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Characterisation of naturally occurring *Erwinia amylovora* strains lacking the common plasmid pEA29 and their detection with real-time PCR

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Abstract *Erwinia amylovora*, the causal agent of fire blight, carries the common plasmid pEA29 of 29 kb. To screen for occurrence of natural strains without plasmid pEA29, we applied PCR analysis with primers from the plasmid and the chromosomal *ams* region. In addition, a described TaqMan probe from pEA29 and newly designed primers from the *ams*-region were used for identification by real-time PCR. One strain isolated in Iran, one strain from Spain and two strains from Egypt lacked plasmid pEA29. From a recent screening series in southern Germany, in 123 *E. amylovora* strains from necrotic fire blight host plants, one strain was found without the common plasmid. The strains without pEA29 were virulent in assays with immature pears and on apple seedlings, but showed a reduced growth level in minimal medium without amino acids and thiamine. Transposon-labelled pEA29 was transformed into the plasmid-free strains resulting in restoration of this growth deficiency. The plasmid was stably maintained in these *E. amylovora* cells. The newly designed chromosomal primers for conventional and for real-time PCR identified *E. amylovora* strains in field samples lacking pEA29. These variants are apparently rare, but were detected in isolates from different regions in the world with fire blight.

Keywords Fire blight · Plasmid pEA29 · rtPCR · TaqMan · Specific detection

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Introduction

Plasmids are ubiquitous in plant pathogenic bacteria and may contribute to the fitness or even virulence of pathogens. *Erwinia amylovora* causes fire blight on pome fruit trees and rosaceous ornamentals (Vanneste 2000). For detection and identification, the indigenous plasmid pEA29 was assumed to occur in all virulent *E. amylovora* strains (Bereswill et al. 1992; Falkenstein et al. 1988; Salm and Geider 2004). Strains without pEA29 required addition of thiamine, when grown in minimal medium (Laurent et al. 1989). The plasmid carries genes involved in thiamine

biosynthesis (McGhee and Jones 2000), and a lack of pEA29 may reduce fitness of *E. amylovora* for colonisation of plant tissue (Falkenstein et al. 1989). Other growth properties did not reveal a difference for *E. amylovora* strains with and without plasmid pEA29. As reported previously, three *E. amylovora* strains from Germany, Ea7/74, Ea1/79 and Ea11/88, were cured from plasmid pEA29 by introduction of a cloned fragment with the replication origin of pEA29 and selection for loss of the competing plasmid (Falkenstein et al. 1989). In other experiments (Bellemann et al. 1990), transposon mutagenesis with Tn4431 produced a series of insertions in genomic DNA of *E. amylovora*. Occasionally, the transposon was delivered into pEA29 visible by band shifts in plasmid fragments and created a convenient label in the plasmid for genetical manipulations. Additional plasmids have been described for *E. amylovora*, and a 70 kb plasmid was assumed to compensate for a lack of pEA29 (Llop et al. 2006). In this study, we screened *E. amylovora* strains from various geographic origins for the presence of pEA29 and describe strains, where neither pEA29 nor any other plasmid could be detected. Novel PCR primers from the chromosomal *ams* region were designed and used to detect those strains by conventional as well as by real-time PCR.

Materials and methods

Bacterial strains and their cultivation

Bacteria used in several experiments are listed in Table 1 and additional bacteria for controls in real-time PCR in Tables 3, 4, 5. When indicated, tetracycline was added to the agar or the liquid medium (StI, Standard I, Merck AG, Darmstadt, Germany; or LB, Luria-Bertani broth) at 20 µg ml^{-1}. Minimal medium MMA contained 6 g Na_2HPO_4x2 H_2O, 3 g KH_2PO_4, 0.5 g NaCl, 1 g NH_4Cl with 0.2 g $MgSO_4x7$ H_2O, 0.1 g $CaCl_2x3$ H_2O, separately autoclaved, and 0.2 g nicotinic acid and 10 g (1%) sorbitol, sterile-filtrated; 0.2 g thiamine was added to 1 l of medium, when indicated. MM2C medium containing asparagine has been described (Bereswill et al. 1998). The strains were pre-grown in LB medium and diluted in 1:100 steps in 5 ml MMA medium in 25 ml Erlenmeyer flasks shaken with 140 rpm at 28°C.

From field samples, *E. amylovora* strains were isolated from a suspension of plant material in water on LB-agar plates with cycloheximide (50 µg ml^{-1}). White colonies were assayed on MM2Cu agar for formation of mucoid yellow colonies (Bereswill et al. 1998) or by serological tests (Falkenstein et al. 1988). They were stored in nutrient broth with 15% glycerol at −80°C.

Virulence assays

For assays with pear, immature pears were sliced, inoculated with bacteria from liquid cultures or agar plates and incubated at 28°C in plastic boxes for several days. Young leaves of apple seedlings were cut with scissors and inoculated with bacteria suspended in water using sterile toothpicks. The plants were incubated in a growth chamber at 25°C. The negative control was mock inoculation with water, and the positive control inoculation with *E. amylovora* Ea1/79.

Plasmid manipulations

Plasmids were isolated and analysed as described in laboratory manuals (Sambrook and Rusell 2001). Plasmid pEA29 was labelled by random transposition with plasmid pUCD623 (Shaw et al. 1988) delivering Tn4431 (a Tn3 type, Tc^r; ref. Bellemann et al. 1990). Tetracycline-resistant cells were screened for the sites of transposon insertion and mutants with altered *Pst*I-patterns of pEA29 were considered as plasmid insertions. Strain Ea7/74(pEA29Tc) was used for preparation of the plasmid purified with commercial columns (Macherey-Nagel, Düren, Germany, NucleoBond PC 20). Plasmid pEA29Tc was transformed into plasmid-free strains by electroporation (Metzger et al. 1992), and transformants selected on agar with tetracycline.

For transfer experiments, strains Ea7/74(pEA29Tc) and EaX1/79Sm (without plasmid pEA29) were suspended in water, mixed 1:1 and 10 µl applied to slices of immature pears. After 4 days 10 µl ooze was diluted in 0.5 ml medium and 0.2 ml plated on selective agar (StI Tc20Sm500, StI agar with 20 µgml^{-1} tetracycline and 500 µg ml^{-1} streptomycin).

PCR assays

DNA templates Bacteria from various sources were grown overnight in LB medium and then diluted to a

Table 1 Bacterial strains used in PCR assays and plasmids with resistance markers

Strain	Origin	Reference
Ea1/79	*Erwinia amylovora*, northern Germany, 1979, apple 'James Grieve'	Falkenstein et al. (1988)
Ea1/79Sm	Ea1/79 with spontaneous Sm-resistance	
EaX1/79Sm	Ea1/79Sm without plasmid pEA29	Falkenstein et al. (1989)
Ea7/74	*Erwinia amylovora*, northern Germany, 1974, *Cotoneaster bullatus*	Falkenstein et al. (1988)
Ea11/88	*Erwinia amylovora*, Germany, 1988, *Cotoneaster salicifolius*	Falkenstein et al. (1988)
Ea11/88Sm	Ea11/88 with spontaneous Sm-resistance	
EaX11/88Sm	Ea11/88Sm without plasmid pEA29	Falkenstein et al. (1989)
Ea63/05	Pfullingen, Germany, *Pyracantha* sp., 2005	This work
EaA4	Behera, Egypt, pear 'Le Conte', 1982	This work and Shoeib (1986)
EaA10	Behera, Egypt, pear 'Le Conte' 1982	This work and Shoeib (1986)
EaIrn2	Shahriar Karaj, Iran, pear leaf, 2000	This work
EaIrn37	Tabriz, Iran, pear, 2001	This work
IVIA1614-2e	*Pyracantha* sp., Segovia, Spain, 1996	Jock et al. (2002)
Plasmid		
pEA29Tc	pEA29::Tn4431-30	Bellemann et al. (1990); Shaw et al. (1988)

density of A600=1.0 ($1x10^9$ colony-forming units (CFU)), further diluted 1:100 in 0.1% Tween 20 and lysed for 15 min at 65°C. From colonies of prescreened field samples, small amounts of cells were transferred from LB agar plates into 0.1% Tween 20, diluted 1:10 and lysed. To 40 µl master mix, 10 µl samples with template DNA were added.

DNA polymerases, buffers and amplificiation In assays without TaqMan probes, a stock solution of SYBR Green I (Roche) was diluted 1:10 in DMSO and then 1:300 in water. Real-time PCR was performed in the iCycler (BioRad) with the programme version 3.1.

Chemically modified HotStar Taq DNA polymerase (Qiagen, Hilden, Germany) was used for the real-time PCR assays. Taq-polymerase for conventional PCR was purchased from Fermentas (Rot-St. Leon, Germany). Conventional and real-time PCR assays were done in 20 mM Tris-Cl, pH 8.7; 20 mM KCl; 10 mM $(NH4)_2SO_4$ and 2.5 mM $MgCl_2$ with 0.5 u Taq-polymerase in 25 µl and 2 µl lysate or in 50 µl with 10 µl lysate and 1 u Taq-polymerase, respectively. The annealing temperature for all primers (Table 2) was 52° C for 20 s, primer extension at 72°C for 30 s and 94°C for denaturation (20 s). The conventional PCR runs were initiated for 60 s at 95°C followed by 35 cycles.

Real-time PCR with SYBR Green was performed as above. When TaqMan probes were used, two-step runs with 56°C annealing and polymerisation (60 s) and 94°C denaturation (30 s) temperature were performed for 41 cycles. The chemically modified hot start Taq-polymerase was activated for 30 min at 95°C instead of 15 min recommended by the manufacturers in order to obtain reliable signals. Usually 10 µl from lysates of 5×10^7 cells ml^{-1} were applied for signal detection.

Other methods

Bacterial motility was determined on soft agar plates (Hildebrand et al. 2006). The hypersensitive response in tobacco leaves (cv. 'Samsun') was done by infiltration of a bacterial suspension of 1 x 10^8 cells in water and evaluated after 2 days. SDS-PAGE protein analysis of cell extracts was done according to Sambrook and Rusell (2001). All assays were repeated at least three times with similar results.

Results

Identification of *E. amylovora* strains without plasmid pEA29

Strains isolated from plants with fire blight symptoms in Iran were identified as *E. amylovora* by various criteria such as colony morphology on agar with sucrose for levan formation, ooze production on immature pear slices, immunologically with *E. amylovora*-specific polyclonal antiserum and by PCR assays with primers P29A and P29B from the common plasmid pEA29

Table 2 PCR primers for qualitative and quantitative detection of *E. amylovora*

P29A	CGGTTTTTAACGCTGGG	position on pEA29: 26711 (Bereswill et al. 1992)
P29B	GGGCAAATACTCGGATT	position on pEA29: 25724 (Bereswill et al. 1992)
AMSJ14258	TTACTGCAGACGTGCTC	from *amsJ*; this work
AMSK14892c	ATCTTCTCCGCCGGACA	from *amsK*; this work
P29TF761	CACTGATGGTGCCGTTG	*position on pEA29: 26469
P29TM782	FAM-TACCTCCGCAGCCGTCATGG-TAMRA	*position on pEA29: 26490
P29TR872	CGCCAGGATAGTCGCATA	*position on pEA29: 26580
AMSK14819	AACGAGTTGCTGCTACC	from *amsK*; this work
AMSK14840Cy5	Cy5-AGCCGTCTGTGGCAGCACAA-BHQ2	from *amsK*; this work
AMSK14840FAM	FAM-AGCCGTCTGTGGCAGCACAA-BHQ1	from *amsK*; this work
AMSK14948c	CATCGCGTAGCTTAAGG from *amsK*;	this work

* Salm and Geider 2004

The numbers in the names of the primers indicate positions of DNA in data banks (accession number X77921 for the *ams*-region and AF264948 for pEA29)

(Bereswill et al. 1992). One strain, EaIrn37, did not produce the expected PCR results. In order to verify the isolate as *E. amylovora* by PCR, chromosomal primers were applied for amplification. The previously described primers AMSL and AMSRc (Bereswill et al. 1995) were not always satisfactory, because they produced weak signals for some *E. amylovora* strains. Therefore, we applied a primer pair originally used for sequence studies of the *amsJ*/*amsK* region (Bugert and Geider 1995). Primers AMSJ14258 and AMSK14892c (Table 2) produced a PCR fragment of 0.6 kb, specific for *E. amylovora* that was not detected in lysates from *Pseudomonas fluorescens*, *P. graminis*, *Pantoea agglomerans*, *Pectobacterium atrosepticum*, or *E. tasmaniensis* cells. In particular, strain EaIrn37 was identified as *E. amylovora* by these primers (Fig. 1). Some recent isolates of *E. billingiae* and mixtures of bacteria from plants without *E. amylovora* produced shadow bands with the primers, so positive signals may require conformation of fire blight with additional PCR primers.

The Spanish strain IVIA1614-2e produced *Xba*I fragments of pattern type Pt3 like other isolates from the same region (Jock et al., 2002). In PCR assays with primers AMSJ14258 and AMSK14892c, a band of 0.6 kb was obtained, but not the expected signal of 1 kb with the plasmid primers P29A and P29B. Consequently, the strain should be devoid of plasmid pEA29.

Based on data from an earlier report on plasmid-free strains, we have re-investigated 10 additional *E. amylovora* strains from Egypt (Falkenstein et al. 1988) with primers AMSJ14258 and AMSK14892c and confirmed these as *E. amylovora* (data not shown). With the plasmid primers P29A and P29B, no signal was observed for strains EaA4 and EaA10, indicating the lack of plasmid pEA29.

To confirm that the strains EaA4, EaA10, EaIrn37 and IVIA1614-2e, which did not amplify a DNA fragment with primers from plasmid pEA29, are *E. amylovora*, we also applied several semi-selective assays for identification. The strains grew as yellow mucoid colonies on MM2Cu-agar, they produced levan on agar with sucrose and did not grow on MM1Cu-agar as expected for *E. amylovora* (Bereswill et al. 1998).

Screening for pEA29 by plasmid DNA isolation resulted in the lack of any DNA bands for strains

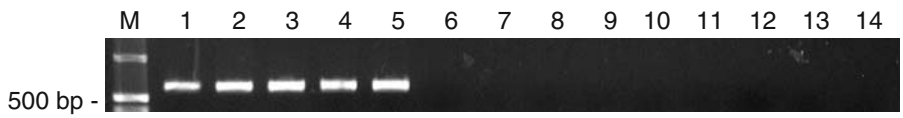

Fig. 1 DNA fragments amplified by primers AMSJ14258 and AMSK14892c from the *amsJ*/*amsK* region of *E. amylovora*. Lanes are:1, Ea1/79Sm; 2, EaA10; 3, EaIrn2; 4, EaIrn37; 5, IVIA1614-2e; 6, A506 (*P. fluorescens*); 7, EhC9-1 (*P. agglomerans*); 8, BK1 (*Kluyvera* sp.); 9, Eca185 (*P. carotovorum*); 10, Eb661 (*E. billingiae*); 11, Eh-SB (*P. graminis*); 12, Et1/99 (*E. tasmaniensis*); 13, Esa13 (*E. tasmaniensis*); 14, water control. M, 1 kb DNA ladder plus (Fermentas)

EaA10 and EaIrn37, but not for strain IVIA1614-2e (Fig. 2). Plasmid pEA29 was isolated from strains Ea1/79 and EaIrn2 and digested with restriction enzyme *Pst*I. The pattern of plasmid DNA from IVIA1614-2e was different from the bands of the common plasmid pEA29 indicating a different plasmid in this strain (Fig. 2).

Comparison of isogenic *E. amylovora* strains with and without plasmid pEA29

From the modified strain Ea7/74, the plasmid pEA29::Tn4431 (pEA29Tc) was isolated and used for transformation of the plasmid-free strains EaA4, EaA10, Eairn37, and IVIA1614-2e. After electroporation of pEA29Tc, the cells were selected on tetracycline and introduction of pEA29Tc was confirmed with primers P29A and P29B.

The isogenic *E. amylovora* strains with and without plasmid pEA29 were cultivated in MMA medium. After four passages with 1:100 dilutions in MMA without thiamine, the cell density was determined by turbidity at 2 days in the culture of the last transfer (Fig. 3). In MM2C medium containing a high concentration of asparagine, no dependence of plasmid-free *E. amylovora* strains on thiamine was seen. The absence of the thiamine in MMA medium did not abolish growth of plasmid-free strains, but caused growth to a significantly lower density for strains without plasmid pEA29 unlike for growth in MMA medium with thiamine. Strains Ea7/74, Ea1/79 and Ea11/88 grew into the stationary phase with a cell density of approx. 0.25 A600 units (Fig. 3). Strains EaA4, EaA10, EaIrn37, and IVIA1614-2e grew only to a density of approx. 0.1 units. Strains cured from plasmid pEA29 also showed low growth performance. The natural strains without plasmid pEA29 increased their growth level to wild-type after introduction of pEA29Tc (Fig. 3).

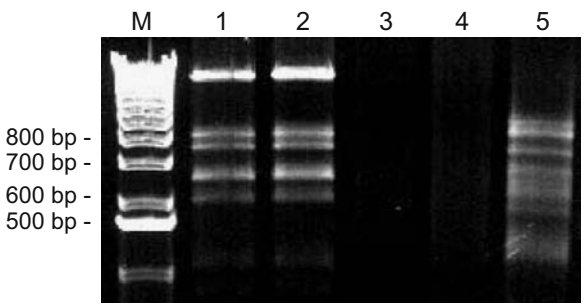

Fig. 2 Agarose gel electrophoresis of plasmid DNA isolated from *E. amylovora* strains and digested with *Pst*I. Lane: 1, Ea1/79Sm; 2, EaIrn2; 3, EaA10; 4, EaIrn37; 5, IVIA1614-2e. Sizes from 1 kb DNA ladder plus (Fermentas). The DNA was isolated after alkaline cell lysis on purification columns (Macherey and Nagel)

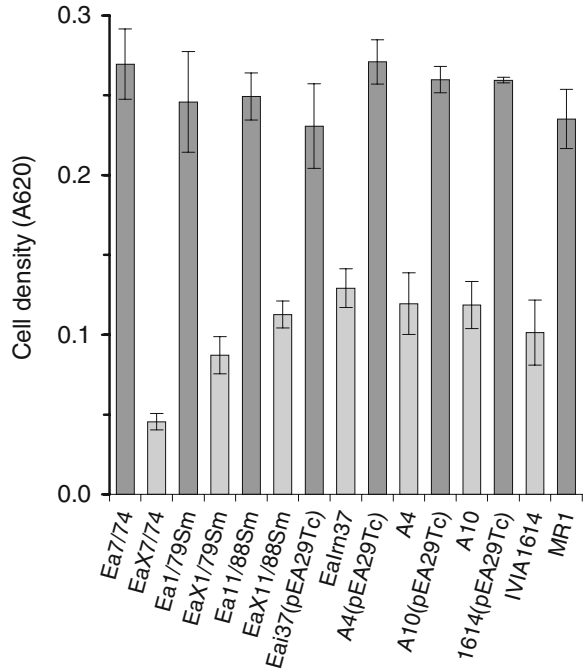

Fig. 3 Growth of *E. amylovora* with and without plasmid pEA29 in MMA medium lacking thiamine. Dark bars represent strains with plasmid pEA29; light bars indicate strains without pEA29. Individual bars refer to Ea7/74 (with pEA29, '+'), EaX7/74 (cured from pEA29, '-'), Ea1/79Sm (+), EaX1/79Sm (−); Ea11/88Sm (+), EaX11/88Sm (−); and natural isolates EaIrn37, EaA4, EaA10, IVIA1614-2e without plasmid pEA29 and with pEA29Tc (indicated); and MR1(+)

Stability of pEA29 and lack of autonomous transfer

Repeated subculturing of strain Ea1/79 did not result in a detectable loss of the plasmid. pEA29 *Erwinia amylovora* strains containing transposon-labelled pEA29 were grown in StI medium, plated on StI agar and single colonies transferred to StI agar with tetracycline to screen for possible loss of plasmid pEA29Tc. Out of 100 colonies of the strains EaA4 (pEA29Tc), EaA10(pEA29Tc), EaIrn37(pEA29Tc) and IVIA1614(pEA29Tc), all 400 colonies grew

under the selective condition, indicating that none had lost plasmid pEA29Tc.

Strains Ea7/74(pEA29T) and EaX7/74Sm were simultaneously grown on pear slices, and 10 µl ooze (approx. 109 bacteria) were spread in 0.2 ml StI medium on selective agar. No growth of double-resistant colonies was observed after 2 days on StI agar with Tc and Sm from diluted ooze. An autonomous transfer of the labelled plasmid pEA29 to Sm-resistant *E. amylovora* cells was not detected in plant tissue with fire blight symptoms in three experiments.

Detection of plasmid-free *E. amylovora* strains by real-time PCR.

Quantitative assays of *E. amylovora* by real-time PCR with a plasmid-borne primer pair and SYBR Green as well as application of TaqMan probes (Salm and Geider 2004) were adapted for *ams*-primers from the chromosome of *E. amylovora*, and the assay conditions were further optimised. The primers AMSK14819 and AMSK14948c (Table 2) produced a fragment of 127 bp which could be seen by conventional PCR. They were applied to real-time PCR assays with SYBR Green (Fig. 4). The signals in area A with primers P29TF761/P29TR872 were shifted to a lower cycle threshold due to the increased copy number of plasmid pEA29 compared to area B with the chromosomal primers.

For *ams* primers AMSK14819 and AMSK14948c and bacterial DNA lysates, a signal was obtained for all *E. amylovora* strains (Fig. 4). In parallel runs, the primer pair P29TF761 and P29TR872 from plasmid pEA29 were applied with SYBR Green for signal detection. In another series, the fluorescence-labelled TaqMan probes AMSK14840Cy5 or AMSK14840-FAM (Table 2) were used for signal detection instead of SYBR Green. In the same assay, these produced identical cycle threshold values with *E. amylovora* DNA. For simultaneous identification of *E. amylovora* strains with and without plasmid pEA29, the described plasmidal and chromosomal primers were applied with TaqMan probes P29TM782 (FAM) and AMSK14840Cy5 (Fig. 5). In this type of assay, strains without plasmid pEA29 gave rise to a steeper increase of the signal with AMSK14840Cy5 in comparison to normal *E. amylovora* strains, but the cycle thresholds were not changed.

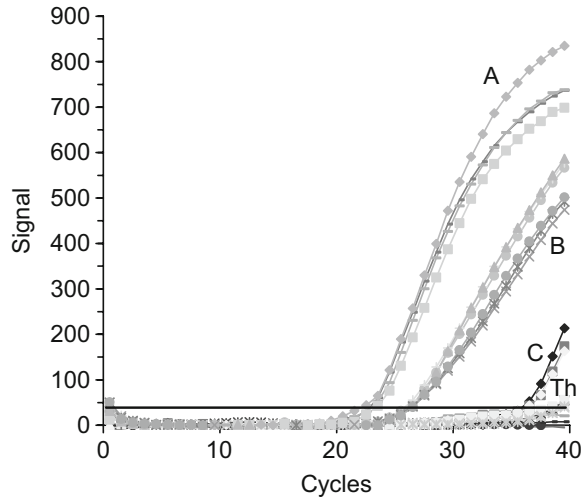

Fig. 4 Detection of *E. amylovora* with real-time PCR using SYBR Green. The primers P29TF761, P29TR872 from plasmid pEA29 (area A) and AMSK14819, AMSK14948c from *amsK* (area B) were used in parallel runs with the same samples and the profiles overlayed. The *E. amylovora* strains include MR1 (USA, from rubus), Ea321 (France), Ea775 (England), CFBP1232 (England, type strain) and Ea4/82 (Egypt). Strain EaX1/79 cured from plasmid pEA29 is only represented in area B arising from chromosomal primers. Area C represents signals for the water control and the lysates from: *P. atrosepticum* 16A-1, *P. carotovorum* Ecc1A-1, *Dickeya chrysanthemi* Ech260, *D. chrysanthemi* Ech3937, *B. subtilis*, *Erwinia* sp. FLA3-2003. Th, cycle threshold

Specificity of the chromosomal real-time PCR primers and sensitivity for detection of *E. amylovora*

In DNA samples from various plant-associated bacteria, PCR signals with the chromosomal primers AMSK14819 and AMSK14948c and SYBR Green or the TaqMan probe AMSK14840Cy5 were only observed for *E. amylovora* strains, but not from other bacteria such as *E. pyrifoliae*, *P. stewartii*, or the epiphytic species *E. billingiae* and *E. tasmaniensis*. Table 3 summarises these data together with assays for strains from additional bacteria.

Titration against decreasing concentrations of lysed *E. amylovora* allowed detection of <500 CFU in a 50 µl assay. Lysis in 0.1% Tween 20 at 65°C was as efficient as lysis in 1% Tween 20 for *E. amylovora*, applied previously (Bereswill et al. 1992) and kept the final Tween concentration low after the addition of a 10 µl sample volume to 40 µl assay mix. The lysed samples could be stored at

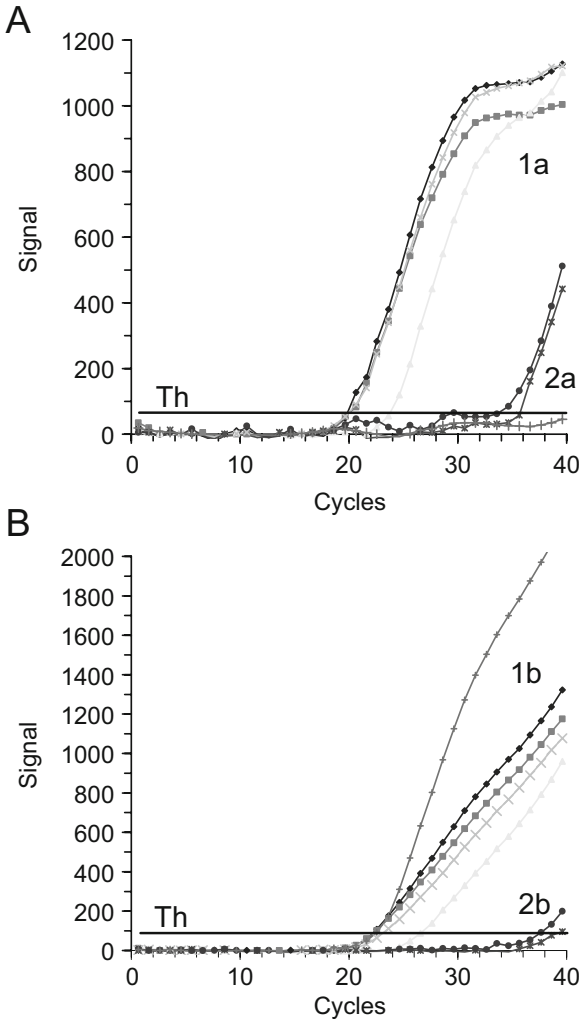

Fig. 5 Simultaneous detection of *E. amylovora* strains with and without plasmid pEA29 by duplex real-time PCR with TaqMan probes from the chromosomal *ams* region and from pEA29. Panel A: PCR with primers P29TF761, P29TM782 (Taqman, FAM), P29TR872. Signals in area 1a from DNA of strains: Ea702/05, Ea725/05, Ea743/05, Ea28/05; in area 2a: nEa41/05, nEa54/05, and Ea63/05. Panel B: PCR with primers AMSK14819, AMSK14840Cy5, AMSK14948c. Signals in area 1b from DNA of strains: Ea63/05 and Ea702/05, Ea725/05, Ea743/05, Ea28/05; area 2b: nEa41/05, nEa54/05. (nEa: not *E. amylovora*)

−20°C for several months without any effect on signal detection.

Screening a series of isolates for plasmid pEA29 with duplex real-time PCR

In a survey for fire blight in the state of Baden-Württemberg in 2005, 125 isolates were obtained from different host plants with fire blight symptoms at various sites (Table 4). The isolates were screened for *E. amylovora* by colony morphology and agglutination assays with a polyclonal antiserum. When lysed colonies from agar plates were analysed with plasmidal and chromosomal TaqMan probes, the latter amplified 123 samples, the former 122. Consequently, two strains proved not to be *E. amylovora*. In addition, one strain Ea63/05, was identified by real-time PCR to lack plasmid pEA29. The colony morphology of this isolate on semi-selective agar plates was identical with other *E. amylovora* strains assayed, but produced little ooze on pear slices. The two isolates without PCR signals with *E. amylovora* primers showed a different colony morphology (Table 5). The plasmid-free strain Ea63/05 was obtained from *Crataegus* x *lavallei* planted in a container of a nursery in Pfullingen (Baden-Württemberg, Germany), where all diseased plants were destroyed after sampling and first confirmation of fire blight.

Virulence assays and additional tests with *E. amylovora* strains lacking plasmid pEA29

Strains EaA4, EaA10, EaIrn37 and IVIA1614-2e did not only show the colony morphology on semi-selective agar as the *E. amylovora* strains in Table 5, but also caused a hypersensitive response in tobacco leaves and were motile in swarming agar with a reduced movement of strains EaA4 and EaA10 compared to the strains from Iran and Spain. In virulence assays on slices of immature pears, these *E. amylovora* strains without plasmid pEA29 produced ooze. On young apple seedlings, they caused wilt and necrosis. In these virulence assays, strain IVIA1614-2e was attenuated. The number of CFU recovered from water suspensions of *E. amylovora* strains with and without plasmid pEA29 did not differ after storage for an extended period of time. With SDS-PAGE analysis, similar protein patterns were observed for plasmid-free strains and for strain Ea1/79Sm as well as for other *E. amylovora* strains from Iran with pEA29 (data not shown). These data, and the assays for virulence, motility and viablility show a concordance of *E. amylovora* strains with or without plasmid pEA29 in several properties.

Table 3 Signals of *E. amylovora* and other bacteria by real-time PCR with SYBR Green or TaqMan probes P29TM782/AMSK14840Cy5

Strains/primers	P29TF761/ P29TR872	AMSK14819/ AMSK14948c
Erwinia amylovora Ea1/79, EaIrn2, EaIrn39 (Iran), MR1 (USA, rubus), Ea321 (France), Ea775 (England), CFBP1232 (type strain), Ea4/82, Ea20/82, Ea25/82, Ea31/82, Ea42/82, Ea43/82, Ea47/82, Ea61/82 (all Egypt), 122 isolates from field samples obtained in 2005 (Table 4)	Yes	Yes
E. amylovora EaA4, EaA10, EaIrn37, IVIA1614-2e, EaX1/79 (cured from pEA29), Ea63/05 (from field samples, Table 4)	No	Yes
Agrobacterium tumefaciens C58	No	No
Bacillus subtilis		
Kluyvera sp. BK1		
Dickeyia chrysanthemi Ech260, 3937		
Erwinia billingiae Eb661		
Erwinia pyrifoliae Ep1/96, Ejp557		
Erwinia tasmaniensis Et1/99, Esa13, FLA2003		
Pantoea agglomerans EhC9-1, EhNZ, Eh252, Eh2b/89, MB96, EhDCA		
P. stewartii DC283		
Pseudomonas fluorescens A506		
P. graminis Eh-SB		
P. syringae U4		
Pectobacterium atrosepticum Eca185, 16A-1 (Poland), Ecc1A-1 (Poland)		
X. campestris pv. campestris Xcc1, Xcc256		
unidentified bacteria: FLA1/00 (apple), FLA2/00 (pear), FLA8/00 (apple), Sf3 (from oozing plum tree), two isolates from prescreened field samples (Table 4)		

Most strains listed in this Table have been described in more detail elsewhere (Falkenstein et al. 1988; Jock et al. 2002).

Discussion

Plasmids often contribute to virulence of plant-pathogenic bacteria. The causal agent of fire blight contains a common plasmid of 29 kb (Falkenstein et al. 1988; Laurent et al. 1989; McGhee and Jones 2000), which may increase fitness of *E. amylovora* during colonisation of plants such as colonisation of young shoots of pear seedlings (Falkenstein et al. 1989). Natural strains may lose plasmid pEA29 at some stages of propagation in a diseased plant or after their isolation in the laboratory. A tendency for spontaneous loss of pEA29 could not be demonstrated here. Strains EaA4, EaA10, EaIrn37 and IVIA1614-2e labelled with plasmid pEA29Tc maintained the tetracycline-resistance and had no tendency for spontaneous loss of the plasmid. The evidence supporting natural occurrence of *E. amylovora* strains without plasmid pEA29 is circumstantial. Strain EaIrn37 was isolated in Iran from an orchard with fire blight, preliminary characterised as *E. amylovora* by microbiological methods in Tehran and then extensively investigated in Germany. No additional strains from the site of isolation were stored. Strain Ea63/05 was screened for plasmid pEA29 in a large collection of field isolates. All plants with fire blight symptoms observed in a nursery with the disease were subsequently destroyed eliminating access to additional strains without pEA29. Strains EaA4 and EaA10 were independently isolated in 1982 in Egypt from diseased pear trees in a narrow region.

Table 4 Host plants and distribution of 125 '*Erwinia amylovora*' isolates obtained in Baden-Württemberg, Germany, in 2005

Host plant	Number of isolates
Pyrus sp.	59
Malus sp.	35
Cydonia sp.	12
Cotoneaster sp.	10
Crataegus sp.	6
Sorbus sp.	1
Pyracantha sp.	1
Mespilus sp.	1

Table 5 PCR and additional identification steps for field isolates from plants with fire blight symptoms lacking pEA29

Strain	LBsuc	MM2Cu	MM	Cf	Cas	PCR:pEA29	*ams*-primers	Conclusion
Ea702	+	ym	Muc	pink	+	+	+	*E. amylovora*
Ea725	+	ym	Muc	pink	+	+	+	*E. amylovora*
Ea743	+	ym	Muc	pink	+	+	+	*E. amylovora*
Ea28/05	+	ym	Muc	pink	+	+	+	*E. amylovora*
nEa41/05	(+)	ng	But	creamy	−	−	−	not *E. amylovora*
nEa54/05	+	ng	But	creamy	−	−	−	not *E. amylovora*
Ea63/05	+	ym	Muc	pink	+	−	+	*E. amylovora*
Ea1/79	+	ym	Muc	pink	+	+	+	*E. amylovora*

MM2Cu agar (Bereswill et al. 1998): ng, no growth; ym, colonies yellow, mucoid; MM1 agar (Bereswill et al. 1998): muc, mucoid; but, butyrious; Cas-medium (Schwyn and Neilands 1987): +, halos indicating release of siderophores; Cf, coliform agar, Rapid Diagnosis medium of Kritzman *et al.* (2003). The first three strains were isolated in Germany 2005 from diseased plants, isolates four to seven are part of strains summarised in Table 4

Considering the rare loss of pEA29 (Jock et al. 2003), two strains without the plasmid and the previous description of a third strain from Egypt (Shoeib 1986) are evidence that these strains can persist in the field. Strain IVIA1614-2e was previously assayed for its PFGE pattern and classified into the pattern type Pt3 (Jock et al. 2002); it was isolated from *Pyracantha* sp. in Spain and is obviously related to strain IVIA1614-2a, which was isolated from *Crataegus* sp. and also described as lacking plasmid pEA29 (Llop et al. 2006). Isolation of these strains without pEA29 from different sites or from different host plants supports their natural existence.

Plasmid pEA29 can affect *E. amylovora* in symptom formation on pear shoots (Falkenstein et al. 1989). In earlier experiments (Laurent et al. 1989) its association with thiamine synthesis was assumed and genes possibly involved in thiamine metabolism were identified by nucleotide sequence analysis (McGhee and Jones 2000). A spontaneous loss of plasmid pEA29 may not completely eliminate the fitness of these strains to colonise host plants. Here we present data on the multiple isolation of the strains in Egypt. In a previous report from Ireland, four strains out of 65 did not produce a PCR signal with plasmid primers (Brennan et al. 2002). In rare cases, PCR with these primers can be negative in an assay, which requires confirmation for the absence of the signal and application of additional methods. The isolate IVIA1614-2b from hawthorn (Llop et al. 2006) and the isolate IVIA1614-2e from *Pyracantha* sp. carry a plasmid different from pEA29. Since strains of *E. amylovora* from Iran and Egypt appeared to have no plasmids (Table 3, Fig. 5), a substitution of pEA29-encoded functions by another plasmid may not be required for natural survival of the plasmid-free pathogen. *Erwinia amylovora* strains without plasmid pEA29 are not common. When attempts to detect the pathogen with PCR primers from the plasmid fail, primers derived from the chromosome can then identify *E. amylovora*.

Chromosomal primers were designed previously from the *ams* genes (Bellemann et al. 1990) or as complex oligonucleotides from rRNA genes (Maes et al. 1995). We have re-investigated primers from the *amsB*/*C* region (Bereswill et al. 1995) and designed oligonucleotides, which amplified a short sequence of the *amsJ*/*amsK* genes. We used these primers for conventional PCR and others from *amsK* for real-time PCR with SYBR Green and for TaqMan probes labelled with FAM or with Cy5 for signal detection. Similar genes occur in other bacterial species, but no signal was obtained from strains classified as *E. pyrifoliae* and *P. stewartii* (Table 3). It is possible to run dual detection by using the published FAM-labelled probes from plasmid pEA29 (Salm and Geider 2004) and the Cy5-labelled chromosomal probes described here. Due to the intermediate copy number of plasmid pEA29, primers derived from the plasmid will be more sensitive than chromosomal primers and might be preferred for screening of low levels of the fire blight pathogen. On the other hand, disease detection in orchards is often based on fire blight symptoms such as wilt, necrosis and sometimes ooze formation, so identification of *E. amylovora* is usually done on

a high bacterial level. Nevertheless, the application of dual TaqMan probes will immediately recognise plasmid-free strains.

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