Mutational Adaptation of *Escherichia coli* to Glucose Limitation Involves Distinct Evolutionary Pathways in Aerobic and Oxygen-Limited Environments

Karen Manché, Lucinda Notley-McRobb and Thomas Ferenci

Department of Microbiology, University of Sydney, Sydney, New South Wales 2006, Australia

Manuscript received March 15, 1999
Accepted for publication May 21, 1999

ABSTRACT

Mutational adaptations leading to improved glucose transport were followed with *Escherichia coli* K-12 growing in glucose-limited continuous cultures. When populations were oxygen limited as well as glucose limited, all bacteria within 280 generations contained mutations in a single codon of the *ptsG* gene. V12F and V12G replacements in the enzyme IIBC^{Glc} component of the glucose phosphotransferase system were responsible for improved transport. In stark contrast, *ptsG* mutations were uncommon in fully aerobic glucose-limited cultures, in which polygenic mutations in *mgl*, *mlc*, and *malT* (regulating an alternate high-affinity Mgl/LamB uptake pathway) spread through the adapted population. Hence the same organism adapted to the same selection (glucose limitation) by different evolutionary pathways depending on a secondary environmental factor. The clonal diversity in the adapted populations was also significantly different. The PtsG V12F substitution under O_2 limitation contributed to a universal "winner clone" whereas polygenic, multiallelic changes led to considerable polymorphism in aerobic cultures. Why the difference in adaptive outcomes? *E. coli* physiology prevented scavenging by the LamB/Mgl system under O_2 limitation; hence, *ptsG* mutations provided the only adaptive pathway. But *ptsG* mutations in aerobic cultures are overtaken by *mgl*, *mlc*, and *malT* adaptations with better glucose-scavenging ability. Indeed, when an *mglA*::Tn*10* mutant with an inactivated Mgl/LamB pathway was introduced into two independent aerobic chemostats, adaptation of the Mgl$^-$ strain involved the identical *ptsG* mutation found under O_2-limited conditions with wild-type or Mgl$^-$ bacteria.

CHEMOSTATS are extremely useful for analyzing the adaptive capabilities of bacterial populations (Dykhuizen 1993). Classic studies recognized that numerous mutational adaptations take place in such cultures (references in Dykhuizen and Hartl 1983). Yet the targets of mutation in chemostat adaptation are still poorly understood even with well-studied glucose- (Helling *et al.* 1987) and lactose-limited (Dean 1989) populations of *Escherichia coli*. Without analysis of the genetic targets and the changes therein, it is impossible to interpret the behavior of evolving populations. Recently, we started a study of the mutational changes involved in improving the fitness of *E. coli* under conditions of glucose limitation (Notley-McRobb and Ferenci 1999a,b). We focus on the transport changes in scavenging nutrients, since these have large fitness contributions and invariably appear during continued selection under limitation conditions.

In the simplest experimental situation, adaptation to a stress like nutrient limitation can be considered as a two-component problem:

$$\text{Organism} + \text{Stress}_1 \rightarrow \text{Organism}_{A1}. \qquad (1)$$

In the system we use, the Organism is *E. coli* and Stress$_1$ is glucose limitation, which imposes gene-regulatory changes (Ferenci 1999) as well as a selection condition. The selection in the chemostat environment is for a genotype that maintains the lowest equilibrium concentration of nutrient (Hansen and Hubbell 1980). The nature of Organism$_{A1}$, the adapted form of *E. coli*, was analyzed recently with six glucose-limited chemostat populations. As expected from earlier studies, these cultures became polymorphic during adaptation (Rosenweig *et al.* 1994) so there was no single genotype representing Organism$_{A1}$. Nevertheless, the same transport loci were repeatedly altered in parallel populations, leading to a similar phenotype (better scavenging of glucose) but with different alleles of genes influencing glucose uptake (Notley-McRobb and Ferenci 1999a,b).

The most common sites of mutation identified within 280 generations of culture were those affecting regulation of the outer membrane LamB glycoporin and an ABC-type, binding-protein-dependent Mgl system (Notley-McRobb and Ferenci 1999a,b). These cellular components constitute the high-affinity glucose transport pathway under nutrient limitation (Ferenci 1996). The regulatory loci *malT*, *mlc*, and *mglD*/O were the main targets for selection, with mutations in two or more of these genes common in all isolates. The effect of the regulatory mutations was to increase scavenging ability by producing more of the LamB/Mgl pathway

Corresponding author: Thomas Ferenci, Department of Microbiology G08, University of Sydney, Sydney, NSW 2006, Australia.
E-mail: t.ferenci@microbio.usyd.edu.au

components. The mutations at these loci were of a diverse nature, but the point mutations were generally transversions at GC base pairs (Notley-McRobb and Ferenci 1999b). This spectrum, typical of DNA damage by reactive O_2 species (Miller 1996), raised the possibility that oxygen radicals were a major source of the mutations in the aerobic cultures. Hence this study was initiated primarily to investigate effects of lowered O_2 tension on mutational processes. But as reported here, the major surprise was not that the mutational spectrum changed, but that the target of transport selection was quite different with additional O_2 limitation.

Perhaps the difference in targets of selection should not have been surprising given that a more accurate description of the selection reported in this article is

$$\text{Organism} + \text{Stress}_1 + \text{Stress}_2 \rightarrow \text{Organism}_{A2}, \quad (2)$$

where Stress_2 is O_2 limitation. We are not aware of an experimental study where long-term adaptation was followed in chemostats with multiple stresses. Yet in the real world a combination of ecological challenges is likely to be commonplace. As reported below, Organism_{A2} is adapted quite differently from Organism_{A1} with respect to glucose transport optimization, revealing alternate pathways and interacting factors in evolutionary outcomes.

All previous work establishing the LamB/Mgl system as the glucose-scavenging pathway was done under aerobic culture conditions (Ferenci 1996). Of course, *E. coli*, like many anaerobic and facultative organisms, also contains the glucose phosphotransferase system (PTS) for transporting glucose (Postma *et al.* 1996). The PTS has a lower sugar affinity compared to Mgl and therefore is less effective under glucose limitation (Ferenci 1996). As noted above, the PTS was not a frequent target for selection in isolates with increased fitness in glucose-limited chemostats. Yet, as shown below, the PTS has a more prominent role than Mgl under microaerophilic conditions and the *ptsG* gene was the main target of selection under O_2 limitation. The *ptsG* gene encodes a glucose-specific recognition component of the PTS (the IIBC^{Glc} protein) in the cytoplasmic membrane (Postma *et al.* 1996). Mutations in *ptsG* are known, but not with enhanced glucose affinity (Buhr *et al.* 1992; Ruijter *et al.* 1992; Begley *et al.* 1996; Lanz and Erni 1998). Two novel single amino acid substitutions described in this study resulted in increased glucose transport at low concentrations. Indeed, a single Val to Phe substitution at residue 12 of PtsG is the most favorable outcome under combined glucose- and O_2-limited conditions, in contrast to the multiple coexisting changes under aerobic glucose limitation.

MATERIALS AND METHODS

Bacterial strains: All bacterial strains used in this study were derivatives of *E. coli* K-12 and are shown in Table 1. Phage P1 transduction (Miller 1972; Notley and Ferenci 1995) with P1 *cml clr100/1000* grown on BW2000 (Muir *et al.* 1985) as donor was used to introduce the *mglA*::Tn10 mutation into isolates. The cotransduction of *ptsG* was accomplished using the linked *zce-726*::Tn*10* marker and transductants differentiated using glucose transport assays.

Growth medium and culture conditions: The basal salts medium used in all experiments was minimal medium A (MMA; Miller 1972; Notley and Ferenci 1995) supplemented with glucose as specified for each experiment. Batch cultures for assaying the properties of isolates contained 0.4% (w/v) sugar and were harvested during midexponential growth.

Glucose-limited chemostats (80 ml) were set up as previously described (Death *et al.* 1993). For cultures additionally subject to oxygen limitation, the sparger was removed so the only air access was from the medium break flushing the surface of the culture. The glucose concentration in the feed medium was 0.08% (w/v) for O_2-limited chemostats and 0.02% for other aerobic chemostats. The continuous cultures were run for up to 4 wk at a dilution rate of 0.3 hr^{-1}. Samples were taken at weekly intervals by plating onto nonselective nutrient agar and 10 randomly separated colonies were purified by streaking, numbered for further testing, and stored in glycerol stocks for assays described below. There was no obvious morphological change in any of the selected isolates.

Culture sampling for the assay of residual glucose concentrations in media using glucose oxidase was as described in Death and Ferenci (1994).

Transport studies: The initial rate of uptake of 0.5 μm [^{14}C]glucose and 1 μm [^{14}C]galactose by glycerol-grown isolates was determined using bacteria resuspended in MMA to an A_{580} of 0.2 (equivalent to 2×10^8 bacteria ml^{-1}) as described previously (Death and Ferenci 1993; Notley and Ferenci 1995). The rate of transport was calculated in units of picomoles sugar transported per minute per 10^8 bacteria. Transport kinetics were measured in the same way except that bacteria were resuspended to different bacterial densities (A_{580} of 0.1 for the *mgl*-con and *ptsG* strains and 0.5 for the wild-type strain) and 60 μl of bacterial suspension was added to 60 μl of glucose solutions to give final concentrations ranging from 0.3 to 4.5 μm.

β-Galactosidase assay: Five-milliliter samples from chemostat (0.02% carbon source) or batch cultures (0.2% carbon source unless otherwise stated) were removed and β-galactosidase activity was measured as described by Miller (1972) using SDS and chloroform-treated cells.

Sensitivity to glucose analogues: Increased sensitivities to the PtsG and PtsM substrates, methyl-α-glucoside and 2-deoxyglucose, respectively, were assayed by plating 0.2% lactate-grown isolates onto glycerol minimal agar plates (Curtis and Epstein 1975) overlaid with 6-mm disks containing 10 μl of 20% (w/v) methyl-α-glucoside or 20% (w/v) 2-deoxyglucose. Zones of inhibition were measured after overnight incubation at 37°.

Mutation analysis: PCR amplification of the 1431-bp *ptsG* sequence and 318 bp of its upstream regulatory region involved two overlapping fragments and two pairs of primers: (5′-CTGTTTCACATCGACGCTTCC-3′) and (5′-GCATGT TATGGCAGAAGCAGG-3′) as forward primers and (5′-CCAG CGCGGATACGCCATCG-3′) and (5′-GCTGCCTTAGTCTCC CCAACG-3′) as reverse primers. The reaction profile consisted of 34 cycles of: denaturation at 94° for 30 sec, followed by annealing at 58° for 30 sec, and extension at 72° for 1.5 min in a DNA thermal cycler (Perkin-Elmer Cetus, Norwalk, CT). PCR products were purified directly with Wizard PCR preps DNA purification system (Promega Corp., Sydney). The nucleotide sequence was determined using the above primers and dye-terminator sequencing reactions on a Catalyst Robotic Workstation. Mutations were located in mutant sequences by

TABLE 1

***Escherichia coli* K-12 strains used in this study**

Strain	Genotype	Parent strain / construction	Selection	Origin / reference
BW2952	F⁻ *araD139* Δ*(argF-lac)U169 rpsL150 deoC1 relA1 thiA ptsF25 flbB5301* Φ*(malG-lacZ⁺)*			Notley and Ferenci (1995)
BW3143	BW2952 *mgl*::Tn*10*			Notley-Mcrobb and Ferenci (1999a)
BW3216	BW2952 *malT-con mgl-con*		Chemostat isolate (originally called 15D2)	Notley-Mcrobb and Ferenci (1999a)
TL212	F⁻ *araD139* Δ*(argF-lac)U169 rpsL150 deoC1 relA1 thiA ptsF25 flbB5301 malT^C-1* Δ*malE444 zce-726*::Tn*10*			Larson *et al.* (1984)
UE26	*glk7 ptsG2 ptsM1 rpsL*			W. Boos
BW3400	BW2952 *ptsG*(V12F)	BW2952	Chemostat isolate (originally called K9D1)	This study
BW3401	BW2952 *ptsG*(V12G)	BW2952	Chemostat isolate (originally called K9C8)	This study
BW3402	BW3400 *zce*-726::Tn*10 ptsG3400*	P1: TL212 into BW3400	Tet^R	This study
BW3403	BW3400 *zce*-726::Tn*10 ptsG⁺*	P1: TL212 into BW3400	Tet^R	This study
BW3404	BW3401 *zce*-726::Tn*10 ptsG3401*	P1: TL212 into BW3401	Tet^R	This study
BW3406	UE26 *zce*-726::Tn*10 ptsG3400*	P1: BW3402 into UE26	Tet^R	This study
BW3407	UE26 *zce*-726::Tn*10 ptsG⁺*	P1: BW3403 into UE26	Tet^R	This study
BW3408	UE26 *zce*-726::Tn*10 ptsG3401*	P1: BW3404 into UE26	Tet^R	This study

aligning with the known *ptsG* sequence in the *E. coli* genome database using software available in ANGIS (Australian National Genomic Information Service, Sydney).

RESULTS

To establish O_2-limited cultures for long-term selection, a simple modification was made to the standard continuous culture conditions previously adopted. The air sparging system was removed from the chemostat so aeration was solely at the air-water interface at the surface of the stirred culture. This level of aeration provided sufficient O_2 access when low cell densities were maintained in the vessel, but the diffusion of O_2 became limiting with higher cell densities. As shown in Figure 1, the effect of the O_2 limitation was apparent from the suboptimal growth yield at higher glucose input levels, in comparison to fully aerated cultures grown on the same medium. The yield at higher bacterial densities in the modified chemostat was still above that from totally anaerobic, fully fermentative cultures grown on the same medium. Hence these cultures were at an inter-

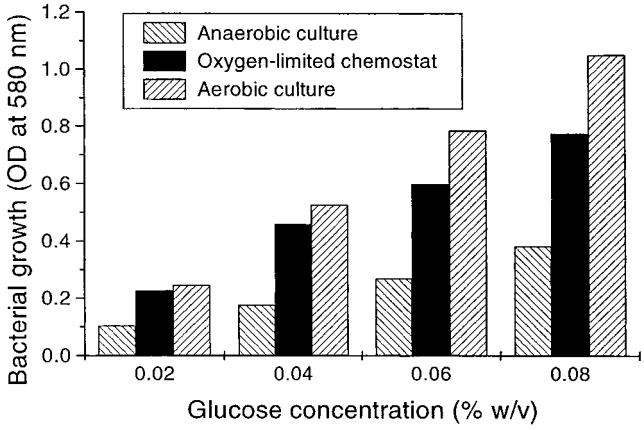

Figure 1.—Bacterial growth yields under aerobic, anaerobic, and oxygen-limited conditions. Strain BW2952 was inoculated into media with different glucose concentrations at 37°. The densities of duplicate cultures were measured until the recorded optical densities reached a constant value; the mean of these determinations is given.

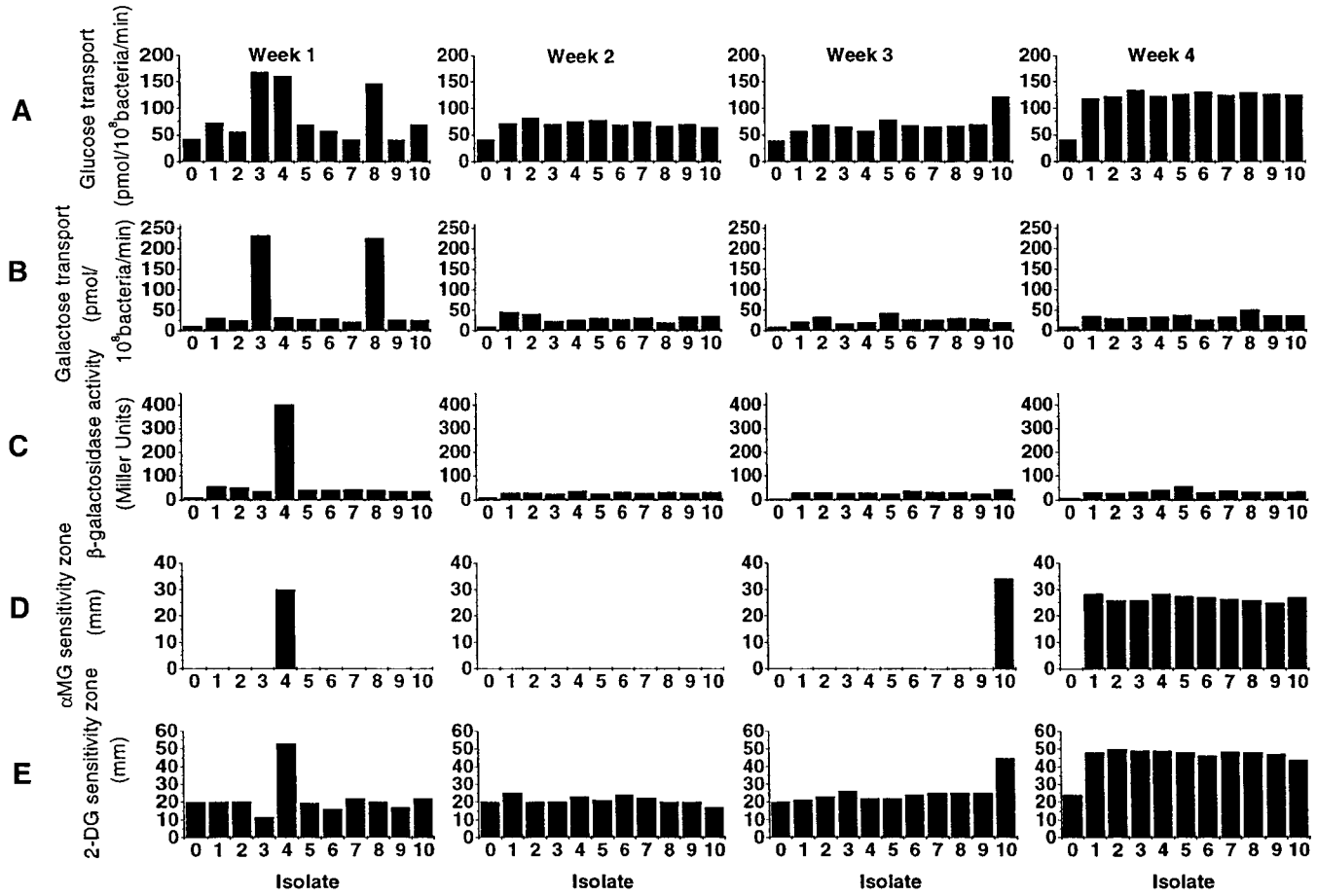

Figure 2.—Profile of chemostat-adapted bacteria isolated after growth on combined O_2 and glucose limitation. The O_2-limited chemostat was inoculated with BW2952, an MC4100 derivative with a *malG-lacZ* transcriptional fusion (Notley and Ferenci 1995). A sample after each week's growth at a dilution rate of 0.3 hr^{-1} was plated onto nonselective nutrient agar and 10 randomly separated colonies were tested for the following phenotypes. (A and B) The initial rates of 0.5 μm [^{14}C]glucose and 1 μm [^{14}C]galactose uptake into isolates grown on glycerol minimal medium as previously described (Death and Ferenci 1993). (C) The β-galactosidase activity due to the *malG-lacZ* fusion was determined with glucose-grown bacteria as previously described (Notley and Ferenci 1995). (D and E) The increased sensitivities to the PtsM substrate, 2-deoxyglucose, and the PtsG substrate, methyl-α-glucoside, were determined by plating lactate-grown isolates onto glycerol minimal agar plates (Curtis and Epstein 1975) overlaid with a 6-mm disk containing 10 ml 20% (w/v) 2-deoxyglucose or 20% (w/v) methyl-α-glucoside. Zones of inhibition were measured after overnight incubation at 37°.

mediate, O_2-limited state. The exact O_2 concentration was not determined but the residual glucose was measured in cultures and all were genuinely glucose limited (<2 μm residual sugar in fully aerobic cultures as well as O_2-limited ones). In the adaptation studies below, the cultures were grown at a glucose supply set at 0.08% (4.4 mm) with the modified chemostat configuration and at a growth rate comparable to earlier aerobic studies (D = 0.3 hr^{-1}; Notley-McRobb and Ferenci 1999a,b).

The properties of a culture grown in the modified chemostat over a period of 4 wk (280 generations) are shown in Figure 2. Each week, 10 randomly isolated colonies were purified from the chemostat by streaking on nonselective media (nutrient agar) and tested for five transport-related phenotypes. The first property, glucose transport at low substrate concentration, increased in all previously assayed glucose-limited populations and was also elevated here in all isolates by 280 generations. This was entirely as expected for an adapting glucose-limited population. But the galactose transport profile (Figure 2B) suggested a very different form of adaptation from other, fully aerobic chemostats. In all previously studied populations, increased glucose transport was accompanied by increased galactose transport (Notley-McRobb and Ferenci 1999a,b). This was because the main target of selection, the Mgl system, transports both glucose and galactose. In the O_2-limited chemostat, galactose transport was elevated only in two isolates in the first week and these types were absent from later samples. In the first week, when the culture grows to its maximal density and is equilibrating to reduced O_2 levels, the Mgl changes were of advantage, but were apparently eliminated from the population on further culture.

A third property assayed in Figure 2C, the *malG-lacZ*

TABLE 2

Mutational changes in *ptsG* in four independent chemostat populations

Starting strain	Aerated / O_2 limited	Codon change	Amino acid change	Found after generation no.	Change present in sequenced isolates
BW2952 Mgl^+	O_2 limited	GTC > GGC	V12G	140	2/2
		GTC > TTC	V12F	280	15/15
BW3413 Mgl^-	Aerated	GTC > TTC	V12F	70	5/5
BW3413 Mgl^-	O_2 limited	GTC > TTC	V12F	110	5/5
BW3413 Mgl^-	Aerated	GTC > TTC	V12F	140	6/6

fusion expression, monitors mutations in *mlc* and *malT* also common in previously studied glucose-limited populations (Notley-McRobb and Ferenci 1999a,b). Increased *malG* expression results from both types of mutation but the *mlc* mutation additionally results in increased sensitivity to nonmetabolizable glucose analogues, as also tested in Figure 2, C and D. The results shown in Figure 2 indicated that only one isolate in the week 1 sample exhibited a characteristic *mlc* phenotype (isolate 4) and subsequent culture resulted in absence of either *mlc* or *malT* mutants among the isolates analyzed.

The results in Figure 2 were unexpectedly distinct from those with aerobic populations. Two other O_2-limited populations after 19 days also showed phenotypic properties such as low *malG-lacZ* fusion expression, indicating they also lacked *mlc* or *malT* mutations common in six aerobic cultures of the same strain by this time (Notley-McRobb and Ferenci 1999a,b). If we take as a starting point that the properties of the anaerobic chemostat population in Figure 2 are a typical result of such experiments, even though only one population was analyzed in detail and two more in a preliminary fashion, it was important to determine the nature of the mutations present in the O_2-limited populations.

By the fourth week, the phenotype of all isolates was a previously uncommon combination of high glucose transport with increased sensitivity to both glucose analogues 2-deoxyglucose (2DG) and methyl-α-glucoside (αMG, Figure 2, D and E). Since 2DG and αMG are both substrates of the PTS, possible mutations in PTS components were investigated. Transduction using bacteriophage P1 was used to map the αMG- and 2DG-sensitivity mutations with linked transposon markers. In all week 4 isolates, transductional incorporation of *zce-726*::Tn*10* resulted in loss of analogue sensitivity in a high percentage of transductants. As confirmed with assays of αMG transport, the transduction resulted in a 70/30 mixture of transductants with either wild-type or isolate level of transport. The transposon in *zce-726*::Tn*10* is within 0.5 min of the *ptsG* locus so further analysis concentrated on sequencing this gene.

The DNA sequence of the whole gene as well as the upstream intergene promoter region was determined for chemostat isolates. As shown in Table 2, no changes were found in the promoter region but all week 4 isolates contained the same single *ptsG* mutation. A base change resulted in a V12F substitution in the IIBC protein. To test the effect of this mutation, a linked transposon was used to shift the *ptsG* mutation into a clean background in strain UE26. This strain lacks both major PTS systems with affinity for glucose ($PtsG^-PtsM^-$) so the introduced *ptsG* is the main transporter for glucose in bacteria grown under nutrient-excess batch culture conditions (Death and Ferenci 1993). As shown in Table 3, the V12F mutation resulted in a considerably improved rate of glucose transport compared to wild-type *ptsG* introduced into the same background. This result explains the selective advantage of the mutation acquired after 280 generations of glucose-limitation selection.

As can be noted from a comparison of glucose transport rates in Figure 2, most week 2 and 3 isolates exhibited elevated levels above that of wild type, even before the V12F mutation became common in the fourth week. Transductional analysis and sequencing of eight of these intermediate isolates revealed another *ptsG* mutation. Interestingly, the sequence change in these intermediate isolates was also in the same codon, as noted in Table 2. Assay of transport with the V12G variant in the UE26 background showed that this change also improved glucose transport, but to a lesser extent (Table 3). Altogether, these results suggest that the chemostat population in Figure 2 adapted by two separate events: the V12G-containing bacteria first took over but were succeeded by the V12F bacteria with even better transport capability.

The question remained: Why did the PtsG pathway

TABLE 3

Transport properties of strains with mutated *ptsG*

Strain	Genotype	Glucose transport (pmol/10^8 bacteria^{-1} min^{-1})
BW2952	$ptsG^+$ $ptsM^+$	37.0
UE26	$ptsG^-$ $ptsM^-$	13.7
BW3407	$ptsG^+$ $ptsM^-$	17.9
BW3406	*ptsG3400*(V → F)$ptsM^-$	99.3
BW3408	*ptsG3401*(V → G)$ptsM^-$	67.5

of adaptation take place under O_2-limited conditions, in contrast to the Mgl/LamB outcome under aerobic conditions? On the basis of published evidence that some transport systems are nonfunctional in *E. coli* K-12 under anaerobic conditions (Muir *et al.* 1985; Mat-Jan *et al.* 1991), a working hypothesis was that the Mgl system did not operate under O_2 limitation and so could not contribute to improvements in transport. If a nonfunctional Mgl system indeed pushed the adaptive pathway toward *ptsG* under anaerobic conditions, it may be expected that a similar alternate evolutionary path would be taken in an aerobic culture in which the Mgl system was inactivated by prior mutation. Hence the nature of adaptive changes was also followed with strain BW3143, which is identical to BW2952 except for an *mglA*::Tn*10* insertion.

Two independent populations of BW3143 grown under fully aerobic, sparged conditions became dominated by isolates with glucose transport changes accompanied by changes in αMG and 2DG sensitivity (initially appearing after 70–140 generations of culture). As shown in Table 2, sequencing of *ptsG* from five independent isolates from each population revealed the same V12F changes found under O_2-limited conditions with BW2952. PtsG was central to improving glucose transport when the Mgl system was inactivated under aerobic conditions; hence the *ptsG* changes were not specifically a consequence of an O_2-limited environment. An O_2-limited population was also initiated with strain BW3143 with very similar outcomes to that found with BW2952. As shown in Table 2, *ptsG* mutations began to be common in this population within 110 generations of culture and all sequenced isolates contained the V12F mutation. The V12G change was not found in the BW3143 experiments, but the number of populations analyzed was so small that the statistical significance of this is not evident.

Why were the *ptsG* mutants not apparent in aerobic Mgl^+ populations? An obvious reason was that the *ptsG* adaptation is less influential on glucose transport than the regulatory mutations commonly observed with aerobic wild-type populations. Figure 3 shows a comparison of the transport kinetics of isolates with either of the two *ptsG* mutations in contrast to aerobic *mgl*/*malT* changes. Glucose transport at the submicromolar level of sugar present in chemostats is faster when the LamB/Mgl pathway is upregulated than when *ptsG* mutations are present. Hence *ptsG* mutants were likely to be outcompeted in adapting aerobic populations. Also, it should be noted that the *mlc* and *mglD* repressor and operator mutations are a variety of loss-of-function changes (Notley-Mcrobb and Ferenci 1999a) rather than the rarer, more specific codon 12 substitutions seen in *ptsG*. Hence the *ptsG* point mutations are likely to be swamped in frequency as well as quality in aerobic evolving populations.

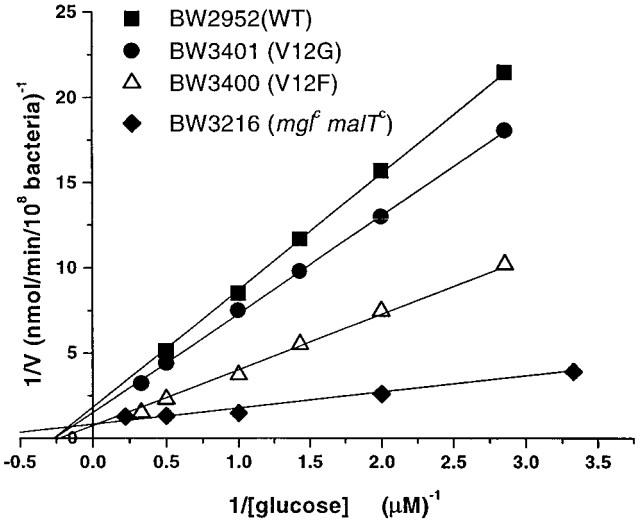

Figure 3.—Kinetics of glucose transport in adapted chemostat isolates. The relationship between glucose transport rates and substrate concentration is presented as a linear double reciprocal plot for wild-type bacteria (BW2952), the two types of *ptsG* mutant with V12F and V12G substitutions (BW3400 and BW3401, respectively), and an isolate BW3216 from an aerobic glucose-limited chemostat with *malT-con* and *mgl-con* mutations (Notley-McRobb and Ferenci 1999a).

DISCUSSION

The results presented in this article illustrate that mutational adaptation is a complex interplay between cellular physiology and the selective stress itself. Even for a well-studied organism like *E. coli*, it was far from obvious that improvements to glucose transport would differ between aerobic and O_2-limited environments.

The sugar transporters of *E. coli* are fairly well defined and glucose can enter *E. coli* via one or more of seven different transport systems (Lengeler 1993). Yet the physiological circumstances governing the relative utility of these systems are still not fully understood (Ferenci 1999). A reasonable conclusion from the current results is that the PtsG pathway is particularly significant under O_2-limited conditions and that the Mgl system is more important in aerobic environments. Further studies are needed to explain why Mgl does not operate under anaerobic conditions—preliminary experiments suggest it is not a transcriptional downregulation of *mgl* expression but is a lack of function (K. Manché, L. Notley-McRobb and T. Ferenci, unpublished results). Possible reasons may be the reduced ATP level under anaerobic conditions (Cole *et al.* 1967) leading to poorer ABC-type activity or the inactivation of one of the MglABC proteins by an unknown mechanism.

The mutations in *ptsG* leading to better glucose transport are encoded in part of the PtsG protein not previously characterized in studies looking at defective or substrate-altered mutations (Buhr *et al.* 1992; Ruijter *et al.* 1992; Begley *et al.* 1996; Lanz and Erni 1998). Residue 12 is in an α-helical segment on the cytoplas-

mic side of the membrane according to a detailed model of PtsG folding and not in a transmembrane segment (Buhr and Erni 1993). Further mutational studies are needed to fully analyze the contribution of this N-terminal helix in transporter function, but the interesting transport differences suggest residue 12 has a significant role in the functioning of the $IIBC^{Glc}$ protein. The substitutions cause considerable changes in sugar affinity for 2DG and αMG and therefore may affect the sugar-binding site (K. Manché, L. Notley-McRobb and T. Ferenci, unpublished results). The finding that only residue 12 mutations appeared in four different independent populations suggests that not too many other mutational options are available for improving PtsG function at low glucose concentration.

The O_2-rich and O_2-limited environments most likely do not differ with respect to the frequency of *ptsG* mutations, judging from the ease of enriching these in both aerobic and anaerobic Mgl^- bacteria. The lack of *ptsG* isolates in aerobic chemostats starting with the wild-type *E. coli* was more likely to be a question of the fitness contributions arising from possible transport changes. An isolate with an *mgl*/*malT* double mutation had superior transport kinetics for glucose compared to an isolate containing the *ptsG* change. Hence the *pts* mutants were likely to be outcompeted over the range of concentrations (below 1 μm) found in aerobic glucose-limited chemostats (Senn *et al.* 1994).

It was interesting that adaptive sweeps containing *ptsG* did not lead to polymorphisms, in that all sequenced mutants from four separate populations contained the same mutation by 280 generations of glucose limitation. Of course, mutations other than transport changes could be more diversifying, but the populations analyzed under O_2 limitation were remarkably homogeneous. As noted above, the PtsG change is presumably the main option in improving transport. Indeed, bacteria with the V12F structural change probably provide the clearest example of a successful mutation sweeping a population in one of the periodic selection events occurring in evolving bacterial populations (Helling *et al.* 1987). Yet the contrast of this result with the same selection under aerobic conditions could not be greater.

Under aerobic conditions, a combination of polygenic mutations in *mlc*, *mgl*, and *malT* resulted in a diversified population (Notley-McRobb and Ferenci 1999a,b). Even when sampling small proportions of the populations, all of six independent populations contained at least 6–10 coexisting and more or less equally fit clones through different combinations of changes at each of these genes. Hence the environment can influence the clonal diversity of a population as well as the targets of selection. It is quite sobering to consider that quite different conclusions about the diversifying effects of adaptive evolution could be drawn from two closely related experimental selections.

These results also highlight the role of secondary features of the environment in evolutionary outcomes. In the general sense of Equations 1 and 2, the adapted populations consisting of $Organism_{A1}$ and $Organism_{A2}$ clearly differed on the basis of $Stress_2$. The cellular response to $Stress_2$, or O_2 limitation, had the effect of changing the target of selection. It remains to be determined whether environmental factors other than O_2 availability, such as temperature and culture pH, also influence adaptive outcomes. In any case, the multifactorial inputs into adaptive strategies undoubtedly complicate prediction of adaptive pathways even with well-studied organisms like *E. coli*.

We thank W. Boos and the *E. coli* Genetic Stock Center for strains and the Australian Research Council for financial support.

LITERATURE CITED

Begley, G. S., K. A. Warner, J. C. Arents, P. W. Postma and G. R. Jacobson, 1996 Isolation and characterization of a mutation that alters the substrate specificity of the *Escherichia coli* glucose permease. J. Bacteriol. **178:** 940–942.

Buhr, A., and B. Erni, 1993 Membrane topology of the glucose transporter of *Escherichia coli*. J. Biol. Chem. **268:** 11599–11603.

Buhr, A., G. A. Daniels and B. Erni, 1992 The glucose transporter of *Escherichia coli:* mutants with impaired translocation activity that retain phosphorylation activity. J. Biol. Chem. **267:** 3847–3851.

Cole, H. A., J. W. Wimpenny and D. E. Hughes, 1967 The ATP pool in *Escherichia coli*. I. Measurement of the pool using a modified luciferase assay. Biochim. Biophys. Acta **143:** 445–453.

Curtis, S. J., and W. Epstein, 1975 Phosphorylation of D-glucose in *Escherichia coli* mutants defective in glucosephosphotransferase, mannosephosphotransferase and glucokinase. J. Bacteriol. **122:** 1189–1199.

Dean, A. M., 1989 Selection and neutrality in lactose operons of *Escherichia coli*. Genetics **123:** 441–454.

Death, A., and T. Ferenci, 1993 The importance of the binding-protein-dependent Mgl system to the transport of glucose in *Escherichia coli* growing on low sugar concentrations. Res. Microbiol. **144:** 529–537.

Death, A., and T. Ferenci, 1994 Between feast and famine: endogenous inducer synthesis in the adaptation of *Escherichia coli* to growth with limiting carbohydrates. J. Bacteriol. **176:** 5101–5107.

Death, A., L. Notley and T. Ferenci, 1993 Derepression of LamB protein facilitates outer membrane permeation of carbohydrates into *Escherichia coli* under conditions of nutrient stress. J. Bacteriol. **175:** 1475–1483.

Dykhuizen, D. E., 1993 Chemostats used for studying natural selection and adaptive evolution. Methods Enzymol. **224:** 613–631.

Dykhuizen, D. E., and D. E. Hartl, 1983 Selection in chemostats. Microbiol. Rev. **47:** 150–168.

Ferenci, T., 1996 Adaptation to life at micromolar nutrient levels: the regulation of *Escherichia coli* glucose transport by endoinduction and cAMP. FEMS Microbiol. Rev. **18:** 301–317.

Ferenci, T., 1999 Regulation by nutrient limitation. Curr. Opin. Microbiol. **2:** 208–213.

Hansen, S. R., and S. P. Hubbell, 1980 Single-nutrient microbial competition: qualitative agreement between experimental and theoretically forecast outcomes. Science **207:** 1491–1493.

Helling, R. B., C. N. Vargas and J. Adams, 1987 Evolution of *Escherichia coli* during growth in a constant environment. Genetics **116:** 349–358.

Lanz, R., and B. Erni, 1998 The glucose transporter of the *Escherichia coli* phosphotransferase system—mutant analysis of the invariant arginines, histidines, and domain linker. J. Biol. Chem. **273:** 12239–12243.

Larson, T. J., D. N. Ludtke and R. M. Bell, 1984 sn-Glycerol-3-phosphate auxotrophy of *plsB* strains of *Escherichia coli*: evidence that a second mutation, *plsX*, is required. J. Bacteriol. **160:** 711–717.

Lengeler, J. W., 1993 Carbohydrate transport in bacteria under environmental conditions, a black box? Antonie Leeuwenhoek **63:** 275–288.

Mat-Jan, F., C. R. Williams and D. P. Clark, 1991 Mutations permitting the anaerobic growth of *Escherichia coli* on trehalose. FEMS Microbiol. **78:** 149–152.

Miller, J., 1972 *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Miller, J. H., 1996 Spontaneous mutators in bacteria—insights into pathways of mutagenesis and repair. Annu. Rev. Microbiol. **50:** 625–643.

Muir, M., L. Williams and T. Ferenci, 1985 Influence of transport energization on the growth yield of *Escherichia coli*. J. Bacteriol. **163:** 1237–1242.

Notley, L., and T. Ferenci, 1995 Differential expression of *mal* genes under cAMP and endogenous inducer control in nutrient stressed *Escherichia coli*. Mol. Microbiol. **160:** 121–129.

Notley-McRobb, L., and T. Ferenci, 1999a Adaptive *mgl*-regulatory mutations and genetic diversity evolving in glucose-limited *E. coli* populations. Environ. Microbiol. **1:** 33–44.

Notley-McRobb, L., and T. Ferenci, 1999b The generation of multiple coexisting *mal*-regulatory mutations through polygenic evolution in glucose-limited populations of *E. coli*. Environ. Microbiol. **1:** 45–52.

Postma, P., J. W. Lengeler and G. R. Jacobson, 1996 Phosphoenolpyruvate:carbohydrate phosphotransferase systems, pp. 1149–1174 in *Escherichia coli and Salmonella: Cellular and Molecular Biology*, Ed. 2, edited by F. C. Neidhardt. ASM Press, Washington, DC.

Rosenweig, R. F., R. R. Sharp, D. S. Treves and J. Adams, 1994 Microbial evolution in a simple unstructured environment: genetic differentiation in *Escherichia coli*. Genetics **137:** 903–917.

Ruijter, G. J., G. van Meurs, M. A. Verwey, P. W. Postma and K. van Dam, 1992 Analysis of mutations that uncouple transport from phosphorylation in enzyme IIGlc of the *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system. J. Bacteriol. **174:** 2843–2850.

Senn, H., U. Lendenmann, M. Snozzi, G. Hamer and T. Egli, 1994 The growth of *Escherichia coli* in glucose-limited chemostat cultures: a re-examination of the kinetics. Biochim. Biophys. Acta **1201:** 424–436.

Communicating editor: R. Maurer