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Products of the *Escherichia coli* Acid Fitness Island Attenuate Metabolite Stress at Extremely Low pH and Mediate a Cell Density-Dependent Acid Resistance[▽]

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***Escherichia coli* has an ability, rare among the *Enterobacteriaceae*, to survive extreme acid stress under various host (e.g., human stomach) and nonhost (e.g., apple cider) conditions. Previous microarray studies have exposed a cluster of 12 genes at 79 centisomes collectively called an acid fitness island (AFI). Four AFI genes, *gadA*, *gadX*, *gadW*, and *gadE*, were already known to be involved in an acid resistance system that consumes an intracellular proton through the decarboxylation of glutamic acid. However, roles for the other eight AFI gene products were either unknown or subject to conflicting findings. Two new aspects of acid resistance are described that require participation of five of the remaining eight AFI genes. YhiF (a putative regulatory protein), lipoprotein Slp, and the periplasmic chaperone HdeA protected *E. coli* from organic acid metabolites produced during fermentation once the external pH was reduced to pH 2.5. HdeA appears to handle protein damage caused when protonated organic acids diffuse into the cell and dissociate, thereby decreasing internal pH. In contrast, YhiF- and Slp-dependent systems appear to counter the effects of the organic acids themselves, specifically succinate, lactate, and formate, but not acetate. A second phenomenon was defined by two other AFI genes, *yhiD* and *hdeD*, encoding putative membrane proteins. These proteins participate in an acid resistance mechanism exhibited only at high cell densities (>10^8 CFU per ml). Density-dependent acid resistance does not require any demonstrable secreted factor and may involve cell contact-dependent activation. These findings further define the complex physiology of *E. coli* acid resistance.**

Enteric microorganisms experience extreme inorganic acid stress (pH 2 or less) as they traverse the stomach en route to the intestine, where conditions are less acidic (pH 5.5 to 8). However, in the intestine the organisms encounter another type of acid stress involving organic acids produced by anaerobic microbial fermentations (e.g., propionic, butyric, and acetic acids). To counter these stresses, pathogenic and commensal strains of *Escherichia coli* possess remarkable systems of acid resistance (AR) rivaling those of *Helicobacter pylori*, a species that has evolved to live in the stomach. *E. coli*, for instance, will survive for hours in a pH 2 environment, whereas organisms such as *Vibrio cholerae* and *Salmonella enterica* typically lose viability within minutes.

Mechanistic and regulatory aspects of *E. coli* acid resistance have been intensively studied over the past decade (14). Research has revealed two general forms of acid resistance. One form is amino acid dependent, while the other is amino acid independent (11, 13, 14, 17, 18, 20, 25, 28, 40, 46). The mechanism of amino acid-independent acid resistance, also known as the glucose-repressed or oxidative acid resistance system, remains enigmatic. However, the amino acid-dependent systems are known to require specific amino acid decarboxylases (GadA/B, AdiA, and CadA) and cognate antiporters (GadC, AdiC, and CadC) that import amino acid substrates (glutamic acid, arginine, or lysine, respectively) in exchange for exporting their respective decarboxylation products (γ-amino butyric acid, agmatine, and cadaverine). The decarboxylation reaction consumes an intracellular proton, which helps maintain a less acidic intracellular pH (39). These systems also require participation by any one of three Cl^-/H^+ antiporters, although their roles are unclear (1, 2, 24).

Despite intensive study, important gaps in our knowledge of acid resistance remain. One of those gaps involves a cluster of 12 protein-encoding genes located at 78.8 min (bp 3652313 to 3665210) on the *E. coli* K-12 genome, shown in Fig. 1, which has been termed an acid fitness island (AFI) (22). These genes (*slpA* through *gadA*) are unique to *E. coli* and the closely related genus *Shigella*, which also exhibits profound levels of acid resistance. Microarray studies have shown that most of the AFI genes in *E. coli* are induced by growth under acidic conditions, and mutations in some members have been associated with an inability to survive pH 2 environments (19, 26, 35, 51). Most notable are *gadA*, encoding an isozyme of glutamate decarboxylase (45, 47), and *gadE*, the essential activator of glutamate-dependent acid resistance (22, 30). Two other genes in this region, GadX and GadW, are involved in modulating *gadE* expression as well as the expression of *gadA* and *gadBC* (16, 32, 33, 44, 48–50, 52). However, the contribution of other AFI genes toward acid resistance has remained unclear.

There are at least three, seemingly contradictory, reports about possible roles for other AFI genes in acid resistance. The confusion stems from the fact that different laboratories have used very different methods to induce or measure acid resistance. One study overexpressed two regulators (EvgA and YdeO) to artificially activate pH 2.5 acid resistance in log-phase, LB-grown cells (35). These log-phase cells, in the ab-

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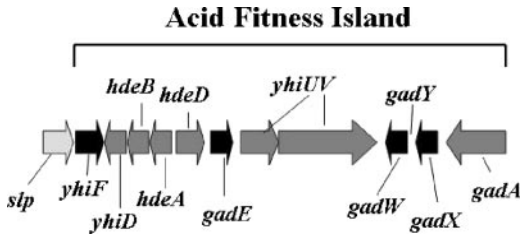

FIG. 1. *E. coli* acid fitness island. This genomic island is situated at centisomes 78.7 to 79.9 (bp 3652706 to 3665603) on the MG1655 chromosome. Black arrows represent regulators, while heavy gray arrows depict other members of the genomic island. The *yhiUV* genes have also been termed *mdtEF*, and their products confer resistance to rhodamine 6S and SDS (21, 36).

sence of overexpression, are normally acid sensitive. The overexpression strategy was used to define, by microarray analyses, a potential acid resistance regulatory network. In the process, they found that a subset of the fitness island genes (*yhiF*, *hdeA*, *hdeD*, and *gadE*) were required for YdeO overexpression-dependent acid resistance. However, the acid-resistant, positive control cultures (i.e., log-phase cells overexpressing these regulators) only showed a modest level of acid resistance (1.5% survival after 1 h in pH 2.5 LB) compared to naturally induced acid resistance, in which stationary-phase cells survive at 50 to 100% after 2 h at pH 2.5. Mutants defective in *hdeB*, *yhiD*, *yhiU*, and -*V* within the fitness island had little to no effect on acid resistance. A contradictory report that used acid-grown, log-phase cells tested in a minimal medium at pH 2.75 did not find an acid resistance phenotype associated with any gene in the area other than with *gadE (yhiE)*, *gadX*, and *gadW* (51).

A completely different strategy was used in a third study. *E. coli* wild-type and *hdeA* mutant cell cultures were grown to stationary phase in LB, at which point the pH of the liquid cultures, containing metabolic products of growth, was directly acidified to pH 2.5 (15). After 1 hour, cells were diluted into fresh LB broth (pH 7), and outgrowth was measured by optical density. Wild-type cells survived this pH 2.5 stress and rapidly grew after dilution. The *hdeA* mutant failed to grow, indicating it did not survive the stress. However, we show in the current report that an *hdeA* mutation has little effect on acid resistance when tested in fresh pH 2.5 minimal medium. These conflicting findings suggested that different AFI genes have conditional influences on acid resistance.

We now report that six AFI genes contribute to two newly described features of acid resistance. First, HdeA (encoding a periplasmic chaperone), YhiF (encoding a putative LuxR family regulator), and the lipoprotein Slp, along with the GadE regulator, are required to protect *E. coli* from its own metabolic products when placed either in pH 2.5-adjusted, spent LB or spent minimal glucose culture filtrates. Second, a new acid resistance phenotype evident only in high-cell-density cultures was discovered that requires two other AFI genes, YhiD (encoding a putative MgtC-family transporter) and a predicted inner membrane protein, HdeD. GadE, which activates these genes, was also required for high cell density-dependent acid resistance. The results underscore the versatility with which *E. coli* can protect itself from extreme acid stress.

MATERIALS AND METHODS

Strains, plasmids, and growth media. The bacterial strains used in this study were derivatives of MG1655 (Table 1). Most strains were constructed earlier; however, the Δ*yhiF*::Km mutant (EF1546) was constructed during this study using the Red recombinase method (12) and oligonucleotides 521 (5′-AAACT GATTATAAAGTTAATGTCCGCACCAGGAGTCGGTTGTGTAGGCTGG AGCTGCTTC) and 522 (5′-CTGTGTGGCAGCGTAGCCAGACTCACCGT AAGCCTGAAATCATATGAATATCCTCCTTAG). Wild-type *yhiF*$^+$ was cloned into pQE80L in a stepwise manner. A PCR fragment containing the YhiF open reading frame was made using oligos 799 (5′-CATCACGGATCCTTTCT TATAATTACC) and 800 (5′-CTAATTAAGCTTTCACACCAGATAATCAA TATGC) and cloned into the pCR4-TOPO vector between the BamH1 and HindII sites. The BamHI-HindIII fragment was then subcloned into pQE80L to make pFW584.

The media used included complex LB medium buffered with 100 mM morpholinepropanesulfonic acid (MOPS) to pH 8.0 (LB pH 8) or 100 mM morpholineethanesulfonic acid to pH 5.5 (LB pH 5.5) and unbuffered LB containing 0.4% glucose. Minimal E medium (53) contained 0.4% glucose (EG). Cultures were grown at 37°C with aeration. Log-phase cells were prepared via a 1:1,000 dilution of 18-h cultures into fresh medium followed by growth to an optical density at 600 nm of 0.3 to 0.4 (approximately 2×10^8 CFU/ml). Stationary-phase cells were collected after 18 h of incubation (approximately 2×10^9 to 4×10^9 CFU per ml). Antibiotics were used at the following concentrations: ampicillin (Ap), 60 μg/ml; kanamycin (Km), 50 μg/ml; tetracycline (Tc), 30 μg/ml; chloramphenicol (Cm), 40 μg/ml.

Acid resistance assays using low-density cultures. Cells were grown for 18 h in LB pH 8 or LB pH 5.5 and diluted 1:1,000 into various acid challenge media. The initial cell density during acid challenge was between 2×10^6 and 4×10^6 CFU per ml. AR system 1 was tested by making dilutions from LB pH 5 and LB pH 8 overnight cultures into EG pH 2.5 challenge medium (no amino acid supplementation). Wild-type cells grown in LB pH 5 are acid resistant, while cells grown in LB pH 8 are acid sensitive. AR system 2, the glutamate-dependent system, is evident in any wild-type culture grown to stationary phase. For this study, stationary-phase cells grown in LB pH 8 were diluted 1:1,000 into pH 2.5 EG medium without (negative control) or with 1.5 mM sodium glutamate. The pH 2.5 cultures were incubated at 37°C without shaking, and samples were collected at intervals to determine viable counts. Aliquots were serially diluted, and duplicates were plated onto LB medium. Colonies were counted after 18 to 24 h. Percent survival was calculated as follows: [(CFU/ml at time X)/(CFU/ml at time zero)] × 100. The results presented are averages of triplicate experiments

TABLE 1. Strains used

Strain	Genotype	Source or reference
EK550	MG1655 Δ*hdeA*	51
EK551	MG1655 Δ*gadE1*::Km	51
EK553	MG1655 Δ*hdeB*	51
EK555	MG1655 Δ*hdeD*	51
EK556	MG1655 *slp*	51
EK592 (MG1655)	Wild type	35
EK613	MG1655 Δ*ydeP*	35
EK616	MG1655 Δ*gadE*	35
EK618	MG1655 Δ*slp-yhiF*	35
EK619	MG1655 Δ*yhiUV*	35
EK692	MG1655 Δ*yhiD*	35
EK720 (MDO1)	AN387 *dctA*::*spc*	
EF962	Δ*gadXW*::Km *crp*::Cm *gadC1001*::Tn*10*dTc	17
EF1240	MG1655 Δ*lac*::cm	31
EF1155	MG1655 *gadE*::*Km*	P1 EK551 X EK592
EF1505	MG1655 *gadC*::Tn*10*dTc	P1 EF962 X EK592
EF1521	MG1655 Δ*slp-yhiF*/pFW254A (pQE80L with *yhiF*$^+$)	This paper
EF1546	MG1655 Δ*yhiF*::km	This paper
EF1548	MG1655 Δ*slp dctA*::*spc*	P1 EK720 X EK556

and include the standard error of the mean. Since time zero values are, by definition, all 100%, error bars were not applied to that time point.

Spent medium acid resistance was tested using spent LB and spent EG medium. Spent medium was prepared by growing MG1655 for 18 h in 100 ml of unbuffered LB in a 125-ml flask, or in 100 ml of EG in a 250-ml flask, at 37°C with shaking (225 rpm). These were low-oxygen conditions in which the final culture pH values were 7.4 and 6.0 for LB and EG, respectively. The pH of filter-sterilized spent medium was then adjusted to pH 2.5 with HCl. Stationary-phase LB pH 8-grown cultures were then diluted 1:1,000 into the pH 2.5 spent medium, and survival was measured as noted earlier.

High-cell-density acid resistance. Cells were grown for 18 h in LB MOPS pH 8 at 37°C with shaking. To test high-density acid resistance, cells from 1 ml of culture were collected by centrifugation at room temperature and washed quickly in 1 ml of pH 5.6 EG to bring the pH closer to the final challenge pH and to remove components of LB. The cells were again collected by centrifugation and finally resuspended in 3 ml of pH 2.1 EG for acid challenge. Initial cell density for acid challenge was between 2×10^8 and 4×10^8 CFU/ml, 100-fold higher than in the standard, low-density tests. The acid-challenged culture was incubated stationary at 37°C, and aliquots were taken at timed intervals to measure viable count. Low-cell-density acid resistance controls were carried out by diluting the pH 5.6 resuspended cells 1:1,000 into pH 2.1 EG (final cell density between 2×10^6 and 4×10^6 CFU per ml). Aliquots were taken at timed intervals, and viable were counts determined as above.

Mixing high-density and low-density cultures. Cultures of wild-type and drug-resistant mutant strains were grown independently in LB MOPS pH 8 and tested for high-density AR. Cells from one drug resistance-tagged strain (taken from the pH 5.6 resuspension) were diluted 1:1,000 into the pH 5.6 resuspension of the other strain. The mixture was collected by centrifugation and resuspended in 3 ml of pH 2.1 EG. Cell viability of each strain was assessed by plating dilutions onto LB agar and LB agar containing appropriate drugs.

RESULTS

Effect of fitness island mutations on system 1 and system 2 acid resistance. Using the defined minimal medium strategy noted above, our laboratory had previously defined three acid resistance systems (14). One system, called AR 1, functions independently of extracellular amino acids. The other two systems, called AR 2 and AR 3, require glutamate and arginine, respectively. To test whether any of the AFI genes have a potential role in amino acid-dependent or -independent acid resistance, mutants defective in each gene were tested for the presence of the amino acid-independent system (AR 1) and for one of the amino acid-dependent systems (AR 2). A mutation in *ydeP*, which does not reside within the AFI locus, was also tested because it was implicated in acid resistance in an earlier study (34). As shown in Fig. 2A and B, the only gene that affected these systems was *gadE*. The *gadA*, *-X*, and *W* genes, with proven roles in acid resistance, were tested in earlier studies (16, 33, 44, 50, 52). Failure to see an effect of the other AFI genes (*yhiF*, *yhiD*, *hdeB*, *hdeA*, *hdeD*, *or yhiUV*) conflicted with some of the earlier studies noted above but suggested that the methodology of testing is important in revealing the roles of these gene products.

Acid resistance in spent LB medium requires YhiF, Slp, and HdeA. The apparent discrepancy between the earlier studies using LB at pH 2.5 and our strategy using pH 2.5 minimal medium suggested the AFI gene products might have a purpose beyond handling proton influx. To determine whether any AFI genes had condition-specific roles, the acid resistance phenotypes of wild-type and mutant cells were examined in pH 2.5 fresh LB and pH 2.5 cell-free spent LB prepared from an MG1655 overnight culture. Figure 3A reveals that most of the fitness island mutants, as well as *ydeP*, had little to no effect on acid resistance when tested in pH 2.5 fresh LB. As expected,

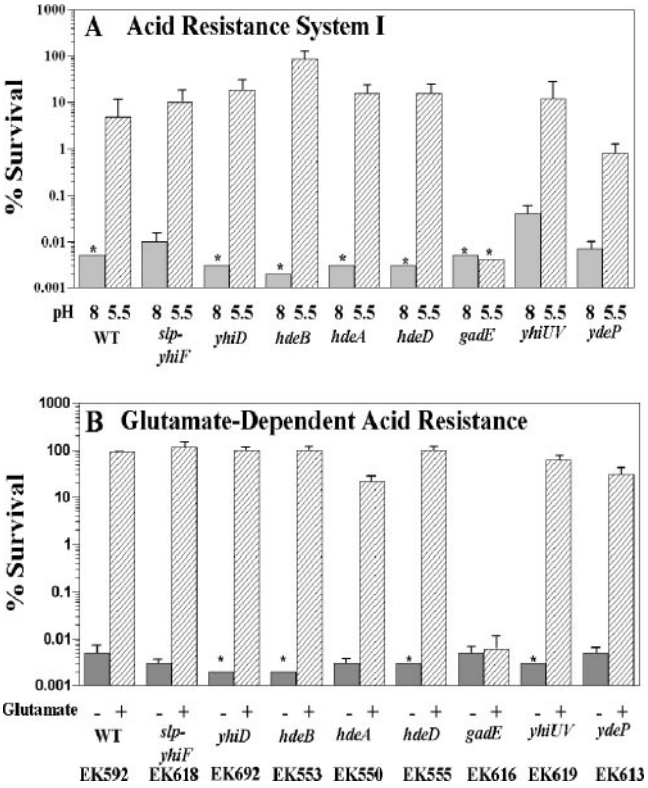

FIG. 2. Effect of acid fitness island mutations on amino acid-independent and glutamate-dependent acid resistance. A. System 1. Cells were grown for 18 h in LB medium buffered to pH 8 (shaded bars) or pH 5.5 (hatched bars) and used to inoculate pH 2.5 EG to a cell density of approximately 1×10^6 to 4×10^6 CFU per ml. Survival was determined at 2 hours. B. System 2. Cells from LB pH 5.5 cultures were used to inoculate pH 2.5 EG without supplementation (shaded bars) or containing 1.5 mM glutamic acid (hatched bars). Results are given as averages with standard errors of the means. Asterisks indicate viability was below the level of detection.

the *gadE* mutant failed to survive, but, in addition, the *hdeA* mutant was found to be acid sensitive. After 4 hours the wild type exhibited 20% survival, whereas the *hdeA* mutant survived at less than 0.1%. This result suggests the HdeA periplasmic chaperone protects the cell from some component of LB that becomes toxic at pH 2.5.

A role for two other members of the fitness island, YhiF (encoding a putative LuxR family regulator) and the lipoprotein Slp, became apparent when spent LB, prepared from stationary-phase cells, was acidified to pH 2.5 and used to test acid resistance. In this situation, the *slp-yhiF* and *hdeA* mutants, along with the *gadE* mutant, failed to survive pH 2.5 (Fig. 3B). Over a 4-h period, these mutants exhibited a 3- to 4-log decrease in survival relative to the wild type, with viability falling below detection after only 2 h. Since the *slp* mutant alone exhibited normal acid resistance, the sensitivity of the *slp-yhiF* mutant was initially thought to be due to the absence of *yhiF*. However, as shown below, it appears that both gene products participate in this acid resistance phenotype. The other AFI mutants remained resistant over this same time frame. These results suggest that metabolic end products secreted by *E. coli* during growth in LB add to the stress experienced at pH 2.5

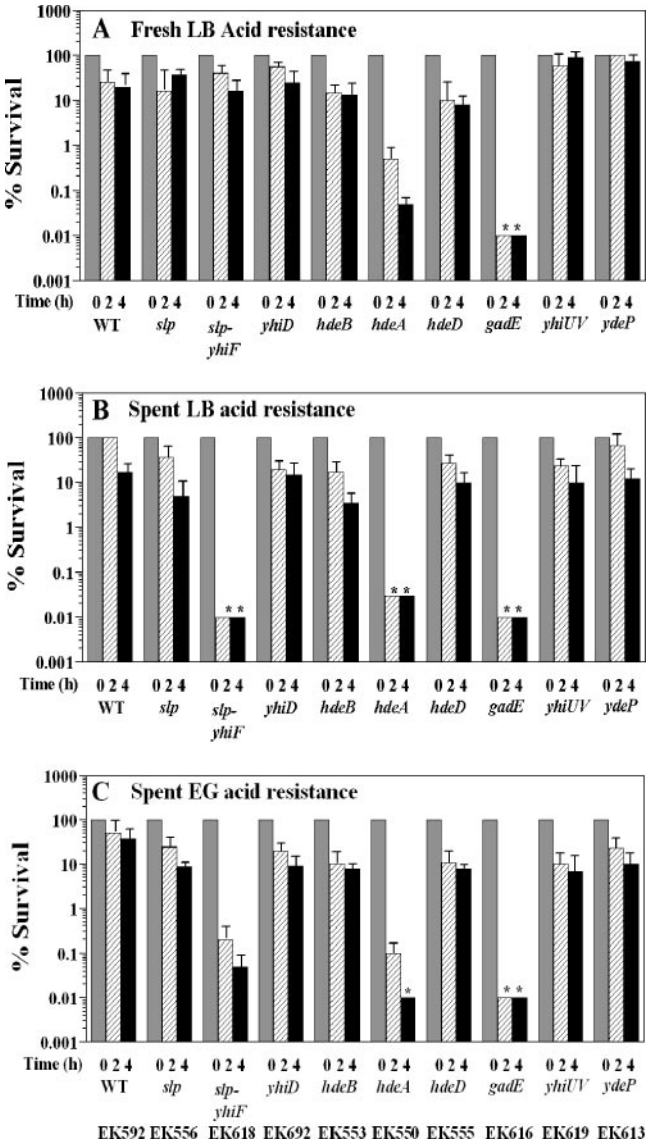

FIG. 3. Effect of fitness island mutations on acid resistance in spent culture media. Cells grown for 18 h in pH 8 buffered LB medium were used to inoculate pH 2.5 fresh LB (A), pH 2.5 spent LB (B), or pH 2.5 spent EG (C) to a cell density of approximately 2×10^6 to 4×10^6 CFU per ml. Survival was determined at 0 (gray bars), 2 (cross-hatched bars), and 4 h (solid bars). Asterisks indicate survival was below the detection limit. Results are given as averages with standard errors of the means.

and that Slp, YhiF, and HdeA are involved in handling that added stress.

***E. coli* secretes acid-protective and toxic compounds during growth in minimal glucose medium.** We repeated the above experiments using spent minimal E glucose medium adjusted to pH 2.5. No glutamic acid was supplied during acid challenge. Because MG1655 dies rapidly in pH 2.5 fresh EG medium without glutamate present, we expected these cells would also quickly succumb when challenged at pH 2.5 in spent EG, as long as glutamate was not added. We were surprised, however, to find that MG1655 survived extremely well in the acidified spent challenge medium (Table 2, MG1655 fresh EG versus MG1655 spent EG). The results suggest that *E. coli* expels one or more compounds during growth that can be used for protection against later acid challenge. The protective effect was observed using spent EG from stationary-phase cultures and conditioned medium prepared from log-phase cultures (data not shown).

One obvious protective factor the cell could excrete to aid acid resistance is glutamic acid, which could later be utilized by the GadA, GadB, and GadC system under acid stress conditions. Previous studies have shown that *E. coli* growing in minimal medium can secrete glutamic acid (8, 9). A *gadC* mutant which lacks the antiporter required for glutamate-dependent acid resistance was used to test this idea. If secreted glutamate were the protective factor, then the *gadC* mutant should not survive in pH 2.5 spent EG. The result shown in Table 2 illustrates that this is the case (EF1505). Thus, glutamate secretion by *E. coli* has a purpose besides providing for metabolic overflow.

On the chance that GadE or a GadE-regulated gene product is also responsible for the secretion of glutamate, spent EG medium prepared from a *gadE* mutant culture was tested for a protective effect on MG1655. The results presented in Table 2 reveal that spent medium from the *gadE* mutant was also able to protect MG1655. Thus, secretion of glutamic acid is independent of GadE and GadE-dependent genes.

Based on the LB results above, we suspected other members of the fitness island might play a role in surviving pH 2.5 spent EG, if, as appears to be the case in LB, metabolic end products become toxic under extreme acid conditions. To explore this, we tested a *gadE* mutant for its ability to survive in MG1655 spent EG adjusted to pH 2.5. Since *gadC* is required for acid resistance in pH 2.5 spent EG and GadE is required to induce *gadC*, the *gadE* mutant also died quickly under this condition (Table 2). To show that loss of viability was not due to general toxicity of metabolic end products regardless of pH, the *gadE* mutant was also tested in spent SG at pH 6.3. Under this condition, the mutant lived and even grew slightly (Table 2).

Since GadE is also required for the expression of other AFI genes, we examined whether any of the AFI gene products also contributed to acid resistance in spent EG (pH 2.5). The results, using spent EG from MG1655, revealed that *slp-yhiF*, *hdeA*, and *gadE* were all required for survival in this medium, as was the case in pH 2.5 spent LB (Fig. 3C).

One potential explanation for the AFI gene requirement in

TABLE 2. Survival of *E. coli* in fresh and spent EG

Strain	Condition	Source of spent EG	% Survival[a] after:		
			1 h	2 h	4 h
MG1655 (EK592)	Fresh EG pH 2.5	NA[b]	<0.006	<0.006	<0.006
MG1655 (EK592)	Spent EG pH 2.5	MG1655	71	52	38
gadC (EF1505)	Spent EG pH 2.5	MG1655	<0.008	<0.008	<0.008
gadE (EK616)	Spent EG pH 2.5	MG1655	<0.006	<0.006	<0.006
gadE (EK616)	Spent EG pH 6.3	MG1655	100	105	110
MG1655 (EK592)	Spent EG pH 2.5	*gadE*	50	26	11
slp (EK556)	Spent EG pH 2.5	MG1655	82	56	9
yhiF (EF1546)	Spent EG pH 2.5	MG1655	95	66	14
slp-yhiF (EF618)	Spent EG pH 2.5	MG1655	1.5	0.23	0.006
slp-yhiF/pQEYhiF+ (EF1521)	Spent EG pH 2.5	MG1655	100	95	35

[a] Averages of triplicate experiments.
[b] NA, not applicable.

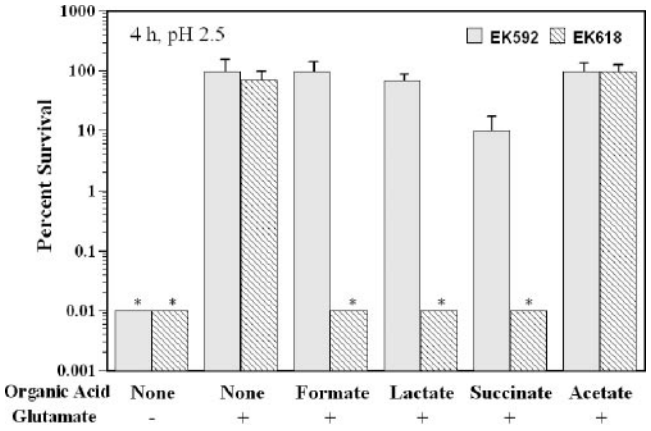

FIG. 4. YhiF-dependent pathways protect against metabolic end products under extreme acid pH. Cells grown to stationary phase in pH 8 buffered LB were used to inoculate pH 2.5 fresh EG containing the additives shown: 1.5 mM glutamate, 20 mM sodium formate, 40 mM sodium lactate, 40 mM sodium succinate, or 40 mM sodium acetate. Cultures were inoculated to 2×10^6 to 4×10^6 CFU per ml and challenged for 4 h. Asterisks indicate survival was below the detection limit.

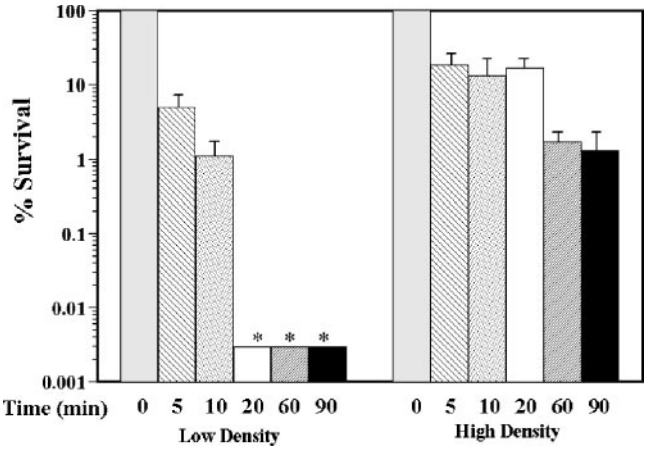

FIG. 5. High cell density-dependent acid resistance. MG1655 (EK592) was grown for 18 h in pH 8 buffered LB. Cells were then washed and resuspended in pH 2.1 EG at low density (2×10^6 to 4×10^6 CFU per ml) or high density (2×10^6 to 4×10^8 CFU per ml). Survival was measured at timed intervals. Asterisks indicate viability was below the level of detection.

spent minimal medium is that the excreted end products of glucose metabolism (for instance, weak acids) simply add to the proton stress *E. coli* must endure while at pH 2.5. Lowering the pH of fresh minimal medium to 2.0 will also increase proton stress. So, if YhiF and HdeA were required to handle the increased intracellular proton stress resulting from end product reentry and dissociation, then mutations in *yhiF* and *hdeA*, which have little effect on survival at pH 2.5 in fresh minimal medium containing glutamate, should become acid sensitive at pH 2.0 in the same medium. When tested at pH 2.0 in minimal medium with glutamate, the *hdeA* mutant actually did become acid sensitive (data not shown). However, the *slp-yhiF* mutant did not. The role of Slp, YhiF, and YhiF-dependent gene products may be to cope more directly with metabolites (for instance, the accumulated anions of dissociated weak acids) rather than simple proton stress.

YhiF and Slp provide redundant protection against metabolite stress at low pH. Since the *slp* mutant seemed proficient at handling metabolite stress, we initially concluded that YhiF was the relevant player. Previous research has shown that transcripts of *slp* and *yhiF* are distinct (3). However, it remained possible that Slp and YhiF define separate, redundant protection pathways. To determine whether YhiF alone was the relevant gene product, a *yhiF* single deletion mutant was constructed and tested. Table 2 reveals that neither *slp* nor *yhiF* alone affected survival, and only the *slp-yhiF* double mutant (EF618) experienced difficulty surviving in spent EG. This suggested that either gene product could mitigate the detrimental effects of the toxic metabolites. In addition, we found that a *yhiF*$^+$-carrying plasmid introduced into the *slp-yhiF* mutant restored resistance to the double mutant (Table 2). The combined results suggest that Slp and YhiF define independent systems that protect the cell against metabolites that are toxic at pH 2.5.

YhiF-regulated gene products and Slp protect cells against lactic, succinic, and formic acids. To test the hypothesis that Slp and YhiF protect the cell against toxic metabolites, wild-type and *slp-yhiF* mutant strains were tested in fresh EG (pH 2.5) containing 1.5 mM glutamic acid that was also spiked with the common fermentation end products acetate, lactate, succinate, or formate. As shown before, the *slp-yhiF* mutant (EF618) was able to survive pH 2.5 in the presence of glutamate just as well as wild-type cells as long as fermentation end products were not included in the challenge medium (Fig. 4, first two bars). However, the *slp-yhiF* mutant survived poorly in the presence of 20 mM formate, 40 mM lactate, or 40 mM succinate, whereas wild-type cells maintained good viability (Fig. 4). Effects were also noted at lower concentrations of these organic acids, but culture viability declined more slowly. It is interesting that the YhiF and Slp systems were not required for survival in acetate up to 40 mM. It is possible that a gene outside of the AFI is required to cope with acetate at extremely low pH.

***E. coli* exhibits cell density-dependent acid resistance.** In addition to their roles in protecting low-cell-density cultures of *E. coli* against metabolic end products that are toxic at pH 2.5, we wondered whether any of the AFI gene products might have a specific benefit toward high-cell-density cultures undergoing extreme acid stress. *E. coli* can achieve high cell densities as biofilms or as planktonic cultures in enclosed spaces that exist in host or nonhost environments. However, acid resistance, as shown above, has generally been studied using low-density cell cultures, approximately 10^6 CFU per ml, in acid challenge medium. Recently, while examining the molecular fate of cellular proteins during acid stress, we subjected high-cell-density cultures ($>10^8$ CFU/ml) to pH 2.1 in order to achieve higher concentrations of protein for analysis. This cell density is 100-fold higher than that normally used to measure acid resistance. Survival assays, however, revealed that cultures predicted to be acid sensitive nevertheless survived at a level 2 or more orders of magnitude higher than low-density cells at the same pH.

Figure 5 shows that MG1655 cells grown overnight (18 h) in

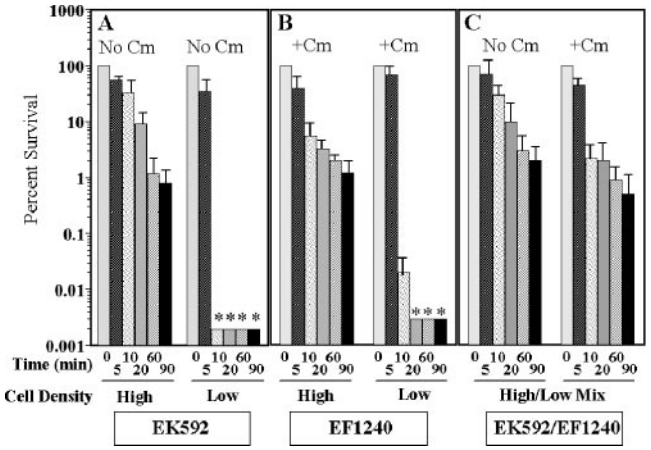

FIG. 6. Cross -rotection of a low cell density strain by a high cell density culture. EK592 (MG1655) and EF1240 (MG1655 Cm^r) were grown for 18 in pH 8 buffered LB and tested for high and low cell density acid resistance (EG pH 2.1) as described for Fig. 4. Survival was determined by plating samples at timed intervals on LB in the absence or presence of chloramphenicol. (A) EK592; (B) EF1240; (C) mixture of EK592 (10^8 CFU per ml) and EF1240 (10^6 CFU per ml). Samples were plated on LB (to detect both strains) and LB plus Cm (to detect EF1240). Asterisks indicate viability was below the level of detection.

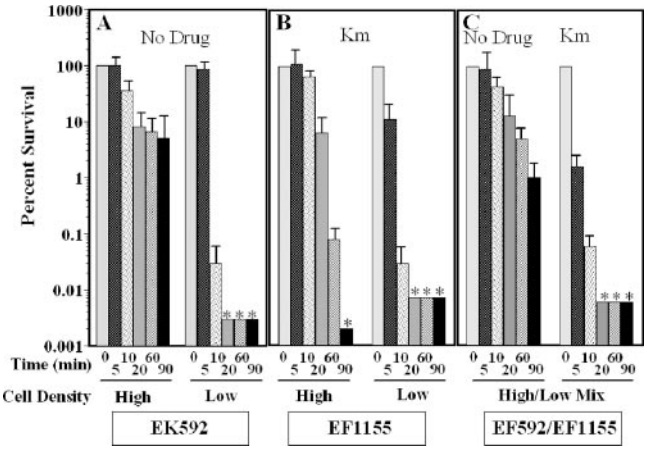

FIG. 7. GadE-regulated gene products are required to implement high cell density acid resistance. Shown are the high cell density and low cell density acid resistance of wild type (EK592) (A), *gadE* mutant (EK1155) (B), and a mixture of high density (10^8 CFU/ml) wild type and low cell density (10^6 CFU/ml) *gadE* mutant (C). Asterisks indicate viability was below the level of detection.

LB pH 8 rapidly died when washed and diluted to 10^6 CFU per ml in pH 2.1 EG without amino acid supplementation. Between 10 and 20 min, viable counts decreased below the level of detection (approximately 0.003%). This was expected based on earlier studies. However, when the same cells were resuspended to over 10^8 CFU per ml in pH 2.1 medium, the viable count only decreased to about 1 to 5% after 20 min and held steady until at least 90 min. The survival margin between high-density and low-density cultures was consistently between 100- and 1,000-fold higher for the high-cell-density suspensions. The survivors did not represent more resistant stable mutants of MG1655, since isolation and retesting of the survivors yielded the same phenotypes at low and high densities (data not shown).

High-density cell cultures do not secrete a stable protective factor. Another possible explanation for these data is that dying cells present in the high-cell-density suspension release a factor (e.g., glutamic acid) that protects a subpopulation of the culture. Glutamic acid, if it were released upon suspension in pH 2.1 medium, could be used by the glutamate decarboxylase/GadC antiporter system and protect a subpopulation of the cells. We, therefore, tested whether cells in high-density cultures released any soluble factors that other cells could use to survive.

We prepared cell-free pH 2.1 EG medium conditioned with high-density cells for 5 or 60 min. One ml of wild-type cells grown overnight in pH 8 buffered LB was washed and resuspended in 3 ml of EG pH 2.1 (final density, approximately 2×10^8 CFU per ml). After 5 min and 60 min of challenge, when viable counts were 100% and 5%, respectively, the conditioned media were collected and filter sterilized. Wild-type cells were diluted to 2×10^6 CFU per ml in this pH 2.1 conditioned medium. If any stable protective factor were released into the pH 2.1 challenge medium, that factor should protect the newly added low-density cells. However, neither the 5-min nor the 60-min high-cell density-conditioned medium was able to protect low-density cells (data not shown). Thus, it appears that at high cell density, one subset of cells does not altruistically release a protective factor into the pH 2.1 medium that saves a smaller subset or, if it does, the factor must be unstable.

MG1655 at high density will protect a second MG1655 strain present at low cell density. The previous data indicated that a stable protective factor is not released by high-density cultures. An alternative possibility is that cell-cell contact at high density may afford protection of a subpopulation of the culture, a mechanism of potential importance in the context of biofilms. To begin to explore this model, we asked whether a high-density culture of one cell type could protect a second, drug-marked, strain added at low cell density in a mixed culture. Cells were mixed immediately before they were resuspended into pH 2.1 medium. Both wild-type strains (EK592 and EF1240), when tested individually, exhibited high cell density-dependent acid resistance (Fig. 6A and B). Low-density cultures died rapidly (<0.002% survival) at pH 2.1, while high-density cultures only declined to approximately 1%. However, when an MG1655 strain marked with Cm^r (EF1240) was mixed at low density with a high-density MG1655 strain (EK592), both cultures survived at approximately 1%. The high-density strain was able to protect the second strain present at low density.

This result, in light of the fact that a secreted factor was not demonstrable, suggests that cell-cell contact in some form may be needed for protection. However, an aggregation mechanism in which peripheral cells might protect cells within the aggregate's center does not appear to be the reason for protection. Cells of pH 2.5 high-density cultures did not visibly aggregate when examined microscopically (not shown).

Mutants defective in *gadE* do not exhibit high-density acid resistance. We wondered if any of the AFI gene products might be required for this high cell density-dependent acid resistance. The *gadE* gene was tested first because it appears to

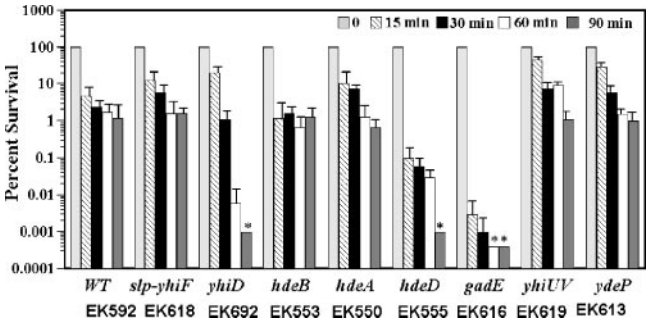

FIG. 8. Effect of fitness island gene mutations on cell density-dependent AR. Cells grown for 18 h in pH 8 buffered LB were washed and resuspended in pH 2.1 EG at high density (2×10^8 to 4×10^8 CFU per ml). Survival was measured at timed intervals. Asterisks indicate viability was below the level of detection.

control most genes within the island. In contrast to the wild type, a *gadE* mutant failed to survive pH 2.1 over 90 min even at high density, suggesting that a GadE-regulated gene product was involved in the phenomenon (Fig. 7A and B). At early time points, the *gadE* mutant did survive somewhat better than at low cell density but was unable to maintain resistance.

A high-density wild-type culture was then tested for its ability to send an acid survival signal to a small number of comixed *gadE* mutant cells. The results, shown in Fig. 7C, indicate that high-density wild-type cells (10^8 CFU per ml) were unable to stimulate acid survival in the low-density *gadE* mutant cells (10^6 CFU per ml). Thus, *gadE* mutants are unable to effectively receive and/or process the acid survival signal from high-cell-density wild-type cells.

HdeD and YhiD are required for high-density acid resistance. Since *gadE* activates the AFI genes, we tested whether or not other AFI genes were important for high cell density-dependent acid resistance. Mutants defective in individual AFI genes were examined for a potential role in high cell density-dependent acid resistance. Two genes without a previously described phenotype, *yhiD* and *hdeD*, proved to be important for this mechanism (Fig. 8). High-density cultures of both mutants, along with *gadE*, progressively lost viability at pH 2.1 over time and eventually fell below detection. All other AFI mutants maintained viability at approximately 1%. The evidence indicates that at least part of the reason *gadE* mutants are defective in high-density AR is a failure to activate HdeD and YhiD. YhiD is a putative ATPase transporter within the MgtC family of transporters, and HdeD is a putative inner membrane protein of unknown function.

Live cells are required to send a survival signal. High-density/low-density mixing experiments were also performed with the *yhiD* and *hdeD* mutant strains to determine if they were defective in receiving or in sending a survival signal. There were two significant findings arising from these experiments. First, high-density wild-type cells could not save either *yhiD* or *hdeD* mutant cultures comixed at low cell density (Fig. 9A and B). These results argue that both gene products must be present to carry out high cell density-dependent acid resistance. YhiD and HdeD are either needed to receive the survival signal from the high-density cells or to implement survival once the signal is received.

The second significant finding from these mixing experiments was that the *hdeD* mutant, when present at high cell density, could at least temporarily send a survival signal to low-density wild-type cells as long as the mutants retained some viability (Fig. 9C). The low-density wild-type culture survived at a significantly higher level when mixed with the *hdeD* mutant than when tested alone. However, the wild-type cells

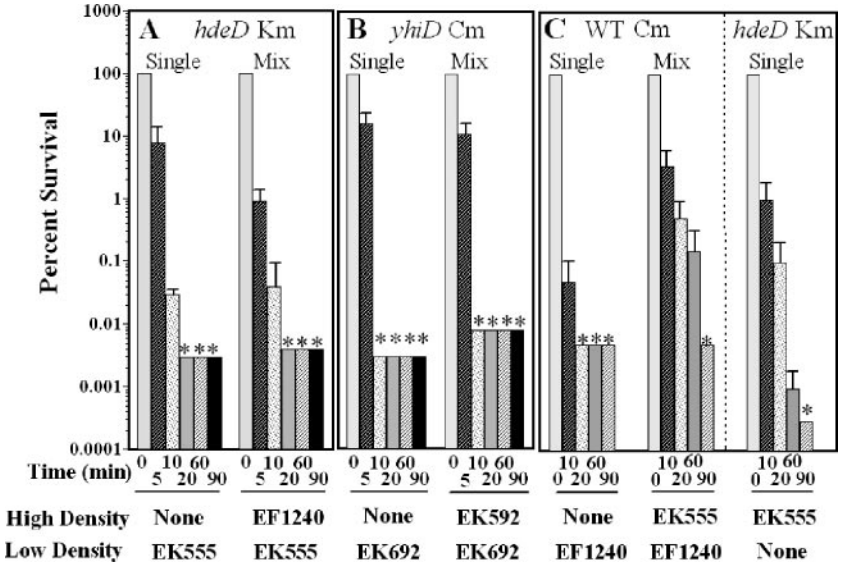

FIG. 9. Ability of mutants to send and receive signals for high cell density AR. EK592 (MG1655), EF1240 (MG1655 Cm^r), EK555 (*hdeD*::Km), and EK 592 (*yhiD*::Cm) were grown for 18 in pH 8 buffered LB. Strains were tested for low cell density acid resistance (EG pH 2.1) and in various high-density/low-density mixtures as described for Fig. 6. Survival was determined by plating samples at timed intervals on LB and LB with chloramphenicol (EK692) or kanamycin (EK555). The figure only reflects results obtained on drug plates and represents the low-density strain in mixes. A. EK555 at 10^6 CFU/ml without and with EK1240 at 10^8 CFU per ml. B. EK692 at 10^6 CFU/ml without and with EK592 at 10^8 CFU per ml. C. Mixture of EK555 (10^8 CFU per ml) and EF1240 (10^6 CFU per ml). Asterisks indicate viability was below the level of detection.

still died by 90 min, soon after the majority of the higher-density *hdeD* cells lost viability (Fig. 9C, last bar set). Control mixtures of the same wild-type strain mixed with another wild-type strain at high density (Fig. 6C) survived at 1 to 5% over the 90-min period (data not shown). When similarly tested, the *gadE* mutant was also able to transiently stimulate survival of wild-type MG1655 (data not shown).

The data suggest that neither HdeD nor GadE is involved in sending the protective signal, only in receiving or implementing it. The results also indicate that the simple presence of a high number of dead cells cannot protect lower-density cells. There must be some communication between live cells.

DISCUSSION

The results presented have revealed two new aspects of acid resistance physiology. The first is that there is a specific mechanism used by *E. coli* to survive in the presence of excreted metabolites that become toxic to cells under extreme acid stress. Slp, YhiF, and HdeA, three AFI gene products, are required to protect the cell against the pH-dependent toxic effects of these metabolites. HdeA, a periplasmic chaperone, appears to protect against the increased proton stress resulting from the presence of organic acids (15, 23, 26). YhiF, a transcriptional regulator, and Slp, an outer membrane lipoprotein, have more specific roles in reducing metabolite stress. Of the known glucose fermentation end products tested, survival in the presence of lactate, succinate, and formate required a Slp- or YhiF-dependent system (54).

HdeA is a chaperone with the unusual property of being able to bind to denatured proteins under acidic conditions (below pH 3) but not at neutral pH (23). It does this by transforming from an ordered conformation at neutral pH into a globally disordered conformation below pH 3. The disordered form allows HdeA to bind to denatured periplasmic proteins and prevent their aggregation. As illustrated here, HdeA appears to play a more important role in acid survival when tested in complex environments (LB) or in the presence of organic acids. HdeA appears to be dispensable in minimal medium at pH 2.5. Why this is is not clear. One possibility is that the chaperone effect of HdeA may protect other proteins required to cope with compounds that become more toxic as the environment acidifies. In the absence of such compounds, HdeA becomes less important. Alternatively, a recent report suggests that HdeB, another periplasmic chaperone, can substitute for HdeA (26). Mutations in both may be needed to see an effect.

YhiF encodes a putative LuxR family regulator; thus, the protection it affords is likely due to a YhiF-activated gene product. The YhiF regulon, however, has not been defined. One study has implicated YhiF as a negative regulator of the gene encoding the C4 transporter DctA and has suggested YhiF be called DctR (7). The DctA family is a subgroup of the dicarboxylate/amino acid:cation symporter or DAACS family. DctA in *E. coli* catalyzes H^+ symport with C4-dicarboxylates (succinate, fumarate, and malate), dicarboxylic amino acids (aspartate and glutamate), or the monocarboxylic acid orotate as a pyrimidine source (5, 29). Enteric bacteria, in fact, can form up to 0.2 mol of succinate per mol of glucose in a mixed acid fermentation (6).

Since two protons enter the cell with each C4-dicarboxylate, it would seem that having the DctA transporter present at pH 2 would be ill advised. YhiF (DctR) repression of *dctA* might eliminate a pathway for proton import and may at least partially explain the selective role of YhiF (DctR) toward acid resistance in spent medium. In this model, one would predict that increased production of DctA in a *yhiF* mutant would increase organic acid and proton influx, thereby increasing cell death. However, introducing a *dctA* mutation into a *slp-yhiF* mutant did not suppress the acid-sensitive phenotype, which suggests that one or more YhiF-dependent gene products other than DctA are involved in acid resistance.

An alternative scenario is that DctA, under extreme acid pH conditions, may actually have a protective effect by exporting toxic organic acids. Formate, lactate, and succinate all have a carboxyl groups with pK_a values less than pH 4.5 (formic acid, pK_a 3.75; lactic acid, pK_a 3.86; succinic acid, pK_a1 4.19 and pKa2 5.57). Thus, all three organic acids will be fully protonated and uncharged at pH 2.5. Uncharged organic acids pass through intact membranes without assistance from a transporter and, once inside the cell, will release protons from side groups whose pKa values are below the pH of the cytoplasm. The internal pH of *E. coli* surviving at pH 2.5 is approximately 4.5; thus, any of these organic acids would be expected to release protons that could drive internal pH even more acidic (39). DctA could help expel these accumulated organic acids. However, when tested, a *dctA* mutant exhibited nearly wild-type levels of resistance to succinate, lactate, and formate at pH 2.5 (data not shown). The same result was obtained when *dctA*::*spc* was introduced into a *slp* mutant. These two lines of evidence argue against a role for DctA in this acid resistance system. The YhiF-dependent gene(s) mediating resistance to organic acids remains undiscovered.

Slp encodes an outer membrane lipoprotein whose role in *E. coli* physiology has remained enigmatic (3, 38). Its role may be to limit penetration of certain organic acids across the outer membrane or as part of a signal transduction mechanism that activates an organic acid protection system. Recent studies have also suggested some lipoproteins serve as signal transducers (10, 37). It remains to be seen whether Slp may serve a similar role.

It is curious that neither the Slp- nor YhiF-dependent pathway was required for protection against acetate (pK_a 4.76). It seems likely, then, that a separate system is used to protect against the toxic effects of this organic acid.

A second significant finding from this study is that *E. coli* expels glutamic acid during growth and subsequently uses it through the glutamate decarboxylase system to ameliorate proton influx. This means *E. coli* doesn't necessarily depend on the exogenous addition of glutamate to survive acid stress: it can place it there itself. So, to survive extreme acid stress in its own spent medium, *E. coli* must seed its medium with glutamate and have YhiF (or Slp) and HdeA, as well as the GadA/BC decarboxylase system, functioning. Loss of any one of these factors means the cell will not survive.

A third finding from this study is that *E. coli* also possesses an acid resistance mechanism that is activated at high cell density and involves at least two members of the acid fitness island, YhiD and HdeD, both of which are predicted membrane proteins. High cell density-dependent acid resistance cannot be explained as a stochastic event in which a subpopu-

lation of the culture randomly acquires a more acid-resistant physiology than its neighbors. If this were so, there should be no difference in percent survival between high- and low-density cultures. Nor can this acid resistance be due to a mutational event leading to a more resistant phenotype. Survivors from one round of high-density acid stress did not acquire increased acid resistance when retested at low cell density nor, for that matter, at high cell density.

We noticed that low-density cells temporarily survive for about 10 min before rapidly losing viability. The reason for survival at early time points may be due to the intracellular pool of glutamate in low-density cultures. Presumably, this glutamate along with protons would be consumed by glutamate decarboxylase over the first 10 min of a pH 2.1 exposure. However, once the glutamate is consumed, the cells rapidly die.

There are examples of high-cell-density phenomena affecting the acid survival of microbes. An earlier study with *Streptococcus mutans* demonstrated that cell density influenced the tolerance of log-phase cells to pH 3.5 acid challenge (27). The study found that placing log-phase cells at high cell density during pH 5.5 adaptation increased tolerance to pH 3.5 by 1 to 2 orders of magnitude. The increase was attributed to a secreted protein factor produced by high-density-adapted cells. This factor was able to induce acid tolerance in log-phase cells. This is distinct from the high-density acid resistance of *E. coli*, which occurs during challenge at pH 2.1, not during an adaptation phase. Also, high-density acid resistance of *E. coli* does not appear to involve a secreted factor. For similar reasons, the phenomenon we describe is different from that reported earlier for *E. coli* acid habituation, which also involved an uncharacterized secreted product (41–43).

There is a recent example describing a cell-cell contact phenomenon for *E. coli*. Certain uropathogenic strains of *E. coli* inhibit the growth of heterologous strains following cell contact. This process, called contact-dependent inhibition, illustrates that cell contact between different strains of *E. coli* can have potent biological effects (4). Contact-dependent inhibition requires a large surface protein (CdiA) and a two-partner secretion family member (CdiB). CdiB is an outer membrane protein that transports CdiA to the bacterial cell surface. Cell contact, presumably via these proteins, inhibits growth of the target strain through an unknown mechanism.

It is unclear whether the high cell density-dependent acid resistance described here truly represents a similar cell contact response system. It is possible that an unstable soluble factor passes between cells without a need for stable cell-cell contact, or that incidental cell-cell contact may be sufficient. Importantly, the finding of mutants defective in this process argues against an artifactual explanation for the phenotype and supports the idea that high-cell-density acid resistance represents a previously unrecognized low-pH survival mechanism.

In addition to focused studies on acid resistance mechanisms, as presented here, several genome-based microarray studies have shown that exposing *E. coli* to moderate levels of acid (e.g., pH 5) has an incredibly broad impact on metabolism beyond just the acid resistance genes unique to *E. coli*. For example, a recent study by Hayes et al. has shown that exposure to acid pH can potentially shift fermentation away from glucose and toward sugars such as sorbitol, gluconate, and glucuronate, which yield less-acidic products (19). This would minimize acidification of the growth medium and prevent acid damage. Other genes found to be induced by acid in that study encode functions ranging from membrane biogenesis, periplasmic proteins, proton transporters, and multidrug resistance pumps. In sum, the mechanisms used by *E. coli* to adapt to, and survive, extreme low pH are varied, complex, and integrated. This broad acid response system helps the cell avoid self-imposed acid stresses that occur as a result of fermentation and enables the cell to survive an extreme low pH should that prove inevitable.

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