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Genetic diversity of *Curtobacterium flaccumfaciens* revealed by multilocus sequence analysis --Manuscript Draft--

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Abstract:	<p>Bacterial wilt caused by <i>Curtobacterium flaccumfaciens</i> pv. <i>flaccumfaciens</i> is among the diseases that affect <i>Phaseolus vulgaris</i> L. This disease has been frequently detected in bean fields and causes severe production losses in Brazil. The aim of this research was to examine the genetic diversity existing among twenty-four isolates of <i>C. flaccumfaciens</i> collected from their native and alternative host, and a collection of sixty strains belonging to four phytopathogenic pathovars preserved at the French Collection for Plant-associated Bacteria (CIRM-CFBP) by multilocus sequence analysis (MLSA) based on six housekeeping genes (<i>atpD</i>, <i>dnaK</i>, <i>gyrB</i>, <i>ppK</i>, <i>recA</i> and <i>rpoB</i>). A phylogenetic tree with the concatenated sequences of six genes showed high genetic diversity among the strains. For instance, strains belonging to <i>C. f. pv. flaccumfaciens</i> do not cluster together within the species. Similar results were obtained with a minimal MLSA scheme using <i>gyrB</i> and <i>recA</i>, which we propose for reliable identification at the species level of <i>Curtobacterium</i> isolates. No correlation was identified between phylogeny and pathogenicity in the <i>Curtobacterium flaccumfaciens</i> strains analyzed in this work. The specific primers CffFOR2 and CffREV4 designed by Tegli et al. (2002) to detect <i>C. f. pv. flaccumfaciens</i> in naturally infected bean seeds proved to be efficient for the detection of bean-pathogenic strains.</p>	
Response to Reviewers:	We'd like to thank the reviewers for their precise and thorough work on our manuscript. Your work greatly helped us to improve the quality of the manuscript.	

Based on your comments we:

- Reviewed deeply the text according to your remarks.
- Ask a native-English speaker to correct the manuscript to enhance the quality of the text.
- Recalculate the phylogenetic network, and modify the associated Figure 2.
- Corrected the tables and figures needing correction (Table 1, Table 2, Figure 1, Supplemental Figure 2).
- Submitted the complete genome sequences for CFBP 4999 and CFBP 2404 to Genbank. The accession numbers are visible in the text. We ask Genbank for the release of the complete genome sequence of CFBP 3418
- Submitted the sequences obtained for the 6 housekeeping genes to GenBank. The accession numbers are listed in supplemental Table 1 (the sequences were not yet displayed no line on November 15th, still waiting to being processed by Genbank).

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1

1 **Genetic diversity of *Curtobacterium flaccumfaciens* revealed by multilocus sequence analysis**

2

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13

14 **Abstract**

15

16 Bacterial wilt caused by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* is among the
17 diseases that affect *Phaseolus vulgaris* L. This disease has been frequently detected in bean fields and
18 causes severe production losses in Brazil. The aim of this research was to examine the genetic diversity
19 existing among twenty-four isolates of *C. flaccumfaciens* collected from their native and alternative host,
20 and a collection of sixty strains belonging to four phytopathogenic pathovars preserved at the French
21 Collection for Plant-associated Bacteria (CIRM-CFBP) by multilocus sequence analysis (MLSA) based
22 on six housekeeping genes (*atpD*, *dnaK*, *gyrB*, *ppk*, *recA* and *rpoB*). A phylogenetic tree with the
23 concatenated sequences of six genes showed high genetic diversity among the strains. For instance, strains
24 belonging to *C. f.* pv. *flaccumfaciens* do not cluster together within the species. Similar results were
25 obtained with a minimal MLSA scheme using *gyrB* and *recA*, which we propose for reliable identification
26 at the species level of *Curtobacterium* isolates. No correlation was identified between phylogeny and
27 pathogenicity in the *Curtobacterium flaccumfaciens* strains analyzed in this work. The specific primers
28 CffFOR2 and CffREV4 designed by Tegli et al. (2002) to detect *C. f.* pv. *flaccumfaciens* in naturally
29 infected bean seeds proved to be efficient for the detection of bean-pathogenic strains.

30 **Keywords:** *gyrB*, MLSA, phylogeny, *recA*, *Curtobacterium*.

31

32 **Introduction**

33 Common bean (*Phaseolus vulgaris* L.) is an important source of nutrients in the human diet. In
34 2013/14, Brazil was the third largest producer, behind Myanmar and India
35 (<http://www.fao.org/faostat/en/#data/QC>). The common bean has adapted to a wide variety of climatic and
36 soil conditions and in Brazil it is cultivated in three different seasons annually. The widespread production
37 contributes to an increase in the incidence of diseases that can cause significant losses in bean production
38 (Schwartz *et al.* 2005).

39 Bacterial wilt disease of bean was first recorded in 1920 on a farm in South Dakota (USA),
40 associated with a 90% crop loss that year (Hedges 1922) and in subsequent years, complete crop failure
41 (Hedges 1926). Formerly classified as *Corynebacterium* (Hedges 1922), the causal agent of bacterial wilt
42 has been reclassified as *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (Hedges 1922; Collins &
43 Jones 1983). Bacterial wilt of common bean was first reported in Brazil in 1995 in the state of São Paulo
44 (Maringoni and Rosa 1997). Since then, this pathogen has become a serious problem in many parts of
45 Brazil, including Santa Catarina, Mato Grosso do Sul, Goiás and the Federal District (Herbes *et al.* 2008;
46 Theodoro *et al.* 2010; Uesugi *et al.* 2003). *Curtobacterium flaccumfaciens* pv *flaccumfaciens* was reported
47 in Germany and Iran (Sammer & Reiher 2012; Osdaghi *et al.* 2016) indicating that this pathogen is likely
48 to spread to other parts of the world in the future.

49 Recently, *C. f.* pv. *flaccumfaciens* was detected in symptomless alternative hosts in Brazil,
50 including barley (*Hordeum vulgare* L.), black oat (*Avena strigosa* Schreb.), canola (*Brassica napus* L.),
51 ryegrass (*Lolium multiflorum* Lam.), wheat (*Triticum aestivum* L.) and white oat (*Avena sativa* L.). When
52 these alternative hosts are grown in succession with bean, they can act as inoculum sources of the
53 pathogen which can infect the bean crop each growing season. (Gonçalves *et al.* 2017).

54 *Curtobacterium flaccumfaciens* is currently divided into five pathovars (Young *et al.* 1978)
55 following the host range of the strains. In addition to pathovar *flaccumfaciens*, pathovar *betae* ((Keyworth
56 *et al.* 1956) Collins & Jones 1983) causes vascular wilt and leaf spot in beetroot (*Beta vulgaris* var.
57 *rubra*); pathovar *oortii* ((Saaltink & Maas Geesteranus 1969) Collins & Jones 1983) causes bulb spot and
58 vascular wilt on the leaves of tulip (*Tulipa* spp.); pathovar *poinsettiae* ((Starr & Pirone 1942) Collins &
59 Jones 1983) causes stem canker and leaf spot in *Euphorbia pulcherrima*; and pathovar *ilicis* ((Mandel *et*
60 *al.*, 1961) Young *et al.*, 2004) is pathogenic to American holly (*Ilex opaca*). There are also two additional

61 pathovars in this species that have been described but not yet taxonomically approved: *Curtobacterium*
62 *flaccumfaciens* pv *beticola* (Chen et al. 2007) causing bacterial leaf spot on sugar beet (*Beta vulgaris* var.
63 *saccharifera*) and *Curtobacterium flaccumfaciens* pv *basellae*, the causal agent of bacterial leaf spot of
64 malabar spinach (*Basella alba* or *B. rubra*) (Chen et al., 2000).

65 The genetic variability of *C. f.* pv. *flaccumfaciens* has been previously studied by pulsed-field
66 gel electrophoresis (PFGE), amplified fragment length polymorphism (AFLP) (Agarkova *et al.* 2012;
67 Guimarães *et al.* 2003), rep-PCR (Souza *et al.*, 2006; Agarkova *et al.*, 2012) and BOX-PCR (Guimarães *et*
68 *al.* 2003). However, these methods suffer from significant drawbacks such as the incorrect identification
69 of PCR patterns based on subjective gel interpretation, and difficulty to reproduce the results in other
70 laboratories (Tancos *et al.* 2015). By contrast, multilocus sequence analysis (MLSA) is a powerful and
71 portable molecular technique for population genetics studies, and assignment of strains to defined species
72 (Bishop *et al.*, 2009). This technique has been successfully used, among others, for characterization of the
73 genetic diversity of *Clavibacter michiganensis* (Jacques *et al.* 2012) and to assess the diversity of
74 *Curtobacterium flaccumfaciens* strains isolated from dry beans and other annual crops in Iran and Spain
75 (Osdaghi *et al.* 2018b).

76 Studies that increase our knowledge of the diversity of *Curtobacterium flaccumfaciens* strains
77 will help to generate a minimal MLSA scheme for *C. f.* pv *flaccumfaciens* identification. This will be
78 useful for plant diagnostic laboratories as well as enabling the improvement of prophylaxis measures and
79 breeding programs to obtain bacterial wilt resistant cultivars of bean.

80 In this study we chose to assess the genetic relatedness of *Curtobacterium flaccumfaciens*
81 strains from different pathovars and from diverse alternative host plants. We analyzed twenty-four strains
82 isolated from their native and alternative host in Brazil and sixty *Curtobacterium flaccumfaciens* strains
83 from the French Collection for Plant-associated Bacteria (CIRM-CFBP) belonging to four
84 phytopathogenic pathovars. The objectives were as follows: 1) to examine the genetic diversity among
85 four pathovars of *C. flaccumfaciens* strains, 2) to determine the relationship among *C. flaccumfaciens*
86 strains isolated from alternative hosts and strains isolated from bean, 3) to develop a molecular tool for
87 identifying *C. flaccumfaciens* strains and 4) to design a molecular tool to test the presence in of bean-
88 pathogenic *C. f.* pv. *flaccumfaciens* strains in bean seeds and plants.

89

90 **Material and methods**

91 **Bacterial strains and preservation**

92 The eighty-four strains analysed in this study are listed in Table 1. Sixty strains were obtained
93 from CIRM-CFBP, the French Collection for Plant-associated Bacteria
94 (https://www6.inra.fr/cirm_eng/CFBP-Plant-Associated-Bacteria). These strains were isolated over a
95 period of 71 years from Brazil, the United States, Japan and Europe. The remaining 24 strains come from
96 the Brazilian collection at the FCA-UNESP (Faculdade de Ciências Agronômicas – Universidade Estadual
97 Paulista “Júlio de Mesquita Filho”, Botucatu, SP, Brazil). These strains were isolated from bean or from
98 diverse alternative hosts in Brazil (Gonçalves et al. 2017) (Table 1). The Brazilian strains were identified
99 as *C. f. pv. flaccumfaciens* using the specific primers PCR described by Tegli *et al.* (2002) and by
100 pathogenicity test in common bean plants (under greenhouse conditions).

101 The bacterial strains were stored as lyophilized cultures and maintained in 40% glycerol stock
102 at -80 °C after cell revival. The strains were checked for purity by cultivation on YPGA (also known as
103 LPGA) culture medium (7 g/L yeast extract, 7 g/L peptone, 7 g/L glucose, and 15 g/L agar, pH 7.2) for
104 two days at 25 °C.

105

106 **Specific detection of *C. f. pv. flaccumfaciens***

107 Bacterial cells, suspended in sterile water, were prepared for PCR by boiling (95 °C/15 min)
108 and stored at -20 °C before use. These cells were used directly as substrate for PCR reactions.

109 The primers *Cff*FOR2 (5'GTT ATG ACT GAA CTT CAC TCC 3') and *Cff*REV4 (5'GAT GTT CCC
110 GGT GTT CAG 3') described by Tegli *et al.* (2002) were used to amplify a 306 bp fragment targeting a
111 conserved region of a *C.f. pv flaccumfaciens* Rep-PCR sequences. The PCR reaction was performed in a
112 mix containing 200µM dNTP, 1.5mM of MgCl₂, 500nM of each primer, 0.75U of GoTaq Flexi DNA
113 polymerase (Promega), and 6µL of the boiled bacterial cell suspension. The thermocycler conditions were
114 as follows: initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 62 °C for 45
115 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min.

116

117 **Primer design for housekeeping gene amplification**

118 Primers designed for *Clavibacter michiganensis* (Jacques *et al.*, 2012) were used for the partial
119 amplification of the housekeeping genes *recA*, *gyrB* and *ppK*. Additionally, new primers were designed in
120 this study for partial amplification of the genes *atpD*, *dnaK* and *rpoB* of *Curtobacterium*. The nucleotide
121 sequences of these genes were retrieved from the complete genome sequences of the following strains:

122 CFBP 4999 (*Clavibacter michiganensis* subsp. *michiganensis*; GenBank accession number:
123 RDQW00000000), CFBP 2404 (*Clavibacter michiganensis* subsp. *insidiosus*; GenBank accession number:
124 RDQV00000000), NCPPB 2581 (*Clavibacter michiganensis* subsp. *nebraskensis*; GenBank accession
125 number: NC020891), R1-1 (*Clavibacter michiganensis* subsp. *insidiosus*; GenBank accession number:
126 CP011043), DSM20149 (*C. f. pv. poinsettiae*; GenBank accession number: AM410867.1), DSM20141 (*C.*
127 *f. pv. betae*; GenBank accession number: AM410869.1), LMG 7042 (*C. f. pv. oortii*, GenBank accession
128 number: KF255551.1), CFBP 3418 (*C. f. pv. flaccumfaciens*; GenBank accession number:
129 PUEZ00000000). The DNA sequences were aligned using MULTALIN
130 (<http://multalin.toulouse.inra.fr/multalin/>). Based on the consensus generated from the alignments, new
131 sets of primers were designed for *Curtobacterium*. Specificity parameters of the primer pairs were
132 checked by the Primer-BLAST program at NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>), the Primer3
133 tool (http://biotools.umassmed.edu/bioapps/primer3_www.cgi) and the AmplifX software (version 1.7.0)
134 (<http://jim.timone.univ-amu.fr/recherche/equipe-t-brue/jullien-nicolas/programmation/amplifx/?lang=fr>)
135 in order to ensure that they amplify only one DNA fragment of the target gene from the *Curtobacterium*
136 genome. Annealing temperatures varying from 52 to 72 °C were tested using a Bio-Rad Gradient
137 Thermocycler to determine the optimal temperature for DNA amplification.

138 The complete genome sequences of strains CFBP 3418, CFBP 2404 and CFBP 4999 were
139 obtained as described in Merda et al. (2017), using the Illumina technology and HiSeq 2500 (Genoscreen,
140 Lille, France) or MiSeq instruments. Libraries of genomic DNA were performed using the Kit NextEra
141 XT (Illumina, USA). Paired-end reads of 2 x 100 bp were assembled in contigs using SOAPDENOV0
142 1.05 (Li et al., 2010) and VELVET 1.2.02 (Zerbino & Birney, 2008). Annotation was performed using
143 EuGene-PP (Sallet, Gouzy, & Schiex, 2014).

144

145 **Amplification and sequencing of housekeeping genes**

146 The primers used for MLSA are listed in Table 2. The PCR reaction mix contained 200 µM
147 dNTP, 1.5 mM of MgCl₂, 500 nM of each primer, 0.75 U GoTaq Flexi DNA polymerase (Promega), and
148 6 µL of boiled bacterial suspension. The PCR program consisted of an initial hold at 94 °C for 5 min
149 followed by 35 cycles of 30" at 94 °C, 30" at annealing temperature (60 or 62°C), extension time of 30" at
150 72 °C, and ending with 7 min at 72 °C. For genes *recA*, *gyrB* and *ppK*, the annealing temperature was 60
151 °C and 62 °C for *dnaK*, *atpD* and *rpoB*. The PCR products were checked on a 1.5% agarose gel, and

152 forward and reverse sequencing was performed directly on the PCR products by GenoScreen company
153 (Lille, France).

154

155 **Sequence analysis**

156 For each individual gene and for the concatenated dataset the DNA Sequence Polymorphism
157 software (version 5.10.01) was used to calculate haplotype numbers (Hap), haplotype diversity (Hd),
158 nucleotidic diversities ($\theta\pi$ and θw), and estimated neutrality (Tajima's D, Fu and Li's and Fu's F). The
159 neutrality estimated analysis revealed the evolutionary forces acting on a particular gene (value "0"
160 meaning neutrality; positive values for diversifying selected genes and negative values for conditions of
161 purifying selection).

162 The consensus sequences for forward and reverse sequences for each strain were extracted
163 using Geneious version Pro v. 4.8.5 (<http://www.geneious.com/>). The sequences were then aligned and
164 trimmed using BioEdit v. 5.0.6. Phylogenetic trees were constructed with MEGA 5.1, using maximum
165 likelihood as the statistical method, and the Jukes-Cantor substitution model with 1000 bootstrap
166 replicates. Trees were constructed for each individual gene, as well as with concatenated alignments of all
167 genes. Sequences of the six genes were extracted from the complete genome sequence of the type strain of
168 *Clavibacter michiganensis* subsp. *michiganensis* (CFPB 4999) were used as outgroups in the analyses.
169 Split decomposition analysis was performed with SplitsTree4 v. 4.13.1 (Huson and Bryant, 2006), which
170 allows for the construction of a tree-like network structure if conflicting phylogeny signals are detected in
171 the data set.

172

173 **Pathogenicity tests on common beans with strains from Brazil**

174 Two different bacterial inoculation methods were tested in the greenhouse. For the needle
175 technique, plants were inoculated at the epicotyl (region between cotyledons and primary leaves) by two
176 punctures with a needle, primed with bacterial suspension as described by Maringoni (2002). For the
177 submersion technique, the first trifoliolate leaf was inoculated by submersion in a bacterial suspension,
178 adjusted to 10^8 CFU/mL (O.D.₅₅₀ = 0.14), followed by an incubation period of 72 h as described by
179 Darsonval *et al.* (2009). In both cases, the bacterial strains were cultivated at 28 °C for 48 h before
180 inoculation. The tests were performed in triplicate using *Phaseolus vulgaris* cv. 'Pérola'. Plants were
181 cultivated in 3-L pots containing autoclaved soil. For both methods, plants were inoculated when the first

182 trifoliate leaf was fully expanded at approximately 12 days after sowing. The maximum and minimum
183 temperatures inside the greenhouse were of 27 °C and 9 °C, respectively. In both methods, plants were
184 inoculated with sterile water as the negative control. As positive control, plants were inoculated using *C. f.*
185 *pv. flaccumfaciens* type strain (CFBP 3418). Thirty days after inoculation, pathogenic strains were
186 classified as positive (+: when symptoms of mosaic or necrotic lesions were observed on leaves) or highly
187 aggressive (+: when the plant growth was very reduced with low number of chlorotic leaves or led
188 common bean plant to death)

189

190 **Results**

191 **Multilocus sequence analysis of *Curtobacterium flaccumfaciens***

192 The concatenated multilocus sequence was constructed by joining six housekeeping genes in
193 alphabetical order 1 – 489: *atpD*; 490 – 957: *dnaK*; 958 – 1686: *gyrB*; 1687 – 2232: *ppk*; 2233 – 2820:
194 *recA*; 2821 – 3483: *rpoB*), resulting in a total of 3483 bp.. These sequences are available on Genbank, the
195 accession numbers are listed in Supplemental Table 1. The phylogenetic tree based on the concatenated
196 gene set using maximum likelihood did not cluster strains according to the host species, geographical
197 origin, year of isolation or pathovar (Figure 1). However, on a finer scale, some interesting smaller
198 clusters can be observed, for instance all strains isolated from beetroot in the United Kingdom were
199 grouped in a cluster supported by a high bootstrap value. All *Curtobacterium flaccumfaciens* strains, with
200 the exception of the strain CFBP 3400, appeared to be clustered in a monophyletic group supported by a
201 100% bootstrap value (Figure 1). Strain CFBP 3400 appears to be highly divergent from the other *C.*
202 *flaccumfaciens* strains and thus was subsequently compared to sequences of strains from other species of
203 *Curtobacterium* in the National Center for Biotechnology Information (NCBI) (Supplemental Figure 1).
204 This analysis showed that this strain actually belongs to the *Curtobacterium flaccumfaciens* species.

205 The phylogenetic tree was composed of three main groups supported by high bootstrap values
206 (Figure 1). While the strains of *C.f. pv. poinsettiae* were all in group G-I and strains from *C.f. pv. betae* and
207 *C.f. pv. ortii* were in group G-III, the strains of *C.f. pv. flaccumfaciens* were present in all three groups. The
208 strain CFBP 3400 (known as *C. f. pv. oortii*) was classified outside of these three groups. Comparing the
209 phenotypic traits from UNESP strains (Table 1) and their distribution along the phylogenetic tree, it was

210 observed that strains with orange colored colonies clustered in the G-I group and strains displaying yellow
211 colonies were distributed in the G-II and G-III groups (data not shown).

212 The genetic diversity within *C. flaccumfaciens* strains is around 10% for the concatenated data
213 (353 polymorphic sites out of 3478 sites in total) and varied considerably from gene to gene as follows:
214 4.29% (*atpD*), 6.94% (*rpoB*), 9.52% (*recA*), 11.95% (*ppk*), 13.25% (*dnaK*) to 14.19% (*gyrB*) (Table 3).
215 The number of alleles at each locus varied from 20 for *atpD* to 42 for *gyrB*. All loci showed
216 polymorphism and polymorphic sites ranged from 21 (*atpD*) to 103 (*gyrB*) (Table 3). Results of neutrality
217 tests (Tajima's, Fu and Li's and Fu's F tests), showed some indication that purifying selection is operating
218 on particular housekeeping genes in *Curtobacterium flaccumfaciens* strains, as indicated by negative
219 values (Table 3).

220 A reduced MLSA scheme using *recA* and *gyrB* data, as proposed for *Clavibacter* by Jacques *et*
221 *al.* (2012) proved to reliably identify the strains of *C. flaccumfaciens* species, with the whole species, and
222 the three internal clusters, being supported by high bootstrap values (Supplemental figure 2).

223

224 **Phylogenetic network**

225 All loci showed a number of significant reticulations with the genes *gyrB*, *ppk* and *recA*
226 showing the greatest numbers of reticulations (Figure 2). The network for *dnaK* showed that *C. f.* pv.
227 *betae*, *C. f.* pv. *oortii* and *C. f.* pv. *flaccumfaciens* share alleles with each other. The *gyrB* gene sequence
228 analysis showed that *C. f.* pv. *poinsettiae* and *C. f.* pv. *flaccumfaciens* share alleles with each other, and
229 that other *C. f.* pv. *flaccumfaciens* strains share alleles with *C. f.* pv. *betae* and *C. f.* pv. *oortii*. The network
230 constructed for *ppk* showed similar results to those of *gyrB*. The network for *recA* revealed strains of *C. f.*
231 pv. *flaccumfaciens* sharing alleles with the other three pathovars. The network made with the concatenated
232 genes also presented a significant number of reticulations.

233

234 **Pathogenicity tests**

235 Symptoms were similar regardless of inoculation techniques used, with mosaic spots appearing
236 on leaves about 10 days after inoculation and developing into necrotic lesions. In the pathogenicity tests,
237 the strains of *C. f.* pv. *oortii*, *C. f.* pv. *betae* and *C. f.* pv. *poinsettiae* were negative for the pathogenicity
238 test (common bean plants were asymptomatic) (Table 1). Symptomatic plants showed a reduced growth

239 compared with asymptomatic plants. Thirty days after inoculation, pathogenic strains were classified as
240 positive (+) or highly aggressive (++) (Table 1). All *C.f. pv flaccumfaciens* strains tested using the
241 “needle” technique were pathogenic. Three of them proved to be highly aggressive (CFBP 8371, 3178
242 UNESP, CFBP 8391), leading to the death of the bean plants. Only these three highly aggressive strains,
243 all isolated from alternative hosts (wheat, oat and cabbage), were capable of causing symptoms on bean
244 using the “submersion” technique.

245

246 **Specific PCR for *Curtobacterium flaccumfaciens* pv. *flaccumfaciens***

247 We tested the accuracy of the PCR primers CffFOR2-CffREV4 described by Tegli *et al.*
248 (2002), designed specifically to detect *C. f. pv. flaccumfaciens* strains in bean seeds. The present results
249 showed that the accuracy of these primers was of 96%. Two *C.f. pv flaccumfaciens* strains isolated in the
250 USA were false negatives (CFBP 3422 and CFBP 3486), confirmed by pathogenicity tests on bean plants.
251 The MLSA analysis showed that these two strains were clustered together in a group of pathogenic strains,
252 which were also positive to PCR amplification assays with the CffFOR2-CffREV4 primers (Table 1). One
253 strain from United Kingdom (CFBP 3401), classified as *C. f. pv. betae*, and grouped with the other *C.f. pv*
254 *betae* strains by MLSA, was false positive, and all other strains of this group gave negative PCR results.
255 Two strains of *C.f. pv betae* had been tested on bean (type strain CFBP 2402 isolated in United Kingdom
256 and strain CFBP 3404 isolated in Brazil) and were non pathogenic (Table 1). The 306 bp fragment
257 amplified by these specific primers was used in blast search against the complete genome sequence of the
258 type strain CFBP 3418 of *C.f. pv. flaccumfaciens* and aligned to the intergenic region between two genes
259 coding for trypsin-like cysteine/serine peptidase (data not shown).

260

261 **Discussion**

262 A MLSA based on six housekeeping genes (*recA*, *gyrB*, *ppK*, *atpD*, *dnaK* and *rpoB*) was used
263 to investigate the phylogenetic relationships among 84 strains of *Curtobacterium flaccumfaciens*
264 distributed into four pathovars (*C.f. pv flaccumfaciens*, *poinsettiae*, *oortii* and *betae*). These strains were
265 isolated from usual or alternative hosts, over a period of 77 years from North America, South America,
266 Europe and Asia. The number of variable sites (3.5 to 12.6%) is comparable to what was found in
267 *Clavibacter michiganensis* (5.0 to 12.8%) (Jacques *et al.*, 2012). Our findings are in agreement with other

268 studies on genetic diversity and phylogenetic relationships in *C. flaccumfaciens* using rep-PCR (Souza et
269 al. 2006), and MLSA (Osdaghi et al. 2018b).

270 The *C. flaccumfaciens* strains were grouped in three main clusters. The only correlation we
271 could make was that all the Brazilian orange-colored strains are grouped in the same group (G-I). Some
272 previous studies stated that diverse techniques (AFLP, PFGE, and rep-PCR) used to explore the genetic
273 diversity in *C. flaccumfaciens*, could separate the strains by their pigments (Agarkova *et al.* 2012; Osdaghi
274 *et al.* 2016; Osdaghi *et al.* 2018a). Colony color aside, the three clusters do not correlate with any of the
275 metadata, that is, pathovar affiliation, isolation date or geographical location, and isolation host. These
276 results are consistent with previous findings. For instance, Souza *et al.* (2006) reported that the diversity
277 they measured in *C. flaccumfaciens* using rep-PCR did not correlate either with the country of isolation.

278 In our study, none of the four pathovars (*pv. betae*, *pv. oortii*, *pv. poinsettiae* and *pv.*
279 *flaccumfaciens*) formed a monophyletic cluster. The cluster G-III contains strains from three pathovars
280 (*pv. betae*, *pv. oortii*, and *pv. flaccumfaciens*), the cluster G-I contains strains from *pv. poinsettiae* and *pv.*
281 *flaccumfaciens*, and the last cluster (G-II) contains only strains from *pv. flaccumfaciens*. Thus, the *pv.*
282 *flaccumfaciens* strains, pathogenic on bean, are as diverse as the species and were scattered among the
283 three clusters. Even inside each cluster, there is no phylogenetic structure; strains from different pathovars
284 cluster together, irrespective of their host of isolation or geographical origin. This means that the six
285 genes used for MLSA analysis were not sufficient to discriminate either *C. flaccumfaciens* pathovars or to
286 distinguish between *C. f. pv flaccumfaciens* strains isolated from bean or from other hosts.

287 These results raised the question about the differences in pathogenicity between the pathovars.
288 As the strains are not genetically different based on a six-genes MLSA, the question was whether they can
289 affect plants other than their isolation host and can they be pathogenic on a much larger host range than
290 initially thought. Representative strains of each pathovar were tested for pathogenicity on common bean.
291 Except for *C.f. pv flaccumfaciens*, which were pathogenic to bean, all strains from other pathovars were
292 non-pathogenic, independent from their hosts. Among the *C.f. pv flaccumfaciens* strains, some isolated
293 from alternative hosts can be hyper-aggressive, even more aggressive than the type strain. Thus our data
294 reveal no correlation between phylogeny and pathogenicity for *C.f. pv flaccumfaciens*. Even if the
295 pathogenicity genes are usually contained in the accessory-genome, discrepancy between phylogeny
296 assessed by MLSA and pathogenicity, even if possible, are uncommon. MLSA, even reduced to two
297 genes, has been found to be sufficient to identify taxa determining specific pathology (Hajri *et al.*, 2012;

298 Fischer-Le Saux *et al.*, 2015; Osdaghi *et al.*, 2018a). However, our results were different from that, with
299 these six genes not being sufficient to differentiate the pathovars. A possible explanation is that the
300 different *Curtobacterium* pathovars could be relatively new and the host-driven selection has been too
301 recent to have an effect strong enough on the phylogenetic structure of the population to be detected by
302 MLSA. This is supported by the fact that the phylogenetic network analysis indicates active gene flux
303 inside the *C. flaccumfaciens* species (shared alleles between pathovars and reticulated network).

304 Our results show that strains isolated from bean or from alternative hosts are both pathogenic
305 on bean and not different based on a MLSA analysis, confirming that the alternative hosts can serve as
306 reservoir for this pathogen (Gonçalves *et al.* 2017). This information should be taken into account when
307 considering management strategies for bacterial wilt disease of bean in the future.

308 Although the different *C. f.* pathovars could not be differentiated using the six housekeeping
309 genes in the MLSA analysis, the method was effective and reliable for identifying strains of *C.*
310 *flaccumfaciens*. Downsizing the MLSA scheme to only two genes proved to be enough for reliable
311 identification of *C. flaccumfaciens* strains to the species level. Thus, the use of *recA* and *gyrB* genes in
312 reduced MLSA scheme for strain identification may be used as rapid procedure for the routine
313 identification of *C. flaccumfaciens* species in biological resources centers.

314 Among the numerous studies carried out to study the diversity of *Curtobacterium*
315 *flaccumfaciens*, only PFGE (Guimarães *et al.*, 2003) was able to differentiate between the four pathovars.
316 However, the development of a simple and portable screening method for rapid identification of *C. f. pv.*
317 *flaccumfaciens* strains among a large number of strains is necessary, for example, for large environmental
318 surveys.

319 The PCR-Based assay developed by Tegli *et al.* (2002) to amplify a 306bp from an original
320 Rep-PCR DNA fragment of 550bp, efficiently detected *C. f. pv. flaccumfaciens* in naturally infected bean
321 seeds, and was also effective to separate bean-virulent from non-virulent strains of *C. f. pv. flaccumfaciens*
322 by amplifying a DNA fragment of the expected size only in pathogenic strains (Osdaghi *et al.*, 2018a). In
323 our study, we have extensively tested this protocol with a large number of strains and the reliability and
324 *validity* of this procedure to accurately detect *C. f. pv. flaccumfaciens* strains was confirmed. This simple
325 PCR, could be the basis to design field tests permitting a better crop management. However, false positive
326 and false negative still occur. Thus, the search to improve the available tools and the development of new
327 approaches for *C. f. pv. flaccumfaciens* identification is still necessary. Further studies using whole-

328 genome sequencing of different *C. f.* pathovars may generate specific and sensitive molecular tools for
329 pathovars discrimination, and PCR-assays targeting the DNA region coding for genes involved in the
330 bean-specificity and bean-pathogenicity.

331 We performed a blast search against the whole-genome of *C. f.* pv. *flaccumfaciens* type strain
332 CFBP 3418 using the pair primers developed by Tegli *et al.* (2002). Like Osdaghi et al., 2018a, we
333 showed that these primers targeted an intergenic DNA region of 306bp between two genes coding for a
334 putative trypsin-like cysteine/serine peptidase (data not shown). Bacterial catalytic proteases are mainly
335 involved in the breakdown of peptide bonds to amino acids required for nutritional purposes or to degrade
336 proteins in the plant cell wall, allowing the bacterial translocation or overcoming plant chemical defenses
337 (Dow et al., 1990; Vignesh et al., 2016). However, the role of these genes and their flanking genes in plant
338 pathogenicity or bean recognition by the pathogen has not been elucidated yet. These findings indicate the
339 necessity of further investigations on the *C. f.* pv. *flaccumfaciens* interactions with the common bean, and
340 the mechanisms involved in *C. f.* pv. *flaccumfaciens* pathogenicity.

341 Our research deepens our understanding about the genetic diversity of *Curtobacterium*
342 *flaccumfaciens*, and confirms the relationship between strains isolated from alternative hosts and bean
343 plants. However, further studies are necessary to better understand the mechanisms involved in *C. f.*
344 pv.*flaccumfaciens* pathogenicity.

345

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351 **Compliance with ethical standards**

352 **Conflict of interest**

353 The authors declare that they have no conflict of interest.

354

355 **References**

356 Agarkova, I. V., Lambrecht, P. A., Vidaver, A. K., & Harveson, R. M. (2012). Genetic diversity among
357 *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* populations in the American High Plains.

- 358 *Canadian Journal of Microbiology*, 58(6), 788–801. doi:10.1139/w2012-052
- 359 Aritua, V., Harrison, J., Sapp, M., Buruchara, R., Smith, J., & Studholme, D. J. (2015). Genome
360 sequencing reveals a new lineage associated with lablab bean and genetic exchange between
361 *Xanthomonas axonopodis* pv. *phaseoli* and *Xanthomonas fuscans* subsp. *fuscans*. *Frontiers in*
362 *Microbiology*, 6, 1080. doi:10.3389/fmicb.2015.01080
- 363 Bishop, C. J., Aanensen, D. M., Jordan, G. E., Kilian, M., Hanage, W. P., & Spratt, B. G. (2009).
364 Assigning strains to bacterial species via the internet. *BMC Biology*, 7(1), 3. doi:10.1186/1741-
365 7007-7-3
- 366 Chen YF, Guo JH and Fang ZD (2000) A new pathovar of *Curtobacterium flaccumfaciens* on malabar
367 spinach. *Acta Phytopathologica Sinica* 30: 171-175
- 368 Chen, Y.-F., Yin, Y.-N., Zhang, X.-M., & Guo, J.-H. (2007). *Curtobacterium flaccumfaciens* pv. *beticola* ,
369 a new pathovar of pathogens in sugar beet. *Plant Disease*, 91(6), 677–684. doi:10.1094/PDIS-91-6-
370 0677
- 371 Collins, M. D., & Jones, D. (1983). Reclassification of *Corynebacterium flaccumfaciens*,
372 *Corynebacterium betae*, *Corynebacterium oortii* and *Corynebacterium poinsettiae* in the genus
373 *Curtobacterium*, as *Curtobacterium flaccumfaciens* comb. nov. *Microbiology*, 129(11), 3545–3548.
374 doi:10.1099/00221287-129-11-3545
- 375 Darsonval, A., Darrasse, A., Durand, K., Bureau, C., Cesbron, S., & Jacques, M.-A. (2009). Adhesion and
376 fitness in the bean phyllosphere and transmission to seed of *Xanthomonas fuscans* subsp. *fuscans*.
377 *Molecular Plant-Microbe Interactions*, 22(6), 747–757. doi:10.1094/MPMI-22-6-0747
- 378 Dow, J. M., Clarke, B. R., Milligan, D. E., Tang, J. L., & Daniels, M. J. (1990). Extracellular proteases
379 from *Xanthomonas campestris* pv. *campestris*, the black rot pathogen. *Applied and environmental*
380 *microbiology*, 56(10), 2994–8.
- 381 Fischer-Le Saux, M., Bonneau, S., Essakhi, S., Manceau, C., & Jacques, M.-A. (2015). Aggressive
382 emerging pathovars of *Xanthomonas arboricola* represent widespread epidemic clones distinct from
383 poorly pathogenic strains, as revealed by multilocus sequence typing. *Applied and environmental*
384 *microbiology*, 81(14), 4651–68. doi:10.1128/AEM.00050-15
- 385 Gonçalves, R. M., Schipanski, C. A., Koguish, L., Soman, J. M., Sakate, R. K., Júnior, T. A. F. S., &
386 Maringoni, A. C. (2017). Alternative hosts of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*,
387 causal agent of bean bacterial wilt. *European Journal of Plant Pathology*, 148(2), 357–365.

- 388 doi:10.1007/s10658-016-1094-4
- 389 Guimarães, P. M., Smith, J. J., Palmano, S., & Saddler, G. S. (2003). Characterisation of *Curtobacterium*
390 *flaccumfaciens* pathovars by AFLP, rep-PCR and Pulsed-Field Gel Electrophoresis. *European*
391 *Journal of Plant Pathology*, 109(8), 817–825. doi:10.1023/A:1026197914417
- 392 Hajri, A., Brin, C., Hunault, G., Lardeux, F., Lemaire, C., Manceau, C., et al. (2009). A «repertoire for
393 repertoire» hypothesis: repertoires of type three effectors are candidate determinants of host
394 specificity in *Xanthomonas*. *PLoS ONE*, 4(8), e6632. doi:10.1371/journal.pone.0006632
- 395 Hajri, A., Pothier, J. F., Fischer-Le Saux, M., Bonneau, S., Poussier, S., Boureau, T., et al. (2012). Type
396 three effector gene distribution and sequence analysis provide new insights into the pathogenicity of
397 plant-pathogenic *Xanthomonas arboricola*. *Applied and environmental microbiology*, 78(2), 371–
398 84. doi:10.1128/AEM.06119-11
- 399 Hedges, F. (1922). A bacterial wilt of the bean caused by *Bacterium flaccumfaciens* nov. sp. *Science*,
400 55(1425), 433–434. doi:DOI: 10.1126/science.55.1425.433
- 401 Hedges, F. (1926). Bacterial wilt of Beans (*Bacterium flaccumfaciens* Hedges), including comparisons
402 with *Bacterium phaseoli*. *Phytopathology*, 16(1), 1–22.
- 403 Herbes, D. H., Theodoro, G. F., Maringoni, A. C., dal Piva, C. A., & Abreu, L. de. (2008). Detection of
404 *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* in seeds of common bean produced in Santa
405 Catarina. *Tropical Plant Pathology*, 33(2), 153–156. doi:10.1590/S1982-56762008000200010
- 406 Jacques, M. A., Durand, K., Orgeur, G., Balidas, S., Fricot, C., Bonneau, S., et al. (2012). Phylogenetic
407 analysis and polyphasic characterization of *Clavibacter michiganensis* strains isolated from tomato
408 seeds reveal that nonpathogenic strains are distinct from *C. michiganensis* subsp. *michiganensis*.
409 *Applied and environmental microbiology*, 78(23), 8388–402. doi:10.1128/AEM.02158-12
- 410 Li, R., Zhu, H., Ruan, J., Qian, W., Fang X, Shi Z, Li Y, Li S, Shan G, Kristiansen K, Li S, Yang H, Wang
411 J, Wang J. (2010), De novo assembly of human genomes with massively parallel short read
412 sequencing. *Genome Res*. 20(2): 265-272. doi: 10.1101/gr.097261.109.
- 413 Marguerettaz, M., Pieretti, I., Gayral, P., Puig, J., Brin, C., Cociancich, S., et al. (2011). Genomic and
414 evolutionary features of the SPI-1 Type III Secretion System that is present in *Xanthomonas*
415 *albilineans* but is not essential for xylem colonization and symptom development of sugarcane leaf
416 scald. *Molecular Plant-Microbe Interactions*, 24(2), 246–259. doi:10.1094/MPMI-08-10-0188
- 417 Maringoni, A. C. (2002). Behaviour of dry bean cultivars to bacterial wilt. *Fitopatologia Brasileira*, 27(2),

- 418 157–162. doi:10.1590/S0100-41582002000200006
- 419 Maringoni, A. C., & Rosa, E. F. (1997). Occurrence of *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*
420 on bean in the state of Sao Paulo, Brazil. *Summa Phytopathologica (Brazil)*.
- 421 Merda, D., Briand, M., Bosis, E., Rousseau, C., Portier, P., Barret, M., et al. (2017). Ancestral
422 acquisitions, gene flow and multiple evolutionary trajectories of the type three secretion system and
423 effectors in *Xanthomonas* plant pathogens. *Molecular Ecology*, 26(21), 5939–5952.
424 doi:10.1111/mec.14343
- 425 Mhedbi-Hajri, N., Hajri, A., Boureau, T., Darrasse, A., Durand, K., Brin, C., et al. (2013). Evolutionary
426 history of the plant pathogenic bacterium *Xanthomonas axonopodis*. *PLoS ONE*, 8(3), e58474.
427 doi:10.1371/journal.pone.0058474
- 428 Osdaghi, E., Taghavi, S. M., Calamai, S., Biancalani, C., Cerboneschi, M., Tegli, S., & Harveson, R.
429 (2018b). Phenotypic and molecular-phylogenetic analysis provide novel insights into the diversity of
430 *Curtobacterium flaccumfaciens*. *Phytopathology*.
- 431 Osdaghi, E., Taghavi, S. M., Hamzehzarghani, H., Fazliarab, A., Harveson, R. M., & Lamichhane, J. R.
432 (2016). Occurrence and characterization of a new red-pigmented variant of *Curtobacterium*
433 *flaccumfaciens*, the causal agent of bacterial wilt of edible dry beans in Iran. *European Journal of*
434 *Plant Pathology*, 146(1), 129–145. doi:10.1007/s10658-016-0900-3
- 435 Osdaghi, E., Taghavi, S. M., Hamzehzarghani, H., Fazliarab, A., Harveson, R. M., Tegli, S., &
436 Lamichhane, J. R. (2018a). Epiphytic *Curtobacterium flaccumfaciens* strains isolated from
437 symptomless solanaceous vegetables are pathogenic on leguminous but not on solanaceous plants.
438 *Plant Pathology*. 67, 388–398 doi:10.1111/ppa.12730
- 439 Sallet, E., Gouzy, J., & Schiex, T. (2014). EuGene-PP: A next-generation automated annotation pipeline
440 for prokaryotic genomes. *Bioinformatics*, 30(18), 2659–2661.
441 <https://doi.org/10.1093/bioinformatics/btu366>
- 442 Sammer, U. F., & Reiher, K. (2012). *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* on Soybean in
443 Germany - A Threat for Farming. *Journal of Phytopathology*, 160(6), 314–316. doi:10.1111/j.1439-
444 0434.2012.01902.x
- 445 Schwartz, H. F. ., Steadman, J. R. ., Hall, R. ., & Forster, R. L. (2005). *Compendium of bean diseases*. St.
446 Paul: APS Press, American Phytopathological Society.
- 447 Souza, V. L. de, Maringoni, A. C., & Krause-Sakate, R. (2006). Genetic variability in *Curtobacterium*

- 448 *flaccumfaciens* isolates. *Summa Phytopathologica*, 32(2), 170–176. doi:10.1590/S0100-
449 54052006000200012
- 450 Tancos, M. A., Lange, H. W., & Smart, C. D. (2015). Characterizing the genetic diversity of the
451 *Clavibacter michiganensis* subsp. *michiganensis* population in New York. *Phytopathology*, 105(2),
452 169–179. doi:10.1094/PHYTO-06-14-0178-R
- 453 Tegli, S., Sereni, A., & Surico, G. (2002). PCR-based assay for the detection of *Curtobacterium*
454 *flaccumfaciens* pv. *flaccumfaciens* in bean seeds. *Letters in Applied Microbiology*, 35(4), 331–337.
- 455 Theodoro, G. de F., Maringoni, A. C., Chumpati, A. A., Correia, H. de C., Theodoro, J. V. C., &
456 Nogueira, R. J. (2010). First report of bacterial wilt of common bean caused by *Curtobacterium*
457 *flaccumfaciens* pv. *flaccumfaciens* in Mato Grosso do Sul. *Journal of Plant Pathology*.
- 458 Uesugi, C. H., Freitas, M. A., & Menezes, J. R. (2003). First occurrence of *Curtobacterium*
459 *flaccumfaciens* pv. *flaccumfaciens* on bean in the State of Goiás and Federal District of Brazil.
460 *Fitopatologia Brasileira*, 28(3), 324–324. doi:10.1590/S0100-41582003000300019
- 461 Vignesh, R.; Prabakar, P.; Jaganathan R., Swathirajan, C. R. Isolation and characterization of extracellular
462 proteases from *Pseudomonas aeruginosa* and *Bacillus subtilis* strains. *Bull. Env. Pharmacol. Life*
463 *Sci.*, Vol 5(8): 40-43, 2016.
- 464 Young, J. M., Dye, D. W., Bradbury, J. F., Panagopoulos, C. G., & Robbs, C. F. (1978). A proposed
465 nomenclature and classification for plant pathogenic bacteria. *New Zealand Journal of Agricultural*
466 *Research*, 21(1), 153–177. doi:10.1080/00288233.1978.10427397
- 467 Young, J. M., Watson, D. R. W., & Dye, D. W. (2004). Reconsideration of *Arthrobacter ilicis* (Mandel et
468 al. 1961) Collins et al. 1982 as a plant-pathogenic species. Proposal to emend the authority and
469 description of the species. Request for an Opinion. *International journal of systematic and*
470 *evolutionary microbiology*, 54(1), 303–305.
- 471 Zerbino DR, Birney E (2008). Velvet: algorithms for de novo short read assembly using de Bruijn graphs.
472 *Genome Res.* 18(5): 821-829. doi: 10.1101/gr.074492.107.
- 473

474 Figure 1. Molecular phylogenetic analysis based on the partial sequences of six housekeeping genes,
475 concatenated (*atpD*, *dnaK*, *gyrB*, *ppK*, *recA*, and *rpoB*). This tree was constructed using a maximum
476 likelihood method based on the Jukes-Cantor model and rooted with the strain CFBP 4999 of
477 *Clavibacter michiganensis* subsp. *michiganensis*. The percentage of trees in which the associated
478 taxa clustered together is shown above the branches. Confidence on nodes was tested with 1000
479 bootstraps replicates. Bootstrap values under 50 are not shown. Highlighted strains with black circle
480 are type or pathotype strains.

481

482 Figure 2. Split graphs of MLSA of two collections of strains of each sequence type (ST) for six genes
483 (*atpD*, *dnaK*, *gyrB*, *ppK*, *recA*, and *rpoB*) and concatenated sequences. Strains from each pathovar
484 (*flaccumfaciens*, *betae*, *oortii*, and *poinsettiae*) are shown in green, red, blue, and yellow,
485 respectively.

486



Figure 1. Molecular phylogenetic analysis based on the partial sequences of six housekeeping genes, concatenated (*atpD*, *dnaK*, *gyrB*, *ppK*, *recA*, and *rpoB*). This tree was constructed using a maximum likelihood method based on the Jukes-Cantor model and rooted with the strain CFBP 4999 of *Clavibacter michiganensis* subsp. *michiganensis*. The percentage of trees in which the associated taxa clustered together is shown above the branches. Confidence on nodes was tested with 1000 bootstraps replicates. Bootstrap values under 50 are not shown. Highlighted strains with black circle are type or pathotype strains. 127x187 mm (427x500 DPI)

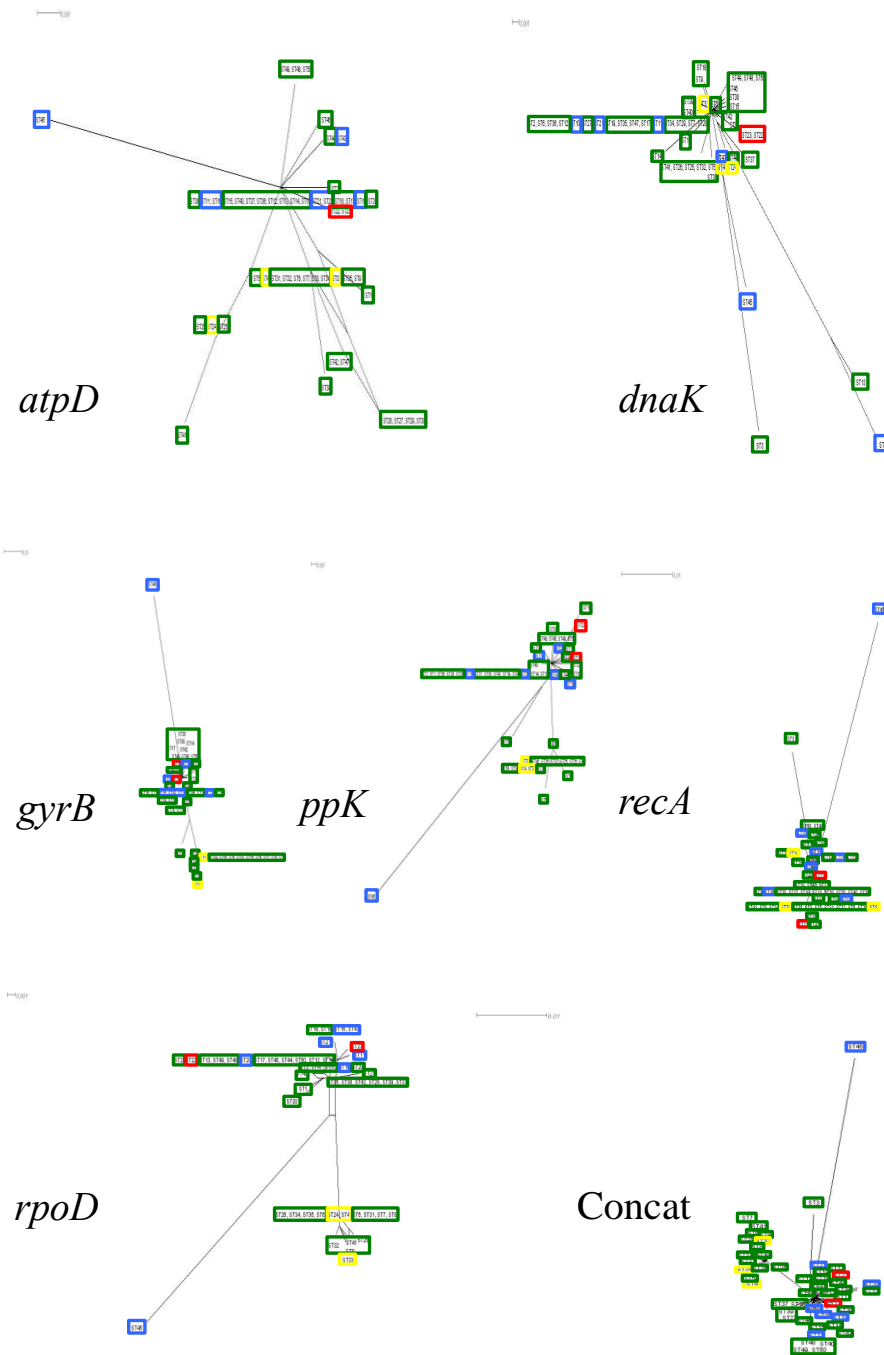


Figure 2. Split graphs of MLSA of two collections of strains of each sequence type (ST) for six genes (*atpD*, *dnaK*, *gyrB*, *ppK*, *recA*, and *rpoB*) and concatenated sequences. Strains from each pathovar (*flaccumfaciens*, *betae*, *oortii*, and *poinsettiae*) are shown in green, red, blue, and yellow, respectively. 186x120 mm (382x283 DPI)

Table 1. Overview of *Curtobacterium flaccumfaciens* strains used in this study.

Code strain	Cf pathovars	Isolation			Inoculation technique		Tegli PCR
		Host	Country	Year	Needle ¹	Submersion ²	
CFBP 1003	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	Hungary	1957	Np ⁴	np	+ ⁵
CFBP 1378	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	USA	1958	np	np	+
CFBP 1379	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	Germany	1958	np	np	+
CFBP 1381	<i>poinsettiae</i>	<i>Euphorbia pulcherrima</i>	USA	1959	np	np	- ⁶
CFBP 1384 ^{PT}	<i>oortii</i>	<i>Tulipa gesneriana</i>	Netherlands	1967	-	-	-
CFBP 1389	<i>flaccumfaciens</i>	<i>Phaseolus sp.</i>	Hungary	1957	np	np	+
CFBP 2402 ^{PT}	<i>betae</i>	<i>Beta vulgaris cv. Rubra</i>	UK	1955	-	-	-
CFBP 2403 ^{PT}	<i>poinsettiae</i>	<i>Euphorbia pulcherrima</i>	USA	-	-	-	-
CFBP 3391	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	USA	-	np	np	+
CFBP 3392	<i>oortii</i>	<i>Tulipa sp.</i>	Netherlands	-	np	np	-
CFBP 3397	<i>oortii</i>	<i>Tulipa sp.</i>	Netherlands	1977	np	np	-
CFBP 3398	<i>oortii</i>	<i>Tulipa sp.</i>	Netherlands	1977	np	np	-
CFBP 3399	<i>oortii</i>	<i>Tulipa sp.</i>	Netherlands	1987	np	np	-
CFBP 3400	<i>oortii</i>	<i>Zantedeschia aethiopica</i>	Netherlands	1990	np	np	-
CFBP 3401	<i>betae</i>	<i>Beta vulgaris</i>	UK	-	np	np	+
CFBP 3402	<i>betae</i>	<i>Beta vulgaris</i>	UK	1972	np	np	-
CFBP 3403	<i>betae</i>	<i>Beta vulgaris</i>	UK	1972	np	np	-
CFBP 3404	<i>betae</i>	<i>Beta vulgaris</i>	Brazil	1980	-	-	-
CFBP 3406	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	-	-	np	np	+
CFBP 3407	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	USA	-	np	np	+
CFBP 3409	<i>flaccumfaciens</i>	<i>Vigna angularis</i>	USA	1919	np	np	+
CFBP 3410	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	USA	-	np	np	+
CFBP 3411	<i>flaccumfaciens</i>	<i>Vigna radiata</i>	USA	1934	np	np	+
CFBP 3412	<i>flaccumfaciens</i>	-	USA	-	np	np	+
CFBP 3414	<i>poinsettiae</i>	<i>Euphorbia pulcherrima</i>	USA	-	np	np	-
CFBP 3415	<i>poinsettiae</i>	<i>Euphorbia pulcherrima</i>	USA	-	-	-	-
CFBP 3416	<i>poinsettiae</i>	<i>Euphorbia pulcherrima</i>	USA	-	-	-	-
CFBP 3417	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	USA	1958	+	-	+
CFBP 3418 ^T	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	Hungary	1957	+	-	+
CFBP 3419	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	-	1954	np	np	+
CFBP 3420	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	Romania	1965	+	-	+
CFBP 3422	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	USA	1956	+	-	-
CFBP 3423	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	USA	1957	np	np	+
CFBP 3424	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	Hungary	1957	np	np	+
CFBP 3425	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	Hungary	1957	np	np	+
CFBP 3427	<i>betae</i>	<i>Beta vulgaris</i>	UK	-	np	np	-
CFBP 3430	<i>betae</i>	<i>Beta vulgaris</i>	-	1955	np	np	-
CFBP 3431	<i>betae</i>	<i>Beta vulgaris</i>	UK	1955	np	np	-
CFBP 3432	<i>oortii</i>	<i>Tulipa gesneriana</i>	Netherlands	1965	np	np	-
CFBP 3433	<i>oortii</i>	<i>Tulipa gesneriana</i>	UK	1969	np	np	-
CFBP 3435	<i>oortii</i>	<i>Tulipa gesneriana</i>	UK	1970	np	np	-
CFBP 3436	<i>oortii</i>	<i>Tulipa gesneriana</i>	Japan	1972	-	-	-
CFBP 3437	<i>oortii</i>	<i>Tulipa gesneriana</i>	Japan	1972	np	np	-
CFBP 3438	<i>poinsettiae</i>	<i>Euphorbia pulcherrima</i>	USA	1958	np	np	-
CFBP 3439	<i>poinsettiae</i>	<i>Euphorbia pulcherrima</i>	USA	1958	np	np	-

CFBP 3440	<i>poinsettiae</i>	<i>Euphorbia pulcherrima</i>	USA	1958	np	np	-
CFBP 3441	<i>poinsettiae</i>	<i>Euphorbia pulcherrima</i>	USA	1959	np	np	-
CFBP 3442	<i>poinsettiae</i>	<i>Euphorbia pulcherrima</i>	USA	1958	np	np	-
CFBP 3443	<i>poinsettiae</i>	<i>Euphorbia pulcherrima</i>	-	1960	np	np	-
CFBP 3444	<i>poinsettiae</i>	-	USA	-	np	np	-
CFBP 3455	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	-	1954	np	np	+
CFBP 3457	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	Germany	1958	np	np	+
CFBP 3459	<i>flaccumfaciens</i>	<i>Vigna radiata</i>	USA	1934	np	np	+
CFBP 3461	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	USA	-	np	np	+
CFBP 3484	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	USA	-	np	np	+
CFBP 3485	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	USA	-	np	np	+
CFBP 3486	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	USA	-	+	-	-
CFBP 3487	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i>	USA	-	np	np	+
CFBP 3509 ^{PT}	<i>betae</i>	<i>Beta vulgaris</i>	UK	1955	np	np	-
CFBP 3513	<i>poinsettiae</i>	-	USA	-	np	np	-
2634 UNESP	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i> L.	Brazil	1996	+	-	+
2910 UNESP	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i> L.	Brazil	2002	np	np	+
CFBP 8394							
3026 UNESP	<i>flaccumfaciens</i>	<i>Phaseolus vulgaris</i> L.	Brazil	2006	np	np	+
CFBP 8393							
3172 UNESP	<i>flaccumfaciens</i>	<i>Triticum aestivum</i> L.	Brazil	2012	++ ⁷	+	+
CFBP 8371							
3173 UNESP	<i>flaccumfaciens</i>	<i>Avena strigosa</i> Schreb.	Brazil	2013	np	np	+
CFBP 8372							
3175 UNESP	<i>flaccumfaciens</i>	<i>Triticum aestivum</i> L.	Brazil	2012	np	np	+
3176 UNESP	<i>flaccumfaciens</i>	<i>Triticum aestivum</i> L.	Brazil	2012	np	np	+
3177 UNESP	<i>flaccumfaciens</i>	<i>Avena strigosa</i> Schreb.	Brazil	2012	np	np	+
3178 UNESP	<i>flaccumfaciens</i>	<i>Avena strigosa</i> Schreb.	Brazil	2012	++	+	+
3179 UNESP	<i>flaccumfaciens</i>	<i>Avena sativa</i> L.	Brazil	2013	np	np	+
CFBP 8373							
3180 UNESP	<i>flaccumfaciens</i>	<i>Avena sativa</i> L.	Brazil	2013	np	np	+
3181 UNESP	<i>flaccumfaciens</i>	<i>Avena sativa</i> L.	Brazil	2013	np	np	+
3183 UNESP	<i>flaccumfaciens</i>	<i>Hordeum vulgare</i> L.	Brazil	2013	np	np	+
CFBP 8374							
3184 UNESP	<i>flaccumfaciens</i>	<i>Hordeum vulgare</i> L.	Brazil	2013	np	np	+
CFBP 8375							
3185 UNESP	<i>flaccumfaciens</i>	<i>Hordeum vulgare</i> L.	Brazil	2013	np	np	+
3186 UNESP	<i>flaccumfaciens</i>	<i>Hordeum vulgare</i> L.	Brazil	2013	np	np	+
3187 UNESP	<i>flaccumfaciens</i>	<i>Lolium multiflorum</i> Lam.	Brazil	2013	np	np	+
3188 UNESP	<i>flaccumfaciens</i>	<i>Lolium multiflorum</i> Lam.	Brazil	2013	np	np	+
3189 UNESP	<i>flaccumfaciens</i>	<i>Lolium multiflorum</i> Lam.	Brazil	2013	np	np	+
3190 UNESP	<i>flaccumfaciens</i>	<i>Lolium multiflorum</i> Lam.	Brazil	2013	np	np	+
3191 UNESP	<i>flaccumfaciens</i>	<i>Brassica napus</i> L.	Brazil	2013	++	+	+
CFBP 8391							
3193 UNESP	<i>flaccumfaciens</i>	<i>Triticum aestivum</i> L.	Brazil	2013	+	-	+
CFBP 8392							
3194 UNESP	<i>flaccumfaciens</i>	<i>Triticum aestivum</i> L.	Brazil	2013	np	np	+
3195 UNESP	<i>flaccumfaciens</i>	<i>Triticum aestivum</i> L.	Brazil	2013	np	np	+

^{PT}Pathotype strains.

^TType strain.

¹Inoculation of Cff by needle technique.

²Inoculation of Cff by submersion technique.

³PCR using specific primers for Cff designed by Tegli et al. (2002).

⁴Not performed.

⁵Positive results.

⁶Negative results.

⁷Highly aggressive strain.

All strains are available at CIRM-CFBP, French Collection for Plant-Associated Bacteria (https://www6.inra.fr/cirm_eng/CFBP-Plant-Associated-Bacteria).

Table 2. Housekeeping gene primers used for MLSA of *Curtobacterium flaccumfaciens* (PCR and sequencing primers).

Gene	Primer code	Sequence	°C ¹	Product size range	Gene target
<i>dnaK</i>	CffdnaKF2	5'-GAC CTC GGA ACC ACC AAC TC-3'	62	689 bp	chaperone Hsp70
	CffdnaKR	5'-CGC TGC TTG GCG ATC TTG TC-3'			
<i>atpD</i>	CffatpDF	5'-AAG CGG AAG ATG TTG TCG AT-3'	62	716 bp	ATP synthase, beta subunit
	CffatpDR	5'-GAC ATC GAG TTC CCC CAC GAC-3'			
<i>rpoB</i>	CffrpoDF	5'-TCG AGT TCG AGA TCG ACA AG-3'	62	862 bp	RNA polymerase, beta subunit
	CffrpoDR	5'-CCG ATC AGG CCG ATG TTC G-3'			
<i>gyrB</i>	gyrB-F18	5'-GGC GTC GGC AGC TCC GTC GTG AA-3'	60	910 bp	DNA gyrase B subunit
	gyrB-R29	5'-GGC AGT CCT TGA GCT TGC CAG G-3'			
<i>recA</i>	recA-F	5'-ACC GCG CTC GCA CAG ATC GAC C-3'	60	722 bp	recombinase A
	recA-R	5'-GCC ATC TTG TTC TTG ACG ACC TTG AC-3'			
<i>ppk</i>	ppk-F	5'-GAG ACC GAG ACC CTC ATC CAG-3'	60	668 bp	Polyphosphate kinase
	ppk-R	5'-CCC GTC CCG ATG TGG CTG TAG TG-3'			

¹Annealing temperature; Primers for genes *recA*, *gyrB*, and *ppk*; Jacques *et al.*, 2012;

primers for genes *dnaK*, *atpD*, and *rpoB*; this study.

Table 3. Sequence variation at the six housekeeping loci among *Curtobacterium* strains.

Locus	No. of sites ^a	GC%	S ^b	Hap ^c	Hd ^d	$\theta\pi^e$	θw^f	Tajima's D ^g	Fu and Li's D ^g	Fu's F ^g
<i>atpD</i>	489	0.66	21	20	0.913	0.00656	0.00853	-0.68320	-0.32567	-5.945
<i>dnaK</i>	468	0.67	62	34	0.932	0.01581	0.02630	-1.56309	0.04095	-9.681
<i>gyrB</i>	726	0.64	103	42	0.971	0.03090	0.02816	-0.24807	-2.10687	-2.268
<i>ppK</i>	544	0.66	65	35	0.948	0.02390	0.02372	-0.37842	-1.24384	-3.525
<i>recA</i>	588	0.67	56	41	0.972	0.01343	0.01891	-1.27094	-1.35764	-16.825
<i>rpoB</i>	663	0.65	46	23	0.893	0.01024	0.01377	-0.97318	-4.36447	-2.097
Concat (h)	3478	0.66	353	49	0.975	0.01746	0.02015	-0.81988	-1.85395	2.343

^a number of analyzed sites.

^b number of polymorphic (segregating) sites.

^c number of haplotypes.

^d haplotype (gene) diversity.

^e level of nucleotide diversity.

^f level of nucleotide diversity from S.

^g results of neutrality tests performed using the method of Tajima, Fu and Li, Fu's F.

^h data for six housekeeping genes, concatenated.

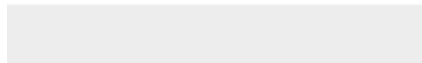



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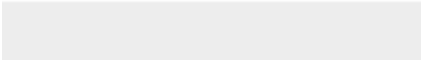



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