Adaptive Mutagenesis at *ebgR* Is Regulated by PhoPQ

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Adaptive mutations are mutations that occur in nondividing or very slowly dividing microbial cells during prolonged nonlethal selection and that are specific to the challenge of the selection in the sense that the only mutations that can be detected are those that provide a growth advantage to the cell. The *phoPQ* genes encode a two-component positively acting regulatory system that controls expression of at least 25 to 30 genes in *Escherichia coli* and *Salmonella typhimurium*. PhoPQ responds to a variety of environmental stress signals including Mg^{2+} starvation and nutritional deprivation. Here I show that disruption of *phoP* or *phoQ* by Tn*10*dCam significantly reduces the adaptive mutation rate to *ebgR*, indicating that the adaptive mutagenesis machinery is regulated, directly or indirectly, by *phoPQ*. The finding that it is regulated implies that adaptive mutagenesis does not simply result from a failure of various error correction mechanisms during prolonged starvation.

Adaptive mutations differ from growth-dependent mutations in two key respects. First, adaptive mutations occur in nondividing or slowly dividing cells which are under selection for a particular phenotype, whereas growth-dependent mutations occur in dividing cells that are not under strong selection. Second, adaptive mutations produce only those phenotypes which allow the cells to grow (13), whereas growth-dependent mutations occur randomly with respect to their effects on fitness. The mechanisms for producing adaptive mutations remain unknown although numerous speculative models have been proposed.

Adaptive mutations are usually observed by spreading a population of bacteria or yeast onto medium upon which growth cannot occur unless a known mutation reverts. The first revertants to appear are presumed to be the result of mutations that were present in the population prior to plating. Typically, additional revertant colonies continue appear for periods of several days up to a month, and it is those late-appearing colonies that are said to result from adaptive mutations.

A variety of experiments have shown that adaptive mutations are specifically beneficial. The clearest demonstration of the specificity of adaptive mutations showed that when selection was applied to a specific nucleotide in the *ebgA* gene to permit lactulose utilization, mutations at that nucleotide accumulated over the course of several days, but in that same population no mutations accumulated at another, equally mutable nucleotide in the same gene where mutations permitted utilization of lactose but not of lactulose (13).

The process or processes that produce adaptive mutations are not well understood. The most widely used system, reversion of the F′-borne *lacI33* frameshift mutation during selection for growth on lactose, led to the conclusion that genes whose products are involved in recombination are necessary for adaptive mutagenesis (1, 15). That conclusion has been cast into doubt by the finding that when the *lacI33* frameshift allele is located on the chromosome, rather than on the F′ episome, adaptive reversion is not affected by lesions in recombination genes (4).

In an effort to identify the genes that are involved in adaptive mutagenesis, I have conducted a large-scale mutant hunt. Here I report that disruption of *phoP* or *phoQ*, which together encode a global two-component regulatory system, significantly reduces adaptive mutagenesis.

MATERIALS AND METHODS

Organisms. *Escherichia coli* K-12 strain SJ134 is F⁻ Δ*lacZ4680 lacY⁺ ebgR⁺ ebgA51 rpsL*. *E. coli* K-12 strain SJ2 is F⁻ Δ*lacZ4680 lacY⁺ ebgR⁺ ebgA⁺ rpsL metC*. *E. coli* K-12 strain FCY2 is F⁻ *trpA46* (10). Bacteriophage λ strain NK1324 is Tn*10*dCam *cI857 Pam80 nin5 b522* att⁻ (18).

Media. The phosphate-buffered minimal medium has been previously described (13). Glucose medium contained 2 g of glucose per liter. Lactulose minimal medium contained 1 g of lactulose (4-*O*-β-D-galactopyranosyl-D-fructo-furanose) per liter, 20 mg of X-Gal (5-bromo-4-chloro-3-indolyl β-galactoside) per liter, and 2 × 10⁻⁴ M IPTG (isopropyl-β-D-thiogalactopyranoside). TAD medium (11) contained 5 μM tryptophan, a concentration that limits *trpA46* strains to grow to about 10⁹ cells per plate. Media were solidified with 15 g of purified agar (Sigma) per liter. L agar consisted of LB agar (20) plus 1 g of glucose per liter. MacConkey lactose medium was prepared from MacConkey agar base (Difco) according to the manufacturer's instructions and contained 2 × 10⁻⁴ M IPTG.

Identification of Tn*10*dCam insertion mutants. Strain SJ134 was infected with λNK1324 as described by Kleckner (18), and chloramphenicol-resistant colonies were selected on glucose minimal medium containing 15 μg of chloramphenicol per ml. Individual colonies were grown as 0.5-ml cultures in minimal medium containing 0.1 g of glucose per liter to limit cell density to about 10⁸/ml, dimethyl sulfoxide was added to 10% (vol/vol), and the cultures were stored at −80°C; 100 μl of each culture was spread onto a lactulose minimal medium plate, and the plates were incubated at 30°C. Four days and seven days after inoculation, any *ebgR*⁻ (lactulose-positive) mutant colonies were marked. Typical cultures produced 5 to 10 colonies on day 4 and by day 7 had accumulated 60 to 80 colonies. Cultures that exhibited unusually high or unusually low (including zero) numbers of colonies were retested. Those that exhibited the same unusual numbers were purified by restreaking, and fresh cultures were again tested. Those that again reproduced the unusual numbers were retained for further study. Isolates that failed to generate any *ebgR* mutants within 7 days were streaked onto MacConkey lactose-IPTG medium. Lactose, although a weak inducer of *ebgR*⁺, permits sufficient synthesis of Ebg β-galactosidase to produce light red colonies in MacConkey lactose, while those in which *ebgAC*, *lacY*, or any other gene required for transport and hydrolysis of β-galactoside sugars has been disrupted produce white colonies. Cultures producing white colonies were discarded.

Reconstruction tests. Reconstruction tests were used to determine the time required for an *ebgR* mutant cell to form a visible colony on lactulose minimal medium. Typically three independent *ebgR* mutants that had arisen on different days from a particular Tn*10*dCam mutant were tested. About 100 cells of the *ebgR* mutant were plated onto lactulose minimal medium in the presence and in the absence of about 10⁸ strain SJ2 scavenger cells. Because strain SJ2 is both *ebgR*⁺ and *ebgA*⁺, two mutations are required in order for that strain to utilize lactulose, and such double mutants do not arise prior to 2 weeks of incubation (12). The population of SJ2 cells mimics the situation when rare *ebgR* mutants arise within a population of the parent cells. The time required for approximately

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the same number of colonies to appear on the plates with SJ2 cells and without SJ2 cells is taken as the typical time required for that strain to form visible *ebgR* mutant colonies.

RESULTS

A library of 19,616 independent mini-Tn*10* (chloramphenicol) (Tn*10*dC*am*) insertions into random sites in the chromosome of *E. coli* K-12 strain SJ134 was screened to determine the effect of the Tn*10*dC*am* insertion on adaptive mutagenesis (Materials and Methods).

Instead of monitoring adaptive mutagenesis by selecting for reversion of a known mutation, the forward (loss-of-function) mutation rate at *ebgR*, a gene that specifies a repressor which controls expression of the *ebgAC*-encoded Ebg β-galactosidase (8), was monitored. The advantages of using *ebgR* as a reporter locus are that (i) adaptive mutation at *ebgR* has already been demonstrated to occur (12) and (ii) selecting for loss of function makes it more likely that any genes so identified will affect adaptive mutations that occur by a variety of events (base substitutions, frameshifts, insertions, etc.), rather than affecting just a single class of mutations as is common in reversion studies.

Strain SJ134 and its derivatives carry the *ebgA51* allele. *ebgA* encodes the α subunit of the Ebg β-galactosidase, but the wild-type Ebg enzyme does not hydrolyze the β-galactoside sugar lactulose at a rate that permits utilization of lactulose as a sole carbon and energy source. The *ebgA51* allele alters the kinetic properties of Ebg enzyme to permit effective lactulose hydrolysis (9). *ebgA* expression is under control of the *ebgR*-encoded repressor that does not respond to lactulose as an inducer (8); thus, *ebgR⁻* mutants of strain SJ134 can utilize lactulose as a sole carbon and energy source.

The mutant hunt has led to the identification of four genes in which Tn*10*dC*am* insertions increase the adaptive mutation rate and seven genes in which the insertions decrease the adaptive mutation rate. The identities and characterizations of the genes other than those discussed below will be reported elsewhere.

To measure the adaptive mutation rate at *ebgR* approximately 10^7 cells are spread onto lactulose minimal medium at 30°C (12). The populations grow at the expense of trace contaminants to a density of about 10^8 cells per plate, after which the population declines slowly (Fig. 1A). *ebgR* colonies appear beginning 3 days after inoculation, and they continue to accumulate for the next several days (Fig. 1B). Each day for 4 days, cells were washed off of two plates, suitably diluted, and plated onto L agar to determine the number of viable cells per plate. Once *ebgR* colonies began to appear, on a subset of plates those colonies were immediately eliminated, with little disturbance of surrounding cells, through the use of a diathermy probe (Hyfrecator Plus model 7-796; Birtcher Medical Supplies), an electrosurgery device that delivers an intense spark which kills the cells in the colony. Those treated plates were used to estimate the number of viable cells.

Reconstruction tests showed that *ebgR* mutants of the wild-type strain SJ134 require 3 days to form detectable colonies on lactulose medium. The plating efficiencies of the *ebgR* mutants in the presence and absence of the scavenger strain SJ2 are indistinguishable. Thus, colonies that appear on day 3 are the result of mutations that were present in the initial population, those that appear on day 4 are the result of mutations that occurred in the population that was present on day 1, etc. The mutation rate each day is the average number of new mutant colonies that appeared 3 days later divided by the number of cells on the plate on that day. Figure 2 shows the cumulative
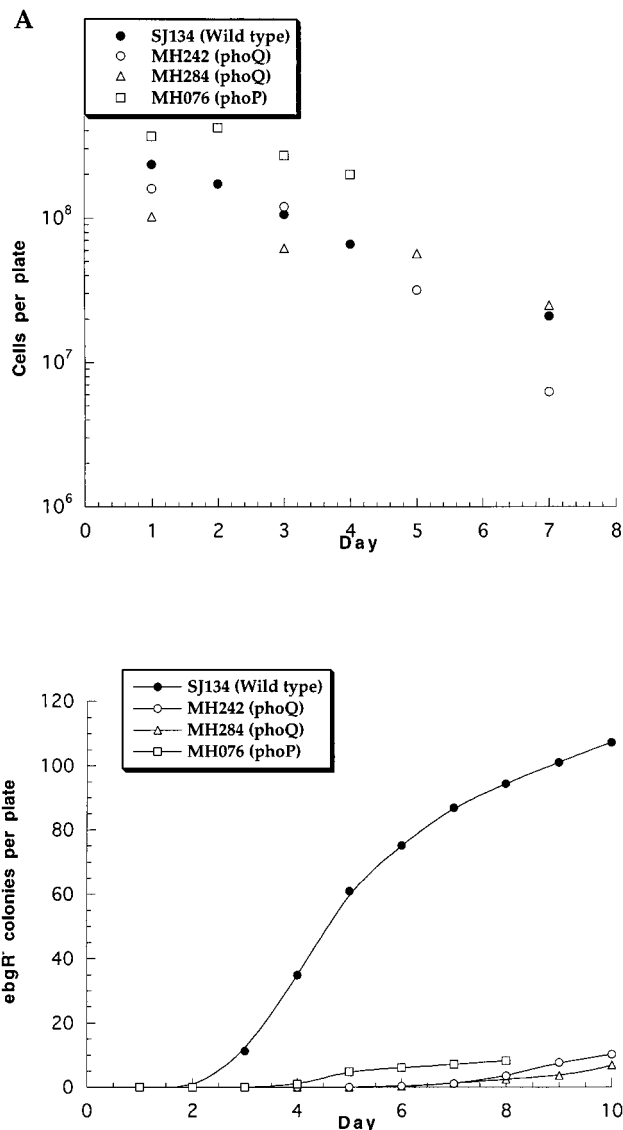

FIG. 1. (A) Population densities on lactulose minimal medium as a function of time at 30°C; (B) appearance of *ebgR* mutant colonies on lactulose minimal medium during incubation at 30°C.

mutations per 10^8 cells, and the slope of the best-fit line is the average mutation rate over the course of the experiment. For the wild-type strain SJ134, the average mutation rate to *ebgR*, over five independent experiments, was $(1.9 \pm 0.2) \times 10^{-7}$ per cell per day (Fig. 2).

Three Tn*10*dC*am* mutants, MH076, MH242, and MH284, yielded mutants at much lower rates than did the wild-type strain, although the population densities were comparable to those of the wild-type strain (Fig. 1). Reconstruction tests showed that *ebgR* mutants of each of the three Tn*10*dC*am* mutants grew slightly more slowly than *ebgR* mutants of the wild-type strain, requiring 4 days to form visible colonies. The mutation rates to *ebgR*, based on 4 days for visible colonies to appear, were 6.3×10^{-9}, 4.8×10^{-8}, and 3.2×10^{-8} per cell per day for MH076, MH242, and MH284, respectively.

Identities of the genes that were disrupted by Tn*10*dC*am* were determined by cloning Tn*10*dC*am* and its flanking regions into plasmid pBluescript KS+ (Stratagene) and sequenc-
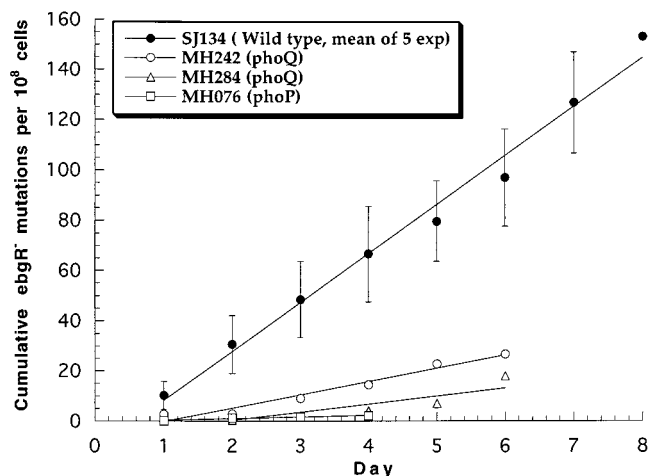
FIG. 2. Cumulative *ebgR* mutations per viable cell during selection on lactulose minimal medium at 30°C. Symbols indicate the days on which the mutations occurred. Mutant colonies appeared either 3 days (SJ134) or 4 days (other strains) after mutations occurred (see text). Lines are least-squares fit to the data points. For strain SJ134, symbols indicate mean values for five independent experiments and error bars indicate the standard errors of those values.

ing. Genomic DNA was digested with one of several restriction enzymes that do not cut within Tn*10*d*C*am, ligated into similarly cut plasmid, and transformed into strain JM109 by electroporation, and transformants were selected on L agar containing 25 mg of chloramphenicol per liter. Plasmid DNA was sequenced on an ABI model 377 automated sequencing apparatus, using primers corresponding to bases 78 to 102 or 1359 to 1383 of Tn*10*d*C*am. Those primers allowed sequencing of about 90 bp of Tn*10*d*C*am outward into the region flanking the insertion. In strain MH076, Tn*10*d*C*am was inserted into *phoP* at bp 676 of the ECOPHOP (Genbank accession no. M81433) sequence. In strains MH242 and MH284, Tn*10*d*C*am was inserted into *phoQ* at bp 2176 and 3029, respectively, of ECOPHOP.

Although the effects of *phoP* and *phoQ* cannot be accounted for on the basis of slow growth, it is possible that the disruptions affect the probability that a newly arisen mutant will survive long enough to begin to grow. The Tn*10*d*C*am::*phoP* and Tn*10*d*C*am::*phoQ* alleles of strains MH076 and MH284 were transduced into strain SJ2 (*metC ebgR*+ *ebgA51*). To mimic the effect of a newly arisen *ebgR* mutant allele in a carbon-starved population, a known *ebgR ebgA51* allele was transduced into cultures of SJ2 and the *phoP* and *phoQ* derivatives that had been carbon starved for 3 days. The transduced populations were plated onto lactulose-methionine medium to select *ebgR* transductants and onto methionine-free glucose medium to select *metC*+ transductants. The recovery of newly arisen *ebgR* mutants was expressed as the transduction efficiency of *ebgR* selected on lactulose medium relative to efficiency of *metC*+ selected on methionine-free medium. For strain SJ2, the relative transduction efficiency was 0.19. For the *phoP* and *phoQ* derivatives, the relative efficiencies were 0.23 and 0.19, respectively; i.e., newly arisen *ebgR* alleles were recovered as efficiently in *phoPQ* as in wild-type backgrounds. Thus, the observed effects on adaptive mutagenesis cannot be attributed either to slow growth or inefficient recovery of mutants, and disruption of *phoP* or *phoQ* must affect adaptive mutagenesis itself.

The observation that three independent Tn*10*d*C*am disruptions of the *phoP* and *phoQ* genes produce the same adaptive

mutation phenotype suggests that it is those insertions themselves, not some possible accompanying random unidentified mutation, that is responsible for the phenotype. The Tn*10*d*C*am::*phoQ* allele of strain MH284 was transduced back into the parent strain, SJ134, and the adaptive mutation rate at *ebgR* was measured. That rate was 4.2×10^{-8} per cell per day, not very different from the rate in MH284 itself, confirming that it is disruption of *phoPQ* that is responsible for the reduced adaptive mutation rate at *ebgR*.

Fluctuation tests (19) were used to determine whether disruption of *phoPQ* affects growth-dependent, as well as adaptive, mutagenesis at *ebgR*. Independent 200-µl cultures of the wild-type strain SJ134 and of MH284 (Tn*10*d*C*am::*phoQ*) were grown in 0.01% (vol/vol) glycerol to yield populations of about 2×10^{7} cells per culture. Five cultures were suitably diluted and plated onto L agar to determine the number of viable cells per culture, and each remaining culture was spread onto a lactulose selection plate. SJ134 colonies were counted 3 days and MH284 colonies were counted after 4 days incubation at 30°C. The Stewart et al. method for analysis of fluctuation tests (23), as implemented by Stewart's DataFit program, permits one to estimate both the average number of mutations that occurred prior to plating during the growth of each culture and the average number of mutations that occurred after plating. For strain SJ134, 127 cultures at 2.4×10^{7} cells per culture produced an average of 1.34 mutations per culture prior to plating to give a growth-dependent mutation rate of 5.6×10^{-8} per cell division. For strain MH284, 40 cultures at an average of 1.5×10^{7} cells per culture produced an average of 0.7 mutation per culture prior to plating, to give a growth-dependent mutation rate of 4.7×10^{-8} mutations per cell division. Thus, disruption of *phoQ* affects adaptive, but not growth-dependent, mutation at *ebgR*.

To determine whether disruption of *phoPQ* would affect adaptive mutagenesis at other loci, adaptive reversion of a *trpA46* missense mutation was examined, and adaptive activation of the *bgl* operon was examined. The adaptive mutation spectra at both *ebgR* (14a) and *bgl* (14) are dominated by insertion element mediated mutations. The Tn*10*d*C*am::*phoQ* allele of strain MH284 was transduced into strain FCY2 (*trpA46*), and the adaptive reversion rates of *trpA46* in strain FCY2 and in FCY2MH284 were compared on TAD medium by monitoring the rate at which Trp+ colonies appeared on the *trpA46* lawns in a manner comparable to that used to measure adaptive mutation rates at *ebgR*. In strain FCY2 the adaptive reversion rate of *trpA46* was 6×10^{-10} per cell per day, whereas it was 6.6×10^{-10} per cell per day in the Tn*10*d*C*am::*phoQ* derivative, strain FCY2MH284.

The adaptive mutation rate at *bgl* was measured in strain MH284 on arbutin selective medium as previously described (14). The *bgl* adaptive mutation rate in strain SJ134 is 3.4×10^{-7} mutations per cell per day (14). In strain MH284 that rate was 2.9×10^{-7} mutations per cell per day, not very different from the wild-type rate.

DISCUSSION

Disruption of *phoPQ* reduces the adaptive mutation rate at *ebgR* by about a factor of 6 but has no effect on the growth-dependent mutation rate at the same locus. That reduction cannot be accounted for either by slow growth or by reduced efficiency of recovery of *ebgR* mutants under the selective conditions used. At the same time, disruption of *phoQ* has no effect on the adaptive mutation rate at either *trpA* or *bgl*. The different responses of the two adaptive mutagenesis reporter systems is consistent with the idea that there are multiple

systems for adaptive mutagenesis and that adaptive mutagenesis at *ebgR* is affected by a system different from those affecting *trpA* and *bgl*. The difference is surprising because adaptive mutagenesis at both *ebgR* and *bgl* is primarily mediated by transposition of insertions elements.

The *phoQ* gene encodes the sensor kinase component, and *phoP* encodes the positive-regulatory component of a two-component regulatory system PhoP/PhoQ that controls expression of at least 30 loci in *Salmonella typhimurium* (21). PhoP, initially described by virtue of its involvement in expression of *phoN*-encoded acid phosphatase (17), is necessary for resistance to defensins, antimicrobial peptides found in macrophages (2, 6). PhoP/PhoQ-activated genes are regulated in response to Mg^{2+} levels (21), and the PhoQ protein includes a Mg^{2+} binding site (24). The system responds to a wide variety of environmental stress signals; indeed, Groisman et al. (5) point out the "involvement of PhoP in response to stress situations such as those which might be present during stationary phase, when a microorganism may face nutritional deprivation, exposure to toxic by-products of metabolism, or both." PhoP/PhoQ regulation is both direct and indirect in that PhoP/PhoQ activates transcription of *pmrAB* (7), which in turn regulates expression of at least seven of the *Salmonella* genes that are under PhoP/PhoQ control (22).

In *E. coli* at least 50 genes are under control of PhoP/PhoQ (16), including genes for resistance to the antimicrobial peptide Magainin 2 (5). Several genes that are induced by starvation for carbon, nitrogen, or phosphorus are thought to be under PhoP/PhoQ control, as are the *pex* genes which are involved in development of the resistant state during entry into stationary phase (5).

Because PhoP/PhoQ is a global regulator that responds to a wide variety of environmental signals, particularly including starvation, and because disruption of *phoP* or *phoQ* significantly reduces the adaptive mutation rate to *ebgR*, it seems likely that adaptive mutagenesis is a regulated process that is subject to positive control by PhoPQ.

Adaptive mutations at *ebgR* appear to require the expression of some gene or genes under control of PhoPQ. This finding is consistent with the view that adaptive mutagenesis is a programmed response to prolonged environmental stress in which a portion of the stressed population transiently enters a hypermutable state as a last desperate effort to find a genetic solution to the current environmental problem (3, 10).

ACKNOWLEDGMENTS

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