

Salmonella enterica Suppresses *Pectobacterium carotovorum* subsp. *carotovorum* Population and Soft Rot Progression by Acidifying the Microaerophilic Environment

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ABSTRACT Although enteric human pathogens are usually studied in the context of their animal hosts, a significant portion of their life cycle occurs on plants. Plant disease alters the phyllosphere, leading to enhanced growth of human pathogens; however, the impact of human pathogens on phytopathogen biology and plant health is largely unknown. To characterize the interaction between human pathogens and phyto-bacterial pathogens in the phyllosphere, we examined the interactions between *Pectobacterium carotovorum* subsp. *carotovorum* and *Salmonella enterica* or *Escherichia coli* O157:H7 with regard to bacterial populations, soft rot progression, and changes in local pH. The presence of *P. carotovorum* subsp. *carotovorum* enhanced the growth of both *S. enterica* and *E. coli* O157:H7 on leaves. However, in a microaerophilic environment, *S. enterica* reduced *P. carotovorum* subsp. *carotovorum* populations and soft rot progression by moderating local environmental pH. Reduced soft rot was not due to *S. enterica* proteolytic activity. Limitations on *P. carotovorum* subsp. *carotovorum* growth, disease progression, and pH elevation were not observed on leaves coinoculated with *E. coli* O157:H7 or when leaves were coinoculated with *S. enterica* in an aerobic environment. *S. enterica* also severely undermined the relationship between the phyto-bacterial population and disease progression of a *P. carotovorum* subsp. *carotovorum* *budB* mutant defective in the 2,3-butanediol pathway for acid neutralization. Our results show that *S. enterica* and *E. coli* O157:H7 interact differently with the enteric phyto-bacterial pathogen *P. carotovorum* subsp. *carotovorum*. *S. enterica* inhibition of soft rot progression may conceal a rapidly growing human pathogen population. Whereas soft rotted produce can alert consumers to the possibility of food-borne pathogens, healthy-looking produce may entice consumption of contaminated vegetables.

IMPORTANCE *Salmonella enterica* and *Escherichia coli* O157:H7 may use plants to move between animal and human hosts. Their populations are higher on plants cocolonized with the common bacterial soft rot pathogen *Pectobacterium carotovorum* subsp. *carotovorum*, turning edible plants into a risk factor for human disease. We inoculated leaves with *P. carotovorum* subsp. *carotovorum* and *S. enterica* or *E. coli* O157:H7 to study the interactions between these bacteria. While *P. carotovorum* subsp. *carotovorum* enhanced the growth of both *S. enterica* and *E. coli* O157:H7, these human pathogens affected *P. carotovorum* subsp. *carotovorum* fundamentally differently. *S. enterica* reduced *P. carotovorum* subsp. *carotovorum* growth and acidified the environment, leading to less soft rot on leaves; *E. coli* O157:H7 had no such effects. As soft rot signals a food safety risk, the reduction of soft rot symptoms in the presence of *S. enterica* may lead consumers to eat healthy-looking but *S. enterica*-contaminated produce.

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The gastrointestinal tract of animals has traditionally been considered the native ecological niche for enteric human pathogens such as *Salmonella enterica* and *Escherichia coli* O157:H7. However, accumulating evidence shows that both *S. enterica* and Shiga toxin-producing *E. coli* colonize and persist on plants in the environment, an important vehicle to humans (1). Over 1 million cases of food-borne illness linked to the consumption of contaminated fruits and vegetables are estimated to occur annually in the United States (2). Large, produce-linked outbreaks of *S. enterica* and Shiga toxin-producing *E. coli* highlight the inadequacy of our understanding of the biology of these food-borne human pathogens outside their animal hosts (3).

The plant phyllosphere is believed to be inhospitable to many microorganisms, such as enteric human pathogens, due to acute fluctuations in environmental conditions, such as temperature, UV radiation, and water and nutrient availability (4, 5). Interactions with plant microflora may contribute to the success of human pathogens on plants by facilitating nutrient and water acquisition and access to the more sheltered plant interior, e.g., through plant cell wall degradation (6), providing protection against environmental stresses in multispecies biofilms (7) or downregulation of plant defenses (8). Previous studies have shown that the actions of phytopathogens improve the survival of human pathogens on plants (4, 5, 9–11). However, information on the impact of enteric

human pathogens on the biology of phyto-bacterial pathogens *in planta* is extremely limited (4, 5, 11). Understanding both the human pathogen and phyto-bacterial components of this interaction is essential to gain a broad understanding of the ecology of human pathogens on plants.

Pectobacterium carotovorum subsp. *carotovorum* is a ubiquitous plant pathogen that causes bacterial soft rot on nearly every type of fruit and vegetable produced in tropical and temperate regions (12–14). Infection by this bacterial phytopathogen causes water-soaked lesions and rapid maceration of plant tissues. *P. carotovorum* subsp. *carotovorum* pathogenesis is mediated by a large repertoire of cell wall-degrading enzymes (CWDEs) secreted by the type II secretion system. The many pectinases, cellulases, and xylanases secreted allow *P. carotovorum* subsp. *carotovorum* to acquire nutrients from a wide range of hosts and are major virulence factors—gene products that increase the amount of disease—of this pathogen (15–17). Mutation of the type II secretion system inhibits plant tissue maceration and the consequent expansion of the infection by *P. carotovorum* subsp. *carotovorum* (18), illustrating the importance of CWDEs in plant pathogenesis (15, 17). In addition to CWDEs, other *P. carotovorum* subsp. *carotovorum* virulence factors include type III effectors (19), a necrosis-inducing protein (20, 21), and the catabolic α -acetolactate synthase BudB (22). BudB catalyzes the first step in the butanediol (*bud*) pathway for environmental alkalinization. Alkaline pH is required for efficient plant tissue degradation by pectate lyases, the main CWDE for *P. carotovorum* subsp. *carotovorum* soft rot progression (15–17, 22, 23). Failure by a *P. carotovorum* subsp. *carotovorum* *budB* mutant to elevate plant tissue pH reduced soft rot by ~70% on potato tubers (22).

Association of *Salmonella* and bacterial soft rot caused by *P. carotovorum* subsp. *carotovorum* was discovered in market surveys (9). Incidence of *Salmonella* ranges from 1 to 10% on “healthy,” unblemished produce but rises to 18 to 20% in the presence of bacterial soft rot. Thus, edible plants are a highly relevant but largely uninvestigated ecological niche for human pathogens. To better characterize the interaction between enteric human pathogens and soft rot bacteria in this environment, we examined the interactions between *S. enterica* or *E. coli* O157:H7 and *P. carotovorum* subsp. *carotovorum* in postharvest lettuce (*Lactuca sativa*). Bacterial populations, soft rot progression, and changes in local pH were observed for up to 96 h postinoculation (hpi). Results from this study highlight distinct differences between *S. enterica* and *E. coli* O157:H7 in their interactions with phytopathogenic bacteria. This study is also the first to examine the impact of enteric human pathogens on plant pathogen growth, phyto-bacterial pathogen-plant interactions, and plant disease progression.

RESULTS

Enhanced growth of *S. enterica* by *P. carotovorum* subsp. *carotovorum* is concurrent with reduced populations of the phyto-pathogen in a microaerophilic environment. Based on previous studies of the growth of human pathogens in the presence of plant pathogens on plants, we hypothesized that (i) *S. enterica* would attain higher populations in the presence of *P. carotovorum* subsp. *carotovorum* and (ii) *S. enterica* growth would reduce *P. carotovorum* subsp. *carotovorum* proliferation. We conducted our experiments in modified atmosphere packaging (MAP) to reflect conditions found in packaged, bagged lettuce. MAP is characterized by low O_2 and high CO_2 concentrations that are maintained by se-

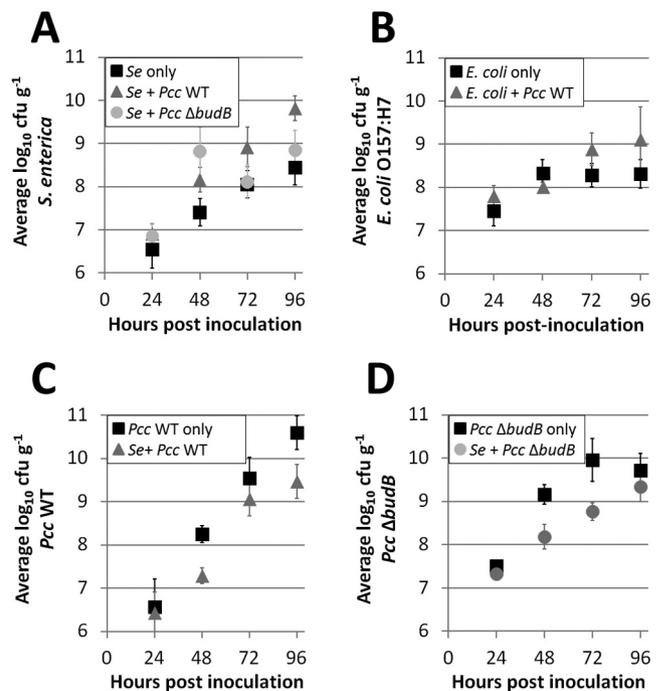


FIG 1 Population dynamics on lettuce in a microaerophilic environment. Growth of *S. enterica* (Se) (A) and *E. coli* O157:H7 (B) was enhanced in the presence of *P. carotovorum* subsp. *carotovorum* (Pcc). In contrast, WT *P. carotovorum* subsp. *carotovorum* (C) and *budB* mutant (D) growth was reduced when lettuce leaves were coinoculated with *S. enterica*. Data shown are the means of nine samples per treatment from a representative experiment. Vertical bars indicate the standard deviations.

lectively permeable polymeric films used for packaging (24). Across five experiments, differences in the mean populations of single- and coinoculated *S. enterica* and *P. carotovorum* subsp. *carotovorum* on lettuce leaves in a microaerophilic environment were seen as early as 24 hpi (Tukey’s honestly significant difference [HSD] test, $n = 135$, $P < 0.0001$) through 96 hpi (Fig. 1A). At 96 hpi, the mean populations of *S. enterica* alone on leaves ranged from 7.58 to 8.45 \log_{10} CFU g^{-1} but reached 8.99 to 9.82 \log_{10} CFU g^{-1} in the presence of wild-type (WT) *P. carotovorum* subsp. *carotovorum*. *E. coli* O157:H7 also attained larger populations when it was coinoculated with WT *P. carotovorum* subsp. *carotovorum* (Fig. 1B) (Tukey’s HSD test, $n = 53$); on average, across all experiments, *E. coli* O157:H7 populations were 1 \log_{10} CFU g^{-1} higher at 96 hpi. Analysis of variance (ANOVA) showed a statistically significant interaction between experiment and treatment for both *S. enterica* populations (degrees of freedom [df] = 8, $F = 3.41$, $P = 1.48e-03$) and *E. coli* populations (df = 4, $F = 5.23$, $P = 1.01e-03$). Growth promotion of enteric human pathogens on plants appears to be a common outcome of their interaction with the soft rot pathogen *P. carotovorum* subsp. *carotovorum*.

We also examined the growth of *S. enterica* coinoculated with a *P. carotovorum* subsp. *carotovorum* *budB* mutant, which is reduced in virulence. Across five experiments, *S. enterica* populations ranged from 7.69 to 9.16 \log_{10} CFU g^{-1} at 96 hpi in the presence of the *P. carotovorum* subsp. *carotovorum* *budB* mutant, an increase of 0.44 to 1.41 \log_{10} CFU g^{-1} over *S. enterica* populations on leaves inoculated with *S. enterica* alone. While significant, this increase was less than the increase seen with *S. enterica* coin-

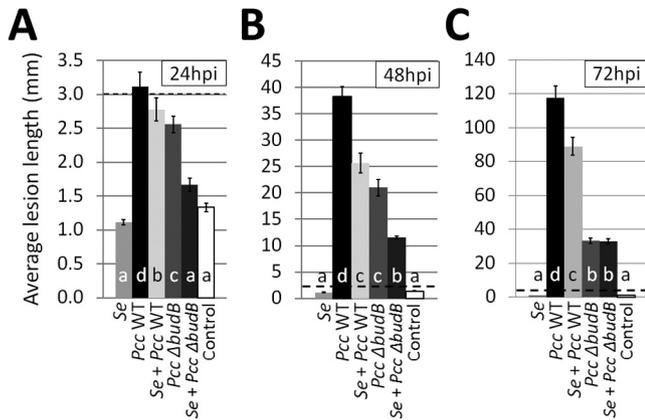


FIG 2 *P. carotovorum* subsp. *carotovorum* virulence was attenuated in the *budB* mutant and in the presence of the human pathogen *S. enterica*. Soft rot lesion length was measured at 24 hpi (A), 48 hpi (B), and 72 hpi (C). Note the difference in scale between graphs. All lesion lengths below 3 mm (black dotted line) were considered to be equal due to variability in puncture wound size. Data shown are the means of nine samples per treatment from a representative experiment. Vertical bars indicate the standard errors.

oculated with WT *P. carotovorum* subsp. *carotovorum* ($P < 0.0001$) at 96 hpi. The increases in population means observed at 96 hpi for *S. enterica* in the presence of either WT *P. carotovorum* subsp. *carotovorum*, the *P. carotovorum* subsp. *carotovorum budB* mutant, or *S. enterica* alone were statistically significant (Tukey’s HSD test, $n = 134$, $P < 0.0001$) (Fig. 1A).

In contrast, *P. carotovorum* subsp. *carotovorum* populations were reduced in the presence of *S. enterica* from 48 to 96 hpi (Fig. 1C and D). The populations of both WT *P. carotovorum* subsp. *carotovorum* and the *P. carotovorum* subsp. *carotovorum budB* mutant on leaves exceeded $9.7 \log_{10}$ CFU g^{-1} at 96 hpi in all five experiments when these bacteria were inoculated alone, and these populations were not statistically different from each other (Tukey’s HSD test, $n = 180$, $P > 0.1$). Across all five experiments, when coinoculated with *S. enterica*, the average populations of WT *P. carotovorum* subsp. *carotovorum* and the *P. carotovorum* subsp. *carotovorum budB* mutant were both reduced by roughly $1 \log_{10}$ CFU g^{-1} at 96 hpi ($P < 0.0001$) (Fig. 1C and D). The means of the two *P. carotovorum* subsp. *carotovorum* strains were not statistically different from each other (Tukey’s HSD test, $n = 180$, $P > 0.05$), indicating that the *budB* mutation has no effect on *P. carotovorum* subsp. *carotovorum* bacterial populations by 96 hpi, regardless of the presence of *S. enterica*. ANOVA showed a statistically significant interaction between experiment and treatment ($df = 12$, $F = 4.24$, $P = 8.69e-06$).

Soft rot disease progression is delayed in the presence of *S. enterica* in a microaerophilic environment. To investigate whether the *P. carotovorum* subsp. *carotovorum* population reduction on leaves coinoculated with *S. enterica* resulted in a decline in soft rot disease, we measured lesion length. Lesions consisting of watery, macerated plant tissue were observed on leaves inoculated with WT *P. carotovorum* subsp. *carotovorum* or the *P. carotovorum* subsp. *carotovorum budB* mutant but not *S. enterica* alone. The average length of lesions produced by WT *P. carotovorum* subsp. *carotovorum* was longer than the average lesion lengths in all other treatments at 24 hpi, 48 hpi, and 72 hpi (Fig. 2A to C). The lengths of lesions were reduced on leaves coinoculated with either *P. carot-*

ovororum subsp. *carotovorum* strain and *S. enterica* and on leaves treated with the *P. carotovorum* subsp. *carotovorum budB* mutant alone. Bacterial soft rot was observed as early as 24 hpi on WT *P. carotovorum* subsp. *carotovorum*-treated leaves, whereas, across five experiments, lesions did not develop until 48 hpi on leaves inoculated with the *P. carotovorum* subsp. *carotovorum budB* mutant or coinoculated with *S. enterica* and either *P. carotovorum* subsp. *carotovorum* strain. Lesion lengths of ≤ 3 mm were considered equal and indistinguishable from the inoculation wound. At 72 hpi, the mean length of lesions observed on leaves inoculated with WT *P. carotovorum* subsp. *carotovorum* was substantially greater than that of leaves coinoculated with WT *P. carotovorum* subsp. *carotovorum* and *S. enterica*, which was significantly greater than the average lesion length on leaves inoculated with the *P. carotovorum* subsp. *carotovorum budB* mutant or coinoculated with the *P. carotovorum* subsp. *carotovorum budB* mutant and *S. enterica* (Tukey’s HSD test, $n = 240$, $P < 0.001$) (Fig. 2C). ANOVA showed a statistically significant interaction between experiment and treatment at 24 h ($df = 20$, $F = 1.65$, $P = 0.044$) and 48 h ($df = 12$, $F = 9.99$, $P = 1.90e-14$) but not at 72 h ($df = 12$, $F = 1.45$, $P = 0.1480$). Lesion lengths are not reported at 96 hpi because entire leaves were typically consumed by soft rot and symptoms caused by *P. carotovorum* subsp. *carotovorum* could no longer be distinguished from those resulting from possible secondary infections.

***S. enterica* suspends the relationship between the *P. carotovorum* subsp. *carotovorum* population and lesion size.** As the population of WT *P. carotovorum* subsp. *carotovorum* on leaves increased, the length of soft rot lesions also increased (Fig. 1C and 2). Surprisingly, a plot of the WT *P. carotovorum* subsp. *carotovorum* population against the length of soft rot lesions on leaves showed only a moderate correlation between the two variables (Fig. 3A). This relationship, described by the slope of the line (β), was weakened by the presence of *S. enterica*. When coinoculated with *S. enterica*, WT *P. carotovorum* subsp. *carotovorum* reached lower population densities and more soft rot bacteria were needed to produce the same size of lesions as in the WT *P. carotovorum* subsp. *carotovorum*-only treatment (Fig. 3B). *S. enterica* also moderated WT *P. carotovorum* subsp. *carotovorum* disease progression such that the slope more closely resembled that of disease progression in leaves singly inoculated with the *P. carotovorum* subsp. *carotovorum budB* mutant. This indicated that *S. enterica* interfered with the ability of *P. carotovorum* subsp. *carotovorum* to rot plant tissues.

***budB* is an important fitness factor for *P. carotovorum* subsp. *carotovorum* in the presence of *S. enterica* at middisease.** Deletion of *budB* did not reduce *P. carotovorum* subsp. *carotovorum* replication on leaves when the bacteria were inoculated alone. However, in the presence of *S. enterica*, the fitness of the *P. carotovorum* subsp. *carotovorum budB* mutant was significantly reduced middisease, as indicated by retarded replication and subsequent soft rot lesion development within 72 hpi. Replication of the *P. carotovorum* subsp. *carotovorum budB* mutant in the first 72 h was severely restricted beyond 2×10^9 CFU g^{-1} compared to WT *P. carotovorum* subsp. *carotovorum* (Fig. 3D). Deletion of the *budB* gene also weakened the relationship, described by the slope of the line (β), between the population of *P. carotovorum* subsp. *carotovorum* and observed lesion length, meaning that a higher *P. carotovorum* subsp. *carotovorum budB* mutant population was necessary to produce the same size of lesions as WT *P. carotovorum*

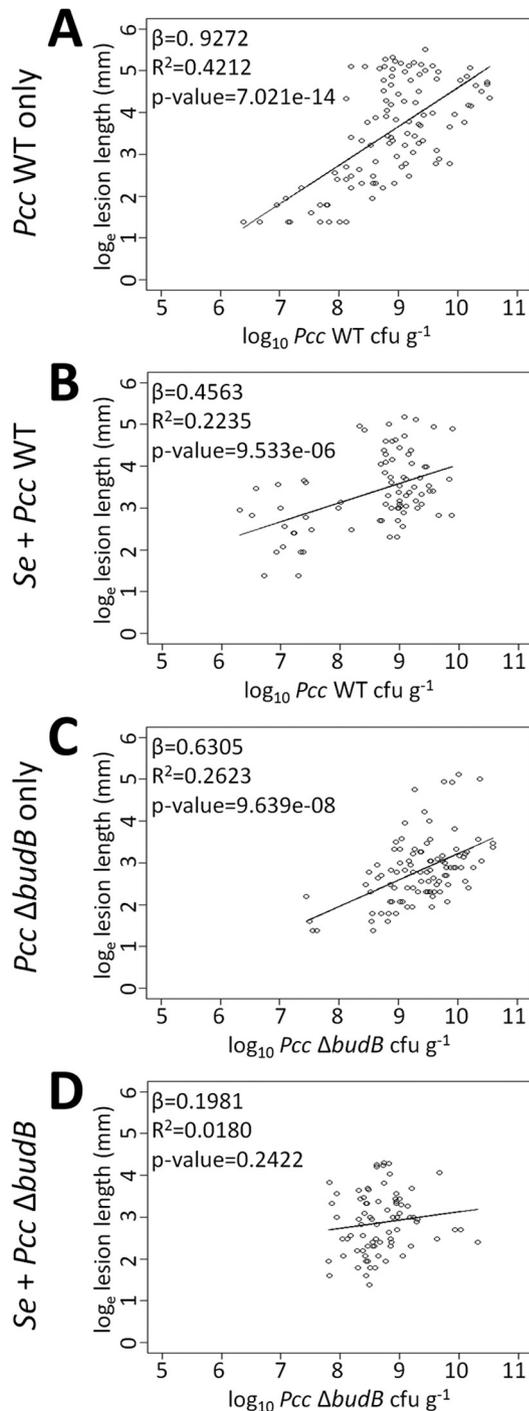


FIG 3 *S. enterica* disrupts the correlation between *P. carotovorum* subsp. *carotovorum* population and lesion size. Populations of WT *P. carotovorum* subsp. *carotovorum* or the *P. carotovorum* subsp. *carotovorum budB* mutant were plotted against the observed soft rot lesion length (\log_e transformed) from 24 hpi to 72 hpi to show the dependency of lesion length on bacterial density. Linear regression lines are shown, with slope (β) and R^2 values indicated. A moderate relationship between the WT *P. carotovorum* subsp. *carotovorum* population and lesion size exists (A); however, the slope of the line changed in the presence of *S. enterica* (B) or deletion of *budB* (C). No relationship between the *P. carotovorum* subsp. *carotovorum budB* mutant population and lesion size can be discerned in the presence of *S. enterica* (D). The P values indicate the probability that the *P. carotovorum* subsp. *carotovorum* population had no effect on lesion length, i.e., the H_0 of $\beta = 0$ is true.

subsp. *carotovorum* (Fig. 3A and C). The *P. carotovorum* subsp. *carotovorum budB* mutant required populations of at least \log_{10} 7.5 CFU g^{-1} for disease development. Regardless of population, the *P. carotovorum* subsp. *carotovorum budB* mutant rarely produced lesions larger than 55 mm (4 \log_e in Fig. 3C). In leaves coinoculated with the *P. carotovorum* subsp. *carotovorum budB* mutant and *S. enterica*, there was little increase in lesion size as the population of the *P. carotovorum* subsp. *carotovorum budB* mutant enlarged and its population was no longer a predictor of lesion size ($P > 0.1$ for the null hypothesis [H_0] of $\beta = 0$ in Fig. 3D). Therefore, the *budB* mutant was compromised in plant colonization by *S. enterica*.

Diminished soft rot symptoms in the presence of *S. enterica* are accompanied by a reduction in pH. In a microaerophilic environment, such as MAP, *S. enterica* uses mixed acid fermentation for energy production, generating organic acids such as acetate, formate, and succinate (25). *P. carotovorum* subsp. *carotovorum* also utilizes acid fermentation in oxygen-limited conditions. Having observed diminished soft rot by the *P. carotovorum* subsp. *carotovorum budB* mutant, which cannot neutralize fermentation acids to elevate local pH, we speculated that *S. enterica* moderates *P. carotovorum* subsp. *carotovorum* soft rot disease progression by acidifying the local pH in leaves, thereby reducing the efficiency of plant tissue maceration by pectate lyases. To test this hypothesis, we determined the pH of leaf tissue surrounding the site of bacterial inoculation. The pH of leaves inoculated with *S. enterica* was significantly lower than the pH of water-inoculated (control) leaves at 96 hpi ($P = 0.04$). In contrast, the pH of leaves inoculated with WT *P. carotovorum* subsp. *carotovorum* steadily increased over 96 h (Fig. 4A to D), concurrent with the observed increase in lesion length (Fig. 2A to C). At 48 hpi, 72 hpi, and 96 hpi, the mean pH of WT *P. carotovorum* subsp. *carotovorum*-inoculated leaves was significantly greater than the pH means of all other treatments. The pH of leaves coinoculated with WT *P. carotovorum* subsp. *carotovorum* and *S. enterica* was similar to that of the *S. enterica*-inoculated leaves from 24 to 72 hpi (Fig. 4A to C) and significantly lower than that of the WT *P. carotovorum* subsp. *carotovorum*-inoculated leaves at all sampling intervals. The pH of the *P. carotovorum* subsp. *carotovorum budB* mutant-treated leaves increased during the first 48 hpi but then declined over the next 48 h to resemble that of the water-inoculated control. Across all five experiments, the pH of leaves coinoculated with the *P. carotovorum* subsp. *carotovorum budB* mutant and *S. enterica* was similar to that of the water control at all sampling intervals (Tukey's HSD test, $n = 240$, $P > 0.05$). ANOVA showed a statistically significant interaction between experiment and treatment at 24 h ($df = 20$, $F = 2.32$, $P = 1.65e-03$), 48 h ($df = 20$, $F = 5.63$, $P = 1.60e-11$), 72 h ($df = 20$, $F = 3.66$, $P = 1.01e-06$), and 96 h ($df = 20$, $F = 3.11$, $P = 2.28e-05$). Taken together, these data suggest that *S. enterica* interferes with the ability of *P. carotovorum* subsp. *carotovorum* to alkalinize plant tissues and thus reduced the activity of pectate lyases, in part by directly acidifying the environment.

***S. enterica* has no effect on the *P. carotovorum* subsp. *carotovorum* population and soft rot disease progression in aerobic conditions.** To test our hypothesis that *S. enterica* interferes with soft rot progression by acidifying the phyllosphere, thereby attenuating alkalinization by *P. carotovorum* subsp. *carotovorum*, we repeated our detached-leaf experiments in aerobic conditions. When oxygen is available, *S. enterica* depends on aerobic pathways

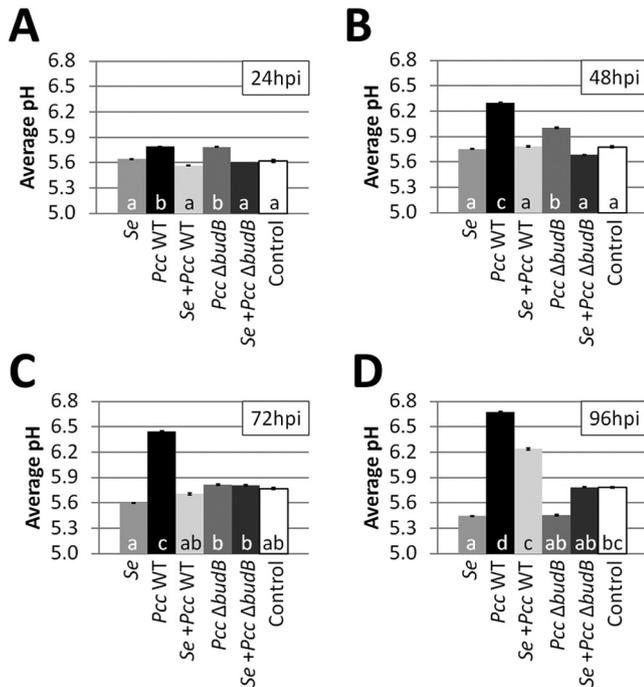


FIG 4 Changes in leaf pH in microaerophilic conditions. The pH of detached leaves inoculated with WT *P. carotovorum* subsp. *carotovorum* or the *P. carotovorum* subsp. *carotovorum budB* mutant and/or *S. enterica* was determined spectrophotometrically every 24 h for 96 h (A to D). The pH of leaf tissue inoculated with WT *P. carotovorum* subsp. *carotovorum* increases toward neutral over time, but this increase was diminished in the presence of *S. enterica*. Though there was a pH increase in the *P. carotovorum* subsp. *carotovorum budB* mutant-inoculated leaves at 24 hpi and 48 hpi (A and B, respectively), the leaf tissue acidified and resembled the water-inoculated control at 72 hpi and 96 hpi (C and D, respectively). At 96 hpi, the pH of leaf tissue inoculated with *S. enterica* was lower than that of the water-inoculated control, presumably due to accumulation of acids produced in anaerobic conditions (D). Data shown are the averages of five experiments. Vertical bars indicate the standard errors.

of energy production, such as through oxidative phosphorylation, and thus does not produce measurable amounts of fermentation acids. In aerobic conditions, *S. enterica* had no effect on the WT *P. carotovorum* subsp. *carotovorum* population, disease progression, or pH (see Fig. S1 in the supplemental material) (24-hpi and 48-hpi data from all three experiments were pooled; Tukey's HSD test, $n_{\text{population}} = 212$ or $n_{\text{lesion and pH}} = 216$, $P > 0.05$). These data support our hypothesis that *S. enterica* restriction of pH elevation plays a significant role in reducing *P. carotovorum* subsp. *carotovorum* soft rot in a microaerophilic environment. The availability of oxygen had no impact on the growth enhancement of *S. enterica* by WT *P. carotovorum* subsp. *carotovorum*. *S. enterica* populations were roughly 1 log₁₀ higher in the presence of WT *P. carotovorum* subsp. *carotovorum*, similar to the enhanced growth of *S. enterica* in the presence of *P. carotovorum* subsp. *carotovorum* that was observed in a microaerophilic environment (Fig. 1A; Fig. S1A).

Reduced soft rot is not due to *S. enterica* proteolytic inactivation of *P. carotovorum* subsp. *carotovorum* CWDEs. Because bacterial soft rot progression is dependent on the activity of *P. carotovorum* subsp. *carotovorum* pectolytic enzymes, we investigated whether the reduction in soft rot in the presence of *S. enterica* was due to proteolytic inactivation of these enzymes by

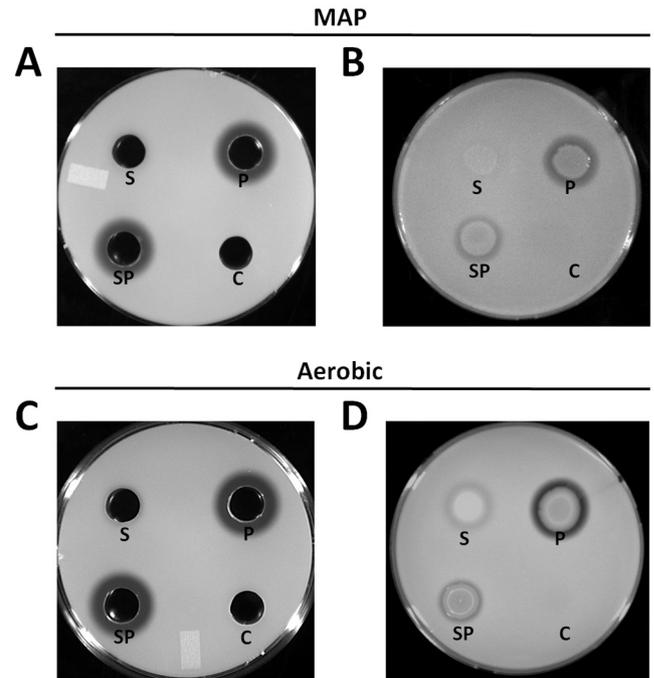


FIG 5 *S. enterica* exhibited no proteolytic activity in microaerophilic conditions (A and B) and minor proteolytic activity in aerobic conditions (C and D). Proteolytic activity of *S. enterica* and WT *P. carotovorum* subsp. *carotovorum* cultured in leaf lysates under microaerophilic (A and B) or aerobic (C and D) conditions. Filtered supernatants from 24-h lettuce lysate cultures of *S. enterica* and WT *P. carotovorum* subsp. *carotovorum* or a coculture of these bacteria were applied to wells in milk agar-lettuce lysate plates to assay cell-free proteolytic activity (A and C). Twenty-four-hour leaf lysate cultures were spotted onto plates to evaluate both cell-associated and cell-free extracellular proteolytic activity (B and D). Proteolytic activity is evident as a zone of clearing around the well or bacterial colony observed after 48 h of incubation at 28°C. Abbreviations: S, *S. enterica*; P, WT *P. carotovorum* subsp. *carotovorum*; SP, coculture of *S. enterica* and WT *P. carotovorum* subsp. *carotovorum*; C, control. Plates are representative replicates from one experiment. There were three replicates per treatment, and the experiment was repeated three times.

S. enterica. Plant cell wall degradation by *P. carotovorum* subsp. *carotovorum* CWDEs leads to lysis of host cells. Leaf lysate is nutritionally analogous to ruptured plant cells that are lysed and have released their contents (26). *S. enterica* had no detectable proteolytic activity in leaf lysate in a microaerophilic environment (Fig. 5A and B). In aerobic conditions, *S. enterica* produced small, faint halos, indicating minor proteolytic activity (Fig. 5C and D), but this did not appear to interfere with soft rot progression (see Fig. S1B in the supplemental material). Under both microaerophilic and aerobic conditions, *P. carotovorum* subsp. *carotovorum* exhibited proteolytic activity (Fig. 5), consistent with reported protease secretion through the type I and II secretion pathways (16, 23). *S. enterica* reduced *P. carotovorum* subsp. *carotovorum* proteolytic activity in aerobic conditions. We posit that the reduction in proteolytic activity was due to a reduction in the *P. carotovorum* subsp. *carotovorum* population grown in leaf lysate (Table S1) which does not occur in the phyllosphere. Due to the reduction in the *P. carotovorum* subsp. *carotovorum* population when cocultured with *S. enterica* in leaf lysate, it would be difficult to distinguish between reduced proteolytic activity due to a reduced *P. carotovorum* subsp. *carotovorum* population and enzymatic inhibition by *S. enterica* following a 1:1 inoculation, as

shown in Fig. 5B and D. We inoculated leaf lysate cocultures with 10× more *P. carotovorum* subsp. *carotovorum* so that at 24 hpi the *P. carotovorum* subsp. *carotovorum* population in the presence of *S. enterica* would be similar to the *P. carotovorum* subsp. *carotovorum* population in the absence of *S. enterica* (Fig. 5A and C). No change in proteolytic activity was observed for the coculture of *P. carotovorum* subsp. *carotovorum* and *S. enterica* grown in leaf lysate, indicating that *S. enterica* does not produce an extracellular protease in the presence of *P. carotovorum* subsp. *carotovorum* and that *S. enterica* does not inhibit *P. carotovorum* subsp. *carotovorum* proteolytic activity. Taken together, our data indicate that the suppression of soft rot by *S. enterica* was not due to proteolytic inactivation of *P. carotovorum* subsp. *carotovorum* CWDEs.

***E. coli* has no effect on the *P. carotovorum* subsp. *carotovorum* population, soft rot disease progression, or pH in a microaerophilic environment.** Having observed that *S. enterica* affects the *P. carotovorum* subsp. *carotovorum* population, disease progression, and local pH modification on leaves in a microaerophilic environment and that *P. carotovorum* subsp. *carotovorum* enhances the growth of *S. enterica*, we asked whether these effects were common outcomes of the interaction between *P. carotovorum* subsp. *carotovorum* and enteric human pathogens. In a microaerophilic environment, coinoculation of leaves with WT *P. carotovorum* subsp. *carotovorum* and *E. coli* O157:H7 enhanced the growth of *E. coli* but had no effect on the WT *P. carotovorum* subsp. *carotovorum* population, disease progression, or pH at 24 hpi, 48 hpi, or 72 hpi (see Fig. S2 in the supplemental material) (data pooled from all three experiments; Tukey's HSD test, $n_{\text{population}} = 53$ or $n_{\text{lesion and pH}} = 90$, $P > 0.05$).

DISCUSSION

In planta growth promotion of enteric human pathogens by the actions of phytopathogenic bacteria in aerobic conditions has been reported (4, 5, 9, 10, 27). In the present study, the *S. enterica* and *E. coli* O157:H7 populations were enhanced 2- to 20-fold and 3- to 15-fold, respectively, at 48 hpi in the presence of WT *P. carotovorum* subsp. *carotovorum* in aerobic conditions, consistent with previous reports of growth with other bacterial soft rot pathogens in aerobic conditions on a variety of plants. *S. enterica* populations in aerobic conditions increased 10-fold on potato, carrot, and pepper disks coinoculated with *Dickeya dadantii* (formerly *Erwinia chrysanthemi*) (9), and *E. coli* O157:H7 populations increased 3.3- to 6.2-fold and 10-fold on lettuce leaves coinoculated with *D. dadantii* (5, 10). The focus in this study was the interaction between human and plant pathogens in a microaerophilic environment in which we found that the average population of *S. enterica* was 6- to 37-fold higher and the average population of *E. coli* O157:H7 was 4- to 85-fold higher at 96 hpi in the presence of WT *P. carotovorum* subsp. *carotovorum*.

Information on the impact of common food-borne pathogens, such as *S. enterica* and Shiga toxin-producing *E. coli*, on plant pathogen growth and virulence is limited to brief mentions in three reports by Aruscavage et al. (4), Noel et al. (11), and Yamazaki et al. (5). Our study is the first to focus on the effect human pathogens have on plant pathogens. We showed that *S. enterica* limits *P. carotovorum* subsp. *carotovorum* population growth and bacterial soft rot development on leaves in a microaerophilic environment and that this was correlated with diminished environmental pH elevation by *P. carotovorum* subsp. *carotovorum*. The reduction in WT *P. carotovorum* subsp. *carotovorum* populations

corresponded to a reduction in the length of soft rot lesions (Fig. 2). If the reduction in the WT *P. carotovorum* subsp. *carotovorum* population and soft rot disease progression was solely the result of competition, similarly sized lesions would have been expected to contain similar populations of WT *P. carotovorum* subsp. *carotovorum* regardless of the presence or absence of *S. enterica*. The reduction in disease progression observed in the presence of *S. enterica* would have resulted only from limitation of WT *P. carotovorum* subsp. *carotovorum* growth, and there would be no effect on the relationship between the WT *P. carotovorum* subsp. *carotovorum* population and soft rot lesion length. Instead, our data revealed that higher populations of WT *P. carotovorum* subsp. *carotovorum* were necessary to produce the same sizes of lesions in the presence of *S. enterica* (Fig. 3A and B), indicating that *S. enterica* likely utilizes strategies other than competition to reduce bacterial soft rot.

pH elevation is especially important in promoting the multiplication of enteric human pathogens in soft rot lesions. Compared to coinoculation with WT *P. carotovorum* subsp. *carotovorum*, a weaker (2- to 18-fold) population increase for *S. enterica* was observed at 96 hpi when *S. enterica* was coinoculated with a *P. carotovorum* subsp. *carotovorum* *budB* mutant defective in environmental pH neutralization, indicating that pH elevation is important for *S. enterica* growth in this niche.

The ability to alkalinize the pH of the plant host, e.g., through the *bud* pathway, is integral to soft rot progression. Pectate lyases, the major enzymes responsible for plant tissue maceration, have pH optima between 8.0 and 8.5 (15, 17, 23). The plant apoplastic pH is typically 5.5 to 6.0, at which the activities of pectate lyases are low (17, 23). The activity of the pectate lyase PelC is 100-fold less at pH 6 than at the pH optimum of 8.5 in *D. dadantii* (28). Accordingly, a *P. carotovorum* subsp. *carotovorum* *budB* mutant was observed to be reduced in virulence, i.e., it produced less macerated tissue, in leaves (this study) and potato tubers (22). Mutation of *budB* in the related phytopathogen *D. dadantii* also reduced its virulence on potato and chicory (29)—confirming the role of *budB* as a soft rot virulence factor. In this study, the presence of *S. enterica* exacerbated the virulence defect of the *P. carotovorum* subsp. *carotovorum* *budB* mutant. Without the *bud* pathway and in the presence of *S. enterica*, soft rot lesion expansion was restricted and more cells were necessary to form lesions. Additionally, when coinoculated with *S. enterica*, replication of the *P. carotovorum* subsp. *carotovorum* *budB* mutant slowed during mid-disease development, indicating that *budB* is also a fitness factor for *P. carotovorum* subsp. *carotovorum*.

Bacterial soft rot progression is dependent on the activity of *P. carotovorum* subsp. *carotovorum* pectinolytic enzymes whose efficiencies peak at alkaline pH. When butanediol production is eliminated in *P. carotovorum* subsp. *carotovorum* or *D. dadantii* *budB* pathway mutants, infected plant tissues are more acidic and disease is reduced compared with those inoculated with the WT (22, 29). The importance of pH in the progression of soft rot caused by *P. carotovorum* subsp. *carotovorum* was also reported by Ni et al. (30), who observed a 50 to 70% reduction in calla lily tuber rot at pH 6.3 compared with that at pH 7.0 and 7.3. We observed a reduction in soft rot lesion size on leaves inoculated with the *P. carotovorum* subsp. *carotovorum* *budB* mutant or coinoculated with WT *P. carotovorum* subsp. *carotovorum* or the *P. carotovorum* subsp. *carotovorum* *budB* mutant and *S. enterica*, a decrease which was accompanied by a reduction in the local pH relative to samples inoculated with

TABLE 1 List of bacterial strains^a

Strain/serovar	Relevant characteristics	Reference(s)
<i>E. coli</i>		
O157:H7 strain 96A	Nal ^r , spontaneous nalidixic acid derivative of outbreak strain clinically isolated from a patient who had consumed unpasteurized apple cider, 1996	43, this work
13466 Nal ^r		
<i>P. carotovorum</i> subsp. <i>carotovorum</i>		
WPP359 (WT)	Chl ^r , <i>gfp::chl</i> -labeled derivative of wild-type strain WPP14, isolated from an infected potato stem in Wisconsin, 2001	18
Δ <i>budB</i>	Kan ^r , Δ <i>budB::kan</i> derivative of wild-type strain WPP14	22
<i>S. enterica</i>		
Saintpaul	Outbreak strain isolated from contaminated peppers from Mexico, 2008	39

^a Nal^r, Chl^r, and Kan^r indicate resistance to nalidixic acid, chloramphenicol, and kanamycin, respectively.

WT *P. carotovorum* subsp. *carotovorum* only. The pH of leaves inoculated with *S. enterica* only was lower than that of the negative control. In contrast, under aerobic conditions in which *S. enterica* does not produce a measurable amount of fermentation acids, the pH of leaves inoculated with *S. enterica* was similar to that of the negative control. Furthermore, coinoculation of leaves with *S. enterica* had no effect on the pH or disease progression by WT *P. carotovorum* subsp. *carotovorum* under aerobic conditions. We interpret these results to mean that *S. enterica* acidifies the phyllosphere in microaerophilic conditions due to production of organic acids during fermentation, thereby reducing the efficiency of plant tissue degradation by pectate lyases. Since soft rot is mediated by CWDEs, we verified that the antagonistic effect of *S. enterica* on *P. carotovorum* subsp. *carotovorum* is not due to proteolytic inactivation of these enzymes. No *S. enterica* proteolytic activity was observed in the microaerophilic environment, and the small amounts of proteolytic activity in aerobic conditions were not accompanied by an effect on *P. carotovorum* subsp. *carotovorum* populations or disease progression. We conclude that the mechanism by which *S. enterica* suppresses *P. carotovorum* subsp. *carotovorum* bacterial soft rot is through attenuation of *P. carotovorum* subsp. *carotovorum* pH environmental alkalization.

In contrast to our results with *S. enterica*, coinoculation with *E. coli* O157:H7 had no effect on the population or soft rot disease progression in a microaerophilic environment. Previous studies by Aruscavage et al. (4) and Yamazaki et al. (5) also showed that coinoculation of leaves with *E. coli* O157:H7 had no effect on the populations of *Xanthomonas campestris* pv. *vitians* and *D. dadantii*, respectively. We found that *E. coli* O157:H7 by itself failed to acidify leaf tissue, unlike *S. enterica*. We conclude that the null effect of *E. coli* O157:H7 on *P. carotovorum* subsp. *carotovorum* is due to the inability of *E. coli* O157:H7 to acidify plant tissue. Without environmental acidification, *P. carotovorum* subsp. *carotovorum* pectate lyase efficiency is not affected and, thus, soft rot development proceeds similarly to that with *P. carotovorum* subsp. *carotovorum* alone. Our observations also indicate that the *E. coli* O157:H7-*P. carotovorum* subsp. *carotovorum* interaction is fundamentally different from the *S. enterica* interaction. Although both *E. coli* and *S. enterica* use mixed acid fermentation in anaerobic conditions, the pH of tissue inoculated with *E. coli* O157:H7 was similar to that of the water control, whereas *S. enterica* acidified the leaves. If the substrates are available in anaerobic conditions, *E. coli* will use anaerobic respiration with nitrate or fumarate as an electron receptor instead of fermentation (31–33). Lettuce leaves have previously been shown to contain significant levels of nitrate (34). It is likely that the nutrients, e.g., nitrate, made available during leaf maceration by *P. carotovorum* subsp. *carotovorum*, lead to *E. coli*

forgoing fermentation for the more energetically favorable anaerobic respiration, which will lead to lower acid production.

We strived to replicate natural conditions in our experimental design. Plant tissue damage resulting from our inoculation technique mimics the mechanical damage associated with handling during and after harvest. Compared to previous studies, our low inocula of *P. carotovorum* subsp. *carotovorum* and *S. enterica* may better reflect the natural populations of these bacteria encountered on leaves in the field or postharvest. The microaerophilic conditions achieved in this study (<1% O₂, >13% CO₂) are similar to conditions found in MAP (0.5 to 3% O₂, 10 to 15% CO₂) for produce (35, 36). Processed fruits and vegetables are frequently placed in MAP to extend shelf life by reducing the rate of plant respiration and ethylene production (senescence) and the growth of microorganisms responsible for spoilage (24). However, MAP has no effect on replication or survival of enteric human pathogens on raw fruits and vegetables (37). Bagged lettuce, packaged in MAP, is the fastest growing segment of lettuce consumption (38). To our knowledge, this is the first study examining the interactions between food-borne enteric human pathogens and bacterial phytopathogens on fresh produce in MAP. By conducting our experiments at 24°C, we illustrate the bacterial interactions that may occur on poorly handled packaged produce.

Soft rot serves as an important cue deterring human consumption of fruits and vegetables mishandled during harvest, during processing, along the supply chain, or by the consumer. While produce may be contaminated by *Salmonella* in the absence of plant pathogens, the presence of phytopathogens doubled the incidence of *Salmonella* on soft rotted produce compared with that on “healthy” (asymptomatic) samples (9) and promoted the growth of *S. enterica* beyond levels needed to cause human disease (this work). Thus, soft rot signals an increased food safety risk to consumers. The reduction in soft rot symptoms in the presence of *S. enterica* is of concern to human health because *S. enterica* obscures the effects of mishandling or spoilage. Produce perceived to be healthy or with little rot may still contain infectious doses of *Salmonella*, and its consumption may lead to human disease. Understanding the interactions between bacterial plant and human pathogens on plants and the factors affecting proliferation and persistence will aid strategies for maintaining food safety.

MATERIALS AND METHODS

Bacterial strains and culture conditions. The bacterial strains used in this study are listed in Table 1. *S. enterica* sv. Saintpaul XbaI pattern JN6X01.0048 caused the largest salmonellosis outbreak in the United States, involving jalapeño and Serrano peppers and possibly tomatoes

(39). Other recent outbreaks of salmonellosis caused by *S. enterica* sv. Saintpaul followed consumption of contaminated alfalfa sprouts (2003, 2008, and 2009) and cantaloupe (2006) (40). Strains were cultured on Luria-Bertani (LB) agar medium at 37°C. When necessary, antibiotics were added at the following concentrations: chloramphenicol (Chl), 50 $\mu\text{g ml}^{-1}$; kanamycin (Kan), 50 $\mu\text{g ml}^{-1}$; nalidixic acid (Nal), 30 $\mu\text{g ml}^{-1}$. *Salmonella-Shigella* (SS) agar, a differential selective medium for *Salmonella*, was used to determine *S. enterica* populations. *E. coli* O157:H7 populations were determined on sorbitol-MacConkey (SMAC) agar, a differential selective medium for pathogenic *E. coli* O157:H7.

Leaf inoculation. Heads of romaine lettuce were purchased from a local grocery store in Madison, WI, and used within 48 h of purchase. Overnight bacterial cultures plated on LB were suspended in sterile deionized water and adjusted to an optical density at 600 nm (OD_{600}) of 0.200 ± 0.005 . The ten outermost leaves of each head of lettuce were detached and stab-inoculated at the midrib with 1 μl of a 10^{-2} dilution of each bacterial suspension ($\sim 10^3$ CFU) using a 10- μl pipette tip. The pipette tip was positioned perpendicular to the leaf surface, and a 1- to 3-mm puncture was made at the midrib halfway along the length of the leaf. Leaves were inoculated with *S. enterica* sv. Saintpaul, *E. coli* O157:H7, WT *P. carotovorum* subsp. *carotovorum*, or a *P. carotovorum* subsp. *carotovorum* *budB* mutant. Alternatively, leaves were coinoculated with 10^3 CFU each of *S. enterica* or *E. coli* O157:H7 and WT *P. carotovorum* subsp. *carotovorum* or the *P. carotovorum* subsp. *carotovorum* *budB* mutant. Control leaves were mock-inoculated with sterile water. Leaves were incubated at 24°C in GasPak 100 system jars containing GasPak EZ anaerobe container system sachets. According to the manufacturer, anaerobic conditions ($\leq 1\%$ oxygen) are achieved within 2.5 h at 35°C, and within 24 h, the carbon dioxide concentration is $\geq 13\%$. These conditions are similar to those reported for lettuce in MAP (41). There were a total of 36 replicates per treatment except in the negative-control group, which contained 12 replicates. The experiment was repeated at least three times.

Alternatively, leaves inoculated with 10^6 CFU of *S. enterica* or *P. carotovorum* subsp. *carotovorum* or coinoculated with 10^6 CFU each of *S. enterica* and *P. carotovorum* subsp. *carotovorum* were incubated aerobically in partially unsealed plastic gallon-size zip-top bags at 24°C. A higher inoculum of *P. carotovorum* subsp. *carotovorum* was necessary to achieve soft rot disease on 100% of samples because *P. carotovorum* subsp. *carotovorum* is less virulent in aerobic conditions (18). Additionally, the plant host is more resistant to phytopathogen infection in aerobic conditions because anaerobiosis impairs plant defenses, such as cell wall lignification, callose deposition, and oxygen-dependent production of phytoalexins, phenolics, and free radicals (18). There were a total of 18 replicates per treatment except in the negative-control group, which contained 6 replicates. The experiment was repeated three times.

Measurement of lesion development and bacterial populations on leaves. Nine bacterium-inoculated and three control leaves were removed and sampled every 24 h up to 96 h. The length of soft rot lesions (i.e., nonintact tissue) was measured using a standard metric ruler. All lesion lengths smaller than 3 mm were regarded as equal because punctures made in the midrib during inoculation ranged from 1 to 3 mm. For lesions of < 3 mm, we were unable to macroscopically differentiate mechanical injury during inoculation and soft rot. Leaf tissue surrounding the point of inoculation ($r = 5$ mm) was removed using a flame-sterilized core borer and manually macerated in a sterile 1.7-ml microcentrifuge tube. Sterile deionized water equivalent to four times (wt/vol) the tissue sample was added to each tube. This suspension was serially diluted and plated onto selective or differential medium for isolation of the inoculated strains. SS and LB+Kan agar plates were incubated overnight at 33°C. SMAC+Nal plates were kept at 42°C overnight. LB+Chl agar plates were maintained at 37°C for 28 h.

Spectrophotometric determination of pH. The pH of each leaf sample was determined spectrophotometrically using a NanoDrop ND-1000 spectrophotometer, based on the method of Braun (42). The absorption maxima of the pH indicator dye bromocresol purple (BCP) (0.1% wt/vol)

were determined to be 430 nm (yellow) and 588 nm (purple). BCP was selected because preliminary data (not shown) indicated that the pH values in the color change interval for BCP (pH 5.2 to 6.8) are similar to the range of pH values expected in the experiment. Four microliters of the described leaf suspension (see above) was combined with 1 μl BCP, and the absorption of this solution was read at 430 nm and 588 nm. The pHs of eight buffered solutions (pH 4.8 to 9.0) were determined both spectrophotometrically and using an Accumet Research AR15 pH/mV/°C meter. These values were fitted using a linear model described by the equation $y = 0.96584x - 0.24483$, where x is the value given by the pH meter and y is the spectrophotometrically calculated pH. The pH values reported in this study reflect the spectrophotometrically determined pH values, adjusted for error using this equation.

Proteolytic activity assay. Lettuce lysate was prepared by crushing the leaves of entire heads of romaine lettuce, collecting the homogenate in 50-ml conical tubes, centrifuging twice at $4,000 \times g$ for 30 min with discard of plant debris in between, and sequentially filtering the supernatant through 0.8- μm and 0.2- μm filters. Fresh leaf lysate was prepared each day.

Bacterial suspensions ($\text{OD}_{600} = 0.5$) of *S. enterica* and *P. carotovorum* subsp. *carotovorum* were prepared from overnight streak cultures and serially diluted 10^{-4} . Lettuce lysate (1 ml) was inoculated with 100 μl of the diluted suspensions or coinoculated with 100 μl of each of the diluted suspensions (1:1 or 1:10 *S. enterica*/*P. carotovorum* subsp. *carotovorum* ratio), and cultures were incubated either aerobically or anaerobically in GasPak jars for 24 h at 28°C with shaking at 200 rpm. Inoculation with sterile water served as the negative control. There were three replicates per treatment.

Bacterial proteolytic activity was assessed on 3% skim milk agar plates containing 10% lettuce lysate. Lettuce lysate was added to cooled autoclaved milk agar, and 25 ml of medium was dispensed into each petri plate. Plates were prepared on the day of inoculation. Ten microliters of each of the lettuce lysate cultures was spotted onto milk agar plates and dried in the hood. Additionally, lettuce lysate cultures were centrifuged at $13,000 \times g$ for 2 min. The supernatant was filtered through a 0.2- μm filter, and 150 μl of the filtrate was applied to wells in milk agar-lettuce lysate plates. Wells were formed by removal of agar plugs using a flame-sterilized core borer ($r = 6$ mm). Plates were incubated either aerobically or anaerobically in GasPak jars, identical to their respective lettuce lysate cultures, at 28°C for 48 h, after which the zones of clearing were measured. Bacterial cultures were also serially diluted and plated onto SS agar or LB+Chl and incubated at 37°C for at least 24 h to enumerate bacterial populations. The experiment was repeated three times.

Statistical analyses. Statistical analyses were performed using R software (version 2.11.1; R Development Core Team, R Foundation for Statistical Computing, Vienna, Austria; <http://www.R-project.org>). Data from all replicated experiments for a particular parameter (i.e., population, disease progression, pH) were pooled, and differences between the means of the various treatments were analyzed using Tukey's HSD test (based on a two-way ANOVA), with significance set at a P value of < 0.05 . Separate Tukey's HSD tests were conducted for *S. enterica*, *E. coli* 157: H7, and *P. carotovorum* subsp. *carotovorum* populations in MAP. Single Tukey's HSD tests were completed at each time point for disease progression and pH in MAP. Data collected at 24 hpi and 48 hpi in aerobic conditions were combined, and each parameter (population, disease progression, pH) was analyzed using a single Tukey's HSD test. Interactions between treatment and experiment were analyzed using a two-way ANOVA.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00557-12/-DCSupplemental>.

Figure S1, TIF file, 0.1 MB.

Figure S2, TIF file, 0.1 MB.

Table S1, PDF file, 0.2 MB.

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