Applied and Environmental Microbiology, May 2005, p. 2493–2500 Vol. 71, No. 5
0099-2240/05/$08.00+0 doi:10.1128/AEM.71.5.2493–2500.2005
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Variation in Acid Resistance among Shiga Toxin-Producing Clones of Pathogenic *Escherichia coli*†

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Received 27 July 2004/Accepted 22 November 2004

Pathogenic strains of *Escherichia coli*, such as *E. coli* O157:H7, have a low infectious dose and an ability to survive in acidic foods. These bacteria have evolved at least three distinct mechanisms of acid resistance (AR), including two amino acid decarboxylase-dependent systems (arginine and glutamate) and a glucose catabolite-repressed system. We quantified the survival rates for each AR mechanism separately in clinical isolates representing three groups of Shiga toxin-producing *E. coli* (STEC) clones (O157:H7, O26:H11/O111:H8, and O121:H19) and six commensal strains from ECOR group A. Members of the STEC clones were not significantly more acid resistant than the commensal strains when analyzed using any individual AR mechanism. The glutamate system provided the best protection in a highly acidic environment for all groups of isolates (<0.1 log reduction in CFU/ml per hour at pH 2.0). Under these conditions, there was notable variation in survival rates among the 30 O157:H7 strains, which depended in part on Mg^{2+} concentration. The arginine system provided better protection at pH 2.5, with a range of 0.03 to 0.41 log reduction per hour, compared to the oxidative system, with a range of 0.13 to 0.64 log reduction per hour. The average survival rate for the O157:H7 clonal group was significantly less than that of the other STEC clones in the glutamate and arginine systems and significantly less than that of the O26/O111 clone in the oxidative system, indicating that this clonal group is not exceptionally acid resistant with these specific mechanisms.

Escherichia coli is an ecologically versatile bacterium that has adapted to a variety of ecological conditions encountered in both animal hosts and the external environment. This organism has evolved multiple mechanisms to survive under low-pH conditions. *E. coli* O157:H7, a food- and waterborne pathogen, has been considered highly acid resistant in nature because of its low infectious dose (17) and ability to survive in acidic foods (32, 36, 45, 49, 50). In the laboratory, *E. coli* O157:H7 was shown to be exceptionally acid resistant compared to other enteric bacteria, such as *Salmonella enterica* (16), especially when exposed to mild acid (pH > 4) prior to exposure to strong acid (pH < 3) (4, 26). Outbreaks of human disease caused by *E. coli* O157:H7 in acidic foods, such as apple juice (13) and salami (12), have stimulated interest in determining the mechanisms behind the acid resistance (AR) of this organism.

Lin et al. developed assays to separate three different AR mechanisms by which *E. coli* can survive in low-pH environments for extended periods of time: two amino acid decarboxylase-dependent systems (arginine and glutamate) and a glucose catabolite-repressed system (27, 28). Their findings suggest that these mechanisms promote survival in low-pH environments, such as those encountered in the stomach and in acidic food products. The AR systems are governed, in part, by the alternate sigma factor RpoS (10, 34), which plays a central role in the regulation and expression of many proteins involved in stationary phase and the stress response (19, 25, 39). Mutant RpoS alleles exist in natural populations of enterohemorrhagic *E. coli* (EHEC) and can lead to acid sensitivity (44).

Studies of AR have focused on *E. coli* O157:H7 because it is one of the most common *E. coli* types associated with outbreaks and sporadic cases of food- and waterborne disease. Diarrheal disease caused by Shiga toxin-producing *E. coli* (STEC) of serotypes other than O157:H7 (non-O157 STEC) has increased worldwide, with recent outbreaks attributable to serotypes O26:H11, O111:H8, and O121:H19 (11, 29, 46). These serotypes mark three genetically distinct clonal groups of STEC: EHEC clonal group 1 consists of O157:H7 and its nonmotile relatives, EHEC clonal group 2 consists of serotypes O26:H11 and O111:H8, and strains of serotype O121:H19 represent a distinct clonal group of STEC (35, 41). Non-O157 STEC are common in animal reservoirs, capable of causing the same severe disease as *E. coli* O157:H7, and are thought to also have a low infectious dose (17); however, these pathogens are associated with disease outbreaks much less frequently than *E. coli* O157:H7. One reason for this disparity in prevalence may result from differences in the inherent acid resistance of the STEC clones and concomitant survival in acidic foods and low infectious dose.

Here we address the question of whether non-O157 STEC strains possess the same ability to survive using the defined AR mechanisms as *E. coli* O157:H7. To address this question, we assembled a collection of clinical isolates representing three clonal groups of STEC (O121:H19, O26/O111, and O157:H7) that have been screened for RpoS expression, and we assessed strain-to strain variation in survival at low pH using the three different AR mechanisms.

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† Supplemental material for this article may be found at http://aem.asm.org/.

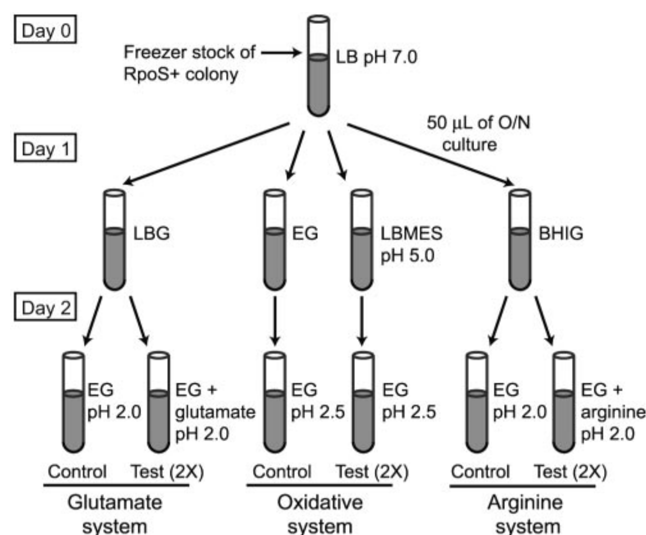

FIG. 1. Diagram of assay conditions for discriminating the three AR mechanisms.

MATERIALS AND METHODS

Bacterial strains. A total of 66 pathogenic strains, including 30 *E. coli* O157:H7 strains, 18 O26:H11 strains, 4 O111:H8 strains, and 14 O121:H19 strains, were used in this study (Table 1). The strains were originally isolated by different investigators from sporadic cases of diarrheal illness in the course of surveillance studies of STEC in the United States (1, 14, 15, 21, 23). We also obtained several additional STEC isolates from James Rudrik of the Michigan Department of Community Health, Lothar Beutin in Germany, and Roger Johnson in Canada. The presence of the Shiga toxin 1 and 2 genes (stx_1 and stx_2) is given in Table 1 as reported in the original publication or to us by the sender. In addition, six nontoxigenic, commensal strains from the ECOR (33) group A (ECOR strains 1, 2, 3, 4, 7, and 10) were used for comparative purposes.

Sequence types and Shiga toxin production. Multilocus sequence analysis of seven housekeeping genes (*aspC*, *clpX*, *fadD*, *icdA*, *lysP*, *mdh*, and *uidA*) was used to characterize the multilocus genotype of each strain. Alleles were identified based on sequence comparisons for each gene, and distinct allele combinations were designated as sequence types (STs). The sequencing methods and ST database are available at the STEC website (http://www.shigatox.net/mlst/index.html). The production of Shiga toxins was determined by enzyme immunoassay using the Premier EHEC test (Meridian Diagnostics Inc., Cincinnati, Ohio) according to the manufacturer's instructions.

RpoS status. Stock cultures of each strain were screened indirectly for the allelic state of *rpoS* with the hydrogen peroxidase II (HPII) assay (7, 48). For each strain, we picked 93 colonies from a Luria-Bertani (LB) agar plate which were then individually inoculated into wells of microtiter plates containing 100 μl of LB broth. Three wells were used for controls. After 3 h of incubation at 37°C, cultures in the 96 wells were transferred to replicate LB agar plates using a 96-pin replicator tool. Plates were incubated for 24 h at 37°C and were then tested for the expression of catalase by dropping 30% (wt/vol) hydrogen peroxide on each colony. Immediate vigorous bubbling indicated positive HPII activity, which is strongly correlated to expression of RpoS (37). Colonies were thus classified as $RpoS^+$ if they exhibited vigorous bubbling in the HPII assay. To confirm RpoS status, $HPII^+$ colonies were subsequently tested for glycogen production, which is also controlled by RpoS (20, 25). $HPII^+$ colonies were streaked onto LB agar and grown overnight at 37°C. Iodine was dropped onto individual colonies, and glycogen accumulation was detected by the formation of a brown color on the colonies (20). For each strain, a single-pick, $RpoS^+$ colony was grown in LB broth, frozen in 10% glycerol at −70°C, and used as stock for all subsequent AR assays.

AR mechanism assays. Methods for measuring AR were modified from those of Lin et al. (27, 28) as diagrammed in Fig. 1. On day zero, an $RpoS^+$ isolate of each STEC strain was inoculated from freezer stocks and grown overnight in LB medium (pH 7.0) at 37°C (Fig. 1). On day 1, 50 μl of overnight culture was used to inoculate 10 ml of LB broth with 0.4% glucose (LBG), LB broth buffered to pH 5.5 with 0.1 M morpholineethanesulfonic acid (LBMES), minimal E medium containing 73 mM K_2HPO_4, 17 mM $NaNH_4HPO_4$, 0.8 mM or 12 mM $MgSO_4$, 10 mM sodium citrate, and 0.4% glucose (EG), and brain heart infusion broth with a final concentration of 0.6% glucose (BHIG). All media were prepared by filter sterilization. Strains were grown in these media for 22 h at 37°C, with shaking at 120 rpm. Optical density at 600 nm was measured for each culture after 22 h of growth, and this measurement was used to determine the inoculum volume into the test environment that would have an initial density of $\sim 10^6$ CFU/ml.

The ability of the $RpoS^+$ isolates to survive acidic conditions was measured for three AR systems (27, 28): the glutamate-dependent (GLU) system, the glucose-repressed oxidative (OXI) system, and the arginine-dependent (ARG) system (Fig. 1). Cultures grown in LBG were tested in the GLU system (EG supplemented with 5.7 mM sodium glutamate at pH 2.0), cultures grown in LBMES were tested in the OXI system (EG at pH 2.5), and cultures grown in BHIG were tested in the ARG system (EG supplemented with 0.6 mM arginine at pH 2.5). Cultures inoculated in EG at pH 2.0 and pH 2.5 without supplements served as controls for the GLU and ARG systems. The control for the OXI system was cells grown overnight in EG pH 7.0 and tested in EG at pH 2.5. A 4 N HCl solution was used to adjust the pH of the test medium, which was then warmed to 37°C before use. All AR tests were conducted at 37°C. Two replicates of each strain were tested per AR system. Samples were taken at 0, 2, and 6 h and plated in duplicate on LB agar using the Autoplate 4000 (Spiral Biotech, Bethesda, MD). Plates were incubated at 37°C for 24 h and then enumerated using the Q Count software (Spiral Biotech).

To determine if Mg^{2+} limitation was contributing to cell inactivation (for example, through changing the permeability of the outer membrane [OM]), strains were also tested in the EG test environments described above, with the addition of 12 mM $MgSO_4$ (high Mg^{2+}) instead of the originally described 0.8 mM $MgSO_4$ (low Mg^{2+}).

Statistical analysis. Plate counts were converted to log CFU/ml values, and log decrease per hour for each assay was determined over the period of the assay. We defined the survival rate, ΔV, as the change in viable cell counts (in $\log_{10}$ CFU/ml) per hour and report the mean and standard deviation for each experiment. Analysis of variance was conducted on the log CFU/ml for each time point and the ΔV values for each assay using SAS (SAS Institute, Cary, NC). Pairwise comparisons were made for each of the clonal groups; significant differences were determined by the Tukey method, adjusted for multiple comparisons. Phylogenetic analysis of the multilocus sequences was conducted with MEGA 2 software (24).

RESULTS

Clonal groups. The STEC strains were sequenced at seven loci (3,753 bp; 1,251 codons) and classified into 27 STs based on the alleles resolved (Table 1). Most (24/30) of the O157:H7 strains belong to the most common EHEC 1 sequence type (ST-66). The close genetic relationship between the O26:H11 and O111:H8 strains is reflected by the fact that both serotypes are associated with the common EHEC 2 clone marked by ST-106 (Table 1). A neighbor-joining tree placed the 27 STEC STs into three distinct clusters (Fig. 2). The genetic diversity between strains within a cluster comprised only a fraction of the nucleotide divergence between clusters (Table 2). The nucleotide diversity within clusters ranged from 0.07% for EHEC 1 to 0.23% for the EHEC 2 group, whereas the divergence between clusters ranged from 1.1% to 2.6% (Table 2).

RpoS status and Stx production. To determine the variability in RpoS expression, we used the HPII catalase assay to examine 93 colony picks from each of 66 STEC strains (Table 1). For 32 of the strains, 100% of the 93 colonies tested were $RpoS^+$. Of the 30 *E. coli* O157:H7 strains tested, 15/30 (50%) had more than 90% $RpoS^+$ colonies. In comparison, 19/22 (83%) and 12/14 (86%) strains of *E. coli* O26/O111 and O121:H19 had more than 90% $RpoS^+$ colonies, indicating significant variation between clonal groups in the proportion of $RpoS^+$ colonies ($G_{adj} = 9.95$; $P < 0.01$, with degrees of freedom = 2). A single $RpoS^+$ colony was selected for each strain and used to create a stock for subsequent experiments.

TABLE 1. STEC strains used in this study

Serotype and strain	Locale (yr)	*stx* gene(s)	% RpoS$^+$	Sequence type	Stx EIA[a]	Source[b]
E. coli O157:H7						
TW07591	Mich. (1997)	stx_2	27	70	+	1
TW07695	Fla. (1997)	stx_1, stx_2	74	60	+	1
TW07700	Calif. (1997)	stx_1, stx_2	91	66	+	1
TW07702	Ohio (1997)	stx_2	95	59	+	1
TW07704	Ohio (1997)	stx_2	88	66	+	1
TW07706	Utah (1997)	stx_1, stx_2	88	66	+	1
TW07928	D.C. (1998)	stx_2	99	66	+	1
TW07937	Mass. (1998)	stx_2	76	66	+	1
TW07938	Mass. (1998)	stx_1, stx_2	100	66	+	1
TW07939	Mass. (1998)	stx_1, stx_2	100	66	+	1
TW07941	Mass. (1998)	stx_1, stx_2	45	66	+	1
TW07943	Mass. (1998)	stx_1, stx_2	96	66	+	1
TW07945	Fla. (1998)	stx_2	94	66	+	1
TW07949	D.C. (1999)	stx_1, stx_2	80	66	+	1
TW07950	D.C. (1999)	stx_1, stx_2	51	66	+	1
TW07952	D.C. (1999)	stx_1, stx_2	82	66	+	1
TW07953	D.C. (1999)	stx_1, stx_2	38	66	+	1
TW07956	D.C. (1999)	stx_1, stx_2	94	66	+	1
TW07957	D.C. (1999)	stx_1, stx_2	63	66	+	1
TW07958	D.C. (1999)	stx_1, stx_2	53	66	+	1
TW07961	Ohio (1998)	stx_1, stx_2	96	66	+	1
TW07962	Ohio (1998)	stx_1, stx_2	82	66	+	1
TW08022	Mont. (2000)	stx_1, stx_2	100	66	+	7
TW08026	Mont. (2000)	stx_1, stx_2	100	71	+	7
TW08027	Mont. (2000)	stx_1, stx_2	100	62	+	7
TW08030	Mont. (2000)	stx_2	45	72	+	7
TW08080	Mont. (2000)	stx_2	61	66	−	7
TW08609	Wash. (2000)	stx_2	100	66	+	7
TW08610	Wash. (2000)	stx_2	100	66	+	7
TW08612	Wash. (2000)	stx_1, stx_2	100	66	+	7
E. coli O26:H11						
TW07594	Ariz. (1997)	stx_1	96	110	+	1
TW07595	Neb. (1998)	stx_1	100	106	+	3
TW07600	Neb. (1998)	stx_1	100	106	+	3
TW07622	Mich. (2002)	stx_1, stx_2	4	106	+	5
TW07814	Idaho (1997)	stx_1, stx_2	100	104	+	6
TW07936	Mass. (1998)	stx_1	100	10	+	1
TW07948	D.C. (1999)	stx_1	97	106	+	1
TW08024	Mont. (2000)	stx_1	100	114	+	7
TW08033	Mont. (2000)	stx_1	100	102	+	7
TW08038	Mont. (2000)	stx_1	98	101	+	7
TW08052	Mont. (2000)	stx_1	76	103	+	7
TW08060	Mont. (2000)	stx_1	100	112	+	7
TW08084	Mont. (2000)	stx_1	99	99	+	7
TW08569	Germany (1998)	stx_2	54	115	−	2
TW08570	Germany (1999)	stx_2	94	113	+	2
TW08571	Germany (2000)	stx_1, stx_2	100	104	+	2
TW08637	Wash. (2000)	stx_1	100	106	+	7
TW09184	Mich. (2003)	*ND*	100	106	+	5
E. coli O111:H8						
TW07598	Neb. (1998)	stx_1, stx_2	100	106	+	3
TW07601	Neb. (1998)	stx_1, stx_2	100	106	+	3
TW08642	Wash. (1999)	stx_1	100	106	+	7
TW08643	Wash. (2001)	stx_1	100	106	+	7
E. coli O121:H19						
TW07615	Mich. (2002)	stx_2	82	182	+	5
TW07927	D.C. (1998)	stx_1, stx_2	100	182	+	1
TW07931	Mass. (1998)	stx_2	100	182	+	1
TW08036	Mont. (2000)	stx_2	100	182	+	7
TW08040	Mont. (2000)	stx_2	100	183	+	7
TW08042	Mont. (2000)	stx_2	100	180	+	7
TW08043	Mont. (2000)	stx_2	90	182	+	7
TW08055	Mont. (2000)	stx_2	88	187	+	7
TW08063	Mont. (2000)	stx_2	99	184	+	7
TW08091	Mont. (2000)	stx_2	100	179	+	7
TW08646	Wash. (2000)	stx_2	100	133	−	7
TW08649	Switzerland (2002)	stx_2	100	185	+	4
TW08650	Switzerland (2002)	stx_2	100	181	+	4
TW08653	Canada (2002)	stx_2	100	182	+	4

[a] Shiga toxin production was tested by enzyme-linked immunoassay on a single RpoS$^+$ colony.

[b] Strains were obtained from the following sources: 1, D. Acheson (1, 43); 2, L. Beutin, Robert Koch Institute, Germany; 3, P. Fey (14); 4, R. Johnson, Laboratory of Foodborne Zoonoses, Canada; 5, J. Rudrik, Michigan Department of Community Health; 6, A. O'Brien (15); 7, P. Tarr (21, 23).

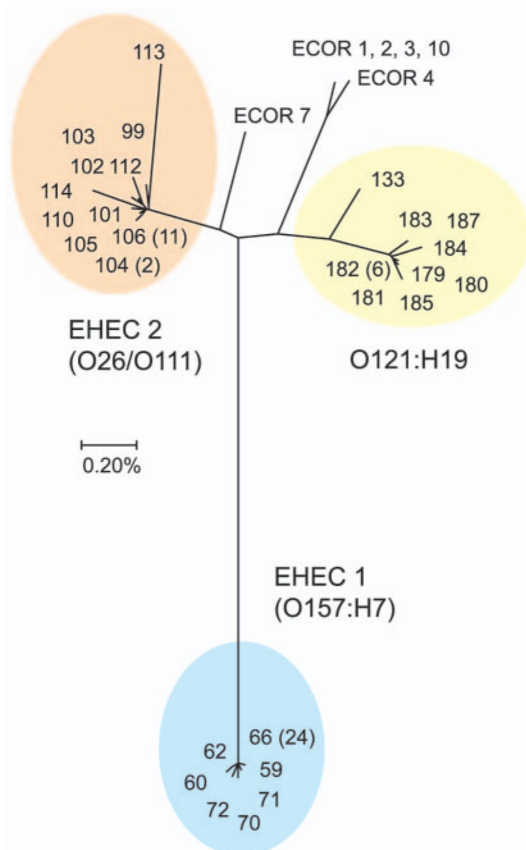

FIG. 2. Neighbor-joining tree showing the clonal groups of STEC based on multilocus STs. Distances were estimated by the Kimura two-parameter model. The number of isolates of each ST is given in parentheses.

The RpoS$^+$ derivatives of the 66 STEC strains were all tested for Shiga toxin production using the Stx enzyme immunoassay; 63 of the 66 RpoS$^+$ isolates were Stx positive (Table 1). We further investigated the three Stx-negative isolates by PCR and found that one had lost the stx_2 gene in comparison to the original STEC strain and one had stx_2 but did not express it. In the third case the original STEC strain was also negative for the stx_2 gene, suggesting that the toxin gene had been lost in a previous transfer.

Variation in AR among *E. coli* O157:H7 strains. Strains of *E. coli* O157:H7 exhibited variation in survival when using each of the AR mechanisms. Final cell densities after 6 h in the GLU system approximated a normal distribution (Anderson-Darling normality test, $P = 0.121$) with a mean $\log_{10}$ CFU/ml of 5.43 ± 0.32. A nested analysis of variance indicated that the among-strain component of the variance was highly significant ($F_{0.05}$ [29, 50] = 75.6; $P < 0.001$) and accounted for 92% of the total variation in cell densities at 6 h. Similar distributions were observed after 6 h for the OXI system (3.09 ± 0.71) and for the ARG system (4.79 ± 0.46) (data not shown).

TABLE 2. Nucleotide diversity within and between three clonal groups of STEC

Clonal group	No. of isolates	No. of STs	Diversity within group (%)	Distance (%) between groups	
				EHEC 1	EHEC 2
EHEC 1	30	7	0.07 ± 0.03		
EHEC 2	22	11	0.23 ± 0.04	2.47 ± 0.28	
O121:H19	14	9	0.19 ± 0.04	2.60 ± 0.26	1.10 ± 0.17

Effect of magnesium on AR of *E. coli* O157:H7. The permeability of the bacterial OM can be disturbed by chelators that disrupt the stabilizing interactions of Mg^{2+}, Ca^{2+}, and lipopolysaccharide molecules (42). Decreased OM stability resulting from chelators could amplify cell inactivation that is perceived to be a consequence of acid stress alone. For example, the citrate present in the test environments could chelate Mg^{2+}, leading to a more permeable OM.

To test for the effects of Mg^{2+} limitation, we challenged strains in both low (0.8 mM $MgSO_4$) and high (12 mM $MgSO_4$) Mg^{2+} concentrations. Under conditions of low Mg^{2+}, ΔV_{GLU} values approximated a normal distribution, with a mean ΔV_{GLU} of -0.09 ± 0.05 (Fig. 3A). ΔV_{ARG} values also approximated a normal distribution, with a mean ΔV_{ARG} of -0.22 ± 0.08 (Fig. 3C). The O157:H7 strains had significantly greater survival rates in the GLU and ARG systems when the $MgSO_4$ levels were increased, and the variation in survival rates decreased. The mean ΔV_{GLU} with high Mg^{2+} was -0.05 ± 0.02. The mean ΔV_{ARG} when $MgSO_4$ was increased was -0.17 ± 0.06. In the GLU system, 22/30 (74%) strains had a significantly greater survival rate with high Mg^{2+} than low Mg^{2+}. In the ARG system, 14/30 (46%) strains had a significantly higher survival rate with high Mg^{2+}. In contrast, the survival rates were not significantly different between high and low Mg^{2+} for the OXI system (Fig. 3B).

Comparison of STEC clones. The glutamate system provided the most protection under acidic conditions, with average ΔV_{GLU} values ranging from 0.004 ± 0.04 for the O26/O111 clone to -0.09 ± 0.05 for the O157:H7 group (Fig. 4A). In the first 2 h of the assay, the O157 strains had a ΔV_{GLU} similar to that of the O26/O111 strains. However, in the 2- to 6-h time period (t_2-t_6), the density of bacteria of the O157:H7 group decreased at a faster rate [$\Delta V_{GLU(t2\text{-}t6)} = -0.10 \pm 0.01$] than strains of the other clonal groups. As a result, the ability of *E. coli* O157:H7 strains to survive using the GLU system was significantly less ($P < 0.001$) than the other clonal groups in the 6-hour assay period. Strains of the non-O157 clonal groups were also tested under high-Mg^{2+} conditions, but there were no significant differences in survival rates for any of the clonal groups with the exception of O157:H7 (see Table S3 in the supplemental material), and so all ΔV values reported are from low-Mg^{2+} test environments.

The oxidative system provided the least amount of protection against acidic environments, with average ΔV_{OXI} values ranging from -0.28 ± 0.08 for the O26/O111 strains to -0.44 ± 0.12 for the O157:H7 strains (Fig. 4B). In this assay, strains in the O157:H7 clonal group decreased in number at a significantly ($P < 0.01$) greater rate ($\Delta V_{OXI} = -0.44 \pm 0.12$) than the O26/O111 clonal group. The greatest variability in survival rate among strains of each clonal group was seen in the OXI system (Fig. 4B). The *E. coli* O121:H19 and ECOR strains had a similar ability to survive acidic conditions and were not significantly different from the O26/O111 or O157:H7 groups

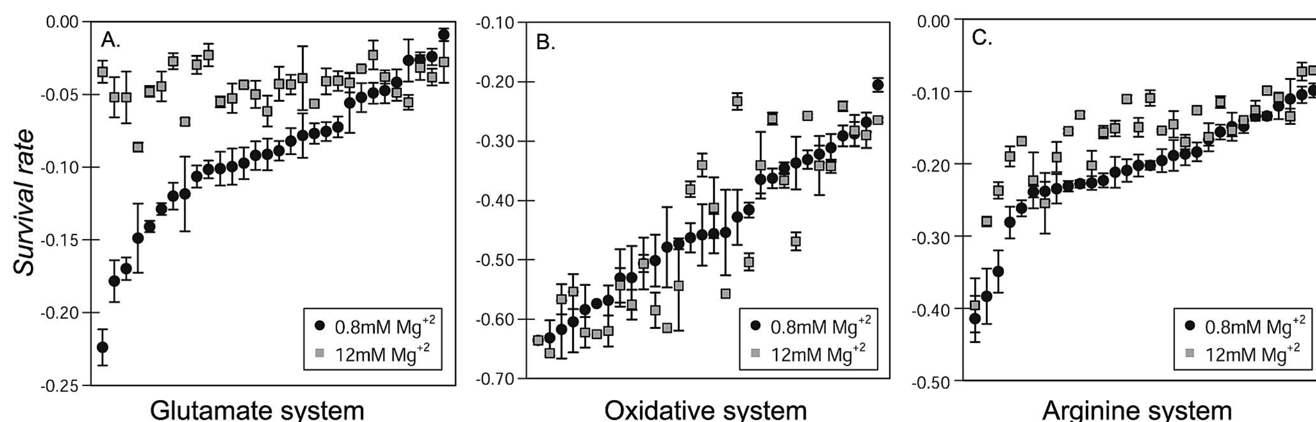

FIG. 3. Variation in survival rates (ΔV = change in the density of viable cells per h) among 30 *E. coli* O157:H7 strains using each AR system. Strains are ranked by the average ΔV from lowest to highest survival and plotted in order from left to right. Error bars indicate standard deviations of two replicate samples. Closed circles indicate survival under low-Mg^{2+} (0.8 mM) conditions, and open squares indicate survival under high-Mg^{2+} (12 mM) conditions. Strains were ranked separately for each AR system. A. ΔV values at pH 2.0 for the glutamate system. B. ΔV values at pH 2.5 for the oxidative system. C. ΔV values at pH 2.5 for the arginine system.

when using the oxidative system as measured by the average ΔV_{OXI} (Fig. 4B).

The arginine system did not provide as much protection as the glutamate system but was more effective than the oxidative system at pH 2.5. ΔV_{ARG} values ranged from -0.11 ± 0.05 for O121 strains to -0.22 ± 0.08 for the O157:H7 strains (Fig. 4C). Pairwise comparisons of the average ΔV_{ARG} value for each clonal group indicated that the O157:H7 strains were significantly different from the O26/O111 and O121 strains ($P < 0.003$) but not the ECOR A group in the arginine system (Fig. 4C). The majority of strains exhibited high survival with the GLU system, whereas the abilities to utilize the OXI and ARG systems were more variable across strains. Strain TW08650 (O121:H19) has high survival rates with all three mechanisms, with $\Delta V_{GLU} = -0.01$, $\Delta V_{OXI} = -0.13$, and $\Delta V_{ARG} = -0.12$. Strain TW07962 (O157:H7) exhibits low survival rates in two of the systems, with $\Delta V_{GLU} = -0.09$, $\Delta V_{OXI} = -0.45$, and $\Delta V_{ARG} = -0.35$. Overall, the survival rate using one mechanism is not significantly correlated with the ability to survive using another (r^2 for GLU versus OXI = 0.42; r^2 for OXI versus ARG = 0.19; r^2 for GLU versus ARG = 0.15). ΔV values for each strain can be found in Table S4 of the supplemental material.

We partitioned the total phenotypic variance in survival rate into within-clonal and between-clonal group components. For the glutamate system, 42% of the variation was due to differences between strains within a clonal group and 52% was due to differences between clonal groups. For the oxidative system, 62% was due to differences between strains within group while 33% was due to differences between clonal groups. For the arginine system, 67% was due to differences between strains within a clonal group and 30% was due to differences between the clonal groups. These results indicate that a substantial fraction of the total variation in acid resistance for a given mechanism is accounted for by the differences between STEC lineages.

$RpoS^+$ versus $RpoS^-$ colonies. Eight strains of *E. coli* O157:H7 (TW07591, TW07695, TW07702, TW07704, TW07928, TW07941, TW07962, and TW08030) were selected, and the $RpoS^+$ and $RpoS^-$ isolates derived from single colo-

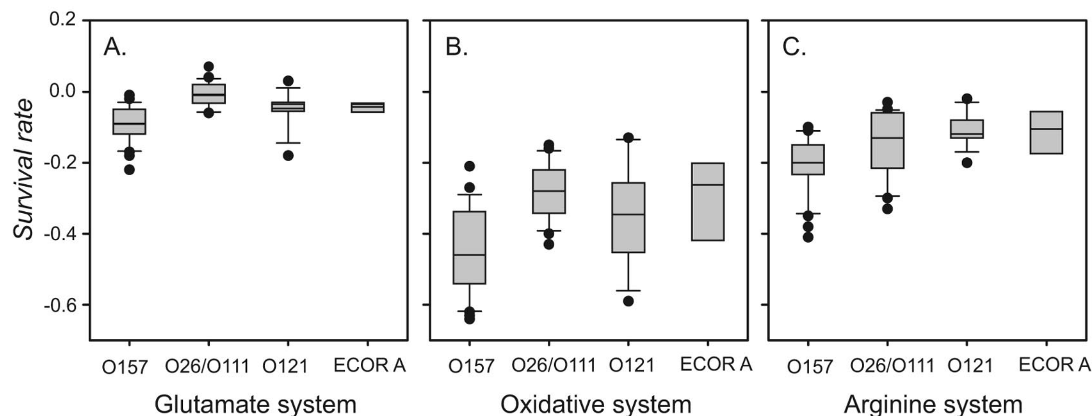

FIG. 4. Box plots of survival rates (ΔV) for strains in each clonal group. The horizontal bar indicates the mean for each group. A. ΔV values for each serogroup in the glutamate system at pH 2.0. B. ΔV values for each serogroup in the oxidative system at pH 2.5. C. ΔV values for each group in the arginine system at pH 2.0.

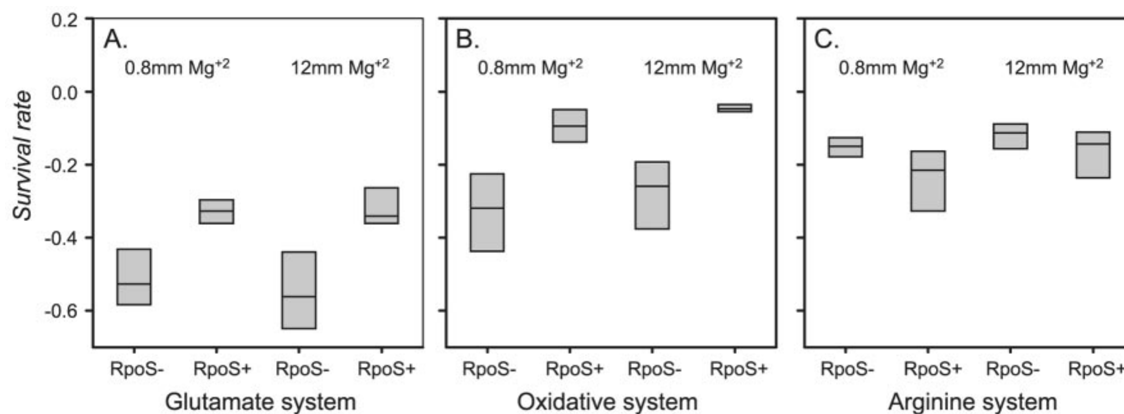

FIG. 5. Box plots of survival rates (ΔV) for paired $RpoS^+$ and $RpoS^-$ isolates from eight strains of *E. coli* O157:H7 in each AR system. Tests were conducted under low-Mg^{2+} (0.8 mM) and high-Mg^{2+} (12 mM) conditions.

nies for each strain were tested for their abilities to survive using the three AR mechanisms. In the glutamate system, the $RpoS^-$ isolates (colonies that exhibited slow bubbling in the HPII assay) all exhibited significantly lower mean survival rates ($t_{[0.05,\ 60]} = 35.97$; $P < 0.001$) than the corresponding $RpoS^+$ isolate at both levels of Mg^{2+}, with the exception of strain TW07962 in low Mg^{2+} (Fig. 5A). In the oxidative system, only strains TW07962 and TW07928 did not exhibit significantly lower survival rates ($t_{[0.05,\ 60]} = 24.39$; $P < 0.001$) for the $RpoS^-$ isolates compared to the corresponding $RpoS^+$ isolate at both levels of Mg^{2+} (Fig. 5B). In contrast, the $RpoS^-$ isolates exhibited similar or greater survival than the $RpoS^+$ isolates in the arginine system (Fig. 5C).

DISCUSSION

E. coli O157:H7 was first thought to have enhanced abilities to resist acid and other environmental stresses because of its low infective dose and ability to survive in and be transmitted by acidic foods. Strain-to-strain variation in AR ability has been reported (albeit with a small number of strains), with a continuum of survival abilities classified from highly resistant to sensitive. For example, Buchanan and Edelson (9) observed that the ability to survive in acidic environments varies substantially among isolates within a single serotype; AR of EHEC strains grown to stationary phase was dependent on the type of acidulant and whether the pH-dependent AR system had been induced (9). Benjamin and Datta (5) compared survival of six O157:H7 strains in LB at pH 2.5 and found that two of the strains were considered highly acid tolerant while the other four strains were moderately and slightly acid tolerant. Leyer et al. (26) measured survival of acid-adapted and nonadapted cells of five strains of *E. coli* O157:H7; although the acid-adapted cells had a better survival rate than the nonadapted, there was variability in survival rates among the strains. Lin et al. (28) assayed 11 strains of *E. coli* O157:H7 for their ability to use three different AR mechanisms to protect against high acidity. Considerable variation existed in the abilities of strains to use a particular AR mechanism, indicating that not all O157:H7 strains had the same capability to survive in a low-pH environment using a specific AR mechanism (28). Our analysis with 30 O157:H7 isolates collected independently from sporadic cases of disease shows continuous variation in ability to survive at low pH using these AR mechanisms and that survival rates fit a normal distribution. In addition, more than half (~50% to 70%) of the total phenotypic variation in acid resistance among STEC clones is a result of between-strain variation within a clonal group. This finding suggests that there are additional factors that contribute to variability in acid resistance of STEC strains.

Understanding the extent of variation among pathogens of a group, for example of *E. coli* O157:H7 strains, is of considerable importance, especially for purposes of predictive modeling. Whiting and Golden have identified the differences among strains of the same species as a major source of variation in growth and survival studies; variations determined for four different parameters (growth, survival, thermal inactivation, and toxin production) of *E. coli* O157:H7 strains were larger than the error calculated from experimental procedures using a single strain or cocktail (47). We also observed that the strain-to-strain variation within a clonal group was larger than variation between replicates or due to experimental error. Thus, drawing conclusions about differences in the acid resistance abilities of a variable group of bacteria will require examination of large numbers of isolates.

The alternate sigma factor RpoS governs gene expression during stationary phase and in response to stressful environments. Variation in the expression of this global regulator can contribute to overall variability in the stress response. It is clear that expression of the alternate sigma factor *rpoS* is required for stationary-phase induction of acid resistance in *E. coli* and *Shigella* (39), and Arnold and Kaspar found that as the growth of *E. coli* O157:H7 neared stationary phase, survival of cells in tryptic soy broth at pH 2.0 increased dramatically (4).

Expression of *rpoS* can vary in natural populations of *E. coli*, possibly because *rpoS* null mutants have a competitive advantage in prolonged exposure under starvation conditions (48). Variation in *rpoS*, ranging from null to full expression, could account for a component of the variability observed in the AR ability of *E. coli* strains. Waterman and Small found during a survey of AR of 58 STEC strains that mutant *rpoS* alleles exist in natural populations of STEC. Complementation with *rpoS*

on a plasmid conferred AR to 9 of 13 acid-sensitive strains (44). We also observed variation in the allelic state of RpoS among STEC, with *E. coli* O157:H7 strains having the most variability. In this study, we attempted to correct for the potential confounding effects of *rpoS* null mutants in our isolates by assaying RpoS activity indirectly through the HPII assay (7) and selecting $RpoS^{+}$ colonies to test AR.

Lin et al. determined that functional *rpoS* is required for a strain to be able to use the oxidative mechanism of acid resistance. A mutant *rpoS* allele resulted in a reduced ability to utilize the arginine and glutamate mechanisms of AR (28). In a comparison of survival using a specific AR mechanism of eight *E. coli* O157:H7 strains that had $rpoS^{+}$ and $rpoS^{-}$ isolates, our results did not agree with those of Lin et al. In the arginine system, mutant *rpoS* isolates survived better than the wild-type isolates. The allelic state of RpoS was confirmed in these strains by testing colonies for HPII activity with hydrogen peroxide and for glycogen production with iodine. Arginine decarboxylase, encoded by *adiA*, is positively regulated by CysB (10, 38) and by AdiY, a transcriptional regulator encoded downstream of *adiA* (40). It is possible that RpoS does not contribute to regulating expression of *adiA*. However, further studies are needed to determine the role of RpoS and other regulators in governing the arginine AR system.

Chelating compounds, as well as organic and inorganic acids, increase the permeability of the OM (2, 18), which has been linked to loss of viability during acid stress in *E. coli* O157:H7 (22). Bacterial inactivation by acid could be aided by the presence of chelators in the test environment. Citric acid has been shown to be a potent OM permeabilizer in O157:H7, and the permeabilization effect can be abolished with the addition of 5 mM Mg (18). Mg^{2+} stabilizes cell membranes (8) by interacting with adjacent lipopolysaccharide molecules (42). Our results indicate that high levels of magnesium in the low-pH environment significantly increase the survival rates of most *E. coli* O157:H7 strains in the glutamate system. The increase in survival is not as dramatic for the arginine system, possibly because this system is tested at a higher pH. Interestingly, bacteria of the other STEC clonal groups, as well as isolates of the ECOR group, were not significantly affected by the increased levels of magnesium in these two AR systems. No differences were observed in survival for the O157 and O26/O111 clones in the OXI system at the two levels of magnesium, but the O121 group showed a significantly lower survival rate at 12 mM Mg in the OXI system. Further work is needed to understand the mechanisms by which Mg^{2+} concentration influences survival in acidic environments.

In vitro studies on AR of *E. coli* O157:H7 strains, other pathogenic *E. coli* strains, and different enteric bacteria have indicated that *E. coli* O157:H7 can exhibit AR, but whether O157:H7 strains have superior ability to survive in acid has not been substantiated. The consensus seems to be that *E. coli* O157:H7 is well suited to adverse conditions even though little direct evidence supports the hypothesis that this pathogen is substantially different from other *E. coli* serotypes in AR abilities (30). Most AR studies are based on only a few non-O157 STEC strains of the same serotype, which reduces the statistical power to detect significant differences between groups. For example, Miller and Kaspar observed survival of two strains of *E. coli* O157:H7 and a single nonpathogenic strain under acidic conditions (32). While a significant difference in survival was reported between the O157:H7 and nonpathogenic strains, their suggestion that resistance to acidic pH is an additional characteristic that distinguishes O157:H7 from other *E. coli* serotypes is not warranted, because other pathogenic *E. coli* serotypes were not tested. Benjamin and Datta compared survival of 14 EHEC strains in LB at pH 2.5 and 3.0 and found that no correlation could be drawn between acid tolerance and serotype (5), but again only a few non-O157 serotypes were evaluated. McKellar and Knight surveyed a set of 19 EHEC strains for ability to survive in tryptic soy broth acidified with HCl (pH 2.0) and with acetic acid (pH 4.0). When strains were grouped by source, they found that survival of outbreak strains in acid was significantly greater than in strains from human or animal sources. When strains were grouped by serotype, however, strains of *E. coli* O157:H7 were among the more acid sensitive, but clear conclusions could not be drawn since other EHEC serotypes were only represented by a single strain each (31). Berry et al. recently reported a comprehensive comparison of AR between 39 isolates of O157 and 20 isolates of non-O157 biotype I *E. coli* and found no significant differences in the ability of strains from either group to survive in BHI at pH 2.5; they concluded that high acid tolerance is not unique to strains of pathogenic *E. coli* (6). Our results indicate that strains of *E. coli* O157:H7 do not have superior AR abilities compared to strains of *E. coli* O121:H19 and O26:H11 using the three AR mechanisms. While *E. coli* O157:H7 clones can survive in low pH using the three described AR mechanisms, there is no evidence that O157:H7 has greater acid resistance in any single system than other clones of STEC in these laboratory test environments.

In a comparison of three clonal groups of STEC and a clonal group of nonpathogenic ECOR strains, we have shown that strains of *E. coli* O157:H7 do not have superior AR abilities using three specific AR mechanisms. Under natural conditions, however, *E. coli* O157:H7 may have the ability to use multiple mechanisms in combination, through some form of epistasis, to achieve higher AR than bacteria of other clonal groups. It is also possible that *E. coli* O157:H7 has evolved alternative mechanisms, yet to be identified, which contribute to acid resistance in nature. This study suggests that there is no evidence for enhanced AR of O157:H7 by a measurable superiority of one of the three major AR mechanisms of *E. coli*. There is also compelling evidence for a substantial strain-to-strain component of variation in survival rate; more than 60% of the total variance in survival rates for the oxidative and arginine systems was attributed to between-strain differences. Our findings demonstrate that because of the high interstrain variability, a large number of isolates should be examined in order to test and draw conclusions about survival of *E. coli* O157:H7 and non-O157 STEC. A narrow focus on a few isolates or a single serotype may seriously underestimate the variability that underlies important phenotypic variation in pathogen populations.

ACKNOWLEDGMENTS

We thank the following people for kindly providing the strains used in this study: David W. Acheson, CFSAN; Lothar Beutin, Robert Koch Institute; Paul D. Fey, University of Nebraska; Alison D. O'Brien, Uniformed Services University of the Health Sciences; Phillip I. Tarr,

Washington University at St. Louis; Roger Johnson, University of Guelph; and James Rudrik from the Michigan Department of Community Health. We also thank Lukas Wick and Shannon Manning for helpful discussion.

This research was supported in part by the Michigan Agriculture Experimental Station.

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