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Technical Advance

Simultaneous Imaging of *Pseudomonas fluorescens* WCS365 Populations Expressing Three Different Autofluorescent Proteins in the Rhizosphere: New Perspectives for Studying Microbial Communities

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To visualize simultaneously different populations of pseudomonads in the rhizosphere at the single cell level in a noninvasive way, a set of four rhizosphere-stable plasmids was constructed expressing three different derivatives of the green fluorescent protein (GFP), namely enhanced cyan (ECFP), enhanced green (EGFP), enhanced yellow (EYFP), and the recently published red fluorescent protein (RFP; DsRed). Upon tomato seedling inoculation with *Pseudomonas fluorescens* WCS365 populations, each expressing a different autofluorescent protein followed by plant growth for 5 days, the rhizosphere was inspected using confocal laser scanning microscopy. We were able to visualize simultaneously and clearly distinguish from each other up to three different bacterial populations. Microcolonies consisting of mixed populations were frequently observed at the base of the root system, whereas microcolonies further toward the root tip predominantly consisted of a single population, suggesting a dynamic behavior of microcolonies over time. Since the cloning vector pME6010 has a broad host range for gram-negative bacteria, the constructed plasmids can be used for many purposes. In particular, they will be of great value for the analysis of microbial communities, for example in processes such as biocontrol, biofertilization, biostimulation, competition for niches, colonization, and biofilm formation.

Additional keywords: fluorescence, triple color imaging.

Biological control of soilborne pathogens by *Pseudomonas fluorescens* is usually based on (i) the production of an antifungal factor (AFF) by the bacterium and (ii) an efficient delivery of this AFF along the root system by root colonization (Lugtenberg et al. 1991, 2000; Thomashow and Weller 1995). However, the efficacy of biocontrol bacteria requires further improvement. Therefore, fundamental knowledge about spatiotemporal interactions between the bacterium, the plant, the phytopathogenic fungus, and the endogenous microbial population are required. In order to visualize and understand *Pseudomonas* root colonization and interactions with other bacteria and fungi in the rhizosphere, we expressed multiple autofluorescent proteins (AFPs).

Green fluorescent protein (GFP), isolated from the jellyfish *Aequorea victoria*, has been the most revolutionary reporter in biology since its application as a marker was published by Chalfie et al. (1994). The major advantage of GFP as a reporter is its noninvasive analysis without the need for exogenous substrates or energy. The GFP is a very suitable marker for studying bacterial behavior at the single cell level in the rhizosphere, such as *Pseudomonas* root colonization (Bloemberg et al. 1997; Normander et al. 1999; Ramos et al. 2000; Tombolini et al. 1997, 1999) and rhizobial nodulation (Gage et al. 1996; Xi et al. 1999). Recently, color variants of the GFP, e.g., enhanced cyan (ECFP), enhanced green (EGFP), and enhanced yellow (EYFP), with shifted excitation and emission maxima have been developed and used for dual color imaging (Ellenberg et al. 1999; Matus 1999; Tsien 1998; Yang et al. 1998). Most recently, the red fluorescent protein (RFP; DsRed) (Matz et al. 1999) isolated from *Discosoma* spp. has broadened the range of AFPs, creating the opportunity for triple color imaging.

We report the construction of four rhizosphere-stable plasmids for constitutive expression of *ecfp*, *egfp*, *eyfp*, and *rfp* (DsRed), respectively, in *P. fluorescens*, rhizobia, and other gram-negative bacteria. Using confocal laser scanning microscopy, we showed that all four plasmids are highly suitable reporter vectors for visualization of bacteria at the single cell level in the rhizosphere. Here, we report, for what we believe is the first time, dual and even triple imaging of mixed *Pseudomonas* populations in the rhizosphere, each expressing a different AFP.

RESULTS

Construction of rhizosphere-stable plasmids for the expression of AFP in *P. fluorescens*.

A set of four rhizosphere-stable plasmids was constructed to express the *egfp*, *ecfp*, *eyfp*, and *rfp* genes under control of the

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lac promoter (Fig. 1), which resulted in plasmids pMP2444, pMP4516, pMP4518, and pMP4661, respectively. Since pBBR1-based plasmids appeared not to be stable in *P. fluorescens* during subsequent subculturing in medium without antibiotic pressure (data not shown), they were fused to the rhizosphere-stable cloning vector pME6010, which is based on the pVS1 replicon (Heeb et al. 2000). This resulted in plasmids pMP4655, pMP4641, pMP4658, and pMP4662, respectively (Fig. 1). The latter plasmids were transformed into the efficient root-colonizing strain *P. fluorescens* WCS365 and tested for stability in the rhizosphere. After testing several hundreds of bacteria for each construct reisolated from the root tip of tomato plants 7 days after seedling inoculation and subsequent plant growth in a gnotobiotic quartz sand system, no loss of the plasmids was observed. The constructs were also positively tested for stability in the rhizosphere bacterium *Rhizobium* spp. (data not shown) (Stuurman et al. 2000); no loss of the plasmid was observed after reisolation from the rhizosphere. Apparently, pME6010 tolerates insertions of pBBR1 in both orientations, without loss of plasmid stability. Expression of the AFP genes in *Escherichia coli* as well as in *P. fluorescens* WCS365 was confirmed by epifluorescence microscopic analysis. However, expression of DsRed could hardly be detected after growth of *E. coli* at 37°C and *P. fluorescens* WCS365 at 28°C. This was probably due to instability at these temperatures, since expression of DsRed in *P. fluorescens* could easily be detected on tomato root surfaces of plants grown at 21°C. Expression of the different AFP genes had no significant effect on the growth rate of *P. fluorescens* WCS365 when the strains were grown in liquid King's medium B (KB) but resulted in a slightly longer lag phase (Fig. 2) in comparison to the wild-type strain WCS365.

Quantification of AFPs expressed in *E. coli* and *P. fluorescens*.

Quantification of AFP expression in *E. coli* and *P. fluorescens* (Fig. 3) was performed to (i) compare the relative intensities of the different AFPs, (ii) compare expression of the AFPs in *E. coli* and *P. fluorescens*, and (iii) analyze the overlap in excitation and emission of the strains expressing *ecfp*, *egfp*, or *eyfp*. Expression of *rfp* (encoding DsRed) in *E. coli*

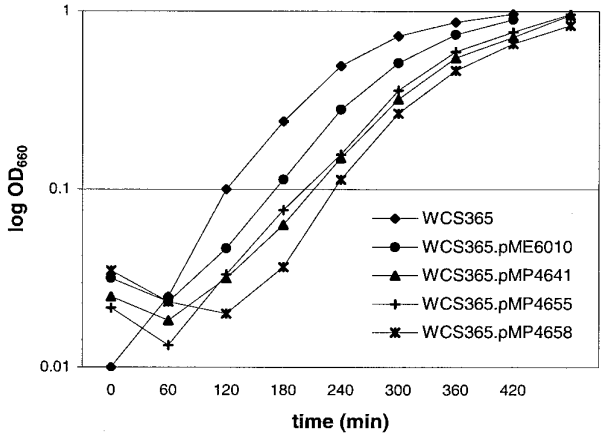

Fig. 2. Growth of *Pseudomonas* strains expressing *afp* genes. Overnight cultures of *P. fluorescens* WCS365 harboring pME6010, pMP4641 (*ecfp*), pMP4655 (*egfp*), and pMP4658 (e*yfp*), respectively, were diluted to an optical density at 660 nm of 0.01 to 0.5 in fresh King's medium B. Optical density of the cultures was measured during growth at 28°C under vigorous shaking. The presented data are mean values of three replicate experiments.

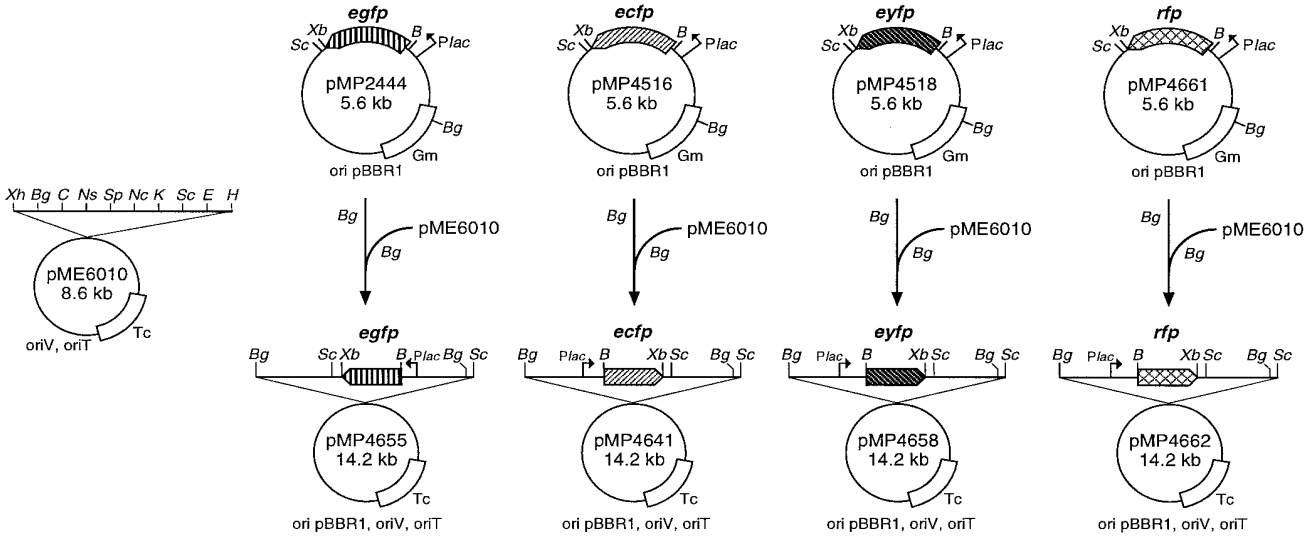

Fig. 1. Construction of plasmids to express autofluorescent proteins in gram-negative bacteria. Plasmid pME6010 (Heeb et al. 2000) was used as a cloning vector to construct rhizosphere-stable reporter plasmids expressing *egfp*, *ecfp*, *eyfp*, and *rfp*, respectively, under the control of the *lac* promoter. Plasmids pMP2444, pMP4516, pMP4518, and pMP4661, all containing an origin of replication of the pBBR1 class, were fused with pME6010 by restriction with *Bgl*II followed by ligation, resulting in pMP4655, pMP4641, pMP4658, and pMP4662, respectively. Orientations of the autofluorescent protein genes are indicated. Abbreviations: B = *Bam*HI, Xh = *Xho*I, Bg = *Bgl*II, C = *Cla*I, Ns = *Nsi*I, Sp = *Sph*I, Nc = *Nco*I, K = *Kpn*I, E = *Eco*RI, H = *Hin*dIII, Sc = *Sac*I, Xb = *Xba*I, Tc = tetracycline, Gm = gentamicin, P*lac* = *lac* promoter, *egfp* = enhanced green fluorescent protein, e*cfp* = enhanced cyan fluorescent protein, *eyfp* = enhanced yellow fluorescent protein, and *rfp* = DsRED protein.

and *P. fluorescens* was not included, since we could not observe expression by epifluorescence microscopy in *E. coli* and *P. fluorescens*. Under the filter conditions of the fluorometer, strains expressing *egfp* show the highest relative fluorescence,

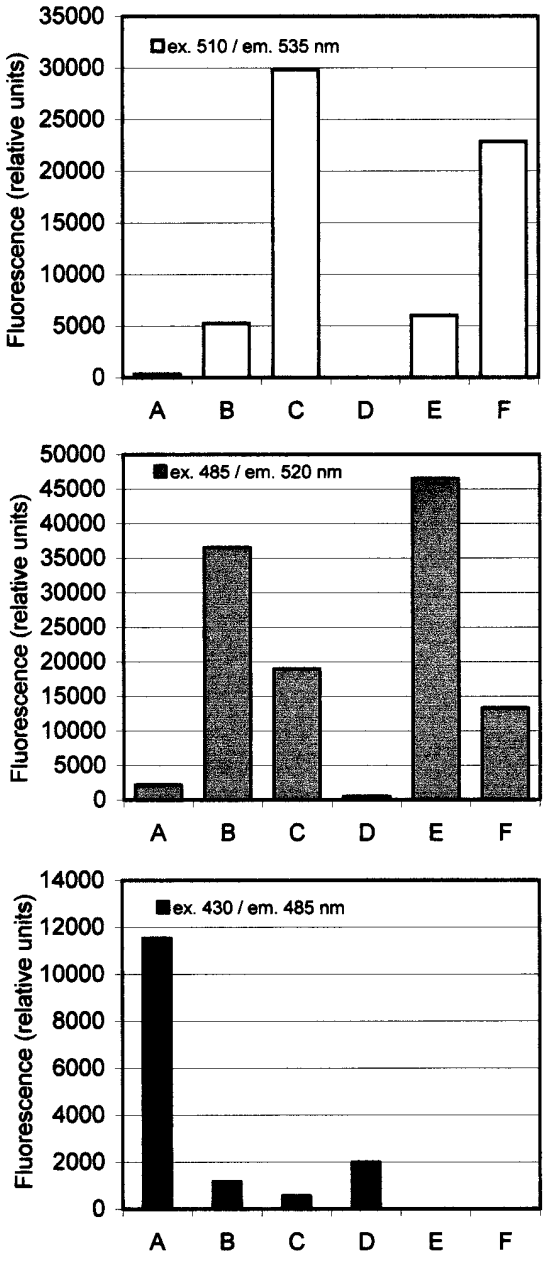

Fig. 3. Quantification of autofluorescent proteins in *Escherichia coli* and *Pseudomonas fluorescens*. Overnight cultures of *E. coli* DH5α and *P. fluorescens* WCS365 harboring plasmids pMP4641 (*ecfp*), pMP4655 (*egfp*), or pMP4658 (*eyfp*), respectively, were diluted into fresh Luria-Bertani medium or King's medium B to an optical density at 660 nm of 0.6. Fluorescence of the diluted cultures was quantified in a 96-well titer plate containing aliquots of 200-µl cultures using a plate reader fluorometer with excitation (ex) and emission (em) filters for analysis of enhanced yellow fluorescent protein (EYFP; ex 510 nm and em 535 nm), enhanced green fluorescent protein (EGFP; ex 485 nm and em 520 nm), and enhanced cyan fluorescent protein (ECFP; ex 430 nm and em 485 nm), respectively. Depicted in this figure are the emission values of strains *E. coli* DH5α harboring **A,** pMP4641; **B,** pMP4655; or **C,** pMP4658 and *P. fluorescens* WCS365 harboring **D,** pMP4641; **E,** pMP4655; or **F,** pMP4658. The presented data were corrected for autofluorescence.

followed by strains expressing *eyfp* and *ecfp*, respectively (Fig. 3). Expression of the *afp* genes could easily be detected in all strains, with relatively small differences between *E. coli* and *P. fluorescens*, except *ecfp* expression was much lower (but still clearly detectable) in *P. fluorescens* than in *E. coli*. Figure 3 shows that excitation and emission for EGFP and EYFP clearly overlap as can be expected from their excitation and emission spectra (Tsien 1998). However, negligible overlap of the emission and excitation data between ECFP and EGFP as well as between ECFP and EYFP was observed.

Confocal laser scanning microscopy analysis of tomato root colonization by *P. fluorescens* expressing various AFPs.

In order to visualize *P. fluorescens* bacteria colonizing tomato roots, 2-day-old germinated tomato seedlings, with roots of approximately 1 cm in length, were inoculated with *P. fluorescens* WCS365 derivatives expressing ECFP, EGFP, EYFP, or DsRed. After inoculation, the seedlings were grown in a gnotobiotic quartz sand system for 5 days and the root systems with an average length of 7 cm subsequently examined for the presence of bacterial cells using confocal laser scanning microscopy. Microcolonies consisting of mixed populations were predominantly observed in the upper half of the root system, whereas colonies in the lower half tended to consist of predominantly one population. However, mixed microcolonies could still be observed in the lower part. Figure 4 shows images of *P. fluorescens* microcolonies on the tomato root surface present in the region located between 3 and 4 cm below the root base. Confocal microscope images of tomato plants inoculated with single *P. fluorescens* populations show that *ecfp* (Fig. 4A), *egfp* (Fig. 4B), *eyfp* (Fig. 4C), and DsRed (Fig. 4D) are expressed in the rhizosphere and are all highly suitable markers for visualization of bacteria at the single cell level in the rhizosphere. The latter observation was remarkable, since expression of *rfp* in *P. fluorescens* was not observed by epifluorescence microscopy when cells were grown at 28°C. Subsequently, tomato seedlings were inoculated with mixed cultures of two differently labeled *P. fluorescens* WCS365 derivatives. *Pseudomonas* cells expressing *ecfp* and *egfp* could clearly be distinguished from each other in microcolonies (Fig. 5A). The same applies for *Pseudomonas* cells expressing *ecfp* and *eyfp* (Fig. 5B and C). Since the emission spectrum of DsRed does not overlap with that of the other AFPs, tomato seedlings were inoculated with a mixture of three *P. fluorescens* derivatives expressing *ecfp*, *egfp*, and *rfp*, respectively. Triple imaging of root surfaces after growth in the gnotobiotic sand system resulted in visualization of microcolonies consisting of all three populations (Fig. 5D). Bacteria expressing the three different AFPs could easily be distinguished from each other.

DISCUSSION

In order to study root colonization and interactions of (biocontrol) pseudomonads in the rhizosphere, a set of reporter vectors was constructed for constitutive expression of *ecfp*, *egfp*, *eyfp*, and *rfp*, respectively, under control of the *lac* promoter (Fig. 1). In *P. fluorescens*, the resulting plasmids were shown to be stable during growth in the rhizosphere (without antibiotic pressure). Since the plasmids are based on

cloning vector pME6010 (Heeb et al. 2000), which contains a pVS1 replicon, the constructed plasmids are presumably stable in many other gram-negative bacteria and highly suitable to visualize bacteria in environments where no antibiotic pressure can be applied. We show here that expression of AFPs under control of the *lac* promoter does not influence the growth rate of *P. fluorescens* in KB, although the lag time was extended (Fig. 2). Expression of AFPs can easily be quantified using a plate reader fluorometer, which provides opportunities for gene expression studies.

Due to (i) highly advanced confocal laser microscopy equipment that allows sequential scanning and detection of emitted light at freely selectable wavelengths and (ii) the recent availability of the DsRed protein (Matz et al. 1999), we

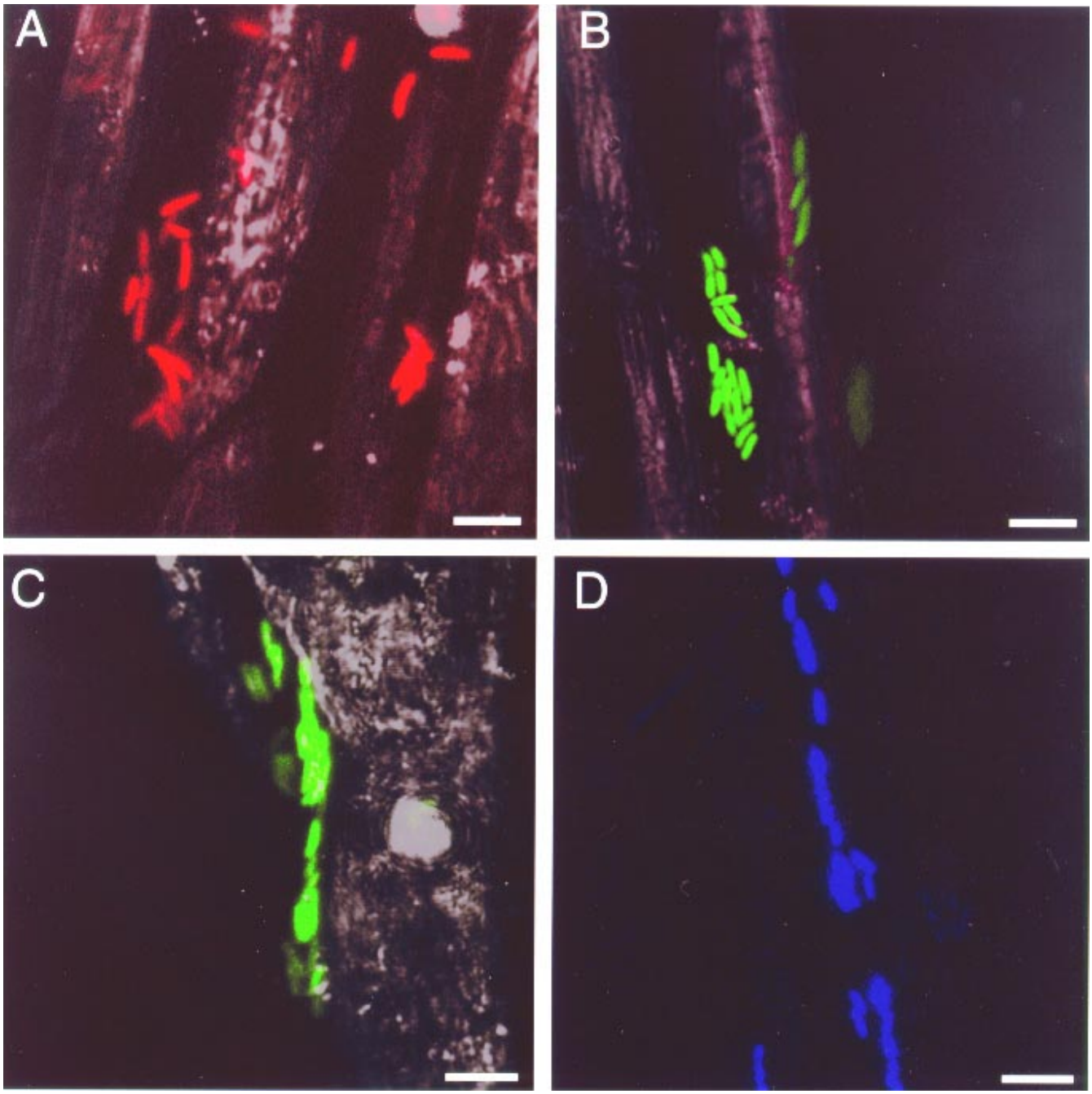

Fig. 4. Confocal laser scanning microscopical analysis of tomato root colonization by *Pseudomonas fluorescens* WCS365 expressing autofluorescent proteins. Two-day-old germinated tomato seedlings were inoculated with *P. fluorescens* WCS365 harboring reporter plasmids expressing various *afp* genes. After inoculation, plants were grown in a gnotobiotic quartz sand system. After 5 days of growth in the gnotobiotic system, roots were examined for the presence of fluorescent bacteria by confocal laser scanning microscopy (details in Materials and Methods). Tomato root surfaces colonized by *P. fluorescens* WCS365 harboring **A,** pMP4641 (*ecfp*); **B,** pMP4655 (*egfp*); **C,** pMP4658 (*eyfp*); or **D,** pMP4662 (*rfp*). Enhanced cyan fluorescent protein (ECFP)-expressing bacteria appear red, enhance green fluorescent protein (EGFP)- and enhanced yellow fluorescent protein (EYFP)-expressing bacteria green, and red fluorescent protein (DsRed)-expressing bacteria blue. The size bar represents 5 μm in all panels.

have been able to tag *Pseudomonas* bacteria with four different AFPs and to visualize them in the rhizosphere (Fig. 4). It was shown that *Pseudomonas* populations expressing *ecfp*, *rfp*, and *egfp* or *eyfp* were clearly distinguishable from each other in the rhizosphere (Fig. 5). We succeeded, for what we believe is the first time, in visualizing and clearly distinguishing three different *Pseudomonas* populations simultaneously in the rhizosphere in a noninvasive way (Fig. 5D). Visualization of mixed *P. fluorescens* populations showed that mixed microcolonies can easily be detected in the upper part

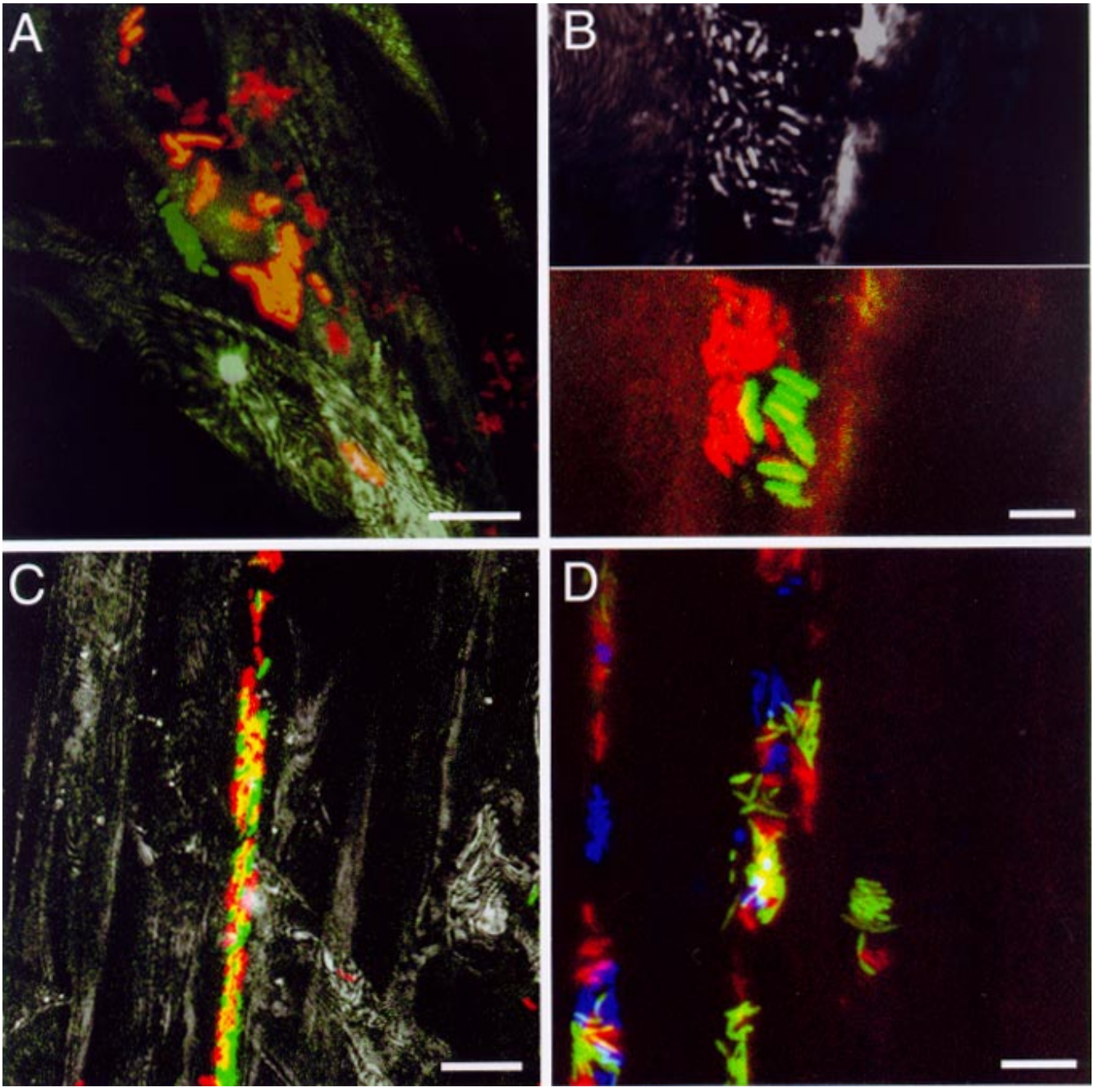

Fig. 5. Confocal laser scanning microscopical analysis of tomato root colonization by *Pseudomonas fluorescens* WCS365 expressing different autofluorescent proteins. **A, B, and C,** Parts of the lower half of tomato roots, the seedlings of which were inoculated with mixtures of two *P. fluorescens* WCS365 derivatives expressing different *afp* genes (details on growth in Fig. 4 caption): **A,** pMP4641 (e*cfp*) and pMP4655 (*egfp*); **B and C,** pMP4641(e*cfp*) and pMP4658 (e*yfp*). **B,** The upper part shows an image obtained by measuring the light reflected by surfaces present in the object, whereas the lower part shows the same area with only the enhanced cyan fluorescent protein (ECFP)-specific fluorescence in red and the enhanced yellow fluorescent protein (EYFP)-specific fluorescence in green. **D,** Part of the root surface of a tomato plant, the seedling of which had been inoculated with a mixture of three *P. fluorescens* WCS365 derivatives harboring pMP4641 (*ecfp*), pMP4655 (*egfp*), and pMP4662 (*rfp*), respectively. In the panels, enhanced cyan fluorescent protein (ECFP)-expressing bacteria appear red, enhanced green fluorescent protein (EGFP) and enhanced yellow fluorescent protein (EYFP) expressing bacteria appear green, and red fluorescent protein (DsRed)-expressing bacteria appear blue. White represents the reflected light. **C and D,** Yellow color is caused by overlap of red and green colors. The size bars represents **A and B,** 5 µm; and **C and D,** 10 µm.

of the root system. In the lower part of the root system, colonies completely consisted of one population, to which sometimes several bacteria (visible as single cells) from the other population are attached. These results suggest the following sequence of events. (i) Microcolonies are started by one bacterium that will divide over time, resulting in the formation of the microcolony. (ii) Other bacteria can reach the same site at a later time point and can become part of the same microcolony. (iii) The site to be colonized is most likely a site of exudation.

Currently, many studies of bacteria in natural environments show that they live in complex communities, predominantly in biofilms, where they interact with other species. The novelty of the results presented in this paper provides many new perspectives for analyzing the formation and function of microbial populations and communities.

MATERIALS AND METHODS

Bacterial strains and growth conditions.

P. fluorescens strain WCS365 (Geels and Schippers 1983; Simons et al. 1996) was routinely cultured in KB (King et al. 1954) at 28°C. When appropriate, tetracycline was added to the culture medium to a final concentration of 80 μg/ml. *E. coli* strain DH5α was routinely cultured in Luria-Bertani medium (LB) (Sambrook et al. 1989) and, when appropriate, supplemented with tetracycline (final concentration of 20 μg/ml) or gentamicin (final concentration of 10 μg/ml).

Construction of plasmids, plasmid stability, and *afp* expression.

Initially, *egfp*, *ecfp*, and *eyfp* (Clontech, Palo Alto, CA, U.S.A.) were cloned into the broad-host-range vector pBBR1MCS-5 (Kovach et al. 1995), resulting in plasmids pMP2444, pMP4516, and pMP4518, respectively (Stuurman et al. 2000). Introduction of these plasmids into *P. fluorescens* strain WCS365 resulted in clear expression of the various AFP genes (data not shown). To obtain rhizosphere-stable plasmids expressing *afp*s, plasmids pMP2444, pMP4516, and pMP4518 were restricted with *Bgl*II and fused by ligation with pME6010 (Heeb et al. 2000), resulting in plasmids pMP4655, pMP4641, and pMP4658, respectively (Fig. 1). In a later stage, the very recently marketed pDsRED vector (Clontech) was used to clone the DsRed gene as a *Bam*HI-*Xba*I fragment into cloning vector pBBR1MCS-5, resulting in plasmid pMP4661. Fusion of pMP4661 with pME6010 using the mutual *Bgl*II site resulted in plasmid pMP4662 (Fig. 1). After transforming these plasmids to *P. fluorescens* WCS365 by electroporation, stability of the plasmids in the rhizosphere (without the presence of antibiotic pressure) was determined after inoculating tomato seedlings, as described below, with the WCS365 derivatives and growth of the seedlings in a gnotobiotic quartz sand system. After 7 days, bacteria were isolated from the root tip as described before (Simons et al. 1996) and plated on KB agar plates without antibiotics. From each WCS365 derivative, 200 colonies were subsequently tested for the presence of the plasmid on KB supplemented with tetracycline. Expression of *egfp*, *ecfp*, and *eyfp* in *E. coli* and *P. fluorescens* was quantified using a HTS7000 Bio Assay Reader (Perkin & Elmer Life Sciences, Oosterhout, The Netherlands). Overnight cultures were diluted in fresh LB or KB to an optical density at 660 nm of 0.6 using a LKB Biochrom Nova Spectrometer (Amersham Pharmacia Biotech, Roosendaal, The Netherlands). Fluorescence of the diluted cultures was quantified using a white, 96-well titer plate containing 200-μl culture aliquots. Fluorescence of the cultures was determined with excitation filters having maxima at 430 (± 35 nm), 485 (± 20 nm), and 510 nm (± 10 nm) and emission filters having maxima at 485 (± 20 nm), 520 (± 10 nm), and 535 nm (± 25 nm) for quantification of ECFP, EGFP, and EYFP, respectively.

Growth of tomato seedlings in a gnotobiotic test system.

Tomato plants were grown in a gnotobiotic sand system as described previously by Simons et al. (1996). Tomato seeds (*Lycopersicon esculentum* Mill. cv. Carmello) were kindly provided by Novartis B.V., Enkhuizen, The Netherlands. To inoculate tomato seedlings overnight, bacterial cultures were diluted to 10^7 CFU/ml. Tomato seeds were sterilized, germinated, and inoculated, and seedlings were grown under conditions described previously (Simons et al. 1996).

Microscopy.

Bacteria harboring plasmids with AFP genes were examined using a Leica MZFLIII stereo microscope equipped with epifluorescence detection (Leica, Bensheim, Germany). Filter sets tailored to the specific chromophores were used (for ECFP, 440/21-nm excitation with 480/36-nm emission; for EGFP, 480/40-nm excitation with 510-nm long pass emission; for EYFP, 500/10-nm excitation with 518/16-nm emission; and for DsRed, 510/20-nm excitation with 560/40-nm emission).

Tomato roots colonized by *P. fluorescens* after 5 days of growth in the gnotobiotic sand system were washed in phosphate-buffered saline (PBS) to remove sand particles and mounted in PBS on an attached coverslip. Samples were examined with an inverted fluorescence microscope (DMIRBE; Leica) equipped with filter blocks with spectral properties matching those of ECFP (440/21-nm excitation with 480/36-nm emission; XF114, Chroma, Brattleboro, VT, U.S.A.), or EGFP and EYFP (470/20-nm excitation with 515-nm long pass emission; I3, Leica), or DsRed (538/22-nm excitation with 590-nm emission long pass emission; N2.1, Leica), to which a Leica SP confocal scanhead was attached. Dual and triple color images were acquired by sequentially scanning with settings optimal for ECFP (excitation with the 457-nm argon laser line, emission detection between 470 and 490 nm), followed by settings optimal for EGFP (excitation with the 488-nm argon laser line, detection of emitted light between 500 and 520 nm) or EYFP (excitation with the 488-nm argon laser line, detection of emitted light between 530 and 550 nm), and finally by settings optimal for DsRed (excitation with the 568-nm krypton laser line, detection of emitted light between 580 and 620 nm). Reflected light images were obtained by detection of light at the wavelength used for excitation. Cross talk between the channels in this setup was always monitored and appeared to be negligible in all cases. The projections of the individual channels were merged in Photoshop 5.0 (Adobe, San Jose, CA, U.S.A.) to facilitate visualization.

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