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Prolonged Stationary-Phase Incubation Selects for *lrp* Mutations in *Escherichia coli* K-12

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Evolution by natural selection occurs in cultures of *Escherichia coli* maintained under carbon starvation stress. Mutants of increased fitness express a growth advantage in stationary phase (GASP) phenotype, enabling them to grow and displace the parent as the majority population. The first GASP mutation was identified as a loss-of-function allele of *rpoS*, encoding the stationary-phase global regulator, σ^S (M. M. Zambrano, D. A. Siegele, M. A. Almirón, A. Tormo, and R. Kolter, Science 259:1757–1760, 1993). We now report that a second global regulator, Lrp, can also play a role in stationary-phase competition. We found that a mutant that took over an aged culture of an *rpoS* strain had acquired a GASP mutation in *lrp*. This GASP allele, *lrp-1141*, encodes a mutant protein lacking the critical glycine in the turn of the helix-turn-helix DNA-binding domain. The *lrp-1141* allele behaves as a null mutation when in single copy and is dominant negative when overexpressed. Hence, the mutant protein appears to retain stability and the ability to dimerize but lacks DNA-binding activity. We also demonstrated that a *lrp* null allele generated by a transposon insertion has a fitness gain identical to that of the *lrp-1141* allele, verifying that cells lacking Lrp activity have a competitive advantage during prolonged starvation. Finally, we tested by genetic analysis the hypothesis that the *lrp-1141* GASP mutation confers a fitness gain by enhancing amino acid catabolism during carbon starvation. We found that while amino acid catabolism may play a role, it is not necessary for the *lrp* GASP phenotype, and hence the *lrp* GASP phenotype is due to more global physiological changes.

Natural microbial populations spend the majority of their lives under nutrient deprivation, due to intense competition for available resources (31, 32). The chemoorganotroph *Escherichia coli* can survive extended periods of carbon starvation; cells isolated from batch cultures starved for several years can still grow when supplied with exogenous carbon (11, 12). Prolonged starvation is a condition in which microbes such as *E. coli* undergo rapid evolution by natural selection: mutants with an increased fitness, termed the growth advantage in stationary phase (GASP) phenotype, grow and displace their wild-type parents as the majority (12, 41, 42, 43). The GASP phenomenon is continuous throughout the starvation period, as multiple rounds of population takeover have been observed (11, 43, 44). The first GASP mutation identified was an allele of *rpoS* (*rpoS819*) (43). Mutants with the *rpoS819* allele are referred to as G_I ($GASP_I$) strains, as they have a GASP phenotype when competed against their wild-type (G_0) parent (43, 44). Strain ZK1141, isolated from an aged culture of the *rpoS819* G_I strain (ZK819), expresses the GASP phenotype when competed against its G_I parent and was thus designated a G_{II} strain (41, 44). The G_{II} GASP phenotype of ZK1141 is due to three mutations, designated *sgaA*, *sgaB*, and *sgaC* (44). In the work reported here, we identified the *sgaB* mutation as an allele of *lrp*, encoding the leucine-responsive regulatory protein, and demonstrate that a published *lrp* null allele also confers a GASP phenotype.

The *sgaB* GASP mutation is an allele of *lrp*. Lrp is a dimeric DNA-binding protein that can act as either an activator or a repressor, depending on the promoter (6, 9, 33). Also depending on the promoter, Lrp's activity can be modulated positively or negatively by intracellular leucine levels. The Lrp regulon is extensive and includes many genes involved in amino acid metabolism and transport. In general, Lrp regulates amino acid metabolism by increasing anabolism and decreasing catabolism. Lrp appears to be a protein widely conserved in microbes, as *lrp* homologs have been detected in other proteobacteria such as *Bradyrhizobium japonicum* and *Klebsiella aerogenes*, in the gram-positive bacterium *Bacillus subtilis*, and even in the archaea *Pyrococcus furiosus* and *Sulfolobus solfataricus* (3, 7, 14, 21, 24).

The *sgaB* locus was mapped roughly to min 20 on the *E. coli* chromosome (44), and further analysis by P1 transduction (29) showed that the *sgaB* locus was closely (89%) linked to the *cydC* locus (Table 1) at min 20.0. The *lrp* locus mapped nearby at min 20.1, and like *sgaB* mutants (44), *lrp* null mutants are more sensitive than the wild type to L-serine during growth on glucose (2). For these reasons, we considered *lrp* a candidate for *sgaB* and determined the sequence of the *lrp* allele of ZK1141 and the alleles of its parents, ZK819 (G_I) and ZK126 (G_0). The *lrp* genes (including 69 upstream bases that contains the minimal promoter [25]) were PCR amplified, and both strands were sequenced. Both alleles from the parental strains ZK126 and ZK819 were identical to the published wild-type allele (Genbank accession number M35869) (40). However, we found a single lesion in the coding region of the *lrp* gene of ZK1141: an in-frame, 3-bp deletion of the bases 5′-GGA-3′, resulting in a protein that lacks the Gly-39 residue of wild-type Lrp (40).

Platko and Calvo (34) identified a putative helix-turn-helix (HTH) DNA-binding motif in the N-terminal region of Lrp (34). Most HTH proteins identified thus far have a glycine at position 9 in the HTH domain (4, 16), corresponding to the Gly-39 of Lrp absent in Lrp-1141. This glycine residue is thought to play a critical role in creating the turn structure, as its lack of a β carbon reduces any steric interference in creating

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TABLE 1. *E. coli* recipient and donor strains used in this study

Strain	Genotype; phenotype	Source or reference
Recipients		
ZK126	W3110 *tna2* Δ*lacU169*; G_0	8
ZK819	ZK126 *rpoS819 rpsL*; Sm^r, G_I	43
ZK1141	ZK819 *lrp-1141 sgaA sgaC*; G_{II}	41
ZK2552	ZK819 *ilvGMEDA*$^+$; Val^r	44
ZK2553	ZK819 *bgl*$^+$; Bgl^+	44
Donors		
ZK1240	ZK126 *cydC*(*surB1*)::miniTn*10*Km^r	35
ZK2621	ZK126 *sdaA94*::Tn*10*Cm^r	This study[a]
BE2	W3110 *lrp-35*::Tn*10*	R. G. Matthews (10)
BE3479	PS2209 *gltB*(*psiQ32*)::*lacZ* (Mu d1-1734)	R. G. Matthews (10)
CAG18467	MG1655 *zfd-1*::Tn*10*	36
CAG18493	MG1655 *zbi-29*::Tn*10*	36
DL39G	λ^- *aspC13 fnr-25 glyA42*::Tn*5 ilvE12 tyrB507*	*E. coli* Genetic Stock Center, Yale University
EC1051	*dadA279*::Mu (Ap^r *lac*) *araD139* Δ*lacU169 met1 thi trp strA*	M. Freundlich (28)

[a] The *sdaA94*::Tn*10*Cm^r insertion mutation was isolated by random insertional mutagenesis of strain ZK126 with λNK1324 (23) and screening among the Cm^r mutants for those that could not grow on serine as described elsewhere (37). The Tn*10*Cm^r insertion of this mutant was mapped by arbitrary PCR (5, 44) to the middle of *sdaA* (nucleotide 654 of 1365). Mutants with this allele showed only background level of L-SD activity (1.4 ± 7.4 versus 121 ± 21 mg of pyruvate formed min^{-1} $OD_{600}{}^{-1}$ for ZK126 [18]).

the left-handed α helix of the turn (4, 16, 30). However, in some HTH proteins other amino acids can be substituted for glycine at position 9 of the motif and still allow proper folding (16, 17). To our knowledge this is the first characterization of an HTH protein deleted for the glycine at position 9. We predicted that the mutant protein has a misfolded DNA-binding motif and would behave as a loss-of-function protein.

The *lrp-1141* GASP allele is a loss-of-function allele. To determine if the *lrp-1141* allele was a loss-of-function mutation, we compared several of its phenotypes with those of the wild-type allele and a published null allele, *lrp-35* (Table 1). All three alleles were assayed in the ZK126 (G_0) genetic background. To control for possible effects of expression from the *tetR* or *tetA* gene of the mini-Tn*10* insertion of the *lrp-35* allele (22, 23), we introduced into the *lrp*$^+$ and *lrp-1141* strains by P1 transduction a mini-Tn*10* transposon linked (22%) to the *lrp* locus. This mini-Tn*10* insertion did not affect the outcome of the assays described below, nor did it affect the GASP phenotype of the G_I *lrp-1141* strain (data not shown).

Strains with *lrp* null alleles are reported to grow more slowly than wild type on glucose and have an increased sensitivity to serine during growth on glucose (2). We confirmed these results and observed that the *lrp-1141* mutant behaves identically to the *lrp-35* null mutant in both assays. As a more sensitive assay for Lrp activity, we measured L-serine deaminase (L-SD) activity in these strains (18). The major L-SD of *E. coli* is encoded by *sdaA*, whose expression is repressed directly by Lrp and activated by leucine and glycine; the leucine activation is due in part to relief of Lrp-mediated repression (26, 37). We assayed L-SD activity as previously described (18) for the wild type and the two *lrp* mutants grown in the presence or absence

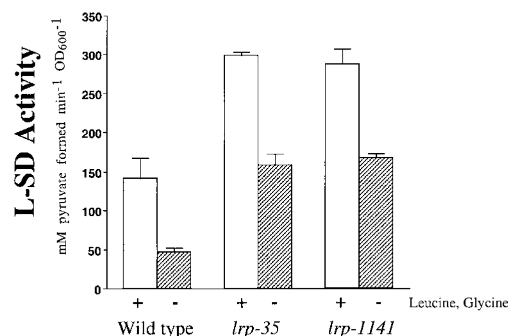

FIG. 1. The *lrp-1141* mutant has the same L-SD activity as the *lrp-35* mutant. L-SD activity was assayed in mid-exponential-phase cells (OD_{600} of 0.3) grown in M63 glucose (0.2%)–valine (0.005%)–isoleucine (0.005%) medium, with (open bars) or without (hatched bars) the L-SD inducers glycine (0.0075%) and leucine (0.005%). Bars indicate standard deviations ($n \geq 4$).

of the L-SD inducers glycine and leucine. In both conditions, the *lrp-1141* mutant showed L-SD activities (milligrams of pyruvate formed per minute per unit of optical density at 600 nm [OD_{600}]) indistinguishable from the *lrp* null allele activity (Fig. 1). Similar results were observed in assays of an Lrp-activated gene, *gltB* (see below). These results indicate that *lrp-1141* is a null allele, as it shows no residual repression or activation activity at these promoters.

The 2-fold-higher L-SD activity in the *lrp* mutants grown in the presence of leucine and glycine is interesting, considering previous reports demonstrating that leucine alone increased L-SD activity only 1.2-fold in *lrp* mutants (26). As glycine and leucine increase L-SD activity in an additive manner (18), these results indicate that unlike leucine, glycine may induce L-SD in an Lrp-independent manner. Alternatively, the different findings may be due differences in strain backgrounds.

The *lrp-1141* allele is dominant negative when overexpressed. Mutations in the DNA-binding domain of *lrp* that disrupt the DNA-binding activity but do not affect its stability or its ability to dimerize act in a dominant-negative manner, as their protein products are stable and can form heterodimers with the wild-type Lrp monomers that cannot bind DNA (34). To determine if the *lrp-1141* allele is dominant negative, we cloned both the mutant and wild-type genes, including 288 bases of upstream sequence, and introduced these clones by transformation into strains with different chromosomal *lrp* alleles. The *lrp*$^+$ and *lrp-1141* alleles of ZK126 and ZK1141 were amplified by PCR with primers engineered with 5′ ends recognizable by the restriction enzyme *Eco*RI, and these *Eco*RI-digested PCR products were ligated into the low-copy-number plasmid pSU2718 (27), creating plasmids pEZ1 (*lrp*$^+$) and pEZ2 (*lrp-1141*). We sequenced both strands of the cloned inserts and confirmed that they were the correct alleles. For both constructs, pEZ1 and pEZ2, the *lrp* gene was inserted in an orientation opposite that of the *lac* promoter of the pSU2718 vector.

To determine the dominant or recessive nature of the *lrp-1141* allele, three assays were performed on the strains carrying various combinations of *lrp* alleles on the chromosome (single copy) and on a plasmid (multicopy). We assayed two growth phenotypes, colony size and serine sensitivity, on M63 glucose plates as described above. We also assayed transcription of the *gltBDF* operon, encoding glutamate synthase, which is positively regulated by Lrp (10). The *gltB*::*lacZ* transcriptional fusion (Table 1) was crossed into our strains by P1 transduction. The results of all three assays were the same: when the *lrp-1141*

TABLE 2. Phenotypes of strains with various alleles of *lrp* on the chromosome and in the pSU2718 vector[a]

lrp allele		Phenotype on M63 glucose (0.2%)		*gltB*::*lacZ* activity[c]
Chromosome	Plasmid	Relative colony size	Growth on serine[b]	
+	None	++	r	286 (10)
+	+	++	r	297 (22)
+	*lrp-1141*	+	s	21.4 (1.0)
lrp-1141	None	+	s	13.6 (1.0)
lrp-1141	+	++	r	265 (24)
lrp-1141	*lrp-1141*	+	s	25.2 (1.3)
lrp-35	None	+	s	27.1 (3.5)
lrp-35	+	++	r	296 (9)
lrp-35	*lrp-1141*	+	s	27.3 (3.2)

[a] All assays included chloramphenicol (30 μg/ml) to ensure plasmid maintenance.
[b] Determined as sensitive (s) or resistant (r) by filter disk method (44).
[c] β-Galactosidase activity of mid-exponential-phase cells grown in M63 medium plus glucose (0.4%), leucine (10 mM), isoleucine (0.4 mM), and valine (0.6 mM) was measured as described elsewhere (10). Activities are reported in Miller units (29); numbers in parentheses are standard deviations ($n \geq 4$).

allele is in multicopy, it is dominant to the single-copy wild-type allele (Table 2). These results indicate that the Lrp-1141 protein is stable and can form inactive heterodimers with the wild-type Lrp monomers. However, the wild-type allele in multicopy is dominant to the *lrp-1141* allele in single copy. Overexpression of the wild-type allele most likely restores Lrp-dependent activity by increasing the total concentration of wild-type monomers such that, in addition to the inactive heterodimers, a sufficient amount of active wild-type homodimers form.

The *lrp-35* null mutation also confers a GASP phenotype. If *lrp-1141* is a null allele, then the *lrp-35* null allele should exhibit the same GASP phenotype as *lrp-1141*. We therefore determined the stationary-phase fitness of the *lrp-35* null allele relative to the lrp^+ allele by competition assays in the G_I (*rpoS819*) background, as described previously (44). We eliminated any possible fitness losses of the *lrp-35* strains due to expression from the mini-Tn*10* insertion by competing the *lrp-35* mutants against two different lrp^+ strains carrying either the *zbi-29*::Tn*10* or *zfd-1*::Tn*10* intergenic insertion (Table 1). Competing strains carried one of two neutral markers to distinguish them: the ability to grow on β-glucosides (Bgl^+) or the ability to grow on glucose in the presence of valine (Val^r) (44). These markers were switched between strains to confirm that they did not affect fitness.

Unlike the lrp^+ control strain (Fig. 2A), the *lrp-1141* strain expresses a GASP phenotype when inoculated as a 1,000-fold minority into a culture of the lrp^+ strain (Fig. 2B) (44). As predicted, the *lrp-35* null mutant exhibited a GASP phenotype essentially identical to that of the *lrp-1141* mutant (Fig. 2C). This result confirmed that loss-of-function mutations in *lrp* confer a GASP phenotype on *E. coli*. This indicates that the Lrp regulon is induced during prolonged carbon starvation, and that activity of this regulon under these conditions results in a fitness loss relative to those cells unable to express this regulon.

The *lrp-1141* and *lrp-35* alleles compete with equal fitness. The possibility remained that there is some undetected activity of the Lrp-1141 protein that plays a role in GASP. In fact, *lrp-1141* mutants, unlike *lrp-35* null mutants, display mucoid growth on glucose at 30°C (44). However, the *lrp-1141* mutants displayed no fitness advantage relative to the *lrp-35* null mutants when competed in stationary phase (data not shown). Hence, the mucoidy phenotype of *lrp-1141* plays no role in stationary-phase competitions, and we conclude that it is the loss-of-function nature of the *lrp-1141* allele that is responsible for the GASP phenotype.

Since there was no detectable fitness difference between the *lrp-1141* and insertion (null) allele, we found it surprising that the GASP allele we isolated from a starved culture was *lrp-1141*. Small, in-frame deletions such as *lrp-1141* should be rare among the total array of spontaneous null mutations possible, as only one-third of all spontaneous deletions are in frame, and deletions themselves would occupy only a small percentage of the null mutations possible. At least two factors independent of relative fitness may have increased the likelihood of isolating a strain with such an unexpected GASP allele.

One possible factor is that *lrp* contains a potential deletion hot spot. The 3-bp deletion of the *lrp-1141* allele overlaps the sequence 5′-GTGG-3′ of the wild-type allele, which has been identified as a hot spot for spontaneous mutation in *E. coli*: a high frequency of mutations was found at or in close proximity to this sequence in wild-type strains (13), and even higher frequencies were found in $PolA^-$ strains (13, 19). Among the classes of spontaneous mutations associated with this sequence was a high frequency of deletion mutations, many of which were small. It is therefore possible that this same (unknown) mechanism acted at the 5′-GTGG-3′ hot spot of *lrp* and facilitated the *lrp-1141* deletion. If so, this may indicate a lowered

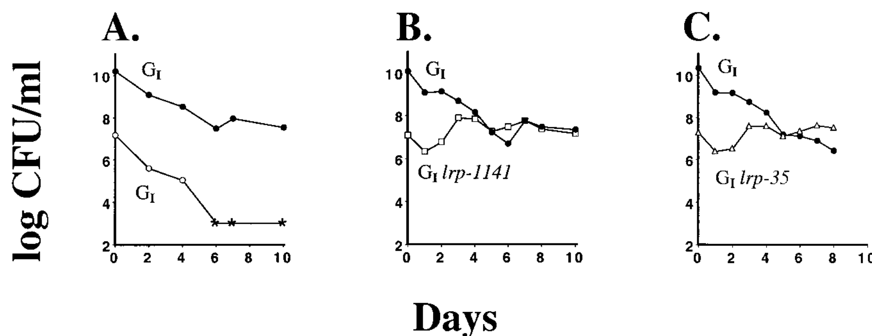

FIG. 2. The *lrp-35* mutation confers a competitive advantage to G_I cells. Into a 1-day-old LB culture of a Val^r G_I mutant (●) was inoculated as a 1,000-fold minority a 1-day-old LB culture of a Bgl^+ G_I mutant (A; ○), G_I *lrp-1141* mutant (B; □), or G_I *lrp-35* mutant (C; △). Viable counts of the two competing populations were determined by titering the mixed culture onto M63-glucose-valine or M63-salicin plates. Asterisks indicate that viable counts fell below detectable levels ($<10^3$ CFU/ml). Similar patterns were observed in six replicate mixtures per strain pairing, including ones when the selectable markers were switched between strains. Data for panel B are from reference 44.

level of DNA polymerase I activity during prolonged starvation, that is, that *polA*$^{+}$ cells may be phenotypically PolA^{-} in stationary phase.

A second possible factor leading to the selection of *lrp-1141* is its dominant nature. While recessive mutations do not change the cell's physiology until the wild-type product disappears (by degradation or dilution resulting from cell division), dominant mutations can potentially change the cell's physiology as soon as its mutant product is made. Hence, dominant GASP mutations (dominant negative or gain of function) may predominate in starved populations because they can change the physiology of the cell more rapidly than recessive mutations. This may be of critical importance to starved cells, if dilution of wild-type protein by cell division is rare or absent and the cells have only a limited time to acquire GASP mutations before they are outcompeted for nutrients and die. Of particular relevance to this model is the finding that starved *E. coli* cells can have multiple copies of their chromosome (1), which would make it even more difficult for a recessive loss-of-function GASP phenotype to be expressed. It would therefore be of great interest to isolate and characterize the nature of *lrp* and other GASP mutations of other starvation survivors to determine the frequency of dominant versus recessive GASP alleles.

Mutations that disrupt alanine, serine, and threonine catabolism do not prevent the *lrp* GASP phenotype. Previously, we demonstrated that the *sgaB* GASP allele (*lrp-1141*) conferred faster growth when serine, threonine, or alanine was used as the sole carbon and energy source (44). Lrp represses the genes encoding the primary catabolic enzymes for these amino acids: *sdaA* (L-SD), *glyA* (serine hydroxymethyltransferase), *dadAX* (D-alanine deaminase and alanine racemase), and *kbl-tdh* (2-amino-3-ketobutyrate coenzyme A lyase and threonine dehydrogenase) (28, 33). Thus, the most likely reason why *lrp* mutants grow more rapidly than the wild type on these amino acids is that the quantity of these enzymes in the wild type is growth rate limiting, and *lrp* mutants produce more of these enzymes.

We proposed a model that the increased catabolism of these amino acids is responsible for the GASP phenotype of the *sgaB* (*lrp-1141*) mutant (44). To address this model, we constructed *sdaA*, *glyA*, and *dadA* insertion mutants of the G_I *lrp-1141* strain by P1 transduction (Table 1) and assayed for a loss of the GASP phenotype. Significantly, G_I *lrp-1141* strains with mutations in any of the three genes, or any combination of the three, were still able to outcompete the G_I strain. These results indicate that enhanced catabolism of alanine, serine, and threonine is dispensable for the *lrp* GASP phenotype. However, while not essential, these catabolic activities may still play a significant role stationary-phase competition, as *sdaA* mutants in the G_0, G_I, and G_I *lrp-1141* backgrounds were outcompeted by their respective *sdaA*$^{+}$ parents (data not shown).

It is of particular note that the two known GASP loci, *lrp* and *rpoS*, are both regulators of many genes. Mutations in regulators such as these make global shifts in metabolism and physiology, often with a coordinated effect (for instance, enhanced catabolism of multiple amino acids in *lrp* and *rpoS* mutants [44]). Therefore, while altering a single activity may increase fitness, altering many activities simultaneously by altering the function of a global regulator may result in an even higher fitness gain. Hence, beneficial mutations in global regulators may be selected over other mutations when bacteria are exposed to new environments. In support of this hypothesis are reports that clinical and soil isolates of *E. coli* and salmonellae have extensive allele variation of *rpoS* (15, 20, 38; E. R. Zinser and R. Kolter, unpublished data) indicating that there is considerable selective pressure acting on the *rpoS* locus in the natural world.

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