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Green Fluorescent Protein as a Marker for *Pseudomonas* spp.

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The development of sensitive methods for observing individual bacterial cells in a population in experimental models and natural environments, such as in biofilms or on plant roots, is of great importance for studying these systems. We report the construction of plasmids which constitutively express a bright mutant of the green fluorescent protein of the jellyfish *Aequorea victoria* and are stably maintained in *Pseudomonas* spp. We demonstrate the utility of these plasmids to detect individual cells in two experimental laboratory systems: (i) the examination of a mixed bacterial population of *Pseudomonas aeruginosa* and *Burkholderia cepacia* attached to an abiotic surface and (ii) the association of *Pseudomonas fluorescens* WCS365 with tomato seedling roots. We also show that two plasmids, pSMC2 and pGB5, are particularly useful, because they are stable in the absence of antibiotic selection, they place an undetectable metabolic burden on cells that carry the plasmids, and cells carrying these constructs continue to fluoresce even after 7 days in culture without the addition of fresh nutrients. The construction of improved *Escherichia coli-Pseudomonas* shuttle vectors which carry multiple drug resistance markers also is described.

Pseudomonas spp., whether playing a role as pathogens or plant-beneficial root colonizers, are found in environmental, clinical, and industrial settings predominantly as biofilms (10, 15, 33). The formation of biofilms has been the focus of intense study, with microscopy as a key tool (11, 18, 20). While electron and light microscopy have been used to visualize cells in a biofilm, preparation of samples often requires techniques which kill the cell or alter the structure of the sample (8, 10). The advent of confocal scanning laser microscopy allows the visualization of living biofilms but requires fluorescently marked or stained cells (20).

The development of sensitive methods for monitoring bacteria in laboratory model systems and natural environments, especially in biofilms or on plant roots, is of great importance. The green fluorescent protein (GFP) of the jellyfish *Aequorea victoria* has proved to be valuable as a tool for studying a variety of biological questions (1, 7, 37). GFP is useful for examining biological phenomena because cells can be studied nondestructively and without addition of exogenous substrates. Additionally, GFP-marked cells can be visualized by using standard microscopes equipped with commonly available fluorescent filter sets (7).

Here we report the construction and evaluation of plasmids which constitutively express a bright mutant of GFP (9) and are stably maintained in *Pseudomonas* strains. We demonstrate the utility of these plasmids to visualize individual cells in two systems: (i) a mixed bacterial population of *Pseudomonas aeruginosa* and *Burkholderia cepacia* attached to an abiotic surface and (ii) the association of *Pseudomonas fluorescens* WCS365 with tomato seedling roots. We also describe the construction of improved *Escherichia coli-Pseudomonas* shuttle vectors which carry multiple drug resistance markers.

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MATERIALS AND METHODS

Bacterial strains, media, and chemicals. The strains and plasmids used in this study are listed in Table 1. All strains were grown on rich medium (Luria-Bertani [LB] medium) at 37°C, except *P. fluorescens* WCS365, which was grown at 28°C. Where appropriate, the culture medium was supplemented with antibiotics at the following concentrations: ampicillin, 500 μg/ml; tetracycline, 150 μg/ml; and carbenicillin, 1 mg/ml. The minimal medium used was standard succinate medium (SSM [24]) (supplemented with biotin [20 μg/ml], thiamine [20 μg/ml], and 1× trace elements) unless otherwise indicated. The 400× stock of trace elements contained $MnSO_4$ (0.61 g/liter), $ZnSO_4 \cdot 7H_2O$ (0.1 g/liter), H_3BO_3 (1.27 g/liter), $Na_2MoO_4 \cdot 2H_2O$ (0.4 g/liter), and $CuSO_4$ (0.04 g/liter). All enzymes for DNA manipulation were purchased from New England Biolabs (Beverly, Mass.). All plasmids were constructed in *E. coli* DH5α by standard protocols (2) and then transferred to *Pseudomonas* by electroporation (see below). Motility assays were performed on 0.05× LB agar plates made with 0.3% agar, and the distance migrated through the agar was measured after 24 h.

Electroporation. Electroporations were performed as reported previously (30) or with a rapid method described below (modified from the method of D. Boyd [3a]). A large clump of cells (~10^{10} cells) of the appropriate organism was scraped from a plate and resuspended in 1 ml of ice-cold double-distilled water (ddH_2O). The cells were washed three times with 1 ml of ice-cold ddH_2O and then resuspended in a small volume (50 to 150 μl) of ice-cold 10% glycerol. Approximately 0.5 to 1 μg of plasmid DNA was mixed with 50 μl of these cells, added to a 2-mm-gap electroporation cuvette, and electroporated at 1.8 kV with an E.coliPulser (Bio-Rad, Melville, N.Y.). The electroporated cells were diluted into 1 ml of LB medium and grown for 2 h before being plated on LB agar supplemented with the appropriate antibiotic. For *Pseudomonas* spp., plating 250 μl of this culture typically yielded 100 to 500 colonies (~500 electroporants/μg of DNA).

Quantitation of fluorescence. To determine the fluorescence intensities of strains carrying various GFP constructs, we utilized a fluorescence spectrometer (model ILS50B; Perkin-Elmer, Beaconsfield, United Kingdom). In all cases, the excitation wavelength used was 480 nm and emission intensity was measured at 511 nm with a 1-s integration time. A slit width of 5 nm was used for both excitation and emission. Cultures were grown in LB or SSM (as indicated for each experiment) and diluted to an A_{620} of 0.25 (in the medium in which they were grown) for subsequent analysis. Emission scans of all bacterial strains used in these studies, from 400 to 560 nm with an excitation wavelength of 480 nm, showed no emission peaks when the strain did not carry a GFP plasmid.

Attachment assay. Minimal M63 salts medium (25) supplemented with glucose (0.2%), $MgCl_2$ (1 mM), and Casamino Acids (0.5%) was inoculated with a 1:100 dilution of an overnight LB culture (~10^9 CFU/ml) in a test tube containing a small tab of polyvinylchloride plastic cut to approximately 3 by 6 mm from a microtiter dish made of this material (Becton Dickson, Oxnard, Calif.). This plastic tab was sterilized with 100% ethanol and served as a surface on which a biofilm was able to form. After 4 h of incubation standing at 37°C, the plastic tab was removed from the test tube, rinsed thoroughly with sterile ddH_2O, blotted gently to remove excess liquid, and mounted on a microscope slide. The plastic tab was then examined with a Standard 16 microscope modified with an epiflu-

TABLE 1. Bacterial strains and plasmids

Bacterial strain or plasmid	Description	Reference(s) or source
Strains		
P. aeruginosa PAO1		14
P. fluorescens WCS365		17, 29
B. cepacia 5116-1		G. Pier
E. coli DH5α		27
E. coli 1164		31
Plasmids		
pBR322	Inc ColE1, cloning vector; Ap^r Cb^r Tc^r	3
pUCP18	Inc ColE1, cloning vector; Ap^r Cb^r	28
pUCP19	Inc ColE1, cloning vector; Ap^r Cb^r	28
pUC181.8	pUC18 containing 1.8-kb *Pst*I fragment for maintenance in *Pseudomonas*; Ap^r Cb^r	16
pWTT2081	Carries origins of pACYC184 and pCS1; Tc^r	36
pGB1	pUCP18 containing 1.8-kb fragment from pWTT2081 carrying Tc^r	This study
pGB2	pUCP19 containing 1.8-kb fragment from pWTT2081 carrying Tc^r	This study
pGB3	pGB1 containing *gfp* from pSMC2; Tc^r Ap^r Cb^r	This study
pGB4	pBR322 carrying the 1.8-kb stabilizing fragment from pUC181.8; Ap^r Cb^r Tc^r	This study
pGB5	Fusion of pWTT2081 and pSMC2; Ap^r Cb^r Tc^r	This study
pmut2	pKEN carrying bright mutant of *gfp*; Ap^r	9
pSMC2	pmut2 containing 1.8-kb stabilizing fragment from pUC181.8; Ap^r Cb^r	This study

orescence illumination kit (Zeiss, Oberkochen, Germany) and equipped with a standard fluorescein isothiocyanate excitation-emission filter set (Zeiss) and a 35-mm camera (Nikon FX-35DX). Photographs were made with Kodak Royal Gold 400-speed color print film (Eastman Kodak Co., Rochester, N.Y.) with an exposure time of 1 to 2 s (phase-contrast micrographs) or 3 to 5 s (fluorescent micrographs) at a magnification of ×1,000.

Root association assay. To test plasmid stability in the rhizosphere, tomato seeds (*Lycopersicon esculentum* Mill. cv. Carmello; S&G Seeds B. V., Enkhuizen, The Netherlands) were sterilized, germinated, inoculated with *P. fluorescens* WCS365 carrying plasmid pGB5, and grown for 9 days in a gnotobiotic system as described in detail by Simons et al. (29). For visualization of bacteria associated with plant roots by microscopy, germinated tomato seeds were prepared and inoculated as described by Simons et al. (29) and subsequently placed on plant nutrient solution (PNS) (19)–1.8% agar plates and grown for 2 days in a vertical position. After 2 days, plant roots had typically grown from a length of 0.4 cm to approximately 3 cm. For growth in the gnotobiotic system or on PNS–1.8% agar plates, inoculated seedlings were incubated in a climate-controlled chamber (18°C and 70% relative humidity with 16 h of illumination per day). To visualize bacteria associated with roots, germinated tomato seeds were inoculated, incubated in liquid PNS for 2 h at room temperature, and then washed five times in fresh PNS solution to remove unattached bacteria.

Root association was observed by microscopy as reported previously (8). We used the PNS-agar plate method described above in place of the gnotobiotic system, because sand particles (yellowish-green in color and rectangular or irregular in shape when viewed by fluorescence microscopy) in the gnotobiotic system can strongly adhere to roots surfaces, and in particular to root hairs, but are distinguishable from the bright green, rod-shaped bacteria. The PNS-agar plate method was utilized to produce high-quality images of cells adhered to the root surface. The microscope used to visualize root surfaces was a Leitz Laborlux D (Leica, Rijswijk, Germany) equipped with a Enst Leitz Wetzler GMBH lamp, type 307-43.003 (Leica) and a 488-nm filter. The video camera used to acquire images of the fluorescent bacteria on root surfaces was a three-color charge-coupled-device video camera, model DXC-930P (Sony Co., Kohda, Japan). Phase-contrast micrographs were taken with the microscope described above equipped with a Photoautomat MPS51 S Spot camera (Wild, Heerbrugg, Switzerland), using 100 ASA film (Ilford) and a 15-s exposure time. Phase-contrast and fluorescent micrographs were taken at magnifications of ×1,000 and ×2,500, respectively.

RESULTS

Construction of plasmids. Figure 1 summarizes the schemes used to construct the cloning vectors and GFP-expressing plasmids. Figure 1A shows the construction of pSMC2, a derivative of pmut2 (9) which is stably maintained in *Pseudomonas*. Plasmid pmut2 carries a mutant form of GFP which fluoresces ~40-fold more brightly than the wild-type protein. pSMC2 was constructed by cloning the 1.8-kb *Pst*I stabilizing fragment of pUC181.8 (16) into the unique *Pst*I site of pmut2.

The upper portion of Fig. 1B shows the construction of a Tc^r derivative of pUCP18 (28). The 1.8-kb *Sal*I-to-*Pst*I fragment of pWTT2081 (36) carrying the tetracycline resistance marker was blunted with T4 DNA polymerase as described previously (2) and cloned into the *Ssp*I site of pUCP18 (digestion with *Ssp*I results in blunt-ended DNA fragments). The resulting plasmid was designated pGB1. An identical approach was used to construct a Tc^r derivative of pUCP19 designated pGB2 (not shown).

A derivative of pGB1 (designated pGB3) carrying GFP also was constructed (Fig. 1B). The *Xba*I-to-*Pst*I fragment of pSMC2 was cloned into pGB1 previously digested with *Xba*I and *Pst*I (these restriction sites are located in the multiple cloning site of pGB1).

Figure 1C illustrates the construction of a derivative of pBR322 competent for replication in *Pseudomonas*. The 1.8-kb *Pst*I stabilizing fragment of pUC181.8 (16) was blunted with T4 DNA polymerase and cloned into the *Ssp*I site of pBR322, generating pGB4. This plasmid can serve as a shuttle cloning vector, as it is stable in the presence of antibiotic selection in both *E. coli* and *Pseudomonas.*

pGB5 is a fusion of pWTT2081 and pSMC2. This plasmid was constructed by digesting both pWTT2081 and pSMC2 with *Hin*dIII (Fig. 1D), ligating the complete plasmids, and confirming the plasmid structure by restriction analysis. Although this plasmid is larger than the constructs described above, it combines the two advantages of pWTT2081 and pSMC2. First, pWTT2081 has been shown to be extremely stable in the rhizosphere (36). Additionally, the promoter driving expression of GFP on pSMC2 allows high expression and easy detection of the cells carrying this construct (see below). On pGB5 and pSMC2, GFP was expressed in *Pseudomonas* without the addition of an inducer by a derivative of the P_{tac} promoter (22), while P_{lac} drives expression of GFP in pGB3 (28).

GFP expression in *Pseudomonas*. The intensities of fluorescence of the various GFP-expressing constructs carried in *P. aeruginosa*, *P. fluorescens* WCS365, and *E. coli* were quantitatively determined (Table 2). In *P. fluorescens* WCS365, plasmid pGB3 is weakly fluorescent at 511 nm but can be observed in liquid culture or on plant roots by fluorescence microscopy with the conditions outlined in Methods and Materials (not shown). Plasmids pSMC2 and pGB5 carried in *Pseudomonas* are significantly (two- to fourfold) more fluorescent than pGB3 and are readily detectable and quantifiable with a fluorescence spectrometer. Interestingly, plasmids pGB3, pGB5, and pSMC2 lead to much more fluorescence in *E. coli* than in the *Pseudomonas* strains (~10-fold), although the reason for this difference is not understood.

To assess GFP-mediated fluorescence during periods of extended incubation without addition of fresh nutrients, we monitored the fluorescences of *P. aeruginosa* carrying pSMC2 and *P. fluorescens* WCS365 carrying pGB5 incubated in stationary phase for 1, 4, and 7 days after overnight growth in glucose minimal medium (Fig. 2). In this medium, cells exhaust the glucose and become carbon starved after overnight incubation. Despite the decrease in absorbance over the 7-day incubation

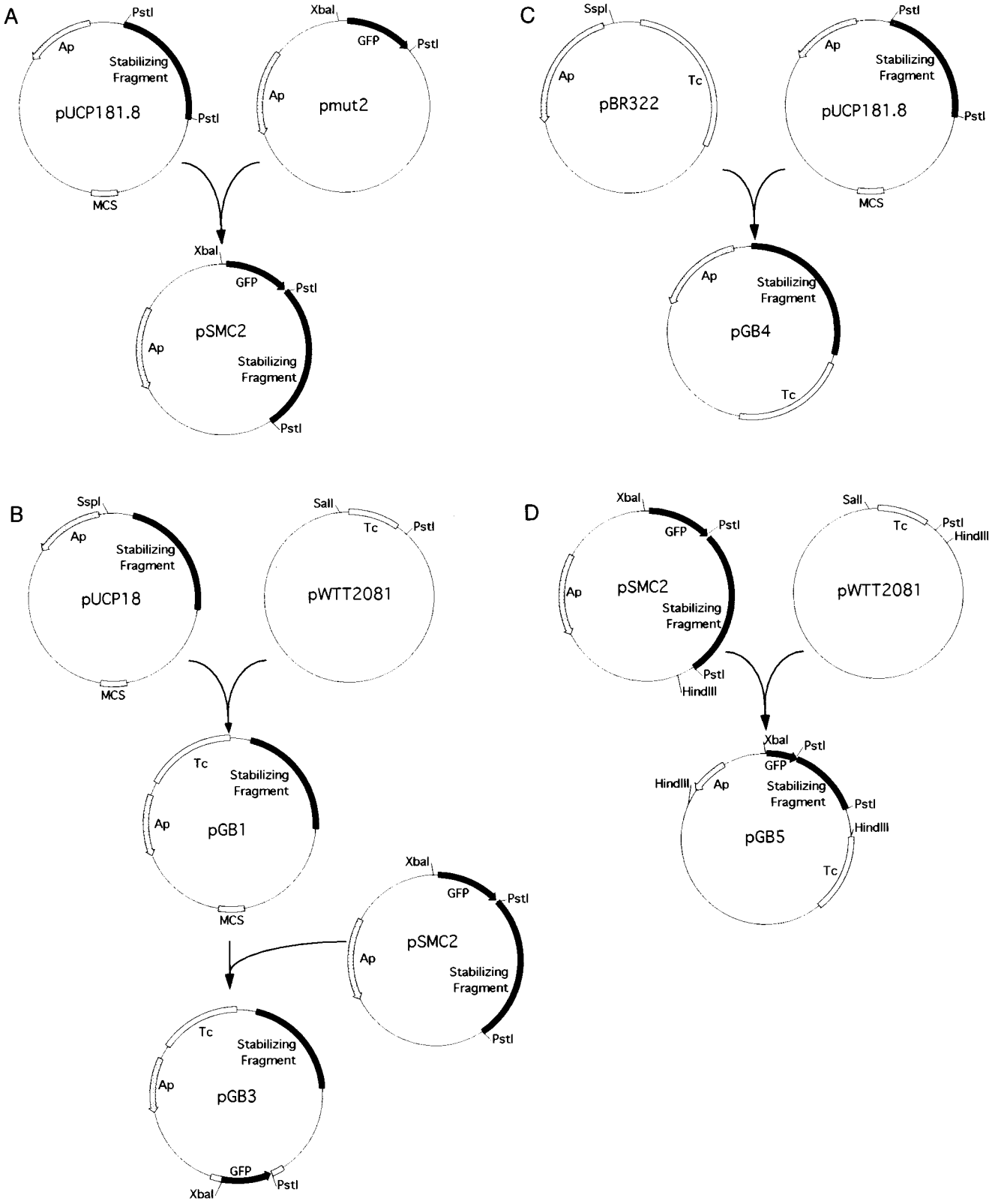


FIG. 1. Construction of pSMC2 (A), pGB1 and pGB3 (B), pGB4 (C), and pGB5 (D). See Results for a complete description of plasmid constructions. Plasmids are not shown to scale. MCS, multiple cloning site.

TABLE 2. Quantitative analysis of fluorescence at 511 nm of *Pseudomonas* and *E. coli* strains carrying various GFP-expressing constructs

Strain and plasmid	Emission (511 nm)[a]
P. fluorescens WCS365	
None	8
pGB1	8
pGB3	10
pWTT2081	9
pGB5	24
E. coli 1164	
None	11
pGB1	13
pGB3	812
pSMC2	585
pWTT2081	11
pGB5	489
P. aeruginosa PAO1	
None	8
pSMC2	43
pGB5	48

[a] Determined as described in Materials and Methods. Cultures were grown overnight in LB medium supplemented with the appropriate antibiotic.

TABLE 3. Stabilities of plasmids in *Pseudomonas* strains[a]

Organism	Plasmid	Stability (%)[b]
P. aeruginosa PAO1	pSMC2	92
P. fluorescens WCS365	pGB1	90
	pGB3	68
	pGB4	74
	pGB5	100[c]

[a] Cells were grown in the presence of the appropriate antibiotics, washed twice and diluted 1:1,000 in LB medium without antibiotics, and grown overnight. The procedure of dilution and growth was repeated four times, resulting in at least 30 generations of growth in the absence of antibiotic selection pressure. Plasmid stability was subsequently evaluated by plating an aliquot of the final culture on LB plates and streaking 100 single colonies on LB agar plates with and without the appropriate antibiotic.

[b] The results shown represent the averages from duplicate experiments.

[c] Stability was assessed after reisolation of bacteria from plant roots grown for 9 days in the gnotobiotic system as reported previously (29).

period for all strains (Fig. 2B), the fluorescence intensity increased slightly for the strain carrying either pSMC2 or pGB5 (Fig. 2A). Therefore, the cells remained fluorescent even after extended periods of starvation. These data suggest that pSMC2 and pGB5 may be valuable tools for marking *Pseudomonas* spp. even after extended periods without fresh nutrients.

Plasmid stability under nonselective conditions. All plasmids described in this study are stable when used under constant selective pressure (data not shown). Because bacteria in biofilms have increased resistance to antibiotics and antibiotic pressure cannot always be applied (such as in root association assays), the stabilities of plasmids pSMC2, pGB1, pGB3, pGB4, and pGB5 under nonselective conditions were assessed.

The results show that after at least 30 generations of growth, a majority of the cells, ranging from 68% (pGB3) up to 100% (pGB5), still carried their respective plasmid (Table 3). However, the relatively high instability of plasmids pGB3 and pGB4 under nonselective conditions suggests that these constructs may not be suitable for long-term experiments without selective pressure.

Plasmid pSMC2 was further analyzed, and its stability in the absence of antibiotic selection was confirmed by two parameters (Table 4). After subculturing and growth for 4 successive days, 95.3% of the cells retained carbenicillin resistance (indicating retention of pSMC2). In addition, these cultures also retained approximately 89.1% of the fluorescence intensity compared to cultures grown in the presence of carbenicillin. The stability of pSMC2 in *P. aeruginosa* suggests that this plasmid could serve as a valuable tool for studies of bacterial communities grown in the absence of antibiotic selection.

The stability of pGB5 was assessed on plant roots. *P. fluorescens* WCS365 carrying pGB5 (Tc^r) was allowed to colonize the roots of tomato seedlings in the absence of added antibiotics. After incubation for 9 days in the gnotobiotic system (29), the seedling roots were vortexed vigorously in minimal SSM to release tightly associated bacteria, plated on LB medium (without tetracycline), and assessed for retention of pGB5 by measuring the percentage of cells retaining tetracycline resistance. Assaying bacteria from the roots of two plants revealed that 100% (200 of 200) of the recovered bacteria were

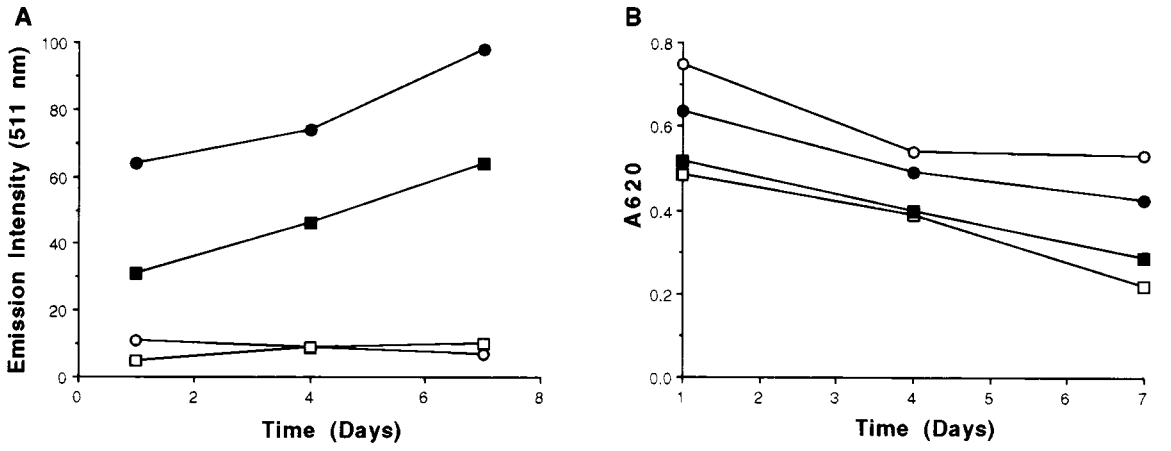


FIG. 2. Expression of GFP under starvation conditions. The emission intensities (emission wavelength, 511 nm; excitation wavelength, 480 nm) (A) and A_{620}s (B) of *Pseudomonas* strains carrying GFP-expressing plasmids grown in minimal SSM supplemented with the appropriate antibiotic are shown for days 1, 4, and 7. ○, *P. aeruginosa*; ●, *P. aeruginosa*/pSMC2; □, *P. fluorescens* WCS365; ■, *P. fluorescens* WCS365/pGB5.

TABLE 4. Stability of plasmid pSMC2 and fluorescence of *P. aeruginosa* carrying pSMC2 in the absence of selection

Day	% Carbenicillin resistant[a,b]	% Emission (511 nm)[a]
1	99.3 (0.33)	93.2 (1.27)
2	98.3 (0.88)	93.3 (2.86)
3	95.3 (0.67)	87.9 (2.42)
4	95.3 (0.67)	89.1 (2.53)

[a] Calculated relative to the value for cells grown in the presence of carbenicillin. Cells were diluted 1:1,000 into fresh LB medium each day and grown overnight. The results presented are averages from triplicate experiments, with standard errors shown in parentheses.
[b] Carbenicillin was added to 1 mg/ml.

Tc^r and thus retained pGB5 (Table 3). The stability of this plasmid in our experiments confirmed previously reported results (36).

The stabilities of pGB5 and pSMC2 also were assessed after extended incubation in minimal medium without antibiotic selection. After 7 days in minimal SSM, cell viability and percent retention of the plasmid were determined with duplicate cultures. The viable counts of *P. aeruginosa* remained constant over the 7-day test period, with 99% (198 of 200) of the strains retaining pSMC2. For *P. fluorescens* WCS365 over the same incubation period, viable counts fell from 5×10^8 to 1×10^7 CFU but 100% (200 of 200) of the viable cells retained pGB5.

Plasmid burden. Two assays were utilized to determine the relative burden of the GFP-expressing plasmids on the growth of the *Pseudomonas* strains. Such a burden could be conferred on the cells because of the presence of the plasmid DNA or the expression of the GFP protein. The first assay assessed the growth of these strains in liquid LB medium, with and without the GFP plasmids (Fig. 3). Under the laboratory conditions tested, the growth rates of strains with and without the GFP-expressing plasmids were identical, suggesting that there is not a significant burden to the cells carrying these plasmids.

We also assessed the motilities of these strains on 0.3% agar as an independent test of the metabolic burden imposed by these plasmids on the bacterial strains. *P. aeruginosa* with and without pSMC2 migrated 2.1 and 1.8 cm, respectively, after a 24-h incubation. *P. fluorescens* WCS365 without any plasmid migrated 2.1 cm, which is comparable to the migrations of the same strain carrying pWTT2081 (2.0 cm), pGB1 (2.0 cm), pGB3 (2.0 cm), and pGB5 (2.5 cm). The distances migrated reported above represent the averages for duplicate experiments and show that there was no significant difference in the motilities of strains in the presence or absence of the GFP-expressing plasmids. Taken together, the data presented in this section suggest that the plasmids described here do not place an excessive metabolic burden on the cells that carry these constructs.

Use of GFP to examine mixed cultures of *P. aeruginosa* and *B. cepacia*. To study the establishment and development of mixed bacterial communities, it might be necessary to mark particular strains for observation in a manner which does not perturb other members of the community. Some examples of tagging a strain in a mixed population have been reported, including the use of luciferase-marked cells (6), strains carrying *lacZ* reporter fusions (12), or labeled rRNA probes (35).

We have adopted the use of GFP-expressing plasmids to observe individual organisms within assemblages of bacteria that have been reconstituted in the laboratory. Figure 4 depicts a mixed culture of *P. aeruginosa* and *B. cepacia*, two organisms commonly isolated from the lungs of patients with cystic fibrosis (18, 23). Shown is a mixture of *B. cepacia* (with no plasmid) and *P. aeruginosa* carrying pSMC2. It is not possible to differentiate these species by simple morphological criteria by phase-contrast microscopy (Fig. 4A); however, by comparing

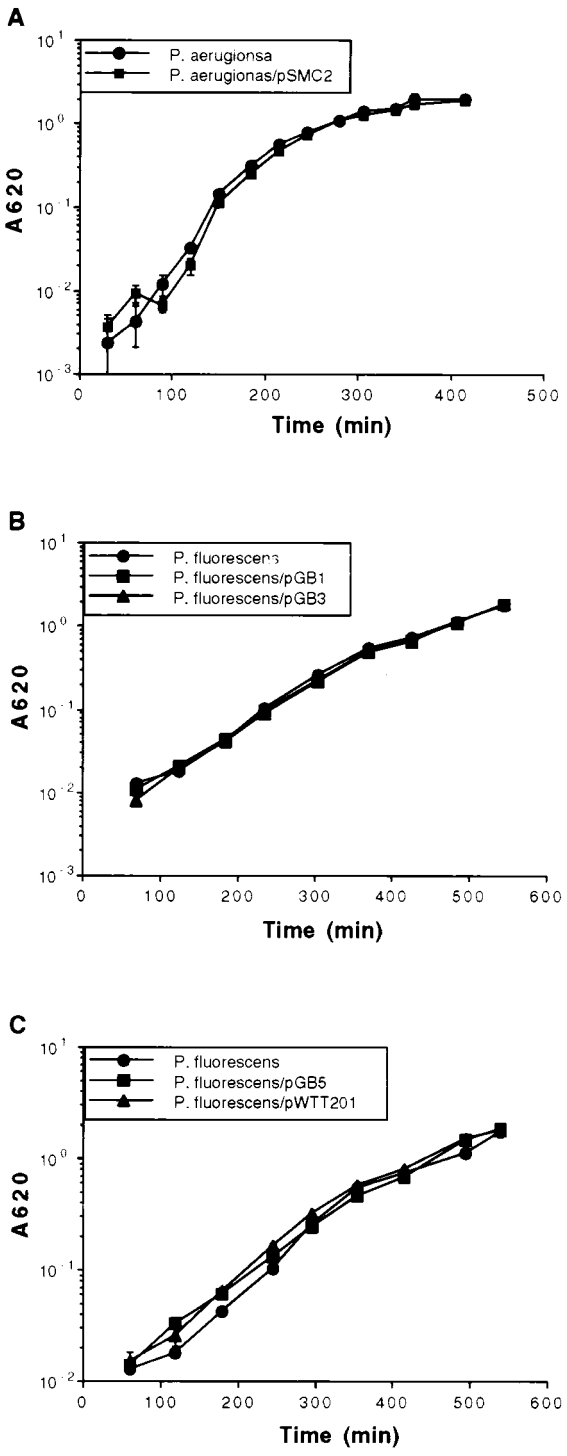


FIG. 3. Growth of *Pseudomonas* strains with and without GFP plasmids. Shown are plots of A_{620} versus time for *P. aeruginosa* and *P. fluorescens* WCS365 with and without the various GFP plasmids. Cells were grown as described in Materials and Methods. The data shown are averages for three replicates; the standard error for each point is also shown.

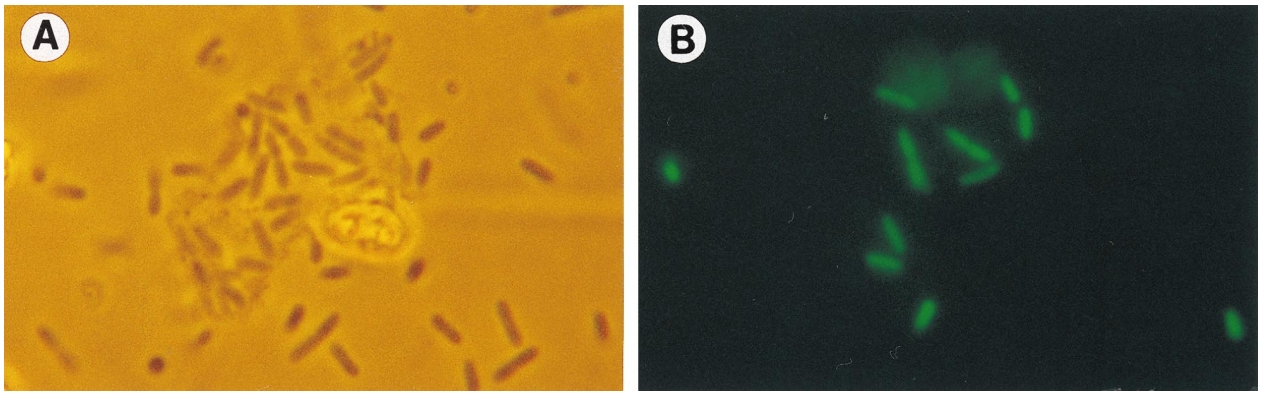


FIG. 4. Expression of GFP in mixed cultures. Shown is a mixture *B. cepacia* and *P. aeruginosa* carrying the GFP-expressing plasmid pSMC2. Cells of both strains can be seen in the photograph taken under phase-contrast microscopy (A). Only the individual cells carrying pSMC2 can be seen under fluorescence microscopy (B).

phase-contrast and fluorescent (Fig. 4B) micrographs, the GFP-marked organism can be easily distinguished. As shown in the micrograph in Fig. 4B, the even distribution of GFP throughout the cytoplasm results in the visualization of clearly delineated cells. It is important to note that *P. aeruginosa* not carrying the GFP-containing plasmids does not fluoresce under these conditions and that we have never visualized green fluorescence outside the cell (not shown). This general approach could be used in laboratory studies to detect marked strains in a reconstituted mixed assemblage without disrupting the cells. We have been able to observe one or a few GFP-marked cells in a background of $>10^3$ unlabeled cells.

The experiments presented above show that pSMC2 is stable in *P. aeruginosa*, suggesting that the fluorescent cells viewed by microscopy represent the vast majority of the *P. aeruginosa* cells present in the assemblage. When pure cultures of *P. aeruginosa* carrying pSMC2 were analyzed, all cells observed by phase-contrast microscopy also were fluorescent (>200 cells observed) under the experimental conditions used in this assay (incubation for 4 h in the absence of antibiotic selection) (data not shown).

GFP as a marker for *P. fluorescens* WCS365 in root association. *P. fluorescens* WCS365 is an efficient colonizer of tomato roots (29). In order to visualize individual cells and assemblages of *P. fluorescens* WCS365 on plant roots, we utilized the stable GFP-expressing construct pGB5 (Fig. 1). Because *P. fluorescens* WCS365 is highly resistant to ampicillin (up to a tested concentration of 1 mg/ml), pSMC2 (Ap^r Cb^r) was not suitable for use with this strain.

Single cells, as well as assemblages of cells expressing GFP, could be easily visualized on the root surface following tomato seedling inoculation (Fig. 5B to D). After 2 days of growth postinoculation, assemblages of bacteria were observed at the root tip by fluorescence microscopy (Fig. 5B and D) but were difficult to see with phase-contrast microscopy (Fig. 5A), although the outline of the root cells could be observed. The fluorescent cells were still visible on the root for up to 9 days after inoculation (data not shown), and association with the root surface was observed after as soon as 2 h after inoculation of the germinated seedling (Fig. 5C).

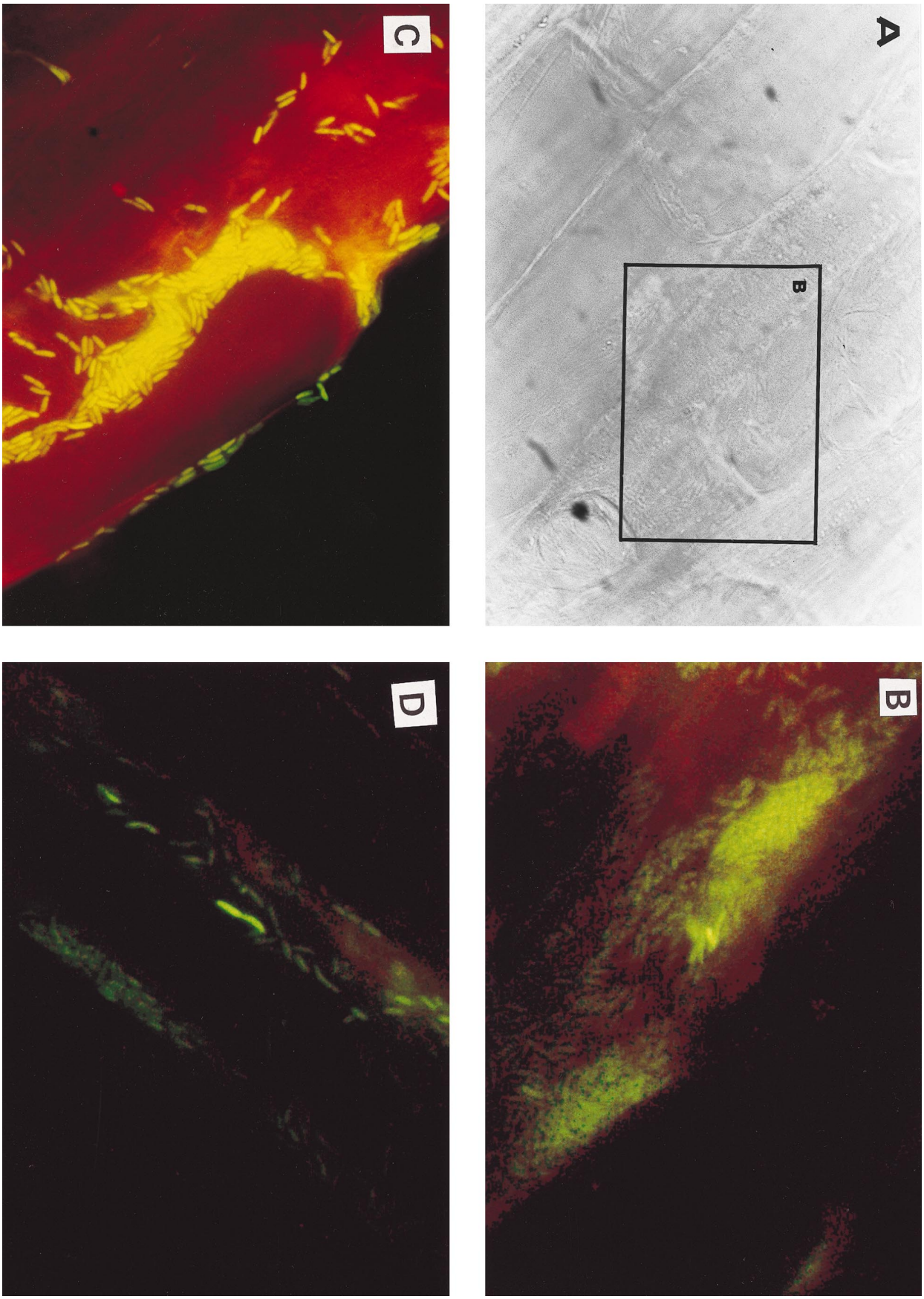
Single cells associated with the root surface (Fig. 5C) appeared brighter and sharper than cells in assemblages of bacteria (Fig. 5B and D). This observation is consistent with scanning electron microscopic studies which have shown that microcolonies of *P. fluorescens* WCS365 on plant roots are covered with a mucoid-like layer (8) which could act to scatter incoming or outgoing light. Most of the bacteria on the root surface were observed in assemblages of bacteria located at the border of adjacent plant cells. These bacterial assemblages were observed as soon as 2 h postinoculation (Fig. 5C), an observation consistent with previous reports (8, 26). We suggest that these GFP-containing plasmids could be used to explore the colonization behavior of wild-type and mutant strains.

DISCUSSION

GFP has proved to be a valuable tool for studying a variety of biological questions with living systems (1, 7, 37). In this report, we describe the construction of new GFP-containing plasmids for use in *Pseudomonas* spp. The extremely stable plasmids pGB5 and pSMC2 carry a bright mutant of GFP (9) which allows easy detection with standard epifluorescence microscopes and filter sets. Our experiments have demonstrated the utility of these plasmids in two model systems, the formation of *P. aeruginosa* biofilms and the association of the plant-beneficial colonizer *P. fluorescens* WCS365 with the roots of tomato seedlings.

GFP adds another weapon to the arsenal of microbiologists studying complex biological systems. In root association experiments, GFP-expressing plasmids can be used to simplify the detection and locate the position of an individual cell on plant roots. As presented here (Fig. 5), under controlled laboratory conditions, GFP-containing cells of *P. fluorescens* WCS365 were observed associated with the roots of tomato seedlings. GFP-containing plasmids can also be used to mark cells in

FIG. 5. Use of GFP to detect *Pseudomonas* in a tomato root association assay. Phase-contrast (A) and fluorescence (B, C, and D) microscopy of the root surfaces of tomato seedlings inoculated with *P. fluorescens* WCS365 containing pGB5 are shown. (A, B, and D) Analyses of the root tip of a tomato seedling grown on PNS agar for 2 days after inoculation and which had a total root length of 3 cm. (A) Phase-contrast analysis of the root surface in the 2- to 2.5-cm region of the root tip. Part of the region shown in panel A (indicated by a box) was studied by fluorescence microscopy as shown in panel B, revealing the presence of fluorescent bacterial cells. Fluorescent assemblages of bacteria were also observed very close to the end of root tip in the 2.5- to 3-cm root region (D). (C) A portion of the root surface of a germinated seed which had been incubated for 2 h postinoculation in PNS at room temperature, revealing the presence of bacteria associated with the root surface.



biofilms. Investigators have generated biofilms in the laboratory on microscope slides for facile and continual monitoring (4, 5, 20). In the investigation of biofilms or other systems with multiple species, the GFP-containing strains can easily be identified without disrupting the microbial community. In the experiments described here (Fig. 4), we could rapidly distinguish individual species in a mixture of *P. aeruginosa* and *B. cepacia* associated with an abiotic surface. Furthermore, the stability of the plasmids in the absence of any selection obviates the need for the addition of antibiotics to these systems for short-term experiments.

The advantages of using GFP as a marker include ease of detection, no requirement for an exogenous substrate or energy source, no processing of the cells, and the ability to monitor individual cells. Light and electron microscopy require fixing and staining, which can dehydrate and perturb biological samples; no fixing or staining is needed to visualize GFP-carrying cells, so these artifacts may be avoided. Furthermore, scanning confocal laser microscopy allows the visualization of fully hydrated biofilms but also requires that cells be fluorescently stained or labeled (20); GFP provides such a fluorescent marker. No sample preparation is necessary to detect GFP activity, as is the case for β-galactosidase activity (13), the *ice* marker (21), or the *lux* system (32). Although the detection limit for ice nucleation activity can be as low as a single cell, the required processing of the sample to assay activity results in a loss of spatial information (e.g., the exact location of the cell[s] expressing such activity on a root). Since GFP activity can be detected directly by microscopy, the position of a single bacterium can be localized. Once GFP is synthesized and properly folded, no energy source is required for its activity, in contrast to the *lux* system, which requires ATP for activity (32). This lack of an energy requirement may allow the detection and visualization of starved cells, as demonstrated in the experiments described here (Fig. 2). The strength of GFP as a marker lies in the detection of individual cells in a nondestructive manner.

There are potential pitfalls to the use of GFP as a bacterial marker which need to be evaluated for individual systems. Marking cells with GFP expressed from a plasmid provides the flexibility to transform a wide range of strains. However, the stabilities of the GFP plasmids described above may not be sufficient for some applications, and the utilization of a system with a stable chromosomal copy of GFP may be necessary (34). The variability of GFP expression in different species (described above) and under various environmental conditions may make it difficult to utilize GFP-derived fluorescence to quantitate cells numbers. Another possible drawback of GFP as a bacterial marker is interference by other fluorescent particles or bacteria in a particular system.

We also have described the construction of shuttle vectors with additional antibiotic selection markers. The two species of *Pseudomonas* described in this report, *P. aeruginosa* and *P. fluorescens* (strain WCS365), have different patterns of resistance to antibiotics. *P. aeruginosa* PAO1 is sensitive to ampicillin, carbenicillin, and tetracycline but has high natural resistance to kanamycin. *P. fluorescens* WCS365 is sensitive to tetracycline and kanamycin but has a high natural resistance to ampicillin and carbenicillin. Therefore, plasmids carrying both ampicillin and tetracycline resistance markers will prove valuable for a wide range of *Pseudomonas* species. Furthermore, the construction of these plasmids was designed so that as much of the original cloning vector as possible was left unaltered. Plasmids pGB1 and pGB2 retain all the advantages of using pUC plasmids (38), in addition to their tetracycline resistance marker.

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