Phylogenetic evidence for extensive horizontal gene transfer of type III secretion system genes among enterobacterial plant pathogens

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This study uses sequences from four genes, which are involved in the formation of the type III secretion apparatus, to determine the role of horizontal gene transfer in the evolution of virulence genes for the enterobacterial plant pathogens. Sequences of *Erwinia*, *Brenneria*, *Pectobacterium*, *Dickeya* and *Pantoea* were compared (a) with one another, (b) with sequences of enterobacterial animal pathogens, and (c) with sequences of plant pathogenic γ and β proteobacteria, to evaluate probable paths of lateral exchange leading to the current distribution of virulence determinants among these micro-organisms. Phylogenies were reconstructed based on *hrcC*, *hrcR*, *hrcJ* and *hrcV* gene sequences using parsimony and maximum-likelihood algorithms. Virulence gene phylogenies were also compared with several housekeeping gene loci in order to evaluate patterns of lateral versus vertical acquisition. The resulting phylogenies suggest that multiple horizontal gene transfer events have occurred both within and among the enterobacterial plant pathogens and plant pathogenic γ and β proteobacteria. *hrcJ* sequences are the most similar, exhibiting anywhere from 2 to 50% variation at the nucleotide level, with the highest degree of variation present between plant and animal pathogen sequences. *hrcV* sequences are conserved among plant and animal pathogens at the N terminus. The C-terminal domain is conserved only among the enterobacterial plant pathogens, as are the *hrcC* and *hrcR* sequences. Additionally, *hrcJ* and *hrcV* sequence phylogenies suggest that at least some type III secretion system virulence genes from enterobacterial plant pathogens are related more closely to those of the genus *Pseudomonas*, a conclusion neither supported nor refuted by *hrcC* or *hrcR*.

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INTRODUCTION

Type III secretion systems (T3SSs) are used by a wide array of plant and animal pathogenic Gram-negative bacteria to secrete and inject virulence factors directly into the cytosol of eukaryotic host cells (Galan & Collmer, 1999; Madden *et al.*, 2001). The T3SSs of *Yersinia*, *Escherichia*, *Salmonella* and *Shigella* have been extensively studied largely due to the potentially devastating health effects that these pathogens pose to human life (Galan & Collmer, 1999; Groisman, 2001; Tampakaki *et al.*, 2004). Interestingly, these same pathogenicity systems are also used by plant pathogens (e.g. *Erwinia*, *Pseudomonas*, *Xanthomonas* and

Ralstonia) to infect numerous agriculturally important plants (Chen, 2006; Shumann, 1991; van der Zwet & Beer, 1995), sometimes leading to millions of US dollars worth of losses each year among certain agricultural commodities.

T3SSs encode approximately 20–25 gene products, nine of which are conserved among plant and animal pathogens and are used in the formation of the needle complex, a unique T3SS structure (Fig. 1a) that is characteristic of this contact-dependent secretion mechanism (Bogdanove *et al.*, 1996; Gophna *et al.*, 2003). Of these nine conserved gene products, one is an outer membrane protein (*hrcC*), one is periplasmic (*hrcJ*), likely serving as a connector of the inner and outer membrane components of the apparatus, four are integral inner membrane proteins (*hrcR*, *S*, *T* and *U*) with periplasmic extensions, participating in the rod formation of the apparatus, and three are peripheral cytoplasmic proteins (*hrcV*, *Q* and *N*) involved in initiating secretion of effectors from the cytoplasm (Tampakaki *et al.*, 2004). These nine structural components are so conserved among plant and animal pathogens that plant pathogenic

Abbreviations: GARLI, genetic algorithm for rapid likelihood inference; HGT, horizontal gene transfer; ILD, incongruence length difference; ML, maximum likelihood; MP, maximum parsimony; T3SS, type III secretion system.

The GenBank/EMBL/DDBJ accession numbers for the *hrcC*, *hrcJ*, *hrcR* and *hrcV* sequences of the strains examined in this study are EU561240–EU561268, EU561269–EU561288, EU561289–EU561316 and EU561317–EU561337, respectively.

Fig. 1. (a) Schematic representation of the type III secretion needle complex used by Gram-negative plant and animal pathogenic micro-organisms. The four genes used to construct the phylogenies presented in this study are integral components of the needle complex, interacting with both the bacterial cell membranes and the chaperone–effector molecules secreted into the host cells. (b) Schematic representation of the characterized *hrp/hrc* operons as described for *Erwinia amylovora* (Oh *et al.*, 2005). For visual clarity, oblique lines were added to the figure to denote the removal of T3SS chromosomal segments not related to the four *hrc* genes analysed here.

T3SS proteins of *Xanthomonas* have been shown to secrete virulence effectors (Yops) of *Yersinia* species *in vitro* (Rossier *et al.*, 1999).

The T3SSs of both plant and animal pathogens are located in general on pathogenicity islands (PAIs), which appear to be horizontally transferred among disparate micro-organisms (Groisman & Ochman, 1996). These PAIs are found mostly on plasmids among the enterobacterial animal pathogens. Conversely, they appear to have been integrated into the bacterial genomes of their enterobacterial plant pathogenic counterparts (Hacker *et al.*, 1997; Oh *et al.*, 2005; Toth *et al.*, 2006). The pathogenicity island of *Erwinia amylovora*, which has been recently characterized (Oh *et al.*, 2005), consists of a 62 kb region with 60 ORFs. A region of approximately 22 kb encodes the proteins of the T3SS in three operons: *hrpA*, *hrpC* and *hrpJ* (Fig. 1b; Oh *et al.*, 2005).

This study focuses on species of the genus *Erwinia* and the closely related genera *Brenneria*, *Pectobacterium*, *Dickeya* and *Pantoea*. This economically important group of enterobacterial plant pathogens is distributed worldwide, and they vary widely in their ecology, host range tropism, and pathogenicity (Krieg & Holt, 1985). The strains used in this study have been well characterized taxonomically and their evolutionary history has been described based on numerous housekeeping genes (Brown *et al.*, 2000; Hauben *et al.*, 1998; Naum *et al.*, 2008; Young & Park, 2007). For example, Fig. 2(a) depicts a *de novo* evolutionary reconstruction of enterobacterial plant pathogenic species based on 16S rDNA gene sequences (Naum *et al.*, 2008). To

understand the role of horizontal gene transfer (HGT) of T3SS genes among the enterobacterial plant pathogens, we analysed sequences from four genes, *hrcC*, *hrcR*, *hrcJ* and *hrcV*. These four genes represent each of the three characterized operons (Oh *et al.*, 2005), and encode proteins which serve different functional purposes (Fig. 1b).

METHODS

Sampling. A total of 29 strains were included in the phylogenetic analysis of the *hrcC* sequences: 28 isolates were included in *hrcR* and *hrcV* analysis, and 52 samples were included in the *hrcJ* analysis (Table 1). Gene sequences for the enterobacterial plant pathogens were generated in our laboratory with the exception of the two *Erwinia pyrifoliae* sequences, which along with the remaining samples were downloaded from GenBank. The *hrcJ* dataset included the broadest sample of taxa because sequences for this gene were alignable across plant and animal pathogenic representatives. Strains of *Erwinia*, *Brenneria*, *Pectobacterium*, *Dickeya* and *Pantoea* were obtained from the American Type Culture Collection (ATCC), United States Department of Agriculture (USDA) (Appalachian Fruit Research Center, Kearneysville, WV), and from Dr Amy Charkowski (University of Wisconsin, Madison) as frozen cultures and Luria–Bertani (LB) slants respectively. Sample authentication was confirmed based on 16S rDNA sequence analysis prior to their use (Naum *et al.*, 2008).

Bacterial DNA extraction and PCR amplification. Total genomic DNA was extracted using a silica-based matrix (Instagene, Bio-Rad) as described in the manufacturer's directions, which allows for total chromosomal isolation from Gram-negative bacteria. Briefly, bacterial cells from 24 h cultures grown on LB agar at room temperature were resuspended in Instagene matrix and heated at 56 °C for 30 min.

Fig. 2. (a) A *de novo* reconstructed model of a 16S rDNA gene phylogeny encompassing the same enterobacterial plant pathogenic strains as those used in this study. To simplify this schematic representation we indicate only the genus and not the species names. (b) Redrawing of previously published relationships of the plant pathogenic enterobacteria based on a comparative analysis of concatenated alignments of *atpD*, *carA* and *recA* sequences using the likelihood algorithm (Young & Park, 2007). (c) ML tree resulting from the phylogenetic analysis of the concatenated *gapA/gyrA* sequence dataset consisting of 899 nucleotide positions, produced in GARLI and viewed in PAUP*. ML bootstrap nodal support was generated in PAUP*, and subsequent values are reported for each node in parentheses. Abbreviations for the taxa used are as follows: *Erwinia*, Er; *Brenneria*, Br; *Pectobacterium*, Pe; *Dickeya*, Di; *Pantoea*, Pa; *Citrobacter*, Ci; *Enterobacter*, En; *Escherichia*, Es; *Ewingella*, Ew; *Hafnia*, Ha; *Klebsiella*, Kl; *Photobacterium*, Ph; *Proteus*, Pr; *Rhanella*, Rh; *Salmonella*, Sa; *Serratia*, Se; *Shigella*, Sh; *Xenorhabdus*, Xe; *Yersinia*, Ye; *Haemophilus*, H. Multiple strains are denoted as 1 or 2 in the order that they have been listed in Table 3. For legibility purposes we have not italicized species names. Additionally, the plant pathogenic genera have been colour-coded for visual clarity, with *Erwinia* in red, *Brenneria* in blue, *Pectobacterium* in green, *Pantoea* in yellow and *Dickeya* in dark green.

The preparations were then vortexed vigorously and boiled for 10 min. Suspensions were pelleted and the supernatant containing the DNA was decanted into a clean microtube. The optimum amount of DNA to be used in PCR amplification reactions was determined to be 2 µl, after an optimization PCR was carried out using 20, 10, 5, 2 and 1 µl of DNA. Oligonucleotide primers were designed for amplification of the *hrcI*, *hrcV*, *hrcC* and *hrcR* genes from conserved sequences flanking variable regions of the genes, using the few available *Erwinia*, *Brenneria* and *Pectobacterium* sequences from GenBank. The primers used were: *hrcCF* (5'-CTG GAG CAC CAT TTC CAG TGG T-3') and

hrcCR (5'-CCT TCC AGC GCC TGG ATA TCG G-3'); *hrcRF* (5'-CCA ATA TGG CGC TGT ACG GCA-3') and *hrcRR* (5'-GCC GTC GAG CAG SCG GGT CCA-3'); *hrcJF* (5'-AAC GAT GCC AAC GAA G-3') and *hrcJR* (5'-GGG ACA AAG ACA ATC GCC A-3'); and *hrcVF* (5'-CAT GAT CAT CAT TCC GCT GCC CA-3') and *hrcVR* (5'-AGC TCT TGC GCC AGC TCT GGC TGT TCG CT-3'). PCR (Ehrlich *et al.*, 1991) was carried out with 2 µl DNA template, 2.5 µl 10 × PCR buffer (Sigma), 1.5 µl 25 mM MgCl₂, 2 µl 2.5 mM dNTPs, 2.5 µl of each 10 µM primer stock, 0.1 µl of 5 U µl⁻¹ *Taq* DNA polymerase (Sigma), and purified distilled H₂O for a total reaction

Table 1. Bacterial strains and accession numbers for gene sequences used in the phylogenetic analyses

Taxon	Strain	Accession number			
		<i>hrcJ</i>	<i>hrcV</i>	<i>hrcC</i>	<i>hrcR</i>
<i>Erwinia</i>					
<i>Er. amylovora</i>	178	EU561269	EU561317	EU561245	EU561289
<i>Er. amylovora</i>	552		L25828		
<i>Er. mallotivora</i>	8645	EU561270	EU561319	EU561240	EU561300
<i>Er. mallotivora</i>	8646	EU561271	EU561320	EU561246	EU561308
<i>Er. rhapontici</i>	1	EU561272	EU561321	EU561243	EU561292
<i>Er. rhapontici</i>	29283*	EU561273	EU561322	EU561244	EU561293
<i>Er. lupinicola</i>	wl31	EU561274	EU561323	EU561241	EU561298
<i>Er. lupinicola</i>	348	EU561275		EU561242	EU561299
<i>Er. psidii</i>	8423	EU561276	EU561324	EU561247	EU561309
<i>Er. psidii</i>	8429	EU561277	EU561325	EU561248	EU561310
<i>Er. tracheiphila</i>	5845			EU561253	EU561306
<i>Er. tracheiphila</i>	33245*				EU561307
<i>Er. pyrifoliae</i>	DSM12163	DQ180962	DQ180962		
<i>Brenneria</i>					
<i>Br. nigrifluens</i>	1391	EU561279	EU561327	EU561251	EU561290
<i>Br. nigrifluens</i>	4789	EU561280	EU561328	EU561252	EU561291
<i>Br. alni</i>	Pfv20*	EU561281		EU561255	EU561303
<i>Br. salicis</i>				EU561254	
<i>Br. rubrifaciens</i>	9136			EU561256	EU561313
<i>Br. rubrifaciens</i>	5950				EU561311
	29291*			EU561249	EU561312
<i>Br. quercina</i>	1846			EU561250	EU561301
<i>Br. quercina</i>	1895				EU561302
<i>Pectobacterium</i>					
<i>Pe. carotovorum</i>					
subsp. <i>carotovorum</i>	4	EU561282	EU561329	EU561257	EU561314
subsp. <i>carotovorum</i>	15713*	EU561283	EU561330	EU561258	EU561297
subsp. <i>brasiliensis</i>	1692	EU561284	EU561331	EU561261	EU561304
subsp. <i>brasiliensis</i>	1695	EU561285	EU561332	EU561262	EU561305
subsp. <i>odoriferum</i>	SCRI 482	EU561286	EU561334	EU561264	EU561294
<i>Pe. betavascularum</i>	Ecb1			EU561259	EU561295
<i>Pe. betavascularum</i>	Ecb2			EU561260	EU56129
<i>Pe. atrosepticum</i>	SCRI 1043	EU561287	EU561333	EU561267	EU561316
<i>Dickeya</i>					
<i>Di. dadantii</i>	11663*		EU561335	EU561265	EU561315
<i>Di. dadantii</i>	EC16			EU561266	
<i>Pantoea</i>					
<i>Pa. agglomerans</i>	33243*		EU561336		
<i>Pa. stewartii</i>	8199	EU561288	EU561337	EU561268	
<i>Pseudomonas</i>					
<i>Ps. syringae</i>	B728a	NC_007005	NC_007005		
<i>Ps. syringae</i> 1	1448A	NC_005773			
<i>Ps. syringae</i> 2	DC3000	AE016853			
<i>Ps. syringae</i> 3	LMG5090	DQ246442			
<i>Ps. syringae</i> 4	ATCC115288	AB049570			
<i>Ps. mendocina</i>	ymp	CP000680			
<i>Ps. cichorii</i>	83-1	DQ168848			
<i>Ps. viridiflava</i>	RMX3.1b	AY597283			
<i>Ps. viridiflava</i>	RMX23.1a	AY597282			
<i>Ps. viridiflava</i>	ME3.1b	AY597281			
<i>Xanthomonas</i>					
<i>Xa. campestris</i>	pv. <i>vesicatora</i>	AM039952	AM039952		
<i>Xa. campestris</i> 1	85-10	NC_007508			
<i>Xa. campestris</i> 2	80004	NC_007086			

Table 1. cont.

Taxon	Strain	Accession number			
		<i>hrcJ</i>	<i>hrcV</i>	<i>hrcC</i>	<i>hrcR</i>
<i>Xa. campestris</i> 3	ATCC 33913	NC_003902			
<i>Xa. axonopodis</i>	306	NC_003919			
<i>Xa. oryzae</i> 1	BLS256	AY875714			
<i>Xa. oryzae</i> 2	MAFF311018	AP008229			
<i>Xa. oryzae</i> 3	KACC10331	AB115081			
<i>Ralstonia</i>					
<i>Ra. solanacearum</i>	GMI1000	AL646053	AL646053		
<i>Ra. solanacearum</i> 1	IPO1609	AJ245811			
<i>Ra. solanacearum</i> 2		NC_003296			
<i>Escherichia</i>					
<i>Es. coli</i>	042	AF200363	AF200363		
<i>Es. coli</i> 1	O157:H7	NC_002695			
<i>Es. coli</i> 2	E2348/69	AF022236			
<i>Es. coli</i> 3	101-1	AJ303141			
<i>Es. coli</i> 4	0181-6/86	AJ633129			
<i>Salmonella</i>					
<i>Sa. enterica</i>	SC-B67	NC_006905			
<i>Sa. enterica</i> 1	CT18	AL627271			
<i>Sa. typhimurium</i>	LT2	NC_003197	NC_003197		
<i>Yersinia</i>					
<i>Ye. pestis</i>	CO92	AL117189			
<i>Ye. enterocolitica</i>	8081	AM286415			

*Indicates the type culture for each of the species represented as originally obtained from ATCC.

volume of 25 µl. PCR was performed in a PTC-200 thermal cycler (MJ Research) under the following conditions: initial denaturation at 94 °C for 2 min, 94 °C for 50 s, 58 °C for 50 s and 72 °C for 45 s (35 cycles), ending with incubation at 72 °C for 8 min. The amplified segments were ~850 bp long for *hrcC*, ~500 bp long for *hrcR*, ~500 bp long for *hrcJ*, and ~1500 bp long for *hrcV*.

Cloning and DNA sequencing. PCR products were purified using QIAquick gel extraction kits (Qiagen) according to the manufacturer's protocol, except that DNA was eluted in 20 µl buffer. Cloning reactions were completed using pGEM T Easy cloning kits (Promega) according to the manufacturer's protocol, except that the ligation and transformation reaction volumes were halved. Cloned inserts were amplified directly from colonies using the original PCR primers and amplification conditions. PCR products were cleaned using exonuclease I and shrimp alkaline phosphatase as in Mason-Gamer (2004). Purified PCR products were sequenced in both directions using an automated Sanger dideoxy-chain-termination method (Amplicon Express). DNA sequences were edited and combined in Sequencher v.4.1 (Gene Codes Corporation).

Sequence alignment and phylogenetic analyses. DNA sequences and resultant computer-translated amino acid sequences (*hrcC* and *hrcR*) were aligned using CLUSTAL W v.1.5 (Thompson *et al.*, 1994), followed by manual adjustments in MacClade v.4.0 (Maddison & Maddison, 2000). Amino acid codon coloration was used in MacClade v.4.0 (Maddison & Maddison, 2000) on the nucleotide datasets to ensure the resulting alignments did not alter the protein sequence. Regions judged to have an ambiguous alignment were excluded (~400 bp for *hrcC*, 45%; ~120 bp for *hrcR*, 21%; and ~590 bp for *hrcV*, 40%) prior to the phylogenetic analyses. All

datasets were first analysed in PAUP* (Swofford, 2001) using the principle of maximum parsimony (MP) under heuristic search methods using tree bisection-reconnection (TBR) branch-swapping, and 100 random taxon addition replicates. For the nucleotide datasets, the shortest trees were then used as the starting topology for the evaluation of 16 nested models of sequence evolution (Frat *et al.*, 1997; Sullivan *et al.*, 1997; Swofford *et al.*, 1996). The best-fit model of substitution for each dataset was determined by the likelihood score of each under a chi-squared approximation of the null distribution (Yang *et al.*, 1995). Parameter space was searched for the best tree with simultaneous estimation for model parameters using a maximum likelihood (ML) search conducted using the Genetic Algorithm for Rapid Likelihood Inference (GARLI) software (Zwickl, 2006). As recommended by Zwickl (2006), multiple runs were performed (~20) to ensure that results were consistent, as the algorithm is inherently stochastic. The log-likelihood values of each run were retained in order to compare the individual runs, and the tree and model parameters corresponding to the best score were used.

Branch support was determined in GARLI by 100 ML bootstrap iterations (Felsenstein, 1985), and with Bayesian posterior probability (MrBayes v.3.1.2) approximation of 1 000 000 generations, discarding 25 % (2500) of the tree samples as recommended in the MrBayes v.3.1.2 manual (Huelsenbeck & Ronquist, 2001). For Bayesian character support methods, parameters of sequence evolution estimated from the final ML tree were used (Table 2). Amino acid character support was determined in PAUP* by 1000 bootstrap iterations and 1 000 000 generations of Bayesian (MrBayes v.3.1.2; (Huelsenbeck & Ronquist, 2001) posterior probability approximation under the principle of MP, again discarding 25 % (2500) of the tree samples.

Table 2. ML model parameters used in the phylogenetic analyses of the nucleotide data

Gene	Model*	Base frequencies	Rate matrix	I + Γ
<i>hrcJ</i>	GTR + Γ	A,C,G,T (0.226665, 0.271862, 0.305593, 0.195877)	AC,AG,AT,CG,CT,GT (1.05305, 2.34379, 0.63654, 0.97192, 3.64812, 1.00000)	NA, 0.692249
<i>hrcV</i>	GTR + I + Γ	A,C,G,T (0.185737, 0.305335, 0.285268, 0.223658)	AC,AG,AT,CG,CT,GT (1.75130, 4.17764, 1.35511, 1.40323, 5.02710, 1.00000)	0.017862, 0.43826
<i>hrcC</i>	GTR + I + Γ	A,C,G,T (0.213890, 0.295173, 0.287391, 0.203006)	AC,AG,AT,CG,CT,GT (1.48399, 3.94858, 0.71867, 1.03759, 7.14812, 1.00000)	0.190209, 0.672602
<i>hrcR</i>	GTR + I + Γ	A,C,G,T (0.212377, 0.305330, 0.243388, 0.238905)	AC,AG,AT,CG,CT,GT (1.48844, 4.45970, 0.79636, 2.22929, 5.79619, 1.00000)	0.346505, 1.601213

*GTR, general time reversible; I, proportion of invariable sites; Γ , number of gamma distribution rate categories; NA, not available.

Nucleotide gene trees were rooted by midpoint rooting, rather than outgroup rooting (Schuettelpelz & Hoot, 2006), because the homologous gene sequences from the closest available outgroups were too divergent to align reliably. The *hrcC* and *hrcR* amino acid sequence trees were rooted with *Escherichia coli* (GenBank accession numbers AJ303141 and AF022236) and *Yersinia pestis* (GenBank accession number NC_004839), respectively. The absence of suitable outgroup taxa impeded the detection of the direction of certain HGT events between and among plant and animal pathogenic taxa. However, in these specific instances, direction was addressed inferentially.

Housekeeping gene analysis for determination of strain evolution. To determine phylogenetic incongruence in relation to HGT we included three housekeeping gene trees in this study in order to provide a vertically inherited evolutionary framework to which *hrc* gene phylogenies were compared. Fig. 2 shows a *de novo* reconstructed model of a 16S rDNA gene phylogeny encompassing the same enterobacterial plant pathogenic strains used in this study, with only the genus names listed to simplify this schematic representation (Naum *et al.*, 2008). Fig. 2(b) shows a redrawing of the concatenated housekeeping gene phylogeny presented by Young & Park (2007) based on *atpD*, *carA* and *recA* gene sequences, with representatives of the same *Erwinia*, *Brenneria*, *Pectobacterium*, *Dickeya* and *Pantoea* species used in this study. Additionally, Fig. 2(c) is the phylogeny resulting from a concatenated analysis of *gapA* and *gyrA* gene sequences including 31 plant pathogen taxa, six animal pathogen representatives and one outgroup taxon (Table 3; Hauben *et al.*, 1998; Lelliott & Dickey, 1985; Starr & Chatterjee, 1972). Primers used for PCR amplification were *gapAF* (5'-TGA AAT ATG ACT CCA CTC ACG G-3'), *gapAR* (5'-TAG AGG ACG GGA TGA TGT TCT G-3'), *gyrAF* (5'-GCT GCG CGY CCW GAG GCT GGA C-3') and *gyrAR* (5'-CCA CGR CTG GCT TCC GGC A-3'), and amplification reactions were carried out following the protocol described above. The amplified segments were ~ 410 bp for *gapA* and ~ 489 bp for *gyrA*. DNA sequencing and phylogenetic analyses were carried out as described above, with the concatenated dataset encompassing 899 nucleotide positions.

Incongruence length difference (ILD) test. Statistical congruence between genes was assessed using the ILD test (Farris *et al.*, 1994) available in PAUP* (Swofford, 2001). Gene partitions were assembled as nexus files in interleaved format using the PAUP* text editor, such that each gene represented a single interleaved portion of the combined data matrix. In this fashion, data partitions were easily defined as the beginning and end of interleaved blocks of sequences. ILD comparisons were performed using the partition homogeneity test on the plant pathogenic strains shared among all eight gene datasets (*hrcJ*, *hrcV*, *hrcC*, *hrcR*, 16S rDNA, *gapA*, *gyrA* and *gapA*/

gyrA), with 1000 data partitions using heuristic searches (Allard *et al.*, 1999)

RESULTS

The *hrcC* DNA alignment consisted of 29 taxa and 855 characters, of which 458 were informative, whereas the amino acid alignment consisted of 31 taxa, including the outgroups, and 291 characters of which 150 were informative. The final ML search of the nucleotide data resulted in one tree (Fig. 3a), and the (most parsimonious) MP analysis of the amino acid data yielded 16 equally most parsimonious trees [272 steps; consistency index (CI)=0.87; rescaled consistency (RC)=0.81], from which the strict consensus was derived (Fig. 3b). The *hrcR* DNA alignment consisted of 28 taxa and 508 characters, of which 384 were informative, while the amino acid alignment consisted of 29 taxa and 170 characters, of which 133 were informative. The final ML search of the nucleotide data resulted in one tree (Fig. 4a), and the MP analysis of the amino acid data yielded two equally most parsimonious trees (175 steps; CI=0.93; RC=0.97), from which a strict consensus was derived (Fig. 4b). The *hrcJ* alignment consisted of 52 taxa and 454 characters after truncating the beginning and end of the dataset in order to eliminate gaps resulting from the absence of data due to varying sequence lengths. The GARLI tree with the best log-likelihood score for *hrcJ* is presented in Fig. 5(a). The *hrcV* alignment consisted of 28 taxa and 1536 characters, of which only the first 910 were used in the phylogenetic analysis because the remaining 630 base pairs could only be meaningfully aligned for the enterobacterial plant pathogens. After the 800th base pair approximately, the sequences of *Pseudomonas*, *Xanthomonas*, *Ralstonia*, *Escherichia*, *Salmonella* and *Yersinia* began diverging from the sequences of *Erwinia*, *Brenneria*, *Pectobacterium*, *Dickeya* and *Pantoea*, and by the 910th nucleotide position, they could no longer be aligned. The GARLI tree with the best score for *hrcV* is presented in Fig. 5(b).

The concatenated *gapA*/*gyrA* alignment consisted of 38 taxa and 899 characters after truncating the beginning and end of the dataset in order to eliminate gaps resulting from the

Table 3. Bacterial strains and accession numbers for gene sequences used in the housekeeping gene phylogenetic analyses

Taxon	Strain number	Accession number	
		<i>gapA</i>	<i>gyrA</i>
<i>Erwinia</i>			
<i>Er. amylovora</i>	(Ea)178	EU340558	EU340596
<i>Er. rhapontici</i>	1, ATCC 29283*	EU340561, EU340562	EU340599 (1)
<i>Er. mallotivora</i>	(Em)8645, (Em)8646	EU340559, EU340560	EU340597, EU340598
<i>Er. psidii</i>	8423, (Ep)8429	EU340565, EU340566	EU340600, EU340601
<i>Brenneria</i>			
<i>Br. rubrifaciens</i>	(Br)5951, 29291*	EU340569, EU340570	EU340607 (1)
<i>Br. nigrifluens</i>	(En)1391, 4789	EU340571, EU340572	EU340604, EU340605
<i>Br. alni</i>	pvf120*	EU340574	EU340606
<i>Br. quercina</i>	(Bq)1846, 1895	EU340575, EU340676	EU340602, EU340603
<i>Pectobacterium</i>			
<i>Pe. carotovorum</i>			
subsp. <i>carotovorum</i>	Eca4, 15713*	EU340579, EU340580	EU340610, EU340611
subsp. <i>brasiliensis</i>	1692, 1695	EU340583, EU340584	EU340614, EU340615
subsp. <i>odoriferum</i>	SCRI482	EU340589	EU340620
<i>Pe. betavasculorum</i>	ECB1, ECB2	EU340581, EU340582	EU340612, EU340613
<i>Pe. atrosepticum</i>	SCRI1043, WPP158	EU340587, EU340588	EU340618, EU340619
<i>Pe. wasabiae</i>	WPP163, SCRI488	EU340585, EU340586	EU340616, EU340617
<i>Pe. cypripedii</i>	29267*, 29269	EU34057, EU340577	EU340608, EU340609
<i>Dickeya</i>			
<i>Di. dadantii</i>	11663*, EC16	EU340590, EU340591	EU340621, EU340622
<i>Pantoea</i>			
<i>Pa. agglomerans</i>	33243*	EU340592	EU340623
<i>Pa. stewartii</i>	8199, 29227	EU340593, EU340594	EU340624, EU340625
<i>Pa. ananatis</i>	11530, 19321	EU340595	EU340626, EU340627
Other			
<i>Shigella flexneri</i>	5 strain 8401	NC_008258	NC_008258
<i>Escherichia coli</i>	UTI89	NC_007946	NC_007946
<i>Salmonella Typhimurium</i>	LT2	NC_003197	NC_003197
<i>Salmonella Typhi</i>	Ty2	NC_004631	NC_004631
<i>Klebsiella pneumoniae</i>	ATCC 13883	M66869	X16817
<i>Serratia marcescens</i>	DB11	M63373	U56906
Outgroup taxon			
<i>Haemophilus influenzae</i>	86-028NP	NC_007146	NC_007146

*Indicates the type culture for each of the species represented as originally obtained from ATCC.

absence of data due to varying sequence lengths. The GARLI tree with the best log-likelihood score for *gapA*/*gyrA* is presented in Fig. 2(c).

Erwinia, *Brenneria* and *Pectobacterium* are non-monophyletic on the *hrcC* and *hrcR* phylogenies, a conclusion strongly supported by clades C and D (Figs 3a and 4a). Both clades contain multiple *Erwinia*, *Brenneria* and *Pectobacterium* taxa with bootstrap support values of 90 and 98% for clades C and D, respectively. In addition, strains of *Erwinia mallotivora* 1 and 2, *Pectobacterium carotovorum* subsp. *carotovorum* 1 and 2, and *Pectobacterium betavasculorum* 1 and 2 are broken up, each being more closely related to other *Erwinia* and *Pectobacterium* species than to each other. We resequenced *hrcC* and *hrcR* for *Erwinia tracheiphila* 1 and *Brenneria salicis* 1 to confirm that the original sequences were not

artefacts, since in the *hrcC* phylogeny, the sequences are indistinguishable and on a long branch (Fig. 3a). Even though the *hrcC* and *hrcR* reconstructions include only enterobacterial plant pathogen representatives, we suggest a monophyletic origin of the virulence determinants comprising their T3SS because of the extensive divergence between the sequences of *Erwinia*, *Brenneria*, *Pectobacterium*, *Dickeya* and *Pantoea* from sequences of the enterobacterial animal pathogens and plant pathogenic γ and β proteobacteria, at both the nucleotide and the amino acid levels.

The *hrcJ* and *hrcV* trees (Fig. 5) are generally congruent (Table 4), indicating a substantial degree of evolutionary and genetic linkage between these loci. The relationships are largely unaffected by the number of representative sequences, which differs between the two phylogenies

Fig. 3. (a) ML tree comprising *hrcC* gene sequences from 29 *Erwinia*, *Brenneria*, *Pectobacterium*, *Dickeya* and *Pantoea* strains. The amplified products encompass the 363rd nucleotide to the 1059th nucleotide based on the *Pectobacterium atrosepticum* genome (NC_004547). ML solutions were generated in GARLI and visualized in PAUP*. ML bootstrap nodal support was generated in PAUP*, and subsequent values are reported for each node in parentheses. Bayesian nodal support values are presented for all nodes without parentheses. (b) Strict consensus tree of the 16 equally most parsimonious trees resulting from the *hrcC* amino acid dataset. Species abbreviations for the taxa used are as follows: *Erwinia*, Er; *Brenneria*, Br; *Pectobacterium*, Pe; *Dickeya*, Di; *Pantoea*, Pa; *Escherichia*, Es; *Yersinia*, Ye. Multiple strains are denoted as 1 or 2 in the order that they have been listed in Table 1. For legibility purposes we have not italicized species names.

because *hrcJ* sequences were more conserved, allowing us to include a greater number of animal pathogen and γ and β proteobacterial plant pathogen representatives. Both trees place *Xanthomonas* and *Ralstonia* as sisters to one another and to the other plant pathogens, and they also group *Escherichia* and *Salmonella* together, and as a sister group to *Yersinia*. Moreover, the trees differentiate the plant and animal pathogens into two well-defined clades. Furthermore, both phylogenies group *Erwinia*, *Brenneria*, *Pectobacterium*, *Dickeya* and *Pantoea* as closest relatives, with *Pseudomonas syringae* as their sister group. Additionally, the enterobacterial plant pathogens form two well-supported clades, designated A and B (Fig. 5a). Clade A consists of *Pectobacterium* and *Brenneria* species, along with two strains of *Erwinia rhapontici*, while clade B is dominated by *Erwinia* and *Pantoea*. However, in the *hrcV* tree (Fig. 5b), *Erwinia lupinicola* 1 is placed outside both clade A and clade B (although this position is not well supported), while *P. carotovorum* subsp. *carotovorum* 1 is found in clade B. Taken together, these results suggest that the *hrcJ* and *hrcV* virulence genes of the enterobacterial

plant pathogens and *Pseudomonas* share a recent common ancestor.

Furthermore, the *hrcJ* and *hrcV* trees are congruent to one another with respect to the resulting relationships between the enterobacterial plant pathogens (Table 4). The trees are also consistent in the placement of *Erwinia*, *Brenneria*, *Pectobacterium*, *Dickeya* and *Pantoea* with the other plant pathogens and not with the enteric animal pathogens, a result in agreement with and expanding on a previous study of *hrcJ* sequences (Brown *et al.*, 1998). It is notable that the *hrcC* and *hrcR* trees are incongruent to one another and to both the *hrcJ* and *hrcV* phylogenies (Table 4), which could be the result of multiple interspecific HGT events among the enterobacterial plant pathogens after the acquisition of the T3SS into this lineage.

On the other hand, all of the housekeeping gene trees (Fig. 2) support multiple independent origins of the plant pathogenic genera within the *Enterobacteriaceae*, and all loci are consistent in their taxonomic classification of the four genera. Additionally, in all three housekeeping gene

Fig. 4. (a) ML tree comprising *hrcR* gene sequences from 28 enterobacterial plant pathogen species representatives. The amplified products encompass the 210th nucleotide to the 630th nucleotide based on the *Pectobacterium atrosepticum* genome (NC_004547). ML solutions were generated in GARLI and visualized in PAUP*. ML bootstrap nodal support was generated in PAUP*, and subsequent values are reported for each node in parentheses. Bayesian nodal support values are presented for all nodes without parentheses. (b) Strict consensus tree of the two equally most parsimonious trees resulting from the *hrcR* amino acid dataset. Species abbreviations for the taxa used are as follows: *Erwinia*, Er; *Brenneria*, Br; *Pectobacterium*, Pe; *Dickeya*, Di; *Pantoea*, Pa; *Escherichia*, Es; *Yersinia*, Ye. Multiple strains are denoted as 1 or 2 in the order that they have been listed in Table 1. For legibility purposes we have not italicized species names.

phylogenies, it is evident that housekeeping gene evolution – taken here to depict the vertical evolution of the strains analysed – contrasts with virulence gene evolution and does not support a single origin of a plant pathogenic ancestor followed by speciation into the numerous plant pathogenic genera and species.

Finally, ILD testing comparing the virulence genes with one another and with several housekeeping loci confirmed the hypothesis of incongruence ($P=0.001$, Table 4). Strikingly, each pairwise comparison of the virulence genes with the housekeeping loci resulted in a statistically significant P value ($P<0.05$, Table 4), leading us to reject the null hypothesis. This result indicates that the virulence determinants examined in this study have all been subject to some level of lateral transfer between the enterobacterial plant pathogens. The only gene dataset comparison for which we could not reject the null hypothesis (i.e. for which the dataset partitions were found to be congruent based on the ILD test) was between the *hrcJ* and *hrcV* partitions. These results support our hypothesis that both ancestral and recent HGT events have been driving the

evolution of virulence in the enterobacterial plant pathogens.

DISCUSSION

The nine proteins involved in forming the needle complex of the T3SS are conserved across multiple animal and plant pathogens and are believed to share a common ancestor with the flagellar export mechanism used by many microorganisms (Gophna *et al.*, 2003). However, it is evident from the results of this study that vertical acquisition (Fig. 2) has been interjected by numerous HGT events, which undeniably have been a driving force in shaping the evolutionary history of the enterobacterial plant pathogen virulence genes (Figs 3, 4 and 5).

Despite the sampling differences between the *hrcJ* and *hrcV* datasets, analyses of the conserved gene segments resulted in congruent phylogenies for the two genes (Fig. 5, Table 4), which are in conflict with the phylogenies generated from *hrcC* and *hrcR* gene sequences (Figs 3 and 4, Table 4). Unlike the *hrcJ* and *hrcV* datasets, the partial *hrcC* and *hrcR*

Fig. 5. (a) ML tree resulting from the phylogenetic analysis of the *hrcJ* sequence dataset including enterobacterial plant and animal pathogenic representatives, and non-enterobacterial plant pathogenic γ and β proteobacterial taxa. The amplified products encompass the 93rd nucleotide to the 414th nucleotide based on the *Pectobacterium atrosepticum* genome (NC_004547). (b) ML tree resulting from the phylogenetic analysis of the *hrcV* sequence dataset. The amplified products encompass the 124th nucleotide to the 1359th nucleotide of the *Pectobacterium atrosepticum* genome (NC_004547), with only the first 910 nucleotides (124–1034) included in the analyses. The resultant maximum trees for these datasets were produced in GARLI and viewed in PAUP*. ML bootstrap nodal support was generated in PAUP*, and subsequent values are reported for each node in parentheses. Bayesian nodal support values are presented for all nodes without parentheses. Species abbreviations for the taxa used are as follows: *Erwinia*, Er; *Brenneria*, Br; *Pectobacterium*, Pe; *Dickeya*, Di; *Pantoea*, Pa; *Pseudomonas*, Ps; *Xanthomonas*, Xa; *Ralstonia*, Ra; *Escherichia*, Es; *Salmonella*, Sa; *Yersinia*, Ye. Multiple strains are denoted as 1 or 2 in the order that they have been listed in Table 1. For legibility purposes we have not italicized species names.

gene sequences we studied were highly variable, much like the C-terminal region of *hrcV*, and only sequences from *Erwinia*, *Brenneria*, *Pectobacterium*, *Dickeya* and *Pantoea* could be meaningfully aligned. In addition, because the taxonomic placement of *Erwinia*, *Brenneria*, *Pectobacterium*, *Dickeya* and *Pantoea* in the family *Enterobacteriaceae* has been confirmed by analysis of multiple housekeeping genes (Fig. 2; Brown *et al.*, 2000;

Hauben *et al.*, 1998; Young & Park, 2007), it is striking that the *hrcJ* and *hrcV* trees (Fig. 5) reveal that the enterobacterial plant pathogens likely acquired their *hrcJ* and *hrcV* genes during a single HGT event from the same ancestor as *Pseudomonas syringae* prior to their speciation. Assessing the evolutionary history of *hrcC* and *hrcR* gene sequences has, however, been a more difficult task. First, there are relatively few *hrcC* and *hrcR* homologues currently in the nucleotide databases (GenBank and EMBL), and obtaining an appropriately broad sample from throughout the family would have been outside the scope of this study. Second, based on available sequences, it is evident that homologous gene sequences from the enterobacterial animal pathogens as well as other plant pathogenic γ and β proteobacteria are so divergent from those of *Erwinia*, *Brenneria*, *Pectobacterium* and *Dickeya* that they cannot be meaningfully compared in a phylogenetic context. This extensive divergence is interpreted here as important, albeit indirect, evidence that the T3SS determinants of the enterobacterial plant pathogens together form a monophyletic group. Virulence sequences from within the enterobacterial phytopathogens are far

Table 4. Pair-wise ILD values among eight virulence loci in 12 representative enterobacterial plant pathogens

Locus	P value (1000 partitions)						
	<i>hrcV</i>	<i>hrcC</i>	<i>hrcR</i>	16S rDNA	<i>gapA</i>	<i>gyrA</i>	<i>gapA/gyrA</i>
<i>hrcJ</i>	0.174	0.001	0.001	0.001	0.001	0.001	0.001
<i>hrcV</i>		0.001	0.001	0.001	0.001	0.001	0.001
<i>hrcC</i>			0.001	0.001	0.001	0.001	0.001
<i>hrcR</i>				0.001	0.001	0.001	0.001

more similar to one another than they are to any available sequences from animal and other plant pathogenic taxa.

A conclusion of monophyly, however, assumes a singular evolutionary origin for the enterobacterial plant pathogens that is not supported by the currently accepted taxonomy of the group (Fig. 2). The 16S rDNA, *atpD/carA/recA* and *gapA/gyrA* phylogenies suggest that the enterobacterial plant pathogenic genera of *Erwinia*, *Brenneria*, *Pectobacterium*, *Dickeya* and *Pantoea* arose independently multiple times within the family. Therefore, if we recognize that the hypothesis of polyphyly accurately reflects the evolutionary history of *Erwinia*, *Brenneria*, *Pectobacterium*, *Dickeya* and *Pantoea* then there must be an alternative explanation for the relationships resulting from the virulence gene phylogenies. For example, if plant pathogenicity evolved independently multiple times and was associated with a reduced rate of virulence gene evolution in the resulting lineages, then we would expect the plant pathogens to exhibit relatively high sequence similarity in spite of their polyphyletic origins. Conversely, if an animal pathogenic lineage has arisen from within a lineage of plant pathogens, and the host switch was associated with an increased rate of sequence evolution, then eventually the plant pathogens will display greater sequence similarity to one another than to the animal pathogen in spite of their paraphyly relative to the derived animal pathogen. However, both of these scenarios would require rate changes that affect *hrcC* and *hrcR* simultaneously and in the same way, while at the same time *hrcJ* and *hrcV* remain unaffected. These hypotheses would be more plausible if *hrcC* and *hrcR* encoded extracellular proteins, as it has been suggested that extracellular protein components undergo bursts of rapid polypeptide evolution to facilitate the mechanical adaptations necessary for the T3SS to interface with different eukaryotic cell types (Dale & Moran, 2006). However, since neither gene product is known to be expressed extracellularly, these explanations seem unlikely.

Alternatively, repeated exchanges of common T3SS alleles among the enteric plant pathogens could provide a similar phylogenetic outcome – that is one of monophyly – among the T3SS genes used in this study. Clearly, the repeated horizontal transfer of a few preferred alleles would result in the homogenization of population structure and subsequently result in the evolutionary divergence of the phytopathogens from closely related animal pathogenic neighbours that lie just beyond the barrier of these extensive allelic exchanges. The effects of continued interspecific HGT are clearly seen in the differing phylogenetic placement of the bacterial strains from within species among the phylogenies (Figs 3, 4 and 5). There are cases where strains of a species group together on one tree, but are separated on the other tree, suggesting that one of the two strains has acquired a phylogenetically distinct gene copy through HGT. For example, the two *E. mallotivora* sequences are nearly identical, and group together, on the *hrcJ* and *hrcV* trees (Fig. 5), but they are widely separated from one another on the *hrcC* and *hrcR*

trees (Figs 3 and 4). The two *P. carotovorum* subsp. *carotovorum* strains, on the other hand, group closely together on the *hrcJ* tree, but are widely separated on the *hrcC* (Fig. 3a), *hrcR* (Fig. 4a) and *hrcV* (Fig. 5b) trees. Conflicts involving interspecific relationships are evident as well. For example, the grouping of *E. tracheiphila* 1 with *B. salicis* 1 at the base of the *hrcC* tree (Fig. 3a) is in conflict with both of their positions on the *hrcR* tree (Fig. 4a) (neither is included on the *hrcJ* or *hrcV* tree). A likely explanation for the conflicting placements of these bacterial strains and species is the acquisition of different gene copies. Since the strains were isolated from different individuals of the same host species, and transfer events are believed to be influenced more by physical proximity of strains than by phylogenetic relatedness (Matte-Tailliez *et al.*, 2002; Toth *et al.*, 2006), it is possible that the bacterial strains acquired their copies of the genes from neighbouring bacteria on the same plant.

Finally, even though the lack of an appropriate outgroup has encumbered our ability to detect the direction of the noted HGT events, we can infer that the enterobacterial plant pathogens probably acquired their T3SS prior to their radiative speciation, from the same ancestor as *Pseudomonas syringae*, a conclusion that is strengthened by the observed % GC content, which is uniform between these four virulence genes and housekeeping genes such as 16S rDNA, *gapA* and *gyrA*. Thus, it is ironic and somewhat unexpected that the virulence genes may have provided an additional and important clue to buttress the hypothesis of a monophyletic plant pathogenic lineage in the family *Enterobacteriaceae*, as originally proposed by Brown *et al.* (2000). Moreover, the differences in the phylogenetic positions of the enterobacterial plant pathogenic species among the *hrcJ*, *hrcV*, *hrcC* and *hrcR* trees are possibly the result of continued HGT events followed by genetic recombination during and possibly after the speciation of *Erwinia*, *Brenneria*, *Pectobacterium*, *Dickeya* and *Pantoea*, as clearly seen in the phylogenetic placement of the enterobacterial plant pathogenic strains. Additionally, the ineffectiveness of the flagellar gene homologues as outgroups corroborates the hypothesis proposed by Gophna *et al.* (2003) stating that the T3SS and the flagellar export mechanism share a common ancestor but have evolved independently of one another since. Taken together, these results have provided important clues about the natural history of acquisition of virulence among a highly significant group of bacterial plant pathogens, and although the precept that HGT has forged virulence evolution is certainly not a novel one (Brown *et al.*, 1998; Cohan, 1996; Comas *et al.*, 2006; Dale & Moran, 2006; Gophna *et al.*, 2003), we know of no other analysis encompassing the enterobacterial plant pathogens which has demonstrated that the diversification of the virulence operons is likely to be ancestral to the radiation of the plant pathogens themselves. The monophyly of the T3SS genes of the enterobacterial phytopathogens is evidenced by the extraordinary observed diversification at the amino acid

level (in *hrcC*, *hrcR* and *hrcV*), which was so advanced that the inclusion of relevant enteric homologues was largely an impossible task. This diversification among the plant and animal pathogens within the *Enterobacteriaceae* has likely persisted for epochs among these unique lineages, and continues to be maintained by ongoing HGT events.

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