

# Seeds of the Wild Progenitor of Maize Possess Bacteria That Antagonize Foodborne Pathogens

Hanan R. Shehata,<sup>1,2</sup> Mansel W. Griffiths,<sup>3,4</sup> and Manish N. Raizada<sup>1</sup>

## Abstract

Endophytes are microorganisms that inhabit plant tissues without causing disease. Some endophytes help their hosts to combat pathogens. Here we explored the hypothesis that the plant-derived foods consumed by humans and other animals host endophytes that also antagonize foodborne pathogens or food-rotting agents. Our laboratory previously cultured a library of bacterial endophytes from different members of the maize/corn family (*Zea*) including wild relatives. Here, 190 of these endophytes were screened for their ability to antagonize four foodborne pathogens (*Escherichia coli* O157:H7, *Listeria monocytogenes*, *Clostridium perfringens*, and *Salmonella enterica* Newport) and a food spoiling agent (*Pseudomonas fluorescens*) using dual culture assays. Two *Paenibacillus polymyxa* endophytes (strains 3C6 and 3G11) were found to inhibit the growth of all five deleterious strains on agar. Using conserved polymerase chain reaction primers and sequencing, both beneficial endophytes were found to encode polymyxin genes, suggesting a potential antibacterial mechanism of action. Polymyxin production by both strains was confirmed using enzyme-linked immunosorbent assay. Strains 3C6 and 3G11 originated, respectively, from the seeds of the wild Central American maize species *Zea diploperennis*, and the wild ancestor of modern maize, *Zea mays* ssp *parviglumis* (Parviglumis). As the latter is the direct ancestor of modern maize, we discuss the role its endophyte(s) may have played in promoting crop domestication by suppressing foodborne pathogens and/or food-spoilage agents.

**Keywords:** endophyte, foodborne, pathogen, antibiosis, *Paenibacillus polymyxa*, Parviglumis

## Introduction

ENDOPHYTES ARE MICROORGANISMS, usually bacteria or fungi, which inhabit plant tissues without causing disease symptoms (Hallmann *et al.*, 1997; Strobel, 2003; Rosenblueth and Martínez-Romero, 2006). The term “endophyte” is derived from the Greek words, endon (meaning within) and phyte (meaning plant) (Sturz *et al.*, 2000). Endophyte colonization has been observed in nearly all plant species tested (Ryan *et al.*, 2008; Hardoim *et al.*, 2015). Endophytes may live latently or grow actively inside plant tissues (Taghavi *et al.*, 2009). Endophytes can colonize a diversity of tissues, of which seed-inhabiting endophytes are more likely to be vertically transmitted across generations (Johnston-Monje and Raizada, 2011; Truyens *et al.*, 2015).

Plant–endophyte associations sometimes form symbiotic (mutualistic) relationships by which the endophytes gain access to a protected habitat and nutrients, whereas the host gains benefits from the endophytes in terms of growth promotion,

nutrient acquisition, or antagonism against plant pathogens (Rosenblueth and Martínez-Romero, 2006; Ryan *et al.*, 2008; Johnston-Monje and Raizada, 2011). Although endophytes can be beneficial, some toxic alkaloid-producing fungal endophytes in grasses such as tall fescue and perennial ryegrass have been linked to health disorders (fescue toxicosis) in cattle and horses that consume these grasses (Guerre, 2015).

An unexplored hypothesis is whether the seeds and other foods selected by humans and other animals possess endophytes that not only help their plant hosts but also indirectly benefit human and animal health by antagonizing foodborne pathogens or food-spoilage organisms, either in the source grain/food or in foods consumed alongside (e.g., meat). Endophytes would be viable in raw foods, whereas some spores may survive cooking.

Many bacteria are causative agents of foodborne illness, of which *Escherichia coli*, *Salmonella enterica*, *Listeria monocytogenes*, and *Clostridium perfringens* are especially problematic, whereas *Pseudomonas fluorescens* causes spoilage to foods (Bach *et al.*, 2002; Brynestad and Granum,

<sup>1</sup>Department of Plant Agriculture, University of Guelph, Guelph, Ontario, Canada.

<sup>2</sup>Department of Microbiology, School of Pharmacy, Mansoura University, Mansoura, Egypt.

<sup>3</sup>Department of Food Science, University of Guelph, Guelph, Ontario, Canada.

<sup>4</sup>Canadian Research Institute for Food Safety, University of Guelph, Guelph, Ontario, Canada.

2002; Pitout *et al.*, 2003; You *et al.*, 2006; Allen *et al.*, 2016; Lin *et al.*, 2016). *E. coli* is a Gram-negative bacterium commonly found in the human intestine as a commensal organism, but some strains of *E. coli* are pathogenic such as enterohemorrhagic *E. coli* (EHEC). The most common strain of EHEC is *E. coli* O157:H7 (Callaway *et al.*, 2009), which is a very important foodborne pathogen found in feed, water, soil, and manure (Bach *et al.*, 2002). In humans, this pathogen causes diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome.

*S. enterica* strains, including *Salmonella enterica* serotype Newport, are Gram-negative bacteria that cause salmonellosis in humans as well as in animals and is associated with gastroenteritis and bacteremia. Disease outbreaks have been associated with consumption of contaminated poultry, eggs, pork, beef, seafood, milk, cheese, chocolate, peanuts, cereal, sprouts, mangoes, and other fruits and vegetables (Van Beneden *et al.*, 1999; Pitout *et al.*, 2003; Sivapalasingam *et al.*, 2003; Zhao *et al.*, 2003).

*L. monocytogenes* is a Gram-positive, nonspore forming, rod-shaped bacterium that can be found in water, dairy products, meats, egg products, seafood, and vegetables (Farber and Peterkin, 1991; Embarek, 1994; Lawrence and Gilmour, 1994; Novoslavskij *et al.*, 2015). Infection symptoms range from moderate gastrointestinal illness to severe septicemia and meningitis, with a mortality rate of 20–30% in the case of invasive listeriosis, which makes it a major food safety concern (Carpentier and Cerf, 2011; Cossart, 2011; Allen *et al.*, 2016). Listeriosis affects cattle, sheep, and goats with the disease associated with encephalitis, septicemia, and uterine infections and abortion (Low and Donachie, 1997).

*C. perfringens* is an anaerobic, Gram-positive, rod-shaped, spore-forming bacterium that causes two types of food poisoning in humans: a mild, self-limiting type A diarrhea and the more severe type C necrotic enteritis. In animals, *C. perfringens* causes diarrhea, enterotoxemia, and necrotic enteritis (Brynesstad and Granum, 2002; Uzal and Songer, 2008; García and Heredia, 2011; Silva and Lobato, 2015; Silva *et al.*, 2015), whereas in poultry, it can cause avian malignant disease, gangrenous dermatitis, or avian necrotic enteritis, the most severe bacterial poultry enteric disease (Cooper and Songer, 2009).

Finally, the food spoiler *P. fluorescens* is a Gram-negative, rod-shaped bacterium that causes major economic losses (Andreani *et al.*, 2015). *P. fluorescens* causes food spoilage by producing extracellular lipases and proteases; the bacterium is common in dairy products, fish products, and leafy greens (Miller *et al.*, 1973; Olanya *et al.*, 2015; Lin *et al.*, 2016).

Our laboratory has previously isolated ~200 bacterial endophytes from seeds, roots, and shoots of different genotypes of the maize/corn family, *Zea*, including wild, ancient, and modern varieties (Johnston-Monje and Raizada, 2011; Johnston-Monje *et al.*, 2014). Among these varieties were wild *Parviglumis tesosinte* (*Zea mays* ssp *parviglumis*), the direct, living ancestor of modern maize from Mexico (Piperno *et al.*, 2009), and *Zea diploperennis*, a wild perennial Central American maize (Iltis and Doebley, 1980). Endophytes from this collection, in particular from wild and ancient maize, were recently found to host microbes with antifungal and nutrient acquisition abilities (Mousa *et al.*, 2015; Shehata, 2016; Shehata *et al.*, 2016a, b, c), perhaps selected by their host plants that grow without synthetic pesticides and fertilizers.

In this study, as a first step toward testing our hypothesis, we ask whether bacterial endophytes from the maize family,

including those isolated from grain, can antagonize, *in vitro*, the mentioned bacterial foodborne pathogens as well as the food spoiler *P. fluorescens*.

## Materials and Methods

### Bacterial strains

The bacterial endophytes used in this study (Fig. 1A; Supplementary Table S1; Supplementary Data are available online at [www.liebertpub.com/fpd](http://www.liebertpub.com/fpd)) were previously isolated from diverse wild, ancient, and modern genotypes of the maize family, *Zea* (Johnston-Monje and Raizada, 2011; Johnston-Monje *et al.*, 2014). The bacterial endophytes were maintained as glycerol stocks in a –80°C freezer.

Foodborne pathogens, *Escherichia coli* O157:H7 (C899), *Listeria monocytogenes* (LJH391), *Clostridium perfringens* (C1354), and *Salmonella enterica* Newport (C407), and food spoiler, *Pseudomonas fluorescens* (C942), were obtained from the Canadian Research Institute in Food Safety culture collection at the University of Guelph.

### In vitro screening for antagonistic activity against foodborne pathogens

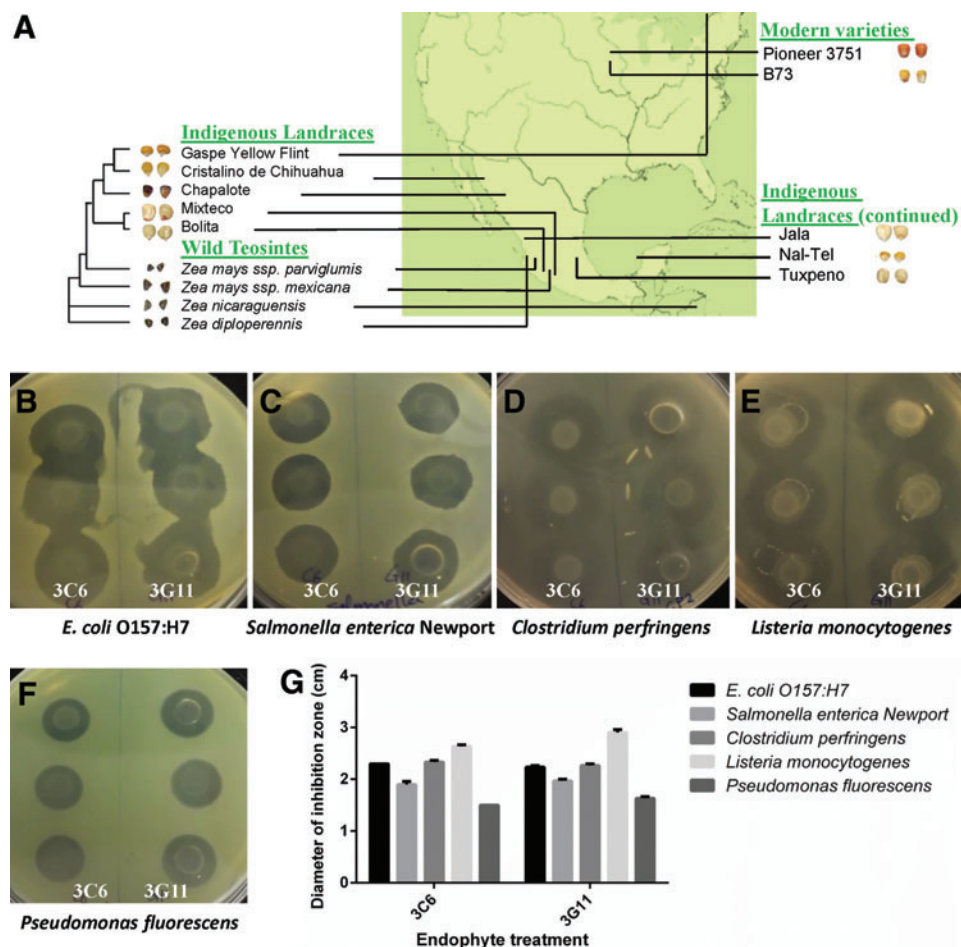
Bacterial endophytes were cultured in Luria-Bertani (LB) medium overnight at 37°C with shaking at 250 rpm. From each bacterial endophyte culture (OD<sub>595</sub> = 1.0), 15 µL were spotted onto 10 cm Petri plates containing 15 mL of solidified LB agar, in triplicate, allowed to dry, then the plates were inverted and incubated overnight at 37°C to allow colonies to develop. In parallel, the foodborne pathogenic strains were cultured in Tryptic Soy Broth overnight at 37°C with shaking at 200 rpm. *C. perfringens* was cultured in Brain Heart Infusion in an anaerobic chamber at 37°C. From each pathogenic strain culture, 10 µL (OD<sub>595</sub> = 1.0) (200 µL for *C. perfringens*, OD<sub>595</sub> = 0.6) were mixed with 10 mL of melted and cooled LB agar, and then poured over LB agar on which the endophyte colonies were growing. After solidification, the plates were incubated at 37°C (in an anaerobic chamber for *C. perfringens*). Inhibition zone diameters were recorded (Bevilacqua *et al.*, 2003).

### Taxonomic identification of antagonistic strains based on 16S rRNA sequencing

To identify the antagonistic endophytes, full length 16S rRNA sequences were used. DNA was extracted using a Bacterial Genomic Miniprep Kit (NA2110; Sigma), and then quantified using a NanoDrop ND-1000 (Thermo Scientific, USA). Polymerase chain reactions (PCRs) were conducted using universal 16S rRNA primers (Frank *et al.*, 2008; Ghyselinck *et al.*, 2013) according to a previously described protocol (Shehata *et al.*, 2016a) and then Basic Local Alignment Search Tool-Nucleotide searched against the 16S ribosomal sequence database at National Center for Biotechnology Information. A phylogenetic tree was constructed using reference 16S sequences from GenBank to confirm strain identity the phylogeny tool (Phylogeny.lirmm.fr) using default parameters (Castresana, 2000; Edgar, 2004; Dereeper *et al.*, 2008).

### Genomic fingerprinting of antagonistic strains

To test whether strains 3C6 and 3G11 are identical or distinct, BOX-PCR followed by gel electrophoresis was



**FIG. 1.** Screening of maize endophytes for their ability to suppress foodborne pathogens and a food spoiling agent. (A) A map showing the geographical origins of the different maize genotypes used in this study as sources of endophytes. Agar plates showing zones of growth inhibition of foodborne pathogens and a food spoiling agent by endophytes 3C6 and 3G11: (B) *Escherichia coli* O157:H7, (C) *Salmonella enterica* Newport, (D) *Clostridium perfringens*, (E) *Listeria monocytogenes*, and (F) *Pseudomonas fluorescens*. (G) Graph showing the ability of endophytes 3C6 and 3G11 to inhibit growth of the deleterious pathogens (diameter of inhibition zones). The map image is licensed under the Creative Commons Attribution-Share Alike 3.0 Unported license. Color images available online at [www.liebertpub.com/fpd](http://www.liebertpub.com/fpd)

conducted. Each reaction consisted of 20  $\mu$ L of GoTaq<sup>®</sup> Green Master Mix (M712C; Promega, Madison, WI), 2  $\mu$ M BOX-A1R primer with sequence CTACGGCAAGGCGA CGCTGACG, 50 ng of DNA, and double-distilled water up to 40  $\mu$ L. BOX-PCR conditions were 7 min at 95°C, then 30 cycles (1 min at 95°C, 1 min at 53°C, and 8 min at 65°C), and a final extension at 65°C for 15 min (Cottyn *et al.*, 2001). The PCR products were run on a 1% agarose gel in Tris-acetate-EDTA at 75 V for 11 h. Banding patterns were visualized and extracted using Image Lab 5.1 software.

#### Detection of polymyxin genes in candidate antagonistic strains

Primers for polymyxin genes *pmxA*, *pmxB*, *pmxC*, *pmxD*, and *pmxE* (Supplementary Table S2) were designed using Primer 3 software based on the reference sequence for *Paenibacillus polymyxa* strain PKB1 (GenBank: JN660148.1). PCR mixtures contained 10  $\mu$ L of GoTaq Green Master Mix (M712C; Promega), 50 ng of DNA, 0.5  $\mu$ L of each of the forward and reverse primers (10  $\mu$ M), and double-distilled

water up to 20  $\mu$ L. The same reaction conditions and gel purification methods were used as in the 16S gene amplification already described. Retrieved sequences were searched using BLASTN against the Genbank nr/nt collection.

#### Detection of polymyxin production

Strains 3C6 and 3G11 were cultured in glucose starch calcium carbonate (GSC) medium for 3 days at 37°C with shaking at 200 rpm. The GSC medium consisted of, per liter, 20 g each of glucose, starch, and ammonium sulfate, 10 g of yeast, 2.6 g of  $K_2HPO_4$ , 0.1 g of  $FeSO_4 \cdot 7H_2O$ , 0.5 g of  $MgSO_4 \cdot 7H_2O$ , 0.25 g of sodium chloride, and 9 g of calcium carbonate (Shaheen *et al.*, 2011). The GSC medium was previously found to be optimal for polymyxin production (Niu *et al.*, 2013). Two milliliters from each culture were centrifuged at 10,000 rpm for 5 min. Two experiments were conducted using GSC culture supernatants. The first experiment was to evaluate the antibacterial activity of the cell-free supernatant. Culture supernatants were passed through 0.22  $\mu$ m filters and from the resultant cell-free supernatants; 15  $\mu$ L were then spotted on LB agar plates and assayed

for activity against *E. coli* as already described. Polymyxin standards prepared from commercial polymyxin solution (81271; Sigma) at concentrations of 500, 250, 125, 62.5, 31.25, 15.6, and 7.8 ng/ $\mu$ L were spotted on agar in parallel. The second experiment was to assay for polymyxin production. In this experiment, the GSC culture supernatants were mixed with methanol at a ratio of 1:4 and allowed to incubate at 4°C for 30 min. The mixtures were then centrifuged at 10,000 rpm for 5 min. The resultant supernatants were used to assay for polymyxin production using a Colistin and Polymyxin ELISA Kit (DEIA042; Creative Diagnostics, USA). *E. coli* DH5-Alpha strain was used as a negative control. The test was run using 50  $\mu$ L of each methanol extract, in triplicate, according to the manufacturer's recommendations. Polymyxin standards (0, 2.5, 7.5, 22.5, 67.5, and 202.5 ppb) were run in parallel, in triplicate, to construct a semilogarithmic standard curve (A% against the log concentration). The absorbance was measured at 450 nm (A<sub>450</sub>). The relative absorbance (A%) was calculated as the mean A<sub>450</sub> of the sample or A<sub>450</sub> of the standard  $\times$  (100/A<sub>450</sub> of the 0 ppb standard).

#### Statistical and graphical analyses

Prism 6 (GraphPad Software, USA) was used for graphical displays and statistical analysis. Pearson *r* was used to measure the linear correlation between the banding patterns of strains 3C6 and 3G11.

## Results

#### *In vitro* screening for antagonistic activity against foodborne pathogens and a food spoiler

Out of 190 bacterial endophytes tested from the maize family (Fig. 1A) for antagonistic activity against four foodborne pathogens, 2 endophytes (strain 3C6 and strain 3G11) were found to show zones of growth inhibition *in vitro* of *Escherichia coli* O157:H7, *Listeria monocytogenes*, *Clostridium perfringens*, and *Salmonella* Newport; the same two endophytes also inhibited the food spoiling agent, *P. fluorescens* (Fig. 1B–G).

#### Taxonomic identification of candidate endophytes based on 16S rRNA

The two candidate strains were identified based on their full 16S rRNA sequences along with their positions on a phylogenetic tree constructed using reference 16S rRNA sequences. Strains 3C6 and 3G11 were found to resemble *Paenibacillus polymyxa* (100% and 99% similarity, respectively) (GenBank: KX015879 and KX015880) and to cluster with this species in a phylogenetic tree (Fig. 2A, B). Genomic fingerprinting showed that 3C6 and 3G11 were very closely related (Pearson correlation of banding pattern,  $r=0.941$ ), but distinct given the extra band in the genomic fingerprint of strain 3G11 (Fig. 2C–E). Strain 3C6 originated from seeds of the wild Central American maize species *Zea diploperennis*, whereas 3G11 originated from seeds of the direct, wild ancestor of modern maize, *Zea mays* ssp *parviglumis* (Parviglumis teosinte) (Fig. 2B).

#### Detection of polymyxin genes and polymyxin metabolite(s) in candidate antipathogen strains

As polymyxins are well-known antibiotics of previously reported *P. polymyxa* strains (Yahav *et al.*, 2012), strains 3C6

and 3G11 were tested for the presence of polymyxin genes. An operon encoding polymyxin synthetase genes was previously identified from a plant growth-promoting *Paenibacillus polymyxa* strain E681 as having five open reading frames, *pmxA*, *pmxB*, *pmxC*, *pmxD*, and *pmxE* (Choi *et al.*, 2009). Whereas *pmxA*, *pmxB*, and *pmxE* were found to encode polymyxin synthetases, *pmxC* and *pmxD* resembled genes that encode transport proteins. Here strains 3C6 and 3G11 were tested using PCR primers for *pmxA–E* followed by sequencing and BLAST analysis of amplicons of the correct size. The genomes of both strains contained sequences with high similarity to *pmxB*, *pmxC*, *pmxD*, and *pmxE*, but apparently not for *pmxA* (Fig. 2F; Supplementary Table S3).

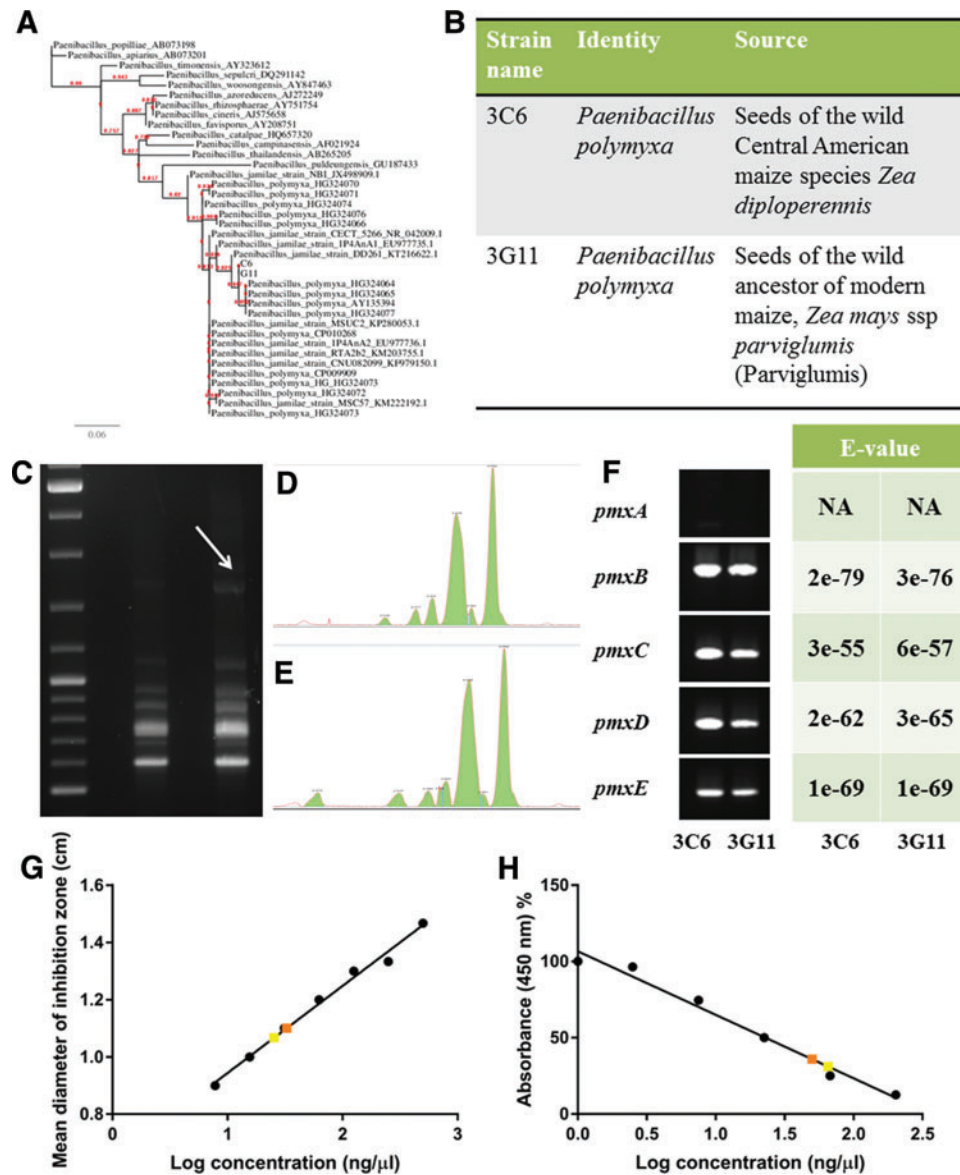
To confirm the production of polymyxin from strain 3C6 and 3G11, first, cell-free supernatants were assayed for antibacterial activity. Cell-free supernatants of 3C6 and 3G11 were found to demonstrate antibacterial activity equivalent to 32.7 and 25.4 ng/ $\mu$ L of polymyxin, respectively (Fig. 2G). Furthermore, polymyxin was detected in methanol extracts of culture supernatants using a polymyxin ELISA Kit. Strains 3C6 and 3G11 were found to secrete 50 and 65.3 ng/ $\mu$ L of polymyxin, respectively, under the conditions used (Fig. 2H).

## Discussion

Seeds of two wild maize genotypes, *Zea diploperennis* and Parviglumis teosinte, were found to possess bacterial endophytes that can suppress four foodborne bacterial pathogens and one food spoiling agent, *in vitro*. It is especially intriguing that Parviglumis teosinte seeds possess an endophyte (strain 3G11, predicted to be *P. polymyxa*) that can suppress the growth of human and livestock pathogens, including those transmitted by fecal matter. Parviglumis (also known as Balsas teosinte) is the extant, wild, direct ancestor of modern maize, selected and domesticated beginning ~10,000 years ago in the Balsas river valley of Mexico (Matsuoka *et al.*, 2002).

One theory of crop domestication holds that nutritious seeds were brought to temporary human settlements by animals, foraging for human food scraps, and left behind in their feces (Diamond, 1997). The animal manure was a rich nutrient source that subsequently promoted germination and growth of the seed-bearing plants. In another theory, crop domestication promoted human settlements that led to food waste, which attracted wild animals, ultimately leading to animal domestication and increased human–animal contact (Larson and Fuller, 2014). Exciting but speculative questions arise from these theories.

First, as it is known that wild animals select foods, in part, for their medicinal value (Huffman, 2003), then perhaps animals in the Balsas river valley habituated to eating Parviglumis teosinte seeds, in part, because their endophytes helped to protect the animals from dangerous bacterial pathogens that originated from other animals or increasing human populations (Harper and Armelagos, 2013). Balsas teosinte seeds are surrounded by a hard fruit case that can help them to escape damage after ingestion. Archaeological evidence has revealed the presence of animal bone fragments, including mammals, near human settlements in this region (Piperno *et al.*, 2007; Ranere *et al.*, 2009). Mammals are the major reservoir of *E. coli* strains such as strain O157:H7 (Bach *et al.*, 2002; Delaquis *et al.*, 2007; Franz and van Bruggen, 2008; Callaway *et al.*, 2009), whereas listeriosis caused by *L. monocytogenes* affects mammals (Low and Donachie, 1997). *C. perfringens* is the



**FIG. 2.** Taxonomic identification of candidate endophytes and detection of polymyxin. (A) Phylogenetic tree predicting the identity of endophyte strains 3C6 and 3G11 as *Paenibacillus polymyxa*. (B) Table showing the candidate identity and host plant source of endophytes 3C6 and 3G11. (C–E) Genomic fingerprinting following BOX-PCR demonstrating that strains 3C6 and 3G11 are distinct from one another: (C) gel electrophoresis of BOX-PCR products, and lane profiles of (D) strain 3C6 and (E) strain 3G11. (F) Detection of polymyxin genes in 3C6 and 3G11. The E-values indicate the count of hits expected by chance during the Basic Local Alignment Search Tool search. The lower the E-value, the stronger is the homology. (G) Detection of antipathogen activity (against *Escherichia coli*) in the supernatants of strains 3C6 (orange) and 3G11 (yellow). (H) Enzyme-linked immunosorbent assay-based detection of polymyxin metabolite(s) in the methanol-extracted supernatants of strains 3C6 (orange) and 3G11 (yellow). BOX-PCR; E-value, expect value; NA, No Amplification. The white arrow in C refers to an extra band in the banding pattern of strain 3G11. Color images available online at [www.liebertpub.com/fpd](http://www.liebertpub.com/fpd)

most serious toxin-producing clostridia that affects both humans and animals (García and Heredia, 2011), and its spore-forming ability imparts longevity and persistence (Brynstad and Granum, 2002).

Alternative to the hypothesis that Parviglumis seed endophyte(s) may have helped ancient animals in the Balsas region of Mexico, perhaps the endophytes protected local humans from bacterial pathogens after direct animal to human transmission or through contaminated meat. Archaeological evidence has revealed that the ancient humans of the Balsas river valley were hunters and that animal protein was

abundant (Piperno *et al.*, 2007; Ranere *et al.*, 2009). *L. monocytogenes* is a human and animal foodborne pathogen that can be transmitted to humans directly from infected animals or contaminated food (Farber and Peterkin, 1991; Embarek, 1994; Lawrence and Gilmour, 1994; Carpentier and Cerf, 2011; Cossart, 2011; Novoslavskij *et al.*, 2015). By contrast, *P. fluorescens* is a food spoiler (Andreani *et al.*, 2015), and hence Balsas teosinte grain may have had the potential to protect ancient peoples when added to foods.

Alternative to the mentioned food-related hypothesis, if humans temporarily penned small animals, leading to a

buildup of dangerous manure, then perhaps *P. polymyxa* protected the animals that were fed Parviglumis grain. *Salmonella enterica* serotype Newport is known to be transmitted through infected manure used in agricultural fields, or from manure to soil to plants (You *et al.*, 2006). In general, it is thought that many current human pathogens have their origins in agricultural domestication, in part because human populations increased, which promoted disease transmission, and in part because humans were in contact with domesticated animals, which were the sources of these pathogens (Diamond, 2002; Pearce-Duvet, 2006; Harper and Armelagos, 2013). The problem with such a theory is that there appears to be no evidence for animal domestication in this region at this time; turkeys were domesticated elsewhere in Mexico thousands of years later (Thornton *et al.*, 2012; Larson and Fuller, 2014). However, humans at the time of maize domestication may have lived with dogs in ancient Mexico (Zeder *et al.*, 2006), and dog-to-human transmission of pathogens has been shown, as has pathogen transmission from rodents that gather at food storage sites (Pearce-Duvet, 2006; Harper and Armelagos, 2013). The corollary theory would be that there might have been a buildup of human feces at the first settlements, which could have led to widespread outbreaks of the bacterial pathogens suppressed by *P. polymyxa*.

Finally, it may be that Parviglumis-associated *P. polymyxa* provided no direct benefits to ancient humans and animals associated with them, but rather that the endophyte, by providing resistance to a wide spectrum of bacteria including plant pathogens, protected its host and thus contributed to making Parviglumis a hardy plant that was attractive for domestication. In fact, here we uncovered the presence of an antibacterial *Paenibacillus polymyxa* endophyte from *Zea diploperennis*, a wild perennial teosinte from Central America (Iltis and Doebley, 1980). We also previously demonstrated that both wild and modern maize possess other *Paenibacillus polymyxa* endophytes that combat diverse fungal pathogens of crops (Mousa *et al.*, 2015). Combined, these data are suggestive of long-term mutualism between the maize family (*Zea*) and its *Paenibacillus polymyxa* endophytes.

In terms of the antibacterial mechanism of action, our data show that strain 3G11 and strain 3C6 from *Z. diploperennis*, both predicted to be *P. polymyxa* strains, encode polymyxin genes and secrete polymyxin *in vitro*. Polymyxins are cationic lipopeptide antibiotics, well known for their antibacterial activity especially against Gram-negative bacteria (Choi *et al.*, 2009; Naghmouchi *et al.*, 2011). The mechanism of action depends on the detergent-like properties of polymyxins; they can bind membrane-associated lipopolysaccharides, resulting in disruptions to the integrity of cytoplasmic membranes and outer membranes (Naghmouchi *et al.*, 2011; Yahav *et al.*, 2012). Polymyxin antibiotics were used as antibiotics to treat bacterial infections, but because of their side effects, their use is currently limited to topical use on surface injuries or wounds, or used systemically in multidrug-resistant infections (Choi *et al.*, 2009; Yahav *et al.*, 2012). Interestingly, Polymyxin E was found to work synergistically with bacteriocins to control foodborne pathogens (*L. monocytogenes* and *E. coli*) (Naghmouchi *et al.*, 2011). The involvement of polymyxin genes in suppressing the five harmful microbes in this study will need to be tested experimentally.

Several other antimicrobials were reported to be produced by *P. polymyxa* strains. Among these antimicrobials are

paenibacillin, a peptide antibiotic that is active against Gram-positive bacteria (He *et al.*, 2007), fusaricidins, a group of peptide antibiotics with activity against Gram-positive bacteria (Kajimura and Kaneda, 1997), and polyxin, a bacteriocin-like compound, with activity against Gram-positive and Gram-negative foodborne pathogens (Piuri *et al.*, 1998).

## Conclusions

Here we hypothesized that the maize family possesses endophytes that can suppress foodborne pathogens and food spoiling agents. We demonstrated, *in vitro*, that seeds of two wild maize relatives possess bacterial endophytes that inhibit the growth of five such harmful microbes, and we have proposed a candidate mechanism of action. One of these wild maize relatives is the direct ancestor of modern maize, raising interesting questions about the role of its endophyte(s) in promoting crop domestication by ancient Mexican farmers. More experiments in the future are needed to test the various hypotheses proposed in this article. With respect to future applications, as both endophyte strains were found to produce polymyxin, they may not be ideal for use as natural food or feed preservatives, as they may contribute to the spread of antimicrobial resistance.

## Acknowledgments

The authors thank William Lachowsky for training and facilitating the work at the Canadian Research Institute in Food Safety at the University of Guelph. This research was supported by a grant to M.N.R. from Natural Sciences and Engineering Research Council of Canada. H.R.S. was supported by a generous scholarship from the Egyptian Government.

## Disclosure Statement

No competing financial interests exist.

## Authors' Contributions

H.R.S. helped to design the study, carried out experiments, and wrote the article. M.W.G. helped to design the study and supplied foodborne pathogens. M.N.R. helped to design the study and to write the article. All authors read and approved the final article.

## References

- Allen KJ, Watecka-Zacharska E, *et al.* *Listeria monocytogenes*—An examination of food chain factors potentially contributing to antimicrobial resistance. *Food Microbiol* 2016;54:178–189.
- Andreani NA, Carraro L, *et al.* A genomic and transcriptomic approach to investigate the blue pigment phenotype in *Pseudomonas fluorescens*. *Int J Food Microbiol* 2015;213:88–98.
- Bach S, McAllister T, *et al.* Transmission and control of *Escherichia coli* O157: H7-A review. *Can J Anim Sci* 2002;82:475–490.
- Bevilacqua L, Ovidi M, *et al.* Screening of *Bifidobacterium* strains isolated from human faeces for antagonistic activities against potentially bacterial pathogens. *Microbiol Res* 2003;158:179–185.
- Brynestad S, Granum PE. *Clostridium perfringens* and foodborne infections. *Int J Food Microbiol* 2002;74:195–202.

- Callaway TR, Carr M, *et al.* Diet, *Escherichia coli* O157: H7, and cattle: A review after 10 years. *Curr Issues Mol Biol* 2009;11:67.
- Carpentier B, Cerf O. Review—Persistence of *Listeria monocytogenes* in food industry equipment and premises. *Int J Food Microbiol* 2011;145:1–8.
- Castresana J. Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol* 2000;17:540–552.
- Choi S-K, Park S-Y, *et al.* Identification of a polymyxin synthetase gene cluster of *Paenibacillus polymyxa* and heterologous expression of the gene in *Bacillus subtilis*. *J Bacteriol* 2009;191:3350–3358.
- Cooper KK, Songer JG. Necrotic enteritis in chickens: A paradigm of enteric infection by *Clostridium perfringens* type A. *Anaerobe* 2009;15:55–60.
- Cossart P. Illuminating the landscape of host-pathogen interactions with the bacterium *Listeria monocytogenes*. *Proc Natl Acad Sci U S A* 2011;108:19484–19491.
- Cottyn B, Regalado E, *et al.* Bacterial populations associated with rice seed in the tropical environment. *Phytopathology* 2001;91:282–292.
- Delaquis P, Bach S, *et al.* Behavior of *Escherichia coli* O157: H7 in leafy vegetables. *J Food Prot* 2007;70:1966–1974.
- Dereeper A, Guignon V, *et al.* Phylogeny.fr: Robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res* 2008;36:W465–W469.
- Diamond J. *Guns, germs, and steel: The fates of human societies*. New York: W.W. Norton & Company, 1997.
- Diamond J. Evolution, consequences and future of plant and animal domestication. *Nature* 2002;418:700–707.
- Edgar RC. MUSCLE: Multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* 2004;32:1792–1797.
- Embarek PKB. Presence, detection and growth of *Listeria monocytogenes* in seafoods: A review. *Int J Food Microbiol* 1994;23:17–34.
- Farber J, Peterkin P. *Listeria monocytogenes*, a food-borne pathogen. *Microbiol Rev* 1991;55:476–511.
- Frank JA, Reich CI, *et al.* Critical evaluation of two primers commonly used for amplification of bacterial 16S rRNA genes. *Appl Environ Microbiol* 2008;74:2461–2470.
- Franz E, van Bruggen AH. Ecology of *E. coli* O157: H7 and *Salmonella enterica* in the primary vegetable production chain. *Crit Rev Microbiol* 2008;34:143–161.
- García S, Heredia N. *Clostridium perfringens*: A dynamic foodborne pathogen. *Food Bioprocess Tech* 2011;4:624–630.
- Ghyselinck J, Pfeiffer S, *et al.* The effect of primer choice and short read sequences on the outcome of 16S rRNA gene based diversity studies. *PLoS One* 2013;8:e71360.
- Guerre P. Ergot alkaloids produced by endophytic fungi of the genus *Epichloe*. *Toxins* 2015;7:773–790.
- Hallmann J, Quadt-Hallmann A, *et al.* Bacterial endophytes in agricultural crops. *Can J Microbiol* 1997;43:895–914.
- Haridoim PR, van Overbeek LS, *et al.* The hidden world within plants: Ecological and evolutionary considerations for defining functioning of microbial endophytes. *Microbiol Mol Biol Rev* 2015;79:293–320.
- Harper KN, Armelagos GJ. Genomics, the origins of agriculture, and our changing microbe-scape: Time to revisit some old tales and tell some new ones. *Am J Phys Anthropol* 2013;152:135–152.
- He Z, Kisla D, *et al.* Isolation and identification of a *Paenibacillus polymyxa* strain that coproduces a novel lantibiotic and polymyxin. *Appl Environ Microbiol* 2007;73:168–178.
- Huffman MA. Animal self-medication and ethno-medicine: Exploration and exploitation of the medicinal properties of plants. *P Nutr Soc* 2003;62:371–381.
- Iltis HH, Doebley JF. Taxonomy of *Zea* (Gramineae). II. Sub-specific categories in the *Zea mays* complex and a generic synopsis. *Am J Bot* 1980;67:994–1004.
- Johnston-Monje D, Mousa W, *et al.* Impact of swapping soils on the endophytic bacterial communities of pre-domesticated, ancient and modern maize. *BMC Plant Biol* 2014;14:233.
- Johnston-Monje D, Raizada MN. Conservation and diversity of seed associated endophytes in *Zea* across boundaries of evolution, ethnography and ecology. *PLoS One* 2011;6:e20396.
- Kajimura Y, Kaneda M. Fusaricidins B, C and D, new depsipeptide antibiotics produced by *Bacillus Polymyxa* KT-8: Isolation, structure elucidation and biological activity. *J Antibiot* 1997;50:220–228.
- Larson G, Fuller DQ. The evolution of animal domestication. *Annu Rev Ecol Evol Syst* 2014;45:115–136.
- Lawrence LM, Gilmour A. Incidence of *Listeria* spp. and *Listeria monocytogenes* in a poultry processing environment and in poultry products and their rapid confirmation by multiplex PCR. *Appl Environ Microbiol* 1994;60:4600–4604.
- Lin H, Shavezipur M, *et al.* Prediction of growth of *Pseudomonas fluorescens* in milk during storage under fluctuating temperature. *J Dairy Sci* 2016;99:1822–1830.
- Low J, Donachie W. A review of *Listeria monocytogenes* and listeriosis. *Vet J* 1997;153:9–29.
- Matsuoka Y, Vigouroux Y, *et al.* A single domestication for maize shown by multilocus microsatellite genotyping. *Proc Natl Acad Sci U S A* 2002;99:6080–6084.
- Miller A, Scanlan R, *et al.* Volatile compounds produced in sterile fish muscle (*Sebastes melanops*) by *Pseudomonas putrefaciens*, *Pseudomonas fluorescens*, and an *Achromobacter* species. *Appl Microbiol* 1973;26:18–21.
- Mousa WK, Shearer CR, *et al.* Bacterial endophytes from wild maize suppress *Fusarium graminearum* in modern maize and inhibit mycotoxin accumulation. *Front Plant Sci* 2015;6:805.
- Naghmouchi K, Belguesmia Y, *et al.* Antibacterial activity of class I and IIa bacteriocins combined with polymyxin E against resistant variants of *Listeria monocytogenes* and *Escherichia coli*. *Res Microbiol* 2011;162:99–107.
- Niu B, Vater J, *et al.* Polymyxin P is the active principle in suppressing phytopathogenic *Erwinia* spp. by the biocontrol rhizobacterium *Paenibacillus polymyxa* M-1. *BMC Microbiol* 2013;13:137.
- Novoslavskij A, Terentjeva M, *et al.* Major foodborne pathogens in fish and fish products: A review. *Ann Microbiol* 2015;66:1–15.
- Olanya OM, Niemira BA, *et al.* Effects of gamma irradiation on the survival of *Pseudomonas fluorescens* inoculated on romaine lettuce and baby spinach. *Food Sci Technol-LEB* 2015;62:55–61.
- Pearce-Duvel JMC. The origin of human pathogens: Evaluating the role of agriculture and domestic animals in the evolution of human disease. *Biol Rev* 2006;81:369–382.
- Piperno DR, Moreno JE, *et al.* Late Pleistocene and Holocene environmental history of the Iguala Valley, Central Balsas Watershed of Mexico. *Proc Natl Acad Sci U S A* 2007;104:11874–11881.
- Piperno DR, Ranere AJ, *et al.* Starch grain and phytolith evidence for early ninth millennium B.P. maize from the Central Balsas River Valley, Mexico. *Proc Natl Acad Sci U S A* 2009;106:5019–5024.

- Pitout JDD, Reisbig MD, *et al.* Association between handling of pet treats and infection with *Salmonella enterica* serotype Newport expressing the ampC  $\beta$ -lactamase, CMY-2. *J Clin Microbiol* 2003;41:4578–4582.
- Piuri M, Sanchez-Rivas C, *et al.* A novel antimicrobial activity of a *Paenibacillus polymyxa* strain isolated from regional fermented sausages. *Lett Appl Microbiol* 1998;27:9–13.
- Ranere AJ, Piperno DR, *et al.* The cultural and chronological context of early Holocene maize and squash domestication in the Central Balsas River Valley, Mexico. *Proc Natl Acad Sci U S A* 2009;106:5014–5018.
- Rosenblueth M, Martínez-Romero E. Bacterial endophytes and their interactions with hosts. *Mol Plant-Microbe Interact* 2006;19:827–837.
- Ryan RP, Germaine K, *et al.* Bacterial endophytes: Recent developments and applications. *FEMS Microbiol Lett* 2008;278:1–9.
- Shaheen M, Li J, *et al.* *Paenibacillus polymyxa* PKB1 produces variants of polymyxin B-type antibiotics. *Chem Biol* 2011;18:1640–1648.
- Shehata HR. Molecular and physiological mechanisms underlying the antifungal and nutrient acquisition activities of beneficial microbes. PhD dissertation, University of Guelph, 2016.
- Shehata HR, Lyons EM, *et al.* Bacterial endophytes from wild and ancient maize are able to suppress the fungal pathogen *Sclerotinia homoeocarpa*. *J Appl Microbiol* 2016a;120:756–769.
- Shehata HR, Lyons EM, *et al.* Relevance of *in vitro* agar based screens to characterize the anti-fungal activities of bacterial endophyte communities. *BMC Microbiol* 2016b;16:1–7.
- Shehata HR, Ettinger CL, *et al.* Genes required for the anti-fungal activity of a bacterial endophyte isolated from a corn landrace grown continuously by subsistence farmers since 1000 BC. *Front Microbiol* 2016c;7:1548
- Silva ROS, Lobato FCF. *Clostridium perfringens*: A review of enteric diseases in dogs, cats and wild animals. *Anaerobe* 2015;33:14–17.
- Silva ROS, Oliveira CA Jr., *et al.* *Clostridium perfringens*: A review of the disease in pigs, horses and broiler chickens. *Ciência Rural* 2015;45:1027–1034.
- Sivapalasingam S, Barrett E, *et al.* A Multistate outbreak of *Salmonella enterica* serotype Newport infection linked to mango consumption: Impact of water-dip disinfection technology. *Clin Infect Dis* 2003;37:1585–1590.
- Strobel GA. Endophytes as sources of bioactive products. *Microb Infect* 2003;5:535–544.
- Sturz AV, Christie BR, *et al.* Bacterial endophytes: Potential role in developing sustainable systems of crop production. *Crit Rev Plant Sci* 2000;19:1–30.
- Taghavi S, Garafola C, *et al.* Genome survey and characterization of endophytic bacteria exhibiting a beneficial effect on growth and development of Poplar trees. *Appl Environ Microbiol* 2009;75:748–757.
- Thornton EK, Emery KF, *et al.* Earliest Mexican turkeys (*Meleagris gallopavo*) in the Maya region: Implications for pre-hispanic animal trade and the timing of turkey domestication. *PLoS One* 2012;7:e42630.
- Truyens S, Weyens N, *et al.* Bacterial seed endophytes: Genera, vertical transmission and interaction with plants. *Environ Microbiol Rep* 2015;7:40–50.
- Uzal FA, Songer JG. Diagnosis of *Clostridium perfringens* intestinal infections in sheep and goats. *J Vet Diagn Invest* 2008;20:253–265.
- Van Beneden CA, Keene WE, *et al.* Multinational outbreak of *salmonella enterica* serotype Newport infections due to contaminated alfalfa sprouts. *JAMA* 1999;281:158–162.
- Yahav D, Farbman L, *et al.* Colistin: New lessons on an old antibiotic. *Clin Microbiol Infect* 2012;18:18–29.
- You Y, Rankin SC, *et al.* Survival of *Salmonella enterica* serovar Newport in manure and manure-amended soils. *Appl Environ Microbiol* 2006;72:5777–5783.
- Zeder MA, Emshwiller E, *et al.* Documenting domestication: The intersection of genetics and archaeology. *Trends Genet* 2006;22:139–155.
- Zhao S, Qaiyumi S, *et al.* Characterization of *Salmonella enterica* serotype Newport isolated from humans and food animals. *J Clin Microbiol* 2003;41:5366–5371.

Address correspondence to:  
 Manish N. Raizada, PhD  
 Department of Plant Agriculture  
 University of Guelph  
 50 Stone Road  
 Guelph, ON N1G 2W1  
 Canada

E-mail: raizada@uoguelph.ca