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Acid Resistance Systems Required for Survival of *Escherichia coli* O157:H7 in the Bovine Gastrointestinal Tract and in Apple Cider Are Different

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***Escherichia coli* O157:H7 is a highly acid-resistant food-borne pathogen that survives in the bovine and human gastrointestinal tracts and in acidic foods such as apple cider. This property is thought to contribute to the low infectious dose of the organism. Three acid resistance (AR) systems are expressed in stationary-phase cells. AR system 1 is σ^S dependent, while AR systems 2 and 3 are glutamate and arginine dependent, respectively. In this study, we sought to determine which AR systems are important for survival in acidic foods and which are required for survival in the bovine intestinal tract. Wild-type and mutant *E. coli* O157:H7 strains deficient in AR system 1, 2, or 3 were challenged with apple cider and inoculated into calves. Wild-type cells, adapted at pH 5.5 in the absence of glucose (AR system 1 induced), survived well in apple cider. Conversely, the mutant deficient in AR system 1, shown previously to survive poorly in calves, was susceptible to apple cider (pH 3.5), and this sensitivity was shown to be caused by low pH. Interestingly, the AR system 2-deficient mutant survived in apple cider at high levels, but its shedding from calves was significantly decreased compared to that of wild-type cells. AR system 3-deficient cells survived well in both apple cider and calves. Taken together, these results indicate that *E. coli* O157:H7 utilizes different acid resistance systems based on the type of acidic environment encountered.**

A particularly virulent form of *Escherichia coli* designated serotype O157:H7 has emerged as an important pathogen over the last two decades (20, 26). Numerous outbreaks of *E. coli* O157:H7-related disease have been recorded since 1982, the largest of which occurred in Japan in 1997 (33, 50). As a member of the enterohemorrhagic group of *E. coli*, this organism causes a variety of diseases, including hemorrhagic colitis, hemolytic uremic syndrome, and thrombotic thrombocytopenic purpura (8, 12, 32, 35, 41).

E. coli O157:H7 is a major food-borne pathogen that threatens many aspects of the food industry (36). The major reservoir of *E. coli* O157:H7 generally is perceived to be the bovine gastrointestinal tract, thus providing ample opportunity for contamination of ground beef products, a common source of infection (21, 40, 43, 49). However, human infections also have been associated with foods other than hamburger. These include acidic foods, such as apple cider and salami, although the source of infection can usually be traced to bovine fecal contamination (1, 5, 23).

Contamination of recreational and drinking water with infected feces has caused waterborne outbreaks of *E. coli* O157:H7 disease, too, although the specific source of the fecal contamination is sometimes unclear. For instance, a well-publicized outbreak at a county fair in 1999 is thought to have resulted from contaminated well water (10). However, it is debatable whether this outbreak resulted from contamination of well water with *E. coli* O157:H7-infected bovine or human feces or a combination of both (6).

E. coli O157:H7 is thought to have a very low infectious dose (<700 organisms) (19, 46) due, in part, to very efficient mechanisms of stress resistance. To colonize or cause disease, an infecting microorganism must breech an impressive array of environmental insults imposed by a host (e.g., acid pH, bile salts, high osmolarity, defensins). Survival is achieved through the induction of defensive microbial stress response systems. Because of its low infectious dose, an extremely important component of *E. coli* O157:H7 pathogenesis is thought to be its outstanding ability to survive in extremely acidic environments, such as the stomach or in areas of the intestine that contain high levels of organic acids (15, 38). A variety of published reports have documented this acid resistance (AR) (2, 4, 7, 16, 25, 27, 42).

Gorden and Small (18) and Small et al. (44), using laboratory strains of *E. coli*, first reported the presence of low-pH-induced, stationary-phase AR that protects cells exposed to extremely low pH (pH 2.5). Their seminal work in this area revealed that there are σ^S dependent and independent systems of AR. Our investigations have determined that AR in *E. coli* is comprised of three efficient systems. Two of these systems are present in *Shigella flexneri*, but none of these systems occur in *Salmonella enterica* serovar Typhimurium (28). Expression of AR system 1 requires growth to stationary phase in acid-

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

Strain	Serotype or genotype[a]	Source or reference
ATCC 43895	O157:H7	48
EK274	ATCC 43895 Na^r Rf^r	39
FRIK 816-3	ATCC 43895 *rpoS*::pRR10 (Ap)	11
EK275	FRIK 816-3 Na^r Rf^r	39
EF484	43895 *gadC*::pRR10 (Ap) Na^r Rf^r	This work
EF489	43895 *adiA*::pRR10 (Ap) Na^r Rf^r	This work
EF501	43895 *gadA*::pRR10 (Ap) Na^r Rf^r	39

[a] Na, nalidixic acid; Rf, rifampin; Ap, ampicillin.

buffered complex media (Luria-Bertani [LB]) and successfully protects cells to pH 2.5 in amino-acid-free minimal medium. Glucose represses induction of this AR mechanism and exposes two other acid survival systems that require the addition of an amino acid during pH 2.5 acid challenge (28). One system is glutamate dependent (AR system 2), while the other requires arginine (AR system 3). We have shown that the alternate RNA polymerase sigma factor σ^S, important to stationary-phase physiology, is not required for the glutamate- or arginine-dependent acid survival systems but is essential for expression of AR system 1 (29). Essential components of AR system 2 include glutamate decarboxylase (GAD; two isoforms), encoded by *gadA* and *gadB*, and a putative glutamate: γ-amino butyric acid antiporter, encoded by *gadC* (9, 22, 24). The product of the *adiA* locus, arginine decarboxylase (3), was proven to be responsible for system 3 arginine-based acid survival (28). These three systems account for the pH 2.5 AR phenotype originally described by Gorden and Small for *E. coli* K-12 (18).

E. coli O157:H7 also possesses these three systems of AR (9, 28, 29). While levels of AR in *E. coli* O157:H7 strains generally are more robust than many laboratory strains of *E. coli*, the levels are equivalent to what can be observed in commensal isolates. In a previous study it was shown that, as with K-12, the *rpoS* gene of *E. coli* O157:H7 also controls expression of AR system 1 (39). In addition, it was shown that a mutant of *E. coli* O157:H7 lacking *rpoS* was shed in lower numbers in experimentally infected mice and calves and that this reduced shedding, at least in mice, was due to decreased AR (39).

In the present work we continued the examination of AR in *E. coli* O157:H7 by selectively removing the three determinants of AR to ask which systems are important for survival in acidic foods and which are required in the bovine gastrointestinal tract. Surprisingly, it was found that different AR systems are needed for survival in these two environments.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* strains used in this study are listed in Table 1. *E. coli* O157:H7 strain ATCC 43895 was used as the parent, wild-type strain throughout the study, and all of the mutants described below were derived from this strain. This strain was originally isolated from contaminated ground beef in a 1982 outbreak of hemorrhagic colitis (strain EDL933) (48), and it has been sequenced (37). Construction of an *rpoS* derivative of this strain involved insertional inactivation with pRR10, which carries the gene encoding β-lactamase (FRIK 816-3 [11]). Mutants of ATCC 43895 disrupted in *adiA*, *gadA*, or *gadC* were constructed in the same way (9). The disruption of these genes does not have polar effects on other genes, and therefore the observed phenotypes are specific to these mutations. Genetic evidence for this has been revealed in *E. coli* K-12, whose operon structure has been shown by genome sequencing to be equivalent to that of *E. coli* O157:H7. This evidence includes the following: (i) the *gadA* mutant is not acid sensitive (AS), indicating that no polar effect affecting AR occurred on downstream genes (39); (ii) Northern blot analysis indicates that the *gadC* gene is contained within the bicistronic operon *gadBC* that forms a transcript of specified length (30, 31)—no genes are found downstream from *gadC* in the operon, and therefore no polar effects could have occurred; (iii) *adiA* is part of the *adiAY* operon as shown by Northern blot analysis (17). AdiY is a putative positive regulator of *adiA*, but an *adiY* deletion mutant showed no alteration in AR, indicating that the acid sensitivity of the *adiA* mutant described in this work was not due to polar effects on the downstream *adiY* gene (17).

Spontaneous mutants of the *adiA*, *gadA*, or *gadC* strains resistant to nalidixic acid and rifampin were generated to aid in recovery from calf fecal specimens. These mutants had survival and growth characteristics similar to those of the parent strain EK274, which was also nalidixic acid and rifampin resistant. Ampicillin (50 μg/ml) was added to cultures of the *rpoS*, *gadA*, *gadC*, and *adiA* mutants to maintain pRR10.

AR assays. Cells were grown overnight in one of several media, including LBG (LB plus 0.4% glucose), BHIG (brain-heart infusion [BHI] plus 0.4% glucose), buffered LB (either 100 mM morpholinepropanesulfonic acid [pH 8] or 100 mM morpholineethanesulfonic acid [MES; pH 5.5]) (34), and minimal E salts glucose (EG) (47). Cultures were grown in 3 ml of the appropriate medium in 13-mm-diameter test tubes with shaking (240 rpm) at 37°C to stationary phase (22 h, 1×10^9 to 5×10^9 CFU/ml). The oxidative system was tested with cells grown overnight in pH 5.5 LB or BHI-buffered medium followed by 1:1,000 dilution into prewarmed (37°C) pH 2.5 EG (adjusted with HCl). The glutamate and arginine systems were tested by using stationary-phase cells grown in LBG or BHIG followed by 1:1,000 dilution into prewarmed pH 2.5 EG supplemented with 1.5 mM glutamate or 0.6 mM arginine, respectively. Viable cell counts were determined at 0, 2, and 4 h post-acid challenge by diluting cells in LB, plating cells onto LB agar, and incubating them for 20 h at 37°C. Results presented are representative of triplicate experiments. Survival in acidic (pH 3.5) apple cider (contains pulp, single lot, pasteurized; Thrifty Maid) was assayed similarly. Cultures were grown in appropriate media to stationary phase and diluted 1:1,000 into cider prewarmed to 25°C, and viable counts were determined as described above.

***E. coli* O157:H7 shedding studies.** Six- to eight-week-old weaned dairy calves were acclimated for 2 weeks before inoculation, the first week outdoors followed by a second week in a climate-controlled BL-2 containment facility. Each calf was fecal cultured three times during acclimation for *E. coli* O157:H7 to assure that only *E. coli* O157:H7-negative calves were inoculated in the study. Pairs of calves were housed together on pine shavings, given water ad libitum, and fed grain and hay twice daily. The calves were euthanized with sodium pentobarbital and incinerated at the conclusion of each experiment. Protocols approved by the Auburn University Institutional Animal Care and Use Committee were followed to ensure the welfare of the calves, and strict containment precautions were followed to prevent the release of *E. coli* O157:H7 and to guarantee the safety of laboratory personnel.

Bacterial strains for the calf inoculations were grown in BHI broth (pH 5.5) to stationary phase (22 h, 1×10^9 to 5×10^9 CFU/ml). Cell pellets were harvested by centrifugation, washed, and suspended in 0.85% NaCl. Four calves were inoculated by gastric lavage with a 50-ml inoculum of 0.85% NaCl containing 10^{10} total CFU of an equal number of EK274 and either EF484 (*gadC*) or EF489 (*adiA*), followed by 500 ml of 0.85% NaCl. In a separate experiment, 10^{10} total CFU of an equal number of stationary-phase-grown EK274 and logarithmic-phase-grown EF501 (*gadA*) (2.5 h; 1×10^8 to 5×10^8 CFU/ml) were inoculated into two calves. Conversely, 10^{10} total CFU of an equal number of stationary-phase-grown EF501 and logarithmic-phase-grown EK274 were inoculated into two calves.

Following inoculation, fecal samples were cultured daily for 16 days for enumeration of *E. coli* O157:H7. Fifty-gram specimens were collected each morning and were immediately transported to the laboratory for culture. Quantitative culture of the samples was performed by adding 1 g of feces to 9 ml of phosphate buffer followed by serial 10-fold dilution in phosphate buffer. A 0.1-ml volume of each dilution was plated in duplicate onto sorbitol-MacConkey agar (Difco, Detroit, Mich.) containing nalidixic acid (35 μg/ml), which selected for the inoculated *E. coli* O157:H7 strains, or agar containing both nalidixic acid and ampicillin (50 μg/ml), which selected for the insertion mutants. The CFU per gram of EK274 present in specimens was calculated by subtracting the combined number of nalidixic acid-resistant and ampicillin-resistant colonies from the number of nalidixic acid-resistant colonies. Serologic confirmation of *E. coli* O157:H7 suspect colonies was made by using a commercial latex agglutination kit (RIM *E. coli* O157:H7; Remel, Inc., Lenexa, Kans.).

TABLE 2. Effect of *rpoS*, *gadC*, and *adiA* on the three AR systems in *E. coli* O157:H7

Adaptation medium[a]	Induced AR system	Challenge medium (pH 2.5)	% Survival[b]			
			EK274	EK275 (*rpoS*)	EF484 (*gadC*)	EF489 (*adiA*)
LB (pH 8)	Negative control	EG	<0.005	<0.004	<0.005	<0.005
LB (pH 5.5)	AR 1 (RpoS-dependent)	EG	38	<0.005	40	10
LBG	Negative control	EG	<0.004	<0.006	<0.005	<0.007
LBG	AR 2 (Glt-dependent)	EG + Glt	45	5	<0.007	73
LBG	AR 3 (Arg-dependent)	EG + Arg	<0.004	<0.004	ND[c]	<0.006
BHIG	Negative control	EG	<0.004	<0.005	<0.004	<0.005
BHIG	AR 2 (Glt-dependent)	EG + Glt	64	20	<0.007	85
BHIG	AR 3 (Arg-dependent)	EG + Arg	2	1	10	<0.005

[a] Adaptation involved overnight growth in the medium indicated. AR 1 is completely inhibited during growth at pH 8, the condition chosen as a negative control. The negative controls for systems 2 and 3 involved adding cells with active systems to pH 2.5 media that lack substrates for the system, i.e., glutamate (Glt) and arginine (Arg).

[b] Percent survival was measured by determining the number of surviving cells after 4 h of acid treatment. Data presented are representative example from triplicate trials.

[c] ND, not determined.

Statistical methods. Data were entered into a computer spreadsheet program (Excel version 5.0; Microsoft) and analyzed by using statistical software (Statistical Analysis System [SAS] version 6.12 software; SAS Institute, Cary, N.C.). Differences between strains (wild-type versus mutant or AR versus AS) were analyzed by analysis of variance for repeated measures at a significance level of $P < 0.05$.

RESULTS

AR mutants of *E. coli* O157:H7. The *E. coli* O157:H7 parent (EK274) and derivative *rpoS* (EK275), *gadC* (EF484), and *adiA* (EF489) mutants were tested for the three AR systems previously identified (29). The data presented in Table 2 show that insertional inactivation of *rpoS* eliminated the oxidative system and partially reduced the arginine- and glutamate-dependent systems when cells were adapted in LB pH 5.5 and BHIG, respectively. These results agree with those reported earlier (39). It is important to note that, although an *rpoS* mutation lowers glutamate- and arginine-dependent AR, these systems do not have an absolute requirement for RpoS. The extent of RpoS involvement varies with growth condition. This was particularly evident, as noted previously, when adaptation of the *rpoS* mutant was made in BHIG rather than LBG. After growth in BHIG, EK275 (*rpoS*) exhibited near wild-type levels of glutamate- and arginine-dependent AR (Table 2), but, when adapted in BHI pH 5.5, it remained defective in oxidative AR (39). The *gadC* and *adiA* mutants, however, retained wild-type oxidative AR but were clearly defective in the glutamate- and arginine-dependent systems, respectively.

Mechanism of *E. coli* O157:H7 AR in apple cider. AR of *E. coli* O157:H7 is believed to contribute to the survival of this microbe in acidic foods, such as unpasteurized apple cider, that have been linked to *E. coli* O157:H7 infection (1, 5). Having assembled mutants deficient in each individual system, we were able to investigate which AR system was most important for survival in this type of environment. In initial experiments, wild-type *E. coli* O157:H7 strains were adapted for 18 h in LB MES pH 5.5, which induces AR system 1, and in LBG or BHIG, both of which repress AR system 1 but induce the other two systems. The adapted cells were diluted to 10^6 CFU/ml in apple cider (pH 3.5), and survival was monitored over time at 25°C. The results immediately suggested that AR system 1 was most important in this environment. Cells adapted in LBG, repressed for AR system 1, did not survive in pH 3.5 apple cider to the same extent as cells grown in LB MES pH 5.5 (Fig. 1). We then tested various mutants for survival. The results clearly demonstrated that an *rpoS* mutant, lacking AR system 1 but exhibiting normal resistance via systems 2 and 3, survived very poorly, becoming undetectable after only 24 h (Fig. 2). In contrast, mutants defective in AR system 2 (*gadC*) or AR system 3 (*adiA*) survived at high levels for at least 3 days. Because *rpoS* mutants are susceptible to a variety of different stresses, we needed to prove that acidic pH was the reason this mutant succumbed to apple cider. This was accomplished by

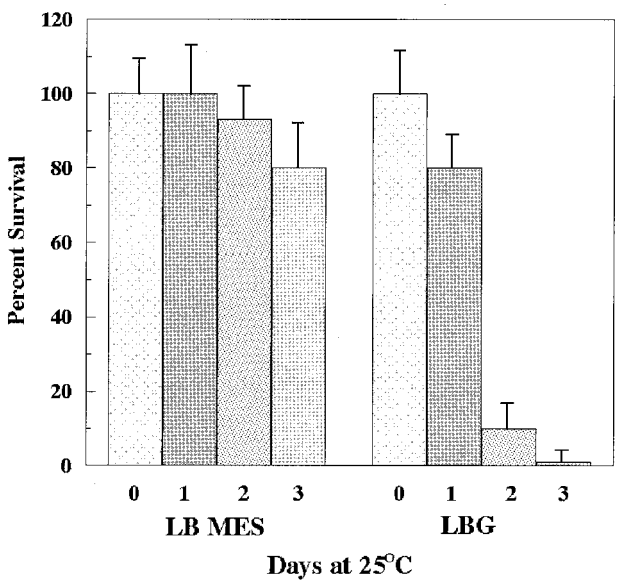

FIG. 1. Survival of *E. coli* O157:H7 in pH 3.5 apple cider. Wild-type *E. coli* O157:H7 (EK274) cells were grown overnight in either LB MES (pH 5.5) or LBG. These stationary-phase cultures were diluted 1:1,000 into Thrifty Maid apple cider with a measured pH of 3.5. The cultures were incubated at 25°C for the times indicated. Survival was measured by diluting the cultures and plating samples onto LB agar. One-hundred percent survival ranged from 1.5×10^6 to 3×10^6 CFU per ml for all cultures.

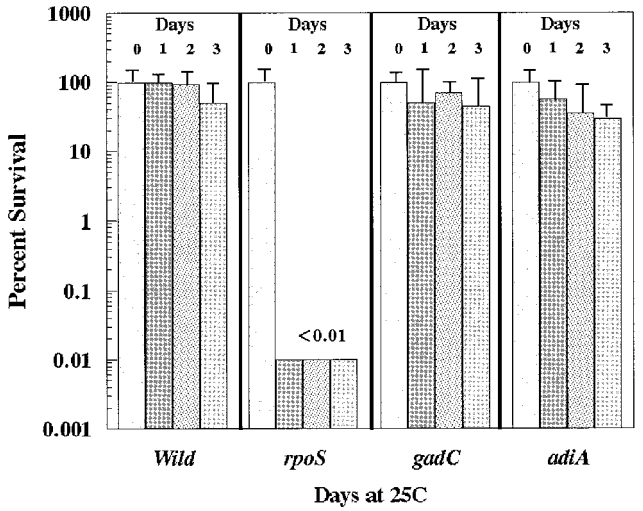

FIG. 2. Survival of *E. coli* O157:H7 mutants in pH 3.5 apple cider. Cells were grown overnight in LB MES (pH 5.5) and diluted 1:1,000 into Thrifty Maid apple cider with a measured pH of 3.5. The cultures were incubated at 25°C for the times indicated. Survival was measured by diluting the cultures and plating samples onto LB agar. One-hundred percent survival ranged from 1.5×10^6 to 3×10^6 CFU per ml for all cultures. Strains tested include wild-type (EK274), *rpoS* (EK275), *gadC* (EF484), and *adiA* (EF489).

showing that the *rpoS* mutant grew if the pH of apple cider was neutralized (data not shown).

Mechanism of *E. coli* O157:H7 AR in the bovine gastrointestinal tract. We speculate that *E. coli* O157:H7 encounters a more severe acid stress in the gastrointestinal tract of cattle; certainly the pH of the abomasum (pH 2 to 2.5) is lower than that of the apple cider tested (pH 3.5). One reason that *E. coli* may have multiple systems of AR is that individual systems may cope with different acid stress environments. Thus, *E. coli* may use one system to protect itself in apple cider and another to survive acid stress in acidic portions of the gastrointestinal tract. To test this hypothesis, quantitative bacteriology was utilized to examine survival of the *E. coli* O157:H7 *gadC* (EF484) and *adiA* (EF489) mutants in the calf intestine. As presented in Materials and Methods, equal numbers of the mutant and wild-type strains were coinoculated into four calves and the fecal quantities of the strains (differentially marked with antibiotic resistance) were monitored for 16 days.

As observed previously with this model, the wild-type strain was shed in large numbers within 24 h of inoculation (14, 39) and continued to be excreted in numbers high enough to be enumerated ($\geq 10^3$ CFU/g of feces) for the 16-day postinoculation period (Fig. 3). In contrast, the *gadC* mutant was shed in significantly lower quantities than the wild-type strain from days 1 to 15 ($P < 0.05$). This shedding phenotype resembles that of an *rpoS* mutant, which also showed reduced shedding compared to that of the wild type, as was reported previously (39).

In contrast to the shedding phenotype displayed by the *gadC* mutant, the *adiA* mutant was shed in amounts comparable to those of the wild type, with no significant differences ($P > 0.05$) observed between the two strains during the 16-day experimental period (Fig. 3). The shedding phenotype of the *adiA* mutant resembled that of the *gadA* mutant, which also showed no reduction in shedding compared to that of the wild type (39).

The decreased shedding exhibited by the *gadC* mutant indicated that this AR determinant is critical to *E. coli* O157:H7 survival through the calf gastrointestinal tract. To confirm the overall role of AR in shedding, four calves were inoculated with equal numbers of stationary-phase (and therefore acid-resistant) wild-type strain (EK274) and logarithmic-phase (and therefore AS) *gadA* mutant (EF501). The *gadA* mutant was chosen for these experiments because previous work had shown it to have both wild-type AR at pH 2.5 and a wild-type shedding phenotype (39). In addition, because the pRR10 insertion confers ampicillin resistance on its recipient, the *gadA* mutant could be easily distinguished from the wild-type strain in fecal culture.

The shedding results from the calves that received the AR-AS mixture of *E. coli* O157:H7 showed that, especially during the first 9 days of shedding, the acid-resistant strain was shed in significantly greater amounts than was the AS strain ($P < 0.05$; Fig. 4). After day 9, the AS cells continued to shed at lower numbers than the AR cells. However, with the exception of day 12, statistical significance was unattainable due to the low numbers of cells being shed.

Finally, to address possible unforeseen effects on shedding manifested by the *gadA* mutant when grown under conditions designed to render *E. coli* O157:H7 AS, we reversed the growth conditions of the two strains and inoculated two calves with equal numbers of the AS wild-type strain (EK274) and the acid-resistant *gadA* mutant strain (EF501). The results confirmed previous findings in that greater numbers of the acid-resistant-cultured *gadA* strain were shed than the AS-cultured wild-type strain (data not shown).

DISCUSSION

The purpose of the present study was to examine how *E. coli* O157:H7 protects itself when exposed to two very different acidic environments relevant to food safety. Apple cider was chosen for one of these environments, because it is naturally acidic (~pH 3.5) and has been the source for human outbreaks of *E. coli* O157:H7 disease (5). Cattle were chosen as the other environment, because they serve as reservoir hosts for *E. coli* O157:H7, which must traverse the low pH (2.0 to 2.5) of the abomasum to reach the colon. By unraveling the AR mechanisms used by *E. coli* O157:H7 to survive in these and other acidic environments, it may be possible to design strategies targeting this survival mechanism as a means of pathogen reduction.

The role played by the *rpoS*-encoded global stress response regulator σ^S in the AR of *E. coli* O157:H7 and in the ability of this organism to survive in cattle has been previously examined (39). An *rpoS* mutant of *E. coli* O157:H7 lacks AR system 1 and is susceptible to other stresses present in the gastrointestinal tract. This mutant was shed from experimentally inoculated mice and calves in significantly lower numbers than its wild-type parent (39). While low gastric pH was the reason for reduced shedding of the *rpoS* mutant from mice, the same conclusion could not be drawn from the calf studies because neutralization of the acid in the calf abomasum was not possible.

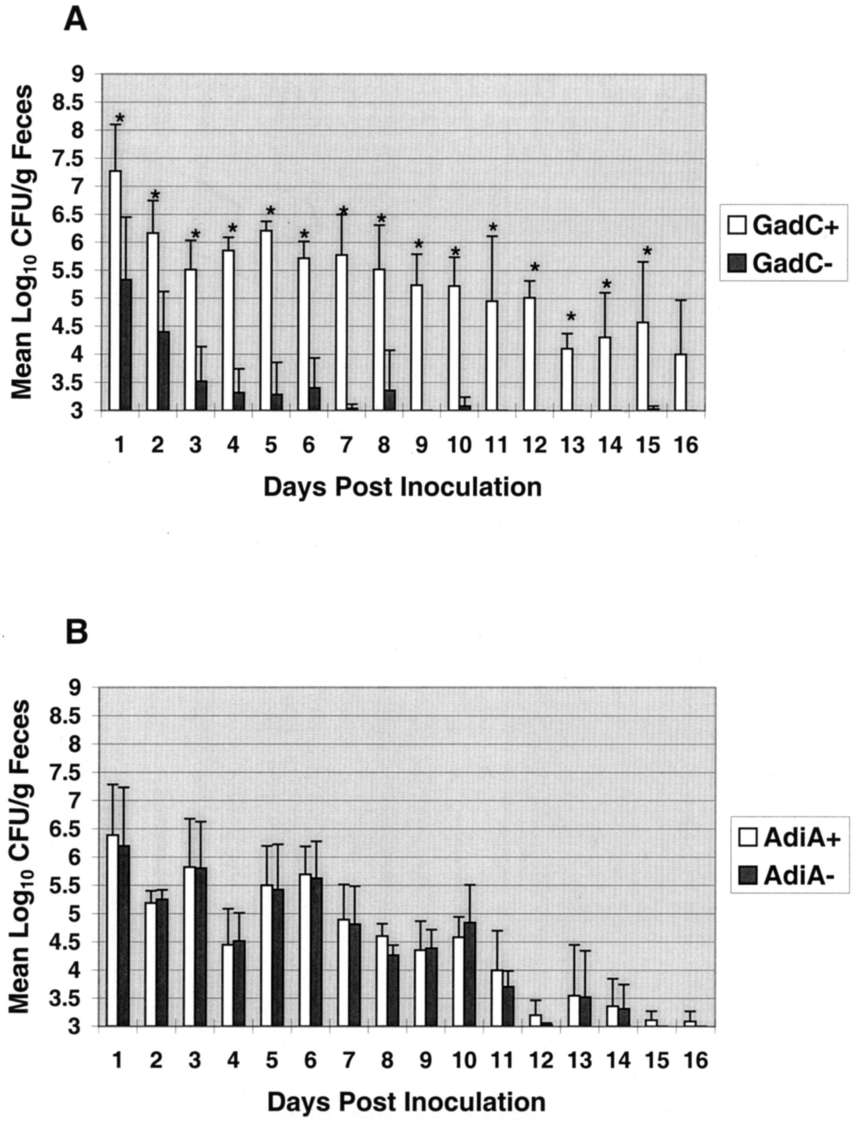

FIG. 3. Shedding levels over time of *E. coli* O157:H7 wild-type (□) and GadC$^-$ (■) (A) or wild-type (□) and AdiA$^-$ (■) strains (B). Calves were inoculated with 10^{10} total CFU containing equal numbers of wild-type and mutant strains on day 0. Fecal samples were cultured daily beginning 1 day postinoculation. Colony counts are displayed as the means for four calves. Bars indicate standard deviation of mean $\log_{10}$ CFU/gram values. Asterisks indicate significant difference in shedding between wild-type and mutant strains at $P < 0.05$ using repeated measures of analysis of variance. Specimens containing $<10^3$ CFU/g of feces were below the level needed for accurate enumeration.

Because *gadC* and *adiA* affect no known stress responses other than AR, testing of these mutants was expected to more definitively characterize a role for AR in surviving the gastrointestinal tract and acidic foods. In the present work, deletion of *gadC* resulted in elimination of the glutamate-dependent AR system 2, while deletion of *adiA* abolished the arginine-dependent AR system 3 (Table 2). The results clearly show that eliminating AR system 1 reduced fecal shedding as well as survival in apple cider. In contrast, removing AR system 2 specifically affected fecal shedding.

One might question why previous results indicated a *gadA* mutant was unaffected with respect to fecal shedding and AR (39), while the present work clearly showed that a *gadC* mutant was shed at very low levels and was AS. The reason for this is that there is only one gene encoding the glutamate:γ-amino butyric acid antiporter (GadC), but there are two isozymic forms of glutamate decarboxylase (*gadA* and *gadB*). As long as one of the decarboxylase genes is active and the GadC antiporter is present, the cells will retain glutamate-dependent AR (9).

In contrast to expectations, results with the *adiA* mutant indicated that the arginine-dependent AR system 3 is neither required for survival in apple cider nor necessary for survival in calves (Fig. 2 and 3). The reason for this may be that sufficient arginine is not available in either the stomach or apple cider environments. Alternatively, in the apple cider pH 3.5 environment the intracellular pH may not be optimum for arginine decarboxylase activity.

One possible reason why neither the glutamate nor arginine system facilitated survival in pH 3.5 apple cider is that the

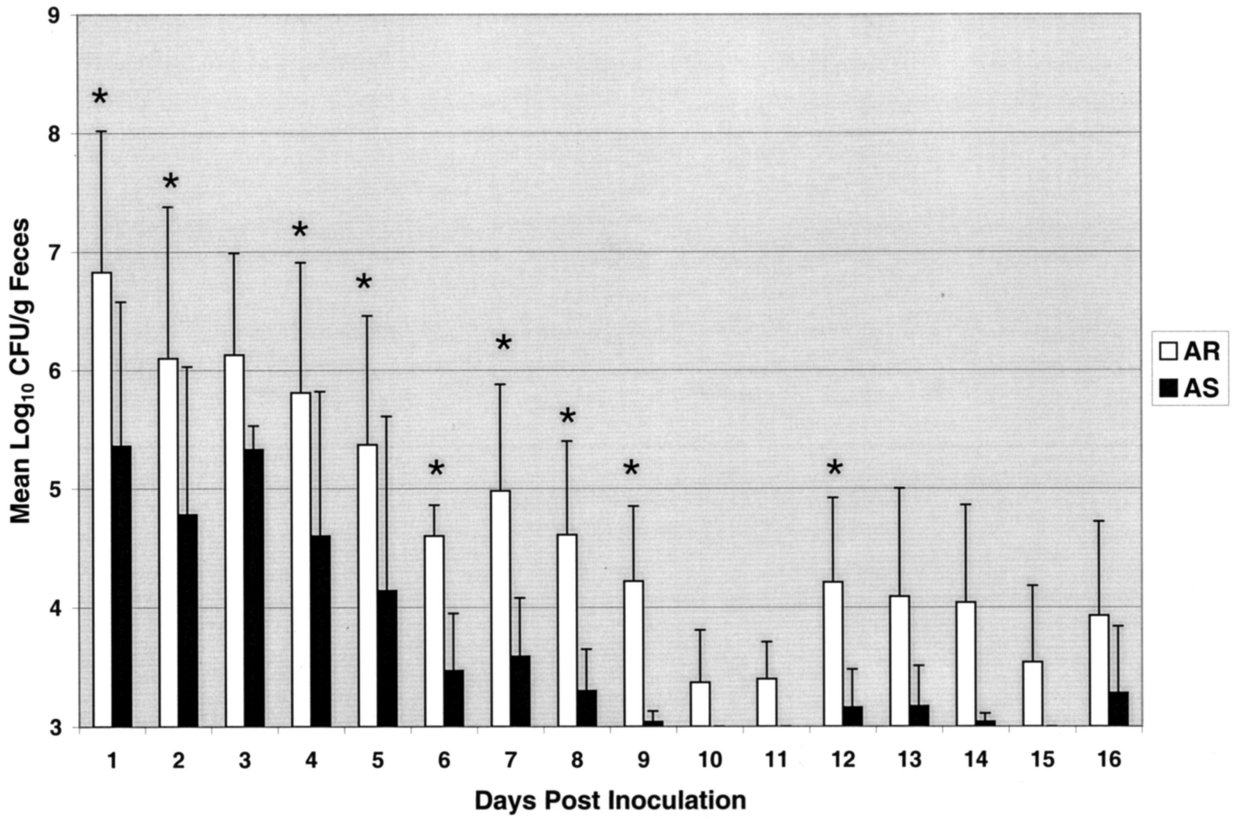

FIG. 4. Shedding levels over time of *E. coli* O157:H7 acid-resistant (□) and AS (■) strains. Calves were inoculated with 10^{10} total CFU containing an equal mixture of stationary-phase-grown EK274 cells (AR) and logarithmic-grown EF501 cells (AS) on day 0. Fecal samples were cultured daily beginning 1 day postinoculation. Colony counts are displayed as the means of four calves. Bars indicate standard deviation of mean $\log_{10}$ CFU/gram values. Asterisks indicate significant difference between shedding of acid-resistant and AS strains at $P < 0.05$ using repeated measures analysis of variance. Specimens containing $<10^3$ CFU/g of feces were below the level needed for accurate enumeration.

intracellular pH of pH 3.5-stressed cells may not be low enough to activate glutamate or arginine decarboxylase (45). The pH optima of these *E. coli* enzymes are pH 4 and 5, respectively. Another possibility is that apple cider might contain concentrations of glutamate and arginine too low for AR 2 and AR 3 to be active, because the activity of these two AR systems is amino acid dependent. This is a less likely hypothesis for the following reasons. A recent report presented the concentration of glutamate in apple juice to be between 0.4 and 1.2 mM (13). Our work has shown that a minimum glutamate concentration of approximately 0.2 mM is needed for in vitro survival at pH 2.5 (data not shown). Thus, it appears that apple juice (and presumably apple cider) contains plenty of glutamate to supply an active glutamate decarboxylase system.

The finding that glutamate-dependent AR system 2 is not required for survival of *E. coli* O157:H7 in apple cider is interesting in light of the findings of Cotter et al., who reported on the role of an analogous GAD system found in another food-borne pathogen, *Listeria monocytogenes* (13). These researchers found that a glutamate decarboxylase double mutant (*gadAB*) of *L. monocytogenes* was more rapidly killed than the wild-type strain in apple cider. Our results indicated that it is the *rpoS*-dependent AR system 1, and not the glutamate-dependent AR system 2, that is critical for survival of *E. coli* O157:H7 in apple cider. As noted above, possible explanations for this apparent contradiction may be a difference in the pH optima of the *Listeria* versus *E. coli* glutamate decarboxylases, a difference in the internal pH of the two organisms at external pH 3.5, or a difference in the organic acid concentration of apple juice versus apple cider. It was previously shown that the glutamate-dependent AR system will protect cells at higher external pH values if the organic acid concentration is elevated (29).

Perhaps the most remarkable finding of the study was that although *gadC* is not required for *E. coli* O157:H7 survival in apple cider, it is required for survival in calves (Fig. 3). In contrast to the acid-resistant wild-type *E. coli* O157:H7, the AS *gadC* mutant is killed in greater numbers during passage through the calf gastrointestinal tract. This difference in survival in these two acidic environments (apple cider versus calf gastrointestinal tract) could be due to the lower pH (2.0 to 2.5) in the bovine abomasum or, more likely, to the distinction between the organic acid found in apple cider and the inorganic acid of the calf abomasum. A mutant carrying a deletion in another AR system 2 gene, *gad*A, was shown previously to retain AR and to be shed normally in calves (39). This caveat may be explained by the fact that the glutamate decarboxylase system is redundant in *E. coli*, with enzyme molecules being produced by two unlinked genes, *gad*A and *gad*B. Thus,

enough decarboxylase is produced in the *gad*A mutant by its intact *gad*B gene to retain its AR phenotype.

The work presented here also illustrated that in the absence of mutations affecting AR, parental cells grown to be acid resistant (stationary phase) survived in the calf gastrointestinal tract better than parental cells grown to be AS (log phase) (Fig. 4). The reason for this is that the *rpoS*-dependent stress response systems (AR system 1) and the glutamate-dependent AR system 2 specific for acid survival are not active in exponentially growing cells (39). This is consistent with stationary-phase cells having greater resistance to acid pH.

This is the first report that AR systems in *E. coli* O157:H7 are differentially utilized based on the type of acidic environment encountered. The fact that the σ^S-regulated AR system 1 is required for *E. coli* O157:H7 survival in both apple cider and cattle highlights the important role played by RpoS in the AR response of this pathogen. Future studies aimed at targeting this global regulatory protein in *E. coli* O157:H7-colonized calves are presently under way.

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