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RESEARCH LETTER

Genetic tools for tagging Gram-negative bacteria with mCherry for visualization *in vitro* and in natural habitats, biofilm and pathogenicity studies

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Received 11 December 2009; revised 14 January 2010; accepted 14 January 2010. Final version published online 18 February 2010.

DOI:10.1111/j.1574-6968.2010.01916.x

Editor: Jeff Cole

Keywords
autofluorescent proteins; GFP; mCherry; pathogenesis; biofilm.

Abstract

Live-cell imaging techniques are essential to gain a better understanding of microbial functioning in natural systems, for example in biofilms. Autofluorescent proteins, such as the green fluorescent protein (GFP) and the red fluorescent protein (DsRed), are valuable tools for studying microbial communities in their natural environment. Because of the functional limitations of DsRed such as slow maturation and low photostability, new and improved variants were created such as mCherry. In this study, we developed genetic tools for labeling Gram-negative bacteria in order to visualize them *in vitro* and in their natural environment without the necessity of antibiotic pressure for maintenance. *mcherry* was cloned into two broad host-range cloning vectors and a pBK-miniTn*7* transposon under the constitutive expression of the *tac* promoter. The applicability of the different constructs was shown in *Escherichia coli*, various *Pseudomonas* spp. and *Edwardsiella tarda*. The expression of *mcherry* was qualitatively analyzed by fluorescence microscopy and quantified by fluorometry. The suitability of the constructs for visualizing microbial communities was shown for biofilms formed on glass and tomato roots. In addition, it is shown that mCherry in combination with GFP is a suitable marker for studying mixed microbial communities.

Introduction

Live cell techniques are essential to gain a better understanding of microbial organization and functioning *in vitro* and in nature. The use of autofluorescent proteins for noninvasive microscopy is nowadays a well-established and valuable tool in biology and biotechnology. For studying microbial communities, multiple autofluorescent proteins can be applied simultaneously for visualization of different populations and intracellular processes. The use of red fluorescent protein (DsRed) in combination with enhanced green fluorescent protein (eGFP) is very suitable as the excitation and emission spectra of these proteins are well separated (Matz *et al.*, 1999). In comparison with GFP, the use of DsRed has been hampered due to its longer maturation time (caused by its tetrameric form) and lower photostability. mRFP1 was the first monomeric derivative of DsRed, which has a shorter maturation time (Bevis & Glick, 2002). Subsequently, improved variants were developed with a more complete maturation and an over 10-fold increased photostability, of which mCherry is considered as one of the best alternatives for mRFP1 (Shaner *et al.*, 2004). Tagging bacteria with marker genes is predominantly based on transformation of plasmids carrying the gene, which require antibiotic pressure for maintenance in the cell. Plasmids are attractive genetic tools for bacterial tagging due to their multicopy number, selective properties and easy handling for cloning strategies. In many natural environments, antibiotics cannot be applied for the efficient maintenance of plasmids (e.g. biofilms). However, cloning vectors that can be maintained without antibiotic selection are scarce. Alternatively, transposons can be used for stable integration in the chromosome, but have the disadvantage of being present as one copy per cell, which will result in a lower production of marker protein(s) in comparison with plasmids when using the same promoter.

Most bacteria form biofilms in their natural habitat (Costerton *et al.*, 1995). Biofilms are defined as bacterial cells attached to a biotic or an abiotic surface, which are encased in an extracellular matrix (glycocalyx) mainly consisting of exopolysacharides. Studying biofilms is important because biofilm formation is commonly involved in bacterial infections, and plays an important role in industrial and agricultural processes. For example, *Pseudomonas* spp. that form biofilms on plant roots can protect plants against microbial diseases (Bloemberg & Lugtenberg, 2001). Microorganisms in a biofilm were shown to be more resistant to biocides, antibiotics and host immune responses (Costerton *et al.*, 1999), which hampers the application of antibiotics for plasmid maintenance.

The aim of this work is to develop a set of genetic tools for tagging Gram-negative bacteria with *mcherry* that is constitutively expressed, can be maintained in the cell without antibiotic selection and is expressed at a level that allows visualization of single cells.

Materials and methods

Bacterial strains, culture conditions and plasmids

The bacterial strains and plasmids used in this study are listed in Table 1. *Pseudomonas* strains were grown at 28 °C in King B broth (King *et al.*, 1954) or in a modified M63 minimal media (Pardee *et al.*, 1959), for which M63 was supplemented with 1 mM $MgSO_4$, 0.2% glucose and 0.5% casamino-acids. Antibiotics were added when required in the following final concentrations: tetracyclin, 40 μg mL^{-1}; gentamycin, 10 μg mL^{-1}; kanamycin, 50 μg mL^{-1}; or streptomycin, 10 μg mL^{-1}. *Escherichia coli* was grown in Luria–Bertani (LB) broth (Sambrook & Russel, 2001) at 37 °C. When appropriate, LB was supplemented with antibiotics in the following final concentrations: tetracyclin, 16 μg mL^{-1}; carbenicillin, 100 μg mL^{-1}; or kanamycin, 50 μg mL^{-1}. *Edwardsiella tarda* was grown at 28 °C in tryptic soy broth (Becton Dickinson and Company, Sparks, MD). When required, the medium was supplemented with gentamycin (30 μg mL^{-1}) or tetracyclin (16 μg mL^{-1}).

Growth curves were obtained by diluting an overnight culture to an $OD_{620\,nm}$ of 0.1 in 20 mL of LB medium. Subsequently, cultures were grown for 24 h at 28 °C at 150 r.p.m.

Construction of plasmids

The *mcherry* gene was amplified with primer oMP1197 (5′-AAAAGGATCC*GGGGAATTCTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGTGAGCGGATAACAATTTTCACACAGGAAACAGCTAA*ATGGTGAGCAAGGGCGAG-3′), including a BamHI site (underlined) and the *tac* promoter (italics) and primer oMP1198 (5′-AAAGGATCCAAAACCGCCCTGCAAGGCGGTTTTTTCGTTTTCTTACTTGTACAGCTCGTCC-3′), including a BamHI site (underlined) and cloned into pGEM®-T Easy Vector System II (Promega Benelux, Leiden, the Netherlands), resulting in pGEM-*mcherry*.

From this construct, a BamHI fragment or a NotI fragment including *mcherry* and the *tac* promoter were cloned into plasmids pME6031 (Heeb *et al.*, 2000), pBBR1MCS-5 (Kovach *et al.*, 1995) and pBK-miniTn*7* (Koch *et al.*, 2001) (Fig. 1), resulting in plasmids pMP7604, pMP7605 and pMP7607, respectively (Fig. 1). Plasmids are publically available and will be supplied on request by the first author.

Transformation of plasmids and the stability of mCherry constructs

Bacterial strains were transformed with plasmids by conjugation according to standard methods (Sambrook & Russel, 2001) Conjugation of plasmids pMP7604 and pMP7605 was accomplished by mixing the donor *E. coli* DH5α containing pMP7604 or pMP7605, the helper *E. coli* strain containing pRK2013 and the recipient strains either *Pseudomonas putida* PCL1445, *Pseudomonas fluorescens* WCS365, *Pseudomonas aeruginosa* PAO1 or *E. tarda* FL60-60. Plasmid pMP7607 was introduced into *P. putida* PCL1445 for transposition via quadripartite mating using *E. coli* DH5α containing pMP7607, *E. coli* DH5α containing helper plasmid pRK2013 and *E. coli* DH5α containing pUX-BF13.

The stability of the *mcherry* containing constructs was analyzed by daily subculturing tagged strains (1 : 100) in liquid medium without antibiotics for approximately 30 generations. Each day, dilutions of the cultures were plated on LB plates without antibiotics. After colony formation, colonies were counted and analyzed for expression of *mcherry* using a Leica MZFLIII stereo fluorescence microscope (Leica, Wetzlar, Germany) (excitation 510/20 nm with 560/40 nm emission). This experiment was performed in triplicate and repeated once.

Quantification of mCherry expression

The production of mCherry in transformed strains was quantified using an HTS 7000 Bio Assay Reader (Perkin Elmer, Waltham, MA). Two hundred microliters of overnight cultures was transferred to a black 96-well flat-bottomed plate (Packard BioScience BV, Groningen, the Netherlands). Fluorescence was quantified by excitation at 590 nm with three flashes and by measuring the emission at 635 nm for 40 μs. The cell density of the cultures was determined by measuring a 1 : 10 dilution of the overnight culture at $OD_{620\,nm}$.

Table 1. Bacterial strains and plasmids

Bacterial strains and plasmids	Relevant characteristics	Reference or source
Pseudomonas putida		
PCL1445	Wild type; excellent colonizer of grass roots. Production of lipopeptides putisolvin I and II	Kuiper *et al*. (2004a)
PCL1477	PCL1445 containing pME6031, Tc^r	This study
PCL1478	PCL1445 containing pBBR1MCS-5, Gm^r	This study
PCL1479	PCL1445 containing pMP7604, Tc^r	This study
PCL1480	PCL1445 containing pMP7605, Gm^r	This study
PCL1481	PCL1445 containing Tn*7* P_{tac}-mCherry after transposition with pMP7607, Km^r, $Strep^r$	This study
PCL1482	PCL1445 containing pMP4655, Tc^r	Kuiper *et al*. (2004b)
Pseudomonas fluorescens		
WCS365	Wild type; excellent colonizer of tomato roots	Geels & Schippers (1983)
PCL1700	WCS365 containing pMP7604	This study
PCL1701	WCS365 containing pMP7605	This study
Pseudomonas aeruginosa		
PAO1	Wild type; clinical isolate	Holloway (1955)
PCA0241	PAO1 containing pMP7604	This study
PCA0242	PAO1 containing pMP7605	This study
PCA0243	PAO1 containing pMP4655	This study
Edwardsiella tarda		
FL60-60	Wild type, isolated from catfish	Pressley *et al*. (2005)
PCA239	FL60-60 containing pMP7604	This study
PCA240	FL60-60 containing pMP7605	This study
Escherichia coli		
DH5α	*endA1 gyrSA96 hrdR17*(*rK-mK-*) *supE44 recA1*; general purpose host strain used for transformation and propagation of plasmids	Boyer & Roulland-Dussoix (1969)
Plasmids		
pRSET-B	Vector for high-level expression of recombinant proteins in *E. coli*, Ap^R	Invitrogen
pGEM-T-easy	Cloning vector for Taq amplified PCR products; Ap^r	Promega, the Netherlands
pRK2013	Helper plasmid for triparental mating, Km^r	Ditta *et al*. (1980)
pUX-BF13	R6K replication-based helper plasmid, providing the PBK-miniTn*7* transposition function in *trans*, Ap^r, mob^+	Bao *et al*. (1991)
pME6031	Broad host-range cloning vector which is maintained in Gram-negative bacteria without selection pressure, Tc^r	Heeb *et al*. (2000)
pBBR1MCS-5	Broad host-range cloning vector for Gram-negative bacteria, Gm^r	Kovach *et al*. (1995)
pBK-miniTn*7*	pUC19-based delivery plasmid for miniTn*7*-KmΩSm1, Ap^r, Sm^r, Km^r, mob^+	Koch *et al*. (2001)
pMP4655	pME6010 derivate harboring the *egfp* gene under the control of the *lac* promoter	Bloemberg *et al*. (2000)
pMP7604	pMP6031 derivate harboring mCherry gene under the control of the *tac* promoter	This study
pMP7605	pBBR1MCS-5 derivate harboring mCherry gene under the control of the *tac* promoter	This study
pMP7607	pBK-miniTn*7*-KmΩSm1 derivate harboring mCherry gene under the control of the *tac* promoter	This study

Fluorescence microscopy and confocal laser scanning microscopy (CLSM)

Planktonic cells of *mcherry*-labeled strains were studied using an Axioplan 2 microscope (Zeiss, Mannheim, Germany), equipped with filterset XF108-2, for which 10 μL of an overnight culture was used. Images were captured using an AxioCam MRc5 camera (Zeiss).

Bacteria attached to tomato roots and glass surfaces were visualized using an Axioplan epifluorescence microscope (Zeiss) coupled to an MRC 1024ES confocal system (Biorad, Hemel Hempstead, UK). Images were obtained using a Krypton/Argon laser using excitation 488 nm-emission 522/35 nm for eGFP and excitation 568–585 nm long pass emission for mCherry. The projections of the individual channels were merged using IMAGEJ 1.38 (Wayne Rasband, National Institutes of Health).

Biofilm formation on glass

Biofilm formation on glass was established by placing a microscopy glass slide in a 50-mL falcon tube containing

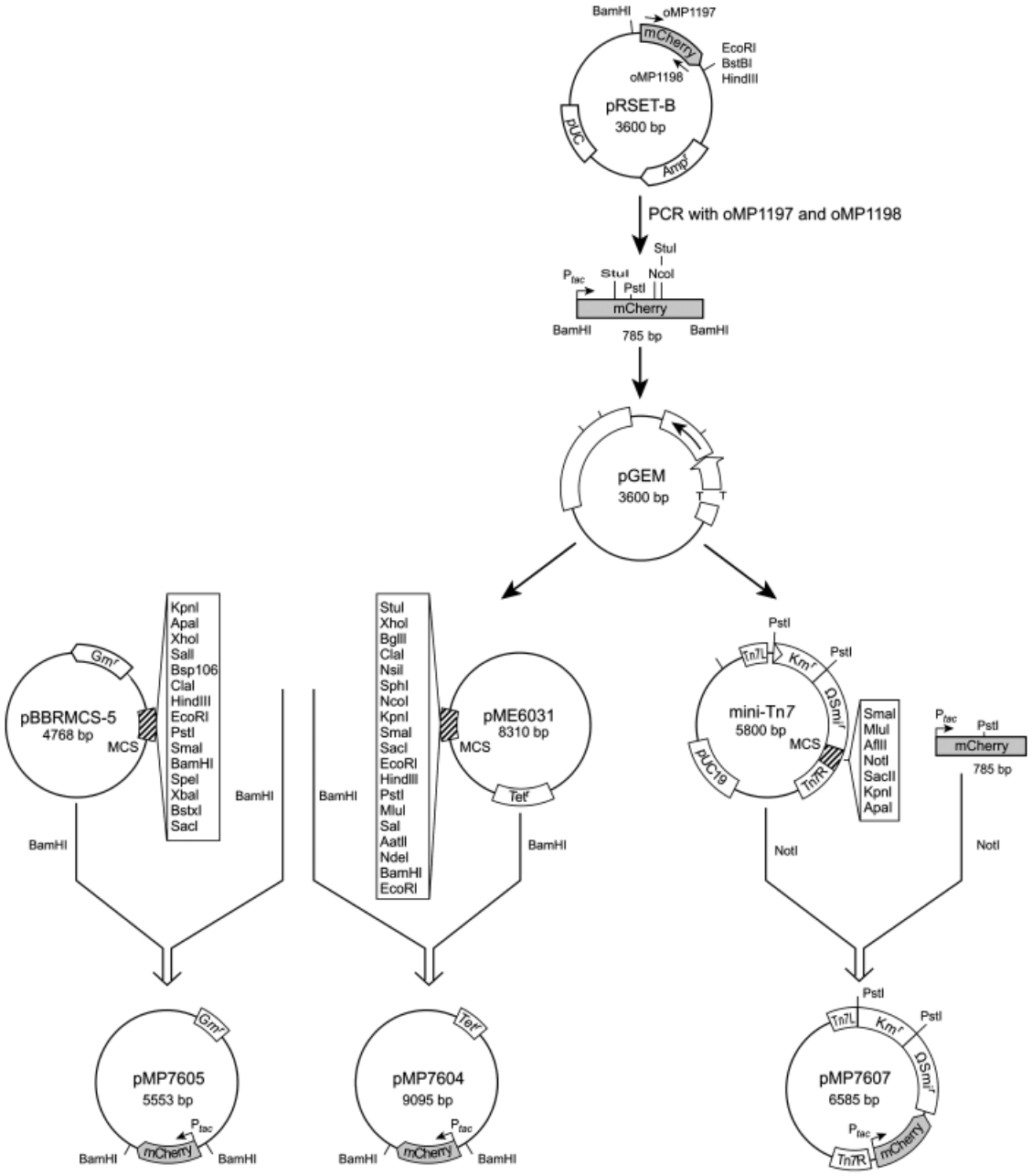

Fig. 1. Schematic representation of the construction of mCherry marker plasmids. For details, see Materials and methods; relevant and unique restriction sites are shown. Plasmids pME6031, pBBR1MCS-5 and pBK-miniTn*7* were used as cloning vectors to construct pMP7604, pMP7605 and pMP7607, respectively, expressing *mcherry* under control of the *tac* promoter. Vector pGEM functioned as an intermediate cloning vector for the PCR product containing *mcherry* using pRSET-B-mCherry as a template. Primer oMP1197 contained the *tac* promoter. Amp^r, ampicillin resistance gene; Tet^R, tetracyclin resistance gene; ΩSm^r, streptomycin resistance gene; Gm^r, gentamycin resistance gene; Km^r, kanamycin resistance gene; P_{tac}, tac promoter; MCS, multicloning site; Tn*7*L, transposon Tn*7* left border; Tn*7*R, transposon Tn*7* right border.

20 mL M63 medium to which 5 μL of an overnight culture was added. Tubes were incubated under nonshaking conditions at 28 °C for 24 h. A biofilm was formed in the middle of the glass slide at the liquid–air interface. Before microscopic analysis, the slide was rinsed carefully and a cover slip was placed on top. The biofilm was analyzed using CLSM as described above.

To establish mixed biofilms, cultures of strains tagged with mCherry and eGFP were mixed in a 1 : 1 ratio.

Tomato root colonization assays

Root colonization assays were performed using the gnotobiotic system as described by (Simons *et al.*, 1996). Coated

tomato seedlings (a 1 : 1 ratio of bacterial strains) were placed in the gnotobiotic quartz sand system, moistened with a plant nutrient solution without a carbon source but with NO_3 as a nitrogen source. After growth for 7 days, plants were removed from the system and were carefully washed with a phosphate-buffered saline solution. Roots were subsequently analyzed for the presence of bacterial biofilms using CLSM as described above.

Results

Construction of Gram-negative strains expressing mCherry

To express *mcherry* in Gram-negative bacteria, the gene was cloned in two broad host-range vectors, i.e. pBBR1MCS-5 (Gm^r) and pME6031 (Tc^r) and in the miniTn*7* transposon (Km^r) located on pBK-miniTn*7* (Fig. 1). Plasmid pRSET-B-mCherry was used as a template for obtaining a PCR fragment of *mcherry* using primers oMP1197 (containing the *tac* promoter) and oMP1198 (Table 1). This resulted in a 785-bp PCR product, which was cloned into pGEM®-T EasyII and subsequently cloned into pME6031, pBBR1MCS-5 and pBK-miniTn*7*, resulting in pMP7604, pMP7605 and pMP7607, respectively (Fig. 1; Table 1). These plasmids were introduced into *P. putida* PCL1445, *P. aeruginosa* PAO1, *P. fluorescens* WCS365 and *E. tarda* FL6-60, which resulted in bright red fluorescent colonies as observed by fluorescence microscopy. One colony from each transformation or transposition event was selected for the following studies.

Growth rate and stability

Growth in liquid LB medium of *P. putida* PCL1445 transformed with pMP7604, pMP7605 and pMP7607 and their corresponding empty vectors was followed. The expression of *mcherry* as well as the presence of the vectors had no significant effect on growth compared with the wild-type strain *P. putida* PCL1445 (data not shown).

The stability of the plasmids and the transposon integration was tested by subculturing in nonselective media (without antibiotic selection pressure) for approximately 30 generations. Samples of the subcultures were plated and colonies were screened for the expression of *mcherry* by fluorescence microscopy. Strain PCL1481 carrying miniTn*7*::*mcherry* did not show any loss of integration. No loss of plasmid was observed for PCL1479 carrying pMP7604, whereas 3% of the colonies of strain PCL1480 carrying pMP7605 had lost fluorescence at day 3 (data not shown).

Qualitative and quantitative analysis of mCherry expressed in *P. putida* PCL1445

A qualitative and quantitative analysis for mCherry production in *P. putida* PCL1445 tagged with pMP7604, pMP7605 and pMP7607 was performed in order to evaluate the

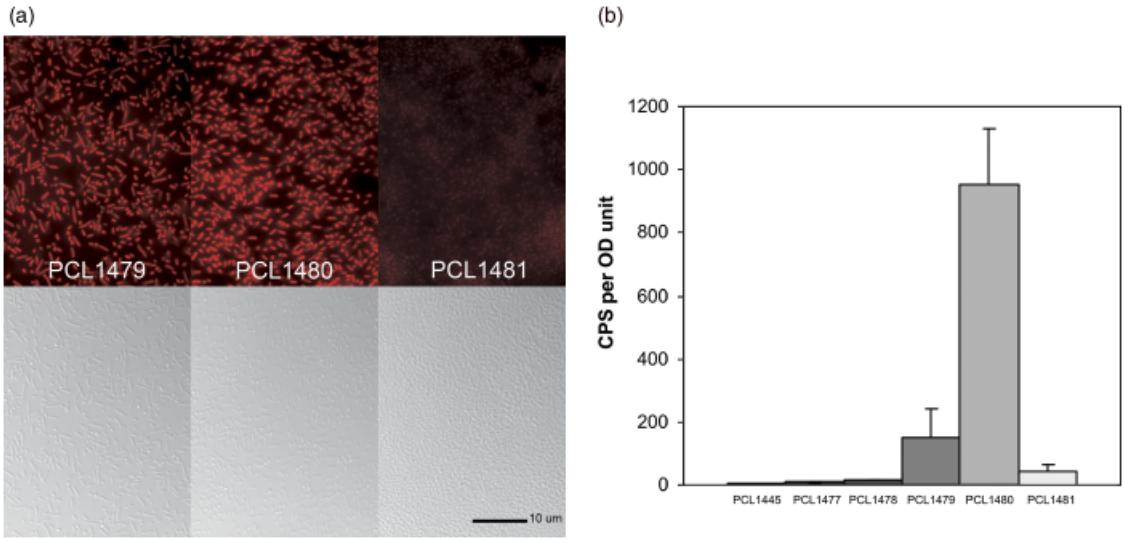

Fig. 2. Qualitative and quantitative analysis of mCherry production in *Pseudomonas putida* PCL1445 strains transformed with different *mcherry*-containing plasmids. Cells were analyzed for mCherry production after overnight growth in liquid King B medium. (a) Microscopic analysis of strains PCL1479 (containing pMP7604), PCL1480 (containing pMP7605) and PCL1481 (containing pMP7607). Upper images were made by fluorescence microscopy and the corresponding lower images (same experiment) by normal light microscopy. Exposure times for fluorescent microscopy images were equivalent to each other for comparison of differences in brightness between strains. (b) Quantitative analysis of mCherry production using a fluorometer with an excitation optimum at 590 nm and an emission optimum at 635 nm. The averages of three independent measurements of PCL1445-WT, PCL1477 (containing pME6031), PCL1478 (containing pBBR1MCS-5), PCL1479 (containing pMP7604), PCL1480 (containing pMP7605) and PCL1481 (transformed with pMP7607) are represented.

resulting brightness of the different constructs. Cells of overnight cultures were visualized using fluorescence and light microscopy (Fig. 3a) and fluorescence was quantified using fluorometry (Fig. 2b). *mcherry* expression was detected at the single-cell level for all tagged strains. Microscopic and fluorometric analyses showed that strain PCL1480 (harboring pMP7605) produced the highest amount of mCherry and strain PCL1481 (containing miniTn*7*-*mcherry*) produced the lowest amount (Fig. 3a and b). The strains PCL1479, PCL1480 and PCL1481 produced mCherry in a ratio of 15 : 95 : 1, respectively. No significant fluorescence was detected for *P. putida* PCL1445 cells and strains PCL1477 and PCL1478 containing the cloning vectors pME6031 and pBBR1MCS-5 (Fig. 2b).

Expression of mCherry in Gram-negative bacteria

To evaluate the applicability of the mCherry marker vectors for tagging Gram-negative bacteria, several other Gram-negative spp., such as *P. fluorescens* WCS365 (an efficient root colonizer), *P. aeruginosa* PAO1 (a model strain for cystic fibrosis research) and *E. tarda* FL6-60 (a fish pathogen and model for zebrafish immunology), were transformed with pMP7604 and pMP7605. This yielded PCL1700, PCL1701, PCA0241, PCA0242, PCA0239 and PCA0240, respectively. Fluorescence microscopy analysis showed the production of mCherry for all transformed strains (data not shown). Single colonies were isolated and overnight cultures were grown for quantitative analysis of mCherry production and comparison with *P. putida* PCL1445 (Fig. 4). Strains containing pMP7605 showed the highest mCherry production. Comparable mCherry production levels were observed among the four strains tested, except for the one carrying pMP7605, which showed a lower level of expression in *E. tarda* FL6-60.

Visualization of mCherry-tagged *P. putida* PCL1445 strains in biofilms formed on glass and tomato roots

To analyze the applicability of the *mcherry*-expressing constructs pMP7604, pMP7605 and pMP7607 in established test systems, which are not suitable for efficient application of antibiotic pressure, *P. putida* PCL1445-tagged strains were allowed to form biofilms on glass (*in vitro* biofilm assay) and on tomato roots (*in vivo* assay used to study root colonization). Using CLSM, the tagged strains were visualized at the single-cell level in both assays (Fig. 4a–f). Biofilms formed on glass consisted of a homogenous spread layer. In contrast, biofilms on tomato roots as formed for 7 days of growth after seedling inoculation were visualized as distinct colonies formed at the interjunctions between the root cells. The brightest fluorescence signal was produced by *P. putida* PCL1480 cells, followed by PCL1479 and PCL1481, which is consistent with the quantitative fluorometric data of these strains (Figs 2 and 3).

Simultaneous visualization of bacterial populations tagged with mCherry or eGFP in biofilms

In order to analyze the use of the *mcherry*-expressing constructs in combination with *egfp* for simultaneous visualization, differentially tagged bacterial populations of the same strain were allowed to form biofilms and were subsequently visualized by CLSM (Fig. 5). Because the *egfp* is cloned in a similar vector as pME6031 and is also expressed under control of the P_{tac} promoter, pMP7604

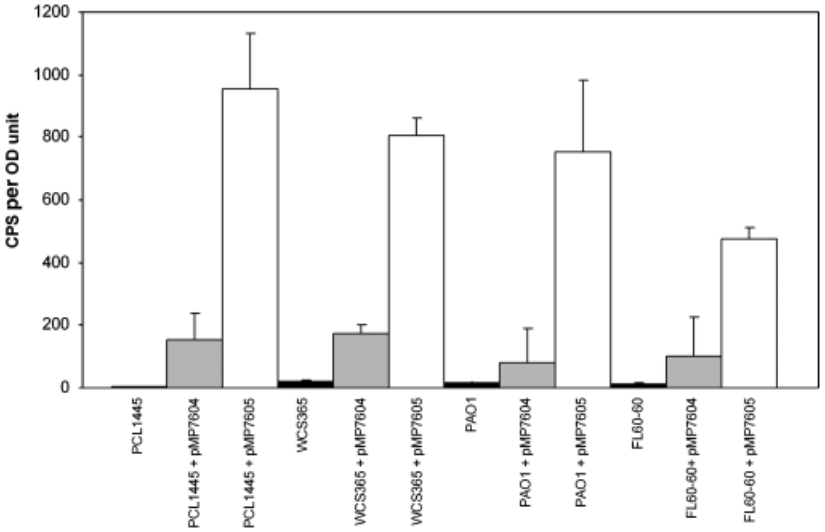

Fig. 3. Quantitative analysis of mCherry production in *Pseudomonas putida* (PCL1445), *Pseudomonas fluorescens* (WCS365), *Pseudomonas aeruginosa* (PAO1) and *Edwardsiella tarda* (FL60-60). Cells were verified for *mcherry* expression after overnight growth in King B medium. Quantitative analysis of mCherry production was determined using a fluorometer with an excitation optimum at 590 nm and an emission optimum at 635 nm. The averages of three independent measurements of 200 μL cultures of *P. putida*, *P. fluorescens*, *P. aeruginosa* and *E. tarda* containing either no plasmid, pMP7604 or pMP7605 are represented.

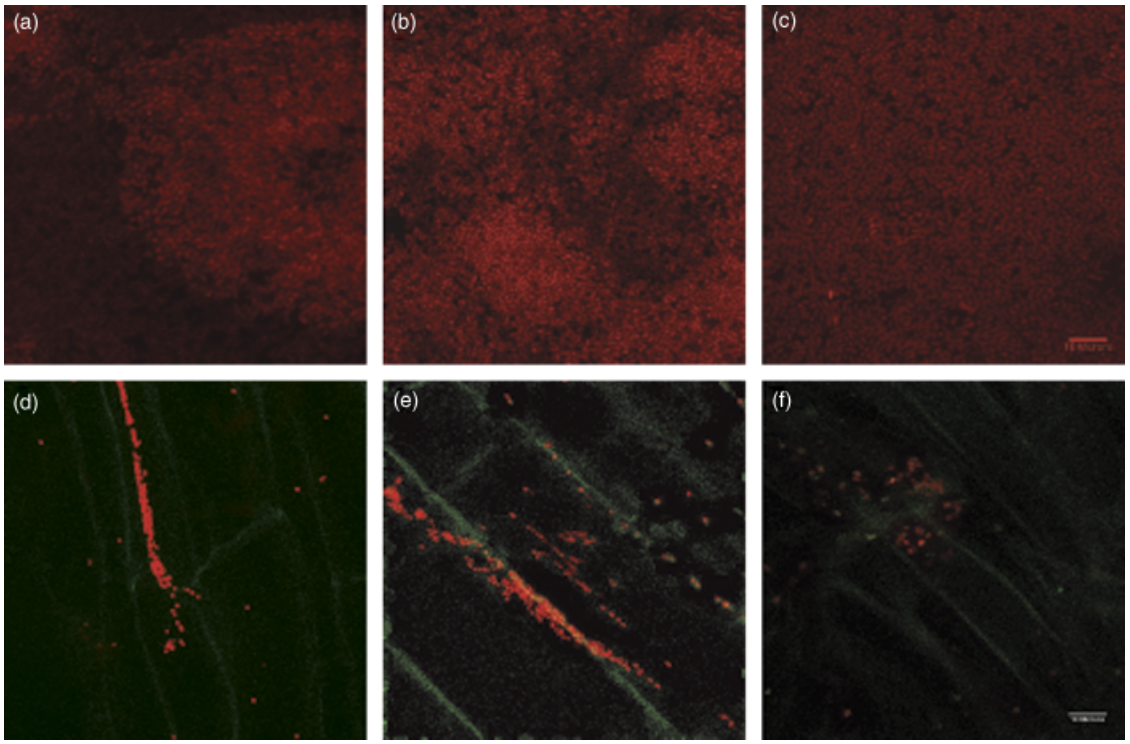

Fig. 4. CLSM analysis of *Pseudomonas putida* strains PCL1479 (containing pMP7604), PCL1480 (containing pMP7605) and PCL1481 (containing pMP7607). (a–c) Biofilms formed on glass after 24 h of incubation. (d–f) Tomato root colonization after 7 days of plant growth and inoculation, performed in a gnotobiotic sand system. Images were processed to projections of Z-series with IMAGEJ. Each scale bar represents 10 μM.

was selected for testing simultaneous visualization. CLSM analysis of the biofilms formed on glass (Fig. 5a and b) showed clearly the presence and distinction between *mcherry*- and *egfp*-tagged bacteria. For tomato root colonization experiments, *P. putida* PCL1445 strains harboring pMP7604 (PCL1479) or pMP7605 (PCL1480) (Fig. 5d) were used for mixed inoculation (1 : 1) of seedlings with *P. putida* PCL1445 tagged with *egfp*. CLSM analysis of the roots after 7 days of growth clearly showed the presence of mixed microcolonies originating from the *mcherry*- and *egfp*-tagged populations (Fig. 5c and d).

Discussion

Nowadays, the use of autofluorescent proteins as markers for the noninvasive microscopic analysis of biological processes is a well-established successful technical approach (Errampalli *et al.*, 1999; Larrainzar *et al.*, 2005; Bloemberg, 2007). Autofluorescent proteins with sufficiently separated excitation and emission spectra are required for simultaneous visualization of (1) interactions between different bacterial populations or various spp. and (2) metabolic processes. GFP has been extensively optimized for codon usage in different organisms (Patterson *et al.*, 1997) and its intrinsic characteristics such as photostability, brightness and excitation/emission spectrum (Shaner *et al.*, 2007). GFP is the most frequently used marker gene in biology and biotechnology. Excitation and emission spectra of GFP and red fluorescent protein (Matz *et al.*, 1999) hardly overlap, which makes their combination suitable for simultaneous application (Tecon *et al.*, 2009). In order to improve brightness, maturation and photostability optimized monomeric forms of red fluorescent protein have been produced recently, of which mCherry is one of the best members (Shaner *et al.*, 2004, 2005). mCherry has been used successfully in several recent studies, as a reporter, and also as a biosensor (Hillson *et al.*, 2007; Lewenza *et al.*, 2008; Malone *et al.*, 2009).

We have cloned *mcherry* under the control of the *tac* promoter, which is expressed constitutively at a low level, into the vectors pBBRMCS-5 (Kovach *et al.*, 1995) and pME6031 (Heeb *et al.*, 2000) and into the transposon vector pBK-miniTn*7* (Koch *et al.*, 2001) (Fig. 1). The performance of these genetic tools for tagging various Gram-negative bacteria was compared. The three different vectors were chosen for their difference in antibiotic selection gene (gentamycin, tetracyclin and kanamycin, respectively) and the opportunities for maintenance as a plasmid (pBBRMCS-5 and pME6031) or integration into the chromosome (pBK-miniTn*7*). In addition, pBBRMCS-5 (a derivative of the general cloning vector pBBR) is assumed to have a higher

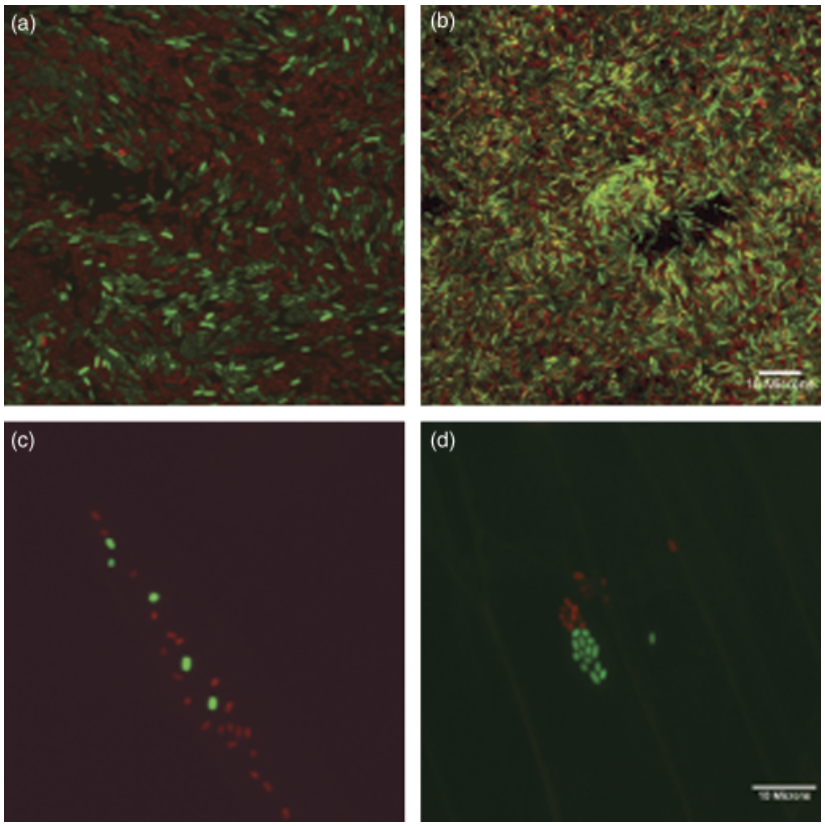

Fig. 5. CLSM analysis of mixed populations of eGFP- and mCherry-labeled *Pseudomonas* strains. Biofilms formed on glass after inoculation with 1 : 1 mixtures of *Pseudomonas putida* PCL1482 (eGFP) and PCL1479 (mCherry) (a), and *Pseudomonas aeruginosa* PCA0243 (eGFP) and PCA0241 (mCherry) (b). Images of tomato root surfaces analyzed after 7 days of growth and inoculation with 1 : 1 mixtures of *P. putida* PCL1482 (eGFP) and *P. putida* PCL1479 (mCherry) (c), and *P. putida* PCL1482 (eGFP) and PCL1480 (mCherry) (d) colonizing the root system. Images were processed with IMAGEJ from projections of Z-series. Each scale bar represents 10 μM.

copy number than pME6031 (containing the pVS1 replicon). pME6031 was described as being maintainable without the selective pressure of tetracyclin (Heeb *et al*., 2000). All vectors were reported to have a broad host range in Gram-negative bacteria.

Pseudomonas putida strain PCL1445, which is an excellent root colonizer and is able to form biofilms on abiotic surfaces such as polyvinylchloride (Kuiper *et al*., 2004a), was selected to examine the new constructs containing *mcherry*. Growth curves of the transformed strains did not show an effect of the constructs and *mcherry* expression on growth (data not shown). However, care should be taken when using these plasmids under other growth conditions. As expected, the pME6031-derived plasmid pMP7604 was maintained without antibiotic pressure (no loss was observed), whereas the pBBRMCS-5-derived plasmid pMP7607 showed a loss of 3% in cells of the population after 3 days of subculturing without antibiotic pressure. Qualitative and quantitative analyses showed that all constructs can be used for visualization at the single-cell level and that the intensity of fluorescence resulting from the use of the different genetic constructs correlates with the copy number of the different plasmids and the transposon used (Fig. 2). The *mcherry* constructs created were shown to be functional in different *Pseudomonas* spp. (i.e. *P. putida* PCL1445, *P. fluorescens* WCS365 and *P. aeruginosa* PAO1) and the fish pathogen *E. tarda*, with comparable mCherry production levels (Fig. 3). In addition, fluorescence was observed during cloning in *E. coli*.

Labeled strains under *in vitro* (biofilm formation on glass) and *in vivo* (tomato root colonization) conditions showed that the constructs are well suited for the visualization at the single-cell level (Figs 4 and 5). In addition, tagging with the *mcherry* plasmid constructs was shown to be useful for the simultaneous visualization with the eGFP-tagged strain of *P. putida* PCL1445 as shown for biofilms formed on glass and tomato roots (Fig. 5). Also, single strains tagged with eGFP and mCherry were recently shown to be useful for bioreporter studies (Tecon *et al*., 2009). The vectors constructed in this study could function as markers to locate bacteria in such studies.

In conclusion, we have developed a set of genetic tools for the expression of *mcherry* in Gram-negative bacteria for studies *in vitro* and in natural environments, especially of value when antibiotic pressure cannot be efficiently applied.

Acknowledgements

We would like to thank Joost van Soest and Merle Eijkhof for their technical assistance. We are grateful to the Tsien lab

(University of California, San Diego) for obtaining pRSET-B-mCherry and Ole Nybroe for providing pBK-miniTn*7*.

Authors' contribution

S.d.W. and G.V.B. contributed equally to this work.

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