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Polymerase chain reaction for verification of fluorescent colonies of *Erwinia chrysanthemi* and *Pseudomonas putida* WCS358 in immunofluorescence colony staining

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J.M.VAN DER WOLF, J.R.C.M. VAN BECKHOVEN, PH.M. DE VRIES, J.M. RAAIJMAKERS, P.A.H.M. BAKKER, Y. BERTHEAU AND J.W.L. VAN VUURDE. 1995. The potential of polymerase chain reaction (PCR) for verifying the identity of colonies stained by the immunofluorescence colony-staining (IFC) procedure was investigated. Using primers directed against conserved sequences of the pectate lyase-genes coding for isozymes PLa, PLd and PLe of *Erwinia chrysanthemi*, the authors confirmed the identity of 96% of 20 fluorescent target colonies, punched from IFC-stained samples with pure cultures. In pour plates with mixtures of *Erw. chrysanthemi* and non-target colonies from potato peel extracts, the identity of 90% of 113 target colonies was confirmed.

Using primers directed against sequences of the ferric-pseudobactin receptor gene *pupA* of *Pseudomonas putida* WCS358, the identity of 96% of 22 target colonies was confirmed in IFC-stained samples with pure cultures. In pour plates with mixtures of *Ps. putida* WCS358 and non-target bacteria from compost extracts, the identity of 59% of 108 fluorescent colonies was confirmed by PCR. It was shown that components from non-target bacteria lowered the threshold level of PCR for *Ps. putida* WCS358 100-fold.

INTRODUCTION

Immunofluorescence colony staining (IFC) was developed for detection of plant pathogenic bacteria in complex substrates (Van Vuurde 1987). IFC combines isolation and serology by staining bacterial colonies grown in pour plates with fluorochrome-labelled antibodies. The threshold level of IFC for detection of *Erwinia carotovora* subsp. *atroseptica* and *Erw. chrysanthemi* in cattle manure slurry was approximately 100 cfu ml^{-1} (Van Vuurde and Roozen 1990). IFC enabled Leeman *et al.* (1991) to monitor the plant growth promoting *Pseudomonas fluorescens* strain WCS374 in non-sterile soil, with a detection limit of 20 cfu g^{-1} of soil. The specificity of IFC in general is higher than that of methods based solely on serology, because it combines the use of specific antibodies with growth in a selective medium. Consequently, non-culturable bacteria do not interfere with the test as may be the case in conventional immunofluorescence methods.

In spite of the combined use of selective media and specific antibodies, for pathogenic *Erwinia* spp. still cross-reactions in IFC were found (Van der Wolf *et al.* 1993, 1994). Due to the low detection level of IFC even low densities of cross-reacting bacteria in the sample can cause false-positive results. For reliable detection of target bacteria, verification of IFC-positive results is therefore required.

The identity of IFC-positive colonies can be determined by puncturing cells from fluorescent colonies with a capillary needle and plating to pure cultures, which subsequently can be characterized. For colonies of pour plates stained with an antiserum against *Erw. carotovora* subsp. *atroseptica*, about 80% of isolations from samples containing naturally-infected potato peel extracts were successful, although laborious plating and identification procedures were necessary to separate

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target bacteria from contaminating non-target bacteria (N. J. M. Roozen, personal communication). Recovery of cells of *Erw. chrysanthemi* from IFC plates stained with a specific antiserum was low due to a relatively poor survival of the target bacteria in agar films (N.J.M. Roozen, personal communication).

The aim of this study was to develop a rapid method to confirm the identity of fluorescent colonies by using the polymerase chain reaction (PCR). This method was tested for the plant pathogen *Erw. chrysanthemi* and for *Ps. putida* WCS358, a strain with plant growth promoting properties (Geels and Schippers 1983).

MATERIALS AND METHODS

Bacterial strains and growth conditions

Strains were grown on slopes of trypticase soy agar (TSA) (BBL) for 24 h at 27°C. *Erwinia chrysanthemi* IPO-DLO strain 917 from *Saintpaulia* (=strain no. 3937 (Kotoujansky *et al.* 1982)) was used as target strain in the first study. Specificity of primers in PCR for *Erw. chrysanthemi* potato strains was checked with IPO-DLO strains 502, 978, 980, 981, 982, 984, 986, 988, 990 and 992 all belonging to serogroup O_1, the most frequently isolated serogroup in Western Europe (Janse and Ruissen 1988). Specificity was also checked with *Erw. carotovora* subsp. *atroseptica* IPO-DLO strain 1064 (serogroup I), *Erw. carotovora* subsp. *carotovora* IPO-DLO strain 139 (serogroup II), four *Pseudomonas* strains (S67, S70, S71 and S93) cross-reacting with various antisera against *Erw. chrysanthemi* (Van der Wolf *et al.* 1993) and seven unidentified bacterial isolates from potato (S10, S64, S103, S109, S128, S129 and S132). The latter were selected for maximum variation of colony morphology and colour on TSA.

Specificity of PCR for *Ps. putida* WCS358 was tested with fluorescent *Pseudomonas* strains RDR4, RDR20 and RDR25, cross-reacting with antiserum 8931 against *Ps. putida* WCS358, with *Ps. fluorescens* WCS374 (Geels and Schippers 1983), and with 34 bacterial isolates from the rhizosphere of radish. The radish isolates were selected for maximum variation of colony morphology and colour on TSA.

All strains were tested in concentrations of 10^7–10^8 cells ml^{-1}. To determine the number of cfu, 10-fold serial dilutions of the bacterial suspensions were pour-plated in TSA in 24-well tissue culture plates and grown for 2 d at 27°C. Colony counting was done using a stereomicroscope (Wild M3) at 10 times magnification.

Determination of bacterial densities with immunofluorescence cell staining

Immunofluorescence cell staining (IF) was performed in a direct format using antibodies conjugated with fluorescein iso-thiocyanate (FITC) (Van Vuurde 1990). Bacteria were diluted in phosphate buffered saline (0·27 mol l^{-1} NaCl, 0·04 mol l^{-1} Na_2HPO_4, 2·9 mmol l^{-1} KH_2PO_4, 5·4 mmol l^{-1} KCl, 0·01% NaN_3 pH 7·2) supplemented with 0·1% Tween 20 (PBST) and fixed on specially designed perspex sample holders to provide an equal distribution of cells over the wells (Van Vuurde and Van den Bovenkamp 1989).

Antiserum production

Polyclonal antisera 9025C and 8931 were prepared in rabbits according to the immunization protocol of Vruggink and Maas Geesteranus (1975). Antiserum 9025C was directed against a crude cell extract of *Erw. chrysanthemi* 502, whereas 8931 was directed against living cells of *Ps. putida* WCS358. Antibodies were purified according to Steinbuch and Audran (1969) and conjugated with FITC as described by Allen and Kelman (1977).

Immunofluorescence colony staining

Erwinia chrysanthemi suspensions were mixed with various dilutions of a potato peel extract, free of bacteria reacting in IFC with antiserum 9025C against *Erw. chrysanthemi*, and pour-plated in TSA in wells of 24-well tissue culture plates (Van Vuurde and Roozen 1990). *Pseudomonas putida* WCS358 suspensions were mixed with various dilutions of a compost extract, free of bacteria reacting with antiserum 8931 against *Ps. putida* WCS358, for pour plating in TSA. Pour plates with pure cultures of the target bacteria and dilutions of the extracts without addition of the target organisms served as controls. After 2 d incubation at 27°C to allow colony growth, plates were dried with circulating air at *ca* 40°C. Agar preparations were stained overnight at 27°C with a 1 : 50 dilution of the anti-target FITC-conjugated antibodies.

Sample preparation for polymerase chain reaction (PCR)

Suspensions of pure cultures for testing in PCR with *Erw. chrysanthemi* primers were prepared in demineralized water. Suspensions of pure cultures for testing in PCR with *Ps. putida* WCS358 primers were prepared in PBST, centrifuged for 2 min at 10 000 *g* and resuspended in 100 μl of PBST.

After IFC staining samples for PCR were taken from pour plates as follows: (1) bacteria were sampled present in the conjugate dilution after overnight incubation; (2) punches were taken of a fluorescent colony, using a Pasteur pipette bent at 120° (i.d. 1 mm); and (3) cells from a fluorescent colony were isolated using a fine capillary glass needle. Non-fluorescent colonies and cells from non-fluorescent colonies were sampled as negative controls. Sampling was done under a stereomicroscope adapted for FITC fluorescence by using

a cold light source (Intralux 6000) suitable for fluorescence, equipped with a 10 mm glass fibre cable and an exciting filter for blue light illumination in combination with a yellow barrier filter in a Wild M3 stereomicroscope.

Further sample preparations for PCR were done for *Erw. chrysanthemi* according to the procedure of Darrasse *et al.* (1993) and for *Ps. putida* WCS358 according to the procedure of W. Bitter (University of Utrecht, personal communication). Punches and cells were transferred into 70 μl of demineralized water for *Erw. chrysanthemi* (Darrasse *et al.* 1993) and 10 μl of PBST for *Ps. putida* WCS358. Samples were boiled for 15 min in case of *Erw. chrysanthemi* and for 5 min for *Ps. putida* WCS358. Boiled samples were centrifuged for 2 min at 10 000 *g* and the supernatant fluids were tested in PCR.

To test the influence of agar medium compounds or non-target colonies in pour plates on the threshold level of PCR, an agar film of 100 mm^2 without bacteria or one containing 8000 non-target colonies from a potato peel or a compost extract was boiled for 3 min in 1 ml of demineralized water (*Erw. chrysanthemi*) or 1 ml of PBST (*Ps. putida* WCS358). One ml of liquified pour plate was added to 1 ml of a 10-fold serial dilution of a target suspension. The same serial dilutions diluted 1 : 1 with water (*Erw. chrysanthemi*) or PBST (*Ps. putida* WCS358) instead of the liquified pour plate served as a control.

Polymerase chain reaction

For *Erw. chrysanthemi*, a total volume of 50 μl per reaction was used. Maximal 37·5 μl of sample supplemented with 10 nmol dNTP (Pharmacia, Roosendaal), 1 unit Taq-polymerase (Boehringer Mannheim) and 50 pmol of each of the primers ADE1 and ADE2 (Darasse *et al.* 1993) were diluted in PCR buffer (final concentration of 10 mmol l^{-1} Tris-HCl, 1·5 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} KCl and 0·01% gelatin (pH 8·3)). ADE1 and ADE2 are directed to conserved sequences of the pectate lyase (*pel*)-genes coding for isozymes PLa, PLd and PLe and did not react with other species of soft-rot *Erwinia* spp. (5). PCR of *Erw. chrysanthemi* total DNA results in a product of 420 bp. A total of 35 PCR cycles was run in a Perkin Elmer 9600 thermocycler under the following conditions: denaturation at 94°C for 1 min, primer annealing and DNA extension for 2 min at 72°C.

For *Ps. putida* WCS358, a total volume of 50 μl per reaction was used. Maximal 5 μl of sample supplemented with 5 nmol dNTP (Pharmacia, Roosendaal), 0·5 unit Taq-polymerase (Supertaq, SphearoQ, HT Biotechnology Ltd, Ditton Walk, UK) and 72 pmol of the primers WB3 (5′-GAGTTCCAGTTAACACCACC-3′) and WB5 (5′-CAACGTGATTCGAAAGCGTC-3′) were diluted with PCR buffer. WB3 and WB5 are homologous to specific regions within the structural gene encoding the ferric-pseudobactin receptor PupA of *Ps. putida* WCS358 and gives a 1400 bp PCR-product (Bitter *et al.* 1991). A total of 30 PCR cycles was run in a Perkin Elmer 480 or a Sensa 949E thermocycler under the following conditions: denaturation at 94°C for 1·5 min, primer annealing at 62°C for 1 min and DNA extension at 72°C for 2 min.

In some experiments, second amplifications were run on 5 μl of the product of the first PCR using a fresh reaction mixture and the same conditions.

Amplification products were separated on 1% agarose gels and stained with ethidium bromide.

Statistical analysis

Analysis of variance and least significant difference calculations were used to evaluate test data at a confidence level of 95%.

RESULTS

Specificity of the primers for *Erw. chrysanthemi*

Like *Erw. chrysanthemi* 917, all 10 *Erw. chrysanthemi* strains, isolated from potato in The Netherlands gave a typical PCR product of 420 bp with the *pel*-primers, while none of the 13 isolates from other species gave PCR products (Fig. 1). The lower bands in the gel are a result of primer dimerization.

Specificity of the primers for *Ps. putida* WCS358

The outer membrane receptor PupA was found to be specific for the uptake of ferric pseudobactin 358; the siderophore produced by *Ps. putida* WCS358 cannot be utilized by many other fluorescent *Pseudomonas* strains (Bakker *et al.* 1990). In accordance, the *pup*A primers did not produce a 1400 kb fragment with any of the 34 isolates from radish rhizosphere extracts. However, DNA fragments with aberrant lengths were produced for all four fluorescent *Pseudomonas* isolates tested (Fig. 2). *Pseudomonas fluorescens* strain WCS374 yielded in PCR a DNA fragment of 600 bp. The fluorescent *Pseudomonas* strains RDR4, RDR20 and RDR25 which cross-react with *Ps. putida* WCS358 antibodies, produced DNA fragments in the range of 300–500 bp in PCR.

PCR threshold levels for bacterial suspensions

The threshold level of PCR for suspensions of pure cultures was determined by pour plating at about 40 cfu per reaction for *Erw. chrysanthemi* (5 μl of $8·3 \times 10^3$ cfu ml^{-1}) and about 100 cfu per reaction for *Ps. putida* WCS358 (5 μl of $1·9 \times 10^4$ cfu ml^{-1}) (Table 1). The number of bacterial cells determined by IF and pour plating were not statistically different for concentrations near to the detection level (Table 1).

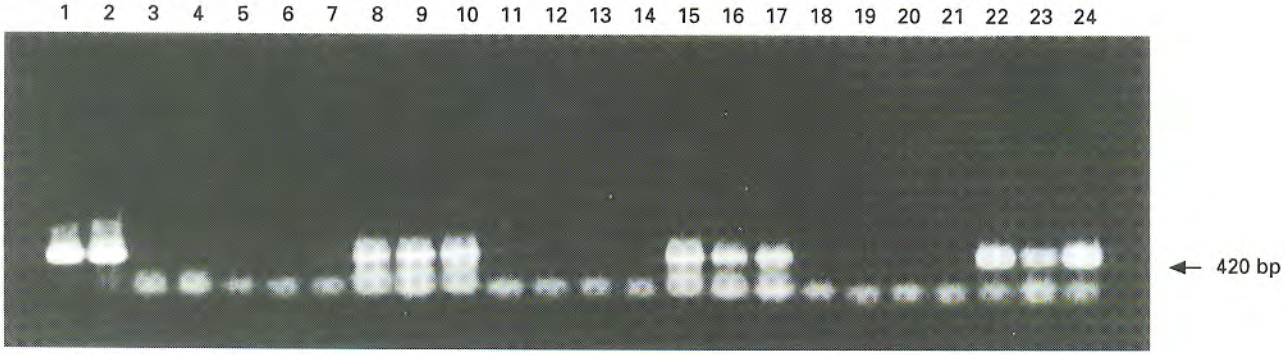

Fig. 1 PCR products of *Erwinia chrysanthemi* strains 917 (lane 1), 502 (2), *Erw. carotovora* subsp. *atroseptica* strain 1064 (3), *Erw. carotovora* subsp. *carotovora* strain 139 (4), S10 (5), S64 (6), S67 (7), *Erw. chrysanthemi* strains 978 (8), 980 (9) and 981 (10), S70 (11), S71 (12), S93 (13), S103 (14), *Erw. chrysanthemi* strains 982 (15), 984 (16) and 986 (17), S109 (18), S128 (19), S129 (20), S132 (21), *Erw. chrysanthemi* strains 988 (22), 990 (23) and 992 (24). S70, S71 and S67 are fluorescent *Pseudomonas* strains cross-reacting with antibodies against lipopolysaccharides of *Erw. chrysanthemi*. Other 'S' numbers are unidentified bacterial isolates from potato peel extracts

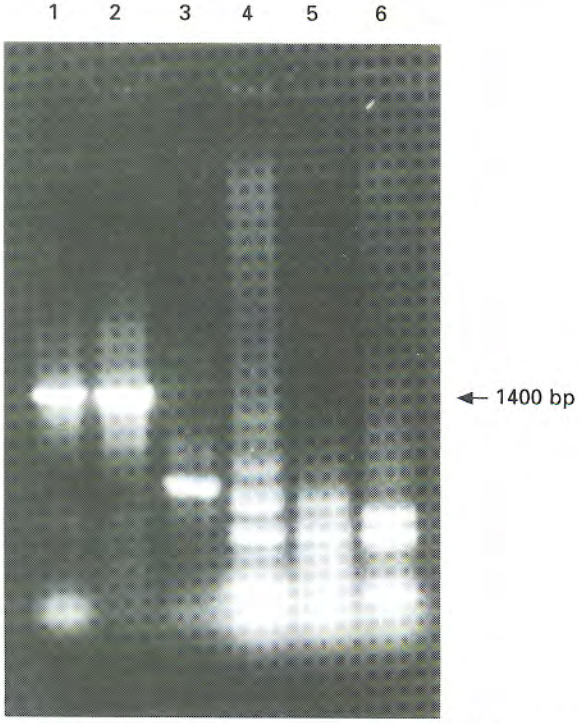

Fig. 2 PCR products of *Pseudomonas putida* WCS358 (lane 1 and 2) and fluorescent *Pseudomonas* strains WCS374 (3), RDR4 (4), RDR20 (5) and RDR25 (6) with primers against *pupA* of *Ps. putida* WCS358

Addition of the liquified TSA pour plates, containing non-target bacteria grown from a compost extract, to suspensions of pure cultures of *Ps. putida* WCS358, decreased the PCR sensitivity from 10^4 to 10^6 cells ml^{-1} (Fig. 3a). Addition of the liquified agar medium without non-target bacteria did not affect the detection level. The detection level of the PCR for *Erw. chrysanthemi* was not affected by the addition of the liquified pour plates containing non-target bacteria or medium without bacteria.

A second PCR on an aliquot of the reaction products of the first PCR did not improve the threshold level for *Erw. chrysanthemi* (results not shown). For *Ps. putida* WCS358, a second PCR improved the detection threshold from 10^6 cells ml^{-1} to 10^5 cells ml^{-1} for *Ps. putida* WCS358 suspensions mixed with compost bacteria, but did not improve the threshold level for pure cultures of *Ps. putida* WCS358 (Fig. 3b). The second amplification resulted for *Ps. putida* WCS358 in an extra band of 800 bp.

Comparison of methods to sample target cells from fluorescent colonies for PCR

A capillary needle, which is commonly used to isolate cells from fluorescent colonies for dilution plating (Van Vuurde 1990), was compared with a Pasteur pipette as an instrument to sample target cells from IFC preparations for PCR. A capillary needle punctures cells from colonies, whereas a Pasteur pipette punches an agar disk with a colony. However, in IFC preparations with high densities of non-target colonies punches with fluorescent colonies may also contain up to 100 non-target colonies. During sampling, effective transfer of fluorescent cells or colonies was watched under a (stereo) microscope with incident blue light.

IFC-positive colonies were sampled from pour plates with pure cultures. For *Erw. chrysanthemi*, sampling with a Pasteur pipette yielded for 19 out of 20 samples the typical 420 bp product in PCR (efficiency 95%). With a capillary needle only nine out of 20 samples (45%) gave the specific PCR product. Similarly, for *Ps. putida* WCS358, the Pasteur pipette procedure yielded in 21 out of 22 punches (96%) the expected 1400 bp PCR product. Using a capillary needle, only eight out of 18 of the samples (44%) gave a specific PCR

Table 1 PCR threshold levels for detection of *Erwinia chrysanthemi* (strain 917) and *Pseudomonas putida* WCS358 determined by immunofluorescence cell (IF) countings and pour plate countings

Erw. chrysanthemi 917			*Ps. putida* WCS358		
Pour plate countings (cfu ml^{-1})	IF countings (cells ml^{-1})	PCR§	Pour plate countings (cfu ml^{-1})	IF countings (cells ml^{-1})	PCR
$9{\cdot}2 \times 10^4$*	$1{\cdot}6 \times 10^5$†	+	$3{\cdot}8 \times 10^4$	$3{\cdot}9 \times 10^4$	+
$(1{\cdot}3 \times 10^3)$‡	$(2{\cdot}0 \times 10^4)$		$(1{\cdot}5 \times 10^3)$	$(1{\cdot}0 \times 10^3)$	
$8{\cdot}3 \times 10^3$	$8{\cdot}7 \times 10^3$	+	$1{\cdot}9 \times 10^4$	$2{\cdot}0 \times 10^4$	+
$(4{\cdot}4 \times 10^2)$	$(1{\cdot}2 \times 10^3)$		$(1{\cdot}0 \times 10^3)$	$(8{\cdot}2 \times 10^2)$	
$6{\cdot}1 \times 10^3$	$6{\cdot}0 \times 10^3$	−	$9{\cdot}0 \times 10^3$	$8{\cdot}9 \times 10^3$	−
$(8{\cdot}5 \times 10^1)$	$(2{\cdot}0 \times 10^3)$		$(8{\cdot}8 \times 10^2)$	$(4{\cdot}0 \times 10^1)$	
$4{\cdot}7 \times 10^3$	$4{\cdot}7 \times 10^3$	−	$8{\cdot}0 \times 10^3$	$8{\cdot}0 \times 10^3$	−
$(2{\cdot}8 \times 10^1)$	$(1{\cdot}2 \times 10^3)$		$(3{\cdot}0 \times 10^1)$	$(8{\cdot}0 \times 10^1)$	

For *Erw. chrysanthemi* 917, primers directed against *pelADE* were used and for *Ps. putida* WCS358 primers directed against *pupA*.
* For pour plate countings $n = 3$.
† For IF, per concentration at least 1 microscope field on 3 wells was counted.
‡ Standard deviations are given in parentheses.
§ In PCR 5 μl per reaction was tested.

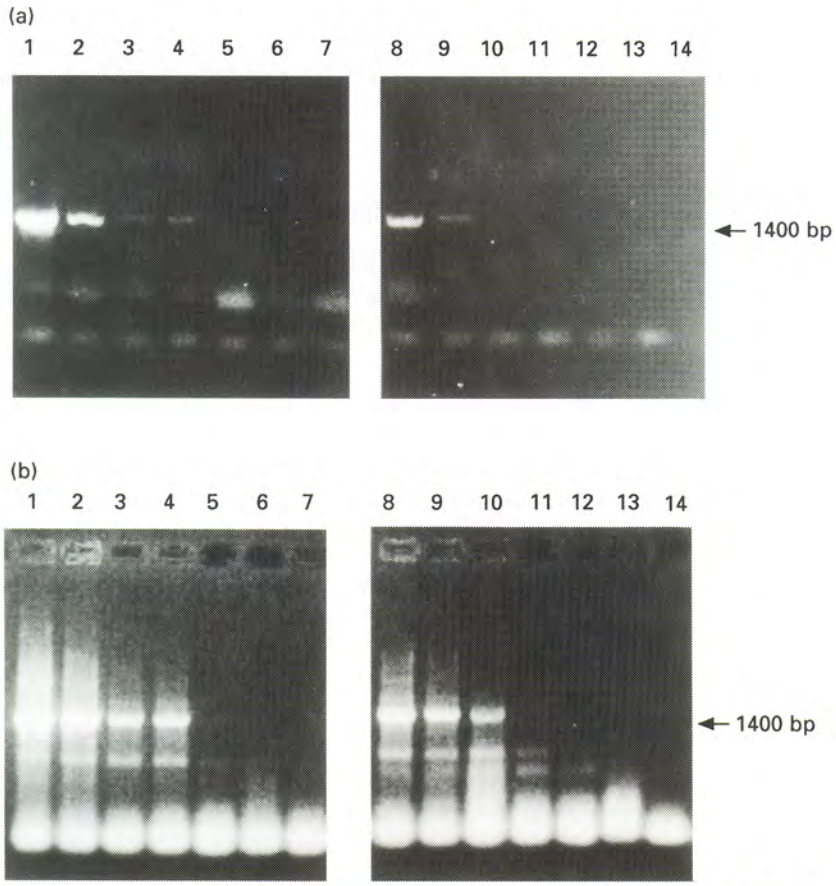

Fig. 3 Influence of compost bacteria on the threshold level of PCR for detection of *Pseudomonas putida* WCS358 using primers directed against the *pupA* gene. (a) PCR products after 30 cycles. (b) Effect of a second PCR of 30 cycles on the PCR products as shown in (a). Lanes: 1–7: 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10 cells ml^{-1} of a pure culture of *Ps. putida* WCS358 in PBST; 8–14: 10^7, 10^6, 10^5, 10^4, 10^3, 10^2 and 10 cells ml^{-1} of *Ps. putida* WCS358 mixed with compost bacteria from colonies grown in pour plates of TSA

product. The efficiency of the capillary needle method was not improved by repeated puncturing of several IFC-positive colonies (data not shown).

Further experiments were done using a Pasteur pipette for sampling.

Efficiency of PCR for verification of IFC-positive plates with mixed cultures

A potato peel extract and a compost extract, selected for the absence of colonies reacting in IFC with *Erw. chrysanthemi* and antisera against *Ps. putida* WCS358, respectively, were pour-plated (Tables 2 and 3).

In five independent experiments, on average 90% of the 113 fluorescent colonies of *Erw. chrysanthemi* punched from pour plates containing different ratios of target and non-target bacteria from potato peel extracts gave a PCR product of 420 bp (Table 2). The efficiency of confirmation ranged for the five individual experiments from 86% to 100%. No products with an aberrant size were found. Non-fluorescent colonies from agar films containing also fluorescent colonies or from agar films with only non-target colonies did not react in PCR.

In three independent experiments, on average 59% of the 108 fluorescent *Ps. putida* WCS358 colonies, taken from pour plates containing different ratios of *Ps. putida* WCS358 and bacterial colonies from extracts, gave a PCR product of 1400 bp (Table 3). The efficiency of confirmation ranged for the three individual experiments from 35 to 70%. When non-fluorescent colonies were punched from agar films also containing fluorescent *Ps. putida* WCS358 colonies, 12% of the 25 punches were positive. PCR reactions were not obtained for punches from non-fluorescent colonies in pour plates containing only non-target colonies. No products with an aberrant size were found.

The effect of the density of background colonies on PCR efficiency was not statistically significant either for *Erw. chrysanthemi* or *Ps. putida* WCS358 (Tables 2 and 3). Furthermore, no clear relationship was found between target colony size and a positive reaction in PCR (data not shown).

Agar films containing *Ps. putida* WCS358 colonies often showed small fluorescent micro-colonies on the surface of the pour plates, as a result of secondary growth during incubation with antibody-conjugate. These micro-colonies were not found for slower growing *Erw. chrysanthemi.*

During the overnight staining period with the FITC-conjugated antibody dilution, cells from colonies of surface-grown bacteria were released and enriched. These suspensions, collected from 10 pour plates, containing various ratios of *Ps. putida* WCS358 and non-target colonies, were tested in PCR. These 10 suspensions were PCR-positive, while no PCR products were found with samples collected from two pour plates only containing 1000–10 000 non-target colonies per well. For *Erw. chrysanthemi*, no PCR products were found with the dilutions of FITC-conjugated antibodies, collected after a 24-h staining period from pour plates containing various ratios of target and non-target colonies.

DISCUSSION

The risk for false-positive results of a procedure in which a serological test is combined with a method based on DNA

Table 2 Efficiency of PCR for verification of fluorescent colonies in IFC using an antiserum against *Erwinia chrysanthemi* (data from five independent experiments)

		Fluorescent colonies*			Non-fluorescent colonies	
Erw. chrysanthemi 917 number/well	Saprophytes number/well	Number of colonies tested	Number of PCR-positive†	Confirmed (%)	Number of colonies tested	Number of PCR-positive†
1–10	10^2–10^3	10	9	90	1	0
1–10	10^4–10^5	19	17	89	0	0
10–100	10^2–10^3	31	30	97	2	0
10–100	10^4–10^5	25	22	88	4	0
100–1000	10^2–10^3	16	13	81	2	0
100–1000	10^4–10^5	12	11	92	0	0
	Total	113	102	90	9	0
0	10^2–10^5				14	0

Colonies were punched with a Pasteur pipette from agar films with different ratios of *Erw. chrysanthemi* and bacteria from potato peel extracts.
* The potato peel extract did not contain bacteria cross-reacting in IFC.
† Specific amplification product of 420 bp.

Table 3 Efficiency of PCR for verification of IFC-positive colonies using an antiserum against *Pseudomonas putida* WCS358 (data from three independent experiments)

		Fluorescent colonies*			Non-fluorescent colonies	
WCS358 number/well	Saprophytes number/well	Number of colonies tested	Number of PCR-positive†	Confirmed (%)	Number of colonies tested	Number of PCR-positive†
1–10	200	26	16	62	5	0
1–10	20 000	18	12	67	5	0
10–100	200	30	20	67	6	2
10–100	20 000	16	7	44	5	0
100–1000	200	18	9	50	4	1
	Total	108	64	59	25	3
0	20 000				4	0

Colonies were punched with a Pasteur pipette from agar films with different ratios of *Ps. putida* WCS358 and compost extract bacteria.
* The compost extract did not contain bacteria cross-reacting in IFC.
† Specific amplification product of 1400 bp.

hybridization is reduced over a procedure based on only one of the detection methods. This is demonstrated both for detection of *Erw. chrysanthemi* and *Ps. putida* WCS358. Using the *pel*-primers for detection of *Erw. chrysanthemi*, no PCR product was found with the fluorescent *Pseudomonas* strains that cross-react in IFC with *Erw. chrysanthemi* antibodies (Fig. 1). Using the *pupA* primers for detection of *Ps. putida* WCS358, PCR products with clearly aberrant lengths were found for RDR4, RDR20 and RDR25, that cross-react in IFC with antibodies against *Ps. putida* (Fig. 2). In addition, the specificity can be further improved by characterization of the PCR product, e.g. by the multiplication of internal sequences in a 'nested' PCR (Kapperud *et al.* 1993), by restriction enzyme analysis or by Southern blotting (Grant *et al.* 1993).

Apart from avoiding false-positive reactions, for reliable confirmation of IFC by PCR, it is further necessary that the DNA primers specifically react with all target strains reacting in IFC and that the diagnostic sensitivity of PCR is high enough to give a positive reaction for samples from target colonies. For *Erw. chrysanthemi* in mixed cultures, on average 90% of the fluorescent colonies were positively confirmed, while none of the IFC-negative colonies reacted in PCR (Table 2). However, for *Ps. putida* WCS358 in mixed cultures only 59% of the target colonies were verified in PCR, compared to 96% in pure cultures (Table 3). This low efficiency may be due to interference of compost residues, such as humic acids (Picard *et al.* 1992) and acidic plant polysaccharides (Demeke and Adams 1992), or due to PCR inhibiting components of non-target bacteria (Fig. 3). The threshold level of PCR for *Ps. putida* WCS358 increased from 10^2 cells per reaction to 10^4 cells after addition of a sample of liquified pour plate with a compost extract. To estimate the number of cells in colonies that develop in agar films, the volume of a spindle-shaped colony was calculated by the formula $4/3 \cdot \pi \cdot r^2 \cdot t$, in which r is the radius and t the thickness of the disk. The disk-shaped colonies measured had an average radius of 50 μm and a thickness of 12·5 μm, which gives a volume of 10^6 μm^3. Assuming a *Pseudomonas* cell to be a cylinder with an average length of 2 μm and a radius of 0·5 μm (Buchanan and Gibbons 1974), the cell volume is 1·5 μm^3. Based on this calculation an average *Pseudomonas* colony will have an estimated number of 7×10^4 bacterial cells. The sensitivity of PCR for target cells free in suspension decreased to the level of 10^5 cells per reaction sample in the presence of interfering components which may be a critical level, especially for smaller colonies. This probably explains the high percentage of false-negative reactions in PCR.

The capillary needle procedure was tested in order to reduce the amount of PCR inhibiting contaminants obtained by sampling with a Pasteur pipette. However, as sampling with a needle resulted in a lower efficiency than punching, the number of target cells recovered in the false-negative samples was probably below the PCR detection level.

Micro-colonies of *Ps. putida* WCS358, which were formed as a result of secondary growth on the surface of the agar medium after overnight staining with FITC-labelled antibodies could be observed using a u.v. microscope at a magnification of 200. Growth of *Ps. putida* WCS358, a fast-growing strict aerobe, is favoured on the surface of the agar films and in the conjugate dilution, where higher oxygen conditions will exist than lower in the agar layer. The 'false-positive' reactions of non-target colonies punched from pour plates containing *Ps. putida* WCS358 may be caused by a

reaction with contaminating cells from these micro-colonies in the preparation after secondary growth. *Erwinia chrysanthemi* did not produce visible micro-colonies on the agar films probably because *Erw. chrysanthemi* grows slower than *Ps. putida* WCS358 and no reaction was found in PCR with the antibody-conjugate dilution.

For *Ps. putida* WCS358, PCR was not suitable for verifying individual fluorescent colonies as a consequence of the relatively low efficiency of the method. The efficiency of the verification can be increased by testing more punches containing IFC-positive colonies per preparation. Assuming that the chance for false-negative results is independent in the same preparation, at least five *Ps. putida* WCS358 colonies per preparation should be tested, to obtain 99% probability of finding one positive reaction in PCR. As an alternative, the antibody conjugate from IFC preparations containing fluorescent colonies can be tested in PCR to confirm the presence of *Ps. putida* WCS358.

To verify the identity of *Erw. chrysanthemi* in IFC, PCR has several advantages over dilution plating followed by standard identification procedures. Viability of bacteria in colonies during storage or staining procedures does not influence PCR results, as long as the target DNA stays intact. Furthermore, some contamination of the target bacteria with other micro-organisms will not affect PCR results. As a relatively fast method, PCR can be performed in one working day. It complements the verification procedure by isolation in pure culture, which is still required for isolation and identification of cross-reacting bacteria or new strains of the target species.

PCR has also been used for direct detection of bacteria in some substrates (Goldsteyn Thomas *et al.* 1991; Grant *et al.* 1993). However, laborious DNA purification methods have to be applied, in order to remove inhibiting compounds from substrates such as soil and several food products (Picard *et al.* 1992; Rossen *et al.* 1992). The inability to distinguish between DNA originating from living and dead cells can also be a drawback of the direct use of PCR. Finally, although reliable methods for quantification of bacterial densities with PCR are available for a few other models, they are not available for these target bacteria yet. The combined use of IFC and PCR enables quantitative, sensitive and specific detection of culturable target bacteria, without the necessity of laborious DNA extractions.

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