Efficient colonization of plant roots by the plant growth promoting bacterium Bacillus amyloliquefaciens FZB42, engineered to express green fluorescent protein

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A R T I C L E   I N F O

Article history:
Received 7 August 2010
Received in revised form 12 December 2010
Accepted 20 December 2010
Available online 13 January 2011

Keywords:
Green fluorescent protein
Bacillus amyloliquefaciens
Plant root colonization
Surfactin

Abstract

A single copy of the gfp gene linked with the P spac promoter and flanked by the terminal FZB42 amyE sequences was stably integrated into the chromosome of plant growth promoting bacterium Bacillus amyloliquefaciens FZB42 via homologous recombination. A spontaneous mutant, FB01mut, emitting bright fluorescence was detected among the transformants and found suitable for colonization experiments.

Competitive rhizosphere colonization is crucial for plant-PGPR interactions (Chin-A-Woeng et al., 2000; Kamilova et al., 2005; Timmusk et al., 2005). Compared to Gram-negative PGPR, mainly Pseudomonas spp. (Lugtenberg et al., 2001; Preston, 2004), relatively little is known about colonization pattern of Gram-positive strains, despite their obvious advantages in practical application due to their ability to produce heat- and desiccation-resistance endospores (Emmert and Handelsman, 1999; Kloeper et al., 2004; Reva et al., 2004).

Bacillus amyloliquefaciens FZB42 is a Gram-positive PGPR which is commercially applied in a broad range of host plants. The whole genome sequence of FZB42 became available in 2007 (Chen et al., 2007) as the first of Gram-positive PGPR and consecutive investigations were performed in order to elucidate its plant growth promoting and biocontrol activities (Butcher and Helmann, 2006; Chen et al., 2006, 2009a,b; Idris et al., 2004; Koumoutsis et al., 2004, 2007; Moldenhauer et al., 2007; Schneider et al., 2007). However, colonization pattern of FZB42 and other PGP bacilli on host plants was not studied in detail, mainly due to lacking of a useful marker system which is stably maintained and permanently expressed in the environment.

Since more than one decade, the green fluorescent protein (GFP) from jellyfish Aequorea victoria has been proved to fulfill the expectations as valuable molecular marker which can be expressed in

1 Introduction

Plant growth promoting rhizobacteria, PGPR (Lugtenberg and Kamilova, 2009), are a heterogeneous group of bacteria associated with the plant rhizosphere, that contribute to increased yield of crops, vegetables and other plants of economical importance. Several mechanisms are involved in plant-beneficiary activities of PGPR; they include synthesizing phyto-hormones (Idris et al., 2007) and volatile organic compounds (Ryu et al., 2003), producing available nutrients for plants (van Loon, 2007), and suppressing phytopathogenic soil bacteria, fungi, viruses and nematodes (Compant et al., 2005; Haas and Defago, 2005). Some PGPR act also beneficial by eliciting plant response reactions directed against biotic (“induced systemic resistance, ISR”, van Loon, 2007) and abiotic stress (“induced systemic tolerance, IST”, Yang et al., 2008).
many environmental organisms (Errampali et al., 1999). As early as 1997, Bloemberg et al. reported about construction of plasmids which are stably maintained in Pseudomonas spp. and constitutively express a bright mutant of GFP (Bloemberg et al., 1997). Itaya et al. (2001) constructed a Bacillus subtilis plasmid encoding GFP allowing detection of fluorescent B. subtilis colonies on agar plates, and Bacillus megaterium and Paenibacillus polymyxa tagged with plasmid-borne gfp were studied for plant root colonization (Liu et al., 2006; Timmusk et al., 2005). Unfortunately, except few representatives of plasmids following theta replication, plasmids, especially their derivatives containing foreign DNA, are notoriously unstable in bacilli (Ehrlich et al., 1986), limiting their use in constitutive expression of marker genes under environmental conditions. No strains engineered for robust GFP expression are currently available for Bacillus spp. and other Gram-positive PGPR.

Here we describe labeling of B. amyloliquefaciens FZB42 with GFP by stable integration of a single gfp gene copy into the chromosome. A mutant emitting bright fluorescence was found suitable for studying plant colonization under environmental conditions as demonstrated by applying gfp+ tagged FZB42 to three different plant species. Furthermore, MALDI-TOF mass spectrometry revealed that surfactin is the only secondary metabolite, which is non-ribosomal synthesized during colonizing plant cells by FZB42.

2. Materials and methods

2.1. Bacterial strains and growth conditions

Bacterial strains B. amyloliquefaciens FZB42 and B. subtilis 168 were cultivated routinely in Luria broth (LB) at 30 °C. FZB42 was deposited as strain 10A6 in the culture collection of the Bacillus Genetic Stock Center (BGSC). For inoculating plant seedlings, bacteria were grown in LB until OD_{600} of 1.0 and diluted by 1000 times before use. The roots of the seedlings were allowed to soak into the bacterial suspension (∼10^5 CFU ml^{-1}) for 2 min.

2.2. Plant material and cultivation

The duckweed clone Lemna minor ST was isolated by Pirson and Seidel, and was delivered from the culture collection of the Botanical Institute of the University of Jena. Lemna was propagated axenically in filter-sterilized Steinberg medium as described previously (Idris et al., 2007). Briefly, one sterile Lemna plantlet bearing two fronds was transferred into a well (16 mm in diameter) of a micro-titer plate containing 2 ml Steinberg medium and 0.2% bacterial culture with an OD_{600} of 1.0. The micro-titer plate was incubated in a growth chamber at 20 °C under continuous light.

Zea mays seeds were obtained from company Saaten-Union, Germany. The seeds were treated with 70% ethanol for 3 min and then with 5% (v/v) sodium hypochlorite for another 3 min and then rinsed with sterile distilled water. After surface sterilization, eight maize kernels, embryo upside, were placed into a Petri dish filled with 7 ml sterile water and then incubated at 30 °C in dark room. The seeds were kept wet until their germination after 40–45 h. Then, the seedlings were transferred onto Murashige Skoog medium solidified with 0.8% agar and further cultivated in a plant growth chamber (24 °C, light 16 h per day).

The seeds of Arabidopsis thaliana ecotype Columbia-0 were surface-sterilized as described above. The sterilized seeds were incubated at 24 °C for seven days onto MS agar (0.6%) containing 1% sterile sucrose until germination. The seedlings were then transferred onto MS agar (0.8%). The square plate (12 cm × 12 cm) with the seedling was kept in vertical position and incubated under same conditions as described for maize seedlings.

2.3. Construction of fluorescence protein-labeled FZB42

Primers were designed according to the information obtained from the whole genome sequence of FZB42 (7). The upstream border sequence of the FZB42 amyE gene (“amy front”) was amplified from FZB42 chromosomal DNA using primers amyFront-1: 5′-AGTTGACGTCCTCTCCGATTTCGCGCAACAC-3′ (Aatr restriction site is underlined) and amyFront-2: 5′-TGGCATCTCTCCAGCAGC-3′. The downstream border sequence of amyE gene (“amy back”) was amplified with primers amyBack-1: 5′-AGCCAAATACCTGACCCAG-3′ and amyBack-2: 5′-AGCTCAAGCTCCGACCTGG-3′. The amplified sequences were inserted into vector plasmid pUC18Em^+ and yielding recombinant plasmid pVF1 containing the two amyE border sequences. The gfp+ gene together with an upstream located P_{spac} promoter element was derived from plasmid pCE149 (BGSC, Kaltwasser et al., 2002; Scholz et al., 2000) and cloned into plasmid pVF1. The resulting integrative plasmid pHF01 was transformed into competent FZB42 cells as described previously (Idris et al., 2007). The amy+ transformants were selected onto LB plates supplemented with 1% starch, 1 µg/ml erythromycin and 25 µg/ml lincomycin. Homologous recombination was confirmed by PCR and fluorescence microscopy. The DsRed gene was cloned from plasmid pECE163 (BGSC), whilst the TdTomato gene was cloned from plasmid pTDTomato (Shaner et al., 2004).

Plasmid pECE163 (BGSC) containing the DsRed gene without promoter was linearized by endonuclease EcoRI and then blunt ended by Klenow fragment. The DsRed gene cassette was subsequently isolated from pECE163 using the second restriction enzyme SpeI and cloned into plasmid pHF01 where the gfp+ gene had been removed by KpnI and SpeI, leaving the P_{spac} promoter and the trpA terminator intact. The cohesive ends of the “empty” pHF01 created by KpnI were also blunt ended by Klenow to ligate it with the DsRed fragment derived from pECE163 yielding pHF03.

Vector pTDTomato was obtained from the lab of Roger Tsien (Shaner et al., 2004) and the TdTomato gene was amplified with the forward primer 5′-GATAATGTGACCAATGTTGTAGCAAGGGG-3′ (KpnI restriction site is underlined) and reverse primer 5′-TTGATTCTCTCTGACCAAGTG-3′ (SpeI restriction site is underlined). The amplified PCR product was cut by KpnI and SpeI and then cloned into plasmid “empty” pHF01lacking gfp+ as described above, but still containing the preceding P_{spac} promoter sequence (pHF04).

The plasmids with red fluorescence gene, pHF03 and pHF04 were transformed into FZB42, yielding strains FB01 (DsRed) and FB04 (TdTomato), respectively.

2.4. Comparison of fluorescence emitted by FB01 and FB01mut

To compare the intensities in fluorescence of FB01 and the spontaneous mutant FB01mut, bacterial cultures grown in LB at 37 °C, until OD_{600} of ∼2.4, were used. The samples for fluorescence measurements were prepared by resuspending bacterial pellets with cell fixation buffer (1× PBS with 0.3% formaldehyde) and subsequent dilution using the same buffer to an OD_{600} of 0.2. 200 µl of the diluted cells in Costar 96 black clear bottom plates (Corning Life Sciences) were analyzed by SpectraMax M2e ( Molecular Device). The relative fluorescence emission was measured at 520 nm after excitation at 485 nm.

2.5. Real-time RT-PCR

Total RNA was isolated by the Nucleo Spin RNA L kit ( Macherey & Nagel) from cells grown in LB-medium at 37 °C and 210 rpm. cDNA was synthesized from 1.5 µg of total RNA with reverse transcriptase (ABI, Life technologies, CA, USA) and the random hexamer primer. A quantitative real-time PCR was performed using a CFX96 Real-Time PCR Detection System (Biorad, Hercules, CA, USA) with 10 µl of 2× SYBR green supermix (Biorad, Hercules, CA, USA) and 0.2 µM of each primer. The primers were designed with Primer3 (Rozen and Skaletsky, 2000) and listed in Table 1. The melting curves were obtained with the CFX Manager 3.1 software (Biorad, Hercules, CA, USA) and the comparative Ct method was used for the quantification of transcripts of target genes (Livak and Schmittgen, 2001). The relative gene expression was calculated with the ΔΔCt method using H37Rv (as a housekeeping gene) as the reference gene.
primers from this kit (High Capacity RNA-to-cDNA) according to the manufacturer instructions. Real-time RT-PCR was performed with the 7500 Fast Real Time System (Carlsbad, CA, USA) by using 1 μl cDNA mixture, gene-specific primers gfp-real-1, and gfp-real-2 (see below) and Power SYBR Green PCR master mix kit (Carlsbad, CA, USA) according to the manufacturer instructions. The PCR primer sequences were: gfp-real-1: TCCATGGCCAAACATGGTC, gfp-real-2: CGGATAACGGGAAAAGCA. As an endogenous control, the highly conserved gyrA gene was used, applying the following primers: gyrA-real-1: GACGGCAAGAAACAAATCATCA, and gyrA-real-2: CCGGATAACGGGAAAAGCA. Three technical replicates were performed. Quantification based on the threshold cycle (Ct) values according to Pfaffl (2001). The absolute RNA expression level was normalized with the values obtained for gyrA expression.

2.6. Monitoring bacterial colonization on plants

After bacterial inoculation, roots of A. thaliana grew along the inner wall of the square plate (12 cm × 12 cm, see above) and samples were taken seven days after root inoculation. Roots were detached from the plantlet, and after rinsing directly monitored by CFLS microscopy. Samples of maize roots were prepared seven days after inoculation by simple scratching a piece of the root surface, around 1 cm in length, from different parts of the root with a sterile razor blade or by cutting cross sections, 50 μm in thickness, using a microtome.

Samples from L. minor ST roots and fronds for light microscopy were withdrawn one day, five and nine days, after inoculation by simple scratching a piece of the root surface, stained with uranyl and Reynolds lead citrate and viewed in a Zeiss EM 900 electron microscope (Carl Zeiss AG Oberkochen, Germany).

For scanning electron microscopy (SEM) the samples, maize roots and L. minor fronds and roots were processed as described above. Dehydration, through a graduated series of ethanol solutions and finally 100% acetone, was followed by critical point drying with liquid carbon dioxide using the CPD 030 (BAL-TEC, Germany). Specimens were then mounted on stubs for SEM, sputtered with gold (Sputter Coater SCF, 005, BAL-TEC, Germany) and examined with a LEO 1430 scanning electron microscope.

2.9. Detection of lipopeptides of FZB42 by MALDI-TOF mass spectrometry

For MALDI-TOF mass spectrometric analysis of lipopeptides produced by FZB42 in its interaction with plant roots, Lemma plantlets, inoculated with FZB42, were grown in two ml Steinberg plant growth medium (Idris et al., 2007) as described above. They were harvested and extracted with 100 μl 70% acetonitrile/0.1% trifluoroacetic acid. Lipopeptides released into the growth medium and adhering to the plant roots were detected mass spectrometrically. Mass spectra were recorded using a bruker Autoflex MALDI-TOF instrument equipped with a 337 nm nitrogen laser for desorption.
and ionization of analytes. Two μl aliquots of the medium and the plant extracts were mixed with the same volume of matrix solution (a saturated solution of α-cyano-4-hydroxycinnamic acid in 70% aqueous acetonitrile containing 0.1% trifluoroacetic acid, by vol.), spotted on the target and measured. Positive ion detection and linear mode were used.

3. Results

3.1. GFP tagging of FZB42

To obtain robust fluorescence signals in long term colonization experiments, we decided to introduce a single copy of the fluorescence protein gene under control of a strong constitutive promoter into the FZB42 chromosome. Fig. 1 summarizes our strategy for constructing an integrative plasmid expressing GFP+ in FZB42. Sequences, covering the border 5′ (amy “front”) and 3′ (amy “back”) regions of the FZB42 amylase gene, were amplified and cloned into a pUC vector plasmid designed for expressing erythromycin resistance in Gram-positive bacteria. This results in integrative plasmid pVBF. The gfp+ gene flanked by the Pspac promoter and the trpA terminator was derived from pECE149 (BGSC) and cloned into plasmid pVBF. The resulting plasmid pBF01 was used after linearization to replace the central part of the FZB42 amyE gene by the erm – Pspac – gfp + – trpAt cassette via double crossover recombination at both terminal amy regions. The amy - em8 transformants (BF01), bearing a single copy of the gfp gene in their chromosome, expressed fluorescence as revealed by epifluorescence microscopy. A similar strategy was used to construct FZB42 derivatives, FB03 and FB04, respectively, expressing the DsRed gene derived from plasmid pECE163 (BGSC) and the TdTomato gene derived from plasmid pTdTomato (Shaner et al., 2004).

3.2. Expression of fluorescence by different FZB42 strains

Confocal laser scanning microscopy (CLSM) was used to prove fluorescence emission of the FZB42 strains harboring a single copy of the three different fluorescence protein (FP) genes in their chromosome. GFP-labeled cells emitted brightest fluorescence, whereas DsRed-labeled cells were the dimmest ones, probably due to its slow maturation rate of around 20 h at 37°C (Shaner et al., 2005) which is even doubled at room temperature (Bevis and Glick, 2002). Moreover, DsRed and even TdTomato labeled bacteria showed a considerable cell-to-cell variation in brightness. Direct optical measurement of brightness of the three proteins expressed in FZB42 was also consistent with the result obtained by CLSM. Unlike the tetrameric DsRed, TdTomato, as an optimized derivative from DsRed, encodes for a tandem dimeric red fluorescence protein which has a faster maturation rate (about 2 h) and good photostability (Shaner et al., 2004). As expected, the fluorescence emitted by TdTomato-labeled FB04 looked much brighter and more uniform than that of the DsRed-labeled FB03, but did not reach the same degree of brightness as the GFP labeled FB01 strain (Fig. 2A and B).

Since plant colonization studies were conducted at relative low temperatures the emission of fluorescence was monitored at 37°C and 20°C as well. Whilst cells of the GFP-labeled BF01 emitted permanently a homogeneous fluorescence independent from growth temperature, it rules out that fluorescence from FB03 (DsRed) and FB04 (TdTomato) varied greatly at both temperatures. The brightness of TdTomato decreased considerably when the bacteria were...
incubated at the lower temperature, possibly because TdTomato was originally optimized for mammalian cells cultured at 37 °C (Shaner et al., 2005).

3.3. Emitting brighter fluorescence by FB01mut does not affect its growth and long term stability

Despite better performance of the gfp+ labeled cells when compared with the DsRed and DsTomato tagged strains, their fluorescence did not fulfill our requirements. Fast photobleaching of only 10 s was registered in LB grown bacterial colonies. To overcome this problem, we tested several approaches including replacing the Ppae promoter by two indigenous promoters derived from FZB42 and used various buffers for specimen preparation. However, we failed to increase brightness and photostability.

A spontaneous mutant of FB01 with enhanced fluorescence was occasionally isolated from LB agar and compared with the parental strain under identical growth conditions. As Fig. 2C and D shows, the mutant strain FB01mut did express higher fluorescence then FB01. Assessment of intensity of fluorescence in LB grown cells (see Section 2) corroborated this result suggesting that FB01mut is able to emit fluorescence exceeding that of FB01 by at least 1.5 times.

Our attempts to identify the site of mutation failed. No nucleotide exchange was detected within the 1500 bp region covering the complete GFP coding region and its flanking promoter and terminator, suggesting that secondary mutation(s) might positively affect fluorescence in FB01mut. In fact, real-time RT-PCR demonstrated that expression of the gfp transcript in FB01mut was 2.5 times higher than that of the original FB01 transformant (Fig. 3).

Long term stability of GFP expression is necessary for monitoring colonization by GFP labeled strains under field conditions. In order to assess this property, FB01 and FB01mut were grown in LB in absence of antibiotic pressure and transferred after diluting 1:1000 every 12 h in fresh medium. After four days, corresponding to 30 generations, colonies obtained after serial dilution of the culture were assessed at UV light 390 nm for emitting fluorescence. All the 400 colonies examined from each strain were still able to emit green fluorescence.

Does production of GFP negatively affects growth of FB01 and FB01mut? In order to address this question, the three strains were cultivated in LB medium at 37 °C and monitored for their growth at OD₆₀₀. No differences between the strains did exist, neither in terms of generation time during exponential phase nor in final density at the end of growth (SM Fig. 1) suggesting, that GFP expression in FB01 and FB01mut does not negatively affect their growth parameters. Therefore, GFP-labeled strain FB01mut was chosen for further plant colonization studies.

Plant colonization studies were performed in a gnotobiotic system, in which FZB42 and its fluorescent derivative FB01mut were applied to seedlings of maize (Z. mays) and A. thaliana, axenically grown in soft agar containing Murashige–Skoog (MS) medium, and to L. minor ST cultivated in Steinberg liquid medium as described in Section 2.

3.4. FZB42 colonizes plant roots in different modes

After eight days of growing in MS soft agar at room temperature, the primary root of Z. mays was grown rapidly up to a length of 20 cm. A segment of the primary root, located around 2–8 cm distant from the root tip, a region especially rich on emerging lateral roots, was found heavily colonized by FB01mut, which formed micro-colonies onto surface of outer epidermis cells of the primary root (Fig. 4A) and at the junctions of primary root and lateral roots (Fig. 4B). In general, GFP labeled bacteria were detected in decreasing density towards root tip. A phenomenon previously reported for tomato root colonization by P. fluorescens (Chin-A-Woeng et al., 1997). Transmission electron microscopy (TEM) from cross sections of maize primary root, revealed that bacterial cells colonized surface of the root as biofilm (Davey and O’Toole, 2000) by forming several cell layers on it (Fig. 4A). Thickness of the layer did not exceed 2–3 µm as revealed by computer aided 3D-analysis of the TEM images.

Whilst FZB42 and FB01mut, respectively, when colonizing maize roots, colonized preferentially base and adjacent parts of primary root including the junction between root epidermis and root hairs (Fig. 4D), the same bacteria, when applied to Arabidopsis plantlets, colonized plant roots in a different mode. Here, primary root tip and the tip area of emerging lateral roots and root hairs were preferred targets of colonization by FZB42 and its derivative FB01mut (Fig. 4C and D). On surface of Arabidopsis roots, a significant portion of colonizing bacteria grew in patches along grooves or niches formed at the border regions of adjacent epidermis cells. Growth within this physically protected area might lead to a more intimate contact to root surface which could result in better utilization of nutrients extruded by the root.

B. amyloliquefaciens colonized too the roots and the ventral side of L. minor fronds. Bacterial colonization started at root tips, and at the junction linking fronds and roots, where micro-colonies became visible 24 h after inoculation. During following days, bacterial micro-colonies spread over whole surface of fronds and roots. Colonization of Lemna culminated in forming of a local biofilm that covered surface of Lemna root (Fig. 4E). Notably, nine days after inoculating, nearly the whole ventral side of Lemna fronds was found colonized by Bacillus cells (Fig. 4F) suggesting that fronds are preferred sites of bacterial colonization.

3.5. An extracellular matrix is formed during biofilm formation at root surfaces

SEM of bacterial associations colonizing plant cells revealed existence of a extracellular polymeric matrix encasing bacterial cells. It contained specific fiber-like structures which are involved in adhesion bacteria onto plant surfaces, the extracellular matrix served as a kind of “molecular glue”, enabling Bacillus cell associations to form multi-cellular aggregations on maize roots (Fig. 4C). Extracellular matrix seemed to have same function in FZB42 cells colonizing Lemna fronds (Fig. 4G and H). Besides its function as attractant which is adhesion bacteria onto plant surfaces, the extracellular matrix served as a kind of “molecular glue”, enabling Bacillus cell associations to form multi-cellular aggregations on maize roots (Fig. 4C). Extracellular matrix seemed to have same function in FZB42 cells colonizing Lemna fronds (Fig. 4G and H). When comparing cell shape of cells colonizing maize and Lemna surfaces, clear differences became visible. Whilst cells colonizing maize roots appeared in SEM as slim or slender rods (Fig. 4B–D), FZB42 colonizing Lemna organs appeared more compact displaying a sausage-like structure (Fig. 4F and G).
Moreover, during prolonged incubation time (around nine days) dense biofilms consisting of shorter, compact cells were formed. During this process cell shape altered from rod- to dumpy barrel or square-like structure (Fig. 4H).

3.6. Surfactin is synthesized when FZB42 is colonizing L. minor

FZB42 is a potent producer of cyclic lipopeptides, such as surfactins, fengycins, and Bacillomycin D, as well as polyketides, such as bacillaene, difficidin, and macrolactin, when growing in Landy medium (Chen et al., 2006, 2007; Koumoutsi et al., 2004). However, only surfactin was detected by MALDI-TOF MS, when FZB42 colonized L. minor plantlets, cultivated in Steinberg medium (Figs. 5 and 6), as demonstrated by the surfactin specific mass peaks at \( m/z = 1044.8; 1046.8; 1058.8; 1060.8 \) and 1074.8 (Koumoutsi et al., 2004). In absence of Lemna plantlets, FZB42 did not propagate in Steinberg medium, and no surfactin specific peaks could be detected in the Steinberg medium and in extracts of Lemna plantlets grown without FZB42 (results not shown).

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**Fig. 4.** CLSM of BF01mut colonizing plant tissues. (A) Surface of maize roots and (B) junction area, adjacent to an emerging root hair. The bacteria grew around the root hair base. Images 3A and B were taken eight days after inoculation. (C) Arabidopsis primary root tip. (D) Arabidopsis root hair. Images 3C and D were taken seven days after inoculation. (E) Lemna root covered by FB01mut biofilm. (F) Lemna frond, ventral side, colonized by FB01mut. Images 3E and F were taken nine days after inoculation. Details see text.
4. Discussion

We describe here labeling of the plant growth promoting and biocontrol strain *B. amyloliquefaciens* FZB42 by chromosomal integration of a single *gfp*+ (Scholz et al., 2000) gene copy into its chromosome and the pattern of plant colonization by the highly fluorescent derivative FB01mut on three different plants. According to the best of our knowledge this is the first study performed with a fluorescent G+ bacterium which contains a chromosomal integrated single *gfp* gene copy. Comparable studies were performed with *B. megaterium* and *P. polymyxa*, but in these cases tagging by plasmid-borne *gfp* gene copies was performed which might affect long term stability of the plasmid bearing strain (Liu et al., 2006; Timmusk et al., 2005). The *gfp*+ harboring FZB42 was proven as being not affected in its growth parameters and to appear suitable for long term studies performed in natural environment. Moreover, due to genetic amenability of FZB42, its *gfp*+ labeled variant is useful to monitor effect of several mutations possibly affecting colonization behavior within a competitive environment.

To obtain high stability and to avoid a genetic burden affecting growth of the labeled strain, we decided not to label FZB42 by the standard method using episomic *gfp*+ gene copies, but to integrate a single copy of the *gfp*+ gene into the bacterial chromosome taking advantage of natural DNA competence and presence of a functional homologous recombination system in FZB42 (Koumoutsi et al., 2004). Disadvantage of this approach is that expression of the single *gfp*+ copy present in bacterial genome results in relative low intensity of green fluorescence, a problem especially apparent in G+ bacteria with cell envelopes consisting of multiple peptidoglycan layers. Indeed, the original *gfp*+ transformants, although appearing brighter than the DsRed and DsTomato tagged cells, emitted a relative dim fluorescence, when grown onto LB agar. Fortunately, the spontaneous mutant FB01mut emitting brighter fluorescence and slightly delayed photobleaching has been proven suitable for
were preferred sites for colonizing by gfp+ tagged FZB42, but the pattern of colonization primary roots was found different. FZB42 colonized preferentially Arabidopsis root tips; but favored sites more distant from the root tip when colonizing maize roots. This could indicate that both plants secrete nutrient rich exudates, attracting rhizobacteria, at different sites. FZB42 was also able to colonize L. minor roots and fronds, thereby forming robust biofilms onto certain surface areas. We have already demonstrated that FZB42 can significantly support Lemna growth, when bacterial cells were added to duckweed fronds (Idris et al., 2007). Due to its small size, aquatic life style and rapid propagation rate, L. minor is an attractive subject for investigating plant–microbe interactions (Lockhart et al., 1989). L. minor, reproduces primarily by vegetative budding, occasionally by flowering. Unlike the roots of most other kinds of plants, Lemna roots contain rich chlorophyll whilst having no root hair. Notably, Lemna emits red autofluorescence from the chlorophyll molecules present in fronds and roots, nicely contrasting the green fluorescent FZB42 bacteria from plant surfaces.

Root colonization by environmental FZB42 is linked with formation of robust biofilm, structured communities of cells adherent to a surface (Watnick and Kolter, 1999). Robust biofilm formation was also detected when wild type B. subtilis and P. polymyxa colonized primary root tips of A. thaliana (Bais et al., 2004; Timmusk et al., 2005). According to our SEM images, FZB42 cells are encased by an extracellular matrix which keeps together bacterial association onto the surface of plant root epidermis cells. Such matrix structure has been described as essential components of biofilms. They consist of a variety of cell surface components, such as wall polysaccharide (capsules), lipopolysaccharide, cell surface agglutinin, and exopolysaccharide (Bais et al., 2004). Notably, fiber-like structures were detected, which adhering bacterial cells at the root surface and linking the cells with each other. Similar structures were also detected, when B. amyloliquefaciens colonized Arabidopsis seeds (Reva et al., 2004). Formulation of biofilm at Lemna surface was found connected with synthesis of surfactin, corroborating an earlier finding performed with undomesticated B. subtilis (Bais et al., 2004). However, other lipopeptides and polyketides produced by FZB42 grown in Landy medium (Chen et al., 2006, 2007; Koumoutsi et al., 2007), were not detected by MALDI-TOF mass spectrometry performed with the cell extract and the growth medium from L. minor, inoculated with FZB42. This implies an important role of surfactin in colonizing plant roots by plant associated B. amyloliquefaciens strains. In contrast to bacillomycin D and fengycin, and the polyketides bacillaene, difficidin and macrolactin, which are all produced by FZB42 under laboratory conditions, antimicrobial action of surfactin is relatively weak. Therefore, it is tempting to speculate, that the biocontrol action of FZB42 observed under environmental conditions (Chen et al., 2007, 2009b), is not necessarily linked with production of antibiotics within rhizosphere. However, more sensitive detection methods have to apply to prove this probability.

Plant colonization by FZB42 was restricted to the rhizosphere. Neither the CFLSM nor the TEM and SEM images indicated endophytic growth of FZB42 at the three model plants suggesting that FZB42 is a true epiphyte. In contrast to FZB42, GFP tagged B. subtilis 168 did not colonize L. minor surfaces (Fan, unpublished results), corroborating earlier reports about inability of domesticated B. subtilis 168 to colonize plant roots (Bais et al., 2004).

Acknowledgment

Financial support by Federal Ministry of Education and Research, BMBF, Germany, is gratefully acknowledged.
Appendix A. Supplementary data


References


