Evaluation of a Biocontrol Preparation Consisting of Enterobacter asburiae JX1 and a Lytic Bacteriophage Cocktail To Suppress the Growth of Salmonella Javiana Associated with Tomatoes

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ABSTRACT

A biocontrol preparation based on a combination of Enterobacter asburiae JX1 and a cocktail of five lytic bacteriophages was evaluated for control of Salmonella Javiana within the rhizosphere of plants and in pre- and postharvest tomatoes. The biocontrol preparation introduced into the rhizosphere of growing tomato plants reduced the persistence of Salmonella, although no synergistic action was observed between E. asburiae JX1 or the bacteriophage cocktail when used in combination. When the biocontrol preparation was coinoculated with Salmonella onto the blossom of tomato plants, the prevalence of the enteric pathogen both on the surface and in internal tissues of the subsequent tomatoes was significantly reduced compared with controls. Tomatoes derived from plants inoculated with Salmonella alone had a prevalence of 92% surface contamination (22 of 24 tomato batches positive for Salmonella) and 43% internal contamination (31 of 72 batches positive). This Salmonella prevalence was reduced to 0% (0 of 38 positive) and 2% (1 of 57 positive), respectively, when the biocontrol preparation was applied. Although bacteriophages reduced the prevalence of internalized Salmonella, the main growth suppressing effect was via the antagonistic activity of E. asburiae JX1. No bacteriophages were recovered from tomatoes despite being introduced at 6 log PFU onto the blossom of plants. The biocontrol preparation was not effective for controlling the growth of Salmonella introduced onto postharvest tomatoes that were stored for 7 days at 15°C. The application of E. asburiae JX1 is a promising approach for controlling Salmonella encountered in tomato production, and there was no evidence to suggest that the antagonistic activity could be enhanced by the coinoculation of bacteriophages.

More than 1.5 million cases of salmonellosis are estimated to occur each year within the United States, with 15,000 people requiring hospitalization and 500 fatalities (34). In addition to the traditional Salmonella vehicles such as poultry and eggs, there has been an increasing incidence of salmonellosis linked to fresh produce (14). Outbreaks of Salmonella infections have been linked to seed sprouts, cantaloupes, unpasteurized fruit juice, watermelons, mango, and (relevant to the current study) tomatoes (4, 18, 50, 51). Contaminated tomatoes have been implicated in several high-profile outbreaks within North America, which have resulted in more than 3,000 reported clinical cases since 1990, although the actual number is likely much higher (10, 23, 38). The most notable suspected outbreak occurred in 2008 from contaminated tomatoes (subsequently linked to peppers) from Mexico (11). The outbreak lasted over 80 days and resulted in more than 1,400 confirmed clinical cases of salmonellosis.

Salmonella serovars implicated in foodborne outbreaks associated with tomatoes are uncommon, and the recurring serovars are primarily Newport, Montevideo, and Javiana (6, 38). The underlying reasons why certain Salmonella serovars are associated with tomatoes remains unclear, although the persistence and interaction with tomatoes clearly is serovar dependent (22, 44). Salmonella can become associated with tomatoes at the primary production level. The enteric pathogen is frequently recovered from soil and irrigation water in tomato-producing areas, leading to direct or indirect contamination of the fruit (20, 38). Salmonella also can persist within the rhizosphere of tomato plants, although internalization into the inner plant tissue is limited (28). A more direct route of contamination is deposition of Salmonella onto the blossom (flowers) of tomato plants, which can allow the pathogen to become established both externally and internally in the subsequent fruit (44). Salmonella also can be internalized when fruits are submerged in contaminated dunk water, especially when warm fruits are immersed in cold water (36). Once internalized, Salmonella cannot be removed by washing and potentially can grow at the temperatures used to ripen green tomatoes during distribution (15 to 25°C) (42, 44).

Because of the risk of internalization, Salmonella should be controlled at the primary production level. Treatment of irrigation water with ozone or chlorine has been considered as a potential approach, although the cost and environmental impact are clear limitations (2, 43).
Biocontrol strategies offer a more practical and cost-effective approach for controlling pathogens in the environment (19, 25, 33, 35). Both antagonistic microbes (15, 33, 35, 49) and bacteriophages (19, 25) have been successfully applied to suppress the activity of phytopathogens. However, the use of biocontrol microbes for controlling human pathogens at the primary production level has received relatively little attention (3, 25, 30).

Bacteriophages have been applied to control the growth of *Salmonella* on cantaloupe, lettuce, and alfalfa sprouts, although studies to date have met with limited success, achieving only 1- to 2-log reductions (5, 17, 21, 25, 30, 39). Antagonistic bacteria with anti-*Salmonella* activity have been applied to control the growth of this pathogen on sprouting seeds. Matos and Garland (35) used *Salmonella* and an undefined mixed culture of bacteria directly recovered from alfalfa sprout batches to coinoculate alfalfa seeds. These researchers reported a >5-log reduction in *Salmonella* counts on the final alfalfa sprouts after a 7-day sprouting period, although a residual population of the enteric pathogen persisted. The coinoculation of *Pseudomonas fluorescens* strain 2-79 with *Salmonella* onto alfalfa seeds reduced but did not eliminate the pathogen on the subsequent sprouts (16, 32, 35). To overcome the limited efficacy of bacteriophages and biocontrol bacteria, there has been interest in using both agents in combination. Hong and Conway (24) found synergistic activity of lytic bacteriophages and a *Glucanobacter asaii* strain coinoculated onto honeydew melon to control the growth of *Listeria monocytogenes*. The authors reported that *G. asaii* alone reduced *Listeria* populations by 3 to 4 log CFU/g and application of bacteriophages alone resulted in a 1-log reduction of the pathogen. However, when the two agents were combined, the final *L. monocytogenes* populations were 6 log lower than those in control samples. In a similar study, Ye et al. (53) used a combination of *Enterobacter asburiae* JX1 and a cocktail of five lytic bacteriophages to suppress the growth of *Salmonella* on sprouting mung beans. The *E. asburiae* JX1 or bacteriophages alone resulted in a 0.9- to 2.5-log reduction of final *Salmonella* populations on sprouts. However, when used in combination, these two biocontrol agents decreased *Salmonella* levels by 5.7 to 6.4 log CFU/g, and the pathogen was detected only after culture enrichment.

In the present study, the same combination of *E. asburiae* JX1 and bacteriophage cocktail described by Ye et al. (53) was evaluated for controlling the growth of *Salmonella* Javiana in the rhizosphere of tomato plants and in pre- and postharvest tomatoes.

**MATERIALS AND METHODS**

**Microorganisms.** *Salmonella enterica* serovar Javiana strain 5913 (originally isolated from chicken manure) was kindly donated by the Public Health Agency of Canada (Guelph, Ontario). *Salmonella Javiana* was chosen for the study because of previous links to salmonellosis outbreaks associated with tomatoes (10). *E. asburiae* JX1 was originally isolated from mung bean sprouts and transformed to carry lux CDABE:kanamycin, as previously described Ye et al. (53). The bacteriophage cocktail used consisted of phages P01, P01, P102, P700, and P800, which were isolated from Ontario pig farms as previously described (53). Based on morphology, two types of phages were identified as belonging to the virus families *Siphoviridae* and *Myoviridae* (53). The host ranges of the individual constituent bacteriophages have been reported (53).

**Preparation of inocula.** *Salmonella Javiana* and *E. asburiae* JX1 were cultivated aerobiologically in tryptic soy broth (TSB; Oxoid, Basingstoke, UK) at 37 or 30°C, respectively, for 24 h. The cells were harvested by centrifugation (5,500 × g for 10 min at 4°C) and washed once in saline. The cell suspension was resuspended in saline to a cell density of 10⁶ CFU/ml (optical density of 2 units at 600 nm).

*Salmonella* bacteriophage lysates were prepared by inoculating 50 μl of an overnight culture of *Salmonella Javiana* into 250 ml of Luria-Bertani broth (Oxoid) with 0.12 g/ml magnesium sulfate and incubating at 37°C for 8 h. The culture was then dispensed into a centrifuge tube, and the cells were removed by centrifugation (7,000 × g at 4°C for 10 min). The supernatant containing phage was decanted and passed through a 0.22-μm pore-size syringe filter. Phage numbers were enumerated using the three-tube most-probable-number (MPN) method (37). Three sets of dilutions of the filtered lysates were prepared in SM buffer (composition per milliliter: 5.8 mg of NaCl, 2 mg of MgSO₄·7H₂O, 50 mM Tris, pH 7.5, and 0.1 mg of gelatin), and 10-μl aliquots were spotted onto cell lawns of *Salmonella Javiana* as described by Ye et al. (53). The plates were incubated overnight at 37°C, and the formation of plaques was considered a positive reaction. The levels of bacteriophages were then derived from MPN tables (37). The MPN method was used in the current study because of the strong lytic action of the bacteriophages, which caused individual plaques to merge, making accurate enumeration problematic. The phage cocktail was prepared by mixing equal levels of the bacteriophages to a final density of 10⁶ PFU/ml.

**Cultivation and inoculation of tomato plants.** Tomato seeds (*Lycopersicon esculentum* varity Abigail VFET) were obtained from the Ontario Seed Company Ltd. (Waterloo, Ontario, Canada). The seeds were planted into plug trays containing commercial grade PGX soil and germinated for 3 to 5 weeks with periodic watering via trickle irrigation (15 s of watering every 15 min). The resultant seedlings were transplanted into 1-gal (3.8-liter) soil microcosms and trickle irrigated with fertilizer solution (20-8-20 high-nitrate all season feed, Evergro Inc., Delta, British Columbia, Canada).

For inoculating the rhizosphere of plants, 100-μl aliquots containing *Salmonella Javiana* 5193 (7 log CFU/ml), *E. asburiae* JX1 (7 log CFU/ml), or the bacteriophage cocktail (7 log PFU/ml) and combinations thereof were dispensed onto the soil surrounding the stem base of tomato plants 7 weeks postgermination (nine plants per treatment). Immediately following inoculation and at days 14 and 28, the roots of three plants per treatment set were removed for microbiological analysis. The roots (with associated soil) were separated from the plant by severing the stem with a sterile scalpel blade and were transferred to a sterile stomacher bag. The roots were weighed, and 100 ml of neutralizing broth (NB; Cole-Parmer, Montreal, Quebec, Canada) was added. The suspended roots were then physically massaged for at least 60 s to release the bacteria and phages. A dilution series was prepared in 0.1% peptone water (Oxoid, Basingstoke, UK), 0.1-ml aliquots were spread plated onto XLT4 agar (Oxoid), and the plates were incubated at 37°C for 24 h for the enumeration of *Salmonella*. Selected typical colonies (black centers with a clear halo) were confirmed using the Oxoid *Salmonella* latex agglutination test. When no colonies were detected on XLT4 plates, 40-ml aliquots of
the root surface rinse were incubated at 37°C for 24 h. A 0.1-ml aliquot of the enriched culture was then inoculated into the center of a semisolid Rappaport-Vassiliadis plate (Oxoid) that was incubated at 42°C for 24 h. Cells from the outer perimeter of the growth halo (presumptive motile Salmonella) were streaked onto XLT4 agar and incubated at 37°C overnight. The Oxoid Salmonella latex agglutination test was used to confirm the identification of presumptive colonies.

*E. asburiae* JX1 was enumerated on tryptic soy agar (TSA; Oxoid) supplemented with 30 μg/ml kanamycin (Sigma-Aldrich, Oakville, Ontario, Canada) (TSAkan) and incubated at 30°C for 48 h. Confirmation of *E. asburiae* JX1 was performed by screening for the bioluminescent phenotype with a Night-Owl image analyzer (E.G.&G. Berthold, Munich, Germany). In parallel, 40 ml of the root rinse solution was enriched in 200 ml of TSBkan and incubated at 37°C for 24 h. Aliquots (100 μl) of the enriched culture were then plated onto TSAkan that was subsequently incubated at 30°C for 48 h.

Total aerobic bacteria were enumerated onto TSA incubated at 30°C for 48 h.

Bacteriophages were enumerated by the addition of 100 μl of chloroform to 1-ml aliquots of the root rinse sample. The bacteriophages were then enumerated using the MPN method as described above.

The tomato blossoms were inoculated in plants that had reached the flowering stage (ca. 7 weeks postgermination). The inoculum was prepared as described above, with a final bacterial cell density of 7 log CFU/ml and a bacteriophage cocktail density of 7 log PFU/ml. Aliquots (100 μl) of the suspension were then introduced onto the flower stamen via a pipette (12 plants per treatment group). The flower was held upright to avoid excessive runoff of the inoculum. The fruits were allowed to develop for 6 to 7 weeks and were harvested at the early breaker stage. The fruits were separated into batches of three, which constituted a single sample.

Ripened (red) tomatoes were purchased from a local supermarket. These fruits were placed in a suspension of Salmonella Javiana (10⁶ CFU/ml) for 10 min and then transferred to a suspension of *E. asburiae* JX1 (3 or 6 log CFU/ml) with and without bacteriophage cocktail (3 or 6 log PFU/ml), and controls were placed in saline. The fruits were removed after 10 min and transferred to a vacuum chamber and subjected to three vacuum-release cycles to facilitate internalization (vacuum applied for 3 min followed by release). The fruits were then separated into batches of three and placed into sterile plastic bags that were loosely closed. The bags were transferred to an environmental chamber maintained at 15°C at a relative humidity of 95%. Tomato samples (three batches) were withdrawn at day 0 (after inoculation) and after 7 days of storage.

**Recovery of Salmonella,* E. asburiae* JX1, and bacteriophages from the surface and internal tissue of tomatoes.** The surface microbiota of tomatoes was recovered by submerging the batch of three fruits sequentially into 30 ml of NB followed by gentle rubbing for approximately 60 s to release the microbial populations. The NB rinse was transferred to a sterile container, and a dilution series was prepared with 0.1% peptone water. The fruits were then surface disinfected by submerging in 500 ml of 2% sodium hypochlorite (9 mg/ml free chlorine as measured using a chlorine test kit; Thermo-Fisher Scientific, Whitby, Ontario, Canada) for 10 min (44). Residual hypochlorite was removed by rinsing the treated fruit three times in 500 ml of sterile distilled water. The inner core (calyx) of each fruit was removed with a sterile scalpel, suspended in NB (1:10 ratio), and stomached for 1 min at 260 rpm (Stomacher 400, Seward, London, UK). A serial dilution series was prepared in peptone water, and enumeration and enrichment were performed as described above.

**Suppression of Salmonella growth in broth culture using a combination of bacteriophages and biocontrol bacteria.** *Salmonella* Javiana suspensions were inoculated into 10 ml of TSB to a final cell density of 3 log CFU/ml along with the test biocontrol bacterium (3 log CFU/ml). A bacteriophage cocktail prepared from five selected phage isolates was introduced into the culture to give a multiplicity of infection (MOI) of 1. The inoculated NB was then incubated at 15 or 30°C for 48 h, and *Salmonella* was enumerated by preparing a dilution series that was subsequently plated onto XLT4 agar and incubated at 37°C for 24 h.

**Deferred assay.** The deferred assay was performed as described by Fett (16). *E. asburiae* JX1 was inoculated into 10 ml of TSB and cultured overnight at 30°C. Aliquots (10 μl) of the culture were spotted onto two TSA plates that were incubated at 15 or 30°C for 48 h. One of the TSA plates was exposed to chloroform vapor for 1 h at room temperature in a fume hood. After exposure, this plate was overlaid with 5 ml of soft agar (TSB with 2.5% agar, w/vol) that was inoculated with 100 μl of an overnight culture of *Salmonella* Javiana. The agar was allowed to set at room temperature, and the plate was incubated at 37°C for 24 h. Anti-*Salmonella* activity was visualized by zones of inhibition (>10 mm) on the agar plates.

**Statistical analysis.** The control of *Salmonella* in the rhizosphere was evaluated in two separate trials with a total of nine plants per treatment. For the blossom inoculation study, 12 plants per treatment were used, and 72 to 120 tomatoes (24 to 40 batches of three fruits each) were harvested for microbiological analysis. Control of *Salmonella* on postharvest tomatoes was assessed from three batches of three fruit per batch at each sampling point. The experiment was repeated twice.

The counts of bacteria or bacteriophage per milliliter were transformed to logarithmic values, and the means were analyzed with an analysis of variance and Tukey’s test (S-Plus, Insightful Corp, New York, NY). The qualitative data from the blossom inoculation trials were analyzed using contingency table analysis (S-Plus). In all cases the significance level was set at *P* ≤ 0.05.

**RESULTS**

**Control of Salmonella on tomato plant rhizosphere.** *Salmonella* Javiana inoculated into the rhizosphere of tomato plants decreased progressively over a 4-week period (Table 1). The coinoculation of *E. asburiae* JX1 with the enteric pathogen into the rhizosphere of plants resulted in significantly lower levels (*P* < 0.05) of *Salmonella* throughout the 4-week trial despite the fact that levels of *E. asburiae* JX1 also decreased with time within the rhizosphere (Table 1).

The introduction of bacteriophages also resulted in a significant decrease (*P* < 0.05) in *Salmonella* levels relative to controls (Table 1). There was no significant (*P* > 0.05) difference in the rate of *Salmonella* decline when the bacteriophage cocktail and *E. asburiae* JX1 were applied in combination. Although the bacteriophages effectively decreased *Salmonella* levels, phage levels were below the level of detection (ca. <2.5 log PFU/g) except after the first sampling point (immediately after inoculation into the tomato plant rhizosphere) (Table 1).
The coinoculation of *E. asburiae* JX1 and bacteriophages did not have a significant effect (*P > 0.05*) on the counts of total aerobic organisms recovered from the rhizosphere of the tomato plants (Table 1), which suggests that the coinoculation of the biocontrol preparation was specific for *Salmonella* and did not affect the general rhizosphere microbiota.

**Control of Salmonella Javiana on the surface and in the internal tissues of developing tomato fruit.** *Salmonella* was inoculated onto the blossom of tomato plants in the presence or absence of the biocontrol preparation. The fruits were allowed to develop for 7 weeks and harvested at the early breaker stage (transition point from green to red tomatoes). Levels of *Salmonella, E. asburiae* JX1, and bacteriophages were determined from surface rinse and internal tissues of the fruits. In all cases, the levels of the introduced microbes were too low to allow direct enumeration but could be detected in enrichment cultures. *Salmonella* was recovered from 92% of the surface rinse solutions taken from tomatoes derived from plants coinoculated with the enteric pathogen alone (Table 2). When *E. asburiae* JX1 was coinoculated with the pathogen onto the blossom of plants, a significant reduction (*P < 0.05*) in the prevalence of *Salmonella* was observed; only 5% of the fruit were positive for the pathogen. The coinoculation of bacteriophages onto the blossoms did not significantly reduce (*P > 0.05*) the prevalence of *Salmonella* on the surface of tomatoes when compared with inoculation of the pathogen alone (Table 2). No *Salmonella* was recovered from the surface rinse of tomatoes derived from plants coinoculated with *E. asburiae* JX1 and bacteriophages, although the prevalence of the pathogen was not significantly different (*P > 0.05*) than that when *E. asburiae* JX1 was coinoculated without the phage (Table 2).

*Salmonella* was found internally in 43% of the fruit derived from plants inoculated with the pathogen alone (Table 2). Coinoculation with either only bacteriophages or only *E. asburiae* JX1 significantly reduced (*P < 0.05*) populations of internalized *Salmonella* compared with the control (Table 2). Coinoculation of both bacteriophages and *E. asburiae* JX1 resulted in a *Salmonella* prevalence of 2%, which was not significantly different (*P > 0.05*) from the prevalence observed when *E. asburiae* JX1 was applied alone (Table 2). These results suggest that the main anti-*Salmonella* action was via the activity of *E. asburiae* JX1 and that the bacteriophage cocktail had less of an impact.

### TABLE 1. Microbial counts from the rhizosphere of noninoculated (control) and inoculated tomato plants

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>Salmonella</th>
<th>Salmonella + <em>E. asburiae</em> JX1</th>
<th>Salmonella + phage</th>
<th>Salmonella + phage + <em>E. asburiae</em> JX1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 days</td>
<td>14 days</td>
<td>28 days</td>
<td>ND A</td>
<td>ND A</td>
</tr>
<tr>
<td>Total viable organisms</td>
<td>3.11 ± 0.16 A</td>
<td>5.11 ± 0.18 A</td>
<td>5.89 ± 0.51 A</td>
<td>ND A</td>
<td>ND A</td>
</tr>
<tr>
<td><em>Salmonella</em></td>
<td>6.08 ± 0.72 B</td>
<td>5.16 ± 0.14 A</td>
<td>6.27 ± 0.104 A</td>
<td>ND A</td>
<td>ND A</td>
</tr>
<tr>
<td><em>Salmonella + E. asburiae</em> JX1</td>
<td>5.99 ± 0.65 B</td>
<td>4.86 ± 0.15 A</td>
<td>6.09 ± 0.94 A</td>
<td>ND A</td>
<td>ND A</td>
</tr>
<tr>
<td><em>Salmonella + phage</em></td>
<td>5.68 ± 0.18 B</td>
<td>5.13 ± 0.13 A</td>
<td>6.28 ± 0.70 A</td>
<td>ND A</td>
<td>ND A</td>
</tr>
<tr>
<td><em>Salmonella + phage + E. asburiae</em> JX1</td>
<td>5.95 ± 0.19 B</td>
<td>5.05 ± 0.17 A</td>
<td>6.01 ± 0.80 A</td>
<td>ND A</td>
<td>ND A</td>
</tr>
</tbody>
</table>

*Salmonella* Javiana (7 log CFU), *Enterobacter asburiae* JX1 (7 log CFU), or bacteriophage cocktail (7 log PFU) and combinations thereof were introduced into the rhizosphere of 6-week-old tomato plants. Periodically, the roots of three plants per treatment were removed for determination of microbial counts. Within each column, means followed by the same letter are not significantly different.

* ND, not detected.

* Positive by enrichment.
E. asburiae JX1 was recovered from 98% of the surface rinse samples screened (Table 2). The bacterium also was recovered from 54 to 79% of the internal tomato tissues. No bacteriophages were detected in any of the tomato samples tested despite being coinoculated with their Salmonella host at relatively high levels (6 log PFU/ml). This finding suggests that the bacteriophages were either being inactivated or sequestered on the developing tomato fruit.

Control of Salmonella on the surface and in the internal tissues of ripened (red) tomatoes during postharvest storage. Ripened tomato fruits were steep inoculated with Salmonella Javiana with or without the biocontrol preparation and stored at 95% relative humidity and 15°C for 7 days. The levels of Salmonella, E. asburiae JX1, and bacteriophages were screened in surface rinses and in homogenates prepared from the tomato pulp (Table 3).

### TABLE 2. Prevalence of Salmonella Javiana and Enterobacter asburiae JX1 on and within tomatoes derived from inoculated tomato plants

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Surface Positive</th>
<th>Surface Negative</th>
<th>Internal Positive</th>
<th>Internal Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0</td>
<td>34</td>
<td>0</td>
<td>36</td>
</tr>
<tr>
<td>Salmonella</td>
<td>22 (92)</td>
<td>2</td>
<td>31 (43)</td>
<td>41</td>
</tr>
<tr>
<td>Salmonella + E. asburiae JX1</td>
<td>2 (5)</td>
<td>38</td>
<td>1 (1)</td>
<td>83</td>
</tr>
<tr>
<td>Salmonella + phage</td>
<td>25 (83)</td>
<td>5</td>
<td>11 (19)</td>
<td>46</td>
</tr>
<tr>
<td>Salmonella + phage + E. asburiae JX1</td>
<td>0</td>
<td>38</td>
<td>1 (2)</td>
<td>56</td>
</tr>
</tbody>
</table>

Salmonella
- Control: 0
- Salmonella: 22 (92%)
- Salmonella + E. asburiae JX1: 2 (5%)
- Salmonella + phage: 25 (83%)
- Salmonella + phage + E. asburiae JX1: 0

*E. asburiae JX1*
- Control: 0
- Salmonella: 0
- Salmonella + E. asburiae JX1: 39 (98%)
- Salmonella + phage: 0
- Salmonella + phage + E. asburiae JX1: 38 (100%)

**Control of Salmonella on the surface and in the internal tissues of ripened (red) tomatoes during postharvest storage.** Ripe tomato fruits were steep inoculated with Salmonella Javiana with or without the biocontrol preparation and stored at 95% relative humidity and 15°C for 7 days. The levels of Salmonella, *E. asburiae* JX1, and bacteriophages were screened in surface rinses and in homogenates prepared from the tomato pulp (Table 3).

### TABLE 3. Effect of Enterobacter asburiae JX1 and bacteriophage cocktail on Salmonella Javiana inoculated onto ripe tomatoes that were subsequently stored at 15°C for 7 days

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Surface Salmonella (log CFU/ml) Mean ± SD</th>
<th>Internal Salmonella (log CFU/ml) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Day 0</td>
<td>Day 7</td>
</tr>
<tr>
<td>Salmonella</td>
<td>5.54 ± 0.60 A</td>
<td>6.98 ± 0.74 B</td>
</tr>
<tr>
<td>Salmonella + E. asburiae JX1 (3 log CFU/ml) + phages (3 log PFU/ml)</td>
<td>5.6 ± 0.28 A</td>
<td>6.93 ± 0.78 B</td>
</tr>
<tr>
<td>Salmonella + E. asburiae JX1 (6 log CFU/ml) + phages (6 log PFU/ml)</td>
<td>6.15 ± 0.09 A</td>
<td>7.04 ± 0.21 B</td>
</tr>
</tbody>
</table>

**Surface populations**
- Salmonella: 5.54 ± 0.60 A
- Salmonella + E. asburiae JX1 (3 log CFU/ml) + phages (3 log PFU/ml): 5.6 ± 0.28 A
- Salmonella + E. asburiae JX1 (6 log CFU/ml) + phages (6 log PFU/ml): 6.15 ± 0.09 A

**Internal populations**
- Salmonella: 2.61 ± 1.18 AD
- Salmonella + E. asburiae JX1 (3 log CFU/ml) + phages (3 log PFU/ml): 0.35 ± 0.68 B
- Salmonella + E. asburiae JX1 (6 log CFU/ml) + phages (6 log PFU/ml): 1.76 ± 1.53 A

### Notes:
- A: 1 log CFU/ml
- B: 2 log CFU/ml
- C: 3 log CFU/ml
- D: 4 log CFU/ml
- E: 5 log CFU/ml
- F: 6 log CFU/ml

* A: 1 log CFU/ml
* B: 2 log CFU/ml
* C: 3 log CFU/ml
* D: 4 log CFU/ml
* E: 5 log CFU/ml
* F: 6 log CFU/ml

- ND, not detected by enrichment (<0.1 log CFU/ml).

**Control of Salmonella on the surface and in the internal tissues of ripened (red) tomatoes during postharvest storage.** Ripe tomato fruits were steep inoculated in a Salmonella Javiana suspension (6 log CFU/ml) and subsequently transferred to a suspension containing *E. asburiae* JX1 and bacteriophages. Within each column and row, means followed by the same letter are not significantly different.

*B* Blossoms of 6- to 7-week-old tomato plants were inoculated with Salmonella Javiana (6 log CFU/ml), *E. asburiae* JX1 (6 log CFU/ml), or bacteriophage cocktail (6 log PFU/ml) and combinations thereof. The subsequent tomatoes were harvested at the breaker stage and then screened for the presence of Salmonella and *E. asburiae* JX1 both externally and internally.

* Each sample consisted of three tomatoes.

* Individual surface-disinfected tomatoes were screened for internalized bacterial populations.
levels on the fruit surface increased significantly \( (P < 0.05) \) during the 7-day storage period. The coinoculation of biocontrol preparation did not significantly \( (P > 0.05) \) affect the growth of \( \text{Salmonella} \) on the surface of tomatoes, and final levels of the enteric pathogen were comparable to those of the controls. Increasing the levels of the biocontrol preparation within the steeping solution from 3 to 6 log CFU and PFU per ml did not have a significant impact on the final \( \text{Salmonella} \) levels on the tomatoes at the end of the storage period.

\( \text{Salmonella} \) grew within the internal tissue of tomatoes, and the presence of the biocontrol preparation had a negligible impact on the final populations of the pathogen. \( \text{E. asburiae} \) JX1 also grew within the internal tissue of tomatoes, and final levels were not significantly different \( (P > 0.05) \) from those of \( \text{Salmonella} \).

Bacteriophages were recovered from the surface rinse solutions of tomatoes at \( 3.01 \pm 0.64 \) or \( 5.28 \pm 0.14 \) log PFU/ml after the initial inoculation. However, no bacteriophages were recovered from the internal tissue or surfaces at the end of the 7-day storage period.

Further experiments revealed anti-\( \text{Salmonella} \) activity of the biocontrol preparation in broth culture. TSB was inoculated with \( \text{Salmonella} \) Javanica plus the biocontrol preparation and incubated at either 15 or 30\(^\circ\)C for 48 h, and \( \text{Salmonella} \) levels were determined. The growth suppressing effect of the bacteriophage cocktail plus \( \text{E. asburiae} \) JX1 was significantly lower \( (P < 0.05) \) in cultures incubated at 15\(^\circ\)C than in those incubated at 30\(^\circ\)C (Fig. 1).

Plaque assays performed by spotting the bacteriophage cocktail onto cell lawns of \( \text{Salmonella} \) that were subsequently incubated at 15\(^\circ\)C for up to 7 days produced only weak (small) plaques. This result suggests that the lytic ability of the bacteriophages against \( \text{Salmonella} \) was compromised by the storage temperature. The antagonistic activity (as measured by the deferred assay) of \( \text{E. asburiae} \) JX1 was not affected by the 15\(^\circ\)C incubation temperature and resulted in zones of \( \text{Salmonella} \) inhibition that were >10 mm. This result suggests that the low efficacy of \( \text{E. asburiae} \) JX1 for control of \( \text{Salmonella} \) growth on tomatoes could not be attributed to the storage temperature used in this study.

**DISCUSSION**

A biocontrol preparation consisting of \( \text{E. asburiae} \) JX1 and a cocktail of lytic bacteriophages for control of \( \text{Salmonella} \) associated with tomato fruits and plants has been evaluated. When introduced into the rhizosphere of growing tomato plants, both \( \text{E. asburiae} \) JX1 and bacteriophages decreased the levels of \( \text{Salmonella} \). However, \( \text{Salmonella} \) levels within the rhizosphere of tomato plants naturally declined in the absence of \( \text{E. asburiae} \) JX1 and bacteriophages. Although \( \text{Salmonella} \) can grow on young seedlings (sprouts), survival on older plants is compromised by nutrient limitation and competitive microbiota (28, 29). However, both bacteriophages and \( \text{E. asburiae} \) JX1 increased the rate of decline of \( \text{Salmonella} \) within the rhizosphere. \( \text{E. asburiae} \) reduced the growth of \( \text{Salmonella} \) on \text{Arabidopsis} \) through a competitive inhibition mechanism (12). In the current study, \( \text{E. asburiae} \) JX1 numbers declined, as did those of \( \text{Salmonella} \), during the 4-week period, which suggests that the mode of inhibition was not competitive inhibition.

Bacteriophages also declined within the rhizosphere of tomato plants and were detected only at the first sampling time directly after inoculation. The decline in bacteriophages could be attributed to the metabolic down-regulation of \( \text{Salmonella} \) host cells, which would restrict phage replication (21). An alternative explanation is that the phages were bound to the soil particles, thereby losing infectivity (47). Bacteriophages strongly bind to clay particles via electrostatic interactions that can prevent contact with the host and eventual loss of infectivity (7, 8, 40, 46, 52). In the current study, the bacteriophages probably infected \( \text{Salmonella} \) cells soon after inoculation into the rhizosphere, as opposed to providing long-term control.

When \( \text{Salmonella} \) was introduced onto the blossoms of tomato plants, the bacterium was recovered from both the internal and external tissues of the subsequent fruits. These results are in agreement with those of other workers, who reported contamination of tomatoes when the pathogen was introduced onto blossoms of plants (22, 44). The coinoculation of \( \text{E. asburiae} \) JX1 significantly reduced the prevalence of \( \text{Salmonella} \) on fruits, both internally and externally. The results support the hypothesis of Shi et al. (45), who reported that tomatoes harboring \text{Enterobacter} \) had a low \( \text{Salmonella} \) prevalence.

The prevalence of \( \text{Salmonella} \) within tomatoes was reduced in the presence of bacteriophages, although the treatment had a negligible effect on surface contamination. The limited efficacy of phages for control of \( \text{Salmonella} \) on
and within tomato fruits may be related to the sensitivity of phages to UV light. Iriarte et al. (26) found that phages introduced into the phyllosphere of plants progressively decreased due to UV radiation, desiccation, and the presence of copper ions applied in pesticide solutions. These authors inoculated tomato leaves with bacteriophages that had lytic activity against the phytopathogen Xanthomonas campestris and periodically assessed phage levels and plant disease symptoms. The level of bacteriophages decreased by 6 log PFU within 10 h of being applied to leaves, principally because of exposure to UV radiation from sunlight. The researchers also observe that disease symptoms of tomato plants infected with X. campestris were delayed by 4 days; therefore, the bacteriophages provided only short-term protection against the phytopathogen. In the current study, Salmonella-infecting bacteriophages probably were rapidly inactivated by exposure to UV light, which may account for the limited efficacy for control of the enteric pathogen compared with the efficacy of E. asburiae JX1. Bacteriophages also are sensitive to acidic environments that denature proteins and thus ultimately limit the ability of phages to control pathogens introduced onto acidic fruit (17, 21, 25, 31). Bacteriophage treatment may have been more successful if a higher MOI had been used. In one study, the ability of bacteriophages to control Campylobacter and to a lesser extent Salmonella was dependent on the MOI (9). In the current study, an MOI of 1 was used. Although this MOI was effective for controlling Salmonella on sprouts (53), when it was used in combination with E. asburiae JX1 in the present study on developing tomato fruit, phage efficacy was limited.

The results indicate that the main anti-Salmonella effect on tomatoes was due to the activity of E. asburiae JX1. These findings are in contrast to those obtained when the same biocontrol preparation was applied to control the growth of Salmonella on sprouting mung beans (53), in which Salmonella growth was suppressed only when a combination of E. asburiae JX1 and bacteriophage cocktail was used (53). However, there are significant differences between sprouting mung beans and developing tomato fruit. Mung beans were sprouted at relatively high temperatures (20 to 30°C) during a short time period (48). Sprouts also are rich in nutrients that can support the growth of Salmonella, providing the bacteriophages with an actively growing host to facilitate phage replication (21). In contrast, the developing tomato fruit represents a relatively harsh environment with exposure to UV light, desiccation, and antimicrobial agents (26). No phages were recovered from the harvested tomatoes, which would explain the lack of an additive anti-Salmonella effect with E. asburiae JX1. Nevertheless, E. asburiae JX1 alone was sufficient to suppress growth of Salmonella on tomatoes and represents a promising approach to control the enteric pathogen at the primary production level.

The efficacy of the biocontrol preparation to suppress the growth of Salmonella on postharvest tomatoes was limited. As previously reported, Salmonella readily grew on tomatoes and reached high levels within a short time period under conditions typically used for ripening the fruit (13, 27, 44). Although the growth of E. asburiae JX1 was comparable to that of Salmonella, there was no significant effect on pathogen levels, possibly because the inactivation of bacteriophages attenuated the anti-Salmonella effect of E. asburiae JX1. In the sprouting mung bean model, E. asburiae JX1 alone had limited efficacy for controlling Salmonella in the absence of bacteriophages (53). The sequestering of bacteriophages on plant material has been reported previously and attributed to binding of the phages to charged polymers (3, 30). However, Abuladze et al. (1) reported that a 1- to 2-log reduction of E. coli O157:H7 on slices of tomato could be achieved using bacteriophages. This finding suggests that phages can retain infectivity when introduced onto tomatoes. However, Abuladze et al. (1) used a high MOI of >1,500, which may have resulted in inundation of the Escherichia coli O157:H7 in contrast to cell lysis by phage replication. The relatively low MOI used in the current study would have led to a negligible inundation effect.

Temperature also may represent a limiting factor given that the biocontrol preparation had a limited effect on the growth of Salmonella in broth culture incubated at 15°C. This temperature effect also may explain why the biocontrol preparation worked more effectively on sprouting mung bean sprouts, which are grown at temperatures >20°C. The actual mode by which E. asburiae JX1 inhibits the growth of Salmonella remains obscure, although a factor or factors released by the bacterium may be involved, as indicated by the plate diffusion assay (53). Results from the current study indicate that E. asburiae JX1 produced the anti-Salmonella factor(s) at 15°C, thus, the low storage temperature cannot explain the lack of effect of the biocontrol bacterium on the enteric pathogen on tomatoes. A more likely reason for the low efficacy of the biocontrol preparation for suppressing Salmonella growth is the low infectivity of the bacteriophage preparation. Phages can infect and replicate in host cells only within a certain temperature range (41). The temperature range that supports phage replication is related to conformational arrangement of the phage nucleic acids and the metabolic flux of the energy-yielding pathways of the host cells (41). Although plaques were formed by the bacteriophage cocktail on Salmonella cell lawns incubated at 15°C, these plaques were weak, indicating poor phage replication.

In conclusion, the results of this study have demonstrated the efficacy of a biocontrol preparation for suppressing the growth of Salmonella in the rhizosphere of tomato plants and on the developing fruit. The contribution of bacteriophages was negligible, and the primary anti-Salmonella effect was the action of E. asburiae JX1. The underlying reasons for the lack of effect of bacteriophages probably is related to the need for an actively growing host to facilitate phage replication, to temperature effects, and also to sequestering via plant constituents.

Although the mode by which E. asburiae JX1 inhibits the growth of Salmonella remains unclear, these results indicate that this Enterobacter strain has potential use as part of a novel biocontrol strategy to enhance the
microbiological safety of tomatoes at the primary production level.

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