Evaluation of a novel endophytic *Pseudomonas lactis* strain for control of boxwood blight[1]

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Abstract

Boxwood blight, caused by *Calonectria pseudonaviculata* (*Cps*) (Synonyms: *Cylindrocladium buxicola*, *Cylindrocladium pseudonaviculatum*), is a devastating disease which affects boxwood (*Buxus*) in private and public gardens worldwide. SW, an endophyte identified as a strain of *Pseudomonas lactis*, was isolated along with seven other bacterial strains from symptom-reversed boxwood leaves infected by *Cps*. SW had the largest population among the 8 isolates. SW inhibited *Cps* culture growth similar to three other antagonistic isolates but was the strongest suppressor of *Cps* conidial germination and germling development. When the cell-free supernatant (CFS) from SW liquid cultures was used to treat boxwood plants 48 h prior to inoculation with *Cps*, it resulted in a 69.4% disease reduction; suggesting involvement of its metabolites in disease suppression. Boxwood blight control efficacy was further evaluated with resuspended SW cell at 10^{8-9} cfu·ml^{-1} at different treatment lead times. When SW was applied 2 or 10 days before inoculation with *Cps*, boxwood blight disease decreased by 72% and 67%, respectively. Further extending the lead time to 20 and 30 days, the efficacy was reduced to 26-27%. However, with its features of moderate control efficacy, high population in plant tissue and safety towards humans, SW shows great potential as a biocontrol agent for boxwood blight.

Index words: Biocontrol agent, endophyte, plant pretreatment, *Calonectria pseudonaviculata* suppression.

Species used in this study: Bacterium strain, SW (*Pseudomonas lactis*). Plant species: *Buxus sempervirens* 'Justin Brouwers'.

Significance to the Horticulture industry

Boxwood blight is a destructive and fast-growing disease affecting *Buxus*. It is a serious concern for the boxwood nursery, landscape and plant trading industries. Economic losses from this disease are enormous due to the lack of resistant cultivars, ineffectiveness of control practices and reliance on costly and potentially environmentally-damaging chemical controls. The discovery of *Pseudomonas lactis* strain SW, a foliage endophyte strongly antagonistic to the boxwood blight pathogen, opens up new avenues for controlling boxwood blight sustainably and effectively. Pretreating plants with SW at high concentrations with lead times of 10 days or less reduced disease incidence by more than 70%. Thus, SW has great potential to be developed into a biofungicide which can fulfill the urgent need for boxwood blight control.

Introduction

Boxwood blight is a destructive, fast-spreading disease caused by *Calonectria pseudonaviculata* (*Cps*), damaging private and public gardens worldwide (LeBlanc et al. 2018). In the United States, the disease has spread to more than 27 states since it was first reported in 2012 (Calabro 2018, Gilson 2018, Ivors et al. 2012). Trying to stop the spread and manage infestations of boxwood blight is an overwhelming challenge for the boxwood production, landscaping and retail industries.

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Economically viable and environmentally sound disease control methods are urgently needed for control of boxwood blight. The current boxwood plant protection paradigm relies largely on repeated fungicide applications which are not only costly, and contingently effective (Baudoin et al. 2015, Bush et al. 2016), but also present significant human health and environmental hazards(Anonymous 2019). Frequently, producers resort to crop destruction when a chemical control fails, resulting in irretrievable losses. Many efforts have been made in search of better control alternatives. Mulching is a readily available and practical measure that provides a physical barrier to prevent infested soil and diseased leaf litter from splashing onto boxwood leaves (Likins et al. 2018). However, it has no effect on aerial or mechanical spread of the disease. Biocontrol agents have also been discussed as potential alternatives to chemical fungicides. A recently identified *Burkholderia* strain (SSG) from boxwood provided nearly perfect control for boxwood blight when used for plant treatment prior to inoculation with *Cps* (Kong and Hong 2019). However, its development may not be acceptable because it is closely related to a bacterial group, *B. cepacia* complex, in which some of members are opportunistic human pathogens causing pneumonia in immunocompromised individuals with underlying lung disease (Mahenthiralingam et al. 2005, Pal 2006).

Endophytes are microorganisms which reside in plant tissue but do not cause disease (Nair and Padmavathy 2014). They have received considerable attention because of their abilities to promote plant growth and suppress plant pathogens. SW was isolated from symptom reversed boxwood leaves that first showed water-soaked lesions three days after inoculation with *Cps* but the symptoms disappeared later. In addition to having the greatest population, it was the second most effective isolate for suppression of growth of *Cps* culture (Kong and Hong

2019). The objective of this research was to identify SW and evaluate its efficacy for control of boxwood blight.

Materials and Methods

SW isolation, culture, cell suspension and cell free supernatants. Six leaves of boxwood plants (*Buxus sempervirens* 'Justin Brouwers') which initially showed boxwood blight symptoms after inoculation with *Cps* but then the symptoms disappeared a few days later were surface sterilized with 70% ethanol, cut into small pieces and homogenized in 6 ml sterile deionized water (SDW). A volume of 0.2 ml of the suspension were spread onto potato dextrose agar (PDA, Sigma-Aldrich, St. Louis, MO, USA) plates. The plates were incubated at 25 C (77 F) for 48 h and then examined for colony populations and morphology. Colonies present on the plate were grouped by size and color and counted. A representative of small white colonies (SW) in the plate was subcultured and maintained on PDA at 25 C (77 F).

To prepare cell suspensions and cell free supernatants of the culture (CFS), a single colony of SW culture on PDA was transferred into tubes containing 4 ml of nutrient broth (NB, Sigma-Aldrich, St. Louis, MO, USA) and grown on a shaker at 180 rpm, 27 C (81 F) for 24 h to make a culture stock. For plant application, 1 ml of the stock was cultured in 150 ml potato dextrose broth (PDB, Sigma-Aldrich, St. Louis, MO, USA) for 40 h under the same conditions. The concentrations of the culture were $10^{8\text{-}9}$ cfu·ml^{-1} that was determined by 10-fold serial dilution of the broth culture followed by plating the diluted suspensions in individual PDA plates and counting colonies after 48 h at 27 C (81 F). The culture was centrifuged at 14210 ×g for 15 min to precipitate bacterial cells. To make the cell suspension, the cell pellet was resuspended in 0.01 % Tween 20 (Sigma-Aldrich, St. Louis, MO, USA). To obtain CFS, the supernatant of the broth culture was passed through a Millipore^{TM} Plus 0.22-µm filter (Millipore, Billerica, MA, USA).

Identification of SW. The stock culture of SW was used for DNA extraction. Polymerase chain reaction (PCR) was performed to amplify the 16S rRNA gene using the universal primers 27F, 968F and 1410R (Nübel 1996). Individual PCR products were sequenced in duplicate from both strands. The sequences were processed and aligned using Mega X (Knyaz et al. 2018). The consensus sequence from the alignment was used to classify SW using EzBioCloud (Yoon et al. 2017) and was deposited into GenBank at http://blast.ncbi.nlm.nih.gov.

Cps and conidial inoculum preparation. A *Cps* isolate Sb, was grown at 25 C (77 F) and maintained at 20 C (68 F) on PDA as described previously (Kong and Hong 2018). The conidia for plant inoculation were produced using a liquid culture in fresh potato broth as described previously (Kong et al. 2017). Conidia were suspended in 0.01% Tween 20. Conidial concentration was determined with a hemocytometer. Conidial concentrations at 2-5 ×10^{4}·ml^{-1} were used for boxwood plant inoculation.

Cps suppression assays. To study antibiotic activity of SW, an assay to determine suppression of *Cps* conidia germination and development was conducted. A 90-mm PDA plate was spread with 500 conidia in SDW. In the center of the plate, a 7-mm sterilized disk of Whatman® filter paper 1 (Sigma-Aldrich, St. Louis, MO, USA) was placed and spotted with 10 µL of either a test isolate cell culture stock (SW, SSG, SP, LW2) or the control PDB. *Cps* colony formation in the plate was examined after one-week incubation at 25C (77F) in the dark. The assay, including three replicate plates, was repeated twice.

Plant pretreatments and inoculation. Buxus sempervirens 'Justin Brouwers' plants in 2.8- liter (6 in diam) pots were used. Plants were maintained on a gravel pad at the Virginia Tech Hampton Roads Agricultural Research and Extension Center until used. Since boxwood blight is a high-impact disease in the USA with very limited distribution in the eastern Virginia, plants were transferred to large plastic storage containers 55.9 × 33 × 44.5 cm (22 × 13 × 17.5 in) in the laboratory at the time of experiment. Plants were kept at 23 C (73 F) with a 9 h light/15 h dark cycle and watered as needed during the experiment. Treatments included SW cell suspension at $10^{8\text{-}9}$·ml^{-1}, CFS, and the control, 0.01% Tween 20. Each treatment was done on three replicate plants by spraying 20 mL of a respective solution. Treated plants were kept in the containers and arranged in a randomized complete block design, one plant per treatment per block. Containers were covered for a day after treatment to maintain moisture and uncovered until inoculation with *Cps*. Treatment lead time before inoculation included 2, 10, 20 and 30 days.

Inoculation was done by spraying the plants with a conidial suspension at $10^{4\text{-}5}$·ml^{-1} until dripping. Inoculated plants were incubated in a closed container to keep moisture as done for pretreatment for 2 days. Total and infected leaves were counted 7 days after inoculation, and both counts were used to calculate disease incidence of each treatment. Disease reduction or efficacy of SW at a lead time was computed by dividing the difference in disease incidence between the control and SW treatments by the control disease incidence. All the experiments were done twice.

Statistics. Analysis of Variance (ANOVA) was used to analyze data homogeneity between the repeated experiments of pretreatment for control of the boxwood blight and significance among treatments of homogenous experiments. Means among treatments were separated with T-test at equal variances according to the least significant difference (LSD) at $\alpha = 0.05$. ANOVA, standard errors and t-tests were done using Excel statistical functions [Microsoft Excel 2016 (v16.0)].

Results and Discussion

SW was a bacterium characterized by small white colonies on PDA. It accounted for more than 60% of the endophytic bacterial population in the isolation plates (~500 colonies per plate) (Fig. 1). The estimated population in a leaf was close to 10,000 cfu, suggesting

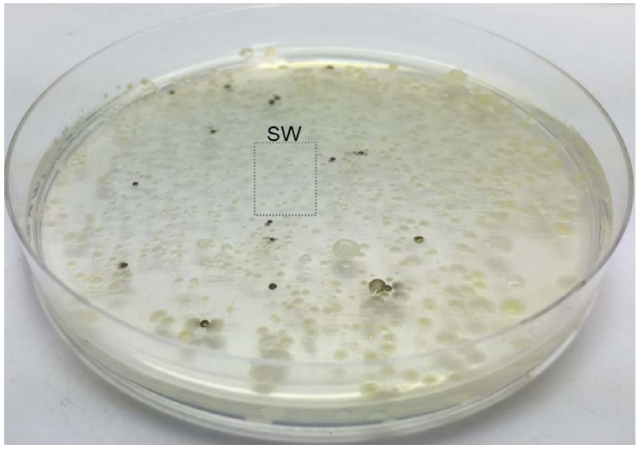

Fig. 1. Colony morphology and population of boxwood endophytic SW strain in an isolation plate. Leaves that showed symptom reversion after infection by boxwood blight pathogen was surface sterilized and homogenized in 1 ml sterile deionized water (SDW) per leaf. Each potato dextrose agar (PDA) plate was spread with 200 µl resultant suspension. Colonies in the plates were examined and counted after 48 h at 25 C (77 F). Typical morphology of SW's small and white colonies is presented in the rectangle box.

that it might have contributed greatly to the symptom reversion of leaves inoculated with *Cps*. The 16S sequence of SW (Accession#:MK423986) matched best with that of a strain of *Pseudomonas lactis* WS4672 (T) at 98.99% identity. Other similar species on the basis of 16S rRNA gene sequences were *P. paralactis*, *P. azotoformansin*, *P. gessardii* and *P. cedrina*. *P. lactis* and *P. paralactis* are recently identified species in *Pseudomonas* which produce antibiotic lipopeptides. *P. azotoformansin*, *P. gessardii* and *P. cedrina* have been placed in the *P. fluorescens* group that is home to many biocontrol agents for plant root pathogens (Haas and Keel 2003). These bacteria produce the antimicrobial compounds pyoluteorin and 2,4-diacetylphloroglucinol which can actively suppress various plant pathogens. It is not clear whether SW may also produce these antibiotics. However, SW strongly suppressed *Cps* colony formation from conidia (Fig. 2), suggesting that the metabolites inhibit conidia germination. SW has been shown to suppress *Cps* culture growth in a dual culture assay (Kong and Hong 2019). These results suggest that SW produced antibiotics that can suppress not only conidia germination but also mycelial growth. Interestingly, two other endophyte isolates from symptom reversed boxwood leaves, LW2 and SP, were also classified as *P. lactis* based on16S rRNA gene sequences (Accession#:MK423984 and MK423985). However, they did not prevent *Cps* conidia formation (Fig. 2), although to some extent they inhibited *Cps* growth in culture (Kong and Hong 2019). This suggests that the SW strain of *P. lactis* may produce unique antifungal compounds that are not produced by other members of *P. lactis*.

SW was further investigated for boxwood blight prevention and showed good efficacy. Plants pretreated with a SW cell suspension and a CFS 2 days before *Cps* inoculation had infection rates of 14.8% and 15.9%, respectively (Fig. 3). Compared to the control (52%), the treatments reduced disease by 72% and 69%. There was no significant difference between the treatments. Compared to other biofungicides tested previously which reduced infection by *Cps* less than 10% (Yang and Hong 2017), efficacies of both SW cells and CFS are substantially higher. The efficacies are also higher than that of recently reported non-indigenous fungal and bacterial biocontrol agents, which provide up to 60% protection when used

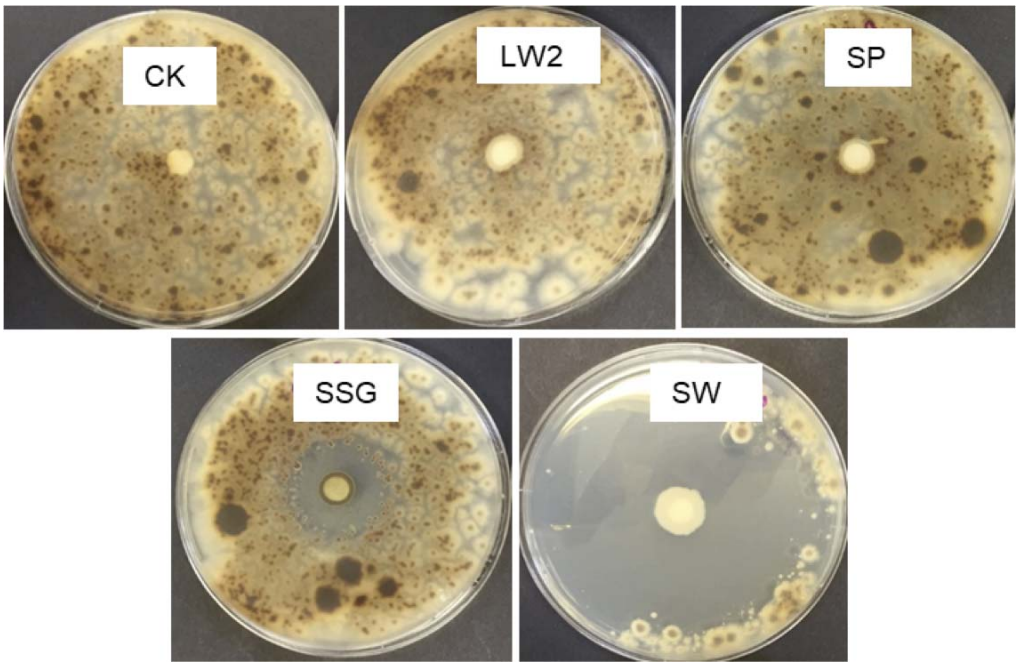

Fig. 2. Growth of *Calonectria pseudonaviculata* (*Cps*) colonies as affected by the addition of boxwood endophytes. In the center of potato dextrose agar plates spread with *Cps* conidia, a drop of a boxwood endophyte isolate (LW2, SP, SSG and SW) or nutrient broth (CK) was placed and incubation at 25 C (77 F). Pictures were taken 7 days after the placement.

Fig. 3. Boxwood blight infection rates and representative symptoms on 'Justin Brouwers' boxwood as affected by endophyte isolate SW. Plants were pretreated with SW resuspension at 10^{8-9} cfu·ml^{-1}, SW cell-free supernatant (SWCFS), and 0.01% Tween control 2 days before inoculation with *Cps* at 2-3×10^4 at 23 C (77 F). Pictures were taken 7 days after inoculation. The infection rate and the standard error, +/-, of respective treatment were placed in parentheses.

with a long lead time and on a weekly basis, respectively (Kong and Hong 2017, Yang and Hong 2018). However, the efficacy of SW was surpassed by SSG, another endophyte recovered from the same boxwood leaves (Kong and Hong 2019).

SW protection of boxwood plants lasted at least for 10 days. Plants received similar protection at pretreatment lead times of 10 days as they did at 2 days before inoculation with *Cps* (Fig. 4). However, the efficacy was significantly reduced when the lead time was extended to more than 20 days. This result suggests that SW may survive poorly on leaf surfaces, like other endophytes (Joy and Parke 1994), or that there may be very limited SW cells entering plant tissue after application. This means that SW may have to be used repeatedly (every 10 days) to maintain its efficacy. Under such a system, an accurate disease forecast system is necessary to reduce frequency of the application. *P. lactis* has not been found to have any adverse interactions or issues with human and environments although one of its close relatives, *P. azotoformans*, has been reported as a pathogen of cereal grains (Iizuka et al. 2006). Additionally, the bacteria may be produced inexpensively due to its origin from milk. SW could be rapidly adopted as a safe and sustainable boxwood blight control practice. It is interesting that there were a large population of SW in boxwood leaves showing symptom reversion (Fig. 1). Research is needed on how SW survives and distributes in healthy boxwood leaves. Exploring the correlation between the distribution and population of antagonistic endophytes and understanding the mechanisms underlying symptom reversion on infected plants are research directions to pursue. Continued efforts are needed in order to develop SW into a biofungicide for boxwood blight control.

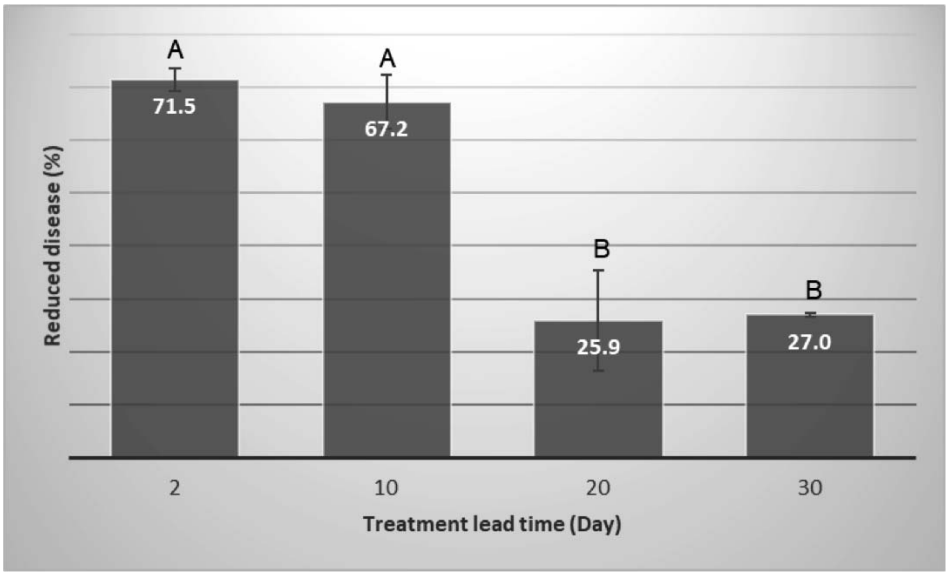

Fig. 4. Effect of lead time in the SW treatment on boxwood blight. Plants were treated with SW cell suspension at 10^{8-9} cfu·ml^{-1}, and the control 2, 10, 20 and 30 days prior to inoculation with *Cps* at 2-3×10^4 conidia·ml^{-1}. Disease reduction was calculated based on disease incidence of the treatment and control at 7 dpi after inoculation. Each column is an average of six plants from two repeated experiments (n=12). Bar indicates standard errors and letter on the top indicate significant difference among treatment lead time (α=0.05).

Literature Cited

Anonymous. 2019. Chlorothalonil fungicide banned in European Union. Vegetable Grower News. https://vegetablegrowersnews.com/news/chlorothalonil-fungicide-banned-in-european-union/. Accessed May 17, 2019.

Baudoin, A., et al. 2015. Evaluation of fungicides for control of boxwood blight, 2014. Plant Disease Management Reports 9:OT006.

Bush, E., et al. 2016. Best management practices for boxwood blight in the virginia home landscape. VT/09-16/PPWS-85NP Virginia Cooperative Extension, https://www.pubs.ext.vt.edu/search-results.html?q=boxwood+blight. Accessed May 17, 2019.

Calabro, J. M. 2018. Reclaiming boxwood from boxwood blight Nursery Management, www.nurserymag.com/article/reclaiming-boxwood-from-blight-calabro/. Accessed 5/2/2018.

Gilson, D. 2018. Boxwood blight found in Wisconsin for the first time. https://datcp.wi.gov/Pages/News_Media/BoxwoodBlightFound.aspx. Accessed 7/27/ 2018.

Haas, D., et al. 2003. Regulation of antibiotic production in root-colonizing Pseudomonas spp. and relevance for biological control of plant disease. Annu. Rev. Phytopathol. 41:117–153.

Iizuka, T., et al. 2006. Miuraenamides A and B, novel antimicrobial cyclic depsipeptides from a new slightly halophilic myxobacterium: Taxonomy, production, and biological properties. J. Antibiot. 59:385–391.

Ivors, K. L., et al. 2012. First report of boxwood blight caused by *Cylindrocladium pseudonaviculatum* in the United States. Plant Dis. 96:1070–1070.

Joy, A. E., et al. 1994. Biocontrol of *Alternaria* leaf blight on American ginseng by *Burkholderia cepacia* AMMD. Pages 93-100 The Challenge of the 21st Century. Proc. Inc. Ginseng Conf, W. G. Bailey, C. Whitehead, J. T. A. Proctor and J. T. Kyle, eds. Simon Fraser University, Vancouver, BC.

Knyaz, C., et al. 2018. MEGA X: Molecular Evolutionary Genetics Analysis across Computing Platforms. Mol. Biol. Evol. 35:1547–1549.

Kong, P., et al. 2017. Biocontrol of boxwood blight by *Trichoderma koningiopsis* Mb2. Crop Protect. 98:124–127.

Kong, P., et al. 2018. Host responses and impact on the boxwood blight pathogen, *Calonectria pseudonaviculata*. Planta 249:831–838.

Kong, P., et al. 2019. Utilization of plant endophytes for control of boxwood blight. Sou. Nur. Assoc Research Conference, F. Baysal-Gurel, ed. SNA. (In press).

Kong, P., et al. 2017. First report of *Pachysandra terminalis* leaf spots by *Calonectria pseudonaviculata* in Virginia. Plant Dis. 101:509.

LeBlanc, N., et al. 2018. Boxwood blight: an ongoing threat to ornamental and native boxwood. Appl. Microbiol. Biotechnol. 102:4371–4380.

Likins, T. M., et al. 2018. Preventing soil inoculum of *Calonectria pseudonaviculata* from splashing onto healthy boxwood foliage by mulching. Plant Dis. https://doi.org/10.1094/PDIS-05-18-0826-RE.

Mahenthiralingam, E., et al. 2005. The multifarious, multireplicon Burkholderia cepacia complex. Nature Reviews Microbiology 3:144–156.

Nair, D. N., et al. 2014. Impact of endophytic microorganisms on plants, environment and humans. The Scientific World Journal 2014:11.

Nübel, U. e. a. 1996. Sequence heterogeneties of gens encoding 16S rRNAs in *Paenibacillus polymyxa* detected by temperature gradient gel electrophoresis. J. Bacteriol. 178:5636–5643.

Pal, K. K. a. B. M. G. 2006. Biological control of plant pathogens. The Plant Health Instructor Am. Phytopath. Soc., 10.1094/PHI-A-2006-1117-02.

Yang, X., et al. 2017. Evaluation of biofungicides for control of boxwood blight on boxwood. Plant Disease Management Reports 11:OT023.

Yang, X., et al. 2018. Biological control of boxwood blight by *Pseudomonas protegens* recovered from recycling irrigation systems. Biol. Control 124:68–73.

Yoon, S.-H., et al. 2017. Introducing EzBioCloud: a taxonomically united database of 16S rRNA gene sequences and whole-genome assemblies. Int. J. Syst. Evol. Microbiol. 67:1613–1617.