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Phenotypic traits of *Burkholderia* spp. associated with ecological adaptation and plant-host interaction

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Running head: Ecological adaptation of *Burkholderia* spp.

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ABSTRACT

Burkholderia species have different lifestyles establishing mutualist or pathogenic associations with plants and animals. Changes in the ecological behavior of these bacteria may depend on genetic variations in response to niche adaptation. Here, we studied 15 *Burkholderia* strains isolated from different environments with respect to genetic and phenotypic traits. By Multilocus Sequence Analysis (MLSA) these isolates fell into 6 distinct groups. MLSA clusters did not correlate with strain antibiotic sensitivity, but with the bacterial ability to produce antimicrobial compounds and control orchid necrosis. Further, the *B. seminalis* strain TC3.4.2R3, a mutualistic bacterium, was inoculated into orchid plants and the interaction with the host was evaluated by analyzing the plant response and the bacterial oxidative stress response *in planta*. TC3.4.2R3 responded to plant colonization by increasing its own growth rate and by differential gene regulation upon oxidative stress caused by the plant, while reducing the plant's membrane lipid peroxidation. The bacterial responses to oxidative stress were recapitulated by bacterial exposure to the herbicide paraquat. We suggest that the ability of *Burkholderia* species to successfully establish in the rhizosphere correlates with genetic variation, whereas traits associated with antibiotic resistance are more likely to be categorized as strain specific.

Keywords: Bacteria-plant interaction; antibiotic resistance; biocontrol; oxidative stresses, pathogenesis

1. Introduction

Burkholderia species are often found in association with plants in the endosphere, phylosphere and rhizosphere (via nodulation or free-living lifestyles) (Eberl and Vandamme 2016). In addition, several *Burkholderia* spp. have been reported to establish antagonistic (via production of potent antifungal compounds) or mutualistic interactions with distinct soil fungi; e.g. *B. terrae* (Warmink and van Elsas 2009) and *B. rhizoxinica* (Partida-Martinez and Hertweck 2005); whereas others can be endosymbionts in insects (Kikuchi et al. 2005). In clinical settings, genetically distinct but phenotypically similar *Burkholderia* spp. comprising the *Burkholderia cepacia* complex (Bcc) have been isolated from chronic infection in immunocompromised patients, especially chronic cystic fibrosis patients, emerging as an opportunistic pathogen that causes severe infection (Mahenthiralingam et al. 2005; Eberl and Vandamme 2016). In same vein, genetically similar strains can be isolated from soil or immunocompromised patients.

Species of the *Burkholderia* genus have well-known biotechnological potential, which include beneficial effects as antagonists to phytopathogens and plant growth promoters (Santos et al. 2001; Perin et al. 2006; Dourado et al. 2013; Araújo et al. 2016). Members of this genus are often capable of nitrogen fixation, phosphorus solubilization, and xenobiotic catabolism (Coenye et al. 2001; Minerdi et al. 2001; O'Sullivan and Mahenthiralingam, 2005; Vu et al. 2013; Shehata et al. 2016). However, the potential to cause disease in humans, especially the immunocompromised, has hampered the generalized use of *Burkholderia* species as biocontrol agents. Recently, Eberl and Vandamme (2016) identified two main clades within this genus, which were consistent with a pathogenic group and an environmental/plant beneficial group. The latter encompasses several species of environmental *Burkholderia* beneficial for plants. The clade of pathogenic strains for humans, animals, and plants include *B. pseudomallei, B. mallei* and *B. glumae*, and the species of the Bcc.

However, the Bcc also includes species that are known as plant growth promoters and biological control agents, such as *B. vietnamiensis, B. ambifaria* (Parke and Gurian, 2001) and *B. cenocepacia* (Chávez-Ramirez et al. 2020). Other species tolerate heavy metals such as cadmium (Abou-Shanab et al. 2007), and, in general, members within the Bcc exhibit high levels of metabolic diversity, being potentially useful as herbicides and for bioremediation of contaminated soils (Coenye et al. 2001; Coenye and Vandamme, 2003). Despite being originally described as a phytopathogen (Li et al. 2010), and isolated from cystic fibrosis patients (Zhu et al. 2016), *B. seminalis* is an example of a Bcc species that can effectively

control the phytopathogenic *B. gladioli* in orchid (Araújo et al. 2016). This phytopathogenic bacterium also causes tobacco and rice leaf necrosis (Furuya et al. 1997), internal corn straw rot (Lu and Henn, 2007), rice panicle rust (Fiori et al. 2011), and is an opportunistic human pathogen (Dursu et al. 2012). Therefore, it is clear that the differentiation between beneficial and pathogenic *Burkholderia* is context-dependent, and as such, cannot be inferred solely based on genetic information.

The ability of a bacterium to colonize distinct environments, such as soil, animals, and plants, depends on genes that are differentially regulated in an environmental specific manner. Also, shifts in the local environment may result in changes in the nature of an ecological interaction, e.g. from mutualistic to pathogenic lifestyles. For instance, the virulence of *B. seminalis* towards *Galleria mellonella* is manifested at 37ºC, while the ability to inhibit phytopathogenic fungi was induced at 28ºC (Gonçalves et al. 2019). This result suggest that the temperature could regulate the virulence in an animal model (37ºC) or the capability to inhibit fungi in the rhizosphere (28ºC). Moreover, the role of DNA methylation and genomic islands in the regulation of iron, trehalose and D-arabitol utilization operons was proposed as an evolutionary signature in the adaptation of *B. seminalis* strains isolated from different environments, such as cystic fibrosis sputum, water, soil, and apricot (Zhu et al. 2016). Therefore, the ecological adaptation of *Burkholderia* may result from strain-specific metabolic features and differential regulation of operons associated with specific niches (Zhu et al. 2016).

In this study, we characterized a group of *Burkholderia* spp. isolated from different environments. to identify correlations between genetic profile (using multilocus sequencing analysis, MLSA) with specific traits (orchid necrosis control, enzyme and antimicrobial production) and host-pathogen interaction (virulence to *Galleria mellonella* and antibiotic resistance). The group of isolates we investigated also included *B. seminalis* TC3.4.2R3, which was cultured from internal sugarcane root tissues (Luvizotto et al. 2010) and was able to control orchid necrosis (Araujo et al. 2016). Our results revealed positive correlations between the genetic profile and phenotypic traits. In contrast, the antibiotic resistance profile did not correlate with the genetic profile, suggesting that antibiotic resistance gene pools could be acquired by horizontal gene transfer. We also identified genes in *B. seminalis* TC3.4.2R3 associated with antibiotic resistance and investigated in more detail the interaction of this endophytic bacterium with orchid plant.

113 **2. Material and Methods**

114

115 *2.1.* Burkholderia *spp. strains and growth, and plant material*

116

117 The *Burkholderia* spp. strains used in this study were obtained mostly from environmental
118 samples, including soil, rhizosphere and roots, as well as isolates obtained from the different
119 parts of the sugarcane plant (*Saccharum* spp.) (for a detailed description see Table 1). In
120 general, independent cultures of each strain were grown in tryptic soy broth (TSB) at 28 °C for
121 24 h in a shaker incubator (150 rpm). To determine growth curves, these cultures were grown
122 as pre-inoculum to an initial optical density (OD_{600nm}) of 0.04 (8 x 10^6 CFU ml^{-1}) in 5% TSB
123 medium. Cultures were incubated in the BioTek plate reader using 425 rpm at 28°C, and the
124 growth was monitored every 2 h during a total of 32 h. These assays were performed for each
125 individual isolate using a total of 4 replicates.

126 For the plant assays, orchids (*Oncidium* Alowa Iwanaga, a hybrid between *Oncidium*
127 goldiana and *Oncidium* Star Wars) were obtained from "Green Plugs Mudas de Flores e
128 Plantas" (Mogi das Cruzes, São Paulo, Brazil).

129

130 *2.2. Multilocus Sequence Analysis (MLSA)*

131

132 Multilocus Sequence Analysis (MLSA) was performed using the *atpD* (ATP synthase β
133 subunit), *gltB* (short-chain glutamate synthase), and *gyrB* (DNA gyrase, B subunit) genes to
134 obtain phylogenetic information on the *Burkholderia* isolates. We used the primer sets
135 previously described by Spilker *et al.* (2009), under the following conditions: initial
136 denaturation at 94 °C for 4 min; 35 cycles of denaturation at 94 ºC for 30 sec, annealing
137 temperature of each primer pair at 53 °C, 55 °C and 53 °C (for the *gltB*, *atpD* and *gyrB* gene,
138 respectively) for 30 sec, extension at 72 °C for 90 sec, and a final extension step at 72 °C for 7
139 min. Amplifications were performed in a final volume of 50 µl containing 12.5 µl of EasyTaq®
140 DNA Polymerase, 2.0 µl (5-20 ng. ml^{-1}) DNA, 10 pmol of each primer, and 33.5 µl milli-Q
141 water. PCR amplicons were checked by electrophoresis, purified using a polyethylene glycol
142 method (PEG 800020%; NaCl 2.5 mM), and sequenced at the Center for Human Genome
143 Studies, Institute of Biology, University of São Paulo. Sequences were analyzed using
144 BLASTn (National Center Biotechnology Information, NCBI) against the GenBank database.
145 Phylogenetic reconstructions were performed using the Neighbor Joining Method (NJ) method

146 with 1000 replicates based on the genetic distance matrices calculated by the Jukes-Cantor
147 model (1969), using MEGA v. 6.
148
149 *2.3. Antagonism assays against pathogenic bacteria and fungi*
150
151 The antagonistic assays were performed using the overlay method. The *Burkholderia* spp.
152 isolates were cultured in TSB medium for 24 h at 28 °C. Ten-µl aliquots were inoculated onto
153 Petri dishes containing TSB agar medium at 28°C for two days, allowing the initial growth of
154 *Burkholderia* spp. and the diffusion of bacterial metabolites in the medium. After that,
155 *Burkholderia* spp. colonies were inactivated by exposure to UV radiation for 1 h. Pathogenic
156 bacteria (*Escherichia coli, Bacillus* sp., *Staphylococcus aureus* and multi-resistant
157 *Pseudomonas aeruginosa*) obtained from the LABMEM/NAP-BIOP (Department of
158 Microbiology, ICB/USP) collection were grown in TSB for 24 h at 28 °C with shaking (150
159 rpm). Then, 5-ml overlay of semi-solid TSB culture medium containing 100 µl of pathogenic
160 bacterial culture was added over the inactive colonies. These flasks were incubated at 28 °C
161 for 48 h, after which the presence of potential inhibition halos was recorded.
162 For antifungal activity, 10 µl aliquots of *Burkholderia* spp. grown as described above were
163 inoculated onto Petri dish plates with PDA medium, and incubated for two days at 28 °C. After
164 growth, 5 mm diameter disks of PDA medium containing the phytopathogenic fungi
165 (*Ceratocystis paradoxa, Fusarium verticillioides, Aspergillus fumigatus, Colletotrichum* sp.,
166 and *Ceratocystis fimbriata*) mycelium were deposited on the surface of the plates in an opposite
167 side as that of the *Burkholderia* spp. isolate. As controls, disks of each fungus were tested on
168 plates containing only the PDA medium. Each individual *Burkholderia* spp. isolate was also
169 grown on PDA to test cell viability and growth. Plates were incubated at 28 °C for 5 days, and
170 the inhibition halos were evaluated. Each treatment (for both antibacterial and antifungal
171 antagonism assays) contained a total of 6 replicates.
172
173 *2.4. Phosphate solubilization, and siderophore and cellulase production*
174
175 The ability of these strains to solubilize inorganic phosphate was quantitatively evaluated
176 by measuring the halo obtained after bacterial growth on a medium supplemented with
177 $Ca_3(PO_4)_2$. Plates were scored after seven days of incubation at 28 °C (Verma et al. 2001).
178 Siderophore production was assayed according to Schwyn and Neilands (1987), using Chromo
179 Azurol S Agar (CAS). A yellow or orange halo around the bacterial colony indicated a positive

180 result for siderophore production. For endoglucanase detection, the bacteria were grown on
181 M9 minimal medium (Sigma) plates containing 0.5 % yeast extract and 1 %
182 Carboxymethylcellulose (CMC) (*w/v*). After microbial growth, 10 ml 0.1% Congo Red
183 solution were added, incubated at room temperature for 15 minutes and washed with NaCl (5
184 M). The presence of a colorless or yellowish halo surrounding the colony indicated the activity
185 of endoglucanase (Teather and Wood, 1982).
186
187 *2.5. Biocontrol of orchid necrosis and interaction with the host plant*
188
189 This assay was performed as described in Araújo *et al.* (2016). Briefly, *Burkholderia* spp.
190 strains were grown, washed with PBS buffer, and resuspended to a cell density of 10^5 CFU ml^{-}
191 ^{1}. To evaluate the suppressive potential of the strains, orchid leaves were punctured with a
192 sterile toothpick and 5 µl of cell suspensions at OD_{600nm} = 1.0 were placed on the resulting
193 wound. All *Burkholderia* spp. were co-inoculated with *B. gladioli* LMG 2216. *B. gladioli* LMG
194 2216 and PBS were inoculated alone as positive and negative controls, respectively. Three
195 biological replicas were carried out per strain. The plants were kept at 25 °C with 85% relative
196 humidity for 5 days. Each individual plant was visually inspected every day for the presence
197 of necrosis around the *B. gladioli* inoculation point.
198 The beneficial interaction between a *Burkholderia* spp. with orchid plants was examined
199 using the *B. seminalis* strain TC3.4.2R3 in experiments assessing the bacterium and plant
200 responses. The strain TC3.4.2R3 was previously showed to effectively control Orchid necrosis
201 (Araújo et al. 2016), to inhibit specific fungi, and had no virulence in mice (Gonçalves et al.
202 2019). For the assay, *B. seminalis* TC3.4.2R3 was inoculated into leaves and pseudobulbs with
203 or without *B. gladioli* and the bacteria and plant responses were monitored for the presence of
204 necrose and further evaluated. In addition, to assess the potential suppressive effect of *B.*
205 *seminalis* against *B. gladioli,* both bacteria were inoculated by infiltration in abaxial leaf
206 tissues. For this, we inoculated *B. seminalis* and incubated for 6 days to guarantee endophytic
207 colonization. After this period, *B. gladioli* was inoculated using the same strategy 2 cm apart
208 from the endophytic strain. The presence of symptoms was evaluated every day for 10 days.
209
210 *2.6. Animal model using* Galleria mellonella *larvae*
211
212 The *Burkholderia* spp. strains were grown in TSB for 24 h at 28 ºC. Cultures with an initial
213 OD_{600} of 1.0 were centrifuged at 13,000 rpm for 5 min and resuspended in PBS. Ten-µl aliquots

214 of each bacterial strain were applied to the pro-leg of fifth to sixth instar stage (approximately
215 between 2 and 2.5 cm in length) *G. mellonella* larvae (250-300 mg). Inoculated larvae were
216 maintained in Petri dishes at 28 °C. Uninoculated larvae and larvae inoculated with PBS were
217 used as negative controls. Twenty-µl of the dilution 10^6 and 10^7 of each inoculum were seeded
218 onto TSB plates to confirm the concentration and purity of the inoculum. *G. mellonella*
219 mortality was evaluated every day for 7 days. The inoculation of bacteria into *G. mellonella*
220 was carried out in triplicate, each replicate containing a total of 10 larvae (Pereira et al. 2015).
221
222 *2.7. Antibiotic resistance*
223
224 Antibiotic resistance was tested by direct inoculating the *Burkholderia* spp. isolates in
225 saline solution with an OD_{600} of 0.5 using the turbidity standard of the McFarland scale. A
226 sterile cotton swab was dipped into the suspension of the standardized culture and spread
227 evenly over the surface of Mueller-Hinton agar. Plates were allowed to dry, and individual
228 antibiotic disks (Cefar Diagnóstica Ltda, Brazil) (Table 2) were placed in each plate. Cultures
229 were incubated at 28 °C for 24 h and the diameters of the growth inhibition haloes were
230 measured.
231
232 *2.8. Evaluating antibiotic resistance genes potentially acquired via horizontal gene transfer*
233
234 The genome of the strain TC3.4.2R3 was used as a model organism to identify genes
235 associated with antibiotic resistance potentially acquired via horizontal gene transfer. The
236 CARD (Comprehensive Antibiotic Resistance Database) database was used to perform
237 qualitative prediction of genes conferring resistance to antibiotics. This database includes 4094
238 Ontology Terms and 2570 reference sequences (Jia et al. 2017). The complete genome of *B.*
239 *seminalis* TC3.4.2R3was analyzed against CARD using BLASTx. Positive genes were inferred
240 with a similarity threshold of ≥60% and an *E*-value cutoff of 10^{-5}.
241 The genomic island locations were predicted using the Alien Hunter software (Vernikos
242 and Parkhill, 2006). The presence of genes that confer resistance to antibiotics in regions of
243 genomic islands was performed manually using the results obtained by Alien Hunter and
244 BLASTx.
245
246 *2.9. Growth and lipid peroxidation of* B. seminalis *TC3.4.2R3 under experimental conditions*
247

248 The strain *B. seminalis* TC3.4.2R3 was grown in 50 ml TSB glucose (10%) under four
249 distinct experimental conditions, as follows: TC3.4.2R3 alone (control), TC3.4.2R3 + paraquat
250 (250 µM), and TC3.4.2R3 + Orchid exudates. Each individual flask was kept shaking (150
251 rpm) at 28 ºC for 72h. Lipid peroxidation was determined by estimating the content of
252 thiobarbituric acid reactive substance – TBARS (Heath and Packer, 1968; Monteiro et al. 2011)
253 in the cells. Malondialdehyde (MDA) was quantified by measuring the absorbance at 535 and
254 600 nm, and the concentration was calculated using an extinction coefficient of 155 mM^{-1} cm^{-}
255 ^{1}.
256
257 *2.10. Total RNA isolation and cDNA synthesis*
258
259 Bacteria cells were harvest by centrifugation (15 min at 6000 rpm) and the RNA was
260 isolated using the PureLink® RNA Mini Kit (Ambion, Foster City, CA, USA). RNA samples
261 were resuspended in 30 µl of water and stored at -80 °C. The integrity and quantity of extracted
262 RNA were verified in a 1.2 % denaturing agarose gel prepared with MOPS 200 mM, sodium
263 acetate 50 mM, EDTA 10 mM, formaldehyde (0.7 %) and SYBR safe gel stain. The RNA
264 concentration was determined using NanoDrop ND-1000 (Thermo Scientific, USA). All
265 materials used for RNA work were treated with DEPC to eliminated RNase. Total RNA (0.5
266 µg) was reverse-transcribed into cDNA using random hexamer primers (Invitrogen, Carlsbad,
267 CA, USA) and 200 U Superscript III RNase H^{-} reverse transcriptase (Invitrogen, Carlsbad,
268 CA, USA), according to the manufacturer's manual. For each RNA sample, a negative RT (no
269 addition of reverse transcriptase) was performed and used as a negative control in subsequent
270 PCRs.
271
272 *2.11. Expression of genes associated with oxidative stress*
273
274 The primer design used to amplify the target genes in this study were carried out using
275 *Primer 3* v. 0.4.0 (http://frodo.wi.mit.edu/), and the draft genome of *B. seminalis* TC3.4.2R3
276 as a reference (Araújo et al. 2016). First, conventional PCR was used to validate the design
277 primers. All amplification products of each of the five genes (one *sod* gene and four *kat* genes)
278 were purified, sequenced and compared to the GenBank data using BLASTn
279 (http://blast.ncbi.nlm.nih.gov/) in order to confirm amplicon specificity.
280 The qPCRs were done on a StepOne Plus (Applied Biosystems, Foster City, CA, USA)

281 thermocycler programmed to an initial denaturation at 94°C for 5 minutes, followed by 40
282 cycles of 15 seconds at 94°C and 1 minute at 60°C. The specificity of qPCR primer sets was
283 evaluated using melting curves with a gradient from 60 to 96°C, ranging 1°C each 30 seconds.
284 Each amplification reaction was performed containing 2 µl of cDNA (ca. 16 ng), 10 µM of
285 each primer and the Platinum SYBR Green master mix (Applied Biosystems, Foster City, CA,
286 USA). The DNA recombination gene *recN* was used as a reference gene. The StepOne Plus
287 Software was used to determine the relative quantification of the target genes in comparison to
288 the reference gene. Gene expression data were statistically compared using the Student *t*-test
289 (*t*-test, α=0.05).
290
291 *2.12. Statistical analysis*
292
293 Statistical differences were tested using one-way analysis of variance (ANOVA), followed
294 by Duncan test. The level of significance was set at $P<0.05$ for all experiments. All statistical
295 analyses were carried out using R software v. 2.15.1. For correlational analysis between MLSA
296 profiles and antibiotic susceptibility data, and MLSA profiles and bacterial traits (i.e., synthesis
297 of antimicrobial compounds, virulence against *G. mellonella*, and control of orchid necrosis),
298 we used a non-parametric Mantel-type test implemented as the RELATE routine in
299 PRIMER6+.
300
301 **3. Results**
302
303 *3.1. Phylogenetic analysis of* Burkholderia *spp.*
304 Our collection of *Burkholderia* spp. strains were obtained from distinct environmental
305 samples (e.g., soil, rhizosphere and roots), most of which arose from sugarcane plants
306 (*Saccharum* spp.) (see Table 1 for details). Only 2 strains, i.e. TC3.4.2R3 and LMG2216, were
307 previously identified as *B. seminalis* (Araújo et al. 2016) and *B. gladioli,* respectively, while
308 the species of the others remain unassigned. To determine phylogenetic relationships and
309 taxonomic characteristics of these strains, we used MLSA based on three genes (*atpD*, *gltB*
310 and *gyrB*), which were previously validated for the analysis of the Bcc group (Baldwin et al.
311 2005). Except for *B. gladioli* LMG2216, all strains clustered into well-supported clades by
312 parsimony analysis within the Bcc species (Fig. 1). The *B. seminalis* strain TC3.4.2R3 – a
313 sugarcane endophytic isolate – clustered in a clade with *B. seminalis* FL-5-4-10-S1-D7 – an

314 isolate obtained from soil. Strains CV3.2.2F5, TC3.4.1R1, TH3.3.2F5, CV3.3.3F2, TC3.4.1F2,
315 TC3.4.2R2 and TC3.3.3F1 – all of which were obtained from sugarcane – clustered into a clade
316 that includes *B. contaminans*, even though these isolates clustered together in a divergent
317 group. The strain CMAA1233 is in a divergent clade with no similarity with any of the
318 identified species used in this study, suggesting it is a possible new species within the Bcc
319 group. The strains AN5.5, 28RZ and 47RZ clustered within a divergent clade related to *B.*
320 *cenocepacia* strains CR318, HI2424 and AU1054, albeit this clade does not include the
321 epidemic strain *B. cenocepacia* J2315. The strains 93RZ and 67SI grouped in a well-supported
322 clade with *B. stabilis* and *B. pyrrocinia.* Overall, the MLSA analysis revealed a total of six
323 groups (MG), as follows: MG1 (strains CV3.2.2F5, TC3.4.1R1, TH3.3.2F5, CV3.3.3F2,
324 TC3.4.1F2, TC3.4.2R2 and TC3.3.3F1), MG2 (strain CMAA1233), MG3 (*B. seminalis* strain
325 TC3.4.2R3), MG4 (strains AN5.5, 28RZ and 47RZ), MG5 (strains 93RZ and 67SI) and MG6
326 (*B. gladioli* LMG2216) (Fig. 1).
327 We also determined differences in growth rate across these isolates. The results revealed
328 that all isolates had similar growth curves, reaching the stationary phase after ca. 12 h of
329 incubation. The only exception was the *B. gladioli* LMG2216, which required 20 h of
330 incubation to reach a stationary phase (Fig. S1).
331
332 *3.2. Antimicrobial susceptibility and correlational analysis*
333
334 The susceptibility of these strains to 16 antimicrobials belonging to nine different classes
335 (Table 2) was examined using disk diffusion assays (Table 3). Overall, all tested strains were
336 sensitive to cephalosporins, ceftazidime and cefepime, levofloxacin (fluoroquinolone),
337 meropenem (carbapenem), tigecycline (glycylcycline), piperacillin + tazobactam (β-lactam),
338 and chloramphenicol. Most of the strains belonging to the MLSA MG1 group were resistant to
339 β-lactams (Piperacillin, Carbenicillin and Ticarcillin + Clavulanate), albeit the strain
340 CV3.3.3F2 was sensitive/intermediate. In the MG5 group, the strain 67SI was
341 sensitive/intermediate to all antibiotics, while 93RZ was resistant to Sulfamethoxazole-
342 Trimethoprim, Piperacillin, Ticarcillin + Clavulanate, Carbenicillin, Aztreonam, Doxycycline
343 and Tetracycline (Table 3). In addition, the strains CV3.3.3F5 and TH3.3.2F5 (MG1),
344 CMA1233 (MG2), and 93RZ (MG4) were resistant to tetracycline. Last, only the strains
345 TC3.4.1F2 and 93RZ were resistant to imipenem and doxycycline, respectively (Table 3).

346 The strain TC3.4.2R3 (MG3) was resistant to the β-lactams, such as Piperacillin,
347 Carbenicillin and Ticarcillin + Clavulanate, but was sensitive to Aztreonam, Imipenem,
348 Sulfamethoxazole-Trimethoprim, Doxycycline, Tetracycline, Minocycline. We further investigated
349 the genome of this strain TC3.4.2R3 (GenBank Accession number LAEU00000000) and
350 successfully identified genes encoding 5 antibiotic efflux pumps and 2 genes (Bsem_05019
351 and Bsem_05476) encoding β-lactamase enzyme, which is known to be associated with
352 resistance to β-lactams and cephalosporin (Table S1).
353 We performed correlational analysis between the MLSA clustering profile and the
354 antibiotic susceptibility data across all 15 *Burkholderia* strains. The result of a pairwise
355 correlation between genetic (MLSA) clustering and sensibility to antibiotics showed that these
356 traits are not significantly correlated (rho=0.05; *P*=0.32). We also used genomic information
357 of the strain TC3.4.2R3 to identify genes potentially associated with antibiotic resistance that
358 could be acquired by horizontal gene transfer. By combining BLASTx against CARD, we
359 identified 76 genes associated with potential antibiotic resistance, of which 25 were in
360 chromosome 1 (CR1), 46 in chromosome 2 (CR2), and 5 in the plasmid (P3) (Table S2). From
361 these, two of the genes found in CR1, in addition to three genes in CR2, and two in P3; were
362 found within genomic islands in the genome. Most interestingly, some of them were associated
363 with protein transport and efflux pumps (see Table S2 in Supporting Material for details).
364
365 *3.3. Synthesis of antimicrobial compounds, virulence against* G. mellonella*, and potential*
366 *control of orchid necrosis*
367
368 The antibacterial and antifungal activities of *Burkholderia* spp. strains were tested on PDA
369 (fungi) and TSA (bacteria) plates after 5 or 2 days, respectively, against several bacterial and
370 fungal species. Overall, the 15 strains successfully inhibited the Gram-positive bacterium
371 *Staphylococcus aureus* and the phytopathogenic fungi *Colletotrichum* sp. and *Ceratocystis*
372 *fimbriata* (Table 4) while none inhibited the multidrug-resistant bacterium *Pseudomonas*
373 *aeruginosa.*
374 The strains within the MG1 displayed a wider inhibitory spectrum than strains from other
375 MLSA MGs, including virulence against *G. mellonella* and the ability to control orchid
376 necrosis. For example, all strains inhibited the bacteria *Escherichia coli, Bacillus* sp., and the
377 fungi *Ceratocystis paradoxa* and *Aspergillus fumigatus,* and only the strain CV3.2.2F5 was not
378 able to inhibit the fungus *Fusarium verticillioides.* In addition, all strains caused greater than
379 60% mortality of *G. mellonella*, and five (out of seven) strains caused 100% of larvae mortality.

380 Five strains within the MG1 were also able to control orchid necrosis (Table 4). In general,
381 strains from MLSA groups MG2, MG3, MG4, MG5 and MG6 were less virulent against *G.*
382 *mellonella* and in the evaluated conditions, the 5 strains from the groups MG2, MG4 and MG6
383 were not able to inhibit *E. coli.* The two strains from MG4 (i.e. 67SI and 93Rz) did not inhibit
384 *Bacillus* sp. but inhibited all tested fungi. Last, we found a significant correlation between
385 MLSA clustering profile and the collection of tested traits (i.e., synthesis of antimicrobial
386 compounds, virulence against *G. mellonella*, and control of orchid necrosis) (rho=0.51;
387 *P*=0.004).
388
389 *3.4. Plant beneficial activities*
390
391 Strains used in the present study were originally isolated from soil, rhizosphere or inside
392 plants tissues (endophytes) and except for the strain LMG 2216, which was identified as *B.*
393 *gladioli*, (a known plant pathogen), all others had no deleterious effect on plant growth.
394 Cellulases and siderophore production and phosphate solubilization were observed for all
395 evaluated strains. In addition, as reported before, 10 strains inhibited orchid necrosis caused by
396 *B. gladioli* (Table 4).
397
398 *3.5.* B. seminalis *TC3.4.2R3 as a model organism for risk assessment and biotechnological*
399 *application*
400
401 We further explored the strain TC3.4.2R3 as a model organism for *B. gladioli* control.
402 Inoculation of *B. gladioli* in orchid leaves induced necrosis (Fig. 2a), while the co-inoculation
403 with *B. seminalis* TC3.4.2R3 suppressed these symptoms (Fig. 2b). This result confirmed the
404 activity of the endophyte *B. seminalis* as a biological control agent against the phytopathogen
405 *B. gladioli.* Plants inoculated with PBS (negative control) and *B. seminalis* TC3.4.2R3 did not
406 display any symptoms of orchid necrosis (Fig. 2c,d). Of key relevance, we also found that the
407 disease was not suppressed when the endophytic bacterium was inoculated 1 cm apart from the
408 *B. gladioli* inoculation point. Moreover, the inoculation of *B. seminalis* in abaxial leaf tissues
409 by infiltration 6 days prior to *B. gladioli* suppressed orchid necrosis. However, the suppression
410 of the disease was observed only in the zone that both endophytic and pathogenic bacteria were
411 in contact (Fig. 2e). Collectively, these results indicate that the plant colonization by *B.*
412 *seminalis* is necessary to guarantee physical contact between the endophyte and the

413 phytopathogen, thus promoting orchid necrosis suppression. Previous results have shown that
414 this strain TC3.4.2R3 is not able to induce orchid systemic resistance (data not shown).
415 We also investigated the bacterial responses to orchid exudates. Since plant defense may
416 induce oxidative stress in plant-associated bacteria, we also compared the bacterial response to
417 the herbicide paraquat. Overall, these results revealed that whereas the plant exudates
418 stimulated bacterial growth, the presence of paraquat was inhibitory to bacterial growth (Fig.
419 3A), in addition, paraquat was found to induced peroxidation (Fig. 3B).
420 The expression of five *B. seminalis* TC3.4.2R3 genes related to antioxidative responses
421 (*sodB, kat1, kat3, kat5* and *kat6*) was evaluated after 3 h and 20 h of exposure to oxidative
422 stress induced by paraquat and orchid root exudates. Overall, the expression of these oxidative
423 stress-associated genes was regulated by orchid root exudates and paraquat in a specific
424 manner. The *sodB* gene expression was only induced by paraquat, while *kat6* was consistently
425 induced by root exudates. At 20 h, the expression of *sodB* and *kat1* was induced by paraquat,
426 but repressed by orchid root exudates, indicating that these genes are likely specific for the
427 protection against oxidative stress induced by paraquat. The *kat3* gene was only induced in the
428 presence of paraquat at 3 h, but by both paraquat and orchid root exudates at 20 h (Fig. 4). The
429 *kat5* gene expression was induced by both conditions at 3 and 20 h (Fig. 4), thus suggesting a
430 potential role in general oxidative stress response of this bacterium.
431
432 **4. Discussion**
433
434 *Burkholderia* species are often isolated from the endosphere, phylosphere and rhizosphere
435 of many plant species, or isolated from chronic infection in immunocompromised patients,
436 especially chronic cystic fibrosis patients (Eberl and Vandamme, 2016). These isolates can be
437 genetically distinct but phenotypically similar and is not possible to discriminate strains from
438 soil, plant or patients. These bacteria present high phenotypic plasticity, determined by
439 differential response to environmental stimulus, which is likely an important, yet unexplored
440 mechanism of *Burkholderia* members to occupy different niches and survive in distinct and
441 often contrasting environments, such as the human lung and the plant rhizosphere.
442 The adaptive mechanisms that allow these bacteria to colonize different host are still
443 uncertain, but Nunvar et al. (2017) observed that the host immune system can modulate *B.*
444 *cenocepacia* evolution during chronic CF infection, since genes that encode proteins involved
445 in the protection against hydrogen peroxide and hypochlorous acid are more frequently

446 mutated in sputum isolates of CF-patients. This indicates that mutation, but not gene transfer,
447 in these genes is likely associated with the bacterial persistence in the airways.
448 In this study, we compared 15 *Burkholderia* spp. isolates obtained from distinct
449 environmental samples to evaluate the association between taxonomic profile and traits
450 associated to virulence to immunocompromised patients (antibiotic resistance and virulence to
451 the model *G. mellonella*) and colonization of the rhizosphere and the host plant. Remarkably,
452 14 (out of 15) strains belonged to the Bcc, confirming previous observations that members of
453 the Bcc group, while potentially pathogenic for immunocompromised people, are highly
454 prevalent as plant-associated organisms.
455 Based on MLSA, these isolates were clustered into 6 groups (MG01 to MG06). The group
456 MG04 clustered with strains CR318, AU1054 and HI2424. A recent study (Wallner et al. 2019)
457 had proposed to split *B. cenocepacia* species in human pathogenic and plant adapted species.
458 *B. cenocepacia* sensu strict including the epidemic strain J2315, and a second species related
459 to plant-adapted strains, suggested to be named *B. servocepacia*, which includes the strains
460 CR318, AU1054 and HI2424. The authors observed that the plant-adapted strains carry genes
461 for the utilization of plant derivatives and to compete in the soil, while *B. cenocepacia* carry
462 genes associated to virulence and survival in humans. In fact, the members of MG04 (strains
463 AN 5.5, 28Rz and 47 Rz) were sensitive/intermediate to all antibiotic used to compare the
464 strains (Table 3) and inhibit most of the microorganisms evaluated, except *E. coli* that is
465 associated to humans (Table 4), suggesting that these bacteria are more adapted to soil-plant
466 than in the human host environment.
467 The clustering based on MLSA analysis showed no correlation with antibiotic sensitivity,
468 thus suggesting that at least some of the antibiotic resistance genes could be potentially
469 acquired by horizontal gene transfer. While 9 out of 76 (i.e., 11.8%) antibiotic resistance-
470 associated genes were present in clearly define genomic islands, we could not rule out that
471 other genes might be present in horizontally acquired sequences in which the classical features
472 of genomic islands had been lost for this strain.
473 In contrast, the ability to produce antimicrobial and antifungal compounds was more
474 prevalent in the MG1 MLSA group. Indeed, these isolates showed a significantly wider
475 inhibitory spectrum than strains from other MLSA groups, including the capacity to control
476 orchid necrosis. Collectively, these results showed that there is a correlation between the
477 production of antifungal and antibacterial compounds and the MLSA clusters, suggesting that
478 most of the genes that control these traits are acquired by vertical gene transfer. The ability to
479 produce antimicrobial compounds by *Burkholderia* spp. is an important feature promoting

480 niche adaptation, since these bacteria must compete in the soil and rhizosphere, before the plant
481 colonization. Using this ability to inhibit other microorganisms, certain strains are known to
482 suppress plant diseases. For example, *B. seminalis* strain R456, which was isolated from the
483 rhizosphere of rice, was shown to reduce the incidence and severity of rice sheath blight under
484 greenhouse conditions (Li et al. 2011), *B. seminalis* TC3.4.2R3 was isolated from sugarcane
485 roots, but shown to be effective in the control of the orchid necrosis (Araújo et al. 2016), while
486 *B. cenocepacia* CACua-24, isolated from sugarcane rhizosphere, produced antifungal and
487 antibacterial compounds against a broad range phytopathogens. In our study, most of the
488 *Burkholderia* strains were able to control orchid necrosis, caused by *B. gladioli,* and to
489 produced antimicrobial compounds against *Escherichia coli, Bacillus* sp., *Ceratocystis*
490 *paradoxa* and *Fusarium verticillioides.*

491 Based on these traits, we selected *B. seminalis* TC3.4.2R3 to further evaluate interactive
492 aspects with orchid plants, especially under oxidative stress conditions. This is of key relevance
493 since the ability to cope with oxidative stress is directly associated with the skill of distinct
494 strains to colonize and thrive across distinct gradients of environmental conditions, including
495 plant and patients. Niche adaptation depends on the capability of organisms to explore and
496 exploit nutrients and to cope with local environmental conditions. In the present study, the
497 orchid root exudate, despite its potential to induce oxidative stress, promoted a significant
498 growth of *B. seminalis* TC3.4.2R3 when compared to the standard culture medium. In contrast,
499 the bona fide pro-oxidant paraquat significantly reduced this bacterium growth (Fig. 3A). The
500 presence of paraquat (but not root exudates) was found to increased membrane lipid-mediated
501 peroxidation. Collectively, these findings suggest that paraquat triggered ROS production
502 leading to membrane damage, and thus inhibition of the bacterial growth. In contrast, root
503 exudates, which are generally composed of sugars (e.g., galactose, mannitol, fructose, arabitol,
504 dulcitol and ribitol) and organic acids (LeFevre et al. 2013; Li et al. 2013), were not found to
505 significantly induce membrane damage, but induced the bacterial growth.

506 Moreover, plant exudates were found to trigger the bacterial antioxidant response. For
507 instance, in *B. glumae* superoxide dismutase (*sod*) and catalase (*kat*) genes are upregulated
508 after 30 h, thus likely playing an important role in pathogenicity and protection against visible
509 light (Chun et al. 2009). Similarly, in *Sinorhizobium meliloti, katA* and *sodC* genes were
510 induced during the interaction with *Medicago* plants (Ampe et al. 2003), suggesting the role of
511 these genes in the bacterium colonization of the host plant. Likewise, the virulence of
512 *Burkholderia* spp. in macrophages is related to oxidative responses (Keith and Valvano, 2007;
513 Vanaporn et al. 2011). Therefore, the ability to cope with oxidative stress is an important

514 strategy of *Burkholderia* species used to colonize different hosts, such as plant and
515 immunocompromised patients. Our findings showed that *B. seminalis* TC3.4.2R3 responded
516 to oxidative stress differentially when challenged by paraquat and root exudate, indicating that
517 this bacterium uses a different strategy for oxidative stress protection provided from diverse
518 sources, which could explain the phenotypic plasticity that allow these bacteria to colonize a
519 broad range of taxonomically different hosts.
520 The adaptation of *B. seminalis* to specific environments is highly associated with its unique
521 metabolic capacity, which according to Zhu et al. (2016) may be strain-specific and generally
522 linked with genomic variants and niche-dependent differential expression of the corresponding
523 genes. Regulating the expression of different genes in response to these environments,
524 *Burkholderia* spp. could present the plasticity that allow them to occupy different niches. This
525 plasticity strategy can promote increasing fitness, and, once coupled with mutation and
526 selection, can result in a wide range of adaptation across contrasting environmental conditions
527 (Nunvar et al. 2017).
528
529 **5. Conclusion**
530
531 In conclusion, we found evidence supporting that the ability of *Burkholderia* spp. to
532 establish and to persist into immunocompromised patients, plants and soil are likely associated
533 with genetic variation. However, the evolution of these traits seems to occur in different ways.
534 Traits associated with antibiotic resistance appear to be more likely strain-specific, with
535 potentially no correlation with taxonomic variations. This lack of correlation could be due to
536 the selection of adaptative mutations during chronic infection that increase the ability of some
537 strains to persist in the host. On the other hand, traits associated to soil and plant colonization
538 correlate with the taxonomic profile, being likely acquired by vertical gene transfer.
539 Collectively, our results indicate that selection of specific strains can be used for plant
540 protection with low risk for immunocompromised patients. We advocate further studies are
541 needed to broaden the spectrum of isolates and environmental samples to corroborate our
542 findings, thus providing knowledge on the ecological niche adaptation and evolution of
543 *Burkholderia* spp. across disparate systems.
544
545
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Declaration of Competing Interest

The authors declare that there is no conflict of interest

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702

703 **Legends**

704

705 **Table 1** Description of the *Burkholderia* sp. strains used in this study.

706

707 **Table 2** Description of antimicrobial compounds and respective disc concentrations used in
708 this study.

709

710 **Table 3** *In vitro* susceptibility of *Burkholderia* strains (*n*=15) tested for 9 antibiotics*

711

712 **Table 4** Virulence to *Galleria mellonella,* control of orchid necrosis, and antimicrobial activity
713 of *Burkholderia* spp.. The synthesis of antimicrobial compounds was evaluated *in vitro* by the
714 presence of inhibition halos.

715

716 **Fig. 1.** Phylogenetic reconstruction based on Multilocus sequence analysis (MLSA) of the
717 *Burkholderia* spp isolates. The figure displays the unrooted neighbor-joining tree (Jukes and
718 Cantor, 1969) based on MLSA (*atpD*, *gltB*, *gyrB*) data. The numbers in the branches indicate
719 bootstrap values calculated using 1000 replications. The scale bar indicates 0.01 substitutions
720 per nucleotide position.

721

722

723 **Fig. 2.** Control of orchid necrosis caused by *Burkholderia gladioli* in leaf fragments of
724 *Oncidium flexuosum* 'Aloha Iwanaga'. (a) symptoms caused by the inoculation of *B. gladioli;*
725 (b) suppression of the symptoms due to the co-inoculation of *B. gladioli* and *B. seminalis*
726 TC3.4.2R3*;* (c) leaf inoculated with *B. seminalis* TC3.4.2R3; (d) negative control, i.e. leaf
727 inoculated with PBS buffer, and (d) inoculation of *B. gladioli* (red circle) 2 cm apart from *B.*
728 *seminalis* TC3.4.2R3 (black circle). All photographs were taken 5 days after inoculation.

729

730 **Fig. 3**. Bacterial growth and lipid peroxidation. (a) *B. seminalis* TC3.4.2R3 growth curve in
731 culture medium (10% TSB), culture medium supplemented with Paraquat (250 μM) or orchid
732 root exudates. (b) Lipid peroxidation of the bacterial membrane after exposure to culture
733 medium (10% TSB), culture medium with Paraquat (250 μM) or orchid root exudates.
734
735 **Fig. 4**. Relative of expression of *sodB, kat1, kat3, kat5* and *kat6* genes *B. seminalis* TC3.4.2R3
736 exposed to paraquat (250 μM) and orchid root exudates. The expression profiles were evaluated
737 at 3 h after bacteria inoculation (top panel) and (b) 20 h after bacteria inoculation (bottom
738 panel). The *recN* (DNA repair protein) was used as a reference gene. The results are shown as
739 the average of three replicates and * indicates statistically significant differences (α= 0.05)
740 based on Student *t*-tests.
741

742 **Table 1.**

Strain	Species	Isolation place	References
CV3.2.2F5	*Burkholderia sp*	Root endophyte	Luvizotto *et al*., 2010
TC3.4.1R1	*Burkholderia sp*	Root endophyte	Luvizotto *et al.*, 2010
TH3.3.2F5	*Burkholderia sp*	Rhizosphere	
CV3.3.3F2	*Burkholderia sp*	Rhizosphere	Luvizotto *et al*., 2010
TC3.4.2R2	*Burkholderia sp*	Root endophyte	Luvizotto *et al*., 2010; Araújo *et al.*, 2016
TC3.3.3F1	*Burkholderia sp*	Rhizosphere	Luvizotto et al., 2010
TC3.4.1F2	*Burkholderia sp*	Root endophyte	Luvizotto *et al*., 2010
67SI	*Burkholderia sp*	Soil	Gonçalves and Motta, 2007
93Rz	*Burkholderia sp*	Rhizosphere	Gonçalves and Motta, 2007
TC3.4.2R3	*B. seminalis*	Root endophyte	Luvizotto *et al.*, 2010
AN 5.5	*Burkholderia sp*	Antarctic isolate	Bioproducts laboratory collection. ICB. USP
28Rz	*Burkholderia sp*	Rhizosphere	Gonçalves and Motta, 2007
47Rz	*Burkholderia sp*	Rhizosphere	Gonçalves and Motta, 2007
CMAA 1233	*Burkholderia sp*	Plant	EMBRAPA Jaguariuna Collection
LMG 2216 (type strain)	*B. gladioli*	Plant pathogen	Institut für Pflanzenbiologie der Universität Zürich collection

743

744

745 **Table 2.**

Class	Antimicrobial	Disc concentration ($\mu g.ml^{-1}$)
Sulfonamides	Sulfamethoxazole-Trimethoprim (SZT)	25
Amphenicol	Chloramphenicol (CHL)	30
Carbapenem	Imipenem (IPM)	10
	Meropenem (MEM)	10
Cephalosporin	Ceftazidime (CAZ)	30
	Cefepime (FEP)	30
fluoroquinolone	Levofloxacin (LVX)	5
Beta-lactams	Piperacillin (PIP)	20
	Piperacillin + Tazobactam (TZP)	110
	Ticarcillin + Clavulanate (TIC)	85
	Carbenicillin (CAR)	100
monobactams	Aztreonam (ATM)	30
Glycylcycline	Tigecycline (TGC)	15
Tetracycline	Minocycline (MIN)	30
	Doxycycline (DOX)	30
	Tetracycline (TET)	30

746

747

748 **Table 3.**

Strain	**MLSA group**	PIP	CAR	TIC	ATM	IPM	SZT	DOX	TET	MIN
CV3.3.3F2	MG1	S**	I	I	S	S	S	I	S	I
TC3.3.3F1		R	R	R	S	S	S	S	I	S
TH3.3.2F5		R	R	R	I	I	S	I	R	S
CV3.2.2F5		I	R	R	R	S	S	S	R	S
TC3.4.2R2		R	R	R	S	S	S	S	S	S
TC3.4.1R1		I	R	R	S	S	S	S	I	S
TC3.4.1F2		R	R	R	I	R	S	S	I	S
CMAA 1233	MG2	R	R	R	R	I	S	S	R	S
TC3.4.2R3	MG3	R	R	R	S	S	S	S	S	S
AN 5.5	MG4	S	I	I	S	S	S	I	S	I
28Rz		S	I	I	S	S	S	S	S	S
47Rz		I	I	I	S	S	S	I	S	I
67SI	MG5	S	S	I	S	S	S	I	S	I
93Rz		R	R	R	R	S	R	R	R	S
LMG2216	MG6	S	S	I	S	S	S	I	S	S

749 * corresponding names are described in table 2.

750 ** S = sensitive, R=resistant, I = intermediate

751

752 **Table 04.**

Strain	MLSA group	Virulence to *G. mellonella*[i]	Control of orchid necrosis[ii]	Eco[iii]	Bsp[iii]	Cep[iv]	Fuv[iv]	Asf[iv]
CV3.3.3F2	MG1	1	+	I[v]	I	I	I	I
TC3.3.3F1		1	+	I	I	I	I	I
TH3.3.2F5		2	+	I	I	I	I	I
CV3.2.2F5		2	+	I	I	I	NI	I
TC3.4.2R2		1	+	I	I	I	I	I
TC3.4.1R1		1	-	I	I	I	I	I
TC3.4.1F2		1	-	I	I	I	I	I
CMAA 1233	MG2	3	+	NI	I	NI	I	NI
TC3.4.2R3	MG3	2	+	I	I	I	I	NI
AN 5.5	MG4	3	+	NI	I	I	NI	I
28Rz		2	-	NI	I	I	I	I
47Rz		1	+	NI	I	I	I	I
67SI	MG5	2	-	I	NI	I	I	I
93Rz		2	+	I	NI	I	I	I
LMG2216	MG6	3	NR	NI	I	I	I	I

753 [i] Mortality at 7th days: 1= >95%; 2= range from 60 to 85%; 3= < 25%
754 [ii] +: control of orchid necrosis caused by *Burkholderia gladioli;* -: no control of orchid necrosis;
755 NR: not tested
756 [ii] *Escherichia coli (*Eco) and *Bacillus* sp (Bsp).
757 [iii] *Ceratocystis paradoxa* (Cep)*, Fusarium verticillioides* (Fuv) and *Aspergillus fumigatus*
758 (Asf).
759 [iv] I: presence of inhibition halo and NI: absence of inhibition halo.