Genetic diversity of Curtobacterium flaccumfaciens revealed by multilocus sequence analysis

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	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 og 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).

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

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Abstract

Bacterial wilt caused by *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* is among the diseases that affect *Phaseolus vulgaris* 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 *C. flaccumfaciens* 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 (*atpD, dnaK, gyrB, ppK, recA* and *rpoB*). A phylogenetic tree with the concatenated sequences of six genes showed high genetic diversity among the strains. For instance, strains belonging to *C. f.* pv. *flaccumfaciens* do not cluster together within the species. Similar results were obtained with a minimal MLSA scheme using *gyrB* and *recA*, which we propose for reliable identification at the species level of *Curtobacterium* isolates. No correlation was identified between phylogeny and pathogenicity in the *Curtobacterium flaccumfaciens* strains analyzed in this work. The specific primers CffFOR2 and CffREV4 designed by Tegli et al. (2002) to detect *C. f.* pv. *flaccumfaciens* in naturally infected bean seeds proved to be efficient for the detection of bean-pathogenic strains.

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

Introduction

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

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

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

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

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

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

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

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

Material and methods

Bacterial strains and preservation

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

The bacterial strains were stored as lyophilized cultures and maintained in 40% glycerol stock at -80 °C after cell revival. The strains were checked for purity by cultivation on YPGA (also known as 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 two days at 25 °C.

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

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

The primers *Cff*FOR2 (5´GTT ATG ACT GAA CTT CAC TCC 3´) and *Cff*REV4 (5´GAT GTT CCC GGT GTT CAG 3´) described by Tegli *et al*. (2002) were used to amplify a 306 bp fragment targeting a conserved region of a *C.f.* pv *flaccumfaciens* Rep-PCR sequences. The PCR reaction was performed in a mix containing 200µM dNTP, 1.5mM of $MgCl_2$, 500nM of each primer, 0.75U of GoTaq Flexi DNA polymerase (Promega), and 6µL of the boiled bacterial cell suspension. The thermocycler conditions were as follows: initial denaturation at 94 °C for 3 min, followed by 30 cycles of 94 °C for 1 min, 62 °C for 45 s, and 72 °C for 30 s, and a final extension at 72 °C for 5 min.

Primer design for housekeeping gene amplification

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

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

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

Amplification and sequencing of housekeeping genes

The primers used for MLSA are listed in Table 2. The PCR reaction mix contained 200 µM dNTP, 1.5 mM of $MgCl_2$, 500 nM of each primer, 0.75 U GoTaq Flexi DNA polymerase (Promega), and 6 µL of boiled bacterial suspension. The PCR program consisted of an initial hold at 94 °C for 5 min followed by 35 cycles of 30" at 94 °C, 30" at annealing temperature (60 or 62°C), extension time of 30" at 72 °C, and ending with 7 min at 72 °C. For genes *recA*, *gyrB* and *ppK*, the annealing temperature was 60 °C and 62 °C for *dnaK*, *atpD* and *rpoB*. The PCR products were checked on a 1.5% agarose gel, and

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

Sequence analysis

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

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

Pathogenicity tests on common beans with strains from Brazil

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

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

Results

Multilocus sequence analysis of *Curtobacterium flaccumfaciens*

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

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

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

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

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

Phylogenetic network

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

Pathogenicity tests

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

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

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

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

Discussion

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

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

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

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

These results raised the question about the differences in pathogenicity between the pathovars. As the strains are not genetically different based on a six-genes MLSA, the question was whether they can affect plants other than their isolation host and can they be pathogenic on a much larger host range than initially thought. Representative strains of each pathovar were tested for pathogenicity on common bean. Except for *C.f.* pv *flaccumfaciens*, which were pathogenic to bean, all strains from other pathovars were non-pathogenic, independent from their hosts. Among the *C.f.* pv *flaccumfaciens* strains, some isolated from alternative hosts can be hyper-aggressive, even more aggressive than the type strain. Thus our data reveal no correlation between phylogeny and pathogenicity for *C.f.* pv *flaccumfaciens*. Even if the pathogenicity genes are usually contained in the accessory-genome, discrepancy between phylogeny assessed by MLSA and pathogenicity, even if possible, are uncommon. MLSA, even reduced to two 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 rapidly 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-

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

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

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

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Compliance with ethical standards

Conflict of interest

The authors declare that they have no conflict of interest.

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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.

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.

line figure Click here to access/download;line figure;Figure 1.docx

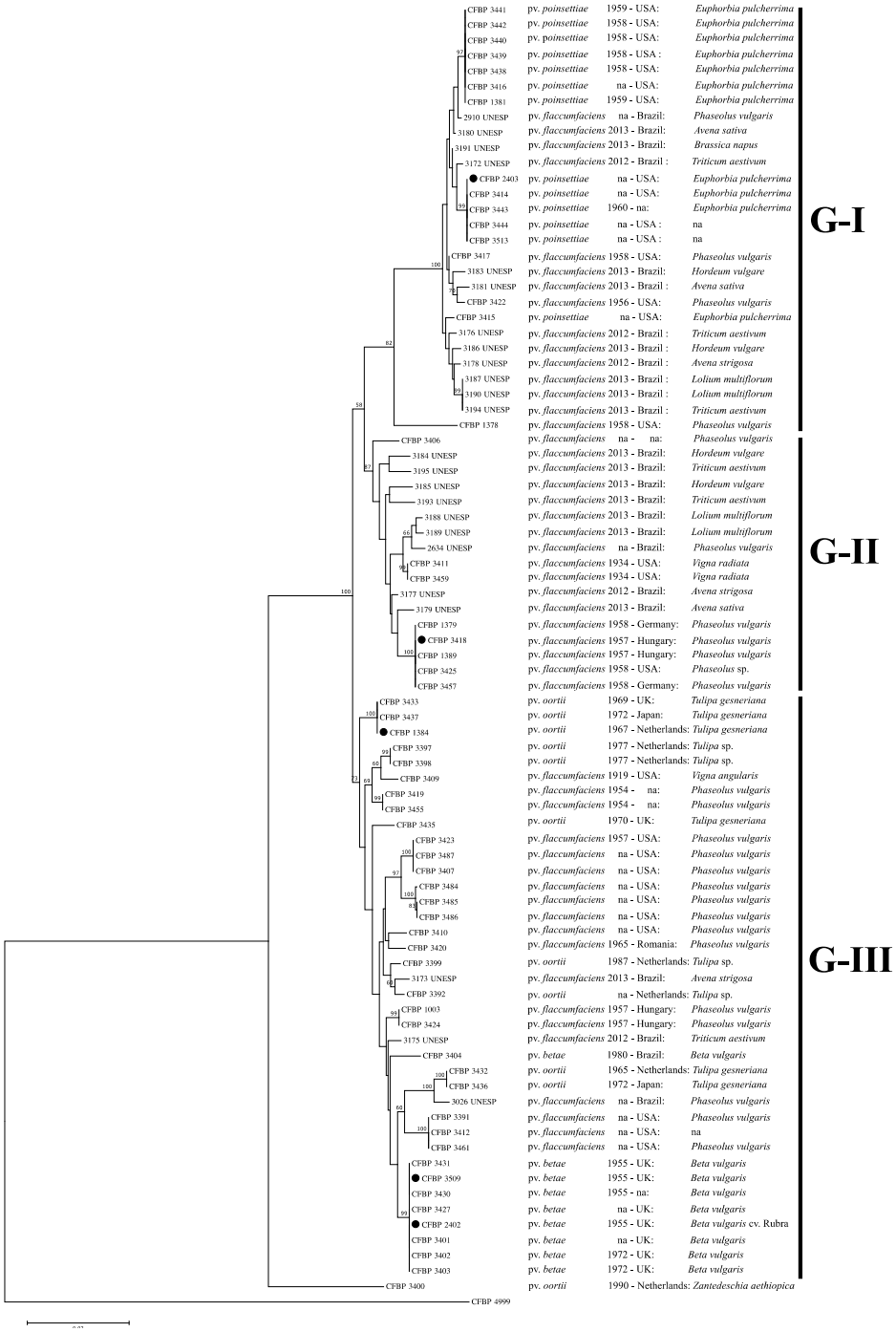

Figure 1. Molecular phylogenetic analysis based on the partial sequences of six housekeeping genes, concatenated (*atpD*, *dnaK*, *gyrB*, *ppK*, *recA*, and *rpoB*). This tree was constructed using a maximum likelihood method based on the Jukes-Cantor model and rooted with the strain CFBP 4999 of *Clavibacter 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)**

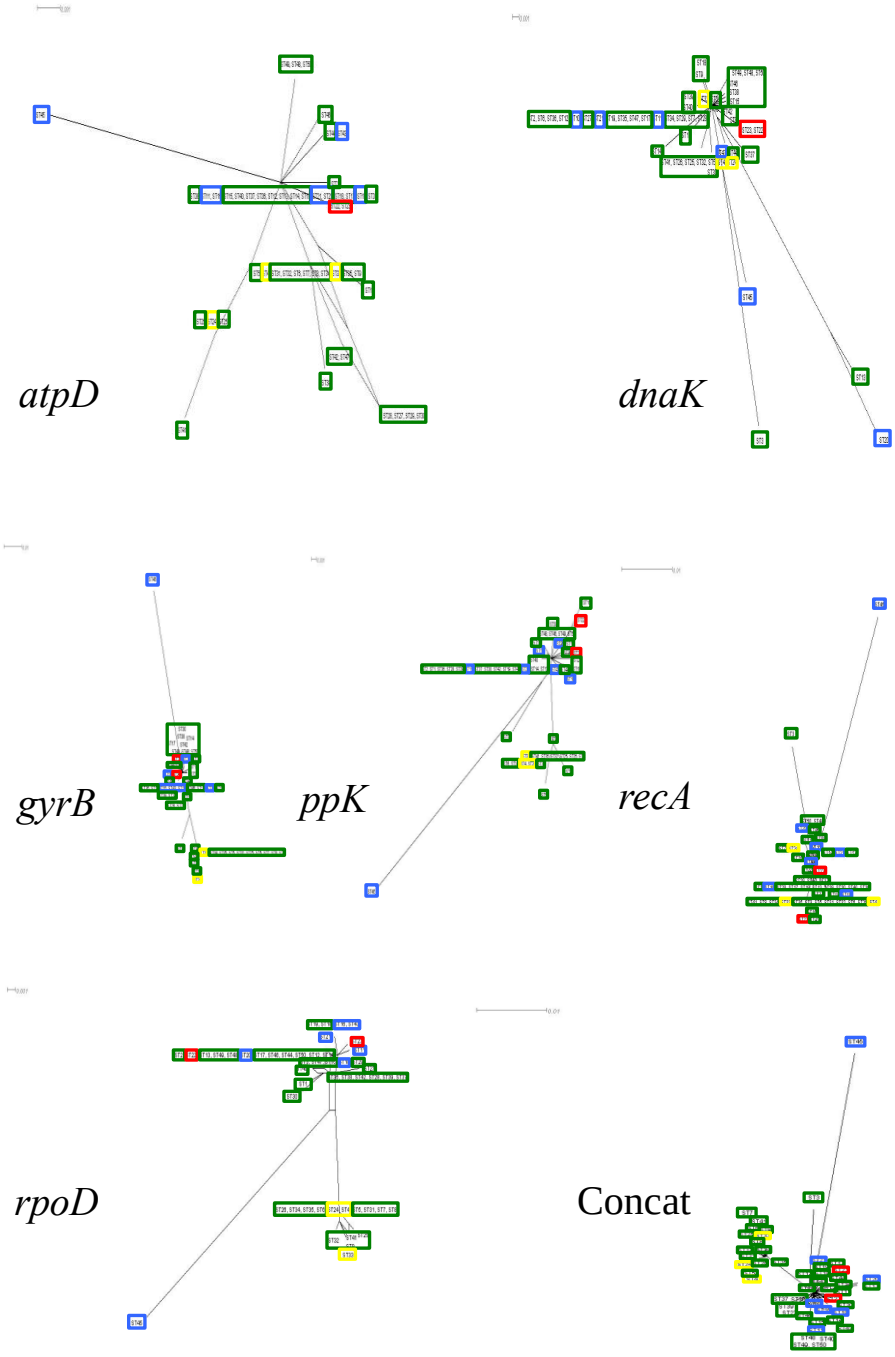

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Table 1. Overview of *Curtobacterium flaccumfaciens* strains used in this study.

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

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

[PT]Pathotype strains.

[T]Type strain.

[1]Inoculation of Cff by needle technique.

[2]Inoculation of Cff by submersion technique.

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

[4]Not performed.

[5]Positive results.

[6]Negative results.

[7]Highly 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
dnaK	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'			
atpD	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'			
rpoB	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'			
gyrB	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'			
recA	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'			
ppK	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'			

[1]Annealing 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]	θπ[e]	θw[f]	Tajima's D[g]	Fu and Li's D[g]	Fu's F[g]
atpD	489	0.66	21	20	0.913	0.00656	0.00853	-0.68320	-0.32567	-5.945
dnaK	468	0.67	62	34	0.932	0.01581	0.02630	-1.56309	0.04095	-9.681
gyrB	726	0.64	103	42	0.971	0.03090	0.02816	-0.24807	-2.10687	-2.268
ppK	544	0.66	65	35	0.948	0.02390	0.02372	-0.37842	-1.24384	-3.525
recA	588	0.67	56	41	0.972	0.01343	0.01891	-1.27094	-1.35764	-16.825
rpoB	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.

line figure

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Conflict of Interest Statement