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Research Paper

Escherichia coli O157:H7 Stationary-Phase Acid Resistance and Assessment of Survival in a Model Vegetable Fermentation System

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ABSTRACT

Escherichia coli O157:H7 (STEC) acid resistance may aid the pathogen's ability to cross the human gastric barrier, which makes it an organism of concern in acidic foods. Our objective was to determine how STEC acid resistance may correlate with survival during vegetable fermentations. Seven *E. coli* O157:H7 strains were screened to assess acid resistance in simulated stomach acid at pH 2. The strains were separated into two groups that differed in acid resistance ($P < 0.05$), with three being acid sensitive and four acid resistant. The growth rates of these strains were measured in a Luria broth at pH values from 4.2 to 6.8. Two strains having similar growth kinetics, B201 (acid sensitive) and B241 (acid resistant), were selected for further analysis. B201 was found to be missing (compared with B241) two glutamic acid decarboxylase regulatory genes required for acid resistance, *gadE* and *gadX*. These strains were challenged in lactic acid (100 mM) solutions, including cucumber juice (CJ) media at pH 3.3. As expected, B201 was more acid sensitive than B241, and a filtered fermented CJ was more inhibitory than similarly acidified CJ. In competitive growth studies with *Lactobacillus plantarum* LA445 in CJ, B201 or B241 grew from approximately 10^4 to 10^8 CFU/mL within 24 h, but the STEC strains were below the limit of detection by 48 h. In all fermentations, *L. plantarum* reached 10^8 CFU/mL by 48 h. However, in three of four independent fermentation experiments, strain B201 survived longer than B241. This was possibly due to buffering in B241-LA445 fermentation brines that had increased lactic acid for a given pH compared with B201-LA445. These data indicate that stationary-phase acid resistance may not accurately predict STEC survival during vegetable fermentations.

HIGHLIGHTS

- *E. coli* STEC strains differed in acid sensitivity under different acid stresses.
- An acid-sensitive STEC strain was found to lack *gad* regulatory genes.
- Acid resistance of STEC did not correlate with fermentation brine survival.

Key words: Acid resistance; Competition; *Escherichia coli* O157:H7; Survival; Vegetable fermentation

Shiga toxin–producing *Escherichia coli* O157:H7 strains (STEC) are pathogenic and have a low infectious dose estimated at 10 CFU/g or lower in foods *(34)*. These strains are typically capable of surviving extended exposure to moderate (pH 3 to 4.5) and extreme (pH 1.5 to 3.0) acid stress *(9, 10)*. To cause disease, pathogenic strains of *E. coli* such as the O157:H7 serotype must first survive the gastric barrier, a powerful defense imposed by the acid in the human stomach. Hydrochloric acid (HCl) is an important bactericidal component, with pH values as low as 1.2 to 2.5 in a fasting stomach *(22, 36, 42)*. Reduced stomach acidity can influence whether this pathogen crosses the gastric barrier and has been associated with an increased risk in developing complications from STEC such as hemolytic uremic syndrome *(15)*. The gastric barrier is not the only source of acid stress that foodborne pathogenic *E. coli* might encounter. Organic acids are commonly found in the digestive tract, as well as in fermented and acidified foods *(14, 27, 30)*.

Organic acids that are present in fermented and acidified foods are inhibitory to bacteria due to pH stress, but they can also cause intracellular acid anion stress and alter cell physiology *(7, 37–39)*. In their protonated (undissociated) form, organic acids in fermented or acidified foods can diffuse across the lipid bilayer of bacterial cells *(8)*. Upon entry into a bacterial cell, the neutral pH of the intracellular environment causes protons to dissociate from the carboxyl groups of weak acids and results in acidification of the cytoplasm and acid anion accumulation

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TABLE 1. *Strains used in this work*

ID[a]	Previous ID	Genus and species	Serotype	Source[b]
B201	SRCC1675	*Escherichia coli*	O157:H7	Apple cider, Silliker
B241	28RCI	*Escherichia coli*	O157:H7	Bovine, Clay Center
B246	3139-98	*Escherichia coli*	O157:H7	Human, Clay Center
B263	RM1242	*Escherichia coli*	O157:H7	Human, WRRC
B271	RM4406	*Escherichia coli*	O157:H7	Human, WRRC
B301	RM5630	*Escherichia coli*	O157:H7	Water, WRRC
B307	RM5875	*Escherichia coli*	O157:H7	Water, WRRC
MOP3	LA445	*Lactobacillus plantarum*	NA[c]	Fermentation, FSRU

[a] ID, culture identification code, USDA-ARS Food Science Research Unit, Raleigh, NC.
[b] Silliker, Silliker, Inc., Chicago, IL; Clay Center, U.S. Meat Animal Research Center, Clay Center, NE; WRRC, Western Regional Research Center, Albany, CA.
[c] NA, not applicable.

(39). STEC cells can mitigate this stress through multiple acid resistance systems, including inducible amino acid decarboxylation enzymes (principally glutamate decarboxylase), which can raise intracellular pH *(21)*. Organic acids vary in antimicrobial activity depending on the pK of the acid and other intrinsic and extrinsic factors such as dissolved oxygen and temperature *(6, 28, 31)*.

There have been outbreaks of disease caused by pathogenic *E. coli* from the consumption of contaminated fermented foods, including kimchi, cheese, and apple cider *(3, 13, 17, 23)*. Whereas STEC strains are known to vary in acid resistance *(26, 33),* it remains unclear how these differences may influence the ability of these strains to survive food fermentation. At the start of a vegetable fermentation, the pH and environmental conditions may be permissive for the growth of STEC in competition with other epiphytic bacteria *(16, 24)*. As pH decreases, organic acids (principally lactic and/or acetic acid) accumulate during fermentation, inhibiting growth and eventually killing STEC strains, although these strains may survive for extended periods *(4)*. To aid in understanding which STEC strains would be the most likely to survive in fermented vegetables, we hypothesized that known differences in the acid resistance of STEC strains, which may aid in passage through stomach acid, would correlate with survival of STEC in model vegetable fermentations in competition with *Lactobacillus plantarum,* the bacterium known to predominate in vegetable fermentations *(18)*. Understanding factors that influence the survival of pathogenic *E. coli* strains in fermented vegetable brines may help define safe processing conditions for fermented foods.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. coli* O157:H7 strains and the *L. plantarum* strain LA445 were obtained from the U.S. Department of Agriculture Food Science Research Unit culture collection, Raleigh, NC (Table 1). The media for preparing cell cultures included de Man Rogosa Sharpe (MRS) broth for culturing *L. plantarum,* or Miller Luria-Bertani (LB) broth (Sigma-Aldrich, St. Louis, MO) supplemented with 1% (w/v) glucose (D+ glucose, Sigma-Aldrich) (LBG), for culturing *E. coli*. A sterile cucumber juice (CJ) medium was prepared as a 50% solution in water with a final concentration of 2% sodium chloride (NaCl), as described by Dupree et al. *(16)*. Briefly, cucumbers were blended into a slurry, frozen, and filtered through cheesecloth; after centrifugation at 4,000 × *g* (Sorvall Superspeed centrifuge, GSA rotor, Dupont Instruments, Newton, CT) for 45 min, the medium was filter sterilized. The CJ medium was stored at 4°C when not in use and was anaerobically equilibrated at room temperature in an anaerobe chamber (COY Laboratory Products Inc., Grass Lake, MI) for 16 h before use. To prepare acid resistance–induced cells *(10),* STEC strains were streaked for isolation from frozen stocks. Next, selected colonies were grown in LBG without aeration at 37°C, and *L. plantarum* was grown in MRS broth at 30°C overnight for 18 to 20 h. The final pH of the LBG was measured to confirm that it was below pH 5 (to help assure that induction of acid resistance occurred). Cells were harvested by centrifugation using a Sorvall SM-24 rotor (10 min, approximately 3,000 × *g,* at 15°C) (Sorvall Superspeed centrifuge, DuPont Instruments) or in 1-mL volumes using a microcentrifuge (12,000 × *g,* 1 min at room temperature) (Spectrofuge 24D, Labnet International, Inc., Edison, NJ). After the cells were resuspended, washed with 0.85% NaCl (saline), and centrifuged as above, they were resuspended in an equal volume of saline or were concentrated 10-fold to give at least 10^9 CFU/mL and then were used as described below.

For all cells subjected to acid challenge or brine fermentation, acid stress was relieved by an initial 10-fold dilution into a 10 mM potassium phosphate or 100 mM 3-(*N*-morpholino)propanesulfonic acid buffer (pH 7.0; Sigma-Aldrich) containing 0.85% NaCl (buffered saline). Cell counts were determined by serial 10-fold dilution followed by spread plating on petri plates or by using the track dilution method *(25, 41)* with rectangular agar plates (100 by 100 mm; Thermo-Fisher Scientific, Waltham, MA). Colonies were enumerated after incubation for 24 h at 37°C (LB agar, STEC) or 48 h at 30°C (MRS, *L. plantarum*). Log reductions were calculated as the difference between initial and final cell counts, using mean log CFU per milliliter counts from at least three independent biological experimental repetitions from initial and final cell samples. For mixed-culture samples, STEC strains were counted on LB agar after incubation for 24 h at 37°C because *L. plantarum* did not form visible colonies under these conditions. *L. plantarum* from mixed cultures was plated on MRS agar modified with the addition of HCl to achieve an agar pH of approximately 6.25. Incubation of this medium at 30°C for 48 h resulted in countable *L. plantarum* colonies, but STEC did not form visible colonies.

Growth rate measurements. Bacterial growth rates were determined using a 96-well microtiter plate assay with 180-μL volumes of LBG medium and 20 μL of cell suspension. Cell

suspensions with each of the seven STEC strains (individually) were prepared as described above. The LBG (at 111% concentration) was adjusted to the indicated pH values (pH 6.85, 5.76, 5.17, 4.85, 4.47, and 4.19) using 5 N HCl and then was filter sterilized using 0.2-μm, 500-mL bottle filters (Thermo-Fisher Scientific). Cells were diluted (approximately 100-fold) and added to the wells to give an initial inoculation optical density at 630 nm of between 0.05 and 0.11 absorbance units above uninoculated control wells, which had 20 μL of sterile saline added instead of cells. The microtiter plate wells were then overlaid with 75 μL of sterile mineral oil to prevent evaporation and were incubated for 24 h at 30°C with automatic shaking prior to reading once an hour in the microtiter plate reader (model ELx808, BioTek Instruments, Inc., Winooski, VT). The growth rates for each strain were estimated from the optical density data using a custom Matlab program (F.B., personal communication) that was based on a sequential processing algorithm, rather than a curve fitting algorithm (Breidt et al. *(7)*), to process nonsigmoidal curves that sometimes occur with automated growth measurements and conditions inhibitory to bacterial growth.

Synthetic stomach acid challenge. *E. coli* O157:H7 strains were challenged in simulated stomach acid aerobically and anaerobically. LB medium was acidified with HCl to pH 2.0 (±0.1) and filter sterilized to simulate stomach acid as described *(29)*. For anaerobic conditions, the medium was then placed in an anaerobic chamber (Coy Laboratory Products Inc.) in 100-mL glass bottles with the caps loose for ≥16 h to allow the dissipation of dissolved oxygen *(31)*. Cell cultures were prepared in LBG as described above, and 200 μL of each washed cell suspension was added to 1.8 mL of the acidified LB in separate wells of a 12-well culture plate (Corning 353043, Sigma-Aldrich), resulting in approximately 10^7 to 10^8 CFU/mL. The culture plates were then incubated for 2 h at 37°C and shaken at 80 rotations per minute (rpm) on a platform shaker (for aerobic conditions) or statically in the anaerobic chamber (anaerobic conditions).

Organic acid challenge. For susceptibility to organic acids, four different solutions were prepared in water or CJ medium made from fresh cucumbers (as described above) for aerobic or anaerobic acid challenge (as described above). Lactic acid challenge solutions with approximately 100 mM sodium L-lactate (Sigma-Aldrich) were prepared with deionized water with a final concentration of 2% NaCl and a final pH adjusted to 3.3 using HCl (acidified water [AW]). The remaining solutions used for anaerobic challenge were preincubated in the anaerobic chamber in bottles with caps loose for at least 16 h to allow oxygen depletion. Anaerobic acidified water (AAW) consisted of the AW medium supplemented with 6 mM acetic acid (glacial acetic acid, Sigma-Aldrich). Acidified cucumber juice (ACJ) was prepared with 100 mM lactic acid, 6 mM acetic acid in 50% CJ with 2% NaCl and was adjusted to pH 3.3. Finally, a fermented CJ medium (FCJ) with 2% NaCl was prepared that contained approximately 100 mM lactic acid and 6 mM acetic acid at pH 3.3. This medium was prepared by inoculating *E. coli* strain B241 and *L. plantarum* strain MOP3 into 150 mL of CJ with 2% NaCl, followed by incubation for 24 h at 30°C. The fermented medium was then centrifuged at 3,000 × *g* for 10 min at 15°C. The supernatant was then decanted and supplemented with lactic acid targeting 100 mM final concentration as confirmed by high-performance liquid chromatography (HPLC, see below). The pH was adjusted to pH 3.3 and the medium was sterilized by filtration through a 0.22-μm filter. After adjustments, the overall composition was 45% FCJ with approximately 100 mM lactic acid, 1.8% (w/v) NaCl, and pH 3.3.

For aerobic acid challenge, *E. coli* O157:H7 cells were prepared by static growth for 20 h at 37°C in 10 mL of LBG or CJ, harvested by centrifugation, washed in saline, and resuspended (as described above). For anaerobic acid challenge, cells were similarly prepared but were grown overnight in CJ with 2% NaCl in the anaerobic chamber for 20 h at 30°C. Acid challenge was done in 12-well culture plates containing 1.8 mL of AW, AAW, ACJ, or FCJ, and each well included 0.2 mL of cell suspension (B201 or B241). The initial cell concentrations were between 10^6 and 10^7 CFU/mL. The plates were covered and incubated aerobically for 1 h or in the anaerobic chamber for 3 h. The samples (0.1 mL) were removed at the start and end of incubation for enumeration as described above.

Biochemical analysis. HPLC analysis of the organic acid challenge solutions and fermentation samples was conducted using a modification of the method of McFeeters and Barish *(32)*. Samples were separated on an Aminex HPX-87H resin column (300 by 7.8 mm; Bio-Rad Laboratories, Hercules, CA) used with a Shimadzu HPLC system (UFLC, Shimadzu Scientific Instruments, Durham, NC) and accompanying software. The mobile phase was 0.01 N H_2SO_4, with a flow rate of 0.9 mL/m and a column temperature of 65°C. Acetic acid, lactic acid, glucose, and fructose were quantified using a refractive index detector (RID-10A, Shimadzu). Organic acid concentrations are reported as total acid (protonated plus dissociated), except where noted. The detector was standardized for each analyte in the range of 0.5 to 100 mM.

Model vegetable fermentations. *E. coli* strains B201 or B241 and *L. plantarum* LA445 overnight cultures were prepared as described above, and saline suspensions were transferred to the anaerobic chamber. Sterile CJ medium (98 mL) in Corning glass jars with 2% NaCl were preincubated with the caps loose for a minimum of 16 h to remove dissolved oxygen. The B201 or B241 cultures along with LA445 were diluted in 0.85% NaCl and were added (1 mL each) to result in an initial cell concentration of approximately 10^4 CFU/mL of each culture. For pure culture controls, 1 mL of sterile saline was added instead of a coculture. The fermentations were incubated at 30°C in the anaerobic chamber incubator (Coy Laboratory Products Inc.) for 48 h, and 2-mL samples were withdrawn for analysis. Sampling times included 0, 8, 24, 25.5, 27, 28.5, 30, 31.5, 33, 34.5, 36, and 48 h. For each 2 mL removed, 500 μL was added to 500 μL of buffered saline (100 mM, pH 7.0, with 0.85% NaCl; Sigma-Aldrich) prior to the removal from the anaerobic chamber for enumeration via track or spread plating as described above. The remaining 1.5 mL from sampling was filter sterilized using a 0.22-μm filter and was frozen for subsequent HPLC and pH evaluation. The competitive growth experiments were replicated four times with independently prepared cultures.

Genome analysis. The software package PATRIC *(44)* was used to analyze genome data from B201 and B241, based on the completed genome sequences published by Baranzoni et al. *(2)*. The sequences were examined to determine differences in known acid resistance systems, including amino acid decarboxylase pathways, such as the glutamate acid resistance system (GAD) *(21, 29)*. The GAD genes examined included the glutamate decarboxylases (*gadA* and *gadB*), the antiporter (*gadC*), and the transcriptional regulators (*gadE, gadX, gadW*).

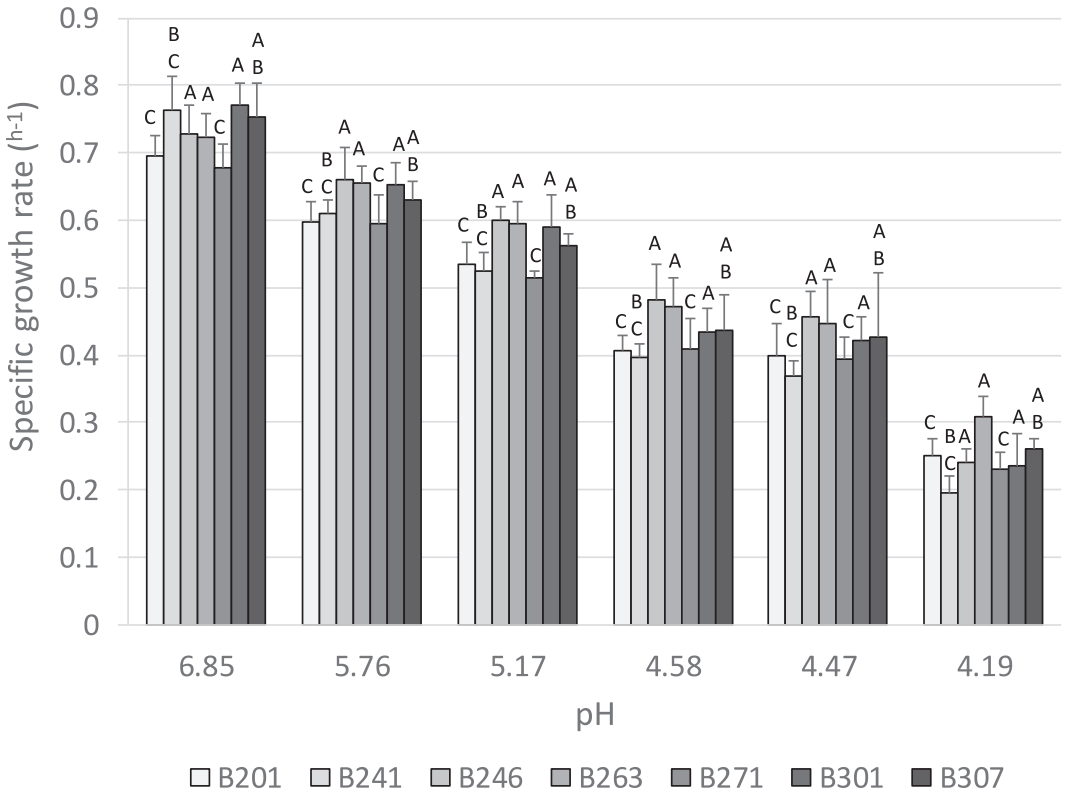

FIGURE 1. *Growth rates of STEC strains at different pH values. The growth rates of seven STEC strains were measured at different pH values. The error bars represent the standard deviations of three or more independent replications. The legend indicates the strain names (Table 1). Strains with the same letter within a pH group were not statistically different (*$P > 0.05$*) from one another by Tukey-Kramer grouping.*

Experimental design and statistical analysis. The experiments were designed to determine the acid resistance of selected STEC strains and then compare the ability of selected strains (acid sensitive and resistant) to survive in a model vegetable fermentation system. The HCl assay and organic acid challenge data were the result of three or more independent repetitions for each strain. SAS (version 9.4 software, SAS Institute Inc., Cary, NC) was used to evaluate statistical differences for growth rate data and acid challenge assays using the general linear models procedure with the Tukey-Kramer adjustment. The competitive growth model fermentation was done with four independent replications. For all experiments, differences were considered significant at the $P < 0.05$ level. Protonated acid calculations were done using the Henderson-Hasselbalch equation with the acid pK_a adjusted for the ionic strength of the CJ and organic acid solutions (for 2% NaCl, the ionic strength was approximately 0.342 M) using the Davies equation *(11)*.

RESULTS AND DISCUSSION

Growth rates of STEC strains. To determine how pH may influence the growth characteristics of pathogenic *E. coli* strains, we investigated seven selected strains (Table 1) that were previously characterized with varying acid resistance *(33)*. In an acetic acid assay system, Oh et al. *(33)* found that strain B241 was acid resistant under conditions typical of acidified vegetables (with acetic acid) compared with most other STEC strains with and without the addition of glutamic acid, indicating a functional GAD system. Strains B201 and B271 were comparatively acid sensitive and apparently lacked GAD based on the observed phenotype. GAD has been shown to be an important acid resistance mechanism *(21)*. The other three strains had varying acid sensitivity and evidence of a functional GAD system *(33)*. Comparison of growth rates for these strains in CJ medium containing 2% NaCl for pH values between 6.85 and 4.19 (Fig. 1) showed that growth rates declined with pH, as expected. STEC strains have been shown to grow in vegetable fermentation brines during the initial stages of fermentation, but they typically die off as pH is reduced below pH 4 and organic acid accumulates *(16)*.

HCl challenge. In previous acid challenge studies with STEC strains primarily focused on conditions relevant to acidified foods *(33),* acetic acid at pH 3.3 was used. To determine whether the acid resistance phenotypes observed by Oh et al. *(33)* would correlate with the ability to survive conditions of simulated stomach acid with HCl as the primary acidulent, a variant of a simulated stomach acid system *(29)* with pH 2.5 was used. Anaerobic and aerobic conditions, which had previously been shown to influence STEC survival in acid challenge studies *(28),* were investigated. Because the strains were prepared in a glucose-containing medium, previous data had indicated that the stationary-phase acid resistance system AR1 would be inactive, although other acid resistance systems, including the glutamate decarboxylase AR2 system and arginine decarboxylase system AR3, would be induced and

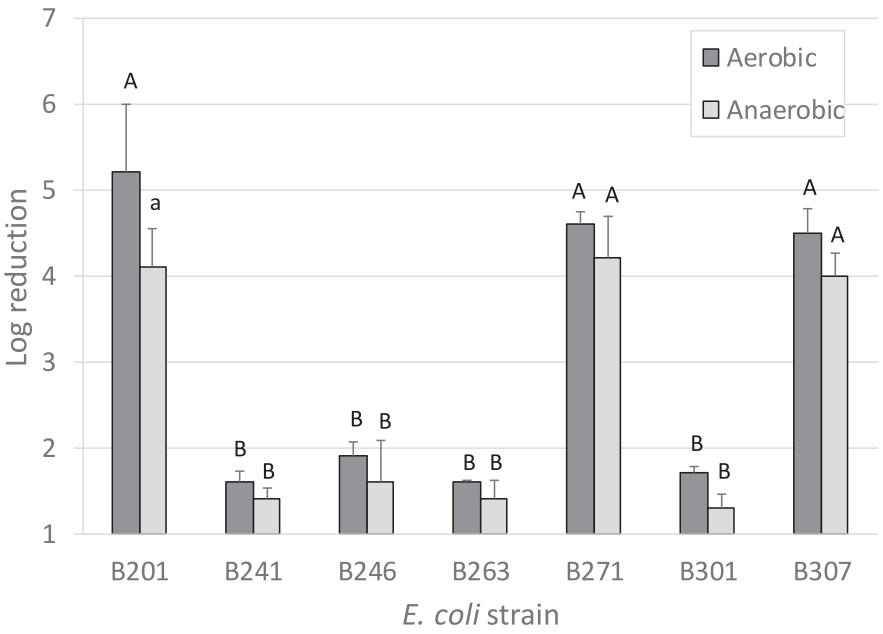

FIGURE 2. *HCl resistance of STEC strains in stationary phase. Acid resistance was measured under aerobic (dark gray bars) or anaerobic (light gray bars) conditions for strains B201, B241, B246, B263, B271, B301, and B307, as indicated with HCl at pH 2 in LB broth. The error bars represent the standard deviations from three or more independent trials. Treatments with the same letter were not statistically different (*$P > 0.05$*) from one another in the Tukey-Kramer grouping. The lowercase letter indicates a statistical difference between aerobic and anaerobic challenge for strain B201.*

active if present *(1, 21)*. However, further work will be necessary to show how these acid resistance systems are expressed in this system. The data showed there was no statistical difference between aerobic or anaerobic conditions, with the exception of strain B201. From these data, the strains could be separated into two classes: strains B201, B271, and B307 were relatively acid sensitive and the others acid resistant, with more than 2 log CFU/mL differences in survival in the HCl system (Fig. 2). Two strains were selected that consistently were identified in both the weak acid study of Oh et al. *(33)* and in the results from Figure 2 as either acid sensitive (B201) or acid resistant (B241) for further study. These strains also had similar growth rates ($P > 0.05$), as shown in Figure 1.

Genome sequence analysis. The completed genome sequences of B201 and B241 *(2)* were used to analyze potential differences related to acid resistance between the two strains. Analysis of the B201 genome showed a deletion of two regulatory genes, *gadE* and *gadW* (Fig. 3), which are involved in the activation of transcription of the AR2 glutamate decarboxylase and transport genes (*gadA* and *gadBC*) *(12)*. This result supports the previous data for B201 that showed no increase in acid resistance with the addition of glutamic acid during acetic acid stress *(33)* and may help explain the differences in acid resistance between B201 and B241 shown in Figure 2.

Lactic acid challenge. To further investigate the acid resistance of B201 and B241 for conditions relevant to vegetable fermentations, the survival of these strains in lactic acid solutions was investigated under aerobic and anaerobic conditions. Whereas fermentation conditions are expected to be anaerobic, commercial manufacturers of fermented cucumbers may purge brined cucumbers with compressed air to help remove carbon dioxide, which can accumulate and cause a bloated cucumber pickle defect *(19)*. For lactic acid challenge studies, acid conditions (approximately 100 mM lactic acid, pH 3.3) typical of commercial brine fermentations were used *(18)*. For preparation of FCJ medium, brine from CJ fermented with a mixed culture of LA445 and B241 for 20 h under anaerobic conditions at 30°C was found to have 23.3 mM lactic acid. Following acid supplementation and pH adjustment to pH 3.3 and culture addition, the acid concentrations measured by HPLC for FCJ were 102.3 mM lactic acid and 4.29 mM acetic acid, which was presumably produced by B241 during fermentation, and not seen in the pure culture fermentations with the homofermentative LA445 (data not shown). Similarly, the ACJ and AAW (nonfermented CJ and water-based media) had lactic acid concentrations of 108.2 and 95.6 mM, and acetic acid concentrations of 4.29 and 5.69, respectively. The AW medium used for aerobic challenge had 92.5 mM lactic acid (no acetic acid was added).

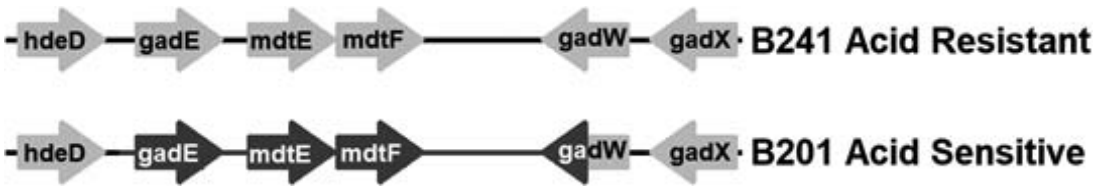

FIGURE 3. *The* gad *regulatory region of B201 and B241. A representation of the sequence data from B201 and B241 with arrows indicating the gene names, size, and orientation on the bacterial chromosome. The dark gray arrow regions indicate gene sequences that were absent in B201 compared with B241.*

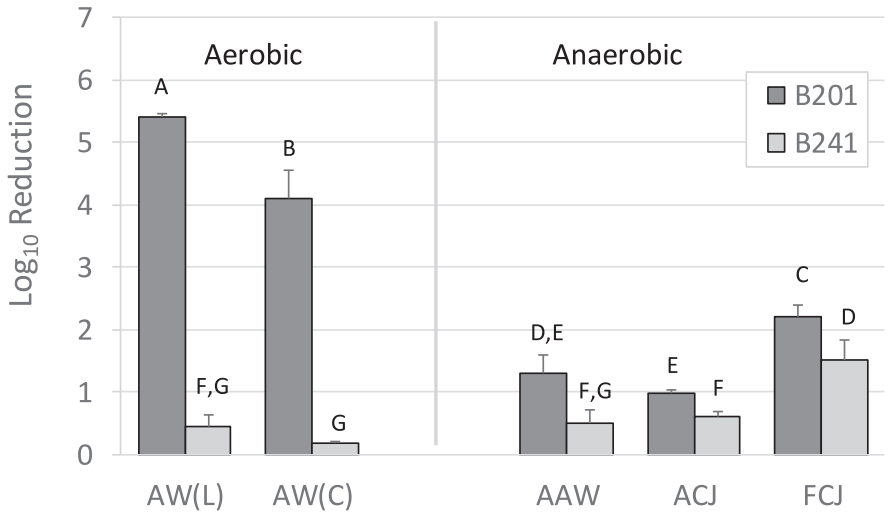

FIGURE 4. *Lactic acid resistance of B201 and B241 in stationary phase. The acid lethality (log reduction) for B201 (dark gray bars) and B241 (light gray bars) is shown for aerobic and anaerobic conditions as indicated. Targeted media acid concentration conditions included acidified water with cells grown in lactic acid (AW[L]): 100 mM lactic acid at pH 3.3, cells grown in LBG; AW(C): similar to AW(L), except cells were grown in CJ; anaerobic acidified water (AAW): 100 mM lactic acid, 6 mM acetic acid, pH 3.3 (in water); acidified cucumber juice (ACJ): 100 mM lactic acid, 6 mM acetic acid, pH 3.3 (in CJ); fermented cucumber juice (FCJ): 100 mM lactic acid, 6 mM acetic acid, pH 3.3. The error bars represent the standard deviations from three or more independent trials. Bars with different capital letters were statistically different (*$P < 0.05$*).*

For all acid treatments, B201 was more acid sensitive than B241 ($P < 0.05$) (Fig. 4). For aerobic treatments of lactic acid in water, B201 cells that were prepared by growth in LB (AW[L]), did not survive as well as B201 ($P < 0.05$) prepared by growth in CJ medium (AW[C]) (Fig. 4, aerobic). The mean log reduction values for these experiments were 5.4 and 4.1 log CFU/mL, respectively. In contrast, B241 had less than 0.5-log reductions, which were similar ($P > 0.05$) regardless of growth media. These data confirmed the results of Kreske et al. *(28),* who showed that under aerobic conditions oxygen increases acid sensitivity, and confirmed previous results that showed the relative sensitivity of B201 compared with B241. It is unclear why cells grown in CJ medium (which was used in subsequent fermentation competition studies, below), had improved survival compared with cells prepared in LBG. Because CJ medium was used in fermentation competition studies (described below), subsequent anaerobic acid challenge studies with AAW, ACJ, and FCJ were done with cells prepared with CJ.

Under anaerobic conditions (Fig. 4, anaerobic) using solutions prepared with lactic acid in water (AAW), lactic acid and acetic acid in CJ (ACJ) had similar mean log reduction values for B201, 1.3 and 0.97, and for B241, 0.5 and 0.61, respectively. For FCJ, B201 and B241 strains had increased ($P < 0.05$) log reduction values of 2.2 and 1.5, respectively, compared with that for the same strain in AAW or ACJ (Fig. 4, anaerobic). These observations are suggestive and may indicate that some medium component in the fermented sample other than the acid concentration and pH may be influencing (increasing) lethality for the FCJ samples compared with the other treatments. Fermentation brines with lactic acid bacteria are known to contain multiple inhibitory compounds in addition to organic acids, including diacetyl, acetaldehyde, bacteriocins, peroxides, and possibly other small molecules *(14, 43)*. Future work will be required to investigate the differences in lethality observed for FCJ and the other anaerobic media tested.

Competitive growth of STEC and *Lactobacillus*. To determine whether the differences observed in acid resistance genotype and phenotype influenced the ability of B201 or B241 to survive in vegetable fermentations, a model fermentation system with a CJ brine containing 2% NaCl was used. The 2% NaCl represented fermented vegetable foods that are not further processed before consumption. CJ medium contains no known antimicrobial inhibitors but may have nutrients representative of a variety of brined and fermented vegetable products. We compared the survival of B201 and B241 in competition with *L. plantarum* LA445, an isolate that was originally from an experimental 11,000-L anaerobic cucumber fermentation tank that was able to outcompete an *L. plantarum* starter culture *(20)*. Selective plating of the STEC strains or LA445 was achieved using LB agar at 37°C or MRS acidified to pH 6.25 at 30°C. The MRS had an initial pH of 6.4 but, without acidification by HCl to pH 6.25, some STEC growth was observed. In all experiments, we found that the STEC strains initially outgrew the *L. plantarum* culture, starting at approximately 10^4 CFU/mL and reaching 10^8 CFU/mL within 24 h (Fig. 5). By 48 h, however, the STEC strains were below the limit of detection, and LA445 was approximately 10^8 CFU/mL. Interestingly, B201, the acid-sensitive strain, survived longer than B241, the acid-resistant strain, in three of the four replicated experiments (Fig. 5, panels A, B, and C), whereas survival of B201 and B241 was similar for the remaining experiment (Fig. 5,

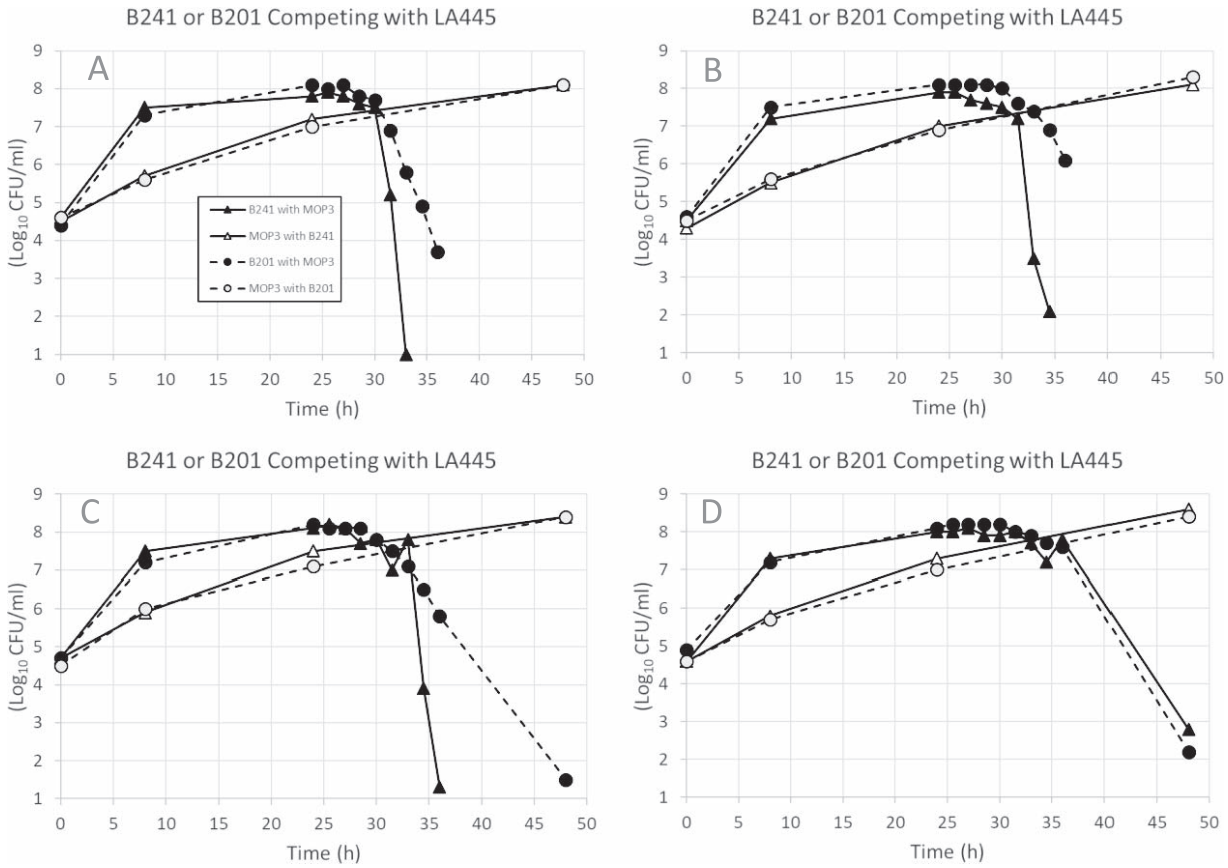

FIGURE 5. *Competitive growth of B201 or B241 with LA445. Four independent replications of the experiments are shown (panels A through D). Competition of B241 (black triangles, solid line) with LA445 (clear triangles, solid line) and competition of B201 (black circles, dashed line) with LA445 (clear circles, dashed line) are shown. All cell counts that are shown below 2 log are estimated from data with 1 to 20 colonies per plate.*

panel D). The LA445 cultures had similar growth patterns in all competitive culture experiments and was approximately 10^8 CFU/mL at 48 h.

The lactic acid concentration was below the limit of detection in the pure cultures until 24 h (Table 2). Acetic acid was not present with pure cultures of LA445 by itself and was not detected in the pure cultures of STEC until 24 h, at which time there was 6.09 ± 0.59 mM for B201 and 5.21 ± 0.86 mM for B241 (data not shown). In the mixed cultures, there was also no detectable lactic acid until the 24-h sampling point, at which time B201-LA445 fermentations had 17.62 ± 1.87 mM and B241-LA445 fermentations had 18.64 ± 3.19 mM (Table 2). The total lactic acid increased to 31.80 ± 3.09 mM in B201-LA445 and 39.41 ± 2.92 mM in B241-LA445 by 36 h. The final concentrations of lactic acid for B201-LA445 and B241-

TABLE 2. *Biochemistry of mixed culture fermentations with B201 and B241 in competition with MOP3*[a]

Time (h)	B201 (mixed culture)				B241 (mixed culture)			
	pH	Lactic	Glucose	Fructose	pH	Lactic	Glucose	Fructose
0	5.58 ± 0.05	ND	25.07 ± 0.73	28.04 ± 0.87	5.59 ± 0.04	ND	25.06 ± 0.75	28.02 ± 0.88
8	5.47 ± 0.03	ND	24.13 ± 1.10	27.19 ± 1.23	5.49 ± 0.03	ND	24.34 ± 1.09	27.42 ± 1.23
24	4.42 ± 0.11	17.62 ± 1.87	17.53 ± 0.84	25.53 ± 0.99	4.44 ± 0.07	18.64 ± 3.19	17.48 ± 1.60	24.43 ± 2.13
25.5	4.33 ± 0.09	19.16 ± 1.86	17.04 ± 0.84	25.25 ± 0.28	4.40 ± 0.06	21.48 ± 2.38	17.47 ± 1.07	24.97 ± 0.59
27	4.26 ± 0.08	20.75 ± 1.92	16.68 ± 0.94	25.2 ± 0.39	4.34 ± 0.07	23.64 ± 2.60	16.65 ± 1.02	24.48 ± 0.56
28.5	4.19 ± 0.08	21.94 ± 2.76	15.84 ± 0.77	24.47 ± 0.82	4.28 ± 0.09	26.04 ± 2.74	15.77 ± 1.09	23.89 ± 0.86
30	4.11 ± 0.09	23.16 ± 2.60	15.12 ± 1.36	23.82 ± 1.21	4.18 ± 0.11	27.74 ± 4.21	14.46 ± 1.41	22.58 ± 1.46
31.5	4.06 ± 0.08	25.11 ± 2.86	14.70 ± 1.41	23.67 ± 1.29	4.08 ± 0.11	30.98 ± 3.22	14.02 ± 1.06	22.6 ± 0.59
33	3.98 ± 0.07	27.73 ± 2.84	14.51 ± 0.90	23.94 ± 0.17	4.01 ± 0.09	33.66 ± 3.84	13.22 ± 0.91	22.04 ± 0.60
34.5	3.91 ± 0.07	29.70 ± 3.02	13.87 ± 0.95	23.47 ± 0.44	3.91 ± 0.08	36.54 ± 3.00	12.56 ± 0.95	21.64 ± 0.70
36	3.86 ± 0.07	31.80 ± 3.09	13.24 ± 0.96	23.05 ± 0.29	3.84 ± 0.09	39.41 ± 2.92	11.91 ± 1.06	21.26 ± 0.78
48	3.57 ± 0.02	48.18 ± 2.77	8.18 ± 1.19	19.11 ± 0.69	3.57 ± 0.06	55.40 ± 3.73	6.66 ± 1.28	16.69 ± 1.20

[a] Acid and sugar concentrations are in mM units (±standard deviation from the mean). ND, not detected.

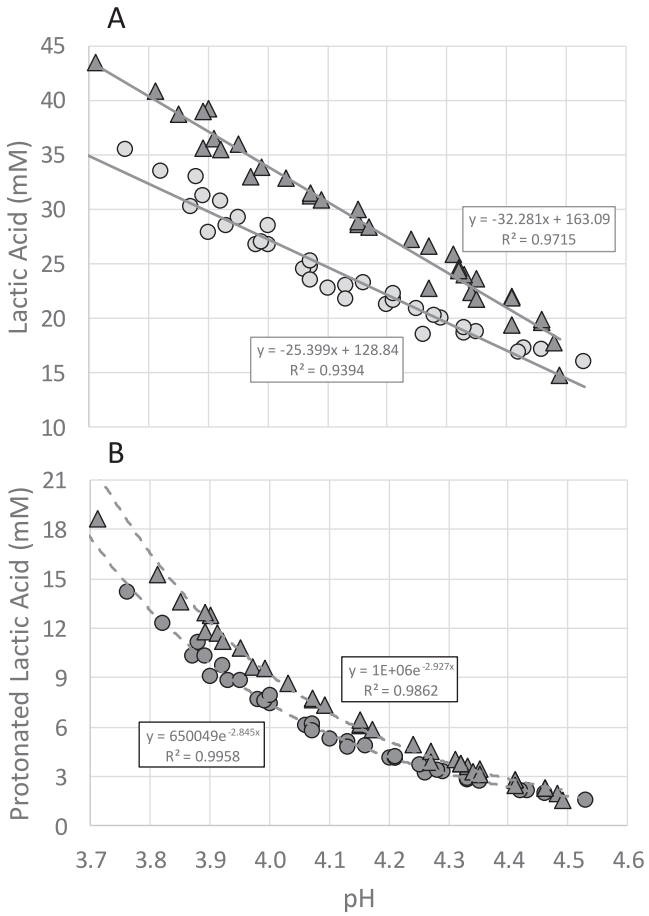

FIGURE 6. *Lactic acid at different pH values. The total lactic acid (A) and calculated protonated lactic acid concentrations (B) are shown at different pH values for all fermentations with B201 in competition with LA445 (circles, dashed line) or B241 in competition with LA445 (triangles, solid line). The lines represent linear (A, solid line) or exponential (B, dashed line) regression for the indicated data set. The boxes indicate the model equations and regression coefficients.*

LA445 cultures at 48 h were 48.18 ± 2.77 and 55.40 ± 3.73 mM, respectively. For all fermentations, the mean glucose concentrations remained above 6 mM at 48 h. More total sugar remained in the B201 mixed-culture fermentations by 48 h compared with the B241 fermentations (27.29 versus 23.35 mM) (Table 2). It was also evident that glucose was the preferred carbon source, with fructose utilization increasing after glucose levels declined at 48 h.

For all mixed-culture fermentations, the pH changes were similar over time (Table 2); however, the total lactic acid and protonated lactic acid concentrations were higher for a given pH value in the B241 competition experiments compared with the B201 competition experiments (Fig. 6A and 6B). These data indicate that the increased mortality of B241 compared with B201 in the competition experiments may be due to increased buffering of the CJ medium, which allowed greater lactic acid production by LA445 during competitive growth with B241 compared with B201 and, therefore, greater protonated acid concentrations (Fig. 6B). Weak acid killing of bacteria in organic acid–containing solutions is mediated by both protonated acid effects and proton (pH) effects *(5),* although quantifying these effects remains difficult due to synergistic interactions. It is possible that buffering due to glutamate decarboxylation *(21)* by B241 in the fermentations may have altered the extracellular pH sufficiently to influence acid production by LA445, although this remains unclear. In pure culture fermentation, B241 brines had a mean pH of 4.55 compared with a pH of 4.39 for B201 at 48 h, although sugar utilization and acid production were similar (data not shown). These differences may be due to decarboxylation reactions or related buffering reactions. It is known that *L. plantarum* also has a *gad* system *(40),* as well as a malic acid decarboxylation enzyme, which can influence growth rates and medium pH *(35)*. Future work will be needed to determine how buffer capacity in fermentation brines changes during fermentation. Understanding the dynamic changes in pH, protonated acid, and buffering in fermentation brines may lead to a better understanding of safe fermentation practices, aiding producers and regulatory agencies.

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