in which wt P$_{otsBA}$ drove the transcription of *gfp*, the gene for the green fluorescent protein. We detected no transcription in any of the ten lines (supplementary fig. S1, Supplementary Material online). Phenotypic evolution differed between strains with and without IS*10*: evolution did not involve upregulation of *otsBA* in strains lacking IS*10*.

One possible reason that no lines evolved expression of *otsBA* is that other mutations that upregulate *otsBA* are not as beneficial as the IS*10* insertion in P$_{otsBA}$, if such mutations exist at all. Testing this hypothesis required examining evolutionary events that did not occur: mutations that upregulate P$_{otsBA}$ independently of IS*10*. To isolate a set of such mutants, we used a strain with a *lacZY* fusion to *otsB*. This strain can only grow with lactose as a sole carbon source when P$_{otsBA}$ promotes transcription. Use of this *otsB-lacZY* transcriptional fusion strain allowed us to isolate a number of mutations in the *otsBA* promoter (fig. 2). These mutations include a point mutation, deletion, duplications, and insertion of mobile element IS*5* into the promoter. In addition, DNA sequencing revealed no mutations in P$_{otsBA}$ of one isolate, suggesting that mutations in *trans* are also able to activate the promoter. Insertion of IS*10* is not the only way to upregulate P$_{otsBA}$ in the absence of RpoS.

To determine how effective these mutations were at upregulating expression of *otsBA*, we measured expression of each of the mutant promoters using a β-galactosidase assay. These experiments revealed that the level of transcription from the mutant promoters varied widely, with some mutations promoting more transcription than an IS*10* insertion (fig. 3). Although IS*10* insertion is not the mutation leading to the highest levels of expression, it could be the most fit if an intermediate level of expression was optimal.
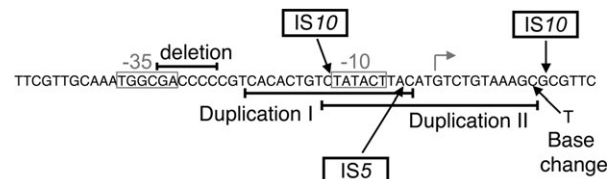

Fig. 2. Mutations recovered by selection with an *otsBA::lacZY* fusion. Mutants above the sequence were isolated in strain DMS2098, a background that contains IS*10*, and were previously reported (Stoebel et al. 2009). Mutants below the sequence were isolated in DMS2191, a background that does not contain IS*10*. All mutants were recovered once, except for the C to T transition at +11 relative to the transcriptional start site, isolated five times, and the IS*10* insertion between bases +12 and +13, which was isolated 19 times. The IS*10* insertion between +11 and +12 is the insertion found in the experimentally evolved lines. The start of translation is at +56. The transcriptional start site is from Becker and Hengge-Aronis (2001), and the −10 and −35 sites are inferred from the data in Shultzaberger et al. (2007).

To test this, we recombined the mutant promoters in front of the wt *otsBA* operon and measured the fitness of each of these up-mutants. We found that just as the IS*10* insertion is in the middle of the spectrum of mutations with respect to expression, it is also in the middle of the spectrum of fitness effects. Of the promoter mutations studied, two were more fit than IS*10* insertion, and three were less so (fig. 4A). The relationship between *otsBA* expression and fitness (fig. 4B) resembles that seen for other metabolic systems (Dykhuizen et al. 1987) where fitness approaches an asymptote as activity increases. We can conclude that P$_{otsBA}$ contains the potential for mutations upregulating *otsBA* that are more adaptive than an IS*10* insertion.

The data presented so far demonstrate that strains lacking IS*10* possess the potential to evolve high levels of *otsBA* expression, and correspondingly increase their fitness, but none of our experimentally evolved lines realized this outcome. This may have occurred because mutations upregulating P$_{otsBA}$ are much more common in strains that possess
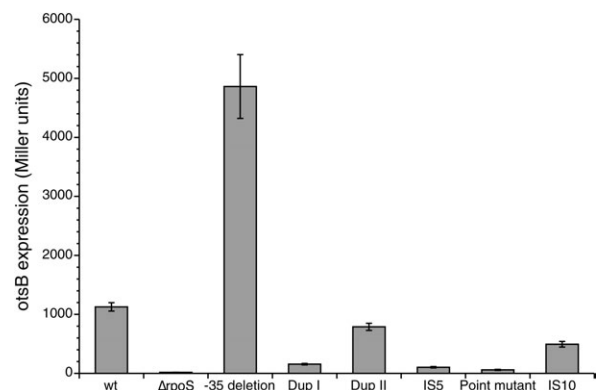

Fig. 3. Activity of the isolated promoter mutations as assayed by β-galactosidase activity. Strains are labeled as seen in figure 2. Wt refers to an *rpoS*+ line (DMS2096) and IS*10* to a strain with IS*10* inserted at the same location as recovered in our experimental evolution. Data are from four independent replicates. Error bars represent the standard error of the mean.
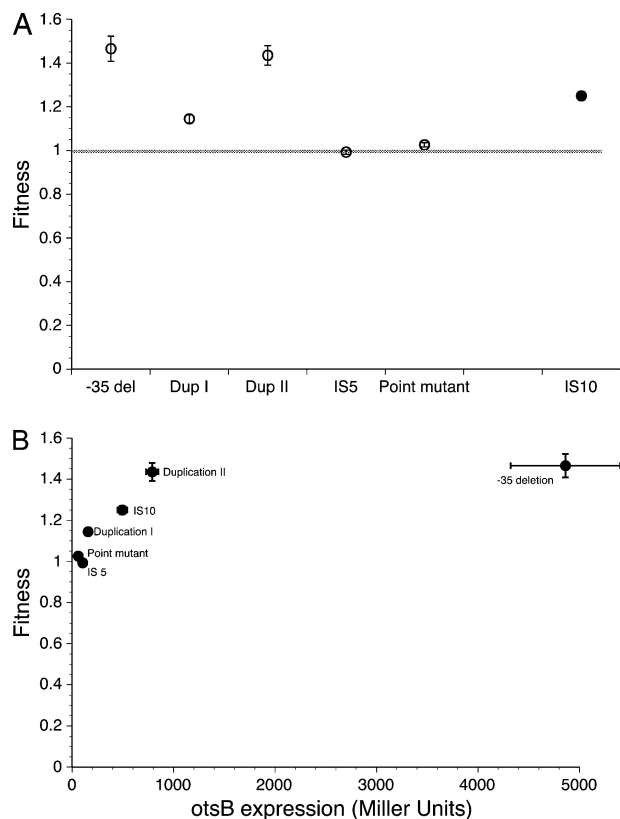
Fig. 4. (A) Fitness effects of mutations in P_{otsBA}. Promoter mutations isolated from the otsB::lacZY fusion were recombined into the wild-type chromosome and competed against a ΔrpoS strain with wild-type P_{otsBA}. Two mutations (the −35 deletion and duplication II) have higher fitness than an IS10 insertion. (B) Relationship between transcription of otsBA and fitness increase. For small increases in transcription, the relationship between otsBA transcription and fitness increase is approximately linear. A very large increase in transcription (the −35 deletion) does not increase fitness much beyond that achievable with less transcription (by duplication II).

Fig. 5. Epistasis between P_{otsBA}::IS10 and other unknown adaptive mutations. Fitness of the ten evolved lines that lack IS10 was measured relative to their ancestor. In addition, P_{otsBA}::IS10 was added to each of these lines, and they were competed against a strain with only P_{otsBA}::IS10. The unknown adaptive mutations were more beneficial on the ancestral background than on the background P_{otsBA}::IS10, significantly so in nine of ten cases ($P < 0.05$, t-test with P values modified by the sequential adjustment method of Holm, 1979). The diagonal line shows equal fitness on the two backgrounds.

IS10. For example, selecting mutants upregulating P_{otsBA} in a background containing IS10 resulted in 20 of 21 mutants possessing IS10 insertions (Stoebel et al. 2009). To test directly if strains that contain IS10 have a higher rate of mutations upregulating otsBA, we employed a fluctuation test (Pope et al. 2008). We found that a strain with IS10 has a 12-fold higher rate (1.8×10^{-9} vs. 1.5×10^{-10} mutations per generation) of mutations that increase P_{otsBA} transcription than a strain that does not contain IS10. Furthermore, only one of eight of the mutations in the IS10-free background conferred a fitness increase greater than or equal to IS10 insertion. When we consider only mutations conferring a fitness increase of this size, the strain with IS10 has a 96-fold higher rate of mutations than the strain without the element. This suggests that IS10 influences evolution by increasing the probability of mutations upregulating of otsBA rather than by creating phenotypes that could not otherwise occur.

When a strain is free of IS10, the first step of adaptation does not involve upregulation of otsBA. When IS10 is in the genome, otsBA becomes the locus of evolution. Is this
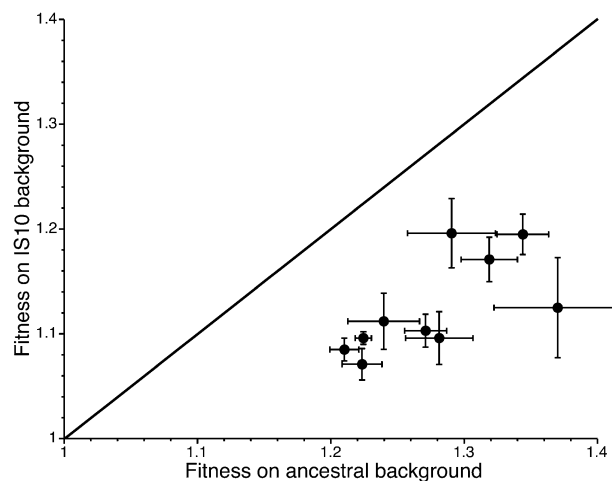
a temporary detour on the path of adaptation or does it influence subsequent adaptive mutations? The insertion of IS10 into P_{otsBA} might alter subsequent evolution if there is epistasis between the IS10 insertion and adaptive mutations that fixed in strains that did not contain IS10. If the fitness effect of these other (unknown) mutations differs on a P_{otsBA}::IS10 genetic background, then evolution may take a different trajectory than it would have if IS10 was present in the genome. To test this, we combined the P_{otsBA}::IS10 mutation with the adaptive mutation(s) of the IS10-free lines. We found that the adaptive mutation(s) of the IS10-free strains had a lower fitness benefit on a P_{otsBA}::IS10 genetic background than on the wt background (fig. 5). There was no evidence for sign epistasis (Weinreich et al. 2005) as the unknown adaptive mutations were beneficial on both backgrounds. Nevertheless, the lowering of the benefit of these adaptive mutations on a P_{otsBA}::IS10 background indicates the potential for an IS10 insertion to alter evolutionary trajectories.

Discussion

We have found that the presence of IS10 in the genome of a strain of E. coli alters the process of adaptation. As this strain, which lacks the global regulator RpoS, adapts to a high-osmolarity environment, the target of evolution depends on the presence of the element IS10. When strains possessed IS10, adaptation always involved insertion of the element into P_{otsBA}, resulting in upregulation of otsBA. In contrast, strains free of the element adapt via mutations at other loci that do not effect otsBA expression. This difference cannot be due to an inability of IS10-free strains to

acquire mutations upregulating *otsBA* because we have shown that such mutations do exist. We propose that these other *otsBA* upregulating mutations did not appear in our evolution experiment because they occur at a nearly 100-fold lower rate than IS*10* insertions into P$_{otsBA}$. We conclude that in this system, IS*10* in the genome changes the path of adaptive evolution because it increases the rate of mutations at certain loci and with certain phenotypic effects not because it creates adaptive mutations with unique phenotypes.

How general is our conclusion that when IS-mediated mutations fix in populations, it is because of the increased rate of mutation at certain loci or to certain phenotypes rather than because the IS-mediated mutations have unique phenotypic effects? To our knowledge, we are the first to have directly tested these alternatives. However, known IS-mediated adaptive mutations appear to have phenotypes that are achievable without mobile elements. For example, many of the previously characterized IS-mediated adaptive mutations involve deletions or duplications (Cooper et al. 2001; Zhong et al. 2004), which can also occur via non–IS-mediated mechanisms. IS elements are frequently involved in evolution by knocking out genes (Moran and Plague 2004; Zhong et al. 2004), a process that can obviously occur independently of mobile elements.

What of IS elements in promoter evolution? IS elements can bring about dramatic changes in patterns of transcription, by bringing entire new promoters (such as elements IS*3* and IS*10*), by introducing a new −35 site to create a novel hybrid promoter (such as elements IS*1* and IS*2*) or changing the conformation of a promoter (such as IS*5* at the *bgl* promoter) (Galas and Chandler 1989). However, IS elements are clearly not the only way to affect regulatory evolution. Patterns of transcription can also be altered by changing interactions between a regulatory protein and its target DNA sequence, either by mutations in the promoter or mutations in the protein. This phenomenon has been seen both in comparisons of different strains or species (Osborne et al. 2009; Perez and Groisman 2009) and in experimental evolution systems (Miller et al. 1988; Treves et al. 1998).

Compensating for the loss of a sigma factor, the particular case under investigation here, is likely to have fewer kinds of mutational alternatives than other kinds of regulatory evolution. In bacteria, transcription absolutely requires a sigma factor to position RNA polymerase properly at a promoter. To rewire a promoter from RpoS dependent to RpoS independent requires changes in the promoter that allow one of the six other sigma factors to recognize it or changes in the sigma factors themselves. Contrast this with the act of repressing or activating transcription: Proteins that do these functions are diverse and more abundant than sigma factors. The loss of a repressor could potentially be compensated for by any other repressor, making for a much larger suite of mutations than those that recruit a new sigma factor. Despite this functional constraint, it is apparent in our data that there are a number of

mutations in P$_{otsBA}$ that can recruit a new sigma factor. Switching sigma factors at P$_{otsBA}$ need not involve an IS element.

Although we have argued that IS*10* is not required for the evolution of *otsBA* transcription without RpoS, this does not mean that IS*10* insertion is entirely equivalent to other promoter mutations. For example, the exact level of transcription differed among the mutants that we examined. The size of the IS*10* insertion might alter local patterns of chromatin structure or DNA supercoiling, with potential pleiotropic effects on gene expression that we did not attempt to measure. Finally, we caution against generalizing from the fitness of different promoter mutations under our experimental conditions to the advantage of such a mutation across multiple environments. We know nothing about how the mutations we studied behave in other environments, and it is entirely possible that IS*10* insertion brings with it unique patterns of gene expression in some other environment. It is possible that across other environments, the relative fitnesses of each mutation would be different, including a possibly detrimental effect of IS*10* insertion.

There are at least two reasons to think that ability of a mobile element like IS*10* to create adaptive phenotypes is likely to be idiosyncratic. First, IS*10*, like many mobile elements, has preferences for the DNA site into which it inserts. Although P$_{otsBA}$ does not contain a sequence that is optimal for insertion, the site where IS*10* inserts during adaptive evolution differs at only one of the strongly important bases (Stoebel et al. 2009). The presence or absence of a good insertion site in a promoter will affect insertion frequency. Other potential targets of adaptive evolution might not contain insertion hot spots, greatly reducing the probability of an IS-mediated beneficial mutation occurring at those loci.

The second reason that IS-mediated evolution may be idiosyncratic is environmental. If transposition frequency is upregulated in a particular environmental conditions, then adaptation to this environment is more likely to involve mobile elements. (This is not to say that this type of regulation is necessarily beneficial for the element or its host.) High osmolarity is known to regulate both levels of transcription and DNA recombination by its effects on the level of DNA supercoiling (Dorman and Corcoran 2009). In the specific case of IS*10*, the regulatory proteins H-NS and IHF are known to play a fundamental role in transposition (Haniford 2006), as is the level of supercoiling (Chalmers et al. 1998). Integration host factor, histone-like nucleoid structuring, and supercoiling levels all respond to environmental changes, providing a possible mechanistic link between environmental changes and transposition (Haniford 2006; Dorman 2009). It is possible that adapting to high-osmolarity conditions is particularly likely to involve IS*10* because the rate of IS*10* transposition is higher than in other conditions.

If an IS-mediated adaptive mutation fixes first, how will subsequent evolution be affected? First, adaptation at the target of evolution may change. We observed that IS*10*

insertion into P$_{otsBA}$ was not the best mutation in the promoter. Fixation of the IS10 insertion may prevent the evolution of a better pattern of *otsBA* expression. Evolution at other loci may also be affected. We found epistasis between IS10 insertion into P$_{otsBA}$ and adaptive mutations at other loci. Because fixation probability depends on the magnitude of benefit of an adaptive mutation in this system (Gerrish and Lenski 1998), the insertion of IS10 into P$_{otsBA}$ decreases the fixation probability for mutations that would have otherwise occurred. The insertion of IS10 may change the trajectory by which evolution proceeds.

In conclusion, we have found that strains that possess IS10 evolve via mutations in different loci and with different phenotypic effects, than strains that do not. This is because IS10 increases the frequency of appearance of mutations that upregulate *otsBA* transcription rather than because possessing IS10 allows strains to evolve in beneficial ways that they otherwise could not. Finally, epistasis between IS10 insertion into P$_{otsBA}$ and mutations that otherwise might have fixed first suggests that once IS10 inserts into P$_{otsBA}$, the trajectory of further adaptation may be altered.

Supplementary Material

Supplementary figure S1 and tables S1 and S2 are available at *Molecular Biology and Evolution* online (http://www.mbe.oxfordjournals.org/).

Acknowledgments

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