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Evolutionary Silence of the Acid Chaperone Protein HdeB in Enterohemorrhagic *Escherichia coli* O157:H7

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The periplasmic chaperones HdeA and HdeB are known to be important for cell survival at low pH (pH < 3) in *Escherichia coli* and *Shigella* spp. Here we investigated the roles of HdeA and HdeB in the survival of various enterohemorrhagic *E. coli* (EHEC) following exposure to pH 2.0. Similar to K-12 strains, the acid protections conferred by HdeA and HdeB in EHEC O145 were significant: loss of HdeA and HdeB led to over 100- to 1,000-fold reductions in acid survival, depending on the growth condition of prechallenge cells. However, this protection was much less in *E. coli* O157:H7 strains. Deletion of *hdeB* did not affect the acid survival of cells, and deletion of *hdeA* led to less than a 5-fold decrease in survival. Sequence analysis of the *hdeAB* operon revealed a point mutation at the putative start codon of the *hdeB* gene in all 26 *E. coli* O157:H7 strains analyzed, which shifted the ATG start codon to ATA. This mutation correlated with the lack of HdeB in *E. coli* O157:H7; however, the plasmid-borne O157-*hdeB* was able to restore partially the acid resistance in an *E. coli* O145Δ*hdeAB* mutant, suggesting the potential function of O157-HdeB as an acid chaperone. We conclude that *E. coli* O157:H7 strains have evolved acid survival strategies independent of the HdeA/B chaperones and are more acid resistant than nonpathogenic K-12 for cells grown under nonfavorable culturing conditions such as in Luria-Bertani no-salt broth at 28°C. These results suggest a divergent evolution of acid resistance mechanisms within *E. coli*.

Enterohemorrhagic *Escherichia coli* (EHEC) strains include a diverse group of Shiga toxin-producing *E. coli* (STEC) strains that cause bloody diarrhea, hemorrhagic colitis, and life-threatening hemolytic-uremic syndrome (HUS). *Escherichia coli* O157:H7 is the most common EHEC serotype and contributes significantly to human infections and outbreaks in the United States. In a recent surveillance study that was largely based upon the data available in 2006, the estimated annual number of domestically acquired food-borne hospitalizations caused by *E. coli* O157:H7 in the United States was almost 8-fold higher than that of non-O157 STEC (41). This predominance of *E. coli* O157:H7 in food-borne illness cannot be explained simply by its virulence determinants, since similar virulence determinants, including genes encoding Shiga toxins, are present in other non-O157 EHEC strains. Thus, these EHEC strains possess a pathogenic potential similar to *E. coli* O157:H7 (35).

EHEC strains naturally reside in ruminants, primarily cows, and are transmitted to humans mainly through food vehicles. Enhanced survival fitness in different environments (inside and outside a host) would increase the environmental prevalence of the pathogen, resulting in increased EHEC infections. To cause infection, EHEC must survive in an extremely acidic environment (pH < 3) within the host's stomach. Similar to *Shigella* spp., *E. coli* can survive at pH 2.0 for several hours, which is attributed to at least four well-studied acid resistance systems: the oxidative acid resistance system (AR1) and the glutamate-, arginine-, and lysine-dependent acid resistance systems (AR2, AR3, and AR4, respectively) (17, 48). Furthermore, *E. coli* encodes chaperone proteins, HdeA and HdeB, known to confer protection at low pH (pH < 3); this protection of periplasmic proteins from the acid-induced damages is particularly important considering the high permeability of outer membrane (25, 32, 46). It is noteworthy that genes encoding these acid resistance systems and chaperone proteins are well conserved in both EHEC and nonpathogenic strains such as K-12, but distinct differences in acid resistance have been reported between commensal *E. coli* and *E. coli* O157:H7, between different serotypes of EHEC strains, between *E. coli* O157:H7 strains from various environmental origins, or even between natural variants isolated from the same *E. coli* O157:H7 strain (4, 6, 7, 9, 26, 36). The enhanced acid resistance in *E. coli* O157:H7 could be explained in part by its genetic repertoire, since strains of this pathogroup contain the largest genome on average among complete *E. coli* genomes (30). *E. coli* O157:H7 strain EDL933 carries an additional 1.34 Mbp of DNA organized as 177 genomic islands (O-islands) compared to the *E. coli* K-12 strain MG1655 (38). Transcriptomic analyses of *E. coli* O157:H7 in response to acid stress revealed upregulation of a large number of O-island genes, suggesting that this pathogroup may be equipped with additional genes/pathways to ensure their survival in acidic environments (5, 26).

The *hdeA* and *hdeB* genes were identified initially by Yoshida et al. in studies characterizing H-NS nucleoid protein-dependent genes in *E. coli* (47). The *hdeAB* genes are conserved in various serotypes and pathotypes of *E. coli*, indicating that they are part of the *E. coli* core genome. In K-12 strains, the *hdeAB* genes are located on a 12-gene island known as the acid fitness island (AFI). Many genes on this island have been shown to play a role in the acid resistance of *E. coli* under various conditions (32). This 12-gene island is conserved also in several non-O157 EHEC strains (O26:H11, O103:H2, and O111:H$^-$); however, in *E. coli* O157:H7, this island is much larger due to the insertion of a nine-gene clus-

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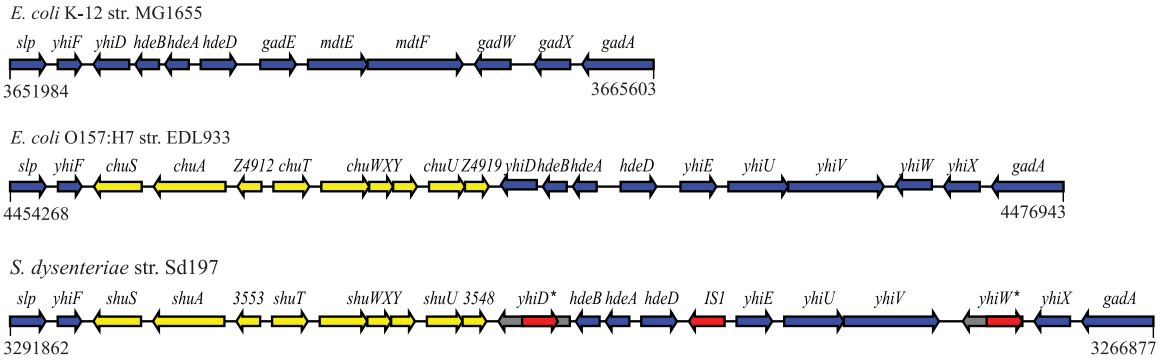

FIG 1 Genomic location of *hdeA* and *hdeB* in *E. coli*. *hdeA* and *hdeB* are located on a 13-kb acid fitness island (AFI) in K-12 strain MG1655 (blue arrows). This island is conserved in the *E. coli* O157:H7 strain EDL933, except that the O-island 140 (yellow arrows) was inserted between the acid fitness genes *yhiF* and *yhiD*. The O-island 140 consists of nine genes involved in iron acquisition (34). Homologs of O-island 140 were present also in *S. dysenteriae* (yellow arrows). *yhiE*, *yhiU*, *yhiV*, *yhiW*, and *yhiX* in strain EDL933 correspond to *gadE*, *mdtE*, *mdtF*, *gadW*, and *gadX* in strain MG1655, respectively. Numbers indicate the genomic position of the AFI in each strain. The AFI in *S. dysenteriae* is highly similar to that of *E. coli* O157:H7, except that a few genes (e.g., *yhiD* and *yhiW* [*gadW*]) were interrupted by insertion sequence elements (red arrows) as indicated by an asterisk. The sequences shown here were retrieved from GenBank (MG1655, NC_000913; EDL933, AE005174.2; *S. dysenteriae*, CP000034). *Escherichia coli* strains that carry an MG1655-type AFI include strains BW2952 (CP001396), K-12 ATCC 8739 (NC_010468), O26:H11 strain 11368 (AP010953), O103:H2 strain 12009 (AP010953), and O111:H$^-$ strain 11128 (AP010960). *Escherichia coli* strains that carry an EDL933-type AFI include O157:H7 strains TW14359 (CP001368), Ec4115 (CP001164), and Sakai (BA000007) and O55:H7 strain CB9615 (CP001846).

ter that has been designated O-island 140 in strain EDL933 (38). A similar AFI is present also in the enteropathogenic *E. coli* strain CB9615 (O55:H7) and in *Shigella dysenteriae* (Fig. 1). Transcription of the *hdeAB* operon is regulated at hierarchical levels by more than 10 master transcriptional regulators, including the transcriptional activators RpoS and GadE, and the repressors H-NS and MarA (40). Expression of *hdeAB* is induced in stationary phase or at acidic pH (21). HdeA and HdeB appear to function in a similar fashion by dissociating, upon exposure to low-pH conditions (pH $<$ 3), into chaperone-active monomers which bind to substrate proteins and prevent the aggregation of acid-denatured proteins (22, 25, 31). The contributions of HdeA and HdeB to cell viability under low pH were identified largely from studies using derivatives of the nonpathogenic K-12 laboratory strain, whereas limited information is available in other serotypes or pathogroups of *E. coli*, including EHEC.

We examined in this study the role of HdeA and HdeB in several *E. coli* strains, including two *E. coli* O157:H7 outbreak strains linked to a 2006 U.S. spinach-associated outbreak and a 1993 hamburger-associated outbreak, one *E. coli* O145 nonmotile clinical isolate linked to the 2010 Yuma romaine lettuce-associated outbreak, and two enteropathogenic Shiga toxin-negative *E. coli* O55:H7 strains. For comparison, we included two K-12 strains, BW25113 and MG1655, which have been widely used for characterization of the HdeA and HdeB functions (25, 31, 32, 46). Our study demonstrates the distinct roles of HdeA and HdeB proteins in various serotypes and pathotypes of *E. coli* and suggests a divergent evolution of acid resistance mechanisms within this species.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth media. The *E. coli* strains and mutants used in this study and their sources are listed in Table 1. Strains were grown routinely in Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, and 5 g NaCl per liter) or modified LB broth containing various concentrations of salt as detailed in each section. LB-full salt (LBFS) contains 10 g of NaCl per liter, whereas LB-no salt (LBNS) contains no NaCl. Antibiotics were used at the following concentrations: ampicillin (Amp), 100 μg ml^{-1}; carbenicillin (Cb), 50 μg ml^{-1}; kanamycin (Km), 50 μg ml^{-1}; and gentamicin (Gm), 15 μg ml^{-1}.

Gene deletion and DNA cloning. In-frame deletion of the individual gene or deletion of the gene cluster was achieved by replacing the target genes with a Km cassette using the standard Lambda Red-mediated gene replacement method (10). Briefly, the Km cassette was amplified directly from the template plasmid pKD4 (Table 1) using the gene knockout primers described in Table S1 in the supplemental material. The PCR products (about 1.6 kb) were agarose gel purified and transformed by electroporation into *E. coli* strains carrying the helper plasmid pKD46 (Table 1). The Km^r transformants were selected on LB agar plates containing 50 μg ml^{-1} of Km. The deletion mutants were verified by colony PCR using primers flanking the target genes (Table S1). Mutants were then subcultured and incubated at 37°C and tested for Amp sensitivity, indicating loss of the helper plasmid pKD46. To eliminate the Km cassette, the plasmid pCP20 (Amp^r) was transformed into each mutant strain and the transformants were patched on LB plates and incubated at 37°C to test for the loss of both Km and Amp resistances.

For complementation analysis, DNA fragments containing *hdeA*, *hdeB*, or the *hdeAB* operon were PCR amplified, digested with the restriction enzymes KpnI and HindIII, and then cloned into the plasmid pBBR1MCS-5 (Table 1). Primers used for cloning *hdeA* from *E. coli* O157:H7 or *hdeB* from *E. coli* K-12 or O157:H7 are described in Table S1 in the supplemental material. For cloning the *hdeAB* operon, two sets of primers were designed to clone the DNA fragment containing only the *hdeAB* structural genes or *hdeAB* plus their native promoter (Table S1). The constructs were verified by DNA sequencing, using primers specific to vector pBBR1MCS-5 and to the cloned genes. The sequence-confirmed clones were then transformed into the target strains by electroporation, and the transformants were selected on Gm-containing plates and verified by colony PCR.

DNA sequencing. Bidirectional DNA sequencing was performed on PCR-amplified DNA fragments or plasmids. Primers used for amplification and sequencing of the *hdeAB* genes are presented in Table S1 in the supplemental material. PCR products were purified by ExoSAP-IT (USB, Cleveland, OH) and then used directly as templates for the cycle sequencing reactions using the BigDye Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA). For sequencing the cloned DNA fragments, plasmids (~100 ng) were used directly as templates for dye incorporation reactions. Following sequencing reactions, the excess dye was removed using a BigDye XTerminator kit (Applied Biosystems) and

TABLE 1 The *E. coli* strains and plasmids used in this study

Strain or plasmid[a]	Antibiotic resistance[b]	Characteristics[c]	Source or reference
Strains			
DH5α	None	Host strains for plasmids pBBR1MCS-5, pKD46, and pKD4 and their derivatives	S. Lory
BW25113	None	Δ*(araD-araB)567* Δ*lacZ4787*(::*rrnB-3*) λ⁻ *rph-1* Δ*(rhaD-rhaB)568 hsdR514*	NBRP (NIG, Japan): *E. coli*
MG1655	None	*E. coli* K-12 strain	ATCC
RM12238	None	Clinical isolate linked to the 2010 Yuma, AZ, lettuce-associated outbreak; EHEC O145	J. Rudrik
RM2027	None	O55:H7, DecA 5B, human, 1979, Florida; CDC 660-79	STEC Center (39)
RM13616	None	O55:H7, enteropathogenic *E. coli*, human, Brazil	L. Riley
RM6067	None	Bagged spinach isolate linked to the 2006 U.S. spinach-associated outbreak; EHEC O157:H7	W. Chmielecki (1)
RM6069	None	Clinical isolate linked to the 2006 U.S. spinach-associated outbreak; EHEC O157:H7	W. Chmielecki (1)
RM6607	None	Clinical isolate linked to the 1993 U.S. hamburger-associated outbreak; EHEC O157:H7	2
RM6608	None	Meat isolate linked to the 1993 U.S. hamburger-associated outbreak; EHEC O157:H7	2
JW3478	Km^r	*hdeA* deletion mutant of BW25113 (Δ*hdeA*::Km)	NBRP (NIG, Japan): *E. coli*
JW5669	Km^r	*hdeB* deletion mutant of BW25113 (Δ*hdeB*::Km)	NBRP (NIG, Japan): *E. coli*
MQC322	None	*hdeA* deletion mutant of BW25113 (Km cassette was eliminated from JW3478)	This study
MQC324	None	*hdeB* deletion mutant of BW25113 (Km cassette was eliminated from JW5669)	This study
MQC307	Km^r	*hdeA* deletion mutant of RM6067 (Δ*hdeA*::Km)	This study
MQC309	Km^r	*hdeB* deletion mutant of RM6067 (Δ*hdeB*::Km)	This study
MQC203	Km^r	*hdeAB* deletion mutant of MG1655 (Δ*hdeAB*::Km)	This study
MQC205	Km^r	*hdeAB* deletion mutant of RM2027 (Δ*hdeAB*::Km)	This study
MQC207	Km^r	*hdeAB* deletion mutant of RM13616 (Δ*hdeAB*::Km)	This study
MQC211	Km^r	*hdeAB* deletion mutant of RM12238 (Δ*hdeAB*::Km)	This study
MQC135	Km^r	*hdeAB* deletion mutant of RM6067 (Δ*hdeAB*::Km)	This study
MQC143	Km^r	*hdeAB* deletion mutant of RM6607 (Δ*hdeAB*::Km)	This study
MQC350	Gm^r	The plasmid pBBR1MCS-5 was transformed into the strain BW25113	This study
MQC348	Gm^r	The plasmid pBBR1MCS-5 was transformed into the strain MQC322	This study
MQC346	Gm^r	The plasmid pXQ20 was transformed into the strain MQC322	This study
MQC374	Gm^r	The plasmid pBBR1MCS-5 was transformed into the strain MQC324	This study
MQC376	Gm^r	The plasmid pXQ22 was transformed into the strain MQC324	This study
MQC378	Gm^r	The plasmid pXQ21 was transformed into the strain MQC324	This study
MQC408	Gm^r	The plasmid pBBR1MCS-5 was transformed into the strain RM12238	This study
MQC410	Gm^r	The plasmid pBBR1MCS-5 was transformed into the strain MQC211	This study
MQC412	Gm^r	The plasmid pXQ20 was transformed into the strain MQC211	This study
MQC414	Gm^r	The plasmid pXQ21 was transformed into the strain MQC211	This study
MQC416	Gm^r	The plasmid pXQ22 was transformed into the strain MQC211	This study
MQC418	Gm^r	The plasmid pXQ25 was transformed into the strain MQC211	This study
MQC420	Gm^r	The plasmid pXQ26 was transformed into the strain MQC211	This study
MQC422	Gm^r	The plasmid pXQ28 was transformed into the strain MQC211	This study
MQC424	Gm^r	The plasmid pXQ27 was transformed into the strain MQC211	This study
MQC426	Gm^r	The plasmid pBBR1MCS-5 was transformed in the strain MG1655	This study
MQC428	Gm^r	The plasmid pBBR1MCS-5 was transformed in the strain MQC203	This study
MQC430	Gm^r	The plasmid pXQ20 was transformed into the strain MQC203	This study
MQC432	Gm^r	The plasmid pXQ21 was transformed into the strain MQC203	This study
MQC434	Gm^r	The plasmid pXQ22 was transformed into the strain MQC203	This study
Plasmids			
pKD46	Amp^r	Red recombinase expression plasmid	AY048746[d] (10)
pKD4	Km^r	Template plasmid for gene deletion	AY048743[d] (10)
pCP20	Amp^r	Flp recombinase expression plasmid for eliminating the inserted Km resistance gene in the mutant strains	B. Wanner (10)
pBBR1MCS-5	Gm^r	Expression vector for complementation analysis	U25061[d] (28)
pXQ20	Gm^r	The *hdeA* along with its native promoter of O157:H7 strain RM6067 was cloned into pBBR1MCS-5	This study
pXQ21	Gm^r	The *hdeB* gene of K-12 strain MG1655 was cloned into pBBR1MCS-5	This study
pXQ22	Gm^r	The *hdeB* gene of O157:H7 strain RM6067 was cloned into pBBR1MCS-5	This study
pXQ25	Gm^r	Both *hdeA* and *hdeB* of K-12 strain MG1655 were cloned into pBBR1MCS-5	This study
pXQ26	Gm^r	Both *hdeA* and *hdeB* of O157:H7 strain RM6067 were cloned into pBBR1MCS-5	This study
pXQ27	Gm^r	Both *hdeA* and *hdeB* along with the *hdeA* native promoter of K-12 strain MG1655 were cloned into pBBR1MCS-5	This study
pXQ28	Gm^r	Both *hdeA* and *hdeB* along with the *hdeA* native promoter of O157:H7 strain RM6067 were cloned into plasmid pBBR1MCS-5	This study

[a] All *E. coli* O157:H7 strains used in this study are natural curli-deficient variants, which exhibit higher acid resistance than their corresponding curli-producing variants (9).
[b] Amp^r, ampicillin resistance; Km^r, kanamycin resistance; Gm^r, gentamicin resistance.
[c] Primers used for gene deletion cloning are described in Table S1 in the supplemental material.
[d] NCBI GenBank accession number.

the purified samples were sequenced using an automated fluorescent sequencer (ABI Prism 3730 DNA analyzer; Applied Biosystems). DNA sequences were assembled in SeqMan (Lasergene 8; DNAStar, Madison, WI), and the consensus sequence was exported in MegAlign (Lasergene 8) for comparison.

Mass spectrometry analyses of HdeA and HdeB proteins. Both HdeA and HdeB were identified by matrix-assisted laser desorption ionization–time of flight-time of flight tandem mass spectrometry (MALDI-TOF-TOF-MS/MS) and top-down proteomic analysis as described previously (12, 13). Briefly, a 1-μl loop of bacterial cells was transferred to a 2-ml O-ring-lined screw-cap microcentrifuge tube containing 300 μl of 67% high-pressure liquid chromatography (HPLC)-grade water (Burdick & Jackson, Muskegon, WI), 33% HPLC-grade acetonitrile (Fisher Scientific, Pittsburgh, PA), 0.2% trifluoroacetic acid (Sigma-Aldrich, St. Louis, MO), and about 100 mg of 0.1 mm zirconia-silica beads (BioSpec Products, Bartlesville, OK). The bacterial cells were lysed by bead-beating for 1 min on a reciprocating shaker (Mini-Beadbeater; Biospec Products, Bartlesville, OK) followed by centrifugation at 16,000 $\times$ *g* for 2 min. An 0.5-μl aliquot of supernatant was spotted onto a 384-well stainless steel target and allowed to air dry at room temperature. The dried sample spot was overlaid with an 0.5-μl aliquot of saturated MALDI matrix solution of either α-cyano-4-hydroxycinnamic acid or 3,5-dimethoxy-4-hydroxycinnamic acid dissolved in 67% HPLC-grade water, 33% HPLC-grade acetonitrile, and 0.2% trifluoroacetic acid. MS and MS/MS data were collected on a 4800 Plus MALDI-TOF-TOF mass spectrometer (AB Sciex, Foster City, CA). MS and MS/MS analysis was performed as described previously (11, 12), except the instrument was calibrated (externally) for MS/MS analysis using the fragment ions of disulfide-reduced/alkylated thioredoxin from *E. coli* (Sigma-Aldrich, St. Louis, MO) prepared with the FOCUS protein reduction-alkylation kit (G-Biosciences, Maryland Heights, MO). HdeA and HdeB were identified by top-down proteomic analysis (12, 13). In order to increase HdeA and HdeB fragmentation efficiency for top-down identification, a disulfide reduction step was included for some samples (14).

Acid resistance assay. The *E. coli* strains were grown in LBFS or LBNS and incubated at either 37°C or 28°C on a shaker (150 rpm) to stationary phase (20 h). The overnight cultures were diluted directly in fresh LB acid challenge broth (pH 2.0, acidified with HCl) to a concentration of 2×10^6 to 5×10^6 CFU ml^{-1} and incubated at 37°C for 2 h on a shaker (80 rpm). At the end of incubation, the cells were immediately diluted in KP buffer (10 mM potassium phosphate buffer, pH 7.0) and the viable cells were recovered by plating them onto LB agar plates using an automated spiral plater (Autoplate4000; Spiral Biotech, Norwood, MA). The concentration of viable cells was compared with that of the initial concentration (immediately after inoculating the pH 2.0 LB broth, prior to incubation at 37°C) to estimate the acid resistance of *E. coli* strains and mutants. The acid resistance was expressed as a percentage of cells that survived following the 2-h acid challenge.

Functional analyses of the *E. coli* O157:H7 *hdeA* and *hdeB*. The cloned *hdeA* gene, *hdeB* gene, and the *hdeAB* operon were transformed into host strains by electroporation. The cloning vector pBBR1MCS-5 was transformed also into the corresponding host strains (wild-type and mutant strains) to serve as controls. The acid challenge was performed as described above, except that the growth broth for prechallenge cells was supplemented with Gm (15 μg ml^{-1}). The acid resistances of the complemented strains were compared with the corresponding wild-type and mutant strains to assess the *E. coli* O157:H7 HdeA and HdeB functions.

Statistical analysis. Statistical analysis was computed with SigmaPlot version 11.0 (Systat Software, Inc.). An unpaired *t* test was performed for two groups comparison, and the analysis of variance (ANOVA) followed by Bonferroni *t* test was performed for multiple comparisons. The effects of temperature and NaCl on acid resistance of both K-12 and O157:H7 were assessed by a two-way ANOVA test. The power of the ANOVA test was computed with *post hoc* tests ($\alpha = 0.05$). For the *t* test, if the equal variance test failed, the Mann-Whitney rank sum test was performed. For the ANOVA, if the normality test or the equal variance test failed, the Kruskal-Wallis one-way ANOVA on ranks was performed followed by Dunn's test for multiple comparisons.

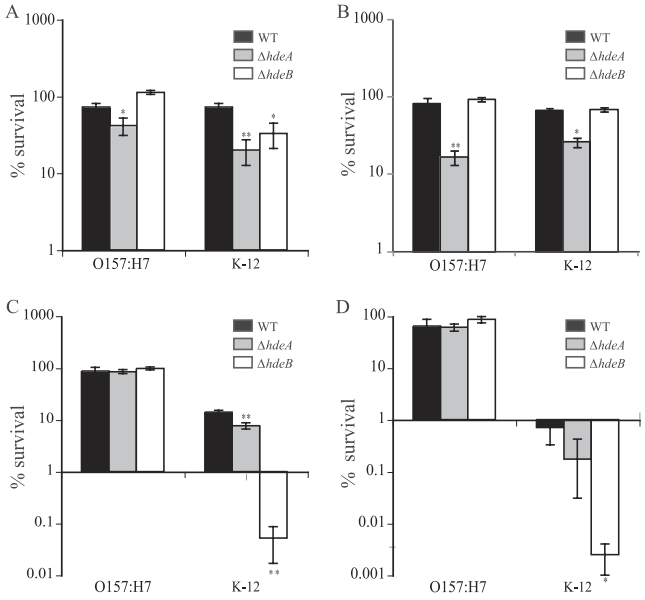

FIG 2 Contributions of HdeA and HdeB to acid resistance in *E. coli* O157:H7 and K-12 strains. Single colonies of *E. coli* wild-type (WT) or mutant (Δ*hdeA* or Δ*hdeB*) strains were grown to stationary phase at 37°C in LBFS (A) or LBNS (B) broth or at 28°C in LBFS (C) or LBNS (D) broth. About 2×10^6 to 5×10^6 cells were inoculated into HCl-acidified LB broth (pH 2.0) and incubated at 37°C for 2 h. The cells were then recovered on LB plates. The number of CFU following the acid challenge was compared with the corresponding CFU at the beginning of the acid challenge to calculate the percent survival. Black columns represent WT strains and gray columns represent the *hdeA* deletion mutants, whereas white columns represent the *hdeB* deletion mutants. The differences in acid resistance between the wild-type and the mutant strains were indicated by *P* value of the Bonferroni *t* test (*, $P < 0.05$; **, $P < 0.001$) (A, B, and C) or the Dunn test (D).

Nucleotide sequence accession numbers. The *hdeAB* sequences from *E. coli* O55:H7 strains RM2027 and RM13616; *E. coli* O157:H7 strains RM6067, RM6069, RM6607, and RM6608; and *E. coli* O145 strain RM12238 were deposited in GenBank with accession numbers from JN705805 to JN705811.

RESULTS

Survival of *hdeA* and *hdeB* deletion mutants following acid challenge. We first investigated the impacts of growth temperature and the osmolarity on the acid resistance of *E. coli* O157:H7. The acid survival of *E. coli* O157:H7 strain RM6067 stationary-phase cells was examined under four culture conditions, LBFS or LBNS at 37°C and LBFS or LBNS at 28°C, and the results were then compared with those of K-12 strain BW25113 (Fig. 2). The pregrowth conditions appeared to have no effect on the acid resistance of RM6067 since there was no significant difference in acid resistance among *E. coli* O157:H7 cells grown under the above four conditions (two-way ANOVA, temperature: $F = 0.9$, $P = 0.783$; NaCl: $F = 0.4$, $P = 0.537$) (Fig. 2, RM6067 WT). In contrast, the pregrowth conditions had a profound impact on the acid resistance of K-12 strain BW25113 (two-way ANOVA, temperature: $F = 2999$, $P < 0.001$; NaCl: $F = 99$, $P < 0.001$). When the growth temperature decreased from 37°C to 28°C, the acid sur-

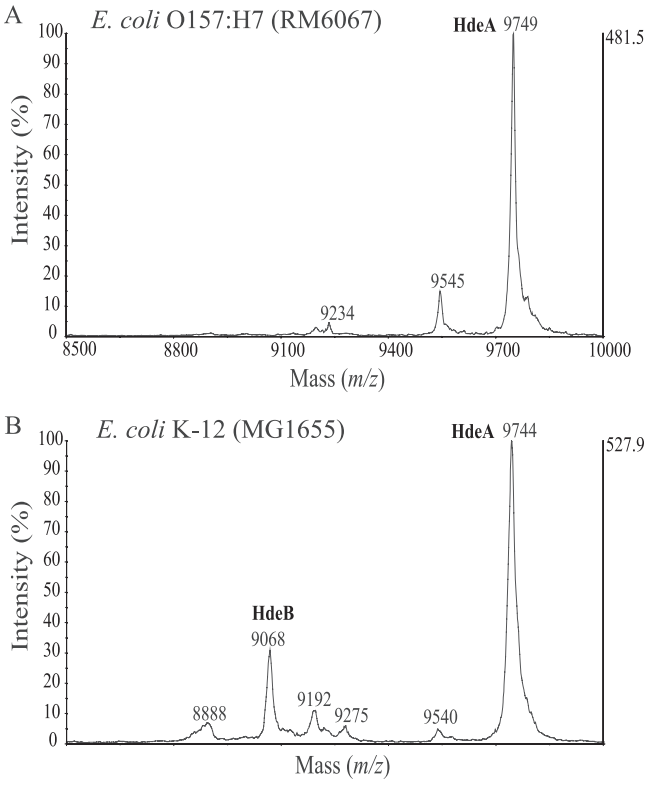

FIG 3 Detection of HdeA and HdeB by mass spectrometry. MS spectra of the extracted cell lysates of *E. coli* O157:H7 strain RM6067 (A) and *E. coli* K-12 strain MG1655 (B) using α-cyano-4-hydroxycinnamic acid matrix. Cells were grown on an LBFS agar plate at 37°C overnight. MS/MS of the protein biomarker at *m/z* 9068 was identified as HdeB protein (see Fig. S1 and Table S2 in the supplemental material), and *m/z* 9744 was identified as HdeA protein. In RM6067 (*E. coli* O157:H7), a distinct HdeA marker was detected and identified, but the corresponding HdeB marker was absent.

vival of wild-type cells decreased 5.9- and 95.6-fold on average for cells grown in LBFS and LBNS, respectively (Fig. 2, BW25113 WT). Removal of NaCl from the growth broth led to a further decrease (~20-fold) in acid resistance of BW25113 grown at 28°C (Fig. 2D, BW25113 WT). We then examined the contributions of the two chaperone proteins, HdeA and HdeB, to the acid survival of *E. coli* O157:H7 and compared those to the K-12 strain BW25113. Loss of HdeA in strain RM6067 resulted in less than a 5-fold decrease in acid survival for cells grown at 37°C and had no impact on the acid survival of the mutant strain grown at 28°C (Fig. 2, RM6067 Δ*hdeA*); loss of HdeB did not affect the acid survival of the mutant strain under any of the four growth conditions examined (Fig. 2, RM6067 Δ*hdeB*). In K-12 strain BW25113, loss of HdeA decreased the acid resistance slightly (<4-fold) for cells grown at either 37°C or 28°C (Fig. 2, BW25113 Δ*hdeA*), whereas loss of HdeB led to a profound decrease (>100-fold) in the acid resistance, but only for cells grown at 28°C (Fig. 2, BW25113 Δ*hdeB*). These data suggest that both HdeA and HdeB are important acid chaperone proteins in the *E. coli* K-12 strain BW25113, but they are not critical for acid survival in *E. coli* O157:H7 strain RM6067.

Detection of HdeA and HdeB proteins by mass spectrometry and top-down proteomics. The production of HdeA and HdeB in *E. coli* O157:H7 was examined by MALDI-TOF-TOF-MS/MS and compared to K-12 strains. The HdeA characteristic peak was detected in both RM6067 and RM6607 grown on LBFS plates at 37°C (Fig. 3A, ion *m/z* 9749, and Table 2); however, the HdeB peak was not observed in either of the O157:H7 strains. In contrast, in K-12 strains MG1655 and BW25113, both HdeA and HdeB proteins were detected under the same growth condition (Fig. 3B, ions at *m/z* 9068 and 9744, and Table 2). Details on the identification of HdeA and HdeB by top-down proteomics are presented in Fig. S1 and Table S2 in the supplemental material. Similar to K-12 strains, HdeA and HdeB were both detected in two enteropathogenic strains of serovar O55:H7 (RM2027 and RM13616) and one non-O157 EHEC strain of serovar O145 (RM12238) (Table 2). Similar results were observed for cells grown on LBNS plates at 28°C (data not shown).

DNA sequence analyses of *hdeA* and *hdeB*. The difference in expression of the HdeA and HdeB proteins in *E. coli* O157:H7 led us to examine the *hdeA* and *hdeB* genes. To obtain a complete picture of *hdeA* and *hdeB* sequence diversity, the *hdeAB* loci of the *E. coli* strains examined above were sequenced. The assembled *hdeAB* sequences were then compared with the *hdeAB* sequences that are available in GenBank, which include the *hdeAB* loci from an additional 30 *E. coli* strains. Detailed information about the strains is presented in Table S3 in the supplemental material.

A total of 10 single nucleotide polymorphisms (SNPs) were identified across the entire *hdeA* gene (333 bp), separating the *E. coli* strains into two large clusters (see Fig. S2 in the supplemental material). The *hdeA* genes from all four of the *E. coli* O157:H7 strains sequenced in this study were identical to the *hdeA* genes of other *E. coli* O157:H7 strains (e.g., EDL933, Sakai, and TW14359), two *E. coli* O55:H7 strains sequenced in this study (RM2027 and RM13616), other non-O157 EHEC strains (RM12238, 11128, 11368, and 12009), and K-12 strains (BW25113, MG1655, and BW2952) (Fig. S2, Table 1, and Table S3 for strain details). In contrast, the *E. coli* O157:H7 *hdeB* genes formed a unique cluster (Fig. 4A) and were nearly identical to the *hdeB* genes of two *E. coli* O55:H7 strains (RM2027 and RM13616). Similarly, the *hdeB* genes of all three K-12 strains and *Shigella flexneri* (strain 2457T) formed a discrete cluster and appeared to have evolved from a common ancestor. The *hdeB* genes of the non-O157 EHEC strains (RM12238, 11128, 11368, and 12009) were identical and formed a cluster distinct from either the O157 or K-12 group (Fig. 4A).

Further examination of SNPs in *hdeB* that separate *E. coli* O157:H7 strains from O55:H7 strains, non-O157 EHEC strains, and K-12 strains revealed three SNPs across the entire *hdeB* gene. The first SNP, a G↔A transition at the +3 position, is unique to the *hdeB* gene of *E. coli* O157:H7 (Fig. 4B). This transition alters

TABLE 2 Detection of HdeA and HdeB proteins in *E. coli* strains[a]

Strain	Serotype	Pathotype[b]	HdeA[c]	HdeB[c]
RM6067	O157:H7	EHEC	+	−
RM6607	O157:H7	EHEC	+	−
MG1655	K-12	Nonpathogenic	+	+
BW25113	K-12	Nonpathogenic	+	+
RM12238	O145:NM	EHEC	+	+
RM2027	O55:H7	EPEC	+	+
RM13616	O55:H7	EPEC	+	+

[a] Detected by MALDI-TOF-TOF-MS/MS.
[b] EHEC, enterohemorrhagic; EPEC, enteropathogenic.
[c] + or −, presence or absence of the characteristic HdeA or HdeB peak (Fig. 3).

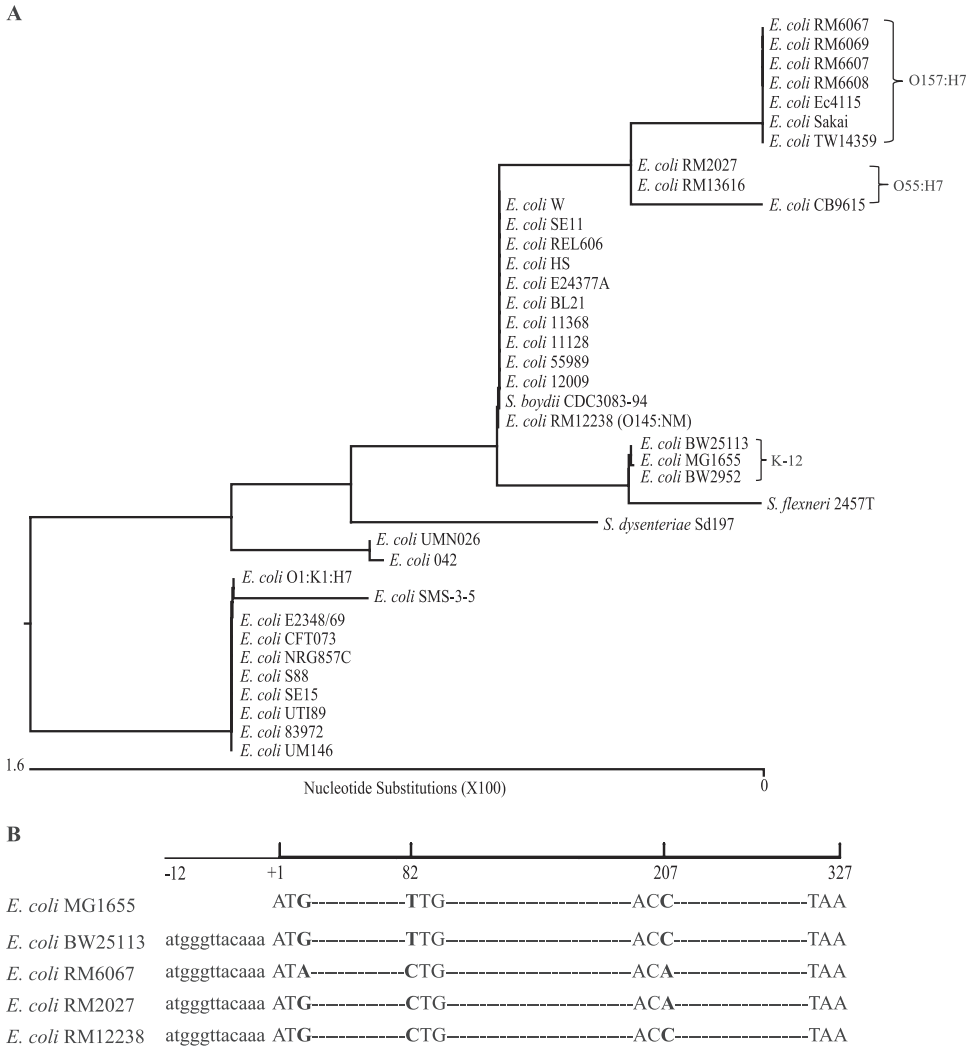

FIG 4 Sequence analyses of the *E. coli hdeB* gene. Clustering analysis of *hdeB* (A) and single nucleotide polymorphisms within *hdeB* (B). Detailed strain information is presented in Table S3 in the supplemental material. The sequences were aligned using the Clustal W module in MegAlign (Lasergene v8; DNAStar).

the putative start codon of the *E. coli* O157:H7 *hdeB* gene to ATA, a rare start codon in both prokaryotic and eukaryotic organisms. Noteworthy is that in several genome-sequenced O157 strains, including EDL933, *hdeB* is annotated as a 339-bp gene and the annotated start codon is the ATG located at the −12 position compared to the *hdeB* gene of the K-12 strain MG1655 (Fig. 4B). Placement of the start codon at −12 is likely incorrect, based upon the *hdeB* gene alignment: the *hdeB* gene in K-12 is 327 bp and its start codon ATG was aligned perfectly with the ATA codon of RM6067 *hdeB* (O157) and the ATG codons of strains RM2027 (O55) and RM12238 (O145) (Fig. 4B). The other two SNPs occurred at the +82 and +207 positions. The SNP at +82, a C↔T transition, is unique to K-12 strains; the SNP at +207 is a C↔A transversion and conserved among *E. coli* O157:H7 and *E. coli* O55:H7 strains. Both SNPs at the +82 and +207 positions are silent mutations as they do not alter the amino acid sequence of the HdeB protein.

The natural mutation at the putative *hdeB* start codon in *E. coli* O157:H7 is a probable explanation for the absence of the HdeB protein in this specific pathogroup of *E. coli* strains (Fig. 3). To investigate how widely this mutation is distributed in O157:H7 strains, the *hdeB* genes in an additional 20 O157:H7 strains and 12 non-O157 strains were sequenced and compared (see Table S4 in the supplemental material). Consistently, the G↔A transition at the +3 position was found only in all 20 *E. coli* O157:H7 strains and not in the other 12 non-O157 strains.

Acid resistance of *hdeAB* deletion mutant in other *E. coli* strains. Because of the distinct contributions of the HdeA and HdeB chaperones to cell acid survival between *E. coli* K-12 strain BW25113 and O157:H7 strain RM6067 (Fig. 2), we further explored the functions of HdeA and HdeB proteins in three other *E. coli* strains that are either phylogenetically or pathogenetically linked to *E. coli* O157:H7. Strain RM12238 is an O145 clinical isolate linked to the 2010 Yuma romaine lettuce-associated outbreak; RM2027 and RM13616 are enteropathogenic O55:H7 clinical isolates, which are considered the immediate ancestors of *E. coli* O157:H7 (15, 16). To avoid bias introduced by a particular strain, we included another *E. coli* O157:H7 strain, RM6607, a

clinical isolate linked to the 1993 U.S. hamburger-associated outbreak, and MG1655, a K-12 strain that has fewer genetic modifications than strain BW25113 (8). The survival of *hdeAB* deletion mutants following acid challenge was examined and compared with that of their corresponding parental strains (Fig. 5). Consistent with our previous observations, loss of HdeA and HdeB in both *E. coli* O157:H7 strains, RM6067 and RM6607, had little effect on their acid survival: there were slight decreases in acid resistance (~2-fold, *t* test, $P < 0.05$) for cells grown in LBFS at 37°C; however, there were no changes in acid survival for cells grown in LBNS at 28° (Fig. 5, RM6067 WT and RM6607 WT). Similar to strain BW25113, the acid survival of strain MG1655 is largely dependent on HdeA and HdeB: there was a significant decrease in acid resistance (>10-fold, *t* test, $P < 0.001$) for cells grown either in LBFS at 37°C (Fig. 5A) or in LBNS at 28°C (Fig. 5B). Loss of the HdeA and HdeB proteins in the *E. coli* O145:NM strain RM12238 led to the greatest decrease in acid resistance for cells grown in LBFS at 37°C (>100-fold, *t* test, $P < 0.001$) as well as for cells grown in LBNS at 28°C (>2,000-fold, *t* test, $P < 0.001$) among the six strains examined (Fig. 5, RM12238). Similar to the O157:H7 strains, loss of the HdeA and HdeB proteins in the two *E. coli* O55:H7 strains resulted in only a 1.5- and 2.0-fold decrease in acid resistance on average for cells grown at 37°C and at 28°C, respectively (Fig. 5, *E. coli* O55:H7).

Functional analysis of the *E. coli* O157:H7 *hdeA* gene. We chose to assess the O157-HdeA function in K-12 and O145 backgrounds since loss of HdeA in either K-12 or O145 strains led to a large decrease in their acid resistance (Fig. 2 and Fig. 5). Because the *hdeA* promoter contains putative binding sites for several transcriptional regulators of the *hdeAB* operon (40), the O157-*hdeA* and its native promoter from strain RM6067 were cloned into pBBR1MCS-5. The function of *E. coli* O157:H7 HdeA was first examined in K-12 mutant strain MQC322 (BW25113Δ*hdeA*). Once the O157-HdeA was provided, the acid resistance of MQC322 increased significantly: the percent survival of the complemented strain (MQC346) was 104.6% and 74.6% of the wild-type strain (MQC350) for cells grown in LBFS at 37°C and 28°C, respectively (Table 3, K-12 BW25113). A similar result was observed in the K-12 strain MG1655 *hdeAB* deletion mutant (MQC203). The plasmid-borne O157-*hdeA* was able to fully restore the acid survival of cells grown in LBFS broth in spite of no HdeB in the complemented strain MQC430: the acid resistance of MQC430 was 110.1% and 92.6% of the wild-type strain (MQC426) for cells grown at 37°C and 28°C, respectively (Table 3, K-12 MG1655). However, for any K-12 mutant strain (MQC322 or MQC203) grown in LBNS at 28°C, supply of the O157-HdeA did not increase the acid resistance of any mutant strain significantly (Table 3, 28°C/LBNS).

We then examined O157-HdeA acid chaperone function in the O145 EHEC strain RM12238. Introduction of plasmid-borne O157-*hdeA* in strain MQC211 (RM12238Δ*hdeAB*) increased the acid resistance for cells grown either in LBFS at 37°C (~5-fold), in LBFS at 28°C (~10-fold), or in LBNS at 28°C (~6-fold) compared with the mutant strain carrying the expression vector only (Table 3, MQC410). Unlike in K-12 strains, the complementation in *E. coli* O145 provided by O157-HdeA was partial under all three growth conditions examined. The acid survival of the complemented strain (Table 3, MQC412) was about 8.3%, 8.9%, and 4.3% of the wild-type strain (Table 3, MQC408) for cells grown

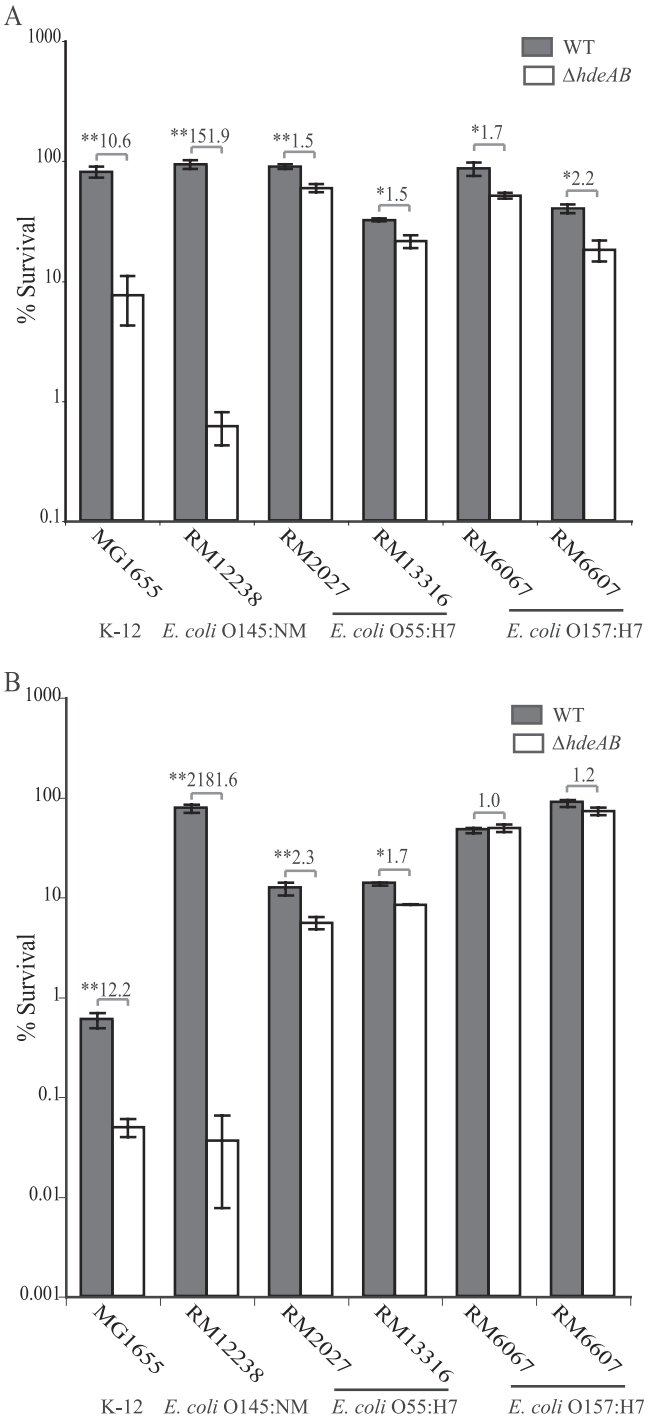

FIG 5 Contribution of HdeA and HdeB to acid resistance of *E. coli* strains from various serotypes and pathotypes. Single colonies of *E. coli* wild-type (WT) strains and their isogenic *hdeAB* deletion mutants (Δ*hdeAB*) were grown to stationary phase either in LBFS broth at 37°C (A) or in LBNS broth at 28°C (B). About 2×10^6 to 5×10^6 cells were inoculated into HCl-acidified LB broth (pH 2.0) and incubated at 37°C for 2 h. The cells were then recovered on LB plates. The number of CFU following the acid challenge was compared to the corresponding CFU at the beginning of the acid challenge to calculate the percent survival. The number on top of the WT strain (dark gray) and the Δ*hdeAB* mutant (white) columns represents the fold decrease in survival of the mutant strain compared with the corresponding WT strain. The difference in acid resistance between the WT and its isogenic mutant was indicated by *P* value of the *t* test (*, $P < 0.05$; **, $P < 0.001$).

TABLE 3 Functional analysis of *E. coli* O157:H7 *hdeA* gene

Strain	Related characteristics[a]	Gene		% survival by growth condition of prechallenge cells[b]		
		hdeA	*hdeB*	37°C/LBFS	28°C/LBFS	28°C/LBNS
K-12 BW25113						
MQC350	BW25113/pBBR1MCS-5	+	+	54.7 ± 9.8*	6.4 ± 0.4*	0.14 ± 0.00*
MQC348	MQC322 (BW25113Δ*hdeA*)/pBBR1MCS-5	−	+	3.2 ± 1.1	0.6 ± 0.2	0.02 ± 0.01
MQC346	MQC322/plasmid-borne O157-P_{hdeA}*hdeA*	+	+	57.2 ± 5.7*	4.8 ± 0.9*	0.04 ± 0.02
K-12 MG1655						
MQC426	MG1655/pBBR1MCS-5	+	+	60.2 ± 6.5*	23.5 ± 5.1*	0.66 ± 0.03*
MQC428	MQC203(MG1655Δhde*AB*)/pBBR1MCS-5	−	−	16.3 ± 5.2	2.5 ± 0.3	0.01 ± 0.01
MQC430	MQC203/plasmid-borne O157-P_{hdeA}*hdeA*	+	−	66.3 ± 3.8*	21.8 ± 4.1*	0.03 ± 0.01
O145 RM12238						
MQC408	RM12238/pBBR1MCS-5	+	+	68.3 ± 19.1*	54.1 ± 6.2**	86.6 ± 2.3*
MQC410	MQC211(RM12238Δ*hdeAB*)/pBBR1MCS-5	−	−	1.1 ± 0.2	0.5 ± 0.2	0.6 ± 0.1
MQC412	MQC211/plasmid-borne O157-P_{hdeA}*hdeA*	+	−	5.7 ± 0.6**	4.8 ± 2.7*	3.7 ± 0.0**

[a] P_{hdeA} indicates the native promoter of *hdeA*.
[b] Total CFU recovered on LB plates following acid challenge as the percentage of initial CFU prior to acid challenge; The difference in acid resistance between the wild type and its isogenic mutant or between a complemented strain and the mutant was indicated by *P* value of the *t* test (*, $P < 0.05$; **, $P < 0.001$).

in LBFS at 37°C, in LBFS at 28°C, and in LBNS at 28°C, respectively.

Functional analyses of the *E. coli* O157:H7 *hdeB* gene. The G↔A transition at the +3 position of the *E. coli* O157:H7 *hdeB* gene leads presumably to the silence of this gene in *E. coli* O157:H7 strains (Fig. 3A). Therefore, we further tested if the *E. coli* O157:H7 *hdeB* gene can be translated and function as an acid chaperone in an *E. coli* strain requiring HdeB for its acid survival. If translated, the O157-HdeB should function as an acid chaperone similar to K-12-HdeB, since the other two SNPs in *hdeB* are silent mutations (Fig. 4B). The *hdeB* genes of strains MG1655 (K-12) and RM6067 (O157:H7) were cloned in the expression vector pBBR1MCS-5, in which the expression of the cloned gene was driven by the *lacZ* promoter. The function of O157-*hdeB* was first examined in K-12 strains and compared with the plasmid-borne K-12-*hdeB* (Table 4). Unlike O157-HdeA, O157-HdeB failed to complement BW25113Δ*hdeB* mutant under any growth conditions examined, although the production of

TABLE 4 Functional analysis of *E. coli* O157:H7 *hdeB* gene

Strain	Related characteristics[a]	Gene		% survival[b]	
		hdeA	*hdeB*	37°C/LBFS[c]	28°C/LBFS or LBNS[c]
K-12 BW25113					
MQC350	BW25113/pBBR1MCS-5	+	+	83.0 ± 9.4*	9.4 ± 3.0*
MQC374	MQC324(BW25113Δ*hdeB*)/pBBR1MCS-5	+	-	47.9 ± 19.7	0.3 ± 0.1
MQC378	MQC324/plasmid-borne K-12-*hdeB*	+	+ (K-12)	94.6 ± 8.3*	1.1 ± 0.3*
MQC376	MQC324/plasmid-borne O157-*hdeB*	+	+ (O157:H7)	77.7 ± 26.5	0.4 ± 0.3
K-12 MG1655					
MQC426	MG1655/pBBR1MCS-5	+	+	60.2 ± 6.5*	23.5 ± 5.1*
MQC428	MQC203(MG1655Δhde*AB*)/pBBR1MCS-5	−	−	16.3 ± 5.2	2.5 ± 0.3
MQC432	MQC203/plasmid-borne K-12-*hdeB*	−	+ (K-12)	19.9 ± 9.0	3.9 ± 0.4
MQC434	MQC203/plasmid-borne O157-*hdeB*	−	+ (O157:H7)	12.3 ± 2.5	2.2 ± 0.3
O145 RM12238					
MQC408	RM12238/pBBR1MCS-5	+	+	68.3 ± 19.1*	86.6 ± 2.3*
MQC410	MQC211(RM12238Δ*hdeAB*)/pBBR1MCS-5	−	−	1.1 ± 0.2	0.6 ± 0.1
MQC414	MQC211/plasmid-borne K-12-*hdeB*	−	+ (K-12)	1.2 ± 0.6	6.0 ± 1.5*
MQC416	MQC211/plasmid-borne O157-*hdeB*	−	+ (O157:H7)	1.9 ± 0.9	1.9 ± 0.5*
MQC418	MQC211/plasmid-borne K-12-*hdeAB*	+	+ (K-12)	3.7 ± 1.6*	5.0 ± 0.5**
MQC420	MQC211/plasmid-borne O157-*hdeAB*	+	+ (O157:H7)	2.6 ± 0.8*	3.1 ± 0.8**
MQC424	MQC211/plasmid-borne K-12-P_{hdeA}*hdeAB*	+	+ (K-12)	5.1 ± 0.5**	18.3 ± 2.4**
MQC422	MQC211/plasmid-borne O157-P_{hdeA}*hdeAB*	+	+ (O157:H7)	2.3 ± 0.2**	13.6 ± 2.6**

[a] P_{hdeA} indicates the native promoter of *hdeA*.
[b] The difference in acid resistance between the wild type and its isogenic mutant or between a complemented strain and the mutant was indicated by *P* value of the *t* test (*, $P < 0.05$; **, $P < 0.001$).
[c] Growth condition of prechallenge cells; for growth at 28°C, K-12 strains were grown in LBFS, whereas O145 strains were grown in LBNS; no complementation was observed for any of the K-12 *hdeB* deletion mutants (MQC324 or MQC203) when cells were grown in LBNS at 28°C.

O157-HdeB was confirmed by MS analysis (Table 4, MQC376). In contrast, K-12-HdeB provided a full complementation for the BW25113Δ*hdeB* mutant for cells grown in LBFS at 37°C (114% of wild-type strain) and a partial complementation for cells grown in LBFS at 28°C (11.8% of wild-type strain) (Table 4, MQC378). Unlike in the mutant BW25113Δ*hdeB*, no complementation for either O157-HdeB or K-12-HdeB was observed in the mutant MG1655Δ*hdeAB* (MQC203) for cells grown under any growth conditions examined (Table 4, MQC432 and MQC434).

The O157-HdeB function was then examined in an O145 EHEC mutant MQC211 (RM12238Δ*hdeAB*). Unlike in K-12 strains, no complementation was observed for cells grown in LBFS at 37°C when either K-12-HdeB or O157:H7-HdeB was provided *in trans*; however, partial complementation was observed for cells grown in LBNS at 28°C when supplied with either O157- or K-12-HdeB (Table 4, MQC414 and MQC416). Greater acid protection conferred by K-12-HdeB (MQC414) was observed compared with that of the O157-HdeB (MQC416). These results were consistent with the MS analysis, which suggested that a greater amount of HdeB was produced in cells carrying the K-12-*hdeB* gene (MQC414) than that of cells carrying the O157-*hdeB* gene (MQC416).

Because the acid chaperone activity of HdeB may be dependent on the HdeA function, we tested whether the acid resistance of MQC211 would be improved when both HdeA and HdeB proteins were provided. A slight increase in acid resistance was observed for cells grown in LBFS at 37°C when both K-12-HdeA/B proteins were provided (Table 4, MQC 418) compared with cells provided with K-12 HdeB only (Table 4, MQC414), but there was no increase in acid resistance for cells grown in LBNS at 28°C. When both O157-HdeA and HdeB proteins were expressed in mutant MQC211 (Table 4, MQC420), there were no significant changes in acid resistance for cells grown under either of the two conditions examined compared with the strain expressing O157-HdeB only (Table 3, MQC416).

We then examined whether the native promoter of the *hdeAB* operon is important for HdeB function in the complemented strains. Interestingly, when the *hdeAB* native promoter together with the *hdeAB* from either K-12 (MG1655) or O157:H7 (RM6067) was cloned and introduced into strain MQC211 (RM12238Δ*hdeAB*), the acid resistance of the mutant strain was improved greatly, but only for cells grown in LBNS at 28°C (Table 4, MQC424 and MQC422). The greatest increase in acid survival was observed for cells carrying the plasmid-borne K-12-*hdeAB* along with its native promoter (MQC424), in which 18.3% of cells remained viable following a 2-h acid challenge at pH 2.0 (Table 4).

DISCUSSION

EHEC strains display higher acid resistance than K-12 strains. Host and nonhost environments are two primary and distinct ecologic niches for enteric pathogens. Improved stress tolerance in enteric pathogens would enhance their environmental survival and correspond possibly to increased infections. One striking feature shared by the enteric pathogens *E. coli* O157:H7 and *Shigella* is their high acid resistance, which was proposed to contribute to, at least in part, their low infective dose (19, 20). Prior to ingestion by an animal host, enteric pathogens often have been living in an environment where biochemical and physiological conditions were not optimal for their growth. Indeed, the non-host-inhabiting pathogens are challenged frequently by adverse conditions, such as limited nutrients, fluctuating environmental temperatures, and oxidative and osmotic stress. In this study, we document that both temperature and the concentration of NaCl to which prechallenge cells were exposed or adapt have a considerable impact on the acid resistance of *E. coli* strains. When grown in LBFS broth at 37°C, there were no significant differences in acid resistance among the four *E. coli* serotypes or three pathotypes examined, regardless of whether it was an EHEC, an EPEC, or a K-12 strain. However, when grown in LBNS broth at 28°C, all three EHEC strains displayed higher acid resistance than both K-12 strains and both enteropathogenic *E. coli* O55:H7 strains (see Table S5 in the supplemental material). Lower temperature (below 37°C) and lower NaCl concentration (below 1%) are characteristics of many nonhost environments, including the phyllosphere, field water, and irrigation water. The three EHEC strains retained the highest acid resistance among all strains examined in this study when prechallenge cells were grown in LBNS at 28°C, which suggests that EHEC strains have evolved the unique capability to thrive in highly stressful nonhost environments.

Functions of HdeA and HdeB are linked to the growth conditions of prechallenge cells. It has been reported previously with both *E. coli* and *S. flexneri* that HdeA is required for cells to survive at pHs below 3.0 (18, 32, 44); however, the acid protection conferred by HdeB varies greatly depending on the acid challenge systems adopted in the studies. For example, Kern et al. reported that HdeB was required for optimal resistance to acid stress at pH 2 or 3 in *E. coli* strain BW25113 (25), whereas Mates et al. reported that HdeB is not required for *E. coli* MG1655 survival at pH 2.5 (32). In our study, HdeA appeared to be more important for acid survival of stationary-phase cells grown at 37°C, whereas at 28°C, it appears to be important only for cells grown in LBFS broth. In contrast, the acid chaperone function of HdeB appeared to be more important for cells grown at 28°C. Interestingly, in a K-12 mutant strain that is deficient in both HdeA and HdeB, once HdeA was provided, the acid resistance of the mutant strain (MG1655Δ*hdeAB*) was restored to the wild-type strain level, suggesting that, under the conditions examined in this study, HdeA was sufficient to protect MG1655 cells from acid-induced damages. This observation agrees with the previous report that deletion of *hdeB* in MG1655 had no impact on its survival at pH 2.5 (32). Unlike HdeA, complementary analysis of *hdeB* was much more complicated than we anticipated. Acid protection conferred by O157-HdeB occurred only in EHEC strain RM12238 (O145). Complementation resulted in a greater increase in acid survival for cells grown in LBNS at 28°C than those grown in LBFS at 37°C (Table 4), which supports our speculation that HdeB is important for acid survival of cells grown in low-salt broth at low temperature. Although production of O157-HdeB was observed in both complemented K-12 *hdeB* deletion mutants, this was not associated with any increased acid survival. Complementation by K-12-HdeB was observed in the BW25113 background but not in the MG1655 background. This strain-specific HdeB function agreed with our previous observation that loss of HdeB had a much larger impact on acid resistance of BW25113 than that of MG1655 when prechallenge cells were grown in LBNS at 28°C. It remains unclear whether the difference described above was due to the distinct genetic background between MG1655 and BW25113.

Acid protection conferred by HdeA and HdeB is serovar dependent in *E. coli*. Our study further demonstrated that the con-

tributions of both HdeA and HdeB to acid resistances of *E. coli* cells are highly serovar dependent. Similar to K-12-derived strains, loss of both chaperones in an *E. coli* O145 strain resulted in a significant decrease in acid survival for cells grown either in LBFS at 37°C or in LBNS at 28°C, suggesting the important roles of HdeA and HdeB in this EHEC strain. However, similar to *E. coli* O157:H7, these two acid chaperones did not appear to be critical for acid survival in either of the two *E. coli* O55:H7 strains. Noteworthy is that, although in both K-12 and O145 strains HdeA/B chaperones are important for cell survival at pH 2.0, the regulation of the *hdeAB* genes in these two serovars is likely different since the complementation provided by either O157-HdeA or O157-HdeB in these two serovars varied largely (Table 3 and Table 4).

***E. coli* O157:H7 strains have evolved to be independent of HdeA/B for acid survival.** Unlike other EHEC strains, *E. coli* O157:H7 strains have evolved to be independent of periplasmic chaperones HdeA and HdeB for their acid survival. This feature appears conserved also in enteropathogenic *E. coli* O55:H7, a clone that is closely related genetically to *E. coli* O157:H7 (15, 16, 45). This phenotypic divergence within the *E. coli* species appears to correlate with the genetic variations of the *hdeB* gene (Fig. 4). *E. coli* O157:H7 strains form a cluster with several *E. coli* O55:H7 strains, whereas other EHEC strains, including serotypes O26, O103, O111, and O145, group together. The *hdeB*-SNP specific to *E. coli* O157:H7 occurs in the start codon, resulting in a change from ATG to ATA, an unusual start codon in both prokaryotic and eukaryotic organisms. Consequently, this change led to the depletion of HdeB naturally in *E. coli* O157:H7, whereas the production of HdeA was not affected (Fig. 3). This loss-of-function mutation in *E. coli* O157:H7 *hdeB* resembles the pathoadaptive mutations reported in various genera of bacterial pathogens (42). For example, knockout of CadA, a pathoadaptive mutation in lysine decarboxylase (LDC) in *Shigella* spp., led to activation of *Shigella* enterotoxin (33). Natural silence of LDC has also been identified in several EHEC strains in which restored LDC activity led to a decrease in adherence to tissue culture cells (23). It remains unclear whether *E. coli* O157:H7 gains any survival fitness or virulence in human host by silencing HdeB selectively. The function of the O157-HdeB appeared to be comparable to that of strain K-12 when it was expressed in a different genetic background (Table 4, O145), although, based upon the MS analyses, the abundance of O157-HdeB in all three complemented strains was uniformly much lower than that of K-12-HdeB (Table 4). The relative lower abundance of HdeB in complemented strains carrying O157-*hdeB* indicates that ATA can be used for initiating the translation of *hdeB*, but with much less efficiency compared with the "normal" start codon ATG (K-12-*hdeB*), a phenomenon that has been reported in *Caenorhabditis elegans* for controlling the DPY-11 activity during differentiation (27).

Summary. Similar to commensal *E. coli* strains, a group of organisms that are highly adapted to the gastrointestinal tract of warm-blooded animals, *E. coli* O157:H7 is equipped with diverse pathways to cope with the extremely acidic environment present in host stomachs (pH < 3) as well as organic acids encountered in host intestines (3, 26, 29). Unlike typical *E. coli* strains, however, *E. coli* O157:H7 appears to have evolved independently of the HdeA and HdeB chaperones for coping with acid stress and displays higher acid resistance than K-12 strains when cells were grown under nonfavorable culturing conditions such as in LBNS at 28°C. Although further studies are required to define the important factors that contribute to this high acid resistance in *E. coli* O157:H7, it is logical to hypothesize that the O157 O-islands confer this unique property. It has been shown that in *E. coli* O157, the master transcriptional regulator GadE regulates the *hdeAB* operon as well as the genes located on the LEE (locus of enterocyte effacement) island (24), suggesting that the acid resistance regulatory network in O157 is different from K-12 strains. It is also known that *E. coli* K-12 strains vary in guanosine tetraphosphate (ppGpp) concentration and RpoS level (43). Variations in RpoS, another key protein regulating *E. coli* acid resistance, were reported to be associated with different levels of acid resistance of *E. coli* O157:H7 (37). However, it remains elusive if the high acid resistance of *E. coli* O157 observed in this study is linked to the RpoS activity directly. Selective silence of *hdeB* in *E. coli* O157:H7 provides evidence of the mutation-driven evolution of the *E. coli* core genome and suggests that, in addition to lateral gene transfer, genetic mutations have played important roles in driving divergent evolution of the acid resistance machinery within the *E. coli* species.

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