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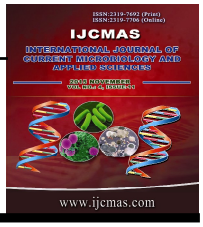

Original Research Article

Modification of Wild Type *Bacillus subtilis* 168 Strain for Single Surfactin Production

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Keywords

Bacillus subtilis, Lipopeptides, Surfactin, Plipastatin, NRPS

A B S T R A C T

Bacillus subtilis 168 a wild type strain harbouring the surfactin (Srf) and plipastatin (Pps) or fengycin (Fen) operons, which are lipopeptides synthesized non-ribosomally by large multienzymatic proteins called the Non-Ribosomal Peptides Synthetases (NRPS). *Bacillus subtilis* 168 *trp* C2 harbours an inactive *sfp* allele (sfp^0) and thereby cannot produce these latter two lipopeptides. Interest in lipopeptides has also been encouraged because of the potential advantages they offer over their synthetic counterparts in many fields spanning agricultural, environmental, food, biomedical, petrochemical and other industrial applications. This work aims to modify *Bacillus subtilis* 168 for single surfactin production in order to facilitate study, extraction and biological specifity of surfactin lipopeptide. The *pps* operon was interrupted in *Bacillus subtilis* 168 after integration of a functional *sfp* gene. The interruption of *pps* operon strongly enhanced surfactin production in the single producer strain BMG02 (1023 mg/L) when compared to the parental strain BMG01 (1482 mg/L) under optimal plipastatin or fengycin production condition, and for strain BMG02 (1758 mg/L) when compared to for strain BMG01 (917 mg/L) under optimal surfactin production condition.

Introduction

Bacillus subtilis strains produce many kinds of bioactive peptides as secondary metabolites. Some of them are synthesized non-ribosomally by a large multifunctional enzyme complex. Among them, surfactins, iturins, and fengycins or plipastatins and kurstakins are the main representatives (Béchet *et al.*, 2012). Non-ribosomal synthesis system NRPS it is an alternative pathway that allows production of poly peptides other than through the traditional translation mechanism. The peptides are created here by multienzymatic complexes called synthetases and the resulting peptides are generally short, 2 to 50 residues. NRPS produces several pharmacologically important compounds, including antibiotics and immunosuppressors (Jacques, 2011). Surfactin was first discovered by Arima *et al.* (1968), as an exo-cellular compound with

an exceptional bio-surfactant activity from the supernatant of a culture of *B. subtilis*. Its structure was elucidated as that of a lipopeptide (Kakinuma *et al.,* 1968). Surfactins are biosurfactant molecules with antiviral, antimycoplasmic and haemolytic activities (Vollenbroich *et al.,* 1997; Peypoux *et al.,* 1999; Deleu *et al.,* 2003). Other strains or species producing surfactin derivatives were identified as *Bacillus coagulans* (Huszcza and Burczyk, 2006) and *Bacillus mycoides* (Athukorala *et al.,* 2009). Related compounds have also been found such as esperin (Thomas and Ito, 1969), halobacillin (Trischman *et al.,* 1994), lichenysin from *Bacillus licheniformis* (Horowitz *et al.,* 1990), pumilacidin from *Bacillus pumilus* (Morikawa *et al.,* 1992) or bamylocin A from *Bacillus amyloliquefaciens* (Lee *et al.,* 2007).

Plipastatins or fengycins are strong antifungal compounds produced after the end of the exponential growth phase, but are less haemolytic than surfactin and inhibit the growth of a large number of fungi (Jacques *et al.,* 1999; Ongena and Jacques, 2008).

Three large Open Reading Frames (ORFs) coding for surfactin synthetases are designated *srfAA*, *srfAB* and *srfAC* (Galli *et al.,* 1994). They present a linear array of seven modules (one module per residue), three modules are present in the products of *srfA-A* and *srfA-B*, *srfA-C* respectively, and the last one in *srfA-D*.

Production of surfactin requires the *srfA* operon encoding the three subunits of surfactin that catalyze the thiotemplate mechanism of nonribosomal peptide synthesis to incorporate the seven amino acids into the surfactin lipopeptide.

The constitutive overproduction of surfactin enhances the invasive growth and the in vitro antagonistic activity of the mutant strain and plipastatin operon disruption increases the surfactin productivity of mutant strains (Coutte *et al.,* 2010).

B. subtilis displays several features, which render this organism an interesting organism not only for the scientific community but also for industrial applications. These features include amongst others the capability to: take up exogenous DNA (so called competence, which facilitates genetic manipulations which are well established for this organism). In addition, it was the first Gram-positive bacterium for which the complete genome sequence became available in 1997 by Kunst *et al.*

In this study, plipastatin operon was interrupted by insertion of plasmid construction with *neomycin* cassette in the transcription direction of plipastatin *pps* operon of *Bacillus subtilis* BMG01 (*Bacillus subtilis* 168 derivative by insertion of sfp^+ gene).

Materials and Methods

Bacterial strains, plasmids and culture conditions

The strains used in this work are presented in table 1. *Bacillus* sp. and Escherichia coli strains were grown aerobically in Luria-Bertani (LB) medium. The strains were grown at 30°C for *Bacillus subtilis* and at 37°C for *Echerichia coli* JM109 in (LB) medium supplemented with ampicillin (50 µg ml^{-1}; Sigma, St. Louis, MO).

Molecular biology methods

Genomic DNA was prepared using the Wizard Genomic DNA Purification Kit and protocol (Promega Corp., Appl Microbiol Biotechnol Madison, USA). DNA concentration was established using a Nanodrop™ 1000 Spectrophotometer. DNA

was used directly as genomic DNA for PCR amplification or stored at -20°C for further utilisation.

Primers; were designed using the published sequence of *Bacillus subtilis* 168 plipastatin operon (accession no. AL009126). Known nucleic sequences of *Bacillus subtilis* strains that are involved in the synthesis of fengycin or plipastatin operons were analysed with Needle online software (Needleman and Wunsch, 1970). All used primers were listed in table 2. The PCR conditions consisted of an initial denaturation step at 94°C for 2 min, followed by 30 cycles of 30 s at 94°C, 30 s at melting temperature for each primer, and extension time depend on fragment size at 72°C. The final extension step was at 72°C for 2 min.

Strains construction

***Bacillus subtilis* BMG01 strain construction**

The *sfp* cassette was generated by PCR using the *sfp* forward and reverse primers and inserted into pGEM-T Easy. The *sfp* cassette was digested by *Eco*RI and ligated into the EcoRI site of pDG1661. This construction named pMG100 and was used to transform *Bacillus subtilis* 168, giving new strain named BMG01.

***Bacillus subtilis* BMG02 strain construction**

This construction was performed using pGEM-T Easy vector. The *ppsA* Cassette was generated by PCR using the *ppsA* forward and reverse primers and inserted into in pGEM-T Easy vector. The ligation mixture was transformed into *E. coli* JM109 cells. The resulting plasmid was named pMG101.

The *dacC* cassette was generated by PCR using the *dacC* forward and reverse primers and inserted into in pGEM-T Easy vector. The ligation mixture was transformed into *E. coli* JM109 cells. The resulting plasmid was named pMG102. Both pMG101 and pMG102 were *Aat*II and *Nco*I double digested, the *dacC* Cassette fragment was then inserted between the *Aat*II and *Nco*I sites of pMG101 to obtain pMG103.

Both pBG106 and pMG103 were *Xba*I and *Ban*II digested. The neomycin cassette was released from pBG106 and inserted between *Xba*I and *Ban*II sites of pMG103 to obtain pMG104, which used to transform *Bacillus subtilis* 168, giving new strain named BMG02.

RNA extraction and RT-PCR

RNA extraction was performed by RNA-later kit of Ambion RNA *later* ® (Applied Biosystems, Courtaboeuf, France). The bacteria were inoculated in Landy MOPS at 37 ºC and under 160 rpm agitation rate. An equivalent volume of 2 x 10^9 cells was obtained at each point of the kinetics previously defined, these volumes were centrifuged at 11,000 g, -9ºC for 5 min. The supernatant was discarded, while the pellet was stored in 1 mL RNA *later* at -20ºC. Then, RNA was purified as mentioned in the kit and the reverse transcription for the RNA into cDNA was performed with RevertAid First Strand cDNA Synthesis Kit (K1621, Fermentas).

Lipopeptides extraction and purification

Cultures were centrifuged at 15,000 *g* for 1 h at 4°C. For lipopeptide extraction, 1 ml samples of supernatants were purified on C18 Maxi-Clean cartridges (Alltech, Deerfield, IL) used according to the recommendations of the supplier.

Lipopeptides were eluted with 5 ml of pure methanol (high-performance liquid chromatography grade; Acros Organics, Geel, Belgium). The extract was dried, and the residue was dissolved in methanol (200 µl) before analysis by high performance liquid chromatography using a C18 column (5 µm; 250 by 4.6 mm; VYDAC 218 TP; VYDAC, Hesperia, CA).

Using High Performance Liquid Chromatography (HPLC), each family of lipopeptides was separately analyzed with the acetonitrile-water-trifluoroacetic acid solvent system (40:60:0.5 [vol/vol/vol] and 80:20:0.5 [vol/vol/vol] for fengycins or plipastatins and surfactins, respectively). Samples (20 µl) were injected, and compounds were eluted at a flow rate of 1 ml min^{-1}. Purified fengycins or plipastatins and surfactins were purchased from Sigma (St. Louis, MO).

The retention time and second derivatives of UV-visible spectra (Waters PDA 996 photodiode array detector; Millenium Software) of each peak were used to identify the eluted molecules. The *Bacillus subtilis* 168 derivatives, BMG01 and BMG02 were grown under two set of different conditions; optimal plipastatin production conditions and optimal surfactin production conditions as described Fahim *et al.* (2012). In all cases, three replicate flasks were used for each strain and the experiment was repeated three times.

α- amylase activity

This activity was determined by growing *Bacillus subtilis* colonies overnight on LB plates containing 1% soluble starch and then staining the plates with a potassium iodide and iodine solution.

Results and Discussion

***Bacillus subtilis* 168 derivatives construction**

To modify *Bacillus subtilis* 168 for single surfactin production firstly, an sfp^+ active gene from the strain *Bacillus subtilis* ATCC 21332 was introduced into the locus *amy*E, as already demonstrated (Coutte *et al.*, 2010) resulting the strain BMG01, the presence of an sfp^+ active gene encode a cofactor which transforme the NRPS involved in the biosynthesis of the two lipopeptides plipastatin and surfactin from apo-enzyme to holo-enzyme.

The strain BMG01was used to construct the single surfactin producer strain BMG02 by the interruption of the plipastatin operon using neomycin cassette. Expression of the plipastatin operon was checked for both BMG02 and its parental strain BMG01. It was measured during the growth in Landy MOPS medium, the transcriptome was blocked and the total RNA was inverse transcribed to cDNA. the plipastatin operon of BMG01 has been amplified a fragment of 760 bp indicates the expression of the plipastatin operon in this strain, while No amplicon was observed for BMG02 with the *ppsA* forward and reverse primers.

Examination of lipopeptide production by the new strain *Bacillus subtilis* BMG02

Cultures were performed with the strain BMG02 and its mother strain BMG01 to verify the interruption of plipastatin operon by the absence of plipastatin production. A lot of studies have pointed out different environmental factors for their effect on lipopeptide production that this effect can be strain-dependent. Carbon and nitrogen sources, mineral requirements (Landy *et al.*,, 1948; Cooper *et al.*,, 1981; Jacques *et al.*,

1999; Guez *et al.*,, 2007; Wei *et al.*,, 2010) as well as oxygen transfer coefficient rate could drastically affect the synthesis (Fahim *et al.*, 2012). To take into account these factors, two set of different cultures conditions (optimal plipastatin production conditions OPPC and optimal surfactin production conditions OSPC) were realized.

Under OPPC, the maximum plipastatin and surfactin production detected were 142 mg/L with standard deviation (SD = 7.3) and 917 mg/L (SD = 15.6), respectively for the coproducer strain BMG01, while maximum surfactin production for the single producer strain BMG02 was 1023 mg/L (SD = 14.8) and as expected no lipopeptides production for *Bacillus subtilis* 168 was detected.

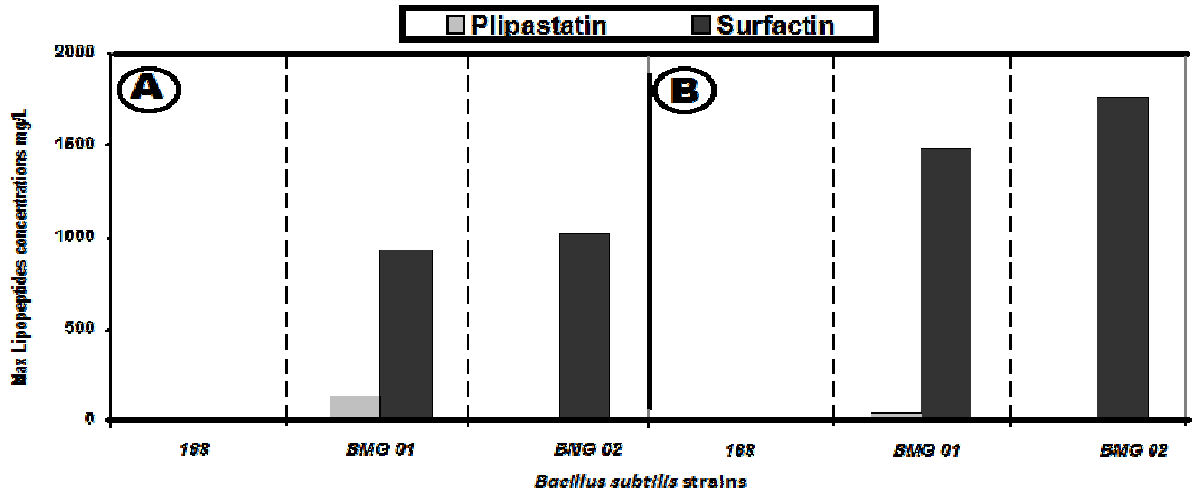
Table.1 Strains and plasmids

Strain or plasmid	Description	Source or reference
Bacterial strains		
Escherichia coli JM109	recA1, endA1, gyrA96, thi, hsdR17 (rK–,mK+), relA1, supE44, Δ(lacproAB), [F´, traD36, proAB, lacIqZΔM15]	Promega
Bacillus subtilis 168	wild type, trpC2, *sfp*^{0}	Lab stock
Bacillus subtilis BMG01	A mutant derivative from 168, trpC2, *sfp*$^+$	This study
Bacillus subtilis BMG02	A mutant derivative from BBG111, trpC$_2$, *amyE*:: *sfp- cat*, (*neo*)::*ppsA*	This study
Plasmids		
pGEM -T Easy	cloning vector Ap^r	Promega
pDG1661	Integration vector, spoVG-lacZ Ap^r, Cm^r, Spc^r	Guéroult-Fleury *et al.* (1996)
pBG106	*neo* fragment, Ap^r, Nm^r	Leclére *et al.* (2005)
pMG100	1.2 kb *Hind*III *sfp* fragment inserted in pDG1661	This study
pMG101	0.63 kb *ppsA* cassette 2 fragment cloned into pGEM-T Easy	This study
pMG102	0.61 kb *dacC* cassette 1 fragment cloned into pGEM-T Easy	This study
pMG103	0.61 kb *Aat*II-*Nco*I *dacC* cassette1 from pMG102 inserted into pBG101	This study
pMG104	1.0 kb *Xba*I *neo* fragment from pBG106 inserted into pMG103	This study

Table.2 Different primers used for the construction of *B. subtilis* BMG02 strain

Primer name	Primer sequence 5′ 3′	Amplified products size (bp)
sfp cassette F	CTGCCTGAATTATGCTGTGG	1100
sfp cassette R	TCGCTGAGGCTACATCAAG	
dacC cassette F	GACGTCAAGACGGGTGAAG	617
dacC cassette R	TCCCATGGAAAACAGGTCTC	
ppsA Cassette F	TGGATTATCTAGACATATAATTTCTTT	
ppsA Cassette R	GAGCTCAAGTAAGAAGGTTCC	634

Figure.1 Plipastatin and surfactin production by mg/L under A (optimal plipastatin production conditions OPPC) and B (optimal surfactin production conditions OSPC)



Under OSPC, the maximum plipastatin and surfactin production detected were 26 mg/L (SD = 8.7) and 1482 mg/L (SD = 16), respectively for the coproducer strain BMG01, while maximum surfactin production for the single producer strain BMG02 was 1758 mg/L (SD = 19.2) and as expected no lipopeptides production for *Bacillus subtilis* 168 was detected.

No plipastatin production was detected for the strain BMG02 led to its operon interruption.

It was observed that the interruption of plipastatin in strain BMG02 led to increasing in surfactin production in the same strain compared to its parental strain BMG01, and the increasing in surfactin production was more significant under OSPC than under OPPC as showed in figure 1.

The interruption of the plipastatin operon obtained here led to increasing surfactin production, in spite of interrupting the surfactin operon reveals no increasing in plipastatin production as mentioned by Ongena *et al.* (2007), which reported that *B. subtilis* 2508 (*B. subtilis* 168 derivative) produced 697 mg/L of surfactin and 434 mg/L of plipastatin in optimalized medium and after the interruption of its surfactin operon, *B. subtilis* 2504 strain was obtained which produced 452 mg/L of plipastatin. Moreover, Coutte *et al.* (2010) was mentioned that the interruption of the plipastatin operon strongly reduced in vitro antifungal properties and, interestingly, enhanced specific surfactin production (1470 mg/L), spreading behaviour and haemolytic activity of the strains.

In the biosyntheses of surfactin and plipastatin, several common precursors are involved as β- hydroxy fatty acid chains and glutamic acid. Also, a high level of ATP for the activation of the different amino acid residues incorporated in their peptide moiety is required. Moreover, the *sfp* gene encodes phosphopantetheine-transferase, responsible for addition of a cofactor, which transformed both NRPS involved in the biosynthesis of these two lipopeptides from apo-enzyme to holo-enzyme. So, these precursors may be redirected to the single surfactin production.

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