Environmental Microbiology Reports (2009) **1**(2), 124–130 doi:10.1111/j.1758-2229.2009.00017.x

# Ecological fitness of *Bacillus subtilis* BGS3 regarding production of the surfactin lipopeptide in the rhizosphere

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## Summary

**Cyclic lipopeptides and particularly surfactins produced by *Bacillus* species retain antibacterial, antiviral, biofilm-forming and plant resistance-inducing activities. In most cases, their role in biological control of plant diseases was evoked on the basis of *in vitro* assays or by using non-producing/overproducing mutants but there is a need for more direct evidence of an efficient lipopeptide biosynthesis in the rhizosphere. In this work, we coupled LC-MS quantification of the lipopeptides secreted by cells colonizing tomato plants with the use of p*srfA*–*lacZ* reporter system integrated within the BGS3 chromosome to study the expression of the surfactin operon *in planta*. Results showed that a higher level of p*srfA* induction was observed upon the establishment of a stable BGS3 population on roots and surfactins extracted from the rhizosphere were produced in biologically significant quantities. Our results also demonstrate that BGS3 efficiently utilizes the main substrates from plant exudates to produce surfactins. This synthesis is also efficient in cells forming colonies and the production may be favoured in bacteria developing slowly in the rhizosphere. This provides a first understanding of how environmental factors may influence lipopeptide production by beneficial *Bacillus* strains.**

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## Introduction

Among the panoply of antimicrobial compounds that can be produced by *Bacillus* species, cyclic lipopeptides of the surfactin, iturin and fengycin families retain potential biotechnological applications (Mulligan, 2005; Mukherjee *et al.*, 2006) but are also tightly involved in most of the mechanisms described to date that explain the beneficial effect of specific strains to control plant diseases (Bais *et al.*, 2004; Ongena and Jacques, 2008). They can facilitate root colonization, act as antagonists by directly inhibiting phytopathogens and reinforce the resistance potential of the host plant via the stimulation of the so-called induced systemic resistance (ISR) phenomenon. This broad range of activities is due to the physicochemical properties of these non-ribosomally synthesized amphiphilic molecules. The *srf* operon encodes genes involved in surfactin biosynthesis. Members of this group are heptapeptides interlinked with a β-hydroxy fatty acid to form a cyclic lactone ring structure. The length of this fatty acid chain may vary from $C_{12}$ to $C_{15}$ and different homologous compounds are thus usually co-produced (Peypoux *et al.*, 1999).

Plant roots exude a large range of potentially valuable small-molecular-weight compounds into the rhizosphere that creates a highly dynamic front for interactions between roots and soil microflora (Bais *et al.*, 2006). Some of these exuded molecules act as chemical signals for motile bacteria to move to the root surface but also represent the main nutrient sources available to support growth and persistence of rhizobacteria (Hirsch *et al.*, 2003). The host plant thus imposes a specific nutritional context but the complexity of microbial communities sharing the ecosystem and the variability of physicochemical parameters inherent to the soil such as mineral content, pH, temperature and oxygen availability may also drastically influence rhizobacterial growth and production of biocontrol determinants as demonstrated with various pseudomonads (Duffy and Défago, 1999; Van Rij *et al.*, 2004; Dubern and Bloemberg 2006; Ongena *et al.*, 2008). Understanding which and how environmental factors may modulate the production of biocontrol determinants is a key to improve the level and reliability of plant disease reduction through the use of rhizobacteria. However, in

the case of *Bacillus* antibiotics and particularly lipopeptides, most of the data suggesting their role in biocontrol derive from antagonism assays performed *in vitro* or by using non-producing or overproducing derivatives and correlation with respective loss or increase in the biocontrol activity (Ongena and Jacques, 2008). Comparatively, studies providing direct evidence through the demonstration of an efficient lipopeptide production *in situ* are scarce (Asaka and Shoda, 1996; Touré *et al*., 2004; Romero *et al*., 2007).

In this context, the study reported here was conducted with the first objective to demonstrate efficient expression of biosynthetic genes and surfactin secretion in the rhizosphere and second to evaluate the influence of some rhizosphere-specific parameters on lipopeptide synthesis. To this end, we used the LacZ gene reporter system in *Bacillus subtilis* BGS3, not only to provide information on the transcriptional activity of specific antibiotics *in situ* but also to easily monitor populations of the introduced strain among the whole root-associated microflora. The BGS3 derivative was generated from the surfactin-overproducing isolate BC25 previously demonstrated to provide a protective effect on tomato via the stimulation of systemic resistance in the host plant (Ongena *et al*., 2007).

## Results and discussion

### *Colonization and surfactin production in the rhizosphere*

Expression of biosynthetic genes and surfactin secretion by the BGS3 derivative was first verified during growth in an optimized medium in batch-type bioreactor with control of fermentation parameters. Surfactin production occurred 5–6 h after the start of bacterial growth and reached a maximum after 18 h of cultivation corresponding to the transition from exponential to stationary growth phase which is in agreement with results from other studies (Fig. S1 in *Supporting information*) (Lin *et al*., 1998; Koumoutsi *et al*., 2004). Biomass and surfactin concentrations were similar to the parental BC25 strain showing that the genetic transformation did not significantly affect the basal metabolic functions or those related to the synthesis of surfactins (not shown).

*In vivo* experiments were conducted on tomato plants grown in a hydroponic culture mode to facilitate colonization study and quantification of surfactins secreted into the surrounding environment. The colonization process observed for BGS3 was quite similar to the one reported for other plant beneficial rhizobacteria (Fig. 1) (Rainey, 1999; Espinosa-Urgel *et al*., 2002) with a first step of bacterial cell attraction/adhesion followed by a efficient colonization phase and by a further decrease of the population to reach a stable level at approximately $5 \times 10^6$ cell per gram of roots (Fig. 1). Such steady-state phase most

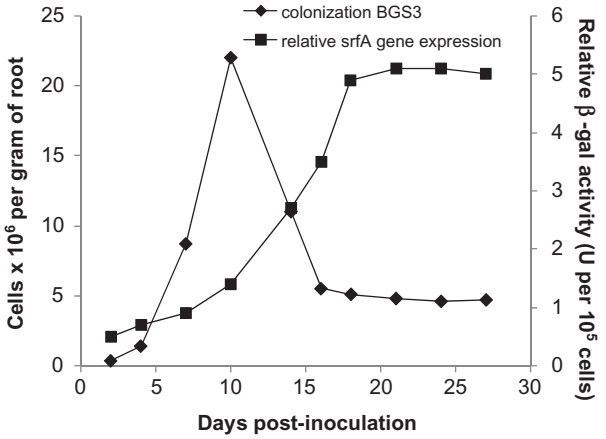


**Fig. 1.** Evolution of population and expression of surfactin operon in *B. subtilis* BGS3 cells colonizing tomato roots. *Bacillus subtilis* BGS3 was constructed as follow: p*srfA* promoter was PCR amplified from *B. subtilis* S499 genomic DNA using primer MO01b (5′-CCT CAT GCC TAT TCT TGA AGC CAT GTA TG-3′) and MO02b (5′-GGA TCC TAT TTC CAT ATT GTC ATA CCT CCC CTA ATC-3′) and cloned into pGEM-T Easy vector (Promega, Madisson, WI) to generate pMO01. After correct sequence verification with universal primer T7 and SP6, p*srfA* promoter was rescued from pMO01 by BamHI–EcoRI digestion and cloned into pDG1663 (Guérout-fleury *et al*., 1996) at the corresponding site to yield pMO02. This final construct was then integrated into *B. subtilis* BC25 (Ongena *et al*., 2007) at the *thrc* locus to give rise to strain BGS3. Ectopic integration was verified by threonine auxothrophy on minimal medium, resistance to erythromycine and sensibility to spectinomycin as described elsewhere (Guérout-fleury *et al*., 1996). Surface-sterilized tomato seeds were germinated for 5 days at room temperature in the dark and in gelified sterile nutrient medium consisting of 5 mM $Ca(NO_3)_2$, 5 mM $KNO_3$, 2 mM $MgSO_4$, 1 mM $KH_2PO_4$ and micronutrients. They were transferred in sterilized 50 ml tubes filled with nutrient solution, inoculated with strain BGS3 and incubated at 25 ± 2°C in the greenhouse with a 16 h photoperiod alternating sunlight and fluorescent light. For inoculum preparation, the bacterium was grown on solidified Luria–Bertani (LB) medium for 24 h and harvested cells were re-suspended in NaCl 0.85% and the concentration was determined by measuring turbidity at 600 nm. BGS3 cells were inoculated at a final concentration of $10^8$ cells $ml^{-1}$ nutrient solution. Root colonization by the BGS3 strain was determined by plate counts on LB medium on the basis of typical morphology of the colonies and blue coloration upon growth on X-Gal-supplemented medium. The use of both criteria was necessary to reliably discriminate BGS3 cells from the contaminating bacterial microflora. Three plants were used to obtain values at each time point and data represent mean values from two experiments. *In situ* surfactin gene expression was evaluated on samples of approximately 0.2 g of root material that was suspended in buffer Z containing lysozyme for lysis of root-adhering cells and further incubated for 60 min at 40°C at pH 7 in the presence of β-gal substrate. *srfA* expression was determined by $A_{420}$ after 60 min of incubation at 40°C. For determination of both colonization and *srfA* promoter gene expression (on the same plants), root samples from three different plants were used at each time point in one assay and data mentioned in the figure represent mean values calculated from two independent experiments that yielded similar results. Relative β-galactosidase activity was obtained by subtracting values obtained for untreated control plants from those measured for BGS3-inoculated tomatoes.

probably corresponds to a resident phase where the population size is restricted by space and/or nutrients availability and is thus limited by plant growth and root exudation rate.

The level of *srfA* gene expression in root-colonizing BGS3 cells remained almost stable during the first 4 days after inoculation, but sharply increased from day 10 to reach a maximum at day 21 (Fig. 1). A high and constant relative expression of *srfA* gene per cell unit in the rhizosphere was then observed until the end of the experiment corresponding to the period where BGS3 cells maintained a stable population. The development of established bacterial populations in the rhizosphere is actually restricted by nutrient availability owing to limited root exudation rate (Lugtenberg *et al*., 2001; Bais *et al*., 2006). *Bacillus* cells on roots can thus be in a nutrient-starved state that would drive cell physiology to slow growth rate that is apparently conducive for surfactin synthesis. Such effect of growth rate on surfactin synthesis was confirmed by experiments in chemostat bioreactors that allows the study of metabolite synthesis upon fixed growth rate ($\mu$) and constant cellular physiological state. Biomass levels obtained at steady state in the different conditions of growth rates were similar at approximately $2.5 \times 10^8$ cells $ml^{-1}$ (Fig. 2). In contrast, *srfA* expression significantly decreased with increasing $\mu$ and the residual β-galactosidase activity measured at $\mu = 0.33\ h^{-1}$ represented only 6% of the activity observed at $\mu = 0.1\ h^{-1}$ (Fig. 2). A similar relationship between $\mu$ and *srfA* expression was observed with another *B. subtilis* isolate derived from the surfactin-overproducing JH642 strain (not shown).

In parallel to the gene expression determination, a reliable recovery and quantification of lipopeptides secreted in the microenvironment by the root-colonizing strain is essential. To this end, we had to combine material extracted from the nutrient solution with the one recovered by repeated extraction of root tissues [1 g of samples extracted with 4 ml of a MeOH/$H_2O$/Triton X-100, 50/50/0.9 (v/v/v) solution]. This could be explained by the fact that secreted surfactins are rapidly embedded in the membrane structure of the target organism as suggested by the rapid disappearance of the lipopeptide from the nutrient solution when supplemented in hydroponically grown tomatoes (not shown). In two different experiments, rhizosphere samples were collected after a period of 20 days post inoculation and LC-MS analyses (see footnote of Table 1 for details) of their content in surfactin revealed a mean value of 320 ± 80 μg per $10^8$ cells. In our conditions, considering an average biomass of $7.5 \times 10^6$ cell per gram of root (Fig. 1) and 2 g of root tissue per plant, it corresponds to approximately 1.8 μM in the nutrient solution. Importantly, such a surfactin concentration is in the range of the one necessary to induce ISR on the same plant (5 μM) (Ongena *et al*., 2007). It indicates that the lipopeptide may be produced in biologically significant quantities in the rhizosphere of the host plant.

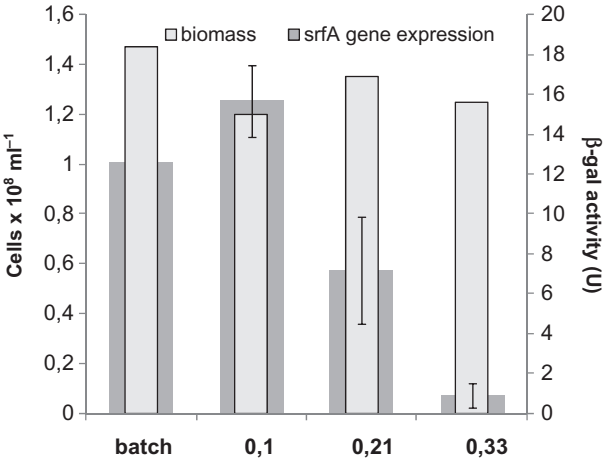


**Fig. 2.** Influence of growth rate of BGS3 cells on expression of surfactin synthetase gene (*srfA*) and biomass production. Chemostat cultures were performed in a 2 l laboratory glass bioreactor with a working volume of 1.5 l and equipped with automated pH control, a dissolved oxygen tension electrode and a magnetic coupled stirrer (BiostatB, B. Braun Biotech. International, Melsungen, Germany). The temperature was maintained at 30°C and pH was regulated at 7 by addition of $H_3PO_4$ (3 N). Aeration rate and agitation were fixed respectively at 0.5 VVM (volume of air per volume of medium per minute) and 600 r.p.m. By contrast with batch culture where the exhaustion of a specific nutrient terminates the exponential growth phase, biomass concentration in the chemostat culture is usually controlled by the permanent limitation of a single defined nutrient (peptone as source of threonine in this case). It allows the study of metabolite synthesis upon fixed growth rate and constant cellular physiological state. Fresh medium (containing per litre bacto-peptone 1 g, $(NH_4)_2SO_4$ 1 g, sucrose 10 g, $KH_2PO_4$ 1.9 g, $CuSO_4$ 0.001 mg, $FeCl_3$ 0.005 mg, $NaMoO_4$ 0.004 mg, $MnSO_4$ 3.6 mg, KI 0.002 mg, $MgSO_4$ 3.6 mg, $ZnSO_4$ 0.014 mg, $H_3BO_3$ 0.01 mg, citric acid 10 mg) was fed into the reactor using a peristaltic pump to control the dilution rate. The volume of the culture was maintained constant by the use of an overflow device. Cultures were inoculated with a 2% volume of 16 h subcultures realized in the same medium and the bioreactor was switched to continuous mode after an initial batch start-up. Silicone antifoam was added to control extensive foam formation. The different growth rates ($\mu$) of 0.1, 0.21, 0.33 $h^{-1}$ were respectively considered as low, intermediate and relatively high in this medium on the basis of the growth kinetic observed in batch culture in the same conditions. Samples were harvested at steady state for cell density determination ($OD_{600}$) and β-galactosidase activity measurement on cell extract prepared by lysozyme treatment and centrifugation (Fickers *et al*., 2008). One unit of β-galactosidase activity was defined as the amount of enzyme that produces 1 nmol of o-nitrophenol $min^{-1}$ at 37°C per $OD_{600}$ unit. Data are average values (± standard error) obtained from surfactin concentrations in three independent cultures performed for the study of one specific $\mu$. Surfactin concentration in every independent culture was calculated on the basis of three measurements at 1 h intervals during steady state.

### *Surfactin biosynthesis in tomato exudates*

We further tested p*srfA* induction and surfactin production upon growth of strain BGS3 in the root exudates collected

**Table 1.** Surfactin biosynthesis upon growth of strain BGS3 in tomato root exudates.[a]

| | Biomass (cells × $10^8$ $ml^{-1}$) | *srfA* expression[b] (β-gal U) | Surfactin production[c] (μg per $10^8$ cells) |
|---|---|---|---|
| Natural exudates,[d] liquid[e] | 0.34 ± 0.09 | 17 ± 3 | 14 ± 4 |
| Recomposed exudates,[f] liquid | 1.40 ± 0.07 | 25 ± 8 | 34 ± 9 |
| Natural exudates, solid[e] | 0.37 ± 0.05 | 14 ± 4 | 4 ± 2 |
| Recomposed exudates, solid | 1.28 ± 0.09 | 18 ± 7 | 5 ± 1 |

**a.** Cultures were realized in 150 ml agitated erlenmeyer flasks (130 r.p.m., 28°C) filled with 50 ml of medium. In all cases, data are mean values and standard error from four independent experiments.
**b.** See legend of Fig. 2 for methods of quantification. Cell densities are those corresponding to the maximal OD measured 48 h after incubation. Values for gene expression also correspond to the maximal β-gal activities measured during the culture, usually 24 h after inoculation.
**c.** Supernatant samples were collected after 48 h of growth, loaded on $C_{18}$ solid-phase extraction cartridges (900 mg, Alltech) and surfactins were desorbed with 100% ACN. The resulting samples were analysed by reverse-phase HPLC coupled with single quad mass spectrometer (HPLC Waters Alliance 2695/diode array detector, coupled with Waters SQD mass analyser) on a X-terra MS (Waters) 150*2.1 mm, 3.5 μm column. Surfactins were eluted in the isochratic mode (78% acetonitrile in water acidified with 0.1% formic acid) at a constant flow rate of 0.3 ml $min^{-1}$ and 40°C. Compounds were first identified on the basis of their retention times compared with purified standards and the amounts were calculated on the basis of the corresponding peak area (max plot). The identity of each homologue was confirmed on the basis of the masses detected in the SQD by setting electrospray ionization (positive ion) conditions in the MS as source temperature, 130°C; desolvation temperature, 250°C; nitrogen flow, 500 l $h^{-1}$; cone voltage, 70 V.
**d.** Filter-sterilized tomato exudates freshly collected from non-inoculated plants grown for 23 days in the greenhouse (see legend of Fig. 1). These natural exudates were supplemented with 2 g $l^{-1}$ $(NH_4)_2SO_4$ to compensate for the lack of available nitrogen.
**e.** Production by BGS3 upon growth as planktonic cells in liquid cultures or as colonies on the same but solidified media (bactoagar at 1.5%).
**f.** Medium composed of exudates artificially recomposed [containing 10 g $l^{-1}$ glucose equivalent in (i) sugars 5 g $l^{-1}$: glucose 34%, fructose 57%, maltose 8%, ribose 0.75%; (ii) organic acids 4.5 g $l^{-1}$: citrate 77%, succinate 19%, malate 2%, fumarate 0.5%; (iii) amino acids 0.5 g $l^{-1}$: glutamate 23%, aspartate 18%, leucine 12%, isoleucine 10%, lysine 9%, (serine + aspartame) 5.5%, (arginine + threonine) 5.5%, (glutamate + glycine) 5.5%, histidine 5.5%, phenylalanine 5.5%] on the basis of data from Kamilova and colleagues (2006).

from tomato plantlets after 23–25 days of growth in nutrient solution under sterile conditions. The β-galactosidase activity and lipopeptide secretion into the medium were also effective under these conditions (Table 1), confirming that exudate components are conducive for *srfA* operon expression in BGS3 cells. This was also demonstrated by cultivating BGS3 in a minimal medium containing as sole carbon sources the sugars, organic acids and amino acids typically found in the tomato exudates and added in the proportions determined by Kamilova and colleagues (2006) (Table 1). Increased levels of biomass and surfactin synthesis compared with natural exudates can be explained by higher concentrations of nutrients in the medium.

When tested individually, the main representatives of typical root exudates supported growth to an almost similar level (data not shown) but both *srfA* gene expression and surfactin secretion were significantly higher in the presence of organic acids and amino acids as unique C sources (Fig. 3A). Aspartate and glutamate are the major amino acids in exudates but quantitatively, they represent minor components. Although it may vary in function of various plant factors, succinic and citric acids are the more abundant substrates released by tomato roots and also represent adequate nutrient sources utilizable by BGS3 for surfactin production (Fig. 3A).

Rhizosphere-colonizing bacteria typically aggregate in microcolonies on the root surface (Ramey *et al*., 2004) and their actual physiology is probably not related to a planktonic state. We therefore performed BGS3 cultures in 12-well microplates on the various media described above but gelified by adding 1.5% bactoagar in order to test for lipopeptide synthesis by immobilized cells. Growth on X-Gal-supplemented media containing either natural or recomposed exudates resulted in both cases in the formation of blue colonies with β-galactosidase-positive phenotype (not shown). This first visual assessment was supported by measurements of the gene expression within the cells scrapped off the wells. The values obtained for surfactin gene expression are in the range of those measured in liquid cultures in the presence of both kinds of exudates (Table 1). In contrast, we observed reduced surfactin production rates. The secreted amounts could be underestimated due to diffusion limitation of the lipopeptide throughout the biofilm into the environment or because of a less efficient recovery from the gelified matrix than from the liquid medium. On the other hand, differential surfactin biosynthesis observed following growth on solid media with individual carbon sources correlates well with those from liquid cultures with higher levels of gene expression in the presence of organic and amino acids than the three sugars tested (Fig. 3B). Globally, these results demonstrate that surfactin production is also readily effective in BGS3 cells developing on solid surface according to the fact that it actually occurs in biofilm-related structures formed on roots (Bais *et al*., 2004; Ramey *et al*., 2004). On another hand, qualitative analyses of lipopeptide patterns by LC-MS revealed that the $C_{14}$ and $C_{15}$ homologues together represent more than 80% of the total surfactins produced in most media.

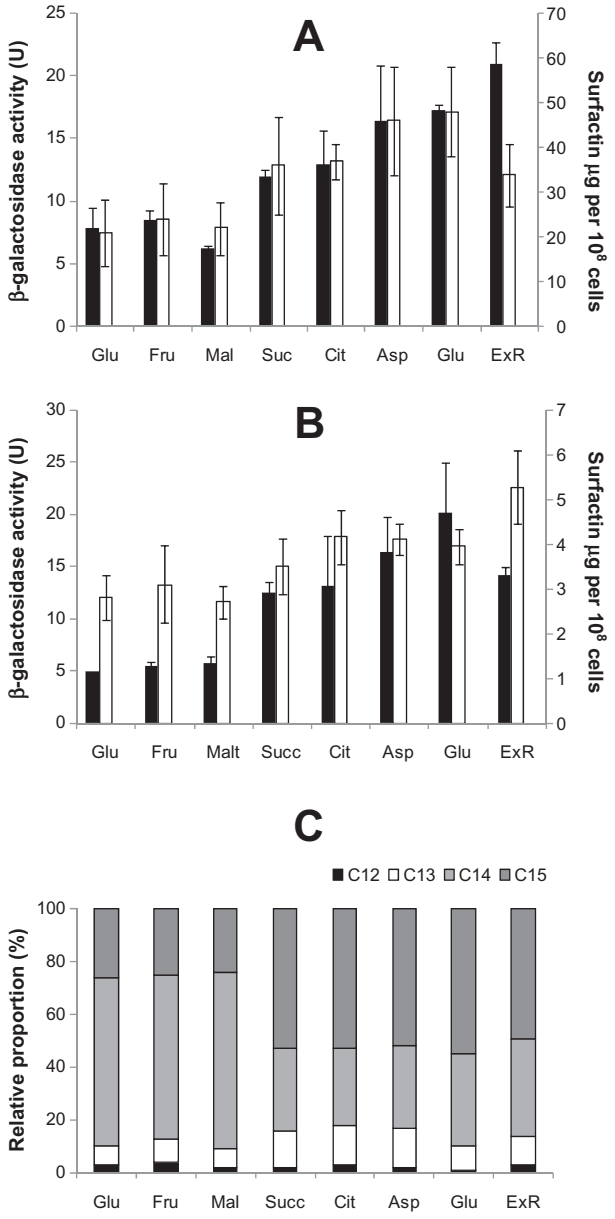


**Fig. 3.** Surfactin synthesis in the presence of various carbon sources typically found in tomato exudates. Lipopeptide gene expression (black columns) and production (white columns) were determined both in liquid cultures (A) in flasks under the conditions described in Table 1 and on the same gelified media (B). The proportions of the various homologues differing in the length of the fatty acid chain from $C_{12}$ to $C_{15}$ and produced in the liquid cultures are shown in (C). The substrates (glucose 10 g $l^{-1}$, fructose 10 g $l^{-1}$, maltose 5 g $l^{-1}$, succinate 15 g $l^{-1}$, citrate 10 g $l^{-1}$, aspartame 10 g $l^{-1}$, glutamate 15 g $l^{-1}$, recomposed exudates containing 10 g $l^{-1}$ glucose equivalent, for details see footnote of Table 1) were tested by adding a concentrated solution to a minimal medium composed by $MgSO_4{\cdot}7H_2O$ 0.5 g $l^{-1}$, $K_2HPO_4$ 1.0 g $l^{-1}$, KCl 0.5 g $l^{-1}$, Yeast extract 1.0 g $l^{-1}$, $Fe_2(SO_4)_3$ 1.2 mg $l^{-1}$, $MnSO_4$ 0.4 mg $l^{-1}$, $CuSO_4$ 1.6 mg $l^{-1}$, $(NH_4)_2SO_4$ 1 g $l^{-1}$. In all cases, pH was adjusted to 7 ± 0.2 with NaOH or HCl 0.1 N before sterilization. Cultures on solid media were performed in 12-well microplates in a volume of 1.5 ml per well. Three wells per plate were used for each carbon source. Bacterial growth in the presence of the various substrates was monitored 24 and 48 h after inoculation by measuring turbidity (OD) at 600 nm after scrapping cells for the surface of the medium and re-suspension to homogeneity. The same cells were used for β-gal activity determination. Surfactin produced in each well was extracted from the gelified medium by adding the same volume of HCl 4 N (1.5 ml) and heating for 10 min at 80°C. The resulting solution was diluted to 10 ml with distilled water and extracted on $C_{18}$ cartridge to yield semi-purified surfactins in pure methanol (1 ml) used for LC-MS analysis (see footnote of Table 1). In every experiment and for each specific substrate, surfactin concentration and β-gal activity were determined in three replicates (wells) in the same plate and first means were calculated from these data. Results presented in the figure are average values (± standard error) calculated from those first means obtained in three independent experiments.

It also clearly appeared that the relative proportions of the different homologues may vary in function of the C source and the synthesis of $C_{15}$ surfactins is seemingly favoured in the presence of organic acids and amino acids compared with the sugar group (Fig. 3C). The $C_{15}$ homologue is also by far (58%) the main form produced by BGS3 in natural exudates. This is of biological relevance since surfactins with the longest fatty acid chains appear to be the more active, not only for antimicrobial activity (Kracht *et al.*, 1999) but also for their potential to trigger plant defence reactions (Jourdan *et al.*, 2009). It partly relies on the fact that a more hydrophobic aliphatic chain in the molecule induces greater surface active properties and membrane destabilization/perturbation (Eeman *et al.*, 2006). These results suggest that the nutritional status encountered in the rhizosphere could also somewhat influence qualitatively the production of surfactin homologues by *B. subtilis*.

## Conclusion

Previous *in vitro* experiments have provided some insights on the effect of various sugars and nitrogen substrates and of some abiotic conditions such as temperature and pH on lipopeptide synthesis by *Bacillus* strains (Peypoux *et al.*, 1999; Kim, *et al.*, 2004; Guez *et al.*, 2008). However, the study of antimicrobial compound synthesis in the rhizosphere is more challenging. To our knowledge, this work provides a first demonstration of efficient lipopeptide gene expression in the rhizosphere during *Bacillus*–plant interaction that is associated with relevant surfactin production in the context of biocontrol both quantitatively and qualitatively. Our data show that important rhizosphere-specific factors such as particular nutritional status, slow growth rate and development in aggregated and immobilized structures are conducive for surfactin synthesis. In other experiments, we also observed that *srfA* expression and surfactin production still remain very effective in oxygen-starved culture conditions (V. Nihorimbere, unpublished) suggesting that the

low oxygen status prevailing in the rhizosphere (Hojberg *et al*., 1999) is not detrimental to lipopeptide synthesis by BGS3 cells evolving on roots. Reduced $O_2$ availability and slow growth were also reported to stimulate the production of the cyclic lipopeptide putisolvin by *Pseudomonas putida* (Dubern and Bloemberg, 2006). Although the influence of many other factors deserves further investigation and other *Bacillus* strains have to be studied, these results contribute to fill the gap in the global knowledge of *Bacillus* fitness in the rhizosphere regarding antibiotic synthesis, a crucial point for optimizing biocontrol strategies using this organism.

## Acknowledgments

This work received financial support from the Program F.R.F.C. No. 2.4624.06 and Crédit aux chercheurs No. 1.5.192.08F (National Funds for Scientific Research, F.R.S.-FNRS, Belgium). V. Nihorimbere is recipient of a grant from the Belgian Technical Cooperation (BTC/CTB). M. Ongena and P. Fickers are respectively research associate and postdoctoral researcher at the F.R.S.-FNRS.

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## Supporting information

Additional Supporting Information may be found in the online version of this article:

**Fig. S1.** Gene expression and production of surfactin in artificial medium. Time-course profiles of cell growth, dissolved oxygen level, *srfA* expression and surfactin production during batch fermentation of *Bacillus subtilis* BGS3 under an aeration rate of 1.0 VVM and an agitation rate of 300 r.p.m.

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