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Development of a simplified NASBA protocol for detecting viable cells of the citrus pathogen *Xanthomonas citri* subsp. *citri* under different treatments

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Nucleic acid sequence based amplification (NASBA) is a method of amplifying RNA, for the detection of RNA viruses and human pathogenic bacteria. Recently, NASBA has also been employed for the detection of plant diseases caused by viruses and quarantine bacteria. A major citrus pathogen, *Xanthomonas citri* subsp. *citri* (Xcc), causal agent of citrus bacterial canker, is being studied in depth due to its economic importance, with recent focus concentrating on its viability and survival under different stress conditions and control treatments. In this work, a NASBA protocol using primers for *gum*D mRNA has been developed to assess the viability of this pathogen under different bacteriocidal treatments. This method is rapid, specific and sensitive, and is able to detect viable bacterial cells, using a hybridization device which allows the visualization of the results in only 30 min. The usefulness of the method has been confirmed with bacterial suspensions subjected to different heat treatments and to sodium orthophenylphenate.

Keywords: citrus bacterial canker, *Citrus* spp., nucleic acid sequence based amplification, RNA hybridization, *Xanthomonas citri* subsp. *citri*

Introduction

Xanthomonas citri subsp. *citri* (Xcc) (Vauterin *et al.*, 1995; Schaad *et al.*, 2006) causes citrus bacterial canker (CBC), a severe quarantine disease of most citrus species and cultivars in many citrus production areas worldwide (Graham *et al.*, 2004). CBC is characterized by erumpent lesions on fruit, foliage and young stems of susceptible citrus cultivars (Civerolo, 1984; Timmer *et al.*, 1996; Schubert *et al.*, 2001; Graham *et al.*, 2004). Unfortunately, most commercial citrus species and varieties are moderately to highly susceptible to CBC. When the disease is severe, defoliation, dieback and fruit drop can occur and infected fruits that stay on are less valuable or entirely unmarketable (Graham & Gottwald, 1991). Wind-driven rain is the primary short- to medium-distance dispersal mechanism for CBC and long-distance dissemination is normally caused by transportation of diseased or exposed citrus plant material and fruits (Schubert *et al.*, 2001; Gottwald *et al.*, 2009). Economic losses are mainly due to the impossibility of marketing and exporting fruits with symptoms to CBC-free areas, forcing costly eradication programmes and expensive chemical treatments. The detection methods already in use have demonstrated their sensitivity and ability to detect the presence of the pathogen from canker lesions in fruit samples (Hartung *et al.*, 1993; Cubero & Graham, 2002; Coletta-Filho *et al.*, 2006; Golmohammadi *et al.*, 2007), but they are limited by the inability to distinguish between live and dead bacterial cells, which is important when dealing with the risk of contamination through plant material. Currently, CBC has not been reported in any citrus producing European countries nor in the Mediterranean area where Spain is the principal fresh fruit citrus producer, although many European countries import fresh fruits from areas where CBC is present. According to EU legislation (Anonymous, 2000), those fruits must be previously disinfected with compounds such as chlorine or sodium orthophenylphenate (SOPP). Limitations of these treatments have been verified by recent work which has revealed that bacterial populations decline in treated fruits, but sometimes not below infective levels (not less than 10^3 c.f.u. mL^{-1}, Verdier *et al.*, 2008). In addition, Xcc isolation from lesions observed on imported fresh fruits subjected to those post harvest compounds has been also reported

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(Golmohammadi *et al.*, 2007). This issue constitutes a strong concern about the possible risk of dissemination of CBC through contaminated fruit with or even without symptoms. Despite the results reported by several authors that state that such risk is very low (Gottwald *et al.*, 2009; Shiotani *et al.*, 2009) the risk still exists, as demonstrated by the recovery of bacteria from symptomless fruit naturally contaminated (Verdier *et al.*, 2008) and, above all, by confirmation of the spread of the disease from infected fruit to healthy material as observed by Gottwald *et al.* (2009).

To deal with the risk of introducing CBC in citrus producing areas where the pathogen is not present, procedures to eliminate the possible inoculum of Xcc in the plant material as well as detection methods addressed to specifically detect viable bacteria are needed. Bacterial isolation methods traditionally used are often slow and may lack sensitivity; moreover, only culturable cells can be detected. PCR-based analyses are highly sensitive, rapid and specific, although both live and dead cells may contribute to positive signals (Josephson *et al.*, 1993; Masters *et al.*, 1994), and DNA may persist in a detectable form long after all viable organisms have been killed (Masters *et al.*, 1994; Hellyer *et al.*, 1999a). For these reasons, RNA has been proposed as a more representative target for assessing bacterial viability (Bej *et al.*, 1991). Although rRNA has been employed in several works as a target for viability testing (McKillip *et al.*, 1998; Villarino *et al.*, 2000; Aellen *et al.*, 2006), the long half-life of rRNA species makes this target a less accurate indicator than messenger RNA (Tolker-Nielsen *et al.*, 1997; Rodriguez-Lázaro *et al.*, 2006). In contrast, mRNA has a short half-life within viable cells and is rapidly degraded by specific enzymes (RNases) which are themselves very stable even in environments outside the cell itself (Sela *et al.*, 1957). For all these reasons, mRNA has been suggested as a better cell viability marker (Bej *et al.*, 1996; Klein & Kuneja, 1997; del Mar Lleò *et al.*, 2000; Rodriguez-Lázaro *et al.*, 2006) and may provide a more accurate indication of the presence of viable bacteria.

The main techniques currently in use to amplify RNA sequences are reverse-transcription PCR (RT-PCR), nucleic acid sequence based amplification (NASBA) and reverse transcriptase-strand displacement amplification (RT-SDA) (Hellyer *et al.*, 1999b). RT-PCR is the most widely employed, whilst implementation of NASBA is progressing slowly in studies with bacterial plant pathogens. Few methods have been published for the detection of plant pathogens using this alternative technique, probably because long protocols or expensive systems to visualize the signal are required (Leone *et al.*, 1998; Bentsink *et al.*, 2002; Van Beckhoven *et al.*, 2002), although the coupling of NASBA with real time detection systems provides a useful option (Leone *et al.*, 1998; Van Beckhoven *et al.*, 2002), and recent developments have simplified the visualization step (Olmos *et al.*, 2007).

Simpkins *et al.* (2000) showed that NASBA can selectively amplify mRNA sequences in a background of genomic DNA, indicating that NASBA amplification of mRNA can be used to specifically detect viable cells. This technique amplifies only RNA, so no previous DNase treatments are needed; it is an isothermal reaction and hence does not require expensive equipment; the use of a hybridization device system (Hybrimax, Hybrio Limited) simplifies the time and reagents employed to develop the amplicons (Olmos *et al.*, 2007); and finally, it is more sensitive than RT-PCR (Birch *et al.*, 2001; Jean *et al.*, 2001).

This paper describes the use of the NASBA technique for the detection of living cells of Xcc using three primer sets designed from 16S rRNA, ITS and *gum*D gene sequences of strain Xcc 306. Primers based on ITS sequences were selected as possible targets for viability because of the potential instability of this RNA region due to RNA processing prior to ribosome formation. The *gum*D gene is involved in xanthan gum synthesis and is related to *Xanthomonas* epiphytic survival (Dunger *et al.*, 2007). In this work, bacterial suspensions were subjected to different heating temperatures and times and to high concentration (3%) of sodium orthophenylphenate (SOPP), primarily to assess the NASBA procedure for specific detection of viable bacteria and secondly to determine the effectiveness of these treatments for CBC control.

Materials and methods

Bacterial strains and growth conditions

Strain 306 of Xcc and other bacterial strains of *Xanthomonas, Pseudomonas* and *Pantoea* species from the Collection of Plant Pathogenic Bacteria of IVIA (Instituto Valenciano de Investigaciones Agrarias, Spain) were used in this study (Table 1). Bacterial strains were cultured routinely at 26°C on Luria-Bertani broth (LB, Oxoid). LB cultures were incubated for 48 h under shaking and bacterial concentrations were monitored by optical density at 600 nm (OD_{600} = 0·3 in LB medium), and adjusted to a concentration of 10^8 c.f.u. mL^{-1}.

Heating and chemical treatments of bacteria

Two methods were used to inactivate the bacteria: high temperature and sodium orthophenylphenate (SOPP) exposure. In order to determine the temperature needed to kill the bacteria, 10 mL of LB exponential phase cultures (10^8 c.f.u. mL^{-1}) were treated at 50°C for 15 min, 80°C for 10 min and 100°C for 10 min. For treatment with SOPP, 1·5, 2, 3 and 5% concentrations were assayed. The minimum concentration necessary to kill the bacteria was found to be 3%, and this concentration was used in the treatment as follows: 1 mL of the bacterial suspension at the same optical density was centrifuged at 10 000 ***g*** for 1 min and the pellet resuspended in 1 mL of 3% (v/v) SOPP. After 2 min the suspension was centrifuged and washed two times with sterile water to remove the product. After heat or SOPP treatments, the culturable condition was evaluated by colony

Table 1 List of bacterial strains used in this study

Species	Strain	Origin	Spot position in Fig. 2
Xanthomonas citri subsp. *citri*	306	NC_003919	(c+)[b]
Pseudomonas syringae pv. *syringae*	IVIA 2083-1	Spain	1
Pseudomonas syringae pv. *syringae*	IVIA 2083-8	Spain	2
Pseudomonas syringae pv. *syringae*	IVIA 3289-2	Spain	3
Pseudomonas syringae pv. *tomato*	IVIA 2469-1	Spain	4
Pseudomonas fluorescens	IVIA 374	Spain	5
Pantoea agglomerans	IVIA 560	Spain	6
Xanthomonas campestris pv. *campestris*	IVIA 2150-1	Spain	7
Xanthomonas sp.[a]	IVIA 3013	Uruguay	8
Xanthomonas sp.[a]	IVIA 3045-4	Uruguay	9
Xanthomonas sp.[a]	IVIA 3052-3	Argentina	10

[a]*Xanthomonas* isolated from citrus fruits, but not pathogenic.
[b]c+: positive control.

enumeration on LB agar medium, plating 100 μL of each suspension in triplicate and incubating for 48 h at 26°C. Viability was examined by a membrane integrity assay using the LIVE/DEAD® BacLight™ Bacterial Viability Kit (Molecular Probes) according to the manufacturer's instructions. Briefly, 100 μL of the treated bacterial suspension were mixed with 50 μL SYTO-9 and 50 μL propidium iodide, and incubated in the dark for 15 min. The viable and non-viable cells were immediately monitored under epifluorescence microscopy (Nikon ECLIPSE E800). Green and red cells were classified as live and dead, respectively (Boulos *et al.*, 1999).

To confirm that the presence of RNA could be exclusively attributed to viable bacteria, RNA extraction was performed prior to and after the treatments and used for NASBA analyses as described below. Isolation of RNA (with and without DNase treatment) was performed immediately after the treatment and after 1, 7 and 24 h. All experiments were performed twice.

Bacterial RNA was extracted using the RNeasy Mini kit (Qiagen Inc.) following manufacturer's instructions and using 9 mL of a bacterial suspension of 10^8 c.f.u. mL^{-1}. An aliquot of the RNA was subjected to DNase treatment using Turbo DNA-free kit (Ambion, Applied Biosystems), following manufacturer's instructions, to confirm that the NASBA results were not affected by the presence of the DNA. Concentration and yield of total RNA after DNase treatment were measured and verified by electrophoresis with ethidium bromide staining on a 1·2% agarose gel and the integrity and purity of every RNA sample was quality tested by measuring the A260/A280 ratio. RNA samples were used or stored at −80°C until required.

Primers and probes

Primer sets from ITS, 16S rRNA and g*um*D genes were designed using Vector NTI Advance 10 (Invitrogen-Life Technologies) from Xcc 306 nucleic acid sequences and synthesized by MWG Biotech AG (Table 2).

NASBA primers bearing the bacteriophage T7 RNA polymerase promoter binding and transcriptional initiation sequence at their 5′ end are indicated with the prefix T7 (Table 2). Digoxigenin (DIG)-labelled oligonucleo-

Table 2 Nucleotide sequences of oligonucleotide primers and probes used in this study. T7 RNA promoter sequence is in italics

Primer or probe	Sequence	Amplicon size (bp)
16S-F[a]	5′-GAGGAACATCCGTGGCGAAGG-3′	410
16S-R[a]	5′-GACAAGGGTTGCGCTCGTTGC-3′	
16S-F probe	5′-ACACTGACACTGAGGCACGAAAG-3′	
T7/16S-R	5′-*AATTCTAATACGACTCACTATAGG*GACAAGGGTTGCGCTCGTTGC-3′	
ITS-F	5′-GTGACGTAGCGAGCGTTTGA-3′	147
ITS-R	5′-CCAAGTTGCCTCGGAGCTATC-3′	
ITS-R probe	5′-CGCCTTAGCCTCAACGACACGT-3′	
T7/ITS-F	5′-*AATTCTAATACGACTCACTATAGG*GTGACGTAGCGAGCGTTTGA-3′	
gumD-F	5′-CGCTGCAGGACAAGATCC-3′	480
gumD-R	5′-TCGTACTGGATACGCTTCTTCATC-3′	
gumD-F probe	5′-GGCGCAGGTGAATGGTTT-3′	
T7/gumD-R	5′-*AATTCTAATACGACTCACTATAGG*TCGTACTGGATACGCTTCTTCATC-3′	

[a]F-R: forward and reverse primers, respectively.

tide probes internal to the nucleotide regions defined by the different primer pairs were also synthesized by MWG Biotech for the detection of amplified target nucleic acid sequences (Table 2).

The NASBA system

For the NASBA, the Nuclisens basic kit (BioMérieux) was used according to the manufacturer's instructions with some modifications. Briefly, 80 μL of diluent, 14 μL of molecular grade water, 16 μL of stock KCl (80 mM) and 5 μL of each required primer (5 pmoles) were added to the lyophilized reagent sphere. Subsequently, 2 μL of purified RNA template were added to 10 μL of the amplification solution and the tubes were incubated for 5 min at 65°C. Finally, 5 μL of enzyme solution were added and the reaction was carried out at 41°C for 120 min.

The NASBA product for each sample was dispensed onto a positively charged nylon membrane (Roche Diagnostics), dried at room temperature and cross-linked by UV for 4 min. To assess the drop volume on the nylon membrane best suited for the detection assay, a comparison was performed using three different volumes (1, 1·5 and 2 μL) of NASBA product. After evaluation of visible spots on the membrane, 1 μL was used for all future analyses. Prehybridization was performed at 50°C using a buffer containing 5× SSC (standard sodium citrate: 300 mM NaCl, 30 mM sodium citrate, pH 7·0); 0·1% (w/v) *N*-lauroyl-sarcosine; 0·002% (w/v) SDS (sodium dodecyl sulphate) and 1% blocking reagent (Roche Diagnostics). Hybridization was performed adding a 3-DIG labelled specific probe at 10 pmol μL^{-1} to prehybridization buffer at 50°C. Membranes were washed twice with 2× SSC supplemented with 0·1% SDS at 25°C. This step was repeated using 0·5× SSC supplemented with 0·1% SDS. Membranes were equilibrated with 100 mM maleic acid, 150 mM NaCl, 0·3% (v/v) Tween 20, pH 7·5 (Roche) and blocked with blocking buffer [100 mM maleic acid, 150 mM NaCl pH 7·5 and 1% (w/v) blocking reagent]. Antidigoxigenin-alkaline phosphatase antibodies (Roche Diagnostics) were subsequently added at 150 mU mL^{-1} to the blocking buffer, and membranes were washed twice as in the previous steps with 100 mM maleic acid, 150 mM NaCl, 0·3% (v/v) Tween 20, pH 7·5 and equilibrated for 2 min with 100 mM Tris/HCl, 100 mM NaCl, pH 9·5 (Sigma). The substrate used for colorimetric detection contained 315 mg mL^{-1} NBT and 175 mg mL^{-1} BCIP in 100 mM Tris/HCl, 100 mM NaCl, pH 9·5. Thorough washing with water stopped the reaction.

The hybridization was carried out using a Hybrimax device (Hybribio Limited) following manufacturer's instructions. This system is based on the principle of flow-through hybridization, in which the solutions employed are all flowed through the membrane automatically by vacuum pump. All steps require small volumes, just enough to cover the membrane (5 mL). Vacuum pressure reduces each hybridization step to 30–60 s, providing results in just 30 min. All the experiments were performed twice. One sample not subjected to any bactericide treatment (positive control) and one DNA extraction sample from Xcc 306 treated with RNase (negative control), or a water sample (negative control), were included in every NASBA analysis.

To estimate the sensitivity of the NASBA detection signal, the results were compared by colorimetric detection, with the signal obtained by using a chemiluminescent substrate (CSPD) (Roche Diagnostics). This method employs the same reagents and steps as the colorimetric system described previously, but uses CSPD instead of the colorimetric substrate (NBT plus BCIP), and the observation is performed in a fluorescence reader (LAS 3000 SYSTEM, Fuji) instead of by visual reading.

Primer and probe specificity tests

To evaluate the specificity of the probe for the target sequences a cross-reaction assay was performed using the designed primers and probes. The 3-DIG labelled probe designed for 16S rRNA gene sequence was used to hybridize samples amplified by NASBA with the *gumD* primers and the 3-DIG labelled probe designed for *gum*D gene sequence was used to hybridize samples amplified by NASBA with the 16S rRNA primers.

At the same time, in order to check the specificity of the NASBA assay, a subset of the most common *Citrus* epiphytic, saprophytic and pathogenic bacteria was analysed (Table 1) using the *gumD* primers, which provided the best results in viability assessment. Bacterial RNA was extracted and NASBA was performed as described previously.

Sensitivity test of NASBA analysis

The detection limit of NASBA was determined by amplifying samples from 10-fold serial dilutions of RNA obtained by *in vitro* transcription from a cloned DNA sample of *gumD* gene and 16S rDNA from Xcc strain 306. The procedure was as follows: PCR products from 16S rDNA and *gumD* genes obtained with primer sets 16S-F/16S-R and gumD-F/gumD-R, were cloned into plasmid vector pCR2-1 (Promega). The resulting plasmids were purified with the plasmid extraction kit (QIAGEN) and each target gene was transcribed *in vitro* to RNA by the Riboprobe® combination system–SP6/T7 (Promega Corporation) following manufacturer's instructions. After spectrophotometric quantification, 10-fold serial dilutions were performed and used in NASBA reactions. Two separate experiments were performed and every transcript dilution of defined RNA was tested in triplicate in each.

Results

Viability analysis of bacteria after treatments

Samples treated by heating at 80°C for 10 min, 100°C for 10 min and SOPP 3% (v/v) exposure, produced only

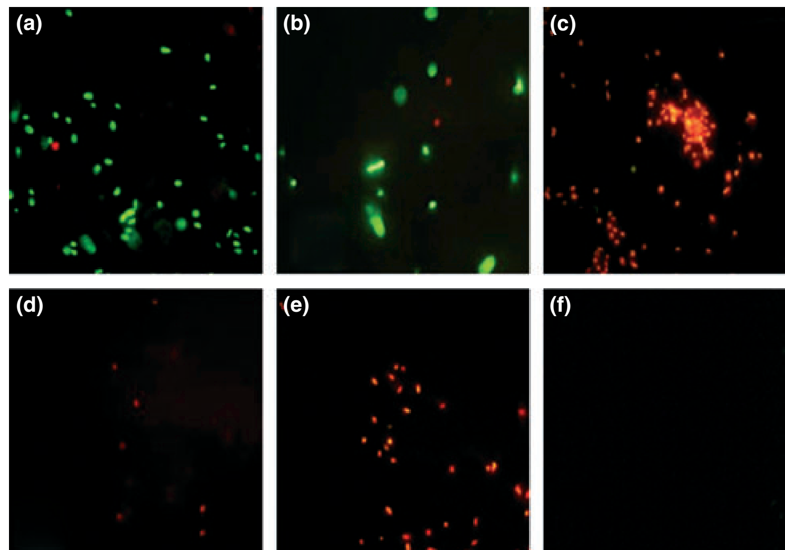

Figure 1 LIVE/DEAD® test performed on *Xanthomonas citri* subsp. *citri* strain 306 bacterial suspensions subjected to the following treatments: (a) control without treatment; (b) 50°C per 15 min; (c) 80°C per 10 min; (d) 100°C per 10 min; (e) Sodium orthophenylphenate 3%; (f) negative control (reaction mix). Viability was examined by a membrane integrity assay and monitored under epifluorescence microscopy. Green and red cells are classified as live and dead, respectively.

red fluorescence cells after staining with the LIVE/DEAD® kit, indicating that no viable cells were present after the treatments (Fig. 1). Green cells were observed after treatment at 50°C for 15 min, but no colonies were obtained in the culturability assays in all the repetitions performed.

Specificity tests

The specificity of the probes employed in the NASBA analyses was confirmed with the cross-reaction assay employing primers for the 16S rRNA gene with *gumD* probe and vice-versa. The results were negative in all samples, demonstrating the specificity of the probes for the target sequences.

Specificity of the NASBA was confirmed by analyses performed on 11 *Citrus* epiphytic, saprotrophic and pathogenic bacteria using primers from *gumD* gene (Table 1). A positive NASBA detection signal was observed only from RNA isolated from the *X. citri* subsp. *citri* strain, whereas RNAs isolated from other bacteria (including water sample) showed negative signals (Fig. 2).

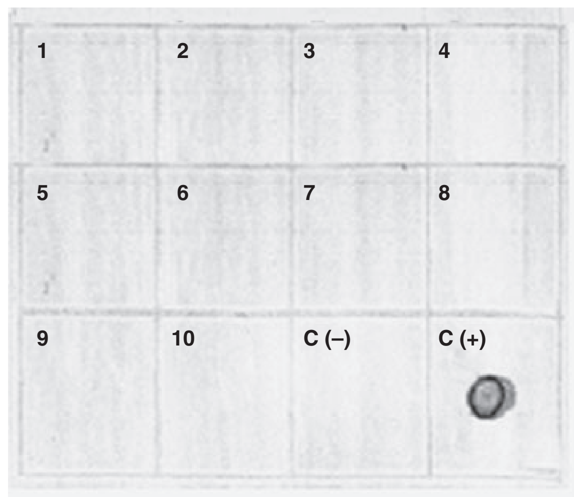

Figure 2 NASBA specificity analysis with several bacterial RNA samples (see Table 1). 1 to 10: saprophytic bacteria isolated from citrus fruits; C(–): negative control; C(+): positive control, *Xanthomonas citri* subsp. *citri* strain 306.

Sensitivity test

NASBA analysis showed a very high sensitivity, being able to detect up to 1 fg of an RNA sample using primers from the *gumD* gene on a RNA dilution series prepared *in vitro* from strain Xcc 306 (cloned fragment of the gene *gum*D and retrotranscription). By comparison, with RT-PCR the sensitivity dropped to 100 pg of RNA. Therefore, the sensitivity of the NASBA assay was three orders of magnitude higher than RT-PCR.

Detection of viable cells by NASBA assay in bacterial suspension after inactivation treatments

NASBA results with primer pairs designed from 16S rRNA and ITS sequences were positive for all treated samples, thus suggesting that the rRNA fragments selected were not good markers for viability analysis (Fig. 3a,b). Amplification with *gumD* primers was negative from samples treated to 80°C or higher for 10 min and 3% SOPP (Fig. 3c), where no viable cells were detected by the LIVE/DEAD® kit. NASBA results from

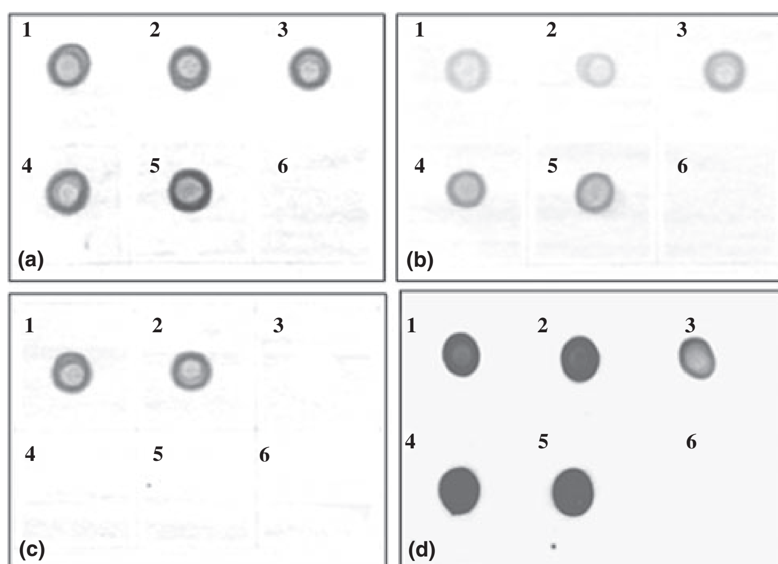

Figure 3 NASBA analysis of RNA samples amplified with primer (a) 16S, (b) ITS, (c) gumD isolated from *Xanthomonas citri* subsp. *citri* strain 306 bacterial suspensions subjected to the following treatments: 2) 50°C per 15 min; 3) 80°C per 10 min; 4) 100°C per 10 min; 5) SOPP 3%. Positive (no treatment) and negative (water sample) controls in boxes 1) and 6), respectively. (d) NASBA analysis of RNA samples amplified with primer 16S and detected by the chemiluminescent method.

samples that showed green fluorescent cells with the LIVE/DEAD® kit were always positive, thus indicating that the *gumD* gene can be a good marker for viability testing. NASBA analyses performed on RNA samples isolated immediately after treatment (t = 0) and after different times (t = 1, 7 and 24 h), showed the same bactericide effectiveness for all samples processed (data not shown).

To confirm that the origin of the NASBA signal was due only to RNA, a NASBA analysis was performed on the same RNA samples but subjected to DNase treatment, as described in the methods. No differences were observed between the two membranes after NASBA detection, thus confirming that DNA does not affect the NASBA technique as reported by Simpkins *et al.* (2000). The comparison between colorimetric and chemiluminescent detection provided similar results regarding the sensitivity and cleanliness of the signals (Fig. 3d).

Discussion

Xanthomonas citri subsp. *citri* is present in several countries of South America from which many EU countries import fresh fruits. An accurate detection method for this quarantine pathogen needs to be optimized for the analysis of samples both with and without symptoms, in particular for samples with no prior information regarding the possible bacterial inoculum present. Consequently, fast and sensitive methodologies for the detection of Xcc are required.

For this purpose, Golmohammadi and co-workers recently assayed several PCR protocols and showed that real time PCR was the most sensitive technique, especially when PCR products generated are detected by the use of TaqMan probes. They also showed the limitations of the different postharvest treatments already in use in citrus fruit exporting countries, thus raising a strong concern about the possible risk of dissemination of the disease (Golmohammadi *et al.*, 2007). Methods based on DNA amplification, however, fail when viable bacteria are specifically required to be detected, e.g. in order to evaluate the real risk posed by an infected sample for citrus bacterial canker spread, to assess the efficacy of bactericide treatments or to determine the survival of the bacteria in plant samples. The recent observation of the existence of the viable but non culturable (VBNC) state in Xcc and its further resuscitation adds more complexity to the estimation of the risk of dissemination of the disease from contaminated material (Golmohammadi, 2009).

The NASBA technique is one of the most promising systems employed for RNA detection of several bacteria and viruses for clinical purposes or environmental studies, but it has not been extensively applied to bacterial plant diseases (Bentsink *et al.*, 2002; Van Beckhoven *et al.*, 2002). The ability to detect viable cells with several primer sets has been evaluated in this work, and NASBA analysis using 16S and ITS primers was positive for all heat-treated and SOPP samples, indicating the failure to discriminate between living and dead cells of these RNA regions, a result confirmed in previous work employing the same RNA regions (Rodriguez-Lázaro *et al.*, 2006), and contrary to that described by other authors (Hellyer *et al.*, 1999a,b; Aellen *et al.*, 2006). Differences among those results could be due to specific features of the fragments targeted such as the RNA stability due to secondary structures or rRNA protein interactions.

mRNA amplification using *gumD* primers was positive with RNA isolated from Xcc control sample (no treat-

ment), and after heat treatment at 50°C, but negative after 80°C, 100°C and 3% SOPP exposure, demonstrating the usefulness of these primers as viability indicators. Culturability and viability of the treated bacteria were evaluated in parallel by plate colony counts, and membrane integrity assay using the LIVE/DEAD® kit, which showed that only red cells (non viable) were present after treatments at 100°C, 80°C and 3% SOPP. Green cells were observed at 50°C, but no colonies were obtained in the plating assays, indicating that this treatment had affected the culturability, inducing the cells to a viable but non culturable state or just being a preliminary step before cell death. These results showed a good correspondence between NASBA and membrane integrity analyses. In samples treated with 3% SOPP and at 100°C or 80°C, viable cells were not observed by membrane integrity assay, and amplification by NASBA using sequences of *gumD* gene were not successful, indicating a total degradation of the RNA target. This assay that indicates directly the viability status of the bacteria, along with the results obtained with the *gum*D gene, suggests that it is a suitable marker for detection of viable cells of Xcc.

The NASBA was three fold more sensitive than a real time RT-PCR performed with the same dilutions and samples, able to detect up to 1 fg of sample, compared to 100 pg with the real time RT-PCR. This difference suggests that the NASBA assay is a robust and reliable method even when low detection limits are required, an important advantage over the RT-PCR technique, because evaluation of a viable bacterial population in a plant sample requires the highest sensitivity. The specificity of the NASBA was improved by using a probe that binds to the internal sequence amplified by the primers at a higher temperature than in the NASBA amplification (50º instead of 41°C), thus adding an increase in stringency, and hence, in specificity. This was confirmed by analysing other bacterial pathogens, epiphytes and saprophytes. Despite the high sensitivity of the NASBA technique, a good specificity resulting in the positive detection of only the RNA isolated from Xcc samples was achieved.

The use of the Hybrimax device for amplified product detection was very convenient because it allowed the use of few reagents and short time periods for the detection of viable bacteria. Target sequences were detected effectively, both quickly and specifically, reducing the detection steps from a laborious, expensive, and time-consuming task (Bentsink *et al.*, 2002) to a faster and easier way to perform the analysis. Other visualization systems take advantage of a real-time approach using molecular beacons in the reaction mix and subsequent detection by fluorescence (Leone *et al.*, 1998; Van Beckhoven *et al.*, 2002). This system, called AmpliDet, shows the advantage of monitoring the analyses in unopened tubes, decreasing the risk of contamination, is sensitive and robust, and could be used for quantification. Some drawbacks of this alternative detection system are the need for expensive equipment (real time machine or fluorescence reader), the possibility of interference of the probe in the NASBA reaction, the need for an accurate design of more expensive primers and probe due to the need for more strict purification (HPLC) for more consistent results, and the difficulty in optimizing assay conditions (Gracias & McKillip, 2007). In this case, a simple system was intended that could be used in routine laboratories providing a good specificity and very high sensitivity. The design of the probe does not affect the reaction because no interference is shown with this system, thus the optimization of conditions is straightforward. Additionally, the colorimetric detection of the nucleic acid was compared with a chemiluminescence system, using the CSPD substrate and reading the membranes with a LAS-3000 system. Results were similar with both systems, with the advantage that chemiluminescence can also be quantitative, measuring the intensity of the signal.

This study has demonstrated that NASBA is a rapid, specific and sensitive technique able to discriminate between living and dead bacterial cells. The system developed is able to study the viability of Xcc using primers from the gene *gumD*, revealing a good correlation with other techniques to monitor the effect of several treatments on bacterial survival. In addition, the NASBA procedure can be used with naturally infected plant material. Several studies have analysed RNA extracted from spiked plant material or naturally contaminated material with the pathogen of interest (bacteria, Van Beckhoven *et al.*, 2002; Van der Wolf *et al.*, 2004; viruses, Leone *et al.*, 1998; Vasková *et al.*, 2004), obtaining good results. The extraction of RNA from plant material introduces some difficulties in the assays in terms of the removal of inhibitors, but these can be ameliorated by the use of commercial reagents and protocols. The efficiency of these protocols has enabled reliable detection of the pathogen in total RNA extracts from naturally infected samples at a level of 10 cells per reaction, equivalent to *ca.* 10^4 cells mL^{-1} (Van der Wolf *et al.*, 2004). Thus, viability testing in plant material like citrus (leaves, fruit, bark, etc.) becomes a real and valuable possibility in field experiments.

A highly sensitive technique like NASBA is necessary to accurately assess the survival, viability and risk of spread of Xcc in field conditions. The low numbers of bacteria able to spread from contaminated material and initiate new infestations, the intrinsic difficulties for RNA isolation from plant material, and the frequent high percentage of amplification failure due to RNA degradation or reaction inhibition suggests that this highly specific and sensitive NASBA protocol may turn into a useful tool for survival studies as well as be included in diagnostic protocols for specific detection of viable cells of Xcc in fresh citrus fruits and plant material.

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References

Aellen S, Que YA, Guignard B, Haenni M, Moreillon P, 2006. Detection of live and antibiotic killed bacteria by quantitative real-time PCR of specific fragments of rRNA. *Antimicrobial Agents and Chemotherapy* **50**, 1913–20.

Anonymous, 2000. Council Directive 2000/29/EC on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community. *Official Journal of the European Union L* **169**, 1–159.

Bej AK, Mahububani MH, Atlas RM, 1991. Detection of viable *Legionella pneumophila* in water by polymerase chain reaction and gene probe methods. *Applied and Environmental Microbiology* **57**, 597–600.

Bej AK, Ng WY, Morgan S, Jones D, Mahububani MH, 1996. Detection of viable *Vibrio cholerae* by reverse transcriptase polymerase chain reaction. *Molecular Biotechnology* **5**, 1–10.

Bentsink L, Leone GOM, van Beckhoven JRCM, van Schijndel HB, van Gemen B, 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**, 647–55.

Birch K, Dawson CE, Cornett JH, Keer JT, 2001. A comparison of nucleic acid amplification techniques for the assessment of bacterial viability. *Letters in Applied Microbiology* **33**, 296–301.

Boulos L, Prevost M, Barbeau B, Coallier J, Desjardins R, 1999. Live/Dead BacLight application of a new rapid staining method for direct enumeration of viable and total bacteria in drinking water. *Journal of Microbiological Methods* **37**, 77–86.

Civerolo E, 1984. Bacterial canker disease of citrus. *Journal of the Rio Grande Valley Horticultural Society* **37**, 127–45.

Coletta-Filho HD, Takita MA, Souza AA *et al*., 2006. Primers based on the *rpf* gene region provide improved detection of *Xanthomonas axonopodis* pv. *citri* in naturally and artificially infected citrus plants. *Journal of Applied Microbiology* **100**, 279–85.

Cubero J, Graham JH, 2002. Genetic relationship among worldwide strains of *Xanthomonas* causing canker in citrus species and design of new primers for their identification by PCR. *Applied and Environmental Microbiology* **68**, 1257–64.

Dunger G, Relling VM, Tondo L *et al*., 2007. Xanthan is not essential for pathogenicity in citrus canker but contributes to *Xanthomonas* epiphytic survival. *Archives in Microbiology* **188**, 127–35.

Golmohammadi M, 2009. Xanthomonas citri *subsp.* citri, *Causal Agent of Citrus Bacterial Canker: Isolation and Molecular Detection Methods, Evaluation of Survival in Fruits*. Valencia, Spain: Polytechnic University of Valencia, PhD thesis.

Golmohammadi M, Cubero J, Peñalver J, Quesada JM, López MM, Llop P, 2007. Diagnosis of *Xanthomonas axonopodis* pv. *citri*, causal agent of citrus canker, in commercial fruits by isolation and PCR-based methods. *Journal of Applied Microbiology* **103**, 2309–15.

Gottwald T, Graham JH, Bock C *et al*., 2009. The epidemiological significance of post-packinghouse survival of *Xanthomonas citri* subsp. *citri* for dissemination of Asiatic citrus canker via infected fruit. *Crop Protection* **28**, 508–24.

Gracias SK, McKillip JL, 2007. Nucleic acid-based amplification (NASBA) in molecular bacteriology: a procedural guide. *Journal of Rapid Methods and Automation in Microbiology* **15**, 295–309.

Graham JH, Gottwald TR, 1991. Research perspectives on eradication of citrus bacterial canker diseases in Florida. *Plant Disease* **75**, 1193–200.

Graham JH, Gottwald TR, Cubero J, Achor DS, 2004. *Xanthomonas axonopodis* pv. *citri*: factors affecting successful eradication of citrus canker. *Molecular Plant Pathology* **5**, 1–15.

Hartung JS, Daniel JF, Pruvost OP, 1993. Detection of *Xanthomonas campestris* pv. *citri* by the polymerase chain reaction method. *Applied and Environmental Microbiology* **59**, 1143–8.

Hellyer TJ, DesJardin LE, Hehman GL, Cave MD, Eisenach KD, 1999a. Quantitative analysis of mRNA as a marker for viability of *Mycobacterium tuberculosis*. *Journal of Clinical Microbiology* **37**, 290–5.

Hellyer TJ, DesJardin LE, Teixeira L, Perkins MD, Cave MD, Eisenach KD, 1999b. Detection of viable *Mycobacterium tuberculosis* by reverse transcriptase-strand displacement amplification of mRNA. *Journal of Clinical Microbiology* **37**, 518–23.

Jean J, Burton Blais B, Darveau A, Fliss I, 2001. Detection of hepatitis A virus by the nucleic acid sequence-based amplification technique and comparison with reverse transcription-PCR. *Applied and Environmental Microbiology* **67**, 5593–600.

Josephson KL, Gerb CP, Pepper IL, 1993. Polymerase chain reaction detection of nonviable bacterial pathogens. *Applied and Environmental Microbiology* **59**, 3513–5.

Klein PG, Kuneja VJ, 1997. Sensitive detection of viable *Listeria monocytogenes* by reverse transcription-PCR. *Applied and Environmental Microbiology* **63**, 4441–8.

Leone G, Van Schijndel H, Van Gemen B, Kramer FR, Schoen CD, 1998. Molecular beacon probes combined with amplification by NASBA enable homogeneous, real-time detection of RNA. *Nucleic Acids Research* **26**, 2150–5.

del Mar Lleò M, Pierobon S, Tafi MC, Signoretto C, Canepari P, 2000. mRNA detection by reverse transcription-PCR for monitoring viability over time in an *Enterococcus faecalis* viable but non-culturable population maintained in a laboratory microcosm. *Applied and Environmental Microbiology* **66**, 4564–7.

Masters CI, Shallcross JA, Mackey BM, 1994. Effect of stress treatments on the detection of *Listeria monocytogenes* and enterotoxigenic *Escherichia coli* by the polymerase chain reaction. *Journal of Applied Bacteriology* **77**, 73–9.

McKillip JL, Jaykus LA, Drake M, 1998. rRNA stability in heat-killed and UV irradiated enterotoxigenic *Staphylococcus aureus* and *E. coli* 0157:H7. *Applied and Environmental Microbiology* **64**, 4264–8.

Olmos A, Bertolini E, Cambra M, 2007. Isothermal amplification coupled with rapid flow-through hybridisation for sensitive

diagnosis of *Plum pox virus*. *Journal of Virological Methods* **139**, 111–5.

Rodriguez-Lázaro D, Hernández M, D'agostino M, Cook N, 2006. Application of nucleic acid sequence-based amplification for the detection of viable foodborne pathogens: progress and challenges. *Journal of Rapid Methods and Automation in Microbiology* **14**, 218–36.

Schaad NW, Postnikova E, Lacy GH *et al*., 2006. Emended classification of xanthomonad pathogens on citrus. *Systematic and Applied Microbiology* **29**, 690–5.

Schubert TS, Rizvi SA, Sun X, Gottwald TR, Graham JH, Dixon WN, 2001. Meeting the challenge of eradicating citrus canker in Florida again. *Plant Disease* **85**, 340–56.

Sela M, Anfinsen CB, Harrington WF, 1957. The correlation of ribonuclease activity with specific aspects of tertiary structure. *Biochimica et Biophysica Acta* **26**, 502.

Shiotani H, Uematsu H, Tsukamoto T *et al*., 2009. Survival and dispersal of *Xanthomonas citri* pv. *citri* from infected Satsuma mandarin fruit. *Crop Protection* **28**, 19–23.

Simpkins SA, Chan AB, Hays JP, Pöpping B, Cook N, 2000. An RNA transcription-based amplification technique (NASBA) for the detection of viable *Salmonella enterica*. *Letters in Applied Microbiology* **30**, 75–9.

Timmer LW, Zitko SE, Gottwald TR, 1996. Population dynamics of *Xanthomonas campestris* pv. *citri* on symptomatic and asymptomatic citrus leaves under various environmental conditions. *Proceedings of the International Society of Citriculture* **1**, 448–51.

Tolker-Nielsen T, Larsen MH, Kyed H, Molin S, 1997. Effects of stress treatments on the detection of *Salmonella typhimurium* by *in situ* hybridization. *International Journal of Food Microbiology* **35**, 251–8.

Van Beckhoven JR, Stead DE, 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**, 840–9.

Van der Wolf JM, Van Beckhoven JR, De Haan EG, Van den Bovenkamp GW, Leone GOM, 2004. Specific detection of *Ralstonia solanacearum* 16S rRNA sequences by AmpliDet RNA. *European Journal of Plant Pathology* **110**, 25–33.

Vasková D, Spak J, Klerks MM, Schoen CD, Thompson JR, Jelkmann W, 2004. Real-time NASBA for sensitive detection of *Strawberry vein banding virus*. *European Journal of Plant Pathology* **110**, 213–21.

Vauterin L, Hoste B, Kersters K, Swings J, 1995. Reclassification of *Xanthomonas*. *International Journal of Systematic Bacteriology* **45**, 472–89.

Verdier E, Zefferrino E, Méndez S, 2008. Survival of *Xanthomonas citri* subsp. *citri* on the surface of citrus fruit after post harvest treatment. *Fitopatología* **43**, 24–31.

Villarino A, Bouvet OM, Regnault B, Martin-Delautre S, Grimont PAD, 2000. Exploring the frontier between life and death in *Escherichia coli*: evaluation of different viability markers in live and heat- or UV-killed cells. *Research in Microbiology* **151**, 755–68.