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METHODS

Detection of recombinant *Pseudomonas putida* in the wheat rhizosphere by fluorescence in situ hybridization targeting mRNA and rRNA

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Abstract A method was developed to detect a specific strain of bacteria in wheat root rhizoplane using fluorescence in situ hybridization and confocal microscopy. Probes targeting both 23S rRNA and messenger RNA were used simultaneously to achieve detection of recombinant *Pseudomonas putida* (TOM20) expressing toluene *o*-monooxygenase (*tom*) genes and synthetic phytochelatin (EC20). The probe specific to *P. putida* 23S rRNA sequences was labeled with Cy3 fluor, and the probe specific to the *tom* genes was labeled with Alexa647 fluor. Probe specificity was first determined, and hybridization temperature was optimized using three rhizosphere bacteria pure cultures as controls, along with the *P. putida* TOM20 strain. The probes were highly specific to the respective targets, with minimal non-specific binding. The recombinant strain was inoculated into wheat seedling rhizosphere. Colonization of *P. putida* TOM20 was confirmed by extraction of root biofilm and growth of colonies on selective agar medium. Confocal microscopy of hybridized root biofilm detected *P. putida* TOM20 cells emitting both Cy3 and Alexa647 fluorescence signals.

Keywords FISH · mRNA · Rhizosphere bacteria · In situ gene expression

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Introduction

The rhizosphere is an area encircling the plant root system, which is characterized by enhanced biomass productivity and symbiosis between microbes and plants. This highly productive region is an important component of carbon, nitrogen, and phosphorus biogeochemical cycling (Singh et al. 2004). The plant–microbe interaction is also important for promoting plant growth and disease suppression (Mark et al. 2005; Morrissey et al. 2004). Researchers have exploited the symbiotic relationship between plants and root bacteria for remediation of pollutants, such as volatile organic carbon contaminants, parathion (Anderson et al. 1993), atrazine (Anderson Coats 1995), trichloroethylene (Shim et al. 2000), polychlorinated biphenyls (Brazil et al. 1995; Villacieros et al. 2005), 2,4-D (Monti et al. 2005),

and heavy metals (Wu et al. 2006). The functional importance of microbial activity in the rhizosphere warrants development of new methods to examine the distribution of rhizobacteria and their gene expression in situ.

In the past two decades, fluorescence in situ hybridization (FISH) has emerged as a powerful tool for identifying species and monitoring microbial activity (Amann et al. 1995; DeLong et al. 1989; Poulsen et al. 1993). Recent reports on the application of in situ hybridization targeting ribosomal rRNA has enabled distinctions between bacteria and non-bacteria species in soil (Christensen et al. 1999; Dorn et al. 2003) and type I and II methanotrophs in lake sediments (Kalyuzhnaya et al. 2006). Previous studies have reported the use of confocal microscopy with FISH to monitor endobacteria on the mycelia of an ectomycorrhizal fungus (Bertaux et al. 2005) and pathogenic bacterial strains of *Salmonella* and *Listeria* on barley roots (Kutter et al. 2005). Watt et al. (2006) have developed a method to quantify native *Pseudomonas* spp. on wheat roots and to determine the spatial distribution of bacteria.

Most of the previous reports target ribosomal RNA (rRNA), which is naturally abundant due to its essential role for protein synthesis. One limitation of solely using rRNA is that the bacterial identification is specific only for the genus and cannot distinguish between species. A remedy for increasing stringency for monitoring specific strains is to use probes targeting both messenger RNA (mRNA) of the specific genes and species-specific rRNA. However, measurement of mRNA has been challenging due to its short lifetime, low abundance, and degradation by RNase (Chen et al. 2004; Pernthaler Amann 2004). A number of studies have reported mRNA detection in bacteria mostly from aqueous environments or food (Bakermans Madsen 2002; Chen et al. 2004; D'Souza Jaykus 2003; Hahn et al. 1993).

This research applied techniques for multi-colored FISH and mRNA hybridization to detect a specific bacterium on wheat roots grown in sandy soil. A recombinant rhizobacterium, *Pseudomonas putida* 06909 (Lee et al. 2001) into which we inserted toluene monooxygenase (*tom*) genes from *Burkholderia cepacia* G4 (Yee et al. 1998) and a synthetic phytochelatin gene (EC20) (Bae et al. 2000), was used as the model microorganism. The *tom* genes enable the microbes to degrade trichloroethylene. The synthetic phytochelatin consists of $(Glu\text{-}Cys)_{20}$-Gly and chelates metals such as mercury, cadmium, and copper. This strain was inoculated into the rhizosphere and detected using a protocol developed for root biofilm samples that targeted both mRNA and 23S rRNA. The steps taken to optimize the detection of the recombinant *P. putida* are (1) designing a probe targeting the *tom* locus, (2) determining the specificity of the probes in pure culture using positive and negative control strains, and (3) detecting the recombinant *P. putida* in the wheat rhizoplane through the optimized hybridization protocol.

Materials and methods

Bacterial strains and growth conditions

Rhizosphere bacterial strains used in this study were (1) recombinant *P. putida* with *tom* genes and EC20 (*P. putida* TOM20), (2) *P. putida* 06909 wild type (Lee et al. 2001), (3) *Pseudomonas fluorescens* (Yee et al. 1998), and (4) *Rhizobium* 10320D with chromosomally inserted *tom* genes (*Rhizobium* TOM) (Yee et al. 1998). All rhizosphere bacterial strains were grown in Luria–Bertani (LB) liquid medium or on M9 medium agar plates at 30°C. *P. putida* TOM20 was grown with 200 μg/ml ampicillin, 100 μg/ml kanamycin, and 40 μg/ml tetracycline. *P. putida* wild type was grown with 200 μg/ml ampicillin. *P. fluorescens* was grown with 10 μg/ml ampicillin. *Rhizobium* TOM was grown with 50 μg/ml kanamycin. *Escherichia coli* strains DH 5α and λ pir, used for cloning purposes, were grown at 37°C in LB medium.

Construction of recombinant *P. putida* expressing TOM

The *tomA012345* genes of *B. cepacia* G4 were integrated into the chromosome using pLANT3, a pCNB4-based suicide plasmid containing the six constitutively expressed genes cloned into a *tn*5 region (Yee et al. 1998); this plasmid replicates only in λ pir strains. Plasmid pLANT3, with a kanamycin resistant gene, was harbored in the triparental-mating donor strain *E. coli* S17 λ pir. Similarly, a suicide plasmid for integrating EC20 under the control of the P_{tac} into the chromosome (pUTPtacM20) was constructed according to the Molecular Cloning Manual (Sambrook and Russell 2001). The *tac* promoter is constitutive in the *P. putida* strain due to the lack of the *lacI* gene. The 1.3 kb P_{tac}-Maltose Binding Protein-EC20 (PtacM20) fragment was obtained by using plasmid pVMC20 (Wu et al. 2006) as template and the following two primers: 5′CGCGGATCCTGAAATCTGTTGACAATTAATCAT3′, and 5′TTTTTCTTGTTATCGCAA3′. To obtain flanking *Not*I sites, this fragment was digested with *Bam*HI and *Hin*dIII and ligated into pUC18Not using DH5α to obtain pUCPtacM20. Plasmid pUCPtacM20 was then digested with *Not*I to obtain the *Not*I flanking PtacM20 fragment. The fragment was ligated into the *Not*I digested vector pUTminiTn5 (Biomedal, Spain) to obtain the plasmid pUTPtacM20 and transformed into the donor strain *E. coli* S17 λ pir.

Chromosomal insertions of the *tom* locus and the P_{tac}-EC20 genes into *P. putida* based on single transposon integrations were performed sequentially using triparental conjugation protocols modified from Yee et al. (1998) and established methods (De Lorenzo and Timmis 1994; Herrero et al. 1990).

Wheat seedling growth and inoculation

Hard red winter wheat (*Triticum sativum* cv. Cal Rojo) seeds were surface-sterilized by washing and shaking in 95% ethanol for 45 min, in 5.25% sodium hypochlorite for 15 min and in sterile water for 10 min three times. Seeds were grown in plastic germination cones (SC10 Super Cell®, Stuewe and Sons, Coravallis, OR, USA) with 3.8-cm diameter opening on top and 21-cm depth containing sandy soil. One seed was transferred, with sterilized forceps, into one germination cone. Each cone contained either autoclaved or non-autoclaved sandy loam soil (20% clay, 80% sand, and 80% silt). The soil was autoclaved four times (Amsco 3011 Sterilizer, Alfa Medical, Hempstead, NY, USA) for 1 h at 120°C using the dry cycle (once every 2 days). The different treatments compared were (1) autoclaved soil with *P. putida* TOM20 inoculation (A/P), (2) autoclaved soil without *P. putida* TOM20 inoculation (A/NP), (3) non-autoclaved soil with *P. putida* TOM20 inoculation (NA/P), and (4) non-autoclaved soil without *P. putida* TOM20 inoculation (NA/NP). There were four replicates for each treatment, and all were grown in a greenhouse with 80% to 90% relative humidity and temperatures between 18 and 25°C. To ensure proper growth, the seedlings were irrigated with plant growth solutions containing essential micro- and macronutrients (Pedler et al. 2000). The seedlings were inoculated with *P. putida* TOM20 when irrigated with the growth solution every 2 days. *P. putida* TOM20 grown in LB medium overnight was centrifuged and washed with plant growth solution once. The pellet was then resuspended in plant growth solution to a cell concentration of 5×10^8 cells/ml for inoculation of the seedlings.

Root slurry extraction

Roots from 24- to 37-day-old seedlings were carefully removed from the germinating cones, and the attached soil was gently shaken off. The roots were then placed in sterile water to remove any remaining soil. The root slurry extraction protocol developed in this study was modified from a previously published soil microbe extraction method (Burmolle et al. 2003). A 3-cm section of the root tip was excised and processed for probe hybridization and confocal microscopy. The rest of the root was transferred to a 50-ml centrifuge tube containing 15 ml TTSP [0.05% Tween80 and 50 mM tetrasodium pyrophosphate (Sigma) at pH 7]. The roots were then vortexed vigorously for 5 min and placed in a sonicating water bath for 30 min. The tubes were centrifuged at 2,000 rpm for 5 min to separate roots and large sand particles from the small clay and bacterial particles. The supernatant containing the rhizoplane bacteria was transferred to a new tube and centrifuged at 15,000 rpm for 10 min. The roots were dried at 105°C for at least 24 h on a pre-weighed aluminum tray, then weighed. The supernatant was discarded, and the pellets were resuspended in 500 μl of TTSP. The cell suspension was carefully placed in 500 μl of 80% Nycodenz® (Accurate Chemical and Scientific, Norway), a non-ionic gradient medium for separation of small particles based on density and buoyancy. Without mixing the two layers, the cells were centrifuged at 13,000 rpm for 10 min. The top and mid-layers were transferred to a new centrifuge tube, where the larger particles were allowed to settle to the bottom. The supernatant containing the cells was filtered through a 30-μm membrane to further remove soil particles. The root slurry was serially diluted (1:100) and plated on LB plates with 32 μg/ml amphotericin B, 50 μg/ml ampicillin, 25 μg/ml kanamycin, and 20 μg/ml tetracycline. Amphotericin B was added to prevent fungal growth. The plates were incubated at 30°C for 18 h, and the number of colony-forming units (CFU) were normalized to dry root weight.

Oligonucleotide probes

An oligonucleotide probe (Ppu: 5′-GCTGGCCTAACCTTC-3′) targeting *P. putida* 23 s rRNA (Schleifer et al. 1992) was labeled with Cy3 at the 5′ end. An oligonucleotide probe (TOM: 5′-GGAATGTAAATGCCTTGGCGC-3′) targeting the *tomA5* gene mRNA was labeled with Alexa647 at the 5′ end. All probes were purchased pre-labeled from Integrated DNA Technology (Coralville, IA, USA).

Probe specificity and hybridization optimization

P. fluorescens 2–79 served as a negative control for both the TOM and Ppu probes. *P. putida* 06909 and *Rhizobium* TOM were used as positive controls for the Ppu and TOM probes, respectively. All four strains, including *P. putida* TOM20, were used for optimization of hybridization temperature. Optimization of hybridization conditions was performed by simultaneously hybridizing both probes to each strain over eight temperatures between the range of 42 to 52°C and post-hybridization temperature ranging between 40 and 50°C. Microcentrifuge tubes containing the samples were hybridized using the temperature gradient function in the BioRad iCycler (BioRad, Hercules, CA, USA). The optimal condition was selected based on confocal microscopy observation of the strongest fluorophore signals with the lowest background signal.

Hybridization of pure culture and root biofilm

The hybridization protocol, based on a previous report (Pernthaler and Amann 2004), was modified for the detection of mRNA and rRNA in pure cultures and in

rhizosphere biofilms. Pure cultures grown overnight in selective liquid medium were used for investigation of probe specificity. The 3-cm root tip section, obtained from the same plant harvested for slurry extraction, was used for root biofilm visualization.

Fixation

Pure cultures were centrifuged and washed once with pH 7.4 phosphate-buffered saline (PBS). Paraformaldehyde (2%) in PBS was added to completely cover the pure culture cell pellets and root tip for 30 min at room temperature (RT). The samples were washed with 2% paraformaldehyde in 50% ethanol twice by gentle pipetting of the solution. The cell pellets/roots were washed with 100% ethanol and air dried. The samples were then immersed in 0.1% active diethylpyrocarbonate (DEPC; Sigma) in PBS for 12 min at RT. The DEPC solution was removed by pipetting, and the cell pellets/roots were washed with PBS and sterile deionized (DI) water. Lysozyme [5 mg/ml in Tris EDTA (TE)] was added for 30 min at RT to permeabilize the cells, and the samples were then washed in sterile DI water. Cell pellets/roots were incubated in proteinase K (1 mg/ml in TE) for 15 min at RT, washed in DI water three times, in 100% ethanol once, and air-dried.

Hybridization

The hybridization procedures were the same for processing pure cultures and root biofilm except for minor differences in sample containment. Freshly harvested wheat root was placed on glass slide in a sterile Petri dish. The procedure for processing root tips was performed with extra care not to wash off the biofilm. Pure cultures were hybridized in microcentrifuge tubes, while the biofilm was left intact on the root and hybridized on the glass slide. The samples were prehybridized in hybridization buffer [50% *v*/*v* formamide (Fisher Scientific), 2× saline-sodium citrate (SSC) (1× SSC is 15 mM sodium citrate and 0.15 M sodium chloride), 10% *w*/*v* dextran sulfate (Sigma), 1X Denhardt's solution (Sigma), and 0.2 mg/ml sheared salmon sperm DNA (QBiogene)] without probes at 47.2°C for 1 h. The cocktail containing both TOM and Ppu probes were denatured at 80°C for 5 min in the hybridization buffer. The probes were added to the samples to a final concentration of 10 mg/ml and incubated at 47.2°C for 16 h. The roots were covered with aluminum foil to prevent evaporation. After the hybridization step, the samples were subjected to post-hybridization washes at 45.2°C. The first wash was with 1× SSC with 50% formamide for 1 h. The second wash was in 0.2× SSC with 0.01% sodium dodecyl sulfate for 30 min.

Confocal microscopy

Samples were visualized using Zeiss LSM 510 confocal microscope with Plan-Neofluar 100X oil objective lens. Images of Cy3-labeled cells were captured with an excitation wavelength of 543 nm and emission wavelength of 570 nm; Alexa647-labeled cells were captured with an excitation wavelength of 633 nm and emission wavelength of 660 nm. Laser intensities and image settings were kept constant for all samples, allowing for direct comparisons of different samples. Each image acquired consisted of fluorescence from the Cy3 channel, the Alexa647 channel, and the brightfield view and a composite of the three previously mentioned layers.

Results

Root colonization of recombinant *P. putida*

Plate counts of root slurry extract were used to obtain a rapid assessment of *P. putida* TOM20 colonization of wheat root. Growth of the bacterium was determined through colonies from slurries plated on selective LB medium agar plates. Results from plate counts indicated that the CFU for the inoculated roots in both autoclaved (A/P) and non-autoclaved (NA/P) soil were significantly higher than the ones in the roots without bacterial inoculation (A/NP and NA/NP; Fig. 1).

Probe specificity and hybridization

To investigate the specificity, three strains of rhizobacteria, *P. putida* 06909 wild type, *P. fluorescens*, and *Rhizobium*

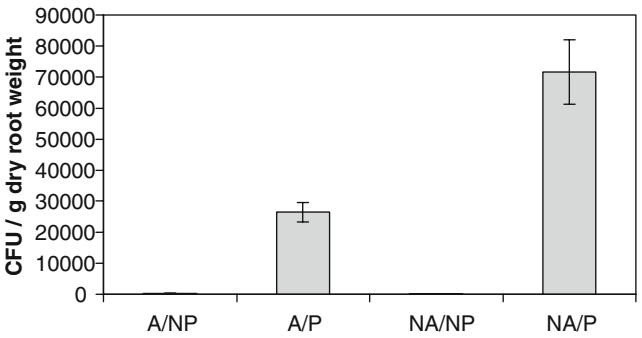

Fig. 1 Plate count data obtained from extracted wheat root slurry. Four soil treatments were compared: autoclaved and inoculated (*A/P*), autoclaved and non-inoculated (*A/NP*), non-autoclaved and inoculated (*NA/P*), and non-autoclaved and non-inoculated (*NA/NP*). Colony-forming units were normalized to dry root weight. Each data point is the mean of three independent samples, and the *error bars* represents standard error of the mean (SEM). The difference between NA/P and A/P was statistically significant (p value=0.05) using the t test with unequal variance

TOM, were simultaneously hybridized with both probes. *P. fluorescens* served as a negative control for both the TOM and Ppu probes. *P. putida* 06909 wild type was the positive control for the Ppu probe and the negative control for the TOM probe. *Rhizobium* TOM was the positive and negative control for the TOM and Ppu probes, respectively.

A cocktail containing Ppu and TOM probes were used for hybridizations. The hybridization efficiency of the probes was determined via confocal microscopy, and the temperature combination with the highest fluorescence signal for the positive controls with the lowest background for the negative controls was found to be at 47.2°C/45.2°C (hybridization/post-hybridization). Figure 2 shows the confocal microscopy images of the pure culture controls. As expected, no fluorescence in the Cy3 and Alexa647 emission ranges was detected for after hybridizing with the *P. fluorescens* cells (Fig. 2a). Hybridized *P. putida* wild-type cells exhibited fluorescence signal in the Cy3 channel and not in the Alexa647 channel (Fig. 2b). *Rhizobium* TOM cells showed fluorescence in the Alexa647 channel but not in the Cy3 channel (Fig. 2c). Hybridized *P. putida* TOM20 cells showed fluorescence in both the Cy3 and Alexa647 channels (Fig. 2d). This experiment was repeated twice with similar results.

Root biofilm imaging

To demonstrate the utility of these probes for the detection of microbes on root biofilm, samples from the inoculated (NA/P) and non-inoculated (NA/NP) plants grown in non-autoclaved soil were examined via confocal microscopy (Fig. 3). In the non-inoculated roots, native bacterial cells without fluorescent signals were observed (Fig. 3a). In the inoculated roots, we observed *P. putida* TOM20 cells attached to the root hair, and strong overlapping Cy3 and Alexa647 signals were detected (Fig. 3b). Four replicates of each treatment were hybridized and examined with confocal microscopy. The representative images are presented here.

Discussion

The root microbial community has important functions in nitrogen and carbon cycling, plant growth, and plant disease resistance (Morrissey et al. 2004; Singh et al. 2004). The symbiosis between plants and bacteria has also been applied for rhizoremediation of toxic chemicals (Anderson Coats 1995; Anderson et al. 1993; Brazil et al. 1995; Monti et al. 2005; Shim et al. 2000; Wu et al. 2006). Given the functional importance of rhizosphere bacteria, methods to examine specific root microbial gene expression and spatial distribution are needed. This research uses FISH to visualize mRNA and rRNA transcripts of a recombinant *P. putida* on wheat root. *P. putida* is a rhizosphere bacteria that has been shown to colonize a variety of plant roots. The recombinant strain is used for inoculation of the artificial microcosm to enable detection of combination of genes that does not naturally exist in the rhizosphere.

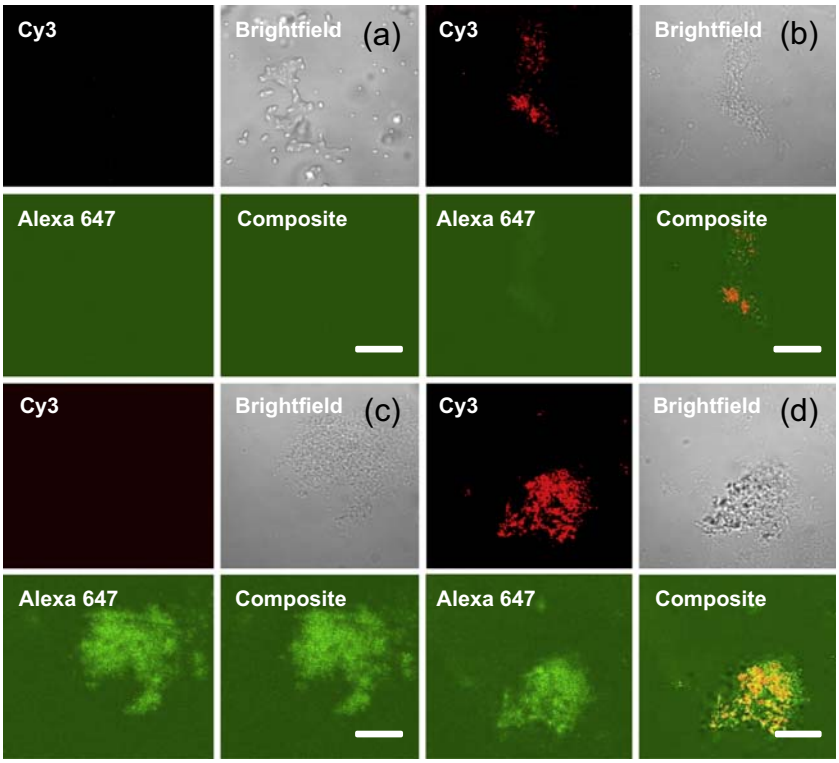

Fig. 2 Confocal microscopy of pure culture strains grown in liquid medium hybridized with the Ppu-TOM probes cocktail. Each image includes fluorescent signals from Cy3 (*top left*) and Alexa647 (*bottom left*), the brightfield (*top right*), and the composite of the three (*bottom right*). *P. fluorescens* (**a**) was used as negative control for both probes. *P. putida* wild type (**b**) was used as positive control for the Ppu probe and the negative control for the TOM probe. *Rhizobium* TOM (**c**) was used as the positive control for the TOM probe, and the negative control for the Ppu probe. *P. putida* TOM20 (**d**) pure culture exhibited signals from both Cy3 and Alexa647 fluorophores. The scale bar represents 20 μm

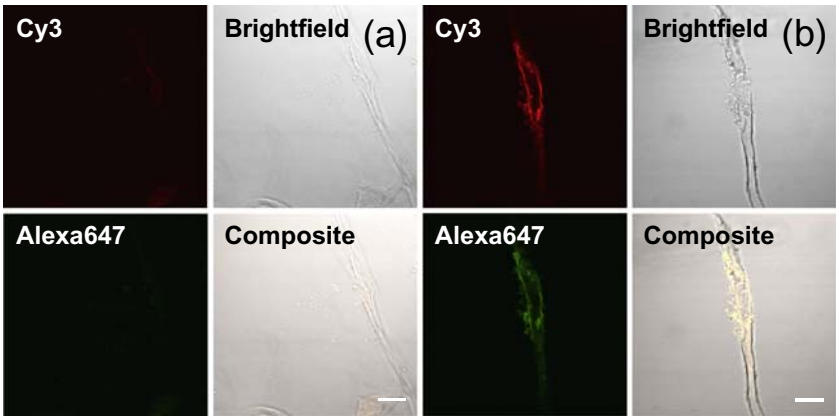

Fig. 3 Confocal microscopy of biofilms hybridized with TOM and Ppu probes on wheat roots grown in sandy soil. The *four panels* are of the Cy3 channel (*top left*), Alexa647 channel (*bottom left*), the brightfield view (*top right*), and the composite of the three layers (*bottom right*). **a** Wheat seedlings grown in non-autoclaved and non-inoculated soil (NA/NP). **b** Wheat seedling grown in non-autoclaved and inoculated soil (*NA/P*). Root from NA/NP (**a**) was used to obtain the background fluorescence, and minimal signal was observed. The image of the root from NA/P (**b**) demonstrated the detection of *P. putida* TOM20 cells exhibiting both Cy3 and Alexa647 signals. The scale bar represents 20 μm

Probe design and hybridization

In the sequence selection for the TOM probe, GC content and melting temperature similarity to the Ppu probe was taken into consideration to achieve simultaneous hybridization of both probes. Combining the hybridization steps of the two probes shortened the sample processing time, preserved sample volume, and reduced possible fluorochrome quenching. A hybridization temperature of 47.2°C was selected based on microscopy of pure cultures hybridized with different temperatures. The temperature selected yielded the highest signals for both Cy3 and Alexa647 fluors and the lowest background fluorescence. The post-hybridization temperature was set at 2°C lower than the hybridization temperature (45.2°C) to further remove non-specifically bound probes.

A GenBank BLASTN (Altschul et al. 1997) search of the TOM probe sequence resulted in significant alignments with *Burkholderia* sp. and *Ralstonia* sp., which was as expected since the *tom* genes were originally from *B. cepacia*. The complete genome sequence of *P. putida* 06909 was not available. Therefore, the 21 base pairs sequence of the TOM probe was compared to the complete genomic sequences of all available *P. putida* strains to determine possible homologous sequences. The highest similarity was 13 out of 21 base pairs with the *P. putida* F1 and KT2440 genome sequences. However, the partial homology resulted in a lower melting temperature, and the post-hybridization washes were designed to remove the partially bound probes. Hybridized pure cultures of three rhizosphere bacteria confirmed the specificity of each probe against the positive and the negative controls (Fig. 2). In addition, it was demonstrated that both probes can be hybridized simultaneously under one optimal temperature. The Ppu probe was highly specific to the species level and did not hybridize to either *P. fluorescens* or *Rhizobium* TOM (Fig. 2a, c). The TOM probe did not hybridize to *P. putida* wild type or *P. fluorescens* (Fig. 2a, b). Hybridization of both probes was demonstrated through detection of both Cy3 and Alexa647 signals in *P. putida* TOM20 pure culture (Fig. 2d).

Root sample processing

Colonies grown on plates were used as an indication of survival of the recombinant *P. putida* (Fig. 1) in the rhizosphere. A soil microbe extraction method using Nycodenz® to separate particles (Burmolle et al. 2003) was adapted and applied for the root slurry extraction. A more recent article also outlined protocols for separating microbes from soil particles using the Nycodenz® density buoyancy gradient (Bertaux et al. 2007). One caveat associated with the use of Nycodenz® is that the microbes extracted are in the fraction that are not attached to larger soil particles and do not represent the total number of bacteria present. Plating results from the large particle fraction of the Nycodenz® density gradient yielded colonies as well, albeit 25% fewer colonies than the counts from the bacterial fraction (data not shown). This result indicated that bacteria were adhering to the larger soil particles and that plate counts of the bacterial particle fraction were only a partial count. Thus, the CFUs were only used for confirmation that *P. putida* TOM20 was present in conjunction with confocal microscopy of the root biofilm and not as direct cell count.

Wheat roots (A/NP and NA/NP) not inoculated with *P. putida* TOM20 resulted in CFU that were at least two orders of magnitude lower than the CFU from the inoculated soil (A/P and NA/P) (Fig. 1). The CFU represents the bacteria that can grow on selective medium

containing kanamycin, ampicillin, tetracycline, and amphotericin B and is representative of the *P. putida* TOM20 population. The original purpose of including autoclaved soil in the treatment was to stunt the growth of the indigenous microbial population to allow the recombinant bacterium to colonize. However, the plate count data (Fig. 1) indicated that autoclaving deterred bacterial growth, which could have been a result of altered soil properties from heating to 120°C.

During the initial stage of this research, the TOM probe was first labeled with the fluorescein fluor, which had an excitation wavelength of 470 nm and emission of 520 nm. However, confocal microscopy indicated that the roots had similar excitation and emission wavelengths, thus the TOM probe was subsequently labeled with a near-infrared fluor (Alexa647) to reduce background signal detection. Autofluorescence interference with the fluorescent signals was still observed in certain areas of the root; however, the intensity was significantly lower than the fluorescence captured at emission wavelength of 520 nm. Background fluorescence was also a main challenge reported by others (Briones et al. 2002; Watt et al. 2006).

On the thinner root hairs, low autofluorescence was observed, and some cells did not overlap with the root, enabling detection of fluorescence signals from the bacteria (Fig. 3). Wheat roots from non-autoclaved soils were compared (NA/NP and NA/P). From the plate count data (Fig. 1), NA/P had significantly more CFU than NA/NP, indicating that the recombinant *P. putida* TOM20 was present in the inoculated roots. Confocal microscopy of NA/P (Fig. 3b) confirmed the presence of *P. putida* TOM20 hybridized to both Ppu and TOM probes. In the control root (NA/NP), without *P. putida* TOM20 inoculation, no fluorescence in the Cy3 and Alexa647 emission ranges was detected (Fig. 3a). In general, individual cells or cell clusters were observed at the root cap, on root hair, embedded in the dead root, or separated from the root tips. The microscopy images demonstrated detection of the recombinant rhizobacterium on wheat root even in the presence of plant mucilage and trace amount of soil organic matter.

This investigation optimizes the hybridization techniques allowing for the detection of the recombinant *P. putida* strain on wheat root by targeting both the mRNA and rRNA. The capability to observe specific bacterial species in situ can lead to numerous potential applications. The survival and cell activity of a bacterium in the rhizosphere can be characterized simultaneously without the need for cultivation. Monitoring of genes associated with carbon, nitrogen, and phosphorus cycling will lead to understanding of the role of plant–microbe interaction in carbon sequestration and nutrient mineralization (Singh et al. 2004). The study of the spatial distribution of bacteria on the root will contribute to our understanding of microbial ecology of rhizosphere biofilms and mechanisms of plant root–microbial symbiosis.

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