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ORIGINAL ARTICLE

Detection of *Erwinia* species from the apple and pear flora by mass spectroscopy of whole cells and with novel PCR primers

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Abstract

Aims: To detect the apple and pear pathogens *Erwinia amylovora* and *Erwinia pyrifoliae* as well as the related epiphytes *Erwinia tasmaniensis* and *Erwinia billingiae*, we created novel PCR primers and also applied them to a series of other plant-associated bacteria as control. To facilitate fast diagnosis, we used matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI–TOF MS).

Methods and Results: The PCR primers were deduced from the *pstS–glmS* regions, which can include the gene for levansucrase, and also from regions encoding capsular polysaccharide synthesis. All primer combinations were specific for their associated *Erwinia* species to detect them with conventional PCR, also in mixed cultures from necrotic plant tissue. Other primers designed for quantitative PCR with SYBR Green or together with TaqMan probes were applied for real-time detection to determine growth of *Erw. amylovora*, *Erw. billingiae*, *Erw. pyrifoliae* and *Erw. tasmaniensis* in apple blossoms. From whole-cell protein extracts, profiles were generated using a Bruker microflex machine and *Erwinia* strains classified according to a score scheme.

Conclusions: The designed PCR primers identified the *Erwinia* species unambiguously and can be applied to qualitative and quantitative tests. MALDI–TOF MS data were in agreement with the PCR assays.

Significance and Impact of the Study: The applied diagnosis methods allow fast and precise monitoring of two pathogenic and two epiphytic *Erwinia* species. They are valuable for population studies with apple and pear flowers and with diseased plant material.

Introduction

Fast identification of bacteria is a crucial step for control of plant pathogens. Reliable discrimination between pathogens and closely related epiphytes applied as antagonists can help to reduce pesticide application while sustaining productive crop production (Montesinos 2003). Fire blight and Asian pear blight caused by *Erwinia amylovora* and *Erwinia pyrifoliae*, respectively, lead to significant losses in the production of apple and pear in many countries of the Northern hemisphere and New Zealand (Bonn and van der Zwet 2000) and of Asian pears in Korea and Japan (Rhim *et al.* 1999; Kim *et al.* 2001). Antagonistic bacteria from the species *Erwinia tasmaniensis*, *Erwinia billingiae* (Jakovljevic *et al.* 2008) or *Pantoea agglomerans* (Stockwell *et al.* 2002) interfere with pathogen growth in plant tissue and have been applied to reduce spread of fire blight. On the other hand, *Erw. tasmaniensis* and to a lesser extent *Erw. billingiae* share molecular properties of their genome sequences with *Erw. amylovora* and *Erw. pyrifoliae* (Kube *et al.* 2010). Many PCR assays have been described to detect *Erw. amylovora* (McManus and Jones 1995; Jock *et al.* 2000; Llop *et al.* 2000; Jones and Geider 2001; Geider 2005; Mohammadi *et al.* 2009; Powney *et al.* 2011), a few

for *Erw. pyrifoliae* (Lehman *et al.* 2008), but specific PCR assays for *Erw. billingiae* and *Erw. tasmaniensis* are still missing. PCR analysis can detect bacteria even in mixed cultures and allows screening of populations for the presence of selected bacterial species.

Recently, methods to identify bacteria by matrix-assisted laser desorption ionization–time-of-flight mass spectrometry (MALDI–TOF MS) analysis have been commercialized for high-throughput identification of clinical pathogens (Bizzini and Greub 2010). Protein profiles generated by MALDI–TOF MS are used for the identification and typing of bacteria and yeasts (Dare 2006; Stevenson *et al.* 2010). This method was successfully adapted for the identification of plant-associated Erwinia species, especially *Erw. amylovora* and *Erw. pyrifoliae* (Sauer *et al.* 2008), and for the detection of other plant-pathogenic and environmental bacteria such as *P. stewartii* and *P. agglomerans* (Wensing *et al.* 2010). We tested direct detection of the fire blight pathogen in host plant tissue.

To identify bacteria from the apple and pear flora, we developed novel PCR primers specific for *Erw. amylovora*, *Erw. pyrifoliae*, *Erw. billingiae* and *Erw. tasmaniensis* and combined specific PCR screening for community analysis with MALDI–TOF MS.

Materials and methods

Bacterial strains

Bacteria used in the experiments are listed in Table 1.

PCR assays and oligonucleotide primers

Primer pairs and TaqMan probes applied in this study are listed in Table 2, focusing on the *pst – glmS* and EPS-encoding regions. Their approximate positions in the gene clusters are indicated in Fig. 1. The exact size of the products can be calculated from the numbers in the primer names in column 2 of Table 2. For conventional PCR (cPCR), the primer annealing temperature was 52°C for 20 s and the samples were analysed after 35 cycles. Similar conditions were used for quantitative PCR (qPCR) with SYBR Green and 40 cycles analysed. With Taqman probes, two-step runs were applied for 60 s at 56°C for annealing and polymerization. Additional details have been described before (Mohammadi *et al.* 2009). For qPCR with SYBR Green, hotstart Taq polymerase (Ampliqon, Skovlunde, Denmark) was used. With TaqMan probes, we applied normal Taq polymerase. The Bio-Rad detection systems iCycler or CFX96 were used for qPCR. Calibration for the primer pairs and TaqMan probes was performed with dilution series of the bacteria (adjusted to 1×10^9 ml^{-1}).

MALDI–TOF mass spectroscopy

Sample preparation was performed as described previously (Sauer *et al.* 2008). Briefly, bacteria were grown on LB agar plates or in 1 ml of LB broth with 1% glycerol in 2-ml reaction tubes for 24 h at 28°C. Cells were harvested by centrifugation, washed with 1 ml water to remove residual components of the growth medium, pelleted again and resuspended in 0·3 ml water and 0·8 ml ethanol. For lysis, cells were pelleted, air-dried to remove ethanol and resuspended thoroughly in 40 μl of 70% formic acid and 40 μl acetonitrile. The cell debris was removed by centrifugation, and the cleared lysates were stored at −20°C. One to 2 μl of the extracts was placed on an MSP 96 polished steel target and co-crystallized with the same amount of matrix (saturated α-cyano-4-hydroxy cinnamic acid in 50% acetonitrile/2·5% trifluoro acetic acid) for analysis with a Bruker microflex machine. For direct measurement of cell material instead of lysates, a thin film of bacteria from fresh colonies was applied to the target position, overlaid with 1 μl of matrix and allowed to dry.

Biotyper analysis

Protein profiles were derived as average of 250 spectra and analysed using the Biotyper software in automation mode (Version 2.0, Bruker Daltonics, Bremen, Germany). Pattern analysis was performed against reference library version 3.0. Results were interpreted according to a log-score scheme (Sauer *et al.* 2008). Values exceeding 2.0 represent a high likelihood for positive identification.

Pear slices, apple flowers and extraction of plant tissue

Immature pear fruits (cv. 'Bartlett') were harvested at walnut size, washed and stored in a cold room. For infection assays, fruits were cut into 5-mm-thick slices, placed in a Petri dish and inoculated with *Erw. amylovora* using a toothpick. To keep high humidity, Petri dishes were sealed with Parafilm and incubated at 28°C for 5 days. To check detection sensitivity, tissue with beginning of necrosis but no significant ooze formation was chosen for further analysis. Pear tissue discs were punched with a cork borer (6 mm diameter), ground with 200 μl acetic acid and cleared by centrifugation. The supernatant was mixed with an equal volume of acetonitrile and analysed using MALDI–TOF MS as described earlier.

Necrotic pear plant tissue was obtained from Carinthia, Austria. Approximately 100 mg bark was extracted with 1 ml water for 15 min at RT. Aliquots were plated on LB agar with cycloheximide (50 μg ml^{-1}), and the colonies were analysed.

Table 1 Bacterial strains used in the assays

Strain	Other name, origin	Source, reference
Erwinia amylovora		
CFBP 1232^T	ATCC 15580^T, S59/5, pear, England	
CFBP 1430	Hawthorn, France	Paulin and Samson (1973)
Ea1/79	DSM 17948, apple, Germany	Falkenstein *et al.* (1988)
Ea1/79del100	Deletion in *ams* region; Cm, Sm	Laboratory collection
Ea1/79Sm*	Streptomycin-resistant mutant of Ea1/79	Laboratory collection
Ea273	ATCC 49946, apple, USA	S. Beer
Ea4/82	Pear, Egypt 1988	Falkenstein *et al.* (1988)
Ea7/74	C6/6, cotoneaster, Germany, 1974	Falkenstein *et al.* (1988)
Ea775	Hawthorn, England	E. Billing
EaRW1/06	Cotoneaster, Rottweil, Germany, 2006	Laboratory collection
EaSp1767-3	Pear, Navarra, Spain	M. Lopez
EaSp1951-5	Cotoneaster, Huesca, Spain	M. Lopez
EaTp3/00	Pear, Canada, 2000	Jock and Geider (2004)
JLVNZ04	New Zealand	J. Vanneste
OR6	Pear, USA, Oregon,	McManus and Jones (1995b)
PFB15	Plum, Idaho, USA	K. Mohan via J. P. Paulin
RKK3	Raspberry, Michigan, USA	McManus and Jones (1995b)
T90	Turkey	W. Zeller
Erwinia billingiae		
Eb660	NCPPB 660, pear, England, isolated by E. Billing	Mergaert *et al.* (1999)
Eb661^T	NCPPB 661^T, pear, England, isolated by E. Billing	Mergaert *et al.* (1999)
Eb661Sm*	Streptomycin-resistant mutant of Eb661	Laboratory collection
Eb7/3E4	Apple leaves, Dossenheim, Germany	Laboratory collection
Eb1261	NCPPB 1261, pear, England, isolated by E. Billing	Mergaert *et al.* (1999)
EbKae2a	Pear, Carinthia, Austria, 2007	Laboratory collection
EbKae9a	Pear, Carinthia, Austria, 2007	Laboratory collection
RIPF gl19	Pear leaves, Skierniewice, Poland, 2006	P. Sobiczewski
RIPF gl3	Pear leaves, Skierniewice, Poland, 2006	P. Sobiczewski
RIPF go2	Pear fruits, Skierniewice, Poland, 2006	P. Sobiczewski
Erwinia tasmaniensis		
Et1/99^T	DSM 17950, apple flower, Tasmania, Australia	Geider *et al.* (2006)
Et1/99Sm*	Streptomycin-resistant mutant of Et1/99	Laboratory collection
Et2/99	DSM 17949, pear flower, Victoria, Australia, 2006	Geider *et al.* (2006)
Et4/99	Apple bark, Queensland, Australia	Geider *et al.* (2006)
Et14	Apple flower, Tasmania, 1999	Laboratory collection
Et88	Pyracantha, Tatura, Victoria, Australia	V. Williamson
Et882	Flower, Victoria, Australia, 2000	V. Williamson
EtDs03	FLA3, apple flower, Dossenheim, Germany, 2003	Jakovljevic *et al.* (2008)
EtDs08	FLA08, apple flower, Dossenheim, Germany, 2008	This work
EtDs09	Apple flower, Dossenheim, Germany, 2009	This work
EtSb13	Esa13, apple flower, Stellenbosch, South Africa, 2002	L. Mansvelt
EtSb41	Esa41, apple flower, Stellenbosch, South Africa, 2002	L. Mansvelt
Erwinia pyrifoliae		
Ep1/96	DSM 12162, Asian pear (*Pyrus pyrifolia*), South Korea	Rhim *et al.* (1999)
Ep1/96Sm*	Streptomycin-resistant mutant of Ep1/96	Laboratory collection
Ep2/97	Asian pear, South Korea, not virulent	Jock *et al.* (2003)
Ep16/96^T	DSM 12163^T, CIP 106111^T, Asian pear, South Korea	Rhim *et al.* (1999)
Ep41/97	Asian pear, South Korea, not virulent	Jock *et al.* (2003)
Ejp556	Asian pear, Japan	Kim *et al.* (2001)
Ejp557	Asian pear, Japan	Kim *et al.* (2001)
Erwinia persicina		
CFBP 3622^T	ATCC 35998^T, tomato, Japan	(Hao *et al.* 1990)
Erwinia rhapontici		
CFBP 3618^T	ATCC 29283^T, rhubarb, England	(Hauben *et al.* 1998)

Table 1 (*Continued*)

Strain	Other name, origin	Source, reference
Pectobacterium and *Dickeya*		
Eca185	*Erwinia carotovora* subsp. *atroseptica*	W. Zeller
Ecc1A-1	*Erw. carotovora* subsp. *carotovora*, potato, Poland	E. Lojkowska
Ecc99B-1	*Erw. carotovora* subsp. *carotovora*, potato, Poland	E. Lojkowska
Ecc568	*Erw. carotovora* subsp. *carotovora*, *Apilum graveolens*, Switzerland	Falkenstein *et al.* (1988)
Ecc582	*Cichorium endivia*, Switzerland	Falkenstein *et al.* (1988)
Ech3937	*Erwinia chrysanthemi*, Saintpaulia (African violet)	N. Hugouvieux-Cotte-Pattat
Tas1-2a	Apple flower, Tasmania, 2000	Laboratory collection
Tas1-6a	Apple flower, Tasmania, 2000	Laboratory collection

*Spontaneous mutant selected on agar with streptomycin (200 μg ml^{-1}).

Table 2 PCR primers for the detection of *Erwinia amylovora*, *Erwinia billingiae*, *Erwinia pyrifoliae* and *Erwinia tasmaniensis* created from nucleotide sequences of the *pstS—glmS* and the EPS-encoding region. Relative positions in the nucleotide sequence of this region are indicated by numbers in column 2 ('c' direction of the complementary strand). HEX and TXR indicate TaqMan probes

Primer number	Other name	Sequence
For conventional PCR		
For *Erw. amylovora*		
#149	EAPSGL3961	CCGAAGAACGATTGCACTAC
#150	EAPSGL4610c	CGGTTAGTTAGCGCAGTCTC
For *Erw. billingiae*		
#153	EBPSGL1341	TCAGCTGGTGATCCTTCAAC
#154	EBPSGL2183c	CTCAACTGGACGCTGAGAAG
For *Erw. tasmaniensis*		
#157	ETPSGL4124	GATTGCTGTTCCGAGGTACG
#158	ETPSGL4760c	ACCGATGCCGATATAACCAC
For *Erw. pyrifoliae*		
#163	EPPSGL1646	CAGCGCATCATAAGTGTACC
#164	EPPSGL2698c	TCTGGAATATCGGCTCCGTA
For quantitative PCR		
For *Erw. amylovora*		
#107	AR14819	AACGAGTTGCTGCTACC
#109	AR14948c	CATCGCGTAGCTTAAGG
For *Erw. billingiae*		
#153	EBPSGL1341	TCAGCTGGTGATCCTTCAAC
#447	EBPSGL1497c	CCATCTGGCCATTGTCGAAG
#117-TXR	EBWZ527TXR	TXR-AACTGGCTGAAGTTGCGAGCGA-BHQ1, located between
#76	EBWZ503	GGAATGTAGGTCCGTATG
#77	EBWZ603c	CCAGTTATCCAGCGTCTT
For *Erw. tasmaniensis*		
#56	ETWN101	CCGACTGGCATATCTATC
#57	ETWN217c	CTCCGCTATTGACCTCAT
#157	ETPSGL4124	GATTGCTGTTCCGAGGTACG
#449	ETPSGL4230Qc	CGGATCGGCAACAATCAGTA
#588-HEX	ETWN396HEX	HEX-ACGGCAAACCGTACCTGAAACAGAA-BHQ1, located between:
#587	ETWN367	TACCAGTCACCCAGCAAGTC
#589	ETWN465c	CGATGAGAAGCAGATACGGAAC
For *Erw. pyrifoliae*		
#454	EPPSGS1089Q	GGTTACCGCGTTCGTATGAT
#455	EPPSGS1228Qc	TTGTTGTCGTGAGCGCATAG

BHQ1, BlackHole quencher 1; HEX, Hexachlorfluorescein; TXR, TexasRed.

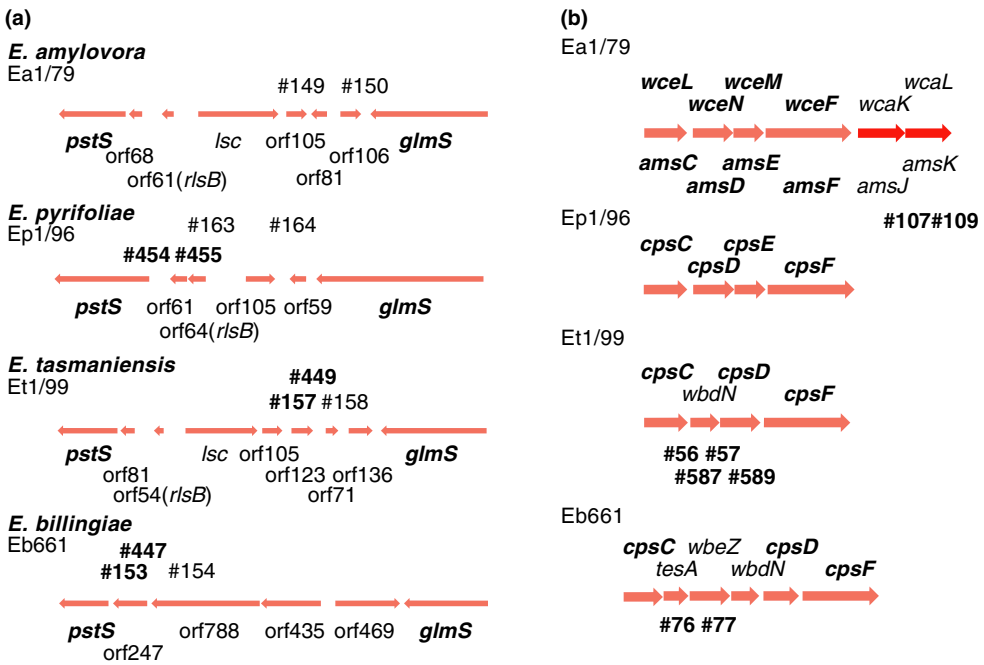

Figure 1 PCR primers applied for the detection of several *Erwinia* species. Panel (a) shows a comparison of the *pstS–glmS* region for *Erw. amylovora*, *Erw. pyrifoliae*, *Erw. billingiae* and *Erw. tasmaniensis*. The numbers # refer to the primers listed in Table 2. Boldface numbers indicate primers for real-time PCR. Panel (b) is the exopolysaccharide (EPS)-encoding region of strains from four Erwinia species. In *Erw. billingiae* genome (FP236843), *wbeZ* is EbC29370; *tesA* is EbC29380. Conserved genes are labelled in bold.

Apple flowers from green house trees (cv. 'Gala') were placed in Eppendorf tubes with water and inoculated with 5000 bacteria in 10 μl water. The flowers were kept at 24/21°C in light/dark for 4 days. They were extracted with 1·5 ml water and aliquots plated on LB agar with streptomycin (500 μg ml^{-1}) and cycloheximide.

Results

Design of novel PCR primers for the detection of several Erwinia species

We have evaluated several chromosomal regions to design primers for the detection of *Erw. amylovora*, *Erw. pyrifoliae*, *Erw. billingiae* and *Erw. tasmaniensis*. The chromosomal regions between *pstS* and *glmS* are divergent for the *Erwinia* species isolated from the apple and pear flora and are completely different for the epiphyte *Erw. billingiae* (Fig. 1a). Another chromosomal region encoding genes for capsular polysaccharide synthesis is related to *Erw. amylovora* and *Erw. pyrifoliae* and carries additional genes for *Erw. billingiae* and *Erw. tasmaniensis*. These are apparently characteristic for the corresponding species (Fig. 1b). Both DNA regions are well suited to design specific PCR primers. Primer pairs amplifying products of 0·6–1 kb for cPCR and approx. 100 bp for qPCR were designed (Table 2). They were assayed for specificity to detect several strains of the species (Table 3). Neither of the designed primer pairs reacted with related species nor with *Erwinia persicina*, *Erwinia rhapontici*, *Pectobacterium carotovorum* (formerly *Erwinia carotovora*) or *Dickeya dadantii* (formerly *Erwinia chrysanthemi*).

Detection of Erwinia species with the novel PCR primers

The primers #149 and #150 from the *lsc* region were positive for all *Erw. amylovora* isolates tested with cPCR including raspberry strains (RKK3) (Fig. 2, Table 3). The primers #153 and #154 were specific for *Erw. billingiae*. Also, recently isolated strains from Austria and Poland were identified and confirmed as *Erw. billingiae*. One isolate, strain Eb660 had a tendency to produce a signal of intermediate strength with primers #153 and #154. Primers #157 and #158 detected all *Erw. tasmaniensis* strains, including isolates from Australia, South Africa and Germany. *Erwinia pyrifoliae* strains from Korea and Japan were identified with primers #163 and #164. While all *Erw. pyrifoliae* isolates including the avirulent *Erw. pyrifoliae* strain Ep2/96 gave positive signals, no cross-reaction

Table 3 PCR and MALDI–TOF MS analyses of various *Erwinia* strains

	Conventional PCR					Quantitative PCR				
	Ea	Eb	Et	Ep	Eb	Eb	Et	Et	Ep	MT
Strain/primers	#149 #150	#153 #154	#157 #158	#163 #164	#76 #77	#153 #447	#56 #57	#157 #449	#454 #455	Score
Erwinia amylovora										
CFBP1232^T	+	–	–	–	≥40	≥40	36·7	29·1	36·4	Ea 2·2
CFBP1430	+	–	–	–	≥40	≥40	≥40	≥40	≥40	Ea 2·1
Ea1/79	+	–	–	–	≥40	≥40	37·8	≥40	36·3	Ea 2·2
Ea273	+	–	–	–	≥40	36·9	37·8	30·2	≥40	Ea 2·2
Ea4/82	+	–	–	–	37·9	≥40	38·5	31·3	36·7	Ea 2·2
Ea7/74	+	–	–	–	37·6	≥40	37·9	≥40	38·1	Ea 2·2
Ea775	+	–	–	–	35·3	≥40	37·6	≥40	≥40	Ea 2·1
EaRW1/06	+	–	–	–	≥40	≥40	36·9	36·8	≥40	Ea 2·1
EaSp1767-3	+	–	–	–	30·5	32	32·2	30·7	38·3	Ea 2·2
EaSp1951-5	+	–	(+)	–	26·8	34·4	27·5	30·7	34·8	Ea 2·3
JLVNZ04	+	–	–	–	≥40	≥40	37·7	33·6	37	Ea 2·2
OR6	+	–	–	–	≥40	≥40	≥40	30·2	≥40	Ea 2·1
PFB15	+	–	–	–	≥40	≥40	≥40	33·8	≥40	Ea 2·3
RKK3	+	–	–	–	36	≥40	34·3	32·1	34·7	Ea 1·9
T90	+	–	–	–	≥40	≥40	≥40	32·8	30·7	Ea 2·2
Tp3	+	–	–	–	≥40	≥40	37·3	≥40	≥40	Ea 2·1
Erwinia billingiae										
Eb7/3E4	–	+	–	–	**20·7**	**22·7**	35·7	33·2	34·9	Eb 2·2
Eb660	–	(+)	–	(+)	**18·6**	**21**	35·3	30	29·7	Eb 2·2
Eb661	–	+	–	–	**18·7**	**19·8**	36·4	34	31·2	Eb 2·0
Eb1261	–	+	–	–	**18·7**	**21·1**	36·7	30·6	37·1	Eb 2·0
Kae2a	–	+	–	–	**19·7**	**16·1**	37·2	35	≥40	Eb 2·0
Kae9a	–	+	–	–	**18·6**	**20·4**	36·4	35·9	37·1	Eb 2·3
RIPFgo2	–	+	–	–	**18·9**	**20·1**	37·4	33·6	33·3	Eb 2·3
RIPFgl3	–	+	–	–	**18·5**	**20·8**	35	34·3	≥40	Eb 2·2
RIPFgl19	–	–	–	–	**18·8**	**20·8**	33·4			Eb 2·3
Erwinia tasmaniensis										
Et1/99	–	–	+	–	31·5	36	**18·2**	**18·4**	35	Et 2·3
Et2/99	–	–	+	–	29·8	34·2	**15·7**	**19·4**	35·8	Et 2·2
Et4/99	–	–	+	–	29·9	36	**21·8**	**23·9**	38·1	Et 2·4
Et14a	–	–	+	–	26·8	32·1	**19·3**	**17·6**	≥40	Et 2·3
Et88	–	–	+	–	30·6	37	**24**	**19·6**	≥40	Et 2·3
Et882	–	–	+	–	29·5	33·7	**20·4**	**21·4**	36·7	Et 2·3
EtDs03	–	–	+	–	30·5	36	**18·8**	**19**	37·8	Et 2·2
EtDs08	–	–	+	–	37	≥40	27·9	**21·1**	37·4	Et 2·3
EtDs09-1	–	–	+	–	≥40	≥40	**19·9**	**22·1**	34·7	Et 2·4
EtSb13	–	–	+	–	31·6	36·4	**20·1**	**20·1**	37·1	Et 2·3
EtSb41	–	–	+	–	29·9	36·3	**19·8**	**21·7**	33·3	Et 2·3
Erwinia pyrifoliae										
Ejp556	–	–	–	+	32·1	34·5	34·5	≥40	**17**	Ep 2·1
Ejp557	–	–	–	+	37·8	≥40	37·4	35·1	**20**	Ep 2·3
Ep1/96	–	–	–	+	38·1	≥40	35·7	31·6	**19**	Ep 2·2
Ep16/96	–	–	–	+	≥40	≥40	36·6	38·5	**17·8**	Ep 2·2
Ep2/96	–	–	–	+	38·3	32·2	35·9	≥40	**16**	Ep 2·3
Ep41/97	–	–	–	+	31·7	36·3	40	≥40	**19·1**	Ep 2·0
Pectobacterium and others										
Eca185	–	–	–	–	≥40	≥40	35·7	30·7	≥40	P. atrosept. 2·3
Ecc1A-1	–	–	–	–	≥40	34·6	36·8	30·7	35·1	P. wasabiae 2·2
Ecc99B-1	–	–	–	–	≥40	37·4	37·4	31	≥40	P. carotov. 2·2
Ecc568	–	–	–	–	≥40	38·6	35·2	31·4	≥40	P. carotov. 2·2
Ecc582	–	–	–	–	≥40	36·9	34·5	28·6	≥40	P. carotov. 2·2

Table 3 (*Continued*)

	Conventional PCR					Quantitative PCR				
	Ea	Eb	Et	Ep	Eb	Eb	Et	Et	Ep	MT
Strain/primers	#149 #150	#153 #154	#157 #158	#163 #164	#76 #77	#153 #447	#56 #57	#157 #449	#454 #455	Score
Ech3937	–	–	–	–	36·6	≥40	36·2	30·8	37·7	D. dadantii 2·2
Tas1-2a	–	–	–	–	36·6	≥40	36	33·9	≥40	Ps. savast:2·0
Tas1-6a	–	–	–	–	≥40	38	36·1	31·9	≥40	Ps. poae 2·2
CFBP 3622	–	–	–	–	≥40	≥40	≥40	34·1	37·2	E. pers. 2·1
CFBP 3618	–	–	–	–	≥40	34	33·5	28·7	≥40	E. rhap. 2·1
Water	–	–	–	–	≥40	≥40	≥40	34·7	≥40	

(), weak or ambiguous signal; significant CT values below 25 are in bold. MT, MALDI–TOF MS.

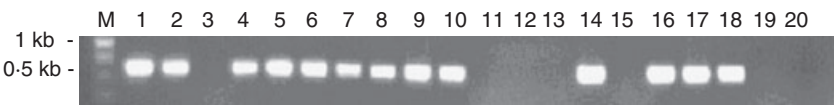

Figure 2 PCR analysis of *Erwinia* strains with *Erwinia amylovora*-specific primers. The primer pair #149/#150 was applied to strains (in lane 1) CFBP1430 (+), (2) Ea273 (+), (3) water (–), (4) Ea7/74 (+), (5) PFB15 (+), (6) Tp3 (+), (7) EaRW1/06 (+), (8) Ea775 (+), (9) OR6 (+), (10) JLVNZ04 (+), (11) EtDs09-1 (–), (12) Eb7/3E4 (–), (13) Ep1/96 (–), (14) RKK3 (+), (15) Ejp557 (–), (16) T90 (+), (17) CFBP1232 (+), (18) Ea4/82 (+), (19) *Erwinia persicina* (–), (20) *Erwinia rhapontici* (–). Positive (+) and negative signals (–) are indicated. M, 1-kb ladder marker.

with *Erw. amylovora* isolates was observed. Therefore, this primer pair is specific for the detection of Asian pear blight.

Similar specificities were obtained for qPCR. The primers #76 and #77 for *Erw. billingiae* were designed from the sequence of gene *tesA* from the EPS-encoding region. Among the investigated *Erwinia* species, the *tesA* gene is only presented in *Erw. billingiae*. For *Erw. tasmaniensis*, another gene of the EPS-encoding region, w*bdN*, was used for the design of primers #56 and #57. Similar specific signals for qPCR were obtained for *Erw. tasmaniensis* applying primers #157 and #449 from the *lsc* region. Primers #153 and #447 detected *Erw. billingiae* in qPCR as specific as #76 and #77 (Table 2). For *Erw. pyrifoliae*, we applied primers #454 and #455, which were specific in qPCR for strains from Korea and from Japan.

TaqMan probes were designed for the detection of *Erw. billingiae* and *Erw. tasmaniensis* (Table 2). The fluorescent labels TexasRed and HEX with the quencher BH1 were both suitable and produced the expected signals with primers #76/#77 and #56/#57, respectively, and not with DNA from other Erwinias (Fig. 3). A cycle threshold of 32 was obtained for a dilution representing 50 cells in the assay. The primer combinations can also be applied for mixed cultures in qPCR (data not shown).

Quantitative and qualitative detection of *Erw. amylovora*, *Erw. pyrifoliae*, *Erw. billingiae* and *Erw. tasmaniensis* strains from apple and pear by qPCR

Apple flowers were inoculated with Ea1/79Sm, Ea1/79del100, Eb661Sm, Et1/99Sm and Ep1/96Sm. After 4 days, the flowers were extracted for bacteria and their amount measured with qPCR as well as by titration on LB agar with streptomycin and cycloheximide. All strains grew on flowers from an initial amount of 5000 cells to a density of approx. 3×10^7 CFU. *Erw. amylovora*, the causative agent of fire blight on apple and pear, as well as *Erw. pyrifoliae*, causing Asian pear blight, grew to this density on apple flowers. The data reveal an inability of apple flowers to discriminate between pathogenic and nonvirulent bacteria (Table 4). Growth of the EPS-deficient nonpathogenic mutant Ea1/79del100 was neither restricted to flowers in contrast to its lack of propagation in host plant tissue. The epiphytic species *Erw. billingiae* and *Erw. tasmaniensis* were also well supported in their growth on apple flowers. The cell titres were determined by qPCR with signals in agreement with their titre on selective agar plates.

Extracts from necrotic pear tissue were diluted and aliquots plated on LB agar with cycloheximide. Randomly selected single white colonies were grown in microtitre plates with LB medium. Cultures were lysed in 0·1% Tween and lysates assayed with qPCR primers #153 and

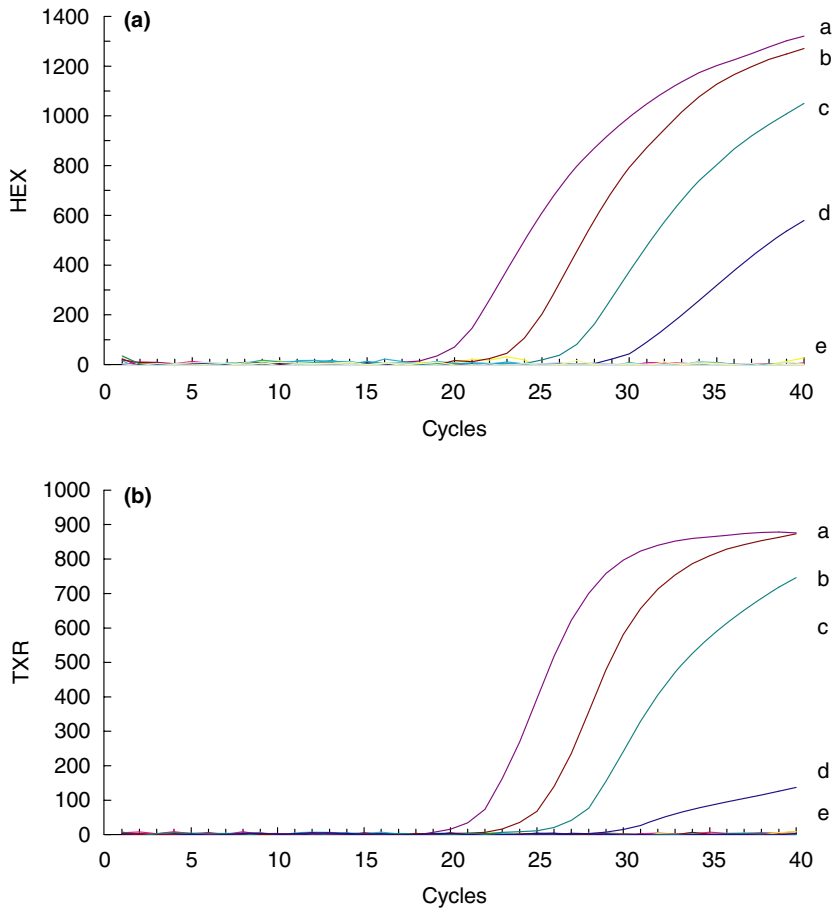

Figure 3 PCR with TaqMan probes for *Erwinia billingiae* and *Erwinia tasmaniensis*. Cultures of strains *Erwinia amylovora* Ea1/79, *Erw. billingiae* Eb661 and *Erw. tasmaniensis* Et1/99 were adjusted to 1×10^9 CFU ml^{-1} and diluted 10^{-2}, $^{-3}$, $^{-4}$ and $^{-5}$ (curves a to d), and 5 μl was applied for PCR assays in 25 μl total volume. The negative control was a sample with water and dilutions of lysed *Erw. amylovora* cells (curves sector e). HEX and TXR refer to the TaqMan probes. (a) Detection with #117-TXR together with #76 and #77, specific for *Erw. billingiae*; (b) Detection with #588-HEX together with #587 and #589, specific for *Erw. tasmaniensis*. The signals correspond to the cell numbers in dilutions.

#447 for *Erw. billingiae* as well as #107 and #109 for *Erw. amylovora* (Table 5). *Erw. amylovora* was confirmed by plating on semi-selective plates and *Erw. billingiae* by MALDI–TOF MS. The colonies of *Erw. amylovora* were mucoid and yellow on MM2Cu minimal agar plates and showed levan formation on LB agar with sucrose (Bereswill *et al.* 1998). The fire blight pathogen was detected in approx. 10% of the propagated colonies and *Erw. billingiae* in approx. 5%.

Identification of strains of the species *Erw. amylovora*, *Erw. pyrifoliae*, *Erw. billingiae* and *Erw. tasmaniensis* by MALDI–TOF mass spectroscopy

All *Erwinia* isolates tested could be clearly identified by score values above 2·0 (Table 3). We compared generation of spectra from cells directly applied to the target with protein extracts. Both methods resulted in species identification, but the scores were generally higher for samples from extractions than for direct application of cells from colonies (data not shown). This difference was most significant for high EPS producers such as *Erw. billingiae*. Closely related species such as *Erw. amylovora* and *Erw. pyrifoliae* were differentiated by specific score values 2·0 and higher. We could confirm species identification of all isolates of *Erw. amylovora*, *Erw. pyrifoliae*, *Erw. billingiae* and *Erw. tasmaniensis*. A characteristic protein pattern for one strain of each species is presented in Fig. 4.

Analysis of symptomatic pear tissue by MALDI–TOF mass spectroscopy

Three days post inoculation pear tissue from regions with water soaking, browning but no visible ooze formation

Table 4 Recovery of *Erwinia* cells after inoculation of apple flowers at 4 days of inoculation. The flowers were inoculated with 5000 CFU of each strain; *Erwinia amylovora* Ea1/79Sm, Ea1/79del100, *Erwinia billingiae* Eb661Sm, *Erwinia tasmaniensis* Et1/99Sm and *Erwinia pyrifoliae* Ep1/96Sm. From 10-fold dilutions, 10 μl lysate were applied for a 50 μl assay with SYBR Green

Primers/strains	Titre/flower	Quantitative PCR (CT)
#107 #109		
Ea1/79Sm	$3{\cdot}0 \times 10^7 \pm 0{\cdot}3 \times 10^7$	27·3 ± 1·72
Ea1/79del100	$2{\cdot}2 \times 10^7 \pm 0{\cdot}2 \times 10^7$	29·1 ± 3·10
#153 #447		
Eb661Sm	$2{\cdot}6 \times 10^7 \pm 0{\cdot}3 \times 10^7$	25·8 ± 2·92
#157 #449		
Et1/99Sm	$3{\cdot}1 \times 10^7 \pm 0{\cdot}7 \times 10^7$	28·2 ± 2·67
#454 #455		
Ep1/96Sm	$2{\cdot}5 \times 10^7 \pm 0{\cdot}7 \times 10^7$	26·6 ± 2·64
CT value for 1×10^4 CFU of each species		27

Table 5 Quantitative PCR screening with SYBR Green and primers #153 and #447 for *Erwinia billingiae* and #107 and #109 for *Erwinia amylovora* in extracts of necrotic pear tissue with fire blight infection. 95 colonies were transferred to microwells with LB medium. The next day, the cultures were lysed in 0·1% Tween and 5 μl assayed for *Erw. amylovora* and *E. billingiae*. Signals (in bold) below 22 are considered to identify either species

Well no.	Ea	Eb
15Ea	**21**	30·8
20Ea	**19·0**	27·4
21Ea	**20·7**	27·9
28Eb	29·4	**18·8**
30Eb	31·9	**19·0**
33Ea	**21·6**	27·6
34Eb	30·5	**18·1**
40Eb	34·8	**17·6**
42Ea	**17·8**	30·6
55Ea	**20·4**	30·6
72Ea	**19·4**	34·7
80Eb	32·3	**18·7**
84Ea	**20·1**	29·7
92Ea	**17·3**	27·5
Ea/Eb*	19·6	20·5

*Reference with 5×10^5 CFU per 25 μl assay.

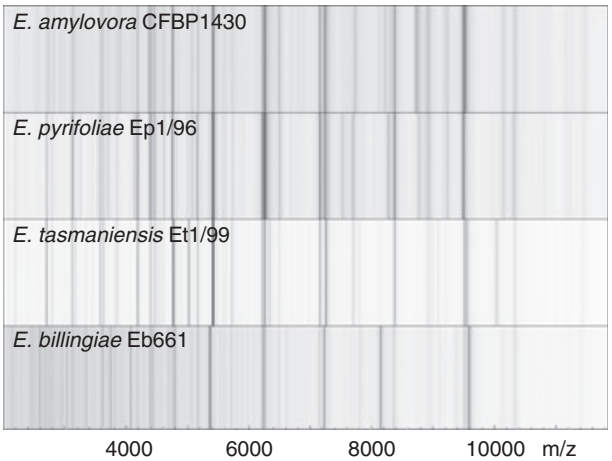

Figure 4 Bar code representation of protein profiles of MALDI–TOF MS spectra. The protein spectra of various *Erwinia* species were visualized in a pseudo-gel representation with peak heights converted to colour intensity.

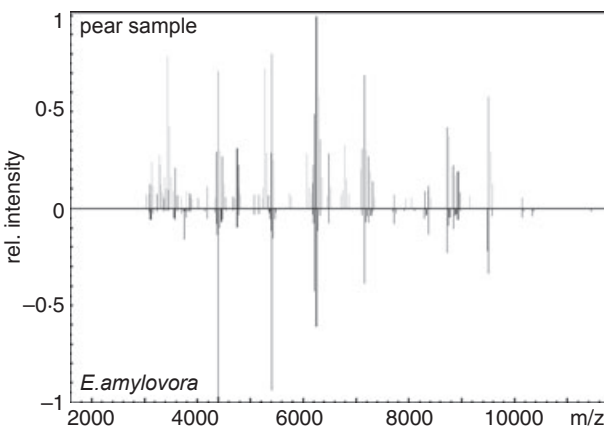

Figure 5 Protein patterns for *Erwinia amylovora* in pear slices and from cell extracts of cultures analysed with MALDI–TOF MS. A spectrum derived from inoculated pear tissue (top) is compared against the *Erw. amylovora* reference (bottom) and analysed in identification view. The *x*-axis displays molecular mass, and *y*-axis indicates signal strength. Darker colours indicate higher peak identity. The figure shows a positive identification of *Erw. amylovora* with an ID score of 2 and above.

was analysed by MALDI–TOF MS. A spectrum of the protein pattern of extracted bacteria is compared to the profile obtained with cells from a pure culture. The upper part of Fig. 5 represents a typical spectrum for *Erw. amylovora* from pear tissue. The pathogen was identified (score values of 2 and above) in a few tissue extracts, whereas the score for the majority of samples was below this threshold, presumably because of contaminations from plant tissue. In these cases, *Erw. amylovora* was still the most likely organism.

Discussion

The development of *Erw. amylovora* on apple and pear flowers or tissue is accompanied by the presence of other bacteria, for example, *Erw. billingiae* and *Erw. tasmaniensis* (Jakovljevic *et al.* 2008). To provide reliable tools to

distinguish between pathogen and these epiphytes, we have created PCR assays for both species to differentiate their populations from *Erw. amylovora*. The Asian pear blight pathogen *Erw. pyrifoliae* has not yet been reported from outside of Korea and Japan, but there and in other countries the species must be discriminated from *Erw. amylovora*. We developed primer pairs specific for both pathogens. Asian pear blight has been described first in South Korea (Rhim *et al.* 1999) supported by communications from a Korean laboratory (Shrestha *et al.* 2003). A similar disease in Japan was named Bacterial Pear Shoot Blight (BPSB) and initially assumed to be caused by *Erw. amylovora* (Beer *et al.* 1996). Several publications and a taxonomic description of Japanese strains classified them as *Erw. pyrifoliae* (Kim *et al.* 2001; Geider *et al.* 2009). Numerous PCR primers and other methods for identification have been described for *Erw. amylovora* (Bereswill *et al.* 1998; Geider 2005; Powney *et al.* 2011). The latter assays comprise semi-selective media with sucrose indicating levan synthesis or minimal agar with copper salt inducing slime production and a yellow colour of colonies. Another assay uses staining of capsules with FITC-labelled lectin, characteristic of *Erw. amylovora*. The occurrence of fire blight was reported in many countries of the Northern hemisphere (North America, Europe) and in New Zealand (Bonn and van der Zwet 2000). Additional specific primers can improve and confirm the detection of *Erw. amylovora* in case of new outbreaks and for quarantine purposes. To our experience with PCR detection of *Erw. amylovora* (Jones and Geider 2001), we rank the novel primer pair from the *pst/glmS* region to be most reliable for the diagnosis of fire blight.

We provide safe identification and eventually discrimination between *Erw. amylovora* and *Erw. pyrifoliae*. Epiphytes have an important impact on modulation of the induced diseases. As control agents, their population dynamics are of high interest. The description of their occurrence and cell densities at various times of application can benefit from cPCR and qPCR primers, which we provide here. *Erwinia billingiae* is a common bacterium invading necrotic plant tissue after fire blight infection. Gene sequences can vary and allow distinction of strains from narrow areas and from different countries (our unpublished data). The high level of capsular EPS can affect PCR signals, but the species is this much uniform that all our designed PCR primers give positive specific signals. *Erwinia tasmaniensis* is a rare epiphyte of apple and pear flowers in German orchards. In Australia, based on our experience with isolation, it is quite common. The species was also isolated in South Africa and occurs rarely on apple flowers (L. Mansvelt, personal communication). *Erwinia tasmaniensis* can contribute to the reduction in fire blight, when applied to orchards (unpublished). The discriminatory primers provided here can be applied to describe occurrence of *Erw. amylovora* and *Erw. pyrifoliae* with *Erw. billingiae* and *Erw. tasmaniensis* and analyse the antagonistic effect of epiphytes.

As *Erw. amylovora* can reach high cell densities in symptomatic tissue, a direct use of MALDI–TOF MS is, in turn, attractive, but reliability of this procedure for samples from heterogeneous environments must be improved. Kits for fast sample preparation from complex tissue are being developed for medical samples (Yan *et al.* 2011). Such enrichment steps might improve the analysis of environmental samples as well. MALDI–TOF MS is less suitable for tracing small amount of bacterial species in mixed populations (Sauer *et al.* 2008). The method is, therefore, best suited to dissect microbial populations with identification of individual colonies on a mixed culture plate.

Depending on the analytical situation, each of the described methods has advantages or disadvantages for screening of bacterial populations of individual colonies. A practical approach to analyse plant tissue is plating of dilution series of extracted bacteria on LB agar with cycloheximide (Geider 2005). This distinguishes yellow colonies of mostly *Pantoea* species from white colonies of other bacteria. Alternatively, the bacterial mixture can be directly analysed by qPCR. The desired species can be recognized, when its cell number exceeds 100 in the assay. A lower content can be safely detected using cPCR. To isolate a species from a mixed population, MALDI–TOF MS is superior for screening suspicious colonies. They can be directly transferred to a steel target for protein profiles.

Our screening data with several *Erwinia* strains and mutants show little if any discrimination for growth on apple flowers. It can be assumed that the nectar with sucrose, glucose and fructose provides a good basis for nutrition. It should be noted that *Erw. billingiae* does not carry genes for sucrose metabolism (Kube *et al.* 2010) and *Erw. tasmaniensis* lacks a sorbitol operon (Kube *et al.* 2008). Plant defence mechanisms do not contribute to selection of growth of bacterial species on apple flowers. Other bacteria and environmental factors such as humidity or UV light can interfere with growth. A complex situation is created by growth competition of bacteria. This includes speed of propagation and production of inhibitory and signalling components.

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References

Beer, S.V., Kim, J.-H., Zumoff, C.H., Bogdanove, A.J., Laby, R.J., Tanii, A., Tamura, O., Gustafson, H.L. *et al.* (1996) Characterization of bacteria that cause ''Bacterial Shoot Blight of Pear'' in Japan. *Acta Hort (ISHS)* **411**, 179–182.

Bereswill, S., Jock, S., Bellemann, P. and Geider, K. (1998) Identification of *Erwinia amylovora* by growth morphology on agar containing copper sulfate and by capsule staining with lectin. *Plant Dis* **82**, 158–164.

Bizzini, A. and Greub, G. (2010) MALDI-TOF MS, a revolution in clinical microbial identification. *Clin Microbiol Infect* **16**, 1614–1619.

Bonn, G.W. and van der Zwet, T. (2000) Distribution and economic importance of fire blight. In *Fire Blight the Disease and Its Causative Agent, Erwinia amylovora* ed. Vanneste, J.L. pp. 37–54 Wallingford, UK: CABI Publishing.

Dare, D. (2006) Rapid bacterial characterization and identification by MALDI-TOF mass spectrometry. In *Advanced Techniques in Diagnostic Microbiology* ed. Tang, Y.W. and Stratton, C.W. pp. 117–133. New York: Springer US.

Falkenstein, H., Bellemann, P., Walter, S., Zeller, W. and Geider, K. (1988) Identification of *Erwinia amylovora*, the fireblight pathogen, by colony hybridization with DNA from plasmid pEA29. *Appl Environ Microbiol* **54**, 2798–2802.

Geider, K. (2005) Molecular detection of fire blight and differentiation of *Erwinia amylovora* strains. *Phytopathol Pol* **35**, 57–68.

Geider, K., Auling, G., Du, Z., Jakovljevic, V., Jock, S. and Völksch, B. (2006) *Erwinia tasmaniensis* sp. nov., a non-phytopathogenic bacterium from apple and pear trees. *Int J Syst Evol Microbiol* **56**, 2937–2943.

Geider, K., Auling, G., Jakovljevic, V. and Völksch, B. (2009) A polyphasic approach assigns the pathogenic Erwinia strains from diseased pear trees in Japan to *Erwinia pyrifoliae*. *Lett Appl Microbiol* **48**, 324–330.

Hao, M.V., Brenner, D.J., Steigerwalt, A.G., Kosako, Y. and Komagata, K. (1990) *Erwinia persicinus*, a new species isolated from plants. *Int J Syst Bacteriol* **40**, 379–383.

Hauben, L., Moore, E.R., Vauterin, L., Steenackers, M., Mergaert, J., Verdonck, L. and Swings, J. (1998) Phylogenetic position of phytopathogens within the *Enterobacteriaceae*. *Syst Appl Microbiol* **21**, 384–397.

Jakovljevic, V., Jock, S., Du, Z. and Geider, K. (2008) Hypersensitive response and acyl-homoserine lactone production of the fire blight antagonists *Erwinia tasmaniensis* and *Erwinia billingiae*. *Microb Biotechnol* **1**, 416–424.

Jock, S. and Geider, K. (2004) Molecular differentiation of *Erwinia amylovora* strains from North America and of two Asian pear pathogens by analyses of PFGE patterns and *hrpN* genes. *Environ Microbiol* **6**, 480–490.

Jock, S., Rodoni, B., Gillings, M., Kim, W., Copes, C., Merriman, P. and Geider, K. (2000) Screening of ornamental plants from the Botanic Gardens of Melbourne and Adelaide for the occurrence of *Erwinia amylovora*. *Australas Plant Pathol* **29**, 120–128.

Jock, S., Kim, W.S., Barny, M.A. and Geider, K. (2003) Molecular characterization of natural *Erwinia pyrifoliae* strains deficient in hypersensitive response. *Appl Environ Microbiol* **69**, 679–682.

Jones, A. and Geider, K. (2001) Gram negative bacteria. *Erwinia amylovora* group. In *Guide for Identification of Plant Pathogenic Bacteria* ed. Schaad, N.W., Jones, J.B. and Chum, W. pp. 40–55. St Paul, MN: APS Press.

Kim, W.S., Hildebrand, M., Jock, S. and Geider, K. (2001) Molecular comparison of pathogenic bacteria from pear trees in Japan and the fire blight pathogen *Erwinia amylovora*. *Microbiology* **147**, 2951–2959.

Kube, M., Migdoll, A.M., Müller, I., Kuhl, H., Beck, A., Reinhardt, R. and Geider, K. (2008) The genome of *Erwinia tasmaniensis* strain Et1/99, a non-pathogenic bacterium in the genus *Erwinia*. *Environ Microbiol* **10**, 2211–2222.

Kube, M., Migdoll, A.M., Gehring, I., Heitmann, K., Mayer, Y., Kuhl, H., Knaust, F., Geider, K. *et al.* (2010) Genome comparison of the epiphytic bacteria *Erwinia billingiae* and *E. tasmaniensis* with the pear pathogen *E. pyrifoliae*. *BMC Genomics* **11**, 393.

Lehman, S.M., Kim, W.S., Castle, A.J. and Svircev, A.M. (2008) Duplex real-time polymerase chain reaction reveals competition between *Erwinia amylovora* and *E. pyrifoliae* on pear blossoms. *Phytopathology* **98**, 673–679.

Llop, P., Bonaterra, A., Penalver, J. and Lopez, M.M. (2000) Development of a highly sensitive nested-PCR procedure using a single closed tube for detection of *Erwinia amylovora* in asymptomatic plant material. *Appl Environ Microbiol* **66**, 2071–2078.

McManus, P.S. and Jones, A.L. (1995a) Detection of *Erwinia amylovora* by nested PCR and PCR-dot-blot and reverse-blot hybridizations. *Phytopathology* **85**, 618–623.

McManus, P.S. and Jones, A.L. (1995b) Genetic fingerprinting of *Erwinia amylovora* strains isolated from tree-fruit crops and Rubus spp. *Phytopathology* **85**, 1547–1553.

Mergaert, J., Hauben, L., Cnockaert, M.C. and Swings, J. (1999) Reclassification of non-pigmented *Erwinia herbicola* strains from trees as *Erwinia billingiae* sp. nov. *Int J Syst Bacteriol* **49**, 377–383.

Mohammadi, M., Moltmann, E., Zeller, W. and Geider, K. (2009) Characterisation of naturally occurring *Erwinia amylovora* strains lacking the common plasmid pEA29 and their detection with real-time PCR. *Eur J Plant Pathol* **124**, 293–302.

Montesinos, E. (2003) Plant-associated microorganisms: a view from the scope of microbiology. *Int Microbiol* **6**, 221–223.

Paulin, J.P. and Samson, R. (1973) Le feu bactérien en France II. Caractéres des souches d'Erwinia amylovora (Burill) Winslow *et al.* 1920, isolées du foyer franco-belge. *Annales de Phytopathologie* **5**, 389–397.

Powney, R., Beer, S., Plummer, K., Luck, J. and Rodoni, B. (2011) The specificity of PCR-based protocols for

detection of *Erwinia amylovora*. *Australas Plant Pathol* **40**, 87–97.

Rhim, S.L., Völksch, B., Gardan, L., Paulin, J.P., Langlotz, C., Kim, W.S. and Geider, K. (1999) *Erwinia pyrifoliae*, an *Erwinia* species different from *Erwinia amylovora*, causes a necrotic disease of Asian pear trees. *Plant Pathol* **48**, 514–520.

Sauer, S., Freiwald, A., Maier, T., Kube, M., Reinhardt, R., Kostrzewa, M. and Geider, K. (2008) Classification and identification of bacteria by mass spectrometry and computational analysis. *PLoS ONE* **3**, e2843.

Shrestha, R., Koo, J., Park, D., Hwang, I., Hur, J. and Lim, C. (2003) *Erwinia pyrifoliae*, a causal endemic pathogen of shoot blight of Asian pear tree in Korea. *Plant Pathol J* **19**, 294–300.

Stevenson, L.G., Drake, S.K., Shea, Y.R., Zelazny, A.M. and Murray, P.R. (2010) Evaluation of Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF) for the identification of clinically important yeast species. *J Clin Microbiol* **48**, 3482–3486.

Stockwell, V.O., Johnson, K.B., Sugar, D. and Loper, J.E. (2002) Antibiosis contributes to biological control of fire blight by *Pantoea agglomerans* strain Eh252 in orchards. *Phytopathology* **92**, 1202–1209.

Wensing, A., Zimmermann, S. and Geider, K. (2010) Identification of the corn pathogen *Pantoea stewartii* by mass spectrometry of whole-cell extracts and its detection with novel PCR primers. *Appl Environ Microbiol* **76**, 6248–6256.

Yan, Y., He, Y., Maier, T., Quinn, C., Shi, G., Li, H., Stratton, C.W., Kostrzewa, M. *et al.* (2011) Improved identification of yeast species directly from positive blood culture media by combining Sepsityper specimen processing and Microflex analysis with the MALDI Biotyper system. *J Clin Microbiol* **49**, 2528–2532.