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François Coutte, Valérie Leclère, Max Béchet, Jean-Sebastien Guez, Didier Lecouturier, et al.. Effect of pps disruption and constitutive expression of srfA on surfactin productivity, spreading and antagonistic properties of *Bacillus subtilis* 168 derivatives. *Journal of Applied Microbiology*, 2010, 109 (2), pp.480-491. 10.1111/j.1365-2672.2010.04683.x . hal-02939416

HAL Id: hal-02939416

<https://hal.science/hal-02939416>

Submitted on 22 Sep 2020

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Effect of *pps* disruption and constitutive expression of *srfA* on surfactin productivity, spreading and antagonistic properties of *Bacillus subtilis* 168 derivatives

Running title: Disruption of *pps* and constitutive expression of *srfA* in *B. subtilis*

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Abstract

Aims : To analyse the effects of plipastatin operon disruption and constitutive expression of surfactin operon in *Bacillus subtilis* 168 on surfactin productivity, *in vitro* invasive growth and *in vitro* antagonism against fungi.

Methods and Results: The *urfA* native promoter was replaced by the constitutive promoter P_{repU} in *B. subtilis* 168 after integration of a functional *sfp* gene. Moreover, the plipastatin synthesis was further disrupted in the *B. subtilis* 168 derivatives. In liquid media, an earlier and higher expression of P_{repU} , than that found with P_{urfA} , led to a specific surfactin production 5-fold higher after 6 hours of culture. On solid media, not only the *in vitro* invasive growth and the haemolytic activity but also the *in vitro* antifungal activity of the constitutive strains were improved as compared to the parental strain BBG111. As expected, the disruption of the plipastatin operon strongly reduced *in vitro* antifungal properties but, interestingly, enhanced specific surfactin production (1.47 g g⁻¹ of biomass), spreading behaviour and haemolytic activity of the strains.

Conclusion: This work demonstrates for the first time the interdependency of surfactin and plipastatin regarding their biosynthesis as well as their influence on the biological activities of the producing strain.

Significance and Impact of the Study: The constitutive overproduction of surfactin enhances the invasive growth and the *in vitro* antagonistic activity of the mutant strain. Both properties are known to play an important role in the biocontrol of plant diseases. Plipastatin operon disruption increases the surfactin productivity of mutant strains. These mutants are interesting for use in continuous bioprocesses for surfactin production or in bioremediation.

Keywords : *Bacillus subtilis*, surfactin, plipastatin, *urfA* operon, constitutive expression, spreading

Introduction

The endospore-forming soil bacterium *Bacillus subtilis* produces up to three families of lipopeptides, *i.e.*, surfactins, iturins and plipastatins or fengycins (Bonmatin *et al.* 2003; Ongena and Jacques 2008). These molecules are amphiphilic cyclic peptides with seven α -amino acids linked to a β -hydroxy fatty acid for surfactins, seven α -amino acids linked to a β -amino fatty acid for iturins and ten α -amino acids linked to a β -hydroxy fatty acid for plipastatins. Surfactins are biosurfactant molecules with antiviral, antimycoplasmic, and haemolytic activities (Vollenbroich *et al.* 1997; Peypoux *et al.* 1999; Deleu *et al.* 2003). It has been shown that these lipopeptides are implicated in the medium colonization capacity of *B. subtilis*, *i.e.*, swarming, spreading or swimming behaviour, by favouring the gathering of the cells into dendrites (Kearns and Losick 2003; Kinsinger *et al.* 2003; Julkowska *et al.* 2004; Julkowska *et al.* 2005; Leclère *et al.* 2006; Hamze *et al.* 2009). They are also necessary for the formation of pellicles at the air/liquid interface (Hofemeister *et al.* 2004; Chollet-Imbert *et al.*, 2009). Moreover, the production of surfactin is essential for biofilm formation and colonization of plant roots, and surfactin formation may be an essential trait for effective *B. subtilis* biocontrol strains (Bais *et al.* 2004).

Plipastatins or fengycins, produced after the end of the exponential growth phase, are strong antifungal compounds and much less haemolytic than surfactin. These antifungal peptides inhibit the growth of a large number of fungi, including *Fusarium* species (Jacques *et al.* 1999; Ongena and Jacques 2008). Moreover, *B. subtilis* lipopeptides were recently mostly studied for their versatile activity in the biocontrol of a wide range of phytopathogens (Souto *et al.* 2004; Leclère *et al.* 2005; Ongena and Jacques 2008). In addition to their direct antifungal activity, these lipopeptides can influence the ecological fitness of the producing

strain in terms of root colonization and also play a key role in the beneficial interaction with plants by stimulating host defence mechanisms.

The production of surfactins is mediated by *urfA*, a 25 kb operon, which is composed of four genes, *urfA-A*, *urfA-B*, *urfA-C* and *urfA-D*. The plipastatin operon (*pps*; 38 kb) consists of five genes: *ppsA*, *ppsB*, *ppsC*, *ppsD* and *ppsE*. These two operons encode the non-ribosomal peptide synthetase (NRPS) subunits, which catalyse the incorporation of the seven amino acids into the surfactin and the ten amino acids into plipastatin (Thimon *et al.* 1992; Marahiel *et al.* 1997; Peypoux *et al.* 1999; Du *et al.* 2001). The genetic locus *sfp*, encoding a phosphopantetheine-transferase, is necessary for lipopeptide production, as it mediates the conversion of the NRPS from the apo form to the holo form (Nakano *et al.* 1988; Quadri *et al.* 1998). The production of surfactins is activated by a quorum-sensing mechanism at the end of the exponential growth phase. The transcription of the *urfA* operon is directly activated by the phosphorylated form of the factor ComA (ComA~P), the phosphorylation of which is controlled by ComP (Cosby *et al.* 1998). Expression of *urfA* is mediated by the promoter P_{*urfA*}, which was recently proved very strong in *B. subtilis* 168, compared with the P_{*urfA*} of *B. subtilis* ATCC 6633 (Duitman *et al.* 2007). Modifications in the regulation of *urfA* directly altered the expression of the competence regulatory gene *comS*, which is nested out of frame, within the *urfA-B* gene, and thus the competence, the sporulation and the motility regulation pathways (Hamoen *et al.* 1995). In fact, ComS causes the release of a competence-specific transcription factor, ComK, from the ClpC-MecA proteolytic complex, resulting in ComK activation (Ogura *et al.* 1999). ComK also activates the transcription of the late competence genes and many genes involved in DNA cleavage, transformability, motility, regulatory factor, arginine biosynthesis and transport [studied through microarray analyses (Berka *et al.* 2002; Hamoen *et al.* 2002; Ogura *et al.* 2002), and reviewed by Hamoen *et al.* (2003)]. This description gives evidence of a complex transcriptional regulation of the *urfA* operon expression.

In this study, genetic modifications of *B. subtilis* 168, whose genome is completely sequenced (Kunst *et al.* 1997), were carried out. The native promoter driving the *surfA* operon was replaced by a constitutive one P_{repU} . Then the plipastatin synthesis was disrupted in the different mutant strains by interruption of the *ppsA* gene. The aim was to study the impact of constitutive expression of the *surfA* operon, with and without the disruption of the plipastatin operon, on the specific production of surfactin but also on phenotypic properties related to the biocontrol abilities of the strains, such as *in vitro* medium colonization capacity or antibacterial and antifungal activities. Positive and negative interactions of both lipopeptides on these phenotypic properties are then discussed.

Materials and Methods

Bacterial strains, plasmids, and growth conditions

Strains of *B. subtilis*, *Escherichia coli* and plasmids used in this study are listed in Table 1. *B. subtilis* strains and *E. coli* JM109 were grown at 37°C with shaking at 130 rpm in Luria-Bertani medium (LB) (Sambrook and Russel 2001). When required, various antibiotics were added: ampicillin, sodium salt (Ap; Sigma, St. Louis, MO, U.S.A., 50 $\mu\text{g ml}^{-1}$), neomycin sulphate (Nm; Sigma, 30 $\mu\text{g ml}^{-1}$), spectinomycin dihydrochloride (Spc; Sigma, 100 $\mu\text{g ml}^{-1}$) and chloramphenicol (Cm; Sigma, 5 $\mu\text{g ml}^{-1}$). For all the *B. subtilis* 168 derivatives, tryptophan was added at a concentration of 16 mg l^{-1} , except in LB medium. The optical density was measured at 600 nm to follow the biomass evolution ($\text{OD}_{600\text{nm}}$) with an Uvikon 940 spectrophotometer (Kontron Instruments, Plaisir, France). Total biomass was determined by dry weight measurement:

5 ml of culture sample were centrifuged for 10 min at $13,000 \times g$, and the pellet was washed once with distilled water. The washed pellet was resuspended in 10 ml of distilled water and dried on small pre-weighed aluminium dishes for 48 h at 105°C .

Molecular biology procedures

Total genomic DNA samples were purified from *B. subtilis* strains 168 and ATCC 21332 using the genomic tips 20/G together with the corresponding buffers purchased from Qiagen (Hilden, Germany). Plasmid DNA samples were prepared from *E. coli* using either the Miniprep Spin or Maxiprep kits (Qiagen). Screening for hybrid plasmids within various *E. coli* transformants was carried out following the “boiling” procedure of Holmes and Quigley (1981). Restriction endonucleases digestions (from Fermentas GMBH, St-Leon-Rot, Germany), ligations and transformations of *E. coli* by the CaCl_2 heat shock, were carried out according to standard procedures (Sambrook and Russel 2001). *B. subtilis* 168 was transformed either by natural competence (Sambrook and Russel 2001) or by electroporation as previously described (Leclère et al. 2005). For PCR amplification, the primers (listed in Table 2a) were designed according to the published sequence of the genome of strain 168 (PubMed Gene NC_000964.2), and supplied by Eurogentec (Angers, France). All the PCR-generated cassettes used in this study were purified from 1% agarose gels using the QIAquick kit (Qiagen).

Strain construction

The following genetic modifications of *B. subtilis* 168 were carried out:

- P_{srfA} and P_{repU} promoters *lacZ* fusions were introduced into *amyE* locus.

-An Sfp^+ mutant was generated by complementation of the defective *sfp*⁰ allele with a wild type copy, inserted ectopically in *amyE* by homologous recombination.

-The P_{srfA} promoter was replaced by the P_{repU} one in the wild-type and in the Sfp^+ mutant by keeping a short sequence located upstream of the RBS site as described by Nakano *et al.* (1991).

-The plipastatin operon was interrupted in the Sfp^+ mutants harbouring the two different promoters. A summary of these different modifications is shown in Fig. 1.

Modified strains were selected after plating on LB agar containing the required antibiotics, followed by incubation at 37°C. The genetic modifications were verified by PCR amplification with *B. subtilis* primers designed outside of the sequence of the plasmid tools used for the homologous recombination (listed in Table 2b). In each modified strain, after the insertion of a DNA fragment within the *B. subtilis* genome, a fragment of larger size was observed, compared with the corresponding fragment obtained from the parental strains (data not shown).

In order to insert the promoter P_{srfA} or P_{repU} with a *lacZ* fusion into *B. subtilis* 168 by homologous recombination within the *amyE* locus, pDG1661 (Guérault-Fleury *et al.* 1996) was first digested by *EcoRI*. The ϵP_{srfA} cassette was generated by PCR using the P_{srfA} forward and reverse primers together with the Taq polymerase “Arrow” from MP Biomedicals (Illkirch, France) and inserted into the pGEM-T Easy vector (Promega, Madison, U.S.A.). On one hand, the ϵP_{srfA} cassette was digested by *EcoRI* and ligated, with T4 DNA ligase (Takara,

Shiga, Japan), into the *EcoRI* site of pDG1661. On the other hand, the $P_{repU-neo}$ fragment from the plasmid pBG133 was extracted by *EcoRI* and ligated into the *EcoRI* site of pDG1661. These constructions named pBG145 and pBG148 were then used to transform *B. subtilis* 168, leading to transformants named BBG127 and BBG128, respectively.

To restore lipopeptide production in *B. subtilis* 168, the *sfp* gene from *B. subtilis* ATCC 21332 was inserted into the *amyE* locus by homologous recombination as demonstrated earlier (Lee *et al.* 2005). The ϵsfp cassette was generated by PCR using the *sfp* forward and reverse primers and inserted into pGEM-T Easy. Then the ϵsfp cassette was digested by *EcoRI* and ligated into the *EcoRI* site of pDG1661. This construction named pBG129 was then used to transform *B. subtilis* 168, giving rise to a transformant named BBG111.

For the construction of the pBG106-derived plasmid (Leclère *et al.* 2005) dedicated to *srfA* promoter exchange by homologous recombination, we proceeded as follows. To generate BBG113 and BBG118, the region located between the end of the *hxlR* gene and the *HaeIII* restriction site situated upstream from the RBS of the first gene of the *srfA* operon, was replaced. The $\epsilon hxlR$ and $\epsilon HaeIII-srfAA$ cassettes were generated by PCR using the *hxlRI* forward and reverse primers and *srfAA1* forward and reverse primers and then inserted into pGEM-T Easy. After digestion by *EcoRI*, the $\epsilon HaeIII-srfAA$ cassette was ligated within the *EcoRI* sites of pBG106, generating pBG124. The $\epsilon hxlR$ cassette was *SalI* and *SphI* double-digested and ligated into the corresponding sites of pBG106, giving rise to pBG127. Then pBG124 and pBG127 were double-digested by *SphI* and *BglIII*, and the longer fragment of pBG124 containing $\epsilon HaeIII-srfAA$ was ligated within the shorter digested fragment from pBG127 containing $\epsilon hxlR$ and $P_{repU-neo}$, leading to pBG133. This plasmid was then used to

obtain mutants by homologous recombination in *B. subtilis* 168 and BBG111 as shown on Fig. 2. The construction of BBG113 was verified by sequencing the $P_{repU-neo-srfAA}$ sequence amplified by PCR using the $P_{repU-neo}$ forward primer and the *srfAA3* reverse primer (listed in Table 2b).

For the construction of the plasmid designed to disrupt the production of plipastatin in *B. subtilis* 168-derived strains by homologous recombination, the $\epsilon ppsA$ cassette was generated by PCR with the *ppsA1* forward and *ppsA1* reverse primers and inserted within pGEM-T Easy, generating pBG146. Then the *spc* cassette from pDG1661 was digested by *NarI* + *XhoI* and introduced into the corresponding sites of pDG146. This construction, named pBG149, was then used to transform *B. subtilis* BGG111 and BBG113. The obtained transformants were named BBG130 and BBG131, respectively.

Lipopeptide production

All *B. subtilis* strains were grown at 37°C with agitation at 130 rpm either in Landy's medium, Landy medium buffered with 0.1 M MOPS as described previously (Guez *et al.* 2008), or in B medium (Julkowska *et al.* 2004). For the preculture, a single colony from overnight plate culture was inoculated into 50 ml of the culture medium and grown overnight at 37°C with agitation at 300 rpm. A second preculture, in 50 ml of the culture medium, was carried out under the same conditions and stopped before the end of exponential growth phase. The cells were centrifuged 10 min at 5,000 × g, washed with physiological water, and centrifuged again. Finally, the pellet was suspended in 100 ml of culture medium in 1 l Erlenmeyer flask to inoculate at an OD₆₀₀ close to 0.05. In all cases, three replicate flasks were used for each strain and the experiment was repeated three times.

Lipopeptide purification and identification

Cultures were centrifuged at $15\,000 \times g$ for 15 min at 4°C. The lipopeptides were quantified in the supernatant and in a cell pellet after lysis. The cell pellet was lysed by speed freezing, resuspension in a lysing solution (20 mM Tris-HCl pH 7.5, 0.2 M NaCl, 1 mM EDTA, 5 mg ml⁻¹ lysozyme, 5 mg ml⁻¹ deoxyribonuclease and 2.5 mg ml⁻¹ ribonuclease A; enzymes were supplied by Sigma) and subjected to ultrasounds during three one-minute-runs at 6 watts using an ultrasonic processor from Bioblock Scientific (Illkirch, France). The lysed pellet was centrifuged at $15,000 \times g$ for 15 min at 4°C and the lipopeptides were extracted from the supernatant. Lipopeptide extraction on C-18 columns and purification by HPLC were carried out as previously described (Leclère *et al.* 2005). Surfactin and plipastatin families were separately analysed using the solvents acetonitrile/water/trifluoroacetic acid (80:20:0.1, vol/vol/vol), and (45:55:0.1, vol/vol/vol) respectively. Purified surfactins used as references were purchased from Sigma. Purified plipastatins were a kind gift from Dr. Magali Deleu (Agricultural University of Gembloux, Belgium). Retention time and second derivatives of UV-visible spectrum (Diode Array Waters PDA 996, Millenium Software) of each peak were used to identify and quantify the eluted molecules.

α -amylase and β -galactosidase assays

α -amylase activity was detected by growing *B. subtilis* colonies overnight on LB plates containing 1% soluble starch and then staining the plates with a potassium iodide and iodine solution.

To analyse the expression of native and constitutive promoters using *lacZ* fusion in *B. subtilis* 168 derivatives, the strains were grown in Landy MOPS medium as for lipopeptide

production, at an initial OD₆₀₀ close to 0.05. 1 ml samples were withdrawn at regular intervals for determination of optical density and β-galactosidase activity as previously described (Miller 1972). In all cases, three replicate flasks were used for each strain and the experiment was repeated twice.

In vitro medium colonization assays

To analyse the invasive properties of the strains, a single colony from overnight plate culture was inoculated in 5 ml of LB medium and grown overnight at 37°C with agitation at 130 rpm. The culture was diluted to get an OD₆₀₀ of about 0.1 and grown again until an OD₆₀₀ of about 0.4 in the appropriate medium was obtained. Finally, this culture was diluted in the appropriate medium to an OD₆₀₀ of about 0.1. The appropriate medium corresponded to the same medium that was used for the assays on agar plates. Three microliters of the latter culture were placed on the center of agar plates. Plates containing exactly 25 ml of either LB or B medium containing 7 g l⁻¹ agar (Julkowska *et al.* 2004) were prepared 90 min before final inoculation and dried open for 15 min in a laminar flow chamber. After inoculation, the plates were left open for 10 min in a laminar flow chamber to dry the inoculum spots. The colonization was monitored for eight days of incubation at 37°C. Each sample was tested three times and a statistical analysis of the results is presented.

Evaluation of haemolytic activity

Broth cultures and plate preparation procedures were carried out in the same way as the medium colonization assays. Plates containing 25 ml of either LB or Landy MOPS medium, supplemented with 5% of defibrinated horse blood (AES Laboratoires, Bruz, France) and 17 g

l⁻¹ agar, were inoculated and incubated at 37°C. Haemolytic activity was checked after one and three day(s) by the development of a clear halo surrounding the colony. In all cases, three replicate plates were used for each strain on both media and the experiment was repeated twice.

Evaluation of *in vitro* antimicrobial and antifungal activities

Antimicrobial activities of the wild-type and modified strains were tested on plate bioassays against *Staphylococcus aureus* and *Micrococcus luteus*, previously grown overnight in LB medium and diluted (10^{-3}). Then 200 µl were inoculated in 4 ml of LB soft agar (7 g l⁻¹). This diluted cell suspension (representing 5×10^{-4} cells) was poured onto LB plates, prepared 60 min before final inoculation, and allowed to solidify.

To test the antifungal activity, different *B. subtilis* strains were co-cultured with *Fusarium oxysporum* on Potato-Dextrose Agar (PDA) medium (Biokar Diagnostics, Beauvais, France) and incubated at 30°C. The same *B. subtilis* inoculum was achieved as for the medium colonization assay, the OD₆₀₀ of the inoculum spot being 0.2. After inoculation, the plates were left open for 10 min in a laminar flow chamber to dry the 5 µl inoculum spots. The antibacterial or antifungal activity was evaluated every day by the development of a growth inhibition area around the colony. Plates were photographed using GelDoc apparatus (Bio-Rad Laboratories, Hercules, CA, U.S.A). In all cases, three replicate plates were used for each strain and the experiment was repeated twice.

Results

Comparison of the promoter strength

In order to confirm that P_{repU} was a suitable promoter to obtain an early expression of *srfA* in *B. subtilis* 168, its strength was compared to that of the natural promoter P_{srfA} during the first 12 h of culture using *lacZ* fusions. Both fusions were inserted into the *B. subtilis* 168 chromosome by homologous recombination within the locus *amyE* and verified by PCR amplification. As expected, the modified strains, named respectively BBG127 and BBG128, no longer displayed amylase activity (data not shown), and were then compared for their β -galactosidase production, in Landy medium buffered with 0.1 M MOPS, to monitor the expression levels of both promoters (Fig. 3). An earlier expression of P_{repU} , which was four times stronger than P_{srfA} after 3 h of culture was observed during the first phase of growth. After 6 h of culture, the expressions of both promoters converged.

Genetic modifications of *B. subtilis* 168

B. subtilis strain 168 contains the surfactin (*srf*) and the plipastatin (*pps*) operons but is unable to produce these lipopeptides because of the lack of a functional *sfp* gene (Sfp^0) (Nakano *et al.* 1988). To study the effect on surfactin synthesis of the constitutive expression of *srfA*, genetic modifications were carried out (Fig. 1). First, a Sfp^+ mutant, named BBG111, was generated by homologous recombination, allowing the restoration of the synthesis of lipopeptides by insertion of an efficient *sfp* gene originating from the *B. subtilis* strain ATCC 21332 into the locus *amyE*, as already demonstrated (Lee *et al.* 2005). This modified strain no longer displayed amylase activity (data not shown). Secondly, the replacement of the native

promoter of the *surfA* operon was effected with the constitutive one, P_{repU} , originating from the staphylococcal plasmid pUB110. These modified strains were named BBG118 and BBG113, respectively (Table 1). As shown in Fig. 2, in these strains, the recombination was carried out upstream from the *Hae*III restriction site, which is itself located at 140 pb upstream from the RBS of *surfA* operon.

Finally, the plipastatin operon was disrupted in the different *B. subtilis* 168-derived strains, generating BBG130 and BBG131.

The modified strains were further compared regarding to their physiological behaviour, biological activities and lipopeptide production.

Lipopeptide production by the *B. subtilis* 168 derivatives

Surfactin production was monitored during two days of cell growth in shaken Erlenmeyer flasks (Table 3, Fig. 4). Although the absolute level of surfactin was different for each modified strain, analysis of lipopeptide production in different media similarly revealed a higher production of surfactin with BBG111 and BBG113 after 48 h incubation in Landy MOPS medium, *i.e.*, 1.5 and 1.2 g l⁻¹ (Table 3), respectively. In this culture medium, the surfactin production is up to 20 times more important than in B medium. The constitutive strain BBG113 produced slightly more plipastatin (44 mg l⁻¹) than its parental strain BBG111. As expected, the strains BBG130 and BBG131 did not synthesize plipastatin, but after 48 h of culture, the surfactin yield was higher in both BBG130 and BBG131, than in their parental strains (Table 4). Moreover, the quantification of lipopeptides in the cellular pellet did not show the presence of high concentration of intracellular surfactin (data not shown).

The kinetics of surfactin yield in Landy MOPS medium, analysed during the exponential growth phase ending at 9 h, showed that the lipopeptide biosynthesis started early in the

constitutive strain (Fig. 4). The observed surfactin production for BBG113 was 5 times higher than for BBG111 after 6 h of culture, BBG113 remaining better during the first 12 h of culture. This result makes the relationship between the early activation of the P_{repU} promoter in the constitutive strain, highlighted by the experiment showed in the Figure 3, and the early production of surfactin showed in the Figure 4.

Haemolytic activity

As surfactin is known to be a strong haemolytic compound (Peypoux *et al.* 1999), *B. subtilis* 168 and its derivatives were cultivated at 37°C on Landy MOPS blood agar (Fig. 5) and on LB blood agar (data not shown). The strains *B. subtilis* 168 and BBG118, which do not produce any lipopeptide (Sfp^0), showed no haemolytic activity (data not shown). For each Sfp^+ strain, haemolytic activity was similar on LB medium and Landy MOPS. The haemolytic halo on both culture media was larger for all Sfp^+ constitutive strains with a maximum value of 3.9 cm, reached after three days for BBG131. Haemolytic activity of the constitutive strain BBG113 was higher than that of BBG111. A very light halo around the BBG111 colony was observed after one day. With BBG113, a similar halo to the one observed with BBG111 was detected after the same delay but on most of the plate surface (Fig. 5). This observation was also verified after two days more. Moreover, in all cases, the plipastatin disruption enhanced the haemolytic activities of the strains BBG130 and BBG131 (Fig. 5).

In vitro invasive growth

Biosurfactants, such as surfactin, mediate surface translocation of bacterial cells by reducing surface tension. Experiments of strain spreading were carried out *in vitro* with all the

B. subtilis 168-derived strains. The diameter of the colonies was evaluated regularly for eight days (Fig. 6). Strain 168, which does not produce lipopeptides, showed a restricted colonization of the medium, as did BBG118. The spreading area was larger for the strain constitutively expressing the surfactin synthetase genes. A refringent halo, which is known to facilitate the colonization by lowering the wettability of the medium, with the appearance of a diffusion circle, was observed surrounding the lipopeptide-producing colonies of BBG111, while it arose earlier for the constitutive strains. The development of the BBG113 colony was twice as large as for BBG111 after two days of incubation and this ratio remained constant during the following days. The strains BBG130 and BBG131, in which plipastatin production was interrupted, presented a colonization capacity 5 to 8 times stronger than their respective parental strains.

In vitro antimicrobial activities

Antibacterial activity was evaluated against *M. luteus* and *S. aureus* by direct confrontation on plates as described in Material and Methods. No antibacterial activity was found against *M. luteus* whatever the bacterial strain. An activity was observed against *S. aureus* for all the *B. subtilis* 168-derived strains, BBG113 showing an activity slightly better than BBG111 (data not shown).

To determine the respective role played by surfactin and plipastatin in the antifungal activities of BBG111 and BBG113, their antagonistic activity *in vitro* against *F. oxysporum* was compared to that obtained with their respective plipastatin non-producer derivatives BBG130 and BBG131 (Fig. 7). A small adverse effect to *F. oxysporum* growth was observed with strains *B. subtilis* 168 (a) and BBG118 (d), probably due to substrate competition or pH modification. This effect was slightly improved when a high level of surfactin was produced

by BBG111 (b) and BBG130 (e). However, a significant and reproducible antifungal activity was observed when the surfactin synthetase gene expression was controlled by the constitutive promoter in BBG113 (c). We observed a high growth inhibition of the fungi when the surfactin synthetase expression was constitutive. This effect disappeared when the plipastatin operon was disrupted in BBG131 (f).

Discussion

The native regulation of the *srfA* operon is complex, especially because it involves quorum-sensing (Cosby *et al.* 1998). In this study, we have replaced the native promoter P_{srfA} of *B. subtilis* 168 by a constitutive one in order to inactivate the natural regulation of the surfactin operon and to check the influence of an early production of surfactin on different phenotypic traits of the strain. This constitutive promoter governs the replication gene *repU* from the *S. aureus* plasmid pUB110 and was previously used with success in *B. subtilis* to increase the production of two other lipopeptides: iturin A (Tsuge *et al.* 2001) and mycosubtilin (Leclère *et al.* 2005). In both cases, the authors observed respectively up to 3-fold and 15-fold more lipopeptides produced by the recombinant strains. Moreover, the authors showed that the constitutive expression of mycosubtilin enhanced the biocontrol activities of the strains (Leclère *et al.* 2005).

In this work, this promoter substitution led to a specific surfactin production 5-fold higher after 6 hours of culture. In addition, the early production of surfactin confers to the producing strain a clear advantage for the colonization of the medium. This early surfactin production is involved in the reduction of the surface tension (Deleu *et al.* 2003) and promotes the invasive behaviour of the strain. This was shown by the spreading experiments performed in this study but also in the haemolytic tests. Earlier, Deleu *et al.* (2003) have

demonstrated that the CMC of surfactin can be correlated to the concentration required to obtain 50% haemolysis.

The low colonization capacity of BGG118, a strain able to overexpress *srfA* and thus *comS*, but unable (Sfp^0) to synthesize the lipopeptides, compared to BBG113, highlighted the minor role played by *comS* in this phenomenon compared to the earlier surfactin production, as reported in a recent study (Hamze *et al.* 2009). Otherwise, since their target has been removed, the complete replacement of P_{srfA} could cause the accumulation within the cell of other *srfA* expression regulators. These factors seem to be implicated in the colonization capacity of *B. subtilis* 168 (Hamze *et al.* 2009).

In this study, we have also disrupted the plipastatin synthesis by interruption of the *ppsA* gene. The resulting mutant strains were used to observe the influence on various phenotypic traits of the synergies and antagonism between surfactin and plipastatin. The production of surfactin, the haemolytic activity and the *in vitro* invasive growth of the strain were increased when the plipastatin operon was interrupted. The colonization and haemolytic capacities were especially drastically amplified in BBG131 where an early high level of surfactin production was observed. Several common precursors like some β -hydroxy fatty acid chains, as well as glutamic acid, are involved in the biosyntheses of surfactin and plipastatin. Both lipopeptide biosyntheses also require high level of ATP for the activation of the different amino acid residues incorporated in their peptide moiety. In addition, both NRPS involved in the biosynthesis of these two lipopeptides need to be transformed from apo-enzyme to holo-enzyme by addition of a cofactor due to the phosphopantetheine-transferase encoded by the *sfp* gene. The knockout of the plipastatin operon may redirect such precursors, needs in ATP and Sfp activity to the single surfactin biosynthesis. On the other hand, the early production of surfactin enhanced the biocontrol abilities of the strain related to the plipastatin production and the *in vitro* invasive growth capacity. A synergistic effect between

lipopeptides had already been clearly demonstrated for surfactin and iturin (Thimon *et al.* 1992; Souto *et al.* 2004; Leclère *et al.* 2005), but never mentioned for surfactin and plipastatin. It is also possible that the early surfactin production improved the diffusion of plipastatin thus amplifying the antifungal activity of the strain. The constitutive strain BBG113 presented a higher *in vitro* antagonistic activity and a rapid colonization of the medium and these properties hold out great promise for biocontrol or bioremediation.

Comparing the production of surfactin and plipastatin by several strains in different culture media brought to light the strong influence of the culture conditions. The highest production of lipopeptides was observed in Landy MOPS medium, with BBG111 and BBG131 for surfactin (1.5 g l^{-1}) and with BBG113 for plipastatin (44 mg l^{-1}), highlighting the importance in controlling the pH. This had already been clearly demonstrated for surfactin operon expression (Cosby *et al.* 1998), but never mentioned for plipastatin biosynthesis. Interestingly, controlling the pH also has a positive effect on surfactin production by BBG113 which is quorum-sensing independent. The B medium, which is used for spreading experiments, was less well-suited for lipopeptide production than the Landy medium. However, the lower amount of surfactin produced in the B medium, which is still considerably higher than the critical micellar concentration of this lipopeptide (10 mg l^{-1}), was sufficient to allow a good invasive growth of the strains. The small differences between the specific surfactin production of strains BBG113 and BBG111 (table 4) after 24 h or 48 h of growth may result from the strong P_{srfA} activity in *B. subtilis* 168 after the end of the exponential growth phase, as demonstrated in our study through *lacZ* fusions and in a recent report (Duitman *et al.* 2007). The high surfactin yield of the strain BBG131 make it worthwhile considering its use in production processes especially ones with total biomass recycling as the modifications carried out on the synthesis of surfactin made it quorum insensitive.

Acknowledgements

This work received financial supports from the Université des Sciences et Technologies de Lille, the Région Nord-Pas-de-Calais, the Fonds Européen pour le Développement de la Recherche and the Ministère de l'Enseignement et de la Recherche. The authors thank M. Ould Ahmed, E. Hamon and T. Oury from ProBioGEM Laboratory for their technical assistance and W. Everett for the re-reading of the manuscript.

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Tables

Table 1 Strains and plasmids used in this study

| Strains or plasmids | Genotype or plasmid description* | Reference or source |
|-------------------------------|---|-------------------------------------|
| Bacterial strains | | |
| <i>Escherichia coli</i> JM109 | <i>recA1, endA1, gyrA96, thi, hsdR17, supE44 Δ(lac-proAB) / F'[traD36, proAB⁺, lacI_q, lacZΔM15]</i> | Promega, Madison, USA |
| <i>Bacillus subtilis</i> 168 | <i>trpC2</i> | Lab stock |
| <i>B. subtilis</i> BBG111 | <i>trpC2, amyE::sfp-cat</i> | This study (derived from 168) |
| <i>B. subtilis</i> BBG113 | <i>trpC2, amyE::sfp-cat, P_{repU}-neo::HaeIII-srfA</i> | This study (derived from BBG111) |
| <i>B. subtilis</i> BBG118 | <i>trpC2, P_{repU}-neo::HaeIII-srfA</i> | This study (derived from 168) |
| <i>B. subtilis</i> BBG127 | <i>trpC2, amyE::P_{srfA}-lacZ cat</i> | This study (derived from 168) |
| <i>B. subtilis</i> BBG128 | <i>trpC2, amyE::P_{repU}-neo-lacZ cat</i> | This study (derived from 168) |
| <i>B. subtilis</i> BBG130 | <i>trpC2, amyE::sfp-cat, ppsA::spc</i> | This study (derived from BBG111) |
| <i>B. subtilis</i> BBG131 | <i>trpC2, amyE::sfp-cat, P_{repU}-neo::HaeIII-srfA, ppsA::spc</i> | This study (derived from BBG113) |
| <i>B. subtilis</i> ATCC 21332 | Wild-type | Lab stock |
| Plasmids | | |
| pGEM-T Easy | Cloning vector, Ap ^r | Promega, Madison, USA |
| pDG1661 | Integration vector, <i>spoVG-lacZ</i> Ap ^r , Cm ^r , Spc ^r | (Guérout-Fleury <i>et al.</i> 1996) |
| pBG106 | P _{repU} -neo fragment, Ap ^r , Nm ^r | (Leclère <i>et al.</i> 2005) |
| pBG124 | 1.1 kb <i>EcoRI</i> HaeIII- <i>srfA-A</i> fragment inserted in pBG106, Ap ^r , Nm ^r | This study |
| pBG127 | 0.7 kb <i>SalI-SphI</i> <i>hxlR</i> fragment inserted in pBG106, Ap ^r , Nm ^r | This study |
| pBG129 | 1.1 kb <i>EcoRI</i> <i>sfp</i> fragment inserted in pDG1661 | This study |
| pBG133 | <i>SphI-BglII</i> <i>hxlR</i> fragment from pBG127 ligated within <i>SphI-BglII</i> HaeIII- <i>srfA-A</i> fragment from pBG124, Ap ^r , Nm ^r | This study |
| pBG145 | <i>EcoRI</i> P _{srfA} fragment ligated within <i>EcoRI</i> fragment from pDG1661, Ap ^r , Cm ^r , Spc ^r | This study |
| pBG146 | 6.5 kb <i>ppsA</i> PCR-generated fragment cloned into pGEM-T Easy, Ap ^r | This study |
| pBG148 | <i>EcoRI</i> fragment from pBG106 ligated within the <i>EcoRI</i> site of pDG1661, Ap ^r , Nm ^r , Cm ^r , Spc ^r | This study |
| pBG149 | <i>NarI-XhoI</i> <i>spc</i> fragment from pDG1661 ligated within <i>NarI-XhoI</i> sites of pBG146, Ap ^r , Spc ^r | This study |

★ Ap^r, resistance to ampicillin; Nm^r, resistance to neomycin; Cm^r, resistance to chloramphenicol; Spc^r, resistance to spectinomycin.

Table 2a PCR primers used for genetic constructions

| Primers | T _m (°C) | Sequence of the primer |
|---------------------------------|---------------------|----------------------------------|
| <i>P_{srfA}</i> forward | | 5'-GACAAAAATGTCATGAAAGAATCG-3' |
| <i>P_{srfA}</i> reverse | 58°C | 5'-GGGTAAAAAGTTATTTCCATATTGTC-3' |
| <i>sfp</i> forward | | 5'-CTGCCTGAATTATGCTGTGG-3' |
| <i>sfp</i> reverse | 55°C | 5'-TCGCTGAGGCTACATCAAG-3' |
| <i>hxlR1</i> forward | | 5'-CTTCCGGGATGTTGACGAGGTC-3' |
| <i>hxlR1</i> reverse | 52°C | 5'-AAAGACACCCTTGC GAAGAGCG-3' |
| <i>srfAA1</i> forward | | 5'-TTTAATAGCGGCCATCTG-3' |
| <i>srfAA1</i> reverse | 55°C | 5'-GAAGTGTCTTCATCAGATCC-3' |
| <i>ppsA1</i> forward | | 5'-CTGGAGGGAATCCGTTGAG-3' |
| <i>ppsA1</i> reverse | 61°C | 5'-TACTGTACCGGCAAAACC-3' |

Table 2b PCR primers used for verification of the genetic constructions

| | | |
|-------------------------------------|------|------------------------------|
| <i>amyE</i> forward | | 5'-CTTCGGTATGTGATTGTGAAGC-3' |
| <i>amyE</i> reverse | 60°C | 5'-GCAGACAATATCAGCATCCTTG-3' |
| <i>hxlR2</i> forward | | 5'-CTGCGCCTTTAATCGTTGC-3' |
| <i>srfAA2</i> reverse | 54°C | 5'-CGTCATGTCCTTACGGTAGCTC-3' |
| <i>P_{repU.neo}</i> forward | | 5'-CGCGAATTCGATTAAGATTAGG-3' |
| <i>srfAA3</i> reverse | 60°C | 5'-TCCTTCATCCGGTCTTTGAC-3' |
| <i>spc1</i> forward | | 5'-TATATACGGTGAGTGGCTCAGG-3' |
| <i>ppsA2</i> reverse | 60°C | 5'-GATATGCTGCAGCTAAATCCTC-3' |

Table 3 Lipopeptide production by BBG111 and its derivatives in different liquid media

| Surfactin production (in mg.l ⁻¹)* | | | | |
|---|----------|------------------|-----------------|-------------------|
| Strain | Time (h) | Landy medium | B medium | Landy MOPS medium |
| BBG111 (<i>sfp</i> ⁺) | 24 | 590 (SD = 14.9) | 90 (SD = 7.1) | 1768 (SD = 31.1) |
| | 48 | 1136 (SD = 13.6) | 214 (SD = 14.7) | 1504 (SD = 67.9) |
| BBG130 (<i>sfp</i> ⁺ , <i>pps</i> ⁻) | 24 | 1066 (SD = 11.3) | ND | 1833 (SD = 51.0) |
| | 48 | 1103 (SD = 45.1) | ND | 1423 (SD = 164.1) |
| BBG113 (<i>sfp</i> ⁺ , <i>P_{repU}-srfA</i>) | 24 | 757 (SD = 102.3) | 178 (SD = 34.1) | 1155 (SD = 105.7) |
| | 48 | 782 (SD = 119.2) | 312 (SD = 24.6) | 1213 (SD = 51.8) |
| BBG131 (<i>sfp</i> ⁺ , <i>P_{repU}-srfA</i> , <i>pps</i> ⁻) | 24 | 693 (SD = 54.8) | ND | 1092 (SD = 8.0) |
| | 48 | 860 (SD = 64.9) | ND | 1540 (SD = 38.2) |
| Plipastatin production (in mg.l ⁻¹)* | | | | |
| Strain | Time (h) | Landy medium | B medium | Landy MOPS medium |
| BBG111 (<i>sfp</i> ⁺) | 24 | 25 (SD = 11.2) | 0 | 32 (SD = 10.0) |
| | 48 | 15 (SD = 6.2) | 0 | 25 (SD = 14.8) |
| BBG113 (<i>sfp</i> ⁺ , <i>P_{repU}-srfA</i>) | 24 | 31 (SD = 6.7) | 0 | 44 (SD = 5.0) |
| | 48 | 26 (SD = 4.1) | 0 | 37(SD = 5.0) |

*Values are means of data from three experiments
SD : Standard deviation.

Table 4 Lipopeptide production yield (in g.g⁻¹ of D.W.) in Landy MOPS medium^{*}

| Strain | Time (h) | Surfactin | Plipastatin |
|---|----------|------------------|------------------|
| BBG111 (<i>sfp</i> ⁺) | 24 | 1.08 (SD = 0.07) | 0.02 (SD = 0.01) |
| | 48 | 0.87 (SD = 0.16) | 0.01 (SD = 0.01) |
| BBG130 (<i>sfp</i> ⁺ , <i>pps</i> ⁻) | 24 | 1.23 (SD = 0.03) | - |
| | 48 | 1.67 (SD = 0.20) | - |
| BBG113 (<i>sfp</i> ⁺ , <i>P_{repU}-srfA</i>) | 24 | 0.88 (SD = 0.21) | 0.03 (SD = 0.01) |
| | 48 | 0.97 (SD = 0.03) | 0.03 (SD = 0.01) |
| BBG131 (<i>sfp</i> ⁺ , <i>P_{repU}-srfA</i> , <i>pps</i> ⁻) | 24 | 0.67 (SD = 0.01) | - |
| | 48 | 1.47 (SD = 0.16) | - |

^{*}Values are means of data from three experiments
SD : Standard deviation.

Figures and legends

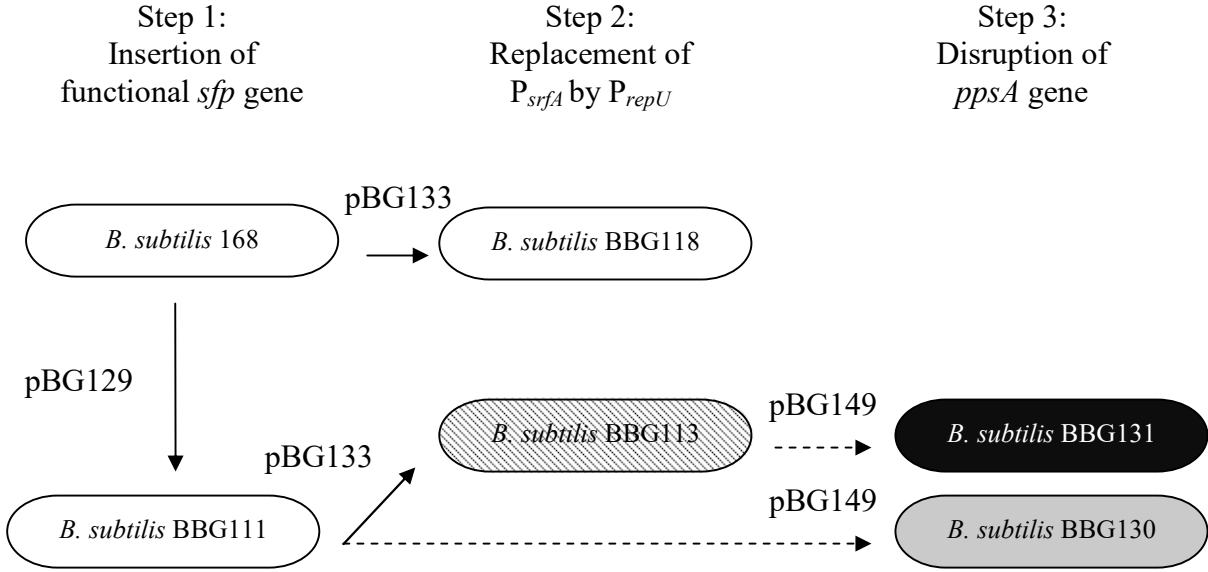


Figure 1 Schematic representation of *B. subtilis* 168 genetic modifications carried out in this study. The plasmids used for homologous recombination are noted on or beside the arrows.

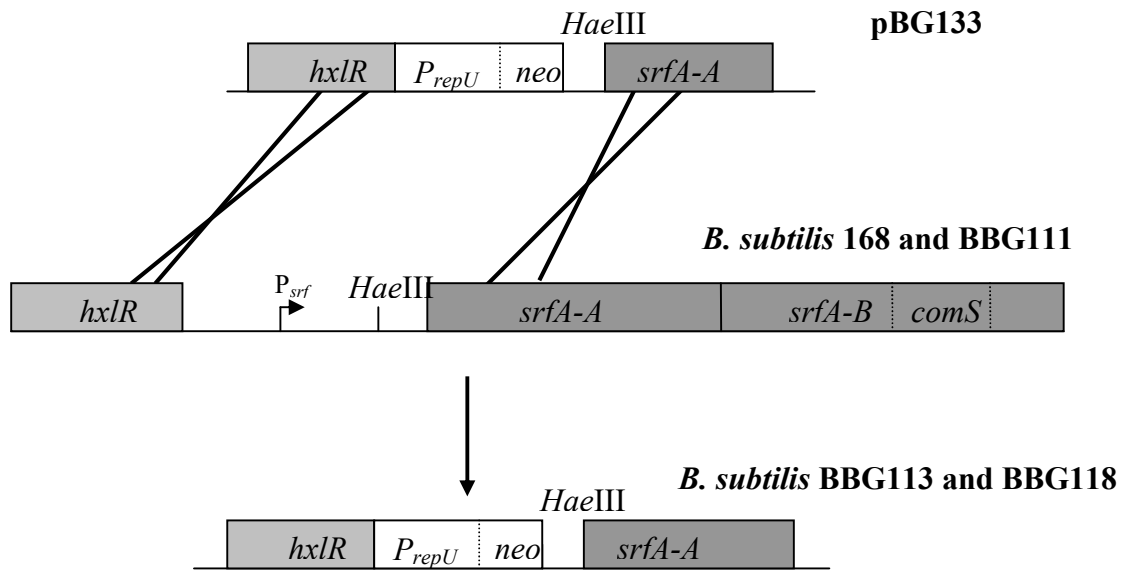


Figure 2 Schematic representation of P_{srfA} promoter replacement in *B. subtilis* 168 and BBG111. The constitutive P_{repU} promoter was integrated by homologous recombination through a double crossing-over to replace P_{srfA} . The replacement in BBG113 and BBG118 was situated upstream of the *HaeIII* restriction site of the *srfA* operon.

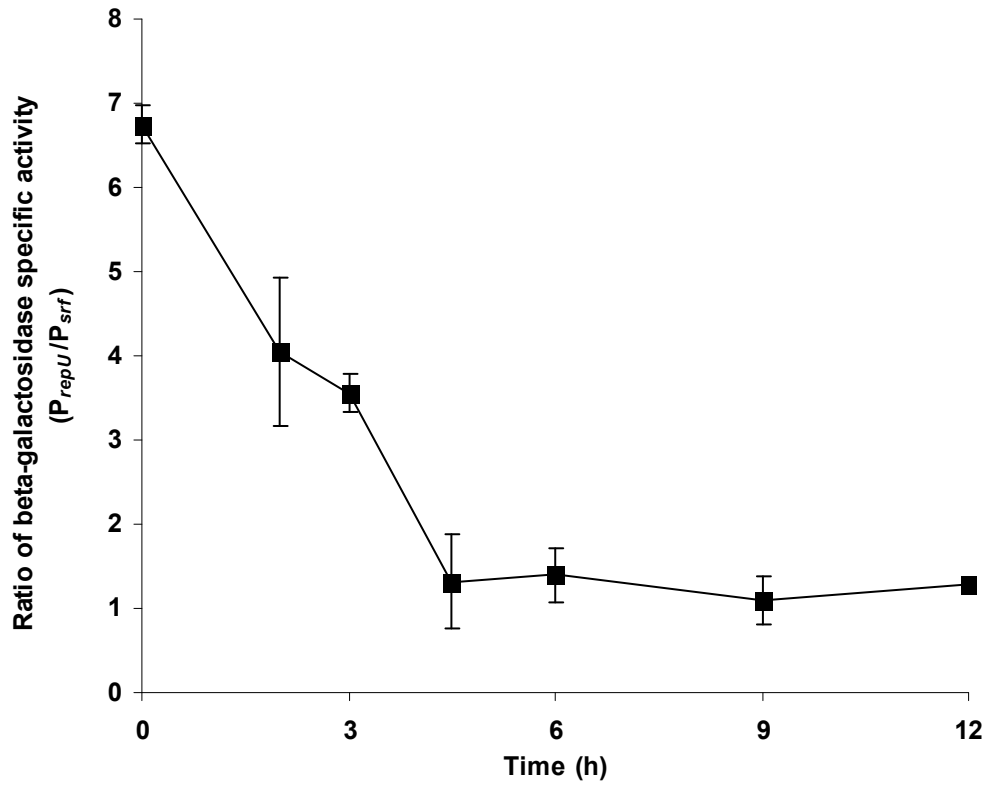


Figure 3 Comparison of the expressions of P_{srfA} and P_{repU} promoters with *lacZ* fusions. *B. subtilis* BBG127 (*trpC2*, *amyE::P_{srfA}-lacZ cat*) and BBG128 (*trpC2*, *amyE::P_{repU}-neo-lacZ cat*) were grown in Landy MOPS medium at 37°C with agitation at 130 rpm. The β -galactosidase specific activity was measured at regular intervals and the ratio between the two β -galactosidase specific activities is presented.

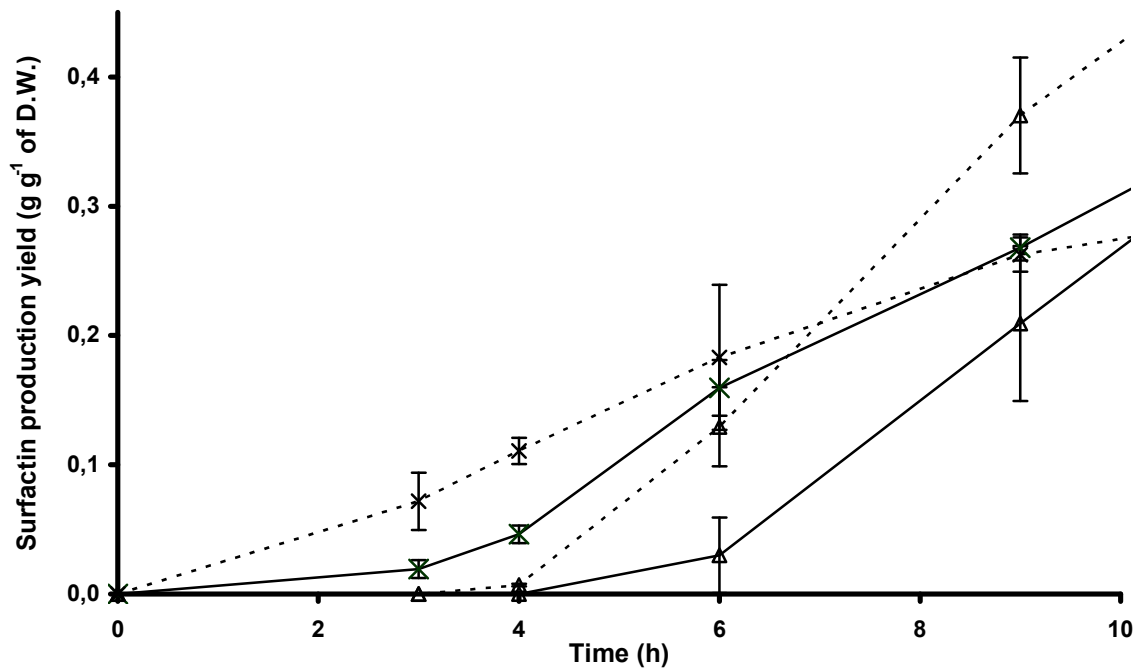


Figure 4 Surfactin production yield by *B. subtilis* BBG111-derived strains cultured in Landy MOPS medium at 37°C with shaking at 130 rpm. The production of surfactin was quantified by HPLC from purified supernatants withdrawn at different times of incubation and divided by the dry weight of biomass. Full lines: (Δ) BBG111 and (\times) BBG113, Dotted lines for the derived strains that do not produce plipastatin: (Δ) BBG130 and (\times) BBG131.

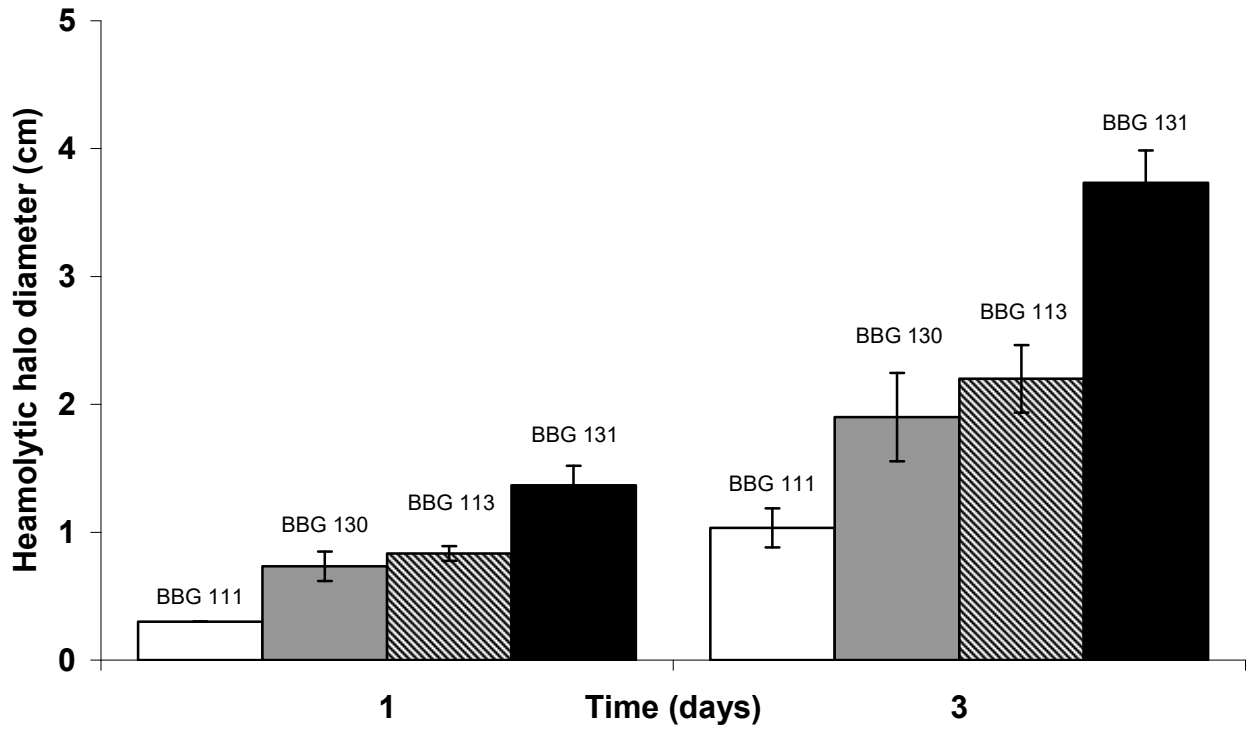


Figure 5 Haemolytic activity on Landy MOPS blood agar of *B. subtilis* BBG111-derived strains. Three microliters of culture of each strain in exponential growth phase were placed using a standardized method onto the center of the plates, then incubated at 37°C.

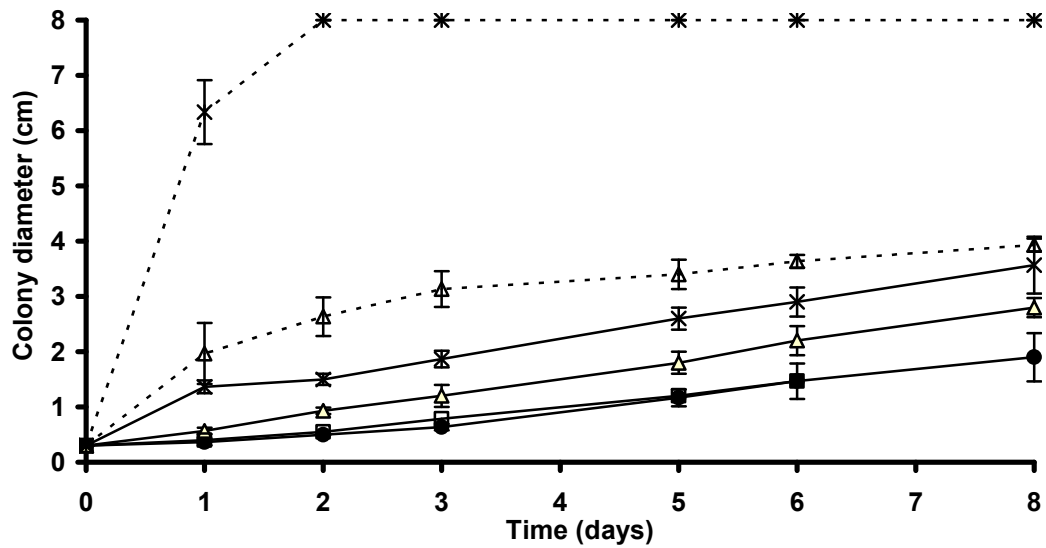


Figure 6 Invasive behaviour of *B. subtilis* 168-derived strains. Strains in exponential growth phase were inoculated as a single colony on 0.7% agar containing B medium at the center of the plate with a standardized method then incubated at 37°C. The diameter of the colonies was measured at regular intervals. Full lines: (●) *B. subtilis* 168, (△) BBG111, (×) BBG113 and (□) BBG118. Dotted lines for the derivatives that do not produce plipastatin: (△) BBG130 and (×) BBG131.

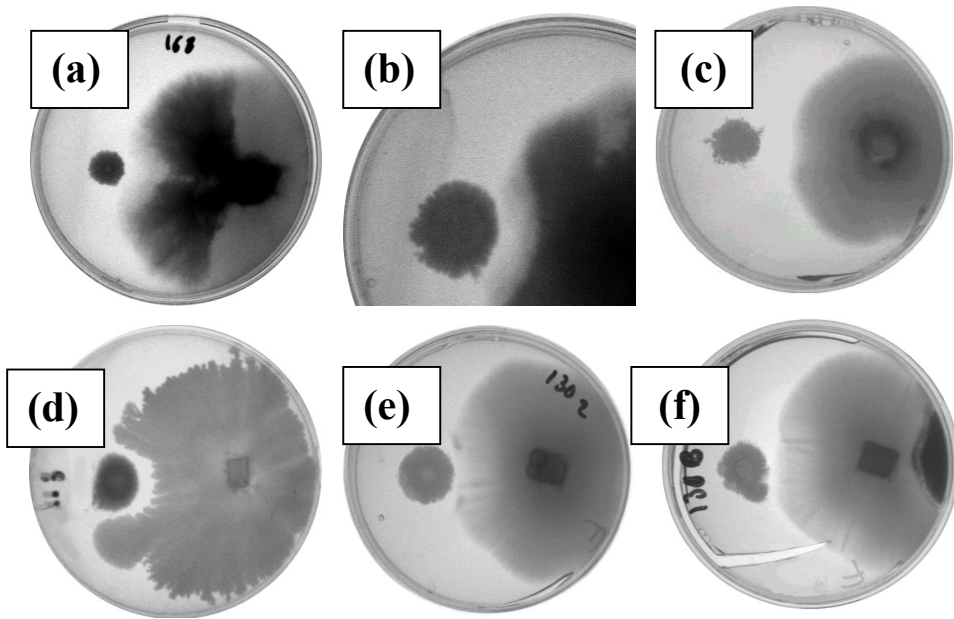


Figure 7 Antifungal effect of *B. subtilis* 168 derivatives against *F. oxysporum*. Five microliters of strain suspension in exponential growth phase were placed using a standardized method (onto the left side of the plate) in front of the fungus (right side of the plate) on PDA medium. The pictures were taken after 6 days of incubation at 30°C. (a) *B. subtilis* 168 (*trpC2*); (b) BBG111 (*trpC2*, *amyE::sfp-cat*); (c) BBG113 (*trpC2*, *amyE::sfp-cat*, $P_{repU-neo}::HaeIII-srfA$); (d) BBG118 (*trpC2*, $P_{repU-neo}::HaeIII-srfA$); (e) BBG130 (*trpC2*, *amyE::sfp-cat*, *ppsA::spc*); (f) BBG131 (*trpC2*, *amyE::sfp-cat*, $P_{repU-neo}::HaeIII-srfA$, *ppsA::spc*).