

Development of a Scar Marker for Pierce's Disease Strains of *Xylella fastidiosa**

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ABSTRACT

The objective of this research was to develop a primer for a polymerase chain reaction specific for *Xylella fastidiosa* strains that cause Pierce's Disease (PD) in grapes (*Vitis vinifera*). The DNA amplification of 23 different strains of *X. fastidiosa*, using a set of primers REP1-R (5'-IIICGICGIATCCIGGC-3') and REP 2 (5'-ICGICTTATCIGGCCTAC-3') using the following program: 94 °C/2 min; 35 X (94 °C/1 min, 45 °C/1 min and 72 °C/1 min and 30 s) 72 °C/5 min, produced a fragment of 630 bp that differentiated the strains that cause disease in grapes from the other strains. However, REP banding patterns could not be considered reliable for detection because the REP1-R and REP 2 primers correspond to repetitive sequences, which are found throughout the bacterial genome. The amplified product of 630 bp was eluted from the agarose gel, purified and sequenced. The nucleotide sequence information was used to identify and synthesize an specific oligonucleotide for *X. fastidiosa* strains that cause Pierce's Disease denominated Xf-1 (5'-CGGGGGTGTAGGAGGGGTTGT-3') which was used jointly with the REP-2 primer at the following conditions: 94 °C/2 min; 35 X (94 °C/1 min, 62 °C/1 min; 72 °C/1 min and 30 s) 72 °C/10 min. The DNAs isolated from strains of *X. fastidiosa* from other hosts [almond (*Prunus amygdalus*), citrus (*Citrus* spp.), coffee (*Coffea arabica*), elm (*Ulmus americana*), mulberry (*Morus rubra*), oak (*Quercus rubra*), periwinkle wilt (*Catharantus roseus*), plums (*Prunus salicina*) and ragweed (*Ambrosia artemisiifolia*)] and also from other Gram negative and positive bacteria were submitted to amplification with a pair of primers Xf-1/REP 2 to verify its specificity. A fragment, about 350 bp, was amplified only when the DNA from strains of *X. fastidiosa* isolated from grapes was employed.

Additional keywords: oligonucleotide, polymerase chain reaction, PCR, REP-PCR.

RESUMO

Desenvolvimento de um marcador scar para *Xylella fastidiosa* causadora do mal de Pierce

O objetivo deste trabalho foi desenvolver um oligonucleotídeo iniciador para reação em cadeia da polimerase (PCR) específico para as estirpes de *Xylella fastidiosa* que causam o mal de Pierce (PD) em videira (*Vitis vinifera*). Amplificações de DNA de 23 diferentes hospedeiros, usando o conjunto de oligonucleotídeos REP1-R (5'-IIICGICGIATCCIGGC-3') e REP 2 (5'-ICGICTTATCIGGCCTAC-3') utilizando o programa: 94 °C/2 min; 35 X (94 °C/1 min, 45 °C/1 min; 72 °C/1 min and 30 s) 72 °C/5 min, produziu um fragmento de 630 pb que diferenciou as estirpes de videiras dos demais. Entretanto, padrões de bandamento REP não são considerados confiáveis para detecção devido ao par de oligonucleotídeos REP 1 e REP 2 corresponderem a seqüências repetitivas encontradas por todo o genoma bacteriano. Desse modo, o produto amplificado de 630 pb foi eluído do gel de agarose, purificado e seqüenciado. A informação da seqüência nucleotídica foi usada para identificar e sintetizar um oligonucleotídeo específico para o isolado de *X. fastidiosa* causadora do mal de Pierce denominado Xf-1 (5'-CGGGGGTGTAGGAGGGGTTGT-3'), que foi utilizado juntamente com o oligonucleotídeo REP-2 nas condições 94 °C/2 min; 35 X (94 °C/1 min, 62 °C/1 min; 72 °C/1 min and 30 s) 72 °C/10 min. Os DNAs das estirpes de *X. fastidiosa* de outros hospedeiros [amêndoa (*Prunus amygdalus*), citros (*Citrus* spp.), café (*Coffea arabica*), olmo (*Ulmus americana*), amora (*Morus rubra*), carvalho (*Quercus rubra*), vinca (*Catharantus roseus*), ameixa (*Prunus salicina*) e ragweed (*Ambrosia artemisiifolia*)] e de bactérias Gram negativas e positivas foram submetidos a amplificação com o conjunto de oligonucleotídeos Xf-1/REP 2. Um fragmento, de aproximadamente 350 pb, foi amplificado apenas com o DNA de *X. fastidiosa* isolada de videira.

Palavras-chave adicionais: oligonucleotídeo, reação em cadeia da polimerase, PCR, REP-PCR.

INTRODUCTION

Pierce's Disease (PD) in grapes (*Vitis vinifera* L.) is endemic to the United States wine producing regions. Initially

found only on the American continent, stories of its presence in Asia and Europe demonstrate a wider global distribution of this bacterium disease. The causal agent, a Gram-negative bacterium *Xylella fastidiosa* Wells *et al.* (Wells *et al.*, 1987) is restricted to the xylem of its host plants and spreads from plant to plant by xylem sap-feeders. In addition to the strain

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that attacks grapes, *X. fastidiosa* also has been detected in a great number of other economically important host plants species, such as alfalfa (*Medicago sativa* L.), citrus (*Citrus* spp.), coffee (*Coffea arabica* L.), peach [*Prunus persicae* (L.) Batsch], plum (*Prunus salicina* Lindl) and other forest and ornamental plants (Hopkins, 1989). Several methods have been used to detect the occurrence of *X. fastidiosa* in contaminated plant tissues, including electron microscopy (Rossetti *et al.*, 1990), serology (Chang *et al.*, 1993) and polymerase chain reaction (PCR) (Qin *et al.*, 2001).

Molecular markers such as random amplified polymorphic DNA (RAPD) (Pooler & Hartung, 1995), arbitrary-primed (AP-PCR) (Costa *et al.*, 2000), restriction fragment length polymorphism (RFLP) (Chen *et al.*, 1992), variable number of tandem repeated (VNTR) (Coleta-Filho *et al.*, 2001), repetitive sequence based-PCR (rep-PCR) (Hendson *et al.*, 2001), PCR-RFLP (Qin *et al.*, 2001), intergenic spacer (ITS) 16S-23S rDNA sequencing (Hendson *et al.*, 2001), clamped homogeneous electric field - CHEF (Hendson *et al.*, 2001), plasmidial profile (Chen *et al.*, 1992) have been used to study the genetic diversity amongst *X. fastidiosa* strains.

Efficient and economical methods for detection of such pathogen are essential for establishing a quarantine program to adequately screen of contaminated plants with low bacterial population (Purcell & Hopkins, 1996). Among other available detection methods, the PCR-based markers have been the most widely used in molecular genetic studies as they are simple, quick, flexible and sensitive. One such marker is the SCAR (sequence characterized amplified region) (Karp *et al.*, 1996), which has the advantage of being less sensitive to the conditions of a standard PCR due to its primer size when compared to RAPD. Moreover SCARs have other desirable characteristics as markers since they require no radioactive isotopes and in addition, detect only a single locus. The development of a straightforward PCR protocol to check grape tissues potentially infected by *X. fastidiosa* causing PD is of fundamental importance to prevent spreading this pathogen in Brazil, by importing propagating plant materials from regions where this phytopathogen is endemic. In the course of previous studies on the genetic diversity between isolates of *X. fastidiosa* carried out in our laboratory, using the repetitive sequences REP, ERIC and BOXA amplified by PCR, it was possible to make a distinction between the *X. fastidiosa* isolated from grapevine and those isolated from plum, coffee and citrus.

As a result, a fragment of nearly 630 bp was amplified by the REP-PCR and used as template for the construction of a specific oligonucleotide primer for detecting *X. fastidiosa* isolated from grapevines.

MATERIALS AND METHODS

Bacterial strains and DNA extraction

The *X. fastidiosa* strains used in this study are listed in Table 1. All *X. fastidiosa* strains were grown in PW broth medium (Davis *et al.*, 1981) for six days on a rotatory shaker

at 28 °C. The DNA samples of *Escherichia coli* (JM 83::pHT 408, MM 294, MM 294::pAMP and MM 294::pKAN), *Xanthomonas axonopodis* pv. *citri* (Hasse) Vanterin *et al.*, *Xanthomonas campestris* pv. *campestris* (Hasse) Gabriel *et al.* and *Erwinia* spp. used for the primer specificity tests were kindly supplied by Dr. Manoel V. F. Lemos (*E. coli* and *Erwinia* sp.) and Dr. Jesus A. Ferro (strains of *Xanthomonas* sp.). The genomic DNA extraction from the *X. fastidiosa* strains followed the method proposed by Ausubel *et al.* (1987) with some modifications, which included the addition of 100 µl of RNase solution (200 µg/ml) soon after the treatment with proteinase K, and incubation at 37 °C for 1 h. The isolated DNA samples were used as template for standard PCR.

PCR amplification procedure

Repetitive sequence based-PCR technology (REP-PCR) was used to identify unique PCR products for *X. fastidiosa* causing the PD. The DNA from *X. fastidiosa* strains were amplified with REP1-R (5'-IIICGICGIATCCIGGC-3') and REP 2 (5'-ICGICTTATCIGGCCTAC-3') primers (Versalovic *et al.*, 1991). The overall reactions were adjusted to a final volume of 20 µl, containing 1 X PCR buffer (50 mM KCl, 200 mM Tris-HCl, pH 8,4), 2 mM MgCl₂, 200 µM of each dNTP (Invitrogen, CA, USA), 1 U of *Taq* DNA polymerase (Invitrogen, CA, USA), 30 ng of each primer (REP1-R and REP 2) and 50 ng of template DNA. The reactions were then taken to a programmable thermocycler (MJ Research, Inc., model PTC 100™) using the following program: 94 °C for 2 min; 35 cycles at 94 °C for 1 min, 45 °C for 1 min and 72 °C for 1 min and 30 s for denaturation, annealing and extension; respectively, and a final extension step at 72 °C for 5 min. The products were visualized in 1.5% agarose gels 1 X TBE buffer containing 0.5 µg/ml ethidium bromide (Sambrook *et al.*, 1989) and photographed using Gel-doc 1000 digital photo-documentation equipment (BioRad).

Sequencing, data analysis and primer design

After electrophoresis, a band of about 630 bp in size from *X. fastidiosa* strain 8935 was cut out of the gel and the DNA recovered and purified using a Concert™ Gel extraction systems kit (Invitrogen, CA, USA). For sequencing, 4 µl of the big dye terminator cycle sequencing ready reaction (PE Applied Biosystems, Foster City, CA, USA), 1.6 pmols of the primers REP1-R and REP 2, and 20 ng of DNA from the purified fragment (not cloned) of the strain 8935 were used to set up a final 10 µl volume sequencing reaction. The reactions were then performed at the same conditions previously described. After fragment precipitation and washing for removal of the ddNTPs unincorporated through sequencing reaction, the samples were sequenced on an ABI PRISM™ 377 automatic DNA sequencer (Applied Biosystems, Inc., Foster City, CA, USA). Sequenced data were analyzed using BLAST – Basic local alignment search tools (version 2.2) program (Altschul *et al.*, 1997) through the network service of the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). The OLIGO 4.0 software (National

TABLE 1 - Strains, hosts, geographic origin and source of *Xylella fastidiosa*

Strain	Host	Geographic Origin	Source
OK	Almond (<i>Prunus amygdalus</i>)	California, USA	-
6746	Almond	Massachusetts, USA	ATCC 35870 ^d
9a5c	Citrus (<i>Citrus sinensis</i>)	São Paulo, Brazil	INRA ^a
10348	Citrus	Paraná, Brazil	IAPAR ^b
10438	Citrus	Paraná, Brazil	IAPAR ^b
11037	Citrus	São Paulo, Brazil	IAPAR ^b
11038	Citrus	São Paulo, Brazil	IAPAR ^b
11066	Citrus	Paraná, Brazil	IAPAR ^b
11067	Citrus	Paraná, Brazil	IAPAR ^b
11347	Citrus	Paraná, Brazil	IAPAR ^b
11348	Citrus	Paraná, Brazil	IAPAR ^b
11380	Citrus	Santa Catarina, Brazil	IAPAR ^b
11399	Citrus	Sergipe, Brazil	IAPAR ^b
11400	Citrus	Sergipe, Brazil	IAPAR ^b
11775	Citrus	Rio Grande do Sul, Brazil	IAPAR ^b
11779	Citrus	Santa Catarina, Brazil	IAPAR ^b
11834	Citrus	Paraná, Brazil	IAPAR ^b
B-14	Citrus	São Paulo, Brazil	CCSM ^c
CVC-5	Citrus	São Paulo, Brazil	CCSM ^c
M2-1	Citrus	São Paulo, Brazil	CCSM ^c
12288	Coffea (<i>Coffea arabica</i>)	Paraná, Brazil	IAPAR ^b
6748	Elm (<i>Ulmus americana</i>)	Washington, USA	ATCC 35873 ^d
6744	Mulberry (<i>Morus rubra</i>)	Massachusetts, USA	ATCC 35868 ^d
6745	Mulberry	Massachusetts, USA	ATCC 35869 ^d
OF	Oak (<i>Quercus rubra</i>)	Florida, USA	-
OG	Oak	Georgia, USA	-
12319	Periwinkle wilt (<i>Catharantus roseus</i>)	-	-
6751	Periwinkle wilt	Florida, USA	ATCC 35878 ^d
9746	Plum (<i>Prunus salicina</i>)	Paraná, Brazil	IAPAR ^b
6747	Prunus	Georgia, USA	ATCC 35871 ^d
6749	Ragweed (<i>Ambrosia americana</i>)	Florida, USA	ATCC 35876 ^d
8935	Grapevine (<i>Vitis vinifera</i>)	Florida, USA	ATCC 35879 ^d
9713	Grapevine	-	IAPAR ^b
6750	Grapevine	California, USA	ATCC 35877 ^d
6752	Grapevine	Florida, USA	ATCC 35879 ^d
6753	Grapevine	Florida, USA	ATCC 35881 ^d
6068	Grapevine	-	FTAT ^e
SLO	Grapevine	California, USA	-

a-INRA, Institut National de la Recherche Agronomique et Université Victor Ségale, Bordeaux, France.; b-IAPAR, Instituto Agronômico do Paraná, Paraná, Brazil.; c-CCSM, Centro Apta Citros "Sylvio Moreira", Instituto Agrônomo, Cordeirópolis - São Paulo, Brazil.; d-ATCC, "American Type Culture Collection", Manassas, Va.; e-FTAT, Fundação Tropical "André Tosello", Campinas, Brazil.

Bioscience, Inc. Plymouth, MN) was used to design a *X. fastidiosa* PD specific 21-mer primer, herein denominated Xf-1 (5'-CGGGGTGTAGGAGGGGTTGT-3') and subsequently synthesized by BIO-SYNTHESIS Inc.

SCAR amplification

The SCAR amplifications of the genomic DNA samples were performed using 50 ng of DNA template in 20 µl reactions containing 1 X PCR buffer (50 mM KCl, 200 mM Tris-HCl, pH 8.4), 2 mM MgCl₂, 200 µM each dNTP (Invitrogen, CA, USA), 1 U of *Taq* DNA polymerase (Invitrogen, CA, USA), 30 ng each primer (Xf-1 and REP 2). The amplification was performed at

the same conditions described before, except annealing was at 62 °C and the final step was for 10 min. The products were visualized and photographed as described before.

Accuracy of the oligonucleotides Xf-1/REP 2

Twenty microliter of each product of a serial dilution of a cell suspension (OD₆₀₀ = 0.92) of the strain 8935 of *X. fastidiosa* in sterile «distilled» water was centrifuged and used to perform PCR, following the same protocol described before. Dilutions (100 µl) were plated on PW medium. After 14 days of incubation at 28 °C, colony-forming units (CFU) were determined.

RESULTS AND DISCUSSION

Identification of a differential fragment of *X. fastidiosa* strain isolated from grapevine using REP-PCR

The genetic diversity of 23 strains of *X. fastidiosa* isolated from four hosts plants (plum, coffee, citrus and grapevine) was analyzed by the amplification of repetitive extragenic palindromic sequences (REP) found in the bacterial genome (Figure 1). The molecular profiles obtained by the REP-PCR amplification showed three distinct groups among the *X. fastidiosa* strains. The first group was outlined only by *X. fastidiosa* isolated from citrus (9a5c, 11347, 10348, 10438, 11066, 11067, 11834, 11380, 11775 and 11779), with amplifying fragments ranging from 300 to 4,000 bp. The second group was composed of seven strains of *X. fastidiosa* from citrus (11037, 11038, B-14, M2-1, 11399, 11400, CVC-5) and one from coffee trees (12288). The distinction of this group was possible due to the presence of a 300 bp amplified fragment. The third group, comprised of strains from grapevine (8935 and 9713) and plum (9746), showed a sufficiently distinct profile from the others, with a 630-bp fragment (Figure 1). The strain 11348 from citrus (line 12) showed a unique profile, differing from the three groups here analyzed. Differences among strains of *X. fastidiosa* isolated from sweet orange [*Citrus sinensis* (L.) Osbeck] and coffee (*Coffea arabica* L.) trees with symptoms of citrus variegated chlorosis and coffee leaf scorch, respectively, were not detected, using the REP1-R and REP 2 oligonucleotides (Qin *et al.*, 2001). The 630-bp product, only amplified from the grapevine strains, could be used to develop a SCAR marker suitable for amplifying specifically strains of *X. fastidiosa* causing PD.

Conversion of a REP-PCR marker into a SCAR marker

The 630-bp fragment was purified and sequenced from both ends (GenBank - <http://www.ncbi.nlm.nih.gov> and access number AY593993). The sequence was compared with those already present in the Genomic Data Bank (GenBank -

<http://www.lbi.ic.unicamp.br>) using the BLASTx, a sub-program of BLAST (Altschul *et al.*, 1997), which compares nucleotide sequences with protein sequences. Similarities were verified between *X. fastidiosa* strain 9a5c from citrus (Simpson *et al.*, 2000) and Temecula strain from grapevine (Van Sluys *et al.*, 2003) with scores and e-values of 259 and $7e^{-71}$ and 280 and $4e^{-77}$, respectively. The 630-bp fragment is present in the intergenic spacer of the *X. fastidiosa* strain 9a5c, covering the ORFs (Open Reading Frame) XF2486 and XF0726 (access number NP 298016) which codifies a hypothetical protein whereas in the "Temecula-1" strain this sequence is present in ORF PD 1095 (access number gi|28197945), which codifies a conserved hypothetical protein.

The Xf-1 oligonucleotide was designed, with 66.7% CG content, limited to the positions 290 to 311 bp. It was used jointly with the REP-2 primer to check the *X. fastidiosa* DNAs isolated from a wide number of host plants (Figures 2 and 3). The amplification yielded a fragment about 350 pb. Concentrations of 50 and 100 ng of DNA samples were tested to optimize PCR conditions. Both DNA concentrations produced indistinguishable amplification fragments for all DNA samples (data not shown). Assays were carried out using four annealing temperatures (50, 52, 55 and 62 °C). A fragment about 350 bp was positively amplified for all *X. fastidiosa* isolates at 50, 52 and 55 °C. However it was present only for the grapevine strains at 62 °C, indicating the primers specificity. Pooler & Hartung (1995) observed that low annealing temperatures resulted in the amplification of identical RAPD bands for all analyzed *X. fastidiosa* strains, including other bacterial strains as well. The Xf-1/REP 2 primer pair showed specificity towards *X. fastidiosa* isolated from grapes and no amplification was observed for the other *X. fastidiosa* strains from almond (*Prunus amygdalus* Batsch), coffee, elm (*Ulmus americana* L.), mulberry (*Morus rubra* L.), periwinkle (*Cathartus roseus* L.), plum (*Prunus salicina* Lindl), oak (*Quercus rubra* L.), ragweed (*Ambrosia artemisiifolia* L.), and *Erwinia* sp., *E. coli*, *X. axonopodis* pv. *citri* and *X.*

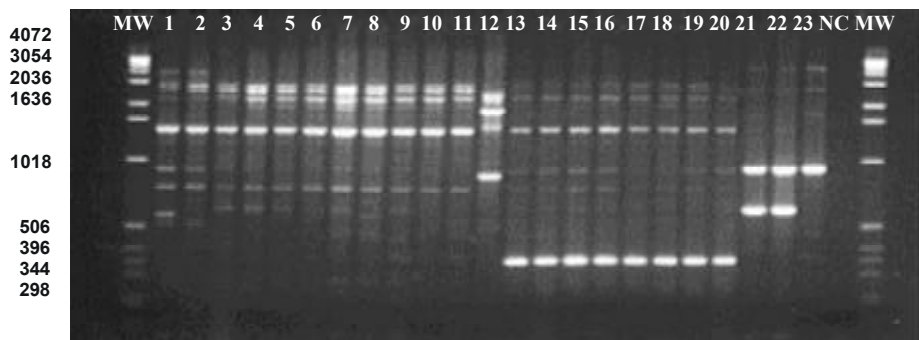


FIG. 1 - Agarose gel electrophoresis of PCR products with primers REP1-R/REP 2 using *Xylella fastidiosa* from different host plants. NC: Negative Control, MW: standard molecular weight of 1 Kb DNA ladder (Invitrogen), 1-19: citrus (*Citrus sinensis*) 31b9a5c, 31b9a5c, 11347, 10348, 10438, 11066, 11067, 11834, 11380, 11775, 11779, 11348, 11037, 11038, B14, M2-1, 11399, 11400, CVC-5 respectively, 20: coffee (*Coffea arabica*) 12288, 21-22: grapevine (*Vitis vinifera*) 8935 and 9713 and 23: plum (*Prunus salicina*) 9746.

campestris pv. *campestris* (Figures 2 and 3). This SCAR fragment generated by Xf-1/REP 2 was not observed for the other bacterial strains tested such as: *Bacillus thuringiensis*, *Bradyrhizobium elkanii*, *Bradyrhizobium japonicum*, *Staphylococcus aureus*, *Staphylococcus* sp. (data not shown).

Sensitivity of Xf-1/REP-2 primers

The 350 bp fragment was detected up to a dilution of 1

$\times 10^{-5}$, corresponding to 130 CFU/ml. However, only 20 μ l were added to the PCR mix, allowing the detection of 1-3 CFU. Due to the sensitivity of the Xf-1/REP 2 primers, they could be useful in the bio-PCR technique by speeding up identification of plant material infected by this phytopathogenic organism. Our results encourage the application of this PCR-based identification method in setting up a reliable diagnostic assay for detection of *X. fastidiosa* in grapevines grown in Brazil.

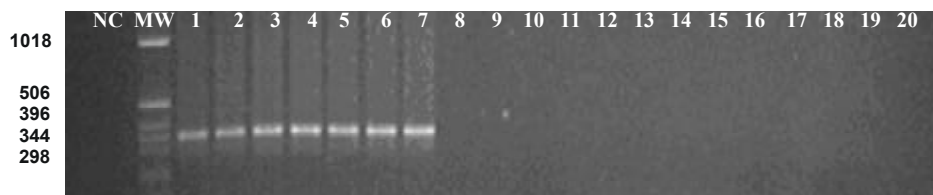


FIG. 2 - Agarose gel electrophoresis of PCR products with primers Xf-1/REP 2. NC: Negative Control, MW: standard molecular weight of 1 Kb DNA ladder (Invitrogen); 1-7: grapevine (*Vitis vinifera*) 8935, 9713, 6068, 6750, 6752, 6753 and SLO, 8-9: citrus (*Citrus sinensis*) 9a5c and 11400, 10-11: mulberry (*Morus rubra*) 6744 and 6745, 12: almond (*Prunus amygdalus*) 6746, 13: plum (*Prunus salicina*) 6747, 14: elm (*Ulmus americana*) 6748, 15: ragweed (*Ambrosia artemisiifolia*) 6749, 16: periwinkle (*Cathartus roseus*) 6751, 17: coffee (*Coffea arabica*) 12888, 18: almond OK, 19-20: oak (*Quercus rubra*) OG and OF.



FIG. 3 - Agarose gel electrophoresis of PCR products with primers Xf-1/REP 2. NC: Negative Control, MW: standard molecular weight of 1 Kb DNA ladder (Invitrogen), 1-2: grapevine (*Vitis vinifera*) 8935 and 9713, 3: plum (*Prunus salicina*) 9746, 4: coffee (*Coffea arabica*) 12888, 5: almond (*Prunus amygdalus*), 6: *Xanthomonas axonopodis*, 7-8: *X. campestris*, 9-12: *Escherichia coli* JN, MM, pAMP, pKAN and 13: *Erwinia* sp.

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