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Mechanisms of Acid Resistance in Enterohemorrhagic *Escherichia coli*

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Enterohemorrhagic strains of *Escherichia coli* must pass through the acidic gastric barrier to cause gastrointestinal disease. Taking into account the apparent low infectious dose of enterohemorrhagic *E. coli*, 11 O157:H7 strains and 4 commensal strains of *E. coli* were tested for their abilities to survive extreme acid exposures (pH 3). Three previously characterized acid resistance systems were tested. These included an acid-induced oxidative system, an acid-induced arginine-dependent system, and a glutamate-dependent system. When challenged at pH 2.0, the arginine-dependent system provided more protection in the EHEC strains than in commensal strains. However, the glutamate-dependent system provided better protection than the arginine system and appeared equally effective in all strains. Because *E. coli* must also endure acid stress imposed by the presence of weak acids in intestinal contents at a pH less acidic than that of the stomach, the ability of specific acid resistance systems to protect against weak acids was examined. The arginine- and glutamate-dependent systems were both effective in protecting *E. coli* against the bactericidal effects of a variety of weak acids. The acids tested include benzoic acid (20 mM; pH 4.0) and a volatile fatty acid cocktail composed of acetic, propionic, and butyric acids at levels approximating those present in the intestine. The oxidative system was much less effective. Several genetic aspects of *E. coli* acid resistance were also characterized. The alternate sigma factor RpoS was shown to be required for oxidative acid resistance but was only partially involved with the arginine- and glutamate-dependent acid resistance systems. The arginine decarboxylase system (including *adi* and its regulators *cysB* and *adiY*) was responsible for arginine-dependent acid resistance. The results suggest that several acid resistance systems potentially contribute to the survival of pathogenic *E. coli* in the different acid stress environments of the stomach (pH 1 to 3) and the intestine (pH 4.5 to 7 with high concentrations of volatile fatty acids). Of particular importance to the food industry was the finding that once induced, the acid resistance systems will remain active for prolonged periods of cold storage at 4°C.

An important property of microbial pathogens associated with oral-fecal routes of transmission is the ability to survive extremely acidic environments as well as moderately acidic environments containing weak acids. Resistance to low pH can be important to food-borne pathogens for survival in specific foods and in the gastrointestinal tract. For example, an acidic pH and weak acids present in certain foods not only confer flavor but also are used to prevent the growth of contaminating organisms (12, 13). In many foods, weak acids are produced by organisms themselves via fermentation, while acidulation is frequently practiced for both fermented foods and acidic foods. Weak acids are also used in food-processing procedures designed to control contaminating pathogens on meat surfaces and in animal feeds (11, 21, 35, 36). Consequently, the ability to resist acid may allow pathogenic microorganisms to survive in acidic foods, animal feed, and food-processing treatments until the organisms are ingested (8, 9, 24).

Once ingested, the successful pathogen must also endure a variety of exposures to acid. The importance of gastric secretions as an early line of defense against enteric pathogens has been recognized for nearly a century, with the primary bactericidal barrier being acid dependent (14, 20, 30). Clearly, the infectious dose (ID) of different enteric pathogens corresponds to their relative abilities to withstand acid. The oral IDs of *Vibrio cholerae*, non-typhi *Salmonella*, spp., and *Shigella flexneri* are approximately 10^9, 10^5, and 10^2, respectively (6, 7). These doses agree with the relative levels of acid resistance of the bacteria, with *V. cholerae* being the least resistant and *S. flexneri* being the most resistant. Enterohemorrhagic *E. coli* strains are believed to have IDs similar to those of *Shigella* spp. Reducing gastric acidity through antacids or other means can significantly reduce the oral 50% IDs (ID_{50}s) for the more acid-sensitive organisms (30). This observation further supports a role for acid resistance as an important virulence feature of successful gastrointestinal pathogens.

With their passage into the small intestine, organisms will encounter a less acidic environment (pH 4 to 6) but one that includes the presence of fermentation end products (weak acids) produced by the normal intestinal flora. Although the pH of the intestinal contents is less acidic than that of the stomach, the presence of weak acids will increase acid stress to potentially lethal levels for enteric bacteria, such as *E. coli* and *Salmonella typhimurium* (31). Nevertheless, these pathogens will survive gastrointestinal acidity and ultimately cause disease (15). All of the available evidence suggests that organisms with inducible mechanisms of acid resistance will be better equipped to survive these acid challenges and cause disease.

In earlier studies with *E. coli*, we identified three distinct low-pH-induced acid survival systems (25). One system is expressed in oxidatively metabolizing bacteria grown in complex

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TABLE 1. *E. coli* strains used in this study

Strain	Relevant genotype	Source (reference)[a]
ATCC 43895 (EK233)	*E. coli* O157:H7 (ATCC 43895)	ATCC
EK227	*E. coli* K-12 wild type	A. Matin (27)
EF285	*E. coli* K-12 *rpoS*::Tn*10*	This study
EF302	*E. coli* O157:H7; patient 1	USAMC
EF303	*E. coli* O157:H7; patient 2	USAMC
EF304	*E. coli* O157:H7; patient 3	USAMC
EF305	*E. coli* O157:H7; patient 4	USAMC
EF306	*E. coli* O157:H7; patient 5	USAMC
EF307	*E. coli* O157:H7; patient 6	USAMC
EF308	*E. coli* O157:H7; patient 7	USAMC
EF309	*E. coli* O157:H7; patient 8	USAMC
EF310	*E. coli* O157:H7; patient 9	USAMC
EF311	*E. coli* O157:H7; patient 10	USAMC
EF312	*E. coli*, commensal, isolated 20 Jan 1996, person A	USACOM
EF313	*E. coli*, commensal, isolated 20 Jan 1996, person B	USACOM
EF314	*E. coli*, commensal, isolated 20 Jan 1996, person C	USACOM
MC4100 (EK220)	F^- *araD139*(*argF-lac*)*U169 rpsL150 relA1 flb-5301 deoC1 ptsF24 rbsR*	G. Bennett (3)
GNB7145 Km (EK198)	MC4100 *adi*::Mud1 1734 (Km^r Lac^+)	G. Bennett (3)
MC4100/pKS015 (EK222)	*adiY* cloned into $pEMBL8^+$	G. Bennett (34)
MDA-4100 (EK221)	MC4100 *cysB trpB*::Tn*10* (60% homology to *cysB*)	G. Bennett (32)

[a] ATCC, American Type Culture Collection; USAMC, University of South Alabama Medical Center; USACOM, University of South Alabama College of Medicine.

media but will protect cells in minimal medium to pH 2.5. This system is not apparent in fermentatively metabolizing cells (cells grown in complex medium containing glucose). However, two other systems of acid survival become evident under these conditions. These two systems will also protect against pH 2.5 in minimal medium but only if that medium is supplemented with arginine (arginine-dependent acid resistance; encoded by *adi*) or glutamate (glutamate-dependent acid resistance).

A regulatory gene, *rpoS*, is also involved in acid resistance. RpoS is an alternate sigma factor (σ^s) involved in regulating the expression of a variety of stress response genes (19, 22, 27, 29). In *S. typhimurium*, RpoS is an acid shock gene that is important for the induction of the acid tolerance response (23). In addition, studies with *E. coli* have implicated RpoS in acid resistance, although the specific systems examined in this study were not tested at that time (17, 33).

All of the acid resistance systems described above were identified in what were essentially laboratory strains of *E. coli*. The goal of the present study was to determine survival characteristics of enterohemorrhagic *E. coli*: during acid stress compared with the survival characteristics of freshly isolated commensal isolates and established laboratory strains. This was done to determine whether the systems identified in the earlier study were uniformly present and whether the enterohemorrhagic strains exhibited superior acid resistance. Parameters examined included absolute pH, the presence of weak acids, and the persistence of acid resistance during cold storage. In addition, the roles of *rpoS* and regulators of *adi* were examined for effects on specific acid resistance systems.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The *E. coli* strains used in this study are listed in Table 1. Strains EF302 to EF311 are *E. coli* O157:H7 strains isolated from 10 patients with bloody diarrhea. They represent separate incidents with no known common source of infection. The minimal medium used was E medium containing 0.4% glucose (EG medium) (37). E medium is composed of 73 mM K_2HPO_4, 17 mM $NaNH_4HPO_4$, 0.8 mM $MgSO_4$, and 10 mM citrate. The complex medium used was Luria-Bertani (LB) broth composed of (per liter) 10 g of Bacto-Tryptone, 5 g of Bacto-Yeast Extract, and 5 g of NaCl (28). LB broth, where indicated, was buffered to 100 mM with either MOPS (morpholinepropanesulfonic acid [pH 8.0]) or MES (morpholineethanesulfonic acid [pH 5.0]). Cultures were grown in 3 ml of media in 13- by 100-mm test tubes at a 45° angle and at 37°C, with shaking at 240 rpm. In all cases, the medium pH was adjusted with HCl and the experiments were conducted at 37°C with preheated media.

Acid resistance assays. The oxidative acid resistance system was assayed after overnight (18 to 20 h) growth in LB broth buffered to pH 5.0 with 100 mM MES (LB-MES). The overnight culture was diluted 1:1,000 into EG medium at pH 2.5 or 2.0 (acid challenge, 37°C). The initial cell density in all cases was 1×10^6 to 3×10^6 CFU/ml. The control for this survival system was a pH 2.0 or 2.5 EG medium acid challenge of cells grown overnight in LB broth containing 0.4% glucose (LBG medium), a condition which represses the oxidative system. The final pH of LBG medium following growth is approximately pH 5. Viable counts were determined immediately before acid challenge (time zero) and at various times after acid challenge. Cells were diluted in pH 7 EG medium, plated to LB agar, and incubated at 37°C for 24 h before being counted. Values given are averages of the results of two to three experiments reproducible to within 50%.

The assay for the arginine-dependent system involved overnight growth of test cells in LBG medium (pH 5.0) followed by a 1:1,000 dilution into EG medium (pH 2.5 or 2.0) containing 0.6 mM arginine (37°C). The glutamate-dependent system was tested in a manner similar to that used for the arginine system. In this case, the 1:1,000 dilution was made into acidified (HCl) EG medium supplemented with 5.7 mM glutamate. The control for these survival systems involved an acid challenge (pH 2.0 or 2.5) in unsupplemented EG medium.

The persistence of acid resistance during cold storage was assayed by adapting the organisms for the various systems described above and then neutralizing the broth cultures to pH 7 with NaOH and statically holding them at 4°C for extended periods (0, 7, 14, 21, and 28 days). At each time point, samples from each culture were diluted 1:1,000 into the following media at pH 2.5: EG medium, EG medium plus arginine, and EG medium plus glutamate. In addition, dilutions were made into LBG medium at pH 2.0.

Resistance to weak acids. The contributions of the three acid survival systems to resistance to weak acids were examined by adapting cultures of *E. coli* at 37°C for the various systems described above and then diluting them 1:1,000 into EG medium, EG medium plus arginine, EG medium plus glutamate, and LBG medium (pH 4.0 or 4.4) containing 20 mM benzoic acid or containing a volatile fatty acid (VFA) cocktail consisting of 90 mM acetic acid, 30 mM butyric acid, and 37 mM propionic acid. The acids were prepared, adjusted to pH 4.4 (with HCl), filter sterilized (0.4-μm-pore-size filter), and combined before being added to sterile media. Viable counts (see above) were determined at time zero of acid challenge and at timed intervals thereafter.

RESULTS

Comparison of acid survival in laboratory, commensal, and enterohemorrhagic strains of *E. coli*. The low ID thought to be associated with enterohemorrhagic strains of *E. coli* could involve extraordinary levels of acid resistance (4, 18). This theory was tested by acid challenging a series of laboratory, commensal, and enterohemorrhagic strains of *E. coli* (25). MC4100 is a common laboratory strain used for molecular studies. EK227 is a wild-type K-12 strain obtained from the culture collection at

TABLE 2. Acid resistance systems in commensal, enterohemorrhagic, and laboratory strains of *E. coli*

Strain	% Survival in[a]:							
	Oxidative system (pH 2.5)[b]		Glutamate/arginine/LBG systems (pH 2.0)[c]					
				Glutamate		Arginine	LBG	
	Control	Test	Control	2 h	6 h		3 h	6 h
Laboratory								
EK220	<0.01	65	<0.01	43	1	0.02	0.04	<0.01
EK227	<0.01	21	<0.01	83	70	1	65	40
Commensal								
EF312	<0.01	12	<0.01	75	69	<0.01	88	47
EF313	0.01	27	<0.01	87	81	<0.01	91	90
EF314	4.1	48	<0.01	80	65	<0.01	66	53
Enterohemorrhagic (O157:H7)								
EK233	0.04	29	<0.01	60	20	2	72	60
EF302	0.06	7	<0.01	46	31	9	95	33
EF303	0.03	15	<0.01	23	15	0.5	33	6
EF304	<0.01	11	<0.01	16	7	0.1	83	53
EF305	<0.01	25	<0.01	44	15	16	98	38
EF306	<0.01	0.2	<0.01	90	14	16	36	7
EF307	<0.01	45	<0.01	74	36	2	53	41
EF308	<0.01	0.2	<0.01	85	22	7	86	63
EF309	<0.01	1	<0.01	88	68	0.4	91	81
EF310	0.01	15	<0.01	60	55	1.5	72	60
EF311	0.02	3	<0.01	91	19	1	49	10

[a] Percent survival is calculated as 100 × the number of CFU per milliliter remaining after the acid treatment divided by the initial CFU per milliliter at time zero. Initial cell densities ranged from 1×10^6 to 3×10^6 CFU/ml. Experiments were repeated two or three times. Variations were within 50% of the stated value.

[b] Controls (acid sensitive) involved growth overnight in EG medium (pH 7) followed by dilution to 1×10^6 to 3×10^6 CFU/ml (1/1,000 dilution) into EG medium (pH 2.5). The acid challenge was for 2 h at 37°C. The test for the oxidative system involved overnight growth in LB-MES medium (pH 5.5) followed by dilution to 1×10^6 to 3×10^6 CFU/ml in EG medium (pH 2.5). Survival (viable counts) was measured after 2 h at 37°C.

[c] Controls (acid sensitive) involved overnight growth in LBG medium followed by dilution to 1×10^6 to 3×10^6 CFU/ml in EG medium (pH 2.0) for 2 h at 37°C. The glutamate and arginine systems also required overnight LBG cultures but were diluted to 1×10^6 to 3×10^6 CFU/ml in EG medium (pH 2.0) containing 5.7 mM glutamate and 0.6 mM arginine, respectively. LBG survival involved dilution into LBG medium (pH 2.0) for 3 and 6 h at 37°C.

Stanford University. EF312, EF313, and EF314 were isolated for this study from laboratory personnel and are used here as commensal strains. The enterohemorrhagic strains included EK233 (ATCC 43895) and independent patient isolates EF302 through EF311 (Table 1). The first set of experiments involved a series of acid challenges at pH 2.5. All strains exhibited similar levels of glutamate-dependent and arginine-dependent acid resistance after 2 h at a challenge pH of 2.5. Survival via the glutamate system was between 80 and 100%, whereas survival attributed to the arginine system was between 10 and 50% (data not shown). Acid resistance (pH 2.5) mediated by the oxidative system did vary depending upon the strain (Table 2). Most strains survived the 2-h exposure at a level of 10 to 70%. However, three enterohemorrhagic strains, EF306, EF308, and EF309, were clearly defective in this system, with a percent survival of no more than 1%.

The same systems were then tested during a pH 2.0 challenge (Table 2). The oxidative system was ineffective for all strains (data not shown). In contrast, all strains proved equally effective at glutamate-dependent acid resistance over the 2-h challenge period. A 6-h challenge period, however, exposed MC4100 as being significantly less resistant in the glutamate assay than either the commensal or enterohemorrhagic strains. The arginine-dependent system was generally less effective than the glutamate-dependent system but did provide acid resistance at pH 2. Arginine failed to protect MC4100 or the commensal isolates but provided fair to good acid resistance for K-12 and the series of enterohemorrhagic strains. Acid resistance was also tested in LBG medium at pH 2 for 3 and 6 h. Once again, MC4100 proved inferior to the other strains in this assay for acid resistance, but no apparent differences were noted between commensal and enterohemorrhagic strains. In sum, all strains possessed the oxidative, glutamate, and arginine systems of acid resistance, although the effectiveness of each system was strain dependent. The strain exhibiting the least acid resistance proved to be the commonly used laboratory strain MC4100.

Effect of weak acids on survival. Weak acids are a frequent threat to bacterial survival in some foods and in the gastrointestinal tract. It was considered that the acid resistance mechanisms effective against extremely acidic pH might also help combat the deleterious effects of weak acids. Benzoic acid is commonly used to test the effects of weak acid on cell viability, and so we examined this compound first. The effect of benzoic acid on the survival of unadapted *E. coli* was significant. Exposure to EG medium (pH 4.0) containing 20 mM benzoate reduced the number of organisms adapted in LBG (pH 5.0) and LB-MES (pH 5.0) media to undetectable levels within 90 min. (Table 3, lines 2 and 6). Organisms grown in LB-MOPS medium (pH 8.0) were even more susceptible to this treatment (lines 9 through 11). The oxidative system (compare lines 2 and 9) was only modestly effective in protecting against benzoate stress, but the arginine-dependent system (compare lines 6 and 7) was ineffective. Of the three known systems, the glutamate-dependent system (compare line 2 and 4 and lines 6 and 8) was the most effective. A level of acid resistance similar to that in the glutamate system was observed when the organisms were challenged in LBG medium (pH 4) with benzoate (line 5).

TABLE 3. Effect of benzoic acid on *E. coli* K-12 acid survival

Adaptation[a]	pH 4.0 challenge[b]	% Survival[c] after challenge time of:		
		30 min	60 min	90 min
1 LB-MES (pH 5.0)	EG	111	87.4	77
2 LB-MES (pH 5.0)	EGBA	38	1.0	<0.002
3 LB-MES (pH 5.0)	EGBA + Arg	46	1.9	<0.002
4 LB-MES (pH 5.0)	EGBA + Glut	78	40.7	14.8
5 LB-MES (pH 5.0)	LBG + BA	87	46	11
6 LBG (pH 5.0)	EGBA	41	0.04	<0.006
7 LBG (pH 5.0)	EGBA + Arg	57	0.38	<0.01
8 LBG (pH 5.0)	EGBA + Glut	79	3.45	0.008
9 LB-MOPS (pH 8.0)	EGBA	1.0	<0.002	<0.002
10 LB-MOPS (pH 8.0)	EGBA + Arg	2.7	<0.002	<0.002
11 LB-MOPS (pH 8.0)	EGBA + Glut	52	2.8	<0.002

[a] Adaptation during overnight growth in the media listed.
[b] BA, 20 mM benzoic acid; Arg, arginine; Glut, glutamine.
[c] Experiments were performed two or three times, with variations less than 50% of the stated value. Initial cell densities were between 1×10^6 and 3×10^6 CFU/ml.

The next test involved the ability of these systems to resist a VFA cocktail containing a ratio of acetic, butyric, and propionic acids similar to what has been measured in human intestines (10) (Fig. 1). MC4100 (EK220), K-12 (EK227), a commensal strain (EK314), and three enterohemorrhagic strains (EK233, EF304, and EF307) were compared in terms of their resistance to VFAs at pH 4.4. The results presented in Fig. 1 illustrate that the arginine-dependent and glutamate-dependent acid resistance systems were very effective at combating VFA stress for at least 7 h. Oxidative resistance to weak acids was not apparent at 7 h of exposure, but some protection was observed at 3 h (data not shown).

Persistence of acid resistance during cold storage. The source of O157:H7 contamination in food may occur days or weeks before consumption. During this period, the tainted food is usually refrigerated, halting the growth of the organism. It is unlikely that acid resistance will develop under these circumstances. However, *E. coli* acid resistance probably develops in the intestinal tracts of cattle to help the organism survive intestinal VFAs. This acid resistance might persist during periods of cold storage following slaughter and processing. Upon ingestion days or weeks after the initial contamination, the acid-resistant organisms will still survive the gastric acidity barrier. To test this hypothesis in vitro, selected strains of *E. coli* were allowed to develop acid resistance (LBG and LB-MES medium grown), afterwhich the medium pH was neutralized and the cultures were placed at 4°C. The refrigerated cells were tested periodically over 28 days for acid resistance at pH 2.5 (oxidative, arginine, and glutamate systems) and pH 2.0 (LBG medium) at 37°C. As a control, unadapted LB-MOPS- or EG medium (pH 7)-grown cells were tested in the same manner. The control cultures remained acid sensitive throughout the experiment (data not shown). The data presented in Fig. 2 illustrate the persistence of acid resistance during cold storage of selected laboratory and enterohemorrhagic strains. The glutamate-dependent (Fig. 2B) and arginine-dependent (Fig. 2C) systems of acid resistance both proved to be remark-

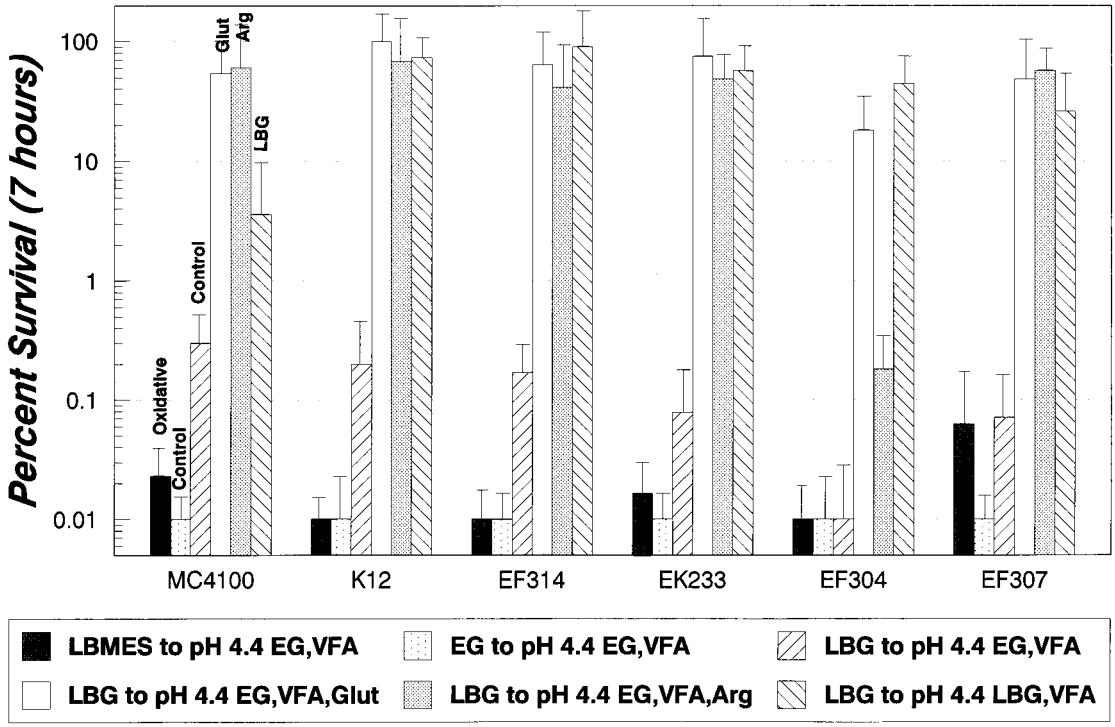

FIG. 1. Resistance of *E. coli* to VFA. *E. coli* strains were tested for their abilities to resist the lethal effects of VFAs at pH 4.4. In the oxidative system, cells were adapted by overnight growth in LB-MES medium and challenged (1×10^6 to 3×10^6 CFU/ml) in EG medium (pH 4.4) containing the following VFA cocktail: 90 mM acetic acid, 30 mM butyric acid, and 37 mM propionic acid. The control for the oxidative system involved cells grown overnight in EG medium that were challenged in the pH 4.4 EG VFA medium. The control for the glutamate, arginine, and LBG medium tests involved cells grown overnight in LBG medium that were challenged in the pH 4.4 EG VFA medium. The glutamate and arginine tests required cells grown overnight in LBG medium challenged to pH 4.4 EG VFA medium supplemented with 5.7 mM glutamate or 0.6 mM arginine, respectively. Survival was measured after 7 h. Values given are means and standard deviations.

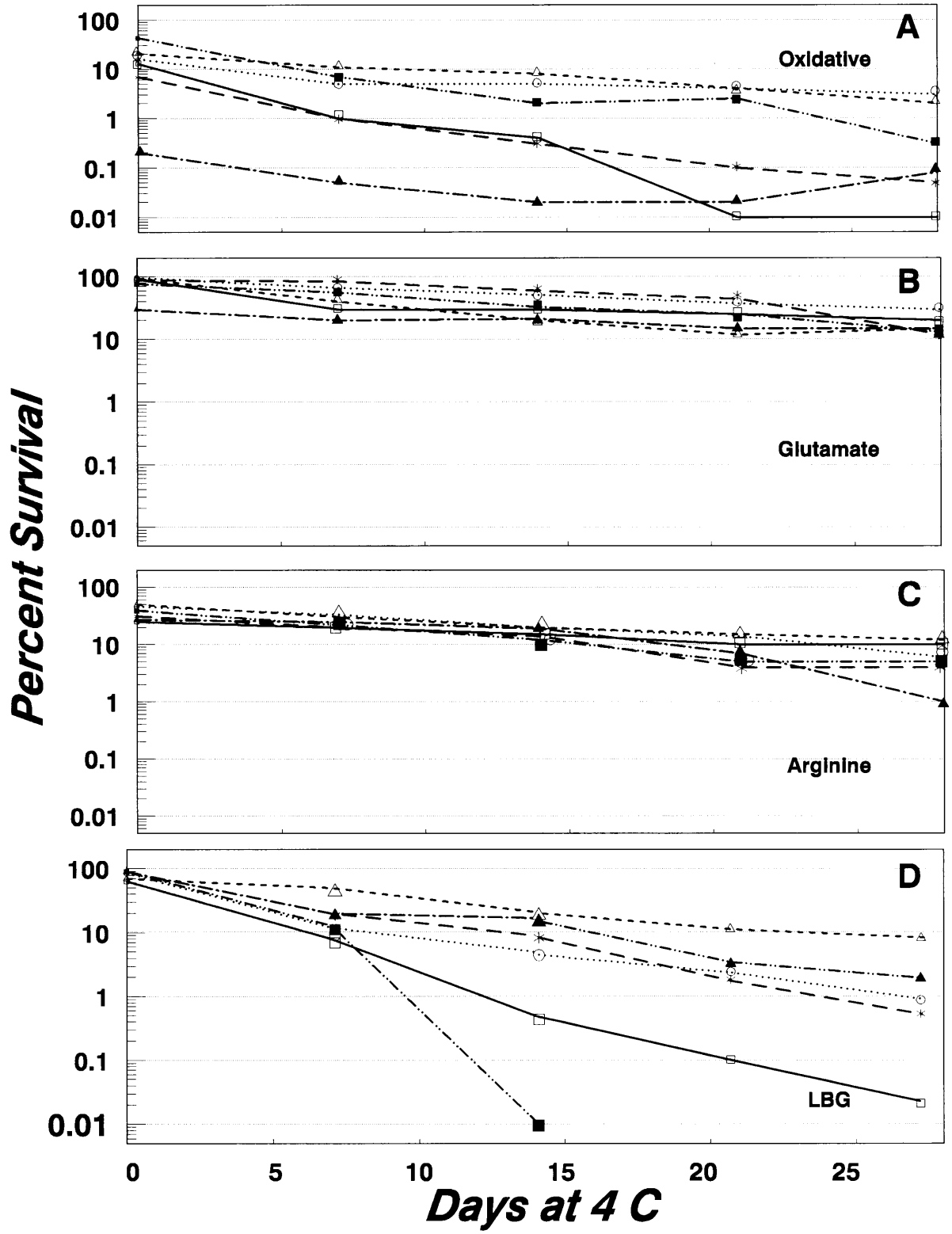

FIG. 2. Persistance of acid resistance systems in cold storage. Strains tested include MC4100 (EK220) (□), K-12 (EK227) (△), EK233 (○), EF302 (*), EF307 (■), and EF308 (▲). (A to C) The oxidative system (A), the glutamate system (B), and the arginine system (C) were measured as described in Table 2, footnotes *b* and *c*. Survival was measured after 2 h at pH 2.5. (D) Survival after 2 h at pH 2.0 in LBG. Values given represent the mean of two experiments. The variation was within 50% of the value given.

TABLE 4. Effect of *rpoS* on survival in acid

pH adaptation	pH 2.5 challenge	% Survival[a] of strain:	
		EK227 (*rpoS*$^+$)	EF285 (*rpoS*)
LBG	EG	<0.001	<0.01
LBG	EG-Arg	19	1.47
LBG	EG-Glut	48	0.38
LB-MES	EG	17	<0.01

[a] Survival after 2 h at pH 2.5. Other conditions were the same as given in Table 2.

ably stable even after 28 days of cold storage. It is important to note that the viable cells representing 100% survival at time zero did not decrease in number during refrigeration. The oxidative system also persisted in most strains (Fig. 2A). The clear exception was the laboratory strain MC4100, which began very acid resistant but steadily became acid sensitive after 2 weeks at 4°C. Even when tested at pH 2.0, the acid resistance of most strains persisted for weeks. The exceptions were MC4100 and EF307, one of the enterohemorrhagic strains tested. However, even those strains developed acid resistance that was stable for at least 1 week of cold storage. The results clearly indicate that acid resistance can persist for long periods during refrigeration, leaving the organism prepared to survive gastric acidity.

Effect of *rpoS* on acid survival. A study to explore whether σ^s contributed to one or more of the acid resistance systems described previously by Lin et al. (25) was undertaken. The data presented in Table 4 show that an *rpoS* mutation eliminated the oxidative system and reduced the arginine- and glutamate-dependent systems 20- to 100-fold after a 2-h challenge at pH 2.5. The results indicate that components of the arginine and glutamate systems, in addition to arginine decarboxylase and the probable glutamate decarboxylase, are required for acid resistance. Neither of these systems is known to be regulated by RpoS (4a, 24a). It is reasonable to predict that the *adi* and glutamate-dependent systems help maintain a less acidic intracellular pH in extremely acidic environments and that the RpoS-dependent systems minimize actual damage to macromolecules.

Effects of *cysB* and *adiY* mutations on the arginine-dependent acid resistance system. The arginine decarboxylase gene (*adi*) is induced during anaerobic growth at acidic pH in rich media (3). The *cysB* and *adiY* regulatory genes play a role in the regulation of *adi* (32, 34). Because the *adi* gene is required for arginine-dependent acid resistance, mutations in the *adiY* and *cysB* genes were tested for predictable effects on arginine-dependent acid resistance. CysB acts as a positive regulator of *adi*, so that one would expect *cysB* mutants to be acid sensitive. The data presented in Table 5 support that prediction. The LBG medium-grown *E. coli cysB* mutant did not survive a 2-h exposure to EG medium (pH 2.5) plus arginine. The effect of *cysB* on acid resistance was specific to the arginine-dependent system; the *cysB* mutation did not affect the glutamate-dependent or oxidative system.

TABLE 5. Effect of *adi* regulatory genes on survival in acid

pH 5.0 Adaptation	pH 2.5 challenge[a]	% Survival of strain:			
		EK220	EK198 (*adi*)	EK221 (*cysB*)	EK222 (pKS015) (*adiY*$^+$)
LBG	EG	<0.01	<0.01	<0.01	<0.01
LBG	EG-Arg	11.6	<0.01	<0.01	73.7
LBG	EG-Glut	34.9	39	29	39
LB-MES	EG	19.2	20	13	18

[a] Cells were challenged for 2 h at pH 2.5. Other conditions were the same as given in Table 2.

A second gene involved in *adi* regulation is *adiY*. The *adiY*$^+$ gene, when cloned on a multicopy plasmid, stimulates the expression of *adi* (34). Consequently, one would expect a strain carrying this clone to have elevated arginine-dependent acid resistance. The data in Table 5 confirmed that prediction and, again, indicated that the effect was specific to arginine-dependent acid resistance.

DISCUSSION

The ID of enterohemorrhagic strains of *E. coli* is apparently very low (18). Pathogens like *S. typhimurium* typically require 10^5 cells for human infection, whereas only a few cells of enterohemorrhagic *E. coli* appear necessary to produce illness. One potential explanation for the low ID of enterohemorrhagic *E. coli* is their ability to survive the acidic conditions of the stomach (2, 4, 9). Indeed, even the ability of commensal strains of *E. coli* to persist in the intestine may be attributable, in part, to their ability to combat the effects of weak-acid stress prevelent in that environment. We previously described three mechanisms of acid resistance in laboratory strains of *E. coli*. These systems could contribute to survival in gastric acid and thus could contribute to the low ID of enterohemorrhagic *E. coli* (25) and its survival in acidic foods (5, 16, 24). The acid resistance mechanisms include oxidative, glutamate-dependent, and arginine-dependent systems. The data presented in this work reveal that the acid resistance of enterohemorrhagic and commensal strains of *E. coli* is superior to that of a common laboratory strain (MC4100) when challenged in LBG broth at pH 2.0. Aside from the strain differences observed for individual systems, the overall pH 2.0 resistances of commensal and enterohemorrhagic strains were similar.

Upon entering the intestine, *E. coli* will encounter weak acids formed as a result of fermentation by the normal intestinal flora. These weak acids help limit the growth of *E. coli*. Thus, mechanisms that confer resistance to weak acids should contribute to *E. coli* survival and colonization in the intestine. This study has confirmed that the presence of weak acids hastened the death of organisms during an acid challenge. However, all strains of *E. coli* tested could effectively use the glutamate-dependent acid resistance system to ward off the lethal effects of weak acids. The oxidative and arginine-dependent systems were not very effective at combating benzoate stress but were effective against a VFA cocktail that mimics the intestinal composition. The results suggest that these acid resistance systems could significantly contribute to the survival of *E. coli* in acidic foods and in the intestine.

Stress proteins enhance the ability of organisms to withstand a number of chemical and physical challenges, e.g., H_2O_2, acid and alkaline pH, heat, and osmolarity. Many of these stress proteins are regulated by the alternate sigma factor, σ^s (26). In a previous study with *S. typhimurium*, the RpoS protein was shown to be induced by acid shock and was required for a sustained acid tolerance response (23). The present study found that an *E. coli rpoS* mutant was completely devoid of the oxidative acid resistance system but was only partially deficient in the arginine-dependent and glutamate-dependent acid resistance systems. Because production of arginine decarboxylase (required for arginine-dependent acid resistance) and glu-

tamate decarboxylase (the enzyme most probably required for glutamate-dependent acid resistance) appears unaffected by *rpoS* mutations, the data suggest that σ^s-dependent gene products collaborate with the decarboxylases in affording maximum acid resistance. This situation is similar to what we have recently discovered for the acid tolerance response of *S. typhimurium*, in which lysine decarboxylase and RpoS-dependent acid shock proteins collaborate for maximum acid tolerance (29a).

One can hypothesize that the gastrointestinal environment of cattle will induce one or more of the acid resistance systems described in this work. Thus, upon ingestion of contaminated food, the acid-adapted organisms are able to survive the gastric acid defense of human hosts and colonize host intestines through competition with commensal organisms. However, there is an interval between the slaughter of the animals and actual ingestion. Could the organisms remain acid resistant over this period even if they are not growing? An important finding from this study is that once activated, the acid resistance systems will remain active over prolonged periods of cold storage. Consequently, enterohemorrhagic *E. coli* contaminating ground beef or other foods will retain acid resistance even when the food is properly refrigerated. These organisms can efficiently survive subsequent encounters with gastric acidity.

Few studies have directly examined the effects of acid pH, VFAs, temperature, and storage on the acid resistance of pathogenic *E. coli*. The studies that have been performed examined this relationship in a very complicated food environment without first understanding the molecular basis for acid resistance (1, 2, 5, 16, 24). Although those studies have provided important information, none considered whether exposure to different growth conditions might influence the ultimate level of acid resistance. Also, they did not address the possibility that multiple systems were involved (4). Understanding the molecular aspects of acid resistance will potentially expose new strategies capable of diminishing the virulence of pathogens in contaminated foods.

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