In Situ Evaluation of *Paenibacillus alvei* in Reducing Carriage of *Salmonella enterica* Serovar Newport on Whole Tomato Plants

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Recently, tomatoes have been implicated as a primary vehicle in food-borne outbreaks of *Salmonella enterica* serovar Newport and other *Salmonella* serovars. Long-term intervention measures to reduce *Salmonella* prevalence on tomatoes remain elusive for growing and postharvest environments. A naturally occurring bacterium identified by 16S rRNA gene sequencing as *Paenibacillus alvei* was isolated epiphytically from plants native to the Virginia Eastern Shore tomato-growing region. After initial antimicrobial activity screening against *Salmonella* and 10 other bacterial pathogens associated with the human food supply, strain TS-15 was further used to challenge an attenuated strain of *S. Newport* on inoculated fruits, leaves, and blossoms of tomato plants in an insect-screened high tunnel with a split-plot design. Survival of *Salmonella* after inoculation was measured for groups with and those without the antagonist at days 0, 1, 2, and 3 and either day 5 for blossoms or day 6 for fruits and leaves. Strain TS-15 exhibited broad-range antimicrobial activity against both major food-borne pathogens and major bacterial phytopathogens of tomato. After *P. alvei* strain TS-15 was applied onto the fruits, leaves, and blossoms of tomato plants, the concentration of *S. Newport* declined significantly ($P \leq 0.05$) compared with controls. Astonishingly, >90% of the plants had no detectable levels of *Salmonella* by day 5 for blossoms. The naturally occurring antagonist strain TS-15 is highly effective in reducing the carriage of *Salmonella* Newport on whole tomato plants. The application of *P. alvei* strain TS-15 is a promising approach for reducing the risk of *Salmonella* contamination during tomato production.

The United States is one of the world's leading producers of tomatoes. Fresh and processed tomatoes account for more than \$2 billion in annual farm cash receipts (<http://www.ers.usda.gov/topics/crops/vegetables-pulses/tomatoes.aspx>). U.S. fresh field-grown tomato production has consistently increased over the past several decades. Concurrently, an increasing number of outbreaks caused by various serovars of *Salmonella enterica* have been associated with the consumption of fresh and fresh-cut tomatoes (1).

Contamination of produce can occur during field production or in the postharvest processing facility. Once contamination occurs, *S. enterica* serovars are able to survive on and in the tomato fruit despite the tomato's acidic interior (2–4). While a wide range of chemical sanitizers and physical treatments have been investigated for killing *Salmonella* on tomatoes postharvest, with various degrees of success (5–7), there is currently no “kill step” in processing that would eliminate *Salmonella* from contaminated tomatoes. At preharvest, there are no cultivars with resistance to other important diseases caused by plant pathogens that are also resistant to colonization by food-borne pathogens such as *Salmonella* (8). Following good agricultural practices (GAPs) (9) is the only available control right now to reduce the risk of tomatoes becoming contaminated with *Salmonella* in the field, indicating that additional interventions, such as biological control, are needed.

Biological control of plant diseases using microorganisms or their metabolites (10–12) offers a safe and effective alternative to the use of synthetic agrichemicals. The aim of this study was to isolate potential bacterial antagonists against *Salmonella*, to examine their modes of action, and to test their effectiveness in reducing carriage of *Salmonella* on whole tomato plants in a high-tunnel setting.

MATERIALS AND METHODS

Isolation and screening of antagonistic bacteria. The native microflora of various plant organs (including leaves, shoots, roots, and blossoms) and soil from various Eastern Shore tomato-growing locations were examined. Simply, 3 g of plant material or soil was mixed for 5 min in 1 ml of phosphate-buffered saline (PBS). An aliquot (100 μ l) was plated onto nutrient yeast glucose agar (NYGA). Ten colonies with unique morphologies that developed within 48 h at 30°C under aerobic conditions were picked for further purification, and a 3% KOH test was done to differentiate the Gram status without staining (13). The pure cultures were then tested for antagonistic activity *in vitro* by using an agar plug method (14). Briefly, pour plates of each test organism were prepared by mixing a 4-ml suspension of a plate culture grown overnight with sterile water in ca. 20 ml of warm tryptic soy agar (TSA). After incubation overnight at 35°C, agar plugs were punched from the agar with a sterile 10-mm stainless steel borer. Plugs were placed onto TSA agar containing a lawn of 10^6 cells of *S. enterica* serovar Newport (15) and incubated at 35°C. Clear zones surrounding the plugs were measured at incubation periods of 24, 48, and 96 h.

Bacterial cultures. Isolates of potential bacterial antagonists and indicator strains (Table 1) were propagated on TSA at 35°C. Stock cultures grown overnight at 35°C on TSA were then resuspended in brain heart infusion (BHI) broth with 25% glycerol and stored at -80°C . Three tomato plant-associated bacterial pathogens, *Erwinia carotovora* subsp.

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TABLE 1 Strains used in the present study

Strain	Source ^a
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Newport 17	CFSAN laboratory collection
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Saintpaul	CFSAN laboratory collection
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Montevideo 42N	CFSAN laboratory collection
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Javiana	CFSAN laboratory collection
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhimurium 368477	CFSAN laboratory collection
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhimurium SAR C 1	SGSC
<i>Salmonella enterica</i> subsp. <i>enterica</i> serovar Typhi SAR C 3	SGSC
<i>Salmonella enterica</i> subsp. <i>arizonae</i> SAR C 5	SGSC
<i>Salmonella enterica</i> subsp. <i>arizonae</i> SAR C 7	SGSC
<i>Salmonella enterica</i> subsp. <i>arizonae</i> SAR C 9	SGSC
<i>Salmonella bongori</i> SAR C 11	SGSC
<i>Salmonella bongori</i> SAR C 13	SGSC
<i>Salmonella bongori</i> SAR C 15	SGSC
<i>Escherichia coli</i> O157:H7 IS O57	CFSAN laboratory collection
<i>Escherichia coli</i> O157:H7 EDL933	CFSAN laboratory collection
<i>Escherichia coli</i> ATCC 51434	ATCC
<i>Escherichia coli</i> ATCC BAA-179	ATCC
<i>Shigella dysenteriae</i> 2457T	CFSAN laboratory collection
<i>Shigella dysenteriae</i> BS103	CFSAN laboratory collection
<i>Cronobacter sakazakii</i> E932	CFSAN laboratory collection
<i>Cronobacter sakazakii</i> E784	CFSAN laboratory collection
<i>Listeria monocytogenes</i> N1-225	CFSAN laboratory collection
<i>Listeria monocytogenes</i> R2-583	CFSAN laboratory collection
Methicillin-resistant <i>Staphylococcus aureus</i> 9	CFSAN laboratory collection
Methicillin-resistant <i>Staphylococcus aureus</i> 12	CFSAN laboratory collection
Methicillin-resistant <i>Staphylococcus aureus</i> 28	CFSAN laboratory collection
Methicillin-resistant <i>Staphylococcus aureus</i> 29	CFSAN laboratory collection
Methicillin-resistant <i>Staphylococcus aureus</i> 30	CFSAN laboratory collection
<i>Staphylococcus aureus</i> NRS70	NARSA
<i>Staphylococcus aureus</i> NRS106	NARSA
<i>Staphylococcus aureus</i> NRS107	NARSA
<i>Staphylococcus aureus</i> NRS271	NARSA
<i>Salmonella enterica</i> serovar Newport 17 Δ tolC::aph	CFSAN laboratory collection
<i>Erwinia carotovora</i> subsp. <i>carotovora</i>	Dilip Lakshman, ARS
<i>Pseudomonas syringae</i> pv. tomato strain dc3000	Dilip Lakshman, ARS
<i>Ralstonia solanacearum</i> race 5	Dilip Lakshman, ARS

^a SGSC, Salmonella Genetic Stock Centre, University of Calgary, Canada; ATCC, American Type Culture Collection, Manassas, VA, USA; NARSA, Network on Antimicrobial Resistance in *Staphylococcus aureus*, Chantilly, VA, USA; ARS, Agricultural Research Service, Department of Agriculture, Beltsville, MD, USA.

carotovora, *Pseudomonas syringae* pv. tomato strain dc3000, and *Ralstonia solanacearum* race 5, were grown on TSA at 25°C (Table 1).

Phenotypic and biochemical characterizations of potential bacterial antagonists. The morphological characteristics of potential bacterial antagonists were observed by Gram and spore stains. These isolates were further tested with the Vitek 2 compact biochemical identification system (bioMérieux, Inc., Durham, NC) and the Biolog Omnilog microbial identification system (Biolog, Hayward, CA) with Gen III MicroPlates for biochemical properties, according to the manufacturers' instructions.

16S rRNA gene amplification and sequencing. Genomic DNA of potential bacterial antagonists was extracted by using the Wizard genomic DNA purification kit (Promega, Madison, WI). A pair of universal primers specific for bacterial 16S rRNA, Eubac27 and R1492 (16), were used to amplify the corresponding gene. PCR amplification of the 16S rRNA gene was performed with a Hotstart Taq Plus DNA polymerase kit (Qiagen, Valencia, CA) under the following conditions: after an initial 5-min incubation at 95°C, the mixture was subjected to 30 cycles, each including 1 min at 95°C, 1 min at 58°C, and 1 min at 72°C. A final extension step was performed at 72°C for 10 min. Primers 4F, 27F, 357F, 578F, 1000R, and 1492R were used for sequencing (16). The BLAST algorithm was used for a homology search against GenBank. Only results from the highest-score queries were considered for phylogeny identification, with 99% minimum similarity (17).

Determination of mode of action and spectrum of antimicrobial activities. To determine the mode of action and antimicrobial spectrum of the bacterial antagonists, both an agar plug assay (using bacterial cultures) and a Bioscreen assay (using culture supernatants) were performed

against a broad spectrum of major food-borne pathogens and bacterial phytopathogens (Table 1). In the agar plug assay, bactericidal effects against pathogenic bacterial strains in the zone of inhibition were confirmed when no viable cells were recovered on TSA plates. In the Bioscreen assay, the antagonist supernatant from a culture grown overnight was filter sterilized with a 0.22- μ m-pore-size cellulose acetate (CA) membrane filter. Each 3-ml TS-15 cell-free culture supernatant (CFCs) was inoculated with 3 μ l of 10⁸ CFU/ml bacterial culture (Table 1). Aliquots (200 μ l) were then dispensed into sterile Bioscreen C microwell plates (Growth Curves USA, Piscataway, NJ) and incubated as described above for the respective bacterial strains. Bacterial growth was determined in five replicates by measuring the optical density at 600 nm (OD₆₀₀) at 20-min intervals for 24 h.

Tomato fruit assay. Red, round, ripe tomato fruits (130 $\pm$ 20 g each) were purchased from a local supermarket and refrigerated for no more than 3 days. Tomatoes were equilibrated to room temperature (RT) before testing and washed with 75% ethanol for surface sterilization and to remove any waxy residue if present. After air drying in a laminar flow hood, tomatoes were aseptically placed onto sterile metal trays with the stem scars facing down. A 20- μ l drop (18) of a suspension of an *S. Newport* culture grown overnight (washed twice with PBS and resuspended in 5 ml of PBS) was placed within a 3-cm-diameter circle on the side of the tomato, equidistant from both ends of the tomato. The *Salmonella* inoculum was allowed to dry before antagonist inoculation. A 40- μ l drop of the antagonist culture suspension (washed twice with PBS and resuspended in 5 ml of fresh tryptic soy broth [TSB]) or 40 μ l of TSB only was then placed on top of the *Salmonella* inoculum. After 1.5 h in the hood, completely air-dried samples were placed into a humidity chamber (i.e., a closed container filled with 1.5 liters of water in a 30°C incubator). After 24 h of incubation, each tomato was placed into a sterile Whirl-Pak filter bag containing 30 ml of PBS and hand rubbed for 5 min to dislodge surface-inoculated *Salmonella*. The wash suspension was diluted 10-fold in PBS, and 0.1-ml aliquots of the appropriate dilutions were spread onto XLD agar (Becton, Dickinson and Company, Sparks, MD) to determine the surviving *Salmonella* populations.

Field trials in a high tunnel. (i) **Plants.** Trials were performed in 2010 (July through September) on tomato cultivar BHN602 in an insect-screened high tunnel at the U.S. Department of Agriculture (USDA) Beltsville Agricultural Research Center (BARC) north farm, Beltsville, MD. Tomato plants were grown from seeds in one of the BARC greenhouses in commercial organic peat mix (Johnny's 512 mix; Johnny's Selected Seeds, Fairfield, ME) and fertilized with Neptune's Harvest Organic Fish/Seaweed Blend fertilizer (Gloucester, MA) before and after transplanting. In the high tunnel, fertilizer was supplied from a single injector through drip tape supplemented with an Organic Materials Review Institute (OMRI)-approved calcium source to prevent blossom end rot. Black plastic mulch was used to cover the 8 planting beds (2 by 20 ft each) over the drip tape. Planting slits were made in the black plastic at 15-in. intervals to accommodate 13 transplants per bed. Plants were staked by using the Florida weave method with nylon support strings when they were 10 in. high. All plants were irrigated immediately after transplanting and at least weekly to achieve 1 to 1.5 in. water and to meet fertility requirements. Soil moisture was monitored by irrometers and digitally on a Hobo weather station that was located in the center of the high tunnel. Temperature, RH (relative humidity), PAR (photosynthetically active radiation), and total SR (solar radiation) were also monitored and recorded during this time.

(ii) **Experimental design.** A split-plot design was used with two treatments (*Salmonella* only and *Salmonella* with an antagonist) as the first-level subplot. Inoculation sites, including leaf, blossom, and tomato fruit, were each assigned a second-level subplot, with each inoculation site as an independent experimental unit and day of harvest postinoculation as a repeated measure. The second level corresponds to harvests used for 0 (2 h after inoculation as a benchmark for percent recovery), 1-, 2-, 3-, and 5- or 6-day persistence trials. Thirteen plants were planted in each plot. One

FIG 1 *In vitro* inhibition of food-borne pathogens and tomato bacterial phytopathogens by *Paenibacillus alvei* A6-6i and TS-15 on tryptic soy agar. The inhibition zones (mm) were measured against strains of *Salmonella* spp., *Escherichia coli*, *Cronobacter sakazakii* (CS), *Listeria monocytogenes* (LM), *Shigella dysenteriae* (SD), methicillin-sensitive *Staphylococcus aureus* (MSSA), methicillin-resistant *Staphylococcus aureus* (MRSA), *Ralstonia solanacearum* race 5, *Pseudomonas syringae* pv. tomato strain dc3000, and *Erwinia carotovora* subsp. *carotovora*. The plot represents the lowest, highest, and average measurements for each of the species listed above. The experiment was repeated twice.

plant on each end of each bed served as an uninoculated border plant, leaving 11 replicates per plot.

(iii) Inoculum preparation. Because of concerns about the safe use of pathogens in the field, an attenuated *S. Newport* 17 $\Delta tolC::aph$ strain was constructed for the high-tunnel study. The *tolC* gene on the *S. Newport* strain 17 chromosome was replaced by a cassette containing a kanamycin resistance gene by using the one-step inactivation method described previously by Datsenko and Wanner (19). TolC is an outer membrane protein important not only for the efflux of small compounds but also for the export of large proteins. Disruption of *tolC* abolished the ability of *S. Typhimurium* to adhere to, invade, and survive in eukaryotic cells (20). An *S. Enteritidis tolC* mutant was shown to be avirulent in the BALB/c mouse model as well (21). TSB suspensions of an *S. Newport* 17 $\Delta tolC::aph$ strain culture grown overnight were washed twice in PBS and then spot inoculated onto three marked leaves (20 μ l each), six to nine blossoms (10 μ l each), and three breaker-to-red tomato fruits (20 μ l each) for a final concentration of $\sim 10^9$ CFU/ml per plant. The inoculation spots were allowed to air dry (~ 1 h) before the antagonist was applied. Antagonist cell suspensions were made from a bacterial lawn. After two washes with PBS, cells were resuspended in 10 ml of TSB. Forty microliters of the antagonist cell suspension or sterile TSB was applied to the same inoculation spot on leaves and fruits, and 10 μ l was applied to *Salmonella*-inoculated blossoms, of each plant in the “with”- or “without”-antagonist group, respectively. Leaves, blossoms, and fruits were harvested at 0, 1, 2, 3, and 5 days postinoculation (dpi) (for blossoms) or at 6 dpi (for leaves and fruits).

(iv) Sample collection. Inoculated leaves, blossoms, and fruits from each plant were removed with sterile scissors and placed into individual plastic zipper bags, which were sealed and transported in an insulated cooler to the laboratory for analysis within 1 h. For leaves and blossoms, each sample bag was filled with 15 ml and 10 ml of PBS, respectively, and hand rubbed for 3 min to dislodge surface populations of *Salmonella*. For fruits, each sample bag was filled with 30 ml of PBS and subjected to sonication at 55 Hz/min for 30 s (22), which has been shown to be harmless to the infecting microorganisms. PBS was diluted or concentrated through filtration (at later time points in the experiment) and surface plated (0.1 ml in duplicate) onto TSA with kanamycin (TSA-Kan) (50 μ g/ml). Plates were incubated at 35°C overnight, and kanamycin-resistant colonies were counted. Two colonies were randomly picked from each TSA-Kan plate and confirmed by PCR using a set of verification primers (19).

(v) Statistical analysis. Estimates of the rate of reduction in bacterial counts were obtained by fitting a robust linear model of the log-transformed CFU onto days (days after inoculation). The slopes of the fitted lines from antagonist-treated and untreated surfaces were compared for differences in the rates of reduction. The analysis was performed by using the R statistical software package, version 2.11.1, with the robust library. The results were tallied for each combination of plant location, antagonist, plant, and day. Within each plant location, both a regression and a rank test compared the effect of using the antagonist with that of not using it. The sum of the counts on all plates was divided by the sum of the volumes (0.1 ml) of the initial sample. An imputation procedure, dis-

FIG 2 Growth inhibition of major food-borne pathogens in *P. alvei* A6-6i cell-free culture supernatants. Brain heart infusion (BHI) broth was used as a control. Bacterial growth of *L. monocytogenes* (LM), *S. dysenteriae* (SD), *E. coli* O157, *C. sakazakii* (CS), and *S. Newport* strains (A) and methicillin-resistant *S. aureus* (MRSA) strains (B) in *P. alvei* A6-6i CFCs and BHI was determined in five replicates by measuring the OD₆₀₀ at 20-min intervals for 24 h. The experiment was repeated twice.

cussed previously by Blodgett (23), accounted for the plates for which the bacteria were too numerous to count.

RESULTS

Isolation and identification of antagonistic bacteria. A large number of environmental isolates from the tomato field were screened for antimicrobial activity against *S. Newport*. Two isolates, one from an epiphytic leaf surface of native Eastern Shore vegetation and the other from Eastern Shore tomato soil, showed distinct inhibition areas on basal TSA. These isolates formed pale colonies and swarmed vigorously on TSA. Morphologically, the isolates were rod-shaped, 0.7- to 0.95- μ m by 3.18- to 3.42- μ m, Gram-positive bacteria. Upon prolonged incubation on an agar medium, cells produced central endospores.

The isolates were positive for oxidase, nitrate reduction, gelatin liquefaction, starch hydrolyzation, casein hydrolysis, glucose fermentation, and urease but negative for catalase, indole production, and H₂S formation. The bacterium grew well in TSB under aerobic conditions. Genomic analysis showed that the 16S rRNA genes of both isolates share >99.0% sequence similarity to that of *Paenibacillus alvei*. Biolog Gen III MicroPlate analysis confirmed

the high level of similarity of both isolates (>99%) to *P. alvei*. Thus, it was concluded that both isolates belong to the species *P. alvei*, and they were given the strain designations A6-6i and TS-15, respectively.

Broad antimicrobial spectrum of *P. alvei* strains A6-6i and TS-15. *In vitro* agar plug assays showed inhibition zones against all the indicator strains, including six major food-borne pathogens and three major tomato bacterial phytopathogens, when challenged with both *P. alvei* isolates (Fig. 1A and B). Notably, the antagonist migrated outward from the plug after forming the inhibition zone with *Shigella dysenteriae* or *Listeria monocytogenes*, and the antagonistic growth ring expanded with time, especially in the case of *Listeria*. Both A6-6i and TS-15 had a wide range of inhibition against methicillin-resistant *Staphylococcus aureus* (MRSA) strains, with zone diameters from 15 to 35 mm and 15 to 20 mm, respectively. It is also interesting to note that strain A6-6i showed strong inhibitory effects on various MRSA strains tested despite the fact that some strains were resistant to up to 14 different antimicrobial drugs.

When supernatants were tested against the panel of Gram-

negative and Gram-positive bacteria by using the Bioscreen assay, both A6-6i (Fig. 2) and TS-15 (not shown) CFCs exhibited a broad spectrum of antimicrobial activity, in which the lag phase was significantly extended in all pathogens tested and the cell density was largely reduced at the end of incubation. Furthermore, the lag phases of *Cronobacter sakazakii*, *S. dysenteriae*, *L. monocytogenes*, and some MRSA strains were extended to almost 24 h in both A6-6i and TS-15 CFCs. The CFCs from TS-15 had a much stronger inhibitory effect than did the CFCs from A6-6i when tested against *S. Newport* (not shown).

Efficacy of *P. alvei* A6-6i and TS-15 on tomato fruit in humidity chambers. *S. Newport* showed significant reductions on the tomato fruit surface by both *P. alvei* strains A6-6i and TS-15. However, compared to an average of a 0.5-log reduction by A6-6i, TS-15 had a 5-log reduction of the *S. Newport* population applied to tomato fruits (Fig. 3). Numbers of *S. Newport* bacteria recovered from tomato surfaces were 100 times lower on average when the antagonist was added prior to the application of *Salmonella* onto the tomato surface. Nevertheless, no significant difference in the rate of population decline was found regardless of whether the antagonist was inoculated before (Fig. 3A) or after (Fig. 3B) *S. Newport* inoculation, pointing to a potential bactericidal mode of action rather than simple competitive exclusion.

Field trials in a high tunnel using *P. alvei* TS-15. Based on the results from the tomato fruit assay, *P. alvei* strain TS-15 was selected for further high-tunnel field trials. During field trials from July through September 2010, the maximum daily temperature and RH varied between 26.7°C and 37.8°C and between 56% and 80%, respectively (available at <http://www.wunderground.com/history/>). At day 0, variations between the group without TS-15 and the group with TS-15 were detected on leaf and blossom but not on tomato in terms of *Salmonella* populations after inoculation (Fig. 4). Taking all the variations into account, the concentration of *Salmonella* was significantly lower ($P \leq 0.05$) on plants with TS-15 on leaves, blossoms, and fruits from 1 to 5 dpi (for blossom) or 6 dpi (for leaf and tomato) (Fig. 4). Notably, nearly 100% of the “*Salmonella*-only” plants still had detectable levels of *Salmonella* at the end of the blossom and leaf trials, whereas only 2 plants (<20%) in the blossom trial and 6 plants (~50%) in the leaf trial had detectable levels of *Salmonella* in the “antagonist group.” Moreover, the rate of decrease in bacterial concentration was significantly higher ($P \leq 0.05$) on leaves and blossoms with TS-15 than on those without TS-15; decreases were 12-fold per day and 2.7-fold per day for leaves and 8.9-fold and 1.4-fold for blossoms, respectively (Fig. 5). Nevertheless, no statistically significant difference in the mortality rates of *Salmonella* on tomato fruits was found.

DISCUSSION

Contaminated tomatoes have been implicated in several high-profile outbreaks in the United States (24, 25), and *Salmonella enterica* serovar Newport is among the most recurrent serovars implicated in food-borne outbreaks associated with tomatoes (26, 27). Extensive research has been done to show that *Salmonella* can contaminate tomato fruit at the primary-production level through soil, irrigation water, and blossoms (4, 28–30), allowing the pathogen to colonize the exterior and interior of developing fruit. Due to the risk of internalization, *Salmonella* needs to be controlled at the farm level. Biological control has been widely applied to suppress plant diseases caused by phytopathogens (31,

FIG 3 Recovery of *S. Newport* from intact tomato fruit surfaces after treatment with antagonist inoculations over 24 h at 30°C in a humidity chamber. (A) Recovery of *S. Newport* with *S. Newport* inoculated first. (B) Recovery of *S. Newport* with the antagonist inoculated first. Error bars represent the standard deviations of the means of two experiments, each with 10 replicates ($n = 20$).

32). However, few control agents have been reported to control human food-borne pathogens on produce, especially at the pre-harvest level. With only 1- to 2-log reductions, limited success was achieved by using bacteriophages as biocontrol agents (33–35). *Enterobacter asburiae* strain JX1 demonstrated a >5-log reduction in the growth of *Salmonella* in the rhizosphere of tomato plants and on developing fruit (34); however, this bacterium can cause an array of diseases in humans itself (36), making it an undesirable candidate for commercial commodities destined for the human food supply. In this study, two new bacterial strains, A6-6i and TS-15, exhibiting substantial antimicrobial efficacy against a broad range of food-borne pathogens and tomato bacterial phytopathogens, were identified as *P. alvei*, a bacterium very rarely associated with human infections (37). Results of the *in situ* tomato plant trials furthermore showed that *P. alvei* strain TS-15 is highly effective in reducing the carriage of *S. Newport* on tomato plants, indicating its potential use as a novel biocontrol agent to mitigate *Salmonella* contamination at the preharvest level.

The antagonist may exhibit competitive exclusion over certain food-borne pathogens; many *Paenibacillus* species are already part of the natural microbial community in soil, water, and the rhizosphere of various plants (38). Results from the Bioscreen and agar plug assays, however, indicate that the inhibitory (i.e., bacteriostatic and bactericidal) effects of this antagonist on food-borne pathogens can be attributed mainly to its antimicrobial activities. Whole-genome sequencing was performed to help identify di-

FIG 5 Rate of decrease in the *S. Newport* concentration postinoculation on leaves, blossoms, and tomato fruits. In a high-tunnel setting, leaves, blossoms, and tomato fruits were harvested at 0, 1, 2, 3, and 5 dpi (for blossoms) or 6 dpi (for leaves and tomato fruits) to recover any remaining *S. Newport* bacteria. The rates of decrease in the *S. Newport* concentration in the control and antagonist treatment groups were calculated and compared. Results are shown as means $\pm$ 2 standard errors. An asterisk indicates that the rate of decrease in the *S. Newport* concentration was significantly higher ($P < 0.05$) than that in the control group.

verse antibiotic biosynthetic genes present in these two isolates (39). A few novel antimicrobial agents with activities against many food-borne pathogens, including *Salmonella* spp., *E. coli* O157: H7, *L. monocytogenes*, *S. dysenteriae*, *C. sakazakii*, and drug-resistant *S. aureus*, were discovered in our laboratory (our unpublished data).

In the high-tunnel trial, *P. alvei* TS-15 was much more effective in suppressing the growth of *Salmonella* on blossoms and on leaves than on tomato fruits. However, this seemed to have more to do with the lack of persistence of *Salmonella* on tomato fruit surfaces rather than with any lack of *Paenibacillus* activity. The mortality rate in the *Salmonella* control group was much higher for tomato fruits than for leaves and blossoms. In general, the smooth, waxy surfaces of developing tomato fruits were associated with reduced survival of microbes (40–42), and this has implications for the application strategy for this biocontrol agent. Despite this caveat of the tomato surface study, the effectiveness of biological intervention has been shown to be greatly impacted by the ratio of antagonist to pathogen in culture (43). Provided that the ratio of *P. alvei* TS-15 to *S. Newport* was only 1:1 in the fruit assay and the high-tunnel trial, and the actual level of *Salmonella* on naturally contaminated produce is much lower than 6 log CFU/g of tissue, application of *P. alvei* TS-15 at 6 log CFU/g of tissue, as used in this study, should be more than sufficient to inhibit the growth of *Salmonella* on tomato to a clinically significant level.

FIG 4 Recovery of an attenuated *S. Newport* strain from tomato plants, including leaves, blossoms, and tomato fruits. In the high-tunnel study, *S. Newport* was recovered from leaves, blossoms, and tomato fruits at 0, 1, 2, 3, and 5 dpi (for blossoms) or 6 dpi (for leaves and tomato fruits) with inoculation with *S. Newport* only or coinoculation with *S. Newport* plus the antagonist. The results were tallied for each combination of plant location, antagonist, plant, and day. The estimated recovery of *S. Newport* from each sample point from log-transformed data in control (–) and antagonist treatment (+) panels was scatter plotted for leaf (A), tomato fruit (B), and blossom (C).

In summary, the results of this study have demonstrated the efficacy of *P. alvei* TS-15 against *Salmonella* on the blossoms and leaves of tomato plants. In order to more successfully apply such an agent, studies will be conducted to determine the efficacy of *P. alvei* TS-15 in suppressing *Salmonella* in the rhizosphere of tomato plants, to develop a formulation of *P. alvei* TS-15 for use against *Salmonella* and other food-borne pathogens on produce crops, and to assess biological safety due to environmental and human exposure to this organism incidentally in the food and feed supply. Such studies will ascertain the suitability of this promising new microbial agent as an early-intervention tool in our battle against *Salmonella* contamination of fresh-cut produce.

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