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Activation of the Cryptic PhnE Permease Promotes Rapid Adaptive Evolution in a Population of *Escherichia coli* K-12 Starved for Phosphate

Mélanie L. Guillemet* and Patrice L. Moreau

Laboratoire de Chimie Bactérienne, Centre National de la Recherche Scientifique, Aix-Marseille University, Marseille, France

***Escherichia coli* K-12 suffers acetic acid stress during prolonged incubation in glucose minimal medium containing a limiting concentration of inorganic phosphate (0.1 mM P_i), which decreases the number of viable cells from 6×10^8 to ≤10 CFU/ml between days 6 and 14 of incubation. Here we show that following two serial transfers into P_i-limiting medium, evolved mutants survived prolonged incubation (≈10^7 CFU/ml on day 14 of incubation). The evolved strains that overtook the populations were generally PhnE$^+$, whereas the ancestral K-12 strain carries an inactive *phnE* allele, which prevents the transport of phosphonates. The switching in *phnE* occurred with a high frequency as a result of the deletion of an 8-bp repeated sequence. In a mixed culture starved for P_i that contained the K-12 ancestral strain in majority, evolved strains grew through PhnE-dependent scavenging of probably organic phosphate esters (not phosphonates or P_i) released by *E. coli* K-12 between days 1 and 3, before acetic acid excreted by *E. coli* K-12 reached toxic levels. The growth yield of *phnE*$^+$ strains in mixed culture was dramatically enhanced by mutations that affect glucose metabolism, such as an *rpoS* mutation inactivating the alternative sigma factor RpoS. The long-term viability of evolved populations was generally higher when the ancestral strain carried an inactive rather than an active *phnE* allele, which indicates that cross-feeding of phosphorylated products as a result of the *phnE* polymorphism may be essential for the spread of mutants which eventually help populations to survive under P_i starvation conditions.**

Bacteria such as *Escherichia coli* have evolved mechanisms to adapt to abrupt environmental changes that may result from the introduction of toxic products (e.g., HCl and H_2O_2) or from the deprivation of nutrients. In fact, cells starved for glucose (Glc), the preferred source of carbon (C), and for inorganic phosphate (HPO_4^-; P_i), the preferred source of phosphorus (P), use specific (the cyclic AMP [cAMP] receptor protein [CRP] and Pho regulons, respectively) and general (the RpoS regulon) adaptive responses. First, cells induce specific responses to Glc and P_i limitation with the aim of maintaining growth. Induced mechanisms allow cells to scavenge low levels of Glc and P_i through induction of the phosphotransferase system (PTS) and phosphate-specific transport (PST) system for Glc and P_i, respectively. These regulons may eventually help cells to grow on secondary sources of C (e.g., acetate previously excreted during growth on Glc or lactose) and of P (e.g., phosphite and phosphonates). The response to Glc starvation occurs primarily through the activation of the CRP-cAMP complex, when the transport of Glc via the PTS decreases and the response to P_i starvation occurs primarily through the activation of the two-component system PhoR-PhoB, when the concentration of P_i in the medium is <4 μM and the transport of P_i via the PST system decreases (13, 27, 36). Second, if cells cannot find C and P sources to maintain growth, they normally enter stationary phase. Starved cells accumulate a new sigma factor (σ^s, RpoS) of the RNA polymerase, which contributes to change the expression of >500 genes: the expression of σ^{70}-dependent genes required for normal growth and for specific responses (including the CRP and Pho regulons) decreases, while σ^s-dependent genes are induced. The latter include genes for defense against extreme pH (e.g., *gadB* and *gabD*) and H_2O_2 (e.g., *katE*, *dps*, *pdhR*, and *poxB*) (20, 29, 34). Thus, the RpoS regulon is induced in advance, while stress is not occurring but could appear during prolonged stationary phase. In fact, there is evidence that RpoS-dependent processes help cells starved of Glc to resist oxidative stress and cells starved of P_i to resist oxidative and eventually acetic acid stresses (20–23).

Surprisingly enough, RpoS$^-$ mutants accumulate rapidly in populations incubated under C- and P-limiting conditions. During growth in Glc-limited chemostats, which normally induces the RpoS regulon because of the low growth rate, loss-of-function *rpoS* mutants may sweep the populations in a few generations. After 50 generations (17 days), the dominant *rpoS* mutant subpopulation may accumulate small-effect mutations that help evolved strains to grow on low levels of Glc. Finally, by 100 generations, *rpoS*$^+$ cells may recover (8). Similarly, a population incubated for 37 days in a P_i-limited chemostat may evolve strains that contain several mutations. However, a single mutation in *rpoS* or in genes (e.g., *spoT* and *hfq*) that decrease RpoS levels is sufficient to provide a fitness benefit under conditions of P_i limitation (31). Thus, populations evolving in Glc- and P_i-limited chemostats appear to favor growth (through notably the enhanced expression of the σ^{70}-dependent genes of the CRP and Pho regulons) at the expense of general stress response (through notably the reduced expression of σ^s-dependent genes). This solution

Received 31 August 2011 **Accepted** 25 October 2011
Published ahead of print 4 November 2011
Address correspondence to Patrice L. Moreau, moreau@ifr88.cnrs-mrs.fr.
* Present address: INRA-MICALIS, Jouy-en-Josas, France.
This article is dedicated to the memory of Benjamin Moreau.
Supplemental material for this article may be found at http://jb.asm.org/.

doi:10.1128/JB.06094-11

may cause no significant problem, since cells are essentially in constant environments (that is, constant nutrient concentrations, osmolarity, partial O_2 pressure [pO_2], and pH).

In contrast, the spread of *rpoS* mutants might cause problems during prolonged incubation in batch cultures, in which environmental conditions may change dramatically as a result of the accumulation of secondary metabolites such as ammonia (during catabolism of amino acids) and acetic acid (during catabolism of Glc) (36). In fact, during prolonged incubation in batch cultures containing amino-acid-rich LB medium, evolved mutants that initially overtake the populations carry low-activity rather than null-activity *rpoS* alleles (38). This may satisfy a tradeoff between enhanced capacity to grow on scarce nutrients and sufficient RpoS-dependent defense against alkaline pH; at the entry into stationary phase, the pH of the LB medium increases from pH 7 up to pH 8.5 as a result of the degradation of amino acids and production of ammonia, which consumes protons ($NH_3 + H^+ \leftrightarrows NH_4^+$) (6, 36).

We explored the phenotypic, metabolic, and genetic characteristics of strains that evolved during serial transfers in P_i-limiting batch cultures, in which cells are exposed to a decrease in internal pH as a result of the excretion, reentry, and dissociation inside the cell of acetic acid, which liberates protons ($CH_3COOH \leftrightarrows CH_3COO^- + H^+$) (21). We show here that *E. coli* K-12 rapidly evolved heterogeneous and dynamic populations that survived prolonged incubation under aerobic, P_i starvation conditions. Many evolved strains that spread through the cultures exhibited an RpoS$^-$ phenotype. At first glance, such an evolution process was in good agreement with the fact that during prolonged incubation in batch monocultures starved for P_i, *rpoS*$^+$ cells lose viability after 6 days of incubation as a result of the accumulation of acetic acid (30 mM at pH 4.8) (20, 21), whereas *rpoS* mutants eventually survive by growing between days 6 and 12 of incubation on low levels of acetate and on high levels of P_i released into the medium by *rpoS* mutants (11). However, we also show that single *rpoS* mutants could not grow in a mixed culture starved for P_i containing *E. coli* K-12 in majority. Two mutations isolated from an evolved strain were sufficient for growing in a mixed culture: a 1-bp deletion in *rpoS* that inactivated RpoS and an 8-bp deletion in *phnE* that removed a frameshift normally present in *E. coli* K-12 (17, 33). Activation of the Pho regulon was required primarily to induce the expression of the *phnE*$^+$ allele. The role of the PhnE permease, which is primarily to transport phosphonates (C-P) (18, 35), was to scavenge probably organic phosphate esters (C-O-P) released by *E. coli* K-12 starved for P_i, thereby allowing growth recovery of evolved strains between days 1 and 3 of incubation before acetic acid excreted by *E. coli* K-12 reached toxic levels. Whereas most evolved strains were PhnE$^+$, the long-term viability of evolved populations was generally higher when the ancestral strain carried an inactive rather than an active *phnE* allele, which indicates that cross-feeding of phosphorylated products, as a result of the *phnE* polymorphism, is essential for the spread of evolved mutants.

MATERIALS AND METHODS

Strains, media, and culture conditions. ENZ strains derived from the *E. coli* K-12 strain ENZ535 (MG1655) (see Table S1 in the supplemental material). JW strains, which were derived from the *E. coli* K-12 strain BW25113, were used as donors to transduce single-gene in-frame deletions (Km^r) (2, 19). To construct Km^s derivatives of deletion mutants, the *kan* cassette flanked by the FLP recombination target sites was removed by introducing the FLP recombinase-expressing plasmid pCP20 at 32°C and purifying clones at 42°C (2); Ap^s and Km^s clones were tested for KatE$^-$ (Δ*rpoS*; ENZ1946), PhoA$^-$ (Δ*phoB*; ENZ2084), and MePn$^-$ (Δ*phnE*; ENZ2274) phenotypes (see below). In order to distinguish the different strains in mixed cultures, we transduced the *lacY*::Tn*10* (Tc^r) and Δ*lacY*::*kan* (Km^r) mutations into the ancestral and evolved strains. To backcross the *rpoS1901* allele into the ancestral strain ENZ535, we transduced the *cysC*::Tn*10* marker (7 kb apart from *rpoS*; frequency of cotransduction of 35%) (38) from ENZ2005 (*cysC*::Tn*10* Δ*rpoS*::*kan*) into ENZ1901, selected a *cysC*::Tn*10 rpoS1901* strain (Tc^r Km^s), retransduced the *cysC*::Tn*10* region into the ancestral strain ENZ535, and determined that 35% (14/40) of the Tc^r transductants exhibited an RpoS$^-$ phenotype. Finally, we transferred the *cysC*$^+$ allele from ENZ535 into an *rpoS1901* mutant strain, thereby giving rise to strain ENZ2041 (ENZ535 *rpoS1901*). The same strategy was used to transfer the *rpoS819* allele from ZK819 (38) into ENZ535 (ENZ2039). The P_i-limiting MOPS (morpholineethanesulfonic acid) minimal medium contained 40 mM MOPS, 86 mM NaCl, 9.8 mM KCl, 9.5 mM NH_4Cl, 40 mM glucose, and 0.1 mM K_2HPO_4 (pH 7.2) (21). Cultures (50 ml in 500-ml Erlenmeyer flasks) were agitated at 150 rpm in a covered water bath rotary shaker. For the experiments involving incubation in spent medium, the supernatants of 1-day-old 50-ml cultures in P_i-limiting medium were filter sterilized into 500-ml Erlenmeyer flasks and inoculated with 0.5 ml of cultures serially diluted into salts (86 mM NaCl, 10 mM KCl, and 9.5 mM NH_4Cl). All incubations were performed at 37°C. The pHs of the media were determined at 25°C (21).

Measurement of cell viability. To assess cell viability, serial dilutions were prepared in M9 buffer (19), and aliquots (20 μl) were spotted in triplicate onto LB medium plates, which were spread with 2,000 U catalase (21) and might contain kanamycin (30 μg/ml) or tetracycline (12 μg/ml). In the figures, the values of 10 CFU/ml in parentheses indicate that no CFU were detected when 5 20-μl portions of the cultures were directly plated.

Levels of glucose and acetic acid. The culture supernatants were adjusted to pH 7, and the concentrations of glucose and acetate were determined by enzymatic tests (R-Biopharm). The values are the means from duplicate assays (standard deviations are ≤10% unless otherwise indicated).

RpoS and Glg phenotypes. Isolated colonies were touched with a toothpick, cells were suspended in M9 buffer (200 μl), and aliquots (4 μl) were spotted onto LB medium plates, which were incubated for 24 h. Patches of cells, which were mostly in stationary phase, were exposed (i) to iodine vapor to determine the cellular levels of glycogen (Glg), estimated through the intensity of the brown color, and (ii) to H_2O_2 (2 μl of a 30% solution) to determine catalase activities ($H_2O_2 \rightarrow O_2 \uparrow + H_2O$), estimated through the time required for O_2 bubbling. Because RpoS strictly controls the expression of *katE*, Δ*rpoS* cells (KatE$^-$ KatG$^+$) bubble O_2 after a delay of 5 s, whereas *rpoS*$^+$ cells bubble O_2 immediately (22). Various mutants (e.g., *glgA*, *glgB*, *glgC*, *pgm*, and *galU* mutants) in addition to Δ*rpoS* mutants produce low levels of glycogen (7). Thus, we used the terms RpoS$^-$ phenotype and Glg$^-$ phenotype for cells exhibiting Glg$^-$ KatE$^-$ (like Δ*rpoS* mutants) and Glg$^-$ KatE$^+$ (like *pgm* mutants) phenotypes, respectively.

MePn phenotype. Isolated colonies were touched with a toothpick, cells were suspended in salts (200 μl), and aliquots (4 μl) were spotted onto glucose-M9 minimal medium plates (19) and onto glucose-MOPS minimal medium plates (0.1 g/liter agarose) containing 0.05 mM methyl phosphonate (MePn) (Fluka) as the source of P_i. PhnE$^+$ cells gave rise to confluent patches on MePn plates in 1 (*rpoS*$^+$) or 2 (*rpoS*) days of incubation. PhnE$^-$ cells gave rise to isolated colonies after 3 to 4 days of incubation.

DNA sequencing. PCR fragments were generated with PrimeSTAR HS DNA polymerase (Takara Bio Inc.). PCR products were purified using a QIAquick PCR purification kit (Qiagen), and sequencing was performed at GATC-Biotech.

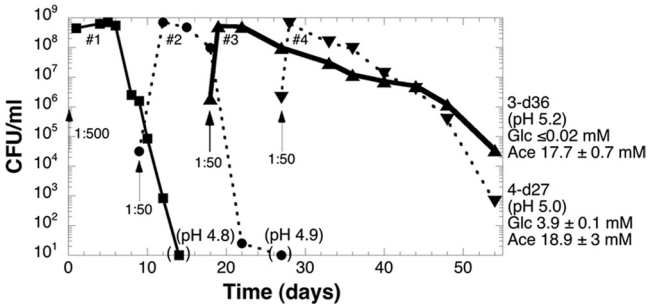

FIG 1 Adaptive evolution of *E. coli* K-12 under P_i starvation conditions. Strain ENZ535 (MG1655) was inoculated (0.1 ml) into 50 ml of P_i-limiting medium (time zero, indicated by the arrow labeled 1:500) and incubated further for 14 days (■). On day 9 of incubation, 1 ml of the culture (culture 1) was inoculated into 50 ml of fresh P_i-limiting medium (indicated by the arrow labeled 1:50), giving rise to subculture 2, which was incubated further for 18 days (●). On day 9 of incubation of culture 2, 1 ml was inoculated into 50 ml of fresh P_i-limiting medium (indicated by the arrow labeled 1:50), giving rise to subculture 3, which was incubated further for 36 days (▲). On day 9 of incubation of culture 3, 1 ml was inoculated into 50 ml of fresh P_i-limiting medium (indicated by the arrow labeled 1:50), giving rise to subculture 4, which was incubated further for 27 days (▼). The pH of the spent medium is indicated in parentheses. The final concentrations of glucose and of acetic acid (Ace) after 36 and 27 days of incubation in subcultures 3 and 4, respectively, are indicated.

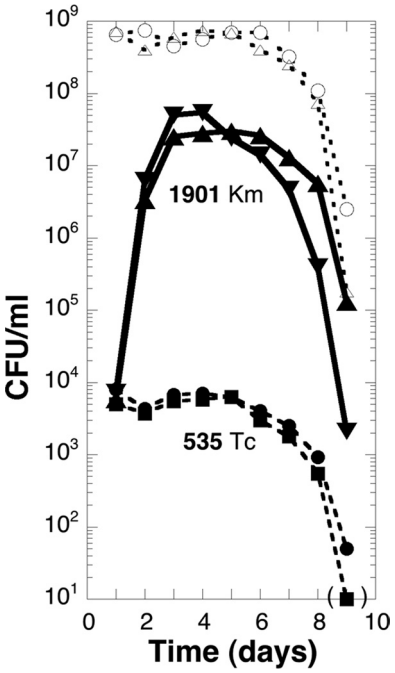

FIG 2 The evolved strain ENZ1901 grows in a culture of *E. coli* K-12 starved for P_i. Both evolved strain ENZ1901 (Km^r, ENZ2000) (▲ and ▼) and ancestral strain ENZ535 (Tc^r, ENZ1797) (● and ■) were grown as monocultures in P_i-limiting medium for 1 day, diluted 10^3-fold, added (0.5 ml of each) into 50 ml of a 1-day-old culture of the ancestral strain ENZ535 in P_i-limiting medium, and incubated further for 8 days. CFU were determined on plates containing LB (○ and △), LB-Km (▲ and ▼), or LB-Tc (● and ■).

RESULTS

Rapid and diverse evolution under P_i starvation conditions. To determine whether the *E. coli* K-12 strain ENZ535 (MG1655) could evolve mutants which could survive prolonged incubation in P_i-limiting batch culture, we inoculated a culture in MOPS minimal medium containing excess glucose (40 mM) and a limiting concentration of P_i (0.1 mM) (21). Cells grew exponentially with a generation time of ≈1 h for 10 h and entered stationary phase when P_i was nearly exhausted (11). Thereafter, cells were diluted 1:50 into fresh P_i-limiting medium every 9 days of incubation and incubated further for up to 36 days. As shown in Fig. 1, the long-term viability of P_i-starved cells increased dramatically after 2 serial transfers: viability reached ≈10^7 CFU/ml after 14 days of incubation in the third and fourth subcultures, whereas viability was ≤10 CFU/ml after 14 days of incubation in the first culture. Similar results were obtained in eight other independent experiments (see Fig. S1 in the supplemental material, and data not shown).

The increase in the long-term viability of the evolved populations appeared to occur concomitantly with an increase in the consumption of glucose and a decrease in the production of acetic acid. For instance, an evolved culture with a high long-term viability contained 10^6 CFU/ml, ≤0.02 mM glucose, and ≤0.02 mM acetic acid at pH 6.9 after 60 days of incubation (data not shown), an evolved culture with a moderate long-term viability contained 3×10^4 CFU/ml, ≤0.02 mM glucose, and 18 mM acetic acid at pH 5.2 after 36 days of incubation (Fig. 1), and the first cultures contained ≤10 CFU/ml, 10 mM glucose, and 30 mM acetic acid at pH 4.8 after 14 days of incubation (11, 21).

Since final evolved populations survived prolonged incubation like *rpoS* mutants (11), we tested evolved cells for characteristic $RpoS^-$ phenotypes, namely, the Glg^- (low production of glycogen) and $KatE^-$ (reduced catalase activity) phenotypes (see Materials and Methods). We found Glg^- $KatE^-$ (like Δ*rpoS* mutants; referred to as $RpoS^-$), Glg^- $KatE^+$ (like *pgm* mutants; referred to as Glg^-), and Glg^+ $KatE^+$ (referred to as Glg^+) cells; the three phenotypes, both in isolation and in association, occurred in proportions that fluctuated during prolonged incubation (see Fig. S1 in the supplemental material), which indicated that evolved populations were highly dynamic and that diverse subpopulations coexisted.

Evolved strains grow in mixed culture starved for P_i. To determine how evolved strains behaved in a population starved for P_i, $RpoS^-$ (ENZ1901), Glg^- (ENZ1902), and Glg^+ (ENZ1903, ENZ1904, and ENZ1905) strains were isolated from two parallel final evolved populations (see Fig. S1B′ in the supplemental material); evolved and ancestral strains were marked with either Tc or Km resistance, grown in isolation for 1 day in P_i-limiting medium, added as a minority into 1-day-old saturated cultures of the ancestral strain ENZ535 grown in P_i-limiting medium, and incubated further for 8 days.

When the $RpoS^-$ evolved strain ENZ1901 (Km^r) and the ancestral strain ENZ535 (Tc^r) were added together as a minority (each to ≈6×10^3 CFU/ml) into a saturated culture of ENZ535 (≈6×10^8 CFU/ml), the cell counts for the evolved strain ENZ1901 (Km^r) increased up to 3×10^7 to 6×10^7 CFU/ml between days 1 and 3 of incubation, whereas the cell counts for the ancestral strain ENZ535 (Tc^r) did not change during this time period (Fig. 2). Similar results were found for mixed cultures containing a single strain in minority and when the Tc^r and Km^r markers were switched between the evolved strain ENZ1901 and the ancestral strain ENZ535 (see Fig. S2 in the supplemental material). To determine more precisely the kinetics of growth of the evolved strain ENZ1901 ($RpoS^-$) in a mixed culture, we added

increasing concentrations of cells (from 10^2 up to 10^6 CFU/ml) into 1-day-old saturated cultures of the ancestral strain ENZ535 ($\approx 6 \times 10^8$ CFU/ml); in all cases, the evolved strain ENZ1901 grew exponentially with a generation time of ≈3 h (versus ≈1 h in fresh medium) between days 1 and 2 to 3 of incubation up to a maximum yield of 3×10^7 to 6×10^7 CFU/ml (see Fig. S3 in the supplemental material).

Expression of the growth-under-P_i-starvation (GPS) phenotype was not limited to RpoS$^-$ evolved strains; the evolved strains ENZ1902 (Glg$^-$) and ENZ1903 (Glg$^+$) also expressed a GPS phenotype, whereas the evolved strains ENZ1904 (Glg$^+$) and ENZ1905 (Glg$^+$) did not (see Fig. S4 in the supplemental material, and data not shown). The fact that evolved strains such as ENZ1901 (RpoS$^-$) and ENZ1902 (Glg$^-$) expressed a GPS phenotype may help explain the rapid spread of such mutants in evolved populations.

The evolved strain ENZ1901 (RpoS$^-$) dies in pure culture starved for P_i. To determine whether changes in metabolism could help explain the behavior of evolved strains that grew in mixed cultures, we focused on studying the metabolic pattern of the evolved strain ENZ1901 (RpoS$^-$). When incubated in isolation under P_i starvation conditions, ENZ1901 consumed higher levels of glucose and excreted lower levels of acetic acid than the ancestral strain ENZ535 (Fig. 3B and C). This pattern may help explain why evolved populations contained low levels of acetic acid. However, the viability of ENZ1901 declined precipitously in pure culture, whereas it increased in mixed culture during the first 3 days of incubation (Fig. 2 and 3A). Thus, intrinsic changes in metabolism and unique interactions with the ancestral strain could account for the GPS phenotype of the evolved strain ENZ1901.

The evolved strain ENZ1901 carries a null-activity *rpoS* allele. Characteristics of the evolved strain ENZ1901, namely, low production of glycogen and reduced catalase activity, low excretion of acetic acid during glucose metabolism, and growth under starvation conditions, have been observed in strains carrying various mutations (e.g., mutations in *rpoS*, *spoT*, and *hfq*) that may somehow decrease the induction of the RpoS regulon (11, 29, 31, 38). To determine whether the evolved strain ENZ1901 carried a low- or null-activity *rpoS* allele, we transferred the *rpoS* gene from the evolved strain ENZ1901 into the ancestral strain ENZ535 using its cotransduction with the *cysC*::Tn*10* marker (see Materials and Methods). The ENZ535 *rpoS1901* reconstructed strain exhibited the same drastic RpoS$^-$ phenotype (Glg$^-$ KatE$^-$) as ENZ535 Δ*rpoS* mutants (data not shown), which indicated that the evolved strain ENZ1901 carried a null-activity *rpoS* allele. Sequencing of *rpoS* (993 bp, 330 amino acids) in ENZ1901 revealed the deletion of 1 bp (G/C) in codon 214.

The low-activity *rpoS819* allele confers a growth-advantage-in-stationary-phase (GASP) phenotype; *rpoS819* single mutants grow and eventually take over a population of wild-type cells during prolonged incubation in rich LB medium (38). In contrast, the null-activity *rpoS1901* and Δ*rpoS* alleles and the low-activity *rpoS819* allele transduced into the ancestral strain ENZ535 could not confer a significant growth advantage in mixed cultures starved for P_i (see Fig. S5 in the supplemental material). Thus, another adaptive mutation(s) beyond the *rpoS1901* mutation was required to account for the GPS phenotype.

PhoB-dependent mechanisms are required for expression of the GPS phenotype. The Pho regulon is induced transiently in

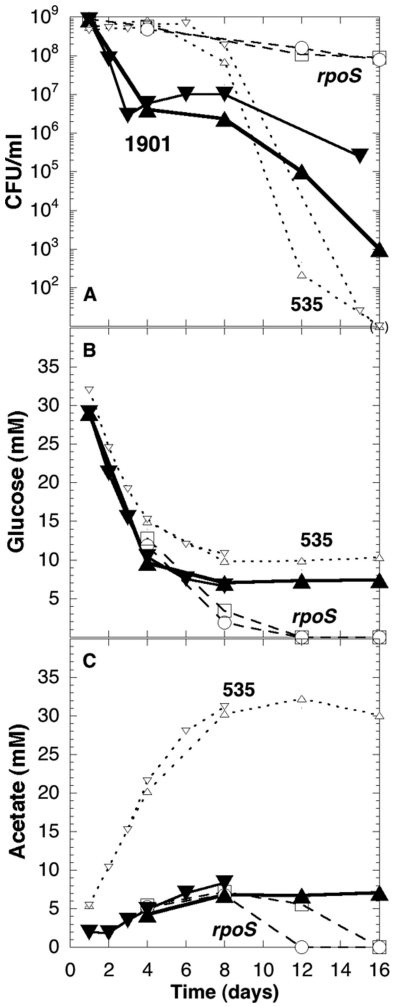

FIG 3 Viability and metabolic pattern of the evolved strain ENZ1901 in pure culture starved for P_i. Strains were inoculated (0.1 ml) into 50 ml of P_i-limiting medium (time zero) and incubated further for up to 16 days. The numbers of viable cells in the cultures (A) and the concentrations of glucose (B) and of acetate (C) in the spent medium were determined. Evolved strain ENZ1901 (Km^r, ENZ2000) (▲ and ▼), ancestral strain ENZ535 (Tc^r, ENZ1797) (△ and ▽), and ENZ535 carrying *rpoS*::Tn*10* (ENZ985) (○) and Δ*rpoS* (ENZ2020) (□) were used.

P_i-starved cells through a reversible phosphorylation of PhoB. Mutants express the Pho regulon constitutively as a result of a stable phosphorylation of PhoB (13, 32). Such mutations might occur in the evolved strain ENZ1901, which could provide an advantage over the ancestral strain to scavenge P-containing compounds during prolonged incubation. To test this possibility, we asked whether the inactivation of PhoB prevented the expression of the GPS phenotype. As shown in Fig. 4A, the evolved strain ENZ1901 carrying a Δ*phoB* (or Δ*phoBR*) mutation did not grow and lost viability in mixed culture. In addition, we found that Δ*phoB* (or Δ*phoBR*) mutants, which exhibit the same viability as the parental strain ENZ535 during prolonged incubation in monocultures starved for P_i (21), could not evolve surviving populations during serially propagated culture in P_i-limiting medium (data not shown). Thus, PhoB-dependent mechanisms might be required to evolve mutants and/or to allow evolved mutants to express a GPS phenotype.

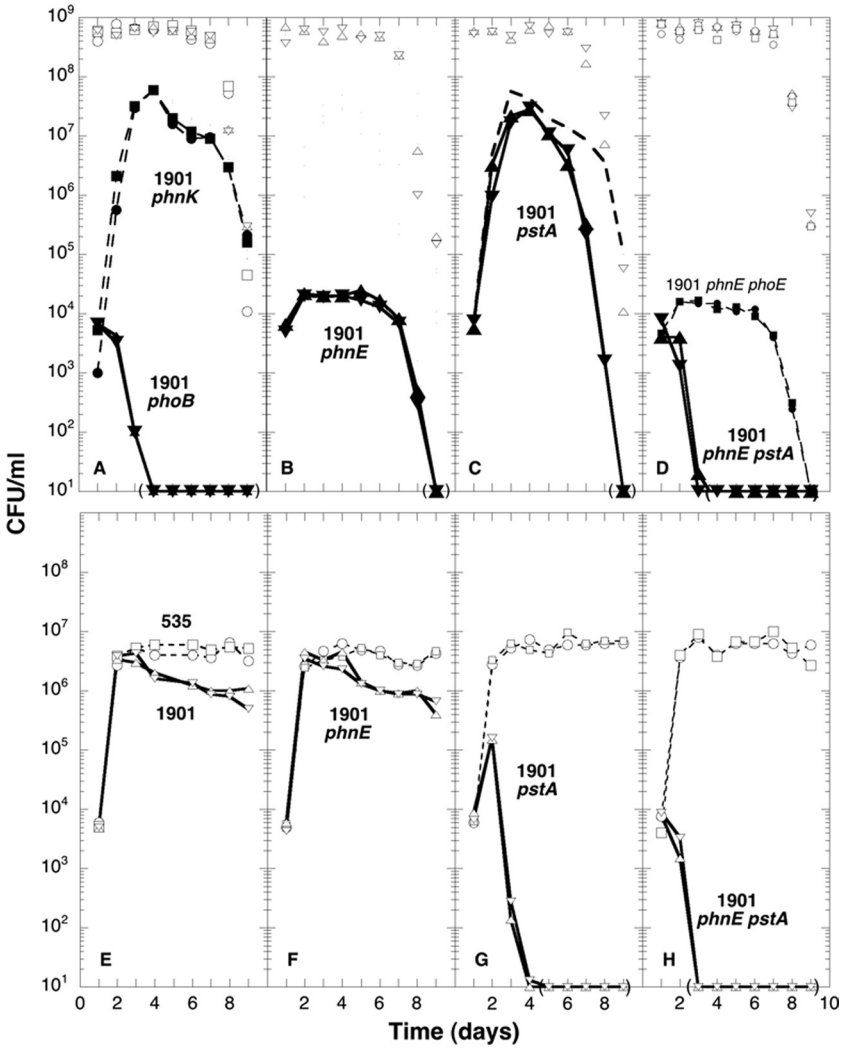

FIG 4 Growth of the evolved strain ENZ1901 (Km^r) in mixed culture and in spent medium. (A to D) Mixed cultures. Derivatives of the evolved strain ENZ1901 (Km^r) (solid symbols) were added into 1-day-old cultures of the ancestral strain ENZ535 (Tc^r, ENZ1797) (open symbols) in P_i-limiting medium. (A) ENZ1901 Δ*phoBR* (ENZ2015) (▲), ENZ1901 Δ*phoB* (ENZ2079) (▼), and ENZ1901 Δ*phnK* (ENZ2268) (● and ■). (B) ENZ1901 Δ*phnE* (ENZ2271) (▲ and ▼). (C) ENZ1901 Δ*pstA* (ENZ2260) (▲ and ▼) and ENZ1901 (broken line) (the values are from Fig. S2A in the supplemental material). (D) ENZ1901 Δ*phnE* Δ*pstA* (ENZ2277) (▲ and ▼) and ENZ1901 Δ*phnE* Δ*phoE* (ENZ2278) (● and ■). (E to H) Spent media. The ancestral strain ENZ535 (Tc^r, ENZ1797) (○ and □) and derivatives of the evolved strain ENZ1901 (Km^r) (△ and ▽) were grown as monocultures in P_i-limiting medium for 1 day, diluted 10^3-fold, and added (0.5 ml of each) into 50 ml of filter-sterilized spent medium obtained from 1-day-old cultures of the ancestral strain ENZ535 in P_i-limiting medium. (E) ENZ1901 (ENZ2000). (F) ENZ1901 Δ*phnE* (ENZ2271). (G) ENZ1901 Δ*pstA* (ENZ2260). (H) ENZ1901 Δ*phnE* Δ*pstA* (ENZ2277).

The permease of the phosphonate utilization system is required for the GPS phenotype. To determine whether the evolved strain ENZ1901 carried mutations in *phoBR* and/or in genes that belong to the Pho regulon, we transduced into ENZ1901 various Km^r markers that were inserted (i) in Pho genes (e.g., *pstA*, *phoE*, *phoA*, and *ugpA*) and (ii) close to Pho operons (i.e., *phoBR* and *phnCDEFGHIJKLMNOP*) (2) and tested Km^r transductants for the GPS phenotype. Only mutations identified by linkage near the beginning of the *phnCDEFGHIJKLMNOP* operon were required for the expression of the GPS phenotype (data not shown). Sequencing of the *phnCDE* genes in ENZ1901 revealed in *phnE* a deletion of 8 bp in tracts of 3 similar sequences of 8 bp called A and B (14), switching from the sequence 5′-ABB-3′ to 5′-AB-3′. Such an 8-bp deletion, which removes a frameshift that normally inactivates *phnE* in *E. coli* K-12, occurs at a high frequency, probably because of a strand slippage event during DNA replication (14, 17, 33).

The *phnCDEFGHIJKLMNOP* operon encodes 14 proteins essential for growth on phosphonates such as MePn: PhnE is the permease subunit in the PhnCDE ABC transporter, and PhnGHIJK form the complex essential for cleaving the C-P bond in phosphonates (15, 26, 35). We confirmed that the evolved strain ENZ1901 (*phnE*$^+$) grew with the same efficiency on MePn or P_i as the sole source of P, whereas the ancestral strain ENZ535 (*phnE*::8 bp) did not grow on MePn but gave rise to variants at a high frequency of ≈2 MePn$^+$ cells per 10^5 total cells (data not shown), compared with a typical frequency of ≈10^{-7} for a single-base substitution (10). Most interestingly, an ENZ1901 Δ*phnK* strain that expressed a GPS phenotype (Fig. 4A) could not grow on MePn as the sole source of P and did not give rise to any MePn$^+$

variants (data not shown). These data indicated collectively that the ABC transporter PhnCDE, but not the C-P lyase PhnGHIJK, was required for the expression of the GPS phenotype.

PhnE scavenges organophosphates released by P_i-starved cells. The PhnCDE system, which transports primarily phosphonates, can also transport organic phosphate esters and P_i (9, 18). Since growth of the evolved strain ENZ1901 in mixed culture did not require the C-P lyase activity, PhnE might be required to scavenge organophosphates, P_i, or both. We have shown previously that cells incubated in P_i-limiting medium (i) consume most of the available P_i during exponential growth, (ii) transiently accumulate organophosphates (e.g., NAD and uridine diphosphoglucose [UDPG]) at the entry into stationary phase, and (iii) release significant levels of P_i after 4 days of incubation (11, 22). Thus, ENZ1901 could scavenge through PhnE residual levels of P_i not consumed during exponential growth or P_i and/or organophosphates released during stationary phase.

To test these possibilities, we assessed the behavior of ENZ1901 Δ*phnE* mutants (i) in mixed culture and (ii) in 1-day-old spent medium (ENZ535 cells grown in P_i-limiting medium were removed on day 1 of incubation). ENZ1901 Δ*phnE* mutants grew normally in spent medium (Fig. 4F) but could barely grow in mixed culture (Fig. 4B), which indicated that the evolved strain ENZ1901 ($PhnE^+$) grew in mixed culture primarily as a result of the PhnE-dependent scavenging of P-containing compounds released by the ancestral strain ENZ535 starved of P_i. However, ENZ1901 Δ*phnE* mutants (Fig. 4B) did not lose viability in mixed culture as rapidly as ENZ1901 Δ*phoB* mutants did (Fig. 4A), which indicated that PhoB should induce another essential gene(s) besides *phnE*. The *phoE* and *pstA* genes were likely candidates: the porin PhoE facilitates the diffusion of P_i and P compounds across the outer membrane, and the PstA permease is the primary transporter of P_i (13). Whereas ENZ1901 Δ*phnE* Δ*phoE* double mutants behaved like ENZ1901 Δ*phnE* single mutants (Fig. 4B and D), Δ*phnE* Δ*pstA* double mutants lost viability rapidly like ENZ1901 Δ*phoB* mutants (Fig. 4A and D), which indicated that the induction by PhoB of both *phnE* and *pstA* was required for the viability of the evolved strain ENZ1901 in mixed culture.

The synergistic effect of the Δ*phnE* and Δ*pstA* mutations was unexpected because ENZ1901 Δ*pstA* single mutants grew normally between days 1 and 3 of incubation in mixed culture (Fig. 4C). However, ENZ1901 Δ*pstA* mutants lost viability more rapidly than ENZ1901 after 4 days of incubation (Fig. 4C), which suggested that PstA might improve the viability of the evolved strain ENZ1901 through scavenging of P_i during prolonged incubation. Similarly, growth of the evolved and ancestral strains in spent medium (up to a total of $\approx 10^7$ CFU/ml) might result primarily from the scavenging through PstA of residual levels of P_i left in the medium on day 1 of incubation (estimated as ≈1% of the initial concentration of P_i, that is, ≈1 μM) (Fig. 4E and G), and the limited growth (up to 2×10^5 CFU/ml) of ENZ1901 Δ*pstA* mutants in spent medium between days 1 and 2 (Fig. 4G and H) might result from the scavenging through PhnE of low levels of organophosphates already released by P_i-starved cells on day 1 of incubation.

These data indicated collectively that the viability of the evolved strain ENZ1901 in a mixed culture starved for P_i required primarily the Pho-dependent activity of the PhnE permease to scavenge probably organophosphates released by the ancestral strain ENZ535 until day 3 of incubation and secondarily the Pho-dependent activity of the PstA permease to scavenge (i) P_i left in the medium on day 1 of incubation and (ii) P_i released by the ancestral strain ENZ535 after day 4 of incubation. The PhnE activity was expressed uniquely by the evolved strain ENZ1901, whereas the PstA activity was expressed by both evolved and ancestral strains, which could compete for P_i in the medium especially at the entry into stationary phase, thereby limiting the growth of ENZ1901 Δ*phnE* mutants in mixed culture containing the ancestral strain in excess (Fig. 4B and F).

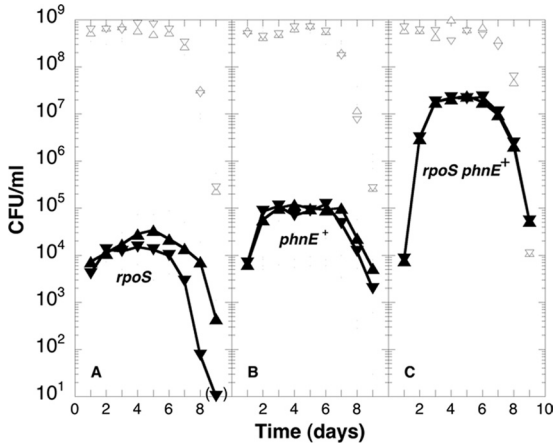

FIG 5 The reconstructed strain ENZ535 *rpoS1901 phnE*$^+$ exhibits a GPS phenotype. Derivatives of the ancestral strain ENZ535 (solid symbols) were tested in mixed cultures with ENZ535 (Tc^r, ENZ1797) (open symbols) as a majority. (A) ENZ535 *rpoS1901* (Km^r, ENZ2265) (▲ and ▼). (B) ENZ535 *phnE*$^+$ (Km^r, ENZ2256) (▲ and ▼). (C) ENZ535 *rpoS1901 phnE*$^+$ (Km^r, ENZ2263) (▲ and ▼); identical results were obtained with ENZ535 Δ*rpoS phnE*$^+$ (Km^r, ENZ2259).

The *rpoS* mutant and *phnE*$^+$ alleles are sufficient for expression of the GPS phenotype. We transferred the *phnE*$^+$ allele from the evolved strain ENZ1901 (by using cotransduction with Δ*proP*::Kan and assaying for the $MePn^+$ phenotype) alone or in combination with the *rpoS1901* allele into the ancestral strain ENZ535 and tested the ensuing reconstructed strains in mixed cultures. Whereas ENZ535 carrying the *rpoS1901* (or Δ*rpoS*) allele alone grew barely from $\approx 5 \times 10^3$ up to $\approx 4 \times 10^4$ CFU/ml (Fig. 5A; see Fig. S5A and B in the supplemental material), ENZ535 carrying the *phnE*$^+$ allele alone grew modestly up to $\approx 10^5$ CFU/ml (Fig. 5B), and ENZ535 carrying both the *rpoS1901* (or Δ*rpoS*) and *phnE*$^+$ alleles grew up to $\approx 2 \times 10^7$ CFU/ml in 3 days (Fig. 5C), which indicated that the *rpoS* mutant and *phnE*$^+$ alleles were sufficient to trigger a full GPS phenotype.

The *phnE* polymorphism is essential for the evolution of a population starved for P_i. Although they exhibited different phenotypes, the five final evolved strains that we isolated, namely, ENZ1901 ($RpoS^-$ GPS^+), ENZ1902 (Glg^- GPS^+), ENZ1903 (Glg^+ GPS^+), and ENZ1904 and -1905 (Glg^+ GPS^-) were $MePn^+$ (data not shown). Thus, the *phnE*$^+$ allele, associated with various adaptive mutations, might be essential for the spread of evolved mutants in a population starved for P_i. To determine whether the *phnE* polymorphism was required before the apparent fixation of the *phnE*$^+$ allele in evolved populations, we conducted evolution experiments by using the *E. coli* K-12 strain ENZ535 and a *phnE*$^+$ derivative. When the *phnE*$^+$ strain was used as the starter strain,

only one of 4 populations survived on day 30 of incubation after 2 serial transfers. In contrast, when *E. coli* K-12 (*phnE*::8 bp) was used as the starter strain, 3 of 4 populations survived after 30 days of incubation (see Fig. S6 in the supplemental material). The four latter evolved populations were diverse; almost all cells (94%) exhibited a PhnE$^+$ phenotype on day 9 of incubation, but this proportion steadily decreased during prolonged incubation to reach 68% on day 30. The RpoS$^-$ phenotype was less abundant and decreased more rapidly, from 49% on day 9 to 14% between days 21 and 30. Thus, the *phnE* polymorphism might be essential for the spread of diverse subpopulations under P_i starvation conditions.

DISCUSSION

The *E. coli* K-12 strain ENZ535 (MG1655) loses viability after 6 days of incubation in a batch culture initially containing 40 mM glucose and a limiting concentration of P_i (0.1 mM), reaching ≤10 CFU/ml on day 14 of incubation, presumably as a result of the excretion of high levels of acetic acid (21). We show here that after generally 3 growth cycles of 9 days under P_i starvation conditions, ENZ535 evolved populations that survived during prolonged incubation (≈10^7 CFU/ml on day 14 of incubation). Evolved populations generally consumed all the glucose yet excreted low levels of acetic acid during prolonged incubation. This is reminiscent of Δ*rpoS* mutants in pure culture, which consume glucose but do not produce acetic acid and thus survive P_i starvation (≈10^8 CFU/ml on day 14 of incubation) (11). It appeared that ≈50% of the evolved populations contained, at least transiently, a majority of RpoS$^-$ cells.

The rapid spread of evolved strains may be accounted for by their unique capacity to grow in a mixed culture starved for P_i containing the ancestral strain ENZ535 in majority. However, the so-called growth-under-P_i-starvation (GPS) phenotype could not be triggered by single *rpoS* mutations. A clue about other mutations required for expression of the GPS phenotype was provided by the findings that both evolution of surviving populations and growth of evolved strains in mixed culture required the induction of the Pho regulon. The induction of two Pho-dependent genes was required for growth of an evolved strain in mixed culture, namely, *phnE* and *pstA*, which encode permeases for P compounds. PhnE and PstA might be primarily required to scavenge, respectively, organophosphates and P_i released until day 3 and after day 4 of incubation by the ancestral strain ENZ535 starved of P_i.

PhnE is normally cryptic in *E. coli* K-12 (14, 17, 18, 33). However, an evolved strain that exhibited a GPS phenotype carried a large deletion of 8 bp in *phnE* that restored an active *phnE* allele; such a deletion, which may result from a strand slippage event during replication of direct repeat motifs, occurred at a high frequency. Although most evolved strains were PhnE$^+$, the polymorphism for *phnE* in evolving populations was essential since the parental *E. coli* K-12 strain evolved more surviving populations than a *phnE*$^+$ derivative did during repeated cycles under P_i starvation conditions. Thus, cross-feeding of organophosphates between the ancestral *E. coli* K-12 strain (*phnE*::8 bp) in stationary phase and *phnE*$^+$ variants might be essential for the spread of evolved mutants in populations starved for P_i. We have shown previously that P_i-starved cells transiently accumulate organophosphates at the entry into stationary phase (22). Organophosphates could be released and then transported inside *phnE*$^+$ variants by the PhnCDE system, thereby providing an alternative source of P.

The two changes characterized in an RpoS$^-$ GPS$^+$ evolved strain, that is, a 1-bp deletion in *rpoS* (conferring an RpoS$^-$ phenotype) and an 8-bp deletion in *phnE* (conferring a PhnE$^+$ phenotype), were sufficient to express a full GPS phenotype. The lack of RpoS activity, which may result from various changes in *rpoS* sequence (8), may primarily maintain growth under P_i starvation conditions through a substantial activity of the tricarboxylic acid (TCA) cycle (11), and the essential role of PhnE may be to provide rapidly a source of P. We have shown previously that *rpoS*$^+$ cells starved for P_i redirect the metabolism of pyruvate through PoxB, which causes the excretion of toxic levels of acetic acid by day 6 of incubation (20, 21). In contrast, *rpoS* mutants maintain the carbon flux through the TCA cycle, which prevents the accumulation of acetic acid and eventually allows the consumption of the low levels of acetic acid that are excreted. In fact, *rpoS* mutants resume growth on acetic acid when high levels of P_i are released by the bulk of *rpoS* mutants after 6 days of incubation (11). However, such a resumption of growth of *rpoS* mutants occurred in a pure culture but not in a mixed culture containing a minority of *rpoS* mutants. In the latter case, excess *rpoS*$^+$ cells excreted high levels of acetic acid by day 6 of incubation, which prevented growth recovery of *rpoS* mutants and eventually killed both *rpoS*$^+$ and *rpoS* cells. In contrast, an evolved strain carrying both the *phnE*$^+$ and *rpoS* mutant alleles could resume growth in a mixed culture starved of P_i as soon as the ancestral strain in majority entered stationary phase and released organophosphates.

Could the PhnE- and RpoS-dependent processes of adaptation of the *E. coli* K-12 strain MG1655 under aerobic, P_i starvation conditions help explain the behavior of *E. coli* in the natural environment? We propose that the population polymorphisms in *phnE* and *rpoS* might help protect commensal *E. coli* strains during initial colonization of the human intestine. Several lines of evidence support this idea.

First, the *E. coli* K-12 laboratory strain MG1655, which is derived from the human isolate *E. coli* K-12 (3), is closely related to commensal gut strains of *E. coli* recently isolated from humans (28). Whereas *E. coli* K-12 contains a *phn* operon, the commensal *E. coli* HS strain contains no *phn* operon (25). Whereas *E. coli* K-12 contains *a phnE::*8 bp mutation, environmental *E. coli* strains isolated in water contain a *phnE*$^+$ allele (14). This may result from the fact that environmental *E. coli* may generally use phosphonates as a source of P_i (35), which may provide a strong selection for the PhnE$^+$ state. Thus, the polymorphism in *phnE* might occur in specific commensal *E. coli* strains such as *E. coli* K-12. In contrast, polymorphism in *rpoS* is common in the natural environment (8).

Second, bacteria might experience repeated P_i starvation periods in the distal part of the small intestine (ileum) and in cecum (21); the concentrations of nutrients, including amino acids, monosaccharides (e.g., glucose), and P_i, may vary considerably between feeds due to absorption in the proximal small intestine (duodenum), when C may be obtained through the metabolism of disaccharides (e.g., lactose) (12, 30). Interestingly, a P_i sensor that recognizes an increase in dietary phosphate concentrations, and consequently increases renal P_i excretion, may exist in the duodenum (4).

Third, the intestine after birth is sterile and contains relatively high oxygen levels (5, 24). Because *E. coli* can grow with and without oxygen (facultative anaerobe), it is generally the first to colo-

nize the infant intestine in the first days after birth (24) and can reach high concentrations in ileum and cecum (5, 12). The spread of enterobacteria was also observed transiently after heavy antibiotic treatment in human (from 2 to 34%) and mouse (from 1 to 71%) gut microbiomes (1, 37). Aerobic respiration is essential for colonization of the mouse intestine by MG1655 (16).

Thus, during the first days of colonization of the ileum and cecum, it is tempting to speculate that *E. coli* K-12 might be periodically starved for P_i but could eventually convert lactose into pyruvate and oxygen into H_2O (36). Such a metabolic activity might eventually decrease the viability of *E. coli* K-12 because aerobic pyruvate metabolism through the pyruvate dehydrogenase (PDH) complex, and eventually through RpoS-regulated pyruvate oxidase (PoxB), might cause the accumulation of toxic levels of acetic acid (21). However, the rapid sweep of the population by, notably, *phnE*$^+$ *rpoS* evolved mutants, which could grow on organophosphates released by *E. coli* K-12 in stationary phase, might eventually allow survival of the whole evolved population during prolonged incubation under aerobic, P_i starvation conditions. Prolonged metabolism by evolved populations, which could consume acetic acid and oxygen in the small intestine, might eventually help in successful colonization of the colon by strictly anaerobic bacteria (16, 36).

ACKNOWLEDGMENT

We express our gratitude to Angélique Chanal (CNRS-LCB) for help in constructing strains and in sequencing evolved mutants.

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