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Analysis of variable short-sequence DNA repeats on the 29 kb plasmid of *Erwinia amylovora* strains

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Abstract

The fire blight pathogen *Erwinia amylovora* has been specifically and sensitively detected by PCR assays with primers derived from plasmid pEa29. The amplified fragment of approximately 1 kb can vary in length for individual strains, easily seen in a digest with restriction enzymes *Sau*3A or *Hpa*II. DNA fragments from this variable region were cloned and DNA sequence analysis revealed short-sequence DNA repeat (SSR) motifs which were reiterated to various extents. The SSR units consisted of eight nucleotides (ATTACAGA), and terminated with ATTA which is part of an SSR. The shortest repetition consisted of four units and the longest one in Austrian *E. amylovora* strains was 15 units. The number of SSR units was remarkably stable during propagation of strains, but was occasionally changed when a strain was stressed by exposure to antibiotics, copper sulphate or storage at low temperature. Changes in the SSR number could be due to adjustment in bacterial fitness to environmental pressure. We designed oligonucleotide PCR primers from DNA sequences adjacent to the SSR region of pEA29 for rapid analysis of SSR length variations. With this PCR assay, more than 130 strains were classified into at least 11 types based on the number of repeats. *E. amylovora* strains isolated in Germany carried mostly six repeats in pEA29, which never changed under laboratory conditions. *E. amylovora* strains from Hungary and the Netherlands were quite divergent for the SSRs and further changes were sometimes observed after plating on agar medium. Homology search of nucleotide sequence data libraries revealed similarities of the SSR motif to partition functions of low copy number plasmids. Amino acid homology searches showed similarity of the deduced amino acid sequence in the ORF adjacent to the SSR motif to replication proteins of plasmids. The SSR may play a role in regulation of plasmid replication and partition as assumed for iterons.

Abbreviations: SSRs – short-sequence DNA repeats; ORF – open reading frame; kb – kilobases; PFGE – pulsed-field gel electrophoresis.

Introduction

Fire blight is a bacteriosis of pome fruit trees and other rosaceous plants (Van der Zwet and Keil, 1979) and has been observed in more than 30 countries (Van der Zwet, 1996). In the Southern hemisphere the disease has been only confirmed in New Zealand. Various methods have been described for detection of the pathogen such as screening on semi-selective plates (Bereswill et al., 1998) or PCR based methods (Bereswill et al., 1992, 1995).

All strains of *Erwinia amylovora* carry a low copy number plasmid of 29 kb (pEa29) (Falkenstein et al., 1988). The nucleotide sequence of a cloned 1 kb *Pst*I DNA fragment was used to design oligonucleotides for specific and sensitive detection of *E. amylovora* by PCR. Nested PCR assays were performed after sequence analysis of the 1 kb DNA

fragment (McManus and Jones, 1995). Small but significant variations in the fragment size were observed for individual strains (Lecomte et al., 1997) and used for a proposal to classify *E. amylovora* strains from Central Europe into three types and will be shown here to rely on short-sequence DNA repeats (SSRs).

Variations in the number of SSRs may result from recombination processes or by slipped-strand mispairing (SSM) during replication (Strand et al., 1993). Bacteria may thus adapt their genetic repertoire in response to environmental pressure. Thus, SSM-mediated variations can have implications for bacterial pathogenesis and evolutionary fitness and allow epidemiological studies on the spread of pathogenic bacteria (Van Belkum et al., 1998).

Examples for bacterial SSRs are the iterons (Filutowicz et al., 1994), which are present at plasmid origins of replication and specifically bind to an initiator protein such as RepA. An initiation complex is obtained upon saturation of the iteron moieties (Brendler et al., 1997).

This report describes the molecular characterization of the SSR motif within the 1 kb *Pst*I DNA fragment on plasmid pEA29 in *E. amylovora* leading to a size variation of the PCR fragment. More than 130 *E. amylovora* strains were investigated and placed into 11 types based on the SSR number.

Materials and methods

Bacterial strains

The *E. amylovora* strains assayed are listed in Table 1. They were grown on Standard-I agar (St.I, Merck AG, Darmstadt, Germany) at 28 °C for two days.

PCR and restriction fragment analysis

The PCR reactions were done in 50 µl with 10 ng of template DNA and 0.5 U of *Tth* polymerase as described previously (Bereswill et al., 1992). The 1 kb PCR fragment was amplified with the pEa29 primers A (5′-CGGTTTTTAACGCTGGG) and B (5′-GGGCAAATACTCGGATT) and subcloned into pGEM-T (Promega) for sequence analysis. For RFLP analysis, the PCR reaction was precipitated with two volumes of ethanol and washed in 70% ethanol. The pellet was dissolved in water and digested with restriction enzymes *Sau*3A or *Hpa*II at 37 °C for 3 h. The products were analyzed on an 8% polyacrylamide gel. PCR primers from the repeat region were RS1 (5′-ACCTCAGTGCGATTACAG) and in the antisense direction of the 1 kb fragment RS2c (5′-GTCCCATTCTGTGTTAAG), derived from DNA adjacent to the repeat region 36 bp left and 61 bp right of the SSR motif.

DNA sequence analysis

Amplified fragments from six strains were eluted from a 0.8% agarose gel with the Nucleotrap kit (Macherey and Nagel, Düren, Germany) and subcloned in pGEM-T. The inserts were sequenced with the A.L.F. sequencer (Pharmacia Biotech AB, Uppsala, Sweden). Fragments amplified from Ea1/79 and EaU-1 were sequenced with primers derived from the T7- and T3-promoter regions. Insertions of other strains were sequenced with the sequencing primer RSeq (5′-CTTAGCCATA ACGCAACA). Sequences were assembled with Align v3.0 and Clone manager v4.1 (S&E Software). Database searches were performed with BLASTX of the Genome Net, Universities of Kyoto and Tokyo, Japan and EBI, Hinxton Cambridge/UK and with emotif (Nevill-Manning et al., 1998).

Stability tests for SSRs in pEA29

E. amylovora strains were propagated from single colonies on various agar plates such as MM2Cu medium which produces a specific colony morphology for *E. amylovora* (Bereswill et al., 1998). Bacterial colonies were stored on St.I agar in the cold room for three months. Fully grown cultures were plated on streptomycin (200 µg/ml) or rifampicin (200 µg/ml). *E. amylovora* from an artificially infected plant was reisolated after 10 days. Afterwards the strains were analyzed by PCR to detect changes in the SSR size.

Results

Detection of a variable short repeat sequence in PCR fragments amplified with plasmid primers

The DNA fragment from plasmid pEA29 of strain Ea1/79 was amplified and cloned into vector pGEM-T and subsequently sequenced (Figure 1). The nucleotide sequence was almost identical with a corresponding sequence published for strain Ca11 by McManus

Table 1. Sources and properties of *E. amylovora* strains

Strain	Other name/origin (source, isolated by) or [ref.]; year of isolation
115.22	Bulgaria, *C. oblonga* (S. Bobev); 1989
202.1	Bulgaria, *C. oblonga* (S. Bobev); 1989
2650-1	Ville de Paris; *Cotoneaster franchetti* (J.P. Paulin); 1995
2651-1	Ville de Paris; *Cotoneaster salicifolia* (J.P. Paulin); 1995
2652-2	Ville de Paris; *Cotoneaster lacteus* (J.P. Paulin); 1995
295/93	Hälrang, Austria; *Cotoneaster salicifolia* (M. Keck); 1993
674/94	Digling, Austria; *Pyrus communis* (M. Keck); 1994
BA109	Cariddi, Italy. pear, cv. 'Bella di Giugno', Bari (Apulia); 1990
BPIC1631	Czechia, *Crataegus*, Middle Bohemia (V. Kudela); 1989
BPIC847	Arkadia, Greece, *P. communis* cv. 'Passe crassane' (P.G. Psallidas); 1984
BPIC913	Crete, Greece, *P. communis* cv. 'de Cayan' (P.G. Psallidas); 1985
BPIC928	Mitilini, Greece, *P. communis* cv. 'Passe crassane' (P.G. Psallidas); 1986
CA3R	California, U.S.A, apple (Mcmanus and Jones); 1995
CFBP1367	Ea321/Lille, France; *Crataegus* sp. (J.P. Paulin via S. Beer); 1978
CFBP1368	Ea322/ France; *Cotoneaster* sp. (J.P. Paulin via S. Beer); 1972
CFBP1430	Lille, France, *Crataegus* sp. (R. Samson and J.P. Paulin); 1972
CR03	Croatia, *P. communis* (B. Cvietkovik); 1996
CR04	Croatia, *Crataegus* sp. (B. Cvietkovik); 1996
CRIV	Croatia, *Crataegus* sp. (B. Cvietkovik); 1996
E242	Ostfildern, Germany, pear (E. Moltmann); 1996
E248	Neustetten, Germany, pear (E. Moltmann); 1996
E263	Ludwigsburg, Germany, apple (E. Moltmann); 1996
E268	Murrhardt, Germany, apple (E. Moltmann); 1996
E269	Murrhardt, Germany, Cotoneaster (E. Moltmann); 1996
E282	Backnang, Germany, pear (E. Moltmann); 1996
E284	Reutlingen, Germany, pear (E. Moltmann); 1996
E284Cu3	propagated on MM2Cu plate from CCT plate
E291	Mössingen, Germany, pear (E. Moltmann); 1996
E298	LRA Calw, Germany, quince (E. Moltmann); 1996
E299	LRA Calw, Germany, pear (E. Moltmann); 1996
E8	spontaneous avirulent mutant of E9, no levan and amylovoran synthesis
E8Sm	spontaneous mutant of E8, Sm^r
E9	USA (R. Goodman via W. Zeller)
Ea1/79	Northern Germany; *Cotoneaster* sp. (W. Zeller); 1979
Ea1/79Cu6	tolerant to copper sulphate in medium
Ea1/79Rif	rifampicin resistant spontaneous mutant of Ea1/79
Ea12/84	Egypt; (via W. Zeller); 1984
Ea1496/66	New Zealand (J. Young); 1966
Ea209	Israel, pear (via S. Manulis); 1996
Ea226	Israel, loquat (via S. Manulis); 1996
Ea22I/83(R)	Egypt; *P. communis* cv. 'Le Conte' (via W. Zeller); 1983
Ea237	Baden-Württemberg, Germany, *Malus* (via K. Richter); 1996
Ea25/82	Egypt; *P. communis* cv. Le Conte (via W. Zeller); 1982
Ea25/82Sm	spontaneous mutant of Ea25/82,Sm^r [Zhang et al., 1997]
Ea250	Sachsen-Anhalt, Germany, *Malus* (via K. Richter); 1996
Ea263	Israel, quince (via S. Manulis); 1996
Ea266	E4001A/ Simcoe, Ontario, Canada; R.I. Greening apple (W. G. Bonn via S. Beer); 1977
Ea267	Israel, apple (via S. Manulis); 1996
Ea27/91	Southern Germany, *Pyrus communis* cv, 'Schweizer Wasserbirne' (W. Zeller); 1991
Ea270	Baden-Württemberg, Germany, *Pyrus* (via K. Richter); 1996
Ea273	New York State, USA; R.I. Greening apple (S. Beer); 1971
Ea28/91	Southern Germany, *Sorbus asia* (W. Zeller); 1991
Ea286	Bayern, Germany,*Pyrus* (via K. Richter); 1996
Ea322A	avirulent mutant of CFBP1368 (Ea322)
Ea331.93	Switzerland, pear cv. 'Josephine von Mechelen', Kanton Zuerich (T. Hasler); 1993

Table 1. Continued

Strain	Other name/origin (source, isolated by) or [ref.]; year of isolation
Ea345.90	Switzerland, *Cotoneaster salicifolius*, Kt. Thurgau (T. Hasler); 1990
Ea4/82	Egypt; *Pyrus communis* cv. 'Le Conte' (via W. Zeller); 1982
Ea4/83	Egypt; *M. sylvestris* (via W. Zeller); 1983
Ea5/84	Egypt; *P. communis* cv. Le Conte (via W. Zeller); 1984
Ea508.94	Switzerland, *Crataegus*, Kt. Aargau (T. Hasler); 1994
Ea595	NCPPB595 (ATCC29850); England, *P. communis*; (J.E. Crosse via W. Zeller); 1958
Ea7/74	Northern Germany; *Cotoneaster* sp. (W. Zeller); 1974
Ea7/74-Bi	Ea7/74; reisolated from ooze of a pear slice
Ea7/74C	Ea7/74; reisolated from a pear tree in an experimental orchard
Ea7/84	Egypt; *P. communis* cv. 'Le Conte' (via W. Zeller); 1984
Ea771	NCPPB771; England, *Cotoneaster melanocarpa*; (E. Billing via W. Zeller); 1959/60
Ea775	NCPPB775; England, *Crataegus* sp. (E. Billing via W. Zeller); 1959/60
Ea8439/83	New Zealand (J. Young); 1983
Ea-AUS02	Germany, from Australian wood sample (*Cotoneaster pannosus*); 1997
Ea-AUS02A	Germany, from Australian wood sample (*Cotoneaster pannosus*); 1997
Ea-AUS02H	Germany, from Australian soft agar sample (*Cotoneaster pannosus*); 1997
Ea-AUS34	Germany, from Australian wood sample (Sorbus); 1997
Ea-Eb	Germany; strawberry (grown near apple orchard with fire blight, diseased fruit obtained from W. Zeller); 1993
Ea-Ki	Münster, Germany; cherry tree (E. Geßner); before 1991
EaM5/87	Germany; *Crataegus* sp. (W. Zeller); 1987
Ea-Rb	Canada; *Rubus idaeus* (raspberry leaf); 1995
EaU-1	Utha (S. Thomson); USA
EaU8/96	Utha, apple; USA; 1996
FB2a	Basque Country, Spain, *M. baccata* (Crab apple), 1995, M. M. Lopez; 1995
H895	Nyarlorinc, Hungary, apple, Golden Dilicious, from purple leaf vein, (J. Nemeth); May 1996
H898	Sarkad, Hungary: pear, from shoot, (J. Nemeth); May 1996
H902	Borota, Hungary: quince, from shoot, (J. Nemeth); May 1996
H902-3P	H902: Colonies stored on St.I-agar plates for 3 months at 4 °C
H909	Zakanyszek, Hungary: apple, Jonathan, from shoot, (A. N. Kovacs); June 1996
H910	Mohacs, Hungary: quince, from shoot, (A. N. Kovacs); June 1996
H931	Mezokovacshaza, Hungary: medlar, from shoot, (J. Nemeth); July 1996
ICMP13293	New Zealand, from Australian wood sample*; 1997
ICMP13294	New Zealand, from Australian wood sample*; 1997
ICMP13295	New Zealand, from Australian wood sample*; 1997
ICMP13296	New Zealand, from Australian wood sample*; 1997
ICMP13297	New Zealand, from Australian wood sample*; 1997
ICMP13298	New Zealand, from Australian wood sample*; 1997
ICMP13299	New Zealand, from Australian wood sample*; 1997
ICMP13300	New Zealand, from Australian wood sample*; 1997
IL6	Illinois, USA *Rubus idaeus* (McManus and Jones); 1995
JLVNZ1	Hawke's bay, New Zealand, [Sm] (J. Vanneste); 1993/94
JLVNZ10	Hawke's bay, New Zealand, [Sm] (J. Vanneste); 1993/94
JLVNZ11	Hawke's bay, New Zealand, [Sm] (J. Vanneste); 1993/94
JLVNZ12	Hawke's bay, New Zealand, [Sm] (J. Vanneste); 1993/94
JLVNZ2	Hawke's bay, New Zealand, (J. Vanneste); 1994/95
JLVNZ3	Hawke's bay, New Zealand, [Sm] (J. Vanneste); 1992/93
JLVNZ4	Hawke's bay, New Zealand (J. Vanneste); 1994/95)
JLVNZ5	Hawke's bay, New Zealand, [Sm] (J. Vanneste); 1993/94
JLVNZ6	Hawke's bay, New Zealand, [Sm] (J. Vanneste); 1993/94
JLVNZ7	Hawke's bay, New Zealand, [Sm] (J. Vanneste); 1994/95
JLVNZ8	Hawke's bay, New Zealand (J. Vanneste); 1994/95)
JLVNZ9	Hawke's bay, New Zealand, [Sm] (J. Vanneste); 1992/93
IPV-BO2906	Albania, pear (U. Mazzucchi); 1995
ISPAVE094	Caserta, Campania, *P. pyrifolia* (L. Corazza); 1996
LE11	Lecce, Apulia, Italy, *P. communis* cv. 'Bella di Giugno' (C. Cariddi); 1990

Table 1. Continued

Strain	Other name/origin (source, isolated by) or [ref.]; year of isolation
NZR5	New Zealand [McManus and Jones, 1995]
OMP-BO 691.2	Bologna, Emilia-Romagna, Italy, *P. communis* (A. Calzolari); 1995
OMP-BO1077.7	Bologna, Emilia-Romagna, Italy, *P. communis* (A. Calzolari); 1994
OMP-BO1160.2/94	Bologna, Emilia-Romagna, Italy, *Crataegus* sp. (A. Calzolari); 1994
OMP-BO1178.1A/94	Bologna, Emilia-Romagna, Italy, *Sorbus* sp. (A. Calzolari); 1994
OMP-BO1204.1/94	Bologna, Emilia-Romagna, Italy, *Pyracantha* sp. (A. Calzolari); 1994
OMP-BO786.1/91	Messina, Sicily, Italy, *P. communi* (A. Calzolari); 1991
OR6	Oregon, USA pear [Mcmanus and Jones, 1995]
PD207	The Netherlands, *Cotoneaster* sp.; 1979
PD350	The Netherlands, *P. communis* (J.D. Janse); 1984
PD350Sm	spontaneous mutant of PD350,Sm^r [Zhang et al., 1997]
PD439	The Netherlands, *Cotoneaster salicifolius* 'Floccosus' (J. D. Janse); 1984
PD439Sm	spontaneous mutant of PD439,Sm^r [Zhang et al., 1997]
PD439R	spontaneous mutant of PD439 with levan synthesis [Bereswill et al., 1997]
PD494	The Netherlands, *P. communis* (J.D. Janse); 1984
PD494Sm	spontaneous mutant of PD494, Sm^r [Zhang et al., 1997]
PD576	The Netherlands, *Crataegus* sp. (J.D. Janse); 1984
PD579	The Netherlands, *Crataegus* sp. (J.D. Janse); 1985
PD579Sm	spontaneous mutant of PD579,Sm^r [Zhang et al., 1997]
PFB15	2655/ plum (K. Mohan via J.P. Paulin)
PFB4	2653/ plum (K. Mohan via J.P. Paulin)
PMV6076	*hrp-dsp*-deletion mutant of CFBP1430
T	South England, *P. communis* (E. Billing); 1960
T90	Turkey (T. Momol); 1990
T91	Turkey (T. Momol); 1991

*Wood sample (stem or branch of *Cotoneaster* sp.) from the Melbourne Royal Botanic Gardens, analyzed by C. Hale and R.K. Taylor: Fire blight on cotoneaster (Report to MAF regulatory authority, May 1997). The 'PD'-strains show low levan synthesis (Bereswill et al., 1997). More properties of strains may be found in previous papers.

and Jones (1995). A motif of eight nucleotides (ATTACAGA) was found at the left side which was reiterated several times. The number of repeat units was different for strains Ea1/79 and Ca11. When the amplified fragments from various *E. amylovora* strains were cleaved with restriction enzymes *Sau*3A or *Hpa*II, a difference in size was observed for the large DNA fragment, whereas other fragments were identical. The variable fragment comprised the repeat structure and could be useful to differentiate strains. On an 8% polyacrylamide gel, strains Ea1/79, Ea273, 674/94 and H902 produced these fragments migrating at different positions (Figure 2). This supported a recent report of Lecomte et al. (1997) that *E. amylovora* strains from Austria and France can be grouped into several types by digestion of the amplified fragments with *Sau*3A.

Nucleotide sequences of the amplified fragments from various E. amylovora *strains and comparison to sequences in nucleotide libraries*

Based on the preliminary results showing an RFLP in the amplified DNA fragment of pEA29, we cloned the entire fragment from strains Ea1/79, EaU-1, H902, 674/95, ICMP13295, EaAUS02, and Ea273 (Figure 1). The shortest SSR number was found for strains Ea273 (four repeats) and the largest size for 674/94 (15 repeats). Strains Ea273 and ICMP13295, as well as Ea1/79 and EaAUS02 had the same SSR size, which was confirmed by sequence analysis.

In all cases the arrays of SSRs were terminated by 'ATTA', representing the first half of the unit 'ATTACAGA'. Its concatenation results in 'AATT', which is recognized by restriction enzyme *Tsp*509I.

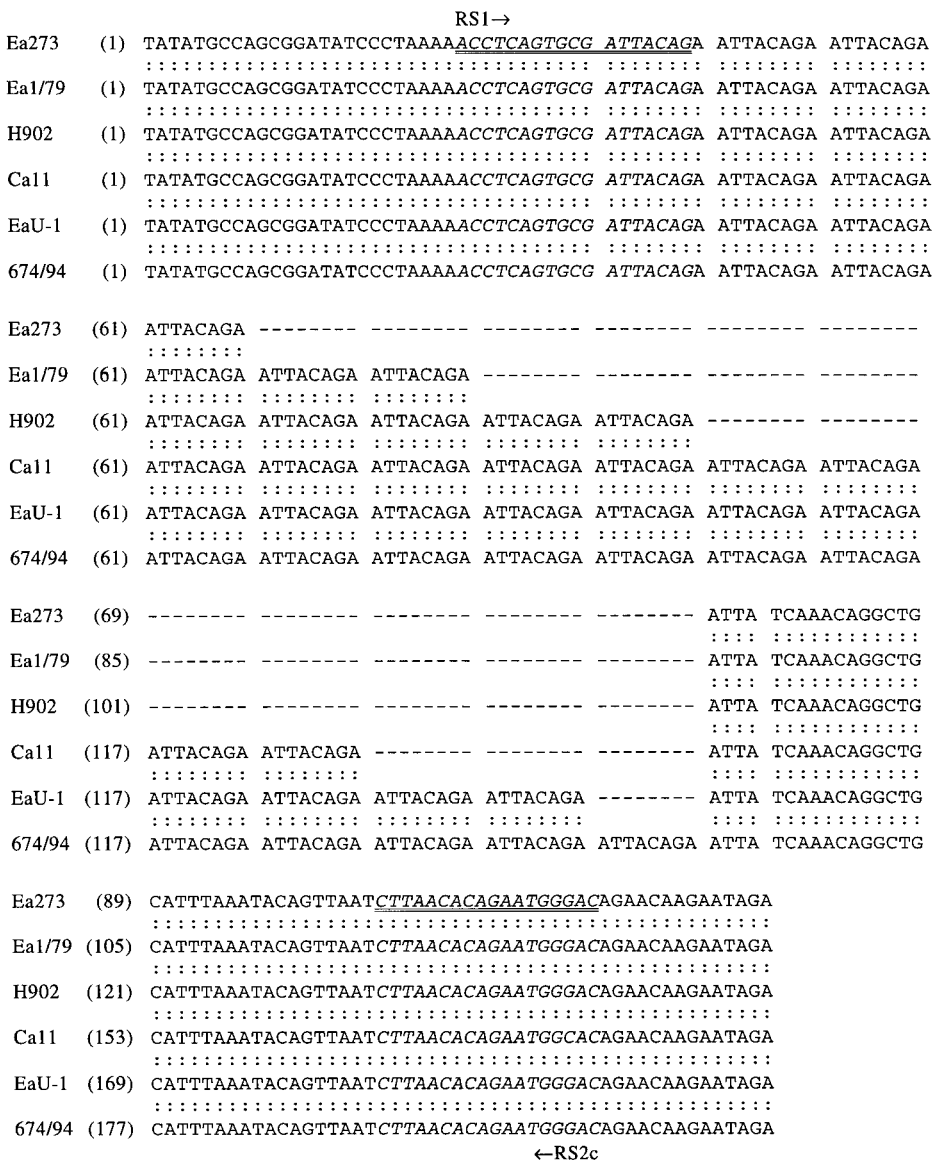

Figure 1. Comparison of the nucleotide sequences in the SSR region of pEA29 for various *E. amylovora* strains. The nucleotides representing primers used to analyze the repeats are in italics and double underlined in the DNA sequence of the first strain (Ea273).

Alignment of this motif results in a stem-loop structure for the DNA sequence with the eight base pair repeats. In the complementary strand 'TAA' encodes for a stop codon within the repeats. Consistent with the size of the SSR, the original fragment varied around 1 kb and contained a *Sau*I (*Aoc*I, *Cvn*I) site similar to the 1.1 kb *Pst*I-fragment F of pEA29 (Falkenstein et al., 1988). An open reading frame (ORF-2, Figure 3) for 108 amino acids was present in the center of the amplified PCR fragment. The encoded protein had significant similarity to RepA, presumable replication proteins of various plasmids, and in particular to ParA of *Agrobacterium tumefaciens* (29%) (Gallie et al., 1987) in a BLASTX search for amino acid alignment. In a motif search, the homology to ParA was confirmed. On the other hand, ORF-2 was only half the size of *parA* and could therefore represent a truncated gene. The repeat sequence could be related

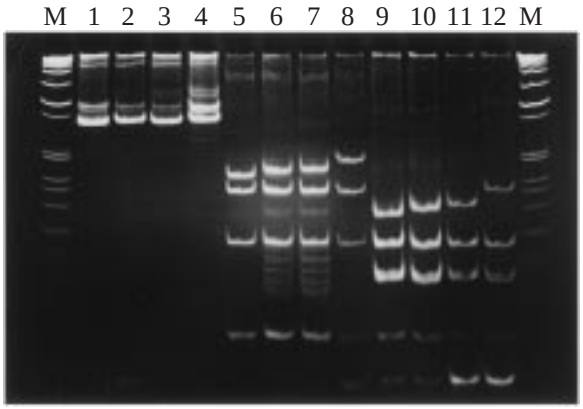

Figure 2. Analysis of short-sequence DNA repeats of *E. amylovora* by restriction enzyme digestion of PCR fragments amplified with plasmid primers. Lanes 1–4: undigested; 5–8: digested with *Hpa*II and 9-12: digested with *Sau*3A. Lanes 1, 5, 9: Ea273; 2, 6, 10: Ea1/79; 3, 7, 11: H902; 4, 8, 12: *E. amylovora* 674/94. M: 1 kb ladder.

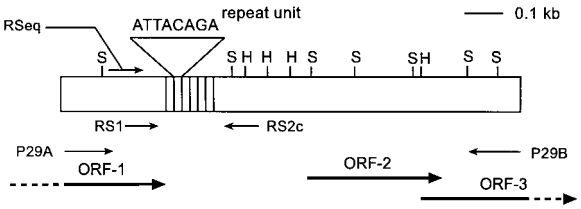

Figure 3. A scheme for elements used in analysis of the amplified PCR fragment from pEA29 of strain Ea1/79. RSeq: primer for nucleotide sequence determinations; RS1, RS2c: primers for amplification of the SSR region; P29A, P29B: primers for amplification of whole DNA fragment; H, *Hpa*II; S, *Sau*3A.

to a replication or partitioning function of plasmid pEA29.

Large scale analysis of short-sequence DNA repeats in E. amylovora *strains*

After nucleotide sequence analysis of cloned fragments with the SSR, we used PCR primers close to the repeated sequences in order to analyze the actual number of repeats in individual strains (Figure 3). The primers were designed from the DNA sequence adjacent to the repeat region (RS1, RS2c), which amplified a DNA fragment of 95–183 bp. A ladder of four to fifteen repeats with representatives for each size indicated arrangements of most possible SSR sizes (Figure 4). More than 130 *E. amylovora*

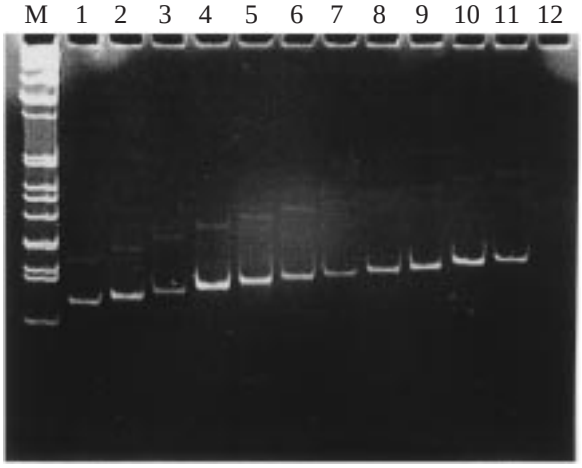

Figure 4. Analysis of *E. amylovora* strains with four to fifteen SSRs after amplification with primers RS1 and RS2c. Lanes 1: Ea273; 2: Ea595; 3: Ea1/79; 4: Ea4/83; 5: H902; 6: BA109; 7: BPIC1631; 8: 202.1; 9: PD579; 10: EaU-1; 11: 674/94; 12: water control; M: 1 kb ladder.

strains from various countries and times of isolation were analyzed. A small or large number of repeats was less frequent than an intermediate number. From the strains analyzed, 42 carried a repeat number of 6 (33%) as the dominant type and 32 strains had five repeats (25%), 22 strains seven repeats (17%), 7 strains four, eight or nine repeats (5.5% each), three strains 10 or 11 repeats (2.5%), 2 strains 12 or 15 repeats (1.5% each) and one strain carried 14 repeats (0.8%) (Table 2, Figure 5). The occurrence of 13 repeats was not observed among the strains assayed. Strains isolated from Australian plant material were not considered in the calculation for reasons discussed below.

The SSR sizes of strains isolated in a single country were divergent especially when at least three strains from one country were assayed. Even in Hungary, where fire blight was only introduced recently, strains with divergent SSR sizes were isolated. All strains which have been derived from Australian wood material originated in the Melbourne Botanic Gardens, but were isolated in New Zealand and Germany. The eight ICMP strains (Table 2) may have been derived from the same plant, and the five Ea-AUS strains, isolated in Germany, were from another Cotoneaster plant and from a Sorbus tree. It is intriguing to find two different SSR-types for *E. amylovora* strains on plants with weak fire blight symptoms in a small area.

Table 2. SSR sizes of *E. amylovora* strains from various origins

Albania		**Germany**	
IPV-BO2906/1995	8 (Pt5)	Ea7/74	6 (Pt1)
(Australia)[$]		Ea7/74-C	6
Ea-AUS02	6 (Pt1)	Ea7/74-Bi	6
Ea-AUS02H	6 (Pt1)	Ea1/79	6 (Pt1)
Ea-AUS02A	6 (Pt1)	Ea1/79Cu6	6
Ea-AUS02B	6	Ea1/79Rif	*5
Ea-AUS34	6 (Pt1)	Ea27/91	6 (Pt1)
ICMP13293	4 (Pt1)	Ea28/91	6 (Pt1)
ICMP13294	4 (Pt1)	EaM5/87	5 (Pt1)
ICMP13295	4 (Pt1)	Ea-Eb	5 (Pt1)
ICMP13296	4 (Pt1)	Ea-Ki	6 (Pt1)
ICMP13297	4 (Pt1)	Ea237	10 (other)
ICMP13298	4 (Pt1)	Ea242	9
ICMP13299	4 (Pt1)	Ea248	6
ICMP13300	4 (Pt1)	Ea250	7 (Pt1)
Austria		Ea263	5
295/93	15 (Pt1)	Ea268	6
674/94	15 (Pt1)	Ea269	6
Bulgaria		Ea270	5 (Pt1)
Ea115.22	7 (Pt1)	Ea282	5
Ea202.1	11 (Pt1)	Ea284	4
Canada		Ea284Cu3	*6
Ea266	7 (Pt1)	Ea286	6 (Pt1)
Ea-Rub	6 (other)	Ea291	5
Croatia		Ea298	5
CR03	8 (Pt2)	Ea299	5
CR04	5 (Pt2)	**Greece**	
CRIV	5 (Pt2)	BPIC847	6 (Pt2)
Czechia		BPIC928	7 (Pt2)
BPIC1631	10 (Pt1)	BPIC913	6 (Pt2)
Egypt		**Hungary**	
Ea4/82	6 (Pt2)	H895	12
Ea4/83	7	H898	7
Ea25/82	6 (Pt2)	H902	8
Ea25/82 Sm	6	H902-3P[§]	*6
Ea22I/83(R)	6 (Pt2)	H909	11
Ea5/84	6 (Pt2)	H910	7
Ea7/84	6 (Pt2)	H931	9
Ea12/84	6 (Pt2)	**Israel**	
England		Ea209	5 (Pt5)
T	8 (other)	Ea267	5 (Pt2)
Ea595	5 (Pt1)	Ea263	6 (Pt2)
Ea771	6 (other)	Ea226	6 (Pt5)
Ea775	7 (Pt4)	**Italy**	
France		OMPO786.1/91	5 (Pt2)
CFBP1367	5 (Pt3)	OMPO1160.2/94	6 (Pt3)
Ea322 (CFBP1368)	5 (Pt3)	OMPO1178.1A/94	6 (Pt3)
Ea322A	5	OMPO1204.1/94	6 (Pt3)
CFBP1430	5 (Pt3)	OMPO1077.7	7 (Pt3)
PMV6076	5 (Pt3)	ISPAVE094	4 (Pt1)
2650-1	6 (Pt3)	OMP-BO691.2	6 (Pt3)
2651-1	5 (Pt3)	BA109	9 (Pt2)
2652-2	8 (Pt3)	LE11	7 (Pt2)

Table 2. Continued

The Netherlands		ILVNZ9	7
PD207	4	ILVNZ10	7
PD350	5	ILVNZ11	7
PD350Sm	5	ILVNZ12	5
PD439	9	**Spain**	
PD439Sm	9	FB2a	5 (Pt4)
PD439R	*10	**Switzerland**	
PD494	7 (Pt1)	Ea345.90	5 (Pt1)
PD494Sm	7	Ea508.94	8 (Pt1)
PD576	6 (Pt1)	Ea331.93	6 (Pt1)
PD579	12 (Pt1)	**Turkey**	
PD579Sm	*9	T90	5 (Pt2)
New Zealand		T91	5 (Pt2)
NZR5	6 (Pt1)	**USA**	
Ea1496/66	5 (Pt1)	CA3R	6 (other)
Ea8439/83	7 (Pt1)	OR6	4 (other)
ILVNZ1	7	Ea273	4 (other)
ILVNZ2	7	E9	6 (other)
ILVNZ3	7	E8	5 (other)
ILVNZ4	5	E8Sm	5
ILVNZ5	7	EaU-1	14
ILVNZ6	7	EaU8/96	4 (other)
ILVNZ7	7	PFB4	11 (other)
ILVNZ8	8	PFB15	9 (other)
		IL6 (rubus)	4 (other)

*Marking a change in comparison to the parent strain.
§The strain was stored for three months on a St.I plate in cold room with a mucoid appearance of colonies. Cells were then further propagated on fresh plates and the SSR size analyzed.
$Strains isolated from Australian wood samples in New Zealand (ICMP) and Germany (Ea-AUS). Pt, PFGE pattern type for *Xba*I digests (Zhang et al., 1998).

Stability of the size comprising the repeated motif in individual E. amylovora *strains*

The stability of the SSR size was assayed after transfer of *E. amylovora* strains to agar medium and plants. Transfer of single colonies to nutrient agar was done several times and the SSR size assayed after 5, 10 and 20 passages. With strains Ea273 (low SSR size), H902 (intermediate), and 674/94 (high) no changes were observed for the initial repeat number. In addition, no change was found, when strain Ea7/74 was reisolated from pears, apple seedlings, and trees in an experimental orchard. On the other hand, selection for spontaneous mutants on agar plates with antibiotics or for resistance to copper sulfate gave rise to changes in the repeat number in several cases, but not in all. Different SSR sizes were found for strain Ea1/79 after selection for rifampicin resistance (6→5), for streptomycin-resistance of strain PD579 (12→9) and for Ea284 after propagation on MM2Cu plates (4→6). Strain PD439 is low in levan synthesis and has a tendency to revert to levan producing variants (Bereswill et al., 1997). The repeat number in a revertant strain was found to have changed to 10 units whereas the parent strain carried nine repeats on the plasmid. Long term storage of H902 on St.I nutrient agar in the cold room (three months) gave rise to the appearance of mucoid colonies, and subsequent propagation on fresh medium showed a shift in the SSR size (8→6) for one colony out of five, all other colonies had normal phenotypes on fresh medium.

Discussion

Repetitive DNA is quite common in eukaryotic genomes. In plant cells more than half of the chromosomal DNA may consist of these elements (Jorgensen et al., 1987). SSRs in prokaryotic genomes have been

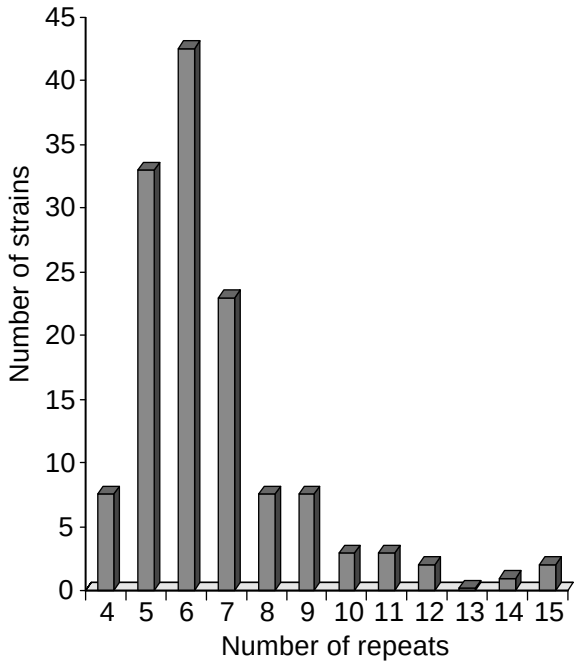

Figure 5. Distribution of SSR sizes among the *E. amylovora* strains investigated.

related to bacterial phase variation and adaptation (Van Belkum et al., 1998). A common mechanism of changing repeat numbers is strand slipping especially during replication. This process is outlined in a scheme (Figure 6) with base pairing in a stem-loop structure occuring exclusively between the bases A and T. The replication origins of bacteria usually carry repeat structures, which seem to attract DNA binding proteins involved in replication steps (Bramhill and Kornberg, 1988). Low copy number plasmids rely on the partition function to become equally distributed between mother and daughter cells. Their partition depends on repeat structures on the plasmids, which has been shown in several cases (Gallie and Kado, 1987; Fu et al., 1996). Plasmid pEA29 of *E. amylovora* also carries repeat structures, possibly to adapt the pathogen to environmental challenges. Stress can result in a change of the repeat number, which is apparently rare during normal propagation. The direction of the change seems to favor intermediate SSR sizes.

The number of repeat units was thought to correlate to the origin of *E. amylovora* strains (Lecomte et al., 1997), but a large diversity of strains isolated in relatively small regions disprove this assumption. This is in clear contrast to identical patterns obtained with PFGE for *E. amylovora* strains isolated from

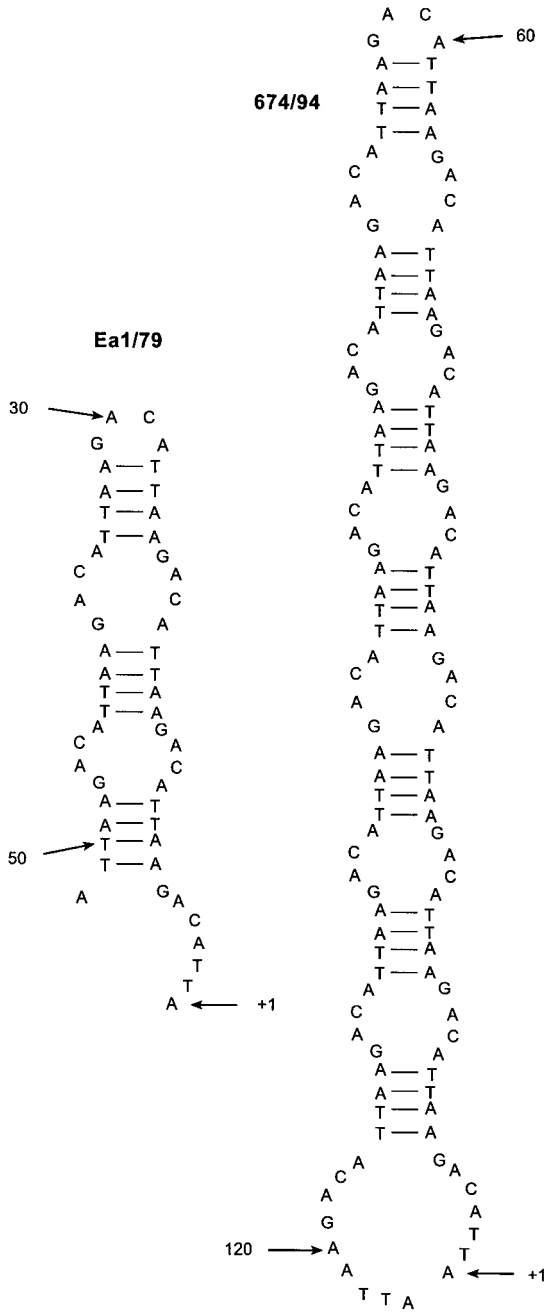

Figure 6. Possible stem-loop formations within the region of repeat units of plasmid pEA29 carried by *E. amylovora* strains Ea1/79 and 674/94.

large geographic regions and analyzed after digestion of their genome with rare cutting restriction enzymes such as *Xba*I (Zhang and Geider, 1997; Zhang et al., 1998). Host plant species or time of isolation did not affect the pattern type. Rare PFGE pattern changes after

introduction of *E. amylovora* to Europe and the Eastern Mediterranean region may have occurred, although differences in pattern type could have existed before introduction of the pathogen. Strains isolated in Central Europe had the PFGE pattern type Pt1, whereas type Pt2 was conserved in the spread of fire blight from Egypt via Israel and Turkey to the Balkans (Zhang et al., 1998). A variation came up in Israel and Bulgaria which may indicate a rare change. Surprisingly, the same pattern type Pt5 was observed in apparently unrelated areas. Within a large number of *E. amylovora* strains assayed from Germany, two strains differed from the standard PFGE pattern type (unpublished). No correspondence of PFGE-pattern type and SSR size was observed. In types Pt1–Pt5, repeat numbers 5, 6, 7, and 8 were represented, repeat numbers exceeding 10 were observed in Austria and Hungary, where fire blight is quite recent.

Strains from the recently infected areas of Hungary were so diverse in SSR sizes that this divergence must have evolved over a short time. Although passaging on agar medium or in plants did not affect the SSR size, selection of spontaneous mutants from antibiotics or copper sulfate containing agar plates did. This was also observed for aging colonies after long storage in the cold room. The semi-stable SSR size could still define individual strains after their isolation providing propagation was performed under mild laboratory conditions.

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Note added in proof. After submission of this manuscript, a paper by Schnabel and Jones (Plant Disease 82: 1334–1336 (1998)) was published describing the SSR region as an 8 bp repeat sequence with 'GAATTACA' as a different frame than 'ATTACAGA' suggested here. The lowest and highest repeat numbers for the former frame were 3 and 14, respectively, (instead of 4 and 15) and were found to be instable with a tendency to form intermediate repeat numbers after passaging individual strains with extreme repeat values.