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Control of *Salmonella* on Sprouting Mung Bean and Alfalfa Seeds by Using a Biocontrol Preparation Based on Antagonistic Bacteria and Lytic Bacteriophages

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ABSTRACT

The following reports on the application of a combination of antagonistic bacteria and lytic bacteriophages to control the growth of *Salmonella* on sprouting mung beans and alfalfa seeds. Antagonistic bacteria were isolated from mung bean sprouts and tomatoes by using the deferred plate assay to assess anti-*Salmonella* activity. From the isolates screened, an *Enterobacter asburiae* strain (labeled ''JX1'') exhibited stable antagonistic activity against a broad range of *Salmonella* serovars (Agona, Berta, Enteritidis, Hadar, Heidelberg, Javiana, Montevideo, Muenchen, Newport, Saint Paul, and Typhimurium). Lytic bacteriophages against *Salmonella* were isolated from pig or cattle manure effluent. A bacteriophage cocktail prepared from six isolates was coinoculated with *E. asburiae* JX1 along with *Salmonella* in broth culture. The combination of *E. asburiae* JX1 and bacteriophage cocktail reduced the levels of *Salmonella* by 5.7 to 6.4 log CFU/ml. Mung beans inoculated with *Salmonella* and sprouted over a 4-day period attained levels of 6.72 $\pm$ 0.78 log CFU/g. In contrast, levels of *Salmonella* were reduced to 3.31 $\pm$ 2.48 or 1.16 $\pm$ 2.14 log CFU/g when the pathogen was coinoculated with bacteriophages or *E. asburiae* JX1, respectively. However, by using a combination of *E. asburiae* JX1and bacteriophages, the levels of *Salmonella* associated with mung bean sprouts were only detected by enrichment. The biocontrol preparation was effective at controlling the growth of *Salmonella* under a range of sprouting temperatures (20 to 30°C) and was equally effective at suppressing the growth of *Salmonella* on sprouting alfalfa seeds. The combination of *E. asburiae* JX1 and bacteriophages represents a promising, chemical-free approach for controlling the growth of *Salmonella* on sprouting seeds.

Sprouted seeds have been implicated in several high-profile outbreaks of foodborne illness *(35, 40, 42)*. *Salmonella* is the most commonly encountered human pathogen associated with foodborne illness outbreaks linked to sprouts, although *Escherichia coli* O157:H7 and other pathogens have also been implicated *(16)*. The most high-profile salmonellosis outbreak linked to mung bean sprouts occurred within Ontario in 2005 and resulted in over 600 reported cases *(9)*. In total, there have been an estimated 36 outbreaks of foodborne illness linked to sprouts, with alfalfa and mung bean sprouts being implicated in the majority of cases *(53),* thereby representing a significant food safety risk.

It is generally acknowledged that the seed used to prepare sprouts is the primary source of pathogens *(20, 40, 56)*. Even when present at low levels (0.1 log CFU/g), pathogens can grow rapidly under the warm (20 to 30°C) and humid conditions used in sprout production *(18, 31)*. In addition, pathogens can become internalized into the developing sprout and consequently cannot be removed by postharvest washing *(53)*. Therefore, the majority of focus to date has been placed on seed decontamination, which aims to inactivate pathogens prior to initiating the sprouting process *(25, 54)*. Despite a diverse range of sanitizing agents being evaluated, only a select few have proven effective at eliminating human pathogens on seeds. For example, seeds treated with a fatty acid– or stabilized sodium chlorite–based sanitizers have been proven to inactivate *Salmonella* introduced onto seeds *(29, 44)*. Yet, because a high proportion of sprouts are destined for the organic market, many sprout produces are reluctant to use chemical sanitizers for seed decontaminating *(48)*. In addition, seed decontamination cannot prevent contamination being introduced during the sprouting process, which can potentially occur if inadequate sanitation is practiced *(33, 40)*. To address such limitations associated with chemical seed decontamination, there has been a sustained interest in using biocontrol methods to reduce or inactivate pathogens on sprouts *(22, 23, 27, 28, 35)*. Not only can biocontrol microbes inhibit the growth of human pathogens, but they can also provide a biological buffer to prevent contamination introduced during the sprouting. To date, biocontrol strategies have been principally involved using antagonistic bacteria *(35)*. Lactic acid bacteria exhibiting antagonistic activity against *Salmonella, E. coli* O157:H7, and *Listeria monocytogenes* are frequently encountered on sprouted seeds *(36)*. Matos and Garland *(35)* used an undefined mixed culture of bacteria directly recovered from alfalfa sprout batches, which was coinoculated onto seeds along

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with *Salmonella*. The researchers reported a >5-log CFU/g reduction in *Salmonella* counts on the final alfalfa sprouts after a 7-day sprouting period. The same group, in addition to others, demonstrated that *Pseudomonas fluorescens* 2-79 (strain originally isolated from the rhizosphere of wheat) coinoculated with *Salmonella* onto alfalfa seeds could also reduce, but not eliminate, populations of the enteric pathogen on the subsequent sprouts *(17, 30, 35)*.

There has also been interest in applying lytic bacteriophages to control human pathogens on foods including fresh produce and sprouts *(6, 19, 23, 27, 28)*. However, studies to date using bacteriophages to control pathogens on sprouted seeds have met with limited success. For example, Kocharunchitt et al. *(28)* coinoculated bacteriophages with *Salmonella* onto alfalfa seed and reported only a 1-log CFU/g reduction of the pathogen on the subsequent sprouts. Pao et al. *(42)* also reported comparable reductions of *Salmonella* when bacteriophages were coinoculated onto mustard seeds. The limited efficacy of bacteriophages to suppress the growth of pathogens on sprouts is unclear, but has been proposed to be through the natural equilibrium that is reached between host cell and phages, which ensures survival of both *(23, 27)*. In addition, the binding of phages to plant material, presence of natural antimicrobial constituents, and generation of resistant mutants has also been highlighted *(42)*.

From studies to date, it is apparent that biocontrol bacteria and bacteriophages used independently can reduce, but not eliminate, *Salmonella* growth on sprouting seeds. However, little work has been undertaken to evaluate the efficacy of combining antagonistic bacteria and bacteriophages to control pathogen growth. Hong and Conway *(26)* reported the synergistic activity of co-inoculating honeydew melon with lytic bacteriophages and a *Gluconobacter asai* strain to control the growth of *L. monocytogenes*. Here, the authors reported *G. asai* applied alone reduced *Listeria* populations by 3 to 4 and bacteriophages by 1. However, when combined, the final *L. monocytogenes* populations were 6 log CFU/g lower compared with control samples *(26)*.

The objective of the following study was to determine the efficacy of combining antagonistic bacteria with lytic bacteriophage to control the growth of *Salmonella* on sprouting seeds. In a previous study, it was noted that tomato fruit derived from plants inoculated with *Salmonella* but that tested negative for the pathogen typically harbored *Bacillus* and *Enterobacter* spp. as part of microflora *(46)*. The result suggested that *Bacillus* and *Enterobacter* might have strong antagonistic activity against *Salmonella,* and at the same time adapted to become established on or within plants. Hence, in the following study, focus was placed at isolating *Bacillus* and *Enterobacter* spp. with antagonistic activity against *Salmonella*.

MATERIALS AND METHODS

Bacteria and preparation of cell suspensions. The *Salmonella enterica* used in the study included serovars Agona, Berta, Enteritidis, Hadar, Heidelberg, Javiana, Montevideo, Muenchen, Newport, Saint Paul, and Typhimurium DT104, all of which were kindly donated by the Public Health Agency of Canada–Guelph (Ontario). The serovars were selected based on previous association with fresh produce outbreaks or of clinical significance. *Salmonella* suspensions were prepared by cultivating individual serovars in 50 ml of tryptic soy broth (TSB; Oxoid, Ltd., Basingstoke, UK) at 37°C for 24 h. The cells were harvested by centrifugation (5,500 × *g* for 10 min at 4°C) and washed once in phosphate-buffered saline (PBS). The pellet was resuspended in PBS to give an optical density (OD) at 600 nm of 0.2 (ca. 7.0 log CFU/ml).

Isolation of antagonistic bacteria. Bacteria exhibiting antagonistic activity against *Salmonella* were isolated from mung bean sprouts ($n = 10 \times 25$-g batches) or tomatoes ($n = 20 \times$ fruit units) purchased from a local supermarket. Batches of sprouts or tomato fruit were suspended in 30 ml of peptone water (0.1% [wt/vol]) and stomached (Seaward, London, UK) at 230 rpm for 30 s. The homogenates were incubated at 30°C for 24 h prior to diluting and plating onto Hektoen enteric agar (Oxoid, Ltd.) plates that were subsequently incubated at 30°C for 24 h. Typical *Enterobacter* colonies (yellow to rose salmon) were subcultured onto tryptic soy agar (TSA) slants, and then incubated at 30°C prior to holding at 4°C until required.

Bacillus spp. were isolated by heating the sample homogenates at 70°C for 10 min, and then incubated at 30°C for 48 h. A dilution series were plated onto *Bacillus cereus* agar (Oxoid, Ltd.) plates, which was incubated at 37°C for 24 h. Colonies were randomly selected and subcultured onto TSA slants, as described above.

Deferred assay. The deferred assay was performed as described by Fett *(17)*. The test bacteria were inoculated into 10 ml of TSB and cultivated overnight at 30°C. Aliquots (10 μl) of the test bacterium culture were spotted onto two TSA plates, which were incubated at 30°C for 2 days. One of the TSA plates was exposed to chloroform vapor for 1 h at room temperature in a fume hood. After exposure, the plate was overlaid with 5 ml of soft agar (TSB with 2.5% [wt/vol] agar), which was inoculated with 100 μl of an overnight culture of *Salmonella* Typhimurium DT104. The agar was allowed to set at room temperature prior to incubation at 37°C for 24 h. Anti-*Salmonella* activity was visualized by zones of inhibition (>10 mm) on the agar plates. When antagonistic activity was detected in an isolate, the corresponding colony on the non-chloroform–treated plate was transferred to 1 ml of nutrient broth (Oxoid, Ltd.) containing 20% (wt/vol) glycerol solution, and then stored at −80°C. Selected isolates exhibiting antagonistic activity were evaluated in terms of inhibiting a range of different *Salmonella* serovars (Agona, Berta, Enteritidis, Hadar, Heidelberg, Javiana, Montevideo, Muenchen, Newport, and Saint Paul). Here, the deferred assay was performed as described above, with *Salmonella* Typhimurium DT104 being substituted with the test serovar.

16S rRNA typing. Selected isolates were taken for 16S rRNA analysis. Here, the isolate was propagated in 10 ml of TSB, and aliquots (1 ml) taken forward for DNA extraction with the QIAamp DNA Stool Mini Kit (QIAGEN, Mississauga, Ontario, Canada). The V2–V3 region of the 16S rRNA genes was amplified with primers HDA1-GC (5′-**CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGG**ACTCCTACGGGAGGCAGCAGT-3′; the GC clamp appears in boldface font) and HDA2 (5′-GTATTACCGCGGCTGCTGGCAC-3′) *(29)*. The reaction mixture (25-μl total volume) consisted of PCR reaction buffer (1×), 1.5 mM $MgCl_2$, a 0.4 mM deoxynucleoside triphosphate mix, 10 pM primer, 50 ng of template DNA, and 2 U of *Taq* DNA

polymerase (Boehringer Mannheim, Mannheim, Germany). PCR was performed in 0.2-ml tubes in a DNA Engine Peltier thermal cycler (PTC-200, Genetic Technologies, Inc., Miami, FL). The thermal cycle consisted of an initial denaturation step at 94°C for 4 min, and then 30 cycles of 94°C for 30 s, 56°C for 30 s, and 72°C for 60 s, with a final extension at 72°C for 10 min.

Denaturing gradient gel electrophoresis (DGGE) analysis of the PCR amplicons was performed with the DCode universal detection system (Bio-Rad Canada, Mississauga, Ontario), as described previously *(51)*. DGGE was performed with 10% [wt/vol] acrylamide gels containing a 35 to 65% gradient of urea and formamide, which increased as electrophoresis progressed. Electrophoresis was conducted in Tris-acetate-EDTA buffer at 100 V, for 18 h at 60°C. DNA bands in gels were visualized by silver staining according to the methods of Van Orsouw et al. *(49)*.

Sequence analysis of the generated band was performed by initially excising the band from the gel, and with the extracted DNA as a template for a further round of PCR amplification and separation on a DGGE gel, as previously described. The band was then excised and extracted with the QIAquick gel extraction kit (QIAGEN), following the manufacturer's instructions. The recovered DNA served as a template for PCR reaction with the HAD primers but without the GC clamp. The products of the PCR reaction were then sequenced by the Laboratory Services Division of the University of Guelph (Guelph, Ontario, Canada). The partial sequences were compared directly with nonredundant nucleotides in the GenBank database by using the Basic Local Alignment Search Tool algorithm hosted by the National Center for Biotechnology Information (Bethesda, MD).

Construction of bioluminescent *E. asburiae* JX1. The isolated *E. asburiae* was genetically modified with antibiotic resistance and a bioluminescence marker to facilitate identification and enumeration. The mini-Tn*5* plasmid (kindly donated by Dr. P. Hill, University of Nottingham, UK) containing the *luxCDABE* gene and a kanamycin resistance gene cassette was maintained within *E. coli* λPir1. Competent *E. coli* S17-1 cells were transformed with a purified plasmid preparation as described by Winson et al. *(55)*. Conjugation between *E. coli* S17-1λpir and recipient cells (*E. asburiae* JX1) was performed on a Whatman cellulose acetate membrane (pore size of 0.45 μm; Thermo-Fisher, Whitby, Ontario, Canada) overlaid onto a Luria-Bertani agar plate, which was subsequently incubated for 14 h at 37°C *(12)*. The selection of *E. asburiae* JX1 exconjugants was based on plating aliquots (50 to 200 μl) of the mating mixture onto Hektoen enteric agar containing kanamycin (30 μg/ml), and incubation overnight at 37°C. Bioluminescent colonies on agar plates were imaged with a Night-Owl image analyzer in conjunction with the manufacturers computer software (E.G. & G. Berthold, Munich, Germany). For automated luminometry and photometry, the Victor 1420 multilabel counter (Perkin-Elmer Instruments, Waltham, MA) and associated software were used. Exconjugants were inoculated into TSB contained within a 96-well microtiter plate, and growth, in addition to bioluminescent expression, was monitored over a 24-h period at 30°C.

Anti-*Salmonella* factor in spent culture medium. Isolates demonstrating anti-*Salmonella* activity were cultured in 40 ml of TSB for 48 h at 30°C. Cells were then removed by centrifugation (5,500 × *g* for 10 min), and the supernatant filter was sterilized by passing it through a membrane with a 0.45-μm pore size. The spent supernatant was diluted in TSB to give a dilution series containing 0 to 50% (vol/vol). Aliquots (180 μl) of the different media were dispensed into a 96-well microtiter plate, and individual wells inoculated with 10 μl of an overnight culture of *Salmonella* Typhimurium DT104. The OD_{450} of each of the wells was measured over 24 h by using a microtiter plate reader (Bio-Rad) maintained at an incubation temperature of 30°C.

Isolation of *Salmonella*-infecting bacteriophages. Effluent lagoon or water samples (450 ml) were collected from 13 pig farms within southern Ontario or from five Alberta beef lot farms. Effluent samples were added to 450 ml of concentrated (2 ×) Luria-Bertani broth (Difco, Becton Dickinson, Sparks, MD) with 10 mM $MgSO_4 \cdot 7H_2O$ (LBM broth), inoculated with 10 ml of *Salmonella* Typhimurium DT104 or *Salmonella* Montevideo culture, and incubated overnight at 37°C, with gentle agitation. Aliquots (10 ml) of the enriched culture were dispensed into a sterile tube containing 0.5 ml of chloroform (Sigma-Aldrich, Oakville, Ontario, Canada) and centrifuged at 2,600 × *g* for 20 min at 4°C. The supernatant was filtered through membrane filters with a pore size of 0.45 μm (Thermo-Fisher), and aliquots (0.1 ml) of the filtrate were transferred to 5 ml of molten soft LBM agar (LBM broth supplemented with 0.5% [wt/vol] agar; Oxoid, Ltd.) inoculated with 0.1 ml of an overnight culture of *Salmonella* Typhimurium DT104 or Montevideo. The contents of the tube were then poured onto the surface of an LBM plate and incubated overnight at 37°C. Plaques (if any) were excised from the LBM plates and transferred to a microcentrifuge tube containing 1 ml of sorbitol–morpholine ethanesulfonic buffer (composition per milliliter was 5.8 mg of NaCl, 2 mg of $MgSO_4 \cdot 7H_2O$, 50 mM Tris [pH 7.5], and 0.1 mg of gelatin) prior to storing at 4°C until required.

Host range specificity was determined by preparing a cell lawn of the test *Salmonella* serovar (Javiana, Montevideo, Heidelberg, Typhimurium, Montevideo, or Newport) on LBM plates. Aliquots (10 μl) of filtered (0.22-μm syringe filter) phage were spotted onto the cell lawn, and plates were incubated overnight at 37°C. The appearance of plaques confirmed the susceptibility of the *Salmonella* serovar to the phage.

Phage stocks of selected isolates were prepared by introducing the appropriate bacteriophage into 50 ml of LBM broth along with 0.1 ml of overnight culture of *Salmonella* Typhimurium DT104, and then incubating them for 24 h at 37°C. Chloroform (0.5 ml) was added to the culture, which was subsequently centrifuged (2,600 × *g* at 4°C for 20 min) and the supernatant filtered through a 0.4-μm-pore-size filter. The phage titer was determined by preparing a dilution series of the filtrate, and spotting 10 μl onto cell lawns of *Salmonella* Typhimurium DT104. The plaques that developed after overnight incubation at 37°C were enumerated, and the phage titer reported as PFU per milliliter.

Suppression of *Salmonella* growth in broth culture by using a combination of bacteriophages and biocontrol bacteria. *Salmonella* Typhimurium DT104 suspensions were inoculated into 10 ml of TSB to a final cell density of either 2 or 3 log CFU/ml, along with the test biocontrol bacterium (2 or 3 log CFU/ml). A bacteriophage cocktail was prepared by mixing equal concentrations (9 log PFU/ml) of the selected six phages. The bacteriophage cocktail was introduced into the culture to give a multiplicity of infection (MOI) of 0.1, 1, or 100. Aliquots (1 ml) were withdrawn from the cultures to verify the initial microbiological levels. The cultures were incubated for 48 h at 30°C, and *Salmonella* levels were enumerated by preparing a dilution series that was subsequently plated onto xylose-lysine-Tergitol 4 (XLT-4; Oxoid, Ltd.) and incubated at 37°C for 24 h.

Suppression of *Salmonella* on sprouting mung beans and alfalfa by using a combination of biocontrol bacteria and bacteriophages. Mung beans (*Vigna radiata*) were donated

by a local sprout producer, and alfalfa (*Medicago sativa*) seeds were purchased from Mumms Seeds, Ltd. (Parkside, Saskatchewan, Canada). Beans (250 g) or seeds (100 g) were soaked in 250 ml (6 log CFU/ml) of *Salmonella* cocktail for 20 min. The *Salmonella* cocktail was prepared from overnight cultures of serovars Agona, Berta, Enteritidis, Hadar, Heidelberg, Javiana, Montevideo, Muenchen, Newport, Saint Paul, and Typhimurium DT104. The cell pellet of individual cultures was resuspended in PBS to an OD_{600} of 0.2, and then combined in equal volumes to form the cocktail that was subsequently diluted to give 6 log CFU/ml.

The beans and seeds inoculated with *Salmonella* were then transferred to a 250-ml suspension of *E. asburiae* JX1 (6 log CFU/ml) and bacteriophage cocktail (6 log PFU/ml). After soaking for 20 min, the beans and seeds were transferred to sterile filter paper within a biological safety cabinet, and allowed to dry overnight at ambient temperature.

Batches (20 g) of inoculated mung beans were placed into a 500-ml container, and soaked in 200 ml of distilled water at 20, 25, or 30°C, for 24 h. The water was removed, and sprouting continued for a further 4 days, with daily watering by a 5-min soak in 300 ml of distilled water.

Alfalfa seeds (20-g batches) were spread onto saturated paper towels on plastic trays. The seeds were sprouted at 25°C, with periodic watering with distilled water.

Sprouts (mung bean or alfalfa) were weighed to determine the yield, with two 25-g batches being taken forward for microbiological testing. The sprout samples were suspended in 225 ml of 0.1% peptone water and stomached for 60 s. A dilution series was prepared from the homogenate and plated onto the appropriate agar. For *E. asburiae* JX1, dilutions were spread plated onto TSA containing 30 μg/ml kanamycin, which was incubated at 30°C for 48 h. *Salmonella* was enumerated on XLT-4 agar incubated at 37°C for 24 h. The sample homogenate was stored at 4°C and subsequently transferred to 37°C for enrichment in the event that no *Salmonella* colonies appeared on plates. After 24 h of incubation, an aliquot (0.1 ml) of the enriched culture was then inoculated into the center of a semi-solid Rappaport-Vassiliadis plate (Oxoid, Ltd.) that was subsequently incubated at 42°C for 24 h. Cells from the outer perimeter of the growth halo (presumptive motile *Salmonella*) were streaked onto XLT-4 agar, and incubated at 37°C overnight. The Oxoid *Salmonella* latex agglutination test was used to confirm the identification of presumptive colonies.

16S rDNA DGGE analysis. Mung bean sprouts derived from seeds inoculated with *Salmonella* cocktail, *E. asburiae* JX1, or six-strain cocktail of bacteriophages and combinations thereof were submerged in 225 ml of peptone water prior to stomaching for 60 s. DNA extraction, PCR, and DGGE was then performed as described above.

Experimental design and statistical analysis. All experiments were repeated at least three times by using duplicate samples in each trial. Bacteria or bacteriophage counts (CFU per milliliter, CFU per gram, or PFU per milliliter) were transformed to log units (log CFU per milliliter, log CFU per gram, or log PFU per milliliter). Means generated were analyzed by analysis of variance and the Tukey test, with the level of significance set at $P < 0.05$.

RESULTS

Isolation and characterization of antagonistic bacteria. Mung bean sprout or tomato isolates (693 presumptive *Enterobacter* and 144 presumptive *Bacillus*) screened for antagonistic activity by using serovar Typhimurium DT104 as the test *Salmonella* identified 2 isolates (derived from mung bean sprouts) that produced zones of inhibition >10 mm. By 16S rRNA sequencing, the isolates were identified as *Enterobacter cancerogenus* and *Enterobacter asburiae*. *E. cancerogenus* is an opportunistic human pathogen that is typically implicated in urinary tract and wound infections *(5)*; hence, it is unsuitable as a biocontrol agent. However, *E. asburiae* is commonly associated with plants, and it has been used as a biocontrol strain for inhibiting the growth of enteric pathogens such as *Salmonella* and *E. coli* O157:H7 *(11)*.

The *E. asburiae* JX1 strain isolated in the current study demonstrated antagonistic activity against a broad range of *Salmonella* serovars (Agona, Berta, Enteritidis, Hadar, Heidelberg, Javiana, Montevideo, Muenchen, Newport, Saint Paul, and Typhimurium DT104) producing zones of inhibition >10 mm with the deferred assay. The anti-*Salmonella* activity of *E. asburiae* JX1 was stable and was retained after 10 successive subcultures onto TSA.

Studies were undertaken to determine the mode by which *E. asburiae* JX1 inhibited the growth of *Salmonella* Typhimurium DT104. Spent culture media from *E. asburiae* JX1 cultures were extracted, filter sterilized, and then supplemented into TSB inoculated with *Salmonella*. No significant ($P > 0.05$) decrease in final cell densities of *Salmonella* Typhimurium DT104 compared with controls was observed even when introduced at 50% (vol/vol) (results not shown). This would suggest that either the antimicrobial agent(s) was/were not produced or they were released into the medium during planktonic growth.

Isolation of *Salmonella*-infecting virulent bacteriophages. *Salmonella*-infecting bacteriophages were isolated from manure effluent sampled from pig or feedlot farms. From the 858 phage isolates recovered, a cocktail was selected based on a broad host range against a panel of different *Salmonella* serovars (Table 1). It was found that no single phage exhibited strong lytic activity against all of the *Salmonella* challenged, with none of the isolates recovered from pig farms able to infect *Salmonella* Montevideo (Table 1). However, two phages (designated FL38 and FL 41) originating from an Alberta feedlot did exhibit lytic activity against the serovar (Table 1).

A cocktail of the six selected bacteriophages (F01, P01, P102, P700, P800, and FL 41) was prepared and evaluated for controlling *Salmonella* in broth culture or sprouting seeds.

Control of *Salmonella* Typhimurium DT104 in broth culture by *E. asburiae* JX1 and bacteriophage cocktail. *Salmonella* was coinoculated with *E. asburiae* JX1 at different ratios into 10 ml of TSB, and then incubated at 30°C for 48 h. The *Salmonella* levels were subsequently enumerated along with the total aerobic count. No significant ($P > 0.05$) difference was found in the *Salmonella* counts in the presence or absence of *E. asburiae* JX1, irrespective of the ratio applied (Fig. 1). In a similar manner, when *Salmonella* was coinoculated with bacteriophages at different MOI (0.1 to 100), no significant ($P >$

TABLE 1. *Host range of selected* Salmonella*-infecting bacteriophages isolated from pig farms or feedlot manure effluent*[a]

Serovar	Phage isolate[b]: F01	P01	P102	P700	P800	FL38	FL41
Javiana	+	+	+	+	+	ND	ND
Heidelberg	+	+	+	+	+	ND	ND
Typhimurium	+	+	+	+	+	ND	ND
Newport	+	−	−	+	−	ND	ND
Montevideo	−	−	−	−	−	+	+

[a] Isolated phages were purified and spotted onto cell lawns of the test *Salmonella* serovar. The plates were incubated at 37°C for 24 h, and plates were visually inspected for host cell lysis (plaques).

[b] +, susceptible to bacteriophage (plaque formation); −, resistant to bacteriophage (no plaque formation); ND, not detected.

0.05) decrease in pathogen levels was observed compared with when no phages were applied. However, when *E. asburiae* JX1 and bacteriophages (MOI = 100) were applied in combination, a significant ($P < 0.05$) reduction (5.7 to 6.4 log CFU/ml) in the final *Salmonella* levels was observed (Fig. 1). However, none of the treatments applied totally eliminated *Salmonella,* and residual populations persisted (Fig. 1).

Control of *Salmonella* on sprouting mung bean sprouts by using a combination cocktail of *E. asburiae* JX1 and bacteriophage. Mung bean batches were steep inoculated with a cocktail of *Salmonella* consisting of serovars Agona, Berta, Enteritidis, Hadar, Heidelberg, Javiana, Montevideo, Muenchen, Newport, Saint Paul, and Typhimurium DT104. A proportion of the inoculated beans were then transferred to a suspension of an *E. asburiae* JX1 and bacteriophage cocktail. The inoculated beans were then sprouted at different temperatures over 4 days, with periodic irrigation with distilled water.

There was no significant ($P > 0.05$) difference in the yield of mung bean sprouts or visible appearance of sprouts derived from the different treatments. Also, no significant ($P > 0.05$) differences were observed with regard to the total aerobic counts (TAC) of sprout; lots indicated that the inclusion of *E. asburiae* JX1 and/or bacteriophage did not affect the total microbial populations attained on sprouts (Table 2). *Salmonella* grew to high levels on sprouting mung beans in the absence of *E. asburiae* JX1 and/or bacteriophages (Table 2). However, coinoculation of *E. asburiae* JX1 or bacteriophage cocktail with *Salmonella* resulted in a significant ($P < 0.05$) reduction in levels of the enteric pathogen (Table 2). There were no significant ($P > 0.05$) differences between the degrees to which *Salmonella* levels were decreased with bacteriophage compared to *E. asburiae* JX1. However, when used in combination, there was an additive effect with *Salmonella* only being detectable by enrichment (Table 2). *E. asburiae* JX1 grew to high levels on sprouting mung beans and was not significantly ($P > 0.05$) different compared with the TAC. The result would suggest that the *E. asburiae* JX1 formed the predominant bacterium associated with the sprouted mung beans. The combination of *E. asburiae* JX1 and bacteriophages cocktail was equally effective at different sprouting temperatures (20 to 30°C), temperatures typically encountered in commercial sprout operations (results not shown).

Via 16S rDNA DGGE, it was apparent that the diversity of microbial populations was relatively low, with only a small number of bands being observed (Fig. 2). The band corresponding to *Salmonella* was found in all the sprout samples except for the noninoculated controls and

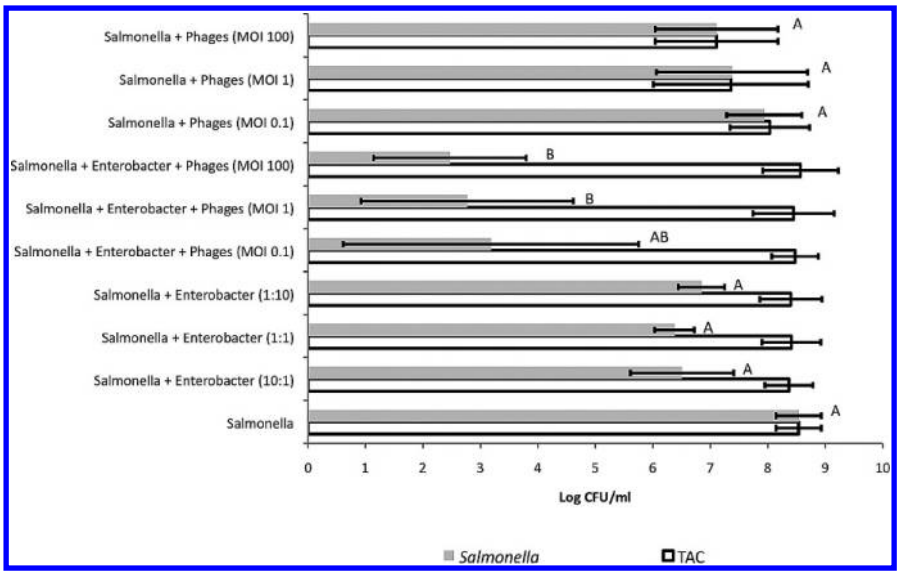

FIGURE 1. *Effect of* Enterobacter asburiae *and bacteriophage cocktail on* Salmonella *Typhimurium DT104 growth in broth culture.* The Salmonella, E. asburiae *JX1, and bacteriophage cocktail was inoculated (2 to 3 log CFU/ml) into TSB at different ratios and incubated at 30°C for 24 h.* Salmonella *and total aerobic counts (TAC) were then enumerated as described in ''Materials and Methods.''* Salmonella *counts followed by the same letter are not significantly (*P > 0.05*) different.*

TABLE 2. *Control of* Salmonella *growth on sprouting mung beans by the coinoculation of* Enterobacter asburiae *JX1 and bacteriophage cocktail, either alone or in combination*[a]

Treatment	Wt of sprouts (g)[b]	TAC	*E. asburiae* JX1 (log CFU/g)	*Salmonella* (log CFU/g)
Negative control	78.20 ± 7.43 A[c]	8.15 ± 0.91 A	ND A[d]	ND A
Salmonella	69.98 ± 7.96 A	8.15 ± 0.46 A	ND A	6.72 ± 0.78 B
Salmonella + *E. asburiae*	72.43 ± 4.87 A	8.48 ± 0.56 A	8.14 ± 0.60 B	1.16 ± 2.14 C
Salmonella + phage	70.60 ± 8.27 A	8.52 ± 0.44 A	ND A	3.31 ± 2.48 C
Salmonella + *E. asburiae* + phage	71.33 ± 9.06 A	8.98 ± 0.41 A	7.91 ± 0.16 B	Positive D[e]

[a] Mung beans were inoculated with a *Salmonella* cocktail along with *E. asburiae* JX1 and bacteriophages. The mung beans were sprouted over a 4-day period at 25°C, and levels of total aerobic count, *E. asburiae* JX1, and *Salmonella* were determined in addition to the yield.
[b] Yield of sprouts per 25 g seeds of sprouted.
[c] Means followed by the same letter within columns are not significantly different.
[d] ND, not detected (negative by enrichment; 25-g sprout sample).
[e] Positive by enrichment (25-g sprout sample).

sprouts derived from beans inoculated with *E. asburiae* JX1. Two bands were common (indicated by the arrows in Fig. 2) to all sprout samples, indicating that the introduction of *E. asburiae* JX1 and bacteriophages does not extensively alter the sprout microflora.

Control of *Salmonella* on sprouting alfalfa by using a combination cocktail of *E. asburiae* JX1 and bacteriophage. *E. asburiae* JX1 and bacteriophages were coinoculated along with *Salmonella* onto alfalfa sprouts, which were subsequently sprouted on trays over a 4-day period. With sprouts derived from seeds inoculated with *Salmonella* alone, the counts of the enteric pathogen attained levels in the order of 7.62 ± 0.21 log CFU/g (Table 3). However, in the presence of *E. asburiae* JX1 and bacteriophages, no *Salmonella* was recovered even when samples (25-g batches) were enriched (Table 3). The TAC of sprouts did not differ between the sprout batches and were insignificantly ($P > 0.05$) different compared with *E. asburiae* JX1 levels. Similar to mung bean sprouts, no significant ($P > 0.05$) difference was observed in the yield of sprouts or visual appearance.

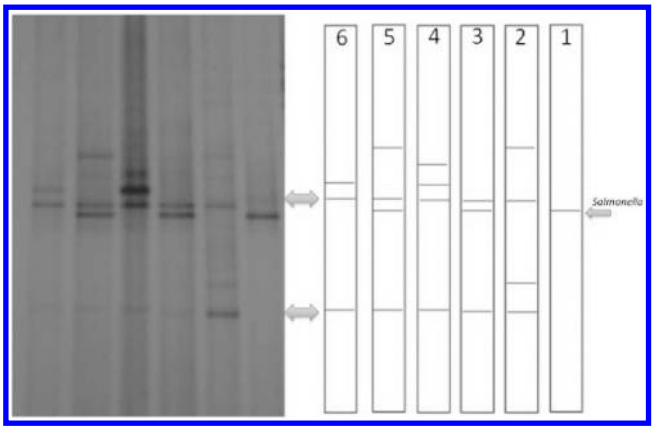

FIGURE 2. *Representative 16S rDNA DGGE profiles of bacterial populations of mung bean sprouts. Profiles represent 1,* Salmonella*; 2, noninoculated control; 3, sprouts derived from mung beans inoculated with* Salmonella *cocktail; 4, sprouts derived from beans inoculated with* Salmonella *and* Enterobacter asburiae *JX1; 5, sprouts derived from beans inoculated with* Salmonella *and bacteriophage cocktail; 6, sprouts derived from beans inoculated with* Salmonella *cocktail, bacteriophages, and* Enterobacter asburiae *JX1. Double-headed arrows indicate common bands found in all sprout samples. Single-headed arrow indicates the band corresponding to* Salmonella.

DISCUSSION

From the *Bacillus* and *Enterobacter* isolates screened, only two of the latter exhibited anti-*Salmonella* activity. Cooley et al. *(11)* also reported that a strain of *E. asburiae* isolated from soil-grown *Arabidopsis thaliana* was effective in inhibiting the growth of *Salmonella* and *E. coli* O157:H7 on growing plants. The mode by which *E. asburiae* JX1 inhibited the growth of *Salmonella* remains unclear. The finding that spent supernatant derived from *E. asburiae* JX1 cultures did not affect the growth of *Salmonella* Typhimurium DT104 in broth culture would suggest that the antimicrobial agent(s) ''such as bacteriocins (cloacins)'' was/were not released into the growth medium *(21)*. The anti-*Salmonella* phenotype of *E. asburiae* JX1 was found to be stable, which would suggest that it was not encoded on plasmids, which is typical for bacteriocin-producing bacteria *(4, 13)*. Cooley et al. *(10)* proposed that the antagonistic activity of *Enterobacter* was through direct competition for nutrients. Given the close physiological similarities between *Salmonella* and *Enterobacter,* this could indeed be the case with *E. asburiae* JX1 *(32)*. The fact that *E. asburiae* JX1 increased to high levels on sprouted seeds would also support the competitive exclusion hypothesis *(36)*. Yet, the fact inhibition zones were observed in the deferred assay would strongly indicate the release of antimicrobial factor(s) from *E. asburiae* JX1, but not evidently in broth culture.

Manure effluent from pig farms proved a rich source of *Salmonella*-infecting bacteriophages. This is in agreement with other researchers who have also reported high prevalence of *Salmonella*-infecting bacteriophages on pig farms *(38)*. However, despite the high number of isolates recovered, none could infect *Salmonella* Montevideo. Yet, a Montevideo-infecting bacteriophage was recovered from an Alberta feedlot farm. It is noteworthy that *Salmonella* Montevideo is highly prevalent within Alberta feedlot

TABLE 3. *Control of* Salmonella *growth on sprouting alfalfa sprouts by the coinoculation of* Enterobacter asburiae *JX1 and bacteriophage cocktail either alone or in combination*[a]

Treatment	Wt of sprouts (g)[b]	TAC	*E. asburiae* JX1 (log CFU/g)	*Salmonella* (log CFU/g)
Negative control	91 ± 11.95 A[c]	8.11 ± 0.09 A	<1.70 A	ND A[d]
Salmonella	102.5 ± 10.27 A	7.89 ± 0.23 A	<1.70 A	7.62 ± 0.21 B
Salmonella + *E. asburiae* + phages	104 ± 10.14 A	8.41 ± 0.27 A	8.40 ± 0.15 B	ND A

[a] Alfalfa seeds were inoculated with a *Salmonella* cocktail along with *E. asburiae* JX1 and bacteriophages. The alfalfa seeds were sprouted on trays over a 4-day period at 25°C and levels of total aerobic count, *E. asburiae* JX1, and *Salmonella* were determined, in addition to the sprout yield.

[b] Yield of sprouts per 25 g of seeds sprouted.

[c] Means followed by the same letter within columns are not significantly different.

[d] ND, not detected (negative by enrichment).

farms, which likely increased the probability of isolating infecting bacteriophages *(47)*.

Because of the different host specificities, it was necessary to use a cocktail of phage isolates *(27)*. In addition, by using a combination of bacteriophages, the potential of generating resistant mutants is also reduced *(27)*. However, despite using a cocktail of bacteriophages, there was only limited efficacy of reducing the levels of *Salmonella* in broth culture. This is in agreement with reports from other researchers who found that phages can retard the growth of *Salmonella,* although they cannot eliminate the pathogen unless the host is in low numbers and MOI is high *(3, 7, 24)*. In this case, the host cell membrane is destabilized by the attachment of multiple phages on the surface, leading to loss of viability, but without bacteriophage replication in a process termed inundation or lysis from without *(3)*. However, in natural environments, equilibrium between host and phage occurs, thereby ensuring mutual coexistence *(19)*. The emergence of phage-resistant mutants is a further aspect that can limit the efficacy of bacteriophages, although this is relatively rare within the natural environment *(1, 6, 8, 15, 23)*.

Bacteriophages have been used to control *Salmonella* on sprouting alfalfa seeds, with limiting success. In one report, the application of bacteriophage to seeds inoculated with *Salmonella* Oranienburg resulted in a 1-log CFU/g reduction *(28)*. The lack of *Salmonella* reduction could not be attributed to the generation of resistant mutations, although the researchers did note a change in colony morphology, suggesting a temporal physiological adaption of *Salmonella* in the presence of infecting phages *(28)*.

Similar to bacteriophages, *E. asburiae* JX1coinoculated with *Salmonella* in broth culture could only reduce the final populations of *Salmonella,* possibly due to the production of antimicrobial factors. Other researchers have also reported the limited efficacy of biocontrol bacteria to eradicate *Salmonella*. For example, Liao *(30)* reported that a *Bacillus* strain or *P. fluorescens* 2-79 could reduce the growth of *Salmonella,* in addition to other human pathogens, by up to 3 log CFU/g on bell peppers, provided the antagonistic bacterium was introduced at a concentration >100× that of the enteric pathogen. *P. fluorescens* 2-79 coinoculated along with *Salmonella* onto alfalfa seeds reduced the final populations of the subsequent sprouts to 3.68 log CFU/g, although it could not eliminate the pathogen.

The additive anti-*Salmonella* activity of *E. asburiae* JX1 and bacteriophage cocktail on sprouts and within broth culture is in agreement with the findings of Hong and Conway *(26),* who worked with listeriophages and *Gluconobacter* to control the growth of *L. monocytogenes* on honeydew melon. It was noteworthy that the combination of *E. asburiae* JX1 and bacteriophages was more effective at inhibiting the growth of *Salmonella* on sprouts compared with in-broth culture. This could be attributed to the additional competitive inhibition of *Salmonella* by the endogenous microflora of sprouts and possibly the greater production of anti-*Salmonella* factor(s) from *E. asburiae* JX1.

The specific mode by which the antagonistic bacteria and bacteriophages collectively inhibit pathogen growth remains unclear. It is possible that by coinoculating antagonistic bacteria, the physiological response of *Salmonella* to phage infection is blocked, thereby making cells more susceptible. A further possibility is the antimicrobial agent expressed by *E. asburiae* JX1 enhances the lytic ability of the bacteriophages. Such a mechanism has been proposed to explain the enhanced antilisterial activity for bacteriocin and bacteriophages combinations. Here, the bacteriocin weakens the cell membrane, thereby enhancing the activity of phage endolysin *(14)*. However, it would be inappropriate to speculate on the underlying mechanism for the additive action, but clearly it is worthy of further study.

The efficacy of the biocontrol preparation worked on both alfalfa and mung bean sprouts, suggesting the treatment could be potentially applied on a diverse range of sprout types. Importantly, the anti-*Salmonella* activity of *E. asburiae* JX1 and bacteriophages was independent of the sprouting temperature (between 20 and 30°C), the range commonly applied in commercial sprout production *(2)*. It has been reported that the infectivity and replication of bacteriophages is temperature dependent *(43, 45, 50)*. This is in part due not only to the need for the host cell to be metabolically active, but also due to the conformation of the phage nucleic acid during the infection process *(19, 50)*. Yet, in the current study, it was evident that such effects did not detrimentally affect the antagonistic action of the biocontrol preparation.

The results from the DGGE analysis agree with those reported by others with respect to the bacterial community associated with sprouts being of relatively low diversity *(33)*. The coinoculation of bacteriophages with *Salmonella* on beans did not have a significant effect on the diversity of bacterial populations on the subsequent sprouts, thereby confirming specificity to *Salmonella*. *E. asburiae* JX1 also did not grossly affect the diversity of populations and was the predominant bacterium making up the microflora of sprouts, given that counts were not significantly different from the TAC. It was interesting to note that the 16S rDNA profiles of sprouts derived from beans inoculated with *Salmonella* and *E. asburiae* JX1 did not contain a band corresponding to the pathogen. This was likely due to the low levels of *Salmonella*-associated sprouts in the presence of the antagonistic bacterium. The inclusion of bacteriophages with *E. asburiae* JX1 resulted in a similar profile when the latter was coinoculated with *Salmonella* alone.

In conclusion, the study has demonstrated that the combination of bacteriophages and *E. asburiae* JX1 collectively acts to inhibit the growth of *Salmonella* on sprouting seeds. By using the combined approach, the limited efficacy of using bacteriophages and antagonistic bacteria alone can be overcome to provide a more effective biocontrol strategy. Such an approach has strong potential as an effective intervention to enhance the microbiological safety of sprouts.

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