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Ugp and PitA Participate in the Selection of PHO-Constitutive Mutants

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ABSTRACT

Mutations that cause the constitutive expression of the PHO regulon of *Escherichia coli* occur either in the *pst* operon or in the *phoR* gene, which encode, respectively, a high-affinity P_i transport system and a histidine kinase sensor protein. These mutations are normally selected on glycerol-2-phosphate (G2P) as the carbon source in the presence of excess P_i. The emergence of early PHO-constitutive mutants, which appear after growth for up to 48 h on selective medium, depends on the presence of *phoA*, which codes for a periplasmic alkaline phosphatase, while late mutants, which appear after 48 h, depend both on *phoA* and on the *ugp* operon, which encodes a glycerophosphodiester transport system. The emergence of the late mutants hints at an adaptive mutation process. PHO-constitutive *phoR* mutants appear only in a host that is mutated in *pitA*, which encodes an alternative P_i transport system that does not belong to the PHO regulon. The conserved Thr^{217} residue in the PhoR protein is essential for PHO repression.

IMPORTANCE

One of the principal ways in which bacteria adapt to new nutrient sources is by acquiring mutations in key regulatory genes. The inability of *E. coli* to grow on G2P as a carbon source is used to select mutations that derepress the PHO regulon, a system of genes involved in the uptake of phosphorus-containing molecules. Mutations in the *pst* operon or in *phoR* result in the constitutive expression of the entire PHO regulon, including alkaline phosphatase, which hydrolyzes G2P. Here we demonstrate that the *ugp* operon, another member of the PHO regulon, is important for the selection of PHO-constitutive mutants under prolonged nutritional stress and that *phoR* mutations can be selected only in bacteria lacking *pitA*, which encodes a secondary P_i transport system.

The PHO regulon of *Escherichia coli* consists of more than 50 genes and operons that respond to orthophosphate (P_i) limitation (1, 2). The best-documented ones are the *phoA* and *phoE* genes, the *pstSCAB-phoU* (or *pst*) and *ugpBAECQ* operons, and the regulatory *phoBR* operon. *phoA* encodes a periplasmic alkaline phosphatase (AP), *phoE* encodes an anion-specific porin, and the first four genes of the *pstSCAB-phoU* operon code for an ABC-type high-affinity P_i transport system which, together with *phoU*, also plays a role in the regulation of the PHO genes. Similar to the *pst* operon, the *ugpBAEC* genes encode an ABC transport system for *sn*-glycerol-3-phosphate and glycerophosphodiesters, while the fifth gene of the operon, *ugpQ*, encodes a phosphodiesterase that hydrolyzes glycerophosphoryl diesters (3, 4). The *phoBR* operon codes for the two-component system that controls the PHO regulon. Null mutations in *phoB* show a PHO-negative phenotype, while *phoR* mutations cause the constitutive expression of the PHO regulon (5).

Instead of the −35 sequences, members of the PHO regulon carry in their promoters a sequence known as the PHO box (6). Under conditions of P_i limitation, the histidine kinase PhoR autophosphorylates and transfers the P_i moiety to the regulatory protein PhoB. The phosphorylated PhoB binds to the PHO boxes, where it interacts with $E\sigma^{70}$ (σ^{70} associated with the core RNA polymerase) (7). Under conditions of P_i excess, PhoR dephosphorylates PhoB, thereby ending the induced expression of the PHO regulon (8). Null mutations, polar and nonpolar alike, in any of the five *pst* genes result in the constitutive expression of the PHO regulon. The mechanism by which the Pst transport system and PhoU repress PHO is still unclear. It has recently been shown that PhoR interacts with PstB and PhoU, suggesting that the external signal of P_i availability is transduced through direct contact between these proteins (9).

In addition to Pst, *E. coli* possesses a constitutive, high-velocity, low-affinity P_i transport system, PitA, that does not belong to the PHO regulon. A third cryptic P_i transporter, PitB, is not functional under P_i starvation or in a PHO-constitutive background (6, 10, 11).

Wild-type *E. coli* is unable to grow on glycerol-2-phosphate

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This article is dedicated to the memory of the late Annamaria Torriani-Gorini, who passed away in May 2013 and whose pioneer work on the regulation of phosphate metabolism inspired many scientists and served as the grounds for the present work.

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TABLE 1 Bacterial strains, plasmids, and DNA oligomers used in this study

Strain, plasmid, or oligomer	Description or sequence	Reference or source
Strains		
MG1655 strains		
MG1655	Wild-type *E. coli*	39
BS7	Δ*pstSCAB-phoU*::Km	40
BS8	*proC*::Tn*10*	41
EG2	*pitA*::Km	This study
RI05	Δ*phoA*::Km	This study
RI06	Δ*ugpBAEC*::Cm	This study
RI07	Δ*ugpBAEC*::Cm Δ*pstSCAB-phoU*::Km	This study
RI08	Δ*phoA*::Cm Δ*pstSCAB-phoU*::Km	This study
RI21	Δ*pitA*::Km Δ*ugpBAEC*::Cm	This study
RI65	Δ*pitA*::Km *phoR217*, high-AP *phoR* mutant	This study
RI81	Δ*pitA*::Km *pst* mutant	This study
TP01	*pitA*$^+$ *phoR129*, transduction from strain JV1 to MG1655 *proC*::Tn*10*	This study
Other strains		
K10	Hfr *garB10 fhuA22 ompF627 fadL701 relA1 pitA10 spoT1 rrnB2 mcrB1 rob-1 creC510*	CGSC
C3	K10 *phoR69* high-AP *phoR* mutant	12
JV1	K10 *phoR129* low-AP *phoR* mutant	This study
JV2	K10 *phoR130* low-AP *phoR* mutant	This study
JV6	K10 *pst* mutant	This study
E2348/69	Enteropathogenic *E. coli* (EPEC) Nal^r	42
KM32	Δ*recBCD*::P*tac-gam-bet-exo cat*	18
KM44	Δ*recBCD*::P*tac-gam-bet-exo kan*	18
Plasmids		
pBS6	*pstSCAB-phoU*$^+$ cloned in the low-copy-no. plasmid pGB2	43
pGEM-T Easy	Cloning vector	Promega
pKD3	Carries the *cat* gene	17
pKD4	Carries the Km^r cassette	17
pUC4K	Carries the Km^r cassette	44
Oligomers		
phoA71	AAGAAGTTATTGAAGCATCCTCGTCAGTAAAAAGTTAATCGTGTAGGCTGGAGCTGCTTC	
phoA1701	TTTCATAGCACCATCCCTCTTCATGTTTTAACCATGAGCGCATATGAATATCCTCCTTAG	
phoA-721	CTTTGGAGATTATCGTCACTG	
phoA-2707	CAGGCAATCACTCATGTAGG	
ugp350	AACGATGAAACCGTTACATTATACAGCTTCAGCACTGGCGGTGTAGGCTGGAGCTGCTTC	
phoR_S_FE	TTTAACGCCTTGCTCATCGG	
phoR_S_RE	CAGCATCGACTGGCTTATGG	
phoR_S_FI	TGAGTTACGTACGCCATTGAC	
phoR_S_RI	CGGCTGCTCATTCATCATCTC	
ugp4480	CCAGATGCAGCCACAGCGTGCTGCCTGCCGTCGGGCGCTCCATATGAATATCCTCCTTAG	
ugp-858	ACCGCCTTGTCATCTTTCTG	
ugp-5210	CTCGTTGTCCTGTTTCACC	

(G2P) as the sole carbon source in the presence of excess P_i. Mutants that grow under these conditions are constitutive for the entire PHO regulon, including AP (12, 13). Most of the mutations appear in *pstSCAB* or in *phoU*, and some are *phoR* mutants, whose constitutive level of AP is usually lower (5). The postulated mechanism for the selection of these PHO-constitutive mutants is associated with the constitutive expression of AP (13), whose high periplasmic concentration hydrolyzes G2P into glycerol and P_i. Glycerol enters the cell with the help of the glycerol facilitator GlpF (14), and P_i is taken up either via PitA, in the case of a *pst* mutation, or via PitA and Pst, if the mutation occurred in *phoR*. It was later reported that besides transporting glycerol-3-phosphate (G3P), the Ugp transport system is also able to take up G2P (4). This observation raises an alternative possibility, namely, that the uptake of G2P by Ugp (which is also constitutively expressed in a PHO-constitutive mutant) is a contributing factor for the selection of the PHO-constitutive mutants. In the present study, the role of Ugp in this process was examined. In addition, in the course of this study it was noticed that *phoR* mutants are considerably less frequent than expected. Investigation of this phenomenon revealed that *phoR* mutants are preferentially isolated in *pitA*-negative strains.

MATERIALS AND METHODS

Bacteria strains and growth conditions. The bacterial strains used in this study are listed in Table 1. LB/L agar was the standard rich medium (15). TGP is a minimal medium composed of T salts (12) supplemented with 0.2% glucose and 1 mM KH_2PO_4. TG2P medium is composed of T salts

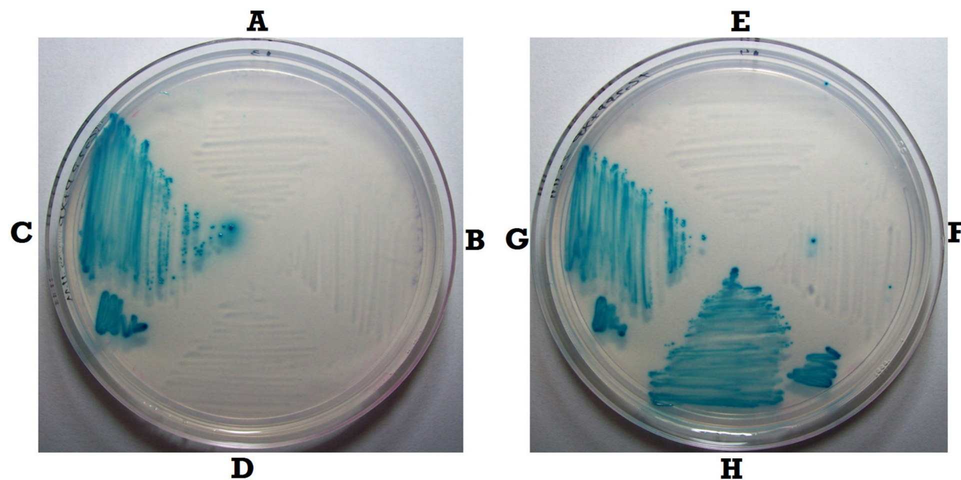

FIG 1 Growth of Δ*phoA* and Δ*ugp* mutants on TG2P plates. Bacteria were streaked on TG2P (minimal medium with G2P as the sole carbon source supplemented with the AP substrate X-P) and grown for 48 h at 37°C. (A) MG1655; (B) Δ*phoA*::Cm; (C) Δ*pst*::Km; (D) Δ*phoA*::Cm Δ*pst*::Km; (E) MG1655; (F) Δ*ugp*::Cm; (G) Δ*pst*::Km; (H) Δ*ugp*::Cm Δ*pst*::Km.

supplemented with 0.2% glycerol-2-phosphate and 1 mM KH_2PO_4. TG2P plates were also supplemented with 40 μg/ml of the AP chromogenic substrate 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine (X-P). Medium A is a semirich P_i-limited medium (P_i concentration, ~0.2 mM) (16). When required, 100 μg/ml ampicillin, 20 μg/ml chloramphenicol, 50 μg/ml kanamycin, or 100 μg/ml spectinomycin was added.

Construction of mutants. The *phoA* and *ugpBAEC* deletions (Δ*phoA* and Δ*ugpBAEC*, respectively) were constructed using the bacteriophage λ-red recombinase system essentially as described previously (17, 18). Primers phoA71/phoA1701 and ugp350/ugp4480 were used to construct the deletion of *phoA* and *ugpBAEC*, respectively. PCR was performed using plasmid pKD3 (carrying a chloramphenicol resistance [Cm^r] cassette) or pKD4 (carrying a kanamycin resistance [Km^r] cassette) as the template. The Cm^r and Km^r cassettes flanked by 40-bp *phoA* or *ugp* DNA sequences were transformed into strains KM32 and KM44, respectively, which carry the bacteriophage λ-red genes in the chromosome, and the recombinants were selected on the appropriate antibiotic plate. The mutations were then transferred into strain MG1655 by P1 transduction. The deletions were confirmed by PCR with primers phoA-721/phoA-2707 for Δ*phoA* and ugp-858/ugp-5210 for Δ*ugpBAEC*. In the case of *phoA*, the deletion was also confirmed by an AP activity assay, as described previously (16). Double mutants were constructed by transferring antibiotic resistance markers between strains using P1 transduction. For the construction of the *pitA*::Km mutant, the *pitA* gene was amplified by PCR with primers pitAF and pitAR and cloned into plasmid pGEM-T Easy. The resulting plasmid was digested with HincII and ligated to a Km^r cassette obtained from plasmid pUC4K digested with HincII. The *pitA*::Km fragment was amplified by PCR using the same primers, and the PCR product was introduced into strain KM32 by recombineering, as described above. The insertion of Km^r into *pitA* was confirmed by PCR.

Selection of PHO-constitutive mutants. PHO-constitutive mutants were selected on TG2P plates essentially as described previously (13). Bacteria were grown overnight in TGP, washed, and suspended in 0.9% NaCl. Approximately 10^9 cells were plated on TG2P medium supplemented with the AP substrate X-P. In parallel, bacterial dilutions were plated on L agar to record the exact cell concentration. The number of blue colonies on the G2P plates was recorded daily. The mutation frequency was calculated by dividing the number of colonies on the G2P plates by the number of CFU on L agar. For each strain, 8 to 20 independent replicates were performed.

Determination of constitutive genotype. PHO-constitutive *pst* mutations were determined by electroporation of the mutant with a plasmid that expresses the entire *pst* operon (pBS6), followed by plating on L agar containing spectinomycin. The loss of AP constitutivity pointed to the presence of a mutation in one of the *pst* operon genes. *phoR* mutations were assessed by transducing the mutant with a P1 lysate of *proC*::Tn*10* cells (strain BS8). Selected tetracycline-resistant colonies on X-P plates that showed approximately similar numbers of blue and white colonies (cotransduction frequency of 60%) indicated a *phoR* mutant genotype.

AP assays. Colony plate assays were performed either by including the chromogenic AP substrate X-P in the plates or by flooding the colonies with a mixture of α-naphthyl phosphate and tetrazotized *o*-dianisidine chloride (Fast Blue) as described previously (19). For quantitative assays, cells were grown overnight in medium A without P_i or medium A supplemented with 1 mM KH_2PO_4 (medium A with P_i). *p*-Nitrophenyl-phosphate (pNPP) was used as a substrate. The reaction was stopped by the addition of 0.25 M Na_2HPO_4, and the AP specific activity was calculated according to the equation $A_{410} \times \text{time (min)}^{-1} \times$ cell density (optical density at 600 nm)$^{-1}$.

P1 transduction. Transductions of chromosomal markers were performed as described previously (15).

DNA sequencing. The *phoR* open reading frame was amplified by PCR using primers phoR_S_FE and phoR_S_RE. PCR products were purified from agarose gels with a Wizard DNA purification system (Promega), and sequencing reactions were performed using the BigDye Terminator (v.3.1; Applied Biosystems) kit, according to the manufacturer's instructions. For the sequencing reactions, oligonucleotides phoR_S_FE, phoR_S_RE, phoR_S_FI, and phoR_S_RI were used. The sequencing products were resolved and analyzed in an automatic sequencer (ABI Prism 3100 genetic analyzer; Applied Biosystems/Hitachi, Warrington, United Kingdom).

RESULTS

To test the roles of *phoA* and of the *ugpBAEC* operon (henceforth referred to as *ugp*) in the selection of PHO-constitutive mutants, we constructed deletion mutants of *phoA* and *ugp* each associated with an antibiotic resistance gene, namely, Δ*phoA*::Cm and Δ*ugp*::Cm (see Materials and Methods). Each construct was introduced into the wild-type strain MG1655. A test of the growth of these mutants along with that of the proper controls was performed by streaking them on TG2P (in which G2P is the sole carbon source and excess P_i is supplemented with the AP chromogenic substrate X-P), which is the same medium used for the se-

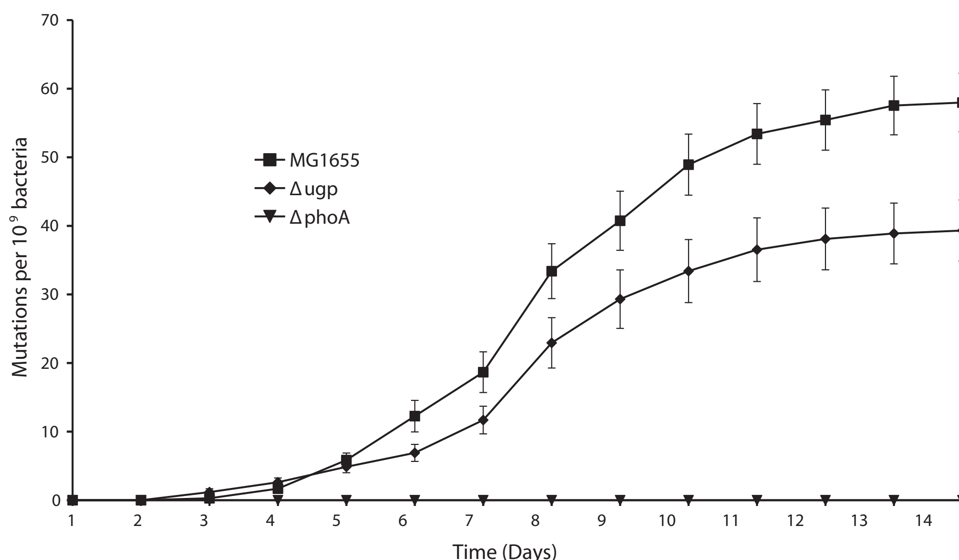

FIG 2 Selection of PHO-constitutive mutants on TG2P. Bacteria were seeded on TG2P plates and incubated for 14 days at 37°C. The appearance of colonies was monitored daily. The data points were calculated by dividing the number of colonies observed on each day by the effective number of CFU seeded on each plate (about 10^9). The data represent the mean ± SEM from 20 independent experiments.

lection of PHO-constitutive mutants (13). Figure 1 shows that, as expected, the wild-type strain MG1655 (Fig. 1A and E) and its *phoA* and *ugp* single mutant derivatives (Fig. 1B and F, respectively) failed to grow, while the PHO-constitutive Δ*pst* mutant derivative grew well on the plate (Fig. 1C and G). The central role of *phoA* in the selection of PHO-constitutive strains became evident from the inability of a Δ*pst* Δ*phoA* double mutant to grow on TG2P (Fig. 1D). On the other hand, the normal growth of the Δ*pst* Δ*ugp* double mutant (Fig. 1H) hinted that the *ugp* operon is apparently not required for the growth of PHO-constitutive mutants on TG2P.

However, these observations do not rule out a function for Ugp during the selection of PHO-constitutive mutations. Since the Ugp system can take up G2P (4), the constitutive expression of Ugp could benefit the bacteria by providing extra glycerol or P_i for the nascent mutant. To test whether *ugp* plays a role in the mechanism of selection of PHO-constitutive mutants, 20 separate cultures each containing 10^9 cells of wild-type strain MG1655, Δ*phoA* mutant RI05, and Δ*ugp* mutant RI06 were plated on TG2P. The number of emerging blue colonies was recorded for the following 14 days (Fig. 2). Both the wild-type strain and the Δ*ugp* mutant showed a similar pattern of accumulation of constitutive mutants on TG2P, but with one important difference; as of day 5, the wild-type strain started to accumulate more PHO-constitutive colonies than did the Δ*ugp* mutant. At the end of 2 weeks, there were, on average, 25% fewer PHO-constitutive colonies derived from the Δ*ugp* mutant than from its wild-type parental strain. No colonies were observed on the plates seeded with Δ*phoA* cells, confirming that the emergence of PHO-constitutive mutants on TG2P is dependent on *phoA*. This agrees with the suggested AP-dependent mechanism for the selection of PHO-constitutive mutants (13). However, the authors of the previous study monitored the appearance of AP-constitutive colonies for only 2 to 3 days, while the present results suggest that the bulk of the PHO-constitutive mutants, which emerged later, are partially dependent on Ugp. It seems that the PHO-constitutive mutants that appeared at later times did not derive from preexistent mutations but emerged in response to the prolonged incubation in the presence of G2P. Their accumulation pattern resembles an inflection curve common in other cases of stress-induced (adaptive) mutations (see Discussion).

When *pst* mutants are selected on TG2P, the supply of P_i is expected to depend only on the low-affinity PitA transporter. However, in the absence of PitA, Ugp, as a G2P transporter, might confer on the emerging PHO-constitutive mutants an advantage by providing an alternative source of P_i. To test this hypothesis, a *pitA*::Km mutation was introduced into the wild-type strain and into the Δ*ugp* mutant. Each organism, along with the wild-type control, was plated on TG2P, and the formation of AP-constitutive colonies was scored as described above. Figure 3 shows that, compared to the findings for the wild type, inactivation of *pitA* alone only slightly reduced the frequency of PHO-constitutive mutants, whereas the mutation frequency of the *pitA*::Km Δ*ugp* double mutant was drastically reduced. Since the majority of the mutants derived from the *pitA*::Km strain had mutations in the *pst* operon (see below), these results strongly suggest that in the absence of a functional P_i transport system, the constitutively expressed Ugp system provides most of the P_i required for growth. In fact, a *pitA*::Km *pst* double mutant was unable to grow on TGP (minimal medium containing 0.2% glucose and 1 mM P_i) (see Fig. S2 in the supplemental material), as both P_i transport systems were eliminated, but it could grow on TG2P (minimal medium containing 0.2% G2P and 1 mM P_i) because G2P serves as a carbon as well as a P_i source (see Fig. S1 in the supplemental material). If this is the case, a Δ*pitA* Δ*pst* Δ*ugp* triple mutant would be unable to grow on G2P. To test this possibility, we pursued the construction of the triple mutant. However, despite many attempts using different combinations of P1 transduction, a triple mutant could not be obtained. The inability to get a Δ*pitA* Δ*pst* Δ*ugp* triple mutant was also reported elsewhere (20).

PHO-constitutive mutations may occur in the *pst* operon or in *phoR* (5). Given the length of the *pst* operon (5 kb) and *phoR* gene

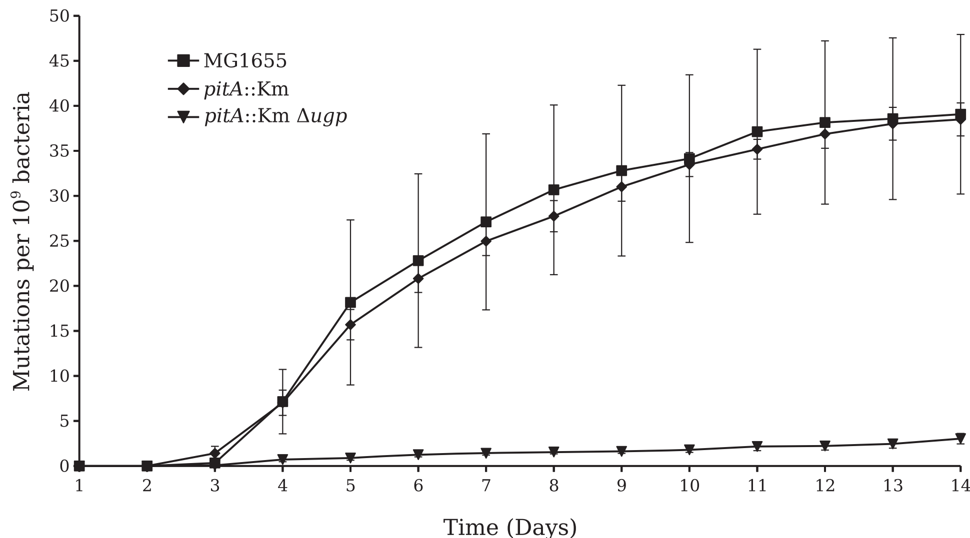

FIG 3 Selection of PHO mutants in a *pitA* background. Bacteria were seeded on TG2P plates as described in the legend to Fig. 2. The data represent the mean ± SEM from 8 independent experiments.

(1.3 kb), 25% of the PHO-constitutive mutations isolated on G2P would be expected to be in *phoR*. However, the vast majority of mutations isolated in this study were in the *pst* operon. A total of 148 spontaneous PHO-constitutive mutants (95 from strain MG1655, 36 from the enteropathogenic *E. coli* strain E2348/69, and 17 from strain K10) were selected and mapped. All MG1655 and E2348/69 derivatives carried mutations in *pst*, and only 2 out of the 17 K10 mutants proved to bear mutations in *phoR*. Sequencing of these two mutants (JV1 and JV2) revealed in one of them a G → T transversion at position 703 and in the other a T → A transversion at position 546, resulting in both cases in premature stop codons and an AP-constitutive genotype.

Strain K10 carries a *pitA* nonsense mutation (11). To test whether the lack of PitA is associated with the emergence of spontaneous *phoR* mutants, 14 PHO-constitutive colonies isolated from MG1655 *pitA*::Km (Fig. 3) were mapped. Nine isolates had mutations in the *pst* operon, while the other five were *phoR* mutants. All five *phoR* mutants carried the *phoR217* allele, a C → A transversion at position 650 resulting in a T217K substitution in the PhoR protein. Even though they carried the same mutation, these five mutants arose independently of each other; i.e., they were not siblings of a bacterial population with a preexisting mutation plated on TG2P. This conclusion derives from the fact that they all appeared at different times on the selective plate, three on day 6, one on day 7, and another one on day 8. The fact that not a single *phoR* mutation was found in almost 100 PHO-constitutive isolates from wild-type strain MG1655 but *phoR* mutants were readily selected from this strain when *pitA* was inactive suggests that the presence of PitA suppresses the occurrence of spontaneous *phoR* mutations (see Discussion).

Another reason for the inability to detect *phoR* mutations in strain MG1655 is that, compared to the results for strain K10, the level of AP as a result of P_i starvation or in PHO-constitutive strains was much lower than that in K10 (Fig. 4). This difference became particularly striking when the *phoR129* mutation was transferred to strain MG1655. The AP activity of K10 *phoR129* (strain JV1) was 11 times higher than that of MG1655 carrying the *phoR129* allele. Apparently, when exposed to the G2P selective medium, the low level of AP and Ugp produced by the constitutive *phoR* nonsense mutants in the MG1655 background was probably insufficient to provide all necessary glycerol and P_i required for their growth.

Hence, *phoR* nonsense mutants derived from MG1655 are not detectable on TG2P. To test this assumption, the MG1655 *phoR129* transductant strain, along with other strains, was streaked on a TG2P plate and in liquid TG2P medium (see Fig. S1 in the supplemental material). As expected, MG1655 and the K10 *pst* mutants as well as K10 *phoR129* grew well, while MG1655 *phoR129* grew very poorly in both liquid and solid media. This clearly shows that *phoR* nonsense mutations, such as *phoR129*, do not cause the production of sufficient AP to allow the growth of strain MG1655 on G2P, but the same mutation promotes the growth of K10 because this strain expresses an intrinsically higher level of AP (Fig. 4). It is also interesting to note that the *pitA*::Km mutant expressed 30% more AP than the wild-type strain, which may contribute to the growth of the nascent *phoR* mutants.

Finally, there still exists the possibility that the *pst pitA*::Km double mutants were able to grow on G2P due to the activation of the cryptic PitB transporter (11). To test this possibility, the 14 PHO-constitutive *pitA*::Km mutants described above (9 *pst* and 5 *phoR* mutants) were grown in liquid TGP (minimal medium containing 0.2% glucose and 1 mM P_i) for 24 h along with some controls. As expected, the parent (MG1655 *pitA*::Km) and its *phoR* derivatives grew well, while the nine isolates that carried mutations in the *pst* operon did not grow in this medium (Fig. S2 in the supplemental material shows one representative result for each isolate), demonstrating that these strains are unable to use P_i as a P source and are thus unlikely to have activated *pitB*. When streaked on a TGP plate, the *pitA*::Km *pst* double mutant grew very poorly, while two different types of *pitA*::Km *phoR* mutants as well as the *pitA*::Km parent grew perfectly well on the plate (see Fig. S2 in the supplemental material). The same growth pattern was observed for all nine *pitA*::Km *pst* mutants (not shown). Hence, none of the PHO-constitutive *pitA*::Km mutants activated *pitB*.

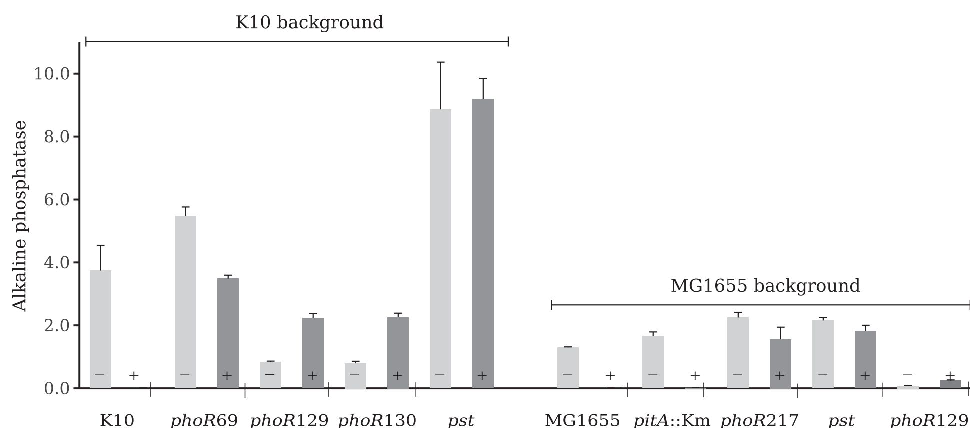

FIG 4 AP activity of the various PHO-constitutive mutants. Bacteria grown overnight in medium A without P_i (−) and medium A with P_i (+) were assayed for AP activity. K10, wild-type K10; *phoR69* (strain C3), K10 high-AP *phoR69* spontaneous mutant; *phoR129* (strain JV1) and *phoR130* (strain JV2), K10 low-AP *phoR* nonsense mutants; *pst* (strain JV6), K10 *pst* spontaneous mutant; MG1655, wild-type MG1655; *pitA*::Km, MG1655 *pitA*::Km (strain EG2); *phoR217* (strain RI65), MG1655 high-AP *phoR* spontaneous mutant; *pst* (strain RI81), MG1655 *pitA*::Km *pst* spontaneous mutant; *phoR129* (strain TP01), MG1655 into which the *phoR129* mutation was transduced. Each bar represents the mean ± SEM from 3 independent experiments.

DISCUSSION

In this study, the rationale behind the selection of PHO-constitutive mutants on G2P was revisited. As expected, the fundamental role of AP as the major contributor in the selection of the constitutive mutants was confirmed (13). However, we showed that the Ugp system also contributes to the emergence of these mutants, apparently by providing the additional phosphorus required for bacterial growth. This conclusion is supported by two findings: the drastic drop in the frequency of PHO-constitutive mutations in a *ugp pitA* background (Fig. 3) and by our and others' inability to construct a Δ*pst* Δ*pitA* Δ*ugp* triple mutant (20). Ugp could enhance the appearance of PHO-constitutive mutants either by taking up G2P in its intact form or by taking up P_i from the medium. It has been shown that two other organophosphate transport systems, GlpT and UhpT, are able to take up P_i as a secondary substrate (5, 21). Though P_i is not a known Ugp substrate (22), the fact that we and others could not obtain a Δ*pst* Δ*pitA* Δ*ugp* triple mutant suggests that the Ugp system may be involved in the transport of P_i. Moreover, the observation that the *pitA*::Km Δ*ugp* double mutant gives rise to very few PHO-constitutive mutants points to a function of Ugp as a P_i transporter. Another possibility would be that G2P is converted into G3P in the periplasm and only then is transported by Ugp. This may be in agreement with a recent report that challenged the notion that Ugp is able to take up G2P *in vitro* (23). However, a periplasmic enzyme that catalyzes the conversion of G2P to G3P is still unknown.

PitA inhibits the formation of *phoR* mutants through a mechanism that is not entirely clear. A *pitA pst* double mutant would leave the bacterium without a proper P_i transport system. Hence, under the conditions used for the selection of PHO-constitutive mutants (G2P as the only carbon source and excess P_i), the selective pressure is tilted toward *phoR*, whose mutation/inactivation would have a lower cost than the inactivation of *pst*.

To the best of our knowledge, *phoR* mutants have thus far been spontaneously isolated only in strain K10 (12, 24). Apparently, K10 possesses two critical characteristics that enable the selection of *phoR* mutants, namely, a *pitA* mutation (11) as well as an intrinsic capacity to synthesize a higher level of AP than most K-12 strains tested in our laboratory (Fig. 4 and data not shown). The reason why K10 synthesizes high levels of AP may be related to the presence of the *creC510* allele, which causes the constitutive expression of CreC (25). CreC is a histidine kinase that can phosphorylate PhoB (via cross talk) when *phoR* is absent (26, 27). Thus, the constitutive expression of CreC might boost *phoA* transcription in nonsense *phoR* mutants, enabling their growth on G2P. Another K10 characteristic is the presence of an *rpoS*(Am) mutation, which produces a less efficient truncated form of RpoS. Bacterial strains that carry this *rpoS* allele express higher levels of σ^{70}-dependent genes, such as *phoA* (28, 29). MG1655 *pitA*::Km displayed a small but statistically significant increase in AP activity ($P = 0.03$, Student's *t* test), possibly because in the absence of PitA there is a less of an influx of P_i that would slightly alleviate the repression of the PHO regulon genes (30). An intrinsic higher AP activity in a strain lacking *pitA* may also contribute to the growth of the nascent *phoR* mutant.

There are two different types of *phoR* mutations: those that confer a high level of AP activity (high-AP mutations) and those that confer a low level of AP activity (low-AP mutations). Strains with nonsense *phoR* mutations exhibit a low level of noninducible AP-constitutive activity, while strains with the other type of *phoR* mutations present a high level of AP activity under excess P_i, which is further induced by P_i starvation (5, 12, 31). The T217K mutant displayed the high-AP phenotype (Fig. 4), which enabled growth on the selective plate by providing high levels of AP and Ugp. Incidentally, the well-characterized *phoR69* mutation is a T220N substitution (31), which is located in the same region in the HisKA domain of PhoR. The *phoR69* mutation confers on K10 high AP activity compared to the level of activity conferred by the *phoR* nonsense mutation in the same strain (Fig. 4). Furthermore, the Thr^{217} residue is highly conserved among sensor histidine kinases similar to EnvZ, such as PhoR (see Fig. S3 in the supplemental material), and it was shown to be critical for the phosphatase activity of EnvZ (32). Since the only high-AP *phoR* mutants hitherto isolated carry mutations in each of two conserved threonine residues in the HisKA domain (the five *phoR* mutants isolated in the present study and the *phoR69* mutant), there are apparently few targets in PhoR that result in high-AP constitutivity. This narrows considerably the spectrum of possible *phoR* mutations in

MG1655, once *phoR* nonsense mutations in this strain result in low levels of AP, and explains why only two high-AP *phoR* mutants were isolated until now.

The bulk of PHO-constitutive mutants appeared on the selective plate between days 5 and 11. Upon restreaking on TG2P, they formed colonies within 48 h, indicating that they are not slow growers and, hence, do not contain preexistent mutations. Furthermore, the pattern of accumulation of PHO mutants in the wild-type, Δ*ugp*, and *pitA*::Km strains resembles the inflection curve common in other models of stress-induced (adaptive) mutations (33–36). Adaptive mutations are not formed under nonselective growth conditions (preexistent mutations), but they appear later, when the bacteria are exposed to the selective conditions. The mechanism through which these mutants arise is still under intense dispute (35, 37, 38).

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