

# Detection of *Clavibacter michiganensis* subsp. *sepedonicus* by AmpliDet RNA, a new technology based on real time monitoring of NASBA amplicons with a molecular beacon

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**Aims:** To develop a procedure for direct detection of viable cells of *Clavibacter michiganensis* subsp. *sepedonicus* (Cms), the causal organism of bacterial ring rot in potato, based on AmpliDet RNA<sup>TM</sup>, in which amplicons generated by nucleic acid sequence based amplification (NASBA) are monitored in real time with a molecular beacon.

**Methods and Results:** Five methods were evaluated and fine-tuned for extraction of RNA from Cms. The most efficient non-commercial RNA extraction method included an enzymatic breakdown of the cell wall followed by a phenol extraction. AmpliDet RNA enabled detection of 10 000 molecules of purified rRNA per reaction and 100 cfu of Cms per reaction in more complex samples. Two primer pairs were tested with DNA and RNA purified from Cms. One primer pair was able to distinguish live from dead cells.

**Conclusions:** An AmpliDet RNA was developed which enabled fast and specific detection of viable cells of Cms in complex substrates at a detection limit of 100 cfu per reaction.

**Significance and Impact of the Study:** This novel AmpliDet RNA is carried out in sealed tubes, thus reducing the risk of carry-over contamination. The method will be particularly suitable for studies on the epidemiology of Cms in which viable cells should be exclusively detected.

## INTRODUCTION

*Clavibacter michiganensis* subsp. *sepedonicus* (Cms) is the causal organism of bacterial ring rot in potato, a quarantine disease that can cause great economic losses (De Boer and Slack 1984). Cms is an insidious pathogen which is difficult to control because it can survive for long periods in a dry state on surfaces of equipment and materials used in potato production (Bonde 1942). Moreover, because the disease often remains latent in plants and tubers, undetected tuber infections are responsible for spreading the disease to clean seed lots and to ring rot-free regions. Therefore, certification by visual inspections of tubers or plants cannot provide the necessary level of disease control (Dijkstra 1942). It is important to have reliable and sensitive methods for detection of Cms in different substrates, especially in

infected tubers, where they can be present in low cell numbers (De Boer and McNaughton 1986).

A broad range of methods based on plating, serology and molecular biology are currently used for detection of Cms. The plating methods are laborious and time consuming as they are preceded by selective enrichment in eggplants. Direct plating generally results in an overgrowth of Cms by more rapidly-growing bacteria. The serological tests include immunofluorescence cell staining (De Boer and Copeman 1980), immunofluorescence colony staining (Roozen and Van Vuurde 1991) and ELISA (De Boer *et al.* 1988). These tests are fairly sensitive, with a detection level of about 10 000 cells ml<sup>-1</sup>, but they may occasionally give false-positive results due to cross-reactions. Methods based on detection of (amplified) nucleic acid sequences can be very rapid, sensitive and specific, but few of the published methods have been widely used, largely because they are difficult to reproduce. For PCR amplification, several primers have been described on the basis of different fragments (Schneider *et al.* 1993; Li and De Boer 1995b).

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Mills *et al.* (1997) selected three primer pairs by genomic subtraction. On the basis of one of these pairs, a TaqMan PCR procedure was developed (Schaad *et al.* 1999). Li *et al.* (1997) developed a fluorescence *in situ* hybridization procedure that allowed double staining of cells with a DNA probe and antibodies. For pure cultures of Cms, detection limits of 1–10 cells per PCR reaction have been reported.

In potato tuber samples, the detection limit of nucleic acid-based methods is dependent on the efficiency of the method used to extract the nucleic acids. The extraction methods described often include a rigorous lysis step to open the dense peptidoglycan cell wall layer that is typical for Gram-positive bacteria (Labischinski and Maidhof 1994). Extraction also requires an effective way of separating nucleic acids from components in the plant extract that can inhibit the amplification reaction.

Recently, AmpliDet RNA<sup>TM</sup>, a novel nucleic acid detection method, has been introduced for the detection of plant pathogens. This method combines Nucleic Acid Sequence Based Amplification (NASBA), an isothermal amplification procedure, with real time monitoring of amplicons using a molecular beacon (Leone *et al.* 1998). NASBA is based on the concurrent activity of the three enzymes AMV reverse transcriptase, RNase H and T7 RNA polymerase for exponential amplification of single-stranded (ss)RNA molecules, and is particularly suitable for RNA amplification. Molecular beacons are single-stranded oligonucleotides containing a stem-loop structure. The loop is complementary to the sequence of the target, and the stem has a double-stranded structure in which one strand is labelled with a fluorophore and the other with a quencher. When the loop hybridizes to the target, the loop and stem will open, thus separating the quencher from the fluorophore and allowing the probe to release fluorescence (Tyagi and Kramer 1996). NASBA appears to be suitable not only for diagnostics but also for indicating biological activities, such as gene expression and cell viability, since RNA molecules tend to degrade rapidly after cell death (Van der Vliet *et al.* 1994).

The objective of this study was to develop an AmpliDet RNA method for the detection of viable cells of Cms in potato tuber extracts, surface water and plant extracts.

## MATERIALS AND METHODS

### Bacterial strains and growth conditions

*Clavibacter michiganensis* subsp. *sepedonicus* (Cms) strain NCPPB 4053 (IPO strain nr. 1831) was used in all experiments. This fluidal strain was isolated from Swedish potato tubers. For specificity studies, several strains of Cms and closely-related bacteria were used (Table 1).

Long-term maintenance of the strains was at  $-80^{\circ}\text{C}$  on beads (Protect, Biotrading Wilnis, Wilnis, the Netherlands)

in 15% glycerol, containing 8 mg ml<sup>-1</sup> Lab Lemco broth (Oxoid).

The experiments were carried out with strains grown on Yeast growth medium (YGM) (De Boer and Copeman 1980; Anon. 1993) or Davis modified (DM) agar (Metzler *et al.* 1992) for 2 days at 21 °C.

### Selection of primers and probes

The primers and probes used were selected from a 16S rRNA sequence alignment of Cms with 16S rRNA sequences from taxonomically-related *Clavibacter* species (Fig. 1; Li and De Boer 1995a; Suzuki *et al.* 1996; Lee *et al.* 1997). The antisense primers consist of a 3' terminal target specific sequence, linked to a 5' terminal T7 promoter sequence, which can be recognized by T7 polymerase. Five different primer combinations were tested: X1f/X1r, X1f/X2r, X3f/X3r, A1f/Ar and A2f/Ar.

The probes CMS1 (acgtgcagagatgtgcg) and CMS2 (gatgtgcgccccca) used for Northern blotting were 5'-biotinylated for detection with a digoxigenin-labelled probe.

The molecular beacons MBcms1 (gccaggAACGTGCA GAGATGTGCGCCcctggc) and MBcms2 (ccgtgcATGT GCGCCCCCAAgcaccg) were coupled at the 3' end with DABCYL, and at the 5' end with FAM (Isogen Bioscience BV, Maarssen, the Netherlands). The structure of the beacons was predicted using the RNA folding package Mfold, developed by Dr M. Zuker (<http://www.ibc.wustl.edu/~zucker/>).

### Sample preparation

Extracts from potato tubers free of Cms were prepared according to Janse (1988) with slight modifications. The extracts were passed through a 40–100 µm filter and the filtrate was concentrated by centrifugation at 10 000 g for 15 min. The pellet was resuspended in 1 ml 0.01 mol l<sup>-1</sup> phosphate-buffered saline (PBS) (2.7 g l<sup>-1</sup> Na<sub>2</sub>HPO<sub>4</sub>·12H<sub>2</sub>O and 0.4 g l<sup>-1</sup> NaH<sub>2</sub>PO<sub>4</sub>·2H<sub>2</sub>O, pH 7.2).

Artificially-contaminated potato tuber extracts were obtained by mixing 900 µl of the extract with 100 µl of bacterial suspension in demineralized water. The potato tuber extracts were centrifuged for 10 s at 255 g. The supernatant fluid was centrifuged for 10 min at 12 000 g. The pellet was resuspended in 1 ml 0.3% (w/v) glycine.

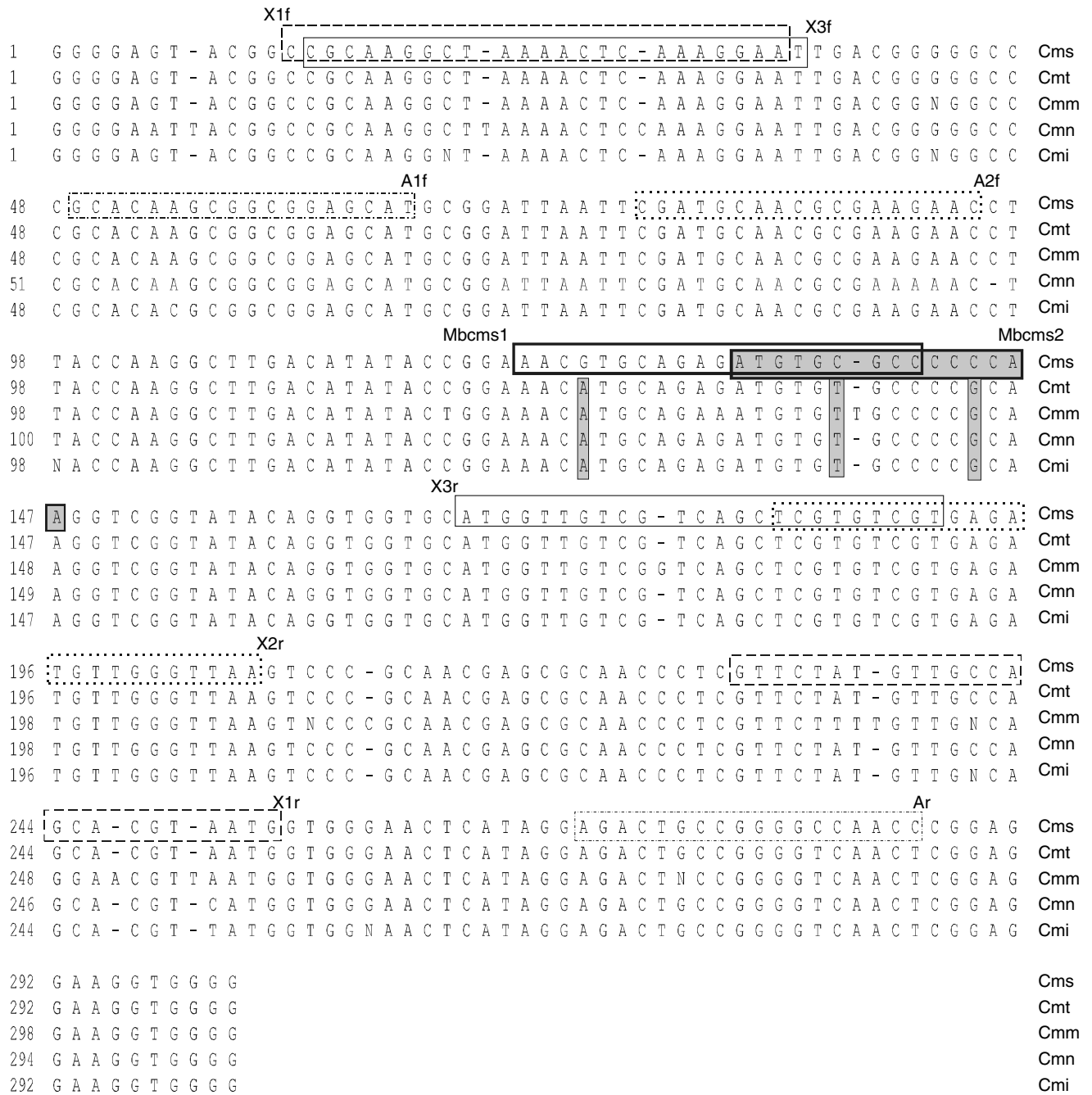
Artificially-contaminated surface water was obtained by adding 20 ml of a bacterial suspension in demineralized water to 180 ml of ditch water free of Cms. A serial dilution of bacteria in demineralized water served as a control. The samples were filtered through an 11 µm 595 filter (Schleicher and Schuell Nederland BV's Hertogenbosch, The Netherlands). The filtrate was further filtered under vacuum through a 0.22 µm GSWP 047 filter (Millipore, Etten-Leur,

**Table 1** Reaction of various bacterial strains in AmpliDet RNA

Species	Origin	Host	PRI number	Original number*	AmpliDet RNA† (Rn value)
<i>Clavibacter michiganensis</i> subsp. <i>sepedonicus</i>	Sweden	Potato	270	PD1381	<b>4.03</b>
<i>C. michiganensis</i> subsp. <i>sepedonicus</i>	Lithuania	Potato	1844	736	<b>2.41</b>
<i>C. michiganensis</i> subsp. <i>sepedonicus</i>	Lithuania	Potato	1845	734	<b>2.36</b>
<i>C. michiganensis</i> subsp. <i>sepedonicus</i>	Lithuania	Potato	1846	697	<b>2.21</b>
<i>C. michiganensis</i> subsp. <i>sepedonicus</i>	?	Potato	498	PD37	<b>3.35</b>
<i>C. michiganensis</i> subsp. <i>sepedonicus</i>	Canada	Potato	924	PD58	<b>4.15</b>
<i>C. michiganensis</i> subsp. <i>sepedonicus</i>	Canada	Potato	929	PD56	<b>4.23</b>
<i>C. michiganensis</i> subsp. <i>sepedonicus</i>	USA	Potato	930	PD323	<b>4.17</b>
<i>C. michiganensis</i> subsp. <i>sepedonicus</i>	Ukraine	Potato	1829	NCPPB3891	<b>4.09</b>
<i>C. michiganensis</i> subsp. <i>sepedonicus</i>	Canada	Potato	1830	NCPPB2140	<b>4.38</b>
<i>C. michiganensis</i> subsp. <i>sepedonicus</i>	Sweden	Potato	1831	NCPPB4053	<b>4.78</b>
<i>C. michiganensis</i> subsp. <i>sepedonicus</i>	Denmark	Potato	1832	DIAS SP204	<b>4.19</b>
<i>C. michiganensis</i> subsp. <i>insidiosus</i>	USA	Soy bean	533	NCPPB1110	0.67
<i>C. michiganensis</i> subsp. <i>insidiosus</i>	UK	Soy bean	534	NCPPB1643	0.66
<i>C. michiganensis</i> subsp. <i>michiganensis</i>	Italy	Tomato	542	NCPPB1064	0.66
<i>C. michiganensis</i> subsp. <i>michiganensis</i>	Hungary	Tomato	544	NCPPB1574	0.38
<i>C. michiganensis</i> subsp. <i>michiganensis</i>	Netherlands	Tomato	630		0.43
<i>C. michiganensis</i> subsp. <i>tritici</i>	India	<i>Triticum aestivum</i>	33	NCPPB471	0.54
<i>C. michiganensis</i> subsp. <i>tritici</i>	Spain	?	1690	IVIA158-2	0.31
<i>Curtobacterium flaccumfaciens</i> subsp. <i>aurantiacum</i>	USA	Bean	550	NCPPB2343	0.61
<i>C. flaccumfaciens</i> subsp. <i>betae</i>	Netherlands	?	36		0.27
<i>C. flaccumfaciens</i> subsp. <i>betae</i>	UK	Beet	37	NCPPB374	0.88
<i>C. flaccumfaciens</i> subsp. <i>flaccumfaciens</i>	?	Bean	237	NCPPB559	0.38
<i>C. flaccumfaciens</i> subsp. <i>flaccumfaciens</i>	?	Bean	238	PD699	0.16
<i>C. flaccumfaciens</i> subsp. <i>flaccumfaciens</i>	Germany	Bean	546	NCPPB567	0.54
<i>C. flaccumfaciens</i> subsp. <i>flaccumfaciens</i>	China	Bean	1628		0.75
<i>C. flaccumfaciens</i> subsp. <i>oortii</i>	Netherlands	?	34		0.33
<i>Rhodococcus fascians</i>	?	Pelargonium	400		0.61
<i>R. fascians</i>	Netherlands	Lily	1611	PD300	0.58
<i>Rathayibacter rathayi</i>	?	?	1882	NCPPB2980	0.27
<i>R. rathayi</i>	?	?	1833	NCPPB3552	0.13
<i>R. iranicus</i>	?	?	1885	NCPPB2253	0.23
<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	?	?	1883	NCPPB2581	0.43
<i>C. michiganensis</i> subsp. <i>nebraskensis</i>	?	?	1884	NCPPB2579	0.34
<i>C. michiganensis</i> subsp. <i>tesselarius</i>	?	?	1886	NCPPB3664	0.45
<i>C. michiganensis</i> subsp. <i>tesselarius</i>	?	?	1887	NCPPB3666	0.42
<i>C. toxicus</i>	?	?	1888	NCPPB3552	0.09
<i>C. toxicus</i>	?	?	1834	NCPPB3808	0.20
<i>C. xyli</i> subsp. <i>cynodontis</i>	?	?	1835	CXC7	0.23
<i>C. xyli</i> subsp. <i>cynodontis</i>	?	?	1836	CXC18	0.30
Negative 1					0.61
Negative 2					0.51
Negative 3					0.69

\*Strains were from the Dutch collection of Plant Pathogenic Bacteria (PD, Plant Protection Service, Wageningen, the Netherlands), the National Collection of Plant Pathogenic Bacteria (NCPPB, Central Science Laboratory, York, UK), from the collection at the Instituto Valenciano de Investigaciones Agrarias (IVIA, Valencia, Spain), from the Danish Institute of Agricultural Sciences (DIAS, Slagelse, Denmark) and from the collection present at Plant Research International (PRI, Wageningen, the Netherlands).

†Positive Rn-values are given in bold and were proven significantly positive by Student *t*-test ( $P = 0.01$ ). Per reaction 30–300 pg of RNA was tested, equivalent to about  $3 \cdot 10^6$ – $3 \cdot 10^7$  copies of RNA.



**Fig. 1** Alignment of 16S rRNA sequences of *Clavibacter michiganensis* subsp. *sepedonicus* (Cms) and of *C. michiganensis* subsp. *tessellarius* (Cmt), *C. michiganensis* subsp. *michiganensis* (Cmm), *C. michiganensis* subsp. *nebraskensis* (Cmn) and *C. michiganensis* subsp. *insidiosus* (Cmi). Sequences of primers and molecular beacons (Mbcms1 and Mbcms2) are marked with boxes. The primer combinations X1f/X1r, X1f/X2r, X3f/X3r, A1f/Ar and A2f/Ar were used in comparative tests. The shaded areas represent differences in sequence of a molecular beacon between the target (Cms) and related sequences

the Netherlands). The filter was washed with 1 ml 0.3% (w/v) glycine to suspend the bacteria.

For preparation of extracts from eggplants, small sections of stems and leaves, weighing approximately 2 g, were homogenized in 8 ml 0.01 mol l<sup>-1</sup> PBS in a strong plastic bag using

a hammer. The supernatant fluid was used for further experiments. For preparation of the artificially-contaminated plant extracts, 900 µl of the plant extract were mixed with 100 µl bacterial suspension in demineralized water and handled as for the artificially-contaminated potato tuber extracts.

## RNA extraction

Variants on five methods for total RNA extraction were compared.

1. The extraction method described by Verreault *et al.* (1988) was modified as follows. The pronase was replaced by proteinase K. The lysis of the bacterial cells was followed by a phenol extraction and an ethanol precipitation at  $-20\text{ }^{\circ}\text{C}$  for 1 h (Maniatis *et al.* 1989).

2. A simplified version of the RNA isolation method according to Verreault *et al.* (1988). In this method, bacteria were suspended in 0.3% (w/v) glycine and concentrated by centrifugation for 10 min at 10 000 *g*. The pellet was resuspended in 30  $\mu\text{l}$  TSE (10 mmol  $\text{l}^{-1}$  Tris/HCl, 10 mmol  $\text{l}^{-1}$  NaCl and 0.8 mmol  $\text{l}^{-1}$  EDTA, pH 7.5) and 30  $\mu\text{l}$  lysozyme (Sigma, 10 mg  $\text{ml}^{-1}$ ) and incubated for 1 h at  $37\text{ }^{\circ}\text{C}$ . Then, 10  $\mu\text{l}$  10% (w/v) SDS and 30  $\mu\text{l}$  proteinase K (Merck Darmstadt, 10 mg  $\text{ml}^{-1}$ ) were added, mixed gently and incubated for at least 2 h at  $37\text{ }^{\circ}\text{C}$ . The RNA was extracted with phenol and chloroform/isoamyl alcohol, and precipitated for 1 h in ethanol at  $-20\text{ }^{\circ}\text{C}$ .

3. A DNA isolation kit for Gram-positive bacteria (Puregene DNA isolation kit, Biozym, Landgraaf, the Netherlands) was used in which the RNase incubation was omitted.

4. The protocol of the RNeasy Minikit (Qiagen, Westburg Leusden, the Netherlands) was adapted as follows. The samples were centrifuged for 10 min at 10 000 *g*, and the pellet was resuspended in 30  $\mu\text{l}$  lysozyme (Sigma, 10 mg  $\text{ml}^{-1}$ ) and 70  $\mu\text{l}$  TE (1 mmol  $\text{l}^{-1}$  EDTA, 10 mmol  $\text{l}^{-1}$  Tris/HCl pH 7.6). This solution was heated for 1 h at  $37\text{ }^{\circ}\text{C}$ . After this step, the standard protocol of the kit was followed.

5. A modification of a method described by Boom *et al.* (1990) was also tested. Samples were centrifuged for 10 min at 10 000 *g*. The pellet was resuspended in 50  $\mu\text{l}$  TE and mixed with 900  $\mu\text{l}$  of the lysis buffer of the Nuclisens RNA isolation kit (Organon Teknika, Boxtel, the Netherlands) and 250 mg glass beads (B. Braun Biotech International, Beesd, the Netherlands; 0.10–0.11 mm). Samples were beaten three times for 15 s with 30 s intervals. They were subsequently mixed with 50  $\mu\text{l}$  silica (Nuclisens RNA isolation kit) and handled according to the Nuclisens protocol.

## NASBA

NASBA was performed according to the protocol described by Leone *et al.* (1998). Real-time measurements with molecular beacons were made with an ABI Prism<sup>TM</sup> 7700 Sequence detector (PE Applied Biosystems, Branchburg, NJ, USA) or a Fluorskan Ascent FL (Labsystems, Breda, the Netherlands). In the Fluorskan, the tubes were placed in a custom-made, 14 mm-thick aluminium tray (Biozym, Landgraaf, the Netherlands) with 96 wells for even heating

of the tubes. Molecular beacon (9 ng per reaction) was added to the mixture. In the ABI Prism, the fluorescence of a sample was measured relative to ROX (1 pmol  $\mu\text{l}^{-1}$ ) (relative fluorescence  $R_n = \text{fluorescence target}/\text{fluorescence ROX}$ ). For measurements within the Fluorskan, no reference fluorophore was added to the samples.

The real-time curves were normalized by subtracting the fluorescence or the relative fluorescence, depending on which fluorimeter was used, of the molecular beacon at the start of the reaction ( $F_{I_0}$ ) from the (relative) fluorescence at the end of a NASBA reaction ( $F_{I_e}$ ). The final value ( $F_{I_n}$ ) was termed the normalized (relative) fluorescence:  $F_{I_n} = F_{I_e} - F_{I_0}$ .

## Post-NASBA analysis

The analysis of the NASBA amplicons by Northern blotting was performed as described by Leone *et al.* (1998).

The NASBA results of the real-time measurements were analysed using the endpoint value of the molecular beacon in the Student *t*-test. A reaction was considered positive when it exceeded the threshold value *V* as calculated by the following formulae: (1) *V* is twice the normalized (relative) fluorescence of the blank, or (2)  $V = A + (\delta A \times T)$ , in which *A* is the average of the blanks,  $\delta A$  the standard deviation and *T* the Student *t*-value at  $P = 0.95$  (two-sided, degrees of freedom  $n-1$ , in which *n* is the number of replicates of the blank).

## Viability assessment

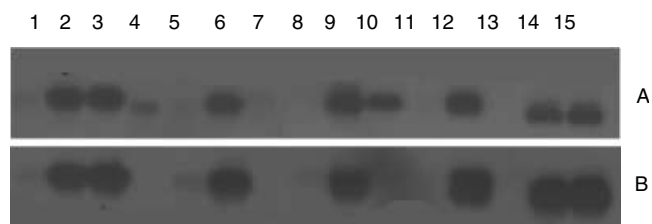
AmpliDet RNA was tested for reactions with heat-killed cells, which were treated for 30 min at  $80\text{ }^{\circ}\text{C}$  in quarter strength Ringers solution. Heat-killed cells were used immediately for RNA extraction, or incubated overnight at room temperature prior to RNA extraction, to allow further RNA degradation.

To assess whether AmpliDet RNA exclusively amplified RNA and not DNA sequences, total RNA extracts contaminated with high molecular weight DNA were treated with 10 U of RNase (Roche Diagnostics) or DNase (Roche Diagnostics) for 30 min at  $37\text{ }^{\circ}\text{C}$  for removal of DNase or RNase. All samples were tested in AmpliDet RNA. Samples were also analysed on a 1% agarose gel stained with  $0.5\text{ }\mu\text{g ml}^{-1}$  ethidium bromide (Sigma-Aldrich).

## RESULTS

### Selection of primers and probes

Five different primer pairs were tested in combination with the biotin-labelled probes, CMS1 and CMS2, in NASBA followed by Northern blotting. All combinations resulted in positive signals on Northern blots, but the limits of



**Fig. 2** Northern blot of NASBA amplicons of *Clavibacter michiganensis* subsp. *sepedonicus*, generated with the primer combinations A2f/Ar (lanes 1, 2 and 3), A1f/Ar (lanes 4, 5 and 6), X3f/X3r (lanes 7, 8, and 9), X1f/X2r (lanes 10, 11 and 12) and X1f/X1r (lanes 13, 14 and 15) and two different biotinylated probes. The primers and probes were tested with  $10^9$  cells  $\text{ml}^{-1}$  Cms (lanes 3, 6, 9, 12 and 15),  $10^5$  cells  $\text{ml}^{-1}$  Cms (lanes 2, 5, 8, 11 and 14) and no Cms present (lanes 1, 4, 7, 10 and 13). The upper panel (A) presents the results obtained with probe cms1, the lower panel (B) the results obtained with probe cms2

detection with the combinations X1f/X1r and A2f/Ar were much lower than those with the primer pairs X1f/X2r, X3f/X3r or A1f/Ar (Fig. 2).

Molecular beacons MBcms1 and MBcms2 were directed to the same target sequences as the biotin-labelled probes CMS1 and CMS2. MBcms2 did not react with any NASBA amplicon (result not shown). MBcms1 reacted with the NASBA products from all primer pairs similarly to the biotin-labelled probe. The use of primer pairs X1f/X1r and

A2f/Ar resulted in the highest normalized fluorescence values (Fig. 3). Primer pair A2f/Ar was selected for further studies.

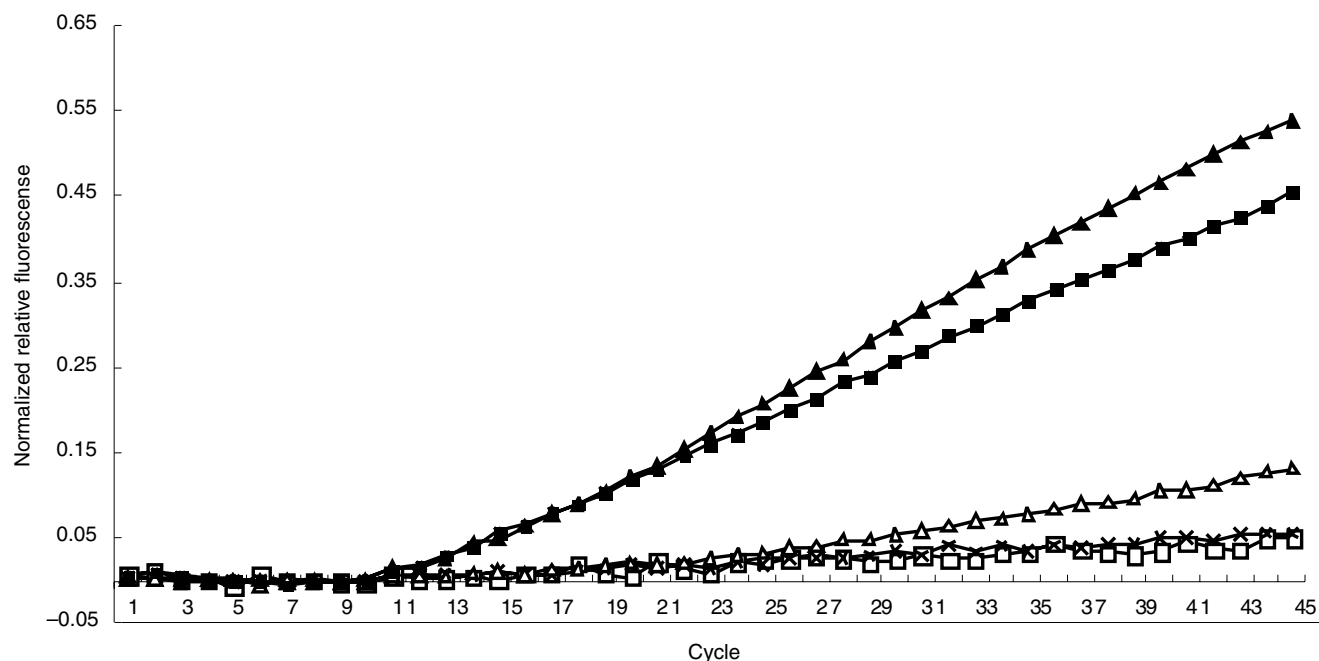
### RNA extraction

Efficiency of RNA extraction from a pure culture of Cms at concentrations of  $10^7$ ,  $10^5$ ,  $10^3$  and  $10^1$  cells  $\text{ml}^{-1}$  in demineralized water was compared by five different methods. The RNA extraction method of Verreault, the modified version of the Verreault isolation method and the RNeasy Minikit, enabled detection of at least  $10$  cells  $\text{ml}^{-1}$  (Table 2). The Nuclisens and Biozym kits resulted in higher limits of detection and were therefore considered less suitable for isolation of RNA from Cms.

### Detection level of AmpliDet RNA

Total RNA of three different Cms strains was isolated and purified by the modified Verreault method. The detection level of the AmpliDet RNA was determined by testing a 10-fold serial dilution series of RNA in a range of  $10^7$ – $10^3$  molecules of RNA per reaction. An amount of  $10^4$  molecules RNA was consistently detected (Table 3).

The sensitivity of the AmpliDet RNA was assessed for detection of Cms in demineralized water, potato tuber extract, surface water and eggplant extract. RNA was



**Fig. 3** Real-time measurement of NASBA amplicons generated with five different primer pairs and detected with molecular beacon MBcms1. A suspension of  $10^5$  cells  $\text{ml}^{-1}$  of *Clavibacter michiganensis* subsp. *sepedonicus* in demineralized water was used. Primer pairs used: X1f/X1r (▲), X2f/X1r (×), X3f/X3r (□), A1f/Ar (△) and A2f/Ar (■)

**Table 2** Comparison of five different RNA extraction procedures for pure cultures of *Clavibacter michiganensis* subsp. *sepedonicus*\*

Cells ml <sup>-1</sup>	Verreault	Verreault modified	Nuclisens	Biozym	Rneasy
10 <sup>7</sup>	11·20†	10·75	9·27	10·29	10·45
10 <sup>5</sup>	10·18	9·91	3·95	4·24	8·67
10 <sup>3</sup>	7·27	7·47	2·21	1·94	7·45
10 <sup>1</sup>	2·16	3·59	0·82	0·87	5·32

\*Extraction procedures are described in the materials and methods section.

†Values are given as the mean ratio between end point measurements of the normalized relative fluorescence (FI<sub>n</sub>) and the blank (water without target bacteria). Positive values are written in bold and were shown to be positive by a Student's *t*-test ( $P = 0·95$ ,  $n = 3$ ). Mean of the blank is 0·82.

**Table 3** Detection levels of AmpliDet RNA for *Clavibacter michiganensis* subsp. *sepedonicus* (Cms) in different substrates

Concentration*	RNA	Pure culture	Potato tuber extract	Surface water	Eggplant extract
10 <sup>5</sup>	3·52†	nd	nd	nd	nd
10 <sup>4</sup>	2·17	nd	nd	nd	nd
10 <sup>3</sup>	1·57	nd	nd	4·60	7·74
10 <sup>2</sup>	nd	4·36	4·05	3·77	3·21
10 <sup>1</sup>	nd	2·56	1·95	1·96	1·50
10 <sup>0</sup>	nd	1·46	1·30	1·26	1·13

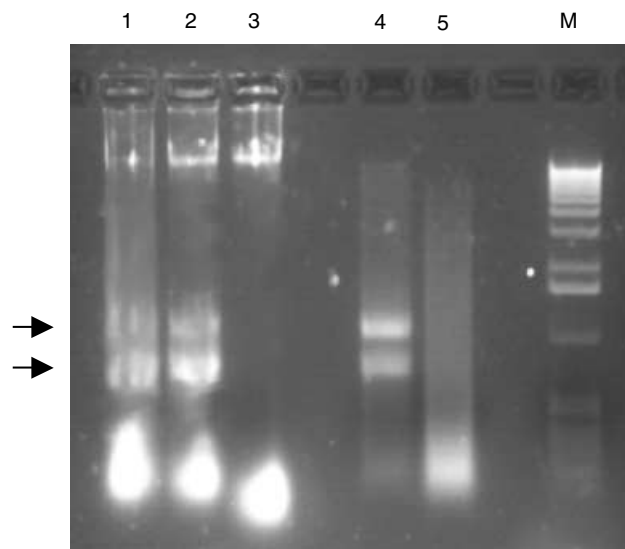
\*For purified total RNA, the concentration is given in molecules per reaction. For pure cultures of Cms and spiked samples, the number of cfu per reaction is given, as determined by plating on YGM.

†Values are given as the mean ratio between the normalized relative fluorescence (FI<sub>n</sub>) of a certain sample and the blank (water or an extract without added Cms). Positive ratios are written in bold and were shown to be positive by Student *t*-test ( $P = 0·95$ ,  $n = 3$ ). nd = not determined. Mean of the blank is 1·08.

isolated and purified by the modified Verreault extraction method. The number of colony-forming units (cfu) added to the diluents was determined by plating on YGM. In pure cultures, 10 cfu per reaction were detected. In potato tuber extract, surface water and eggplant extract the detection level was 100 cfu per reaction. This corresponds to 5000 cfu ml<sup>-1</sup> extract or 5000 cfu 200 ml<sup>-1</sup> surface water. The higher limit of detection in plant and potato tuber extracts could be due to inhibiting substances that were not removed from samples during RNA extraction.

### Specificity of AmpliDet RNA

To determine the specificity of AmpliDet RNA, 12 potato strains of Cms originating from different regions in the



**Fig. 4** Effect of treatment with nucleases and heat on total nucleic acid extracts from *Clavibacter michiganensis* subsp. *sepedonicus* analysed by gel electrophoresis. Treatments are indicated as (1) DNase treatment, (2) no treatment, (3) RNase treatment, (4) heat treatment and (5) heat treatment plus 24 h incubation at room temperature. M is a 100 bp marker. Arrows indicate 23S rRNA and 16S rRNA bands

northern hemisphere were tested together with 26 taxonomically-related strains, which included strains belonging to other subspecies within *Clavibacter michiganensis*. Significant positive reactions were obtained with all Cms strains, but with none of the non-target strains (Table 1).

### Viability assessment

AmpliDet RNA was designed to detect exclusively 16S rRNA and not 16S rDNA sequences. To confirm this, cells of Cms were heat killed and treated with RNase to destroy RNA. Heat-killed cells were also treated with RNase-free DNase, which destroys only DNA and should deliver positive reactions in NASBA. Total nucleic acids from treated samples were extracted by the modified Verreault method and analysed by agarose gel electrophoresis. No RNA was present in the RNase-treated sample or in the heat-killed cells that were incubated overnight at room temperature (Fig. 4). In the heat-killed cells extracted immediately after the heat treatment, RNA was still present. The DNase treatment did not sufficiently break down all high molecular DNA and a small amount of DNA was still visible on the gel. Samples were tested in AmpliDet RNA with primer pairs X1f/X1r and A2f/Ar using an equivalent of 10<sup>6</sup> molecules RNA per reaction. With primer pair X1f/X1r, all nuclease- and heat-treated samples gave a positive signal, although normalized fluorescence values of RNase-treated sample were lower than untreated samples (Table 4). In contrast,

**Table 4** AmpliDet RNA of total nucleic acid extracts from  $10^7$  cfu ml<sup>-1</sup> *Clavibacter michiganensis* subsp. *sepedonicus* after nuclease and heat treatment: two different primer pairs were used, X1f/X1r and A2f/Ar

Treatment	Nucleic acid expected to be present after treatment	Primer pair X1f/X1r	Primer pair A2f/Ar
DNase	RNA	<b>12·8*</b>	<b>5·4</b>
RNase	DNA	<b>6·1</b>	<b>1·2</b>
Heat	DNA and RNA	<b>12·8</b>	<b>4·3</b>
Heat and overnight room temperature incubation	DNA	<b>15·2</b>	<b>1·4</b>
No treatment	DNA and RNA	<b>12·5</b>	<b>5·1</b>
RNase treatment, additional RNA added after treatment	DNA and RNA	<b>11·4</b>	<b>5·0</b>

\*Values are given as the mean ratio between end point measurements of the normalized relative fluorescence ( $Fl_n$ ) and the blank (water without target RNA). Positive values are written in bold and were shown to be positive by a Student's *t*-test ( $P = 0.95$ ). Mean of the blank of the primer pair X1f/X1r is 0.81 and 1.30 for the primer pair A2f/Ar.

with primer pair A2f/Ar, RNase-treated samples and heat-treated samples incubated for 24 h at room temperature did not react in AmpliDet RNA.

## DISCUSSION

In this study, the potential of AmpliDet RNA for real-time detection of Cms, the causal organism of bacterial ring rot, was demonstrated. AmpliDet RNA combines NASBA amplification and real-time monitoring of RNA amplicons in a closed system by the use of a molecular beacon (Leone *et al.* 1998). Other methods for detection of NASBA amplicons, such as Northern blotting and the electrochemiluminescence technique, require opening of tubes after amplification, which carries a substantial risk of carry-over contamination.

The AmpliDet RNA, based on amplification of 16S rRNA sequences, was specific for Cms and did not detect other *C. michiganensis* subspecies (Table 1). The selection of specific sequences to design primers and probes is rather limited, as the dissimilarity in the 16S rRNA genes of *C. michiganensis* subsp. *michiganensis*, subsp. *insidiosus*, subsp. *nebraskensis* and subsp. *sepedonicus* was found to be less than 1%. A high homology at the sequence level was also found between *C. michiganensis* and the plant pathogenic *Clavibacter xyli* group (Li and De Boer 1995a), which has now been proposed for transfer to the genus *Leifsonia* as *Leifsonia xyli* (Evtushenko *et al.* 2000). Of the five selected primer pairs, only two gave sufficient amplification (Fig. 2). The selected NASBA forward primer X1f was directed against a highly conserved region, showing hardly any variation within the eubacteria. The reverse primer X1r was directed against a region showing a slight variation between *Clavibacter* species, but not at the *C. michiganensis* subspecies level. The molecular beacon therefore essentially

determined the specificity of the AmpliDet RNA. Two molecular beacons, MBcms1 and MBcms2, designed within the same sequence region as that formerly used for the design of an oligonucleotide probe, allowed specific detection of Cms in whole-cell *in situ* hybridization (Li *et al.* 1997). This region comprised two mismatches between Cms and the other *C. michiganensis* subspecies (Li *et al.* 1997). Both beacons showed the typical S-shaped melting curves in a temperature range between 30 and 75 °C, indicating that the beacons were well designed (results not shown). However, MBcms2 was unable to hybridize with the NASBA amplicons. This could be due to steric hindrance by the native amplicons as a biotinylated linear probe, targeting the same sequence as MBcms2, was able to react with denatured amplicons on Northern blots.

In previous studies, molecular beacons allowed recognition of even a single nucleotide difference in the target sequence, making them a powerful tool in diagnosis of pathogenic variants (Giesendorf *et al.* 1998; Marras *et al.* 1999). The specificity of the molecular beacon can be adjusted depending on the target. For example, if targets are genetically heterogeneous, the length of the target-specific site of the molecular beacons can be extended to allow for one or more mismatches (Vet *et al.* 1999).

The AmpliDet RNA for Cms was able to detect about  $10^4$  molecules of purified 16S rRNA per reaction, which is equivalent to what is minimally present in one viable cell (Olsen *et al.* 1986). However, for pure cultures of Cms, the limit of sensitivity of detection was 10 cfu per AmpliDet RNA reaction, probably due to loss of some rRNA during extraction of cells. When RNA was extracted from cells present in substrates such as potato tuber extract, surface water or eggplant extract, the sensitivity of detection was further decreased to 100 cfu per reaction. This corresponds to 5000 cfu ml<sup>-1</sup> eggplant or potato tuber extract, and 5000 cfu

200 ml<sup>-1</sup> surface water. Extracted RNA may be contaminated with compounds that can inhibit NASBA, or co-amplification of rRNA extracted from saprophytic bacteria may interfere with the reaction through competition for reagents, as the NASBA primers are not entirely specific for *Cms*.

A great variety of methods and commercial kits for extraction of RNA from bacteria is available, but not all of these methods can be used to extract RNA from Gram-positive bacteria efficiently because these bacteria possess a sturdy cell wall composed of peptidoglycan and teichoic acid. Boom *et al.* (1990) reported earlier that they were able to purify nucleic acids from Gram-negative but not from Gram-positive bacteria with their silica-based method. For the extraction of RNA from *Cms*, it appeared to be necessary to include a thorough mechanical and/or enzymatic lysis step in the extraction protocol. Of the five extraction methods compared, the Biozym and Nuclisens methods were rejected because of insufficient yield of RNA (Table 2). Probably, the cell walls of the bacterial cells were not lysed adequately to obtain acceptable amounts of RNA. The remaining methods include a more thorough lysis step, and resulted in low detection limits of 10 cells ml<sup>-1</sup>. Of these, the modified Verreault method is the least expensive, whereas the RNeasy Minikit is less cost-effective but was the most rapid and most user-friendly method tested. Moreover, the kit is also available in a 96-well format and is therefore suitable for large-scale extractions.

It has been shown in previous studies that NASBA based, in particular, on detection of mRNA sequences can be used to study viability of cells. Enzymes easily degrade mRNA molecules, resulting in a very short half-life in viable cells. Therefore, mRNA was a reliable target for detection of viable cells of *Salmonella enterica* (Simpkins *et al.* 2000). Van der Vliet *et al.* (1994) has shown that the NASBA signal derived from amplification of 16S rRNA sequences coincided with a loss in viability of mycobacteria after exposure to bactericidal antibiotics, whereas PCR based on 16S rDNA primers was not related to viability. In general, for both prokaryotic and eukaryotic organisms, a direct relationship has been found between rRNA amounts and the viability, metabolic activity or integrity of cells, due to a relatively short half-life of RNA (Thompson *et al.* 1987; Lamattina *et al.* 1988; Hahn *et al.* 1992).

Using primers A2f/Ar, AmpliDet RNA detected viable, but not dead cells, in which RNA had been degraded. These primers detected RNA exclusively, not DNA, and consequently, degradation of RNA in heat-killed cells stored for 24 h at room temperature resulted in negative reactions (Table 4). RNA, which was extracted immediately from heat-killed cells and still showed intact RNA bands on gel (Fig. 4), resulted in positive AmpliDet RNA signals.

In contrast, primers X1f/X1r did not distinguish dead from living cells. Reactions with heat-treated cells stored

overnight were significantly positive. RNase-treated extracts of total nucleic acids also resulted in positive reactions. Most likely, these primers also amplified 16S rDNA sequences, even without denaturation of the double-stranded DNA. It has been reported previously that homologous double-stranded plasmid DNA of pSP64-pol could be amplified in NASBA if present in a high copy number (Voisset *et al.* 2000). The single-stranded replicate intermediates of the plasmid appeared to be suitable templates for NASBA amplification.

AmpliDet RNA appears to be a sensitive, specific and robust method for detection of *Cms* in pure cultures as well as in more complex samples such as potato tuber extract, eggplant extract or surface water. The method may also be useful for sample analysis in high throughput, as well as routine scale, if the appropriate RNA extraction methods are used. Use of primers A2f/Ar allows specific detection of viable cells only, which makes AmpliDet RNA very useful in studies on the epidemiology of bacterial ring rot.

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