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Mutations That Activate the Silent *bgl* Operon of *Escherichia coli* Confer a Growth Advantage in Stationary Phase

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Wild-type strains of *Escherichia coli* are unable to utilize aromatic β-glucosides such as arbutin and salicin because the major genetic system that encodes the functions for their catabolism, the *bgl* operon, is silent and uninducible. We show that strains that carry an activated *bgl* operon exhibit a growth advantage over the wild type in stationary phase in the presence of the *rpoS819* allele that causes attenuated *rpoS* regulon expression. Our results indicate a possible evolutionary advantage in retaining the silent *bgl* operon by wild-type bacteria.

In *Escherichia coli*, the primary genes involved in the uptake and utilization of the aryl-β-glucosides salicin and arbutin are encoded by the *bgl* operon (22). The operon comprises three genes, *bglG*, *bglF*, and *bglB* (18, 28). BglG is a positive regulator of the operon acting as an antiterminator, BglF is the *bgl*-specific component of the phosphotransferase that is also a negative regulator of the operon, and BglB is the phospho-β-glucosidase responsible for cleaving the phosphorylated substrate. The *bglR* locus comprises the promoter and other *cis*-regulatory elements. Most wild-type strains are phenotypically Bgl^-, as the *bgl* genes are not transcribed even in the presence of aryl-β-glucosides. Various *cis*-acting elements that include DNA structural elements and *trans*-acting factors, such as the global regulator H-NS, are responsible for the silencing of the *bgl* promoter (16, 20, 25, 26, 31). However, Bgl^+ mutants arise spontaneously in the population at a detectable frequency (6, 9, 13, 23, 24, 29, 33). Once activated, *bgl* expression is specifically regulated by aryl-β-glucosides (1, 2, 3, 17, 27) and is subject to catabolite control (10, 11).

The fact that the *bgl* operon, in spite of being silent, has been maintained without accumulating deleterious mutations is intriguing. One explanation for the retention of such cryptic genes by the organism is that these genes, although silent under the conditions of observation, may be expressed under specific physiological conditions, providing a selective advantage to the organism (21). Thus, it is worthwhile to explore different physiological conditions that could affect the expression of such cryptic genes and thereby gain insights into their evolutionary significance. In the present study, an attempt has been made in this direction with regard to the *bgl* operon of *E. coli*.

Microorganisms such as *E. coli* undergo rapid evolution during prolonged incubation in stationary phase (35). Under these conditions, while the majority of the population dies, a small minority can grow and take over the culture (7, 8, 34, 35). These survivors carry mutations that confer a growth advantage in stationary phase, or GASP. GASP is a continuous phenomenon, i.e., during prolonged incubation in stationary phase, various population takeovers occur as different mutants with increased fitness outgrow the rest of the population (7, 35). As the cultures age, they increase in diversity as several genetically distinct subpopulations coexist. The first GASP mutations identified were in the *rpoS* locus. The *rpoS* gene product is an alternative sigma factor, which regulates the expression of several genes involved in stress response during stationary phase (12, 14, 15, 30, 32). One of the well-characterized *rpoS* mutations that confers the GASP phenotype is the *rpoS819* allele that has a 46-bp duplication at the 3′ end of the gene, causing a frameshift that replaces the last four amino acids with 39 new residues, resulting in attenuated *rpoS* regulon expression (35). Only *rpoS* mutations that result in attenuated expression and not deletions are known to confer the GASP phenotype. The exact physiological basis for the GASP phenotype of strains that harbor the *rpoS819* allele is not clearly understood, but the strains are able to grow faster in the presence of certain amino acids (36).

The present study was undertaken to investigate the possible role of the *bgl* operon in conferring a GASP phenotype. Here, we show that mutants that harbor an activated *bgl* operon accumulate among the survivors of prolonged starvation and that an activated *bgl* operon confers a growth advantage to the organism in stationary phase in strains that carry the *rpoS819* allele.

MATERIALS AND METHODS

Bacterial strains. *The E. coli* strains used in this study are shown in Table 1.

Media and growth conditions. Cells were grown in Luria broth with aeration at 37°C. For long-term cultures, the tubes were topped up with sterile distilled water to compensate for evaporation whenever necessary. Antibiotics were added at the following concentrations: tetracycline (15 μg/ml), kanamycin (50 μg/ml), streptomycin (25 μg/ml), and nalidixic acid (20 μg/ml).

Mixed cultures. Cultures were grown in 3 ml of Luria broth in glass test tubes under aeration at 37°C in a New Brunswick shaker. Mixed cultures were started after 24 h of growth by transference of appropriate volumes of the two cultures in a fresh tube with a total final volume of 3 ml. The titers of the cultures were determined by plating different dilutions on LB plates containing the appropriate antibiotics. Markers used to distinguish the strains in mixed cultures (either for streptomycin resistance and nalidixic acid resistance or for tetra-

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TABLE 1. *E. coli* strains used in this study

Strain	Relevant genotype or phenotype	Reference or source
ZK819	ZK126 *rpoS819* (Str^r Bgl$^-$)	35
ZK820	ZK126 *rpoS819* (Nal^r Bgl$^-$)	35
ZK819-97	ZK819 *bglR* (Bgl$^+$)	This study
ZK819-97T	ZK819 *tna*::Tn*10 bglR* (Bgl$^+$)	This study
ZK819-97Δ*bgl*	ZK819-97T Δ*bglRGFB* (Bgl$^-$)	This study
ZK819-97ΔB	ZK819-97T *bglB104* (Sal$^-$ Arb$^+$)	This study
ZK819Tn*10*	ZK819 Tet^r	This study
ZK819Tn*5*	ZK819 Kan^r	This study
AE328	RV *tna*::Tn*10 bglR*::IS*1* (Bgl$^+$)	A. Wright
MG RpoS$^+$ Cm^r	MG1655 *rpoS*$^+$ Cm^r	J. Gowrishankar
ZK819-IS1	ZK819 *tna*::Tn*10 bglR*::IS*1* (Bgl$^+$)	This study
ZK819 Tn*5* RpoS$^+$	ZK819 Tn*5 rpoS*$^+$ Cm^r	This study
ZK819-97T RpoS$^+$	ZK819-97T *rpoS*$^+$ Cm^r	This study

cycline resistance and kanamycin resistance) were found to be neutral in our experimental conditions.

Genetic manipulations. Phage P1 transduction was performed as described by Miller (19).

Construction of the *bgl* deletion strain. The *bgl* deletion was created using the procedure of Datsenko and Wanner (5). Hybrid primers with 36-bp extensions homologous to the *bgl* sequences spanning positions −110 (*bglR*) and +3920 (*bglB*) were designed for the template plasmid pKD4 carrying the kanamycin resistance gene that is flanked by FLP recognition target sites (forward primer, 5′ ATATAACTTTATAAATTCCTAAAATTACACAAAGTTGTGTAGGCT GGAGCTGCTTCG 3′; reverse primer, 5′ AATGCTTTCGTCGTGGGAAAC ACAACCAGTCATGTACATATGAATATCCTCCTTA 3′]. The PCR product generated using the primers carried a deletion of *bglR*, *bglG*, *bglF*, and a large segment of *bglB*. The deleted sequences were replaced by the kanamycin resistance gene. The deletion was recombined at the *bgl* locus in the strain ZK819-97T using the helper plasmid pKD46 expressing the phage lambda red recombinase, with kanamycin resistance serving as a selectable marker. The resistance gene was then eliminated using the helper plasmid pCP20 that expresses the FLP recombinase, leaving a scar of about 80 bp at the *bgl* locus. The 80-bp sequence carries an ideal ribosome binding site and an initiation codon to eliminate polar effects on downstream sequences. The deletion was confirmed by PCR analysis and the inability of the strain to papillate to Bgl$^+$.

Construction of the *bglB* mutant strain carrying a disruption of *bglB*. The *bglB* mutant strain was created using the same procedure described above. Hybrid primers with a 36-bp extension homologous to the sequences spanning positions +314 and +315 of the *bglB* open reading frame were designed for the template plasmid pKD3 carrying a chloramphenicol resistance gene that is flanked by FLP recognition target sites (forward primer, 5′ CGACGAAGTCGAACCGAATGA AGCGGGGTTAGCGTTGTGTAGGCTGGAGCTGCTTCG 3′; reverse primer, 5′ CCCGCCTGCGCCATTTCATCAAACAGCCGATCGTAACATATGAA TATCCTCCTTA 3′). The PCR product generated was used to disrupt the *bglB* gene in the strain ZK819-97T. The resistance gene was then eliminated, leaving an 80-bp scar that disrupts the 5′ end of the *bglB* gene after residue 104. The disruption was confirmed by PCR analysis, the strain's Arb$^+$ Sal$^-$ status, and its inability to papillate to Sal$^+$ on MacConkey-salicin medium.

RESULTS

Bgl$^+$ mutants are found among the survivors present in 28-day-old cultures. One hundred and fifty survivors from 10 Luria broth cultures of the strain ZK819 maintained at 37°C for 28 days with aeration were randomly chosen for analysis. Of these, five isolates were found to be Bgl$^+$. In four of the five Bgl$^+$ isolates, namely, ZK819-66, ZK819-67, ZK819-71, and ZK819-73, all obtained from the same culture, the activating mutation was in the *hns* locus, as indicated by their phenotypic reversion to the Bgl$^-$state in the presence of a plasmid carrying the wild-type *hns* gene. The remaining Bgl$^+$ isolate, ZK819-97, was Bgl$^+$ even in the presence of the *hns* plasmid, suggesting that the mutation was not within *hns*. Transduction using P1 phage grown on a strain carrying a Tn*10* transposon within the *tna* locus (80% linked to the *bgl* operon) indicated that the activating mutation in ZK819-97 is linked to the *bgl* operon.

The *bgl* regulatory region in ZK819-97 was amplified by PCR to determine the nature of the activating mutation. The size of the fragment obtained was similar to that of the wild-type parent strain, indicating the absence of insertion sequences within the regulatory region, which is a common activating mutation. Nucleotide sequence analysis of the regulatory region revealed the presence of a point mutation, a C-to-T transition, at the cyclic AMP (cAMP) receptor protein (CRP)-cAMP binding site in the regulatory region of the operon. This transition, which brings the site closer to the CRP binding consensus sequence, is known to activate the *bgl* operon (16, 24). Up to now, this point mutation has been only rarely observed among natural Bgl$^+$ isolates and has normally been obtained only after mutagenesis.

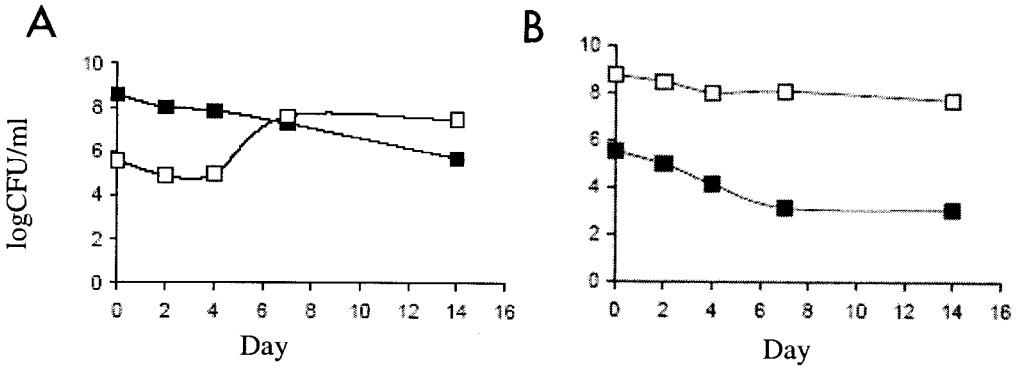

FIG. 1. Competition experiments between ZK819-97 (Bgl$^+$) (□) and ZK820 (Bgl$^-$) (■). One-day-old cultures of the two strains were mixed in ratios of 1:1,000 (A) and 1,000:1 (B).

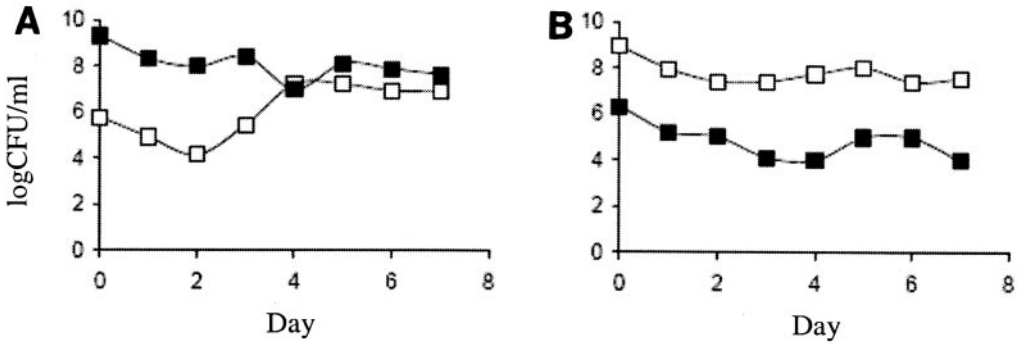

FIG. 2. Competition experiments between ZK819-97T (Bgl^+) (□) and ZK819Tn5 (Bgl^-) (■). One-day-old cultures of the two strains were mixed in ratios of 1:1,000 (A) and 1,000:1 (B).

The Bgl^+ strain ZK819-97 shows a GASP phenotype when competed against the parent. As the Bgl^+ strain ZK819-97 is a survivor of prolonged starvation, it is conceivable that the strain exhibits a GASP phenotype. To test this possibility, ZK819-97 was competed against the Bgl^-strain, ZK820, which is isogenic to the parent strain ZK819 but carries a different antibiotic resistance marker. These studies showed that the Bgl^+ strain was able to take over the culture in stationary phase even when it was initially present in a minority (Fig. 1).

ZK819-97 is likely to carry other mutations in addition to the one responsible for its Bgl^+ phenotype. To determine whether the GASP phenotype is related to the Bgl status, the *bgl* allele from ZK819-97 was transferred by P1 transduction to its parental strain, ZK819, using the Tn*10* transposon within *tna* that is linked to the *bgl* operon. The resulting strain, ZK819-97T, thus is isogenic to the parental strain ZK819, except for containing an activated *bgl* allele. To determine whether the activated *bgl* allele in this strain confers upon it the GASP phenotype, competition experiments were carried out between ZK819-97T carrying the transposon Tn*10* and the parent (Bgl^-) strain ZK819 carrying the transposon Tn*5*. The transposon insertions were used as selective markers and were shown to be neutral in control experiments. The Bgl^+ strain was mixed with the parent strain in different ratios, and the

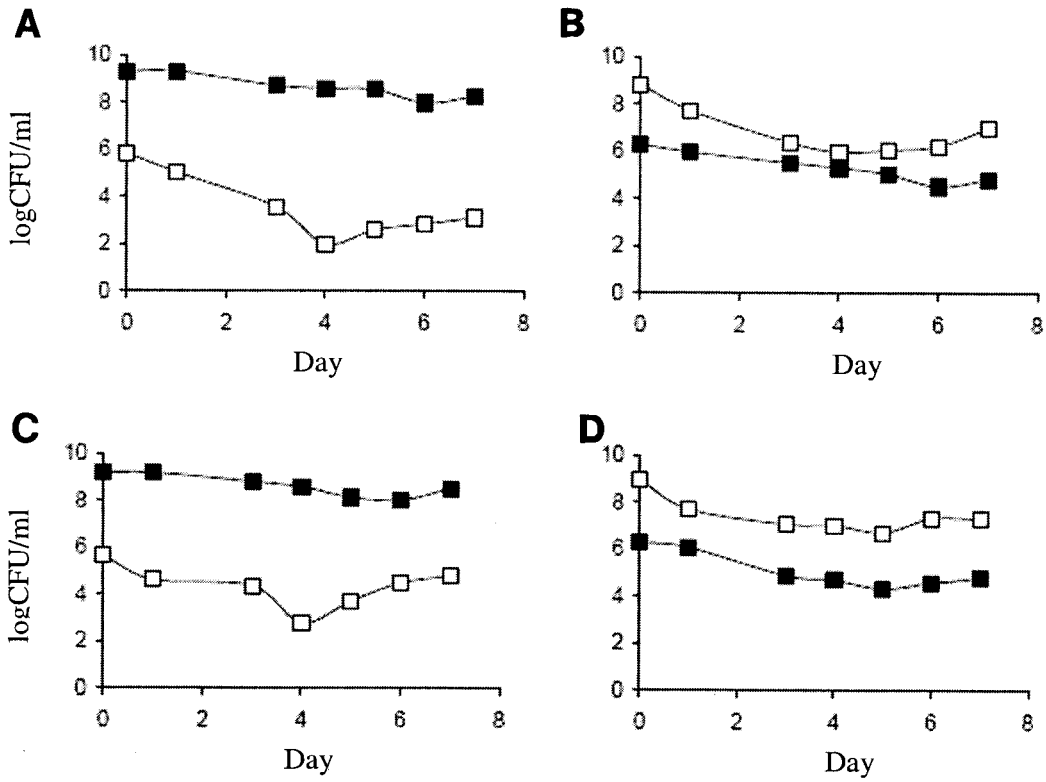

FIG. 3. (A and B) Competition assays of ZK819-97Δ*bgl* (□) with the wild-type strain ZK819Tn5 (■). One-day-old cultures of two strains were mixed in ratios of 1:1,000 (A) and 1,000:1 (B). (C and D) Competition assays of ZK819-97ΔB (□) with the wild-type strain ZK819Tn5 (■). One-day-old cultures of two strains were mixed in ratios of 1:1,000 (C) and 1,000:1 (D).

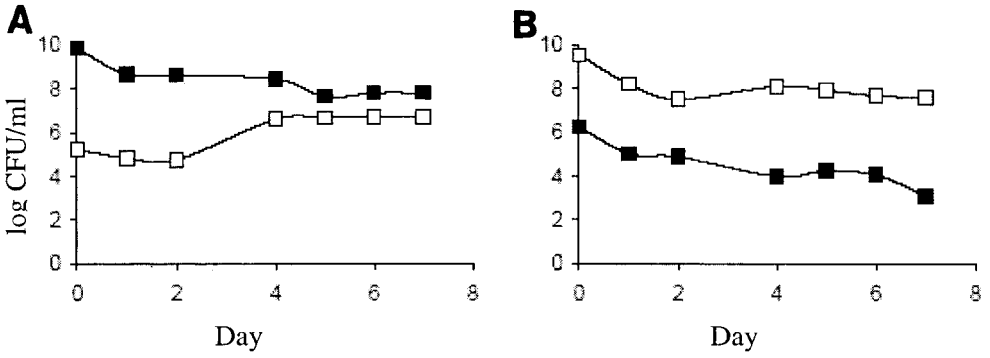

FIG. 4. Competition assays of ZK819-IS1 (□) with ZK819Tn5 (■). One-day-old-cultures of the two strains were mixed in ratios of 1:1,000 (A) and 1,000:1 (B).

population numbers of the two strains were followed during prolonged incubation in stationary phase. One-day-old cultures of the two strains grown in LB were mixed reciprocally at a 1:1,000 ratio, and the populations were monitored by viable count assay. Each competition was performed at least four times. Results from representative mixtures are shown in Fig. 2. As can be seen from the results, the Bgl^+ strain, when added as a minority in the mixed cultures, was able to grow and in about 4 days reach numbers that nearly equal those of the Bgl^-strain. When the Bgl^-strain was added in the minority, its viable counts did not increase. These results indicate a growth advantage for the Bgl^+ strain compared to its parent.

Deletion of the *bgl* locus from the Bgl^+ strain results in the loss of the fitness advantage. For the competition experiments between Bgl^+ and Bgl^-strains, the activated *bgl* allele was transduced from the Bgl^+ survivor ZK819-97 to its parental strain ZK819 by means of P1 transduction. During P1 transduction, a large fragment of DNA (about 90 kb) was transferred to the recipient that may include additional mutations. To associate the GASP phenotype of the strain ZK819-97T specifically with its activated *bgl* allele, the *bgl* locus was deleted from ZK819-97T. If the GASP phenotype of ZK819-97T is associated with the activated *bgl* allele, deletion of the *bgl* locus should result in the loss of its GASP phenotype. To test this, a deletion of the *bgl* locus in ZK819-97T was constructed using the lambda red recombination system (5). This strain, ZK819-97Δ*bgl*, was competed against ZK819. As shown in Fig. 3A, the strain ZK819-97T lost the GASP phenotype when the *bgl* locus was deleted, indicating that the GASP phenotype is specifically associated with the activated *bgl* allele.

Disruption of the *bglB* locus in the Bgl^+ strain results in the loss of the GASP phenotype. To test whether the β-glucosidase function encoded by the *bglB* gene of the *bgl* operon is essential for the GASP phenotype of the strain ZK819-97T, the *bglB* gene was disrupted in this strain. The resulting strain was unable to utilize salicin due to the absence of BglB, although it was still capable of utilizing arbutin, as the constitutive *bglA* locus was present in the strain. This strain, ZK819-97ΔB, was competed against ZK819. As shown in Fig. 3B, the strain ZK819-97TΔB in which the *bglB* locus was disrupted lost the GASP phenotype, indicating that a functional BglB is required for the GASP phenotype.

The GASP phenotype of Bgl^+ strains is independent of the nature of the activating mutation. The activating mutation in the strain ZK819-97T is a point mutation within the CRP-cAMP binding site. The more commonly seen mutations that activate the *bgl* operon are insertions of IS*1* and IS*5* in *bglR*, upstream of the promoter. To determine if the GASP phenotype of ZK819-97T is related to the specific *bgl* allele, the *bgl* operon from a strain carrying an activating IS*1* insertion in *bglR* was transduced into ZK819. The resulting Bgl^+ strain, ZK819-IS1, was competed against the parent ZK819. These results, shown in Fig. 4, indicate that the activation of the *bgl* operon by IS*1* also confers a GASP phenotype to the strain.

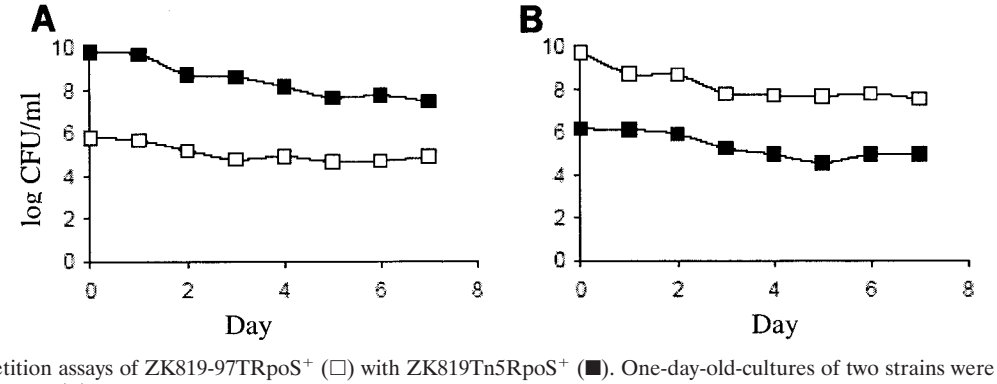

FIG. 5. Competition assays of ZK819-97TRpoS$^+$ (□) with ZK819Tn5RpoS$^+$ (■). One-day-old-cultures of two strains were mixed in ratios of 1:1,000 (A) and 1,000:1 (B).

The *rpoS819* allele is necessary for the GASP phenotype conferred by *bgl* activation. In the experiments described above, the activated *bgl* allele was always associated with the *rpoS819* allele that can independently confer a GASP phenotype. To test whether the GASP phenotype of ZK819-97T can persist in the absence of the *rpoS819* allele, the wild-type *rpoS* allele was transduced into ZK819-97T. The resulting strain, ZK819-97TRpoS$^+$, failed to show the GASP phenotype when competed against the Bgl$^-$ *rpoS*$^+$ parent (Fig. 5). These results indicate that the selective advantage conferred by the Bgl$^+$ state in stationary phase requires the presence of the *rpoS819* mutation.

DISCUSSION

The retention of cryptic genes such as the *bgl* operon, without the accumulation of deleterious mutations, remains an evolutionary puzzle. Here, we have shown that Bgl$^+$ mutants were found among the survivors of prolonged incubation in stationary phase; these strains exhibited the GASP phenotype compared to their parent. These results suggest that expression of the *bgl* genes confers a fitness advantage during stationary phase. Interestingly, this advantage manifests only in the presence of the *rpoS819* allele that has been shown to be a strong GASP locus (35). Though the mechanism involved in the growth advantage conferred by the *rpoS819* allele is unknown, one possibility is that the mutation provides additional metabolic capabilities (36). The activation of the *bgl* genes may help augment this capability.

The precise physiological reasons for the advantage conferred by the activated *bgl* allele under these conditions are not clear at present. The growth medium used in these studies is LB with no added aryl-β-glucosides. However, it is possible that the debris from the dying cells, such as the breakdown products of the peptidoglycan cell wall, contains β-glucosides that provide a catabolizable source of energy. The loss of the GASP phenotype in the strain carrying a disruption of *bglB* encoding the phospho-β-glucosidase B is consistent with this possibility. Another possibility is that the *bgl* operon, besides allowing the utilization of aryl-β-glucosides, might provide an additional function(s) to the organism. For instance, the signaling system involving the BglG-BglF regulators could be utilized in the regulation of other genetic systems that may have a role during stationary phase. This is consistent with the observation that orthologues of BglG and BglF are involved in the regulation of pathogenicity in *Listeria monocytogenes* (4). Though the present studies do not allow quantification of the GASP effect, the loss of the GASP phenotype in the Δ*bgl* strain could be due to the cumulative effect of the loss of both functions. Until the putative downstream targets are identified, this is speculative.

In our analyses, we confined ourselves to mutations that are directly linked to the *bgl* operon. In four other survivors that showed a Bgl$^+$ phenotype (probably siblings), the activating mutation was in the *hns* locus. Since H-NS is a global regulator, it will be difficult to evaluate whether possible fitness effects of the mutation are due to the activation of the *bgl* operon or to a pleiotropic effect of the *hns* mutation.

If the active *bgl* allele provides a fitness advantage in the stationary phase, why is the *bgl* operon silent in most wild-type cells? One possibility is that there is a fitness cost associated with the active allele under specific environmental conditions. The relative ease with which the operon can be activated by insertion elements and other mutations provides a mechanism for the generation of the active allele. This is reminiscent of the selection for the attenuated alleles of *rpoS* as the primary GASP mutations under prolonged incubation in stationary phase. Our studies show that additional mutations in the *bgl* locus enhance this advantage. Thus, rather than being a "cryptic" genetic element, the *bgl* operon appears to be a dynamic component of the bacterial genome.

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