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The fitness effects of a point mutation in *Escherichia coli* change with founding population density

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Abstract

Although intraspecific competition plays a seminal role in organismal evolution, little is known about the fitness effects of mutations at different population densities. We identified a point mutation in the cyclic AMP receptor protein (CRP) gene in *Escherichia coli* that confers significantly higher fitness than the wildtype at low founding population density, but significantly lower fitness at high founding density. Because CRP is a transcription factor that regulates the expression of nearly 500 genes, we compared global gene expression profiles of the mutant and wildtype strains. This mutation (S63F) does not affect expression of *crp* itself, but it does significantly affect expression of 170 and 157 genes at high and low founding density, respectively. Interestingly, acid resistance genes, some of which are known to exhibit density-dependent effects in *E. coli*, were consistently differentially expressed at high but not low density. As such, these genes may play a key role in reducing the *crp* mutant's fitness at high density, although other differentially expressed genes almost certainly also contribute to the fluctuating fitness differences we observed. Whatever the causes, we suspect that many mutations may exhibit density-dependent fitness effects in natural populations, so the fate of new mutations may frequently depend on the effective population size when they originate.

Keywords

Natural selection; Fluctuating fitness; Effective population size; *E. coli*

Introduction

Mutations are the fundamental source of genetic novelty within populations, and as such are integral for evolution. New mutations can span the selective spectrum, conferring positive, neutral, or negative fitness effects relative to the wildtype allele. These fitness effects are important because they not only determine a mutation's fixation probability (Kimura 1962; Patwa and Wahl 2008; Engen et al. 2009; Houchmandzadeh and Vallade 2011; Waxman

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2011), but also in part shape the evolutionary trajectories of populations (Gerrish and Lenski 1998; Weinreich et al. 2006; Desai et al. 2007; Kao and Sherlock 2008). Importantly, the fitness effects of mutations are often context dependent (Korona 1999; Lynch et al. 1999; Fry and Heinsohn 2002; Chang and Shaw 2003; Martin and Lenormand 2006), so estimates of a mutation's relative fitness can differ under different assay conditions (Lind et al. 2010).

One largely under-studied factor that may affect the fitness of some mutations is population density. Although density-dependent selection has been analyzed in numerous natural and laboratory populations, these studies have primarily focused on the relative fitness of alternate phenotypes and life history strategies (Mueller 1997; Travis et al. 2013). Comparatively little is known about the relative fitness of discrete mutations at different population densities. Indeed, the only two pertinent findings we are aware of are in vesicular stomatitis virus, wherein the relative fitness of a mutant genotype differs at high and low density compared to the wildtype (Novella et al. 2004), and in *Drosophila melanogaster*, wherein the intensity of sexual selection on mutant and wildtype males at three different loci (out of eight analyzed) differs at high and low density (Sharp and Agrawal 2008). Although sexual selection is just one component of fitness for sexually reproducing organisms like *D. melanogaster* (others include viability and fecundity, for example), these two studies suggest that fitness may often vary in differently sized populations.

Bacterial pathogens often have relatively small effective population sizes (N_e) (Fraser et al. 2007). Natural selection is relaxed in small populations, largely because slightly deleterious mutations have a relatively high likelihood of becoming fixed just by chance (Ohta 1973). This relaxed natural selection may be the reason why transposable elements (TE) often proliferate in bacterial pathogens (Moran and Plague 2004), which we tested in a laboratory evolution experiment with *Escherichia coli* (Plague et al. 2011). In short, we quantified the TE loads of six large populations ($N_e \approx 1.2 \times 10^9$) and six small populations ($N_e \approx 2.0 \times 10^2$) over 4000 generations. In order to detect potential cross contamination among our experimental populations, we initiated half of the populations with a clone that can metabolize lactose (Lac^+) and half with a clone that cannot (Lac^-). Although we did not find support for our hypothesis about TE proliferation in pathogens (Plague et al. 2011), we wanted to track the neutral versus adaptive evolution within our experimental populations, which first required us to assess the relative fitnesses of the Lac^+ and Lac^- ancestors. When grown in the absence of lactose, the ability (and inability) to metabolize lactose should be selectively neutral. However, we report here the surprising discovery that our Lac^+ and Lac^- clones are not selectively equivalent when grown in the absence of lactose, and that their relative fitnesses depend on the founding population size. We discovered that a co-segregating mutation in the *crp* gene, which encodes cyclic AMP receptor protein (CRP; also known as catabolite activator protein, CAP), is responsible for this fitness asymmetry. CRP is a transcription factor that directly regulates the expression of 495 genes in *E. coli*, and indirectly regulates over 2700 additional genes (Keseler et al. 2011). We found that this *crp* mutation causes differential expression of hundreds of genes at high and low founding population density (though *crp* itself is not differentially expressed at either density), which in turn leads to differential fitness at each density.

Materials and methods

Bacterial strains

Our experimental Lac⁺ strain is *E. coli* K-12 MG1655 substrain FB21284 (obtained from Dr. Frederick Blattner, Univ. Wisconsin), which is derived from *E. coli* K-12 MG1655 but with a kanamycin resistance (Kan^R) cassette inserted into the sole IS150 transposase in the genome (Kang et al. 2004). Also, we PCR amplified and sequenced the complete *crp* gene in this strain, confirming that it has the wildtype sequence. Hereafter we refer to this strain as CRP⁺/Lac⁺.

Our experimental Lac⁻ strain was isolated as a spontaneous mutant of the Lac⁺ strain (Plague et al. 2011). It was originally identified by its inability to metabolize lactose, which is due to an IS5 insertion in the *lacY* gene (GenBank accession no. JF300162). After conducting preliminary fitness assays between our experimental Lac⁺ and Lac⁻ strains and discovering that they are not selectively equivalent, we identified one other co-segregating mutation in the Lac⁻ strain by sequencing its genome on an Illumina Genome Analyzer IIx system at the Virginia Bioinformatics Institute (Blacksburg, VA): a C to T transition at nucleotide 188 in *crp*, which changes serine to phenylalanine at codon 63 (S63F). In short, we obtained a total of 30.4 M sequencing reads with lengths >29 bp, and an average 220-fold coverage. We compared this genome sequence with the published *E. coli* K-12 MG1655 genome sequence (GenBank accession no. U00096.2), and we verified all fixed differences between the two genomes using PCR amplification and sequencing (Table S1). This strain (hereafter CRP⁻/Lac⁻) differs from the CRP⁺/Lac⁺ strain only at the *crp* and *lacY* genes, and both differ from the *E. coli* MG1655 reference genome at two additional loci: *glpR* and the *ppiC-yifN* intergenic region (Table S1).

We also created a third strain with an intermediate genotype to the first two (CRP⁻/Lac⁺) by restoring the wildtype *lacY* gene in the CRP⁻/Lac⁻ strain, using the recombineering protocol of Thomason et al. (2007). To do this, we first transformed CRP⁻/Lac⁻ with an ampicillin resistant pSIM6 plasmid, which contains the Lamda red recombination genes (Datta et al. 2006). After growing a transformed clone to exponential phase (OD₆₀₀ = 0.4–0.6) at 37 °C and 120 rpm, we placed the culture in a 42 °C and 220 rpm shaking water bath for 15 min to express the Lamda red recombination genes. We immediately induced cell competence by putting the cultures in ice-cold water, and then introduced by electroporation a synthetic 70-mer oligonucleotide, each half of which corresponds to the wildtype *lacY* sequence (non-coding strand) flanking the inserted IS5. After electroporation, we transferred cells to fresh Luria-Bertani (LB) medium for outgrowth for 2 h at 32 °C. We then pelleted and washed the cells three times with Ringer's solution before adding them to fresh MOPS Minimal medium (Neidhardt et al. 1974) (Teknova, Inc., Hollister, CA) supplemented with 0.2 % lactose. After overnight growth, the culture was diluted and spread on tetrazolium lactose (TL) indicator plates, on which Lac⁺ colonies (including recombinants) appear white and Lac⁻ colonies appear red (Miller 1972). We isolated one white colony, and confirmed its wildtype Lac genotype by sequencing the *lacY* locus.

Fitness assays

We hypothesized that the preliminary fitness asymmetry we observed between the CRP⁺/Lac⁺ and CRP⁻/Lac⁻ strains in lactose-free medium was due to the *crp* mutation, and not the *lacY* mutation. To test this, we conducted a series of head-to-head competition assays at both high and low founding population density among the three *E. coli* strains above. These competition assays entailed growing two competitors together in a single flask containing fresh medium (Lenski et al. 1991). Therefore, a selectable marker was required to enumerate each competitor at the start and end of each assay, which allowed us to calculate the number of elapsed generations of each competitor, and thus the relative fitness of each. For this, we relied on the Lac phenotype and TL indicator plating to differentiate between each pair of competitors.

All competition assays mimicked the culture conditions in our evolution experiment (Plague et al. 2011), with one exception (noted below). Prior to every competition assay, each competitor was revived in LB broth for 24 h from a frozen glycerol stock, then conditioned for 24 h in MOPS Minimal medium supplemented with 0.2 % glucose and 50 µg/mL kanamycin, both times shaking at 37 °C and 120 rpm. We then added an equivalent volume of each competitor to 12 replicate 50-mL flasks containing MOPS Minimal medium supplemented with 0.2 % glucose and 50 µg/mL kanamycin. For the high founding population density assays, 50 µL of each conditioned competitor (which was $\sim 1.0 \times 10^8$ CFU) was added to 9.9 mL of fresh medium. For the small founding population density assays, the population bottleneck in our evolution experiment (Plague et al. 2011) was too small for competition assays (~ 10 CFU, which would preclude the possibility of enumerating each competitor at the start of the assay), so we instead added 150 µL of a 2×10^{-6} dilution of each conditioned competitor ($\sim 6.0 \times 10^2$ CFU) to 5 mL of fresh medium. We then propagated the competition assays at 37 °C and 120 rpm for 24 h. Although the stationary phase population densities were comparable for the high and low density assays ($\sim 2.0 \times 10^9$ CFU/mL; Fig. S1), the dramatically different founding population sizes led to very different amounts of time spent in exponential phase between the high and low density assays (Fig. S1), and to very different N_e : $\sim 1.2 \times 10^9$ and $\sim 2.5 \times 10^4$ (Wahl and Gerrish 2001), respectively, both of which are comparable to naturally occurring bacterial populations [specifically, free-living *E. coli* (Ochman and Wilson 1987) and intracellular symbionts (Mira and Moran 2002), respectively].

In every competition assay, each competitor was enumerated at the start and end of the assay by spreading a sample of the culture on TL indicator plates. This allowed us to calculate the number of elapsed generations of each genotype, which we in turn used to calculate their relative fitness (Lenski et al. 1991). On average, cells in the high and low founding density competition assays went through about 6.7 and 25 generations, respectively, during 24 h of competition. To compensate for this discrepancy in the number of generations, we performed high density competition assays over 4 days (transferring 100 µL of the 24 h cultures to fresh medium on days 2–4), which on average resulted in a total of 27 generations (comparable to the 25 generations experienced at low density).

For each competition experiment, we compared the fitness of the two competitors using a two-tailed one sample *t* test, testing the null hypothesis that their fitnesses were equivalent

(i.e., that the relative fitness of the Lac^+ competitor was 1.00; Table 1). All t tests were computed using SPSS software (IBM Corp. 2013).

Gene expression microarray analysis

To identify genes that are potentially differentially expressed between CRP^+ and CRP^- at high and low founding population densities, we revived and acclimated the $\text{CRP}^+/\text{Lac}^+$ and $\text{CRP}^-/\text{Lac}^+$ strains, as described above. We then inoculated three replicate flasks with each strain at each population density ($N=12$ total flasks), also as described above. Mid-log phase cells were then harvested from each culture, which differed by ~ 15 h for the high and low founding density populations (Fig. S1), and added into Qiagen RNeasy Protect Bacteria Reagent (Qiagen, Valencia, CA). We extracted total RNA using a Qiagen RNeasy Mini Kit with on-column DNase digestion, and we analyzed gene expression profiles using GeneChip *E. coli* Genome 2.0 Arrays (Affymetrix, Santa Clara, CA) at the Virginia Bioinformatics Institute.

After hybridization and scanning, resulting files were imported into GeneSpring software (Agilent Technologies Inc. 2010) and the Robust Multichip Average was computed for all arrays as a means of summarization and normalization. We compared the expression of each gene in CRP^+ versus CRP^- using an unpaired Student's t test (IBM Corp. 2013). Genes were considered differentially expressed if the resulting P value (after Benjamini-Hochberg adjustment) was < 0.05 . Overlapping genes (i.e., genes that were similarly differentially expressed at high and low density) were identified using a custom script in R software (R Core Team 2011) (see Supplementary Material for this script).

We then mapped the differentially expressed genes to *E. coli* operons according to EcoCyc annotation (Keseler et al. 2013). Molecular functions of these genes were also enriched using DAVID (Huang et al. 2009) and subsequently mapped to KEGG pathway maps using an R package treemap (Bederson et al. 2002).

Results

Because we wanted to use the Lac phenotype to differentiate each competitor in our competition assays (using TL indicator plating; see above), our first goal was to assess whether the lac^+ and lac^- genotypes are selectively equivalent under our competition assay conditions. To test this, we performed competition assays between the $\text{CRP}^-/\text{Lac}^+$ and $\text{CRP}^-/\text{Lac}^-$ strains at both low and high founding population density. As expected, we found that the lac^+ and lac^- genotypes are selectively equivalent at both densities (one sample t tests, $P > 0.05$ for both; Table 1A).

Since the lac^+ and lac^- genotypes are selectively equivalent, we could compare the fitness of CRP^+ to CRP^- by simply competing $\text{CRP}^+/\text{Lac}^+$ against $\text{CRP}^-/\text{Lac}^-$, knowing that any fitness differences must be due to the *crp* genotype. Interestingly, we found that CRP^+ is significantly less fit than CRP^- at low founding population density (one sample t test, $P < 0.001$), but significantly more fit at high founding population density (one sample t test, $P < 0.001$; Table 1B). However, the drastic difference in the inoculation sizes between the low and high density assays leads to two potentially confounding factors in our fitness estimates,

both of which could be responsible for these observed fitness asymmetries. First, because populations in the low and high density assays completed about 27 and 6.7 generations over each 24 h growth cycle, respectively, we conducted the original low and high founding density fitness assays over one and four growth cycles to roughly equalize the number of elapsed generations. Therefore, we also performed high density fitness assays over just one growth cycle (6.7 generations), and found that this fitness estimate was comparable to the original 27 generation fitness assay: CRP⁺ is significantly more fit than CRP⁻ (one sample *t* test, $P < 0.001$; Table 1B). Second, the low and high density competitors spent ~1 and ~16 h, respectively, at stationary phase (Fig. S1) during each growth cycle, so we repeated the high density, four growth cycle assay with each cycle lasting 12 h instead of 24 h. Again, this fitness estimate was comparable to the original: CRP⁺ is significantly more fit than CRP⁻ (one sample *t* test, $P < 0.001$; Table 1B). Therefore, the number of growth cycles and the amount of time spent at stationary phase are not confounding factors responsible for the fitness asymmetries between CRP⁺ and CRP⁻ at low and high founding population densities.

CRP directly or indirectly controls the expression of >3000 genes in *E. coli* (Keseler et al. 2011). Therefore, after confirming that the S63F substitution in *crp* confers differential fitness in *E. coli* at high and low founding population density (Table 1B), we compared the global gene expression profiles of the CRP⁺/Lac⁺ and CRP⁻/Lac⁺ strains at each density using GeneChip *E. coli* Genome 2.0 Arrays. We found that, although *crp* itself was not differentially expressed in the CRP⁺ and CRP⁻ strains at either high or low founding population density, these strains did exhibit significant differential expression of 170 (Table S2) and 157 (Table S3) genes at high and low founding density. These genes come from 131 and 118 operons, respectively (Tables S2 and S3). Overall, 96 and 109 genes were up-regulated in CRP⁻ relative to CRP⁺ at high and low founding density, while 74 and 48 were down-regulated (Fig. 1). Of these total 327 differentially expressed genes, 70 were differentially expressed in the same direction at both high and low founding density (61 up-regulated and nine down-regulated; Fig. 1 and Table S4). Therefore, we observed 187 unique differentially expressed genes in the CRP⁺ and CRP⁻ strains across both population densities.

Discussion

We discovered a non-synonymous point mutation in the *crp* gene in *E. coli* K-12 that confers significantly lower fitness than the wildtype at high founding population density but significantly higher fitness at low founding density (Table 1B). The CRP protein is a dimer comprised of two identical subunits (Busby and Ebright 1999), and it regulates transcription initiation of 261 operons in *E. coli* (Keseler et al. 2011). It does this by first binding to the allosteric effector cAMP, and then binding to DNA near promoter regions, which bends the DNA and facilitates RNA polymerase binding to the promoter (Busby and Ebright 1999). The mutated amino acid residue 63 is on the fifth β -sheet of the protein, which plays a role in cAMP binding as well as dimer formation (McKay et al. 1982). We are uncertain how the S63F mutation affects these processes, but this mutation clearly does affect the expression of many genes across the *E. coli* genome (Fig. 1), though the expression of *crp* itself is not affected. In attempting to identify which of these differentially expressed genes are responsible for the fluctuating fitness differences we observed between CRP⁺ and CRP⁻

strains at high and low founding population density (Table 1B), we can probably first rule out the 70 overlapping genes (61 up-regulated and nine down-regulated) that are differentially expressed at both high and low density (Fig. 1), especially since all exhibit relatively similar expression patterns under both conditions (only six genes exhibit a >25 % difference in relative expression between conditions, and none exhibits a >50 % difference) (Table S4).

One group of candidate genes that may be responsible for the observed fitness asymmetries between CRP⁺ and CRP⁻ (Table 1B) are those involved in the transport and metabolism of sugars other than glucose, because these are the genes that CRP primarily regulates (Kolb et al. 1993). Not surprisingly, this is the Clusters of Orthologous Groups (COG) category with the greatest representation of differentially expressed genes between CRP⁺ and CRP⁻ strains (Fig. 2). Since we performed the competition assays in minimal medium with glucose as the sole carbon source, we suspect that relatively low expression levels of these genes would be selectively advantageous, simply because energy would not be wasted expressing unneeded genes (Stoebe et al. 2008). As such, if these genes are indeed playing a seminal role in driving the observed fitness asymmetries between CRP⁺ and CRP⁻, we would expect them to be down-regulated in CRP⁻ at low founding population density (wherein CRP⁻ had a higher relative fitness), but up-regulated at high founding population density (wherein CRP⁻ had a lower relative fitness). However, the differentially expressed sugar transport and metabolism genes were overwhelmingly up-regulated in CRP⁻ at both densities: 29 and 36 genes were up-regulated at high and low founding density, while only six genes were down-regulated at each density (Tables S2 and S3). Even after eliminating the overlapping genes that were differentially expressed at both high and low founding density (see above), more genes in this COG category were up-regulated than down-regulated at both densities: eight versus five genes at high founding density, and 14 versus five genes at low founding density. Therefore, the expression patterns of these genes do not provide a clear and concise explanation for the asymmetric fitnesses at low and high founding density (Table 1B).

We then turned to the 22 genes involved in homeostasis and stress resistance that were differentially expressed at either high (Table S2) or low founding population density (Table S3). Among these 22 genes, 12 are components of the acid fitness island in *E. coli*, which are organized in five operons: *slp-dctR* (also known as *yhiF*), *hdeAB-yhiD*, *gadE-mdtEF* (*yhiUV*), *gadY*, and *gadAXW* (Hommais et al. 2004). Two other genes, *gadBC*, are also involved acid resistance (Castanie-Cornet et al. 1999). These encoded proteins reduce proton concentration within cells through different mechanisms (De Biase et al. 1999; Ma et al. 2002; Hommais et al. 2004; Mates et al. 2007). Interestingly, all 14 of these acid resistance genes were significantly down-regulated in CRP⁻ at high but not low founding population density in our study (Table S2), and two of them (*yhiD* and *hdeA*) have previously been shown to confer density-dependent acid resistance in *E. coli* (Mates et al. 2007). We suspect that in our experiment, as the pH of the medium dropped from the time we initiated the cultures to when we harvested cells during mid-log phase (Fig. S2), the activation of acid resistance genes became necessary. However, at high founding density, this adaptive response was not adequately triggered in CRP⁻ (Ma et al. 2003), thus leading to significantly lower expression of acid resistance genes (Table S2) and in turn, significantly lower fitness than the CRP⁺ clone (Table 1B). At low founding density, the acid resistance genes were not

differentially expressed in CRP⁻ relative to CRP⁺ (even though the CRP⁻ clone has a slightly accelerated effect on the pH reduction of the medium (Fig. S2)), possibly because these genes exhibit some density-dependent effects (Mates et al. 2007). As such, we suspect that the differential expression of other genes (Table S3) is responsible for the CRP⁻ growth advantage over CRP⁺ at low founding density (Table 1B).

Another possible suite of genes that may be responsible for the CRP⁺ versus CRP⁻ fitness asymmetries at high and low founding population density (Table 1B) are those involved in quorum sensing (Miller and Bassler 2001). Indeed, a quorum sensing operon, *qseBC*, was down-regulated in CRP⁻ at high founding population density (Table S2) but unchanged at low founding density. Therefore, the down-regulation of these genes may be at least partly responsible for the relatively low fitness of CRP⁻ at high founding density (Table 1B). However, our microarray data do not support this hypothesis. Specifically, the QseBC protein controls the master regulator *flhDC* (Sperandio et al. 2002), which in turn primarily controls flagellum synthesis and motility (Claret and Hughes 2002; Stafford et al. 2005). When *qseBC* is down-regulated, as it was in CRP⁻ at high founding density, then *flhDC* should also be down-regulated (Sperandio et al. 2002). However, the *flhDC* genes were up-regulated in CRP⁻ at both high and low density (Table S4). The expression of *flhDC* can be controlled by other factors (e.g., Adler and Templeton 1967; Shin and Park 1995), including cAMP-CRP (Yokota and Gots 1970; Silverman and Simon 1974). Therefore, the constitutive up-regulation of *flhDC* in CRP⁻ is apparently independent of the quorum sensing genes *qseBC*, and instead is probably dependent on the S63F mutation in *crp*. Furthermore, we suspect that *flhDC* up-regulation, and thus presumably increased flagella biosynthesis, was selectively disadvantageous in our competition assays because disabling flagellum synthesis increases *E. coli*'s fitness when growing under continuous shaking (Edwards et al. 2002). If so, then CRP⁻ enjoys a higher fitness than CRP⁺ at low density (Table 1) despite the fitness cost of flagella over-production. This underscores the likelihood that the relative fitnesses of the *crp* genotypes (Table 1B) result from the sum fitness effects of all differentially expressed genes, with some genes conferring a fitness advantage and others a fitness cost (and still others having negligible fitness effects). Moreover, identifying the genes with the greatest fitness effects is probably an impossible task without altering the expression levels of individual genes (e.g., with RNAi), although even then the real cause may still be not evident considering the prevalence of epistasis in bacteria (de Visser et al. 2011). Also, the list of differentially expressed genes we identified (Tables S2–S4) is not exhaustive as all were expressed during mid-log phase and did not include differentially expressed genes in lag or stationary phase, which can also dramatically affect fitness (Vasi et al. 1994). Nonetheless, this microarray analysis reveals that a single point mutation in a global regulatory gene can have substantial effects on gene expression (Fig. 1), and ultimately fitness (Table 1B).

Although biologists have long appreciated the seminal role that intraspecific competition plays in effecting natural selection (Darwin 1859), relatively little is known about the fitness effects of mutations at different population sizes. Of course, our *E. coli crp* fitness measurements were conducted in a specific and constant environment (i.e., minimal medium supplemented with glucose, shaking at 120 rpm and 37 °C), and as such may not apply to many wild *E. coli* populations. Even so, these results and others (Novella et al. 2004; Sharp

and Agrawal 2008) underscore the possibility that population size may often influence the fitness effects, and thus the fate, of new mutations. Therefore, investigating the causes and consequences of such fitness effects may provide valuable insight into the adaptive evolution of natural populations.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.
The numbers of significantly differentially expressed genes in the *E. coli* CRP⁻ strain relative to the CRP⁺ strain, at both high and low founding population density

Fig. 2.

Quantities of differentially expressed genes at high and low founding population density according to COG functional category. For each COG category, the *dark* (*top*) and *light* (*bottom*) shaded bars represent the number of significantly differentially expressed genes at high and low founding population density, respectively, between the *E. coli* CRP⁺ and CRP⁻ strains. The COG categories are organized into four groups: red represents information storage and processing genes, *blue* represents cellular processes and signaling genes, *black* represents metabolism genes, and *green* represents poorly characterized genes

Table 1

Results of competition assays conducted among three *Escherichia coli* CRP/Lac strains at low and high founding population density

Competitors	Founding population density ^a	Number of generations	Number of growth cycles	Relative fitness of CRP ⁺ b
A. CRP ⁻ /Lac ⁺ versus CRP ⁻ /Lac ⁻	Low	25	1	1.00 (±0.02)
	High	27	4	1.03 (±0.04)
B. CRP ⁺ /Lac ⁺ versus CRP ⁻ /Lac ⁻	Low	25	1	0.93 (±0.02)***
	High	27	4	1.07 (±0.03)***
	High	6.7	1	1.07 (±0.05)***
	High	27	4 ^c	1.07 (±0.03)***

The relative fitness values (±sd) are those of the Lac⁺ clone (relative fitness is >1.0 if Lac⁺ grows faster than Lac⁻, and <1.0 if Lac⁺ grows slower than Lac⁻). (A) Selective equivalency analyses of the *lac*⁺ and *lac*⁻ genotypes. (B) Relative fitness analyses of CRP⁺ versus CRP⁻

^aInoculation sizes of each competitor; low density: ~600 CFU, high density: ~1.0 × 10⁸ CFU

^bAsterisks indicate significant *P* values: ****P* 0.001

^c12 h growth cycle duration (all others were 24 h)