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Formation of escherichia coli O157: H7 Persister Cells in the Lettuce Phyllosphere and Application of Differential Equation Models to Predict Their Prevalence on Lettuce Plants in the Field

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ABSTRACT *Escherichia coli* O157:H7 (EcO157) infections have been recurrently associated with produce. The physiological state of EcO157 cells surviving the many stresses encountered on plants is poorly understood. EcO157 populations on plants in the field generally follow a biphasic decay in which small subpopulations survive over longer periods of time. We hypothesized that these subpopulations include persister cells, known as cells in a transient dormant state that arise through phenotypic variation in a clonal population. Using three experimental regimes (with growing, stationary at carrying capacity, and decaying populations), we measured the persister cell fractions in culturable EcO157 populations after inoculation onto lettuce plants in the laboratory. The greatest average persister cell fractions on the leaves within each regime were 0.015, 0.095, and 0.221%, respectively. The declining EcO157 populations on plants incubated under dry conditions showed the largest increase in the persister fraction (46.9-fold). Differential equation models were built to describe the average temporal dynamics of EcO157 normal and persister cell populations after inoculation onto plants maintained under low relative humidity, resulting in switch rates from a normal cell to a persister cell of 7.7×10^{-6} to 2.8×10^{-5} h^{-1}. Applying our model equations from the decay regime, we estimated model parameters for four published field trials of EcO157 survival on lettuce and obtained switch rates similar to those obtained in our study. Hence, our model has relevance to the survival of this human pathogen on lettuce plants in the field. Given the low metabolic state of persister cells, which may protect them from sanitization treatments, these cells are important to consider in the microbial decontamination of produce.

IMPORTANCE Despite causing outbreaks of foodborne illness linked to lettuce consumption, *E. coli* O157:H7 (EcO157) declines rapidly when applied onto plants in the field, and few cells survive over prolonged periods of time. We hypothesized that these cells are persisters, which are in a dormant state and which arise naturally in bacterial populations. When lettuce plants were inoculated with EcO157 in the laboratory, the greatest persister fraction in the population was observed during population decline on dry leaf surfaces. Using mathematical modeling, we calculated the switch rate from an EcO157 normal to persister cell on dry lettuce plants based on our laboratory data. The model was applied to published studies in which lettuce was inoculated with EcO157 in the field, and switch rates similar to those obtained in our study were obtained. Our results contribute important new knowledge about the physiology of this virulent pathogen on plants to be considered to enhance produce safety.

KEYWORDS foodborne pathogen, STEC, produce, leaves, dormancy, mathematical modeling, prediction

The occurrence of outbreaks of *Escherichia coli* O157:H7 (EcO157) infection associated with the consumption of leafy vegetables, predominantly, those associated with the consumption of lettuce, has prompted several studies on the survival of this human pathogen on plants in the field. These studies commonly revealed a biphasic decay of the EcO157 population after its inoculation onto leaf surfaces (1–4). Similar trends were observed for the survival of EcO157 on spinach (5) and *Listeria monocytogenes* on parsley (6) in the field. Biphasic population decay was also reported from greenhouse and laboratory studies in which inoculated plants were incubated under dry conditions that promoted overall EcO157 and *Salmonella enterica* population declines (7–9). The subpopulations of human pathogens that survive the numerous environmental stresses prevailing in the phyllosphere are of utmost importance to food safety since they may cause outbreaks of foodborne disease associated with leafy vegetables and are the target of extensive sanitization efforts. However, the location of these EcO157 cells on leaves and the physiology enabling their survival remain unknown.

Bacterial immigrants face an uncertain fate after their arrival in the phyllosphere due to the heterogeneity of its physicochemical and microbial landscape at the microscale (10, 11). Nutrients and free water are distributed unevenly and not always concomitantly across the phylloplane, thus restricting the number of microsites that may support bacterial multiplication (12–18). Successful bacterial colonization further depends on the microbial context at the site of immigration (19, 20). *Salmonella enterica* cells inoculated onto lettuce plants had a greater probability of surviving low-humidity conditions in the phyllosphere when located in aggregates of plant-associated bacterial species that had precolonized the leaves than when located at uncolonized sites (21). Additionally, plant microbial colonists are affected by temporal variations in physicochemical stresses at multiple scales, from the weather in the field to the level of single bacterial cells at microscopic sites.

Cell dormancy contributes to the intrastrain phenotypic heterogeneity of bacteria in clonal populations and is part of the bet-hedging strategies by which microbial species ensure the survival of a subpopulation under stress conditions and the species fitness over time in unpredictable environments (22). Although the exact underlying molecular mechanisms are still the topic of much discussion (23), this transient slow metabolism allows the bacterial cells to tolerate the effect of antibiotics without harboring antibiotic resistance genetic determinants; thus, these cells have been termed persister cells, due to their ability to survive antibiotic treatment (24). Dormant cells also have an enhanced ability to tolerate sanitizers, such as chloramine (25), and to avoid detection by the host immune system (26).

The phenotypic characteristics of dormant bacterial cells that differ from those of normal cells include quantitative and compositional changes in cellular components, altered micro- and ultrastructures, as well as reduced cell size (22). Monier and Lindow reported that a large portion of the *Pseudomonas syringae* population showed a reduced cell size after inoculation onto leaves compared with that in culture and, importantly, that the cell size was heterogeneous across the leaf-inhabiting population (27). This heterogeneity suggested again the presence of spatially variable conditions at the scale of single cells, and the increase in the number of small cells over time may indicate that cells have entered an inactive state in response to stresses in the phyllosphere.

In the study described herein, we used an antibiotic-based assay to measure the prevalence of persister cells in a population of culturable EcO157 bacteria after the pathogen was inoculated into the phyllosphere of lettuce plants in three different experimental regimes: (i) an EcO157 population increase promoted by low inoculum levels, warm temperature, and free water availability on the leaves, (ii) a steady EcO157

population due to inoculation at the carrying capacity of the leaves and a high relative humidity (RH), and (iii) an EcO157 population decline caused by low RH and limited free water on leaf surfaces. We further used the data resulting from the last regime to build ordinary differential equation (ODE) models that describe the dynamics of persister and normal EcO157 cells on lettuce plants. Our model for biphasic population decay, based on our laboratory studies, was then applied to field data from several published studies on the survival of EcO157 on lettuce in fields located in various geographical and climatic areas to assess its relevance as well as provide insight toward conducting experiments intending to measure the prevalence of persister cells on preharvest lettuce in the field.

RESULTS

Abundance of EcO157 persisters in the lettuce phyllosphere. (i) Growing population. Lettuce plants were inoculated at low cell densities in order to examine the abundance of persister cells in EcO157 populations when conditions are conducive to multiplication. In our study, these conditions were generated by incubating the inoculated plants under a high RH, at a warm temperature, and in the presence of free water on the leaves. The temperature ranged from 24 to 30°C with day and night oscillations, and the RH was 100% throughout the incubation period (Fig. 1A). The leaves were inoculated with EcO157 at 10^5 cells/leaf, and the population size increased 6.5-fold within 48 h; EcO157 multiplication was greater in the first 24 h of colonization and then slowed within the following 24 h but was still significantly different from that at 24 h (Tukey's multiple-comparison test, $P < 0.05$) (Fig. 1B). By 24 and 48 h, the proportion of the persister subpopulation size over the total culturable EcO157 population was 0.005% and 0.015%, respectively, reflecting 2.4- and 7.7-fold increases, respectively, compared with that in the inoculum population (Fig. 1C). The percentage of persister cells over the total culturable cell population at 48 h was significantly greater than that at 0 and 24 h (Tukey's multiple-comparison test, $P < 0.05$).

(ii) Stationary population at carrying capacity. Lettuce plants were inoculated at high EcO157 densities to approximate the carrying capacity of the leaves and then were incubated at high RH (90 to 100%) to prevent bacterial population die-off (Fig. 2A). The latter conditions resulted in EcO157 population sizes remaining constant overall at about 5×10^6 CFU/leaf after inoculation onto the lettuce leaves (one-way analysis of variance [ANOVA], $P = 0.86$), likely due to a limitation in nutritional resources in the phyllosphere (Fig. 2B). The persister population size represented 0.095% and 0.035% of the total culturable population at 24 and 48 h, respectively, corresponding to increases of 14.7- and 5.3-fold, respectively, compared with that immediately after inoculation (Fig. 2C). Tukey's multiple-comparison test revealed that the percentage of persisters at 48 h was not significantly different from that at the time of inoculation ($P = 0.06$). However, the means were shown to be different by Student's t test ($P < 0.01$) (the variances were equal per the F test; $P = 0.41$).

(iii) Declining population. Given that field experiments have shown that EcO157 population sizes decline after their inoculation onto lettuce leaves, we measured the persister subpopulation of EcO157 cells on overall dry leaf surfaces of lettuce plants exposed to low relative humidity, which caused the pathogen population size to decrease after inoculation. The RH started at values of 70 to 72% when the lettuce plant surfaces were left to dry under the constant airflow in a biosafety cabinet for 3 h. After transfer to a small plant chamber, the RH was maintained at about 55 to 65% over the course of the 3-day incubation of the plants and the temperature ranged from 25 to 27°C with day and night oscillations (Fig. 3A). Under the given conditions, the total EcO157 population size declined significantly 210-fold during the 48-h pathogen adaptation phase to overall dry conditions in the phyllosphere and stabilized during the last 24 h of incubation (Tukey's multiple-comparison test, $P < 0.0001$ and $P = 0.83$, respectively) (Fig. 3B). The percentage of persisters in this regime showed the greatest increase among all three regimes (46.9-fold), reaching 0.221% at 48 h after inoculation (Tukey's multiple-comparison test, $P < 0.0005$) (Fig. 3C). While the average percentage

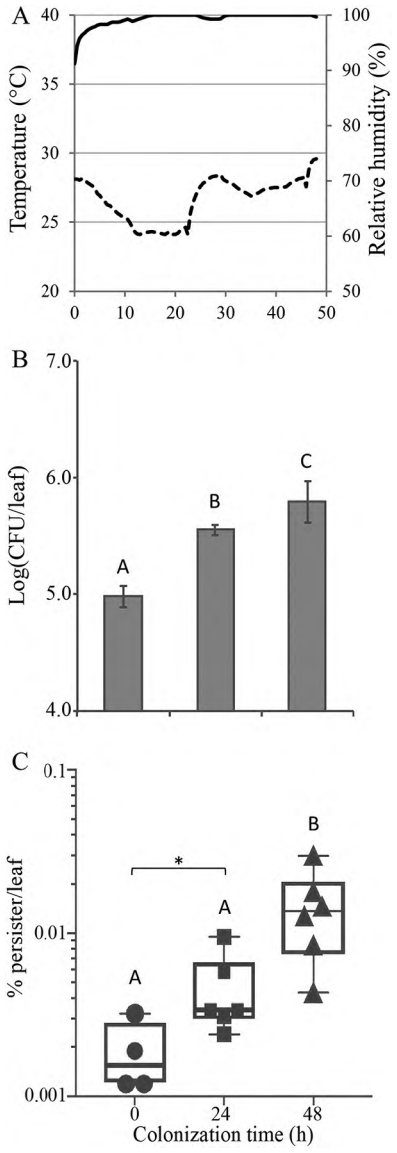

FIG 1 Total culturable population and persister subpopulation of STEC O157:H7 during population increase on the leaves of romaine lettuce plants. (A) EcO157-inoculated plants were incubated with the presence of free water on the leaves and under high RH and warm temperature; percent RH (solid line) and temperature (dotted line) were recorded with a sensor probe placed in the plant incubator. (B and C) The EcO157 total culturable population (shown as the mean and SD) (B) and the persister subpopulation (shown as a percentage of the total population) (C) were monitored over time after inoculation. The different letters above the columns and the whisker boxes indicate significant differences determined by Tukey's multiple-comparison test ($P < 0.05$), using $\log_{10}$(number of CFU per leaf) transformation of the total population size and arcsine(square root of the persister proportion over total EcO157 population) transformation of persister subpopulations (in percent). *, significant difference in the transformed percentage of the persister population at 0 and 24 h by Student's t test ($P < 0.05$).

of persisters per leaf did not change significantly during the subsequent period, in which the population size stabilized, a high variation in the persister fraction of the population was observed among the leaf samples, ranging from 0.0162 to 0.4457%, with an average value of 0.139%.

Model fitting for lab-scale decay regime. In order to describe the average temporal dynamics of culturable normal (nondormant) and persister (dormant) cell populations on lettuce leaf surfaces under dry conditions, we developed the following ordinary differential equation model:

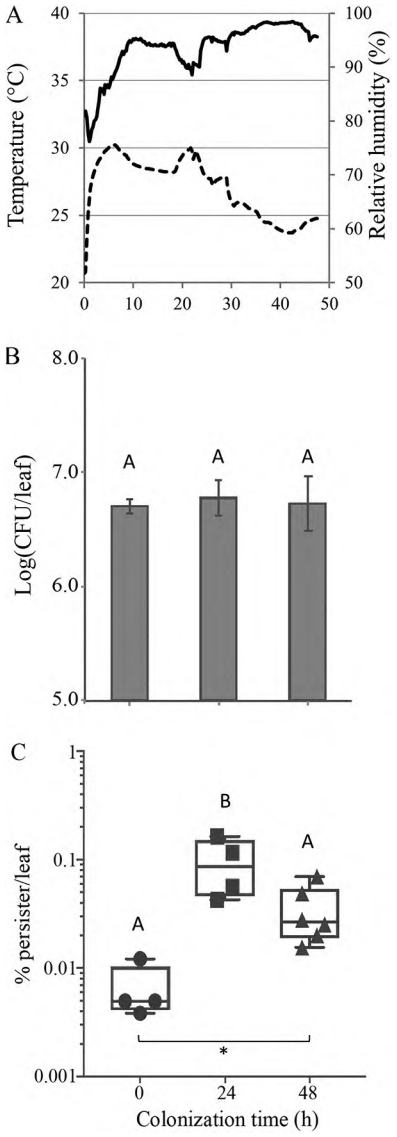

FIG 2 Total culturable population and persister subpopulation of STEC O157:H7 during overall stationary population dynamics at the leaf carrying capacity on romaine lettuce plants. (A) EcO157-inoculated plants were incubated under high RH and warm temperature; percent RH (solid line) and temperature (dotted line) were recorded with a sensor probe placed in the plant incubator. (B and C) The EcO157 total culturable population (shown as the mean and SD) (B) and the persister subpopulation (shown as a percentage of the total population) (C) were monitored over time after inoculation. The different letters above the columns and the whisker boxes indicate significant differences determined by Tukey's multiple-comparison test ($P < 0.05$), using $\log_{10}$(number of CFU per leaf) transformation of total population size and arcsine(square root of the persister proportion over the total EcO157 population) transformation of persister subpopulations (in percent). *, significant difference in the transformed percentage of the persister population at 0 and 48 h by Student's *t* test ($P < 0.01$).

$$\frac{dn_1}{dt} = -\theta_{n_1} n_1 - \alpha_d n_1 \tag{1a}$$

$$\frac{dn_2}{dt} = -\theta_{n_2} n_2 - \alpha_d n_2 \tag{1b}$$

$$\frac{dp}{dt} = (-\theta_p - \beta_d)p + \alpha_d(n_1 + n_2) \tag{1c}$$

$$n_1(0) = n_{10}, n_2(0) = n_{20}, p(0) = p_0 \tag{1d}$$

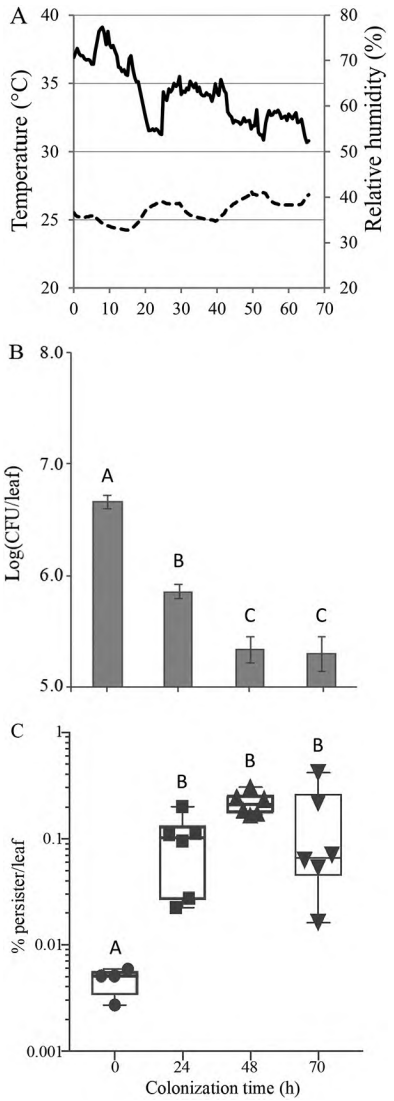

FIG 3 Total culturable population and persister subpopulation of STEC O157:H7 during population decline on romaine lettuce plants. (A) EcO157-inoculated plants were incubated under low RH and warm temperature to promote dry conditions on the leaves; percent RH (solid line) and temperature (dotted line) were recorded with a sensor probe placed in the plant incubator. (B and C) The EcO157 total culturable population (shown as the mean and SD) (B) and the persister subpopulation (shown as a percentage of the total population) (C) were monitored over time after inoculation. The different letters above the columns and the whisker boxes indicate significant differences determined by Tukey's multiple-comparison test ($P < 0.05$), using $\log_{10}$(number of CFU per leaf) transformation of the total population size and arcsine(square root of the persister proportion over the total EcO157 population) transformation of persister subpopulations (in percent).

where, based on Fig. 3B and C, because the persister population is a small fraction of the total population, we assumed that the normal population is composed of two subpopulations, characterized by fast (n_1) and slow (n_2) decay (see the Materials and Methods section for more details regarding model construction). In addition, θ_{n_i} (in 1/hours) is the death rate of the normal cells (fast and slow decay), p (in numbers of CFU per leaf) represents the average persister cell population at time t (in hours), θ_p (1/hours) is the death rate of the persister population, α_d (in 1/hours) is the switch rate from the normal state to the persister state, and β_d (in 1/hours) is the switch rate from the persister state back to the normal state.

Note that in the model presented in equations 1a to 1d, because θ_p and β_d cannot be determined independently, we set $\theta_p + \beta_d$ equal to μ_p in equation 1c. Requiring the

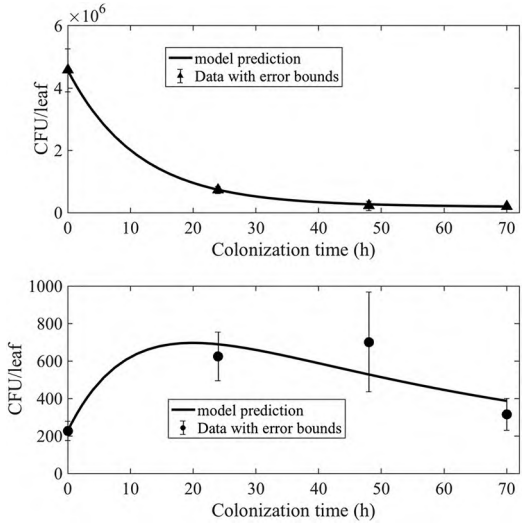

FIG 4 Model prediction for the decay regime. Model (equations 1a to 1d) predictions of the EcO157 total (top) and persister (bottom) populations under experimental decay conditions for inoculated leaves. Setting $\theta_p + \beta_d$ equal to μ_p in equation 1c, requiring the persister population $p(t)$ to remain within the experimental error bounds for the persister population at the respective time points, for a value of μ_p of >0, we found that $\alpha_d \in [7.7 \times 10^{-6}, 2.8 \times 10^{-5}$ 1/h]. Within this region of parameter space, the best model fit corresponds to α_d equal to 1.6×10^{-5} and μ_p equal to 0.022, with R^2 equal to 0.75.

persister population $p(t)$, as predicted by the model presented in equations 1a to 1d, to remain within the experimental error bounds for the persister population at the respective time points, we determined that for $\mu_p > 0$, $\alpha_d \in [7.7 \times 10^{-6}, 2.8 \times 10^{-5}$ 1/h]. Using this range, the condition that μ_p is >0, and the error bounds for the persister population for the respective time points, the best model fit corresponded to α_d equal to 1.6×10^{-5} and μ_p equal to 0.022 (Fig. 4). Remarkably, the model captured the total population dynamics very well ($R^2 = 0.99$; Fig. 4, top). Due to the large average persister population observed at t equal to 48 h (Fig. 3B and C), the model fit to describe the persister population dynamics was not as good ($R^2 = 0.75$) as that for the total population.

Model application. From our modeling perspective, we assumed that the time that a cell remains in an active state is exponentially distributed, and thus, on average, the switch rate from a normal cell to a persister cell can be approximated by the reciprocal of the expected time that a cell stays in the normal cell state. Our parameter analysis with respect to the experimental decay regime revealed the following bounds on the average switch rate from normal to persister cells: $\alpha_d \in [7.7 \times 10^{-6}, 2.8 \times 10^{-5}$ 1/h].

We applied our model framework to EcO157 field studies to determine the possible range of the switch rate (α_d) from a normal to a persister cell state. While phyllosphere EcO157 populations may be comprised of many subpopulations, culturable EcO157 populations inoculated onto plants in the field generally exhibit a biphasic type of decay (see the work of McKellar et al. [28] and references therein). It is unclear if within such population dynamics the tail of the population includes persisters and what fraction of that population they represent. Using a two-subpopulation model presented in equations F1 to F3 in Text SA.1 in the supplemental material, adapted from the model presented in equations 1a to 1d of the decay regime, we estimated model parameters for field trials from the four following studies: those of Bezanson et al. (1), Moyne et al. (3), Moyne et al. (29), and Erickson et al. (2).

(i) Range for switch rate from normal to persister state from field trials. Using field trial data from the four studies mentioned above, our modeling approach, and the fitting procedure outlined in Text SA.4, we determined that α_d ranged from 1.1×10^{-6} to 4.8×10^{-5} 1/h, with only one instance in which α_d was 8.3×10^{-7} 1/h (outside the 10^{-6} to 10^{-5} order-of-magnitude range), which was for the spring trial of Moyne at al.

(29) (Text SA.5). Remarkably, this estimated range for α_d in the field was only slightly wider than the range under our controlled conditions in the experimental decay regime, where α_d ranged from 7.7×10^{-6} to 2.8×10^{-5} 1/h.

(ii) Approximation of persister populations and experimental implications. Assuming that a two-subpopulation model is sufficient to describe population dynamics, utilizing the model presented in equations F1 to F3 in Text SA.1, the maximum size of the persister population (p_{max}) can be approximated simply as

$$p_{\max} \approx (1 + \eta)\frac{\alpha_d n_0}{\theta_n} \tag{2}$$

where the derivation of equation 2 is given in Text SA.6 and where η is a constant chosen so that the initial growth rate of the persister fraction is proportional to the switch rate α_d. Furthermore, using equation T1 from Text SA.6, we can determine the time at which the persister population is predicted to be at a maximum, denoted by t_{max}. Equation 2 can be used to guide experiments seeking to quantify persister formation in the field. Although the field experiments used in this study to determine α_d varied by locale, abiotic factors, bacterial strain, and experimental procedure, they all resulted in overall EcO157 population decay over time. Under these premises, equation 2 can be used to provide an initial population threshold (relative to the limit of detection corresponding to the enumeration method at hand) under which persister detection will be unlikely. If we let $\hat{L}$ denote the limit of detection and if

$$\frac{\hat{L}}{(1 + \eta)\frac{\alpha_d}{\theta_n}} = T > n_0 \tag{3}$$

where T is an initial population threshold, then, from equation 2 we see that p_{max} is $< \hat{L}$; that is, the maximum persister population remains, on average, under the limit of detection.

We discuss these population thresholds in the context of the regions in North America where these field trials were performed. For the studies by Moyne et al. (3, 29), administered in the Salinas Valley in California, T (from equation 3) ranged from 4.7×10^5 to 1.8×10^7 (using the parameter information in the corresponding tables in Text SA.5). Because the limit of detection utilized in these trials was 10 CFU/plant, using the upper range for T, equation 3 suggests that if n_0 is $<\log_{10}$ 7.26 CFU/plant, the persister population may not be detected. Furthermore, using equation T1 (Text SA.6), we estimate that the sample window to observe the maximum persister population (using the parameter variations across the trials in these studies) is $t_{max} \in$ [1.4, 2.4 h].

For the study by Bezanson et al. (1), conducted in Kentville, Nova Scotia, Canada, and Summerland, British Columbia, Canada, T ranged from 1.2×10^5 to 1.3×10^5 and from 2.26×10^4 to 2.3×10^4, respectively (Text SA.5). The Kentville location is characteristic of a maritime climate zone, whereas the Summerland location reflects a semiarid, temperate zone (1). In these cases, again using the upper range for T, equation 3 suggests that if n_0 is $<\log_{10}$ 5.1 CFU/plant for Kentville and if n_0 is $<\log_{10}$ 4.4 CFU/plant for Summerland, the persister population likely may remain below the detection limit of 10 CFU/plant at either location. Using equation T1 in Text SA.6, we estimated $t_{max} \in$ [24, 27 h] and $t_{max} \in$ [11, 14 h] for Kentville and Summerland, respectively.

In the study by Erickson et al. (2), performed in Georgia, EcO157 populations were measured on both the adaxial and abaxial surfaces of lettuce leaves for sunny or shady conditions. Due to data limitations, we applied our models and, hence, parameter fitting to data from only adaxial/sunny and adaxial/shady conditions (Text SA.5). The range for T was 9.3×10^5 to 1.1×10^6 and 5.8×10^5 to 7.7×10^5 for adaxial/sunny and adaxial/shady, respectively. Considering that the detection limit of 10 CFU/g, if n_0 is $<\log_{10}$ 6.04 CFU/g, then the persister population may not be measurable for adaxial/sunny conditions. Additionally, if n_0 is $<\log_{10}$ 5.9 CFU/g, then the persister population may not be detected for adaxial/shady conditions. Lastly, equation T1 in

Text SA.6 indicates that $t_{max} \in$ [12, 14 h] for adaxial/sunny conditions, whereas $t_{max} \in$ [9, 10 h] for adaxial/shady conditions.

In addition to providing guidelines for initial inoculum levels for field trials concerned with quantifying persisters on preharvest lettuce, our modeling approach can provide insight into sample timing in terms of informing associated predictive modeling. Note that equation 2 illustrates that the persister population is sensitive to the fast decay rate θ_n. Thus, characterizing the order of magnitude and variation of θ_n within a set of experimental conditions is crucial for predicting persister dynamics.

DISCUSSION

The physiology of EcO157 cells that survive the many stresses encountered on plant surfaces and that may cause outbreaks of enteric disease linked to fresh fruit and vegetables remains mostly unexplored. We hypothesized that a fraction of these EcO157 cells are in a low metabolic state that affords them protection from unfavorable conditions. Bacterial cells that phenotypically adapt to environmental fluctuations through a low metabolic, nonreplicating state also have increased tolerance to antibiotics (24, 30). The formation of such persister cells has recently been described in EcO157 cells exposed to field water and spinach leaf washes (57). We have demonstrated here the presence of EcO157 persister cells in the lettuce phyllosphere under three different bacterial population dynamics regimes and have provided a mathematical framework to assess the size of these subpopulations that are recovered from plants in the field. The three regimes are representative of conditions experienced by immigrant EcO157 cells on plant surfaces, resulting in (i) an opportunity for the population to grow because of warm temperatures and the availability of water and substrates, (ii) a lack of resources due to arrival at sites where resources are insufficient to promote the overall growth of the population, and (iii) the necessity for the bacteria to rapidly adapt to broad stressful conditions or die.

As expected, the smallest EcO157 persister fraction (0.015%) and the smallest increase in the persister fraction (7.7-fold) after inoculation of the pathogen onto lettuce plants were observed during population growth, which was promoted by the presence of free water on the leaves. This small subpopulation may consist of EcO157 cells at microsites where low metabolic activity is induced by a physical or chemical stress or the limitation of nutrients or/and water availability, despite the overall wetness of the leaf surfaces in this regime. During incubation on moist bean plants, a bacterial biosensor of osmotic stress reported that the *Pantoea agglomerans* population experienced a decreased water potential compared with that for the cultured cells only 5 min after their inoculation onto the leaves (12). A green fluorescent protein-based bioreporter was used to assess the multiplication of *P. agglomerans* at the single-cell level in the bean phyllosphere and revealed that during a 10-fold increase in population size under environmental conditions similar to those used in our study, 3% of the cells had not divided once, indicating that a small number of individual cells did not experience growth-conducive conditions, despite the wetness of the leaves (16). Hence, the small EcO157 persister fraction detected after 24 h of active colonization on wet lettuce leaves likewise may reflect the environmental heterogeneity experienced by the human pathogen at the scale of individual cells in an overall expanding population in the lettuce phyllosphere. It is noteworthy that the fraction of persister cells increased further as the population growth slowed between 24 and 48 h after inoculation, possibly because of an increasing number of cells that faced diminished resources due to colonization of the leaves.

Unlike the intestine, the phyllosphere is a nutrient-poor environment in which limited quantities of substrates, such as fructose, glucose, and sucrose, are available for bacterial multiplication and are heterogeneously distributed (14, 15, 17, 32). The overall carrying capacity of the leaf for epiphytic bacteria is in the range of 10^6 and 10^7 cells per g of leaf, depending on the plant species (32, 33). Inoculation of EcO157 onto lettuce at high densities in this study resulted in a nearly constant mean population size per leaf over a 48-h incubation of the plants under high RH. The fraction of persister

cells in the total EcO157 population after 24 h (0.095%) was significantly greater than that at the time of inoculation (0.0065%), as many cells probably entered a dormant state caused by a lack of substrates available to a bacterial population at carrying capacity. It is noteworthy that the initial increase in the persister fraction in an overall stable population was nearly 1 order of magnitude greater than that under population growth conditions. This finding supports the hypothesis that cells located at sites that are at carrying capacity are more likely to be induced into dormancy. The cause of the decline in the percentage of persisters at between 24 and 48 h after inoculation at carrying capacity is unclear but may reflect the adaptation of a portion of the persister (dormant) cell population to the utilization of more complex substrates and, thus, a reversion to a normal state.

Phyllosphere microbes frequently experience extensive water deprivation and, thereby, limited access to soluble nutrients (10, 34), which may induce a quiescent state in the cells. In our study, among the three test conditions, the persister cell fraction of the total culturable EcO157 population in the lettuce phyllosphere was the highest when plants were incubated under low RH, as it reached 0.22% during population decay. The average persister fraction per leaf did not change significantly after 24 h; however, once the population size stabilized, the leaf-to-leaf variation increased considerably. This increased variation seen once the average population reached equilibrium was also observed in replicate experiments (data not shown) and may indicate that EcO157 populations sustain different environmental pressures among individual leaves over prolonged colonization.

Using relatively simple ordinary differential equation models, we were able to characterize the average switch rate (α_d) of cells from the normal to the persister state with respect to the above-described experimental decay regime. Our modeling approach primarily stemmed from research conducted by Balaban et al., who quantified the switching mechanisms for bacterial persistence via simple ODE models in the context of microfluidic data (35), and by Malik and Smith, who later focused on a general linear ODE model to determine whether dormancy confers bacterial fitness advantages in variable environments (36). Following these works, many other studies have taken similar modeling approaches addressing bacterial kinetics in a variety of environmental settings (exposure to antibiotics, water, soil, etc.) (37–39), attempting to elucidate the mechanisms of persister formation (40, 41), as well as addressing the importance of including the biphasic dynamics of pathogens in the context of risk analysis for public health (42).

In an attempt to model the fate of enteric pathogens on plants in the field, recent studies have primarily utilized empirical models, such as the piecewise log-linear, the biexponential, and the Weibull models, in order to explain the frequently observed biphasic bacterial population decay patterns (28, 43–46). While the decay models developed in our current study (equations 1a to 1d and equations F1 to F3 in Text SA.1 in the supplemental material) exhibit biphasic kinetics similar to those seen in the biexponential model, the main difference is that our models arose from a mechanistic assumption about the formation of a persister subpopulation. That is, the biphasic pattern resulting from our models relies on the quantification of the switch rate of cells from the normal to the dormant state. The power of this approach is that these models take an average viewpoint of the underlying stochastic processes that may govern the switch rates between normal and persister cell types and provide a benchmark by which to compare relatively small time series data sets collected at vastly different scales (in the laboratory versus the field).

The model fits in our study showed that the order of magnitude of the average switch rate from normal to persister cells, $\log_{10} \alpha_d$, ranged (for all but one fit) from -6 to -5 with regard to both laboratory- and field-scale data. This finding suggests that experiments to study persisters conducted at a laboratory scale may be useful for attempting to elucidate the mechanisms of persister formation at the field level. Building from this observation, we applied the model presented in equations F1 to F3 in Text SA.1 to provide guidelines for sufficient initial inoculum levels as well as relevant

sampling windows for the quantification of persister cells on lettuce leaves in the field. Importantly, however, our model guidelines do not apply across the board. For instance, in the study by Islam et al. (47), the time scale for EcO157 population measurements on lettuce is on the order of weeks, whereas the context for our model results is on the order of hours to days. While we showed that our model is a useful tool by applying its parameters to published field population data, it will be informative to further validate it in field trials.

We demonstrate here the formation of EcO157 persister cells on a crop plant that has been implicated in numerous outbreaks of enteric disease and provide models for their quantification on plants in the field. However, it is evident that the persister subpopulations measured under the conditions of our study represented only a small fraction of the total culturable EcO157 population on the lettuce leaves. This suggests that strategies other than the dormancy pathways leading to antibiotic tolerance, such as toxin-antitoxin module activity (30), are additionally at play in EcO157 as mechanisms of survival in the phyllosphere. The lack of an inverse correlation between the total number of culturable EcO157 cells and the fraction of EcO157 persister cells among individual leaves (data not shown) supports the hypothesis that the populations surviving on plant surfaces are composed of diverse subpopulations, perhaps as diverse as the properties of the microsites that they inhabit. Under these spatially heterogeneous and unstable conditions, one could predict that phenotypic variability in metabolic activity among clonal populations of enteric pathogens on leaves is a winning strategy. However, EcO157 cells may also benefit from plant-derived factors, such as the presence of solutes that are utilized as osmoprotectants during conditions of water limitation (48, 49), a cuticle composition that modulates the distribution of free water on drying plant surfaces (50), or a compromised integrity of the plant tissue at certain locations or microsites, which promotes EcO157 multiplication and survival (9, 51), to name only these few. Furthermore, the presence and density of plant microbiota at the site of EcO157 immigration may enhance its survival under dry conditions, as demonstrated for *S. enterica* (21) and for *P. agglomerans* and *Pseudomonas* spp. (20).

Viable but nonculturable (VBNC) cells map on the spectrum of cell dormancy, and the molecular mechanisms of their formation shares similarities with the mechanisms of formation of persisters; moreover, several human-pathogenic bacteria were reported to also be more resistant to antibiotics in their VBNC state (52). The common plant-associated bacterial species *P. syringae* is known to form VBNC cells on leaves (31), while VBNC EcO157 and *L. monocytogenes* cells have been observed on leafy vegetables in the field (29, 53, 54). The EcO157 persister population sizes assessed herein did not include VBNC cells since our approach did not involve the resuscitation-promoting step necessitated to culture them on nutrient agar (55). Further studies probing the physiological state of EcO157 cells on plants are needed to shed light on the mechanisms that allow for their survival in this highly fluctuating environment and that may help them evade the sanitizers and other stresses employed to improve produce safety. This knowledge, if acquired at a range of scales to account for the heterogeneity of the leaf habitat, may additionally inform models to predict the prevalence of foodborne pathogens that are in a protective state and approaches to induce their reversion to a stress-susceptible state.

MATERIALS AND METHODS

Bacterial strain, reagents, and growth media. *E. coli* O157:H7 strain TW14588, a clinical isolate from a 2006 outbreak attributed to Iceberg lettuce served at a Taco John's restaurant (56) and kindly provided by the Thomas Whittam STEC Center, MI, was used in this study. In order to assess the *E. coli* O157:H7 persister cell abundance in the lettuce phyllosphere and recover the pathogen from lettuce leaf washes selectively on agar plates, we used a chromosomally marked derivative of strain TW14588. The marked strain, MB1168, harbors a gentamicin resistance cassette (*aacC1*) that was integrated at the neutral Tn*7* transposon insertion site (*att*Tn*7*) by mini-Tn*7*, as we described previously (49). This strain has a level of resistance to ciprofloxacin similar to that of *E. coli* K-12 and other O157:H7 strains tested in our previous study (57), with a MIC of 0.03 μg/ml, as measured based on the established protocol by Wiegand et al. (58). The strain was routinely maintained and cultured in Luria-Bertani half salt (LBHS; NaCl concentration, 5 g/liter) from a colony grown on LBHS agar containing gentamicin at 15 μg/ml. Ciprofloxacin (Sigma-

Aldrich) was prepared according to the manufacturer's instructions and used at 0.3 μg/ml in the persister assay detailed below. Phosphate-buffered saline (PBS) solution was prepared at 10× (containing, 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 was used as the assay buffer at a 1× concentration.

Growth and maintenance of lettuce plants. Romaine lettuce (*Lactuca sativa* cv. Parris Island) plants were grown in pots in Supersoil potting mix with 150 mg of Osmocote Plus (15-9-12 [N-P-K]) at 22°C with a 14-h photoperiod in a plant growth chamber (Percival Scientific Inc.) and fertilized weekly with 20-20-20 (N-P-K) liquid fertilizer past the five-expanded-leaf growth stage. The plants were used for inoculation at the 8- to 10-leaf stage.

Plant inoculation. The lettuce leaves were inoculated by immersing the aerial part of the plant in a suspension of EcO157 strain MB1168 in potassium phosphate buffer (1 mM, pH 7.0) prepared from a culture grown in LBHS broth to the early stationary phase at 28°C on a roller drum. The bacterial cell concentration in the inoculum suspension was adjusted based on the optical density at 600 nm. The inoculum levels varied depending on the target starting population size and the desired population trends on the leaves: (i) for EcO157 population growth on the leaves, which was achieved by incubating the plants under conditions of high-RH conditions and the presence of free water on the leaves, the inoculum concentration was approximately 2×10^5 cells/ml; (ii) for EcO157 population levels that would remain steady after inoculation, the inoculum suspension was 5×10^6 cells/ml, which resulted in densities maintained at about the carrying capacity of the leaf under high RH; and (iii) for EcO157 population size decline under low RH and low water availability on the leaf surfaces, the inoculum suspension was 5×10^6 cells/ml in order to obtain detectable and measurable population sizes by plate counts throughout the sampling time course. In the last set of experiments with plant colonization under low RH, the plants were air dried in a biosafety cabinet for 3 h immediately after inoculation and before incubation to prevent the prolonged presence of water from the inoculum suspension on the leaf surfaces. The temperature and RH during plant incubation were monitored using a Hobo data logger and probe (Onset, Bourne, MA) (see Fig. 1A, 2A, and 3A for the recorded temperature and RH data under each of the three conditions described above).

Bacterial recovery from leaves and population measurements. The population sizes of strain MB1168 on the leaves were measured immediately and at day 1, day 2, and day 3 after inoculation by placing each leaf in 20 ml 1× PBS in a Whirl-Pak bag and massaging the adaxial and abaxial sides of the leaf vigorously for 1 min to dislodge the bacterial cells. Only the middle leaves in the rosette were used. The leaf wash at each sampling time was dilution plated onto LBHS agar with gentamicin (15 μg/ml) with a Spiral Biotech Autoplate 4000 spiral plater (Advanced Instruments, Norwood, MA), and colonies were counted after incubation for 24 h at 37°C.

Assessment of *E. coli* O157:H7 persister population sizes on lettuce plants. The assay for persister cell enumeration was adapted from published methods using the antibiotic lysing method (59, 60). Exposure of ciprofloxacin-sensitive EcO157 to concentrations of the antibiotic at 10× the MIC in PBS buffer was used to enrich for cells that exhibited phenotypic tolerance to the antibiotic while lacking ciprofloxacin resistance genes. Because of the transient state of cell dormancy, the number of EcO157 cells that survived antibiotic treatment was quantifiable by their ability to form colonies when plated onto nutrient agar. It is important to note that the spontaneous rate of mutation that confers reduced susceptibility to ciprofloxacin in *E. coli* is 3.6×10^{-9} (61). Therefore, given the total population sizes of EcO157 that we measured on leaves and the range of the percentage of persister cells that we detected with the ciprofloxacin tolerance assay in our study, it is highly unlikely that these persister cells originated via spontaneous mutations conferring ciprofloxacin resistance among cells in the EcO157 population.

Immediately after the leaf washes were prepared, ciprofloxacin was added to the 20-ml leaf wash in PBS at 0.3 μg/ml in glass tubes and the culture was incubated at 37°C with shaking at 150 rpm for 5 h, based on our method previously described for Shiga toxin-producing *E. coli* (STEC) (57). In order to remove ciprofloxacin from the cells, the suspensions were filtered through a 10-μm-pore-size TCTP Isopore filter to remove any plant debris and the filtrate was processed through a 0.2-μm-pore-size GTTP Isopore filter to collect the bacterial cells on the filter. The filter was transferred into 0.5 ml 1× PBS in a microcentrifuge tube for the recovery of the cells by vortexing. Bacterial counts were obtained by dilution plating of the resulting suspension in 1× PBS onto LBHS agar with gentamicin (15 μg/ml) and a 24-h incubation of the plates at 37°C. The resulting number of CFU represented the subpopulation of MB1168 cells on the leaves that survived ciprofloxacin challenge. Using this approach, the minimum detection level of persisters was two cells per leaf, which was measured only for the experimental results presented in Fig. 1 at 0 h due a combination of the low inoculum densities on the leaves (in order to ensure population growth) and the low rate of occurrence of persister cells in the inoculum.

Statistical analyses. In each experiment, middle leaves were sampled at random from 15 inoculated plants (1 plant per pot); four replicate leaves were sampled immediately after inoculation, and six replicate leaves were sampled at various times of incubation postinoculation. Each leaf was processed for quantification of the total bacterial population size and the percentage of persister cells individually. Bacterial population sizes were $\log_{10}$ transformed, and the percentage of persister cells was transformed with arcsine(square root of the proportion of persisters) to compare the means statistically. The Brown-Forsythe test, to verify that variances among populations were not significantly different ($P < 0.05$), and one-way ANOVA were performed before the means at different incubation times were compared using Tukey's multiple-comparison test. The unpaired Student's *t* test was used to compare pairs of means in a few instances. Statistical analyses were performed with GraphPad Prism (version 7.04) software (GraphPad Software, San Diego, CA, USA).

Model for population decay regime. Following the regime under dry experimental conditions, we built basic ordinary differential equation models to describe the average temporal dynamics of culturable normal (nondormant) and persister (dormant) cell populations on lettuce leaf surfaces. Our modeling approach is similar to the perspective taken by Balaban et al. (35) and Malik and Smith (36), in that we assumed that cells may switch back and forth between the persister and normal cell states.

Under dry conditions (Fig. 3A), the total EcO157 population exhibited decay dynamics, whereas the persister subpopulation showed an initial increase followed by a decrease in population. Comparing the results presented in Fig. 3B and C, it is clear that after 48 h of incubation the persister population is still a very small percentage of the total population. That is, while Fig. 3B exhibits biphasic decay phenomena (see the work of McKellar et al. [28] and references therein), the data indicate that the tail of the population decay is primarily composed of cells other than persisters. We assume, therefore, that the normal population is composed of two subpopulations, characterized by fast (n_1) and slow (n_2) decay, leading to the following model:

$$\frac{dn_1}{dt} = -\theta_{n_1} n_1 - \alpha_d n_1 + \beta_d (1-\sigma) p \tag{4a}$$

$$\frac{dn_2}{dt} = -\theta_{n_2} n_2 - \alpha_d n_2 + \beta_d \sigma p \tag{4b}$$

$$\frac{dp}{dt} = -\theta_p p - \beta_d p + \alpha_d (n_1 + n_2) \tag{4c}$$

$$n_1(0) = n_{10},\ n_2(0) = n_{20},\ p(0) = p_0 \tag{4d}$$

where θ_{n_1} (in 1/hours) is the death rate of the normal cells (fast and slow decay), p (in numbers of CFU per leaf) represents the average persister cell population at time t (in hours), θ_p (in 1/hours) is the death rate of the persister population, α_d (in 1/hours) is the switch rate from the normal state to the persister state, β_d (in 1/hours) is the switch rate from the persister state back to the normal state, and σ is a constant with a value of between 0 and 1. Note that in our model construction for equations 4a and 4b, we assume that times between switching states are exponentially distributed, using the expected values for $1/\alpha_d$ (in hours) and $1/\beta_d$ (in hours) of the respective distributions. Thus, with respect to metabolic state switching, our model represents an averaged viewpoint of an assumed underlying stochastic process. However, this model can be simplified by dropping the terms involving p in equations 4a and 4b, given the relative order of magnitude of the persister population data and that of the normal cells. The model then becomes that presented above in equations 1a to 1d.

Using data from Fig. 3B and C and our fitting procedure (see Text SA.4 in the supplemental material), we determined parameter values for the model presented in equations 1a to 1d. Note that as an alternative to dividing the normal population into two subpopulations each with a different decay rate (as modeled above), one may hypothesize that these populations are the same and thus may be modeled with a die-off rate that decreases as a function of population density. However, given that an inverse relationship between the bacterial population density and the die-off rate of bacterial cells in the phyllosphere has been reported (31), it is unlikely that this alternative model would accurately describe population decay in this study.

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