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Specific detection of *Ralstonia solanacearum* 16S rRNA sequences by AmpliDet RNA

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Abstract

The potential of AmpliDet RNA for specific detection of *Ralstonia solanacearum* in potato tuber samples and surface water was demonstrated. AmpliDet RNA is a procedure based on nucleic acid sequence based amplification (NASBA) of RNA sequences and homogeneous real time detection of NASBA amplicons with a molecular beacon. The procedure is carried out in sealed tubes, thus reducing the risks for carry-over contamination. AmpliDet RNA enabled reliable detection of specific 16S rRNA sequences of *R. solanacearum* in total RNA extracts from potato tuber samples in 90 min at a level of 10 cells per reaction, equivalent to ca. 10^4 cells ml^{-1} of sample. In surface water, AmpliDet RNA allowed detection of *R. solanacearum* at a level of 10 cfu ml^{-1}, after concentrating bacteria from 200 ml of surface water into 1 ml of surface water by centrifugation.

All strains of *R. solanacearum* and a strain of *R. syzygii* were positive in AmpliDet RNA, but not other (related) bacterial species.

Ralstonia solanacearum (race 3, biovar 2) could be detected reliably in 18 naturally infected potato tuber samples containing varying concentrations of cells. Ninety-one negative tuber samples, from which no *R. solanacearum* was isolated, were tested in AmpliDet RNA, including 23 samples containing bacteria (cross-) reacting with antibodies against *R. solanacearum* in immunofluorescence (IF) cell-staining. Only one negative sample, containing high numbers of IF-positive cells, was positive in AmpliDet RNA.

Introduction

Ralstonia solanacearum is the causative agent of bacterial wilt in many different economically important food crops and ornamentals (Hayward, 1991). The use of healthy plant propagation material is still the most effective way to avoid introduction and dissemination of this harmful plant pathogenic bacterium in pathogen-free areas. For plant testing, reliable diagnostic methods are indispensable and over the years, many assays have been developed based on pathogenicity tests, dilution plating on selective media, serology and amplification of nucleic acid sequences, such as PCR-amplification (Seal and Elphinstone, 1994; Elphinstone et al., 1996; Weller et al., 2000b).

The introduction of nucleic acid sequences based methods has remarkably increased the possibilities for rapid, specific and sensitive detection of *R. solanacearum*. Moreover, they allow to distinguish variants of the pathogen at a taxonomic level, such as the two divisions of *R. solanacearum* in PCR-amplification, which cannot be resolved by any other means (Boudazin et al., 1999).

The implementation of nucleic acid sequence based amplification (NASBA) methods in routine testing programmes, however, has often been impeded by their

lack of robustness. False-positive results can occur by carry-over contamination due to aerosols released during, in particular, analysis of amplicons by electrophoresis techniques, in spite of strict measures to avoid these (Kwok and Higuchi, 1989). On the other hand, false-negative results can be caused by the presence of sample compounds inhibiting amplification (Demeke and Adams, 1992; Rossen et al., 1992).

Carry-over contamination can be avoided largely if the amplification procedure, including detection of amplicons, is performed in sealed reaction vessels. Several homogeneous, real time fluorescence-based amplification procedures in a closed system are currently available. These include detection of double stranded DNA molecules in PCR-amplification by ethidium bromide or SYBR® green (Higuchi et al., 1992). Non-specific amplification products and primer dimers however, will not be distinguished from target amplicons with a general DNA staining procedure. More specific detection of PCR products is allowed in a TaqMan® assay, which has also been developed for several plant pathogenic bacteria, including *R. solanacearum* (Weller et al., 2000a,b). In TaqMan PCR, amplicons are quantified by the use of an internal labelled TaqMan® probe, which subsequently hybridises with the amplicons and is degraded by the 5′–3′ nuclease activity of Taq DNA polymerase thus releasing a fluorescent reporter (Holland et al., 1991).

Double stranded DNA amplicons can also be detected in closed vessels in the LightCycler™ (Livak et al., 1995, Wittwer et al., 1997). In the LightCycler, fluorescence is generated according to the Fluorescence Resonance Energy Transfer. Two different linear probes are used, hybridising adjacently with specific target sequences. One probe is bearing a donor fluorophore and the other an acceptor fluorophore. Hybridisation to the target, results in a fluorescence signal, which is monitored in the closed vessels by fluorometry.

For detection of single stranded nucleic acids in homogeneous real time assays, molecular beacons are particularly suitable. Molecular beacons are single stranded oligonucleotides that contain a stem-loop structure with a quencher at the 3′-end and a fluorophore at the 5′-end (Tyagi and Kramer, 1996; Leone et al., 1998; Vet et al., 1999; Szemes et al., 2001). The stem structure keeps the fluorophore and quencher in close proximity, avoiding fluorescence, as the energy is transferred to the quencher and released as heat. When the probe hybridises to its target, the loop opens and the fluorophore is released from the vicinity of the quencher, allowing the probe to emit fluorescence.

Recently, a NASBA procedure for detection of *R. solanacearum* has been described, in which target specific 16S rRNA sequences were subsequently amplified and detected by Northern blotting (Bentsink et al., 2002). RNA molecules disintegrate rapidly upon death of the bacterial cell and detection of RNA coincides with viability. Consequently, NASBA enables specific detection of living cells. This may be particularly of interest for studies on population dynamics of *R. solanacearum*. DNA, in contrast, can persist for a long time in the environment, and PCR-amplification of DNA sequences will result in positive reactions long after cell death (Van der Vliet et al., 1994; Bentsink et al., 2002).

Northern blotting, however, is not suitable for routine detection, as the procedure is laborious and carries a substantial risk for cross-contamination. For detection of the plant pathogens such as potato leafroll virus and *Clavibacter michiganensis* subsp. *sepedonicus* in potato tubers, assays have been described, designated as AmpliDet RNA, based on a combination of NASBA amplification and homogeneous real time detection of amplicons with molecular beacons (Leone et al., 1998; Van Beckhoven et al., 2002). Real time detection of amplicons reduced labour input and contamination risks drastically (Leone et al., 1998).

In this study, the development and evaluation of AmpliDet RNA is described for detection of *R. solanacearum* in potato peel extracts and surface water, based on amplification of specific 16S rRNA sequences by NASBA and monitoring of NASBA amplicons with a molecular beacon.

Materials and methods

Bacterial strains and plant material

Ralstonia solanacearum IPO strain no. 1609 (=Plant Protection Service no. 2763), a biovar 2 strain isolated from Dutch potato tubers, was used in all experiments. Strains of *R. solanacearum* and various other bacteria used for specificity studies are listed in Table 1. Strains were maintained at −80 °C on beads (Protect Biotrading, Wilnis, the Netherlands) in 15% glycerine and 8 mg ml^{-1} Lab Lemco broth (Oxoid).

Ralstonia solanacearum was isolated from plant extracts using the modified SMSA medium (Engelbrecht, 1994; Elphinstone et al., 1996) and

grown for up to five days at 27 °C. Pure cultures of bacteria were cultured at 27 °C on trypticase soy agar (Oxoid, Unipath Ltd, Hampshire).

Selection of oligonucleotides

The characteristics of the NASBA primers, the biotinylated probe and the molecular beacons MBsol1F and MBsol2F are listed in Table 2. The molecular beacons were designed basically according to the guidelines as described by Tyagi and Kramer (1996). The molecular beacons were analysed with the DNA-folding programme Mfold version 3.0 (Dr. M. Zuker, Macfarlane Burnet Centre, USA). The sequences specific for 16S rRNA of *R. solanacearum* of the probe and molecular beacons were selected upon an alignment with taxonomically related *Ralstonia* 16S rRNA sequences (Li et al., 1993; Seal et al., 1993; Bentsink et al., 2002).

Immunofluorescence (IF) cell-staining

An indirect IF was performed as described by Janse (1988). A polyclonal rabbit antiserum (IPO-DLO no. 9023), for which a titer was estimated of 12 800, was used as a primary antibody in a dilution of 3200, 6400 and 12 800. Secondary FITC-labelled pig anti-rabbit antibodies (Nordic) were used in a 100-fold dilution. Stained cells were examined by UV-microscopy (Zeiss Axioskop, 63 : 1 objective, 10× ocular).

Table 1. Reaction of various bacterial strains in AmpliDet RNA

Family or species	Origin	IPO number[1]	Original number[2]	NASBA-reaction[3]
R. solanacearum (biovar 2, race 3)	Netherlands	1609	PD2763	+
R. solanacearum (biovar 2, race 3)	Netherlands	1661	PD426	+
R. solanacearum (biovar 2, race 3)	Netherlands	1662	PD1254	+
R. solanacearum (biovar 2, race 3)	Chile	1664	PD1408	+
R. solanacearum (biovar 1, race 2)	Costa Rica	1666	PD507	+
R. syzigii	Indonesia	S302	PD2093	+
R. picketti	United Kingdom	1720	Pr1150	–
Burkholderia cepacia	United Kingdom	1702	NCPPB945	–
B. cepacia	United Kingdom	1703	NCPPB946	–
B. cepacia	Unknown	1718	R5d-1	–
Rahnella aquaticus	Spain	1695	1435.D	–
Enterobacteriaceae	Netherlands	S342		–

[1]Strains S302, 1720, 1695 and S342 showed cross-reactions with polyclonal antiserum 9523 in IF.
[2]Strains were obtained from J.D. Janse (Plant Protection Service, Wageningen, The Netherlands), J.G. Elphinstone (Central Science Laboratory, York, UK), A.C. le Roux (Institut National de la Recherche Agronomique, Rennes, France) and M.M. Lopez (Instituto Valenciano de Investigaciones Agrarias, Valencia, Spain).
[3]NASBA reactions were considered positive when FAM/ROX ratio's exceeded twice the ratio of the negative control (water). RNA was tested in an amount of 100 pg per reaction (is ca. 10^7 copies of RNA).

Table 2. Characteristics of primers, biotinylated probe and molecular beacons used in this study

Name	Sequence (5′ → 3′)	Length	Position[a]	Type
P1D	*AATTCTAATACGACTCACTATAGGG*AGAGGCCTTGCGGTCCCCCACT[b]	47	195–215	Antisense primer
P2A	TGGCGGCATGCCTTACACA	19	37–55	Sense primer
PrOLI1	GGGGTAGCTTGCTACCTGCC	21	75–91	Probe
Mbsol1F	<u>CCAGGC</u>CAGCGGGGGTAGC<u>GCCTGG</u>[c]	25	69–82	Molecular beacon
Mbsol2F	<u>CCAGGC</u>AAAGACTAGCTAATA<u>GCCTGG</u>	27	159–173	Molecular beacon
Probe 1	CAGCGGGGGTAGC	13	69–82	Probe
Probe 2	AAAGACTAGCTAATA	15	159–173	Probe

[a]*R. solanacearum* sequence (Li et al., 1992; EMBL/GenBank/DDBJ databases).
[b]The T7 promotor sequence is given in italics.
[c]The sequence for the stem structure is underlined. The molecular beacons are labelled with fluorescein at the 5′ end and with DABCYL at the 3′ end.

Pathogenicity assay

The pathogenicity test by stem inoculation of young tomato plants was performed as described by Janse (1988).

Sample preparation

Extracts from composite samples of 200 potato tubers were prepared according to Janse (1988) with slight modifications as described by Bentsink et al. (2002). The method included filtration through a 40–100 μm filter and concentration of sample components by centrifugation at 10.000g for 15 min. The pellet was finally resuspended in 1 ml of 0.01 M phosphate buffer (2.7 g l^{-1} $Na_2HPO_4 \cdot 12H_2O$ and 0.4 g l^{-1} $NaH_2PO_4 \cdot 2H_2O$, pH 7.2). In case of spiked samples, 900 μl of the prepared tuber extract, free of *R. solanacearum,* was supplemented with 100 μl of a serial dilution of *R. solanacearum* in demineralised water.

Naturally infected samples and samples considered pathogen-free were selected on the basis of IF results. The presence of *R. solanacearum* in naturally infected samples was confirmed by isolation and a pathogenicity assay.

Extracts from spiked surface water were prepared by adding 20 ml of a bacterial suspension in sterile demineralised water to 180 ml of ditch water, free of *R. solanacearum*, thus creating a 10-fold serial dilution of 10^1–10^4 cells ml^{-1}. A 10-fold dilution in sterile demineralised water served as a positive control. Samples were filtrated through a paper tissue and centrifugated for 20 min at 10.000g. The pellet was resuspended in 1 ml of demineralised water.

RNA extraction

Three different ways to extract RNA from tuber and water samples were compared: (1) a method based on the protocol of Boom et al. (1990) with minor alterations as described by Bentsink et al. (2002) using silica as matrix for selective binding of RNA; (2) a similar protocol, but using celite instead of silica as a matrix for binding RNA; (3) a phenol/chloroform extraction procedure as described by Verwoerd et al. (1989).

To extract RNA from serially diluted pure cultures of bacteria, 1 ml of sample was centrifuged for 10 min at 10.000g and resuspended in 50 μl of 10 mM TRIS/HCl (pH 8.0), 1 mM EDTA (TE). To extract RNA from spiked potato tuber extracts, 1 ml of sample was concentrated by centrifugation for 10 min at 3000g. The pellet was discarded and the supernatant was centrifuged for 10 min at 10.000g. The pellet was resuspended in 50 μl of TE. For studies on naturally infected potato tuber samples, 50 μl of sample was used.

NASBA and post NASBA analysis

NASBA and analysis of NASBA amplicons by Northern blotting was done as described by Bentsink et al. (2002). Real time monitoring by measuring fluorescence released by a molecular beacon upon hybridisation with amplicons was performed essentially as described by Leone et al. (1998). Unless stated otherwise, the molecular beacon was used in an amount of 9 ng per reaction (0.45 μg ml^{-1}) and the fluorescent signal was measured in closed tubes for 90 min at 41 °C, using an Applied Biosystems ABI Prism™ 7700 Sequence detector. Fluorescence was expressed in R_n values, calculated as the ratio between the signal of fluorescein and the signal of the fluorophore ROX, supplied to every sample in a concentration of 1 pmol μl^{-1}. In order to be able to compare samples from different measurements, R_n values were normalised to ΔR_n values by subtraction of the mean R_n value recorded at the first minute of the NASBA reaction.

NASBA results were analysed on the basis of the ΔR_n values. For pure cultures of bacteria values were considered positive when they exceeded twice the R_n value of the blank (water). For peel extracts they were considered positive when they exceeded $x \cdot (\Delta x \cdot T)$, in which x = the average of the blanks, Δx the standard deviation and T the Student T value (P = 0.95). For calculation of detection levels, negative R_n values of blanks, which occurred incidentally, were considered zero.

Results

Characteristics of the molecular beacons

Both MBsol1F and MBsol2F showed a typical stem-loop structure, although in MBsol1F an internal hybridisation of two nucleotides in the loop structure was predicted (Figure 1). A thermal transition profile of Mbsol1F and MBsol2F, tested in the NASBA reaction mix showed a better performance of MBsol2F (Figure 2). Both molecular beacons were closed at 41 °C, the temperature at which NASBA

was performed, but in an open conformation MBsol2F showed a maximum R_n values ca. 1.8 times higher than MBsol1F. MBsol2F opened entirely at ca. 77 °C, 10 °C lower than MBsol1F.

Despite the superior characteristics of MBsol2F, amplification of ca. 1 pg purified RNA of *R. solanacearum* by NASBA, resulted in a high R_n value with MBsol1F, but not with MBsol2F (Figure 3A). On Northern blot similar results were obtained, probe 1 but not probe 2, targeting similar 16S rRNA sequences as MBsol1F and MBsol2F, respectively, resulted in a positive signal (Table 2, Figure 3B).

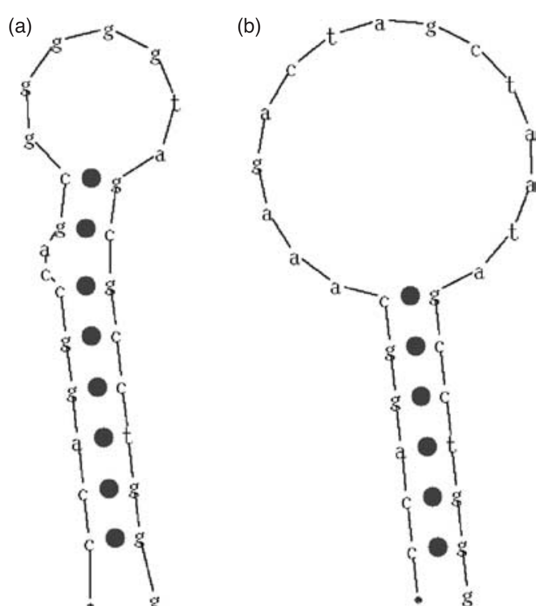

Figure 1. Schematic presentation of MBsol1F (a) and MBsol2F (b) using the DNA-folding programme Mfold (version 3.0).

Extraction of RNA

RNA was extracted from a *R. solanacearum* serially diluted in water or potato tuber samples by a phenol–chloroform treatment or according to the method described by Boom et al. (1990), using silica or celite as a matrix for selective RNA binding. The extraction procedure based on celite resulted in the highest sensitivity, with a detection level of 10^3 cells ml^{-1} in water and 10^4 cells ml^{-1} in peel extract (Table 3). For the two other methods a 10–1000-fold higher detection level was found. This level of detection in peel extracts is similar to that found for *C. michiganensis* subsp. *sepedonicus*, the causal organism of bacterial ring rot of potato (Van Beckhoven et al., 2002).

Specificity of AmpliDet RNA

Specificity was tested on total RNA extracts from pure cultures of four *R. solanacerum* biovar 2 race 3 strains, one biovar 1 race 2 strain, one *R. syzygii*

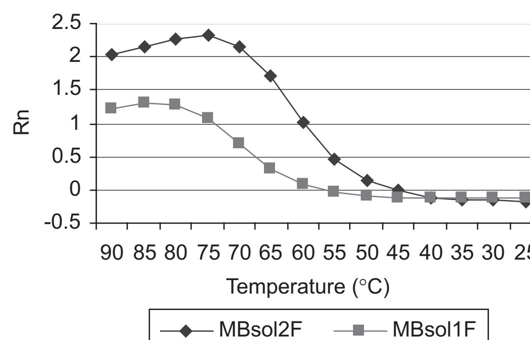

Figure 2. Thermal transition profiles of molecular beacons MBsol1F and MBsol2F.

(a)

Molecular Beacon	Sample[1]	Rn-values
MBsol1F	positive	31.15
MBsol1F	negative	0.47
MBsol2F	positive	0.76
MBsol2F	negative	0.47

[1] Positive samples contained c. 1 pg RNA extracted from 10^5 cells of *R. solanacearum* per reaction. Negative samples contained water

(b)

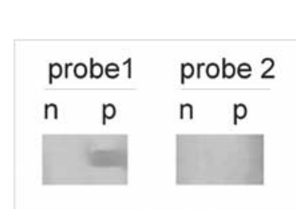

Figure 3. (A) Reaction of molecular beacons MBsol1F and MBsol2F in AmpliDet RNA. R_n values were determined by a post NASBA read. (B) Reaction of biotinylated probe 1 and probe 2, targeting the same 16S rRNA sequences as MBsol1F and MBsol2F, respectively, on Northern blot. Negative samples (n) contained amplicons of water, positive samples (p) contained amplicons of RNA extracted from 10^5 cells of *R. solanacearum* per reaction.

strain, four other taxonomically related bacteria, and two bacteria cross-reacting with polyclonal antibodies in IF (Table 1). Even at a relative high amount of 100 pg total RNA, containing ca. 10^7 copies of 16S rRNA, positive reactions were found only with *R. solanacearum* strains and the *R. syzygii* strain, but not with six other non-target bacteria.

Table 3. Effect of the RNA-extraction procedure on detection for *R. solanacearum* in water and potato tuber extracts with AmpliDet RNA. A phenol–chloroform extraction procedure was compared with procedures based on selective binding of RNA on silica or celite[1]

Bacterial concentration	Phenol–chloroform	Boom (silica)	Boom (celite)
In water			
10^7	**3.2**[2]	**3.1**	**3.7**
10^6	**3.0**	**2.2**	**3.0**
10^5	**2.2**	**3.1**	**3.3**
10^4	0.1	0.1	**3.0**
10^3	0.1	0.1	**2.4**
10^2	0.1	0.0	0.0
0	0.2	0.4	0.2
In tuber extracts			
10^7	**4.5**	**4.3**	**4.4**
10^6	**4.2**	**3.0**	**2.6**
10^5	**1.5**	0.5	**3.4**
10^4	0.2	0.4	**1.9**
10^3	0.2	0.3	0.5
10^2	0.0	0.0	0.0
0	0.1	0.0	0.2

[1]Methods described by Boom et al. (1990).
[2]Results expressed in R_n values. In bold, values significantly positive ($P = 0.95$).

Amount of molecular beacon

The effect of the concentration of molecular beacon MBsol1F on the detection level of AmpliDet RNA was tested, using concentrations of 3, 6, 9 and 12 ng of MBsol1F per reaction (Table 4). The lowest detection level of 10^4 copies of RNA per reaction (equivalent to 1.2 pg of RNA) was found at 6 ng of MBsol1F. However, R_n values for amounts of RNA exceeding 10^4 copies, were significantly higher at 9 and 12 ng than at 6 ng of MBsol1F. An amount of 6 ng of beacon was used in further experiments. On Northern blot, using the biotin labelled probe 1, a similar detection level of 10^4 copies of total RNA (1.2 pg of RNA) was found as in AmpliDet RNA (Figure 4).

Detection in potato tuber extracts

AmpliDet RNA was evaluated for detection of *R. solanacearum* in 18 extracts of composite samples of

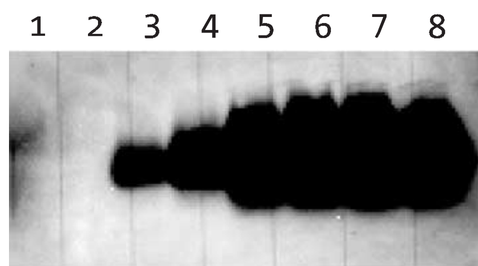

Figure 4. Chemiluminescent detection by Northern blotting with a biotinylated probe 1: lane 1, negative control (water); lane 2, 10^3; lane 3, 10^4, lane 4, 10^5; lane 5, 10^6; lane 6, 10^7; lane 7, 10^8; lane 8, 10^9 copies of total RNA.

Table 4. Reaction of purified total RNA of *R. solanacearum* in AmpliDet RNA, tested with 3, 6, 9 and 12 ng of molecular beacon MBsol1F per reaction ($n = 2$)

Copies RNA	3 ng		6 ng		9 ng		12 ng	
	Average R_n value	s.d.	Average R_n value	s.d.	Average R_n value	s.d.	Average R_n value	s.d.
10^9	**12.1**	0.4	**22.7**	0.1	**33.7**	0.5	**40.3**	0.7
10^8	**10.9**	0.4	**21.9**	0.5	**33.6**	0.0	**36.9**	0.9
10^7	**10.4**	0.2	**17.7**	0.7	**27.2**	0.7	**31.6**	1.3
10^6	**5.8**	2.4	**9.4**	6.2	**12.3**	7.6	**15.9**	6.9
10^5	**2.8**	1.3	**3.0**	0.8	**4.5**	1.9	**4.4**	1.0
10^4	0.3	0.0	**0.8**	0.4	1.4	0.2	2.3	0.4
$10^{3\#}$	0.2		0.4		1.0		2.0	
0	0.2	0.0	0.4	0.0	1.0	0.1	1.9	0.1

#Not duplicated.
In bold: R_n values exceeding twice the average value of the negative control (water).

Table 5. Evaluation of AmpliDet RNA for detection of *R. solanacearum* (biovar 2) in naturally infected (positive) and uninfected (negative) potato tuber extracts

Samples[1]	Number of cells in IF[2]	Number of samples tested	Number of samples positive in AmpliDet RNA
Positive[3]	1–5	1	1
	6–50	9	9
	51–500	7	7
	>500	1	1
Negative[4]	0	68	0
	1–5	10	0
	5–50	10	0
	50–500	3	1
Control	Negative	13	0
	Positive	4	4

[1]Extracts of composite samples of 200 tubers in ca. 15% glycerin were stored for two months at −80 °C prior to analysis by AmpliDet RNA.
[2]Immunofluorescence cell-staining was performed after washing extracts by centrifugation to remove glycerin prior to NASBA.
[3]Samples were considered as positive or negative on the basis of a first screening by IF, and verification of IF-positive samples by isolation and full identification using a pathogenicity assay on tomato and fatty acid profiling.
[4]Extracts of in particular negative samples often contained (cross-reacting) fluorescent cells with an aberrant size or staining intensity.

200 tubers from which *R. solanacearum* had been isolated previously. The extracts had been stored for two months, frozen at −80 °C. In addition, 91 extracts were selected that were considered free of *R. solanacearum* on the basis of dilution plating on SMSA and a pathogenicity assay on tomato. In 23 of these samples, however, IF-positive cells had been found. Samples were tested at least in duplicate in AmpliDet RNA and once more in IF. All 18 samples contaminated with *R. solanacearum* were consistently positive in all tests (Table 5). From 91 negative samples, 1 sample, containing high number of IF-positive cells was consistently positive, whereas all other samples were negative in AmpliDet RNA. No relation was found between density of cells in sample extract and the Ct-value in AmpliDet RNA (data not shown).

Detection in surface water

AmpliDet RNA enabled detection of 10 cfu ml^{-1} in surface water and 1 cfu ml^{-1} in demineralised water (Table 6). RNA was extracted from 200 ml of

Table 6. Detection of *R. solanacearum* with AmpliDet RNA in ditch water and demineralised water

Cells per reaction	Ditch water		Demineralised water	
	Average R_n value	s.d.	Average R_n value	s.d.
10.000[1]	**8.88**	2.57	**13.91**	3.13
1000	**5.61**	1.70	**9.74**	4.72
100	2.52	1.72	**5.96**	4.04
10	1.54	0.76	1.96	0.63
0	1.6	0.85	0.94	0.62

[1]Bacterial cells from 200 ml were concentrated into 1 ml by centrifugation. The RNA from the concentrated cells was extracted into 5 µl, from which 1 µl was analysed in AmpliDet RNA. Consequently, 10,000 cells per reaction was equivalent to 5×10^4 cells ml^{-1}.
In bold: average values significantly positive ($P \geq 0.05$). Results corresponded with those exceeding twice the average value of the negative control.

contaminated water samples, after removing coarse particles by filtration followed by centrifugation to concentrate bacteria 200-fold times.

Discussion

In this study the potential of AmpliDet RNA for homogeneous, real time detection of *R. solanacearum*, the causal organism of bacterial wilt was demonstrated. AmpliDet RNA enabled reliable detection of rRNA sequences of *R. solanacearum* in total RNA extracts from potato tuber samples in 90 min at a level of 10 cells per reaction, equivalent to ca. 10^4 cells ml^{-1}.

The technique was proven to be reliable for detection of *R. solanacearum* (race 3, biovar 2) in naturally infected potato tuber samples containing varying concentrations of cells. Moreover, AmpliDet RNA largely reduces the risks on false-positive results. From 23 IF-positive samples, that were negative in the pathogenicity assay and also negative in a dilution plating assay on SMSA, 22 samples were negative in the AmpliDet RNA, which strongly suggests that in IF cross-reacting cells have been detected. The occurrence of bacteria cross-reacting with polyclonal antibodies against *R. solanacearum* has been described previously (Griep et al., 1998). Only one sample was positive in both IF and in AmpliDet RNA and negative in the dilution plating assay and in the pathogenicity test. Because it is unlikely that bacterial cells are found with similar antigenic determinants as *R. solanacearum*, which also has a high 16S rRNA homology, it is assumed

that this particular sample has contained viable but non-culturable cells, not detectable by plating or in a pathogenicity assay. Insufficient sample material was left to repeat the experiments and to confirm results.

The specificity of AmpliDet RNA was also proven by testing individual bacterial strains. The specificity of the assay is primarily based on the target specific sequences of molecular beacon MBsol1 that partly cover those of the OLI-1 primer used in a specific PCR-amplification procedure for *R. solanacearum* (Seal et al., 1993). The sequences were derived from a variable part of the 16S rDNA, that differentiates *R. solanacearum* from other phylogenetically related bacterial species, namely *R. pickettii* and *Burkholderia cepacia* but not from *R. syzygii* (Seal et al., 1993). Reactions with *R. syzygii*, however, a pathogen highly specific for clove, will not affect specificity of assays on bacterial pathogens in potato. The PCR based on the OLI-1 has been evaluated extensively with large numbers of target strains, including different races and biovars and many non-target strains, and was found to be highly specific for the bacterial wilt pathogen (Seal et al., 1993; Stead, pers. comm.). The primers used for amplification by NASBA were selected from conserved regions, which will amplify at the relatively mild annealing conditions many different bacterial species. The presence of high amounts of non-target RNA in sample extracts may therefore adversely affect the sensitivity of the assay.

In Europe, there is strong evidence that potato and tomato crops have been contaminated by the use of infected surface water for irrigation (Elphinstone, 1996; Wenneker et al., 1999). Concentrations of *R. solanacearum* in surface water were found to vary between 10^3 and 10^6 cfu l^{-1}. Rapid and reliable detection of *R. solanacearum* in water is considered as an important instrument to avoid transfer of the pathogen to potato plants via irrigation. AmpliDet RNA is suitable for testing *R. solanacearum* in water, because it was able to detect 10 cfu per reaction in 100–1000-fold concentrated surface water samples, equivalent to a detection level of ca. 1000 cfu l^{-1}.

In theory, AmpliDet RNA allows an estimation of the concentration of the target RNA in the sample extracts, as a positive relationship was found between the concentration of purified RNA from pure cultures and Ct-value. Similar results were previously found for purified viral RNA (Leone et al., 1998). However, with naturally infected potato extracts the correlation was absent, possibly due to unpredictable losses during the RNA purification or the presence of NASBA inhibiting components left over after purification.

An advantage of amplification of rRNA sequences by AmpliDet RNA compared to PCR-amplification of DNA sequences is its potential to distinguish viable from non-viable cells (Bentsink et al., 2002). Ribosomal RNA is considered to be an indicator for viability as it is sensitive to degradation and will rapidly decay upon cell death (Hahn et al., 1992). PCR-amplification targets are relatively stable DNA sequences, which can result in positive signals even long after cell death. The specific detection of viable cells can be particularly important when studying the persistence of *R. solanacearum* in its natural ecosystem. It may also be of importance for inspection of plant material, as confirmatory assays often involve a pathogenicity test on a sensitive indicator plant or plating on a selective medium, both only responding if viable cells are present. The stability of ribosomal RNA allows storage of tuber samples for minimally eight weeks at $-80\,^{\circ}$C, but at temperatures above zero, the molecules are rapidly degraded (Hahn et al., 1992).

In conclusion, the AmpliDet RNA will allow truly homogeneous specific detection of low concentrations of viable cells of *R. solanacearum* in unopened reaction vials. The assay may be employed for different applications, including survival studies, testing of potato tubers and surface water in disease management strategies.

References

Bentsink L, Leone GOM, Van Beckhoven JRCM, Van Schijndel H, Gemen B and Van der Wolf JM (2002) Amplification of RNA by NASBA allows direct detection of viable cells of *Ralstonia solanacearum* in potato. Journal of Applied Microbiology 93: 647–655

Boom R, Sol CJA, Salimans MMM, Jansen CL, Wertheim-van Dillen PME and Van der Noordaa J (1990) Rapid and simple method for purification of nucleic acids. Journal of Clinical Microbiology 28: 495–503

Boudazin G, Le Roux AC, Josi K, Labarre P and Jouan B (1999) Design of division specific primers of *Ralstonia solanacearum* and application to the identification of European isolates. European Journal of Plant Pathology 105: 373–380

Demeke T and Adams RP (1992) The effects of plant polysaccharides and buffer additives on PCR. BioTechniques 12: 332–334

Elphinstone JG (1996) Survival and possibilities for extinction of *Pseudomonas solanacearum* (Smith) Smith in cool climates. Potato Research 39: 403–410

Elphinstone JG, Hennessy J, Wilson JK and Stead D (1996) Sensitivity of different methods for the detection of *Ralstonia*

solanacearum in potato tuber extracts. Bulletin OEPP/EPPO Bulletin 26: 663–678

Engelbrecht MC (1994) Modification of a selective medium for the isolation and quantification of *Pseudomonas solanacearum.* ACIAR Bacterial Newsletter 10: 3–5

Griep RA, Van Twisk C, Van Beckhoven JRCM, Van der Wolf JM and Schots A (1998) Development of specific recombinant monoclonal antibodies against the lipopolysaccharides of *Ralstonia solanacearum* race 3. Phytopathology 88: 795–803

Hayward AC (1991) Biology and epidemiology of bacterial wilt caused by *Pseudomonas solanacearum*. Annual review of Phytopathology 29: 65–87

Higuchi R, Dollinger G, Walsh PS and Griffith R (1992) Simultaneous amplification and detection of specific DNA sequences. Bio/Technology 10: 413–417

Hahn D, Amann RI, Ludwig W, Akkermans ADL and Schleifer KH (1992) Detection of micro-organisms in soil after *in situ* hybridization with rRNA-targeted, fluorescently labelled oligonucleotides. Journal of General Microbiology 138: 879–887

Holland PM, Abramson RD, Watson R and Gelfand DH (1991) Detection of specific polymerase chain reaction product by utilizing the $5'{-}{>}3'$ exonuclease activity of *Thermus aquaticus* DNA polymerase. Proceedings of the National Academy of Sciences of the United States of America 88: 7276–7280

Janse JD (1988) A detection method for *Pseudomonas solanacearum* in symptomless potato tubers and some data on its sensitivity and specificity. Bulletin OEPP/EPPO Bulletin 18: 343–351

Kwok S and Higuchi R (1989) Avoiding false positives with PCR. Nature 339: 237–238

Leone G, van Schijndel H, van Gemen B, Kramer FR and Schoen CD (1998) Molecular beacon probes combined with amplification by NASBA enable homogeneous, real-time detection of RNA. Nucleic Acids Research 26: 2150–2155

Li X, Dorsch M, Del Dot T, Sly LI, Stackebrandt E and Hayward AC (1993) Phylogenetic studies of the rRNA group II pseudomonads based on 16S rRNA gene sequences. Journal of Applied Bacteriology 74: 837–842

Livak KJ, Flood SJA, Marmaro J, Giusti W and Eetz K (1995) Oligonucleotides with fluorescent dyes at opposite ends provide a quenched probe system useful for detecting PCR product and nucleic acid hybridization. PCR Methods Application 4: 357–362

Rossen L, Norskøv P, Holmstøm K and Rasmussen OF (1992) Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. International Journal of Food Microbiology 17: 37–45

Seal SE, Jackson LA, Young JPW and Daniels MJ (1993) Differentiation of *Pseudomonas solanacearum*, *Pseudomonas syzygii*, *Pseudomonas pickettii* and the blood disease bacterium by partial 16S rRNA sequencing: Construction of oligonucleotide primers for sensitive detection by polymerase chain reaction. Journal of General Microbiology 139: 1587–1594

Seal SE and Elphinstone JG (1994) Advances in identification and detection *of Pseudomonas solanacearum*. In: Hayward AC and Hartman GL (eds) Bacterial Wilt: The Disease and its Causative Agent, *Pseudomonas solanacearum* (pp 35–38) CAB International, Wallingford, Oxon, UK

Szemes M, Schoen CD and Van der Wolf JM (2001) Molecular beacons for homogeneous real time monitoring of amplification products Section 2.3.2. In: Akkermans ADL, Van Elsas JD and De Bruijn FJ (eds) Molecular Microbial Ecology Manual (pp 1–17) Kluwer Academic Publishers, the Netherlands

Tyagi S and Kramer FR (1996) Molecular beacons: Probes that fluoresce upon hybridization. Nature Biotechnology 14: 303–308

Van Beckhoven JRCM, Stead DE and Van der Wolf JM (2002) Detection of *Clavibacter michiganensis* subsp. *sepedonicus* by AmpliDet RNA, a new technology based on real time monitoring of NASBA amplicons with a molecular beacon. Journal of Applied Microbiology 93: 840–849

Van der Vliet GME, Schepers P, Schukkink RAF, van Gemen B and Klatser PR (1994) Assessment of mycobacterial viability by RNA amplification. Antimicrobial Agents and Chemotherapy 38: 1959–1965

Verwoerd TC, Dekker BMM and Hoekema A (1989) A small-scale procedure for the rapid isolation of plant RNAs. Nucleic Acid Research 17: 2362

Vet JAM, Majithia AR, Marras SAE, Tyagi S, Dube S, Poiesz J and Kramer FR (1999) Multiplex detection of four pathogenic retroviruses using molecular beacons. Proceedings of the National Academy of Sciences of the United States of America 96: 6394–6399

Weller SA, Elphinstone JG, Smith NC and Stead DE (2000a) Detection of *Ralstonia solanacearum* from potato tissue by post-enrichment TaqMan PCR. Bulletin OEPP 30: 381–383

Weller SA, Elphinstone JG, Smith NC, Boonham N and Stead DE (2000b) Detection of *Ralstonia solanacearum* strains with a quantitative, multiplex, real-time fluorogenic PCR (TaqMan) assay. Applied and Environmental Microbiology 66: 2853–2858

Wenneker M, Verdel MSW, Groeneveld RMW, Kempenaar C, Van Beuningen AR and Janse JD (1999) *Ralstonia (Pseudomonas) solanacearum* race 3 (biovar 2) in surface water and natural weed hosts: First report on stinging nettle (Urtica dioica). European Journal of Plant Pathology 105: 307–315

Wittwer C, Ririe K, Andrew R, David D, Gunry R and Balis U (1997) The LightCycler™: A microvolume multisample fluorimeter with rapid temperature control. BioTechniques 22: 176–181