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Relaxed natural selection alone does not permit transposable element expansion within 4,000 generations in *Escherichia coli*

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Abstract Insertion sequences (ISs) are transposable genetic elements in bacterial genomes. IS elements are common among bacteria but are generally rare within free-living species, probably because of the negative fitness effects they have on their hosts. Conversely, ISs frequently proliferate in intracellular symbionts and pathogens that recently transitioned from a free-living lifestyle. IS elements can profoundly influence the genomic evolution of their bacterial hosts, although it is unknown why they often expand in intracellular bacteria. We designed a laboratory evolution experiment with *Escherichia coli* K-12 to test the hypotheses that IS elements often expand in intracellular bacteria because of relaxed natural selection due to (1) their generally small effective population sizes (N_e) and thus enhanced genetic drift, and (2) their nutrient rich environment, which makes many biosynthetic genes unnecessary and thus selectively neutral territory for IS insertion. We propagated 12 populations under four experimental conditions: large N_e versus small N_e, and nutrient rich medium versus minimal medium. We found that relaxed selection over 4,000 generations was not sufficient to permit IS element expansion in any experimental population, thus leading us to hypothesize that IS expansion in intracellular symbionts may often be spurred by enhanced transposition rates, possibly due to environmental stress, coupled with relaxed natural selection.

Keywords IS element · Genetic drift · Experimental evolution · Pathogenic bacteria

Introduction

Transposable insertion sequence (IS) elements are common residents in bacterial chromosomes. Since IS elements can make copies of themselves, they replicate faster than non-transposable genes and therefore can potentially multiply after colonizing a genome. However, ISs are usually relatively rare within free-living bacteria (Moran and Plague 2004; Newton and Bordenstein 2011), probably because of their negative fitness effects of disrupting host genes and promoting homologous recombination (Nuzhdin 1999). Interestingly, IS elements often proliferate shortly after bacteria transition from a free-living to an intracellular lifestyle (e.g., Duchaud et al. 2003; Lee et al. 2005; Plague et al. 2008; Schmitz-Esser et al. 2010; Woyke et al. 2006), which seems to be a general trend among these species (Newton and Bordenstein 2011). Although Touchon and Rocha (2007) found no IS load difference between pathogenic and nonpathogenic bacteria, their analysis also included ancient intracellular pathogens and symbionts,

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which are often devoid of IS elements (see Newton and Bordenstein 2011).

IS elements can profoundly influence the genomic architecture of their bacterial hosts, particularly when they are profuse in a genome (Parkhill et al. 2003; Song et al. 2004; Yang et al. 2005). However, it is unknown why they often expand in recently evolved intracellular pathogens and symbionts. Moran and Plague (2004) hypothesized that IS expansion is due to relaxed natural selection in the intracellular environment, and that two ecological aspects of intracellularity are responsible for this relaxed selection. First, intracellular bacteria generally have small effective population sizes (N_e) relative to free-living bacteria (Fraser et al. 2007). In small populations, the efficacy of natural selection is decreased, partly because slightly deleterious genetic changes have a greater likelihood of becoming fixed just by chance (Ohta 1973). Consequently, IS elements may be able to multiply in small intracellular populations simply because natural selection cannot efficiently purge progressively higher IS load genotypes. Second, intracellular symbionts are often bathed in a nutrient-rich environment (Russell 2005), so they do not have to synthesize many of their own required metabolites (e.g., amino acids, vitamins, nucleotides). Therefore, some biosynthetic pathway genes in recently evolved intracellular symbionts may be superfluous and thus selectively neutral territory for IS occupancy.

We tested these hypotheses in a 4,000 generation laboratory evolution experiment with *Escherichia coli*, propagating 12 populations in batch culture under four experimental conditions (each with three replicate populations): (1) minimal culture medium/large N_e, (2) minimal medium/small N_e, (3) rich medium/large N_e, and (4) rich medium/small N_e. Although we did not fully replicate the intracellular bacterial lifestyle, we did propagate populations under nutritional and demographic conditions that are analogous to those experienced by intracellular (treatment #4) and free-living bacteria (treatment #1), and that have been hypothesized to be important factors in the expansion of IS elements in intracellular symbionts (Moran and Plague 2004).

Materials and methods

Bacterial strains

We used *Escherichia coli* K-12 MG1655 strain FB21284 (obtained from Dr. Frederick Blattner, Univ. Wisconsin) to initiate the 12 experimental populations. Strain FB21284 contains a kanamycin-resistance (Kan^R) gene inserted into locus b3558, which is the only IS*150* transposase in the genome (Kang et al. 2004). We chose this strain because it is a Gammaproteobacterium (this clade contains many intracellular pathogens and symbionts (Moran and Wernegreen 2000), including the very closely related *Shigella* spp., which harbor prolific IS loads (Yang et al. 2005)), its genome has been fully sequenced (to facilitate genomic analyses) (Blattner et al. 1997), and it can be grown in the presence of an antibiotic (to help minimize outside contamination).

We first isolated a single ancestral clone from a stab culture of *E. coli* strain FB21284, which can utilize lactose (Lac^+). To facilitate cross contamination checks during the experiment (see below), we then isolated a spontaneous Lac^- mutant by growing $\sim 10^7$ CFU of the Lac^+ ancestor in selective medium containing the galactoside TONPG (1 mM 2-Nitrophenyl 1-thio-β-D-galactopyranoside [TONPG], 0.5 mM IPTG, 50 μg/mL kanamycin, and 0.2% succinate in MOPS Minimal medium) (Miller 1972). TONPG is toxic when cleaved by β-galactosidase, so Lac^- mutants preferentially survive. When grown on tetrazolium lactose (TL) indicator plates, Lac^+ and Lac^- colonies are white and red, respectively. We sequenced the *lac* operon in the Lac^- mutant (GenBank accession no. JF300162), and discovered that an IS*5* element transposed into the lactose permease gene (*lacY*), thus disabling its ability to import lactose.

Culture conditions

We used the defined media MOPS Minimal and MOPS Rich (Neidhardt et al. 1974) (Teknova, Inc., Hollister, CA), both supplemented with 0.2% glucose and 50 μg/mL kanamycin. MOPS Rich has four nucleotides, five vitamins, and 20 amino acids that are lacking in MOPS Minimal. Therefore, the rich medium simulates the nutritionally rich environment of intracellular symbionts, while the minimal medium simulates the environment of free-living bacteria. All populations were grown in 50 mL Erlenmeyer flasks, shaking at 120 rpm and 37°C. At 100-generation intervals, we stored 15% glycerol stocks of every population at −70°C.

For each large population, we transferred 2.5×10^8 CFU every 24 h to 10 mL of fresh medium. For each small population, we transferred 10 CFU every 48 h to 2 mL of fresh medium. To ensure that we transferred an appropriate number of CFU at each bottleneck (N_0), and to estimate the number of generations between bottlenecks (calculated as $\log_2[N_{24}/N_0]$ and $\log_2[N_{48}/N_0]$ for the large and small populations, respectively), we regularly estimated the stationary phase census size of each population throughout the course of the experiment (N_{24} and N_{48} for the large and small populations, respectively). Specifically, we plated three replicate dilutions from each population every ~ 300 generations, which was every 3 and 6 weeks

for the small and large populations, respectively. Using equation (15) from Wahl and Gerrish (2001), the large population bottleneck equated to an N_e of $\sim 1.2 \times 10^9$, which is comparable to free-living *E. coli* populations (Ochman and Wilson 1987; Whittam and Ake 1993). Conversely, the small population bottleneck equated to an N_e of $\sim 2.0 \times 10^2$. Although we generally have a poor understanding of the N_e of most pathogenic bacteria (Fraser et al. 2007), the N_e divergence between our large and small populations is presumably great enough to mediate differential genomic evolution by genetic drift (Lynch and Conery 2003). To support this assertion, our large N_e populations all became ≥40% more fit over the course of this experiment (as assessed in head-to-head competition assays with the generation 0 ancestor), while every small N_e population remained selectively equivalent to the ancestor (H.C., unpublished data). Therefore, the large N_e populations clearly experienced more intense selection, and thus less intense genetic drift, than the small N_e populations.

Contamination checks

To maximize the likelihood of detecting cross contamination among populations, we initiated half the populations with the Lac^+ progenitor and half with the Lac^- progenitor. We then always alternated the daily transfers between Lac^+ and Lac^- populations, assuming that the most likely cross contamination would occur between consecutively transferred populations (after Lenski et al. 1991). We regularly plated samples from each evolving population (every 100 and 300 generations for the large and small populations, respectively) on TL indicator plates to confirm their Lac color phenotype. No cross-contamination occurred during this study, although colonies of the inappropriate color were occasionally detected on the TL plates at low frequency (<1% of the colonies). However, these never appreciably increased in frequency, and in most cases they were absent upon re-plating, suggesting plate contamination.

Because *E. coli* strain FB21284 has a Kan^R gene, we added kanamycin to the culture media to minimize the likelihood of outside contamination. We also checked each experimental population every 500 generations for outside contamination by PCR screening for this specific Kan^R gene, using primers flanking the insert (see Table S1 in the Supporting Information for primer sequences). Because outside contaminant bacteria are unlikely to have a Kan^R gene at the same locus (i.e., inserted within an IS*150*), the PCRs should amplify a single, specific product; any deviations from this would confirm culture contamination (i.e., alternate bands of a different size, additional bands, or no bands). We never detected outside contamination.

IS load quantification

The Lac^+ and Lac^- ancestral clones have 41 and 42 annotated IS elements, respectively. We estimated total IS loads in each evolving population at 1,000-generation intervals (plus generations 500 and 1,500) using real-time quantitative PCR (qPCR), following the protocol in Dougherty and Plague (2008). For each different IS element, we used a primer pair within the element to estimate the IS copy number per chromosome, using the single copy chromosomal gene *rpoB* as the reference (see Table S1 in the Supporting Information for primer sequences). We ran every reaction in triplicate, and we used equation (1) from Pfaffl (2001) to calculate each IS load relative to our *E. coli* Lac^+ ancestor. This equation requires calculating the amplification efficiency of each amplicon, which we did by running qPCRs on three independently derived 10-fold serial dilutions (each of which spanned four orders of magnitude) of the *E. coli* Lac^+ ancestral DNA. For each of these standard curves, the relationship between log DNA concentration and calculated threshold cycle was linear ($R^2 > 0.98$), and each amplification efficiency fell within the expected range of 1.6–2.1 (Pfaffl 2004). We estimated the experimental qPCR error for each amplicon using four replicate DNA extractions of the Lac^- ancestor. We arbitrarily chose one of these extractions as the control template for the relative quantification calculations (Pfaffl 2001), and we calculated a 95% confidence interval for each IS element estimate.

Genome sizing

The genomes of intracellular bacteria often shrink following host restriction (Ochman and Moran 2001). Since some populations evolved under ecological conditions that at least partially simulate an intracellular environment, they too may have experienced genome shrinkage. If so, then simply quantifying IS loads will underestimate the actual contribution of ISs in these populations. Therefore, we used pulsed-field gel electrophoresis (PFGE) (CHEF-DR II pulsed-field electrophoresis system, Bio-Rad Laboratories, Hercules, CA) to size the genomes of five isolates from each population at generation 4,000. We prepared agarose plugs, digested the plugs with the restriction endonuclease I-*Ceu*I, and resolved the restriction fragments as described by Bergthorsson and Ochman (1995, 1998), with several minor amendments: (1) we made plugs by mixing 0.5 mL of washed cell suspension with 1.5 mL of 1.2% Certified Megabase agarose (Bio-Rad), and dispensing this into 75 μL plug molds, (2) we digested plugs overnight at 37°C with 0.6 U of I-*Ceu*I (New England Biolabs, Ipswich, MA) in 100 μL of restriction enzyme buffer, (3) we usually loaded and electrophoresed half of each plug, although for

some samples we loaded whole plugs in order to visualize each I-*Ceu*I fragment, (4) to resolve the largest I-*Ceu*I fragment, we electrophoresed samples in 0.75% agarose gels, ramping pulse times from 10 to 15 min for 96 h at 60 V, (5) to resolve the remaining fragments, we used 0.9% agarose gels, ramping pulse times from 30 to 120 s for 24 h at 150 V. For all isolates that deviated from the ancestor, we also performed PFGE on *Avr*II (New England Biolabs) digested plugs (Bergthorsson and Ochman 1995) in order to corroborate and gain better resolution on the I-*Ceu*I genome size estimates.

Results and discussion

IS elements can generate beneficial mutations in bacteria (e.g., Conlon et al. 2004; Safi et al. 2004), and have even mediated adaptive evolution in *E. coli* evolution experiments (Cooper et al. 2001; Treves et al. 1998), including a putatively adaptive IS*150* expansion in a large N_e population after 10,000 generations (Papadopoulos et al. 1999). However, because IS elements are generally considered genomic parasites (Doolittle and Sapienza 1980; Orgel and Crick 1980), our goal was to assess the role of relaxed natural selection in the IS expansion that frequently occurs in recently evolved intracellular bacteria.

We found that global IS loads did not substantially deviate from the ancestral quantity in any population during the 4,000 generation experiment (Fig. 1), nor did any individual IS element (data not shown). Indeed, only one population at one time point (Rich/large N_e population #3 at generation 1,500) had a global IS load estimate outside the 95% CI of the qPCR experimental error. Furthermore, 57 of the 60 analyzed clones from generation 4,000 had identical I-*Ceu*I restriction site banding patterns to the ancestor, and thus retained the ancestral 4.6 Mb genome size (Table 1). The three variant clones were from the same minimal culture medium/large N_e population (i.e., a "free-living" population); two of these isolates were only 0.5% larger than the ancestor, and the third was 8% larger (Table 1). Therefore, with the exception of this one isolate, all populations essentially retained the ancestral IS load and genome size.

Although no population experienced a net IS increase (Fig. 1), IS elements nonetheless may have had distinct genomic effects among populations or treatments by displaying differential transpositional activity. IS elements transpose either conservatively (i.e., cut-and-paste) or replicatively (i.e., copy-and-paste) (Chandler and Mahillon 2002). Since IS loads did not appreciably expand in any population (Fig. 1), replicative transposition was probably negligible in this experiment. Therefore, potential IS mobility must be primarily relegated to conservative transposition, which we can detect by simply assessing whether IS elements continue to inhabit their ancestral locations in the evolved populations. To this end, we performed diagnostic PCRs on the 60 clones isolated at generation 4,000 ($N = 5$ clones from each population), using primers flanking each ancestral multicopy IS locus ($N = 37$ and 38 loci [of the 41 and 42 total] for the Lac^+ and Lac^- populations, respectively; see Table S1 in the Supporting Information for primer sequences). We found no evidence of transposition out of ancestral loci (Table 2). Therefore, IS elements were either generally quiescent in all 12 populations during the course of this experiment, or natural selection effectively purged most new, and presumably deleterious, IS genotypes from each population.

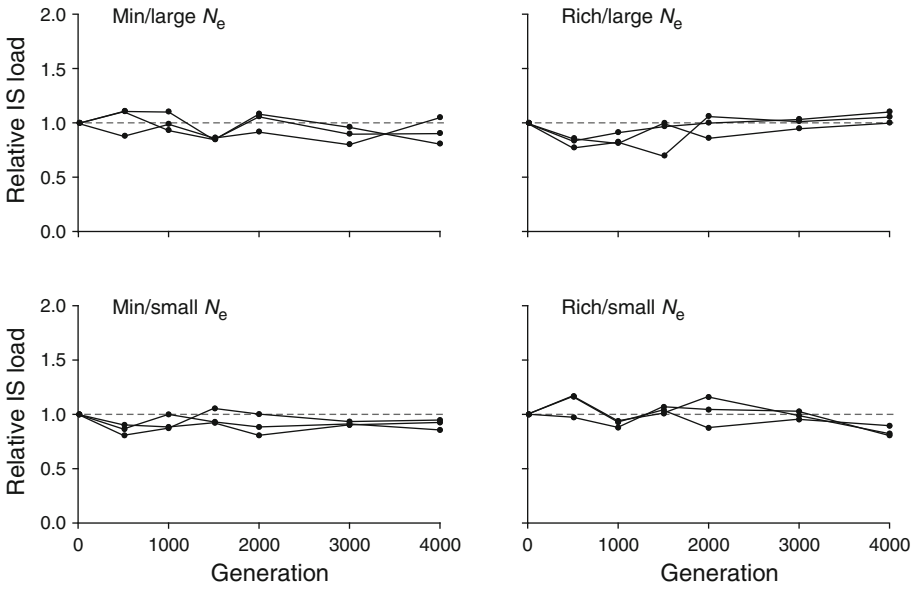

Fig. 1 Global IS load relative to the ancestor in each evolving *E. coli* population over 4,000 generations. Each *dotted line* represents the ancestral IS load

Table 1 I-*Ceu*I restriction fragment sizes and total genome size of clones isolated from the evolving populations at generation 4,000

Treatment	Clones	I-*Ceu*I ancestral fragment sizes (kb)							
		A 2,500	B 700	C 510	D 100	E 130	F 41	G 660	Total size 4,640
Min/large N_e	GP391–395, GP398–399, GP401–405	–	–	–	–	–	–	–	–
	GP396, GP397[a]	–	–	540	–	–	–	–	4,670
	GP400[a]	2,870	–	–	–	–	–	–	5,010
Min/small N_e	GP676–690	–	–	–	–	–	–	–	–
Rich/large N_e	GP361–375	–	–	–	–	–	–	–	–
Rich/small N_e	GP661–675	–	–	–	–	–	–	–	–

I-*Ceu*I recognition sequences occur once within each rRNA operon. Dashes indicate identical sizes to the ancestral clone. *Min* minimal medium, *Rich* rich medium

[a] Clones GP396, GP397, and GP400 were all isolated from Min/large N_e population #2

Clearly, we did not find support for the hypotheses that we set out to test; i.e., that IS elements often proliferate in intracellular pathogens and symbionts because of relaxed natural selection due to enhanced genetic drift and/or a nutrient rich environment (Moran and Plague 2004). Consequently, this may mean that our hypotheses are wrong, and that instead IS expansion confers some selective benefit for bacteria that are transitioning to live within eukaryotic cells, e.g., by generating adaptive genetic variation to help evade host cell defenses (Chao and McBroom 1985; Edwards and Brookfield 2003; Leavis et al. 2007). However, failing to find support for a hypothesis does not necessarily invalidate the hypothesis, simply because the experiment may not have adequately tested the hypothesis. There are several possible reasons why our experiment may not have adequately tested our hypotheses. One possibility is that *E. coli* K-12 is not an ideal bacterium to study the evolution of IS element expansion, perhaps because of its genome size. K-12 is a member of *E. coli* subgroup A (Herzer et al. 1990), which contains strains with the smallest chromosomes (Bergthorsson and Ochman 1998). Since IS load positively correlates to genome size in bacteria (Newton and Bordenstein 2011; Touchon and Rocha 2007), maybe the K-12 chromosome is too small to allow substantive IS expansion. However, this positive correlation is largely driven by ancient intracellular symbionts (Newton and Bordenstein 2011), which usually have small genomes and few or no mobile elements (Ochman and Davalos 2006). Furthermore, the K-12 chromosome is very comparable in size (4.6 Mb) to five of its close *Shigella* spp. relatives (4.4–4.8 Mb), all of which have high IS loads (Yang et al. 2005). Therefore, unless K-12 lost some integral genomic component during its ~90 years as a lab strain (Lederberg 2004) that precludes IS expansion, its chromosome size should not have inhibited IS expansion.

A second possible reason why this experiment may not have adequately tested our hypotheses is that maybe natural selection was not sufficiently relaxed in our rich culture medium or small N_e populations to permit IS expansion. In MOPS Rich medium, ~200 biosynthetic genes are superfluous in *E. coli* K-12 (Riley 1993). This equates to ~5% of the *E. coli* K-12 genome, which seems fairly substantial and also adequate for testing this hypothesis, but may not be equivalent to the number of superfluous genes in some recently evolved intracellular symbionts (e.g., some pathogens scavenge fatty acids from their hosts (Brinster et al. 2009), which are not present in MOPS Rich medium). Also, the efficacy of natural selection positively correlates to a population's N_e (Kimura 1983). Specifically, genotypes with selection coefficients (s) less than about $1/N_e$ are selectively neutral in haploid populations, and thus evolve by genetic drift. Since our large populations ($N_e \approx 1.2 \times 10^9$) were ~6,000,000-fold bigger than our small populations ($N_e \approx 200$), the efficacy of selection was undoubtedly vastly different in each. However, this difference is meaningless if our small populations were still too large to allow any new IS genotypes to evolve neutrally. In these populations, mutations with $s \leq 0.005$ would have been selectively neutral, which is very close to the lowest s value that can be detected in bacterial fitness assays (Dykhuizen 1990; Lind et al. 2010). Most new IS insertions probably have $s > 0.005$ (Elena et al. 1998), although Elena et al. (1998) found that 19% of the 226 random Tn*10* transposon insertions that they tested have statistically insignificant fitness effects. This 19% is almost certainly an overestimate of the new IS genotypes that would evolve neutrally in our small N_e populations (see Elena et al. 1998), although it does suggest that numerous insertion mutations potentially have very small selection coefficients.

Table 2 Ancestral multicopy IS loci that differ in generation 4,000 clones

Treatment	Clones	IS (loci) that differ from the ancestor					
		IS*1* (b0264/5)	IS*1* (b0274/5)	IS*1* (b1893/4)	IS*3* (b0372)	IS*5* (b0259)	IS*30*/IS*911* (b0256/4587)
Min/large N_e	GP391–394	–	–	–	–	–	–
	GP395	–	–	–	–	N	N
	GP396–398, GP400	–	–	–	–	–	–
	GP399	–	–	L[a]	–	–	–
	GP401–404	–	–	S[b]	N	–	–
	GP405	–	–	S[b]	N	N	N
Min/small N_e	GP676–680	–	–	–	–	–	–
	GP681–685	–	–	–	–	–	–
	GP686–690	–	–	–	–	–	–
Rich/large N_e	GP361–365	–	–	–	–	–	–
	GP366–370	–	–	–	–	–	–
	GP371, GP373	–	–	–	–	–	–
	GP372, GP374-375	–	–	L[a]	–	–	–
Rich/small N_e	GP661–665	–	–	–	–	–	–
	GP666–670	N	N	–	–	–	–
	GP671–675	–	–	–	–	–	–

Only diagnostic PCRs that produced smaller (S), larger (L), or no (N) products in at least one clone are shown (the 32 other multicopy IS loci only generated PCR products that were identical to the ancestor). S and L PCR products potentially indicate transposition out of or into a locus, respectively, so we sequenced these PCR products to assess these possibilities (see footnotes for results); N PCR products suggest either chromosomal rearrangement or deletion at the locus, mutation at a priming site, or an insertion between the primers. Dashes indicate positive PCR results that were identical to the ancestor. All PCRs that differed from the ancestor were run at least twice. Bracketed clones are from the same population. *Min* minimal medium, *Rich* rich medium

[a] PCR product due to non-specific primer annealing; as such, this should most likely be labeled as "N" because the ancestral locus did not amplify

[b] 274 bp deletion in intergenic region between IS*1* and *flhD*

Furthermore, it seems unlikely that most pathogen populations are substantially smaller than $N_e \approx 200$ because many have minimal infective doses >200 cells (Kothary and Babu 2001; Todd et al. 2008), so they are probably rarely even bottlenecked to sizes below the N_e of our small experimental populations.

Indeed, a third possible reason why this experiment may not have adequately tested our hypotheses is that perhaps our small N_e bottlenecks were too severe, thus effectively purging too much neutral transpositional variation to achieve IS accumulation. If so, then 4,000 generations may not be long enough for the combined effects of replicative transposition and genetic drift to achieve IS proliferation. Since drift is a stochastic process, it does proceed relatively slowly: on average, a new selectively neutral allele in a haploid population drifts to fixation in about $2N_e$ generations (Kimura and Ohta 1969). However, this estimate only applies to the relatively few new neutral mutations that escape loss; specifically, the probability that a new neutral allele will drift to fixation is equal to its initial frequency in the population, which is $1/N$. Even in our experiment, in which N of the small populations fluctuated between about 10 and 10^{10} every 48 h, the great majority of new IS variants went extinct, no matter when they occurred during the population growth cycle. Therefore, the proliferation of neutral IS variation may require many more generations than transpired in our experiment, so we may need to continue this experiment indefinitely. However, this experiment would accelerate with higher transposition rates, and thus a higher quantity of selectively neutral transpositional variation within each population. This alternative is a fourth possible reason why our experiment may not have adequately tested our hypotheses: we may have overlooked some integral aspect of the intracellular bacterial lifestyle that enhances transposition rates (at least relative to those in this experiment) and thus IS expansion rates. Interestingly, some IS elements display elevated transposition under stressful conditions (Capy et al. 2000), and one stressor that may be of particular importance is thermal stress. Many pathogens probably naturally experience a wide range of temperatures. For example, mammalian pathogens transmitted in feces may get deposited into relatively cool bodies of water or relatively hot feedlots. Even the temperature within a host can change over time if the host is able to elevate its body temperature as an immune response to bacterial infections. Importantly, a number of IS elements exhibit different transposition rates at different temperatures (Cornelis 1980; Haren et al. 1997; Kretschmer and Cohen 1979; Ohtsubo et al. 2005; Pfeifer and Blaseio 1990). By propagating our experimental populations at a static and stress-free 37°C, many IS elements may have remained relatively quiescent, thus preventing any potential IS expansion. Indeed, it is clear that IS elements exhibited very little activity during our experiment (Fig. 1; Table 2), and that without enhanced transpositional activity, IS expansion is highly unlikely or even impossible within 4,000 generations. In contrast, IS loads can vastly differ among individual isolates within intracellular symbiont species (Chain et al. 2006; Dougherty and Plague 2008; Yang et al. 2005), suggesting that IS load variation may sometimes evolve relatively quickly. Although many free-living bacteria may also experience temperature fluctuations and thus elevated transposition rates (e.g., nonpathogenic enterics that are also transmitted in feces), natural selection probably efficiently purges high IS load genotypes from their generally large populations (Lynch 2007). In small intracellular bacterial populations,

enhanced transposition rates would simply hasten neutral evolution caused by enhanced genetic drift and/or a nutrient rich environment, thus potentially hastening the rate of IS accumulation.

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