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Lipopolysaccharide biosynthesis genes discriminate between *Rubus*- and Spiraeoideae-infective genotypes of *Erwinia amylovora*

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SUMMARY

Comparative genomic analysis revealed differences in the lipopolysaccharide (LPS) biosynthesis gene cluster between the *Rubus*-infecting strain ATCC BAA-2158 and the Spiraeoideae-infecting strain CFBP 1430 of *Erwinia amylovora*. These differences corroborate *rpoB*-based phylogenetic clustering of *E. amylovora* into four different groups and enable the discrimination of Spiraeoideae- and *Rubus*-infecting strains. The structure of the differences between the two groups supports the hypothesis that adaptation to *Rubus* spp. took place after species separation of *E. amylovora* and *E. pyrifoliae* that contrasts with a recently proposed scenario, based on CRISPR data, in which the shift to domesticated apple would have caused an evolutionary bottleneck in the Spiraeoideae-infecting strains of *E. amylovora* which would be a much earlier event. In the core region of the LPS biosynthetic gene cluster, Spiraeoideae-infecting strains encode three glycosyltransferases and an LPS ligase (Spiraeoideae-type *waaL*), whereas *Rubus*-infecting strains encode two glycosyltransferases and a different LPS ligase (*Rubus*-type *waaL*). These coding domains share little to no homology at the amino acid level between *Rubus*- and Spiraeoideae-infecting strains, and this genotypic difference was confirmed by polymerase chain reaction analysis of the associated DNA region in 31 *Rubus*- and Spiraeoideae-infecting strains. The LPS biosynthesis gene cluster may thus be used as a molecular marker to distinguish between *Rubus*- and Spiraeoideae-infecting strains of *E. amylovora* using primers designed in this study.

INTRODUCTION

Erwinia amylovora is a bacterial pathogen that causes fire blight, a destructive disease that affects rosaceous plants worldwide (Bonn and van der Zwet, 2000), producing substantial economic losses to apple and pear production. Thus, *E. amylovora* is mainly recognized as a serious pathogen of *Malus* and *Pyrus* spp. Fire blight, however, has been described as a disease of other taxa of the Spiraeoideae subfamily (Potter *et al*., 2007), such as *Prunus*, *Crataegus*, *Pyracantha* and *Amelanchier* (Momol and Aldwinckle, 2000), as well members of the Rosoideae subfamily belonging to the genus *Rubus*, such as raspberry or blackberry (Evans, 1996; Ries and Otterbacher, 1977; Starr *et al*., 1951) (Table S1, see Supporting Information). To date, *Rubus*-infecting isolates have only been reported from North America, although it is unclear how thoroughly surveys for these strains have been conducted elsewhere.

A number of studies have demonstrated very limited cross-infectivity between Spiraeoideae- and *Rubus*-infecting isolates of *E. amylovora*: *Rubus* isolates are mostly unable to cause fire blight symptoms when inoculated into apple trees or immature pear fruits, whereas Spiraeoideae-infecting isolates generally elicit a limited local response in raspberry when administered at high doses (Braun and Hildebrand, 2005; Evans, 1996; Giorgi and Scortichini, 2005). Nonetheless, cross-infected isolates survive on and can be recovered from the nonhost plant, whilst maintaining their ability to infect their original host (Braun and Hildebrand, 2005; Evans, 1996; Giorgi and Scortichini, 2005).

Erwinia amylovora has long been considered a genetically very homogeneous species (Momol and Aldwinckle, 2000), but recent molecular approaches based on the study of repetitive elements, such as Multiple Loci Variable Number of Tandem Repeats Analysis (MLVA) (Dreo *et al*., 2011) or sequencing of Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) (Rezzonico *et al*., 2011), have shown considerable diversity, especially among strains isolated from *Rubus* plants and, to a lesser extent, in Spiraeoideae-infecting strains. Differences between *Rubus*- and Spiraeoideae-infecting strains have been observed previously using DNA fingerprinting methods (Jock and Geider, 2004; McManus and Jones, 1995; Rico *et al*., 2008) and for the deduced protein sequences of the type III secretion system (T3SS) ATPase

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HrcN (Jock and Geider, 2004) and effector DspA/E (Giorgi and Scortichini, 2005). The T3SS is a major determinant for virulence and symptom development in pome fruit trees (Oh and Beer, 2005). The only factor that has been demonstrated so far to alter the virulence of the fire blight pathogen in a host-specific manner is the T3SS effector Eop1, a member of the YopJ/AvrRxv family of type III secreted proteins. This protein from a Spiraeoideae-infecting isolate is essential for pathogenicity in immature pear and apple shoots, but does not alter pathogenicity to raspberry when transformed into a *Rubus*-infecting strain of *E. amylovora* (Asselin *et al*., 2011). The structure of the exopolysaccharide (EPS) amylovoran (Zhao *et al*., 2009) constitutes another distinguishing feature of the two *E. amylovora* types (Maes *et al*., 2001) but, as for the aforementioned elements, the extent to which it contributes to differential host specificity is unknown. Altogether, only very few distinguishable genes have been identified between the *Rubus*- and Spiraeoideae-infecting isolates using subtractive hybridization (Triplett *et al*., 2006).

Recently, complete genomes of the Spiraeoideae-infecting strain *E. amylovora* CFBP 1430 (Smits *et al*., 2010b) and the *Rubus*-infecting strain *E. amylovora* ATCC BAA-2158 (syn. IL-5, Bb1, Ea246; Powney *et al*., 2011b) have been published. Comparative analysis of these genomes revealed a multiple-gene substitution in the lipopolysaccharide (LPS) biosynthetic gene cluster (CFBP 1430, EAMY_0089–0092 vs. ATCC BAA-2158, EAIL5_0082–0084), but no difference in the amylovoran biosynthetic gene cluster. The LPS can cover over 90% of the cell surface in Gram-negative bacteria and is directly involved in host contact whilst acting as a physical barrier against the host antimicrobial response (Rosenfeld and Shai, 2006). LPS is a factor that has been described recently to be involved in the virulence of *E. amylovora*. Berry *et al*. (2009) described a Spiraeoideae-infecting *E. amylovora* strain harbouring a transposon insertion in the *waaL* gene (EAMY_0091). This gene encodes an LPS O-antigen ligase responsible for the attachment of the O-antigen polysaccharide to the lipid A unit. The strain carrying the transposon insertion and hence partial loss of function of the LPS cluster was less virulent in the detached pear test, but also less resistant to reactive oxygen stress, and showed impaired motility (Berry *et al*., 2009). Differences have been observed in LPS serology between *Rubus*- and Spiraeoideae-infecting strains of *E. amylovora* (Mizuno *et al*., 2002).

In this work, we confirm that the genetic differences in the LPS biosynthesis gene cluster are consistent across a wide range of *Rubus*- and Spiraeoideae-infecting *E. amylovora* strains (including most of the published *Rubus* strains), and present a simple multiplex polymerase chain reaction (PCR) protocol that may be used to discriminate between these two host-specific genotypes independently from the source of isolation. Our diagnostic approach is well suited to the detection of nonhost strains of *E. amylovora* in asymptomatic plant material. Furthermore, our results confirm previously established *E. amylovora* phylogeny based on partial *rpoB* sequences.

RESULTS AND DISCUSSION

Selection of *E. amylovora* strains

To enable comparison with previously published phenotypic and genotypic data, we selected as many *Rubus*- (and Spiraeoideae)-infecting strains as possible that had been tested in a pathogenicity cross-test on both host plant types. Furthermore, care was taken to select strains belonging to all CRISPR types (Rezzonico *et al*., 2011) in order to cover the maximum achievable diversity in Spiraeoideae-infecting strains. Several strains with available genomic data [CFBP 1430 (Smits *et al*., 2010b), ATCC 49946 (Sebaihia *et al*., 2010), ATCC BAA-2158 (Powney *et al*., 2011b), CFBP 1232^{T}, MR-1, Ea644 (R. A. Mann *et al*., unpublished)] were also included in the analysis.

Phylogeny of *E. amylovora* based on *rpoB*

Comparative analysis on the regions of housekeeping genes commonly used for the phylogenetic analysis of Enterobacteriaceae (*atpD*, *gyrB*, *infB* and *rpoB*) (Brady *et al*., 2008) was performed using data from the complete genome sequences available (Fig. S1, see Supporting Information). We selected a fragment of the RNA polymerase β-subunit-encoding gene *rpoB* as it displayed the highest diversity among *E. amylovora* strains [up to 33 single nucleotide polymorphisms (SNPs) in 962 bp]. The amplicons for the other genes only displayed little diversity (*atpD*, two SNPs in 642 bp; *gyrB*, four SNPs in 742 bp) or could not separate *Rubus*- from Spiraeoideae-infecting strains (*infB*, 10 SNPs in 615 bp) (Fig. S1). The concatenated tree shows the same topology as the *rpoB* tree which, however, exhibits deeper branches. The topology is also very similar to a core genome tree of the sequenced *E. amylovora* strains (R. A. Mann *et al*., unpublished). On the basis of these data, the *rpoB* gene was chosen for further work.

A minimum evolution tree constructed on the basis of the partial *rpoB* sequences enabled the separation of the *E. amylovora* isolates into four different groups (Fig. 1). The first group (S) contained all Spiraeoideae-infecting isolates, except strain PD 2915, an isolate from *Amelanchier* with a host range limited to this plant (Giorgi and Scortichini, 2005), which clustered within the main cluster of *Rubus*-infecting isolates. *Rubus*-infecting strains were divided into three different branches: a major group (R1) containing all Canadian (including PD 2915) and some US isolates; a smaller set (R2) containing three US isolates (PD 103, ATCC BAA-2158 and Ea 515), which were more closely related to Spiraeoideae-infecting strains; and MR-1, which formed a single-strain group (R3) and showed the most divergence from all other *E. amylovora*. Within each of the *rpoB* groups, the strains showed

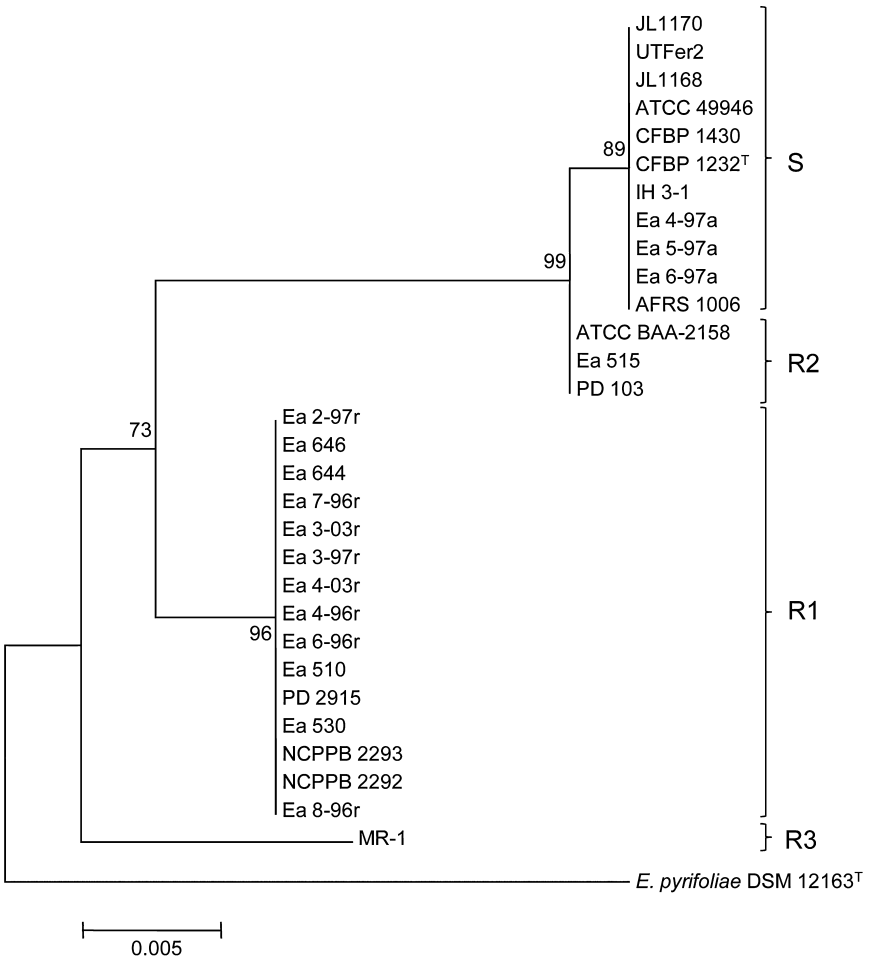

Fig. 1 Relationship between *Erwinia amylovora* isolates based on a 952-bp region of the *rpoB* housekeeping gene inferred using the minimum evolution method. Distances were computed implementing the maximum composite likelihood model and are in units of the number of base substitutions per site. Bootstrap values (1000 replicates) are shown next to the branches. S, Spiraeoideae-infecting isolates; R1–R3, *Rubus*-infecting isolates.

no sequence variability over the 952-bp region covered. This is in sharp contrast with a recent study in which we assessed genetic diversity among strains based on an analysis of the CRISPR regions (Rezzonico *et al*., 2011). We found considerable genetic variation among the studied strains, with variation among isolates of the *Rubus rpoB* cluster R1 being much higher than for the Spiraeoideae-infecting isolates (Rezzonico *et al*., 2011). Nevertheless, the *rpoB*-based phylogeny remains largely concordant with the results obtained using repetitive-sequence PCR (Barionovi *et al*., 2006) and whole-genome phylogeny (R. A. Mann *et al*., unpublished), which place the *Rubus*-infecting strains PD 103 and ATCC BAA-2158 near the Spiraeoideae-infecting strains (Fig. 1). This analysis also confirms the phylogenetic relatedness of PD 2915 within *Rubus*-infecting isolates, despite the fact that this strain was originally isolated from a host (*Amelanchier*) belonging to the Spiraeoideae (Giorgi and Scortichini, 2005).

Detection of *Rubus*-infecting strains using Ea AgriStrip immunoassays

In a previous study (Braun-Kiewnick *et al*., 2011), the Ea AgriStrip lateral-flow immunoassay was developed for the specific detection of *E. amylovora* in field samples. This assay is based on polyclonal antibodies raised against whole cells of five Spiraeoideae-infecting strains. Unfortunately, no *Rubus*-infecting isolates of *E. amylovora* were used in the development, although differences were shown in the detection of different *Erwinia* species (Braun-Kiewnick *et al*., 2011).

We examined whether the Ea AgriStrip immunoassays were able to differentiate between Spiraeoideae- and *Rubus*-infecting isolates of *E. amylovora*, based on the differences in LPS structure predicted from the genome sequences. The Ea AgriStrip immunoassays were positive for all *Rubus*-infecting isolates (Table 1), indicating that this test is not suited to the differentiation between *Rubus*- and Spiraeoideae-infecting *E. amylovora*. This result is probably caused by the polyclonal nature of the antibodies used in the assay, which results in targeting of multiple epitopes on the bacterial cell.

Comparative sequence analysis

Comparative genomics performed using EDGAR (Blom *et al*., 2009) revealed gene arrangements in the LPS clusters of the Spiraeoideae-infecting *E. amylovora* strains CFBP 1430 and ATCC

Table 1 *Erwinia amylovora* strains used in this study, characteristics and results of *waaL* polymerase chain reaction (PCR) and lateral-flow immunoassays.

Strain name (synonyms)*	Isolated from	Origin	CRISPR type†	PCR data *waaL* type‡	Immunoassay§	Pathogenic on	Nonpathogenic¶ on	Reference
Erwinia amylovora								
AFRS 1006 (BB89-FR42)	*Malus domestica* cv. Westland (apple)	Alberta, Canada	I	S	+	Apple	Raspberry	Evans (1996)
CFBP 1232^{T} (NCPPB 683^{T})	*Pyrus communis* (pear)	UK, 1959	I	S	+	Apple, pear	Raspberry, serviceberry	Giorgi and Scortichini (2005)
CFBP 1430	*Crataegus* sp.	France, 1972	I	S	+			Paulin and Samson (1973); Smits *et al*. (2010b)
Ea4-97a	*M. domestica* cv. Gloster	Nova Scotia, Canada, 1997	I	S	+	Apple	Raspberry	Braun and Hildebrand (2005)
Ea5-97a	*M. domestica* cv. Gloster	Nova Scotia, Canada, 1997	I	S	+	Apple	Raspberry	Braun and Hildebrand (2005)
Ea6-97a	*M. domestica* cv. Cortland	Nova Scotia, Canada, 1997	I	S	+	Apple	Raspberry	Braun and Hildebrand (2005)
ATCC 49946 (Ea 273)	*M. domestica*	New York, USA, 1973	I	S	+	Apple	Raspberry	Asselin *et al*. (2011); Sebaihia *et al*. (2010)
JL1168	*P. communis*	Washington, USA	I	S	+			Loper *et al*. (1991)
UTFer2	*M. domestica*	Utah, USA	II	S	+			Foster *et al*. (2004)
JL1170	*P. communis*	Washington, USA	III	S	+			Loper *et al*. (1991)
IH 3-1	*Rhaphiolepis indica* (Indian hawthorn)	Louisiana, USA, 1998	IH	S	+			Holcomb (1998)
ATCC BAA-2158 (BB-1, Ea 246, IL-5, BC 204)	*Rubus idaeus* (raspberry)	Illinois, USA, 1972	R	R	+	Raspberry	Apple	Asselin *et al*. (2011); Powney *et al*. (2011b); Ries and Otterbacher (1977)
Ea03-03r	*R. idaeus* cv. Boyne	Alberta, Canada, 2003	R	R	+			G. Braun (from I. R. Evans)
Ea04-03r	*R. idaeus* cv. Nova	New Brunswick, Canada, 2003	R	R	+			G. Braun
Ea2-97r	*R. idaeus* cv. Boyne	Nova Scotia, Canada, 1997	R	R	+	Raspberry	Apple	Braun and Hildebrand (2005)
Ea3-97r	*R. idaeus* cv. Boyne	Nova Scotia, Canada, 1997	R	R	+	Raspberry	Apple	Braun and Hildebrand (2005)
Ea4-96r	*R. idaeus* cv. K81-6	New Brunswick, Canada, 1996	R	R	+	Raspberry	Apple	Braun and Hildebrand (2005)
Ea8-96r	*R. idaeus* cv. K81-6	New Brunswick, Canada, 1996	R	R	+	Raspberry	Apple	Braun and Hildebrand (2005)
Ea 510 (BR89-FR41, CUCPB 3367, BC201)	*R. idaeus*	Alberta, Canada	R	R	+	Raspberry	Apple	Evans (1996)
Ea 515 (Eab3, CUCPB 3404)	*R. idaeus*	Wisconsin, USA	R	R	+			Heimann and Worf (1985)
Ea 530 (ICMP 1841, ICPB EA131, NCPPB 1859, AFRS 1639, CUCPB 3575)	*R. idaeus*	Maine, USA, 1949	R	R	+			Starr *et al*. (1951)
Ea 592 (IE-R(3))	*R. idaeus***	1995	R	R	+			Asselin *et al*. (2011); Evans (1996)
Ea 644	*R. idaeus* cv. Polana	Massachusetts, USA, 2003	R	R	+			Asselin *et al*. (2011)
Ea 646	*R. idaeus*	Quebec, Canada	R	R	+			S. V. Beer
MR-1 (Ea 574)	*R. idaeus*	Michigan, USA	R	R	+			McManus and Jones (1995)
Ea 6-96r (Ea 625)	*R. idaeus*	Canada, 1996	R	R	+			McGhee and Jones (2000)
Ea 7-96r	*Rubus* sp.	Canada, 1996	R	R	+			McGhee and Jones (2000)
NCPPB 2292	*R. idaeus*	USA, 1949	R	R	+	Raspberry	Apple, pear, serviceberry	Giorgi and Scortichini (2005)
NCPPB 2293	*R. idaeus*	USA, 1949	R	R	+	Raspberry	Apple, pear, serviceberry	Giorgi and Scortichini (2005)
PD 103	*R. idaeus*	USA, 1978	R	R	+	Raspberry	Apple, pear, serviceberry	Giorgi and Scortichini (2005)
PD 2915	*Amelanchier* sp. (serviceberry)	Canada, 1996	R	R	+	Serviceberry	Apple, pear, raspberry	Giorgi and Scortichini (2005)
E. pyrifoliae								
Ep1/96	*Pyrus pyrifoliae* (Chinese pear)	South Korea, 1996	EP	S	+			Kim *et al*. (1999)
E. tasmaniensis								
Et1/99	*M. domestica*	Tasmania, Australia, 1999	ET	S††	+			Geider *et al*. (2006)
LA540	*M. domestica*	Oregon, USA, 1994	ET	S††	+			Pusey *et al*. (2009)
E. piriflorinigrans								
APA 3959 (CFBP 5884)	*P. communis* var. Ercolini	Spain, 2000	n.d.	–	(+)			López *et al*. (2011)
IVIA 2045 (CFBP 5882)	*P. communis* var. Tendral	Spain, 2000	n.d.	–	(+)			López *et al*. (2011)
E. billingiae								
BE21	*M. domestica*	Queensland, Australia, 1999	n.d.	–	–			Powney *et al*. (2011a)
E. aphidicola								
JCM 21239	*Acyrthosiphon pisum* (pea aphid)	Japan, 1996	n.d.	–	–			Harada *et al*. (1997)
JCM 21242	*A. pisum*	Japan, 1996	n.d.	–	–			Harada *et al*. (1997)

*Strains can accumulate alternative names across research collections and, where known, these are given in parentheses.
†CRISPR types as in Rezzonico *et al*. (2011); n.d., not determined.
‡R, *Rubus*-type *waaL*; S, CFBP 1430-type *waaL*.
§Symbols indicate: +, positive detection; (+), intermediate detection (weakly positive test line); –, no detection.
¶Cross-inoculation of apple- or pear-infecting isolates on raspberry plants has been shown to cause necrotic streaks around the infection point (Braun and Hildebrand, 2005) or initial wilting (Giorgi and Scortichini, 2005) in a limited number of plants only, whereas inoculation of apple or pear plants with raspberry-infecting isolates results in either no infection at all (Braun and Hildebrand, 2005) or slight necrosis at the entrance site of the bacterium when inoculating with medium and high bacterial doses (Giorgi and Scortichini, 2005). In both cases, the infected plants did not present the complete range of symptoms and recovered completely.
**Re-isolated from raspberry plants artificially inoculated with *Rubus*-infecting strain Ea510.
††Weak amplification only.

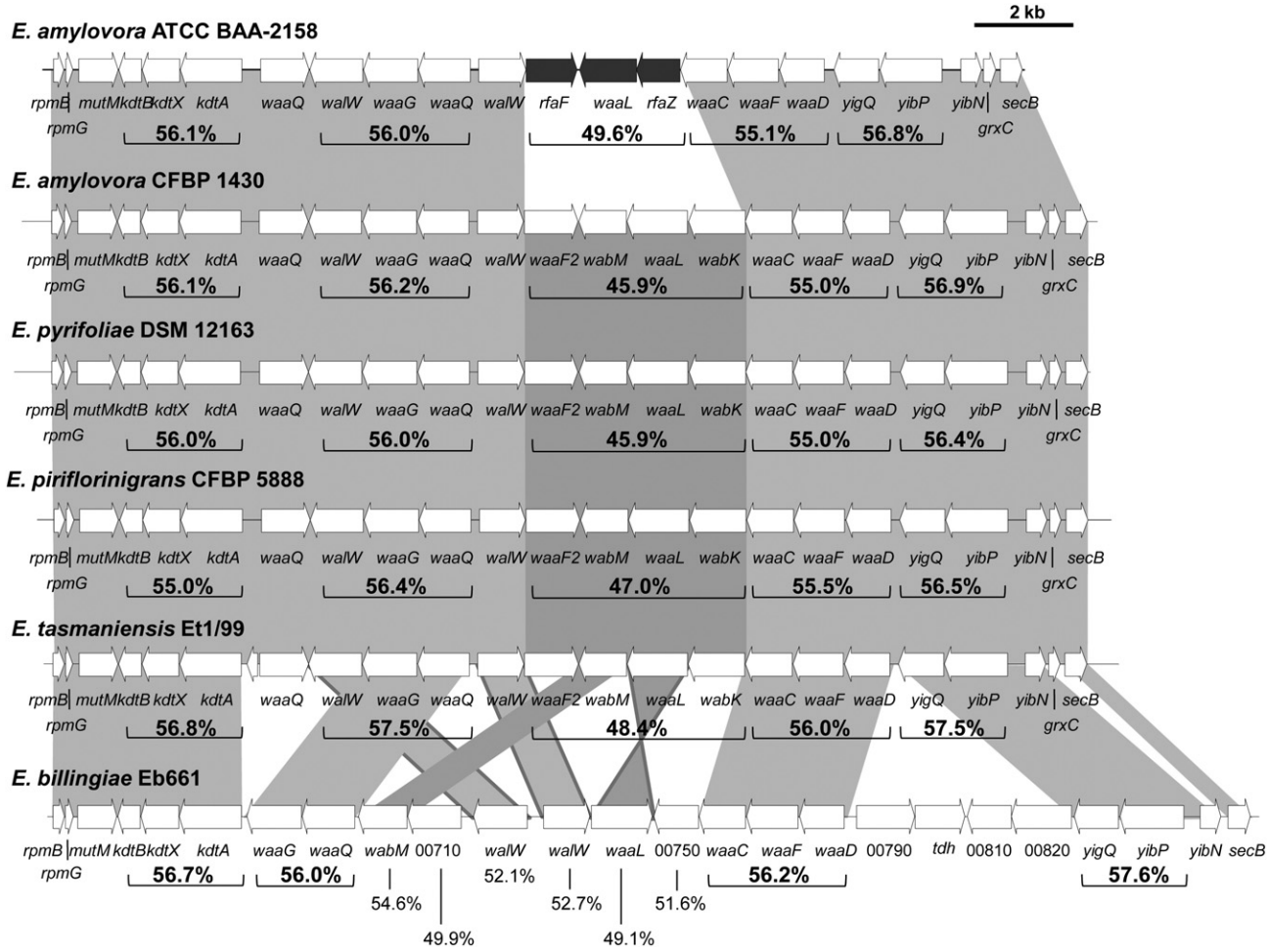

Fig. 2 Maps of the lipopolysaccharide (LPS) biosynthetic gene cluster of different *Erwinia* spp. Conserved genes are indicated with grey shading. The genes in the differential region in the *E. amylovora Rubus*-infecting strain ATCC BAA-2158 are indicated in dark grey. The G + C contents for the differential region and the contiguous genes *kdtAXB*, *waaQG-walW*, *waaDFC* and *yibP-yigQ* are indicated below the respective operons. Identical set-ups within species were omitted.

49946 that were similar to those found in *E. pyrifoliae* strains DSM 12163^T and Ep1/96 (Smits *et al*., 2010a), *Erwinia* sp. Ejp617 (Park *et al*., 2011), *E. piriflorinigrans* CFBP 5888^T (Smits *et al*., 2012, submitted) and *E. tasmaniensis* Et1/99 (Kube *et al*., 2008), including the low G + C region (Fig. 2). In contrast, the LPS biosynthetic gene cluster of the nonpathogenic epiphyte *E. billingiae* Eb661 showed a distinctly different arrangement resembling that found in *Pantoea* species genomes (De Maayer *et al*., 2010, 2012; Smits *et al*., 2010c).

Within *E. amylovora*, a major difference in the organization of the LPS biosynthetic genes was observed between the genome sequences of the Spiraeoideae-infecting strains CFBP 1430 and ATCC 49946 and the *Rubus*-infecting strains ATCC BAA-2158, Ea644 and MR-1 (Fig. 2). The LPS cluster of the Spiraeoideae-infecting strains (from *waaQ* to *waaD*; locus tags for CFBP 1430: EAMY_0083–EAMY_0095) contains 12 genes, whereas the cluster from the three *Rubus*-infecting strains (locus tags for ATCC BAA-2158: EAIL5_0077–EAIL5_0087) contains only 11 genes. Within the latter group, the order of the genes was identical, although sequence identities were more variable (Table 2).

The variation in the LPS biosynthetic gene cluster is restricted to the core region. The Spiraeoideae-infecting strains of *E. amylovora* have three genes encoding glycosyltransferases and a LPS ligase-encoding gene (Spiraeoideae-type *waaL*), whereas the *Rubus*-infecting strain of *E. amylovora*, ATCC BAA-2158, has only two genes encoding different types of glycosyltransferases and one gene encoding a different LPS ligase (*Rubus*-type *waaL*). There is low or no overall sequence identity at the amino acid sequence level between the proteins in the nonconserved regions of the Spiraeoideae-type and *Rubus*-type gene clusters, so that an estimation of the evolutionary relatedness in this region was possible only between isolates infecting the same host plant subfamily (Table 2). With the exception of the almost complete sequence identity between the two *Rubus*-infecting strains Ea644 and

Table 2 Estimates of evolutionary relatedness within *Erwinia amylovora* and related *Erwinia* spp. among host-specific genes of the lipopolysaccharide (LPS) biosynthetic gene cluster (***rfaF-waaL-rfaZ*** and *waaF2-wabM-waaL-wabK* for *Rubus*- and Spiraeoideae-infecting strains, respectively) and in concatenated housekeeping genes *gyrB-rpoB-atpD-infB*. Sequence similarity is expressed as the percentage of identical residues in the pairwise alignment.

	rfaF-waaL-rfaZ and *waaF2-wabM-waaL-wabK*						
		MR-1	Ea 644	ATCC BAA-2158	CFBP 1430	Epyr	Epir	Etas
gyrB-rpoB-atpD-infB	MR-1	–	**99.97**	**98.04**	n.a.	n.a.	n.a.	n.a.
	Ea 644	99.36	–	**98.00**	n.a.	n.a.	n.a.	n.a.
	ATCC BAA-2158	98.56	99.00	–	n.a.	n.a.	n.a.	n.a.
	CFBP 1430	98.46	98.89	99.83	–	91.99	84.47	82.77
	Epyr	95.45	95.38	95.36	95.31	–	85.00	83.23
	Epir	94.35	94.33	94.29	94.19	95.48	–	88.08
	Etas	94.24	94.14	94.15	94.08	95.19	95.53	–

n.a., not applicable, direct pairwise comparison is not possible among *Rubus*- and Spiraeoideae-infecting strains because of the complete divergence of the two LPS biosynthetic gene sequences.

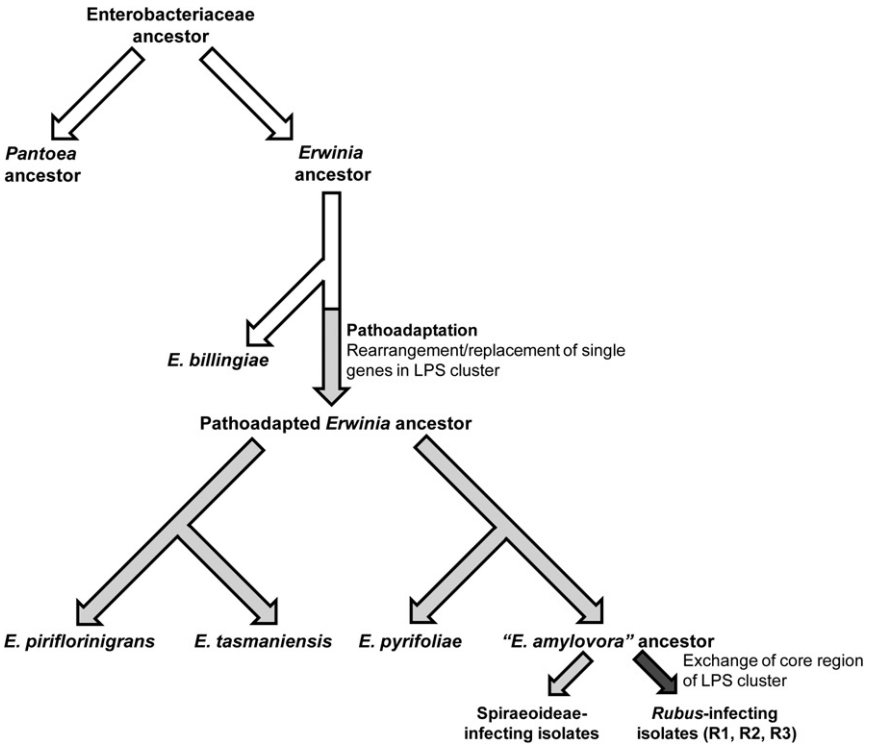

Fig. 3 Hypothesis for an evolutionary history of genome-sequenced *Erwinia* spp. based on the different set-ups of the lipopolysaccharide (LPS) clusters (Fig. 2). The different set-ups are indicated by different arrow colours: white for *E. billingiae* and *Pantoea* spp., light grey for all pathoadapted *Erwinia* spp., including the Spiraeoideae-infecting *E. amylovora* strains, and dark grey for *Rubus*-infecting *E. amylovora* strains.

MR-1, the sequence identities in the LPS biosynthetic gene cluster core were consistently lower than that of the concatenated sequence of housekeeping genes *gyrB-rpoB-atpD-infB* (Table 2). Similarly distant values were also found in the adjacent operons *walW-waaG-waaQ* and *waaC-waaF-waaD*, whereas the more distantly located *kdtB-kdtX-kdtA* and *yigO-yibP* displayed a higher level of sequence identity (Fig. S2, see Supporting Information). The variable regions have 49.6% G + C in the *Rubus*-infecting *E. amylovora* ATCC BAA-2158 and 45.9% G + C in the Spiraeoideae-infecting *E. amylovora* CFBP 1430, whereas flanking regions in both strains have a 55.0%–56.2% G + C content, slightly higher than the average G + C content of *E. amylovora* strains (Powney *et al*., 2011b; Smits *et al*., 2010b). Although variations in G + C content are probably attributable to horizontal gene transfer events, gene rearrangements and exchange within this region (Fig. 2) probably originate from the pathoadaptation process within the ancestor of the pathoadapted *Erwinia* spp. after separation of the saprophytic *E. billingiae* Eb661 (Kamber *et al*., 2011) (Fig. 3).

Differences in the *waaL* gene from *Rubus*- and Spiraeoideae-infecting *E. amylovora* isolates

Oligonucleotide primers were developed to specifically detect either the Spiraeoideae-type (CFBP_lps-fw/-rev) or *Rubus*-type (IL5_lps-fw/-rev) *waaL* gene of *E. amylovora* using PCR. With the exception of the *Amelanchier*-infecting strain PD 2915,

amplification of the *Rubus*-type *waaL* gene was only obtained for the *Rubus*-infecting isolates, whereas the Spiraeoideae-type *waaL* gene was only detected in Spiraeoideae-infecting isolates (Table 1). As the selection of strains used in this work includes most of the *Rubus*-infecting isolates described in the literature so far (thus the broadest geographical, biological and molecular diversity available), it is possible that the 11-gene LPS biosynthetic cluster containing the *Rubus*-type *waaL* is a general trait for *Rubus*-infecting isolates.

Both PCR primer sets were tested on a wide range of *Erwinia* spp. (Table 1). The *Rubus*-type *waaL* primers yielded no amplicons with this broader group. A strong amplicon for *E. pyrifoliae* Ep1/96 and a weak amplicon for strains of *E. tasmaniensis* were obtained with the primer set for the Spiraeoideae-type *waaL*, but not with strains of *E. piriflorinigrans*, *E. billingiae* and *E. aphidicola* (Table 1). This confirms the close relationship between the LPS biosynthetic genes in the three species *E. amylovora*, *E. pyrifoliae* and *E. tasmaniensis* (Fig. 2) (Braun-Kiewnick *et al*., 2011; Smits *et al*., 2011), but also indicates a level of sequence divergence for the necrogenic, narrow-host-range *E. piriflorinigrans* (López *et al*., 2011; Smits *et al*., 2012, submitted), which resulted in the degeneration of the CFBP 1430-type *waaL* primer binding sites (Smits *et al*., 2012, submitted).

Significance of variation of LPS biosynthesis on the evolution of *Erwinia*

In this work, we have analysed the LPS biosynthetic gene cluster of a number of *Rubus*- and Spiraeoideae-infecting strains of *E. amylovora* whose host range has been defined experimentally (Braun and Hildebrand, 2005; Evans, 1996; Giorgi and Scortichini, 2005). The observed differences in LPS and EPS (this study; Maes *et al*., 2001; Mizuno *et al*., 2002) may contribute to this differential host range (Ries and Otterbacher, 1977; Starr *et al*., 1951). However, LPS is hardly the sole host specificity factor, as demonstrated by isolate PD2915, which has a *Rubus*-type *waaL*, but whose pathogenicity is restricted to *Amelanchier* (Giorgi and Scortichini, 2005).

The data obtained herein suggest that *Rubus*-infecting *E. amylovora* underwent a process of adaptation to the new host that also involved a gene replacement in the central region of their LPS biosynthetic gene cluster (Fig. 2). On the basis of the current dataset, we hypothesize that the critical event for adaptation to *Rubus* spp. must have taken place after species separation of *E. amylovora* and *E. pyrifoliae* (Fig. 3), as the Spiraeoideae-infecting isolates of *E. amylovora* and *E. pyrifoliae* (including Japanese strains), as well as *E. tasmaniensis* and *E. piriflorinigrans*, all share the Spiraeoideae-type LPS biosynthetic cluster. This hypothesis is supported by the findings of Asselin *et al*. (2011), who reported that Eop1 from Spiraeoideae-infecting strains ATCC 49946 and Ea110 more closely resembled Eop1 of *E. pyrifoliae* Ep1/96 and *Erwinia* sp. Ejp617 than Eop1 of the *Rubus*-infecting isolates ATCC BAA-2158, Ea510 and Ea644. These observations contradict the hypothesis based on CRISPR spacer analysis, where narrow diversity within the CRISPR repeat regions of Spiraeoideae-infecting strains (compared with *Rubus*-infecting strains) was interpreted as the outcome of an evolutionary bottleneck that occurred through selective enrichment of the Spiraeoideae genotype of *E. amylovora*, caused by the arrival of the domesticated apple (*Malus domestica*) in North America, from the broader genetic pool of *Rubus*-infecting strains (Rezzonico *et al*., 2011). By contrast, the distribution of LPS types in pathoadapted *Erwinia* spp. rather suggests that *Rubus*- and Spiraeoideae-infecting types of *E. amylovora* evolved from a common ancestor with Spiraeoideae-type LPS. These contrasting hypotheses require further study including a more diverse set of strains. Furthermore, the organization of the *E. billingiae* Eb661 LPS biosynthetic cluster, more related to the *Pantoea* spp. LPS biosynthetic cluster (De Maayer *et al*., 2010, 2012; Smits *et al*., 2010c), indicates that the Spiraeoideae-type cluster may have resulted from gene rearrangements at the level of the last common ancestor of the pathoadapted *Erwinia* species (Kamber *et al*., 2011; Smits *et al*., 2011).

The LPS biosynthetic gene cluster is one of the relatively few genetic differences observed between *Rubus*- and Spiraeoideae-infecting genotypes of *E. amylovora* (Powney *et al*., 2011b). Other differential factors, such as the presence and composition of an integrative conjugative element associated with the Hrp T3SS, have been described recently (Mann *et al*., 2012). However, these factors do not change the phylogenetic position of the *Rubus*-infecting strains that remain within the species *E. amylovora* (McManus and Jones, 1995; Powney *et al*., 2011b; Starr *et al*., 1951). This study shows that the LPS biosynthesis genes can be used as a diagnostic marker to distinguish *Rubus*-infecting strains of *E. amylovora* from Spiraeoideae-infecting isolates and other *Erwinia* spp., independent of their plant of origin.

EXPERIMENTAL PROCEDURES

Selection of *E. amylovora* strains

Nineteen strains of *E. amylovora* isolated from *Rubus* spp. across the USA and Canada, and 12 Spiraeoideae-infecting strains of *E. amylovora* representing all of the described CRISPR groups (Rezzonico *et al*., 2011) and genome-sequenced strains, were used for analysis in this study. Additional *Erwinia* species were included as outgroups in comparative analyses (Table 1). All strains were routinely grown and maintained on Luria–Bertani agar plates at 28 °C.

Lateral-flow immunoassays

Bacteria were grown overnight at 28 °C on King's B medium (King *et al*., 1954) agar and detected with the Ea AgriStrip (BIOREBA AG, Reinach, Switzerland) lateral-flow immunoassay using the protocol developed and

validated previously (Braun-Kiewnick *et al*., 2011). This assay is designed in a simple dip-stick format and is based on polyclonal antibodies raised against Spiraeoideae-infecting *E. amylovora*. Both test and control lines become visible after a few minutes with extracts containing the antigen, whereas negative samples produce the upper control line only.

DNA extraction, PCR amplification and sequencing

DNA was extracted from 1.5-mL aliquots of cultures grown overnight at 28 °C in LB broth with the Wizard® Genomic DNA Purification Kit (Promega, Dübendorf, Switzerland). Duplex PCR targeting the *waaL* gene was performed in a total volume of 10 μL using 0.3 mM of each of the four primers [*Rubus*-type *waaL*: IL5_lps-fw (5′-GTCCAGGCGATTAGTGAACA GATG-3′) and IL5_lps-rv (5′-CAGAATGGATGCCAGGTTCGCTCA-3′); CFBP 1430-type *waaL*: CFBP_lps-fw (5′-TATGCACGGTCAGGTAGCGTTTGG-3′) and CFBP_lps-rv (5′-GACGATAGTCGCCTATCTGCTTAC-3′)] in a final concentration of 1 × master mix of the HotStarTaq Master Mix Kit (Qiagen, Basle, Switzerland). Cycling conditions included an initial denaturation and activation of the HotStarTaq enzyme for 15 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and 90 s of elongation at 72 °C, ending with a final elongation for 10 min at 72 °C. Positive amplification and the size of the PCR amplicons obtained were verified by loading 5 μL of each reaction on a 1.8% agarose gel. Products of 442 bp and 506 bp were expected for *Rubus*- and Spiraeoideae-infecting strains, respectively.

A 1086-bp region of the *rpoB* gene was amplified in all *E. amylovora* isolates with primers CM7-F (5′-AACCAGTTCCGCGTTGGCCTG-3′) and CM31b-R (5′-CCTGAACAACACGCTCGGA-3′) (Brady *et al*., 2008) using the same PCR conditions as described above, except that the annealing temperature was set to 55 °C. PCR products were purified using a MultiScreen PCR plate (Millipore, Molsheim, France) and sequenced directly employing an ABI Prism BigDye Terminator v1.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with the same primers as used for amplification.

Sequence analysis

Comparative analysis of the genome sequences of *E. amylovora* strains CFBP 1430 (GenBank accession number: FN434113), ATCC 49946 (FN666575), ATCC BAA-2158 (FR719181 to FR719212), Ea644 and MR-1 (R. A. Mann *et al*., unpublished) was performed with Mauve in progressive mode (Darling *et al*., 2004) and EDGAR (Blom *et al*., 2009) using the settings described previously (Smits *et al*., 2010b). The genomes of related species *E. pyrifoliae* DSM 12163^T (FN392235), *E. tasmaniensis* Et1/99^T (CU468135) and *E. billingiae* Eb661^T (FP236843) were included in the analysis as outgroups. Sequence manipulations were conducted with multiple subroutines of the LASERGENE package (DNASTAR, Madison, WI, USA).

The phylogenetic tree was generated on the basis of a 952-bp fragment of the *rpoB* amplicon. DNA sequences were aligned with CLUSTALW (Thompson *et al*., 1994). Sites presenting alignment gaps were excluded from analysis. The Molecular Evolutionary Genetics Analysis (MEGA) program, version 4.0 (Tamura *et al*., 2007), was used to calculate evolutionary distances and to infer a tree based on the minimum evolution method with the maximum composite likelihood model. Nodal robustness of the tree was assessed by 1000 bootstrap replicates.

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SUPPORTING INFORMATION

Additional Supporting information may be found in the online version of this article:

Fig. S1 Evolutionary relationship between genome-sequenced *Erwinia amylovora* isolates based on multilocus sequence typing fragments for the *atpD* gene (642 bp) (A), *gyrB* gene (742 bp) (B), *infB* gene (615 bp) (C), *rpoB* gene (962 bp) (D) and a concatenated sequence of all four genes (2961 bp) (E). The evolutionary history was inferred using the minimum evolution method. Distances were computed implementing the maximum composite likelihood model and are in units of the number of base substitutions per site. Bootstrap values (1000 replicates) are shown next to the branches. S, Spiraeoideae-infecting isolates; R1–R3, *Rubus*-infecting isolates.

Fig. S2 Estimates of evolutionary relatedness within *Erwinia amylovora* and in related *Erwinia* spp. in the lipopolysaccharide (LPS) operons *waaC-waaF-waaD* and *walW-waaG-waaQ* (top table),

and *yigQ-yibP* and *kdtB-kdtX-kdtA* (bottom table). Sequence similarity is expressed as the percentage of identical residues in the pairwise alignment.

Table S1 Taxonomic position of *Erwinia amylovora* natural host plants within the Rosaceae family (Potter *et al*., 2007) and the infecting *E. amylovora waaL* genotypes. Although around 200 species in 40 rosaceous genera have been reported (van der Zwet and Keil, 1979), these are the major hosts for natural infections (Momol and Aldwinckle, 2000). S, Spiraeoideae-infecting isolates; R1–R3, *Rubus*-infecting isolates. Bold letters indicate that isolates from these taxa were included in this study.