Enumeration, Resuscitation, and Infectivity of the Sublethally Injured *Erwinia* Cells Induced by Mild Acid Treatment

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ABSTRACT

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Current knowledge about microbial injury was derived mostly from studies with bacteria associated with processed foods. Demonstration of injury and repair in phytopathogenic bacteria and its implication on pathogen detection and disease ecology have not been reported. The objective of this study was to investigate the lethal and sublethal effects of acetic acid (AA) on soft-rot *Erwinia* spp. and the limitation of using selective media for isolation of injured cells. Following exposure to 0.3% AA for 6 min, 90 to 99% of *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroseptica*, and *E. chrysanthemi* cells were killed. When AA-treated samples were plated on agar media, the number of bacteria recovered on nonselective media such as brain heart infusion

agar was 3 log units higher than that recovered on selective media such as crystal violet pectate (CVP). Lethal and sublethal actions of AA on *Erwinia* spp. were affected greatly by acid concentration, exposure time, and bacterial strains tested. Injured *Erwinia* cells were able to repair and regain the ability to grow on CVP when placed in nutrient broth but not in selective broth containing crystal violet and sodium dodecyl sulfate. Injured cells also were able to resuscitate on cut surfaces of cucumber fruit and to induce soft rot on green bell pepper. Together, these results suggest that direct use of selective media for isolation of *Erwinia* spp. could limit the recovery of injured cells in samples that have been exposed to chemical or physical stresses. Enrichment of these samples in nutrient broth before plating on CVP is expected to improve the detection of injured *Erwinia* spp.

Additional keywords: bacterial injury.

The widespread use of selective media for isolation of bacteria began in approximately 1920, but the limitation of using these media for detection of foodborne pathogens was not realized until quantitative studies revealed the poor recovery of viable bacteria following heating, drying, or freezing (1). It now is generally believed that, following exposure to a variety of physical or chemical stresses, bacteria can be killed, injured, or unaffected (27). Hartseli (13) was among the first to define “injured bacteria” as those capable of forming colonies on nonselective but not on selective media. Previous studies of microbial injury were focused almost exclusively on bacteria that were associated with food or drinking water (7). Demonstration of injury and repair in phytopathogenic bacteria, including soft-rot *Erwinia* spp., has not yet been reported.

As pre- and postharvest pathogens, soft-rot *Erwinia* spp. frequently are found in soil, irrigation water, and plant debris (19, 24). Several selective media, including crystal violet petate (CVP) (8), D3 (14), and pectate Tergitol (PT) (6), have been developed for isolation of soft-rot *Erwinia* spp. from plant and environmental samples. Plating efficiencies of these media for recovery of *Erwinia* spp. were evaluated, usually using bacteria propagated in the laboratory. Limitation of these media for isolation of injured *Erwinia* cells from the environment or sanitized produce samples has not been considered or investigated. Meneley and Stanghellini (21) reported that isolation of *Erwinia* spp. from soil could be improved by incubating the samples in a semiselective broth

before plating on CVP medium. However, it was not determined if this semiselective broth enhanced the recovery of potentially injured *Erwinia* cells in the samples.

Several sanitizers, including chlorine (2), chlorine dioxide (31), and hydrogen peroxide (28), have been tested for their efficacies in eliminating harmful microorganisms from fresh produce. None of these sanitizers, however, is sufficiently effective to completely remove microorganisms from fruits and vegetables (3). Nevertheless, an increasing number of reports have shown that washing fresh produce and sprouting seed with acetic acid (AA) was effective for suppressing fungal decay (10,29), *Escherichia coli* O157:H7 (32,33), *Salmonella typhimurium* (11), and *Yersinia enterocolitica* (15) on fresh produce or meat products. AA is generally recognized as safe (GRAS) and has been approved by the United States Food and Drug Administration for use as a food additive or as a disinfectant for animal carcasses (12). Although antimicrobial activities of AA against *E. coli* K-12 (26) and *S. bareilly* (5) have been demonstrated, its effect on phytopathogenic bacteria has not been examined. The objectives of this study were to (i) investigate the lethal and sublethal actions of AA on *Erwinia* spp. as affected by acid concentration, exposure time, and bacterial strains; (ii) examine the condition and time frame required for recovery of injured cells in broth media and on cut surfaces of fruit; and (iii) determine the soft-rotting ability of acid-injured *Erwinia* cells on bell pepper fruit.

MATERIALS AND METHODS

Bacterial strains and culture media. Five *Erwinia* strains, including two strains (SR319 and 1) of *E. carotovora* subsp. *carotovora*, two strains (EC16 and 120A) of *E. chrysanthemi*, and one strain (SR8) of *E. carotovora* subsp. *atroseptica*, were used. *E. carotovora* subsp. *carotovora* strain 1 was isolated from pre-peeled baby carrot (*unpublished data*). *E. carotovora* subsp.

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carotovora strain SR319, *E. carotovora* subsp. *atroseptica* strain SR8, and *E. chrysanthemi* strain 120A were obtained from A. Kelman (North Carolina State University) and *E. chrysanthemi* strain EC16 from A. Chatterjee (Kansas State University). They were grown routinely on brain heart infusion agar (BHIA; BD Systems/Difco, Sparks, MD) at 28°C and maintained on the same medium at 4°C. For enumeration of injured cells, acid-treated samples were plated on BHIA and on four selective agar media, including CVP (8), PT (6), D3 (14), and violet-red bile agar (VRBA) (BD Systems/Difco). These media were prepared as previously described (6,8,14) with slight modification. In modified CVP, the medium was supplemented with sodium dodecyl sulfate (SDS) and yeast extract to a concentration of 0.1 mg/ml and 0.2% (wt/vol), respectively. In addition, the concentration of crystal violet in modified CVP was reduced to 1.0 µg/ml. For 1 liter of modified PT medium, Tergitol7 was replaced with 0.6 ml of XLT4 supplement (BD Systems/Difco) containing 27% of Tergitol4. In modified D3, the concentration of LiCl was reduced to 0.7 g/liter. Polypectate for CVP was purchased from M. Burger, Inc. (Madison, WI).

Preparation of bacterial suspensions and acid treatment.

Unless otherwise indicated, bacterial suspensions were prepared from cultures grown on BHIA at 28°C for 24 h. Cell masses were collected from culture plates using a sterile loop and subsequently dispersed in 75 µM of phosphate-buffered saline (PBS, pH 7.1; Invitrogen Corp., Carlsbad, CA) to make a suspension with an optical density of 1.00 ± 0.03 at 600 nm, which was equivalent to a cell density of 1.2 to 1.7×10^9 CFU/ml. The optical density was measured using a spectrophotometer (Beckman Instruments, Fullerton, CA). A microfuge tube containing 1 ml of cell suspension was spun at 12,000 rpm for 1 min by a refrigerated microcentrifuge (Brinkmann Instruments, Westbury, NY). After centrifugation, the supernatant was discarded and the cell pellet was suspended in an AA solution prepared from glacial AA (Mallinckrodt Baker, Inc., Paris, KY) and allowed to incubate at 20°C for 0 to 6 min. AA-treated cells then were collected by centrifugation (12,000 rpm for 1 min) and resuspended in 1 ml of PBS after washing twice in the same buffer. Total exposure time of bacteria to AA included the actual incubation time plus 2 min required for removal of AA following the incubation. Processed samples were plated on nonselective BHIA and also on selective CVP, PT, D3, or VRBA media by spreading. Culture plates then were incubated at 28°C for 48 h. The killing rate was calculated based on the difference in bacterial counts of AA-treated and water-treated samples as determined on BHIA. The injury rate was calculated based on the difference in bacterial count of AA-treated sample determined on selective agars and on bacterial count determined

on BHIA. The plating efficiency of each selective medium was calculated from the difference in plate counts of water-treated samples determined on BHIA and on selective agar. Following exposure to 0.3% AA for 6 min, the suspension containing *E. carotovora* subsp. *carotovora*, *E. carotovora* subsp. *atroseptica*, or *E. chrysanthemi* was plated on nonselective BHIA and on selective CVP and VRBA to compare the killing and injury effect of AA on five different species or strains of *Erwinia*. To determine the effect of acid concentration and exposure time on killing and injury, *E. carotovora* subsp. *carotovora* strain SR319 cells were treated with different concentrations (0.06 to 0.60%) of AA for 6 min or with 0.3% AA for 2 to 10 min and subsequently plated on BHIA and VRBA.

Repair of bacterial injury in nutrient broth containing or lacking selective agents. Nutrient broth (NB; BD Systems/Difco) and NB supplemented with sodium dodecyl sulfate (SDS; 100 µg/ml) and crystal violet (1 µg/ml) were inoculated with AA-treated *E. carotovora* subsp. *carotovora* (strain SR319) cells to make an initial cell concentration of approximately 3 log CFU/ml. Inoculated flasks were incubated at 20°C undisturbed for up to 8 h. The samples were taken at 2-h intervals to monitor the changes in the proportions of injured and recovered cells by plating the samples on BHIA and CVP. Injured cells were prepared by exposing *E. carotovora* subsp. *carotovora* cells from 24-h cultures to 0.3% AA for 6 min. The steady decline in the number of injured cells during the incubation period was assumed to result from the resuscitation of injured cells.

Resuscitation of acid-injured *Erwinia* cells on apple and cucumber fruit. Unwaxed apple (Golden Delicious) and cucumber were purchased from the packer or local grocers, respectively. They were cleaned with laboratory detergent (Liquid-Nox; Alconox Inc., NY) and tap water and surface sanitized with 85% ethanol (18). Injured *E. carotovora* subsp. *carotovora* strain SR319 cells were prepared by exposing the bacteria from 24-h cultures to 0.3% AA for 6 min. A sterile nitrocellulose membrane (0.45 µm; 25 mm in diameter; Millipore Inc., Bedford, MA) was placed on cut surface of apple or cucumber fruit and then inoculated with 50 µl of AA-treated bacterial suspension containing approximately 10^4 CFU of injured cells. The inoculated membrane then was covered with apple or cucumber slices to ensure close contact between the membrane and fruit tissue. After incubation at 20°C for 0, 4, 8, and 16 h, two membranes at each sampling time were removed and each membrane was immersed in 5 ml of PBS followed by vigorous shaking for 30 s. Aliquots (50 µl) of the suspension then were plated on BHIA and CVP to determine the changes in the populations of the injured and uninjured cells during the incubation.

TABLE 1. Recovery of *Erwinia carotovora* subsp. *carotovora* cells on selective and nonselective agar media following exposure to 0.3% acetic acid (AA) or to water (as control) for 6 min^w

Media ^x	Water treated		Acid treated	
	Plate count (log CFU/ml)	Plating efficiency ^y	Plate count (log CFU/ml)	Log CFU killed (K) or injured (I) ^z
Nonselective				
BHIA	9.1 ± 0.0 a	...	7.7 ± 0.1 b	1.4 (K)
Selective				
CVP	9.1 ± 0.2 a	92	3.8 ± 0.1 c	3.9 (I)
PT	9.1 ± 0.1 a	100	5.3 ± 0.2 d	2.4 (I)
D3	9.0 ± 0.3 a	77	3.9 ± 0.3 cc	3.8 (I)
VRBA	9.0 ± 0.1 a	74	3.4 ± 0.2 cc	4.3 (I)

^w *E. carotovora* subsp. *carotovora* strain SR319 cells were exposed to 0.3% AA or to water (as control) for 6 min. Treated cells were washed twice with phosphate-buffered saline to remove residual AA and then plated on selective and nonselective agars. Inoculated agar plates were incubated at 28°C for 48 h prior to the counting. Values represent the average of three experiments with two duplicates in each experiment ± standard deviation. Within a column, numbers not followed by the same letter are significantly different ($P < 0.05$) by the Bonferroni least significant difference separation technique (22).

^x BHIA = brain heart infusion agar, VRBA = violet-red bile agar, CVP = crystal violet pectate medium (8), PT = pectate tergitol medium (6), D3 = medium (14).

^y Plating (recovery) efficiency was calculated according to the formula (plate count of water-treated sample on selective agar/plate count of water-treated sample on BHIA) × 100.

^z Log CFU killed = log CFU count of water-treated sample as determined on BHIA-log CFU count of acid-treated sample as determined on BHIA; log CFU injured = log CFU count of acid-treated sample as determined on BHIA-log CFU count of acid-treated sample as determined on selective agar.

Infectivity assays. Green bell pepper fruit purchased from local grocers were cleaned and surface sanitized with 85% ethanol as previously described (16). Injured *E. carotovora* subsp. *carotovora* cells were prepared by exposing the bacteria from 24-h cultures to 0.3% AA for 6 min; bacteria treated with sterile distilled water were used as controls. The killing and injuring rates of AA treatment were determined as described above. Ten-fold serial dilutions of AA- or water-treated samples were prepared and 5- μ l aliquots of each dilution were deposited on the wounded surface of green bell pepper (17). Wounding was made by puncturing the fruit with a 26-G syringe 15 times to generate a site of approximately 2 to 3 mm in diameter. In each experiment, water- or AA-treated samples from each dilution were inoculated at three different sites on one fruit and the experiment was repeated three times. Inoculated bell pepper fruit were placed in a moist chamber and kept at 20°C for 2 days. Development of soft rot was recorded and percent of infectivity was calculated based on the formula number of sites developing soft rot/number of sites inoculated ($n = 9$) $\times$ 100.

Fig. 1. Bactericidal and injury effects of acetic acid (AA) on *Erwinia carotovora* subsp. *carotovora* SR319 as affected by acid concentration (top) and exposure time (bottom). *E. carotovora* subsp. *carotovora* cells from a 18-h culture were treated with various concentrations (0.0 to 0.6%) of AA for 6 min or treated with 0.3% AA for different periods of time (2 to 10 min). After removal of AA, treated samples were plated on brain heart infusion agar (BHIA) and violet-red bile agar (VRBA) to determine the killing and injury effect of AA on *E. carotovora* subsp. *carotovora*. Percent survival = (CFU count of the sample treated with AA as determined on BHIA/CFU count of sample treated with water – CFU count of the sample treated with AA) $\times$ 100. Log injury = log CFU count of AA-treated sample as determined on BHIA – log CFU count of the same sample as determined on VRBA. The percent survival (■) and log injury (●) rates were determined as described in the text.

Statistical analysis. Resuscitation responses in broth or on cut surfaces of fruit were analyzed by performing analysis of variance to determine the effect of incubation time or environments. Differences between incubation times or environments were performed using the Bonferroni least significant difference mean separation procedure at the $P = 0.05$ significance level (22).

RESULTS

Detection and enumeration of acid-injured cells. Bacterial counts in the samples treated with AA as determined on BHIA were $\approx 96\%$ or 1.4 log units lower than those treated with water (Table 1). In addition, colony sizes of AA-treated cells were smaller, in general, than those formed by water-treated cells after incubation at 28°C for 48 h. The reduction in plate count on BHIA was assumed to be caused by the bactericidal action of AA and the reduction in colony size was due to the extended lag phase required for recovery of injured cells. When AA-treated samples were plated on both selective and nonselective agar media, the number of *E. carotovora* subsp. *carotovora* cells recovered on selective agars, including CVP, PT, D3, and VRBA, was 2.4 to 4.3 log units ($>99\%$) lower than those recovered on BHIA (Table 1), indicating that injured cells failed to recover and form colonies on selective media. A significantly ($P < 0.05$) higher injury rate was obtained when the sample was plated on VRBA, D3, or CVP than on PT (Table 1). Bacterial counts of water-treated samples as determined on VRBA or D3 were lower, in general (23 to 26%), than those determined on PT or CVP medium, indicating that higher selectivity or lower recovery efficiency as observed with VRBA and D3 media also impeded or delayed the recovery of injured cells.

Antimicrobial action of AA on *Erwinia* spp. as affected by acid concentration and exposure time. A positive correlation was observed between the killing or injury rate and the concentration of AA tested (Fig. 1). Upon exposure to 0.6% AA for 6 min, 99.5% (or more than 2 log units) of *E. carotovora* subsp. *carotovora* cells were killed. The number of viable *E. carotovora* subsp. *carotovora* cells as determined on BHIA was reduced from 1.5×10^9 to 8.0×10^6 CFU/ml following acid treatments. Among surviving *E. carotovora* subsp. *carotovora* cells, only a small fraction (4.2×10^2 CFU/ml) was able to form colonies on VRBA, indicating that 99.99% (or more than 4 log units) of surviving cells were injured. A positive correlation was observed between the exposure time and killing or injuring effects (Fig. 1). The longer the exposure time, the higher death rates were observed. For examples, following exposure to 0.3% AA for 10 min, the number of surviving *E. carotovora* subsp. *carotovora* cells as determined on BHIA decreased from 1.7×10^9 to 8.0×10^6 CFU/ml, but the number of surviving cells as determined on VRBA decreased very sharply from 1.4×10^9 to 1.8×10^3 CFU/ml. Similarly, following exposure to 0.3% AA for 6 min, more than 90% of cells were killed and more than 99.9% (or 3 log units) of the surviving cells were injured.

Variation in responses of *Erwinia* spp. or strains to AA antimicrobial action. Five representative strains of soft-rotting *Erwinia* spp., including two strains each of *E. carotovora* subsp. *carotovora* and *E. chrysanthemi* and one strain of *E. carotovora* subsp. *atroseptica*, were treated with 0.3% AA for 6 min and subsequently plated on nonselective BHIA and selective CVP and VRBA media to compare the relative susceptibility of bacterial strains to AA actions. The responses of soft-rotting *Erwinia* spp. to AA treatments varied among the strains or species examined (Fig. 2). Two *E. chrysanthemi* strains were significantly ($P < 0.05$) more susceptible to injuring action of AA than *E. carotovora* subsp. *carotovora* and *E. carotovora* subsp. *atroseptica* strains. For example, exposure of *E. carotovora* subsp. *carotovora* (strains SR319 and 1) to 0.3% AA for 6 min resulted in the reduction in plate count on BHIA by 1.4 and 1.9 log units, respectively. By

comparison, exposure of *E. chrysanthemi* strains to AA under the same condition resulted in the reduction in plate count by more than 2 log units. Regardless of the strain tested, >99.9% (or 3 log units) of surviving cells were injured as indicated by their inability to form colonies on CVP or VRBA.

Resuscitation of acid-injured *E. carotovora* subsp. *carotovora* cells in nutrient broth. When nonselective NB was inoculated with AA-treated *E. carotovora* subsp. *carotovora* cell suspension and incubated at 20°C for up to 8 h, a steady increase in colony count on CVP and a steady decrease in the difference of bacterial counts as determined on CVP and on BHIA were observed (Fig. 3). Some recovery already is evident after 2 h of incubation in NB and the recovery continued to increase for up to 8 h (Fig. 3). Total bacterial count on BHIA decreased slightly during the first 8 h of incubation from 6.5 to 6.2 log units, a steady increase in bacterial counts on CVP resulted more likely from the recovery of injured cells, but not from the multiplication of uninjured cells. Furthermore, the population of untreated cells increased by less than 0.4 log units after incubation in NB for 8 h (data not shown). However, when injured *E. carotovora* subsp. *carotovora* cells were placed in NB containing crystal violet and SDS, a sharp decline in bacterial count (2.2 log CFU/ml) was observed on BHIA during the first 2 h of incubation. Plate counts as determined on CVP showed little change throughout the 8-h incubation period. This result indicated that injured cells failed to resuscitate in NB containing crystal violet and SDS and were more vulnerable to bactericidal activities of selective agents present in selective media.

Resuscitation of acid-injured *E. carotovora* subsp. *carotovora* cells on cut surfaces of cucumber fruit. To compare the resuscitation of injured cells on cut surfaces of cucumber and apple fruit, bacterial counts on selective (VRBA) and nonselective (BHIA) media were determined for injured cells that had been incubated on cucumber or apple for 4, 8, and 16 h. Although little change in bacterial counts as determined on BHIA was found with samples that had been incubated on cucumber for up to 8 h, bacterial counts on VRBA continued to increase during the same period (Table 2). This result indicated that the proportion of

injured cells in the samples declined from 96 to 59% during the first 8 h of incubation. A marked increase in the population of *E. carotovora* subsp. *carotovora* (1.7 log units) and a marked decrease in the population of injured cells were observed with samples which had been incubated on cucumber for 16 h. However, the number of bacteria recovered on VRBA was still 20% fewer than those recovered on BHIA, possibly due to the lower plating efficiency of VRBA as revealed in Table 1. When filter membranes containing injured *E. carotovora* subsp. *carotovora* cells were incubated on cut surfaces of apple fruit, total bacterial counts

Fig. 3. Comparison of the recovery of acid-injured *Erwinia carotovora* subsp. *carotovora* cells in nutrient broth and in selective broth containing crystal violet (1 µg/ml) and sodium dodecyl sulfate (100 µg/ml). Both selective and nonselective nutrient broths containing injured cells at an initial concentration of 2.9 log CFU/ml were incubated at 20°C for up to 8 h. Samples were collected at 2-h intervals and plated on brain infusion agar and crystal violet pectate to monitor the number of injured and recovered cells during the incubation. Number of dead cells = total number of cells at T₀ (2.9 CFU/ml – number of cells injured – number of cells recovered).

Fig. 2. Comparison of the recovery of two strains of *Erwinia carotovora* subsp. *carotovora* (Ecc), two strains of *E. chrysanthemi* (Ech), and one strain of *E. carotovora* subsp. *atroseptica* (Eca) on selective (BHIA) and nonselective (CVP and VRBA) media following exposure to 0.3% acetic acid (AA) for 6 min. BHIA = brain heart infusion agar; CVP = crystal violet pectate; and VRBA = violet-red bile agar. The cell suspension of each strain at an initial concentration of 9.2 ± 0.1 log CFU/ml was treated with 0.3% AA for 6 min. Total plate count of treated sample as determined on BHIA was compared with those determined on CVP or VRBA.

on both BHIA and VRBA showed slight decline during the incubation and the percentage of injured cells in the population showed little change (Table 2). No sign of recovery was observed when injured cells were placed on apple fruit. Injured *E. carotovora* subsp. *carotovora* cells were able to repair the injury only on cut surfaces of cucumber fruits but not cut surfaces of apple fruit.

Soft-rotting ability of acid-injured *E. carotovora* subsp. *carotovora* cells. Green bell pepper fruit were inoculated with AA- or water-treated *E. carotovora* subsp. *carotovora* cell suspensions at the concentrations ranging from 10^9 to 10^3 CFU/ml. The minimal concentration of water-treated (or uninjured) and AA-treated cells required for induction of soft rot was estimated to be 3.8×10^5 and 3.2×10^6 CFU/ml, respectively. Only 5 μ l of bacterial suspension was deposited on the inoculation site; therefore, the total number of uninjured *E. carotovora* subsp. *carotovora* cells (from water-treated samples) required for induction of soft rot on bell pepper fruit was estimated to be 1.9×10^3 CFU. By comparison, the minimal number of acid-treated cells required for induction of soft rot on bell pepper fruit was estimated to be 1.6×10^4 CFU. Because >99.9% of viable *E. carotovora* subsp. *carotovora* cells in AA-treated sample were injured, it was estimated that only one uninjured cell was present in 5 μ l of inoculum. Therefore, induction of soft rot by AA-treated samples was not due to the presence of uninjured cells but due to the resuscitation of injured cells.

DISCUSSION

Data presented here show that exposing *Erwinia* cells to low concentrations of AA can cause death and injury in all of five *Erwinia* strains examined. Injured cells could be detected and enumerated based on their differential abilities to form colonies on nonselective medium such as BHIA but not on selective agar media such as CVP, D3, PT, and VRBA. Those cells capable of forming colonies on BHIA but not on selective agars were considered “injured” as previously defined (7,27). Injury rate determination was dependent on the type of selective medium used and also on the physiological stage of bacteria tested (4). We found that, after exposure to 0.3% AA for 6 min, the number of *E. carotovora* subsp. *carotovora* cells recovered on BHIA was 10^2 - to 10^4 -fold higher than those recovered on selective agars (Table 1). Poor recovery of injured *Erwinia* cells on selective media indicates that direct plating of samples on CVP is not suitable for recovering injured *Erwinia* cells possibly present in the samples that have been treated with heat, irradiation, or chemicals. The limitation of using selective media for detection of specific bacteria in processed foods has long been recognized (1). However, detection of injured *Erwinia* spp. in the environment or sanitized produce samples has not yet been reported. Based on our knowledge about injury in *Escherichia coli* K-12 (26) and *S. typhimurium* (11), it is not unreasonable to predict the presence of injured *Erwinia* cells in soil, irrigation water, or dehydrated plant debris. However, bacteria that are injured could be differentiated

from those defined as viable but nonculturable (VNC) dormant cells (23). The VNC cells are considered nonculturable by the normal microbiological methods, whereas injured cells are considered culturable if they are placed on nonselective agar media containing no inhibitory substances or conditions.

In this study, we demonstrated that injured *Erwinia* cells would resuscitate in nonselective nutrient broth but not in broth containing SDS or crystal violet. Pre-enrichment of the samples in nonselective broth before plating them on selective agar such as CVP should aid the recovery of injured bacteria and improve the detection of *Erwinia* spp. This enrichment step is a standard practice for isolation of foodborne pathogens from clinical or food samples (1). Although such enrichment steps may not be necessary for isolation of *Erwinia* spp. from newly infected plant tissue, it should be considered when isolating *Erwinia* spp. from samples that have been exposed to physical or chemical stresses. An enrichment technique for improving the isolation of *Erwinia* spp. from soil has been described (21) and later applied to study the ecology of pathogens in potato fields (25). Semiselective broth, as described in these reports, was used mainly to increase the proliferation of pectolytic bacteria and to suppress the growth of aerobic native bacteria. It has not been determined if incubation of soil in this semiselective broth improves the recovery of injured *Erwinia* cells. It also is not known if soil extracts added to this nutritionally limited broth (21) may impede the repair of injured cells. Inability of the heat-injured *Salmonella* sp. or *Escherichia coli* cells to resuscitate on minimal medium has been demonstrated previously (27). Enrichment of root or soil samples in PT broth (6) before plating on indicator media also has been shown to improve the recovery of *Erwinia* spp. (9,20). The surfactant Tergitol in PT broth is toxic and expected to inhibit the resuscitation of injured *Salmonella* cells (1) and possibly *Erwinia* cells, as shown in this study. Therefore, the advantage of incubating the samples in PT broth possibly is not due to the enhancement of recovery of injured cells, but to the suppression of untargeted bacteria.

Although the clinical importance of injured gastrointestinal pathogens in humans cannot be easily determined, Sorrells et al. (30) reported that freezing-injured *S. gallinarum* cells showed no loss of virulence in chickens. In this study, we also found that acid-injured *Erwinia* cells were able to resuscitate and to induce soft rot on bell pepper fruit. An ≈ 10 -fold higher number of injured cells (3.2×10^6 CFU/ml) than the uninjured counterpart (3.8×10^5 CFU/ml) is required to induce soft rot. The requirement of a higher number of injured cells to induce soft rot possibly is due to the fact that severely injured cells might eventually die or fail to revive on fruit (Fig. 3). The ability of injured *Erwinia* cells to repair probably is dependent on the type of fruit and environmental conditions tested. We have observed (data not shown) that acid-injured *Erwinia* cells are unable to recover in PBS, in apple juice (pH 3.41), or at refrigeration temperature (4°C). Washing fresh produce with organic acids such as AA or storage at low temperatures may be effective in controlling soft rot caused by uninjured and injured *Erwinia* spp.

TABLE 2. Resuscitation of acid-injured *Erwinia carotovora* subsp. *carotovora* cells on cut surfaces of cucumber and apple fruit^y

Incubation time (h)	Cucumber, plate count (log CFU/membrane) on			Apple, plate count (log CFU/membrane) on		
	BHIA	VRBA	Injury (%) ^z	BHIA	VRBA	Injury (%) ^z
0	5.3 ± 0.2 a	3.9 ± 0.1 a	96 a	4.9 ± 0.3 a	3.8 ± 0.4 a	93 a
4	5.3 ± 0.1 a	4.2 ± 0.2 b	91 b	4.7 ± 0.1 a	3.7 ± 0.2 a	94 a
8	5.3 ± 0.3 a	4.9 ± 0.3 c	59 c	4.3 ± 0.2 b	3.0 ± 0.3 b	95 a
16	7.0 ± 0.4 b	6.5 ± 0.2 d	20 d	4.5 ± 0.1 b	2.9 ± 0.4 b	97 a

^y Filter membranes containing acetic acid-treated *E. carotovora* subsp. *carotovora* strain SR319 cells (0.3%, 6 min) were placed on cut surfaces of cucumber or apple fruit. The changes in the number of injured *E. carotovora* subsp. *carotovora* cells in the population were monitored after incubating the membranes containing acid-treated cells on either fruit for 4, 8, and 16 h. BHIA = brain heart infusion agar and VRBA = violet-red bile agar. Values represent the average of three experiments with two duplicates in each experiment ± standard deviation. Within a column, the numbers not followed by the same letter are significantly different ($P < 0.05$) by the Bonferroni least significant difference separation technique (22).

^z Percent injury = [(plate count on BHIA – plate count on VRBA)/plate count on BHIA] × 100%.

Because of its bactericidal activity, AA has been used for food preservation and for decontamination of animal carcasses (11). A number of studies already have shown that exposure to low concentrations of AA can suppress postharvest rot of fruit (29) and eliminate gastrointestinal pathogens from apple (32,33), parsley (15), and mung bean seed (10). Results from a separate study (data not shown) showed that a mixture of AA and H₂O₂ is more effective than other common sanitizers in removing *Salmonella* cells attached to cut surfaces of apple slices. By considering the minimal concentration of AA and exposure time required to achieve the same level of death or injury rate, *Erwinia* spp. are much more susceptible to AA action than *S. bareilly* (5) and *Escherichia coli* K-12 (26). Although AA is less toxic than other sanitizers, it is able to cause sublethal injury in an extremely large proportion of cells even at relatively low concentrations (<0.1%). Thus, washing fresh produce with AA, possibly in combination with other sanitizers, provides effective means for inactivating harmful bacteria on surfaces of plants.

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LITERATURE CITED

- Andrew, W. H. 1990. Methods for recovering injured "classical" enteric pathogenic bacteria (*Salmonella*, *Shigella* and enteropathogenic *Escherichia coli*) from foods. Pages 55-113. in: Injured Index and Pathogenic Bacteria: Occurrence and Detection in Foods, Water and Feeds. B. Ray, ed. CRC Press, Inc. Boca Raton, FL.
- Bartz, J. A. 1999. Washing fresh fruits and vegetables: Lessons from treatment of tomatoes and potatoes with water. Dairy Food Environ. Sanit. 19:853-864.
- Beuchat, L. R. 1998. Surface decontamination of fruits and vegetables eaten raw: A review. Food Safety Unit, WHO/FSF/FPS/98.2.
- Beuchat, L. R., and Lechowich, R. V. 1968. Survival of heated *Streptococcus faecalis* as affected by phase of growth and incubation temperature after thermal exposure. J. Appl. Bacteriol. 31:414-419.
- Blankenship, L. C. 1981. Some characteristics of acid injury and recovery of *Salmonella bareilly* in a model system. J. Food Prot. 44: 73-77.
- Burr, T. J., and Schroth, M. N. 1977. Occurrence of soft-rot *Erwinia* spp. in soil and plant material. Phytopathology 67:1382-1387.
- Busta, F. F. 1976. Practical implications of injured microorganisms in foods. J. Milk Food Technol. 39:138-145.
- Cuppels, D., and Kelman, A. 1974. Evaluation of selective media for isolation of soft-rot bacteria from soil and plant tissue. Phytopathology 64:468-475.
- De Boer, S. H., Allan E., and Kelman, A. 1979. Survival of *Erwinia carotovora* in Wisconsin soils. Am. Potato J. 56:243-252.
- Delaquis, P. J., Sholberg, P. L., and Stanich, K. 1999. Disinfection of mung bean seed with gaseous acetic acid. J. Food Prot. 62:953-957.
- Dickson, J. S. 1992. Acetic acid action on beef tissue surfaces contaminated with *Salmonella typhimurium*. J. Food Sci. 57:297-301.
- Food and Drug Administration (FDA). 1982. GRAS status of acetic acid, ammonium acetate, sodium acetate, and sodium diacetate. Fed. Regis. 47:27813.
- Hartse, S. E. 1951. The longevity and behavior of pathogenic bacteria in frozen food, the influence of plating media. Am. J. Public Health 41:1072-1075.
- Kado, C. I., and Heskett, M. G. 1970. Selective media for isolation of *Agrobacterium*, *Corynebacterium*, *Erwinia*, *Pseudomonas*, and *Xanthomonas*. Phytopathology 60:969-976.
- Karapinar, M., and Gönül, S. A. 1992. Removal of *Yersinia enterocolitica* from fresh parsley by washing with acetic acid or vinegar. Int. J. Food Microbiol. 16:261-264.
- Liao, C.-H., and Cooke, P. H. 2001. Response to trisodium phosphate treatment of *Salmonella* Chester attached to fresh-cut green pepper slices. Can. J. Microbiol. 47:25-32.
- Liao, C.-H., Hung, H.-Y., and Chatterjee, A. K. 1988. An extracellular pectate lyase is the pathogenicity factor of the soft-rotting bacterium *Pseudomonas viridiflava*. Mol. Plant-Microbe Interact. 1:199-206.
- Liao, C.-H., and Sapers, G. M. 2000. Attachment and growth of *Salmonella* Chester on apple fruits and *in vivo* response of attached bacteria to sanitizer treatments. J. Food Prot. 63:876-883.
- Lund, B. M. 1983. Bacterial spoilage. Pages 219-257 in: Postharvest Pathology of Fruits and Vegetables. C. Dennis, ed. Academic Press, Inc., New York.
- McCarter-Zorner, N. J., Harrison, M. D., Franc, G. D., Quinn, C. E., Ann Sells, I., and Graham, D. C. 1985. Soft-rot *Erwinia* bacteria in the rhizosphere of weeds and crop plants in Colorado, United States and Scotland. J. Appl. Bacteriol. 59:357-368.
- Meneley, J. C., and Stanghellini, M. E. 1976. Isolation of soft-rot *Erwinia* spp. from agricultural soils using an enrichment technique. Phytopathology 66:367-370.
- Miller, R. G. 1981. Simultaneous Statistical Inference, 2nd ed. Springer-Verlag, New York.
- Montville, T. J. 1997. Principles which influence microbial growth, survival, and death in foods. Pages 13-29. in: Food Microbiology-Fundamentals and Frontiers. M. P. Doyle, L. R. Beuchat, and T. J. Montville, eds. ASM Press, Washington, DC.
- Pérombelon, M. C. M., and Kelman, A. 1980. Ecology of the soft rot *erwinias*. Annu. Rev. Phytopathol. 18:361-387.
- Powelson, M. L., and Apple, J. D. 1984. Soil and seed tubers as sources of inoculum of *Erwinia carotovora* subsp. *carotovora* for stem soft rot of potatoes. Phytopathology 74:429-432.
- Przybylski, K. S., and Witter, L. D. 1979. Injury and recovery of *Escherichia coli* after sublethal acidification. Appl. Environ. Microbiol. 37:261-265.
- Ray, B. 1979. Methods to detect stressed microorganisms. J. Food Prot. 42:346-355.
- Sapers, G. M., and Simmons, G. F. 1998. Hydrogen peroxide disinfection of minimally processed fruits and vegetables. Food Technol. 52:48-52.
- Sholberg, P., Haag, P., Hocking, R., and Bedford, K. 2000. The use of vinegar vapor to reduce postharvest decay of harvested fruit. HortScience 30:1271-1275.
- Sorrells, K. M., Speck, M. L., and Warren, J. A. 1970. Pathogenicity of *Salmonella gallinarum* after metabolic injury by freezing. Appl. Microbiol. 19:39-43.
- Tsai, L. S., Huxsoll, C. C., and Robertson, G. 2001. Prevention of potato spoilage during storage by chlorine dioxide. J. Food Sci. 66:472-477.
- Wisniewsky, M. A., Glatz, B. A., Gleason, M. L., and Reitmeier, C. A. 2000. Reduction of *Escherichia coli* O157:H7 counts on whole fresh apples by treatment with sanitizers. J. Food Prot. 63:703-708.
- Wright, J. R., Sumner, S. S., Hackney, C. R., Pierson, M. D., and Zoecklein, B. W. 2000. Reduction of *Escherichia coli* O157:H7 on apples using wash and chemical sanitizer treatments. Dairy Food Environ. Sanit. 20:120-126.