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Alternative methods to describe virulence of *Erwinia amylovora* and host-plant resistance against fireblight

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The evaluation of host-plant susceptibility to *Erwinia amylovora* and of colonization of host-plant tissue by individual strains was facilitated by labelling the pathogen with green fluorescent protein (GFP). Colonization of apple leaves assayed with a fluorescence microscope was associated with visual disease ratings on plants to describe virulence (= aggressiveness) of the fireblight pathogen. Resistance induced with 2,6-dichloro-isonicotinic acid (INA) and benzo(1,2,3-)thiadiazol-7-carbothioic acid-S-methyl ester (BTH, the active component of BION™) restricted colonization by the pathogen to an area adjacent to the inoculation site. Migration in leaves was associated with symptom formation on pear slices and host plants of mutant strains. Non-virulent *E. amylovora* mutants did not migrate into the leaf veins and strains with intermediate-to-low virulence moved slowly. To compare the migration efficiency of individual wild-type strains in apple and plum cultivars, a blend of five wild-type *E. amylovora* strains with specific numbers of short-sequence DNA repeats (SSRs) in the common plasmid pEA29 was applied to distinguish them by PCR. Fast-moving strains identified in the GFP assays were dominant, independent of the apple cultivar. When apple shoots, pear slices or leaves of apple plants were coinoculated with streptomycin (Sm)-resistant strains and the corresponding parent strains, Sm-resistant mutants were able to dominate the wild-type strain for tissue colonization.

Keywords: BION, green fluorescent protein, leaf assay, short-sequence DNA repeats, virulence

Introduction

Fireblight, in some areas causing severe losses in apple and pear production (Van der Zwet & Keil, 1979; Vanneste, 2000), could be better controlled by understanding virulence (efficiency to cause disease symptoms, 'aggressiveness') of *Erwinia amylovora* strains and responses of host-plant cultivars. Colonization of host plants by *E. amylovora* has been studied by electron microscopy (Suhayda & Goodman, 1981b), by radioactive labelling of the pathogen (Suhayda & Goodman, 1981a) and, more recently, with the green fluorescent protein (GFP) from *Aequorea victoria* (Bogs *et al*., 1998). Spread within an infected plant has been described as occurring in vascular bundles (Suhayda & Goodman, 1981b) or in the intercellular space of the parenchyma (Eden-Green, 1972). After artificial inoculation, a rapid migration of the pathogen through xylem vessels and slow colonization of the parenchyma were observed (Bogs *et al*., 1998). Application of the pathogen to leaf surfaces resulted in bacterial movement through the epidermis into the intercellular space of parenchyma and into the vascular system.

GFP has been expressed in eukaryotic and prokaryotic cells, yielding bright green fluorescence when the cells were exposed to UV light. GFP fluorescence is largely independent of other proteins, cofactors or substrates, allowing its expression to be followed *in situ* and in real time (Cody *et al*., 1993; Chalfie *et al*., 1994). Based on GFP expression, plant–microbe interactions have been studied for a variety of bacteria and fungi, such as *Pseudomonas fluorescens* (Tombolini *et al*., 1997), *Rhizobium meliloti* (Gage *et al*., 1996), *Ustilago maydis* (Spellig *et al*., 1996) and *Fusarium oxysporum* (Di Pietro *et al*., 2003). The use of GFP, expressed from high-copy-number plasmids in *E. amylovora*, facilitated the screening of plants using young leaves (Bogs *et al*., 1998). Blue and red fluorescent variants have been created, which can be simultaneously applied with GFP, but background fluorescence of plant tissue interferes particularly with the red fluorescent versions.

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To follow the recent worldwide spread of fireblight (Bonn & Van der Zwet, 2000), the isolated strains were differentiated in their genomic structure by pulsed-field gel electrophoresis (PFGE) analysis (Zhang & Geider, 1997; Jock *et al*., 2002). However, this technique is too laborious for mass screening. Another approach to distinguish individual strains is PCR amplification of a DNA fragment from plasmid pEA29, which carries a variable region with a short-sequence DNA repeat (SSR) of eight nucleotides (5′-ATTACAGA-3′) (Schnabel & Jones, 1998; Kim & Geider, 1999). PCR primers were designed for rapid analysis of variations in SSR numbers, and 130 strains were classified to carry four to 15 repeats in the DNA segment of pEA29 (Kim & Geider, 1999), which vary slightly in an *E. amylovora* population by decreasing down to three repeats (Jock *et al*., 2003).

Efforts to manage fireblight have included spraying of chemicals, such as the antibiotics streptomycin (Sm), oxytetracycline and oxolinic acid (Psallidas & Tsiantos, 2000). At present, monitoring programmes are used to estimate the probability of fireblight outbreaks, and Sm is often applied in the event of a high-risk prediction (Billing, 2000), when permitted. Sm-resistant strains may accumulate, especially after repeated applications. A low level of resistance to Sm can be quickly distributed by transposon Tn5393 transmitted via conjugated plasmids (Chiou & Jones, 1993) and high Sm resistance mainly relies on a change in the gene encoding the 30S ribosomal subunit S12. In case of a sudden increase in frequency of Sm-resistant strains, application of the antibiotic is ineffective. Sm-resistant strains often show an elevated level of amylovoran production (Bellemann *et al*., 1994) and may therefore increase their efficiency in host-plant colonization in comparison to the Sm-sensitive parent strain.

Plants react to pathogen infection by induction of local and systemic defence mechanisms. Local defence of a non-host plant is associated with the hypersensitive response (HR), which causes a necrotic zone at the infection site. After pathogen attack, host-plant tissue can develop systemic acquired resistance (SAR) against the same and unrelated pathogens by activating the expression of a large number of pathogenesis-related proteins. Salicylic acid (SA) is a signal molecule involved in local defence reactions and SAR (Ryals *et al*., 1996; Durner *et al*., 1997). The chemicals 2,6-dichloro-isonicotinic acid (INA) and benzo(1,2,3-) thiadiazole-7-carbothioic acid S-methyl ester (BTH, the active component in BION) have also been shown to increase plant resistance by activating SAR (Vernooij *et al*., 1995; Friedrich *et al*., 1996). Both compounds appear to be functional analogues of SA, because they elicit the same set of defence responses, and they inhibit the H_2O_2-scavenging enzymes, catalase and ascorbate peroxidase (Durner & Klessig, 1995; Wendehenne *et al*., 1998). Another approach to expose *E. amylovora* to plant defence mechanisms has been attempted with a viral EPS-depolymerase gene (Kim & Geider, 2000), which encodes an enzyme degrading the amylovoran capsules of the bacterial cells. Uncapsulated cells are not able to colonize the tissue of host plants (Bellemann & Geider, 1992; Bugert & Geider, 1995).

Resistance to fireblight in plants was induced with chemicals such as prohexadion-Ca (Roemmelt *et al*., 2002) or BION (Norelli *et al*., 2003). In breeding programmes, plants with increased resistance were selected by crossing natural resistance properties into susceptible cultivars and by the creation of transgenic plants expressing foreign resistance genes. Apples and pears were transformed with the genes encoding the antibacterial proteins attacin and cecropin found in the haemolymph of insects. Plants that expressed these genes showed an increased resistance to fireblight (Norelli *et al*., 1994; Reynoird *et al*., 1999).

Rapid methods to estimate the susceptibility of plants at a young growth stage would facilitate tests based on symptom development with inoculated shoots of potted trees. Three techniques – GFP labelling, Sm-resistant strains, and PCR analysis of the SSR region – were used to distinguish five genetically unmodified strains in the shoots of host plants. The methods provide tools to determine virulence of individual strains, defined as their capacity to cause fireblight symptoms such as ooze formation on plants and necrosis, which were compared in terms of their speed to colonize plant tissue. In plant breeding, the assays can be applied to determine the susceptibility of host plants to *E. amylovora*.

Materials and methods

Bacterial strains

Properties of wild-type and mutated bacteria, as well as the plasmids used, are listed in Table 1. When appropriate, antibiotics were added to the agar or the liquid medium (Standard I, Merck AG, Darmstadt, Germany; or LB, Luria-Bertani broth) at 20 μg mL^{-1} [chloramphenicol (Cm) and kanamycin (Km)] or 500 μg mL^{-1} (Sm) and grown at 28°C.

Apple seedlings and treatment with inducers of resistance

Seeds from open-pollinated apples (cv. Golden Delicious) were kept in iced water for 3 days and then in sand for 2 weeks at 10°C. The germinating seedlings were planted into pots (5 cm diameter, 50 mL) and maintained at 22°C for 3 weeks with a 16 h photoperiod. For induction of resistance, they were watered with 1 mg 2,6-dichloro-isonicotinic acid (INA, CGA41394) or 120 mg BION (BTH, content 50%), dissolved in 20 mL water, which was added to the soil of a potted plant. The plants were incubated for 4 days and then inoculated with *E. amylovora*.

The other apple and plum cultivars used were from the collection of the Bundesanstalt für Züchtungsforschung an Kulturpflanzen in Aschersleben, Germany. Growing shoots were inoculated by cutting the two youngest unfolded leaves with scissors dipped into a suspension of an *E. amylovora* strain at 1×10^9 cfu mL^{-1}. The resulting lesions on the stem were measured 4 weeks after inoculation, and the severity of infection was calculated from the

Table 1 Strains and plasmids employed in this study

Strains/plasmids	Properties	Source or reference
Strains		
Ea1/79	Wild type, isolated from *Malus* sp., Germany	Falkenstein *et al.* (1988)
Ea1/79Sm	Ea1/79, spontaneous Sm-resistant mutant	Bellemann *et al.* (1994)
Ea1/79-D50	Ea1/79, *amsD*::Tn5, EPS-negative	Bernhard *et al.* (1993)
Ea7/74	Wild type, from *Cotoneaster* sp., Germany	Falkenstein *et al.* (1988)
Ea7/74-S1	Ea7/74, *scrY*::Tn5seq1, Km^r	Bogs & Geider (2000)
Ea7/74Sm	Spontaneous Sm-resistant mutant, selected on agar with Sm (200 μg mL^{-1})	Bellemann *et al.* (1994)
Ea7/74-LS6	Ea7/74, *lsc*::Tn5, levan-negative, Km^r	Geier & Geider (1993)
Ea7/74-A64	Ea7/74Sm, leucine-deficient	Bellemann & Geider (1992)
Ea7/74-S14	Ea7/74, *srlE*::Tn5, Km^r	Aldridge *et al.* (1997)
Ea7/74-A72	Ea7/74Sm, *dsp*, Km^r	Bellemann & Geider (1992)
EaX7/74	Ea7/74 without pEA29	Falkenstein *et al.* (1989)
Ea11/88	Wild type, from *Cotoneaster* sp., Germany	Bellemann *et al.* (1994)
Ea11/88Sm	Spontaneous Sm-resistant mutant	Bellemann *et al.* (1994)
Ea77	(Ea266, E4001a), from *Malus* sp., Canada	G. Bonn via collection BAZ Aschersleben
Ea237	From *Malus* sp., Baden-Württemberg, Germany	E. Moltmann
Ea250	Apple, Sachsen-Anhalt, Germany	Jock *et al.* (2002) BAZ, Aschersleben
Ea270	From *Pyrus* sp., Baden-Württemberg, Germany	E. Moltmann
Ea282	Isolated from *Pyrus* sp., Backnang Baden-Württemberg, Germany 1996	E. Moltmann
Ea284	From *Pyrus* sp., Reutlingen Baden-Württemberg, Germany 1996	E. Moltmann
Ea286	From *Pyrus* sp., Bavaria, Germany	E. Moltmann
PD350	Levan-negative isolate, Netherlands	Bereswill *et al.* (1997)
PD579	Levan-negative isolate, Netherlands	Bereswill *et al.* (1997)
PMV6076	CFBP1430 with a deletion of *hrp* and *dsp*	Barny *et al.* (1990); Zhang & Geider (1997)
Plasmids		
pfdC1Z′	fd ori; with fd 2 gene, *lacZ′*; Km^r, 3·55 kb	Geider *et al.* (1995)
pfdC1Z′-gfp	*gfp* in pfdC1Z′, fd ori, Km^r	Bogs *et al.* (1998)
pfdC4	fd ori; with fd 2 gene′; Cm^r; 3·5 kb	Geider *et al.* (1995)
pfdC4Z′-gfp	1·4 kb *Hae*II-fragment from pfdC1Z′-gfp with *lacZ′-gfp* inserted into pfdC4, fd ori, Cm^r	This work

Cm^r, chloramphenicol resistance (20 μg mL^{-1}); Km^r, kanamycin resistance (20 μg mL^{-1}); Sm, streptomycin.

necrotized lesion as a percentage of the total shoot length. The standard deviation for the disease rating on shoots was determined to be 60% for a large series of shoot assays (a total of 30) with strains Ea237 and Ea286.

Virulence assays with GFP-labelled *E. amylovora* strains

To track *E. amylovora* in plant tissue, the bacteria were labelled with GFP from *A. victoria* by transfer of plasmid pfdC1Z′-gfp (Km^r) (Bogs *et al.*, 1998) or plasmid pfdC4Z′-gfp (Cm^r) into the cells by electroporation. The reporter gene was expressed via the *lac*-promoter in the high-copy-number plasmid. To stabilize the plasmid in the cells, the transformants were passaged at least four times on LB agar with Cm or Km.

For inoculation of plant tissue, sterile scissors or toothpicks were dipped into a suspension of GFP-labelled bacteria grown in LB broth (diluted in water to 5×10^6 cfu mL^{-1}) and young leaves of apple seedlings were inoculated at a cut 0·5 cm from the tip 1 week after foliation. The plants were covered with plastic bags to increase local humidity. After 4 days (5 days in some sets of experiments) at 22°C in an illuminated growth chamber, leaf tissue was cut at least 2 cm from the inoculation site, and the strips were mounted in tap water between two glass slides. The samples were examined with a Zeiss Axiovert epifluorescence microscope (405 M or S100; Carl Zeiss, Oberkochen, Germany) using the filter combination BP450-490/FT510/LP520 (excitation filter/dichroic-emission filter) and 10× magnification lens. The distance of bacterial migration from the inoculation site through the midrib of a leaf was determined. The standard deviation for these assays was determined in several independent experiments. Negative controls included a GFP-labelled *E. amylovora* mutant without EPS synthesis (*amsD*) and

fluorescent latex particles with a diameter of 0·2 μm. Standard deviations were estimated from conditions resulting in intermediate migration distances and calculated to be approximately 60%.

Immature pears were cut with a knife into slices 3–5 mm thick, inoculated with 10 μL of a bacterial suspension, and incubated in small air-tight plastic boxes at 28°C for 1 week.

Strain differentiation by PCR

Erwinia amylovora strains used in simultaneous inoculation were distinguished by PCR based on their SSRs in plasmid pEA29 (Kim & Geider, 1999). The plants were inoculated by cutting young leaves at shoot tips. *Erwinia amylovora* was reisolated for the PCR assays from stem sections at the transition zone between necrotic and healthy bark. Two plants of a cultivar or more than one shoot tip of a plant were inoculated.

PCR assays

Strains Ea237, Ea250, Ea282, Ea284 and Ea286 (PFGE pattern type Pt1) can be distinguished by their SSR numbers (Kim & Geider, 1999) of 10, 7, 6, 5 and 4, respectively, within a 1-kb PCR fragment of plasmid pEA29 used for detection of *E. amylovora* (Bereswill *et al*., 1992). The five strains were inoculated alone or as a blend into leaves of shoot tips of the apple (*Malus* spp.) and plum (*Prunus cerasifera*) cultivars, which are listed in Table 2. Sterile scissors were dipped into suspensions of bacteria (1×10^9 cfu mL^{-1} to ensure symptom formation) and unfolding leaves were transversely cut in the centre. The plants were further maintained for 2–4 weeks in the glasshouse. Bark of stem sections from the transition zone between healthy and necrotic tissue was sliced into pieces (approximately 100 mg), which were put into an Eppendorf tube, and extracted for 30 min with 1 mL of sterile water. The bacterial cells were lysed for PCR analysis (Bereswill *et al*., 1992), the SSR region amplified with primers RS1 and RS2c (Kim & Geider, 1999), and the products analysed on an 8% polyacrylamide gel.

Assays with Sm-resistant strains

Spontaneous chromosomal Sm-resistant mutants of *E. amylovora* strains Ea7/74, Ea1/79 and Ea11/88 were selected on LB plates with Sm at 200 μg mL^{-1} (Bellemann *et al*., 1994). No difference was found for growth of these mutants in nutrient broth when compared with the wild-type strain. Equal amounts of wild-type and mutant strains were mixed and inoculated by cutting unfolding leaves at shoot tips of apple seedlings with scissors dipped in the bacterial suspension, or using toothpicks dipped in the suspension to inoculate the cut. After 4 weeks, 50 mg bark samples were removed from stem sections in the transition zones between necrotic and healthy bark, and bacteria were extracted in 1 mL of water for 15 min. In each assay, 25 colonies from LB agar were confirmed as *E. amylovora* on MM2Cμ agar (Bereswill *et al*., 1998) and assayed in parallel on LB containing Sm (500 μg mL^{-1}).

Table 2 Migration of simultaneously applied strains of *Erwinia amylovora* in shoots of host plants distinguished by their short-sequence DNA repeat (SSR) patterns of plasmid pEA29

Plant cultivar/signal from strain	Ea237	Ea250	Ea282	Ea284	Ea286
Malus domestica cv. Idared					++
M. yunnanensis var. *veitchii*				+	+
Plant or shoot 2				+	+
M. domestica MM106	++	+		+	++
Plant or shoot 2	+			+	++
M. domestica Pi AS 18,19	+			+	++
Plant or shoot 2	+			+	+
Prunus cerasifera Myrobalanen	++				
Plant or shoot 2		+	++		
M. domestica Rebella	+	++		+	
Plant or shoot 2		++			
M. domestica Remo	+			+	++
M. domestica Renora	+			++	+
M. domestica Resi	+	+		+	+
Plant or shoot 2	++		+		+
M. fusca	++	+		+	+
Plant or shoot 2	+			+	++
M. domestica Gloster (long necrosis)				+	+
M. domestica Gloster (short necrosis)				+	+
Plant or shoot 2	+				+
M. domestica Relinda	+				+
Plant or shoot 2	+			+	+
Frequency of observed bands[a]	19	8	3	16	24

[a]Strong bands (++) were counted twice in the calculations assuming dominant colonization.

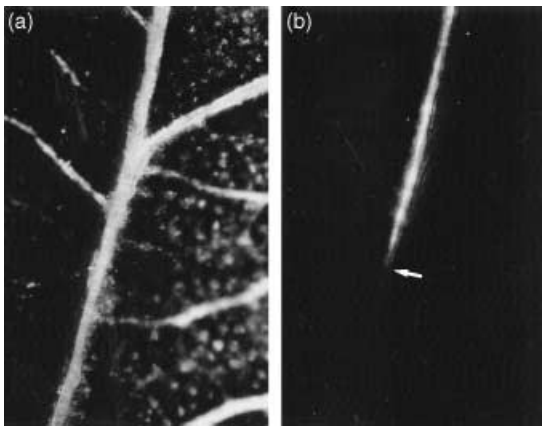

Figure 1 Migration of Ea1/79(pfdC1Z′-gfp) in the midrib of an apple leaf of cv. Golden Delicious seedling 4 days after inoculation. The cut-leaf-tip method was used to inoculate the bacteria into the leaf tip (above top of figure) and a section of the leaf with fluorescence is shown. (a) Sample in bright light, phase contrast; leaf section at approximately 10× magnification. (b) Sample in (a) in UV light, with fluorescent bacteria in the central vein of the leaf. The transition zone between infected and uninfected vascular tissue is marked by an arrow.

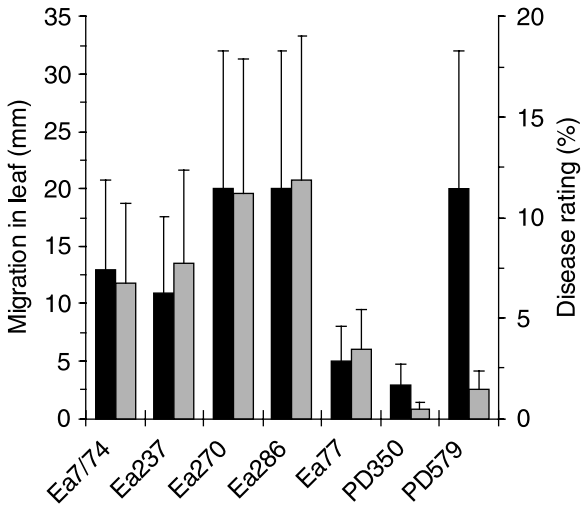

Figure 2 Correlation of the migration distance of *Erwinia amylovora* in the central vein of leaves (4 days after inoculation; black bars) and virulence determined by disease rating (4 weeks after inoculation; light bars). Bacteria were labelled with pfdC1Z′-gfp or pfdC4Z′-gfp to measure the migration distance. Rating was performed by determining the percentage of infection (amount of shoot necrosis divided by the length of the shoot distance × 100). Values are means obtained from at least five inoculated leaves or shoots. Standard deviation (lines on top of bars) was estimated as described in 'Materials and methods'.

Results

Comparison of *E. amylovora* strains for migration in leaf tissue

To measure movement of GFP-labelled *E. amylovora* strains, the inoculated leaves were cut into 2 cm cross-sections starting at the inoculation site, and the distance of bacterial migration was measured using an epifluorescence microscope at low magnification. A typical zone of spread in leaves of apple seedlings is shown in Fig. 1. Parts of the central vein of the leaf were filled with fluorescent bacteria from the inoculation site to some distance towards the petiole. The transition zone from fluorescence to darkness was used to evaluate pathogen migration in plant tissue (Fig. 1). In relation to each other, the fastest migration was found for strains Ea286, Ea270 and PD579, followed by strains Ea7/74 and Ea237 (Fig. 2), whereas the migration rates of strains Ea266 (Ea77) and PD350 were lower (Fig. 2).

Comparison of migration distances and symptom development on shoots

The migration assay with GFP-labelled bacteria was compared with disease ratings based on progression of the necrotic zone of inoculated shoots, showing a relationship for all tested strains except strain PD579 (Fig. 2). Judged from the extent of the necrotic zone in the bark, Ea270, Ea286 and Ea1/79 migrated fast and were rated as highly virulent. Ea237 and Ea7/74 migrated more slowly in leaves and had intermediate virulence according to symptom formation on shoots. The strains Ea77 and PD350 migrated slowly and barely produced symptoms. Strain PD579 caused slow necrosis, but migrated quite rapidly in veins of apple leaves. The low distance of migration of PD350 reflects its deficiency in levan production and reduced amylovoran synthesis (Bereswill *et al*., 1997). No migration was observed for the two negative controls: the amylovoran-deficient strain Ea1/79-D50 (Bernhard *et al*., 1993; Bugert & Geider, 1995) and fluorescent latex particles.

Migration of *E. amylovora* mutants in apple leaves

Various chromosomal *E. amylovora* mutants with decreased virulence showed retarded movement in the central vein of apple leaves compared with the parent strains (Fig. 3). Ea7/74-LS6, a levansucrase-mutant, and Ea7/74-S14, a sorbitol-mutant, showed slow migration (< 8 mm), and EaX7/74, which does not contain plasmid pEA29, showed intermediate migration (14 mm). The migration distances of the *ams*-mutant Ea1/79-D50, the sucrose mutant Ea7/74-S1, the *hrp* mutant PMV6076, the leucine-mutant Ea7/74-A64 and the *dsp*-mutant Ea7/74-A72 were less than 4 mm at 5 days after inoculation, and no symptoms were detected on the plants even 20 days after inoculation. These mutants were previously described as nonpathogenic (Barny *et al*., 1990; Bellemann & Geider, 1992; Bugert & Geider, 1995; Bogs & Geider, 2000).

Induction of plant resistance by plant activators

Young apple seedlings were watered with BION or INA before inoculation with labelled bacteria and the migration of Ea1/79(pfdC1Z′-gfp) in apple leaves was measured.

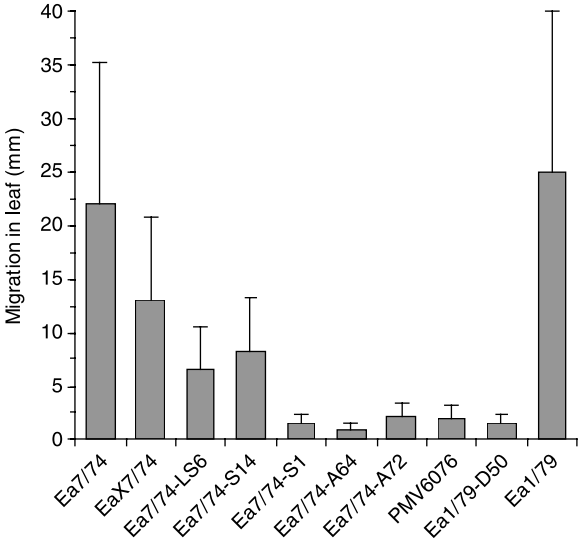

Figure 3 Migration distances of Ea1/79 and Ea7/74 mutants in the midribs of apple leaves at day 4 after inoculation. Bacteria were labelled with plasmid pfdC1Z′-gfp, kanamycin-resistant strains with pfdC4Z′-gfp. Values are means obtained from at least five inoculated leaves. Standard deviation (lines on top of bars) was estimated as described in 'Materials and methods'.

Table 3 Migration of *Erwinia amylovora* strain Ea1/79(pfdC1Z′-gfp) in the midribs of apple leaves (cv. Golden Delicious). Five apple plants were treated as described in 'Materials and methods'. Three untreated plants were used as controls

Plant	Distance from inoculation point (mm)	
	Leaf 1	Leaf 2
Control[a]	≥ 20	≥ 20
BION		
1	1	3
2	5	1
3	≥ 20	≥ 20
4	3	8
5	4	2
INA		
1	1	1
2	3	1
3	1	≥ 20
4	1	1
5	2	8

BION, active component = benzo(1,2,3-) thiadiazole-7-carbothioic acid S-methyl ester; INA. 2,6-dichloro-isonicotinic acid.
[a]Untreated plants.

In about 80% of inoculated leaves no significant migration (< 5 mm) of *E. amylovora* was observed after 5 days (Table 3). In the three control plants without treatment, Ea1/79(pfdC1Z′-gfp) migrated 20 mm into the midrib of each inoculated leaf. INA had a phytotoxic effect on the plants when applied at a concentration of more than 1 mg per pot (50 mL soil). BION did not cause phytotoxic effects at a BTH concentration of 120 mg per pot (50 mL soil).

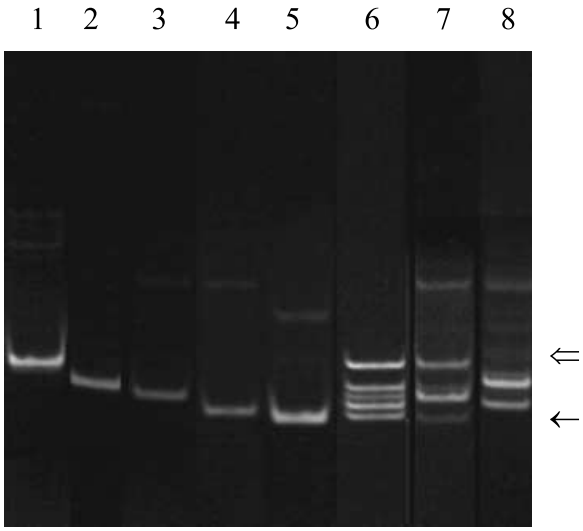

Figure 4 Migration of *Erwinia amylovora* strains in apple and plum tree tissues. Individual strains were identified by the length of their short-sequence DNA repeats (SSRs) in *E. amylovora* plasmid pEA29. The strains were inoculated into shoots separately or as a blend and recovered from the transition zone between necrotic and healthy bark 3 weeks after inoculation. Lanes: 1, Ea237; 2, Ea250; 3, Ea286; 4, Ea282; 5, Ea284 (lanes 1–5 were separately inoculated into shoots of *Malus domestica* cv. MM106); 6, blend of the five strains inoculated into MM106; 7, inoculated as a blend into shoots of *M. fusca*; 8, inoculated as a blend into shoots of *M. communis* cv. Rebella. Arrows: ←, position of PCR fragment with four SSRs; ⇐, position of PCR fragment with 10 SSRs.

Migration of *E. amylovora* strains in plant tissue differentiated by short-sequence DNA repeats in pEA29

Strains Ea237, Ea250, Ea286, Ea282 and Ea284 were inoculated into various apple cultivars and a plum cultivar. In assays with pure strains, the reisolated bacteria showed a single band with the same repeat number as the cells used for the inoculum (Fig. 4). The SSR number was thus stable for strains during a single passage of plant inoculation, as observed by others (Schnabel & Jones, 1998). In experiments with mixed strains, Ea286 was the most virulent strain on the tested plants scored by the frequency of appearance of the corresponding PCR band, followed by Ea237, Ea284, Ea250 and Ea282 (Fig. 4, Table 2). Ea286 was detected in the transition zone between healthy and diseased tissue of most tested plants, but not in *P. cerasifera* and apple cv. Rebella. Strain Ea237 was not found in the transition zones of apple cv. Idared and *M. yunnanensis* var. *veitchii*, and Ea284 was not detected in the transition zone between healthy and diseased bark of *P. cerasifera*. Ea250 was detected in the transition zone of *M. domestica* cvs MM106, Rebella and Resi, *M. fusca* and *P. cerasifera*. The strain with the lowest detection frequency was Ea282. It was only found in the transition zone of *P. cerasifera* and apple cv. Resi.

Table 4 Inoculation of several shoots of a single apple cultivar (Pi AS 18, 19). Plants were assayed as described in 'Materials and methods'

	Signal from *E. amylovora* strain				
Shoot no.	Ea237	Ea250	Ea282	Ea284	Ea286
1		+		+	
2		+		+	+
3		+			+
4		+		+	+
5					+
6	++[a]				
7					+
8		+	+		
9		+		++	
10	+	+		+	+
11		+		+	+
12	+			+	+
13	+	+			++
14	+				++
15	+				+
Frequency of observed bands[a]	7	9	1	8	13

[a]Strong bands (++) were counted twice in the calculations assuming dominant colonization.

Table 5 Distribution of wild-type *Erwinia amylovora* strains and the corresponding streptomycin (Sm)-resistant mutants after simultaneous inoculation into shoots of apple cv. Prima

Mixed strains inoculated	Number of shoots	% Streptomycin-resistant colonies	Mean value[a]
Ea1/79:Ea1/79Sm	10	50, 96, 50, 92, 75, 71, 54, 67, 96, 100	75 ± 20%
Ea7/74:Ea7/74Sm	10	13, 0, 4, 0, 25, 8, 29, 17, 25, 4	13 ± 11%
Ea11/88:Ea11/88Sm	16	76, 96, 64, 68, 56, 48, 63, 56, 44, 84, 72, 60, 64, 84, 40, 88	66 ± 16%

[a]The average percentage of Sm-resistant colonies in three sets of experiments was 51%.

Although none of the strains applied was isolated from plum, Ea282 and Ea237 could, based on these data, have some preference for this uncommon fireblight host. On the other hand, the highly virulent strain Ea286 was not detected in tissue extracted from the transition zone of necrotic plum shoots. From the apple cultivars assayed in Table 2, it was concluded that individual *E. amylovora* strains colonized with similar efficiency.

To determine the variability of strain distribution within a single cultivar, shoots of five young plants of the apple genotype Pi AS 18,19, which was developed in a breeding programme for fireblight resistance, were inoculated with a mixture of strains Ea237, Ea250, Ea286, Ea282 and Ea284 (Table 4). Strain Ea286 was the most prevalent strain, and Ea250 was as fast as Ea237 and Ea284, whereas Ea282 showed the slowest migration.

Migration of wild-type and Sm-resistant strains

Three *E. amylovora* strains were inoculated into shoot tips and pear slices together with the same amount of the corresponding Sm-resistant mutant. In shoot tips, inoculated with a blend of both Ea7/74 strains, the wild-type strain dominated the Sm-resistant mutant, in contrast to the blend of strains Ea1/79 and Ea11/88, where the Sm-mutants dominated the population extracted from the transition zone between necrotic and healthy stem tissue (Table 5). Similar results were obtained from pear ooze and from leaves of inoculated apple plants (Fig. 5). The Sm mutants were therefore in some cases more virulent than the corresponding wild-type strain, especially in colonization of stems and leaves.

Discussion

Virulence of *E. amylovora* strains and susceptibility of host plants to fireblight have been largely evaluated by disease rating of symptom formation (Van der Zwet & Keil, 1979). The present work describes convenient methods to assay the movement of *E. amylovora* in plant tissue by applying genomic strain markers or by labelling the bacteria with the *gfp* gene. This approach revealed a correlation with virulence of individual strains, but there was barely any effect of plant cultivar, except in plum. *Erwinia amylovora* has been reported to migrate in the vascular system of host plants (Suhayda & Goodman, 1981a,b), although in other reports (Eden-Green, 1972) the parenchyma was described as a major site for host-plant colonization. It was recently demonstrated that the xylem is primarily colonized, even when inoculation is performed without damaging plant tissue, but colonization of the parenchyma was also observed (Bogs *et al.*, 1998). The advantages of the GFP system are detection of *E. amylovora* in plant tissue even without disease symptoms, the

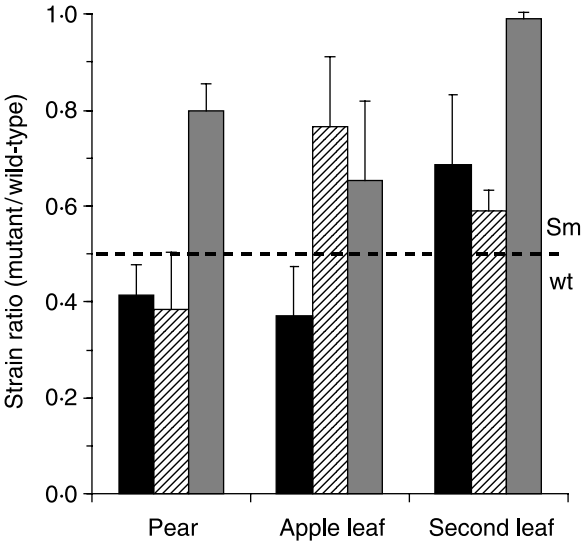

Figure 5 Distribution of wild-type (wt) *Erwinia amylovora* strains and the corresponding streptomycin (Sm)-resistant mutants after simultaneous inoculation into slices of immature pears and into leaves of apple cv. Golden Delicious seedlings. Black bars, Ea7/74-Ea7/74Sm; hatched bars, Ea1/79-Ea1/79Sm; grey bars, Ea11/88-Ea11/88Sm. Bacteria were extracted from pear ooze 1 week after inoculation, from the lower part of an apple leaf used for inoculation (at the tip) and the adjacent leaf of the same plant with symptoms 2 weeks after inoculation of the apple seedling. The part of a bar above the dashed line indicates dominance of an Sm-resistant strain. Standard deviations were calculated from three to five assays.

ability to measure susceptibility of host plants to fireblight in early growth stages, and a means of evaluating virulence of individual *E. amylovora* strains. The GFP method uses genetically altered bacteria applied in a controlled environment. Detached leaves were not as efficient as leaf assays on intact plants. Leaf trichomes of field-grown trees can interfere with high background fluorescence. Hence, the approach is best suited for apple plantlets from the glasshouse.

Inoculation into the interveinal region of a leaf should expose the pathogen more efficiently to plant cell recognition and was used to demonstrate reduced virulence of an *E. amylovora* protease-deficient mutant in comparison to the parent strain (Zhang *et al*., 1999). The assay is not as quantitative as the vein assay. Sometimes, inoculations into the interveinal region can fail to result in visible pathogen invasion, a problem that is even more pronounced for application of bacterial suspensions to the leaf surface (Bogs *et al*., 1998). Exceptionally high or low responses can occur in virulence assays. An additional problem is the amount of inoculum applied. In most cases, high cell densities for inoculation produce the best results in terms of symptom formation. On the other hand, natural infection usually occurs at low inoculum densities. Under laboratory conditions, and especially for potted plants in the glasshouse, the bacterial concentrations must be increased for a reliable response in the assays. Despite differences in the amount of bacteria used for inoculation, migration in the veins can be compared by rating disease symptoms formed after shoot inoculation. Mutants for levansucrase expression, sorbitol metabolism and a strain without plasmid pEA29 exhibited reduced virulence with minimal symptom formation on apple seedlings or on pears (Falkenstein *et al*., 1989; Geier & Geider, 1993; Aldridge *et al*., 1997) and slow migration in leaves.

Short-sequence DNA repeats (SSRs) in prokaryotic genomes have been related to bacterial phase variation and adaptation (Van Belkum *et al*., 1998). When *E. amylovora* is identified with PCR using primers from plasmid pEA29 (Bereswill *et al*., 1992), the length of the amplified DNA fragment can vary (Lecomte *et al*., 1997). A large number of *E. amylovora* strains were differentiated by the SSR region on the plasmid, with primers designed for rapid analysis of SSR length variations (Schnabel & Jones, 1998; Kim & Geider, 1999). Although instability of the SSR number was described after propagation of strains especially under stress conditions (Jock *et al*., 2003), such changes were not found in the assays in the present work, and the repeat number of a strain can be considered to be constant for this purpose. The SSR number is therefore a natural strain marker to distinguish individual strains after their simultaneous inoculation. Although the SSR number may depend on the history of the pathogen population, it was not correlated with the virulence of a strain, unlike levan production (Bereswill *et al*., 1997) or the obligate requirements for amylovoran synthesis (Bugert & Geider, 1995).

Application of Sm for control of fireblight can result in selection of strains resistant to the antibiotic. In Sm-treated orchards, resistant mutants can be selected by the antibiotic, but even after long-term applications Sm-sensitive strains dominate the *E. amylovora* populations in the fields (Jones & Schnabel, 2000). Low resistance is often related to transfer of a transposon with genes inactivating Sm by phosphorylation (Chiou & Jones, 1993). Chromosomal changes disable the ribosomes for binding of Sm. The latter mutants were investigated for their fitness and found to be slightly different from the wild type. Spontaneous resistant mutants showed increased amylovoran synthesis (Bellemann *et al*., 1994) and these strains could outnumber the corresponding wild-type strains in colonizing plant tissue under laboratory conditions. As found in the assays in the present study, some, but not all, Sm mutants showed greater colonization of shoots or pear slices than the corresponding *E. amylovora* wild-type strain.

Assays for plant resistance are critical steps in the survey of new substances or plant cultivars developed to control diseases. Therefore, alternative methods to visual symptom inspection are important for rapid screening of fireblight in early growth stages of host plants and are especially valuable for mass screening of resistance in plant breeding and monitoring other treatments for controlling fireblight.

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