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The Lysine Decarboxylase CadA Protects *Escherichia coli* Starved of Phosphate against Fermentation Acids▽†

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Conflicting results have been reported for the rate and extent of cell death during a prolonged stationary phase. It is shown here that the viability of wild-type cells (MG1655) could decrease ≥10^8-fold between days 1 and 14 and between days 1 and 6 of incubation under aerobic and anaerobic phosphate (P_i) starvation conditions, respectively, whereas the cell viability decreased moderately under ammonium and glucose starvation conditions. Several lines of evidence indicated that the loss of viability of P_i-starved cells resulted primarily from the catabolism of glucose into organic acids through pyruvate oxidase (PoxB) and pyruvate-formate lyase (PflB) under aerobic and anaerobic conditions, respectively. Weak organic acids that are excreted into the medium can reenter the cell and dissociate into protons and anions, thereby triggering cell death. However, P_i-starved cells were efficiently protected by the activity of the inducible GadABC glutamate-dependent acid resistance system. Glutamate decarboxylation consumes one proton, which contributes to the internal pH homeostasis, and removes one intracellular negative charge, which might compensate for the accumulated weak acid anions. Unexpectedly, the tolerance of P_i-starved cells to fermentation acids was markedly increased as a result of the activity of the inducible CadBA lysine-dependent acid resistance system that consumes one proton and produces the diamine cadaverine. CadA plays a key role in the defense of *Salmonella* at pH 3 but was thought to be ineffective in *Escherichia coli* since the protection of *E. coli* challenged at pH 2.5 by lysine is much weaker than the protection by glutamate. CadA activity was favored in P_i-starved cells probably because weak organic acids slowly reenter cells fermenting glucose. Since the environmental conditions that trigger the death of P_i-starved cells are strikingly similar to the conditions that are thought to prevail in the human colon (i.e., a combination of low levels of P_i and oxygen and high levels of carbohydrates, inducing the microbiota to excrete high levels of organic acids), it is tempting to speculate that *E. coli* can survive in the gut because of the activity of the GadABC and CadBA glutamate- and lysine-dependent acid resistance systems.

Escherichia coli adapts to changes in the amounts of nutrients available in the growth medium. For example, decreases in the concentration of glucose (the preferred carbon source), in the concentration of ammonium (the preferred nitrogen source), and in the concentration of inorganic phosphate (P_i) (the preferred phosphorus source) induce the Crp, GlnG/Nac, and PhoB regulons, respectively. The responses to nutrient limitation allow cells to scavenge for traces of the preferred nutrient and for alternative nutrients in order to maintain growth (6, 34, 59). However, when a nutrient is totally depleted, starved cells enter the stationary phase (26).

Besides the specific responses to nutrient limitation, the RpoS (σ^S) regulon is also induced when cells enter the stationary phase (9, 35). Several of the genes that are strongly induced through the activity of the σ^S-containing RNA polymerase (i.e., *gadA*, *gadBC*, *poxB*, *katE*, *ostA*, *psiF*, *dps*, *gadW*, *gadX*, *pdhR*, etc.) (61) can help stationary-phase cells resist exogenous toxic agents that may be encountered while the capacity of the cell to synthesize proteins is decreased. For instance, starved *E. coli* cells that infect the gastrointestinal tract of a host may require the induction in advance of acid resistance mechanisms, such as the glutamate-dependent GadABC system, in order to tolerate HCl (pH 2 to 3) produced in the stomach (47).

However, *E. coli* cells starved for a nutrient(s) must first survive endogenous stresses that may be generated during a prolonged stationary phase. There is evidence that the viability of starved cells incubated under aerobic conditions may be threatened by the production of reactive oxygen species, such as toxic hydroxyl radicals produced through Fenton chemistry ($H_2O_2 + Fe^{2+} \rightarrow HO\cdot + OH^- + Fe^{3+}$) (18, 22, 27, 42). It has been suggested that starved cells may progressively die during prolonged incubation as a result of the accumulation of protein oxidative damage (4, 41).

Conflicting results concerning the rate and extent of cell death during prolonged incubation under aerobic, nutrient starvation conditions have been reported. The viability of *E. coli* K-12 strain D10 ($RNase^-$ Met^-) markedly decreased during incubation in minimal media containing 62 mM P_i buffer, 0.36 mM $FeSO_4$, and a limiting concentration of glucose, ammonium, or P_i (the last medium contained 0.23 mM P_i, 0.1 M Tris-HCl buffer [pH 7.6], and 20 mM glucose); the viability decreased earlier during P_i starvation, decreasing ~10^5-fold

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† This paper is dedicated to the memory of Benjamin Moreau (1977-2000).

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TABLE 1. Bacterial strains

Strain	Genotype	Source	Reference(s)
ENZ535	F$^-$ *rph-1 ilvG rfb-50*	CF1648; MG1655	22
ENZ985	As ENZ535 but *rpoS359*::Tn*10* (null)	P1.ENZ485	16
ENZ1222	As ENZ535 but *poxB*::*kan*	P1.YYC908	13
ENZ1224	As ENZ535 but *poxB176*::*lacZ* Cm^r (PoxB$^-$)	P1.YYC912	13
ENZ1565	As ENZ535 but Δ(*pflBA*25::*cat*)	P1.RM201	50
ENZ1584	As ENZ535 but *gadBC*::Km	P1.EF522	10
ENZ1586	As ENZ535 but *appY*::*aphA* (Km)	P1.TC3617	2
ENZ1588	As ENZ535 but *gadC*(*xasA*)*18*::Tn*10*	P1.JLS9318	25
ENZ1592	As ENZ1584 but *gadA*::pRR10(Ap)	P1.EF522	10
ENZ1607	As ENZ535 but *cadBA*::MudI1734(*kan lacZ*) (CadA$^-$)	P1.GNB8385K	3
ENZ1734	As ENZ1735 but Pro$^+$	P1.ENZ535	
ENZ1735	As ENZ535 but Δ*lacIZ proC*::Tn*5*	P1.QC2456 (D. Touati)	45
ENZ1755	As ENZ535 but Δ(*phoBR*)*kan*	P1.ENZ490	16
ENZ1756	As ENZ535 but *mgsA*::*kan*	P1.MJF397	58
ENZ1766	As ENZ1734 but *poxB176*::*lacZ* Cm^r	P1.YYC912	13

between days 1 and 3 of incubation (15). In stark contrast, the viability of *E. coli* K-12 strain MG1655 (*rph rpoS*$^+$) (24) was almost not affected after 7 days of incubation at 30°C on 4-morpholinepropanesulfonic acid (MOPS) minimal medium plates containing 40 mM MOPS buffer (pH 7.2), 0.01 mM $FeSO_4$, and no glucose, ammonium, or phosphate (55). Finally, the viability of strain MG1655 decreased moderately after 6 days of incubation in M9 minimal medium containing 64 mM P_i buffer (pH 7), Fe as an adventitious contaminant (40), and a limiting concentration of glucose (~50% viability), ammonium (~30% viability), or phosphate (~60% viability in a medium containing 0.12 mM P_i, MOPS buffer, and 20 mM glucose) (4).

Because the differences in previously published data for cell viability under starvation conditions may be accounted for by differences in the experimental conditions used, such as the genotype of the strains and the composition of the medium (buffer composition, concentration of the limiting nutrient, concentration of Fe^{2+}, etc.), the question of the viability of starved cells was reinvestigated under conditions that are generally used in physiological studies (12, 34), namely, by using the common "wild-type" (wt) K-12 strain MG1655 (and its derivatives) (24) and the convenient MOPS minimal medium designed by Neidhart et al. (40) that may contain a limiting concentration of glucose, ammonium, or P_i.

In this study I found that during incubation the viability of P-starved cells could decrease $\geq 10^8$-fold between days 1 and 14 under aerobic conditions and between days 1 and 6 under anaerobic conditions, whereas the viability of N- and C-starved cells decreased moderately. Several lines of evidence indicate that the death of P-starved cells results primarily from the rapid catabolism of glucose into weak organic acids. However, P-starved cells could tolerate and even enhance the production of organic acids when inducible amino acid decarboxylases that consume intracellular protons were active. Besides the glutamate-dependent GadABC system that protected P-starved cells incubated under both aerobic and anaerobic conditions, the lysine-dependent CadBA system, which was thought to have low activity in *E. coli*, dramatically increased the viability of P-starved cells shifted to anaerobiosis. I propose that the processes that occur when cells are starved of P_i in the presence of excess carbon might mimic processes that normally occur during long-term colonization of the human colon by *E. coli*.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* K-12 strains used are listed in Table 1. Transduction by P1*vir* was performed as described by Miller (37).

Media and culture conditions. The minimal medium used for liquid cultures was essentially the MOPS (pK_a 7.2) medium described by Neidhardt et al. (40), which contained 40 mM MOPS (pH 7.4), 4 mM Tricine plus 10 μM $FeSO_4$, 86 mM NaCl, 9.5 mM NH_4Cl, 5 mM K_2HPO_4, and 20 mM glucose, supplemented with five vitamins (0.02 mM thiamine, 0.02 mM calcium pantothenate, 0.02 mM *p*-aminobenzoic acid, 0.02 mM *p*-hydroxybenzoic acid, and 0.02 mM 2,3-dihydroxybenzoic acid) unless indicated otherwise (final pH ~7.2). The P_i-limiting medium contained 0.1 mM K_2HPO_4 plus 9.8 mM KCl, the glucose-limiting medium contained 3 mM glucose, and the ammonium-limiting medium contained 2 mM NH_4Cl, unless indicated otherwise. When acetic, pyruvic, formic, L-lactic, L-glutamic, and L-aspartic acids (sodium salts) were added into the incubation media, the final concentration of Na^+ in the media was adjusted to 86 mM with NaCl. L-Arginine (guanidium-NH_2^+; pK_a 12.5), L-ornithine (δ-NH_3^+; pK_a 10.8), and L-lysine (ε-NH_3^+; pK_a 10.5) (52) were added to media in their monohydrochloride forms.

Cultures incubated under aerobic conditions (50 ml in 500-ml Erlenmeyer flasks) were agitated at 150 rpm in a covered water bath rotary shaker (Aquatron Infors HT). To shift aerobic cultures (optical density at 600 nm [OD_{600}], ~0.2) to anaerobiosis, 13-ml fractions were dispensed into 15-ml screw-cap centrifugation tubes; the tubes were incubated horizontally with gentle agitation to prevent cells from settling. All incubations were performed at 37°C. Culture optical densities were determined spectrophotometrically at 600 nm (Jasco V-530) using cells with a 1-cm path length. The pHs of the media were determined with an InLab410 electrode (Mettler Toledo) at ~25°C.

Measurement of cell viability. To assess cell viability, serial dilutions were prepared in M9 buffer, and aliquots (20 μl) were spotted at least in triplicate onto LB medium plates (37, 39). Beef liver catalase (2,000 U; Sigma) was spread on the LB medium plates, which were incubated under aerobic conditions. Catalase scavenges H_2O_2 adventitiously produced in rich plating medium, which helps "fragile" cells (i.e., cells damaged during prolonged incubation in liquid [Fig. 1C]) form colonies (8). Colonies were counted after overnight incubation at 37°C, and plates were checked after a further 24 and 48 h of incubation for additional colonies, which could appear when the survival rate was low.

Levels of glucose and by-products. The concentrations of pyruvate (Roche), glucose, D- and L-lactate, acetate, formate, and succinate (Roche Boehringer Mannheim/R-Biopharm) were determined by enzymatic tests performed according to the instructions of the manufacturers.

Measurement of β-galactosidase activity. β-Galactosidase levels were determined as described by Miller (17, 37).

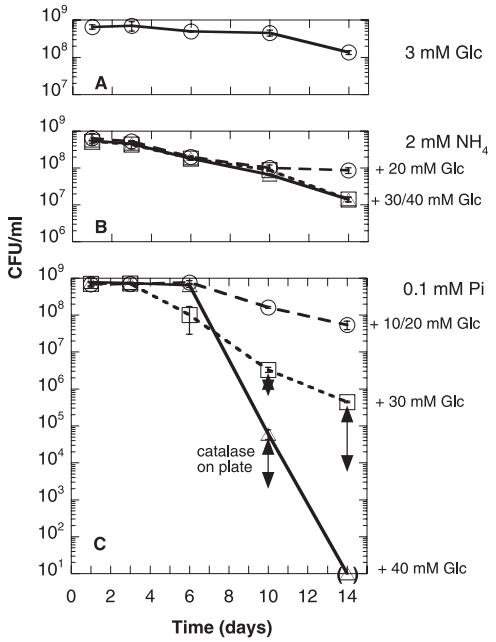

FIG. 1. Viability of starved cells incubated with aeration. Strain ENZ535 (= MG1655) (wt) was inoculated 1:500 (time zero) into MOPS medium with a limited amount of glucose (A), ammonium (B), or P_i (C) and incubated with aeration for 14 days. The media with limited amounts of ammonium and P_i contained 10, 20 (○), 30 (□), or 40 (△) mM glucose. The numbers of CFU were determined on LB medium plates containing catalase; the double-headed arrows indicate the increases in the number of CFU/ml due to the inclusion of catalase. Parentheses indicate that no CFU were detected when 20-μl portions of the cultures were directly plated. The values are the means ± standard deviations for two independent cultures representative of several determinations.

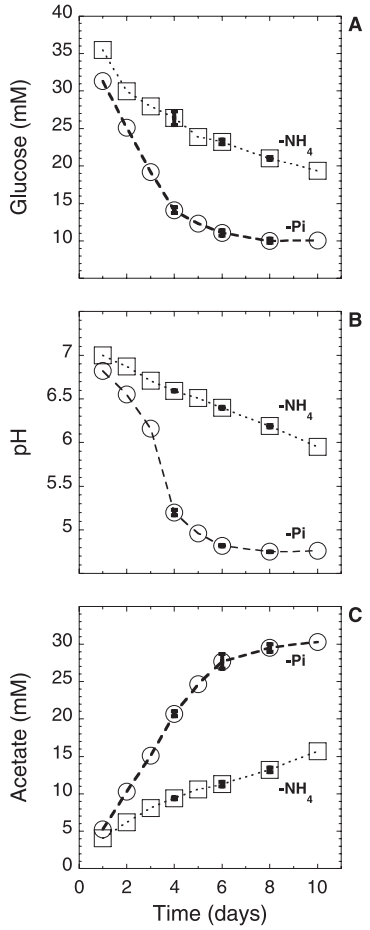

FIG. 2. Metabolic patterns of P- and N- starved cells. Strain ENZ535 (wt) was inoculated 1:500 (time zero) into MOPS medium with a limited amount of ammonium (□) or P_i (○) containing 40 mM glucose and incubated with aeration for 10 days. The pHs of the culture supernatants were determined (B) and adjusted to pH 7 with KOH, and the concentrations of glucose (A) and acetate (C) were determined. The values for days 4, 6, and 8 of incubation are the means ± standard deviations for four independent cultures, and the values for the other days are values for one culture.

RESULTS

Cells starved of P_i exhibit lower viability than cells starved of ammonium or glucose exhibit. To determine the viability of *E. coli* cells that were starved for a specific nutrient, wild-type strain MG1655 was first grown overnight in MOPS medium containing 20 mM glucose, 5 mM K_2HPO_4, and 9.5 mM NH_4Cl and then was diluted 1:500 into MOPS medium containing a limiting concentration of glucose (3 mM), ammonium (2 mM), or P_i (0.1 mM) (40). The cultures were incubated under aerobic conditions and entered the stationary phase (OD_{600}, ~0.6) as a result of C, N, or P starvation after ~10 h of incubation. The numbers of CFU were maximal on day 1 of incubation (~6×10^8 CFU/ml; defined as 100% viability).

Between days 1 and 6 of incubation, the viabilities of C-, N-, and P-starved cells decreased moderately (Fig. 1A, B, and C); the viability of C-starved cells decreased to ~50%, whereas the viability of N-starved cells was lower (~30%) and the viability of P-starved cells (0.1 mM P_i plus 20 mM glucose) was higher (~100%). These data are in good agreement with those of Ballasteros et al. (4) but at odds with those of Davis et al. (15), who found that the viability of C-, N-, and P-starved cells decreased dramatically in a couple of days. Preliminary results suggest that the latter data might be accounted for by the high levels of Fe^{2+} present in the medium used (36-fold higher than the levels in MOPS medium), which could dramatically increase the generation of toxic hydroxyl radicals through Fenton chemistry.

When incubation was continued for up to 14 days, the viability of cells starved of glucose, ammonium (with 20 mM glucose), and P_i (with 20 mM glucose) steadily decreased to ~10% (Fig. 1A, B, and C). However, when the concentration of glucose initially added to the N- and P-limiting media was increased to 40 mM, the viability of starved cells decreased further; the viability decreased moderately in the case of N starvation (Fig. 1B) and markedly in the case of P starvation (Fig. 1C). On day 14 of incubation in a medium that initially contained 0.1 mM P_i plus 40 mM glucose, no viable counts were measured on LB medium plates when 20-μl aliquots of cultures (OD_{600}, ~0.9) were spotted directly or after washing in M9 buffer (Fig. 1C).

These results suggest that different levels of a toxic agent(s) may be produced during prolonged starvation; the highest levels could be produced during P_i starvation in the presence of

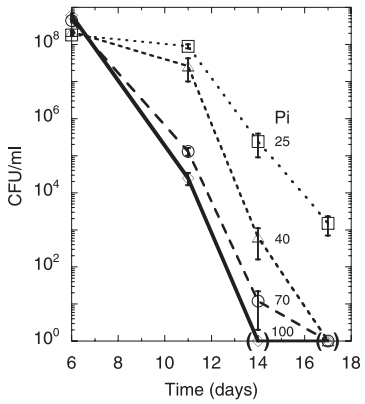

FIG. 3. Life span of P-starved cells depends upon the limiting concentration of P_i in the medium. Strain ENZ535 (wt) grown overnight in complete MOPS medium containing 5 mM K_2HPO_4 was inoculated 1:500 (time zero) into MOPS media containing 40 mM glucose and various limiting concentrations of K_2HPO_4, including 25 μM (□), 40 μM (△), 70 μM (○), and 100 μM (◇), plus KCl to maintain the K^+ concentration at 10 mM. The cultures were incubated with aeration for 17 days. Parentheses indicate that no CFU were detected when 20-μl portions of the cultures were directly plated. The values are the means ± standard deviations for two independent cultures. On day 17 of incubation, the OD_{600} (pHs) of the cultures were 0.32 (pH 4.97), 0.43 (pH 4.83), 0.63 (pH 4.74), and 0.84 (pH 4.70) with 25, 40, 70, and 100 μM P_i, respectively.

high concentrations of glucose, much lower levels could be produced during ammonium starvation, and the lowest levels could be produced during glucose starvation.

P-starved cells may die as a result of glucose catabolism. The concentration of glucose in the medium clearly plays a key role in the loss of viability of P-starved cells during prolonged incubation under aerobic conditions. However, the death of starved cells was due not merely to the inclusion of excess glucose in the medium because the viability of P-starved cells was ~10^6-fold lower than that of N-starved cells after 14 days of incubation in media that initially contained 40 mM glucose (Fig. 1B and C).

The total amount of glucose consumed by P-starved cells after 14 days of incubation was higher (31.3 ± 0.1 mM glucose) than the amount consumed by N-starved cells (23 ± 0.1 mM glucose). Moreover, it appeared that the rate of consumption of glucose (Fig. 2A) and the rate of excretion of acetic acid into the medium (Fig. 2C) were twofold higher in P-starved cultures than in N-starved cultures between days 1 and 4 of incubation. Accordingly, the pH of the medium decreased more rapidly in P-starved cultures than in N-starved cultures (Fig. 2B).

When the initial concentration of glucose was maintained at 40 mM but the limiting concentration of P_i was decreased from 100 to 25 μM (Fig. 3), the yield of cells on day 6 of incubation decreased, and the trigger for cell death was delayed by up to 5 days (from day 6 with 100 μM P_i to day 11 with 25 μM P_i), which suggests that the density of P-starved cells may affect the rate of production of toxic products.

Overall, these results suggest that key factors in the rate of death of P-starved cells may be the total amount of glucose catabolized in the culture and, eventually, the concentration of a toxic end product(s) excreted into the medium.

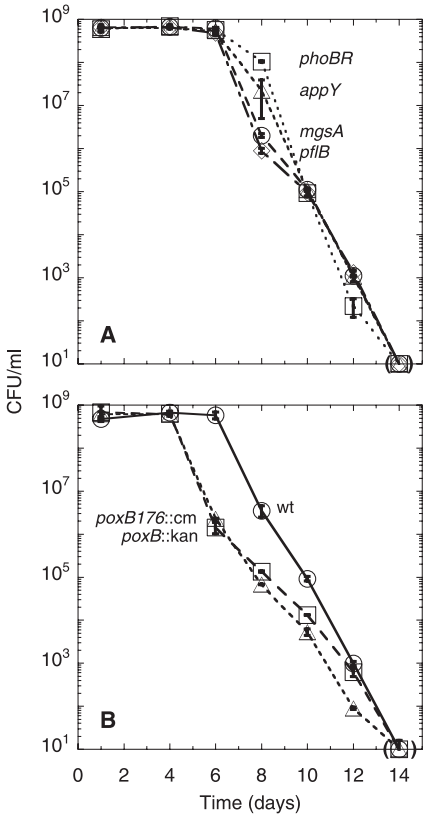

FIG. 4. Lack of PoxB activity affects the viability of P-starved cells. Strains were inoculated 1:500 (time zero) into P_i-limiting medium containing 0.1 mM P_i and 40 mM glucose and incubated with aeration for 14 days. Parentheses indicate that no CFU were detected when 20-μl portions of the cultures were directly plated. The values are the means ± standard deviations for two independent cultures representative of several determinations. (A) Concentrations of ENZ1565 (Δ*pflAB*) (◇), ENZ1586 (*appY*) (△), ENZ1755 (Δ*phoBR*) (□), and ENZ1756 (*mgsA*) (○). (B) Concentrations of ENZ535 (wt) (○), ENZ1222 (*poxB*::*kan*) (□), and ENZ1224 (*poxB176*::*lacZ* Cm^r) (△). The survival curves for the *mgsA* mutant strain (A) and the wt strain (B) are identical.

MG does not affect the survival of P-starved cells. What kind of toxic agent(s) may be excreted during glucose catabolism by P-starved cells? A primary candidate is methylglyoxal (MG), a toxic by-product of glucose metabolism (21). MG inhibits the cellular metabolism and eventually kills growing cells when the concentration in the medium passes critical thresholds (0.3 and 0.6 mM MG, respectively) (58). Because the activity of MG synthase (MgsA) is repressed by P_i, it has been suggested that MgsA activity could increase under P_i starvation conditions (14). However, it is unlikely that MG plays a significant role in the death of P-starved cells incubated with excess glucose because *mgsA* null mutants lost viability like wt cells during prolonged incubation (Fig. 4A and B).

PoxB plays a key role in the viability of P-starved cells. Other potential toxic agents that are excreted during glucose catabolism by P-starved cells are weak organic acids, such as acetic acid (Fig. 2C). It is known that addition of weak organic acids to a culture at a moderately acidic pH can stop growth and eventually kill cells (33, 49). For example, acetate toxicity at pH ≤6 results from the fact that undissociated acetic acid

TABLE 2. Metabolic patterns of strains starved of P_i for 14 days[a]

Strain	OD_{600}	pH	Concn (mM) of:				
			Glucose	Pyruvate	Acetate	D-Lactate	Formate
wt	0.84	4.70	10.1 ± 0.2	0.02 ± 0.01	31.4 ± 0.3	0.19 ± 0.01	0.09 ± 0.02
pflB	0.82	4.76	9.7 ± 0.3	0.01 ± 0.01	27.4 ± 0.5	0.13 ± 0.01	0.06 ± 0.01
poxB	0.91	4.76	10.6 ± 0.3	0.88 ± 0.14	13.6 ± 0.1	1.07 ± 0.01	0.11 ± 0.02

[a] The viabilities of wt, *pflB*, and *poxB* strains are shown in Fig. 4. On day 14 of incubation, the OD_{600} and the pH of the cultures and the concentrations in the culture supernatants (adjusted to pH 7) of glucose, pyruvate, acetate, D-lactate, and formate were determined. The concentration data are the means ± standard deviations for two independent cultures.

(CH_3COOH; pK_a 4.76) equilibrates freely across the cytoplasmic membrane and dissociates preferentially inside the cell into acetate anions and protons because of the initially high internal pH (pH_i) (pH_i ~7.8 in cells grown at pH 7; pH_i ~7.6 in stationary-phase cells) (48, 49).

Glucose can be metabolized into acidic end products through different pathways; it can be metabolized into acetate (via acetyl coenzyme A) through the pyruvate dehydrogenase complex (PDHc), it can be metabolized into formate and acetate (via acetyl coenzyme A) through pyruvate-formate lyase (PflB), and it can be metabolized directly into acetate through pyruvate oxidase (PoxB) (7, 13, 21, 30). Although PDHc and PflB are used preferentially during growth under aerobic and anaerobic conditions, respectively, it has been shown that PDHc and PflB (probably with the help of cytochrome *bd* oxidase and YfiD) may be used simultaneously during growth under microaerobic conditions (1, 62). When P-starved cultures reached a high cell density (~6×10^8 CFU/ml with 0.1 mM P_i), the concentration of dissolved oxygen in the medium could decrease (40). If microaerobic conditions rather than fully aerobic conditions were attained, PDHc and PflB could be active simultaneously. Moreover, the AppY regulon, which controls the expression of a cytochrome *bd* oxidase, is induced under P_i starvation and microaerobic conditions (2). However, PflB and AppY might play no significant role in the viability of P-starved cells because *pflB* and *appY* mutants behaved like wt cells during prolonged incubation under aerobic, P_i starvation conditions (Fig. 4A and B). Likewise, the PhoB regulon should play no significant role (Fig. 4A). The conclusion that PflB may play no significant role in P-starved cells is supported by the fact that wt and *pflB* strains exhibited similar metabolic patterns after 14 days of incubation under aerobic conditions (Table 2).

The use of strain ENZ1766, which carries a *poxB176-lacZ* fusion, showed that the production of PoxB was fourfold higher in P-starved cells (406 ± 7 U of β-galactosidase) than in N-starved cells (101 ± 2 U of β-galactosidase; means for four independent cultures) after 24 h of incubation. Because *poxB* is strictly regulated by RpoS (13), the greater production of PoxB in P-starved cells than in N-starved cells may reflect the greater accumulation of RpoS in P-starved cells than in N-starved cells (35). These data might help explain the findings that the loss of viability (Fig. 1) and the rate of catabolism of glucose into acetic acid (Fig. 2) were greater in P-starved cultures than in N-starved cultures.

However, the role of PoxB may be more complex than merely the production of acetic acid. Previous data have indicated that PoxB may play a key role in P-starved cells by changing the metabolic flux, which may increase the production of acetic acid but decrease the production of oxidative agents between days 1 and 4 of incubation (38). In fact, the lack of PoxB activity changed the kinetics of death of P-starved cells in a complex manner (Fig. 4B). First, between days 4 and 6 of incubation, the viability of *poxB* mutant cells decreased 10^3-fold, whereas the viability of wt cells was not affected. It is likely that the premature death of *poxB* cells is due primarily to oxidative stress because *poxB* mutants accumulate more oxidized molecules (thiobarbituric acid-reactive substances) than wt cells accumulate between days 3 and 4 of incubation (38). Second, between days 6 and 14 of incubation, the viability of *poxB* mutants decreased steadily, whereas the viability of wt cells decreased sharply, which suggests that high levels of a toxic agent(s) were eventually reached in wt cells after 14 days of incubation.

The concentrations of the possible end products of glucose catabolism (acetate, lactate, formate, and pyruvate) were determined on day 14 of incubation in cultures of the wt and *poxB* strains (Table 2). The levels of organic acids excreted were significantly different in the wt and *poxB* strains; the levels of acetate were ~2.3-fold lower and the levels of pyruvate and lactate were ~44- and ~5.6-fold higher, respectively, in cultures of *poxB* mutants than in cultures of wt cells. In toto, *poxB* cells excreted twofold less organic acids (15.6 mM) than wt cells excreted (31.6 mM), and acetate was the most abundant product excreted by both strains (Table 2). Therefore, the findings that *poxB* mutants accumulate ~4-fold more oxidized molecules (thiobarbituric acid-reactive substances) after 4 days of incubation (38) and excrete ~2-fold less organic acids after 14 days of incubation than wt cells (Table 2) support the hypothesis that *poxB* mutants might be exposed early to a stronger oxidative stress and late to a weaker acetic acid stress than wt cells.

Overall, these data suggest that a primary factor in the death of wt cells incubated under aerobic, P_i starvation conditions might be acetic acid. The hypothesis that acetic acid that accumulated during 14 days of incubation (~30 mM acetic acid at pH 4.7 to 4.8) (Fig. 2 and Table 2) could eventually kill P-starved cells is supported by the fact that the viability of starved cells was markedly decreased during incubation at pH 4.8 in the presence of 40 mM acetate, whereas cell viability was barely affected during incubation at pH 4.8 in the absence of acetate. In practice, cells were incubated in P_i-limiting medium for 28 h, centrifuged, resuspended at the same density, and incubated further in MOPS medium lacking P_i and glucose at pH 7.2 or pH 4.8, with or without acetate. After 6 days of incubation under aerobic conditions, the cell viability at pH 4.8

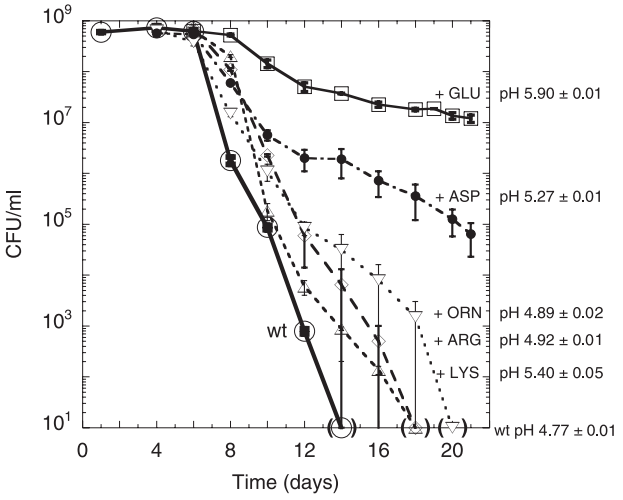

FIG. 5. Glutamate protects P-starved cells. Strain ENZ535 (wt) was inoculated 1:500 (time zero) into P_i-limiting medium (0.1 mM P_i plus 40 mM glucose) that was not supplemented (○) or was supplemented with 30 mM glutamate (GLU) (□), 30 mM aspartate (ASP) (●), 30 mM ornithine (ORN) (▽), 30 mM arginine (ARG) (◇), or 30 mM lysine (LYS) (△). Parentheses indicate that no CFU were detected when 20-μl portions of the cultures were directly plated. The values are the means ± standard deviations for two independent cultures representative of several determinations. The final pHs determined on day 21 are indicated on the right.

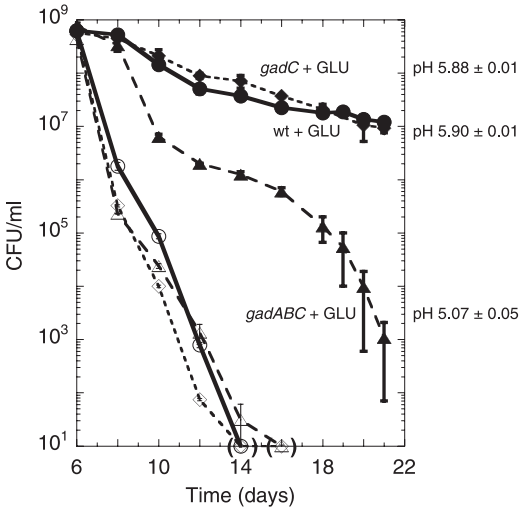

FIG. 6. Effect of *gad* mutations on the protective role of glutamate. Strains ENZ1588 (*gadC*) (◇ and ◆) and ENZ1592 (*gadA gadBC*) (△ and ▲) were inoculated 1:500 (time zero) into P_i-limiting medium (0.1 mM P_i plus 40 mM glucose) without glutamate (GLU) (open symbols) or with 30 mM glutamate (solid symbols) and incubated further under aerobic conditions. Parentheses indicate that no CFU were detected when 20-μl portions of the cultures were directly plated. The values are the means ± standard deviations for two independent cultures or for one culture (◇) representative of several determinations. The data for the wt strain with no glutamate (○) and with 30 mM glutamate (●) are from Fig. 5. The final pHs of the cultures on day 21 of incubation with glutamate are indicated on the right.

decreased ~2-fold in the absence of acetate and up to 10^3-fold in the presence of 40 mM acetate compared to the viability at pH 7.2 (data not shown).

Glutamate protects P-starved cells. *E. coli* possesses five amino acid decarboxylases induced by low pH that may contribute to acid resistance: the ornithine decarboxylase SpeF, the lysine decarboxylase CadA, the arginine decarboxylase AdiA, and the glutamate decarboxylase isozymes GadA and GadB. The decarboxylases exchange one intracellular proton with the α-carboxyl groups of the substrates, and the decarboxylated products putrescine, cadaverine, agmatine, and glutamate:γ-aminobutyric acid (GABA) are returned to the medium by the antiporters PotE, AdiC, CadB, and GadC, respectively. The consumption of protons in the cytoplasm by the decarboxylases increases the pH_i, whereas the leakage of protons into the cell and the consumption of protons in the medium by GABA may increase the external pH (3, 10, 25, 28, 29, 47). However, the inducible amino acid decarboxylases provide different levels of protection during a pH 2.5 challenge for 30 min; GadA and GadB provide the highest level of protection (survival increases from <0.1 to 100% and the internal pH increases from pH 3.6 to pH 4.2), AdiA provides a lower level of protection (75% survival), CadA provides the lowest level of protection (<10% survival), and SpeF provides no protection (48). Furthermore, Lin et al. (33) have shown that expression of the glutamate- and arginine-dependent systems can protect stationary-phase cells against the bactericidal effect of a cocktail consisting of short-chain fatty acids (containing 90 mM acetate) included in a medium at pH 4.4.

If the death of cells incubated under aerobic, P_i starvation conditions were a consequence, at least in part, of the excretion of acetic acid, the inclusion of glutamate in the incubation medium would protect P-starved cells. In fact, the level of survival was ~5% after 21 days of incubation in the presence of 30 mM glutamate (OD_{600}, ~0.7; pH 5.9), whereas all cells were killed after 14 days of incubation in the absence of glutamate (OD_{600}, ~0.8; pH 4.8) (Fig. 5).

Addition of 30 mM ornithine, 30 mM arginine, or 30 mM lysine barely affected the viability of P-starved cells during prolonged incubation (Fig. 5). It has been shown previously that arginine added to P-limiting medium is rapidly degraded by the constitutive SpeA decarboxylase rather than by the inducible AdiA decarboxylase (22, 23).

Protective effect of glutamate results primarily from the H^+-consuming activity of the Gad decarboxylases. To determine whether the protective effect of glutamate (Fig. 5) results from the activity of the inducible GadABC acid resistance system, *gadA* and *gadBC* mutations were introduced into strain MG1655. After 21 days of incubation in a medium that initially contained 0.1 mM P_i, 40 mM glucose, and 30 mM glutamate, the viability of *gadABC* mutants was 10^4-fold lower (OD_{600}, ~0.8; pH ~5.1) than the viability of wt cells (OD_{600}, ~0.7; pH ~5.9) (Fig. 6). The expression of the *gadA* gene and the *gadBC* operon was strongly induced in P_i-starved cells; when *gadA*- and *gadB-lacZ* fusion strains (11) were used, the β-galactosidase activity increased ~100- and ~30-fold, respectively, at the onset of P_i starvation (data not shown).

A single *gadC* mutation had no effect on the viability of P-starved cells incubated with glutamate (Fig. 6), which suggests that exchange of GABA for glutamate was not required to protect P-starved cells. GadC is required for glutamate-

dependent resistance at pH 2.5 when cells are grown at pH 7 but not when cells are grown at pH 6 (25).

The weak protection afforded by addition of 30 mM glutamate to *gadABC* mutants (Fig. 6) was similar to the protection afforded by addition of 30 mM aspartate to wt cells (Fig. 5). Considering the pK_a values of glutamate (γ-COOH, pK_a 4.2) and aspartate (β-COOH, pK_a 3.9) (52), it is possible that glutamate alone and aspartate could buffer the medium to pH 5.1 to 5.3, when the buffering capacity of 40 mM MOPS (pK_a 7.2) was overwhelmed by the excretion of acetic acid (pK_a 4.76), and the pH of the medium decreased to pH 4.7 to 4.8 in the absence of any amino acid. Moreover, the transport of glutamate and aspartate inside the cells (36) might contribute to internal buffering, as suggested previously in the case of citrate (43); acid resistance may occur when the internal pH increases from pH 3.6 to pH 4.2 (48).

Overall, these results support the hypothesis that the primary cause of cell death during prolonged incubation under aerobic, P_i starvation conditions may be the excretion and eventually the reentry into the cells of acetic acid; this process could trigger a decrease in the internal pH that could be efficiently prevented through the H^+-consuming activity of the Gad decarboxylases, provided that glutamate is present in the medium.

Shift to anaerobiosis decreases the life span of P-starved cells. During growth under anaerobic conditions, pyruvate is metabolized through PflB into formate and acetate, which decreases the pH of the medium and eventually triggers induction of *ldhA* and coexcretion of D-lactate with protons (7). Moreover, a critical threshold level of formate in the medium (>10 mM) (62) triggers expression of the formate-hydrogen lyase complex, which degrades formic acid into CO_2 and H_2 (7). LdhA and formate-hydrogen lyase activities may therefore contribute to internal pH homeostasis during growth under anaerobic conditions, thereby reducing the potentially toxic effect of fermentation acids. However, it is not clear whether PflB could play a significant role in starved cells because the expression of the *focA-pflB* operon dramatically decreases in stationary-phase cells grown in rich medium under anaerobic conditions (51).

To determine the viability of starved cells following a shift to anaerobiosis, cultures grown under aerobic conditions to an OD_{600} of ~0.2 were dispensed into screw-cap centrifugation tubes (13 ml in 15-ml tubes). The tubes were tightly closed and incubated with gentle agitation; addition of resazurin to control tubes indicated that cultures were anaerobic after 1 day of incubation. As shown in Fig. 7A, the viable counts in C-starved cultures were maximal on day 1 of incubation and remained roughly constant for 6 days, whereas the viability of C-starved cells steadily decreased under aerobic conditions (Fig. 1A). These results confirm that a shift to anaerobiosis may somehow protect C-starved cells (18). In contrast, the viability of N-starved cells declined to lower levels and more rapidly under anaerobic conditions (~1,000-fold decrease in cell viability between days 1 and 6 of incubation) (Fig. 7B) than under aerobic conditions (~100-fold decrease in cell viability between days 1 and 14 of incubation) (Fig. 1B). Finally, the loss of viability in P-starved cultures occurred more rapidly under anaerobic conditions than under aerobic conditions; no viable cell counts were obtained after only 6 days of incubation under anaerobic conditions with 20 mM glucose (OD_{600}, ~0.6) (Fig. 7C), compared to the ~14 days of incubation required under aerobic conditions with 40 mM glucose (OD_{600}, ~0.8) (Fig. 1C).

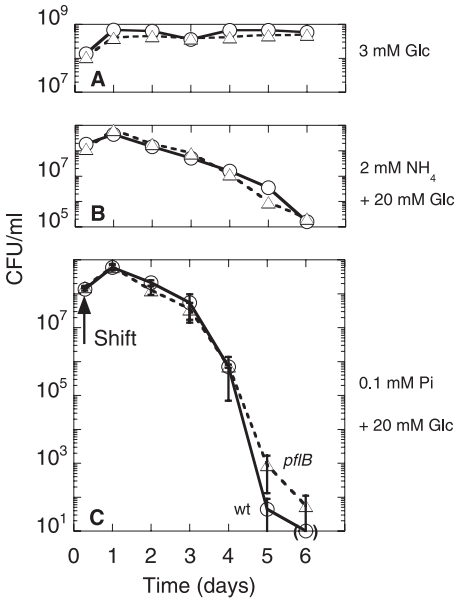

FIG. 7. Viability of starved cells shifted to anaerobiosis. Strains ENZ535 (wt) (○) and ENZ1565 (*pflB*) (△) were inoculated 1:500 (time zero) into MOPS medium with a limited amount of glucose (A), ammonium (B), or P_i (C). The media with limited amounts of ammonium and P_i contained 20 mM glucose. The cultures were incubated with aeration for 7 h until the OD_{600} was ~0.2, and then 13-ml fractions were transferred into 15-ml tubes, which were tightly closed and incubated with gentle agitation. Parentheses indicate that no CFU were detected when 20-μl portions of the cultures were directly plated. The data for glucose and ammonium starvation are from one culture, and the data for P_i starvation are the means ± standard deviations for two independent cultures representative of several determinations.

Therefore, P-starved cells were again more sensitive than N-starved cells and especially C-starved cells after a shift to anaerobiosis, and N- and P-starved cells died more rapidly under anaerobic conditions than under aerobic conditions.

Metabolism of pyruvate switches from PoxB to PflB and LdhA after a shift to anaerobiosis. Whereas the viability of P-starved cells grown aerobically with 20 mM glucose decreased 10^8-fold between days 1 and 6 of incubation following a shift to anaerobiosis (Fig. 7C), the viability of P-starved cells grown aerobically with 60 mM glycerol did not decrease during a 5-day incubation period following a shift to anaerobiosis (data not shown). Because *E. coli* is unable to grow fermentatively on glycerol (7), this result suggests that fermentative metabolism may be required to kill P-starved cells under anaerobic conditions.

To try to determine the metabolic pathways that were used by P-starved cells following a shift to anaerobiosis, the viabilities of *poxB* and *pflB* mutants were compared to the viability of wt cells during prolonged incubation under anoxic conditions. Inactivation of *poxB* weakly reduced (~10-fold) the viability of P-starved cells shifted to anaerobiosis (data not shown). One would expect PoxB to play only a minor role in the viability of P-starved cells because PoxB is a respiratory

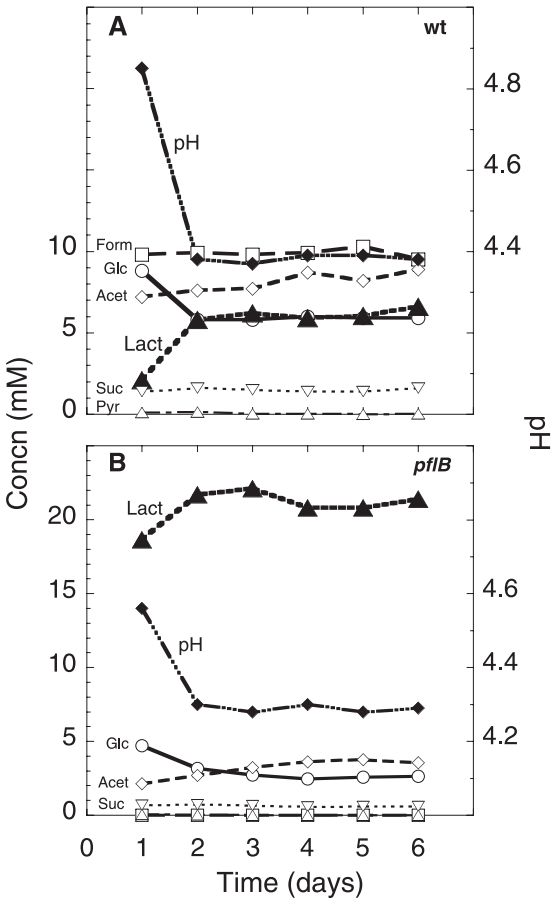

FIG. 8. Metabolic patterns of P-starved cells shifted to anaerobiosis. The viabilities of wt and *pflB* strains are shown in Fig. 7C. The pHs of the culture supernatants were determined (◆) and adjusted to pH 7 with KOH, and the concentrations of formate (Form) (□), acetate (Acet) (◇), D-lactate (Lact) (▲), succinate (Suc) (▽), pyruvate (Pyr) (△), and glucose (Glc) (○) in one culture were determined.

enzyme, which may rapidly stop working after a shift to anaerobiosis (see below).

Inactivation of PflB slightly increased the viability of P-starved cells (Fig. 7C). This result may indicate that the level of activity of PflB is normally low in P-starved cells and/or that other activities compensate for the lack of PflB activity following a shift to anaerobiosis. To determine what may occur, the concentrations of the possible end products of glucose metabolism in cultures of wt and *pflB* strains were determined. As shown in Fig. 8A, wt cells eventually consumed ~14 mM glucose (20 mM glucose was included at time zero) and excreted ~10 mM formate, ~9 mM acetate, ~6 mM D-lactate, and ~1.4 mM succinate. Production of formate and acetate stopped by 1 day of incubation (pH 4.8), whereas production of D-lactate could occur until day 2 of incubation, and the pH of the medium eventually decreased to pH 4.4 (Fig. 8A). As expected, *pflB* mutant cells excreted no formate, low levels of acetate (~3.5 mM acetate probably produced through PoxB activity before anoxic conditions occurred), and low levels of succinate (~0.7 mM) (Fig. 8B). However, *pflB* mutants consumed larger amounts of glucose than wt cells consumed (~18 mM compared to ~14 mM), while the pH of the medium eventually decreased to pH 4.3. In fact, introduction of a *pflB* mutation markedly increased the level of D-lactate from ~6 to ~22 mM (Fig. 8A and B). These results suggest that the carbon flux may shift from PflB to LdhA in *pflB* mutants starved of P_i under anaerobic conditions.

The fact that *pfl* and *pfl*$^+$ strains excreted similar total amounts of fermentation acids (≥26 mM at pH 4.3 to 4.4) (Fig. 8A and B) might simply explain the finding that *pfl* and *pfl*$^+$ strains had similar rates of death (Fig. 7C). This hypothesis is supported by the fact that addition of fermentation acids markedly increased the loss of viability of starved cells incubated at pH 4.4 under anaerobic conditions. In practice, cells were incubated aerobically in glucose-limiting medium until the onset of the stationary phase, centrifuged, and resuspended at the same density in MOPS media lacking glucose and P_i at pH 7.2 or 4.4, with or without organic acids. After 4 days of incubation at pH 4.4 under anaerobic conditions, the viability of starved cells decreased moderately (~20-fold decrease in viable counts) in the absence of organic acids and markedly (>10^7-fold decrease in viable counts) in the presence of 10 mM formate, 30 mM acetate, 30 mM succinate, or 40 mM L-lactate compared to the viability at pH 7.2 (data not shown).

GadBC acid resistance system protects P-starved cells shifted to anaerobiosis. If the excretion and reentry of fermentation acids at pH 4.4 were implicated in the death of P-starved cells shifted to anaerobiosis, one would expect expression of the glutamate-dependent acid resistance system to protect P-starved cells.

Addition of glutamate to the medium when the cultures were shifted to anaerobiosis dramatically increased the viability of P-starved cells after 7 days of incubation; the viable counts reached ~2×10^8 CFU/ml in the presence of 20 mM glutamate, whereas no viable counts were obtained in the absence of glutamate (Fig. 9A). The protective effect of glutamate was essentially dependent upon the activities of the decarboxylase GadB and the antiporter GadC, since the viabilities of *gadABC* (Fig. 9C), *gadC* (Fig. 9D), and *gadABC*/*gadC*$^+$ strains (data not shown) were ~10^4-fold lower than the viabilities of the wt strain (Fig. 9A) and *gadA* strain (data not shown) after 7 days of incubation with 20 mM glutamate. It is known that both GadB and GadC are necessary and sufficient to protect cells shifted from pH ~8 to pH 2.5 in the presence of glutamate, whereas GadA is required only when cells are challenged at pH 2 (10). The viability of *gadABC* mutants observed in the presence of 20 mM glutamate (~1×10^4 CFU/ml) (Fig. 9C) was somewhat lower than that of wt cells incubated with 10 to 20 mM aspartate (~4×10^5 CFU/ml) (Fig. 9A), which suggests that the contributions of glutamate and especially aspartate to buffering may slightly increase the viability of P-starved cells incubated under anaerobic conditions.

Overall, these results support the hypothesis that the primary cause of cell death during prolonged incubation under anaerobic, P_i starvation conditions may be the excretion and eventually the reentry into the cells of formic and acetic acids, which could trigger a decrease in the internal pH that could be efficiently prevented through the activity of the GadBC glutamate decarboxylase system.

Lysine decarboxylase CadA protects cells starved of P_i under anaerobic conditions. Next, the potential role of arginine,

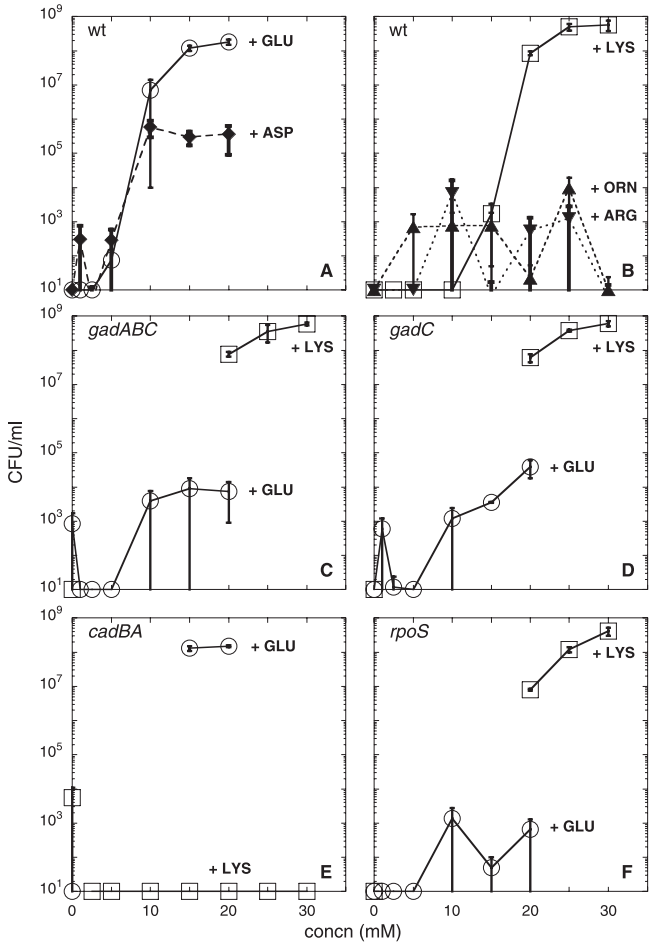

FIG. 9. Glutamate- and lysine-dependent acid-resistant systems increase the viability of P-starved cells shifted to anaerobiosis. Strains ENZ535 (wt) (A and B), ENZ1592 (*gadA gadBC*) (C), ENZ1588 (*gadC*) (D), ENZ1607 (*cadBA*) (E), and ENZ985 (*rpoS*) (F) were inoculated 1:500 (time zero) into MOPS medium with a limited amount of P_i containing 20 mM glucose and 86 mM NaCl or 66 mM NaCl (cultures that were supplemented with glutamate and aspartate). Cultures were incubated with aeration for ~7 h until the OD_{600} was ~0.2 and supplemented with different concentrations (indicated on the abscissa) of glutamate (+ GLU), aspartate (+ ASP), lysine (+ LYS), ornithine (+ ORN), or arginine (+ ARG), and 13-ml fractions were transferred into 15-ml tubes. The numbers of CFU were determined after 7 days of incubation. Most of the data are the means ± standard deviations for two independent cultures representative of several determinations; the exceptions are the data for the cultures with aspartate, ornithine, and arginine, which are data for three independent cultures.

lysine, and ornithine in the viability of P-starved cells shifted to anaerobiosis was examined. It is known that anoxic conditions can significantly increase expression of the *adiA* and *cadA* genes (3).

Inclusion of arginine and ornithine (5 to 30 mM) had no significant effect on cell viability (Fig. 9B). Use of an *adiA*::*lacZ* fusion (3) revealed that *adiA* was not expressed in P-starved cells but was strongly induced in cells grown in rich medium at pH 5.5 under anaerobic conditions (data not shown) (3).

Whereas lysine provides only weak protection to cells challenged with HCl at pH 2.5 (28, 48), addition of 25 to 30 mM lysine to P_i-limiting media when the cultures were shifted to anaerobiosis dramatically increased the viable counts from ≤ 10 to $\sim 6 \times 10^8$ CFU/ml after 7 days of incubation (Fig. 9B). Inactivation of *cadBA* totally prevented the protective effect of lysine (Fig. 9E), which indicates that the protection afforded to P-starved cells by lysine was strictly dependent on expression of the inducible CadBA acid resistance system. Accordingly, the *cadBA-lacZ* fusion was strongly induced in P-starved cells shifted to anaerobiosis; the level of β-galactosidase increased from zero during growth under aerobic conditions to 1,700 U after 1 day of incubation following a shift to anaerobiosis in the presence of both 40 mM lysine (required to induce *cadBA*) (3) and 20 mM glutamate (data not shown). Glutamate should be added together with lysine in order to protect cells against acid stress because *cadBA-lacZ* cells are defective for Cad activity.

CadA protects P-starved cells independent of expression of the RpoS regulon. CadA activity plays a key role in the homeostasis of the internal pH in *Salmonella* (43). However, CadA alone provides a low level of protection at pH 3.0 in *Salmonella*; acid tolerance requires simultaneous expression of CadA and acid shock proteins (ASPs) that are under control of the RpoS regulon (43). In contrast, the lysine-dependent protection of *E. coli* cells starved of P_i was nearly normal in an *rpoS* null strain (Fig. 9F). Therefore, RpoS is not required either for induction of the *cadBA* operon (31, 60) or for activity of the Cad system in *E. coli* starved of P_i. In contrast, the lack of RpoS activity reduced the viability of P-starved cells incubated with glutamate by $\geq 10^4$-fold (Fig. 9F). Whether this effect reflected primarily a low level of expression of *gad* genes (47) remains to be determined.

CadA activity helps cells tolerate the production of fermentation acids. Inclusion of a suboptimal concentration of lysine (20 mM) in the medium markedly increased the viable counts and the pHs of cultures incubated under anaerobic, P_i starvation conditions (Fig. 10). Moreover, it appeared that P-starved cells that were protected by the Cad system consumed more glucose after 7 days of incubation (15 to 20 mM; i.e., all the glucose included in the medium) and excreted more formate into the medium than cells incubated in the absence of lysine consumed and excreted (Fig. 10). These results indicate that the H^+-consuming activity of the lysine-dependent Cad system helps P-starved cells tolerate the production of fermentation acids, notably formic acid, during prolonged incubation under anaerobic conditions.

DISCUSSION

There are conflicting data about the rate and extent of cell death during a prolonged stationary phase. The viabilities of C-, N-, and P-starved cells could decrease dramatically in a few days (15), were not significantly affected (55), or were affected differently; C-starved cells were apparently more sensitive than P-starved cells as a result of accumulation of protein oxidative damage (4). Employing experimental conditions different than those previously used to study the viability of starved cells (notably lower concentrations of iron, higher concentrations of glucose, and longer incubation times), I found that the viability of wt cells (MG1655) could decrease $\geq 10^8$-fold between days 1 and 14 and between days 1 and 6 of incubation under aerobic

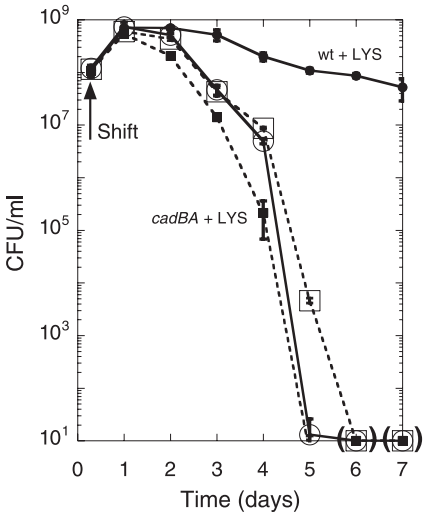

FIG. 10. CadA activity protects P-starved cells shifted to anaerobiosis. Strains ENZ535 (wt) (○ and ●) and ENZ1607 (*cadBA*) (□ and ■) were inoculated 1:500 (time zero) into MOPS medium with a limited amount of P_i containing 20 mM glucose. The cultures were incubated with aeration for ~7 h until the OD_{600} was ~0.2 and then supplemented (solid symbols) or not supplemented (open symbols) with 20 mM lysine (+ LYS), and 13-ml fractions were transferred into 15-ml tubes, which were tightly closed and incubated with gentle agitation. Parentheses indicate that no CFU were detected when 20-μl portions of the cultures were directly plated. The data are the means ± standard deviations for two independent cultures representative of several determinations. On day 7 of incubation, the cultures of the wt strain that were not supplemented with lysine (○) contained 5.1 ± 0.1 mM glucose and 9.3 ± 0.4 mM formate (pH 4.39 ± 0.01), whereas the cultures of the wt strain that were supplemented with 20 mM lysine (●) contained 0.01 ± 0.01 mM glucose and 14.5 ± 0.5 mM formate (pH 5.18 ± 0.01).

and anaerobic P_i starvation conditions, respectively, whereas the viability of N- and C-starved cells decreased moderately.

Several lines of evidence indicate that organic acids rather than oxidative agents cause death of P-starved cells. First MG, a very toxic end product of glucose metabolism that forms specific oxidation products with proteins, the so-called advanced glycation end products (5), played no role in the viability of P-starved cells, although MG production may increase in P-starved cells (14).

Second, induction of PoxB activity, which decreased the levels of oxidative agents (38) and increased the levels of acetic acid produced by P-starved cells (up to ~30 mM acetic acid at pH 4.8), first slowed the loss of viability and eventually accelerated the loss of viability of P-starved cells. Moreover, the level of expression of PoxB, the rate of catabolism of glucose into acetic acid, and the loss of viability were greater in P-starved cultures than in N-starved cultures.

Third, a shift to anaerobic conditions increased the viability of C-starved cells but markedly decreased the viability of N-starved cells and especially P-starved cells, which excreted up to ~10 mM formic acid and ~10 mM acetic acid at pH 4.4.

Fourth, inclusion of glutamate in the medium increased the viability of P-starved cells, as well as the pH of the medium. It has been shown previously that inclusion of glutamate protects stationary-phase cells against acetic acid added to a medium at pH 4.4 (33). I show here that the glutamate-dependent protection of P-starved cells incubated under aerobic and anaerobic conditions was dependent primarily upon the activity of the inducible GadABC acid resistance system. However, the activity of the GadC glutamate antiporter was required under anaerobic conditions but not under aerobic conditions, which suggests that acid stress may be stronger under anaerobic conditions than under aerobic conditions.

Finally, I show that inclusion of lysine in the medium dramatically increased the viability of cells incubated under anaerobic, P_i starvation conditions. When the ability of glutamate and arginine to protect stationary-phase cells against organic acids was examined previously, lysine was apparently not tested (33, 48). I show here that the protection afforded by lysine was strictly dependent upon expression of the *cadBA* operon but was totally independent of expression of the RpoS regulon, whereas both CadA and RpoS activities are required to protect *Salmonella* challenged at pH 3 (43). Expression of the lysine-dependent CadBA system increased the pH of the medium and helped cells tolerate higher levels of formate produced during prolonged incubation under anaerobic, P_i starvation conditions.

The data presented here together with previous data (22, 38, 39) provide the outlines for understanding how toxic agents are produced and detoxified during the catabolism of glucose in P-starved cells. At the onset of the stationary phase under aerobic conditions, oxidative agents reach a maximal level and after this are prevented from accumulating through the combined activities of PoxB and AhpCF (38, 39). In fact, the increase in the activity of the global regulator RpoS increases the expression of *pdhR* and *poxB*, which contributes to a shift in the metabolic flux from PDHc (required for growth) to PoxB; this rerouting decreases the production of NADH and thus the adventitious production of superoxide and H_2O_2 by NADH dehydrogenase II in the aerobic respiratory chain (38). Eventually, the basal levels of AhpCF scavenge the residual low levels of H_2O_2 generated inside the cell (38, 39), and SodC may scavenge superoxide generated in the periplasm (32). Acetic acid that is directly produced from pyruvate by PoxB is excreted and progressively accumulates in the medium. The difference between the pH of the medium (pH 4.8 after 14 days of incubation) and the internal pH (pH_i ~7.6 at entry into the stationary phase) favors reentry of acetic acid and its dissociation, thereby resulting in a substantial accumulation of acetate anions and protons in the cytoplasm, which eventually triggers cell death (48, 49). However, the *gadA* and *gadBC* genes, which are regulated by RpoS (47), are induced concomitant with *poxB* at the onset of the stationary phase. Provided that glutamate is available in the medium, the H^+-consuming activity of Gad decarboxylases can efficiently protect P-starved cells against acetic acid. Furthermore, if P-starved cells are shifted to anaerobiosis at the beginning of the stationary phase, the metabolic flux switches from PDHc to PoxB and then to PflB and LdhA, which in turn triggers excretion of organic acids (formic, acetic, and D-lactic acids), a rapid decrease in the pH of the medium to pH 4.4, and eventually the death of P-starved cells. The H^+-consuming activity of the GadB decarboxylase and especially the CadA decarboxylase helps P-starved cells tolerate the production of organic acids provided that glutamate and lysine, respectively, are available in the medium.

How do decarboxylases protect P-starved cells against weak organic acids? Decarboxylases may protect P-starved cells against weak organic acids by contributing to the homeostasis of both the internal pH and the internal ionic strength. In fact, the decarboxylation of glutamate and lysine consumes one proton and removes one intracellular negative charge, which may compensate for the accumulated protons and weak acid anions, respectively (46, 48, 49). The possible effect on the internal ionic strength is in good agreement with the observation that the shrinkage of P-starved cells that occurred during prolonged incubation was totally prevented by the activity of decarboxylases. However, preliminary results indicated that inclusion of osmoprotectants, which increased the amount of free cytoplasmic water without affecting the internal pH (46), increased the viability of P-starved cells moderately compared to inclusion of glutamate and lysine. In the absence of any protection, a decrease in the internal pH and an increase in the internal ionic strength may contribute to the aggregation of cytoplasmic proteins in nonfunctional assemblies (20, 46). The fact that the rate of cell death increased when the rate of glucose catabolism decreased markedly during incubation under aerobic and anaerobic, Pi starvation conditions supports the hypothesis that inhibition of survival metabolism may trigger cell death (44).

Why does the glutamate-dependent protection afforded by Gad decarboxylases to P-starved cells require the activity of the antiporter GadC under anaerobic conditions but not under aerobic conditions? Because P-starved cells die in the absence of glutamate in 14 days under aerobic conditions, compared to 6 days under anaerobic conditions, a simple hypothesis is that GadC may be dispensable when organic acids slowly enter P-starved cells under aerobic conditions, whereas GadC may be required to boost the activity of the decarboxylases when organic acids enter the cells more rapidly under anaerobic conditions.

The same reasoning may help explain the fact that lysine provides P-starved cells exposed to organic acids at pH 4.4 with stronger protection than glutamate provides, whereas glutamate provides cells challenged at pH 2.5 with stronger protection than lysine provides (28, 48). In fact, protons may enter cells more slowly in the former case (death may occur in 6 days) than in the latter case (death may occur in a few hours). Therefore, CadA, which has a pH optimum of approximately 5.7, may be better adapted to stabilize the internal pH close to neutrality in P-starved cells, whereas glutamate decarboxylases, which have lower pH optima, may be better adapted to stabilize the internal pH between pH 4 and 5 during exposure to pH 2 to 3 (48).

Why can CadA efficiently protect *Salmonella* challenged at pH 3 (43)? The protection of *Salmonella* requires both CadA activity and the synthesis of ASPs that are produced under the control of RpoS (43). In stark contrast, CadA could efficiently protect *E. coli* cells starved of P_i independent of expression of *rpoS*, *phoBR*, and *asr* (Fig. 9F and data not shown). Asr is an ASP produced under strict control of PhoBR in *E. coli* grown at pH 4 to 5 (53, 56). If the Cad system alone could allow *E. coli* cells to resist the slow production of weak organic acids during P_i starvation, it would be tempting to speculate that a role for ASPs in *Salmonella* might be to act as a buffer preventing a rapid decrease in the internal pH, thereby favoring CadA activity during an acid shock at pH 3.0.

The protective effect of CadA on cell viability was detected in P-starved cells incubated under anaerobic conditions but not in P-starved cells incubated under aerobic conditions, probably because the level of expression of *cadA*, which is increased by a shift to anaerobiosis (3), was too low under aerobic conditions. However, the fact that addition of lysine did increase the pH of the medium from pH 4.7 to pH 5.4 under aerobic conditions might reflect a low level of CadA activity. Activity of AdiA was not detected in P-starved cells probably because induction of *adiA* requires anaerobic conditions and that cells be grown at a low pH in rich medium (3, 47).

Could the behavior of P-starved cells help explain the adaptation of *E. coli* to stress in nature? It has been suggested that *E. coli* cells that grow in the proximal part of the human colon may enter the stationary phase as a result of P_i starvation (57). In fact, nutrients, including P_i and glucose, are efficiently absorbed in the small intestine. However, *E. coli* can acquire high levels of C indirectly from dietary polysaccharides degraded by anaerobic bacteria in the microbiota and/or directly from mucus (12, 54). The amount of C available in the colon may reach a concentration equivalent to approximately 40 mM glucose (19). Moreover, it is generally thought that bacteria in the colon are progressively deprived of oxygen and shift their metabolism to fermentation, thereby excreting short-chain fatty acids (mainly acetic, propionic, and butyric acids) and lactic acid (12). Although organic acids produced in vivo are absorbed and may account for up to 10% of the daily harvest of calories (54), the concentrations of weak organic acids in the colon have been estimated to be approximately 90 mM acetic acid, 30 mM propionic acid, and 30 mM butyric acid at pH ~4.4 (33). Such concentrations of weak organic acids should be bactericidal. However, *E. coli* is thought to survive such a toxic environment for ≥14 days (12). Because the conditions thought to prevail in the human colon are strikingly similar to the conditions that can trigger the death of P-starved cells shifted to anaerobiosis, it is tempting to speculate that *E. coli* can survive in the gut because of the activity of the GadB and CadA decarboxylases.

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