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Opportunistic Pathogenic Bacteria Colonize Thorns of Native Rio Grande Valley Plants

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ABSTRACT

Thorns serve as mechanical defenses of plants against herbivory. However, plant thorns harbor microorganisms that are potentially pathogenic. These pathogens may be transferred to herbivores and other animals and provide an additional defense for the plants. Thorns from 5 plant species native to the Rio Grande Valley of South Texas were collected and used to isolate bacteria colonizing the thorn surface. Thorns, leaves and stems of plants were visualized using Scanning Electron Microscopy (SEM) to observe any bacteria on the plant surface. Isolated bacteria were tested for their ability to grow in aerobic versus anaerobic environments, to produce hemolysis, carbon source utilization, and were gram-stained. Bacteria were identified by sequencing of the 16S rRNA gene. A total of 69 pure cultures were obtained of which 61 were positively identified. The majority of the bacteria were gram-positive and facultative (i.e., able to grow both aerobically and anaerobically). Many (23%) were hemolytic suggesting that they were opportunistic blood-borne pathogens. All isolated organisms metabolized a collection of 31 tested organic substrates and metabolic activity was observed to be more efficient among anaerobically-isolated microorganisms compared to microorganisms isolated aerobically. SEM revealed that bacteria were found on the thorn surface but also on the stems and leaves of the plants. Identification using the 16S rRNA gene revealed the presence of 10 species representing 7 genera. The most commonly identified organisms were *Bacillus cereus*, *Serratia marcescens*, and *Pseudomonas aeruginosa*, all of which are opportunistic pathogens.

Additional Index Words: Bacillus cereus, Serratia marcescens, Pseudomonas aeruginosa, hemolysis, semi-arid

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Plants use several mechanisms to reduce predation by herbivores. Defense mechanisms may include the production of secondary metabolite chemicals that are toxic to herbivores or structural defenses such as thorns, spines, or bristles that physically deter or injure herbivores. In some environments, the degree of plant defense may be directly related to the availability of limited resources such as water (Coley et al., 1985). In arid and semi-arid environments with limited water, thorny plants such as succulents and others may be the sole source of water for many herbivores.

Pathogenic bacteria have been found on the thorns of some plant species (DeChamps et al., 2000; Francis et al., 2011; Duerinckx 2008). Some general characteristics of pathogens include possessing a facultative respiration (i.e., able to grow in either aerobic or anaerobic environments), hemolytic activity, and an opportunistic, generalist metabolism (Freeman 1985; Mishra and Agrawal 2013). Halpern et al. (2007) suggest that pathogenic bacteria present on the thorns of plants could represent an additional defense mechanism for the plants against herbivory. They suggest that bacteria are transferred to an herbivore, such as a mammal, that is pricked or otherwise injured by a thorn. This could lead to a potential infection that would be more deleterious to the herbivore than just the physical injury alone. Presumably this would deter the herbivore from further predation of the plant, especially if the infection resulted in serious illness.

In the semi-arid Rio Grande Valley (RGV) of South Texas, many native plant species possess thorns or similar structures. Some examples of native RGV thorny plants include Agave (*Agave americana*), Granjeno (*Celtis pallida*), Brasil (*Candalia hookeri*), Honey Mesquite (*Prosopis glandulosa*), and Retama (*Parkinsonia aculeata*). Agave plants possess long, terminal, black thorns typically longer than 2.5 cm. Granjeno plants possess thorns that grow up to 2.5 cm long with 2 thorns per node. Brasil plants have branches with small leaves that terminate in a sharp, black

thorn. Honey mesquite possesses sharp thorns that are 5 – 7 cm in length and located at each node. Retama plants possess double or triple-pointed thorns at each node that are either red-orange in color (new growth) or grey-brown (old growth).

In this study, thorns from native RGV plant species were collected and cultured for surface bacteria to determine if the thorns were colonized by pathogenic bacteria. Isolated bacteria were assayed for general pathogen characteristics and subsequently identified using DNA sequencing. Several potential pathogens were isolated and compared to those detected on thorns of other plant species.

MATERIALS AND METHODS

Sampling. Thorny plant species native to the Rio Grande Valley (RGV) of Texas were sampled during October 2012. Three sampling locations were selected in or near the city of Edinburg, TX. The sampling locations were: Schunior Road (26° 18′ 21″ N, 98° 8′ 49″ W), North Road (28° 18′ 13″ N, 98° 9′ 32″ W), and UTPA (26° 18′ 23″ N, 98° 10′ 25″ W). Five (5) thorny plant species were used in the study. Thorns from new and old growth plants were sampled in all cases. Agave (*Agave americana*) were located and sampled from the Schunior Road site. Thorns from Granjeno (*Celtis pallida*), Brasil (*Candalia hookeri*), and Honey Mesquite (*Prosopis glandulosa*) were collected from the North Road site. Retama (*Parkinsonia aculeata*) thorns were collected from the UTPA site. Thorns were visually examined for physical damage (e.g., cracks, cuts, holes, color irregularities) and only thorns that did not display visible exterior damage were collected. Thorns were cut from plants using sterile scissors. Cuts were made where the thorn met the branch or leaf terminus. The collected thorns were placed in sterile test tubes containing thioglycolate medium (see below). One thorn was collected from 5 individual plants of the same species following the protocol prescribed by Gilman (1999). Test tubes containing thorns were kept on ice for transport back to the lab (approximately 2 h).

Isolation and gram-staining of bacterial cultures. Tubes of thioglycolate medium were used to isolate bacteria from the surface of the plant thorns. Thioglycolate medium consisted of 7.71 g sodium thioglycolate ($C_2H_3NaO_2S$; Sigma-Aldrich, St. Louis MO), 1.65 g dextrose ($C_6H_{12}O_6$; Sigma-Aldrich, St. Louis MO) and 300 ml of deionized water. The medium was dispensed in 10 ml aliquots into glass test tubes, capped and sterilized by autoclave for 30 min. Thioglycolate medium allowed for the isolation of microorganisms from both an aerobic environment (top of the medium tube) and from an anaerobic environment (bottom of the medium tube). Test tubes of thioglycolate medium that were inoculated with plant thorns were incubated at 30°C for 2 d.

A subsample of 100 µl was removed from the aerobic portion of the medium and spread onto LB Agar plates (Becton-Dickinson, Sparks MD). A second 100 µl subsample was removed from the anaerobic portion of each tube and spread onto a separate LB Agar plate. Aerobically-collected subsamples on LB Agar were incubated in an aerobic environment at 30°C for 5 d. Anaerobically-collected subsamples on LB Agar were incubated in anaerobic candle jars at 30°C for 5 d. Colonies growing on the LB Agar plates were picked at random and subcultured 5 times to achieve pure cultures. Pure cultures were then gram-stained using a standard protocol and viewed with a light microscope to determine whether the microorganisms displayed a gram-positive or gram-negative cell type.

Hemolysis testing. A single colony of each pure culture was transferred and streaked onto Trypticase Soy Agar (Becton-Dickinson, Sparks MD) plates supplemented with 5% sheep's blood (Carolina Biological, Burlington NC). Plates were incubated at 30°C for 2 d then visually observed for either alpha or beta hemolysis. Alpha hemolysis was the partial lysis of the red blood cells in the agar and was characterized by a green or brown color in the agar surrounding the bacterial growth. Beta hemolysis was the complete lysis of the red blood cells and was characterized by a clear zone surrounding the bacterial colonies.

Respiration testing. Isolated bacterial cultures were tested to determine whether they were obligate aerobes, obligate anaerobes, or facultative (i.e., able to grow either aerobically or anaerobically). Cultures originally isolated in an aerobic environment were transferred to fresh LB plates and incubated in anaerobic candle jars at 30°C. Cultures originally isolated in an anaerobic environment were transferred to fresh LB plates and incubated at 30°C. Cultures were scored for growth or no growth after 5 d.

EcoPlate™ characterization. EcoPlate™ commercial test plates (BIOLOG, Hayward CA) were used to assess the carbon metabolism of the isolated organisms. EcoPlates™ were micro-well plates impregnated with 31 different carbon sources and a dye. The carbon sources were: β-Methyl-D-Glucoside, D-Galactonic Acid γ-Lactone, L-Arginine, Pyruvic Acid Methyl Ester, D-Xylose, D-Galacturonic Acid, L-Asparagine, Tween 40, i-Erythritol, 2-Hydroxy Benzoic Acid, L-Phenylalanine, Tween 80, D-Mannitol, 4-Hydroxy Benzoic Acid, L-Serine, α-Cyclodextrin, N-Acetyl-D-Glucosamine, γ-Hydroxybutyric Acid, L-Threonine, Glycogen, D-Glucosaminic Acid, Itaconic Acid, L-Glutamic Acid, D-Cellobiose, Glucose-1-Phosphate, α -Ketobutyric Acid, Phenylethylamine, α-D-Lactose, D,

L-α-Glycerol Phosphate, D-Malic Acid, and Putrescine. If the organism could metabolize the carbon source, the dye was released resulting in a purple color of varying intensity. The more easily the carbon substrate was metabolized, the darker the resulting purple color.

Cells from each of the isolated organisms were suspended in 0.85% sterile saline (8.5 g NaCl in 1 L H_2O) and normalized to a cell density which corresponded to an absorbance reading of 0.5 in a spectrophotometer set at 600 nm. A 100-µl aliquot of the cell suspension was inoculated into each well of the EcoPlate™ and incubated either aerobically or anaerobically for 72 h at 30°C. The EcoPlates™ were then placed in a microplate reader (λ=595) to quantify the intensity of the purple color in each well. Absorbance values were corrected for background interference.

Absorbance values from the EcoPlate™ microplate reader were used 3 ways: (i) the number of carbon sources used was determined by the number of positive tests (presence of any purple color) in each well; (ii) the preferred substrate was designated as the carbon substrate that gave the greatest absorbance value, and (iii) the Total Activity was determined by summing all the positive absorbance values for each set of 31 carbon sources.

16S rRNA gene identification. The identity of each organism was determined by 16S rRNA gene sequencing. Pure colonies of the isolated bacteria were transferred to a sterile tube containing a reaction mixture required for the Polymerase Chain Reaction (PCR). The PCR reaction mixture contained 12.5 µl of GoTaq™ Master Mix (Promega, Madison WI), 1 µl of a 100 µM solution of the forward primer and 1 ml of the reverse primer, and 10.5 ml of nuclease-free sterile water. Primers used were 005Forward (5′ – TGG AGA GTT TGA TCC TGG CTC AG – 3′) and 531Reverse (5′ – TAC CGC GGC TGC TGG CAC – 3′). PCR was performed using a MiCycler Thermocycler (BioRad, Hercules CA). Reaction tubes containing cells were first heated to 95°C for 5 min to lyse the cells. This was followed by 40 cycles of PCR with the following conditions: denaturing at 95°C for 30 s, primer annealing at 52°C for 30 s, and elongation at 72°C for 30 s. A final extension of 1 min at 72°C was performed and the samples held at 4°C. PCR products were purified using a Wizard SV96 PCR Clean Up System (Promega, Madison WI). The concentration and purity of the DNA was measured using the Nanodrop 1000 (Thermo Scientific, Wilmington DE). The purity of the DNA was calculated as the ratio of the Nanodrop absorbance at 260 nm and 280 nm (260/280). A ratio of 1.8 or greater indicated that the sample was mostly nucleic acid and not protein. All samples gave a 260/280 ratio of 1.8 or higher.

The PCR products were sequenced using the DTCS Quick Start Kit (Beckman Coulter, Fullerton CA). The 005Forward primer was diluted to 1 pmol μl^{-1} for the sequencing reaction. The sequencing reaction mixture contained 0 – 9.5 µl dH_2O, 0.5 – 10 µl of DNA, 2 µl of the sequencing primer, and 8 µl of the DCTS Quick Start Master Mix. The total reaction volume was 20 µl. The remainder of the sequencing protocol was performed using the manufacturer's protocol. Reaction mixtures were loaded into the CEQ 8000 Genetic Analysis System (Beckman Coulter, Fullerton CA) to sequence a ~500 bp section of the 16S rRNA gene. Returned sequences were queried using the Basic Local Alignment Search Tool for nucleotides (BLASTn) to identify the microorganisms (Altschul et al., 1990).

Electron Microscopy. Uncultured plant thorns were collected and examined by Scanning Electron Microscopy (SEM) using a Zeiss Evo LS10 microscope (Carl Zeiss LLC, Thornwood NY) to determine if bacterial cells and/or biofilms could be visualized on the thorn surfaces. Thorns of old growth and new growth plants were examined. Thorns were fixed for 1 h at room temperature in phosphate buffer (0.1 M, pH 7.0) containing 2.5% Glutaraldehyde + 2% Formaldehyde. The fixative was replaced with fresh phosphate buffer (0.1 M, pH 7.0) for 20 min at room temperature; this step was repeated two more times. Post-fixation was done overnight at 4°C using 1% Osmium tetroxide in phosphate buffer (0.1 M, pH 7.0). Osmium fixative was replaced with double-distilled water for 5 min and samples were then serially dehydrated by transferring into 25%, 50%, 75%, 95% and 100% ethanol for 20 min in each concentration. Dehydrated samples were transferred to fresh 100% ethanol for 20 min two more times. Samples were then dried at the critical point using ethanol and liquid CO_2. After drying, samples were etched for 5 sec and sputter coated with gold-palladium for 30 sec. A set of plant leaves and stems were also prepared and visualized by SEM as described above. All samples were observed using SEM procedures as prescribed by the manufacturer.

RESULTS

A total of 69 pure cultures were obtained but only 61 were positively identified. The majority (58%) of organisms was gram-positive and most displayed a facultative respiration. There were 8 cultures that were obligate aerobes (data not shown). Only 1 culture was an obligate anaerobic organism; however, this organism was not successfully identified and was not included in the final results. Fourteen of the identified cultures demonstrated some form of hemolysis. Four

cultures displayed alpha hemolysis, the partial lysing of the red blood cells, when grown on blood agar plates. The remaining hemolytic cultures displayed beta hemolysis, the complete lysing of the red blood cells (Tables 1 and 2).

EcoPlates™ were used to assess the carbon usage activities of the isolated organism. All 61 identified cultures were able to metabolize all 31 carbon sources available on the EcoPlates™. The preferred substrate (highest absorbance value) for many organisms was Tween 40, N-acetyl-D-glucosamine, D-Mannitol, or L -Asparagine (data not shown). The average Total Activity (sum of all positive absorbance values) was 22.84. Most of the aerobically-isolated cultures had Total Activity values below the overall average (Figure 1) whereas the anaerobically-isolated organisms displayed Total Activity values above the group average (Figure 2) even in cases where the organisms were identified as the same species. For example, cultures D2(1) and D2(2) were both identified as *Bacillus cereus*. However, D2(1) was isolated aerobically and D2(2) was isolated anaerobically. Both were isolated from the same Honey Mesquite thorn yet the anaerobic isolate displayed twice the Total Activity as the aerobic isolate.

Sequencing of the 16S rRNA gene positively identified the isolated organisms (Tables 1 and 2). With the exception of 2 isolates, the identification of the isolates showed >99% match to the BLASTn database and were within the percent range accepted as the same species. The 2 aforementioned isolates each matched the BLASTn database at 98.47% (data not shown). The most commonly identified bacterial species were *Bacillus cereus* (25 cultures), *Serratia marcescens* (16 cultures), *Pseudomonas aeruginosa* (7 cultures), *Bacillus pumilus* (6 cultures), and *Brevibacillus laterosporus* (2 cultures). The remaining organisms (5 cultures) were only identified once.

SEM results showed that the thorn surfaces were colonized by several types of microorganisms. Cell shapes varied and included cocci, short rods, long rods, and filamentous forms (Figure 3A, Figure 3C). Microorganisms were also viewed on other plant surfaces, such as stems and leaves (Figure 3B, Figure3D). Bacterial cells were observed on new growth and old growth plants (data not shown).

DISCUSSION

Several pathogens were isolated from thorns of RGV plants and identified. These included *Bacillus cereus*, *Pseudomonas aerginosa*, and *Serratia marcescens*. This does not appear to be a region-specific phenomenon as pathogens were also isolated from thorns of plant species in the Middle East, including the same pathogen species, *B. cereus* (Halpern et al.,

Table 1. Identification of bacterial cultures colonizing plant thorns of the Rio Grande Valley. Cultures were isolated from the aerobic portion of thioglycolate-tubed media. The plant host and the strain's hemolytic characteristics are shown. Note that the same species may be found on a single plant or multiple plants, and may show different hemolysis activity. Abbreviations: A = Agave; B = Brasil; G = Granjeno; H = Honey Mesquite; and R = Retama.

Species	Plant Host	Hemolysis	Number Isolated
Achromobacter piechaudi	B		1
Bacillus cereus	A, B, G, H, R		20
Bacillus cereus	G	Alpha	1
Bacillus pumilus	A, G		4
Bacillus pumilus	A, B	Alpha	2
Bacillus pseudomycoides	B		1
Brevibacillus laterosporus	R		1
Total			30

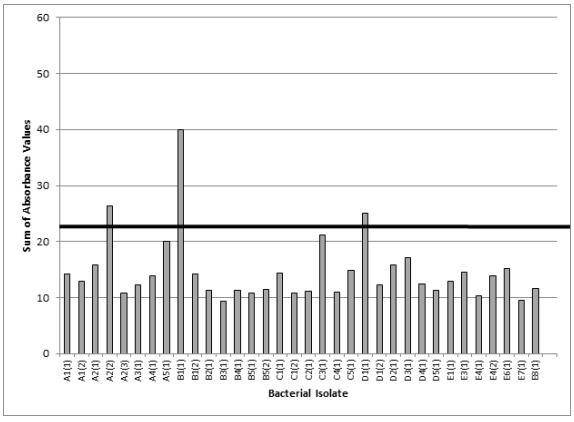

Fig.1. Total Activity of aerobically-isolated bacteria from thorns of 5 plant species in the Rio Grande Valley. The Total Activity was determined by summing the positive absorbance values from EcoPlates™ containing 31 organic substrates. The Average Total Activity for all organisms (22.84) is shown as the solid horizontal black line.

Table 2. Identification of bacterial cultures colonizing plant thorns of the Rio Grande Valley. Cultures were isolated from the anaerobic portion of thioglycolate-tubed media. The plant host and the strain's hemolytic characteristics are shown. Note that the same species may be found on a single plant or multiple plants, and may show different hemolysis activity. Abbreviations: A = Agave; B = Brasil; G = Granjeno; H = Honey Mesquite; and R = Retama.

Species	Plant Host	Hemolysis	Number Isolated
Bacillus cereus	G, H		3
Bacillus cereus	A	Beta	1
Brevibacillus laterosporus	R	Alpha	1
Enterobacter pyrinus	R		1
Escherichia hermanii	B		1
Exiguobacterium acetylticum	G		1
Pseudomonas aeruginosa	A, B, H		5
Pseudomonas aeruginosa	G	Beta	2
Serratia marcescens	A, B, H, R		9
Serratia marcescens	A, G, R	Beta	7
Total			31

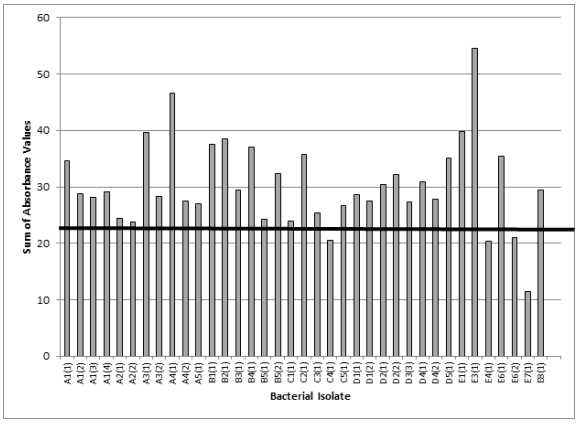

Fig.2. Total Activity of anaerobically-isolated bacteria from thorns of 5 plant species in the Rio Grande Valley. The Total Activity was determined by summing the positive absorbance values from EcoPlates™ containing 31 organic substrates. The Average Total Activity for all organisms (22.84) is shown as the solid horizontal black line.

2007). Several pathogens from RGV thorns displayed characteristics typical of many pathogens, such as facultative respiration and hemolytic activity. Hemolytic activity suggests that these organisms could be bloodborne or may be fastidious and require blood and/or its components for nutrition. However, the organisms were able to metabolize a variety of carbon substrates, especially when grown in anaerobic conditions, which suggests that they are opportunistic, not fastidious, and able to adapt to new environments.

In this study, the most frequently identified bacterium was *Bacillus cereus*, which is a Gram-positive, spore-forming, facultative organism found in many environments (Bottone 2010). Gastrointestinal ill-

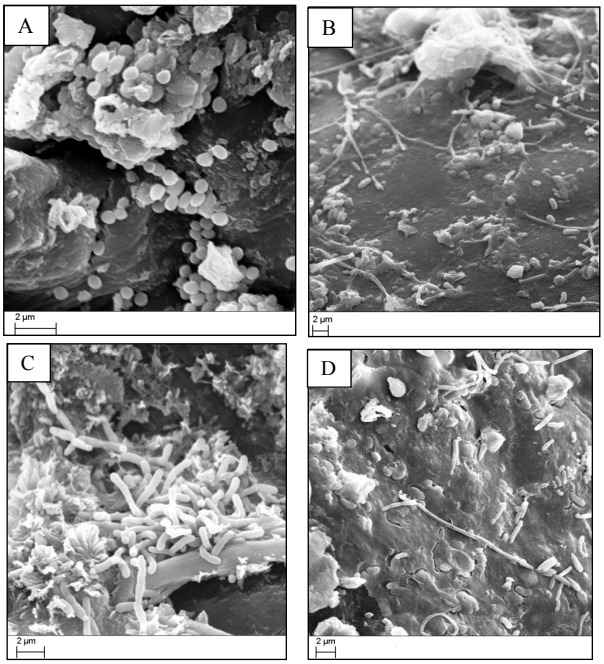

Fig.3. Representative Scanning Electron Microscopy (SEM) of plant thorns and other plant structures. Panel A: Granjeno plant (*Celtis pallida*) thorn; Panel B: Granjeno leaf; Panel C: Brasil plant (*Condalia hookeri*) thorn; Panel D: Brasil leaf. Bars = 2 µm.

nesses, such as food poisoning, as well as meningitis, eye infections, brain abscesses, pneumonia, endocarditis, and gangrene-like infections have been attributed

to *B. cereus* (Arnesen et al., 2008; Barrie et al., 1992; Bessman and Wagner 1975; Block et al., 1978; Bottone 2010). Not all strains of *B. cereus* are pathogenic, although some strains of *B. cereus* are problematic hospital pests that cause secondary infections in compromised patients (Åkesson et al., 1991; Auger et al., 2009; Bryce et al., 1993). Strains of *B. cereus* have been reported to produce hemolysins, which are compounds that cause hemolysis (Ghelardi et al., 2007).

Pseudomonas aeruginosa is another common hospital pest and was isolated from thorns of RGV plants. This bacterium is gram-negative and facultative. Blood stream infections, pneumonia, urinary tract infections, and other infections have been reported most commonly in immune-compromised patients (Peleg and Hooper 2010). Moreover, many strains of P. *aeruginosa* are multi-drug resistant making them especially problematic.

The second most frequently identified organism in this study was *Serratia marcescens*. This is a gram-negative, opportunistic pathogen that often displays a characteristic dark red color (Hejazi and Falkiner 1997). Skin diseases, including ulcers, abscesses and necrotizing fasciitis (a.k.a. flesh-eating disease) have been documented as caused by S. marcescens infections (Liangpunsakul and Pursell 2001; Friedman et al., 2003; João et al., 2008). Other diseases such as urinary tract infections have also been attributed to opportunistic strains of *S. marcescens* (Son et al., 2008).

Other identified organisms included *Bacillus pumilus*, *B. pseudomycoides*, *Brevibacillus laterosporus*, *Escherichia hermanii*, *Achromobacter piechaudi*, *Exiguobacterium acetylticum* and *Enterobacter pyrinus*. Of these, *E. hermannii* is the most pathogenic to animals and had been shown to cause eye infections and brain infections (Dahl et al., 2002). *Achromobacter piechaudi* is generally considered non-pathogenic, although infections have been reported in compromised individuals (Kay et al., 2001). *Bacillus pumilus* and *B. pseudomycoides* are common soil bacteria that are rarely pathogenic. *Brevibacillus laterosporus* and *Exiguobacterium acetylticum* display pathogenicity to insects and fungi, respectively, and have potential biocontrol uses (Saikia et al., 2011; Selvakumar et al., 2009). *Enterobacter pyrinus* causes leaf disease in some plant species but is typically not pathogenic to animals (Chung et al., 1993).

There are several documented cases of thorns serving as the source of infections in humans. Cases of septic arthritis of the knee have been attributed to bacteria housed on plant thorns and transferred to humans by thorn pricks (DeChamps et al., 2000; Francis et al., 2011). Synovitis of the knee caused by bacteria found on plant thorns has also been described (Duerinckx 2008). It is possible that other cases of thorn-transferred infections have occurred but were improperly diagnosed. This may be because the symptoms associated with thorn-related bacterial infections are often non-specific and common (e.g., pain, swelling, stiffness, fever) and difficult to distinguish from other illnesses.

Since the transfer of pathogens can occur via plant thorns and since several opportunistic pathogenic bacteria were isolated from thorns, it is possible that this could be a defense mechanism for plants as suggested by Halpern et al. (2007). Although this is an interesting supposition, there is a lack of information that demonstrates that pathogens can be transferred to animals or that this could deter herbivory. Studies will need to be performed to demonstrate this empirically. Furthermore, bacteria and other microorganisms are also present on other plant structures (e.g., leaves), not just the thorns. In some cases, leaves appeared to harbor a greater density of bacterial cells compared to thorns but this was most likely due to the greater surface area of a leaf relative to the surface area of a thorn. Thus, it is possible that the bacteria present on the thorns are not the result of a specific or mutualistic interaction between the plant thorn and the microorganisms. It is conceivable that the bacteria are non-specific colonizers of plant surfaces. However, due to the presence of potential pathogens on plants thorns, horticulturalists and agriculturalists should use necessary precautions when working with thorny plants.

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