Complete Genome Sequence of the Fire Blight Pathogen *Erwinia amylovora* CFBP 1430 and Comparison to Other *Erwinia* spp.

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Fire blight, caused by the enterobacterium *Erwinia amylovora*, is a devastating disease of rosaceous plants that has global economic importance for apple and pear production and trade. The complete genome of *E. amylovora* CFBP 1430 was sequenced, annotated, and compared with the genomes of other *Erwinia* spp. Several singleton and shared features of the *E. amylovora* CFBP 1430 genome were identified that offer a first view into evolutionary aspects within the genus *Erwinia*. Comparative genomics identified or clarified virulence and fitness determinants and secretion systems. Novel insights revealed in the genome of *E. amylovora* CFBP 1430 hold potential for exploitation to improve the design of more effective fire blight control strategies.

Erwinia amylovora (Enterobacteriaceae) causes fire blight, the most important threat to pome fruit production (i.e., apple, pear, and quince) globally, and it affects a wide variety of *Rosaceae* (primarily *Maloideae*), including ornamental and native forest species important in rural economies, cultural heritage, and landscape ecosystems (Duffy et al. 2005). *E. amylovora* was the first bacterial plant pathogen described (in the late 1790s) and originated in North America, from where it has relatively recently spread first to New Zealand in the 1910s, to the United Kingdom and Northern Europe in the late 1950s, and more widely in Europe and the Middle East in the 1960s (Bonn and van der Zwet 2000). *E. amylovora* has quarantine status outside North America, and it is a contentious trade issue in fruit with fire-blight-free countries such as Australia, Japan, and all areas of the Southern Hemisphere aside from New Zealand (Calvin and Krissoff 1998; Roberts et al. 1998). This invasive pathogen continues to spread across Europe and the Middle East, with advance eastward threatening the native origin of apple germplasm resources in Central Asia (Jock et al. 2002), adding to the urgency for better understanding the disease and pathogen in order to develop more effective control and containment strategies.

Fire blight is so named because epidemics can rapidly develop, resulting in scorched symptoms, with kill of trees or entire orchards within a single growing season leading to devastating economic losses (Bonn and van der Zwet 2000). Typical symptoms include flower necrosis, immature fruit rot, shoot recurvature (syn. shepherd's crook), profuse bacterial ooze, and cankers on woody tissues. *E. amylovora* enters hosts through natural openings (e.g., flower nectaries and leaf hydathodes) and wounds (e.g., mechanical injury and insect or hail damage). The disease develops as blossom, shoot, or rootstock blight depending on the plant tissues infected and affected, and each syndrome requires specific control strategies (Thomson 2000). *E. amylovora* population growth is highly dependent on weather conditions, which has been used to develop disease forecasting models for blossom blight, and it is efficiently vectored by flower-foraging insects, especially honeybees, in a presumably passive association (Johnson and Stockwell 1998). *E. amylovora* is considered to have poor ecological fitness outside host or surrogate plants (Thomson 2000).

Current control efforts rely primarily on antibiotic applications (e.g., streptomycin and oxytetracycline) to protect flowers (with major controversy and outright bans in many countries due to concerns about possible antibiotic resistance development) (McManus et al. 2002), moderately effective biocontrol applications (Johnson and Stockwell 1998), or plant growth regulators (i.e., prohexadione-Ca) (Halbwirth et al. 2003). Commercial cultivars of apple and pear are, for the most part, highly susceptible, and continued efforts to breed high-level resistance have been inadequate thus far (Norelli et al. 2003). Understanding the genetic mechanisms for virulence may lead to the design of improved disease control strategies. Although important insights have been garnered regarding this cardinal phytopathogenic bacterium relative to other pathogens, much remains uncertain about the genetics of *E. amylovora* (Oh and Beer 2005). Recently, complete genome sequencing of a key species representing the related pectolytic-group of ex. *Erwinia* (*Pectobacterium atrosepticum*SCRI 1043) has enhanced design of novel control strategies (Toth et al. 2006). Availability of a published complete genome for the preeminent species of necrotrophic true *Erwinia* is anticipated to have a similar practical impact, as well as provide basic scientific information on evolution and relatedness of the species within the *Erwinia* genus. Here, we present the complete genome sequence of *E. amylovora* and provide comparison with related phytopathogenic and non-pathogenic species.

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RESULTS AND DISCUSSION

Complete genome of *E. amylovora* CFBP 1430.

E. amylovora CFBP 1430 was isolated in 1972 from a *Crataegus* sp. (Paulin and Samson 1973) and later used as a standard strain in many genetic and biochemical studies (Gaudriault et al. 1997; Dellagi et al. 1998). The complete genome sequence of *E. amylovora* CFBP 1430 (Fig. 1) was obtained using Illumina sequencing technology and assembled against the unpublished sequence of *E. amylovora* Ea273

(Bocsanczy et al. 2008). EMBL accession numbers for the genome sequence of *E. amylovora* CFBP 1430 are FN434113 (chromosome) and FN434114 (pEa29).

Overall, the genome sequences of the European (CFBP 1430) and U.S. (Ea273) strains were found to be nearly identical (>99.99%), with only a low number of nucleotide differences. This low number of nucleotide changes reinforces prior indications of low diversity within this pathogen and suggests that minimal evolution has occurred since *E. amylovora* global dispersal. The *E. amylovora* CFBP 1430 chromosome is 301 bp

Fig. 1. Circular representation of the **A**, chromosome and **B**, plasmid pEa29 (not to same scale) of *Erwinia amylovora* CFBP 1430. Circles (from outside to inside): first, scale bar in kilobases; second and third, predicted coding sequences of *E. amylovora* CFBP 1430 chromosome on the leading and lagging strand, respectively (colors according to COGs); fourth (A only), coding sequences of the *hrp/hrc* type III secretion system (T3SS) (red), the *imv/spa* T3SS (green), the type VI secretion system clusters 1, 2 (blue), and 3 (orange), the flagellar genomic island (yellow), and dispersed flagellar gene clusters (purple); fifth, G+C content; sixth, G+C skew.

Table 1. General characteristics of the genome of *Erwinia amylovora* CFBP 1430 compared with *E. amylovora* Ea273

Strain	<i>E. amylovora</i> CFBP 1430		<i>E. amylovora</i> Ea273		
	Chromosome	pEa29	Chromosome	pEa72	pEa29
Size (bp)	3,805,573	28,259	3,805,874	71,487	28,243
Average coverage	39.28	77.04	...	...	...
Calculated copy number	1	1.96	...	...	...
G+C content (%)	53.60	50.24	53.60	55.70	50.24
Predicted no. of CDS	3,706	28	...	...	...
No. of rRNA operons	7	0	...	...	...
No. of tRNAs	78	0	...	...	...

smaller than that of *E. amylovora* Ea273 (Table 1), and this is largely due to intergenomic intergenic transcribed spacer (ITS) variation (discussed below).

Comparative genomic analysis of *E. amylovora* strains CFBP 1430 and Ea273.

MAUVE alignment of both *E. amylovora* strains CFBP 1430 and Ea273 (Fig. 2) revealed a large-scale rearrangement of the genome. Two independent recombination events appear to have occurred within copies of the ribosomal (r)RNA operon. The recombination events resulted in a position exchange of two sizable regions of the chromosome (Fig. 2). This rearranged chromosomal organization would explain differences observed in the pulsed-field gel electrophoresis (PFGE) patterns when the genomic DNA of these strains is digested using the restriction enzyme *Xba*I (Zhang and Geider 1997). In silico analyses predicted the presence of the exact fragment sizes used to classify *E. amylovora* CFBP 1430 as a Pt-3 genotype pattern and the band profile of *E. amylovora* Ea273 as a Pt-X or Pt-4 genotype (Zhang and Geider 1997). Thus, other described PFGE Pt genotypes will probably be similarly clarified after genome sequencing of their representative strains.

Plasmids.

The *E. amylovora* CFBP 1430 genome lacks plasmid pEa72, in contrast to the genome of *E. amylovora* Ea273 (Fig. 2; Table 1). It is described that *E. amylovora* CFBP 1430 carries only a single plasmid (Llop et al. 2006), and this has been confirmed with our complete genome sequencing. Plasmid pEa29 is found in all but a few anomalous strains of *E. amylovora* (Llop et al. 2006) and primers targeting pEa29 sequences are routinely used for specific pathogen detection and diagnosis of fire blight (Llop et al. 2000; Salm and Geider 2004).

In *E. amylovora* CFBP 1430, the known 8-bp repeat region of pEa29 contained five repeats, unlike the four repeats identified in Ea273 (Schnabel and Jones 1998; Kim and Geider 1999). At a different location on pEa29 in *E. amylovora* CFBP1430, another 8-bp insertion was identified in a designated repeat region (McGhee and Jones 2000), rendering the sequence of pEa29 in this strain a total of 16 bp longer than in *E. amylovora* Ea273. The pEa29 variant in *E. amylovora* CFBP 1430 is considerably more divergent from the variant in *E. amylovora* Ea88 (McGhee and Jones 2000).

Inter- and intragenomic variation within rRNA operons.

Seven rRNA operons were amplified from the genome of *E. amylovora* CFBP 1430 and completely sequenced using the Sanger method to preclude biases that may occur within these regions (McGhee et al. 2002). Several obvious differences between the seven individual rRNA operons were detected. Based on a sequence alignment, it was observed that the ITS regions

between the 16S and 23S rRNA genes of three operons of *E. amylovora* CFBP 1430 were composed of ITS regions having the transfer (t)RNA-Ala and tRNA-Ile genes, and of four operons containing the tRNA-Glu. This is different from the *E. amylovora* Ea273 genome, where four tRNA-Ala/tRNA-Ile-operons and three tRNA-Glu-operons were found, a situation that deviates from the described variation in ITS regions for other *E. amylovora* strains (McGhee et al. 2002). When using the ITS allele designations of McGhee and associates (2002), *E. amylovora* CFBP 1430 has the ITS type X⁺; X⁻; X⁺ 139; X⁻ 139; Y⁺ 139, (Y⁻ 139)₂, that is comparable to strain Ea110 although, in that strain, the seventh copy was not determined.

Five of seven copies of the rRNA operon have a 99-bp insertion within the 23S rRNA gene, mapped within helix 45 and probably representing an intervening sequence (IVS), as already described within many species within the *Enterobacteriaceae* family and other bacteria (Pronk and Sanderson 2001). We confirmed with polymerase chain reaction (PCR) that this corresponds to a 5:2 ratio between 23S rRNA gene copies with and without this IVS. BlastN analysis showed that the IVS sequences are present in all seven copies of the 23S rRNA gene of *E. pyrifoliae* DSM 12163^T but are absent in the *E. tasmaniensis* Et1/99 (Kube et al. 2008) and *P. atrosepticum* SCRI 1043 (Bell et al. 2004) genomes. The ITS2 region between the 23S and the 5S rRNA genes varies also in sequence and length between individual copies of the rRNA operons within the *E. amylovora* CFBP 1430 genome. Two different versions of 213 and 219 bp were found with a 4:3 ratio in *E. amylovora* CFBP 1430 whereas, in *E. amylovora* Ea273 genome, this ratio is 5:2.

Clustered regularly interspaced short palindromic repeats.

Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated sequence (CAS) proteins constitute a putative prokaryotic RNA-interference-based immune system (Makarova et al. 2006; Sorek et al. 2008) protecting against bacteriophages or plasmids (Mojica et al. 2005). The annotation of the genome of *E. amylovora* CFBP 1430 revealed eight genes with homology to so-called *cas*-genes (EAMY_2813 to EAMY_2820) (Haft et al. 2005). Using the CRISPR-Finder (Grissa et al. 2007), three regions were identified containing the CRISPR with 36, 34, and 5 CRISPR units (consisting of repeat and spacer). Some of the spacers have homology to known sequences. Three of the spacers have high sequence identity to regions in plasmid pEt35 of *E. tasmaniensis* Et1/99, while one matches the ColE1-type replication region of many plasmids, including pSW100 of *Pantoea stewartii* subsp. *stewartii* SW2. A few others have homology to regions in genomes of *Salmonella* spp. that encode phage-derived genes. The total number of identified spacers (9 of 75) is in the range of currently described CRISPR systems in other bacteria (Mojica et al. 2005; Makarova et al. 2006) but higher than the average.

Fig. 2. MAUVE alignment of the genome sequences of *Erwinia amylovora* CFBP 1430 and *E. amylovora* Ea273. Vertical lines indicate ribosomal (r)RNA operons. Numbers indicate the rRNA operon numerator, as used in the alignments. Arrows indicate the transcriptional direction of the rRNA operons.

The repeats of CRISPR regions 1 and 2 are 29 bp in length with spacers of 32 to 33 bp while CRISPR region 3 has repeats of 26 and 34 to 35 bp spacers. The repeats from CRISPR region 1 are only slightly different from the repeats in CRISPR region 2 but differ significantly from those of CRISPR region 3. The number of repeats in *E. amylovora* CFBP 1430 deviates from the number of repeats found in the genome sequence of *E. amylovora* Ea273. Here, CRISPR region 1 contains one more CRISPR unit. From BLASTN searches, the additional spacer region is 100% identical to bases 19506 to 19475 in plasmid pEU30 of *E. amylovora* UTRJ2 (Foster et al. 2004) complementary to the *virB4* gene. Within CRISPR region 2, two additional CRISPR units were found. One CRISPR unit (spacer 14) has no homology to database sequences, while spacer 31 is 100% identical to the complement of bases 13305 to 13336 in plasmid pEa72 of *E. amylovora* Ea273.

Whole-genome structure comparison of the genomes of *E. amylovora* CFBP 1430, *E. pyrifoliae* DSM 12163^T, and *E. tasmaniensis* Et1/99.

Currently, complete genome sequences are available for three true *Erwinia* spp.: *E. amylovora* CFBP 1430, *E. pyrifoliae* DSM 12163^T (Smits et al. 2010), and *E. tasmaniensis* Et1/99 (Kube et al. 2008). We compared these using MAUVE in the progressive mode. A comprehensive analysis of this comparison indicating significant and relevant dissimilarities in gene content is provided in Figure 3.

Overall, colinearity was observed for the chromosomes of all three species (Supplementary Fig. 1), despite notable large-scale chromosomal reorganizations (Fig. 3). This is consistent with our results from the manual annotation process (i.e., orthologous coding sequences [CDS] in *E. amylovora* CFBP 1430 have approximately 95% sequence identity to *E. pyrifoliae* DSM 12163^T and approximately 90% sequence identity to *E. tasmaniensis* Et1/99). In contrast to these other two species, sizable phage-gene-containing regions were not detected in the *E. amylovora* CFBP 1430 genome, although remnants of such were identified. Only a few large-scale chromosomal reorganizations appear to have occurred since the divergence of these three species.

Plasmids in these ecologically distinct *Erwinia* spp. have low sequence similarity. The only two plasmids where, to a cer-

tain degree, colinearity was observed are the *E. amylovora* CFBP 1430 plasmid pEa29 and *E. pyrifoliae* DSM 12163^T plasmid pEa36, as previously reported (McGhee et al. 2002). The five plasmids of *E. tasmaniensis* Et1/99 (Kube et al. 2008) and the three small plasmids of *E. pyrifoliae* DSM12163^T (Smits et al. 2010) are not related to these plasmids or to each other. However, large segments of pEa29 were found in the chromosome of *E. tasmaniensis* Et1/99 (Fig. 3), with genes that encode the thiamine biosynthesis proteins ThiOSGF, the high-affinity choline transporter BetT, the peptide methionine sulfoxide reductase MsrA, the histone-like nucleotide structuring protein H-NS, the putative aldehyde dehydrogenase AldD, and a LysR-family transcriptional regulator. Using plasmid-cured strains (Llop et al. 2008) and deletion mutants (McGhee and Sundin 2008), these genes have been shown to play a minor role in the virulence of *E. amylovora*.

Gene content comparison among *E. amylovora*, *E. pyrifoliae*, and *E. tasmaniensis* genomes.

We compared complete genomes of three *Erwinia* spp. in order to i) resolve the *Erwinia* core genome and ii) identify differences that clarify the basis of *E. amylovora* pathogen success in contrast to *E. pyrifoliae*, which has a considerably limited host range on pome fruit, and *E. tasmaniensis* Et1/99, which is a pome fruit epiphyte and not reported to be a phytopathogen of other species.

A Venn diagram (Fig. 4) was calculated using the program EDGAR, which employed a reciprocal BLAST on all CDS of the three species with a cut-off of 70% of protein sequence identity over at least 70% of the protein. This Venn diagram revealed 2,474 core CDS, corresponding to approximately two-thirds of all CDS in these genomes. Analysis of the singletons in each genome revealed a large majority that could only be annotated as hypothetical proteins. Just a fraction of predicted proteins were shared between any two of the three species. *E. amylovora* CFBP 1430 and *E. pyrifoliae* DSM 12163^T had many more shared genes in comparison with the putative nonpathogen *E. tasmaniensis* Et1/99 and the other two *Erwinia* spp. Shared genes between the two phytopathogens had a higher sequence identity (approximately 95% for core genes) compared with *E. tasmaniensis* Et1/99 (approximately 90%). Relevant singletons of *E. amylovora* CFBP 1430

Fig. 3. MAUVE alignment of the genomes of *Erwinia amylovora* CFBP 1430, *E. pyrifoliae* DSM 12163^T, and *E. tasmaniensis* Et1/99. Selected features have been indicated with arrows and important features in *E. amylovora* CFBP 1430 are indicated with numbers. Colors represent different types of features: black, singleton features in *E. amylovora* CFBP 1430 (Supplementary Table 1); blue, selected features shared between *E. amylovora* CFBP and either *E. pyrifoliae* DSM 12163^T or *E. tasmaniensis* Et1/99 (Supplementary Table 2); red, genomic islands; and green, phage-gene-containing regions. Number 14 represents the shared regions between *E. amylovora* CFBP 1430 plasmid pEa29, *E. pyrifoliae* DSM 12163^T plasmid pEP36, and the chromosome of *E. tasmaniensis* Et1/99.

and relevant features shared between *E. amylovora* CFBP 1430 and *E. pyrifoliae* DSM 12163^T or *E. tasmaniensis* Et1/99 are discussed in detail below.

Multiple type III secretion systems.

The genome of *E. amylovora* CFBP 1430 encodes three type III secretion systems (T3SS). The hypersensitive response and pathogenicity (Hrp) T3SS, encoded by the so-called pathogenicity island 1 (PAI-1), is an established pathogenicity factor in *E. amylovora* that functions in delivery of effector proteins into the eukaryotic host (He et al. 2004; Oh et al. 2005). Two *inv/spa*-type T3SS islands (PAI-2 and PAI-3), more similar to the SPII T3SS of *Salmonella typhimurium* LT-2 (McClelland et al. 2001) and the *inv/spa* T3SS of the insect endosymbiont *Sodalis glossidinius* str. *morsitans* (Dale et al. 2001), respectively, were identified in the genomes of both *E. amylovora* strains CFBP 1430 and Ea273 (Bocsanczy et al. 2008).

The Hrp T3SS of *E. amylovora* CFBP 1430 is composed of 27 genes (EAMY_0525-EAMY_0551), arranged in three transcriptional units plus regulatory genes (Oh et al. 2005). *E. amylovora* CFBP 1430 carries two notable singletons (ORFU1 and ORFU2, EAMY_0540-EAMY_0541) located between the *hrpA* and *hrpS* genes (Kube et al. 2008; Smits et al. 2010). The *hrp* region in *E. amylovora* CFBP 1430 is located between the *hrp* effector and elicitors regions (HEE) (EAMY_0552-EAMY_0558) and the Hrp-associated enzymes (HAE) (EAMY_0519-EAMY_0524) region (Oh et al. 2005). The HEE region includes the two harpin genes (*hrpN* and *hrpW*), a gene (*dspA/E*) coding for a secreted effector essential for *E. amylovora* pathogenicity (Boureau et al. 2006), and a set of chaperone genes (ORFA, ORFB, ORFC, and *dspB/F*). The HAE region of *E. amylovora* CFBP 1430 (and of Ea273) comprises the *hrp*-associated systemic virulence genes (*hsvABC*), the *hrpK* gene encoding for a putative T3SS translocator (Petnicki-Ocwieja et al. 2005), and two genes encoding proteins of unknown function. A large segment of the HAE region is absent in *E. tasmaniensis* Et1/99 (Fig. 3).

Two *inv/spa*-type T3SSs, PAI-2 (EAMY_0771-EAMY_0792) and PAI-3 (EAMY_1573-EAMY_1593), both located on low G+C regions of the genome and consisting of 22 and 21 genes, respectively, were identified in the genome of *E. amylovora* CFBP 1430. PAI-3 is closely related to the complete *inv/spa* systems recently found in both *E. tasmaniensis* Et1/99 and *E. pyrifoliae* DSM 12163^T (Kube et al. 2008; Smits et al. 2010), whereas PAI-2 shows higher homology to the second, incomplete *inv/spa*-cluster of *E. tasmaniensis* Et1/99 (Kube et al. 2008) and is absent in *E. pyrifoliae* DSM 12163^T. This suggests that both PAI-2 and PAI-3 were acquired prior to the divergence of these three species within the *Erwinia* genus (both are absent in pectolytic *Pectobacterium carotovorum*), whereas PAI-2 was lost entirely in *E. pyrifoliae* DSM 12163^T and partially in the putatively nonpathogenic species *E. tasmaniensis* Et1/99.

The function of these two *inv/spa*-type T3SSs is still unknown, although it was demonstrated that they may not directly be involved in virulence in plants (Zhao et al. 2009). Considering the resemblance of PAI-2 and especially PAI-3 to the T3SS of the insect endosymbiont *S. glossidinius* str. *morsitans* (Dale et al. 2001), these *inv/spa*-type T3SSs may contribute to as-yet-unknown associations between *E. amylovora* and insect vectors.

Additional T3SS effectors.

Genome-wide comparison of *E. amylovora* CFBP 1430 with *E. pyrifoliae* DSM 12163^T (Smits et al. 2010) and *E. tasmaniensis* Et1/99 (Kube et al. 2008) revealed three singleton genes identified as putative T3SS effectors that may contribute to the

broader host range of *E. amylovora*. The *eop2* gene (EAMY_0653) encodes the T3SS helper protein Eop2, which has a C-terminal pectate lyase domain (Grant et al. 2006; Nissinen et al. 2007) and is secreted but apparently not translocated in *Pseudomonas syringae* (Chang et al. 2005), where it elicits a hypersensitive response in tobacco apoplasts (Alfano and Collmer 2004). The effector HopPtoC (EAMY_0744) is an ortholog of the secreted papain-like cysteine proteases YopT in *Yersinia* spp. (Grant et al. 2006) and HopC1 in *P. syringae* pv. *tomato* DC3000 (Buell et al. 2003). The effector encoded by EAMY_3175 is homologous to AvrRpt2, another cysteine protease (Grant et al. 2006), which renders virulent strains of *P. syringae* avirulent on selected lines of *Arabidopsis thaliana*, *Glycine max*, and *Phaseolus vulgaris* (Innes et al. 1993). The protein is cleaved and activated upon entry in the eukaryotic cell (Jin et al. 2003). In *E. amylovora*, *avrRpt2* contributes to the ability of the pathogen to infect immature pear fruit (Zhao et al. 2006).

Flagellar genes.

Two sets of genes encoding flagellar biosynthesis and chemotaxis-related proteins were found in the genome of *E. amylovora* CFBP 1430. One set is tightly clustered (EAMY_2647-EAMY_2702) and matches an analogous region found in *E. pyrifoliae* DSM 12163^T (Smits et al. 2010) but is absent in *E. tasmaniensis* Et1/99 (Kube et al. 2008). This also explains the absence of the quorum-sensing genes *expRI* in *E. amylovora* CFBP 1430 and *E. pyrifoliae* DSM 12163^T which are present in *E. tasmaniensis* Et1/99, in the region between *smgB* (ETA_09720) and *tsx* (ETA_09440). The latter two genes flank the insertion site of the flagellar gene cluster in *E. amylovora* CFBP 1430 and *E. pyrifoliae* DSM 12163^T, revealing the gene replacement that occurred in the latter two species. Furthermore, with respect to *E. amylovora* CFBP 1430, the region upstream of this flagellar gene cluster differs in *E. pyrifoliae* DSM 12163^T, where the insertion of a low-G+C genomic island (EPYR_1033-1040) between the *smgB* and *kpsC* is obvious. This would suggest that, during the evolution from the last common *Erwinia* progenitor to *E. pyrifo-*

Fig. 4. Venn diagram generated with EDGAR representing the pan genome of *Erwinia amylovora* CFBP 1430, *E. pyrifoliae* DSM 12163^T, and *E. tasmaniensis* Et1/99.

liae, two distinct insertion events have taken place at this hot spot.

A second complete set of flagellar genes, which closely resembles those found in *E. tasmaniensis* Et1/99 and *E. pyrifoliae* DSM 12163^T, is split in different clusters throughout the genome of *E. amylovora* CFBP 1430 (EAMY_1450-EAMY_1463, EAMY_1499-EAMY_1515, EAMY_2083-EAMY_2100, and EAMY_2138-EAMY_2144). Compared with the other flagellar assembly apparatus, this second gene cluster appears to be ancestral in the genus *Erwinia* and contains two extra genes encoding FlhZ (EAMY_2138), a putative alternative sigma factor regulatory protein, and CheV (EAMY_1515), a hypothetical chemotaxis signal transduction protein, respectively.

Exopolysaccharide biosynthesis.

Two different exopolysaccharides (EPS), amylovoran and levan, are produced by *E. amylovora*. *E. pyrifoliae* DSM 12163^T only produces amylovoran (Kim et al. 2002), whereas *E. tasmaniensis* Et1/99 does not produce either type of EPS (Kube et al. 2008). Two genes, *amsD* and *amsE* (EAMY_2247-EAMY_2246), of the amylovoran biosynthetic gene cluster (EAMY_2253-EAMY_2241) encoding glycosyltransferases, are only shared in *E. amylovora* CFBP 1430 and *E. pyrifoliae* DSM 12163^T (Smits et al. 2010). This is in agreement with previous reports (Kube et al. 2008; Smits et al. 2010), indicating that the *E. tasmaniensis* Et1/99 EPS cluster is more similar to the stewartan biosynthetic cluster of *Pantoea stewartii* subsp. *stewartii* DC283 (Coplin et al. 1996). The gene encoding levansucrase (EAMY_3695), responsible for the biosynthesis of levan (Gross et al. 1992), is absent from *E. pyrifoliae* DSM 12163^T (Smits et al. 2010) but present in the other species.

Type I secretion.

The type I secretion system *prtADEF* encodes an excreted protease (PrtA) and its export system (PrtDEF) plays a minor role at most in the virulence of *E. amylovora* (Zhang et al. 1999). The *prtADEF* system is only present in the genome of *E. amylovora* CFBP 1430 (EAMY_3577-EAMY_3581) but not in the other species. For *E. tasmaniensis* Et1/99, the lack of this system was interpreted to be one factor in the lack of virulence on pome fruit trees of this strain (Kube et al. 2008) but its absence in the related fire blight pathogen *E. pyrifoliae* DSM12163^T suggests it may be a specific contributor to host-range virulence in *E. amylovora* rather than an absolute virulence determinant.

Type II secretion.

A type II secretion system (*outCDEFFHJKLMNOS-chiV*; EAMY_2865-EAMY_2878) (T2SS), induced during an immature pear assay (Zhao et al. 2005), was found in the genomes of all three *Erwinia* strains, including the nonpathogen *E. tasmaniensis*, indicating that it may not be a virulence factor. Indeed, mutational analysis with *E. amylovora* Ea1189 has demonstrated that this T2SS system has no role in pathogenicity on immature pear or apple seedlings (Zhao et al. 2009). The presence of a chitin-binding domain (pfam03067) in the putative ChiV protein (EAMY_2873) suggests that the T2SS system may contribute to a yet-to-be-identified interaction with insect vectors of *E. amylovora* CFBP 1430.

Type VI secretion.

Three type VI secretion system (T6SS) gene clusters were identified in *E. amylovora* CFBP 1430. They differ in composition and organization of core and hypothetical genes. T6SS clusters 1 (EAMY_3028-EAMY_3000) and 2 (EAMY_1623-EAMY_1620) of *E. amylovora* CFBP 1430 are highly similar

to the T6SS cluster of *E. pyrifoliae* DSM 12163^T and *E. tasmaniensis* Et1/99 (Smits et al. 2010), whereas T6SS cluster 3 (EAMY_3228-EAMY_3201) is absent in both *E. pyrifoliae* DSM 12163^T and *E. tasmaniensis* Et1/99.

The first T6SS cluster of *E. amylovora* CFBP 1430 is composed of 27 genes (Supplementary Fig. 2), of which 15 genes were identified as T6SS-core genes (3 putative effectors and 2 putative signal transducers), 4 were identified as conserved genes between species, and 6 as hypothetical genes. In T6SS clusters 1 and 2, blocks of conserved genes between bacterial species are observed, interspaced by hypothetical genes. The first T6SS cluster of *E. amylovora* CFBP 1430 is organized similarly to the T6SS cluster of *E. pyrifoliae* DSM 12163^T and *E. tasmaniensis* Et1/99, with three critical differences: the genes between DUF877 and COG3456 as well as the genes between *vgrG1* and *vgrG2* differ and *E. pyrifoliae* DSM 12163^T and *E. tasmaniensis* Et1/99 have two additional genes between COG3520 and *clpV*. Three hypothetical genes in *E. amylovora* CFBP 1430 (EAMY_3015, EAMY_3001, and EAMY_3002) and *E. pyrifoliae* DSM 12163^T (EPYR_00659, EPYR_00672, and EPYR_00673) are observed which are absent in *E. tasmaniensis* Et1/99. *E. amylovora* CFBP 1430 and *E. tasmaniensis* Et1/99 share two hypothetical genes (EAMY_3017-EAMY_3018 and ETA_06210-ETA_6230) which are absent in *E. pyrifoliae* DSM 12163^T. In T6SS cluster 2 of *E. amylovora* CFBP 1430, three core genes and one conserved gene were identified. The proteins encoded by this cluster show higher similarities to the corresponding proteins of *E. pyrifoliae* DSM 12163^T than to *E. tasmaniensis* Et1/99.

The third T6SS cluster was identified only in *E. amylovora* CFBP 1430. This cluster is located within a putative genomic island and, therefore, might be acquired by horizontal gene transfer. This cluster consists of 31 genes, 15 genes of which were identified as core genes (three putative effectors) and 16 as hypothetical genes. Putative signal transducers are not present. The cluster differs significantly in gene composition and organization from T6SS cluster 1. The proteins encoded in T6SS cluster 3 have only low sequence identities compared with the corresponding proteins of T6SS cluster 1.

Quorum-sensing elements.

Two main quorum-sensing systems are known in gram-negative bacteria and are defined by the chemical structure of the autoinducer (AI) signal molecule involved (Miller and Bassler 2001). For both systems, the gene endowment of *E. amylovora* is analogous to the one found in *E. pyrifoliae* DSM 12163^T (Smits et al. 2010) and *E. tasmaniensis* Et1/99 (Kube et al. 2008).

The protein encoded by gene EAMY_1412 shows 45% identity with the DNA-binding transcriptional activator SdiA that controls cell division in *Escherichia coli* and *Salmonella typhimurium* (Wang et al. 1991), and which responds to AI-1 signals (i.e., AHL) generated by other microbial species. Similar to the situation found in *E. coli* and *S. typhimurium* (Michael et al. 2001), *Erwinia amylovora* lacks a corresponding signal-generating enzyme. This contradicts previous reports for AHL-signaling in *E. amylovora* (Venturi et al. 2004; Molina et al. 2005). Nearby gene EAMY_1411 has low sequence identity (29%) with the AI-1 synthesis protein PhzI of *Pseudomonas chlororaphis*. Thus, the *E. amylovora* gene set composed by EAMY_1411 and EAMY_1412 either may function in the production and detection of a yet-unknown extracellular signal (not necessarily related to known AI molecules) or EAMY_1412 may be an orphan receptor utilized by *E. amylovora* to eavesdrop on quorum-sensing signals of other organisms. Such functions would explain observations of AHL-biosensor cross-feeding by *E. amylovora*.

The *luxS* gene (EAMY_0812), which is the central component implicated in the generation of the AI-2 quorum-sensing signal, was located in *E. amylovora* CFBP 1430. However, the essential accessory genes involved in signal response (Taga et al. 2003; Reading and Sperandio 2006; Rezzonico and Duffy 2008) are lacking. Without these, despite a weak signaling activity observed using biosensors (Rezzonico and Duffy 2007), the function of LuxS in *E. amylovora* CFBP 1430 must be restricted to its metabolic role of enzyme involved in the activated methyl cycle (Winzer et al. 2003; Rezzonico and Duffy 2008).

Iron uptake determinants.

Iron is an essential nutritional factor for every living organism, as a cofactor for numerous proteins. The regulation of iron uptake in bacteria is under control of global regulation by the ferric uptake receptor Fur, which specifically regulates biosynthesis and uptake of iron-affinity siderophores (Hantke 1981; de Lorenzo et al. 1987; Dellagi et al. 1998). The *fur* gene (EAMY_1148) is found in all three *Erwinia* spp. (Supplementary Table 3).

E. amylovora is known to produce only the hydroxamate siderophore desferrioxamine E (DFO E) (Feistner et al. 1993; Kachadourian et al. 1996) and a transposon mutant within the biosynthetic gene *dfoA* is strongly affected in its ability to colonize the flowers of apple trees as well as in its ability to initiate necrosis, leading to a lower level of virulence (Dellagi et al. 1998). The biosynthetic genes for desferrioxamine E were recently identified in *E. pyrifoliae* DSM 12163^T (Smits et al. 2010) and orthologs for all of these are present in *E. amylovora* CFBP 1430 (EAMY_3238 to EAMY_3240) and *E. tasmaniensis* Et1/99. Despite prior efforts (Expert 1999; Expert et al. 2000), the *dfoJAC* gene cluster identified from the genome sequence only now reveals first insights into the biosynthetic pathway of DFO in *E. amylovora* CFBP 1430, that is essentially similar to the DFO biosynthesis pathway of *Streptomyces coelicolor* A3(2) (Challis 2005).

Resorption of iron (III)-loaded siderophores requires a TonB-dependent receptor that transports the siderophores into the periplasmic space. The genome of *E. amylovora* CFBP 1430 contains four TonB-dependent receptors. One encodes a putative copper receptor OprC (EAMY_1821). A second encodes the ferrioxamine receptor FoxR (EAMY_3241) (Kachadourian et al. 1996; Dellagi et al. 1998). A third and fourth are previously unknown TonB-dependent receptors that cannot be classified completely by the TCDB system (EAMY_1761 and EAMY_1080). With the exception of one of the unclassified receptors that is inactivated in *E. pyrifoliae* DSM 12163^T (Smits et al. 2010), all are shared by *E. pyrifoliae* DSM 12163^T and *E. tasmaniensis* Et1/99.

In the genome of *E. amylovora* CFBP 1430, there are remnants present for two further TonB-dependent receptors. One encodes the ferrichrome receptor FhuA, for which intact orthologs exist in *E. pyrifoliae* DSM 12163^T and *E. tasmaniensis* Et1/99, and one encodes the ferric aerobactin receptor IutA, that is absent from the other two strains. The genome of *E. tasmaniensis* Et1/99 encodes two additional TonB-dependent receptors, of which one is the ferric dicitrate receptor FecA (Kube et al. 2008). For both receptors, ABC transporters, necessary for the uptake of iron over the cytoplasmic membrane, are present as well. The genome of *E. amylovora* CFBP 1430 contains only three iron-uptake ABC transport systems (*fhuCDB*, *hmuSTUV*, and *sitABCD*), all shared by *E. pyrifoliae* DSM 12163^T and *E. tasmaniensis* Et1/99.

The limited genetic basis for iron uptake in *E. amylovora* CFBP 1430 is also reflected by a limited utilization of iron siderophores by this organism (Kachadourian et al. 1996). The

fact that ferric dicitrate is not utilized is confirmed by the absence of the complete *fec* system, whereas the inactivation of *fhuA* will abolish ferrichrome uptake. In contrast to *Pectobacterium atrosepticum*SCRI 1043 (Bell et al. 2004), all sequenced *Erwinia* spp. do not contain the biosynthetic cluster for enterobactin, the catecholate siderophore produced by many enteric pathogens.

Conclusions.

Comparison of all three currently sequenced *Erwinia* genomes has revealed several interesting features that may explain observed phenotypical differences between the species. Although many of the virulence factors found in *E. amylovora* (Oh and Beer 2005) are present in the genomes of one or both of the other species, our comparative analysis demonstrated that a number are specific for *E. amylovora* and, therefore, may contribute to the broad-spectrum and severe-disease phenotype of this species. Our analysis of these *Erwinia* genomes raises questions concerning host range, virulence, and other host interactions. Currently, the major factors considered critical for pathogenicity or virulence on Rosaceous hosts are the Hrp T3SS-related genes and amylovoran biosynthesis (Bernhard et al. 1996; Oh and Beer 2005; Oh et al. 2005; Zhao et al. 2009). Our comparative genomic analysis indicates that *E. amylovora* and *E. pyrifoliae* share a basic repertoire of virulence factors. Their genetic similarity, causation of essentially indistinguishable symptoms, and reported *E. pyrifoliae* pathogenicity on apple (*Malus domestica*) and pear (*Pyrus communis*) cultivars as well as Asian pear (*P. pyrifolia*) (Duffy and Dandekar 2008; Kim et al. 2001) justify reference to both species as fire blight pathogens, despite their different host range and virulence and without minimizing the higher phytosanitary relevance of *E. amylovora*.

Potentially, new virulence-related factors were revealed in *E. amylovora* CFBP 1430, the closely related pathogen *E. pyrifoliae* DSM 12163^T, and the presumably nonpathogenic *E. tasmaniensis* Et1/99. The two T3SSs on genomic islands PAI-2 and PAI-3 have high homology with the insect pathogen *Sodalis glossinidius* str. *morsidans* and to the mammalian pathogens *Salmonella* and *Yersinia* spp. (Zhao et al. 2009). A novel gene encoding a protein with a chitinase-binding domain was found directly downstream of the T2SS in *E. amylovora* CFBP 1430 (*chiV*; EAMY_2878). *E. amylovora* CFBP 1430 and *E. pyrifoliae* DSM 12163^T chromosomes both have genes annotated as β -hexosaminidase (EAMY_0363 and EPYR_00390, respectively), and two plasmids of *E. pyrifoliae* DSM 12163^T encode downstream of the *hcp* genes a putative endochitinase (EPYR_04028 and EPYR_04035) (Smits et al. 2010), absent in *E. amylovora* CFBP 1430. This suggests a possibly closer insect association beyond the passive dispersal known to be critical in fire blight epidemiology (Johnson and Stockwell 1998).

Comparison of our complete genome for *E. amylovora* CFBP 1430 with that of *E. amylovora* Ea273 confirmed the high genetic-homogeneity observed within this species (>99.99%). This level of conservation between a European and U.S. strain reflects the relatively recent dispersal of the species and indicates a low rate of significant evolution. However, our analysis revealed a major genomic reorganization, with two inversions of large genomic regions based on crossovers in two different 16S rRNA operons. These reorganizations explain the different PFGE patterns observed in these and perhaps other genotypes (Zhang and Geider 1997; Jock et al. 2002). The physiological role of this genomic reorganization remains to be examined in more detail using different strains having different PFGE patterns.

Novel insights from the genome of *E. amylovora* CFBP 1430 such as the identification of the T6SSs, the NPRS, and

the PKS cluster (Supplementary information) warrant evaluation of the role of these systems in the virulence, host range, or ecological behavior of *E. amylovora* on Rosaceous plants. This and, particularly, potential new determinants of pathogen–insect interactions may facilitate design of more effective control strategies against fire blight.

MATERIALS AND METHODS

Whole-genome sequencing.

Genomic DNA of an early European isolate of *E. amylovora* (CFBP 1430, France, 1972, *Crataegus*) was isolated using the Wizard Genomic DNA Purification Kit (Promega Corp., Madison, WI, U.S.A.). Whole-genome sequencing was performed by GATC (Konstanz, Germany) using an Illumina sequencer. In total, 4,469,394 high-quality filtered sequence reads were generated, with an average 36-bp read length and an average coverage equivalent to 42×. Quality filtered sequences were aligned to the draft genome sequence of the U.S. strain *E. amylovora* Ea273, available as a fasta file at the Sanger center (Bocsanczy et al. 2008) using the program NGen (DNASTAR, Madison, WI, U.S.A.) and were manually checked for inconsistencies. All reads were additionally de novo assembled using the program EDENA (Hernandez et al. 2008) with manually optimized settings. This yielded 2,627 contigs ($N_{50} = 2,617$, mean length = 1,399, maximum length = 12,053, minimum length = 100). The assembled contigs were aligned to the sequence using the LASERGENE package (DNASTAR). Differences and large gaps were manually checked against the original assembly. Additionally, the reads that were not included in the initial assembly were assembled as well using EDENA using a 60-bp minimal fragment size yielding two fragments of 645 and 91 bp, respectively.

Based on identified differences between the CFBP 1430 and Ea273 genome sequences and areas with known sequence variation, several regions were checked by PCR using standard conditions (Innis et al. 1990) and sequenced using the Sanger method on a 3130XL sequencer (Applied Biosystems, Rotkreuz, Switzerland). Final assembly was done using the LASERGENE package. The rRNA operons were covered by PCR fragments amplified from primers in both flanks of the rRNA regions, and completely sequenced.

Genome annotation.

Genes were predicted using a combined strategy (McHardy et al. 2004) based on the CDS prediction programs Glimmer (Salzberg et al. 1998) and Critica (Badger and Olsen 1999). Subsequently, the potential function of each predicted gene was automatically assigned using the GenDB annotation pipeline (Meyer et al. 2003). The resulting genome annotation was manually curated, and metabolic pathways were identified using the KEGG pathways tool (Kanehisa et al. 2002) in GenDB.

Comparative analysis.

The genome sequences of three ecologically distinct *Erwinia* spp. were compared to identify the set of common genes composing the core genome for this genus and the set of genes unique to each species, referred to as singletons. For this purpose, an “all-against-all” comparison of the genes was accomplished using the BLAST alignment tool (Altschul et al. 1990). The genes were aligned based on the protein sequence (BLASTP) with an initial *e*-value cut-off of 1E-5 using the BLOSUM62 scoring matrix. Genes were considered orthologous when a reciprocal best BLAST hit was found between two genes, and when both BLAST hits were based on alignments exceeding 70% sequence identity spanning over at least 70% of the query gene length (Blom et al. 2009). The *Erwinia*

genus core genome was calculated as the set of genes of a reference strain for which an orthologous gene could be found in each of the compared genomes. In contrast, genes of one strain were considered to be singleton genes when they had no BLAST-hits comparability with any of the other genomes that satisfied the given criteria. A Venn diagram was created using the plotting routines of EDGAR (Blom et al. 2009). Synteny plots were generated as alignments of the complete genome nucleotide sequences using MUMMER 3.0 (Kurtz et al. 2004).

Additional analysis.

Routine sequence manipulations were completed using several subroutines of the LASERGENE package. Comparative analysis of the genome sequences of *E. amylovora* CFBP 1430, *E. pyrifoliae* DSM 12163^T, and *E. tasmaniensis* Et1/99 was performed using MAUVE (Darling et al. 2004) in the standard and progressive mode.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

CRISPRFinder database:
crispr.u-psud.fr/crispr/CRISPRdatabase.php?page=own
 The Sanger Institute *E. amylovora* BLAST server:
www.sanger.ac.uk/cgi-bin/blast/submitblast/e_amylovora
 The Sanger Institute *Erwinia amylovora* webpage:
www.sanger.ac.uk/Projects/E_amylovora
 Transport Classification database (TCDB): www.tcdb.org