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

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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 performed with *Zea mays*, *Arabidopsis thaliana* and *Lemna minor*. Real-time RT-PCR revealed that FB01mut expressed 2.5 times more of the *gfp* transcript than the original GFP-labeled strain. Confocal laser scanning microscopy of plant roots infected with *gfp+* tagged FZB42 revealed that the bacterium behaves different in colonizing surfaces of plant roots of different species. In contrast to maize, FZB42 colonized preferentially root tips when colonizing *Arabidopsis*. FZB42 colonized heavily *Lemna* fronds and roots by forming biofilms consisting of extracellular matrix and cells with altered morphology. Surfactin, but no other lipopeptide or polyketide synthesized by FZB42 under laboratory conditions, was detected in extracts of *Lemna* plantlets colonized by FZB42. Due to its stable and long-lasting emission of bright fluorescence without antibiotic pressure FB01mut is an excellent tool for studying plant colonization under competitive, environmental conditions.

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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).

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; Kloepper 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 *Aequoria victoria* has been proved to fulfill the expectations as valuable molecular marker which can be expressed in

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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 OD₆₀₀ of 1.0 and diluted by 1000 times before use. The roots of the seedlings were allowed to soak into the bacterial suspension (~10⁵ 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 OD₆₀₀ 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'-AGTTTGACGTCCTCCGATTCGCCGACAACAC-3' (AatII restriction site is underlined) and amyFront-2: 5'-TCGATTGT-TGCAGTTTCAGCG-3'. The downstream border sequence of *amyE* gene (“amy back”) was amplified with primers amyBack-1: 5'-AGCGAAATTACCTGACGGCAG-3' and amyBack-2: 5'-AGCTCAAGTTCCGTCACACCTG-3'. The amplified sequences were inserted into vector plasmid pUC18Em^r, yielding recombinant plasmid pVBF containing the two *amyE* border sequences. The *gfp+* gene together with an upstream located P_{spac} promoter element was derived from plasmid pECE149 (BGSC, Kaltwasser et al., 2002; Scholz et al., 2000) and cloned into plasmid pVBF. The resulting integrative plasmid pFB01 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 pFB01 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” pFB01 created by *KpnI* were also blunt ended by Klenow to ligate it with the DsRed fragment derived from pECE163 yielding pFB03.

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'-GATAATGGTACCAATGGTGAGCAAGCGCG-3' (*KpnI* restriction site is underlined) and reverse primer 5'-TCCATTA^rACTAGTCTTACTGTACAGCTC-3' (*SpeI* restriction site is underlined). The amplified PCR product was cut by *KpnI* and *SpeI* and then cloned into plasmid “empty” pFB01 lacking *gfp+* as described above, but still containing the preceding P_{spac} promoter sequence (pFB04).

The plasmids with red fluorescence gene, pFB03 and pFB04 were transformed into FZB42, yielding strains FB03 (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 OD₆₀₀ 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 OD₆₀₀ 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

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 μ l 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: TCCATGGCCAACACTTGTC, gfp-real-2: CCGGATAACGGGAAAAGCA. As an endogenous control, the highly conserved *gyrA* gene was used, applying the following primers: *gyrA*-real-1: GACGCAAGAAACAAATCATCA, and *gyrA*-real-2: CCGGATAACGGGAAAAGCA. Three technical replicates were performed. Quantification based on the threshold cycle (Ct) values according to Paffl (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 \times 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, taken from different parts of the root with a sterile razor blade or by cutting cross sections, 50 μ m 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 and directly used for light microscopy. All specimens were thoroughly rinsed with 5 ml distilled water and then transferred into saline for microscopic observation.

2.7. Microscopy

GFP fluorescence was viewed with an epifluorescence microscope Zeiss Axiophot XIOPHOT using a filter set of 450–490 nm excitation filter and LP520 emission filter. DsRed and TdTomato fluorescence was viewed by using a BP546 excitation filter and a LP590 emission filter.

Confocal laser scanning microscopy was performed with Leica DM IRE2&DM IRB system (Leica) using an excitation laser of 488 nm (Argon laser) and collecting the emission band of 500–550 nm for GFP fluorescence. For DsRed/TdTomato fluorescence NeHe laser of 543 nm was used for excitation and the emission band of 575–655 nm was collected. Transmission light was collected to visualize root structure and was designated as red color in later image reconstruction in order to manifest the contrast with green color. Images were acquired and reconstructed by Leica Confocal Software (LCS 2.6).

2.8. Electron microscopy

For transmission electron microscopy (TEM) a 10 mm segment from the main root of *Z. mays* seedlings was taken 25 mm below the caryopses. The 10 mm segment was divided into two pieces 5 mm in length and further processed for electron microscopy. The sample was fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.4 for 24 h at 4 $^{\circ}$ C. Afterwards the samples were rinsed three times for 1 h each with 4 $^{\circ}$ C cold 0.1 M sodium cacodylate buffer, pH 7.4. For secondary fixation, 2% osmium tetroxide in sodium cacodylate buffer solution was used for 5 h at 4 $^{\circ}$ C. The specimen were subsequently rinsed in cold 0.1 M sodium cacodylate buffer, post-stained with 1% uranyl acetate in 0.05 M maleate buffer solution, pH 5.2 for 5 h at 4 $^{\circ}$ C, dehydrated in ethanol, infiltrated and embedded in Spurr's epoxy resin and polymerized for 24 h at 70 $^{\circ}$ C. Ultrathin-sections were cut with a diamond

knife using an Ultracut S-microtome (Leica, Vienna, Austria). The sections were transferred to uncoated 300 mesh thin-bar-grids, 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 graded 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). Specimen were then mounted on stubs for SEM, sputtered with gold (Sputter Coater SCD, 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, *Lemna* 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 μ l 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

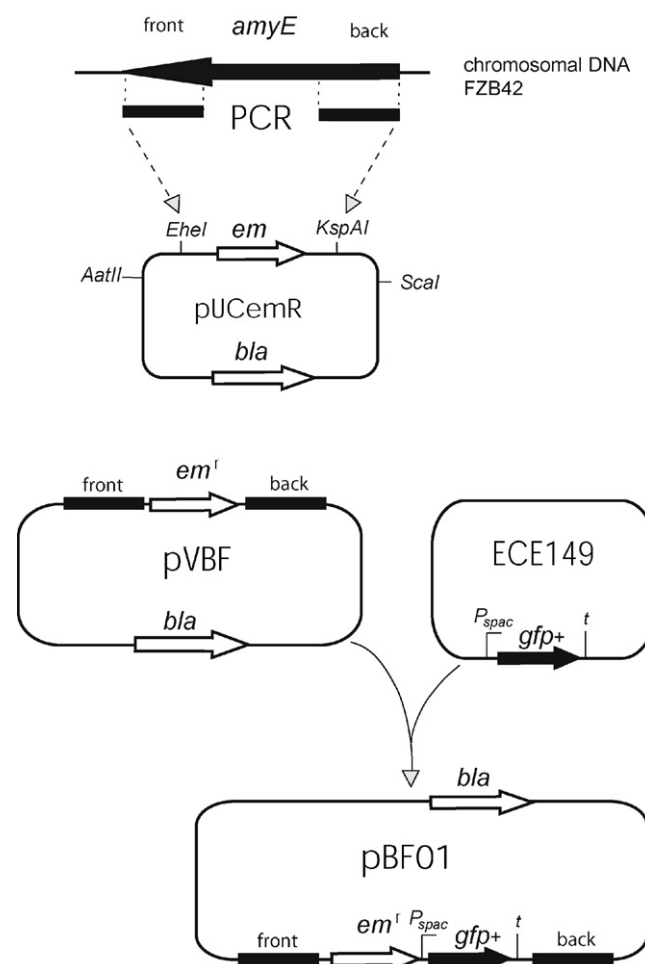


Fig. 1. Construction of integrative plasmid pBF01. See Section 3 for a complete description of plasmid construction. Plasmids are not shown to scale.

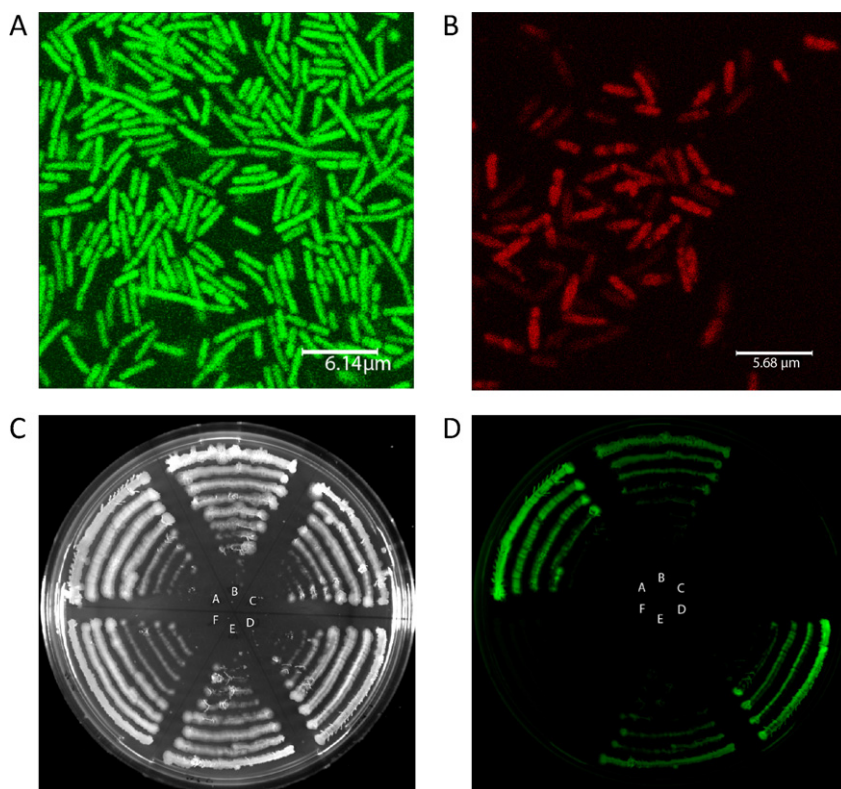


Fig. 2. CLSM of *B. amyloliquefaciens* FZB42 and derivatives. FP-tagged strain FB01 was grown in liquid LB overnight at 24 °C (A), and FB04 was grown at 37 °C (B). Comparison of fluorescence intensity: FZB42 (c and f), FB01 (b and e), and FB01mut (a and d) were grown overnight onto LB agar at 24 °C (C) and monitored for their fluorescence at 390 nm (D). Details of the experiment are described in Section 2.

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 P_{spac} 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* - P_{spac} - *gfp+* - *trpA* cassette via double crossover recombination at both terminal *amy* regions. The *amy*⁻, *em*^R 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

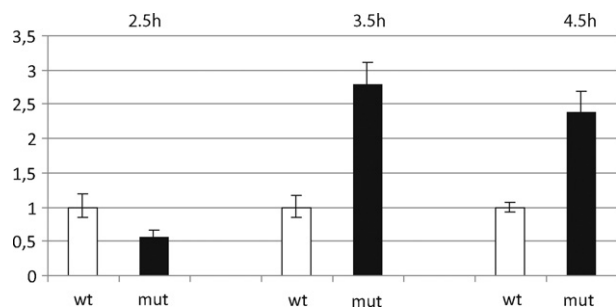


Fig. 3. Transcription of the *gfp* gene in FB01(wt) and FB01mut (mut) after 2.5, 3.5, and 4.5 h growth in LB medium validated by real-time RT-PCR (see Section 2). The relative expression of the *gfp* transcript RNA of FB01mut was calculated as the fold change in comparison to FB01(wt). Maximum absolute deviation was used for statistical analysis.

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 DtTomato 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 P_{Spac} 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 than 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 excreted 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 an extracellular polymeric matrix encasing bacterial cells. It contained specific fiber-like structures which are involved in adhering bacteria at surfaces of roots (Fig. 4C) and root hairs (Fig. 4D). The fiber-like structures were clearly visible also in FZB42 cells forming micro-colonies at the ventral side of *Lemna* fronds (Fig. 4G and H). Besides its function as attractant which is adhering 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).

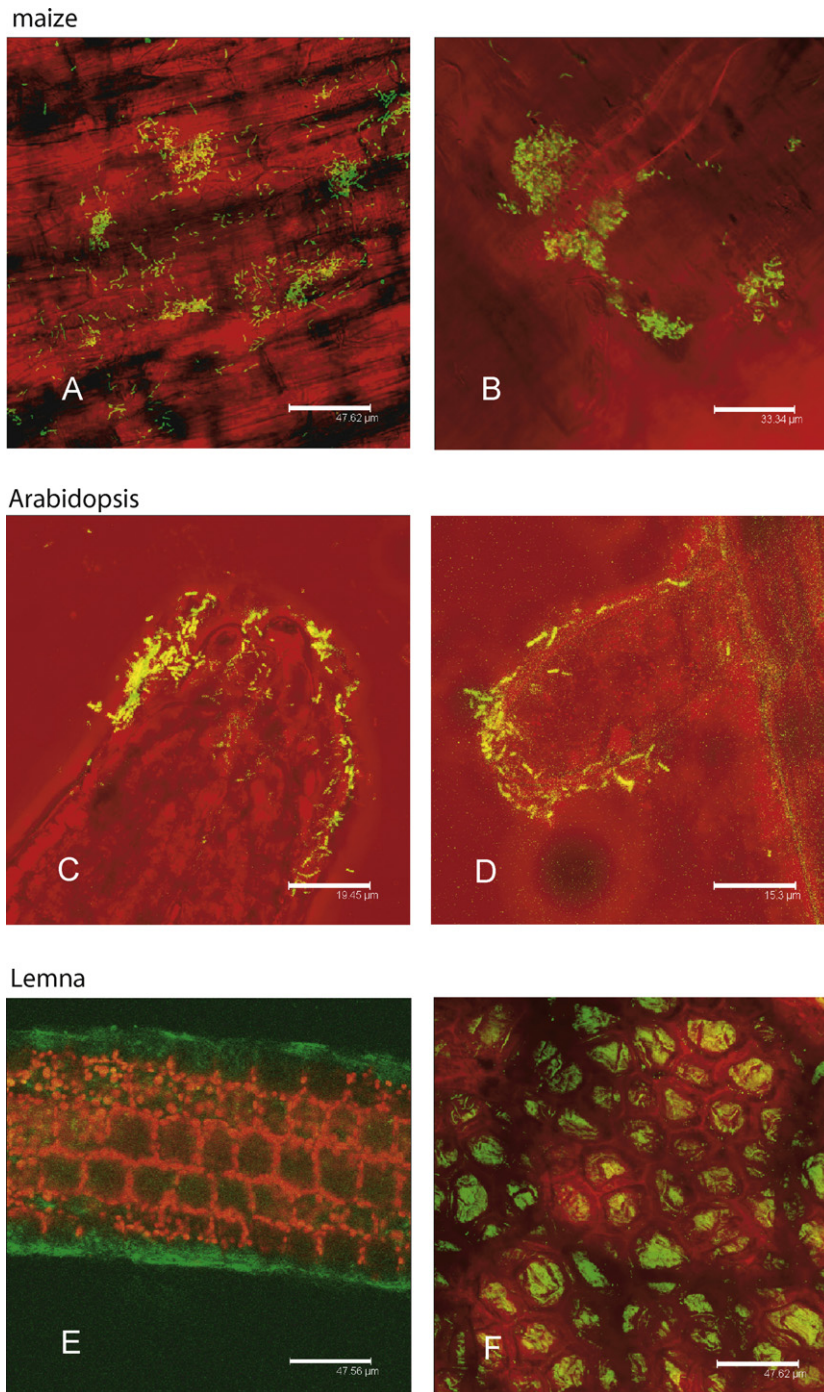


Fig. 4. CLSM of BF01mut colonizing plant tissues. (A) Surface of maize roots and, (B) junction area, adjacent to an emerging root hair. The bacteria grew around the root hair base. Images 3A and B were taken eight days after inoculation. (C) *Arabidopsis* primary root tip, (D) *Arabidopsis* root hair.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

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).

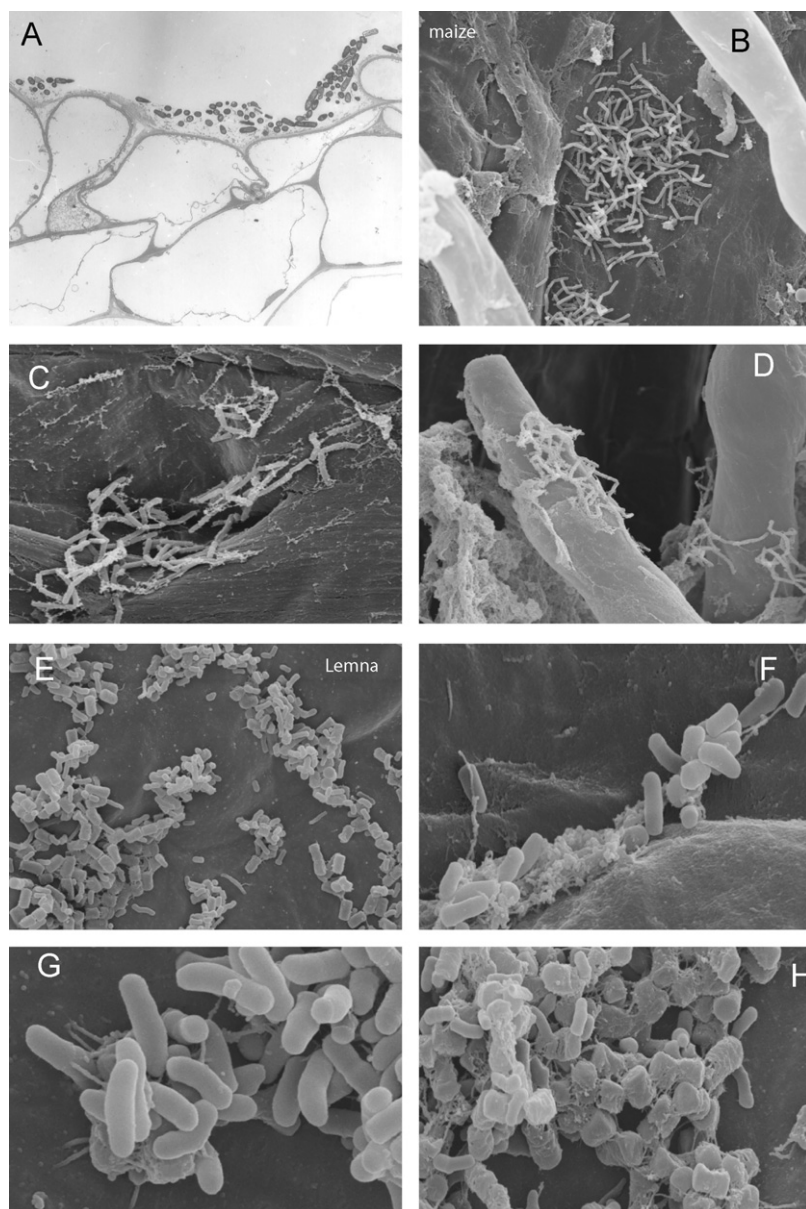


Fig. 5. Electron microscopy of *B. amyloliquefaciens* FZB42, colonizing maize and *Lemna* plants. (A) Transmission electron microscopy, TEM, of FZB42 colonizing maize root. The image was taken from a cross section around 5 cm distant from the root base. (B and C) Scanning electron microscopy (SEM) of FZB42 colonizing maize roots, (D) SEM of FZB42 colonizing maize root hair. Maize images (A–D) were taken eight days after bacterial inoculation. (E and F) SEM of FZB42 colonizing *Lemna* root surface. (G and H) SEM of FZB42 cells colonizing the ventral side of *Lemna* fronds. The images from *Lemna* roots and fronds, respectively, were taken four (E and F) or nine days (G and H) after adding the bacteria. For further explanations 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 (Koumoutsis 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 DtTomato 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

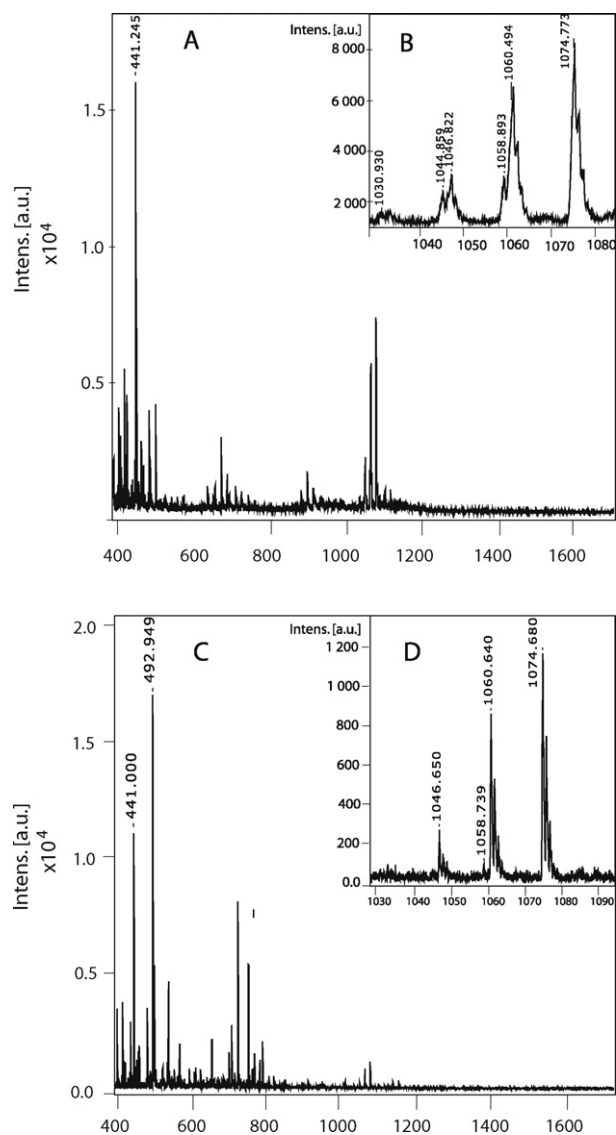


Fig. 6. MALDI-TOF mass spectra of metabolites produced by *B. amyloliquefaciens* FZB42. Metabolites were detected in the Steinberg growth medium (A and B) and in extracts of *Lemna* plantlets with 100 μ l 70% acetonitrile/0.1% trifluoroacetic acid (C and D) in the mass range between m/z = 400–1600. Two μ l aliquots of the growth medium and the plant extracts were mixed with the same volume of CCA matrix solution, spotted on the target and measured as described under Section 2. Surfactin was detected when FZB42 was grown at *Lemna* surfaces (see expanded spectra in the mass range of m/z = 1030–1080, B and D).

plant colonization studies. Surprisingly, *gfp*⁺ tagged FZB42 cells were found to emit more bright fluorescence with prolonged photobleaching period when grown onto plant surfaces than when grown onto LB agar plates. Therefore, green fluorescence emitting FB01mut cells could be easily distinguished from auto-fluorescent plant tissues, allowing plant colonization studies with this strain. Whilst our attempts to detect mutations within the *gfp* coding region and its adjacent promoter region failed, we could demonstrate by real-time RT-PCR a higher level of expression of the *gfp* transcript in strain FB01mut suggesting that brighter fluorescence of this strain was due to an unknown mutation favoring transcription of the *gfp* gene.

As reported for other Gram-positive and Gram-negative rhizobacteria (Liu et al., 2006; Rovira, 1956; Timmusk et al., 2005), FZB42 colonized only a small part of the rhizoplane, mainly the junctions between epidermal cells and areas where lateral roots appeared. Root hairs of both, maize and *Arabidopsis* seedlings,

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).

Formation 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; Koumoutsis 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 rhizoplane. 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).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2010.12.022.

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