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Phylogenetic position and virulence apparatus of the pear flower necrosis pathogen *Erwinia piriflorinigrans* CFBP 5888^T as assessed by comparative genomics

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ABSTRACT

Erwinia piriflorinigrans is a necrotrophic pathogen of pear reported from Spain that destroys flowers but does not progress further into the host. We sequenced the complete genome of the type strain CFBP 5888^T clarifying its phylogenetic position within the genus *Erwinia*, and indicating a position between its closest relative, the epiphyte *Erwinia tasmaniensis* and other plant pathogenic *Erwinia* spp. (i.e., the fire blight pathogen *E. amylovora* and the Asian pear pathogen *E. pyrifoliae*). Common features are the type III and type VI secretion systems, amylovoran biosynthesis and desferrioxamine production. The *E. piriflorinigrans* genome also provided the first evidence for production of the siderophore chrysobactin within the genus *Erwinia sensu stricto*, which up to now was mostly associated with phytopathogenic, soft-rot *Dickeya* and *Pectobacterium* species. Plasmid pEPIR37, reported in this strain, is closely related to small plasmids found in the fire blight pathogen *E. amylovora* and *E. pyrifoliae*. The genome of *E. piriflorinigrans* also gives detailed insights in evolutionary genomics of pathoadapted *Erwinia*.
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Introduction

Fire blight, caused by the invasive enterobacterium *Erwinia amylovora*, is a major disease threat to pome fruit production globally, for which it has profound socio-economic impact [7,15]. Epidemics can develop rapidly, resulting in scorched-like symptoms, with death of trees or entire orchards within a single season. Typical symptoms include flower necrosis, immature fruit rot, shoot recurvature, profuse bacterial ooze and cankers on woody tissues. The phytopathogen *E. amylovora* enters hosts through natural openings such as nectarthodes and wounds. The disease develops as blossom, shoot, trunk, or rootstock blight depending of the plant tissue affected by the organism.

The closely related species *Erwinia pyrifoliae* [25] is a pathogen of Nashi or Asian pear (*Pyrus pyrifolia*). *E. pyrifoliae* causes fire blight disease symptoms essentially indistinguishable from those of *E. amylovora*, but the level of virulence is lower [26]. This *Erwinia* species has up to now a restricted geographic distribution in Korea and Japan [4,41]; isolates from these nations are genetically and phenotypically closely related [19,36]. In addition, very recently, *Erwinia uzenensis* causing bacterial black shoot disease of European pear in Japan was described as a new species within the genus *Erwinia* [35,42].

To understand the biology of *E. amylovora*, the genome of *E. amylovora* CFBP 1430 was analyzed in detail [61] and since then more strains of this species were sequenced [34,53]. This yielded information on the presence of virulence factors and the intra-species diversity [54,61]. Above that, comparative genomics with *E. pyrifoliae* DSM 12163 and *Erwinia tasmaniensis* Et1/99 has yielded more information on the evolution of virulence factors in these species [32,60]. With the emergence of the *Erwinia billingiae* Eb661 genome sequence, the evolutionary model was refined [23,28]. Nevertheless, large gaps are still present in the evolutionary scheme, and genome sequences of other *Erwinia* spp. will have to be determined to fill these gaps.

The species *Erwinia piriflorinigrans* was described to cause necrotic pear blossoms [31,55]. The only symptoms observed were the necrosis of infected blossoms. Infection rates have been

Abbreviations: CRISPR, clustered regularly interspaced short palindromic repeats; CRR, CRISPR repeat region; EPS, exopolysaccharide; ICE, integrative conjugative element; NRPS/PKS, non-ribosomal peptide synthases/polyketide synthases; T1SS, type I secretion system; T2SS, type II secretion system; T3SS, type III secretion system; T6SS, type VI secretion system.

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reported between 10% and 80% infected blossoms per tree and trees produce significantly fewer fruits. This species has a geographical distribution thus far known to be confined to two pear varieties in the fire blight-free region around Valencia in Spain [55]. Other common fire blight hosts in the same region did not show any symptoms of infection with this *Erwinia* species.

The genetic basis for the peculiar symptom expression and host-range limitation of this new pathogen is unclear. The objectives of this study were to sequence the complete genome of the of *E. piriflorinigrans* type strain CFBP 5888^T in order to provide new information on the taxonomy of this new *Erwinia* species, described previously based on standard taxonomic procedures and to investigate its specific virulence determinants. Comparative genomic analysis indicate that *E. piriflorinigrans* belongs to a true, pathoadapted species of the genus *Erwinia* and sheds light on related virulence machineries of other phytopathogenic species [23,32,60].

Materials and methods

Whole-genome sequencing and assembly

Genomic DNA of *E. piriflorinigrans* CFBP 5888^T, isolated from necrotic tissue of flowers of the pear tree (*Pyrus communis*) in Spain [31,55], was isolated from liquid cultures using a standard phenol–chloroform DNA isolation method [12]. Whole-genome sequencing was performed by GATC (Konstanz, Germany) using an Illumina HiSeq 2000 sequencer. Two libraries were generated: a standard paired-end genomic library with a 200-bp average insert length, and a 3-kb mate pair genomic library. A total of 30,718,294 high-quality filtered sequence reads were generated with an average 51-bp read length, and a 391 times average coverage, based on an estimated 4 Mb genome. To reduce the number of reads for subsequent assembly, raw read files were split in smaller units using a custom Perl script.

Complete *de novo* assembly was done using 3,000,000 long-insert-library and 2,800,000 short-insert-library pairs of each of the libraries (total 11,600,000 reads) using the Velvet short read assembler plugin of the Geneious Server (Biomatters Ltd., Auckland, New Zealand). This yielded 48 scaffolds and contigs with many small gaps that were resolved *in silico* afterwards. By repeatedly mapping 5,600,000 reads from the 200-bp insert library using NGen v2.0 (DNASTAR, Madison, WI, USA) to the resulting assembly, unresolved gaps within the scaffolds were manually checked, closed and verified. After the third repetition, remaining scaffolds were split at gaps into single contigs. A total of 26 contigs with a total genome size of 3,968,031 bp (N_{50} = 359,859 bp) remained after this final assembly.

Genome annotation and analysis

Genes were predicted using a combined strategy [38] based on the CDS prediction programs Glimmer [57] and Critica [1]. Subsequently, the potential function of each predicted gene was automatically assigned using the GenDB annotation pipeline [40]. The resulting genome annotation was manually curated, and metabolic pathways were identified using the KEGG pathways tool [24] in GenDB. Comparative genomics analyses were performed using the program EDGAR [6]. Species were compared with all currently sequenced *Erwinia* spp. and *Pantoea* spp. Venn diagrams were drawn based on comparison data from EDGAR. The phylogenetic tree was created with the neighbor joining algorithm on a Kimura distance matrix as implemented in the PHYLIP package version 3.57c. The core genome tree is based on 51,000 protein sequences, 2040 for each of the analyzed 25 strains, which comprise 17,262,448 residues in total (~690,000 per genome). Due to the huge size of the core alignment and the long resulting calculation time for a tree, bootstrapping was not performed. Mauve in the progressive mode [11] was used for whole genome comparison. Annotations of transport proteins were checked against the Transport Classification DataBase [56]. The average amino acid identity (AAI) was calculated as described previously [27]. The non-ribosomal peptide synthases/polyketide synthases (NRPS/PKS) were analyzed using antiSMASH [39], which can discriminate many types of non-ribosomal peptide synthases/polyketide synthases.

The genome sequence of *E. piriflorinigrans* CFBP 5888^T has been deposited at EBI and received the accession numbers CAHS01000001–CAHS01000025 (chromosome) and HE792893 (pEPIR37). Although additional genome sequences are available for *E. amylovora* [34,53] and *E. pyrifoliae* [28,50], we used the genomes of *E. amylovora* CFBP 1430 [61] and *E. pyrifoliae* DSM 12163^T [59] as reference sequences for these species throughout the text. This does, however, not exclude that other genome sequences were used for comparisons.

Results and discussion

The genome of E. piriflorinigrans CFBP 5888^T

The massive amounts of data from Illumina genome sequencing are a challenge for the correct assembly of a bacterial genome [51]. We have used only a fraction of the reads generated from genome sequencing of *E. piriflorinigrans* CFBP 5888^T for the assembly. Finally, a total of 26 contigs with a total genome size of 3,968,031 bp (N_{50} = 359,859 bp) remained after this final assembly (Table 1). Main problems observed were within highly repetitive genes and tandem repeats. Additionally, the rRNA regions were

Table 1
Sequence statistics for *E. piriflorinigrans* CFBP 5888^T and comparison to the genomes of pathoadapted *Erwinia* spp.

	E. piriflorinigrans CFBP 5888^T	*E. tasmaniensis* Et1/99	*E. pyrifoliae* DSM 12163^T	*E. amylovora* CFBP 1430
Genome size*	3,968,031 bp	4,067,864 bp	4,072,827 bp	3,833,832 bp
Number of contigs	26	6	5	2
N50	359,859 bp	–	–	–
Number of CDS	3857	3622	4038	3734
G + C content (%)	52.9	53.7	53.4	53.6
Common plasmid†	pEPIR37	–	pEP36	pEA29
Size	37,376 bp	–	35,901 bp	28,259 bp
Number of CDS	42	–	40	28
G + C content (%)	49.8	–	49.9	50.2
Other plasmids (size)	–	pET49 (48,751 bp); pET46 (46,159 bp); pET45 (44,694 bp); pET35 (35,494 bp); pET09 (9299 bp)	pEP5 (4960 bp); pEP3 (3070 bp); pEP2.6 (2610 bp)	–
Reference	This study	[29]	[59]	[61]

* Total genome size, including plasmids.
† Plasmids related to pEPIR37 (see also Fig. 6).

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