JOURNAL OF BACTERIOLOGY, July 1995, p. 4097–4104 Vol. 177, No. 14
0021-9193/95/$04.00+0
Copyright 1995, American Society for Microbiology

Comparative Analysis of Extreme Acid Survival in *Salmonella typhimurium*, *Shigella flexneri*, and *Escherichia coli*

JYHSHIUN LIN,[1]† IN SOO LEE,[1] JAMES FREY,[2] JOAN L. SLONCZEWSKI,[2] AND JOHN W. FOSTER[1]*

Department of Microbiology and Immunology, University of South Alabama College of Medicine, Mobile, Alabama 36688,[1] and Department of Biology, Kenyon College, Kenyon, Ohio[2]

Received 9 January 1995/Accepted 27 April 1995

Several members of the family *Enterobacteriaceae* were examined for differences in extreme acid survival strategies. A surprising degree of variety was found between three related genera. The minimum growth pH of *Salmonella typhimurium* was shown to be significantly lower (pH 4.0) than that of either *Escherichia coli* (pH 4.4) or *Shigella flexneri* (pH 4.8), yet *E. coli* and *S. flexneri* both survive exposure to lower pH levels (2 to 2.5) than *S. typhimurium* (pH 3.0) in complex medium. *S. typhimurium* and *E. coli* but not *S. flexneri* expressed low-pH-inducible log-phase and stationary-phase acid tolerance response (ATR) systems that function in minimal or complex medium to protect cells to pH 3.0. All of the organisms also expressed a pH-independent general stress resistance system that contributed to acid survival during stationary phase. *E. coli* and *S. flexneri* possessed several acid survival systems (termed acid resistance [AR]) that were not demonstrable in *S. typhimurium*. These additional AR systems protected cells to pH 2.5 and below but required supplementation of minimal medium for either induction or function. One acid-inducible AR system required oxidative growth in complex medium for expression but successfully protected cells to pH 2.5 in unsupplemented minimal medium, while two other AR systems important for fermentatively grown cells required the addition of either glutamate or arginine during pH 2.5 acid challenge. The arginine AR system was only observed in *E. coli* and required stationary-phase induction in acidified complex medium. The product of the *adi* locus, arginine decarboxylase, was responsible for arginine-based acid survival.

Many bacteria must endure or survive transient encounters with extremely low or high pH outside the range suitable for growth. Members of the family *Enterobacteriaceae*, for example, can encounter low-pH stress in the natural "nonhost" environment as well as during passage through the stomach en route to the intestine. Fecal material after defecation presents another problem for the enterics, as the material acidifies because of increased fermentation. Several laboratories have initiated studies designed to examine how microorganisms cope with this form of environmental stress and have referred to the acid survival systems as the acid tolerance response (ATR), acid resistance (AR), and acid habituation (12, 16, 17, 29, 30, 34).

A major problem is encountered when attempting to compare acid survival results among various laboratories that differ in the use of complex versus minimal medium, log-phase versus stationary-phase cells, and acid challenge at various pHs and temperatures. For example, we have used log-phase and stationary-phase cells (*Salmonella typhimurium*) grown in minimal broth at neutral pH and 37°C. Acid tolerance is induced by shifting cells to pH 4.3 (acid shock) for various time periods. There is no cell growth during this adaptation. The adapted cells are then challenged at pH 3.3 and 37°C (8). Two other groups, who refer to their system as AR, grow cells (*Escherichia coli* and *Shigella flexneri*) to stationary phase in a complex medium under moderate acid conditions (pH 5.0). Cells thus adapted are challenged at pH 2.5 and 25°C (17, 34). A fourth group (acid habituation), also using *E. coli*, employs a very low phosphate-based complex medium, nutrient broth, and brief exposures to pH 5 (10 min) to induce acid habituation (16, 30). However, this last group has found that the acid habituation phenomenon does not occur at high phosphate concentrations (31), distinguishing it from the ATR and the AR phenomena, which both occur in high-phosphate medium (12). It has been difficult, if not impossible, to determine from the literature if the systems described are truly different or simply reflect different ways of measuring the same system.

One factor known to be associated with acid survival is the stationary-phase-associated sigma factor σ^S, the product of the *rpoS* locus (18). Studies with this sigma factor serve as an example of how difficult it is to compare acid survival systems measured by different approaches. Small et al. (34) essentially find that *E. coli* (MC4100) and *S. flexneri* (3136) exhibit an RpoS-dependent, stationary-phase-induced, pH 2.5 acid resistance that is induced by growth in complex medium at any pH (pH blind induction). They also report an acid-induced but RpoS-independent acid survival observed under anaerobic conditions. Similar pH 2.5 acid survival was not observed with *S. typhimurium* in that study. Acid survival studies with *S. typhimurium* using minimal medium show two acid-inducible log-phase ATR systems protective at pH 3, one of which is RpoS independent and one of which is RpoS dependent (22). This is in contrast with the poor log-phase systems reported for *S. flexneri* and *E. coli* (34). The *S. typhimurium* studies also revealed two stationary-phase ATR systems; one that is acid inducible and RpoS independent, and one that is unresponsive to pH but RpoS dependent. The RpoS-dependent system appears to be the stationary-phase general stress resistance system (18), which includes resistance to low pH (8, 22, 23). We have asked whether acid survival in minimal versus complex medium utilizes similar mechanisms or whether distinct systems that are not available in minimal medium become available in complex medium to some organisms.

In addition to questions of methodology, there are apparent contradictions that occur in the literature with respect to the

* Corresponding author. Phone: (334) 460-6323. Fax: (334) 460-7931. Electronic mail address: fosterj@sungcg.usouthal.edu.

† Present address: Pig Research Institute of Taiwan, P.O. Box 23, Chunan 35099, Miaoli, Taiwan.

TABLE 1. Bacterial strains

Species and strain	Relevant genotype	Source (reference)
S. typhimurium		
UK1 (SF530)	Virulent wild type	R. Curtiss (6)
LT2 (SF1)	Attenuated	K. Sanderson
SL1344 (SF529)	Virulent, *hisG col xyl rpsL*	R. Curtiss (21)
14028s (SF358)	Virulent, wild type	F. Heffron (American Type Culture Collection)
SF1005 (SF500)	14028s *rpoSΩAp*	F. Fang
SR-11 (SF544)	Wild type	R. Curtiss
SF366	*S. typhimurium*, Delaware	R. Ziprin
LB5000 (SF152)	*ilv-452 metA22 metE551 trpD2 leu hsdLT hsdSA hsdSB*	L. Bullas (3)
JF2560	LT2 *rpoS*::*Ap*	SF1005 × LT2
JF2690	UK1 *rpoS*::*Ap*	SF1005 × LT2
JF2691	SL1344 *rpoS*::*Ap*	SF1005 × SL1344
JF2878	UK1/pKS29 *adi*$^+$	EK201 × UK1
E. coli		
H10407	Toxigenic, heat-labile and heat-stable toxins	M. Lawlor (5)
CU4 (EK5)	F$^-$ *galT12*λ$^-$	S. Rosenfeld
MC4100.S (EK180)	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flb-5301 deoC1 ptsF24 rbsR*	J. Slonczewski (34)
MC4100.GNB (EK220)	F$^-$ *araD139* Δ(*argF-lac*)*U169 rpsL150 relA1 flb-5301 deoC1 ptsF24 rbsR*	G. Bennett (1)
GNB7145 Km (EK198)	MC4100 *adi*::Mu*d*1 1734 (Km^r Lac$^+$)	G. Bennett (1)
EK201	JM101/pKS29 *adi*$^+$	G. Bennett (26)
EF276	GNB7145Km$_{22}$/pKS *adi*$^+$	EK201 × GNB7145Km
S. flexneri		
3136	Wild type	J. Slonczewski (34)
ShF6	3136 *rpoS*::Tn*10*	J. Slonczewski (34)

relative abilities of *S. typhimurium* and *E. coli*/*S. flexneri* to survive acid stress. Some reports of studies with complex medium suggest that *E. coli* and *S. flexneri* survive more extreme acidity than *S. typhimurium* (17, 34). Other investigators, using minimal medium, suggest that *S. typhimurium* may survive low pH more efficiently than *E. coli* (12, 20). We have addressed many of the questions raised from the previous studies by directly comparing the different acid survival strategies of *S. typhimurium*, *S. flexneri*, and *E. coli* using the same strains employed earlier. While resolving the differences between these organisms, we have defined new components of acid survival.

Two key terms used throughout this work are ATR and AR. The ATR encompasses acid survival systems evident in log-phase or stationary-phase cells that can function in minimal medium to protect cells from acid to pH 3.0. AR encompasses acid survival systems evident in stationary-phase cells that protect cells to pH 2.5 and below.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The strains of *S. typhimurium*, *E. coli*, and *S. flexneri* used are listed in Table 1. The minimal media used included E (36), noncitrate E (24), and M9 (27). The complex medium used was Luria-Bertani (LB) (27). Glucose (0.4%), casamino acids (Difco), and various amino acids (0.012%) were added to various media where indicated. Nicotinamide (10^{-5} M) was added to minimal media cultures of *S. flexneri*. Cultures were grown in semiaerobic conditions; 3 ml of medium in 13 100-mm test tubes, shaking at 240 rpm, 45° angle, and 37°C. LB, where indicated, was buffered to 100 mM with either MOPS (morpholinepropanesulfonic acid, pH 8.0) or MES (morpholineethanesulfonic acid, pH 5.0) as described by Slonczewski et al. (33).

Log-phase ATR. Log-phase ATR was measured in the present study essentially as described earlier but with modification to ensure that log-phase cells were fully depleted of stationary-phase systems (8, 22). Log-phase cells were obtained as follows. A starter culture (3.0 ml of minimal glucose) was inoculated with a single colony of the strain to be tested. Eight hours later (37°C, shaking), this mid-log-phase culture was diluted 1:10,000 in 3.0 ml of pH 7.7 minimal medium. When the cultures reached a cell density of 2×10^8 cells per ml (approximately 16 generations of log-phase growth having elapsed since stationary phase), unadapted cells were acid challenged to pH 3.3 by adjusting the growth medium pH with HCl. Adapted cells were prepared by acid shock treatment at pH 4.3 for 20 or 90 min before acid challenge or by growth for one cell doubling at pH 5.8. Viable-cell counts were determined for adapted and unadapted cells at 0, 2, 4, and 6 h post-acid challenge. Each experiment was performed a minimum of two and usually three times. The data shown represent average percent survival values, with variability for each time point not exceeding 50% of the stated value (i.e., a 50% average survival value ranged between 25 and 75% upon repetition).

Stationary-phase ATR. Stationary-phase ATR was measured as described earlier (23). Cells were initially grown to stationary phase in minimal glucose medium (pH 8) and harvested. Unadapted cells were directly resuspended to 2×10^8 cells per ml in pH 3.0 minimal glucose, whereas adapted cells were prepared by resuspension in pH 4.3 minimal glucose for 2 h and then transferred to pH 3.0 for acid challenge. Two to three repetitions were performed for each experiment, with percent survival values reproducible to within 50% of the stated value.

AR. Cells were grown overnight in one of several media, including LBG (LB plus 0.4% glucose), buffered LB (either 100 mM MOPS [pH 8.0] or 100 mM MES [pH 5.0]), and minimal E glucose. The overnight (24-h) stationary-phase cultures were diluted 1:1,000 into warmed pH 2.5 LB or minimal glucose medium (37°C). The pH of the challenge medium did not waver at this dilution. Viable-cell counts were determined at 0 and 2 h post-acid challenge. In a departure from the procedure of Small et al. (34), we used 37°C rather than 25°C for the acid challenge procedure to avoid possible complications of temperature downshift. Two to three repetitions were performed for each experiment, with percent survival values reproducible to within 50% of the stated value.

Acid growth limit. Cells were initially grown overnight and diluted 1:1,000 into LBG or minimal NCE glycerol (40 mM) medium (24) adjusted to pH 4.0 and 4.3, respectively. Growth (optical density at 600 nm [OD_{600}]) was then determined after 72 h at 37°C.

RESULTS

Minimum growth pH in minimal and complex media. Extreme acid survival is defined as survival at a pH below the growth range; therefore, it was important to determine the acid limit for growth of each species under comparison. We first compared the abilities of *E. coli* and *S. typhimurium* to grow at low pH in buffered LB (Fig. 1). *S. typhimurium* (LT2) grew at pH 4.0 with a generation time of about 2.5 h, while *E. coli* (K12 and MC4100) was unable to grow below pH 4.4. *S. flexneri* did not grow below pH 4.7. Similar results were obtained in min-

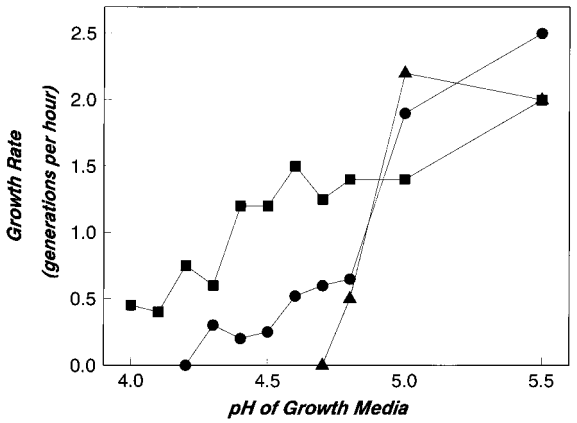

FIG. 1. Growth rate as a function of external pH in buffered LB. Cultures were grown overnight at 37°C in LB with 100 mM MES (pH 6.0) and then diluted 1:100 in LB buffered at the pH indicated. The buffers used were 100 mM sodium citrate (pH 4.0 to 5.0) and 100 mM MES (pH 5.5). Cultures were incubated at 37°C with vigorous shaking. Growth rates were determined from doubling times based on optical density over the approximate range of OD_{600} = 0.03 to 0.3. ■, *S. typhimurium*; ●, *E. coli*; ▲, *S. flexneri*.

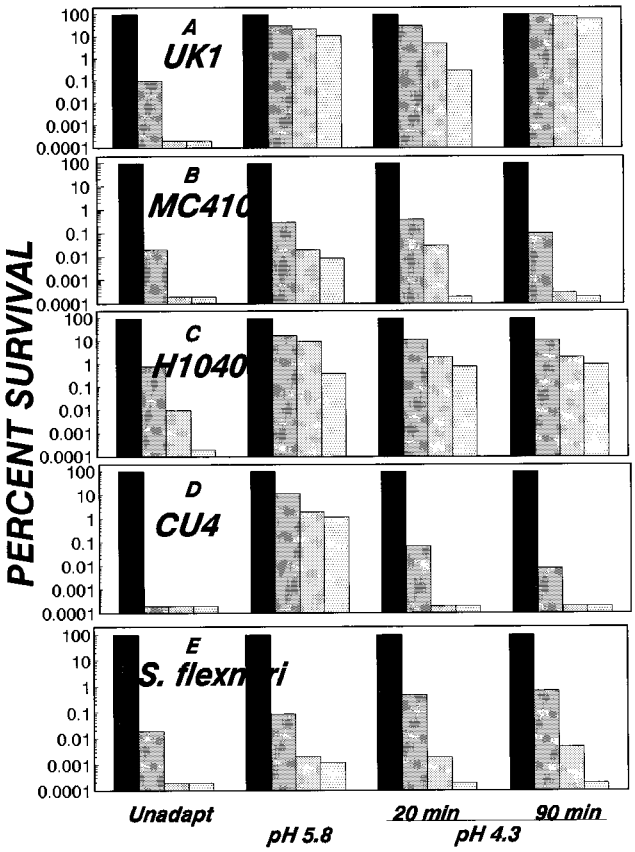

FIG. 2. Log-phase acid tolerance responses. Cells grown to mid-log phase in minimal glucose medium were subjected to various adaptation protocols prior to acid challenge at pH 3.3. Unadapted cell cultures were adjusted directly to pH 3.3. Adaptation to pH 5.8 was allowed to occur for one cell doubling prior to acid challenge. Acid shock adaptation at pH 4.3 was conducted for 20 or 90 min, as indicated, before acid challenge. (A) *S. typhimurium* strain; (B to D) different *E. coli* strains; (E) *S. flexneri*. The bars represent average percent survival (± 0.5 times the stated value) at pH 3.3 after 0, 2, 4, and 6 h (darkest to lightest shaded bars, respectively).

imal glycerol medium, but pHs in all cases were 0.3 units higher (data not shown).

For further comparative analyses, a variety of strains were grown overnight and diluted 1:1,000 into LBG or minimal NCE glycerol (40 mM) medium adjusted to pH 4.0 and 4.3, respectively. Growth was then determined after 72 h at 37°C. *S. typhimurium* consistently grew at the lower pH, whereas most of the other enterics did not (data not shown). This included *Citrobacter freundii*, *Enterobacter cloacae*, *Enterobacter gergoviae*, *Klebsiella oxytoca*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Proteus vulgaris*, *Serratia marcescens*, and *Shigella sonnei*. The one exception was *Enterobacter aerogenes*, which did grow at pH 4.0. The trend was the same in minimal glycerol at pH 4.3 (data not shown). The pH 4.0 acid growth of *S. typhimurium* did not require RpoS and appeared to be distinct from the ATR system, since various *atr* mutants grew well at low pH (data not shown).

ATR for log-phase cells in minimal medium. *S. typhimurium* possesses two acid-inducible ATR systems that are effective in minimal medium and depend on the physiological status of the cell. The two systems are designated log-phase ATR and stationary-phase ATR (22). Log-phase ATR can be induced in two ways, adaptation at pH 5.8 for one doubling or a 20- to 90-min acid shock at pH 4.3. Figure 2 illustrates the results of pH 5.8 and 4.3 adaptations, comparing a virulent *S. typhimurium* strain with *E. coli* (three strains) and *S. flexneri*. All strains except *S. flexneri* exhibited induction of an ATR at pH 5.8. However, while *S. typhimurium* showed clear log-phase acid shock (pH 4.3) adaptation, two of the *E. coli* strains and *S. flexneri* either did not demonstrate this response or adapted poorly. The toxigenic strain of *E. coli*, H10407, did adapt well at pH 4.3, although not quite as well as *S. typhimurium* UK1. It was considered possible that the other two *E. coli* strains and *S. flexneri* might respond better to an acid shock at a less acidic pH. However, acid shock adaptations of *S. flexneri* and *E. coli* CU4 or MC4100 at pH 4.8 and 4.5 for 20 or 90 min did not improve acid tolerance induction (data not shown). Clearly, some strains of *E. coli* (H10407) can induce a minimal medium ATR, while others do so only poorly (MC4100 and CU4). Thus, the poor log-phase acid resistance noted by Small et al. (34) may reflect their use of MC4100. The fact that *E. coli* is capable of inducing a log-phase ATR is reasonable, since *E. coli* has been shown previously to induce acid shock proteins (19). It is not clear why *E. coli* CU4 could induce an ATR via pH 5.8 but remain acid sensitive following pH 4.3, 4.5, or 4.8 acid shock exposures.

ATR of stationary-phase cells grown in minimal medium. Stationary-phase ATR in minimal medium was also compared among the various strains (Fig. 3). The stationary-phase ATR protocol involved transferring stationary-phase cells to pH 4.3 medium for 2 h of acid shock adaptation followed by transfer to pH 3 acid challenge medium. *S. typhimurium* exhibited a typical stationary-phase ATR, demonstrating an acid-induced 500-fold increase in pH 3.0 survival after a 4-h exposure. *S. flexneri* did not appear to exhibit a significant stationary-phase ATR at pH 3.0, while *E. coli* H10407 was very acid tolerant in this assay. However, H10407 was tolerant even when unadapted. On the other hand, MC4100 was more acid sensitive than H10407 but did exhibit an acid-inducible stationary-phase ATR (data not shown). The high degree of acid tolerance even when grown at pH 8.0 suggests that H10407 is much better at inducing the RpoS-dependent general stress resistance system during stationary phase than *S. typhimurium*, *S. flexneri*, or *E. coli* MC4100.

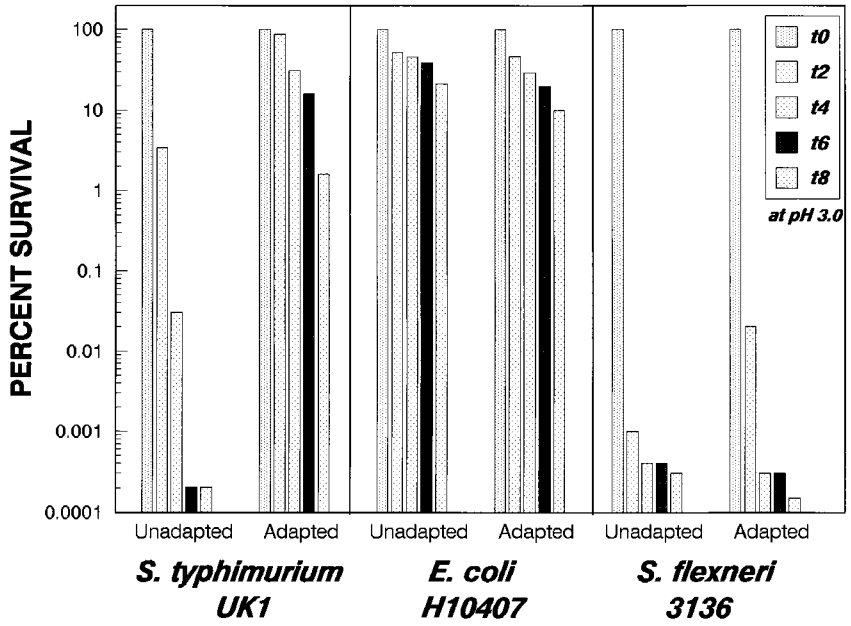

FIG. 3. Stationary-phase acid tolerance responses. Cells were grown overnight in minimal medium (pH 7.7), harvested, and resuspended to 2×10^8 cells per ml directly at pH 3.0 (unadapted) or to pH 4.3 (adapted) for 2 h prior to resuspension at pH 3.0. Bars represent average percent survival (± 0.5 times the stated value) at 0 to 8 h post-acid challenge.

AR of cells undergoing oxidative metabolism and grown in complex medium. The results above indicated that *S. typhimurium* grows at a lower pH than *E. coli* or *S. flexneri* and, in general, displays greater acid survival in minimal medium at pH 3.3 or 3.0. However, Gorden and Small (17) have reported that *E. coli* and *S. flexneri* survive more extreme acid exposure (pH 2.5) in complex medium than *S. typhimurium* despite their higher pH limit for growth. Using procedures similar to that described by Small et al. (33), we confirmed the results of the earlier studies (Table 2). For these studies, pH 8- or pH 5-grown stationary-phase cells were diluted 1:1,000 into various media at pH 2.5. Both *E. coli* and *S. flexneri* survived pH 2.5 in complex medium, whereas the virulent strain of *S. typhimurium* did not. We also observed a modest acid induction of AR (Table 2, line 1 versus 2, and line 3 versus 4) with semiaerobically grown cultures. Small et al. (34) observed a similar acid induction of AR in anaerobically grown cells. However, we suspect from the results presented below that it may not be the level of oxygen that is important but whether the cells are undergoing oxidative or fermentative metabolism that influences which acid-induced systems are expressed.

Consistent with the ATR results above, the toxigenic *E. coli* strain (H10407) proved more resistant to pH 2.5 than MC4100. This was true whether buffered LB or LBG was used for adaptation and LB was used for acid challenge (Table 2, lines 1 to 4). However, no pH 2.5 AR was observed when minimal medium was used for both adaptation and acid challenge (Table 2, line 5). We then discovered that different AR systems were induced in *E. coli* and *S. flexneri* depending upon whether they were undergoing oxidative or fermentative metabolism. Table 2 (line 6) reveals that oxidatively grown (LB-MES, pH 5) *E. coli* H10407 produced a low-pH-inducible acid survival component effective in unsupplemented minimal medium at pH 2.5. However, cells undergoing fermentation in LBG at pH 5 could not protect themselves in pH 2.5 minimal medium (Ta-

TABLE 2. AR systems differ following oxidative versus fermentative growth

Line	Medium used for:		% Survival[c]				
	Adaptation[a]	pH 2.5 challenge[b]	*S. typhimurium* UK1	*E. coli* H10407	*E. coli* MC4100.S	*E. coli* CU4	*S. flexneri*
1	LB-MOPS (pH 8)	LBG	<0.002	19	12	<0.002	13
2	LB-MES (pH 5)	LBG	<0.002	86	89.0	<0.002	93.0
3	LB (pH 8)	LBG	ND[d]	42	20.0	0.1	
4	LBG (pH 5)	LBG	<0.002	74	30.0	14.0	95.0
5	EG (pH 5.5)	EG	<0.07	<0.03	<0.01	ND	<0.03
6	LB-MES (pH 5)	EG		10	2.0		22
7	LBG (pH 5)	EG	<0.005	0.44	<0.02	<0.01	<0.006

[a] Adaptation involved overnight growth in the medium indicated. LB, Luria-Bertani broth; LBG, LB supplemented with 0.4% glucose; EG, minimal E salts glucose (36).
[b] Acid challenge involved a 1:1,000 dilution from the overnight culture to the pH 2.5 medium indicated for 2 h.
[c] Results are the averages of two or more experiments. Variability was not greater than 50% of the stated value [% survival ± 0.5(% survival)].
[d] ND, not determined.

TABLE 3. Amino acid-based AR systems

Line	Medium used for:		% Survival[c]				
			S. typhimurium UK1	*E. coli*			*S. flexneri*
	Adaptation[a]	pH 2.5 challenge[b]		H10407	MC4100.S	CU4	
1	EG	EG	<0.01	<0.03	<0.02	<0.01	<0.007
2	LBG (pH 5)	EG	<0.005	0.44	<0.02	<0.01	<0.006
3	EG (pH 5.5)	LBEG		80	0.3	0.8	40.0
4	LBG (pH 5)	EG + Arg		73	9.7	4.6	0.15
5	EG (pH 5.5)	EG + Arg		<0.01	<0.01	<0.01	<0.01
6	EG-CAA (pH 5.5)	EG + Arg		<0.01	<0.01	<0.01	<0.01
7	EG-Arg (pH 5.5)	EG + Arg		<0.01			
8	LBG (pH 5)	EG + partial amino acid pool[d]	<0.002	<0.002	<0.01		<0.006
9	LBG (pH 5)	EG + Glt	<0.002	107	2.9	1.0	66
10	EG (pH 8)	EG + Glt		74	0.4		0.018
11	EG (pH 5.5)	EG + Glt	<0.01	79	2.9		17
12	M9G (pH 5.5)	M9			<0.01		
13	M9G (pH 5.5)	M9 + Glt			3.7		
14	LBG (pH 5)	EG + Lys	<0.008	1.59	0.05	0.29	<0.007
15	LBG (pH 5)	EG + Orn		3.38	<0.02		
16	LB (pH 8)	EG + Arg		<0.003	<0.01		

[a] Adaptation involved overnight growth in the medium indicated. LB, Luria-Bertani broth; LBG, LB supplemented with 0.4% glucose; EG, minimal E salts glucose (36); M9G, minimal M9 glucose medium (27); CAA, casamino acids; Glt, glutamate.
[b] Acid challenge involved a 1:1,000 dilution from the overnight culture to the pH 2.5 medium indicated for 2 h. Amino acids were added at 0.012%.
[c] Results are the averages of two or more experiments. Variability was not greater than 50% of the stated value [% survival ± 0.5(% survival)].
[d] The partial amino acid pool lacked lysine, arginine, and glutamate.

ble 2, line 7) even though they protected themselves well in pH 2.5 complex medium (Table 2, line 4).

AR of cells undergoing fermentation in complex medium. The results suggested that one or more components of LB were required for pH 2.5 protection in *E. coli* and *S. flexneri* when grown under fermentative conditions (LBG). We discovered that overnight growth in complex medium was not essential. Minimal medium-cultured cells would display AR if complex medium was added to the pH 2.5 acid challenge medium (Table 3, line 1 versus 3). Subsequent attempts to identify the component(s) of LB responsible for pH 2.5 survival were made by adding a variety of amino acid pools to pH 2.5 minimal medium. The results indicated that either arginine (0.6 mM) or glutamate (0.9 mM) was capable of protecting *E. coli* during pH 2.5 acid challenge in minimal medium, but only glutamate worked well for *S. flexneri* (Table 3, line 2 versus lines 4 and 9). Amino acid pools lacking these amino acids would not confer acid protection (Table 3, line 8). The protection was not caused by an increase in medium pH, as the measured pH did not vary more than 0.05 units during the course of acid challenge.

The glutamate and arginine AR systems differed in several respects. First, the glutamate system did not require complex medium induction during overnight growth (Table 3, line 11), but the arginine system did require complex medium adaptation (Table 3, line 4 versus 5). In addition, neither arginine alone nor casamino acids induced the system during overnight growth in minimal medium, suggesting that a complex pattern of induction is needed (Table 3, compare lines 6 and 7 with 4). The second difference between the arginine and glutamate systems in *E. coli* is that the glutamate system was not induced by low pH (Table 3, line 10 versus 11), while the arginine system was acid inducible (Table 3, line 4 versus 16). A striking difference was also noted when comparing the glutamate systems of *S. flexneri* and *E. coli*. The system in *S. flexneri* was clearly acid inducible, in contrast to the pH-independent system in *E. coli* (Table 3, line 10 versus 11). We cannot determine unequivocally if the amino acid-based AR systems inducible during fermentation were also induced under oxidative conditions. The addition of arginine or glutamate during the pH 2.5 challenge improved the survival of LB-MES (pH 5)-grown cells only twofold (data not shown).

Acid survival at pH 3.0. Although essential for pH 2.5 survival in minimal medium, the two amino acid-based AR systems described for fermentatively grown cells were not required for *E. coli* survival at pH 3.0. Acid survival at pH 3.0 rather than pH 2.5 did not require complex medium for either adaptation or challenge (Table 4, lines 1 and 2). At pH 3.0 and above, it seems that the minimal medium stationary-phase ATR and/or the general stress resistance system is sufficient for protection in *E. coli*. The amino acid-based AR systems described here must augment the minimal medium stationary-phase systems, allowing acid survival at lower pH levels. The situation for *S. flexneri* appeared to be somewhat different. Our results suggest that this organism lacks or is deficient in the minimal medium acid protection systems (ATR). This conclusion is based not only upon the results in Fig. 1 and 2 but also on those in Table 4 (lines 1 and 2). The glutamate-based system in *S. flexneri* compensated for this deficiency, allowing 1,000-fold-better survival at pH 3.0 (Table 4, line 2 versus 3). It was considered possible that *S. typhimurium* might possess glutamate- or arginine-based systems but that pH 2.5 was too severe for the organism. Therefore, the presence of these AR systems was tested at pH 3. The data in Table 4 illustrate that neither arginine (lines 4 and 5) nor glutamate (lines 2 and 3) improved the pH 3.0 survival of *S. typhimurium*.

Arginine-based AR system of *E. coli* involves the *adi* arginine decarboxylase. The presence in *E. coli* of an arginine-based AR system immediately suggested the involvement of the acid-inducible arginine decarboxylase, the product of the *adi* locus (1, 35). Examination of an *adi* mutant revealed the absence of the arginine system but the presence of the glutamate-dependent AR system (Table 5). We noted variation in the degree of acid resistance of MC4100 depending upon the source of the clone (compare Table 4, MC4100.S, with Table 5, MC4100.GNB). Consequently, we used the direct parent of the

TABLE 4. Acid survival in minimal medium at pH 3.0 with the acid resistance protocol

Line	Medium used for:		% Survival[c]				
			S. typhimurium UK1	*E. coli*			*S. flexneri*
	Adaptation[a]	pH 3.0 challenge[b]		H10407	MC4100.S	CU4	
1	EG (pH 8)	EG	<0.02	47.0	<0.007	<0.003	<0.003
2	EG (pH 5.5)	EG	42.0	122.0	1.6	<0.007	0.06
3	EG (pH 5.5)	EG + glutamate	44.0	—	3.3	—	45.0
4	LBG (pH 5)	EG	67	—	—	—	—
5	LBG (pH 5)	EG + Arg	67	—	—	—	—

[a] Adaptation involved overnight growth in the medium indicated. LB, Luria-Bertani broth; LBG, LB supplemented with 0.4% glucose; EG, minimal E salts glucose (36).
[b] Acid challenge involved a 1:1,000 dilution from the overnight culture to the pH 3.0 medium indicated for 2 h. Amino acids were added at 0.012%.
[c] Results are the averages of two or more experiments. Variability was not greater than 50% of the stated value [% survival ± 0.5(% survival)].
[d] —, not done.

adi mutant, obtained from G. Bennett, for comparative analysis. In addition to the data from the *adi* mutant, a cloned *adi*$^+$ locus was also shown to complement the *adi* acid-sensitive defect by restoring arginine-based acid resistance to both *S. flexneri* and *S. typhimurium* (Table 5). It is not yet clear why the plasmid-borne *adi*$^+$ gene did not fully complement the AR defect of the *adi* mutant. It is possible that the plasmid-borne *adi*$^+$ is not as active as the chromosomal gene or that the cloned fragment is missing a gene downstream of *adi* that collaborates in the AR mechanism.

DISCUSSION

The enteric group of microorganisms have proven diverse in the ways that they handle acid stress. Acid survival strategies among these organisms can be classified into two general categories, based on whether a system can be induced and function in unsupplemented minimal medium (ATR systems) or requires some form of supplementation for either induction or function (AR mechanisms). The results establish that *S. typhimurium* possesses only the ATR systems, *S. flexneri* utilizes only supplementation-dependent AR mechanisms, while *E. coli* has both types of systems. Table 6 summarizes the various acid survival systems identified. These studies also revealed that the minimum growth pH of *S. typhimurium* is lower than that of the other two organisms, but this property appears to be unrelated to the ATR and AR systems. It should be noted that strain differences may occur within a genus (e.g., *E. coli* CU4). While we have not made an exhaustive screening of natural isolates, we have tested between three and five isolates of each species and found essentially the same pattern (data not shown).

The AR mechanisms that require medium supplementation are found in *E. coli* and *S. flexneri* but are missing from *S. typhimurium*. Three AR systems have been identified so far. There is a low-pH-inducible system, possibly composed of many subsystems, that is expressed when cells undergo oxidative metabolism. Two other AR systems are discernable during fermentative metabolism; one requires glutamate, and the other is arginine dependent. None of the other amino acids tested provided acid resistance. The mechanisms by which arginine and glutamate protect *E. coli* and *S. flexneri* from acid are not clear. The amino acids could serve as precursors of some molecule, such as an amine, that would protect the interior of the cell in some way from acid stress. Arginine has already been implicated in the acid resistance of *Streptococcus sanguis* (4). As little as 2.9 mM arginine protected these cells from acid death at pH 4.0. The mechanism of acid resistance in *S. sanguis* is proposed to be an arginine deiminase that produces ammonia. The ammonia produced may serve as an internal buffer that protects the cell without increasing medium pH. However, our studies have shown that arginine-based acid survival in *E. coli* is due to decarboxylation by the product of the *adi* locus and so is clearly different from the *Streptococcus* system. The discovery that the *adi* product clearly provides an acid survival advantage to *E. coli* validates earlier predictions that arginine decarboxylase might play a role in acid resistance. However, it is curious that a similar prediction made for lysine decarboxylase could not be proven by this strategy.

TABLE 5. Effect of *adi* on arginine-based acid survival[a]

Strain	Relevant genotype	% Survival after pH 2.5 challenge			
		LBG	EG	EG + Arg	EG + glutamate
E. coli					
MC4100.GNB	*adi*$^+$	50.3	<0.001	36.3	39.6
EK198	*adi*::Mu*d*	83	<0.005	0.003	38
EF276	*adi*::Mu*d*/pKS29 *adi*$^+$	30	<0.004	3.5	6.3
S. typhimurium					
UK1		<0.002	<0.001	0.18	<0.004
JF2878	UK1/pKS29 *adi*$^+$	12.5	<0.001	5.3	<0.006
S. flexneri					
3136		90	<0.01	0.15	60
ShF1	3136/pKS29 *adi*$^+$	48	<0.03	2.7	18.75

[a] Cells were grown in LBG to stationary phase and diluted 1:1,000 into various pH 2.5 media as indicated for 2 h.

TABLE 6. Acid survival components

System[a]	Acid induced	Present in:		
		S. typhimurium	*E. coli*	*S. flexneri*
Minimal medium				
Log-phase ATR (pH 3.3)	Yes	Yes	Yes	No
Stationary-phase (pH 3.0)				
ATR	Yes	Yes	?	No
GSR	No	Yes	Yes	Yes
Complex medium (pH 2.5)				
Oxidative	Yes	No	Yes	Yes
Fermentative				
Glutamate	No	No	Yes	No
	Yes	No	No	Yes
Arginine (*adi*)	Yes	No	Yes	No

[a] Log-phase ATR challenge was at pH 3.3. Stationary-phase ATR challenge was at pH 3.0. GSR is the general stress resistance component of acid survival. Complex medium challenge was at pH 2.5.

The results with *E. coli* CU4 are curious. CU4 did not demonstrate an ATR, nor did it exhibit oxidative AR, but it did possess arginine- and glutamate-based AR systems, although at reduced levels relative to other strains of *E. coli*. The more acid-sensitive nature of CU4 suggests a general defect that affects the efficacy of all acid survival mechanisms.

Many acid-inducible genes require coinduction by low pH and a molecule that presumably serves as a substrate for the gene product. Examples are mannose for *aniG* (9), lysine for *cadBA* (1, 14, 27), and formate for *hyd* (2, 32). Melnykovych and Snell (25) found that the addition of arginine, methionine, tyrosine, asparagine, glutamate, and iron to minimal medium would allow full induction of arginine decarboxylase in *E. coli*. However, the same combination of amino acids did not induce *adi* to the point that acid survival occurred (data not shown). Why a component of LB other than arginine is required for full induction of the arginine system is not known. In contrast to the *adi* system, the glutamate-dependent AR system did not require exogenously supplied glutamate for induction in either *E. coli* or *S. flexneri*. Full induction of the glutamate system by stationary-phase cells occurred in minimal medium alone. It is possible that if glutamate is required for induction, the internal pool of glutamate may be sufficiently high in minimal medium-grown cells to serve as an inducer.

The role of σ^S in acid survival seems to be quite complex. Lee et al. (23) have demonstrated that the minimal medium stationary-phase ATR of *S. typhimurium* is not dependent upon the alternate sigma factor RpoS. Similarly, Small et al. (34) have shown that the anaerobic stationary-phase AR system of *E. coli* and *S. flexneri* is also independent of RpoS. Since this latter system is probably composed of both minimal medium ATR and amino acid-based AR components, one can also conclude that at least some of the pH 2.5 amino acid-based AR systems must be RpoS independent. Nevertheless, we can confirm, in part, the original conclusion by Small et al. (34) that *S. typhimurium* does not survive pH 2.5 because a gene system regulated by RpoS was missing from this organism. We have confirmed that *S. typhimurium* does not have the amino acid-based systems and found that the *S. flexneri* glutamate system depends upon *rpoS* (data not shown). However, one could suggest that the difference between *S. typhimurium* and the other enterics may be more profound than the loss of one system. Since we have identified several complex medium-based AR systems in *E. coli*, one can conclude either that *S. typhimurium* does not possess any of the amino acid-based systems or, if it does possess these systems, that they cannot overcome some basic defect (e.g., membrane structure) that makes *S. typhimurium* succumb to pH 2.5. Our results suggest that there is no basic defect in *S. typhimurium*, since transfer of a single gene, *adi*, into *S. typhimurium* afforded significant pH 2.5 acid resistance.

The evidence presented from this comparative analysis suggests that as close as these organisms are evolutionarily, they have developed strikingly different acid survival strategies. The reason for these differences may reflect different needs of the organisms based upon their ecological niches.

ACKNOWLEDGMENTS

We thank G. Bennett for kindly providing the *adi* strains and plasmids used and P. Couling for her careful preparation of the manuscript.

This work was supported by an award to J.W.F. (GM48017) from the National Institutes of Health and to J.L.S. (MCB-9305354) from the National Science Foundation.

REFERENCES

1. **Auger, E. A., K. E. Redding, T. Plumb, L. C. Childs, S.-Y. Meng, and G. N. Bennett.** 1989. Construction of *lac* fusions to the inducible arginine and lysine decarboxylase genes of *Escherichia coli* K-12. Mol. Microbiol. **3:**609–620.
2. **Birkman, A., and A. Böck.** 1989. Characterization of a *cis* regulatory DNA element necessary for formate induction of the formate dehydrogenase gene (*fdhF*) of *Escherichia coli*. Mol. Microbiol. **3:**187–195.
3. **Bullas, L. R., and J.-I. Ryu.** 1983. *Salmonella typhimurium* LT2 strains which are $r^- m^+$ for all three chromosomally located systems of DNA restriction and modification. J. Bacteriol. **156:**471–474.
4. **Casiano-Colón, A., and R. E. Marquis.** 1988. Role of the arginine deiminase system in protecting oral bacteria and an enzymatic basis for acid tolerance. Appl. Environ. Microbiol. **54:**1318–1324.
5. **Clements, J. D., D. C. Flint, and F. A. Klipstein.** 1982. Immunological and physicochemical characterization of heat-labile enterotoxins isolated from two strains of *Escherichia coli*. Infect. Immun. **38:**806–809.
6. **Curtiss, R., III, S. B. Porter, M. Munson, S. A. Tinge, J. O. Hassan, C. Gentry-Weeks, and S. M. Kelly.** 1981. Nonrecombinant and recombinant avirulent Salmonella vaccines for poultry, p. 169–198. *In* L. C. Blankenship, J. H. S. Bailey, N. A. Cox, N. J. Stern and R. J. Meinersmann, (ed.), Colonization control of human bacterial enteropathogens in poultry. Academic Press, New York.
7. **Fields, P. I., R. V. Swanson, C. G. Haidaris, and F. Heffron.** 1986. Mutants of *Salmonella typhimurium* that cannot survive within the macrophage are avirulent. Proc. Natl. Acad. Sci. USA **83:**5189–5193.
8. **Foster, J. W.** 1991. *Salmonella* acid shock proteins are required for the adaptive acid tolerance response. J. Bacteriol. **173:**6896–6902.
9. **Foster, J. W.** 1993. The acid tolerance response of *Salmonella typhimurium* involves transient synthesis of key acid shock proteins. J. Bacteriol. **175:** 1981–1987.
10. **Foster, J., and Z. Aliabadi.** 1989. pH-regulated gene expression in *Salmonella*: genetic analysis of aniG and cloning of the *earA* regulator. Mol. Microbiol. **3:**1605–1615.
11. **Foster, J. W., and B. Bearson.** 1994. Acid-sensitive mutants of *Salmonella typhimurium* identified through a dinitrophenol selection strategy. J. Bacteriol. **176:**2596–2602.
12. **Foster, J. W., and H. Hall.** 1990. Adaptive acidification tolerance response of *Salmonella typhimurium*. J. Bacteriol. **172:**771–778.
13. **Foster, J. W., and H. Hall.** 1991. Inducible pH homeostasis and the acid tolerance response of *Salmonella typhimurium*. J. Bacteriol. **173:**5129–5135.
14. **Foster, J. W., and H. Hall.** 1992. The effect of *Salmonella typhimurium* ferric uptake regulator (*fur*) mutations on iron- and pH-regulated protein synthesis. J. Bacteriol. **174:**4317–4323.
15. **Foster, J. W., Y. K. Park, I. S. Bang, K. Karen, H. Betts, H. K. Hall, and E. Shaw.** 1994. Regulatory circuits involved with pH-regulated gene expression in *Salmonella typhimurium*. Microbiology **140:**341–352.
16. **Goodson, M., and R. J. Rowbury.** 1989. Habituation to normal lethal acidity by prior growth of *Escherichia coli* at a sublethal acid pH value. Lett. Appl. Microbiol. **8:**77–79.
17. **Gorden, J., and P. L. C. Small.** 1993. Acid resistance in enteric bacteria. Infect. Immun. **61:**364–367.
18. **Hengge-Aronis, R.** 1993. Survival of hunger and stress: the role of *rpoS* in early stationary phase gene regulation in *E. coli*. Cell **72:**165–168.
19. **Heyde, M., and R. Portalier.** 1990. Acid shock proteins of *Escherichia coli*. FEMS Microbiol. **69:**19–26.
20. **Hickey, E. W., and I. N. Hirshfield.** 1990. Low-pH-induced effects on patterns of protein synthesis and on internal pH in *Escherichia coli* and *Salmo-*

nella typhimurium. Appl. Environ. Microbiol. **56:**1038–1045.
21. **Hoiseth, S. K., and B. A. D. Stocker.** 1981. Aromatic dependent *Salmonella typhimurium* are non-virulent and effective as live vaccines. Nature (London) **291:**238–239.
22. **Lee, I. S., J. Lin, H. K. Hall, B. Bearson, and J. W. Foster.** The stationary phase sigma factor σ^S (RpoS) is required for a sustained acid tolerance response in virulent *Salmonella typhimurium*. Mol. Microbiol., in press.
23. **Lee, I. S., J. L. Slonczewski, and J. W. Foster.** 1994. A low-pH-inducible stationary-phase acid tolerance response in *Salmonella typhimurium*. J. Bacteriol. **176:**1422–1426.
24. **Maloy, S. R., and J. R. Roth.** 1983. Regulation of proline utilization in *Salmonella typhimurium*: characterization of *put*::Mu*d*(Ap*lac*) operon fusions. J. Bacteriol. **154:**561–568.
25. **Melnykovych, G., and E. S. Snell.** 1958. Nutritional requirements for the formation of arginine decarboxylase in *Escherichia coli*. J. Bacteriol. **76:**518–523.
26. **Meng, S.-Y., and G. Bennett.** 1992. Nucleotide sequence of the *Escherichia coli cad* operon: a system for neutralization of low extracellular pH. J. Bacteriol. **174:**2659–2669.
27. **Miller, J. H.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
28. **Neeley, M. N., C. L. Dell, and E. R. Olsen.** 1994. Roles of LysP and CadC in mediating the lysine requirement for acid induction of the *Escherichia coli cad* operon. J. Bacteriol. **176:**3278–3285.
29. **O'Hara, G. W., and A. R. Glenn.** 1994. The adaptive acid tolerance response in root nodule bacteria and *Escherichia coli*. Arch. Microbiol. **161:**286–292.
30. **Raja, N., W. C. Goodson, C. Chui, D. G. Smith, and R. J. Rowbury.** 1991. Habituation to acid in *Escherichia coli*: conditions for habituation and its effects on plasmid transfer. J. Appl. Bacteriol. **70:**59–65.
31. **Rowbury, R. J., M. Goodson, and D. A. Wallace.** 1992. The PhoE porin and transmission of the chemical stimulus for induction of acid resistance (acid habituation) in *Escherichia coli*. J. Appl. Bacteriol. **72:**233–243.
32. **Schlensog, V., and A. Böck.** 1990. Identification and sequence analysis of the gene encoding the transcriptional activator of the formate hydrogenlyase system of *Escherichia coli*. Mol. Microbiol. **4:**1319–1326.
33. **Slonczewski, J. L., T. N. Gonzalez, M. Bartholomew, and N. J. Holt.** 1987. Mu*d*-directed *lacZ* fusions regulated by acid pH in *Escherichia coli*. J. Bacteriol. **169:**3001–3006.
34. **Small, P., D. Blankenhorn, D. Welty, E. Zinser, and J. L. Slonczewski.** 1994. Acid and base resistance in *Escherichia coli* and *Shigella flexneri*. J. Bacteriol. **176:**1729–1737.
35. **Stim, K. P., and G. N. Bennett.** 1993. Nucleotide sequence of the *adi* gene, which encodes the biodegradative acid-induced arginine decarboxylase of *Escherichia coli*. J. Bacteriol. **175:**1221–1234.
36. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218:**97–106.