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Enhanced formation of shiga toxin-producing *Escherichia coli* persister variants in environments relevant to leafy greens production

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A B S T R A C T

Bacterial persistence is a form of phenotypic heterogeneity in which a subpopulation, persisters, has high tolerance to antibiotics and other stresses. Persisters of enteric pathogens may represent the subpopulations capable of surviving harsh environments and causing human infections. Here we examined the persister populations of several shiga toxin-producing *Escherichia coli* (STEC) outbreak strains under conditions relevant to leafy greens production. The persister fraction of STEC in exponential-phase of culture varied greatly among the strains examined, ranging from 0.00003% to 0.0002% for O157:H7 strains to 0.06% and 0.08% for STEC O104:H4 strains. A much larger persister fraction (0.1–11.2%) was observed in STEC stationary cells grown in rich medium, which was comparable to the persister fractions in stationary cells grown in spinach lysates (0.6–3.6%). The highest persister fraction was measured in populations of cells incubated in field water (9.9–23.2%), in which no growth was detected for any of the STEC strains examined. Considering the high tolerance of persister cells to antimicrobial treatments and their ability to revert to normal cells, the presence of STEC persister cells in leafy greens production environments may pose a significant challenge in the development of effective control strategies to ensure the microbial safety of fresh vegetables.

1. Introduction

Shiga toxin-producing *Escherichia coli* (STEC), one of the most important causal agents of foodborne illness linked to fresh leafy vegetables (Rangel et al., 2005), is spread mainly from cattle into the environment by fecal shedding (Smith et al., 2014). STEC can be further dispersed and disseminated by runoff into watersheds. Contamination of leafy greens can occur either in pre- or postharvest environments, via contaminated water, manure, wildlife, and even insects in the leafy greens-growing fields (Berger et al., 2010; Brandl, 2006; Cooley et al., 2014; Strawn et al., 2013) or in the water used for washing and processing leafy greens, from workers, or cross-contamination from other food at the postharvest level (Beuchat and Ryu, 1997; Gomez-Lopez et al., 2013; Lynch et al., 2009). STEC can cause severe disease in humans at a low infection dose; thus, the presence and dissemination of STEC in leafy greens production environments may pose a risk to public health given that the leafy greens are often consumed raw.

STEC can survive for prolonged periods of time in natural environments including soil, manure, field water, plants, and various processed foods (Franz et al., 2008; Fremaux et al., 2007; Ma et al., 2014; Moyne et al., 2011), where the pathogen will encounter stressful conditions such as nutrient limitation, UV radiation, fluctuating temperatures and water availability, and the presence of antimicrobial compounds (Brandl, 2006; Fremaux et al., 2008). Bacteria including STEC have evolved various mechanisms to cope with challenging environments. Genetic variants produced by adaptive mutations, genome rearrangements, or gene acquisitions can confer upon a subpopulation of cells enhanced fitness in a particular niche (Brzuszkiewicz et al., 2009; Carter et al., 2012, 2014; Wisniewski-Dye and Vial, 2008). Similarly, phenotypic variants, resulting from epigenetic regulation or stochastic gene expression (Avery, 2006), may have the potential to survive better under stressful conditions or to adapt rapidly to new niches (Davidson and Surette, 2008).

Unlike genetic modification, the bacterial persister state is a form of dormancy; thus persister cells are able to survive exposure to antibiotics or other environmental stresses including nutrient limitation and oxidative stress, better than the bulk of the population (Balaban et al., 2004; Lewis, 2010; Norton and Mulvey, 2012). This phenomenon, first described in *Staphylococcus aureus* (Bigger, 1944), is known to be present in diverse bacterial species and may have evolved as a general environmental stress response in bacteria (Ayrapetyan et al., 2015; Kussell et al., 2005). Persister cells are considered as a major cause of the recurrence of chronic infectious disease and contribute to the emergence of antibiotic resistance (Fauvart et al., 2011; Lewis, 2012). Unlike mutation-mediated antibiotic resistance, the persister state is non-genetic and transient, likely brought about by individual cells that can enter a transient dormant-like state where metabolism is slowed and growth is arrested (Balaban et al., 2004; Kint et al., 2012). Persister cells share several physiological characteristics and overlap in molecular mechanisms with that of viable but non-culturable cells (VBNC)

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(Ayrapetyan et al., 2015). However, VBNC and persister cells differ in their detectability on nutrient media. Whereas persisters are able to grow on media shortly after the antibiotics treatment, VBNC cells require a relatively prolonged resuscitation-promoting treatment before they can be cultured (Li et al., 2014).

Persister cells were initially thought to be produced stochastically (Balaban et al., 2004; Levin-Reisman and Balaban, 2016). Recent studies suggest that the ability to form persisters could be genetically controlled and induced in response to antibiotics and environmental stresses (Dorr et al., 2009; Johnson and Levin, 2013; Lewis, 2012; Maisonneuve et al., 2013; Nguyen et al., 2011; Wu et al., 2012). Disruption of genes involved in cell metabolism has frequently been found to alter persister population, implying a link of bacterial metabolism with the formation of persisters (Amato and Brynildsen, 2014; Amato et al., 2013, 2014). Because persister cells have higher tolerance to host immune defenses, antibiotics, and other general stresses (Fisher et al., 2017), such persistence would affect bacterial pathogenesis, antibiotic tolerance, and the environmental persistence of human pathogens. Therefore, persisters may represent subpopulations of enteric pathogens capable of surviving postharvest conditions and antimicrobial treatment of fresh vegetables and fruits, and further infect human hosts.

Although formation of persister cells in nonpathogenic *E. coli* strains, e.g. K-12, has been studied extensively, information about this phenomenon in STEC is scarce. Compared with *E coli* K-12, STEC carries a larger genome (Hayashi et al., 2001; Perna et al., 2001) and more genes encoding toxin-antitoxin (TA) modules or homologs of TA, which are known to play a role in formation of bacterial persisters (Lewis, 2010). In this study, we assessed the persister populations of several STEC strains in three model systems relevant to leafy greens production: 1 > Field water: field surface water in agricultural areas is a common nonhost habitat for enteric pathogens in preharvest environments. A surveillance study on the prevalence of STEC at public access watershed sites in a California central coast agricultural region revealed that nearly 11% of the water samples were positive for STEC (Cooley et al., 2014). Contaminated surface water near produce-growing fields may serve as a source of preharvest contamination; 2 > Spinach leaf lysates: the chemical conditions in spinach leaf lysates serve as a proxy for the microenvironment where leaf tissue is damaged biologically or mechanically. Such sites are known to promote rapid proliferation of enteric pathogens (Brandl, 2008; Simko et al., 2015) while also causing oxidative, osmotic, and antimicrobial stress in enteric pathogens (Kyle et al., 2010; Scott et al., 2017); 3 > Spinach leaf wash waster: we previously reported that STEC O157:H7 was capable of proliferating in spinach wash water and attaching to and forming biofilms on leaf and abiotic surfaces (Carter et al., 2016, 2019); thus contaminated spinach leaf wash water could serve as a source of contamination in the postharvest leafy greens production chain. Field water is generally limited in nutrients, thus we hypothesized that the persister population of STEC in field water would be higher than that in other ecological niches with less restricted resources.

2. Material and methods

2.1. Bacterial strains, reagents and growth media

All *E. coli* strains used in this study are listed in Table 1. The strains were routinely maintained and cultured in LB broth (Lennox). Ciprofloxacin (Sigma-Aldrich) was prepared according to the manufacturer's instructions. Phosphate buffered saline (PBS) solution was prepared at 10X (per liter: 80 g NaCl, 2 g KCl, 14.4 g Na_2HPO_4 and 2.4 g KH_2PO_4; adjusted to pH 7.4), and used as assay buffer at a 1X concentration.

2.2. Determination of minimal inhibitory concentration (MIC)

The MIC for ciprofloxacin for *E. coli* strains was determined using the established guidelines for the broth microdilution method (Wiegand et al., 2008), except that LB broth was used. Briefly, each strain was grown in LB broth to the early exponential phase (OD_{600} of 0.2–0.3). The culture was further diluted in fresh LB broth at 100-fold for inoculation. An aliquot of 50 µl of the inoculum was added to each well of a 96-well microtiter plate containing 50 µl of ciprofloxacin at various concentrations. The wells containing 50 µl of water were used as growth control. The microtiter plate was incubated at 37 °C for 18 h and the MIC was determined to be the lowest concentration at which no detectable growth was observed.

2.3. Quantification of E. coli persisters

The assay for persister enumeration was adapted from previously described methods using the antibiotic-based lysing method (Keren et al., 2004; Theodore et al., 2013). Ciprofloxacin was selected due to its ability to kill growing, slow- and non-growing cells (Eng et al., 1991; Zeiler, 1985). To determine the population of persisters in exponentially growing *E. coli*, an overnight culture in LB broth at 37 °C was inoculated into fresh LB broth at a concentration equivalent to an OD_{600} of 0.005 (~10^6 cells/ml) and grown at 37 °C with shaking at 150 rpm to an OD_{600} of 0.5 (~10^8 cells/mL). The actual cell concentration was determined by plate count. Ciprofloxacin was added to each culture at 10 times the MIC and the culture returned to 37 °C with shaking. The population size of viable *E. coli* cells (persisters) was determined by plate count at 1, 3, and 5 h following addition of the antibiotic. Briefly, the aliquot of culture at each sampling time was centrifuged at 8000 × *g* for 3 min; the cells were washed once with an equal volume of 1X PBS (pH 7.4), and then resuspended in an equal volume of 1X PBS for plating. The plates were incubated at 37 °C for 16–20 h. The CFUs on LB agar plates represent the persisters that survived the ciprofloxacin challenge. To quantify the persister population in stationary phase of culture, the OD_{600} of an overnight culture in LB broth at 37 °C was assessed and an aliquot of appropriate volume was spun down at 8000 × *g* for 3 min. The cells were resuspended in 5 ml of 1X PBS to an OD_{600} of 0.5 (~10^8 cells/mL). The actual cell concentration was determined by plate count. The ciprofloxacin was then added at a concentration of 10X MIC and the persister population was enumerated as described above.

2.4. E. coli persisters in spinach lysates and spinach leaf wash water

Spinach leaf lysates were prepared as described previously using an Omega 8003 juicer (Omega Products) for homogenization (Carter et al., 2016; Kyle et al., 2010). The homogenized samples were centrifuged twice at 20,000 × *g* for 10 min to remove debris and the spinach lysates filter-sterilized, first through a 0.45 µm pore size filter then a 0.2 µm filter. Sterility was verified by plating samples onto LB agar and incubation at 26 °C for two days. One ml of the overnight culture was centrifuged at 8000 × *g* for 3 min and the cells were resuspended in one ml potassium phosphate buffer (KPB; 10 mM; pH = 7.0). The OD_{600} of the cell suspension in KPB was assessed and cells were inoculated in 3 ml sterile spinach leaf lysate at a concentration of around 10^6 cells/ml based on OD_{600}. The cultures were incubated statically at 26 °C for 24 h. The growth of *E. coli* in spinach lysates was monitored by plate enumeration. At the end of incubation, the cells were collected by centrifugation and resuspended in 1X PBS buffer to an OD_{600} of 0.5 (~10^8 cells/ml). The actual cell concentration was determined by plate count. Quantification of persisters was carried out as describe above.

Spinach leaf wash water was prepared as described previously (Carter et al., 2016). Briefly, approximately 50 g of store-bought, ready-to-eat organic baby spinach were placed in 250 ml of KPB, sonicated in an ice-water bath for 10 min followed by incubation on an undulating orbital shaker at room temperature for 15 min. The leaf wash water was filtered through a double layer of cheesecloth, and then further filtered using a 0.2 µm filter. The macronutrient contents and turbidity of the sterile spinach leaf wash water were determined at an analytical

Table 1
Strains used in this study.

Strain	Phylogroup	Serotype	Pathotype[a]	MIC of Ciprofloxacin (μg/ml)[b]	Characteristics
RM6654	E	O157:H7	STEC/EHEC	0.03	Clinical isolate linked to the 2006 U.S. spinach-associated outbreak
RM6535	E	O157:H7	STEC/EHEC	0.03	Lettuce isolate linked to a 2006 U.S. iceberg lettuce-associated outbreak
RM15012	B1	O104:H4	STEC/EAEC	0.25	Clinical isolate linked to the 2011 German outbreak of hemorrhagic infection
RM15013	B1	O104:H4	STEC/EAEC	0.25	Clinical isolate associated with the 2011 German outbreak of hemorrhagic infection
042	B1	O44:H18	EAEC	0.03	Isolate from child with diarrhea. Peru, 1983
55989	B1	O104:H4	EAEC	0.50	Isolated from the diarrheagenic stools of a HIV-positive adult suffering from persistent watery diarrhea in Central African Republic

[a] STEC, Shiga toxin-producing *Escherichia coli*; EHEC, enterohemorrhagic *Escherichia coli*; The two STEC O104:H4 strains were isolated from different patients. EAEC, enteroaggregative *Escherichia coli*.
[b] MIC, Minimum Inhibitory Concentration.

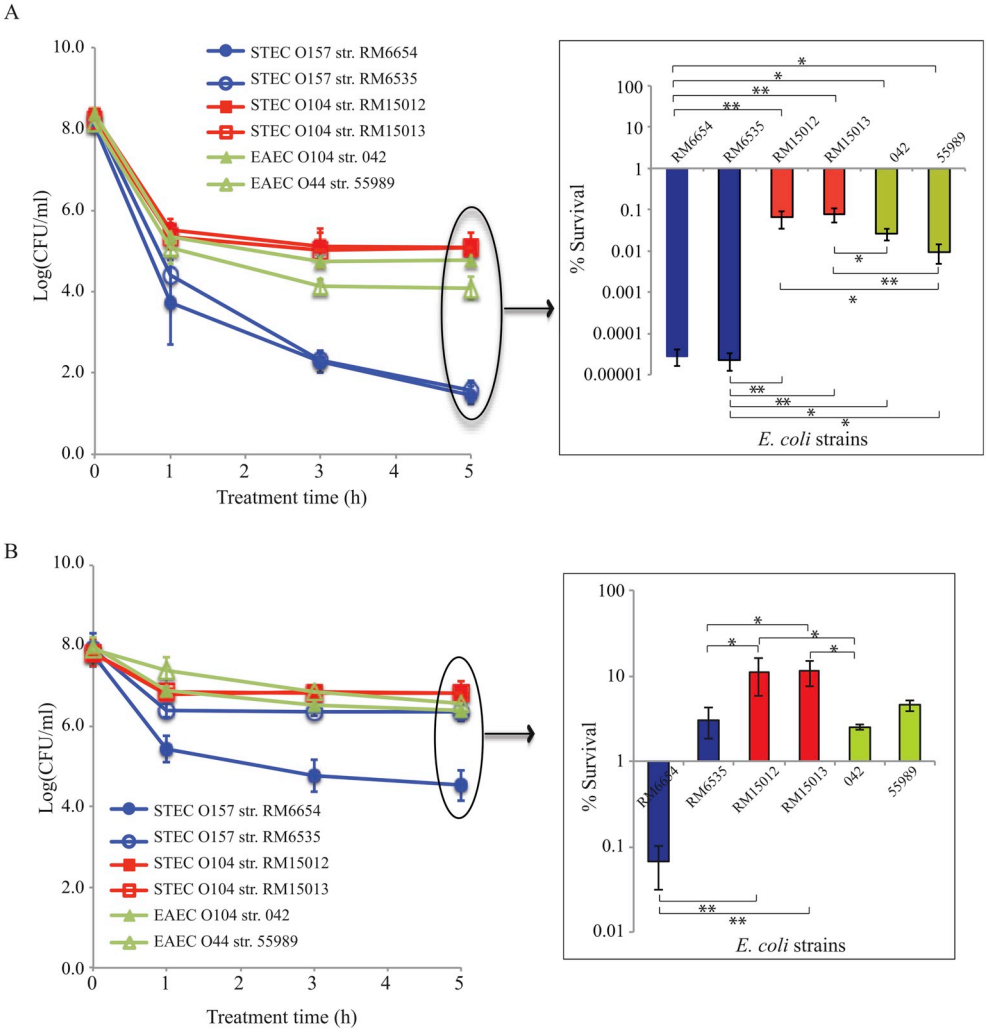

Fig. 1. Survival of various pathogenic *E. coli* strains grown in LB medium following ciprofloxacin treatment. **A**, Cells grown to exponential phase in LB broth at 37 °C. **B**, Cells grown to stationary phase in LB broth at 37 °C and then centrifuged and then resuspended to equal level in PBS. Data represent the surviving cells following various times of treatment with ciprofloxacin. The value presented here is the mean ± s.d. from three experiments performed on different days. The survival rate (%) was calculated by comparing the number of CFU following the ciprofloxacin treatment with the corresponding CFU prior to the addition of ciprofloxacin. The differences in ciprofloxacin tolerance among the *E. coli* strains are indicated by the *P*-value of the One-way ANOVA followed by Turkey's multiple comparisons test (*$P \leq 0.05$; **$P \leq 0.01$) or of the unpaired *t*-test between each of the STEC O157:H7 strains and each of the EAEC strains (*$P \leq 0.05$; **$P \leq 0.01$).

laboratory (http://anlab.ucdavis.edu/). For inoculation, an aliquot of overnight culture was centrifuged at 8000 × *g* for 3 min, and cells were resuspended in 5 ml sterile spinach leaf wash water to an OD_{600} of 0.5 (~10^8 cells/ml). The cultures were incubated statically at 26 °C for 48 h. The total population of *E. coli* was monitored by plate enumeration. At the end of incubation, the spinach leaf wash water was removed by centrifugation at 8000 × *g* for 15 min and the cells were resuspended in 5 ml 1X PBS to perform the persister assay.

2.5. E. coli persisters in field water

Field water was surface water collected in a channel running between fields in a leafy greens-growing region in the Salinas Valley, California (GPS Coordinates: 36.760,436, −121.754,738) as previously reported (Cooley et al., 2007). The water was first filtered through two layers of sterile cheesecloth to remove large particles and plant debris. The filtered water was then autoclaved twice at 121 °C for 1 h, and stored at 4 °C. The macronutrient contents and turbidity of the sterile field water were determined at an analytical laboratory (http://anlab.ucdavis.edu/). Overnight cultures of *E. coli* grown in LB broth were used to inoculate the sterile field water. Briefly, an aliquot of overnight culture was collected by centrifugation at 8,000 g for 3 min and the cells were resuspended in 5 ml of sterile field water to an OD_{600} of 0.5 (~10^8 cells/ml). The total population of *E. coli* was quantified by plate

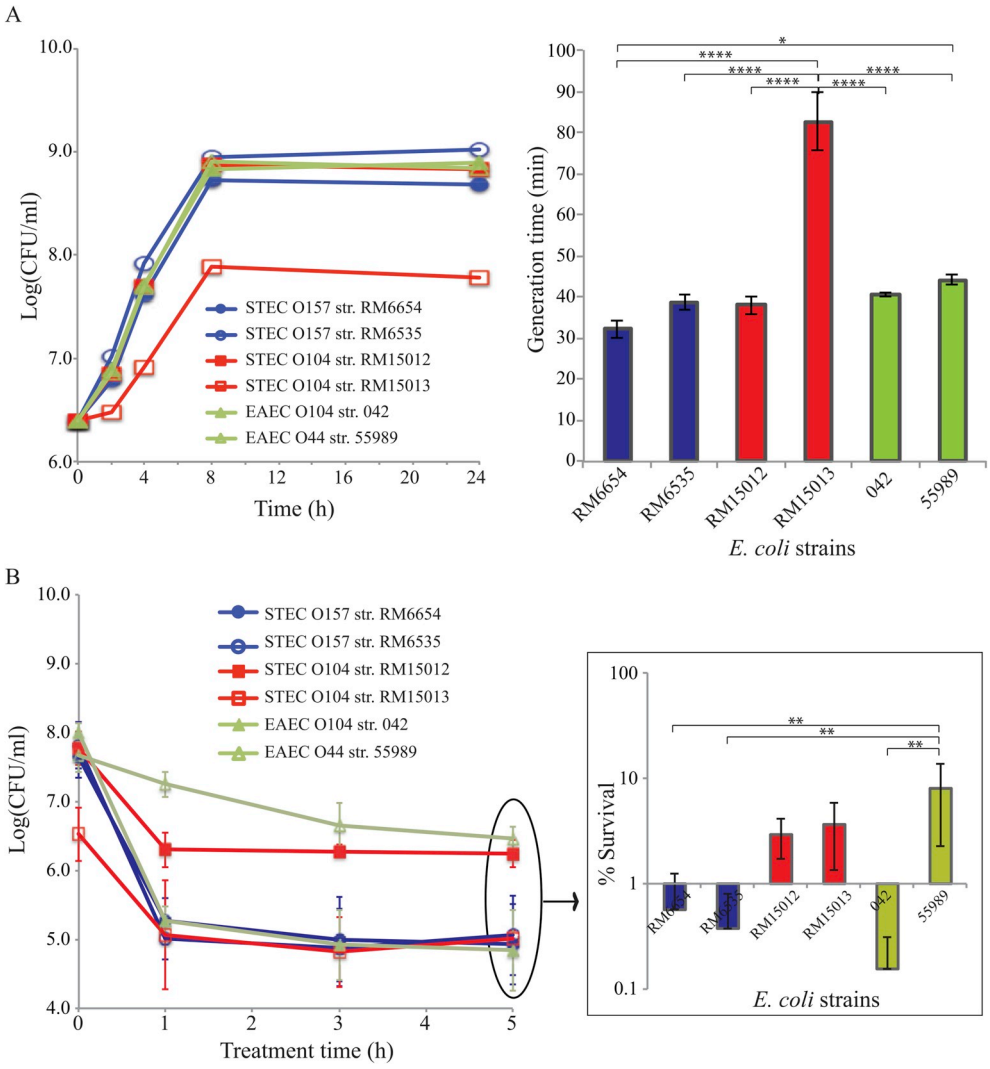

Fig. 2. Growth and persister populations of various pathogenic *E. coli* strains in spinach lysates. A, Growth (left panel) and generation time (right panel) of *E. coli* strains incubated statically in spinach lysates at 26 °C. Data represent the total number of *E. coli* cells recovered on LB plates at various times during 24-h incubation. The generation time was calculated using the population sizes of each strain at 2 h and 4 h following the inoculation. The value presented here is the mean ± s.d. from three independent experiments. **B,** Survival of *E. coli* cells from stationary cultures in spinach lysates following ciprofloxacin treatment. The data represent the average number of surviving cells on LB plates following various times of treatment with ciprofloxacin from four experiments performed on different days. The error bars represent the standard deviation. The survival rate (%) was calculated by comparing the number of CFU following the ciprofloxacin treatment with the corresponding CFU prior to the addition of ciprofloxacin. The differences in ciprofloxacin tolerance among the *E. coli* strains are indicated by the *P*-value of the One-way ANOVA followed by Turkey's multiple comparisons test (*$P \leq 0.05$; **$P \leq 0.01$; ****$P \leq 0.0001$).

enumeration at various times during incubation and the population size of persisters in each sample was determined as described above.

2.6. Statistical analysis

Statistical analysis was computed with Prism 7.0 (GraphPad Software). One-way Analysis of Variance (ANOVA) followed by Tukey's multiple comparisons test (ANOVA/Tukey's test) with $\alpha = 0.05$ was used to assess if the difference in persister population was significant among the *E. coli* strains tested at each treatment time, or among various treatment times for each strain. An unpaired *t*-test with 95% confidence interval was used to assess if the difference in persister population was significant between two *E. coli* strains.

3. Results

3.1. Formation of E. coli persisters in culture media

The minimum inhibitory concentration (MIC) for ciprofloxacin was determined for each *E. coli* strain (Table 1). Among the four STEC strains examined, the two STEC O104:H4 strains were much more resistant to ciprofloxacin (MIC, 0.25 μg/ml) than either of the two O157:H7 strains (MIC, 0.03 μg/ml). EAEC strain 042 displayed similar susceptibility to ciprofloxacin as the STEC O157 strains. In contrast, the EAEC strain 55989 exhibited the highest resistance to ciprofloxacin (MIC, 0.5 μg/ml). A concentration of ciprofloxacin at 10X MIC was used to assess the antimicrobial efficacy of ciprofloxacin at 37 °C for both exponential- and stationary-phase cells grown in LB broth (Fig. 1). For exponential-phase cultures, a rapid decline in cell concentration was observed for all strains after 1 h of exposure to ciprofloxacin (Fig. 1A). Following 3 h treatment, a further decline in cell concentration was observed for both STEC O157 strains; however, the cell population of the other four strains did not change significantly. The survival fractions of both STEC O104:H4 strains were significantly higher than those of the two STEC O157:H7 strains and that of the EAEC strain 55989 (ANOVA/Tukey's test, adjusted $P < 0.05$). After 5 h of treatment, the survival fractions of both STEC O157:H7 strains were significantly lower than that of any of the other four strains (ANOVA/Tukey's test, adjusted $P < 0.01$ for both STEC O104:H4 strains; Unpaired *t*-test, $P < 0.05$ for both EAEC strains) (Fig. 1A, inset). For all strains, the surviving fractions at 5 h post-treatment were at a level similar to that at 3 h (ANOVA/Tukey's test, adjusted $P > 0.99$). However, an apparent continued decline in surviving fractions of both STEC O157 strains when treatment time increased from 3 h to 5 h may indicate the presence of ciprofloxacin-tolerant cells in these surviving populations, despite the fraction values lacking significant differences between these two incubation times.

For stationary-phase cells, a similar killing curve was observed for most strains as for exponential-phase cells following the ciprofloxacin treatment, although the surviving fractions were much higher than those of the corresponding exponential-phase cells (Fig. 1B). The percent survival for strain RM6654 following 1 h-exposure was

significantly lower than that of strains RM15012, RM15013, and 55,989 (ANOVA/Tukey's test, adjusted $P = 0.03$, $= 0.01$, and < 0.0001, respectively). In contrast, the surviving fraction of strain 55989 following 1 h-exposure was significantly higher than that of any other *E. coli* strains examined (ANOVA/Tukey's test, adjusted $P < 0.001$). A further decline in percent survival was observed only for strain RM6654 when treatment time was increased to 3 h and 5 h, indicating the possible presence of ciprofloxacin-tolerant cells in RM6654 surviving populations although the surviving fraction was not significantly different between the two treatment times (ANOVA/Tukey's test, adjusted $P = 0.93$). At 5 h post-treatment, the surviving fractions of both STEC O104:H4 remained the greatest, which were significantly larger than those of both STEC strains O157:H7 and of the EAEC strain 042 (ANOVA/Tukey's test, adjusted $P < 0.01$ for RM6654; $P < 0.05$ for both RM6535 and 042) (Fig. 1B, inset).

3.2. Persister formation in stationary cultures of E. coli in spinach leaf lysates

Similarly to their culture in the nutrient-rich growth medium LB, all *E. coli* strains displayed active growth following inoculation and their cultures reached stationary phase after approximately 8 h of incubation in spinach lysates (Fig. 2A). The generation time of STEC strain RM15013 was significantly greater in spinach lysate than that of any other strains examined (ANOVA/Tukey's test, adjusted $P < 0.0001$). Furthermore, the generation time of strain RM6654 was significant lower than that of the strain 55,989 (ANOVA/Tukey's test, adjusted $P = 0.03$).

Similar to cells grown in LB broth, a biphasic killing curve was observed for all strains grown in spinach lysates following exposure to ciprofloxacin (Fig. 2B). After 1 h treatment, a rapid decline in surviving cells was observed for all strains except 55989, of which the surviving fraction (38.9%) was significantly greater than that of any other strains (ANOVA/Tukey's test, adjusted $P < 0.0001$) (Fig. 2B). When ciprofloxacin treatment was increased to 3 h, the surviving fractions of all strains remained stable, except for EAEC strain 55,989, of which the survival decreased overall relatively more slowly. After 5 h treatment, the percent survival of strain 55,989 was very similar to that of both STEC O104:H4 strains but significantly greater than that of both STEC O157:H7 strains and the EAEC strain 042 (ANOVA/Tukey's test, adjusted $P < 0.01$) (Fig. 2B, inset). Although a larger persister fraction of STEC strain RM6654 and EAEC strain 55989 was detected in the stationary cells grown in spinach lysates than those of the stationary cultures in LB broth, the difference was not statistically significant. The persister population sizes of all the other four strains in stationary culture in spinach lysates was significantly smaller than that of the corresponding stationary culture in LB broth (un-paired *t*-test, $P < 0.05$ for strains RM6535, RM15012, and RM15013; $P < 0.0001$ for strain 042).

3.3. Persister formation during incubation in spinach leaf wash water

Unlike spinach lysates, spinach leaf wash water contains limited nutrients (Table 2) and has been used as a system relevant to post-harvest leafy greens-processing environment (Carter et al., 2016). Nevertheless, the population sizes of all *E. coli* strains increased steadily during the 48-h incubation, with an increase ranging from 0.3 to 1.0 log (Fig. 3A). STEC O104 strain RM15012 exhibited the greatest growth while the population size of EAEC strain 042 increased the least.

The persister population of each strain was examined after incubation in spinach leaf wash water at 26 °C for 48 h, by which time the population of each strain was still increasing and had not reached its maximum size (Fig. 3B). The surviving fraction of strain 55989 was significantly greater than that of any other strains following 1 h treatment (ANOVA/Tukey's test, adjusted $P < 0.0001$). A further significant decrease in the surviving fraction was observed for the two STEC O157:H7 strains (ANOVA/Tukey's test, adjusted $P < 0.01$) and two EAEC strains (ANOVA/Tukey's test, adjusted $P < 0.05$) after 3 h ciprofloxacin treatment. At 5 h post-treatment, the surviving fraction of EAEC strain 55989 remained the highest among all strains examined (37.2%) (ANOVA/Tukey's test, adjusted $P < 0.001$ for each of the STEC strains; adjusted $P < 0.0001$ for EAEC strain 042) (Fig. 3B, inset). There was no significant difference in sizes of the persister population among the four STEC strains when they were incubated in spinach leaf wash water.

Table 2
Chemical analysis of spinach leaf wash water and field water.[a].

	Spinach wash water (SWW)	Field water (FW)
Total carbon (mg/L)	29.4	144.8
Total organic carbon (mg/L)	22.2	75.6
Total nitrogen (mg/L)	7.0	42.6
Ammonium nitrogen (mg/L)	2.4	4.6
Nitrate nitrogen (mg/L)	1.4	33.6
Phosphorus (soluble) (mg/L)	290.4	0.5
Turbidity (NTU)	< 0.1	282
pH	7.5	8.6

[a] Chemical analysis expect pH were conducted at University of California at Davis (http://anlab.ucdavis.edu/). Total carbon and total organic carbon were determined using SOP 822; total nitrogen was determined using SOP 855; ammonium nitrogen and nitrate nitrogen were determined using SOP 847; soluble phosphorus was determined using SOP 835; turbidity was determined using SOP 810. The pH of spinach wash water was measured at 26 °C for spinach wash water and at 15 °C for field water.

3.4. Persister formation during incubation in field water

Unlike spinach leaf wash water, field surface water used in this study contained a very low concentration of soluble phosphorus (Table 2) and was alkaline at 15 °C. The survival of six *E. coli* strains in field water at 15 °C was examined. Unlike the cells inoculated in spinach lysates or spinach leaf wash water, all strains exhibited a slight decline in total population following a one-week incubation, with a decrease ranging from 0.2 to 0.7 log compared with the initially inoculated population size (Fig. 4A).

The persister populations of *E. coli* strains after one-week incubation in field water were further investigated. After 1 h exposure to ciprofloxacin, there was no significant difference in survival among the *E. coli* strains examined except the EAEC strain 55989, of which the surviving fraction was significantly greater than any of the other strains (ANOVA/Tukey's test, adjusted $P = 0.01$, 0.04, 0.03, 0.03, and 0.01 for strains RM6654, RM6535, RM15012, RM15013, and 042, respectively). A similar trend was observed when the treatment time was increased to 3 h and 5 h. Additionally the surviving fraction of strain RM6535 was significantly greater than that of strain RM6654 (ANOVA/Tukey's test, adjusted $P = 0.02$) and of strain 042 (ANOVA/Tukey's test, adjusted $P = 0.03$) at 5 h post-treatment (Fig. 4B, inset). Increasing the ciprofloxacin treatment time did not alter the fractions of surviving cells significantly for any of the strains examined.

4. Discussion

Our study reveals that persister formation varies considerably among STEC strains. While the surviving fractions of exponential phase-cells of the two STEC O157 strains were not statistically different at 3-h compared with 5-h exposure to ciprofloxacin, the apparent different trends between the latter strains and the other STEC strains tested in our study suggests that the surviving fractions for the STEC O157 strains may include both tolerant and persister cells (Kim and Wood, 2017). However, the increased cipro-survival rate of the STEC O157 strains when cells were tested in the stationary phase of culture, which is expected since a greater proportion of the population would be in a

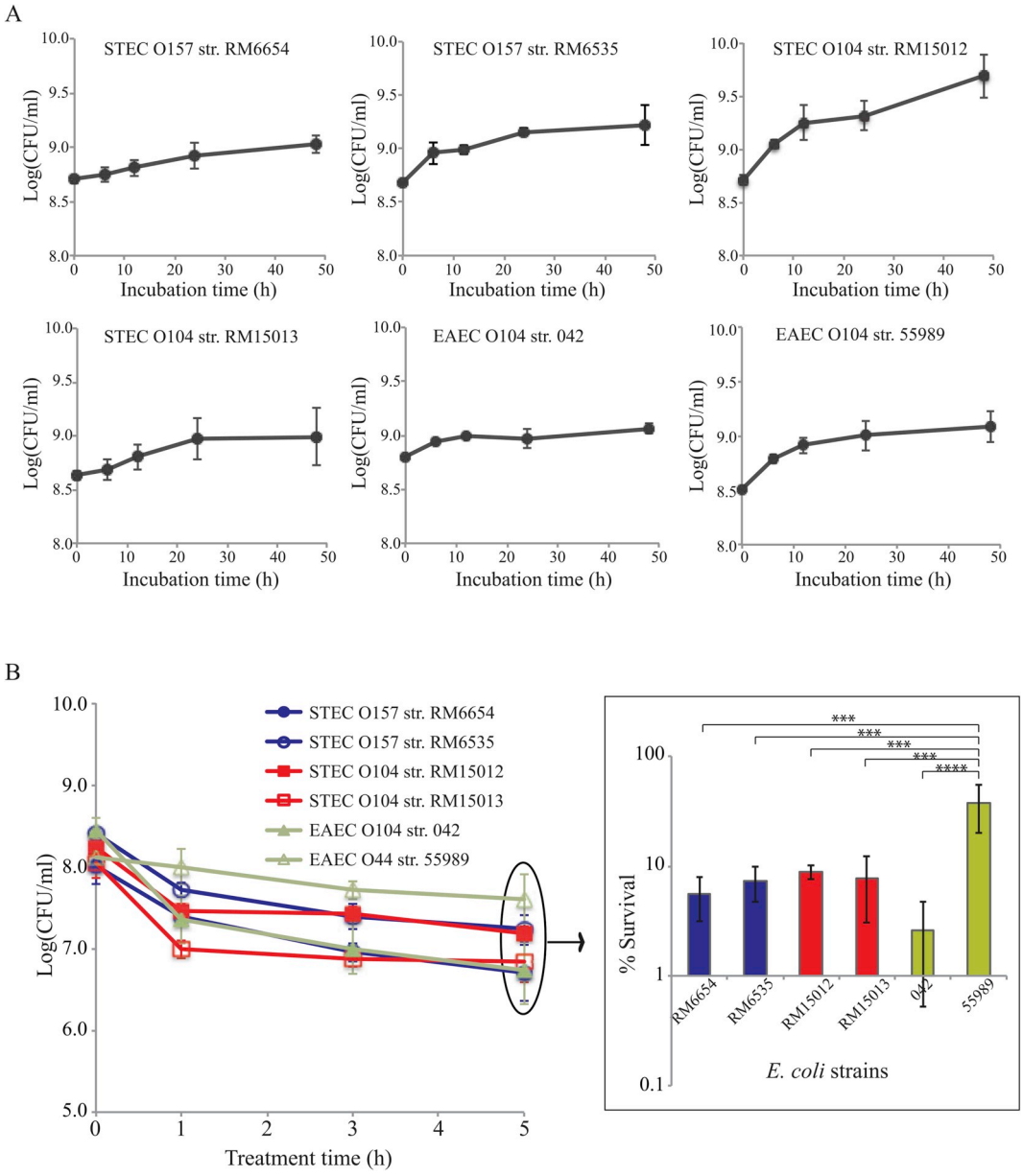

Fig. 3. Growth and persister subpopulations of various pathogenic *E. coli* strains in spinach leaf wash water. A, Population dynamics of *E. coli* strains in spinach leaf wash water incubated at 26 °C for 48 h. Data represent the average number of *E. coli* cells recovered on LB plates at various times during 48-h incubation from three independent experiments. **B,** Survival of *E. coli* strains following ciprofloxacin treatments. The data represent the average number of surviving cells on LB plates following various times of treatment with ciprofloxacin from four experiments performed on different days. The error bars represent the standard deviation. The survival rate (%) was calculated by comparing the number of CFU following the ciprofloxacin treatment with the corresponding CFU prior to the addition of ciprofloxacin. The differences in ciprofloxacin tolerance among the *E. coli* strains are indicated by the *P*-value of the One-way ANOVA followed by Turkey's multiple comparisons test (***$P \leq 0.001$; ****$P \leq 0.0001$).

dormant state, suggests that this serovar indeed forms persister cells in nutrient-rich medium, albeit at strain dependent-levels. Under the exponential growth condition, both STEC O157 strains produced the lowest persister subpopulation among the six strains examined, which was approximately 1000-fold lower than that of the STEC O104 strains. Persister cells were initially thought to result from a stochastic switch between the exponential growing cells and slow or non-growing cells; however, recent studies suggest that this epigenetic switch has a genetic basis that evolved to enhance bacterial survival in changing environments (Kussell et al., 2005). Such persistence-related genetic loci include genes encoding toxin-antitoxin modules (TA), protease Lon, and (p)ppGpp synthetase I SpoT/RelA (Germain et al., 2015). Therefore, differences in persister formation among the STEC strains may have resulted from genetic differences as well as from stochastic variations in expression of the persistence-related loci. Comparative analyses of TA modules between STEC O157:H7 and STEC O104:H4 strains indeed revealed differences in both content and abundance of TA loci (Table S1). For example, a total of 29 TA loci were identified in the STEC O104:H4 strain linked to the 2011 German outbreak of hemorrhagic infection (GenBank accession number NC_018658) in comparison with the 20 TA loci in STEC O157:H7 strain Ec4115 (GenBank accession number, NC_011,353). Furthermore, variants derived from the same STEC strain can differ considerably in stress resistance and catabolic potential (Carter et al., 2011). Therefore, variation in persister formation among STEC strains may have resulted also from strain variations in nutrient uptake and energy production, given that the metabolic state of bacteria is a key player in the formation of persister cells.

Persister cells can be induced in response to various environmental

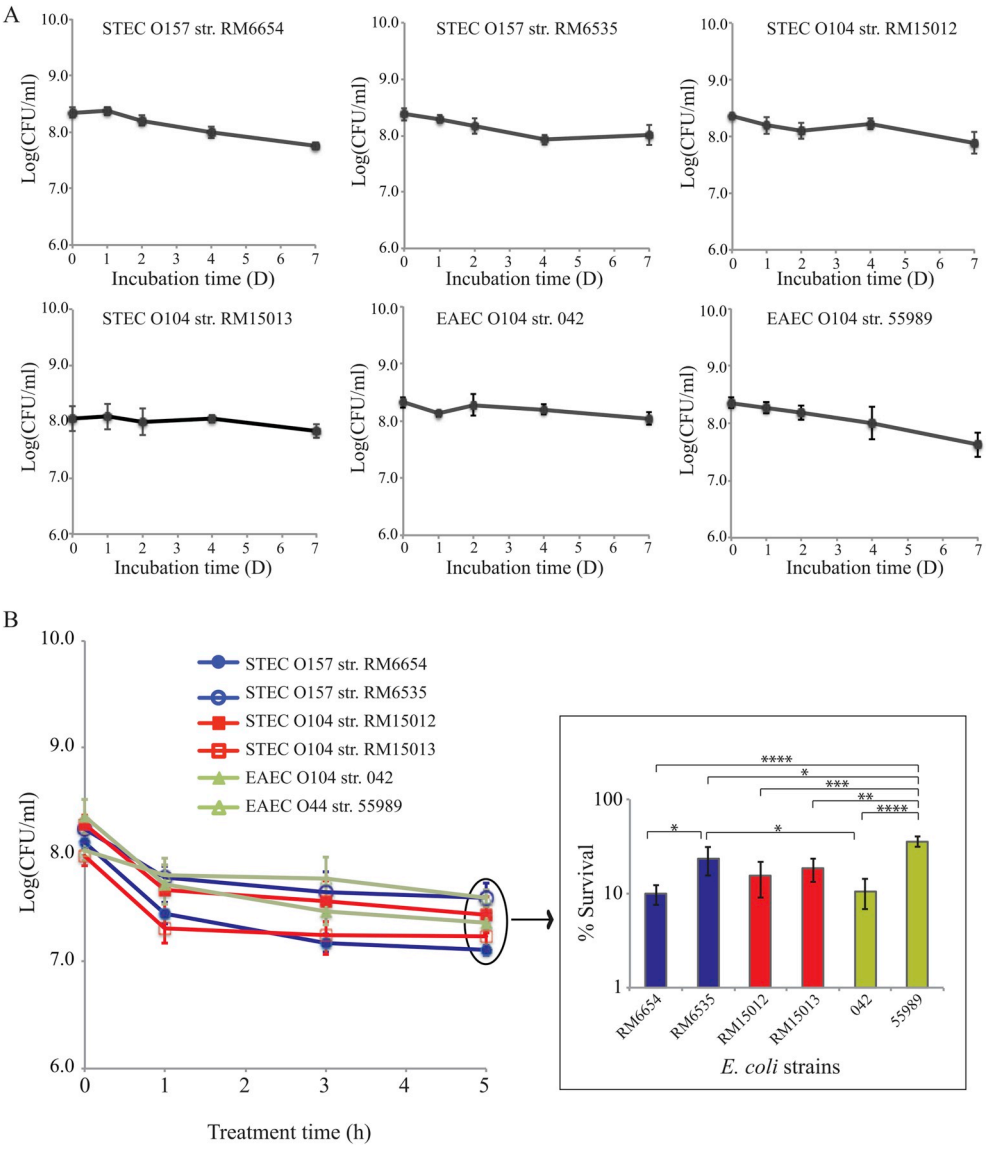

Fig. 4. Growth and persister subpopulations of various pathogenic *E. coli* strains in field water. A, Population dynamics of *E. coli* strains incubated in field water at 15 °C for one week. Data represent the average number of *E. coli* cells recovered on LB plates at various times during the one-week incubation from three independent experiments**. B,** Survival of *E. coli* strains following ciprofloxacin treatment. The data represent the average number of surviving cells on LB plates following various times of treatment with ciprofloxacin from four experiments performed on different days. The error bars represent the standard deviation. The survival rate (%) was calculated by comparing the number of CFU following the ciprofloxacin treatment with the corresponding CFU prior to the addition of ciprofloxacin. The differences in ciprofloxacin tolerance among the *E. coli* strains are indicated by the *P*-value of the One-way ANOVA followed by Turkey's multiple comparisons test (*$P \leq 0.05$; **$P \leq 0.01$; ***$P \leq 0.001$; ****$P \leq 0.0001$).

stresses including nutrient limitation, stringent response, DNA damage, extreme pH, and host immune response and are regulated by several global regulators via multiple pathways (Fisher et al., 2017; Lewis, 2010). The stationary-phase culture has been used as a common model to examine starvation-induced persisters, in which persister levels can increase 100–1000 fold in various bacterial species (Keren et al., 2004; Lewis, 2007). In our study, we investigated the formation of persisters in stationary phase in nutrient-rich medium and in spinach lysates. In both systems, all strains displayed active growth following inoculation and were in stationary phase after 8 h incubation. As expected, a much higher level of persisters was observed in stationary-phase cultures compared with exponential-phase cultures for cells grown in rich medium. The fold increase was between 1000–100,000 for STEC O157:H7 strains and between 96 and 473 for STEC O104 and the EAEC strains. The persister fraction in stationary-phase cells grown in spinach lysates for STEC strains RM6535, RM15012, RM15013, and the EAEC strain 042 was significantly lower than the corresponding one in stationary culture in rich medium (Unpaired *t*-test, $P < 0.05$). This suggested the presence of other factors besides stationary-like conditions and related starvation stress that may affect the formation of persisters cells in spinach lysates. No correlation was observed between the persister level and *E. coli* serotype, or between the persister level and *E. coli* pathotype. The vast difference in starvation-induced persister level among the STEC strains may result from variations in underlying mechanisms in response to starvation, supporting a previous report that no single physiological change can determine the persistence level in a population of cells (Hofsteenge et al., 2013).

Our data indicate that high persister levels are generally present in cultures of strains that exhibit low growth rates. For all six *E. coli* strains no or very slow growth was observed in sterile field water and sterile spinach leaf wash water, respectively, over the period of time examined. As expected, incubation in either field water or spinach leaf wash water increased the persister level significantly compared with the other systems investigated in this study. This high level of persisters of STEC and EAEC in field water suggests that a large number of bacterial cells in agricultural environments, where water is ubiquitous, may be in a persister state. A recent study revealed the presence of STEC O157:H7 persisters in soils as the inoculated pathogen exhibited multiphasic inactivation kinetics in various types of manure and soils (Wang et al., 2018). Mathematical modeling revealed that the survival pattern of *E. coli* O157:H7 on field-grown lettuce follows a biphasic pattern (McKellar et al., 2014), supporting that a subpopulation of *E. coli* O157:H7 is in the dormant and/or VBNC state (Moyne et al., 2013). Similarly, the high level of STEC persisters in spinach wash water implies that STEC could enter the persister-state in postharvest leafy greens processing environments. Considering the high tolerance of

persister cells to environmental stresses and antimicrobial treatments (Kussell et al., 2005; Vega et al., 2012), the formation of STEC persister cells may improve the survival fitness of this pathogen in pre- and postharvest leafy greens production environments thus posing significant challenges in the reduction of microbial contamination in the produce production chain.

5. Conclusions

The persistence of STEC in leafy greens production environments poses a potential threat to public health. These surviving cell populations may be composed at least partly of persister cells, which have the ability to survive for prolonged periods of time and are resistant to antimicrobial treatment due to their high tolerance to antibiotics and other stresses. Our study reveals the enhanced formation of STEC persisters in spinach wash water and in field surface water, where they underwent limited multiplication, indicating that STEC persisters are likely common at diverse points throughout agricultural production. Mitigation approaches aimed at lowering these subpopulation of persister cells may be an important strategy to minimize microbial contamination of produce, such as the reversion of persister cells to metabolically active cells with the fatty acid signaling molecule, *cis*-2-decenoic acid (Marques et al., 2014). Considering the complexity of molecular pathways underlying persister formation, a better understanding of the physiology of persisters induced under each relevant condition would provide important information on molecular cues that can break dormancy and would aid in the development of effective control strategies for elimination of persisters on leafy greens and in their production environments.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.fm.2019.103241.

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