Pectobacterium carotovorum subsp. *brasiliense* Causes Soft Rot and Death of *Neobuxbaumia tetetzo* in Zapotitlan Salinas Valley, Puebla, Mexico

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Abstract

Neobuxbaumia tetetzo (Coulter) Backeberg (tetecho) is a columnar cactus endemic to Mexico. Tetecho plants, flowers, fruits, and seeds play an important role in the semiarid ecosystem, as they serve as a refuge and food for insects, bats, and birds, and are widely used by ethnic groups since pre-Hispanic times. Tetecho is affected by a soft rot that damages the whole plant and causes its fall and disintegration. Eight bacterial colonies of similar morphology were isolated from plants showing soft rot and inoculated in healthy tetecho plants, reproducing typical symptoms of soft rot 9 days after inoculation. Ten representative isolates

were selected for phenotypic and genetic identification using 16S rDNA, IGS 16S-23S rDNA, and *rpoS* genes and for pathogenicity tests on several members of the cactus family and other plants. Based on the results, these bacterial isolates were identified as *Pectobacterium carotovorum* subsp. *brasiliense*. Inoculation of this bacteria caused soft rot in different cacti, fruits, leaves, and roots of other plants. This is the first report of the subspecies *brasiliense* of *P. carotovorum* causing soft rot and death in cacti in the world and the first report of this subspecies in Mexico.

Mexico is considered the country with the highest Cactaceae diversity with 913 taxa described, of which 80% are endemic to the country (Leonor and Sierra 2011). The Tehuacan-Cuicatlan Biosphere Reserve in Mexico is a semiarid region with the greatest diversity of endemic species of animals, plants, and microorganisms. An important group are columnar cacti (Leonor and Sierra 2011) where *Neobuxbaumia tetetzo* (Coulter) Backeberg stands out because of its abundance in Zapotitlan Salinas Valley, Puebla (18°20'N, 97°28'W) (Arias et al. 2017).

Tetecho occupies areas of up to 400 km² and population densities of 1,200 to 1,800 plants per hectare (Ruedas et al. 2006). The average lifespan of tetecho is 110 to 120 years. This cactus plays a fundamental ecological role within the ecosystem because from mid-April to June, the apex of the plant produces flowers, nectar, and pollen that feeds birds, insects, and bats that pollinate and disperse seeds throughout the area (Godínez 2011). Tetecho is an important source of food for ethnic groups that use flower buds, fruits, and seeds to prepare approximately 50 regional dishes. The Tetecho Regional Fair is held annually in May to show the diversity of uses of the plant. Even the dead plants are used as fuel and wood to build rustic huts (Godínez 2011).

One of the main factors that threatens tetecho is soft rot disease. During field trips to Zapotitlan Salinas Valley, Puebla, it was observed that tetecho plants showed areas of yellowish tissue and soft rot, stem rupture, and exudates. Soft rot expands throughout the plant and causes disintegration of tissues, collapse, and death; young plants may be affected. Due to the characteristics of these symptoms, the soft rot, collapse, and death of the tetecho could be caused by bacteria. The only information regarding this problem in tetecho in Mexico was contributed by Tovar (2011), who associated this symptom to borer insects and bacteria that accelerate tetecho death; however,

there are no descriptive records of insects nor bacteria that cause death of this cactus.

Considering the ecological importance of tetecho, the goal of this research was to identify the pathogen that causes soft rot and death of tetecho in Zapotitlan Salinas Valley, Puebla, Mexico.

Materials and Methods

Isolation. Tetecho plants in Zapotitlan Salinas Valley, Puebla, Mexico, were surveyed from 2015 to 2017. Eighty samples of tetecho plants that showed symptoms of soft rot were collected. Tissue sections of approximately 0.5 cm² from the transition zone between rotten and healthy tissue were disinfested using 1% sodium hypochlorite (v/v) for 1 min and then washed with autoclaved distilled water. To isolate bacteria, disinfested tissue was placed into nutrient broth (NB) and casaminoacid-peptone-glucose (CPG) media, as well as in buffer solution (Tris-EDTA). Additionally, exudates from diseased tetecho stems were directly collected in the field and placed in tubes that contained 5 ml of autoclaved distilled water, and 20 µl of this mix were inoculated in the laboratory into NB culture medium; inoculated media was incubated at 18°C for 48 h. Twenty microliters of each culture media were transferred to King's B (KB), Reasoner's 2A agar (R2A), and nutrient agar (NA) media. Suspensions were distributed on the media using a Drigalsky spreader and incubated as described above. Eighty bacterial isolates with similar morphology were recovered. Pure bacterial colonies were preserved in CPG medium at –20°C (Schaad et al. 2001).

Pathogenicity test. The 80 isolates were inoculated on potato slices (*Solanum tuberosum* L. var. Fiana) and tetecho plants (two slices of tuber and two cacti per isolate). Potato tubers were disinfested using 1% sodium hypochlorite for 1 min and washed with autoclaved distilled water; 4-cm diameter and 5-mm thick slices were cut and placed in a Petri dish that contained a piece of sterile Whatman #1 filter paper and water. Twenty µl of an aqueous bacterial suspension with an approximate cell density of 1.5×10^6 CFU ml^{–1} (0.2 absorbance at 580 nm) were placed on the center of slices and incubated at 28°C for 72 h. Controls were inoculated with autoclaved distilled water. One hundred and sixty tetecho plants that were 5 years old and 8 cm in height produced in certified nurseries in Zapotitlan, Puebla, were inoculated by injecting 20 µl of the bacterial suspensions as described previously (Stommel et al. 1996). The surface of the plants was disinfested using 70% ethanol (v/v) and washed with autoclaved distilled water prior to inoculation. Plants were maintained under

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greenhouse conditions (25 to 30°C, RH 80%, with natural light, for 10 days). Thirty tetecho plants that were inoculated with 20 μ l autoclaved distilled water were used as a control. From the 80 isolates that induced soft rot on potato tubers and tetecho plants, 10 selected bacterial isolates (Tet5, Tet16, Tet24, Tet36, Tet52, Tet60, Tet61, Tet72, Tet75, and Tet77) were randomly selected and inoculated by injecting 20 μ l of the bacterial suspension (1.5×10^6 CFU ml^{-1}) into seven cactus species (surface disinfested with alcohol as described above): *Myrtillocactus geometrizans*, *Mammillaria magnimamma*, *Mammillaria discolor*, *Gymnocalycium horstii*, *Pachocereus marginatus*, *Opuntia rufida*, and *Opuntia ficus-indica*. Plants inoculated with autoclaved distilled water were used as a control. The cacti were maintained under the greenhouse conditions described above. To test the ability to macerate tissue from other plant species, fruits, leaves, or roots, the isolates were also inoculated by injection (20 μ l; 1.5×10^6 CFU ml^{-1}) on disinfested (2% sodium hypochlorite for 2 min and washed with autoclaved distilled water) fruits of *Capsicum annuum* var. *annuum*, *Solanum lycopersicum*, *Cucumis sativus*, leaves of *Lactuca sativa*, and roots of *Daucus carota* subsp. *sativus* and *Ipomoea batatas* and placed in a moisture chamber at 28°C for 4 days.

Phenotypic characterization. The isolates were characterized and identified according to Schaad et al. (2001) and Czajkowski et al. (2015). Sensitivity to 12 antibiotics was evaluated (Bio-Rad,

CA, U.S.A.) and biochemical profiles were determined using API20 E (Biomérieux, Durham, NC, U.S.A.).

Molecular characterization. *DNA extraction.* DNA from the isolates was obtained from single colonies that grew 24 h in CPG medium at 28°C, using PureLink Genomic DNA Kits (Invitrogen Life Technologies, Carlsbad, CA, U.S.A.) following manufacturer's instructions.

16S rDNA sequencing. Universal primers 8F (5'-AGAGTTTG ATCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACG ACTT-3') were used to amplify and to sequence 16S rRNA gene from the isolates. Polymerase chain reaction (PCR) was carried out in a final volume of 50 μ l, with 1 $\times$ PCR Buffer, 1.5 mM MgCl_2 , 0.2 mM of each dNTP, 0.6 μ M of each oligonucleotide, 1.5 U of Taq polymerase (Invitrogen), and 10 ng of template DNA. PCR conditions were initial denaturation at 95°C for 5 min, followed by 30 cycles of denaturation at 94°C for 50 s, annealing at 57°C for 30 s, extension at 72°C for 90 s, and final extension at 72°C for 5 min (Eden et al. 1991). Aliquots of 5 μ l were analyzed by electrophoresis in 1.5% (w/v) agarose (Promega, Madison, WI, U.S.A.) in TBE buffer. Gels were stained with ethidium bromide and visualized in a gel documentation (DNR minibus-pro). Amplicons were purified from the gel using Wizard SV gel and PCR Clean-Up System Kit (Promega), following manufacturer's instructions, and sequenced

Fig. 1. Symptoms of soft rot and exudate in tetecho plants in Zapotitlán Salinas Valley, Puebla, Mexico: (a) plants showing soft rot, (b) plants showing exudates, (c) collapse and disintegration of tissue. Symptoms in inoculated tetecho plants under greenhouse conditions: (d) soft rot and exudates 7 days after inoculation (dai), (e) tetecho collapse 9 dai, (f) control plants without symptoms.

in a Genetic Analyzer 3100 (Applied Biosystems Corp., Foster City, CA, U.S.A.).

IGS 16S-23S rDNA sequencing. Intergenic spacer region (IGS) 16S-23S rDNA from the isolates was tested using specific primers for *Pectobacterium carotovorum* subsp. *brasiliense* BR1f (5'-GCGTGCCGGGTTTATGACCT-3') and L1r (5'-CAAGGCATC-CACCGT-3'). BR1f was designed from IGS region based on restriction enzyme SEXA1 and L1r was designed from the 5' end of the 23S rRNA gene (Duarte et al. 2004). PCR was carried out in a final volume of 50 μ l with 1 $\times$ PCR buffer, 2 mM MgCl₂, 0.1 mM of each dNTP, 0.3 μ M of each oligonucleotide, 1.5 U Taq polymerase (Invitrogen), and 10 ng template DNA. PCR conditions were initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 94°C for 30 s, 62°C for 45 s, 72°C for 90 s, and final extension at 72°C for 7 min (Choi and Kim 2013). Amplified products were visualized by electrophoresis in a 2% agarose gel (Promega). Amplicons were purified from the gel using Wizard SV gel and PCR Clean-Up System kit (Promega) and sequenced in a Genetic Analyzer 3100 (Applied Biosystems Corp.). Sequences were analyzed and compared with similar sequences available at the National Center for Biotechnological Information (GenBank-EMBL) (Altschul et al. 1990). Primers BR1f and L1r were used for amplification on DNA extracted from tetecho tissue that showed symptoms of soft rot collected in Zapotitlan Salinas Valley. Negative controls included *P. carotovorum* subsp. *carotovorum*, *P. atrosepticum*, and *Dickeya chrysanthemi* strains obtained from the Plant Pathology Bacteria Laboratory at the College of Postgraduates.

rpoS sequencing. Identity of the isolates was confirmed by sequencing the σ subunit of the RNA polymerase factor 38 (*rpoS*) using primers rpoS1 (ATGAGCCAAAGTACGCTGAA) and rpoS2 (ACCTGAATCTGACGAACACG) (Waleron et al. 2002). PCR was carried out in a final volume of 50 μ l with 1 $\times$ PCR buffer, 2.5 mM MgCl₂, 0.2 mM of each dNTP, 0.3 μ M of each oligonucleotide, 2 U Taq polymerase (Invitrogen), and 10 ng of template DNA. PCR conditions were initial denaturation at 95°C for 3 min, followed by 32 cycles of denaturation at 94°C for 1 min, 55°C for 50 s, 72°C

for 2 min, and final extension of 72°C for 6 min (Waleron et al. 2002). Amplicons were visualized by electrophoresis in 2% agarose gel (Promega). Amplicons were purified using Wizard SV gel and PCR Clean-Up System Kit (Promega), following manufacturer's instructions and sequenced in a Genetic Analyzer 3100 (Applied Biosystems Corp.). Sequences were compared using the BLAST tool at the National Center for Biotechnology Information (GenBank-EMBL) (Altschul et al. 1990). Consensus sequences obtained in this research were deposited in GenBank. The *rpoS* sequences from the isolates were compared with sequences obtained from NCBI (accession nos. KP762578, KP762579, KJ818457, JF926832, KJ818464, and JN600353) and analyzed using the maximum-likelihood method and the neighbor-joining model in MEGA 6 (Tamura et al. 2013).

Results

Symptoms and pathogenicity. Typical symptoms of soft rot, collapse, and death were observed in tetecho plants in Zapotitlan Salinas Valley, Puebla, Mexico (Fig. 1a, b, and c). From tetecho plants that showed typical symptoms of soft rot and exudates, 80 bacterial strains with similar morphology (Tet1–Tet80) were isolated. Bacteria grew on NA, CPG, and KB media. Isolates showed pectinolytic activity on potato slices. Soft rot was observed on inoculated tetecho plants under greenhouse conditions (Fig. 1d and e). In these tetecho plants, exudates were observed 2 days after inoculation (dai), and soft rot was observed in the whole plant 7 and 9 dai when plants collapsed and died. Inoculated bacteria were successfully reisolated from rotten, inoculated tetecho plants showing the same morphological characteristics, fulfilling Koch's postulates. Identity was confirmed by phenotypic characterization, and PCR using specific primers BR1f and L1r (Duarte et al. 2004). No symptoms were observed in control plants (Fig. 1f).

The isolates caused soft rot on cacti *Myrtillocactus geometrizans* (Fig. 2a), *Opuntia rufida*, *Opuntia ficus-indica*, *Gymnocalycium horstii* (Fig. 2b), *Pachicereus marginatus* (Fig. 2c), *Mammillaria magnimamma*, *Mammillaria discolor*, and on fruits of *Capsicum annuum* var. *annuum* (Fig. 2d), *Solanum lycopersicum* (Fig. 2e), *Cucumis*

Fig. 2. Soft rot symptoms observed 2 days after inoculation on (a) *Myrtillocactus geometrizans*, (b) *Gymnocalycium horstii*, (c) *Pachicereus marginatus*, fruits of (d) *Capsicum annuum* var. *annuum*, (e) *Solanum lycopersicum*, and leaves of (f) *Lactuca sativa*.

sativus, leaves of *Lactuca sativa* (Fig. 2f), and roots of *Daucus carota* subsp. *sativus* and *Ipomoea batatas*. Soft rot was observed 2 dai and collapse occurred 4 dai. Fruits of *Cucurbita pepo* and *Physalis ixocarpa* and leaves of *Brassica oleracea* var. *capitata* did not show symptoms. None of the controls showed symptoms and the inoculated bacteria were successfully reisolated from inoculated plants and organs.

Phenotypic characterization. The isolates formed circular colonies, gray in color, convex and wet in appearance. Cells were bacillar, mobile, and Gram negative. Isolates were facultative anaerobes, did not fluoresce on KB medium, grew at 37°C in NB and CPG culture media, did not produce sucrose-reducing substances and indole, and produced acid from lactose, maltose, α -methyl glucoside, and trehalose, but not from sorbitol. Isolates tolerated 7% NaCl in NB and CPG and they were sensitive to amikasin, ampicillin, cephaletin, cefinaxone, chloramphenicol, enonaxin, gentamicin, notilmicin, and

trimethoprim-sulfatomeoxazol. Isolates were not sensitive to penicillin, erythromycin, and dicloxacillin. No differences were observed in the phenotypic characterization.

Molecular characterization. Sequences of the 16S rRNA gene from the isolates (consensus sequence with accession numbers MF403054, MG818833–MG818841) indicated 99% similarity to *P. carotovorum* subsp. *brasiliense* (accession no. CP020350). Using specific primers BR1f and L1r (Duarte et al. 2004) it was possible to amplify a 381-bp fragment from the isolates and tissue samples from the field that showed typical symptoms. Similar results were reported by Choi and Kim (2013). It was not observed in amplicons using the reference strains *P. carotovorum* subsp. *carotovorum*, *P. atrosepticum*, and *D. chrysanthemi* (Fig. 3).

Amplified sequences from the IGS 16s-23S rDNA region and σ subunit of RNA polymerase factor 38 (*rpoS*) obtained from the isolates showed from 99 to 100% (accession nos. MG825334–MG825343

Fig. 3. Amplicons of 381 bp observed using BR1f and L1r primers from the IGS 16S-23S region obtained from 10 selected isolates (2–11) and field tissue samples that show typical symptoms (12). No amplicons were observed on *Pectobacterium carotovorum* subsp. *carotovorum* (13), *P. atrosepticum* (14), and *Dickeya chrysanthemi* (15) strains. 100 bp ladder lane 1.

Fig. 4. Phylogenetic analysis (maximum-likelihood method) using *rpoS* sequences of 10 isolates from tetecho and available sequences of the same gene of *Pectobacterium carotovorum* subsp. *brasiliense*, *P. carotovorum* subsp. *carotovorum*, and *P. atrosepticum*.

and MG963906–MG963915) similarity to *P. carotovorum* subsp. *brasiliense* (accession nos. AF448592 and JF926836).

Phylogenetic analysis (maximum-likelihood method) using *rpoS* sequences of the isolates showed a compact cluster with *P. carotovorum* subsp. *brasiliense* (accession nos. KP762578 and KP762579), separated from *P. carotovorum* subsp. *carotovorum* and *P. atrosepticum*. Variability was observed within tetecho isolates using the *rpoS* gene (Fig. 4).

Based on pathogenicity, phenotypical, and molecular characterization, the causal agent of soft rot, collapse, and death that affects tetecho plants in Zapotitlan Salinas Valley, Puebla, Mexico, is *P. carotovorum* subsp. *brasiliense*.

Discussion

P. carotovorum subsp. *brasiliense* was identified as the causal agent of soft rot, collapse, and death of tetecho in Zapotitlan Salinas Valley, Puebla, Mexico. *P. carotovorum* subsp. *brasiliense* was first described in Brazil causing “black leg” on potato (*Solanum tuberosum* L.) (Duarte et al. 2004). *P. carotovorum* subsp. *brasiliense* has been reported as an emerging bacterium causing “black leg” of potato in New Zealand (Panda et al. 2012), Canada (De Boer et al. 2012), South Africa (van der Merwe et al. 2010), and Holland (Nunes Leite et al. 2014). *P. carotovorum* subsp. *brasiliense* has a wide host range, and the bacterium has been reported on cucumber (*Cucumis sativus* L.) (Onkendi et al. 2016), tomato (*Solanum lycopersicum* L.) (Jaramillo et al. 2017), beetroot (*Beta vulgaris* L.), cabbage (*Brassica oleracea* var. *capitata*), zucchini (*Cucurbita pepo* L.) (Waleron et al. 2015), and pepper (*Capsicum annuum* var. *annuum*) (Gillis et al. 2017). In this research, *P. carotovorum* subsp. *brasiliense* isolated from tetecho that was inoculated on *Cucurbita pepo* and *Brassica oleracea* var. *capitata* did not induce soft rot. Similar results have been reported by Waleron et al. (2015). Also, Alcorn et al. (1991) found differences in the pectinolytic activity of 108 bacteria (*P. carotovorum* subsp. *brasiliense* not included) isolated from cacti from distinct geographical origin. They observed different abilities of the bacteria to produce soft rot within the same genetic group when inoculated in tomato fruit, potato tuber, and carrot root (*Daucus carota*).

P. carotovorum subsp. *brasiliense* was described as a new subspecies of *P. carotovorum* due to its phenotypic and genetic characteristics, and higher virulence and aggressiveness compared with *P. carotovorum*, *P. atrosepticum*, *P. betavascularum*, *P. wasabiae*, and *Dickeya* spp. (Duarte et al. 2004; van der Wolf et al. 2017). The pathogenicity of *P. carotovorum* subsp. *brasiliense* is based on the ability to produce a greater range of enzymes, such as pectate lyase, polygalacturonase, cellulase, and proteases, that confers virulence factors to degrade structural components of the cell wall and infect the host (Lee et al. 2014; Toth and Birch 2005). The main goal of this research was to identify the causal agent of tetecho rot. Future studies should focus on the genetic variability and differences in virulence and aggressiveness of these isolates.

Phenotypical characterization allowed us to differentiate the bacteria isolated from tetecho from the most common pectinolytic Enterobacteriaceae and identify them as *P. carotovorum* subsp. *brasiliense* (Czajkowski et al. 2015; Schaad et al. 2001). In addition, this research provides information on sensitivity of *P. carotovorum* subsp. *brasiliense* to antibiotics and its ability to tolerate 7% NaCl in NB and CPG medium. To verify phenotypical identification, 16S rDNA, IGS 16S-23S rDNA, and *rpoS* genes were amplified and sequenced. The 16S-23S intergenic region and *rpoS* gene have been successfully used to separate *P. carotovorum* subsp. *brasiliense* from other related pectinolytic bacteria (Duarte et al. 2004; Waleron et al. 2002). Analysis of the *rpoS* gene sequences of these isolates showed them to be in the same cluster with *P. carotovorum* subsp. *brasiliense* (Fig. 4). The same gene also shows diversity among these 10 tetecho isolates that may be useful for future research focused on the analysis of the diversity of this pathogen.

The genus *Pectobacterium* is considered a heterogeneous group with genetic diversity. Phylogenetic analysis of eight conserved genes using strains of *P. carotovorum* subsp. *brasiliense* identified

two clusters that were explained based on their geographical origin (Lee et al. 2014; Nabhan et al. 2012). This heterogeneity was also detected in the bacteria as a pathogen, since different virulence and aggressiveness has been detected in pathogenicity tests in potato plants (Marquez-Villavicencio et al. 2011).

Few studies have addressed soft rot in columnar cacti in the world. Alcorn et al. (1991) studied pectinolytic bacteria that cause soft rot of saguaro cactus (*Carnegiea gigantea*) in Arizona and Texas, U.S.A., and northern Mexico. Using fatty acid profiles, Alcorn et al. (1991) were able to identify *Pectobacterium* (*Erwinia*) *cacticida* as a new species within most common pectinolytic bacteria such as *P. atrosepticum* and *P. carotovorum*. Martínez et al. (2011) identified *Pectobacterium* by sequencing total DNA extracted from necrotic tissue of the columnar cactus *Isolatocereus dumortieri* and the intestine of *Copestylum* larvae (Diptera, Syrphidae) associated with this necrotic tissue. Martínez et al. (2011) suggested that bacteria and yeast colonize cacti that are damaged, stressed (mainly due to environmental factors), or senescent. Bacteria and yeast cause lysis of plant cells, exposing cellular content and creating an environment that attracts insects.

Pectobacterium may establish a close relationship with insects (Basset et al. 2000; Muniz et al. 2007). The necrotic tissue of *Stenocereus gummosus* serves as a food and reproduction substrate for *Drosophila mojavensis*, an endemic fly in the Mexican Sonoran desert (Fogleman and Foster 1989). Further studies showed that four endemic species of *Drosophila* fed and reproduced on necrotic tissue of five columnar cactus species. A high frequency of facultative anaerobic bacteria within the Enterobacteriaceae family in the necrotic tissue was also observed (Fogleman and Danielson 2001).

In Zapotitlan Salinas Valley, Puebla, Mexico, the pathosystem tetecho–*P. carotovorum* subsp. *brasiliense* involves at least three insect orders: two Coleoptera and one Diptera (unpublished data). It has been suggested that insects introduce and disperse the pathogen (Tovar 2011); however, there is no information about the role of insects as possible vectors of *P. carotovorum* subsp. *brasiliense*. Further research exploring the role of insects in the development of soft rot in tetecho is necessary to understand the pathosystem and to design an integrated disease management program.

To the best of our knowledge, this is the first report of *P. carotovorum* subsp. *brasiliense* as a cactus pathogen in the world. It is possible that *P. carotovorum* subsp. *brasiliense* and tetecho have coevolved over a long period of time in Zapotitlan Salinas Valley and that the bacterium has been in Mexico for a long time. Pathogenicity in many hosts of *P. carotovorum* subsp. *brasiliense* represents a risk for agriculture and cactacea biodiversity in Mexico.

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