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By

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FENGYCIN PRODUCTION BY STRAINS OF *BACILLUS*: MOLECULAR AND PHYSIOLOGICAL ASPECTS

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Soutenue le 11 Juillet 2016 devant le Jury composé de :

Djamel Drider, Professor (University of Lille 1, France)	Supervisor
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Université Lille 1 Sciences et Technologies

Thèse

Presentée par **Yazen Mohlab YASEEN**

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Pour l'obtention du titre de
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I dedicate this work to my beloved country "Iraq."

Résumé

Ce travail a pour objectif d'analyser les facteurs génétiques et environnementaux qui influencent la production de fengycine chez *Bacillus.* Dans un premier temps, une caractérisation physiologique et moléculaire de la souche *B. subtilis* BBG21, mutant spontané de *B. subtilis* ATCC 21332 surproducteur de fengycine a été menée. Le fonctionnement du promoteur P*fen* a permis de démontrer que la modification d'un seul nucléotide dans l'élément UP est en partie responsable de la surproduction de fengycine. Dans un second temps, l'influence de conditions biotiques et abiotiques sur la synthèse de surfactines et de fengycines et sur l'expression du promoteur P_{fen} a été étudiée. Les sources de carbone disponibles dans le milieu de culture orientent la synthèse vers une famille de lipopeptides alors que la plupart des sources d'azote permettent la cosynthèse à haut niveau des deux molécules étudiées. Une forte expression du promoteur P_{fen} couplée à une synthèse importante de fengycines a été mise en évidence lorsque l'urée ou le mélange urée ammonium sont utilisés comme source d'azote et le mannitol comme source de carbone. L'influence des facteurs température, pH et oxygénation a également été testée. Non seulement ces facteurs modulent la synthèse des lipopeptides mais les conditions optimales de production des surfactines et fengycines sont de plus dépendantes des sources de carbone ou d'azote présentes dans le milieu. Enfin, nous avons étudié l'influence de mutations *srfA* chez *B. subtilis* BBG 21 et celle de la mutation *pnpA* chez *B. subtilis* BBGG21 et dans des dérivés *de B.s.* 168. Les résultats montrent que l'interruption de *srfAA* affecte significativement la production de fengycines alors qu'il n'y pas d'effet lors d'une interruption de *srfAC*. Chez les mutants *pnpA* une forte diminution de la synthèse de la surfactine et de la fengycine est observée. Les expériences réalisées dans ce travail nous permetttent de proposer un schéma partiel de régulation des deux lipopeptides chez *Bacillus subtilis.*

Abstract

This work aimed at analyzing the genetics and the environmental factors that can influence the fengycin production in *Bacillus subtilis.* Firstly, physiological and molecular characterization of the strain *B. subtilis* BBG21 (a spontaneous mutant fengycin overproducer strain) was caryed out .Study the promoter of this strain has been shown that the change of a single nucleotide on the UP element sequence is partially responsible for the overproduction of fengycin in the strain. Then, the influence of certain biotic and abiotic conditions on the surfactin and fengycin synthesis as well as the expression of P_{fen} promoter has been studied. The result demonstrated that some carbon sources appeared to orient synthesis of one family of lipopeptides, while most of nitrogen sources allowed high level of both lipopeptides co-synthesis. A strong expression of promotor P_{fen} and an important synthesis of fengycins were obtained with urea or urea ammonium mixture used as nitrogen source and with mannitol as carbon source. Temperature, pH and filling volume are important for fengycin synthesis and theoptimal conditions are carbon and nitrogen sources dependent. Finally we studied fengycin synthesis in surfactin and *pnpA* mutants derived from Bs 168. The result showed that knock-out of *srfAA* gene decreased fengycin synthesis whereas knock-out in *srfAC* gene has no significant effect. Notably, an important decrease in surfactin and fengycin was observed for *pnpA* mutant strains. The results of this work allowed us to propose a partial regulator schimatic for the two lipopeptides in *Bacillus subtilis*.

Publications:

1) **Yazen Yaseen**, Frédérique Gancel, Max Béchet, Djamel Drider and Philippe Jacques. **Influence of promoters on the production of fengycin in *Bacillus* spp.** *Research in Microbiology*, 2016
2) **Yazen Yaseen**, Frédérique Gancel, Max Béchet, Djamel Drider and Philippe Jacques. **Regulation of fengycin regulation: the promoter expression and gene transcription are dependent on different culture conditions** *Journal of Applied Microbiology*. (Submited)
3) **Yazen Yaseen**, Frédérique Gancel, Max Béchet, Djamel Drider and Philippe Jacques. **Fengycin produced by *Bacillus* spp.: from gene regulation to managment of production.** *Journal of Microbiology*. (under preparation)

- **Oral communications:**

1) **Yazen Yaseen**, Frédérique Gancel, Max Béchet, Djamel Drider and Philippe Jacques. **Comparative study of fengycin promoters using green fluorescent protein (GFP) fusion marker**. Bacell, Annual conference, Amesterdam-Netherland, 2015**.**
2) **Yazen Yaseen**, Frédérique Gancel, Max Béchet, Djamel Drider and Philippe Jacques. **Regulation of fengycin production in *Bacillus* spp.** Journée des doctorants, Lille – France, 2014.

- **Posters:**

1) **Yazen Yaseen**, Frédérique Gancel, Max Béchet, Djamel Drider and Philippe Jacques. **Fengycin regulation between the promoter expression and gene transcription responding to different culture conditions.** Society of General Microbiology annual conference, Liverpool- England, 2016.
2) **Yazen Yaseen**, Frédérique Gancel, Max Béchet, Djamel Drider and Philippe Jacques. **Study of various regulatory factors for the overproduction of fengycin in Bacillus subtilis.** 8th International Conference on Gram-Positive Microorganisms /18th International Conference on Bacilli. Tuscany, Italy, 2015.
3) **Yazen Yaseen**, Frédérique Gancel, Max Béchet, Djamel Drider and Philippe Jacques. **Study of the regulation of fengycin biosynthesis in *Bacillus* spp.** Society of General Microbiology annual conference, Birmingham- England, 2015.

Abrevations

ACP: acyl carrier protein

A-domain: adenylation domain

BBG: Bacillus strain collection of ProBioGEM Laboratory

C-domain: condensation domain

CoA: coenzyme A

DNA: deoxyribonucleic acid

E-domain: epimerization domain

EDTA: ethylene di-amine tetra- acetate

GFP: green fluorescence protein

HEPES: 4-(2- hydroxylethyl)-1- piperazineethane sulfonic acid

HPLC: high performance liquid chromatography

IPTG: isopropyl β-D- thiogalactopyranoside

LB: Luria broth

MALDI-TOF: matrix-assisted laser desorption/ionization-time of flight

MOPS: 3-(N-morpholino)-propane sulfonic acid

mRNA: messenger RNA

MS: mass spectrometry

NRPS: non ribosomal peptide synthetase

OD: optical density

ORF: open reading frame

PCR: polymerase chain reaction

PKS: polyketide synthase

PnpAse: polyneocleotide phosphorylase

PTG: isopropyl β-D-thiogalactoside

Sfp: 4'-PP cofactor 4'-phosphopantetheine cofactor

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Chapter 1
Literature review

Part 1: General introduction and objectives

B. subtilis strains produce a set of bioactive lipopeptides with great potential for biotechnological and biocontrol of plant diseases applications. This group of compounds is classified into five main families, which are: the surfactins [Nakano and Zuber, 1991], the iturins [Duitman *et al,* 1999], the fengycins/plipastatins [Vanittanakom *et al.,* 1986; Nishikiori *et al.*, 1986], the kurstakins [Hathout *et al,* 2000; Béchet *et al.,* 2012], and the locillomycins [Luo *et al.,* 2015]. Each family of lipopeptides is constituted of several variants, which can differ in their fatty acid chain and their peptide moiety. In each family, the peptidic moiety is composed of various amino acids (from 5 to 10) and the length of the lipidic chain is also varying from 10 to 19 carbon atoms [Jacques, 2011].

Fengycin was described to be endowed with a strong antifungal activity, especially against filamentous fungi [Vanittanakom *et al.,* 1986; Wise *et al.,* 2014; Farace *et al.,* 2015] and antibacterial activity against Gram-positive *Staphylococcus epidermidis* and Gram negative *Escherichia coli* [Huang *et al.*, 2006; Roy *et al.*, 2013]. Notable, antiviral activity against Newcastle disease virus and bursal disease virus has also been reported [Huang *et al.,* 2006]. More recently an antitumor activity against human tumor cells has been attributed to this lipopeptide [Sivapathasekaran *et al.,* 2010; Yin *et al.,* 2013; Ditmer 2014]. Fengycins/plipastatins can influence the ecological fitness of the producing strain in terms of root colonization. Thereby their persistence in the rhizosphere, is also playing a key role in the beneficial interaction with plants by stimulating host defence mechanisms (Ongena *et al.,* 2005 and 2007). According to all these biological properties, these lipopeptides have been considered as promising biocontrol agents [Waewthongrak *et al.,* 2014].

Furthermore, fengycin owing to their properties are being increasingly favored in the place of chemically synthesized surfactants for industrial applications such as emulsifiers in food and pharmaceutical industries, in mobilization of heavy oil spills, bioremediation of oil-contaminated soil and enhanced oil recovery [Banat *et al.,* 2010].

Regulation of surfactin and mycosubtilin (a member of Iturin family) operons have been described in details in literature. It involves a set of pheromones from the quorum sensing. On the contrary, there is little available information on the fengycin operon.

The global objective of this work is to increase the knowledge about the parameters which influence fengycin production at molecular and cellular levels.

For this purpose, several fengycin-producing strains have been used: one *B. amyloliquefaciens* strain (FZB42) and five *B. subtilis* strains (*B. subtilis* BBG21, *B. subtilis* ATCC 21332, *B. subtilis* BBG111, *B. subtilis* BSBN1 and *B. subtilis* BBG258) *B. amyloliquefaciens* FZB42 can produce three types of lipopeptides including fengycin, surfactin and bacillomycin. The *B. subtilis* strains produced only surfactin and fengycin. One of these strains, the strain *B. subtilis* BBG21 is a spontaneous mutant of *B. subtilis* ATCC 21332. Compared to its mother strain, BBG21 is able to overproduce fengycin up to 500 mg/L [Fahim *et al.,* 2010; Coutte *et al.,* 2010]

This work **firstly** aimed to check the influence of fengycin operon promoter on the overproduction of this lipopeptide by BBG21. In order to do this, we compared the sequence and the strength of this promoter under several environmental conditions using a gene reporter system to those of two other strains, *B. subtilis* 168 and *B. amyloliquefaciens* FZB42**.**

The second aim was to study the effect of biotic or abiotic conditions on fengycin operon expression and fengycin production.

The **third aim** was to study the effect of specific genes knock-out on fengycin operon expression and fengycin production. As the tested strains produce concomitantly surfactins and fengycins, we checked the influence of the knock-out of srfA genes. The knock-out of polynucleotide phosphorylase (PNPase) gene known to play a key and pleiotropic role in *B. subtilis* kingdom, was also studied.

Part 2: Fengycin from its production to its applications

Bacillus genus

Microorganisms have remarkable biosynthetic abilities to produce metabolites with different biological activities. Soil is the main biotope for species belonging to the genus *Bacillus*, which are Gram-positive and rod-shaped bacteria [Nakano and Zuber 1998]. *Bacillus* cells have variable size of 0.5 µm x 1.2 to 2.5 x 10 µm. They are usually catalase positive and give a variable response to the oxidase test. This bacterium is aerobic, although anaerobic growth seems to occur in complex media, mainly in the presence of nitrate [Logan *et al.*, 2006]. The optimum growth temperature is between 25-35 °C [Movahedi and Waites, 2002].

A common bacilli characteristic is their ability to form endospores allowing them to survive for long periods under different environmental conditions. Their ability to be spore-forming added to metabolic diversity constitutes significant elements that lead to their successful colonization of a widespread diversity of environments. Indeed *Bacillus sp.* strains can be found in soil, water, and air [Aislabie *et al.*, 2013]. *Bacillus sp.* uses their flagella for a swarming motility on surfaces [Nakano and Zuber 1998]. In order to swarm, cells need to secrete a slime layer which includes lipopeptides, a surface tension-reducing molecule, as one of its components [Schaechter 2006; Leclere *et al.*, 2006].

The *Bacillus sp.* group includes the most intensively studied bacilli, and is composed of species such as *B. subtilis* itself, *B. licheniformis* and *B. amyloliquefaciens*, which are of industrial and agricultural relevance [Kumar *et al.*, 2012; Sicuia *et al.*, 2015]. The *B. subtilis* 168 genome is 4.2 Mbp in size and comprises 4,100 protein-coding genes [Wipat and Harwood 1999; Sinchaikul *et al.*, 2002]. Most of the genes are involved in the metabolism and, an average of 4−5% of the genome was shown to be dedicated to secondary metabolites production [Stein 2005; Chen *et al.*, 2007].

Cells are anticipated to grow rapidly, reach high cell densities and excrete a large number of molecules such as amylase, alkaline protease, alkaline phosphatase or esterase. The amount produced (g per liter) render *Bacillus sp.* an important source for industrial enzymes [Nigma, 2013]. *Bacillus spp.* is not considered as pathogen for humans, but can occasionally contaminates food and causes food poisoning, if strains belong to *B. cereus* or *B. anthracis* species [Setlow, 2006].

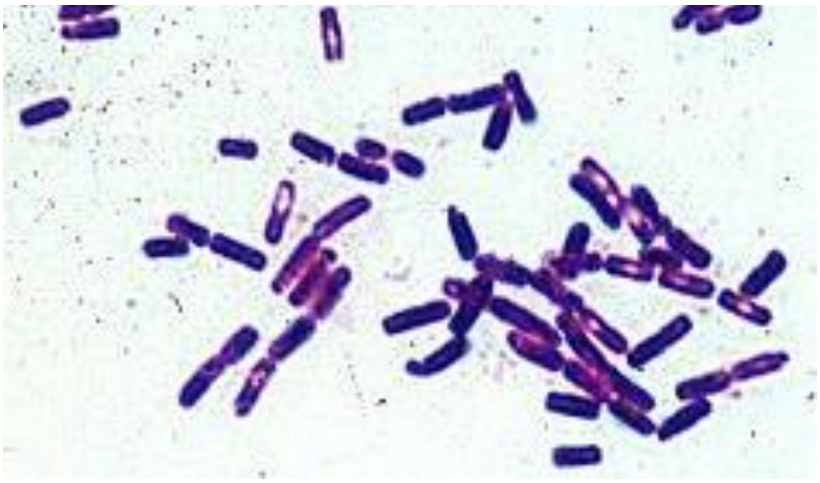

Figure 1 *Bacillus subtilis* Gram staining

Fengycin structure

Fengycins are cyclic lipodecapeptides containing a β-hydroxy fatty acid chain that may be saturated or unsaturated. Fengycins are composed of a peptide chain of ten amino acids linked to a fatty acid chain which varies from 14 to 19 carbon atoms, resulting thereof in a diverse homologous compounds and isomers. Eight amino acids (Tyr, Thr, Glu, Ala, Pro, Gln, Tyr, and Ile) are involved in the formation of a peptide ring via lactone between the hydroxyl group of L-Tyr3 and the C-terminal carboxyl group of L-Ile (Figure 2) [Steller *et al.*, 1999; Chen *et al.*, 2007]. Recently, the cyclized structure of fengycin was shown to play a critical role in its antifungal activity [Kim and Chae 2015].

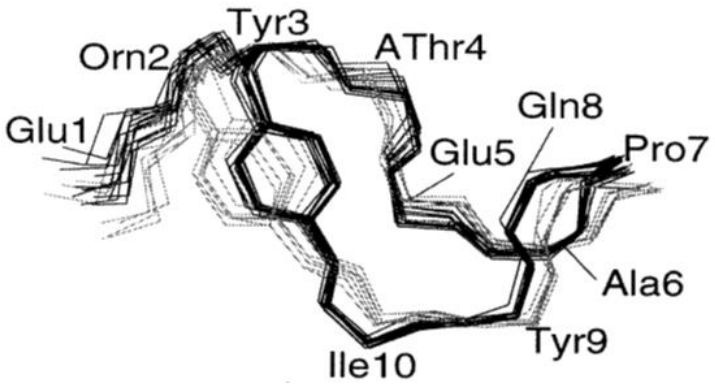

Figure 2: Fengycins/Plipastatins structures adapted from Wang *et al.* (2004)

Members of fengycin family exhibit heterogeneity at the 6th position in peptide moiety as well as in chain length of β-hydroxyl fatty acid. Most of studies revealed the presence of cyclized fengycin homologs A and B, which are distinguishable by the presence of either alanine or valine, respectively, at position 6 of the peptide sequence (Figure 3) [Vanittanakom *et al.*, 1986; Jacques 2011; Kim and Chae 2015; Plaza *et al.*, 2015]. It should be noted that further fengycin types were reported in independent studies. Related to that, Sang Cheol *et al.* [2010] reported a lipopeptide of a molecular mass of 1491.2 which was designed as fengycin S and differed from fengycin B and fengycin A by a substitution of serine for the threonine residue in position 4, and the amino acid residue in position 6 was equal to that of fengycin A. Villegas-Escobar *et al.* [2013] portrayed a new fengycin isoform named fengycin C, with the amino acid sequence Glu-Orn-Tyr-Thr-Glu-Val-Pro-Gln-Thr-Ile. The peptidic moiety differs from fengycin B at position 9 and from fengycin A at positions 6 and 9. Separately, based on the variation of Glu/Gln at position 8, a new subclass of fengycin was also identified using high resolution mass spectrometry [Pathak and Keharia 2013]. Moreover, fengycin also referred to as plipastatin when Tyr3 and Tyr9 are present as the L- and D-form, respectively [Roongsawang *et al.*, 2010; Jacques 2011]. There is a real confusion between the two names plipastatin and fengycin. Fengycins could be named plipastatins according to the place of L- and D- forms of tyrosine within the peptide moiety [Steller *et al.*, 1999; Jacques *et al.*, 1999; Wei *et al.*, 2010]. However, many reports mentioned that they are identical compounds, and this has been proven by Honma *et al.* [2012].

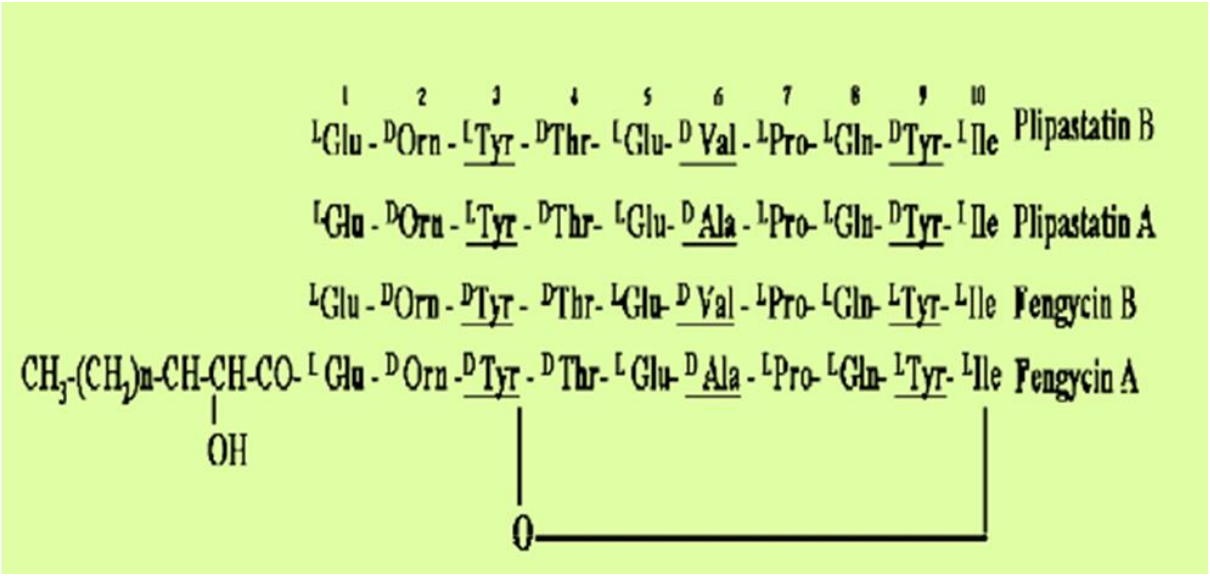

Figure 3 Primary structure of fengycin homologues [Pathak and Keharia, 2013]

Fengycins as NRP compounds

Fengycins are synthesized non-ribosomally by multifunctional complex enzymes system consisting of an arrangement of modules. Each module is responsible for the integration of a specific monomer into the growing polypeptide chain and can be therefore decomposed in specific domains. In non-ribosomal peptide biosynthesis each elongation cycle needs the cooperation of at least three basic domains and one terminal module (Figure 4) [Steller *et al.*, 1999; Wu *et al.*, 2007; Luo *et al.*, 2015].

(1) The **A** domain (adenylation domain) selects its cognate monomer and generates an enzymatically stabilized aminoacyl adenylate. This mechanism bears a resemblance to the aminoacylation of tRNA synthetases during ribosomal peptide biosynthesis [Stachelhaus *et al.*, 1999].

(2) The **T** domain, also called peptidyl -carrier protein (PCP). At this step the adenylated monomer substrate is covalently bound to its 4'-phosphopantetheinic (4'-PP) cofactor as thioester. This occurs by tethering the phosphopantetheinyl moiety of the co-substrate coenzyme A (CoA) in phosphodiester linkage to the hydroxymethyl side chain of the conserved active serine residue in the CP domains. The 4'-PP cofactor acts as a flexible arm to allow the bound amino acyl and peptidyl substrate to travel between different catalytic centers [Strieker *et al.*, 2010].

(3) The **C** domain (condensation domain): Catalyses the peptide bond formation between the amino acyl thioester attached to the PCP in the same module and this of the preceding module [Challis and Naismith, 2004].

(4) The **TE** domain is the last domain. It contains the terminal enzyme that allows the release of the peptide and is also frequently involved in the formation of a macrocyclic product (lactones and lactams) or the oligomerisation of peptide units [Mootz and Marahiel, 1997].

Additional domains can be involved in the biosynthesis of the peptide either to modify its structure or to add some external compounds to the peptide. Among these secondary domains, cyclisation (Cy), methylation (Me), oxidation (Ox), glycosylation, epimerization (E) domains and addition of fatty acid chain have been shown. The last two domains (epimerization and addition of fatty acids) are involved in lipopeptides biosynthesis in *Bacillus spp.*, the E-domain catalyses the epimerization of the PCP-bound L-amino acid of the growing polypeptide chain. The addition of the fatty acid chain to the first amino acid of the peptide moiety is catalyzed by a first specific condensation domain [Finking and Marahiel, 2004].

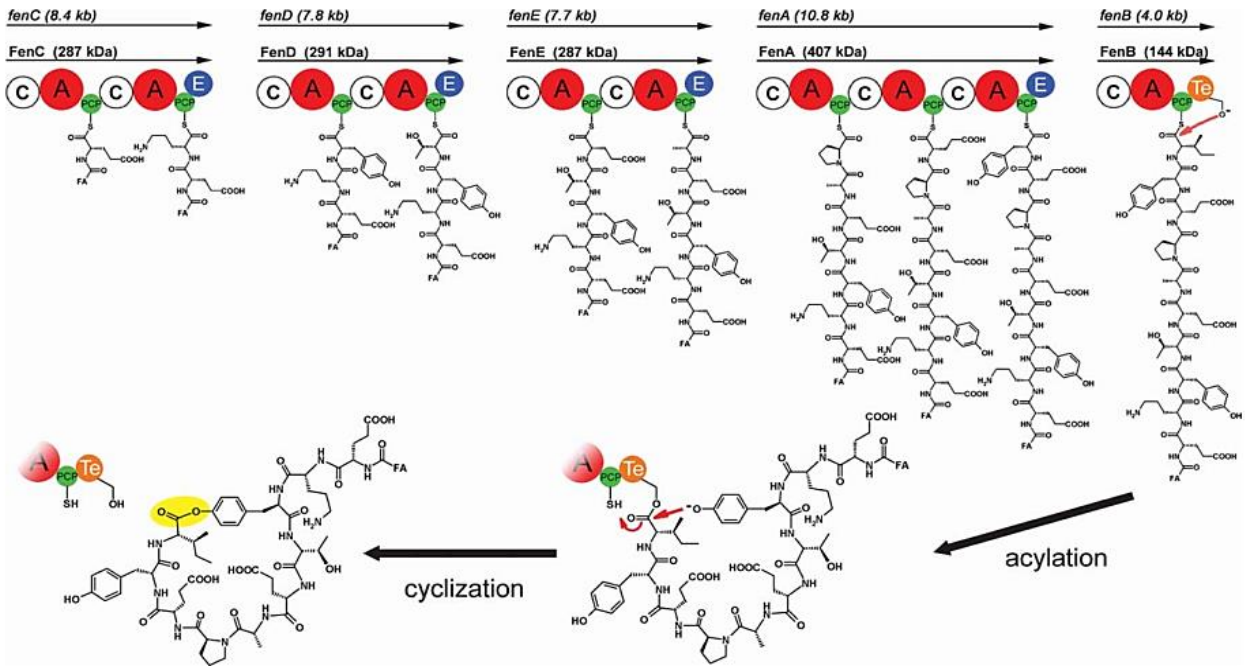

Figure 4: NRPS modules and domains of fengycin operon. (Samel *et al.*, 2006)

Fengycin applications

According to their physicochemical properties such as the amphiphilic character and the affinity for lipid bilayers [Steller *et al.*, 1999], the fengycins family produced by *Bacillus* spp. is being highly studied for their antimicrobial activity, low toxicity for animals and plants and high biodegradability [Mandal *et al.*, 2013], moreover, their role as anti-adhesive agents [Rodrigues *et al.*, 2006], as well as their environmental friendly characteristics [Liu *et al.*, 2015].

Several studies permitted to elucidate the fengycin mechanism of action as antimicrobial agent [Deleu *et al.*, 2008; Nicoli *et al.*, 2010; Horn *et al.*, 2013; Wise *et al.*, 2014]. Fengycins are supposed to act by making the plasma membrane of the target cell more permeable [Eeman *et al.*, 2009]. At sufficiently high concentrations, fengycin aggregates, lead to large sustainable pores which induce a complete efflux of the cellular contents, and the cell death [Wise *et al.*, 2014]. Besides, fengycins are able to inhibit phospholipase A2 and aromatase [Steller *et al.*, 1999; Volpon *et al.*, 2000; Wei *et al.*, 2010]. To show the important role of fengycin in the different fields each application domain was covered separately in this part (Figure 5).

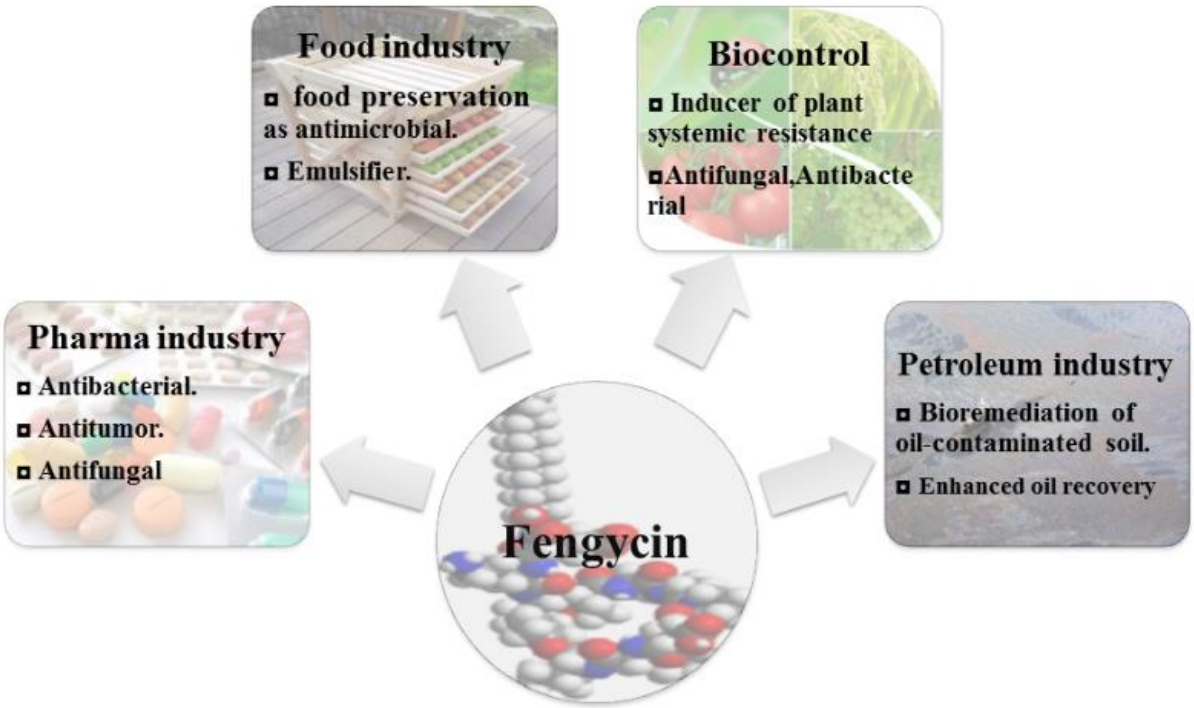

Figure 5. Wide border use of fengycin in the different domains

Biomedical and therapeutic applications of fengycins

As previously mentioned fengycins are well-known for their antifungal activity but, continuing reports underline their antibacterial and antiviral activities especially in combination with surfactin. Huang *et al.*, [2007] reported that a lipopeptidic antimicrobial activity of *B. subtilis* strain fmbj was ascribed to concomitant synthesis of surfactin and fengycin. These substances inactivate the endospores of *B. cereus* through damaging the surface structure of the spores. Related to this, Huang *et al.*, [2006] showed that surfactin and fengycin produced by *B. subtilis* strain fmbj were able to inactivate the cell-free virus stocks of porcine parvovirus, pseudorabies virus, newcastle disease virus and bursal disease virus and could effectively inhibit infections and replication of these viruses. In a recent study, the formation of fengycin self-assembled structure onto a hydrophobic surface showed a clear improvement of antibacterial activity against *Staphylococcus epidermidis* and *Escherichia coli* as well as against the pathogenic yeast *Candida albicans* [Roy *et al.*, 2013]. On the other hand, the ability of fengycin to modulate the surface properties and molecular organization of stratum, by preferentially interacting with cholesterol, makes this lipopeptide a potential candidate for enhancing the penetration of topically applied drugs through the skin [Nicoli *et al.*, 2010]. The antitumor activity of fengycin was tested *in vitro* and *in vivo* using the human lung cancer cells line 95D, and the study showed that fengycin stands as potential anticancer issue, inhibiting the growth of the aforementioned cell line by regulating the cell cycle and promoting apoptosis [Yin *et al.*, 2013].

This anticancer activity was also investigated *in vivo* and *in vitro* against human cancer cell, validating the anti-proliferative potential of fengycin and its ability to inhibit these cells [Ditmer 2014]. Similar anti-proliferative activity was reported for lipopeptides (namely isoforms of surfactins and fengycins) derived from the marine *B. circulans* strain DMS-2, showing interesting cytotoxic activity against cancer cell lines HCT-15 [Sivapathasekaran *et al.*, 2010]. Indirect effects of fengycin and surfactin were shown to negatively influence biofilm formation by *Staphylococcus aureus* ATCC 29213 and *E. coli* CFT073. These effects were observed either by coating the polystyrene surface with these compounds or by adding the biosurfactant to the inoculum [Pecci *et al.*, 2010]. Correlatively, *B. subtilis* filtrate containing a mixture of surfactin, iturin and fengycin, demonstrated significant anti-adhesive and antibiofilm activities. These compounds also had an influence on the planktonic growth of the tested uropathogens [Moryl *et al.*, 2014].

Biocontrol

Several investigations reported fengycin families worthy of being used as biocontrol agents, by exploring the potential use of fengycin and its phytopathogen-antagonistic activities against a plethora of plant pathogens [Kumar *et al.*, 2012; Wise *et al.*, 2014; Chowdhury *et al.*, 2015]. These lipopeptides were considered to be promising biocontrol agents for postharvest diseases of fruit and vegetables, regarding their ability to inhibit fungal pathogens and/or elicit defense responses in plant tissues [Waewthongrak *et al.*, 2014]. Guo *et al.*, [2014] showed that fengycin is an excellent biocontrol agent for cotton soil-borne diseases, by inhibiting the growth of *Rhizoctonia solani in vitro*. Another study indicated that fengycin-like lipopeptides produced by *B. subtilis* CPA-8 play a major role in the biological control against peach brown rot by showing strong antifungal activities against *Monilinia laxa* and *Monilinia fructicola* [Desoignies *et al.*, 2013]. In addition, fengycin and iturins have been shown to be the major *Bacillus* metabolites involved in the antagonism against *Fusarium oxysporum, Cladosporium cucumerinum* and *Botrytis cinerea* [Farace *et al.*, 2015]. However, the fengycin negative mutants retained certain inhibitory capabilities against the bacterial strains *Xanthomonas campestris* pv. *cucurbitae* and *Pectobacterium carotovorum* subsp. *carotovorum* the causative agents of cucurbit powdery mildew [Asari 2015].

Beside direct antagonism, some beneficial bacteria can protect plants indirectly through the stimulation of inducible defense mechanisms that render the host more resistant to further pathogen ingress [Ongena *et al.*, 2007]. Waewthongrak *et al.*, [2014] showed that fengycins act as elicitors of defense-related gene expression in ‘‘Valencia’’ fruit following infection, by eliciting defense-related gene transcription and activity of the defense-related enzymes glucanase and chitinase, which have a direct effect on the pathogen. In addition, surfactin activated the genes coding for POX and LOX proteins that play important roles in generating signal molecules for activating ISR (induced systemic resistance) mechanisms. Desoignies *et al.*, [2013] showed that the systemic resistance induced by *Bacillus spp.* lipopeptides, drastically reduces the infection of sugar beet by *Polymyxa betae*. According to Ongena *et al.*, [2007], *B. subtilis* 168 derivatives overexpressing surfactin and fengycin genes were able to induce resistance in tomato cells. Farace *et al.*, [2015] reported that besides its direct antagonism on the necrotrophic pathogen, *B. cinerea* which infect grapevine leaves, fengycin perception by grapevine cells resulted in early signaling activation. *B. amyloliquefaciens* strain UCMB 5113 produces a linear form of fengycin found to promote growth of plants and to improve the survivability of plants exposed to biotic stress challenges. Fengycin acts by priming stress tolerance, beside the direct antagonistic effect of this strain on several *Brassica*

phytopathogens (*Botrytis cinerea*, *Alternaria brassicae*, A*lternaria brassicicola*, *Verticillium longisporum*, and *Sclerotinia sclerotiorum*), as demonstrated by bacteria and root exudates *in vitro* [Luo *et al.*, 2015].

Food industry

In the food industry, the non-ribosomally synthesized lipopeptides are well characterized in the terms of their anti-adhesive, antimicrobial or antitumor activities [Meena and Kanwar 2015]. One of the greatest causes of loss in the food industry are the postharvest diseases of fruits and vegetables, and according to estimations in the U.S.A., this loss reaches 20 to 25% [Gordillo and Maldonado 2012]. The surface of the fruit or vegetables is covered with fungal spores, bacterial cells and yeasts, fungi being the principal decaying agents in fruit kept in cold storage chambers for long periods [Gordillo *et al.*, 2009]. Arrebola *et al.*, [2010] indicated the antagonistic role of fengycin produced by *B. amyloliquefaciens* strain PPCB004 against five species of fungal postharvest pathogens of *Citrus*. Kim and Chae [2015] showed that treatment of postharvest apples with either the cell culture or a cell-free culture supernatant of *B. subtilis* HM1 reduced disease severity resulting from *Colletotrichum acutatum* of 80.7% and 69.4%, respectively, and fengycin was shown to play a critical role in antifungal activity. Tang *et al.*, [2014] suggested that lower concentrations of fengycin may be effective for food preservation. Fengycins could down-regulate the transcription of some key genes involved in the production of mycotoxins such as fumonisin B1 (FB1), and impair its synthesis by *Fusarium verticillioides*, warranting the role of fengycins as potential candidates to control FB1 contamination in crops and food [Hu *et al.*, 2009].

Bioremediation

Surfactants are surface-active compounds with a pronounced ability to reduce the surface or interface tension of the solvent. Consequently, they are therefore widely used in the fields of environmental remediation. Different experiments with laboratory-scale sand-packed columns and field trials have successfully revealed the effectiveness of biosurfactants in oil recovery. This activity can be realized in two different ways as either an *ex situ* biosurfactant inoculation or *in situ* biosurfactant production to reach the augmentation in oil recovery from subsurface reservoirs [Banat *et al.*, 2010]. In both cases, biosurfactants or their producing microorganisms may resist to the strict environmental conditions. The microbial biosurfactants stand as promising candidate in the petroleum industry field with extremely advantageous features, including lower toxicity, higher biodegradability and their stability

under extreme conditions (temperature, salinity and pH), as well as its high interfacial activities [Liu *et al.,* 2015].

Cheng *et al*., [2013] reported that a mixture of glycolipid and lipopeptide, including rhamnolipid, surfactin, and fengycin produced by *B. subtilis* strain TU2 showed 86% of oil-washing efficiency and acceptable emulsification activity on crude oil, suggesting its potential application in enhanced oil recovery. While, the application of blend of surfactin, iturins and fengycin produced by *B. subtilis* strain K1 on laboratory scale sand pack column saturated with four stroke engine oil resulted in 43 % enhanced oil recovery [Pathak and Keharia 2013].

Sang-Cheol *et al.,* [2010] demonstrated that fengycin S produced by *B. amyloliquefaciens* LSC04 may potentially be used, either directly on oil spills in contaminated environments, or for the biotechnological production of biosurfactants. Furthermore, the ability to produce biosurfactants using oil industry remnants was clearly detected in several *Bacillus* strains isolated from oil spoiled soil. Using oil spreading technique and by emulsification activity, the best emulsification activity and stability was observed in the strain OS17 against kerosene or kerosene mixed with 20% diesel [Violeta *et al.,* 2011].

Fengycin production

Several studies related to the influence of environmental factors on lipopeptide biosynthesis indicated that carbon and nitrogen sources in the media influence the type and yield of the produced lipopeptides. Growth conditions such as temperature, pH or oxygen rate are also importants factors [Volpon *et al.,* 2000; Singh *et al.,* 2014; O'Connor *et al.,* 2014]. The presence of amino acids acting as lipopeptide precursors in the medium may play a role in the biosynthesis of the molecule by modifying the amount of lipopeptides produced and also their structure [Besson *et al.,* 1987; Islam *et al.,* 2012]. Regarding fengycin specifically, three culture parameters were evidenced for their influence. These include source of nutriments, oxygen transfer rate and growth temperature [Besson *et al.,* 1987; Hu *et al.,* 2009; Cameotra *et al.,* 2010; Jacques 2011; Singh *et al.,* 2014]. The inoculum size and physiological age of cells are other factors which may play a role [Varadavenkatesan *et al.,* 2013]. Previous study showed that a first inoculum age of 23 h followed by a second inoculum age and size of 4 h and 1% respectively, gave the maximum biosurfactant production [Willenbacher *et al.,* 2015].

Medium composition

It is well known that the production of most antibiotics (as secondary metabolites) is dependent on the composition of medium. The type and the concentration of carbon, nitrogen sources, phosphate regulation and other nutrients markedly influence the regulatory mechanisms that device the onset of lipopeptides synthesis [Besson *et al.,* 1987; Violeta *et al.,* 2011; Ghribi *et al.,* 2012; Mnif and Ghrib, 2015]. Thus, the selection of appropriate carbon and nitrogen sources is one of the most critical stages in the development of an efficient and economical production process [Mukherjee and Das, 2005]. Moreover, modification of the nutrient conditions, may change the type and the activity of the produced lipopeptides; the best nutrients production condition appeared to be strain-dependent [Volpon *et al.,* 2000; Fonseca *et al.,* 2007].

Islam *et al.,* [2012] found that mannitol and soytone were the best carbon and nitrogen sources for the production of fengycin by *B. subtilis* strain C9. The type of fengycin produced can be also modified according to the culture substrate conditions. Singh *et al.* [2014] suggested that the carbon sources provided for the growth and biosurfactant production not only influence the yield but also the type of biosurfactant. In a medium containing dextrose, sucrose and glycerol, *B. subtilis* strain AR2 produced lipopeptides as a mixture of surfactin, iturin and fengycin. However, in the presence of maltose, lactose and sorbitol, only iturin was produced from the same strain. O'Connor *et al.* [2014] showed that feeding 3-fluoro-L-tyrosine to cultures of *Bacillus spp.* CS93 resulted in the biosynthesis of modified fluorinated fengycins.

Many substrates were tested to find a low cost production medium. The use of alternative raw materials, such as petrol or agro-industrial residues, is a possible strategy to reach this goal. Raw glycerol which is a byproduct of biodiesel production with negative value was successfully used to produce fengycin from the strain *B. subtilis* LSFM-05 [de Faria *et al.,* 2011]. *B. subtilis* LAMI005 was able to grow and produce biosurfactant in an alternative culture media, prepared by using clarified cashew apple juice as carbon and energy source [de Oliveira *et al.,* 2013].

In direct line, biosurfactant production by *B. subtilis* 573 was evaluated using corn steep liquor as culture medium [Gudiña *et al.,* 2015]. Recently, it was suggested that the indirect contact with the target organism can stimulate the lipopeptides synthesis by the producing cells. The study showed that the lipopeptide synthesis can be enhanced in *B. subtilis/amyloliquefaciens* species by responding to some chemical signal emitted (without contact) by the pathogens. Indeed, a higher production of iturins and fengycins was observed

upon incubation in the presence of *Pythium* and *Fusarium* compared with *Botrytis* for which no increase in lipopeptides accumulation could be noticed [Cawoy *et al*., 2015]. Leães *et al*. [2015] mentioned that the fengycin gene expression of *B. amyloliquefaciens* P11 increased twice and higher antimicrobial activity was observed in the presence of inactivated cells of *Staphylococcus aureus* and *Aspergillus parasiticus* compared to the other conditions tested. The same phenomenon was also observed by Sajitha and Dev [2016] with the strain *B. subtilis* B1.

Culture conditions

Various physical and chemical parameters such as agitation, pH and inoculum age and size, could enormously influence the production yield and cost. Enhancement of biosurfactant production could be achieved through the optimization of these different parameters [Mnif *et al*., 2012; Mnif and Ghribi, 2015].Numerous researches were carried out on the physiological conditions influencing the lipopeptides production from *Bacillus spp*. Several bioreactor types were also tested to optimize the lipopeptides production, such as light carriers designed for a three phase fluidized bed biofilm reactor [Gancel *et al*., 2009], standing cultures [Chollet-Imbert *et al*., 2009], bubbleless membrane bioreactors [Coutte *et al*., 2010b], biofilm reactor [Chtioui *et al*., 2010], modified inverse fluidized bed bioreactor [Fahim *et al*., 2012] modified rotating discs bioreactor and non-foaming film reactor [Chtioui *et al*., 2014]. All these studies confirmed the important role of the environmental conditions on fengycin production.

Overall, the increase in oxygen rate positively enhances surfactin production contrary to the fengycin one, which increases with a low oxygen rate. Moreover, the best fengycin production is achieved at 30°C, and the production decreases with the increasing of the temperature, whereas no fengycin production is recorded almost at 37°C [Fahim *et al*., 2012].

It was demonstrated that k_La (oxygen transfer coefficient) is the key parameter controlling the productivity and the selectivity of the bioreaction. By varying the oxygen transfer conditions, the synthesis could be oriented to mixed production or to surfactin/fengycin mono-production. The fraction of surfactin towards total lipopeptides produced and the maximal surfactin production both increased with k_La increase (surfactin concentration about 2 g/L at kLa = 0.04–0.08 s^{-1}), while the maximal fengycin production (fengycin concentration about 0.3 g/L) was obtained at moderate oxygen supply (kLa = 0.01 s^{-1}) [Chtioui *et al*., 2014]. Pryor *et al*. [2007] showed that maximum fengycin production was observed at low incubation temperatures and high substrate moisture contents. The aeration has a strong

positive effect on lipopeptide production. Optimal aeration rates for the production of the two known lipopeptides were 0.1 and 1.5 L/min, respectively. Mnif *et al*. [2012] also reported, using an automated full-controlled 2.6 L fermenter, that aeration of the medium strongly affected the growth and regulated biosurfactants. Therefore, according to these studies, low or high aerations lead to a decrease in surfactin and fengycin synthesis yields.

Yaseen *et al*. [2016] reported that the pH level remarkably influences fengycin production. The minimum production was recorded when the culture started at pH 7.5, leading to elevate the pH in the culture until 8.9 at the end of incubation. The same negative influence was noticed with pH 5.4 at the end of incubation. However, the ideal pH for *B. subtilis* growth is between 5.5 and 8.5 [Fonseca *et al.*, 2007], while the ideal pH for fengycin production is between 6.5 and 7.5 [Fahim *et al*., 2012; Varadavenkatesan *et al.*, 2013].

Part 3

Fengycin produced by *Bacillus subtilis*: regulation and biosynthesis

Abstract

Fengycin produced by *Bacillus spp.* is a naturally bioactive antibiotic produced non-ribosomally by a large complex of enzymes. Owing to its biosurfactant, biodegradable and non-toxic properties, it showed high potential applications in industrial, agricultural and pharmaceutical domains. This review aimed at giving an update on the regulation with a specific focus on the quorum sensing regulators, reported to affect the fengycin operon expression. We correlate all the factors that might influence fengycin regulation to have better understanding as regards its interaction. This review underlines the potential applications of this lipopeptide as biocontrol and human anticancer agent. Scale-up production of fengycin is a prerequisite for the aforementioned applications. For this purpose, fengycin synthesis is tightly dependent on the environmental factors including the nutritional conditions. Study the regulatory factors involve in production will not only help to increase productivity whilst reducing the cost of production, but also help to understand the mechanism by which the fengycin operon is regulated.

Introduction

Bacillus subtilis is known to be a provider of many important biologically active substances via its secondary metabolism machinery [Sinchaikul *et al.,* 2002]. About 4-5% of this bacterial genome is thought to be used for the biosynthesis of antimicrobial substances [Stein 2005; Chen *et al.,* 2007], including the lipopeptide group. Indeed, lipopeptides are antimicrobial peptides produced non-ribosomally by *B. subtilis* using multifunctional complex enzymes called Non-Ribosomal Peptide Synthetases (NRPS). To date, many families of lipopeptides were discovered such as surfactins [Nakano and Zuber, 2009], iturins [Duitman *et al.,* 1999], fengycins/plipastatins [Vanittanakom *et al.,* 1986; Nishikiori *et al.,* 1986], kurstakins [Hathout *et al.,* 2000; Béchet *et al.,* 2012], and locillomycins [Luo *et al.,* 2015].

Fengycin or plipastatin contains 10 amino acids, which are synthesized by five NRP Synthetases (Fen1 to Fen5) [Steller *et al.,* 1999; Jacques 2011]. The corresponding five NRPS subunits FenA (287 kDa), FenB (290 kDa), FenC (286 kDa), FenD (406 kDa), and FenE (146 kDa) [Wei *et al.* 2010] are encoded by an operon composed of five open reading frames (ORFs) named *fenA-E* (or *ppsA-E*) [Tosato *et al.,* 1997; Steller *et al.,* 1999; Jacques *et al.,* 1999; Koumoutsi *et al.,* 2004]. The expression of lipopeptide operons involves a complex regulatory machinery linked to a quorum sensing similarly to that reported for surfactin operon [Hamoen *et al.,* 2003 ; Hayashi *et al.,* 2006] or mycosubtilin, a member of iturins family [Duitman, PhD thesis, 2003] but not for fengycin. The little information mentioned in literature related to the regulation of fengycin operon pointed out the importance of *degQ* gene, which positively regulates the expression [Tsuge *et al.,* 1999; Tsuge *et al.,* 2007], and the recent identification of a nucleotide sequence in the promoter region, located between positions -55 and -39 and known as “UP element”, resulted to be crucial for fengycin synthesis in *B. subtilis* F29-3 [Ke *et al.,* 2009; Yaseen *et al.,* 2016].

The antimicrobial and anticancer activity of fengycin suggests its usage in pharmacological applications [Roy *et al.,* 2013; Yin *et al.,* 2013]. Furthermore, potential commercial applications of fengycin start to develop in several other industries including cosmetics, agrochemical, bioremediation and food industries [Banat *et al.,* 2000; Perfumo *et al.,* 2010].

In spite of its high efficiency, as antibiotic, biosurfactant, biocontrol agent..., the use of this lipopeptide remains limited because of its high cost production. This drawback is

attributed to the substrate sources which usually supplemented in the production media [Pathak and Keharia 2013; Banat *et al.,* 2010]. According to Gudiña *et al.,* (2015), the culture medium used to produce biosurfactants might represent up to 30–50% of the overall production cost. Attempts to diminish the production cost of these substances were undertaken; they consisted to optimize the production by using low cost substrate sources or genetically modified strains [Fonseca *et al.,* 2007].

In terms of regulation and gene expression, the data available on fengycin remain rare compared to other lipopeptide families such as surfactins and iturins. This review provides information regarding the regulation and production of fengycin and its potential application.

Non-ribosomally synthesized peptides

Fengycin is synthesized non-ribosomally by a multifunctional complex enzymes system consisting in the arrangement of different modules. Each module is responsible for the integration of new basic unit into the growing polypeptide chain. These modules can be categorized in a specific domain such as the A domain or adenylation domain, the T domain or thiolation domain, also called Peptidyl-Carrier Protein (PCP), the C domain or condensation domain and the TE domain or thioesterase domain. Each elongation cycle in the non-ribosomal peptide biosynthesis needs the interaction of three basic domains and one terminal module as well as it has been broadly described in figure 4 and 6 [Steller *et al*., 1999; Stein 2005; Chen *et al.,* 2007; Jacques 2011; Wu *et al*., 2007; Luo *et al.,* 2015]. It should be noted that additional domains can be involved in the biosynthesis of the peptide either to modify its structure or to incorporate some external compounds such as the fatty acids.

Role of *Sfp* in NRPS synthesis

Phosphopantetheinyl transferases (PPTases), which are called Sfp, play an essential role in priming non-ribosomal peptide synthetases by converting peptidyl carrier protein (PCP) domain within those multidomainal enzymes from inactive apo-forms to active holo-forms [Nakano *et al*., 1992; Quadri *et al.,* 1998]. This is performed by the addition of the phosphopantetheinate group of coenzyme A on a serine residue. Thus the *sfp* gene is requested by *B. subtilis* to produce the fengycin [Tsuge *et al.,* 1999]. Different *sfp* types may play a role in the level of lipopeptide production [Kim *et al.,* 2000]. Nevertheless, there is no evidence showing that overexpression of *sfp* gene resulted in the enhancement of the

lipopeptide production [Nakano and Zuber, 1991]. On the other hand, the *sfp* activity appeared to be significantly influenced by the modification of pH level in the culture and the ideal pH level for expression has to be between 6-8, while a scale down of expression occurs when the pH is below or up of this interval. According to Quadri *et al.,* [1998] and Duckworth *et al.* [2010], there is no difference in the *sfp* expression using a temperature comprised between 20 to 30C°. Recently, Leães *et al.,* [2015] showed that the *sfp* expression is modulated by inactivated cells of target microorganisms such as *Staphylococcus aureus* and *Aspergillus parasiticus*.

Fengycin operon

The first description of fengycin or plipastatin operon in *B. subtilis* 168 was in performed by Tosato *et al.* [1997] who showed that this operon was composed of five ORFs *ppsA-E*. Then, Lin *et al.* [1999] cloned and sequenced five fengycin synthetases genes (*fenC-B*; unusual order) in *B. subtilis* F29-3. Afterwards, several strains were found to harbor this operon [Tosato *et al.,* 1997; Steller *et al.,* 1999; Płaza *et al.,* 2015]. Generally, the five fengycin ORFs encode for the five fengycin synthetases Fen1-Fen5 [Tapi *et al.,* 2010]. Notably, Fen1 is responsible for the synthesis of the side chain of the peptidic moiety of fengycin by incorporating a β-hydroxy fatty acid chain and by activating and combining glutamate in position 1 and ornithine in position 2. Tyrosine in position 3 and allo-threonine in position 4 are activates and incorporates by Fen2. Fen3 activates and joins glutamate in position 5 and alanine or valine (fengycin B) in position 6. While, Fen4 consists of three modular enzyme, which catalysis the activation and combination of proline, glutamine and tyrosine in position 7, 8 and 9, respectively. And finally, Fen5 allows the incorporation of the last amino acid residue isoleucine in position 10. The last module catalyses the release of the peptide and the construction of an ester link between the carboxylic group of Ile (the last amino acid) and the hydroxyl group of a tyrosine in position 3. D-form of the corresponding amino acid residues is observed in the final product, in the presence of epimerization domains in modules 2, 4, 6 and 9 [Tosato *et al.,* 1997; Steller *et al.,* 1999; Tapi *et al.,* 2010; Płaza *et al.,* 2015].

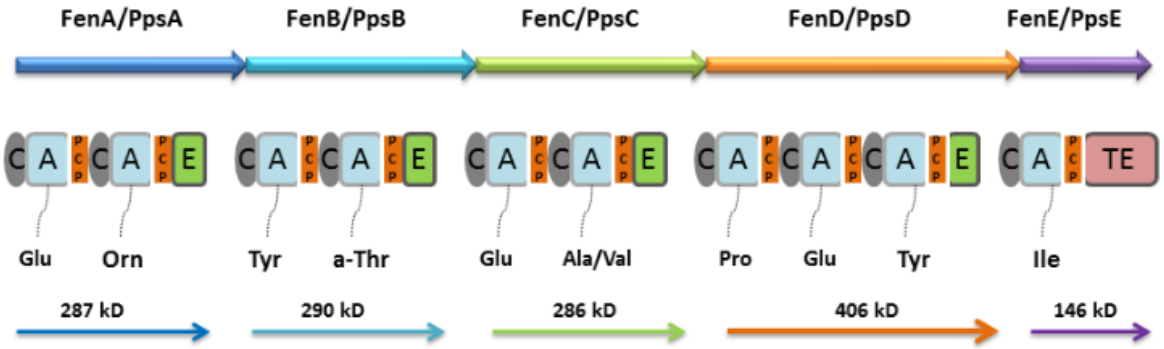

Figure 6. Schematic diagram of the fengycin operon adapted from [Jacques, 2011].
A: Adenylation domain, **C**: Condensation domain, **PCP**: Peptidyl Carrier protein domain
E: Epimerization domain, **Te**: Thioesterase domain

Regulation factors:

UP Element

The expression of fengycin operons could involve complex regulatory machineries. Remarkably, the promoter is located 86 nucleotides upstream from the translation initiation codon of *fenA* or from the start site of *ppsA* [Lin *et al.*, 1999]. Another sequence located between positions -55 and -39, known as "UP element", is crucial for fengycin synthesis in *B. subtilis* F29-3 [Ke *et al.*, 2009].

Usually, the bacterial promoters consist of at least three RNA polymerase (RNAP) recognition sequences: the −10 element, the −35 element, and the UP element, which is commonly composed of a poly-A (–51 to –54) and a poly-T (- 47 to - 50) sequence [Estrem *et al.*, 1999]. The UP element region was shown to increase the transcription by interacting with the C-terminal domain of α subunit of RNA polymerase [Aiyar *et al.*, 1998]. The importane of UP element sequence was shown not only with the fengycin promoter but also with other promoters in *B. subtilis*. Caramori and Galizzi [1998] indicated that the replacement of the UP element region in two different promoters by the flagellin promoter stimulates the transcription activity in *B. subtilis*. In addition, Meijer and Salas [2004] reported that the change in the UP element sequence located upstream from the -35 boxes enhanced the promoter activity *in vivo* as well as *in vitro* in three *B. subtilis* 168 promoters. In a previous study carried out on *B. subtilis* F29-3 fengycin promoter, it was established that

UP element sequence was critical for the promoter activity and mutations realized in this sequence decreased the production of fengycin until 85% [Ke *et al.,* 2009].

Recently, we observed that the differences in the promoter sequence between *B. subtilis* BBG21 mutant strain and its mother strain ATCC 21332 strains, represented by the change of one nucleotide in the UP element increased the fengycin production of *B. subtilis* BBG21 of about 10-fold [Yaseen *et al.,* 2016]. All these studies unveiled the important role of the promoter on the *fen* transcription activity and confirmed that the use of strong promoter can enhance significantly the production of fengycin [Ongena *et al.,* 2007].

***DegQ* gene**

degQ is a pleiotropic regulatory gene which encodes a small polypeptide of 46 amino acids and controls the production of degradative enzymes : an intracellular protease and several secreted enzymes (levansucrase, alkaline proteases and metalloproteases, α-amylase, β-glucanase, and xylanase) [Msadek *et al.,* 1990; Yang *et al.,* 1986]. The overexpression of *degQ* enhanced the production of plipastatin in *B. subtilis* 168 *sfp*+ until 10-fold [Tsuge *et al.,* 1999]. Similar results were obtained with *B. subtilis* NCD-2 strain, when a significant decrease in the antifungal activity as well as the fengycin expression was observed with its *degQ*$^{-}$ derivative mutant [Wang *et al.,* 2015]. Many studies reported that a single base substitution within the -10 region of the *degQ* promoter (TACACT instead of CACACT), allowed overexpression of the intact *degQ* gene, and this led to both increased expression of this gene [Msadek *et al.,* 1990; Yang *et al.,* 1986; Koumoutsi *et al.,* 2007; Xu *et al.,* 2014] and overproduction of plipastatin/fengycin [Tsuge *et al.,* 1999; 29; Wang *et al.,* 2015]. Most of the wild-type *Bacillus* strains that produce antibiotic peptides showed elevated *degQ* expression compared to that of the laboratory strain *B. subtilis* 168 [Yang *et al.,* 1986; Koumoutsi *et al.,* 2007]. Expression of *degQ* was shown to be subjected to catabolite repression and DegS-DegU-mediated control, allowing an increase in the rate of synthesis of DegQ under conditions of nitrogen starvation [Msadek *et al.,* 1990; Do *et al.,* 2011; Tanaka *et al.,* 2015]. On the other hand, DegQ stabilizes phosphorylated DegS in the presence of DegU, indicating a complex interaction of these three proteins [Do *et al.,* 2011]. Moreover, ComA was also reported as regulator factor for the lipopeptide production either directly in the case of surfactin or indirectly by activating *degQ,* which increases the production of fengycin [Deng *et al.* 2013]**.** According to Do *et al.* [2011] *comA* encodes a global transcriptional regulator in the stationary phase and is strongly induced under dense-cell

conditions. Msadek *et al.* [1991] noticed that the expression of *degQ'-'lacZ* was strongly influence by ComA, since it was decreased about 50- to 100-fold in a strain disrupted in *comA* gene. In addition, the deletion of *comP* decreased *degQ* gene expression about 20-fold. ComA likely promotes the expression of *degQ* in *Bacillus* spp., indicated by the two ComA boxes which were found in the *degQ* promoter in *B. subtilis* that are conserved in *B. amyloliquefaciens* FZB42 [Koumoutsi *et al.,* 2007; Raaijmakers *et al.,* 2010]. The DegU-DegS control system responds to an environmental signal such as limitations of nitrogen, carbon, or phosphate sources. In this system, one component accepts an environmental signal and transduces the information to the other component, resulting in activation of the target gene or cell machinery [Louw *et al.,* 1994; Gabdrakhmanova *et al.,* 2005]. Similarly, the ComA-ComP system started under certain environmental conditions. The response regulator ComA is primarily activated by ComP, a histidine kinase that mediates response to nutrient conditions and cell density [Kim *et al.,* 2001; López *et al.,* 2010].

An indirect regulatory effect was reported for the global transcriptional regulators CodY and CcpA on *degQ* expression and activity via their effects on the two *degQ* regulatory system (DegU-DegS and ComA-ComP) [Molle *et al.,* 2003; Ogura and Tsukahara, 2010]. The level of expression of *degQ* was low during the exponential growth phase and was shown to increase substantially in the stationary phase [Msadek *et al.,* 1991]. Similarly but curiously, the fengycin promoter appeared to be active during the log phase, and its activity reached the highest level during the late of the log phase. The highest fengycin production occurred during the late lag and stationary phases [Koumoutsi *et al.,* 2004; Yaseen *et al.,* 2016]. Whether DegQ directly influences the transcriptional regulation of the antibiotic operon or controls the expression of a posttranscriptional regulator involved in the fengycin synthesis (for example, Sfp) remains to be elucidated.

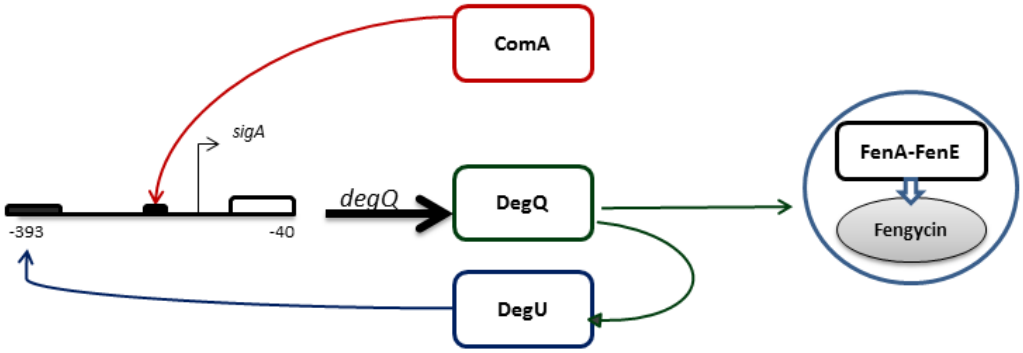

Figure 7. Regulatory network governing DegQ activity in *Bacillus amyloliquefaciens* strain FZB42 (adapted from Koumoutsi *et al.*, 2007)

PhoP-PhoR system

PhoP is one of the positive factors that influence fengycin production in *B. subtilis*. The deletion of this gene led to a significant decrease in fengycin production, while this production was restored by complementation of *phoP* in the deletion mutant strain [Dong *et al.*, 2014]. The antifungal activity of *B. subtilis* NCD-2 decreased significantly by disrupting the *phoR* gene, whereas restoration of this activity was observed with the complementation of *phoR* [Guo *et al.*, 2010]. PhoP-PhoR system was found to be one of the fengycin regulator factors in the new sequenced strain *B. subtilis* BAB-1 [Guo *et al.*, 2014a; Guo *et al.*, 2014b]. PhoP/PhoR is an important two-component regulatory system in *B. subtilis* and other gram-negative and gram-positive bacteria. In response to phosphate deficiency, PhoR, a histidine sensor kinase, phosphorylates its cognate response regulator PhoP (PhoP~P) [Salzberg *et al.*, 2015]. In *B. subtilis*, more than 30 genes were classified as belonging to the Pho regulons and being controlled by the PhoR/PhoP system [Martín, 2004] and by genome-wide transcriptional analysis increasingly more genes were identified as being subjected to the Pho regulon [Allenby *et al.*, 2005].The carbon source was reported to affect the PhoP/PhoR regulation via a transcriptional regulator known as CcpA which operates as a DNA binding protein, either activating or repressing a number of genes in the presence of a preferred carbon source [Puri-Taneja *et al.*, 2006].

Another regulon called SigB can provide a general response to the resulting energy stress, and also to the phosphate starvation condition [Allenby *et al.,* 2005; Antelmann *et al.,* 2000]. SigB mutant showed to induce significantly *ppsA* expression in *B. subtilis* [Allenby *et al.,* 2005]. Meanwhile, SigB mutant induced significantly the PhoP/PhoR system; consistently, the PhoP mutant increased significantly *sigB* gene expression [Paul *et al.,* 2004]. Based on this, we can deduce that the PhoP plays certainly an important role in the fengycin regulation. However, this role has to be elucidated.

AbrB regulator

AbrB is a global transcriptional regulator in *B. subtilis* that represses the expression of various genes; this factor usually represses the stationary phase genes during exponential growth phase [Strauch *et al.,* 1989; Strauch and Hoch 1993; Jakobs *et al.,* 2014]. The genes involved directly in the antibiotic production represent more than 100 genes by the repression effect of this regulator, and other hundreds genes affected indirectly [Kobir *et al.,* 2014]. AbrB protein was found to repress the expression of the *ppsABCDE* genes responsible for the plipastatin production in *B. subtilis* 168 [Chumsakul *et al.* 2010]. The disruption of *abrB* in *B. amyloliquefaciens* SQR9 improves the biocontrol activity for this strain (which produces three lipopeptide families) against cucumber wilt disease [Xu *et al.,* 2014]. During the transition from exponential growth phase into stationary phase, the Spo0A protein (sporulation transcription factor) is phosphorylated by a complex phosphorelay system, resulting in an increase in Spo0A levels which repress the *abrB* transcription, thus exciting AbrB-dependent repressive effects upon the production of the various antimicrobials, antibiotic resistance determinants [Qian *et al.,* 2002; Chumsakul *et al.,* 2013]. Indirectly, AbrB was found to reduce of 20% the PhoP expression [Hulett *et al.,* 1994], or via ScoC (former name Hpr; belonging to the group of transition state regulators) which is activated by *abrA* [Caldwell *et al.,* 2001; Kaushal *et al.,* 2010], resulting in a decrease of fengycin production (as described above for the positive role of PhoP in fengycin production). In addition, Nagórska *et al.* [2008] suggested that the presence of AbrB also facilitates the binding of other negative regulators to the UP region of the target genes, which is a very important region in the fengycin promoter as described above.

Vargas-Bautista *et al.* [2014] showed that Spo0A was required to activate *pks* gene (responsible for the NRPS bacillaene) expression through repression of the transition state regulator AbrB. The *abrB*$^{-}$ derivative of *B. amyloliquefaciens* resulted in a significant

increase in the production of bacilysin (an NRPS product), whereas the *abrB*$^{-}$ mutation suppressed the bacilysin-negative phenotype of a *spo0A*$^{-}$ mutant [Karatas *et al*., 2003]. In harmony, the biofilm of *B. subtilis spoIIGB* mutant was found to induce *ppsADE* expression, suggesting that products of these genes may help the biofilm cells to enhance their chances for survival by inhibiting the growth of nutrient competitors [Ren *et al*., 2004]. Another regulatory protein recently renamed LutR (formerly named YvfI) was found to impact positively on the fengycin gene expression, and the EMSA analysis demonstrated that LutR directly binds to the regulatory region of *ppsABCDE* operon [İrigül-Sönmez *et al*., 2014]. The same study indicated that SinR also directly interacts with the regulatory region of *pps* operon, knowing that LutR and SinR have a close target overlap between their regulators. LutR and SinR are pleiotropic regulators involved in the regulation of a wide variety of cellular processes associated with the onset of stationary phase by effecting, either negatively or positively, degradative enzyme production, antibiotic production and resistance, carbohydrate utilization, sporulation, and biofilm formation [Köroğlu *et al*., 2008]. Similar actions for LutR and SinR were also identified as genes required for production of the polyketide dipeptide antibiotic bacilysin in *B. subtilis* [İrigül-Sönmez *et al*., 2014; Kim *et al*., 2010].

Cross-talk between the fengycin and other lipopeptides

Most of *Bacillus* strains which produce fengycins contain one (or more) other NRPS operon(s) responsible for production of other lipopeptides. Surfactin is the most often found lipopeptide accompanying fengycin [Galli *et al*., 1994; Roongsawang *et al*., 2002; Roongsawang *et al*., 2010]. Four large ORFs coding for surfactin synthetases are designated *srfAA*, *srfAB*, *srfAC* and *srfAD* [Hamoen *et al*., 1995; Lee *et al*., 2007]. A small gene, designated *comS*, is located within the coding region of the fourth amino acid-activation domain of *srfAB* and thus co-expressed with the *srfA* operon. The ComS protein, which contains 46 amino acid residues, is not directly involved in the biosynthesis of surfactin but required for competence development in *B. subtilis* [Nakano and Zuber 1991; Jacques 2011; Ogura *et al*., 1999]. ComS is required for the auto-activation of *comK* by binding of the N-terminal domain of ComS with MecA [Turgay *et al*., 1998]. Thus, ComS release ComK from degradation by the ClpC/ClpP proteolytic complex which, instead, targets the newly formed MecA/ComS complex for degradation [Berka *et al*., 2002]. Besides the important role of ComK in the competence development in *Bacillus* spp. [Ogura *et al*., 2002], the expression of

at least 165 genes are upregulated in the presence of ComK. 16 ComK-directly dependent genes were identified using microarray technique with more other genes which were indirectly dependent [Ogura and Tanaka 1997]. Ogura *et al.* [2002] also found that ComK negatively regulates *degR* expression by preventing σD-driven transcription of *degR*. Surfactin-deficient mutants were found to influence production of the other non-ribosomally synthesized lipopeptides. The production of bacillomycin was blocked, as well as a significant decrease in the production of fengycin was observed in a *B. subtilis* 916 *srf* mutant, while the disruption of bacillomycin increased the fengycin yield to 25% more than in the wild-type strain [Luo *et al.*, 2015]. Zeriouh *et al.* [2014] showed that the biocontrol activity resulting from the act of the bacillomycin and the fengycin was significantly reduced in *B. subtilis sfrAB* mutant. Similarly, the production of bacilysin from *B. subtilis* ATCC 21332, which also produces surfactin, was reduced to 12.5% of that produced by the wild-type strain [Karatas *et al*., 2003]. In contrast, the deletions of *srfAB, srfAC* and *srfAD* had no effect on the fengycin yield in *B. subtilis* 168 [Ongena *et al.*, 2007]. Previous work in our laboratory showed that the *srfAA* mutation in the surfactin operon influences negatively mycosubtilin production by *B. subtilis* ATCC 6633 [Béchet *et al.*, 2013]. The same result was obtained in our previous study in which disruption of the *srfAA* gene resulted in a significant decrease in fengycin production by *B. subtilis* ATCC 21332 (data not published). On the other hand, the inactivation of fengycin operon was shown to influence surfactin production positively. Coutte *et al.* [2010] demonstrated that this disruption clearly increases the surfactin yield in *B. subtilis* 168 *sfp*+. A similar result was obtained with *B. subtilis* BBG21 in our previous work by disrupting the fengycin operon, together with the *gfp* marker to observe the fengycin promoter activity. Under these conditions, surfactin production increased about 30% comparing to the wild-type strain [Yaseen *et al.*, 2016]. Luo *et al.* [2015] also mentioned that the block of fengycin operon increased the surfactin production about 20% more than the wild-type strain. Whatever the interaction between the lipopeptides regulation which can be direct or indirect influence on the expression of the genes implied in synthesis of these lipopeptides, this relation still remains unclear and needs more study.

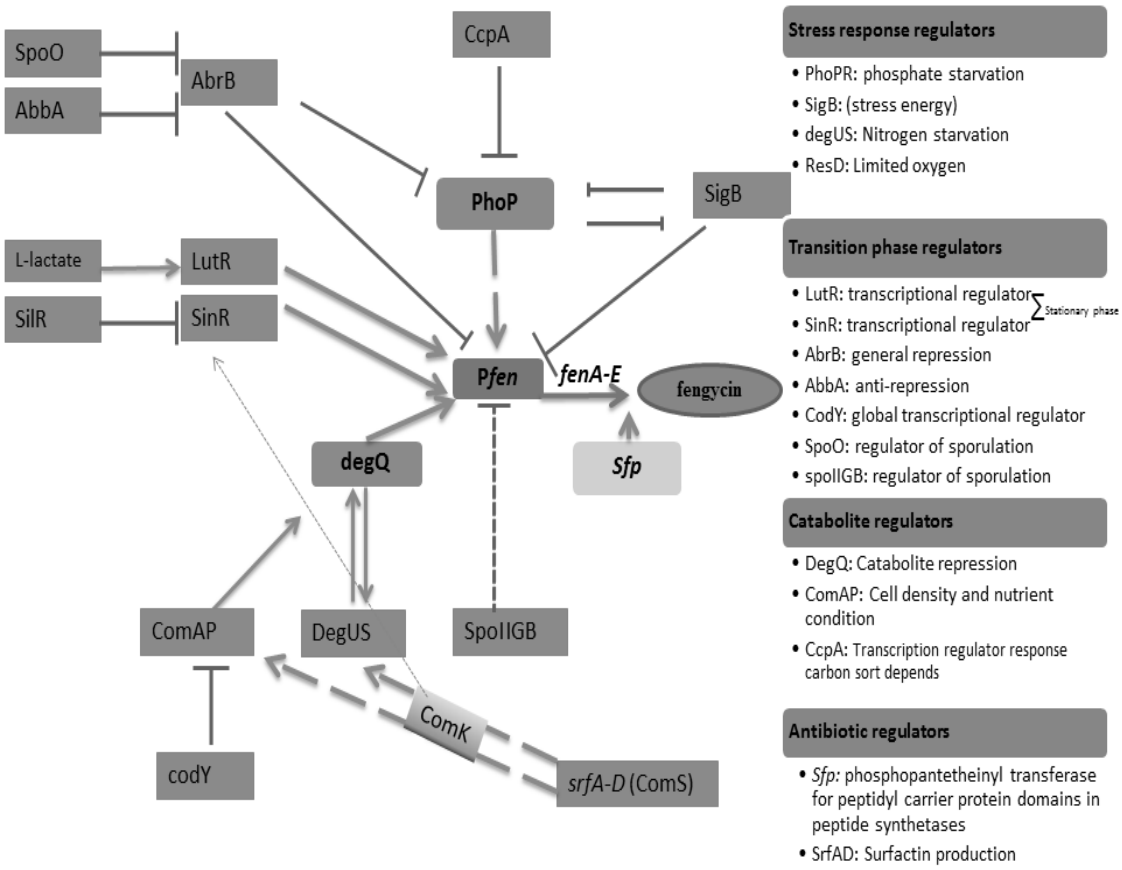

Figure 8. The model of gene regulation involved in fengycin biosynthesis. Closed-head arrows indicate positive regulation whereas line-head arrows indicate negative regulation.

Conclusion

Depending on the culture conditions, many regulators could interact with fengycin production. Several external factors lead to activation of genes which may affect fengycin operon expression either negatively or positively. However, in most cases, their role was been clearly determined. It was found that factors influencing fengycin synthesis depend on the availability and nature of nutrient sources, as well as environmental conditions. Moreover, the interaction in the same strain between lipopeptides belonging to different families could influence the quantity of lipopeptides produced directly or indirectly. Fengycins with all their characteristics could be used as biological pesticide, for oil recovery agent, as non-toxic antibiotic or antitumor agent. Using the industrial residues may be a good solution for reducing the cost of production of this lipopeptide.

Part 4: References

Aislabie, J., Deslippe, J., & Dymond, J. (2013). Soil microbes and their contribution to soil services. *Ecosystem services in New Zealand: conditions and trends*, *143161*.

Aiyar, S. E., Gourse, R. L., & Ross, W. (1998). Upstream A-tracts increase bacterial promoter activity through interactions with the RNA polymerase α subunit. *Proceedings of the National Academy of Sciences*, *95*(25), 14652-14657.

Allenby, N. E., O'Connor, N., Prágai, Z., Ward, A. C., Wipat, A., & Harwood, C. R. (2005). Genome-wide transcriptional analysis of the phosphate starvation stimulon of *Bacillus subtilis*. *Journal of Bacteriology*, *187*(23), 8063-8080.

Antelmann, H., Scharf, C., & Hecker, M. (2000). Phosphate starvation-inducible proteins of *Bacillus subtilis*: proteomics and transcriptional analysis. *Journal of bacteriology*, *182*(16), 4478-4490.

Arrebola, E., Jacobs, R., & Korsten, L. (2010). Iturin A is the principal inhibitor in the biocontrol activity of *Bacillus amyloliquefaciens* PPCB004 against postharvest fungal pathogens. *Journal of applied microbiology*, *108*(2), 386-395.

Asari, S. Y. (2015). Studies on plant-microbe interaction to improve stress tolerance in plants for sustainable agriculture.

Banat, M., Franzetti, A., Gandolfi, I., Bestetti, G., Martinotti, G., Fracchia, L., & Marchant, R. (2010). Microbial biosurfactants production, applications and future potential. *Applied Microbiology and Biotechnology*, *87*(2), 427-444.

Banat, M., Makkar, S., & Cameotra, S. (2000). Potential commercial applications of microbial surfactants. *Applied microbiology and biotechnology*, *53*(5), 495-508.

Béchet, M., Castéra-Guy, J., Guez, J. S., Chihib, N. E., Coucheney, F., Coutte, F., ... & Jacques, P. (2013). Production of a novel mixture of mycosubtilins by mutants of *Bacillus subtilis*. *Bioresource technology*, *145*, 264-270.

Berka, M., Hahn, J., Albano, M., Draskovic, I., Persuh, M., Cui, X. & Dubnau, D. (2002). Microarray analysis of the *Bacillus subtilis* K-state: genome-wide expression changes dependent on ComK. *Molecular microbiology*, *43*(5), 1331-1345.

Besson, F., Chevanet, C., & Michel, G. (1987). Influence of the culture medium on the production of iturin A by *Bacillus subtilis*. *Journal of general microbiology*, *133*(3), 767-772.

Caldwell, R., Sapolsky, R., Weyler, W., Maile, R. R., Causey, C., & Ferrari, E. (2001).

Correlation between *Bacillus subtilis* scoC phenotype and gene expression determined using microarrays for transcriptome analysis. *Journal of bacteriology*, *183*(24), 7329-7340.

Cameotra, S. S., Makkar, R. S., Kaur, J., & Mehta, S. K. (2010). Synthesis of biosurfactants and their advantages to microorganisms and mankind. In *Biosurfactants* (pp. 261-280). Springer New York.

Caramori, T., & Galizzi, A. (1998). The UP element of the promoter for the flagellin gene, hag, stimulates transcription from both SigD-and SigA-dependent promoters in *Bacillus subtilis*. *Molecular and General Genetics MGG*, *258*(4), 385-388.

Cawoy, H., Debois, D., Franzil, L., De Pauw, E., Thonart, P., & Ongena, M. (2015). Lipopeptides as main ingredients for inhibition of fungal phytopathogens by *Bacillus subtilis/amyloliquefaciens*. *Microbial biotechnology*, *8*(2), 281-295.

Chai, Y., Kolter, R., & Losick, R. (2009). A widely conserved gene cluster required for lactate utilization in *Bacillus subtilis* and its involvement in biofilm formation. Journal of bacteriology, 191(8), 2423-2430.

Chen, H., Koumoutsi, A., Scholz, R., Eisenreich, A., Schneider, K., Heinemeyer, I & Junge, H. (2007). Comparative analysis of the complete genome sequence of the plant growth–promoting bacterium *Bacillus amyloliquefaciens* FZB42. *Nature biotechnology*, *25*(9), 1007-1014.

Chen, H., Vater, J., Piel, J., Franke, P., Scholz, R., Schneider, K., ... & Gottschalk, G. (2006). Structural and functional characterization of three polyketide synthase gene clusters in *Bacillus amyloliquefaciens* FZB 42. *Journal of bacteriology*, *188*(11), 4024-4036.

Cheng, F. Y., Yang, H., & Chen, Y. (2013). Characterization of a blend-biosurfactant of glycolipid and lipopeptide produced by *Bacillus subtilis* TU2 isolated from underground oil-extraction wastewater. *Journal of microbiology and biotechnology*, *23*(3), 390-396.

Chollet-Imbert, M., Gancel, F., Slomianny, C., & Jacques, P. (2009). Differentiated pellicle organization and lipopeptide production in standing culture of *Bacillus subtilis* strains. *Archives of microbiology*, *191*(1), 63-71.

Chowdhury, S. P., Uhl, J., Grosch, R., Alquéres, S., Pittroff, S., Dietel, K. & Hartmann, A. (2015). Cyclic Lipopeptides of *Bacillus amyloliquefaciens* subsp. plantarum colonizing the Lettuce Rhizosphere Enhance Plant Defense Responses toward the Bottom Rot Pathogen Rhizoctonia solani. *Molecular Plant-Microbe Interactions*.

Chtioui, O., Krasimir, D., Gancel, F., Nikov, I., & Dhulster, P. (2010). Biosurfactants production in biofilm reactor and their recovery by pertraction [abstract]. *Biotechnologie, Agronomie, Société et Environnement*, *14*(s2), 583-583.

Chtioui, O., Dimitrov, K., Gancel, F., Dhulster, P., & Nikov, I. (2014). Selective fengycin production in a modified rotating discs bioreactor. *Bioprocess and biosystems engineering*, *37*(2), 107-114.

Chumsakul, O., Nakamura, K., Kurata, T., Sakamoto, T., Hobman, L., Ogasawara, N., & Ishikawa, S. (2013). High-resolution mapping of in vivo genomic transcription factor binding sites using in situ DNase I footprinting and ChIP-seq. *DNA research*, dst013.

Chumsakul, O., Takahashi, H., Oshima, T., Hishimoto, T., Kanaya, S., Ogasawara, N., & Ishikawa, S. (2010). Genome-wide binding profiles of the *Bacillus subtilis* transition state regulator AbrB and its homolog Abh reveals their interactive role in transcriptional regulation. *Nucleic acids research*, gkq780.

Gordillo, A., Navarro, R., Benitez, M., De Plaza, T., & Maldonado, C. (2009). Preliminary study and improve the production of metabolites with antifungal activity by a Bacillus sp strain IBA 33. *Microbiology Insights*, *2*, 15.

Gordillo, A., & Maldonado, M. C. (2012). Purification of peptides from Bacillus strains with biological activity. *Chromatography and Its Applications*, *11*, 201-225.

Coutte, F., Leclère, V., Béchet, M., Guez, J. S., Lecouturier, D., Chollet-Imbert, M., ... & Jacques, P. (2010). 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*, *109*(2), 480-491.

Coutte, F., Lecouturier, D., Yahia, S. A., Leclère, V., Béchet, M., Jacques, P., & Dhulster, P. (2010b). Production of surfactin and fengycin by *Bacillus subtilis* in a bubbleless membrane bioreactor. *Applied microbiology and biotechnology*, *87*(2), 499-507.

de Faria, A. F., Stéfani, D., Vaz, B. G., Silva, Í. S., Garcia, J. S., Eberlin, M. N., ... & Durrant, L. R. (2011). Purification and structural characterization of fengycin homologues produced by *Bacillus subtilis* LSFM-05 grown on raw glycerol. *Journal of industrial microbiology & biotechnology*, *38*(7), 863-871.

de Oliveira, F., França, L., Félix, N., Martins, L., Giro, M. E. A., Melo, M., & Gonçalves, B. (2013). Kinetic study of biosurfactant production by *Bacillus subtilis* LAMI005 grown in clarified cashew apple juice. *Colloids and Surfaces B: Biointerfaces*, *101*, 34-43.

Deleu, M., Paquot, M., & Nylander, T. (2008). Effect of fengycin, a lipopeptide produced by

Bacillus subtilis, on model biomembranes. *Biophysical journal*, *94*(7), 2667-2679.

Deng, X., Tian, Y., Niu, Q., Xu, X. E., Shi, H., Zhang, H., ... & Huang, X. (2013). The ComP-ComA Quorum System Is Essential For "Trojan horse" Like Pathogenesis in *Bacillus nematocida*. *PloS one*, *8*(10), e76920.

Desoignies, N., Schramme, F., Ongena, M., & Legrève, A. (2013). Systemic resistance induced by Bacillus lipopeptides in Beta vulgaris reduces infection by the rhizomania disease vector Polymyxa betae. *Molecular plant pathology*, *14*(4), 416-421.

Ditmer, M. (2014). Evaluation of the anti-proliverative effect of thelipopeptides iturin A and fengycin of *Bacillus* spp. and the viral fusionprotein PTD4-VP3 on human cancer and normal cells.

Do, H., Suzuki, Y., Abe, N., Kaneko, J., Itoh, Y., & Kimura, K. (2011). Mutations suppressing the loss of DegQ function in *Bacillus subtilis* (natto) poly-γ-glutamate synthesis. *Applied and environmental microbiology*, *77*(23), 8249-8258.

Dong W., Li S., Lu X., Zhang X., Wang P., Ma P., Guo Q. (2014). Regulation of fengycin biosynthase by regulator PhoP in the *Bacillus subtilis* strain NCD-2, Acta Phytopathologica Sinica *44*(2), 180-187.

Duckworth, P., & Aldrich, C. (2010). Development of a high-throughput fluorescence polarization assay for the discovery of phosphopantetheinyl transferase inhibitors. *Analytical biochemistry*, *403*(1), 13-19.

Duitman EH, Hamoen LW, Rembold M, Venema G, Seitz H, Saenger W, Bernhard F, Reinhardt R, Schmidt M, Ullrich C, Stein T, Leenders F, Vater J (1999) The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: a multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase. Proc Natl Acad Sci USA 96:13294–1329.

Eeman, M., Francius, G., Dufrêne, Y. F., Nott, K., Paquot, M., & Deleu, M. (2009). Effect of cholesterol and fatty acids on the molecular interactions of fengycin with stratum corneum mimicking lipid monolayers. *Langmuir*, *25*(5), 3029-3039.

Estrem, T., Ross, W., Gaal, T., Chen, S., Niu, W., Ebright, H., & Gourse, L. (1999). Bacterial promoter architecture: subsite structure of UP elements and interactions with the carboxy-terminal domain of the RNA polymerase α subunit. *Genes & development*, *13*(16), 2134-2147.

Fahim, S., Dimitrov, K., Gancel, F., Vauchel, P., Jacques, P., & Nikov, I. (2012). Impact of energy supply and oxygen transfer on selective lipopeptide production by *Bacillus*

subtilis BBG21. *Bioresource technology*, *126*, 1-6.

Farace, G., Fernandez, O., Jacquens, L., Coutte, F., Krier, F., Jacques, P., ... & Dorey, S. (2015). Cyclic lipopeptides from *Bacillus subtilis* activate distinct patterns of defence responses in grapevine. *Molecular plant pathology*, *16*(2), 177-187.

Finking, R., & Marahiel, A. (2004). Biosynthesis of nonribosomal peptides 1. *Annu. Rev. Microbiol.*, *58*, 453-488.

Fonseca, R., Silva, R., De França, P., Cardoso, L., & Sérvulo, C. (2007). Optimizing carbon/nitrogen ratio for biosurfactant production by a *Bacillus subtilis* strain. *Applied biochemistry and biotechnology*, *1Luo et al. 2015*(1-12), 471-486.

Gabdrakhmanova, L., Vishniakov, I., Sharipova, M., Balaban, N., Kostrov, S., & Leshchinskaya, I. (2005). Salt stress induction of glutamyl endopeptidase biosynthesis in *Bacillus intermedius*. *Microbiological research*, *160*(3), 233-242.

Galli, G., Rodriguez, F., Cosmina, P., Pratesi, C., Nogarotto, R., de Ferra, F., & Grandi, G. (1994). Characterization of the surfactin synthetase multi-enzyme complex. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, *1205*(1), 19-28.

Gancel, F., Montastruc, L., Liu, T., Zhao, L., & Nikov, I. (2009). Lipopeptide overproduction by cell immobilization on iron-enriched light polymer particles. *Process Biochemistry*, *44*(9), 975-978.

Ghribi, D., Abdelkefi-Mesrati, L., Mnif, I., Kammoun, R., Ayadi, I., Saadaoui, I., & Chaabouni-Ellouze, S. (2012). Investigation of antimicrobial activity and statistical optimization of *Bacillus subtilis* SPB1 biosurfactant production in solid-state fermentation. *BioMed Research International*, *2012*.

Gordillo, A., & Maldonado, C. (2012). Purification of peptides from *Bacillus* strains with biological activity. *Chromatography and its Applications*, *11*, 201-225.

Gudiña, J., Fernandes, C., Rodrigues, I., Teixeira, A., & Rodrigues, R. (2015). Biosurfactant production by *Bacillus subtilis* using corn steep liquor as culture medium. *Frontiers in microbiology*, *6*.

Guo, Q., Dong, W., Li, S., Lu, X., Wang, P., Zhang, X., & Ma, P. (2014a). Fengycin produced by *Bacillus subtilis* NCD-2 plays a major role in biocontrol of cotton seedling damping-off disease. *Microbiological research*, *169*(7), 533-540.

Guo, Q., Li, S., Lu, X., Li, B., & Ma, P. (2010). PhoR/PhoP two component regulatory system affects biocontrol capability of *Bacillus subtilis* NCD-2. *Genetics and molecular*

biology, *33*(2), 333-340.

Guo, Q., Li, S., Lu, X., Zhang, X., Wang, P., & Ma, P. (2014b). Complete genome sequence of *Bacillus subtilis* BAB-1, a biocontrol agent for suppression of tomato gray mold. *Genome announcements*, *2*(4), e00744-14.

Hamoen, L. W., Eshuis, H., Jongbloed, J., Venema, G., & Sinderen, D. (1995). A small gene, designated comS, located within the coding region of the fourth amino acid-activation domain of srfA, is required for competence development in *Bacillus subtilis*. *Molecular microbiology*, *15*(1), 55-63.

Hathout, Y., Ho, P., Ryzhov, V., Demirev, P., & Fenselau, C. (2000). Kurstakins: A New Class of Lipopeptides Isolated from *Bacillus thuringiensis*. *Journal of natural products*, *63*(11), 1492-1496.

Honma, M., Tanaka, K., Konno, K., Tsuge, K., Okuno, T., & Hashimoto, M. (2012). Termination of the structural confusion between plipastatin A1 and fengycin IX. *Bioorganic & medicinal chemistry*, *20*(12), Luo et al. 201593-Luo et al. 201598.

Horn, J. N., Cravens, A., & Grossfield, A. (2013). Interactions between fengycin and model bilayers quantified by coarse-grained molecular dynamics. *Biophysical journal*, *105*(7), 1612-1623.

Hu, B., Zhang, T., Yang, M., Zhou, W., & Shi, Z. Q. (2009). Inhibition of fengycins on the production of fumonisin B1 from *Fusarium verticillioides*. *Letters in applied microbiology*, *48*(1), 84-89.

Huang, X., Lu, Z., Bie, X., Lü, F., Zhao, H., & Yang, S. (2007). Optimization of inactivation of endospores of *Bacillus cereus* by antimicrobial lipopeptides from *Bacillus subtilis* fmbj strains using a response surface method. *Applied microbiology and biotechnology*, *74*(2), 454-461.

Huang, X., Lu, Z., Zhao, H., Bie, X., Lü, F., & Yang, S. (2006). Antiviral activity of antimicrobial lipopeptide from *Bacillus subtilis* fmbj against pseudorabies virus, porcine parvovirus, newcastle disease virus and infectious bursal disease virus in vitro. *International Journal of Peptide Research and Therapeutics*, *12*(4), Luo et al. 20153-Luo et al. 20157.

Hulett, F. M., Lee, J., Shi, L., Sun, G., Chesnut, R., Sharkova, E., & Kapp, N. (1994). Sequential action of two-component genetic switches regulates the PHO regulon in *Bacillus subtilis*. *Journal of bacteriology*, *176*(5), 1348-1358.

İrigül-Sönmez, Ö., Köroğlu, E., Öztürk, B., Kovács, Á. T., Kuipers, O. P., & Yazgan-

Karataş, A. (2014). In *Bacillus subtilis* LutR is part of the global complex regulatory network governing the adaptation to the transition from exponential growth to stationary phase. *Microbiology*, *160*(Pt 2), 243-260.

Islam, M., Jeong,, T., Lee, S., & Song, H. (2012). Isolation and identification of antifungal compounds from *Bacillus subtilis* C9 inhibiting the growth of plant pathogenic fungi. *Mycobiology*, *40*(1), 59-66.

Jacques P., Hbid C., Destain J., Razafindralambo H., Paquot M., De Pauw E., *et al*.(1999). Optimization of biosurfactant lipopeptide production from *Bacillus subtilis* S499 by Plackett-Burman design. *Appl Biochem Biotechnol*, 77-79:223–33.

Jacques, P. (2011). Surfactin and other lipopeptides from *Bacillus* spp. In *Biosurfactants* (pp. 57-91). Springer Berlin Heidelberg.

Jakobs, M., Hoffmann, K., Grabke, A., Neuber, S., Liesegang, H., Volland, S., & Meinhardt, F. (2014). Unravelling the genetic basis for competence development of auxotrophic *Bacillus licheniformis* 9945A strains. *Microbiology*, *160*(Pt 10), 2136-2147.

Karatas, A. Y., Çetin, S., & Özcengiz, G. (2003). The effects of insertional mutations in comQ, comP, srfA, spo0H, spo0A and abrB genes on bacilysin biosynthesis in *Bacillus subtilis*. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*, *1626*(1), 51-56.

Kaushal, B., Paul, S., & Hulett, F. M. (2010). Direct regulation of *Bacillus subtilis* phoPR transcription by transition state regulator ScoC. *Journal of bacteriology*, *192*(12), 3103-3113.

Ke J., Chang Y., Lin P., Liu T. (2009). Activation of the promoter of the fengycin synthetase operon by the UP element. J Bacteriol 191:4615–23.

Kim, S., Kim, B., Park, H., Oh, M., Park, I., Kim, K., ... & Yoon, D. (2000). Expression of sfp gene and hydrocarbon degradation by *Bacillus subtilis*. *Biotechnology letters*, *22*(18), 1431-1436.

Kim, H., & Chae, J. (2015). Review: Postharvest Biological Control of *Colletotrichum acutatum* on Apple by *Bacillus subtilis* HM1 and the Structural Identification of Antagonists. *Journal of microbiology and biotechnology*, *25*(11), 1954-1959.

Kim, I., Ryu, J., Kim, H., & ChI, T. (2010). Production of biosurfactant lipopeptides Iturin A, fengycin and surfactin A from *Bacillus subtilis* CMB32 for control of Colletotrichum gloeosporioides. *J Microbiol Biotechnol*, *20*(1), 138-145.

Kim, B., Shin, S., Choi, K., Kim, K., & Park, H. (2001). Involvement of acetyl phosphate in

the in vivo activation of the response regulator ComA in *Bacillus subtilis*. *FEMS microbiology letters*, *195*(2), 179-183.

Kobir, A., Poncet, S., Bidnenko, V., Delumeau, O., Jers, C., Zouhir, S., ... & Mijakovic, I. (2014). Phosphorylation of *Bacillus subtilis* gene regulator AbrB modulates its DNA-binding properties. *Molecular microbiology*, *92*(5), 1129-1141.

Köroğlu, E., Kurt-Gür, G., Ünlü, C., & Yazgan-Karataş, A. (2008). The novel gene yvfI in *Bacillus subtilis* is essential for bacilysin biosynthesis. *Antonie van Leeuwenhoek*, *94*(3), 471-479.

Koumoutsi A, Chen X, Henne A, Liesegang H, Hitzeroth G, Franke P, Vater J, Borriss R. (2004), Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. J Bacteriol 186:1084–1096.

Koumoutsi, A., Chen, H., Vater, J., & Borriss, R. (2007). DegU and YczE positively regulate the synthesis of bacillomycin D by *Bacillus amyloliquefaciens* strain FZB42. *Applied and environmental microbiology*, *73*(21), 6953-6964.

Kumar, P., Khare, S., & Dubey, R. C. (2012). Diversity of bacilli from disease suppressive soil and their role in plant growth promotion and yield enhancement. *New York Sci J*, *5*(1), 90-111.

Leães, L., Velho, V., Caldas, G., Ritter, C., Tsai, M., & Brandelli, A. (2015). Expression of essential genes for biosynthesis of antimicrobial peptides of Bacillus is modulated by inactivated cells of target microorganisms. *Research in microbiology*.

Leclère, V., Béchet, M., Adam, A., Guez, J. S., Wathelet, B., Ongena, M., ... & Jacques, P. (2005). Mycosubtilin overproduction by *Bacillus subtilis* BBG100 enhances the organism's antagonistic and biocontrol activities. *Applied and environmental microbiology*, *71*(8), 4577-4584.

Lee, K., Yoon, D., Yoon, H., Lee, G., Song, J., Kim, G., ... & Kim, S. (2007). Cloning of srfA operon from *Bacillus subtilis* C9 and its expression in E. coli. *Applied microbiology and biotechnology*, *75*(3), 567-572.

Lin, P., Chen, L., Chang, K., Tschen, M., & Liu, T. (1999). Functional and transcriptional analyses of a fengycin synthetase gene, fenC, from *Bacillus subtilis*. *Journal of bacteriology*, *181*(16), 5060-5067.

Liu, F., Mbadinga, M., Yang, Z., Gu, D., & Mu, Z. (2015). Chemical Structure, Property and Potential Applications of Biosurfactants Produced by *Bacillus subtilis* in Petroleum Recovery and Spill Mitigation. *International journal of molecular sciences*, *16*(3), 4814-48Luo et al. 2015.

Logan, A., Popovic, T., Hoffmaster, A., Murray, R., Baron, J., Jorgensen, H., & Pfaller, A. (2006). *Bacillus* and other aerobic endospore-forming bacteria. *Manual of clinical microbiology Volume 1*, (Ed. 9), 455-473.

López, D., & Kolter, R. (2010). Extracellular signals that define distinct and coexisting cell fates in *Bacillus subtilis*. *FEMS microbiology reviews*, *34*(2), 134-149.

Louw, E., Reid, J., James, M., & Watson, G. (1994). Cloning and sequencing the degS-degU operon from an alkalophilic *Bacillus brevis*. *Applied microbiology and biotechnology*, *42*(1), 78-84.

Luo, C., Liu, X., Zhou, H., Wang, X., & Chen, Z. (2015). Nonribosomal Peptide Synthase Gene Clusters for Lipopeptide Biosynthesis in *Bacillus subtilis* 916 and Their Phenotypic Functions. *Applied and environmental microbiology*, *81*(1), 422-431.

Mandal, M., Barbosa, E., & Franco, L. (2013). Lipopeptides in microbial infection control: scope and reality for industry. *Biotechnology advances*, *31*(2), 338-345.

Martín, F. (2004). Phosphate control of the biosynthesis of antibiotics and other secondary metabolites is mediated by the PhoR-PhoP system: an unfinished story. *Journal of bacteriology*, *186*(16), 5197-5201.

Meena, R., & Kanwar, S. (2015). Lipopeptides as the Antifungal and Antibacterial Agents: Applications in Food Safety and Therapeutics. *BioMed research international*, *2015*.

Meijer, W. J., & Salas, M. (2004). Relevance of UP elements for three strong *Bacillus subtilis* phage ϕ29 promoters. *Nucleic acids research*, *32*(3), 1166-1176.

Mnif, I., Chaabouni-Ellouze, S., & Ghribi, D. (2012). Optimization of the nutritional parameters for enhanced production of *B. subtilis* SPB1 biosurfactant in submerged culture using response surface methodology. *Biotechnology research international*, *2012*.

Mnif, I., & Ghribi, D., (2015). Lipopeptide surfactants: Production, recovery and pore forming capacity. *Peptides*, *71*, 100-112.

Molle, V., Nakaura, Y., Shivers, R. P., Yamaguchi, H., Losick, R., Fujita, Y., & Sonenshein, A. L. (2003). Additional targets of the *Bacillus subtilis* global regulator CodY identified by chromatin immunoprecipitation and genome-wide transcript analysis. *Journal of bacteriology*, *185*(6), 1911-1922.

Mootz, D., & Marahiel, A. (1997). Biosynthetic systems for nonribosomal peptide antibiotic assembly. *Current opinion in chemical biology*, *1*(4), 543-551.

Moryl, M., Spętana, M., Dziubek, K., Paraszkiewicz, K., Różalska, S., Płaza, G. A., & Różalski, A. (2014). Antimicrobial, antiadhesive and antibiofilm potential of lipopeptides synthesised by *Bacillus subtilis*, on uropathogenic bacteria. *Acta biochimica Polonica*, *62*(4), 725-732.

Movahedi, S., & Waites, W. (2002). Cold shock response in sporulating Bacillus subtilis and its effect on spore heat resistance. *Journal of bacteriology*, *184*(19), 5275-5281.

Msadek, T., Kunst, F., Klier, A. & Rapoport, G. (1991). DegS-DegU and ComP-ComA modulator-effector pairs control expression of the *Bacillus subtilis* pleiotropic regulatory gene degQ. *Journal of bacteriology*, *173*(7), 2366-2Luo et al. 20157.

Msadek, T., Kunst, F., Henner, D., Klier, A., Rapoport, G., & Dedonder, R. (1990). Signal transduction pathway controlling synthesis of a class of degradative enzymes in *Bacillus subtilis*: expression of the regulatory genes and analysis of mutations in degS and degU. *Journal of bacteriology*, *172*(2), 824-834.

Mukherjee, K., & Das, K. (2005). Correlation between diverse cyclic lipopeptides production and regulation of growth and substrate utilization by *Bacillus subtilis* strains in a particular habitat. *FEMS microbiology ecology*, *54*(3), 479-489.

Nagórska, K., Hinc, K., Strauch, M. A., & Obuchowski, M. (2008). Influence of the óB stress factor and yxaB, the gene for a putative exopolysaccharide synthase under óB control, on biofilm formation. *Journal of bacteriology*, *190*(10), 3546-3556.

Nakano MM, Zuber P (1991), The primary role of ComA in stablishment of the competent state in *Bacillus subtilis* is to activate expression of srfA. J Bacteriol 173:7269-7274.

Nakano MM, Zuber P (1998), Anaerobic growth of a strict aerobe (*Bacillus subtilis*). *Ann Rev Microbiol*, 52:165-190.

Nakano, M., Corbell, N., Besson, J., & Zuber, P. (1992). Isolation and characterization of sfp: a gene that functions in the production of the lipopeptide biosurfactant, surfactin, in *Bacillus subtilis*. *Molecular and General Genetics MGG*, *232*(2), 313-321.

Nicoli, S., Eeman, M., Deleu, M., Bresciani, E., Padula, C., & Santi, P. (2010). Effect of lipopeptides and iontophoresis on acyclovir skin delivery. *Journal of Pharmacy and Pharmacology*, *62*(6), 702-708.

Nigam, S. (2013). Microbial enzymes with special characteristics for biotechnological applications. *Biomolecules*, *3*(3), 597-611.

Nishikiori T, Naganawa H, Muraoka Y, Aoyagi T, Umezawa H (1986) Plipastatins: new inhibitors of phospholipase A2, produced by *Bacillus cereus* BMG302-fF67. III. Structural elucidation of plipastatins. J Antibiot 39:755–761

O'Connor, K., Hudson, S., Cobb, L., O'Neil, D., Robertson, J., Duncan, V., & Murphy, D. (2014). Novel fluorinated lipopeptides from Bacillus sp. CS93 via precursor-directed biosynthesis. *Amino acids*, *46*(12), 2745-2752.

Ogura, M., & Tanaka, T. (1997). Bacillus subtilis ComK negatively regulates degR gene expression. *Molecular and General Genetics MGG*, *254*(2), 157-165.

Ogura, M., & Tsukahara, K. (2010). Autoregulation of the *Bacillus subtilis* response regulator gene degU is coupled with the proteolysis of DegU-P by ClpCP. *Molecular microbiology*, *75*(5), 1244-1259.

Ogura, M., Liu, L., LaCelle, M., Nakano, M., & Zuber, P. (1999). Mutational analysis of ComS: evidence for the interaction of ComS and MecA in the regulation of competence development in *Bacillus subtilis*. *Molecular microbiology*, *32*(4), 799-812.

Ogura, M., Yamaguchi, H., Kobayashi, K., Ogasawara, N., Fujita, Y., & Tanaka, T. (2002). Whole-genome analysis of genes regulated by the *Bacillus subtilis* competence transcription factor ComK. *Journal of bacteriology*, *184*(9), 2344-2351.

Ongena M, Jourdan E, Adam A, Paquot M, Brans A, Joris B, Arpigny JL, Thonart P (2007) Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. *Environm Microbiol* 9:1084-1090.

Pathak, V., & Keharia, H. (2013). Characterization of fungal antagonistic bacilli isolated from aerial roots of banyan (*Ficus benghalensis*) using intact-cell MALDI-TOF mass spectrometry (ICMS). *Journal of applied microbiology*, *114*(5), 1300-1310.

Paul, S., Birkey, S., Liu, W., & Hulett, F. M. (2004). Autoinduction of *Bacillus subtilis* phoPR operon transcription results from enhanced transcription from EσA-and EσE-responsive promoters by phosphorylated PhoP. *Journal of bacteriology*, *186*(13), 4262-4275.

Pecci, Y., Rivardo, F., Martinotti, M. G., & Allegrone, G. (2010). LC/ESI-MS/MS characterisation of lipopeptide biosurfactants produced by the *Bacillus licheniformis* V9T14 strain. *Journal of mass spectrometry*, *45*(7), 772-778.

Perfumo, A., Rancich, I., & Banat, I. M. (2010). Possibilities and challenges for biosurfactants use in petroleum industry. In *Biosurfactants* (pp. 135-145). Springer New York.

Phan, P., Nguyen, D., & Schumann, W. (2012). Development of a strong intracellular expression system for *Bacillus subtilis* by optimizing promoter elements. *Journal of biotechnology*, *157*(1), 167-172.

Płaza, G., Chojniak, J., Rudnicka, K., Paraszkiewicz, K., & Bernat, P. (2015). Detection of biosurfactants in *Bacillus* species: genes and products identification. *Journal of applied microbiology*, *119*(4), 1023-1034.

Pryor, W., Gibson, M., Hay, G., Gossett, M., & Walker, P. (2007). Optimization of spore and antifungal lipopeptide production during the solid-state fermentation of *Bacillus subtilis*. *Applied biochemistry and biotechnology*, *143*(1), 63-79.

Puri-Taneja, A., Paul, S., Chen, Y., & Hulett, M. (2006). CcpA causes repression of the phoPR promoter through a novel transcription start site, PA6. *Journal of bacteriology*, *188*(4), 1266-1278.

Qian, Q., Lee, Y., Helmann, D., & Strauch, A. (2002). AbrB is a regulator of the σW regulon in *Bacillus subtilis*. *FEMS microbiology letters*, *211*(2), 219-223.

Quadri, E., Weinreb, H., Lei, M., Nakano, M., Zuber, P., & Walsh, T. (1998). Characterization of Sfp, a *Bacillus subtilis* phosphopantetheinyl transferase for peptidyl carrier protein domains in peptide synthetases. *Biochemistry, Luo et al. 2015*(6), 1585-1595.

Raaijmakers, M., De Bruijn, I., Nybroe, O., & Ongena, M. (2010). Natural functions of lipopeptides from Bacillus and Pseudomonas: more than surfactants and antibiotics. *FEMS microbiology reviews*, *34*(6), 10Luo et al. 2015-1062.

Raza, W., Hussain, Q., & Shen, Q. (2012). Production, Regulation and Transportation of Bacillibactin in *Bacillus Subtilis*. *Journal of the Chemical Society of Pakistan*, *34*(4), 996-1002.

Ren, D., Bedzyk, A., Setlow, P., Thomas, M., Ye, W., & Wood, K. (2004). Gene expression in *Bacillus subtilis* surface biofilms with and without sporulation and the importance of yveR for biofilm maintenance. *Biotechnology and bioengineering*, *86*(3), 344-364.

Rodrigues, L., Banat, I. M., Teixeira, J., & Oliveira, R. (2006). Biosurfactants: potential applications in medicine. *Journal of Antimicrobial Chemotherapy*, *57*(4), 609-618.

Roongsawang, N., Thaniyavarn, J., Thaniyavarn, S., Kameyama, T., Haruki, M., Imanaka, T., & Kanaya, S. (2002). Isolation and characterization of a halotolerant *Bacillus subtilis*

BBK-1 which produces three kinds of lipopeptides: bacillomycin L, plipastatin, and surfactin. *Extremophiles*, *6*(6), 499-506.

Roongsawang, N., Washio, K., & Morikawa, M. (2010). Diversity of nonribosomal peptide synthetases involved in the biosynthesis of lipopeptide biosurfactants. *International journal of molecular sciences*, *12*(1), 141-172.

Roy, A., Mahata, D., Paul, D., Korpole, S., Franco, O. L., & Mandal, S. M. (2013). Purification, biochemical characterization and self-assembled structure of a fengycin-like antifungal peptide from *Bacillus thuringiensis* strain SM1. *Frontiers in microbiology*, *4*.

Sajitha, L., & Dev, A. (2016). Quantification of antifungal lipopeptide gene expression levels in *Bacillus subtilis* B1 during antagonism against sapstain fungus on rubberwood. *Biological Control*, *96*, 78-85.

Salzberg, I., Botella, E., Hokamp, K., Antelmann, H., Maaß, S., Becher, D., & Devine, M. (2015). Genome-Wide Analysis of Phosphorylated PhoP Binding to Chromosomal DNA Reveals Several Novel Features of the PhoPR-Mediated Phosphate Limitation Response in *Bacillus subtilis*. *Journal of bacteriology*, *197*(8), 1492-1506.

Samel, S. A., Wagner, B., Marahiel, M. A., & Essen, L. O. (2006). The thioesterase domain of the fengycin biosynthesis cluster: a structural base for the macrocyclization of a non-ribosomal lipopeptide. *Journal of molecular biology*, *359*(4), 876-889.

Sang-Cheol, L., Kim, H., Park, H., Chung, Y., Chandra, S., & Yong-Lark, C. (2010). Isolation, purification, and characterization of novel fengycin S from *Bacillus amyloliquefaciens* LSC04 degrading-crude oil. *Biotechnology and Bioprocess Engineering*, *15*(2), 246-253.

Setlow, P. (2006). Spores of *Bacillus subtilis*: their resistance to and killing by radiation, heat and chemicals. *Journal of applied microbiology*, *101*(3), 514-525.

Shivers, P., Dineen, S., & Sonenshein, L. (2006). Positive regulation of *Bacillus subtilis* ackA by CodY and CcpA: establishing a potential hierarchy in carbon flow. *Molecular microbiology*, *62*(3), 811-822.

Sicuia O.A., Constantinescu F., Dinu S., Cornea C.P., 2015. Biodiversity of *Bacillus subtilis* group and beneficial traits of Bacillus species useful in plant protection. Romanian Biotechnology Letters, 20(5): 107Luo et al. 2015-10750.

Sinchaikul, S., Sookkheo, B., Topanuruk, S., Juan, H. F., Phutrakul, S., & Chen, S. T. (2002). Bioinformatics, functional genomics, and proteomics study of *Bacillus sp. Journal of Chromatography B*, *771*(1), 261-287.

Singh, K., Rautela, R., & Cameotra, S. (2014). Substrate dependent in vitro antifungal activity of Bacillus sp. strain AR2. *Microb Cell Fact*, *13*, 67-77.

Sivapathasekaran, C., Das, P., Mukherjee, S., Saravanakumar, J., Mandal, M., & Sen, R. (2010). Marine bacterium derived lipopeptides: characterization and cytotoxic activity against cancer cell lines. *International Journal of Peptide Research and Therapeutics*, *16*(4), 215-222.

Sousa, D., Dantas, I. T., Felix, N., Sant'Ana, D., Melo, M., & Gonçalves, B. (2014). Crude glycerol from biodiesel industry as substrate for biosurfactant production by *Bacillus subtilis* ATCC 6633. *Brazilian Archives of Biology and Technology*, *57*(2), 295-301.

Stachelhaus T., Mootz HD., Marahiel MA. (1999) The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases. Chem Biol 6:493–505.

Stein, T. (2005). *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. *Molecular microbiology*, *56*(4), 845-857.

Steller, S., Vollenbroich, D., Leenders, F., Stein, T., Conrad, B., Hofemeister, J. & Vater, J. (1999). Structural and functional organization of the fengycin synthetase multienzyme system from *Bacillus subtilis* b213 and A1/3. *Chemistry & biology*, *6*(1), 31-41.

Strauch, A., & Hoch, A. (1993). Transition-state regulators: sentinels of *Bacillus subtilis* post-exponential gene expression. *Molecular microbiology*, *7*(3), 3Luo et al. 2015-342.

Strauch, A., Spiegelman, B., Perego, M., Johnson, C., Burbulys, D., & Hoch, A. (1989). The transition state transcription regulator abrB of Bacillus subtilis is a DNA binding protein. *The EMBO journal*, *8*(5), 1615.

Strieker, M., Tanović, A., & Marahiel, A. (2010). Nonribosomal peptide synthetases: structures and dynamics. *Current opinion in structural biology*, *20*(2), 234-240.

Tanaka, K., Iwasaki, K., Morimoto, T., Matsuse, T., Hasunuma, T., Takenaka, S., & Yoshida, K. I. (2015). Hyperphosphorylation of DegU cancels CcpA-dependent catabolite repression of rocG in *Bacillus subtilis*. *BMC microbiology*, *15*(1), 43.

Tang, Q., Bie, X., Lu, Z., Lv, F., Tao, Y., & Qu, X. (2014). Effects of Fengycin from *Bacillus subtilis* fmbJ on Apoptosis and Necrosis in Rhizopus stolonifer. *Journal of Microbiology*, *52*(8), 675-680.

Tapi, A., Chollet-Imbert, M., Scherens, B., & Jacques, P. (2010). New approach for the detection of non-ribosomal peptide synthetase genes in Bacillus strains by polymerase chain reaction. *Applied microbiology and biotechnology*, *85*(5), 1521-1531.

Tosato V, Albertini AM., Zotti M., Sonda S., Bruschi CV. (1997) Sequence completion, identification and definition of the fengycin operon in *Bacillus subtilis* 168. Microbiology 143:3443–3450

Tsuge K, Ano T, Hirai M, Nakamura Y, Shoda M, (1999). The genes *degQ*, *pps*, and *lpa-8* (*sfp*) are responsible for conversion of *Bacillus subtilis* 168 to plipastatin production. Antimicrob Agents Chemother; 43:2183–92.

Tsuge K, Matsui K, Itaya M, (2007). Production of the non-ribosomal peptide plipastatin in *Bacillus subtilis* regulated by three relevant gene blocks assembled in a single movable DNA segment. J Biotechnol.;129(4):592-603.

Turgay, K., Hahn, J., Burghoorn, J., Dubnau, D. (1998) Competence in *Bacillus subtilis* is controlled by regulated proteolysis of a transcription factor. EMBO J 17: 6730–6738.

Vanittanakom N, Loeffler W, Koch U, Jung G (1986) Fengycin – a novel antifungal lipopeptide antibiotic produced by *Bacillus subtilis* F-29–3. J Antibiot (Tokyo) 39:888–901

Varadavenkatesan, T., & Murty, V. R. (2013). Production of a lipopeptide biosurfactant by a novel Bacillus sp. and its applicability to enhanced oil recovery. *ISRN microbiology*, *2013*.

Vargas-Bautista, C., Rahlwes, K., & Straight, P. (2014). Bacterial competition reveals differential regulation of the pks genes by *Bacillus subtilis. Journal of bacteriology*, *196*(4), 717-728.

Villegas-Escobar, V., Ceballos, I., Mira, J. J., Argel, L. E., Orduz Peralta, S., & Romero-Tabarez, M. (2013). Fengycin C produced by *Bacillus subtilis* EA-CB0015. *Journal of natural products*, *76*(4), 503-509.

Violeta, O., Oana, S., Matilda, C., Maria, C. D., Catalina, V., Gheorghe, C., & Petruta, C. C. (2011). Production of biosurfactants and antifungal compounds by new strains of *Bacillus* spp. isolated from different sources. *Romanian Biotechnological Letters*, *16*, 84-91.

Volpon, L., Besson, F., & Lancelin, J. M. (2000). NMR structure of antibiotics plipastatins A and B from *Bacillus subtilis* inhibitors of phospholipase A 2. *FEBS letters*, *485*(1), 76-80.

Waewthongrak, W., Leelasuphakul, W., & McCollum, G. (2014). Cyclic Lipopeptides from *Bacillus subtilis* ABS–S14 Elicit Defense-Related Gene Expression in Citrus Fruit.

Wang, P., Guo, Q., Ma, Y., Li, S., Lu, X., Zhang, X., & Ma, P. (2015). DegQ regulates the production of fengycins and biofilm formation of the biocontrol agent *Bacillus subtilis* NCD-2. *Microbiological research*, *178*, 42-50.

Wei YH, Wang LC, Chen WC, Chen SY, Production and characterization of fengycin by indigenous *Bacillus subtilis* F29-3 originating from a potato farm. Int J Mol Sci, 2010;11(:4526–38.

Willenbacher, J., Rau, J. T., Rogalla, J., Syldatk, C., & Hausmann, R. (2015). Foam-free production of Surfactin via anaerobic fermentation of Bacillus subtilis DSM 10T. *AMB Express*, *5*(1), 21.

Wipat, A., & Harwood, C. R. (1999). The *Bacillus subtilis* genome sequence: the molecular blueprint of a soil bacterium. *FEMS microbiology ecology*, *28*(1), 1-9.

Wise, C., Falardeau, J., Hagberg, I., & Avis, T. J. (2014). Cellular lipid composition affects sensitivity of plant pathogens to fengycin, an antifungal compound produced by *Bacillus subtilis* strain CU12. *Phytopathology*, *104*(10), 1036-1041.

Wu, Y., Chen, L., Lee, H., Cheng, C., Wu, C., Shu, Y., . & Liu, T. (2007). Nonribosomal synthesis of fengycin on an enzyme complex formed by fengycin synthetases. *Journal of Biological Chemistry*, *282*(8), 5608-5616.

Xu, Z., Zhang, R., Wang, D., Qiu, M., Feng, H., Zhang, N., & Shen, Q. (2014). Enhanced control of cucumber wilt disease by *Bacillus amyloliquefaciens* SQR9 by altering the regulation of its DegU phosphorylation. *Applied and environmental microbiology*, *80*(9), 2941-2950.

Yánez-Mendizábal, V., Zeriouh, H., Viñas, I., Torres, R., Usall, J., de Vicente, A., ... & Teixidó, N. (2012). Biological control of peach brown rot (Monilinia spp.) by *Bacillus subtilis* CPA-8 is based on production of fengycin-like lipopeptides. *European journal of plant pathology*, *132*(4), 609-619.

Yang, M., Ferrari, E., Chen, E., & Henner, D. J. (1986). Identification of the pleiotropic sacQ gene of Bacillus subtilis. *Journal of bacteriology*, *166*(1), 113-119.

Yaseen, Y., Gancel, F., Drider, D., Béchet, M., & Jacques, P. (2016). Influence of promoters on the production of fengycin in *Bacillus* spp. *Research in microbiology*.

Yin, H., Guo, C., Wang, Y., Liu, D., Lv, Y., Lv, F., & Lu, Z. (2013). Fengycin inhibits the growth of the human lung cancer cell line 95D through reactive oxygen species production and mitochondria-dependent apoptosis. *Anti-cancer drugs*, *24*(6), 587-598.

Zeriouh, H., Romero, D., García-Gutiérrez, L., Cazorla, F. M., de Vicente, A., & Pérez-García, A. (2011). The iturin-like lipopeptides are essential components in the biological control arsenal of *Bacillus subtilis* against bacterial diseases of cucurbits. *Molecular Plant-Microbe Interactions*, *24*(12), 1540-1552.

Zeriouh, H., Vicente, A., Pérez-García, A., & Romero, D. (2014). Surfactin triggers biofilm formation of *Bacillus subtilis* in melon phylloplane and contributes to the biocontrol activity. *Environmental microbiology*, *16*(7), 2196-2211.

Zouari, R., Ellouze-Chaabouni, S., & Ghribi-Aydi, D. (2015). Optimization of *Bacillus subtilis* SPB1 Biosurfactant production under solid-state fermentation using by-products of a traditional live mill factory. *Achievements in the Life Sciences*.

Chapter 2
Bioinformatics analysis

Brief description of the bioinformatics analysis of *B. subtilis* BBG21 whole genome

The whole genome of the strain BBG21 has been sequenced in our laboratory by Dr. V. Leclere, yet the result of this sequencing was not analyzed. In this part of the work we firstly compared the genome of this strain with the strain *B. subtilis* 168 genome. The result of this comparison showed that the identity ratio between the two strains was 99% (data not shown).
In order to discover novel secondary metabolites, several bioinformatics tools are available to accomplish genome mining. Among these tools, antiSMASH, allows BLAST and search on the expected cluster to identify closest homologous sequence in the database. AntiSMASH allows the analysis of fragmented genomes and metagenomes making it a powerful prediction tool [Medema *et al.*, 2008]. Predicted peptides can be queried on NORINE database containing more than 1000 non-ribosomal peptides to find similar structures [Caboche *et al.*, 2008]. Another useful prediction tool is the NRPS/PKS substrate predictor. This software focuses on the specificity of A and AT domains (from NRPS and PKS respectively), this is useful to narrow the ambiguity of A domains specificity that occur in other prediction tools [Chen *et al*., 2006; Boddy *et al.*, 2014].

Analyzing the BBG21 genome using AntiSMASH software showed that the strain contain 6 clusters. Four of them are NRPS clusters including the well-known families of surfactin and fengycin, a catecholate type siderophore (bacillibactin) and, the bacilysin. Figure 1 shown two other clusters: a polyketide (bacillaene) and a bacteriocin (subtilosin) clusters.

Bacillibactin is an antibacterial and anti-oxidative molecule produce non- ribosomally in most of *Bacillus spp.* strains under iron-limited growth conditions. Bacillibactin is part of a specific transport system enabling *Bacillus* cells to accumulate and take up limited iron from their natural environment [May *et al.*, 2001; Raza *et al.*, 2012].

The peptide/polyketide bacillaene is produced by many *Bacillus* strains. This antibiotic is active against a broad spectrum of bacteria [Patel *et al.*, 1995; Müller *et al.*, 2014]. Bacilysin is a non-ribosomally synthesized di-peptide antibiotic that comprises L-alanine residue at the N terminus attached to a non-proteinogenic amino acid: L-anticapsin. The antimicrobial activity of bacilysin is initiated by the proteolysis of the di-peptide by a peptidase which release L-anticapsin. This molecule is active against a wide range of bacteria and some fungi [Hilton *et al.*, 1988; Rajavel *et al.*, 2008].

BBG21 strain also contains the subtilosin cluster, a macrocyclic bacteriocin with three intramolecular bridges. Subtilisin which shows a strong bactericidal activity is produced by *B. subtilis* and other *Bacillus* as *B. amyloliquefaciens*. It acts against a variety of Gram-positive bacteria, including *Listeria* [Stein *et al.*, 2004; Baruzzi *et al.*, 2011].

Identified secondary metabolite clusters

Cluster	Type	From	To	Most similar known cluster	MIBiG BGC-ID
The following clusters are from record sequence:					
Cluster 1	Nrps	315673	380807	Surfactin_biosynthetic_gene_cluster (82% of genes show similarity)	BGC0000433_c1
Cluster 2	Terpene	1039053	1059877	-	-
Cluster 3	Otherks-Nrps-Transatpks	1642744	1751619	Bacillaene_biosynthetic_gene_cluster (92% of genes show similarity)	BGC0001089_c1
Cluster 4	Nrps	1803168	1885216	Fengycin_biosynthetic_gene_cluster (100% of genes show similarity)	BGC0001095_c1
Cluster 5	Terpene	1946476	1966970	-	-
Cluster 6	T3pks	2089857	2131005	-	-
Cluster 7	Nrps	3036160	3086070	Bacillibactin_biosynthetic_gene_cluster (92% of genes show similarity)	BGC0000309_c1
Cluster 8	Other	3355868	3396623	-	-
Cluster 9	Sactipeptide	3574191	3595537	Subtilosin_A_biosynthetic_gene_cluster (100% of genes show similarity)	BGC0000602_c1
Cluster 10	Other	3597908	3639326	Bacilysin_biosynthetic_gene_cluster (100% of genes show similarity)	BGC0001184_c1

Figure 1. Overview of the antiSMASH analysis for BBG21 complete genome

The four NRPS clusters in BBG21 and *B. subtilis* 168 strains (surfactin, fengycin, bacillibactin and bacillaene) have been compared using antiSMASH software. Interestingly the identity between the two clusters of surfactin is 100%, whereas 5% of dissimilarety exists between the fengycin clusters. The analyses show 94% of identity between the two clusters of bacillaene while the bacillibactin clusters showed only 64% of identity (Fig. 2).

a-

b-

c-

d-

Figure 2. Cluster blast result between *B. subtilis* BBG21 with *B. subtilis* 168 of each NRPS gene cluster: a- surfactin gene cluster. b- Fengycin gene cluster. c- Bacillibactin gene cluster. d- Bacillaene (hybrid NRPS/PKS protein) gene cluster.

References

Baruzzi, F., Quintieri, L., Morea, M., & Caputo, L. (2011). Antimicrobial compounds produced by *Bacillus* spp. and applications in food. *Science against microbial pathogens: communicating current research and technological advances*, *2*, 1102-1111.

Boddy, N. (2014). Bioinformatics tools for genome mining of polyketide and non-ribosomal peptides. *Journal of industrial microbiology & biotechnology*, *41*(2), 443-450.

Caboche, S., Pupin, M., Leclère, V., Fontaine, A., Jacques, P., & Kucherov, G. (2008). NORINE: a database of nonribosomal peptides. *Nucleic acids research*, *36*(suppl 1), D326-D331.

Chen, H., Vater, J., Piel, J., Franke, P., Scholz, R., Schneider, K., ... & Gottschalk, G. (2006). Structural and functional characterization of three polyketide synthase gene clusters in *Bacillus amyloliquefaciens* FZB 42. *Journal of bacteriology*, *188*(11), 4024-4036.

Hilton, D., Alaeddinoglu, G., & Demain, L. (1988). Synthesis of bacilysin by *Bacillus subtilis* branches from prephenate of the aromatic amino acid pathway. *Journal of bacteriology*, *170*(1), 482-484.

May, J., Wendrich, M., & Marahiel, A. (2001). The dhb operon of *Bacillus subtilis* encodes the biosynthetic template for the catecholic siderophore 2, 3-dihydroxybenzoate-glycine-threonine trimeric ester bacillibactin. *Journal of Biological Chemistry*, *276*(10), 7209-7217.

Medema, H., Blin, K., Cimermancic, P., de Jager, V., Zakrzewski, P., Fischbach, A., & Breitling, R. (2011). antiSMASH: rapid identification, annotation and analysis of secondary metabolite biosynthesis gene clusters in bacterial and fungal genome sequences. *Nucleic acids research*, *39*(suppl 2), W339-W346.

Müller, S., Strack, N., Hoefler, C., Straight, D., Kearns, B., & Kirby, R. (2014). Bacillaene and sporulation protect *Bacillus subtilis* from predation by *Myxococcus xanthus*. *Applied and environmental microbiology*, *80*(18), 5603-5610.

Patel, S., Huang, S., Fisher, S., Pirnik, D., Aklonis, C., Dean, L. & Mayerl, F. (1995). Bacillaene, a novel inhibitor of procaryotic protein synthesis produced by *Bacillus subtilis*: production, taxonomy, isolation, physico-chemical characterization and biological activity. *The Journal of antibiotics*, *48*(9), 997-1003.

Rajavel, M., Mitra, A., & Gopal, B. (2009). Role of *Bacillus subtilis* BacB in the synthesis of bacilysin. *Journal of biological chemistry*, *284*(46), 31882-31892.

Raza, W., Hussain, Q., & Shen, Q. (2012). Production, Regulation and Transportation of Bacillibactin in *Bacillus Subtilis*. *Journal of the Chemical Society of Pakistan*, *34*(4), 996-1002.

Stein, T., Düsterhus, S., Stroh, A., & Entian, D. (2004). Subtilosin production by two *Bacillus subtilis* subspecies and variance of the sbo-alb cluster. *Applied and environmental microbiology*, *70*(4), 2349-2353.

Chapter 3
Influence of promoters on the production of fengycin in *Bacillus* spp.

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Original article

Influence of promoters on the production of fengycin in *Bacillus* spp.

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Abstract

Fengycin is a promising antifungal lipopeptide from *Bacillus* spp. synthesized by non-ribosomal peptide synthetases (NRPS). In this work, fengycin production of a spontaneous fengycin overproducing strain, *Bacillus subtilis* BBG21, was first compared to those of *B. subtilis* BBG111 (a 168 derivative), *B. subtilis* ATCC 21332 and *Bacillus amyloliquefaciens* FZB42 under two different experimental conditions. In both conditions, very high fengycin yields were obtained from strain BBG21 (480 mg/L) in comparison to its counterparts. The high efficiency of the fengycin promoter (P_{fen}) of BBG21 compared to the promoter of BBG111 and FZB42 was confirmed using a GFP reporter gene. Under all tested conditions, this promoter showed highest expression in comparison to the other strains. The highest fluorescence rate was obtained with mannitol as carbon source. In addition, when the P_{pps} promoter from *B. subtilis* BBG111 was replaced by promoter P_{fen} from BBG21, fengycin production increased about 10-fold, while no fengycin overproduction was observed when replacement was performed with P_{fen} from ATCC 21332. Comparative sequence analysis of these different promoters revealed one nucleotide modification in the UP element known for its importance in the regulation process. This point mutation is thus responsible for overproduction of fengycin in BBG21.

Keywords: *Bacillus subtilis*; Fengycin; Plipastatin; Promoter role; GFP expression

1. Introduction

Gram-positive bacteria belonging to the genus *Bacillus* can produce more than twenty antibiotics with various structures [1], including non-ribosomally-synthesized lipopeptides. These lipopeptides are gathered into five families: surfactins, fengycins, iturins, kurstakins and locillomycins [2–4].

Fengycin displays strong widespread antifungal activity [5,6], but also antibacterial and antiviral activities [7,8]. A recent report unveiled its anti-cancer activity inhibiting, growth of human lung cancer cells [9]. This finding confirms the strong potential of this bioactive compound.

Fengycins are cyclic lipodecapeptides containing a β-hydroxy fatty acid chain of 16–19 carbon atoms. They are produced as a mixture of isoforms that vary in length and branching of the fatty acid moiety, as well as in the amino acid composition of the peptide moiety [10,11]. Two main forms of fengycin are produced, which differ in their amino acid composition by only one residue in position 6, that is, D-Ala in fengycin A and D-Val in fengycin B. Fengycins could also be termed "plipastatins" based on the place of L- and D-forms of tyrosine [5,10,12]. Non-ribosomal peptide synthetases responsible for fengycin biosynthesis are encoded by five genes, including *fenA* to *fenE* (named *ppsA/ppsE* in *Bacillus subtilis* 168) [13,14]. These synthetases are transformed from apoproteins to holo-proteins by the addition of the 4′-phosphopantetheinic cofactor. This reaction is catalyzed by a phosphopantetheinyl transferase encoded by the *sfp* gene. The expression of lipopeptide operons involves a complex regulatory machinery linked to quorum sensing, also described at a molecular level for surfactin or mycosubtilin (a member of the

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Abstract

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1. Introduction

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Several studies about the influence of environmental factors on lipopeptide biosynthesis indicated that both the carbon and nitrogen sources in the media and growth conditions such as temperature, pH or oxygen rate influence the type and yield of the produced lipopeptides [18,19]. The presence of amino acids acting as lipopeptide precursors in the medium composition may also play a role in the biosynthesis of the molecule by modifying the amount of the produced lipopeptides but also in some cases their structure [20,21]. Regarding

fengycin specifically, three cultivation parameters have been highlighted for their influence on its production: source of nitrogen, oxygen transfer rate and temperature [2,22].

Four fengycin-producing strains have been used in this study: *B. amyloliquefaciens* FZB42, *B. subtilis* BBG111, *B. subtilis* ATCC 21332 and *B. subtilis* BBG21. *B. amyloliquefaciens* FZB42 can produce three types of lipopeptides including fengycin, surfactin and bacillomycin [23,24]. *B. subtilis* BBG111 is a Sfp^+ *B. subtilis* 168 derivative which produces high amounts of surfactin but a weak amount of fengycin [25]. *B. subtilis* BBG21 is a spontaneous mutant of *B. subtilis* ATCC 21332, a co-producer of surfactin and fengycin [26]. Compared to its mother strain, BBG21 is able to overproduce fengycin up to 500 mg/L [27,28]. This work aimed at understanding the influence of fengycin operon promoter on the overproduction of this lipopeptide by BBG21 under several environmental conditions.

2. Materials and methods

2.1. Bacterial strains, plasmids and primers

Plasmids and strains used in this study are listed in Table 1. All the primers used for genetic construction or inspection are shown in Table 2.

2.2. Bacillus *transformation*

B. subtilis BBG111 was transformed by natural competence pathway [29]. *B. subtilis* BBG21 was firstly transformed by pMMComK plasmid carrying *comK* gene under control of the P_{xyl} promoter, by protoplast electroporation protocol [30]. The resulting strain named *B. subtilis* BBG201 was then transformed using induced competence transformation procedure in the presence of 0.125 to 0.25% of xylose [26]. A modified electroporation method using trehalose was used for transformation of *B. amyloliquefaciens* FZB42 [31].

2.3. Culture conditions

For transformation experiments, *Bacillus* strains *and Escherichia coli* JM109 were grown at 37°C under shaking at 130 rev min^{-1} in Luria–Bertani medium (LB) [29], supplemented with antibiotics (Sigma-Aldrich, St. Louis, MO, USA): spectinomycin di-hydrochloride (100 µg/mL); chloramphenicol (5 µg/mL); tetracycline hydrochloride (6 µg/mL) and erythromycin (1 µg/mL) when required. The α-amylase activity was detected by growing *Bacillus* colonies overnight on LB plates containing 1% soluble starch and staining plates with iodine [32]. Microbial growth was monitored by optical density (OD) at 600 nm with an Uvikon 940

spectrophotometer (Kontron Instruments, Plaisir, France). Landy medium [33] containing 100 mM 3-(N-morpholino) propanesulfonic acid (MOPS) was used as the basic medium for lipopeptide production with 10% filling ratio of Erlenmeyer, 30°C and 160 rpm (Condition "a"). A modified Landy medium containing 100 mM MOPS, 2.2 g/L of ammonium sulphate and 2.5 g/L of glutamic acid with 33% filling ratio of Erlenmeyer, 30°C and 160 rpm was used in condition "b" [34].

2.4. DNA manipulation

Polymerase chain reaction (PCR) was done using the PCR Master Mix (2X) (Thermo Scientific Fermentas, Villebon sur Yvette, France) as a mixture of Taq DNA polymerase. DNA extraction was performed using the Wizard® Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA). Ligation of PCR products was done into pGEM-T Easy vector (Promega Corp.). Plasmid extraction was carried out using GeneJET Plasmid DNA Purification Kit (Thermo Scientific Fermentas). Restriction endonucleases were supplied by Thermo Scientific Fermentas. Ligation of inserts to different vectors was effected using the DNA Ligation Kit <Mighty Mix> from Takara (Ozyme, Saint Quentin en Yvelines, France). Recovery of DNA from agarose gels was performed with GeneJET Gel Extraction kit (Thermo Scientific Fermentas). In all cases, the instructions of the suppliers were followed. All the construction sequences were verified by DNA sequencing (Eurofins Genomics, Ebersberg, Germany).

2.5. GFP integration in Bacillus *strains with pMUTIN-GFP+ vector*

Three fragments of *dacC*-P_{pps}/P_{fen} were amplified by PCR using respectively the primers Terminator-P_{fen} of *B. subtilis* BBG21 and *B. amyloliquefaciens* FZB42, and Terminator-P_{pps} of *B. subtilis* BBG111. The fragments were cloned in pGEM-T Easy vector. The resulting three plasmids were named pBG306, pBG308 and pBG307 respectively. Plasmids pMUTIN-GFP+, pBG306, pBG307 and pBG308 were *Kpn*I and *Cla*I double digested. The *dacC*-P*pps/*P*fen* fragments were inserted between the *Kpn*I and *Cla*I sites of pMUTIN-GFP+ to obtain pBG309, pBG311 and pBG310, respectively.

2.6. Constructions with pFB01 vector

Fragments of *dacC*-P_{fen}, were amplified by PCR using the primers *dacC*-P_{fen}/*B. subtilis* BBG21 and *dacC*-P_{fen}/*B. amyloliquefaciens* FZB42. Two plasmids were obtained after cloning in pGEM-T Easy vector (pBG312 and pBG313 respectively). Then double digestions were realized with *Kpn*I and *Xma*III for these two plasmids and pFB01. For each plasmid, the *dacC-ppsA*/*fenA* fragments were inserted between the *Kpn*I and *Xma*III sites in pFB01, giving pBG314 and pBG315 respectively. Integration was confirmed by both PCR and the loss of ability to use starch. Green fluorescence under fluorescence microscope was observed for all mutants and comparison in the fluorescence intensity between the mutants and their mother strains was carried out using microplate wells containing Landy MOPS medium and incubated in the BioLector device (m2p-labs GmbH, Baesweiler, Germany).

2.7. GFP expression

Each mutant was tested through the high-throughput system of fermentation BioLector. Precultures were realized at 30°C in 500 mL Erlenmeyer flasks containing 50 mL Landy medium. Then, test cultures were carried out in 48 wells flower plate (triplet for every condition) designed for the BioLector (containing pH, pO_2, fluorescence and biomass optodes). Growth and production analyses were carried out in Landy medium at 30°C, under shaking at 1,100 rpm and at pH 7.0. Different nitrogen sources, i.e., tyrosine, threonine, valine (5 g/L each) or urea + NH_4Cl (2.5 g/L each) were investigated as alternatives to glutamic acid in Landy medium. Mannitol (20 g/L) was used as a carbon source to replace glucose (20 g/L) in the medium. The influence of oxygen rate was tested using 1.2 mL of medium instead of 1 mL (80% instead of 66% filling volume) The media were supplemented with 100 mM MOPS except in one experiment designed to follow the change in pH. Samples were withdrawn after 72 h of culture. Biomass and optical density measurements for BioLector calibration were carried out as described previously [35].

2.8. P_{pps} replacement of B. subtilis *BBG111 by promoter P_{fen} from* B. subtilis *BBG21 and ATCC 21332*

The expected P_{fen} DNA fragments were amplified by PCR using the primers *dacC* fwd and *fenA* rev from BBG21 and ATCC 21332 (Table 2), and then cloned in pGEM-T Easy. The resulting plasmids pBG302 and pBG322 were *MfeI-SacI* double digested. The two fragments were inserted between *MfeI* and *SacI* sites (replacing the old *dacC-fenA* fragment carried by pBG193), generating the plasmid pBG303 and pBG223 respectively. The latter were successfully transferred into the strain BBG111 by natural competence transformation, leading to the strain BBG203 and BBG216 respectively.

2.9. Lipopeptide purification and quantification

1 mL of supernatant was extracted using C18 cartridges (Extract-clean SPE 500 mg, Grace Davison-Alltech, Deerfield, IL, USA). Lipopeptide production was quantified by HPLC (Waters Corporation, Milford, MA, USA) using a C18 column (5 µm, 250 × 4.6 mm, VYDAC 218 TP, Hesperia, CA, USA). Analyses of lipopeptides were performed as previously described [26-28].

Table 1 Strains and plasmids used in this study

Plasmid or bacterial strain	Genotype (Phenotype) or plasmid description	Reference
pGEM-T Easy	*bla*^{R}	Promega Corp.
PMMComK	*tet*^{R} , P*xyl-comK*	Hoffmann *et al.*, (2010)
pFB01	*bla*^{R} , *erm*^{R} , *amyE*-front and *amyE*-back cassettes , *gfp* ,(7526 bp)	Fan *et al.* (2012)
pMUTIN-GFP+	*bla*^{R}, *erm*^{R} , *gfp,* (6192 bp)	Bacillus genetic stock center, USA
pBG193	340 bp *Eco*RI P*fen*/BBG21 fragment from pBG185 cloned into pBG191 (5,453 bp); *bla*^{R} , *spc*^{R}	Hussein, ProBioGEM
pBG302	pGEM-T Easy , 991 bp *Mfe*I-*Sac*I P*fen* amplicon from *B. subtilis* BBG21; *bla*^{R} (4008 bp)	This study
pBG303	991 bp *Mfe*I-*Sac*I, P*fen*/BBG21 amplicon from pBG302 cloned into pBG193; *bla*^{R} , *spc*^{R} (5610 bp)	This study
pBG306	pGEM-T Easy, *Kpn*I-*Cla*I, P*fen* amplicon from BBG21; *bla*^{R}, (3683 bp)	This study
pBG307	pGEM-T Easy, *Kpn*I-*Cla*I, P*pps* amplicon from *B. subtilis* BBG111; *bla*^{R}, (3849 bp)	This study
pBG308	pGEM-T Easy, *Kpn*I-*Cla*I P*fen* amplicon from *B. amyloliquefaciens* FZB42; *bla*^{R}, (3916 bp)	This study

pBG309	*bla*^{R}, *erm*^{R}, *Kpn*I-*Cla*I P*fen* amplicon from *B. subtilis* BBG21, (*gfp*), (6858 bp)	This study
pBG310	*bla*^{R}, *erm*^{R}, *Kpn*I-*Cla*I P*pps* amplicon from *B. subtilis* BBG111, (*gfp*), (7001 bp)	This study
pBG311	*bla*^{R}, *erm*^{R}, *Kpn*I-*Cla*I P*fen* amplicon from *B. amyloliquefaciens* FZB42, (*gfp*), (7045 bp)	This study
pBG312	pGEM-T Easy, *Kpn*I-*Xma*III P*fen* amplicon from *B. subtilis* BBG21; *bla*^{R}, (3590 bp)	This study
pBG313	pGEM-T Easy, *Kpn*I-*Xma*III P*fen* amplicon from *B. amyloliquefaciens* FZB42; *bla*^{R}, (4001 bp)	This study
pBG314	*bla*^{R}, *erm*^{R}, *Kpn*I-*Xma*III P*fen* amplicon from *B. subtilis* BBG21, pFB01, (8058 bp)	This study
pBG315	*bla*^{R}, *erm*^{R}, *Kpn*I-*Xma*III P*fen* amplicon from *B. amyloliquefaciens* FZB42, pBF01, (8473 bp)	This study
pBG322	pGEM-T Easy, 991 bp *Mfe*I-*Sac*I P*fen* amplicon from *B. subtilis* ATCC 21332; *bla*^{R}, (4008 bp)	This study
pBG323	991 bp *Mfe*I-*Sac*I P*fen* amplicon from *B. subtilis* ATCC 21332, pBG322 cloned into pBG193; *bla*^{R}, *spc*^{R}, (5610 bp)	This study
B. subtilis ATCC 21332	Wild-type strain, *fen+*, *srf+*	Lab stock

B. subtilis BBG21	Spontaneous mutant of *B. subtilis* ATCC 21332, *fen*+, s*rf*+	Fahim *et al.* (2012)
BBG201	*tet* ^{R} , *B. subtilis* BBG21 PMM ϖ *comK*+	This study
BBG205	*erm* ^{R} , *B. subtilis* BBG21 ΔP*fen*::(P*fen* BBG21 –*gfp*- *erm*^{R})	This study
BBG208	*erm*^{R}, BBG21 Δ*amy*E:: (P*fen* BBG21 –*gfp*- *erm*^{R})	This study
B. subtilis BBG111	*cat*^{R}, *B. subtilis* 168 *sfp*+, *pps*+, *srf*+	Coutte *et al.* (2010)
BBG203	*cat* ^{R} , BBG111 *Ppps* ::(P*fen* BBG21:: *spc* ^{R})	This study
BBG216	*cat*^{R} , BBG111 *Ppps* ::(P*fen* ATCC21332:: *spc* ^{R})	
BBG206	BBG111 ΔP*pps*::(Ppps BBG111 –*gfp*- *erm*^{R})	This study
B. amyloliquefaciens FZB42	Wild-type strain, *fen*+, *srf*+, *bmy*+	Koumoutsi *et al.* (2004)
BBG207	*erm*^{R}, *B. amyloliquefaciens* FZB42 P*fen*/FZB42::*gfp*, Δ *fen*	This study
BBG209	*erm*^{R}, *B. amyloliquefaciens* FZB42 Δ *amy*E::P*fen*/FZB42-*gfp*	This study
Escherichia coli JM109	*recA1, endA1,gyrA96, thi, hsdR17, supE44* Δ(*lac-proAB)*/F'[*traD36, proAB*+, *lacIq, lacZ*ΔM15]	Promega Corp.

Ap^{R}, resistance to ampicillin; Spc^{R}, resistance to spectinomycin ; Erm^{R}, resistance to erthromycin; Cm^{R}, resistance to chloramphenicol; Tc^{R}, resistance to tetracycline.

Table 2 PCR primers used for genetic constructions and their verification

PRIMER	PRIMER SEQUENCE (5'-3')
dacC/Bs. BBG21 *Mfe*I F	AACAATTGCGGCAGACCTGTTTT
fenA/*Bs.* BBG21 *Sac*I R	GTATTCCAACGGGAGCTCAAAG
tr-*ppsA*/*Bs*. F	ATCGGGGTACCTAATAACGCTTTG
ppsA/Bs. R	ATCGATGCGGGAGATGGAATGAT
Tr-*ppsA Bs*. F2	GGGGTACCCAGAAACAAAGAGC
ppsA/Bs. R2	ATCGATGCGGGAGATGGAATGAT
dacC/Bam. F	GGTTCAGAAAGAAAAGTGGTACCC
fenA/Bam. R	GCAGTACGTCAAAATCGATGTC
GFP+ F	TGGCTAGCAAAGGAGAAGAACT
GFP+ R	CTCATCCATGCCATGTGTAATC
dacC F	GTTGGTTCGCGGCTTATCTA
pMUTIN-GFP R	AGCATCACCTTCACCCTCTC
dacC-gfp F2	TGACATTCAGGATCAGAGTTGG
pMUTIN-GFP+ R2	TAAGGGTAAGCTTTCCGTATGT
dacC/*Bs*.21332 D	TATCGGGGTACCCAGAAACA

ppsA/*Bs*. 21332 R	GTATCTGGCTCCGGCCGTTC
dacC/*Bam*. F	AAAAGTGGTACCCGGCTTTC
fenA/*Bam*. R	AGATACGGCCGGGGTTCT
dacC/*Bam*. F2	AGAAAAGTGGTACCCGGCTTT
fenA/*Bam*. R2	AGATACGGCCGGGGTTCTGAT
amyE/*Bam*. F	CTTGCCGGCTTTTTATACG
GFP+ R	TTTGTAACTGCTGCTGGGATTA
pMUTIN-GFP+-*ermB* F	TTGAGTACCTTTTCATTCGTTAAAAA
amyE back *Bam*. R	GATATTGGCGTGACTCCATACA
amyE front *Bam*. F	CTTGCCGGCTTTTTATACG
amyE back *Bam*. R	GATATTGGCGTGACTCCATACA

3. Results

3.1. Lipopeptide production by the Bacillus *spp.*

B. subtilis BBG21, ATCC 21332 and BBG111 and *B. amyloliquefaciens* FZB42 were compared in their lipopeptide synthesis (fengycin and surfactin). Based on previous results [34], two experimental sets of conditions were tested by modifying the nitrogen source available in the medium (glutamic acid or glutamic acid and ammonium sulphate) and the oxygenation rate (expressed as 10 or 33 % filling volume). The Table 3 clearly shows the significant difference between BBG21 and the three other strains as fengycin specific production was between 2.4 to 33.5 times higher in BBG21 under the two tested conditions. For the three *B. subtilis* strains, the low oxygen rate and presence of glutamic acid allowed enhancing about twice the specific production of fengycin but no difference could be observed for *B. amyloliquefaciens* FZB42. Regarding the specific surfactin production, the strains *B. subtilis* ATCC 21332, BBG111 and *B. amyloliquefaciens* FZB42 clearly overproduced this molecule independently of the conditions tested. In the case of *B. subtilis* BBG21, surfactin was overproduced under condition "a", while surfactin and fengycin were synthesized at similar rate under condition "b".

3.2. GFP mutant construction

Several mutant strains were constructed in order to compare the efficiency of the fengycin promoters using GFP+ fused marker. Firstly, pMMComK carrying the *comK* competence gene was introduced into *B. subtilis* BBG21, generating the strain *B. subtilis* BBG201, within which both tetracycline resistance and the presence of the plasmid were confirmed. The three strains (*B. amyloliquefaciens* FZB42, *B. subtilis* BBG201 and BBG111) were successfully transformed by the corresponding vector (pBG309, pBG310 and pBG311) carrying the fused P*fen/*P*pps-ppsA::gfp* integrated at the promoter of plipastatin/fengycin operon. The new strains were named BBG205 (from BBG201), BBG206 (from BBG111) and BBG207 (from FZB42). Constructions were verified by PCR and the disruption of fengycin/plipastatin production was shown by the lack of these lipopeptides by HPLC and MALDI-TOF mass spectrometer analyses. In all these strains, GFP-generated fluorescence was emitted (data not shown).

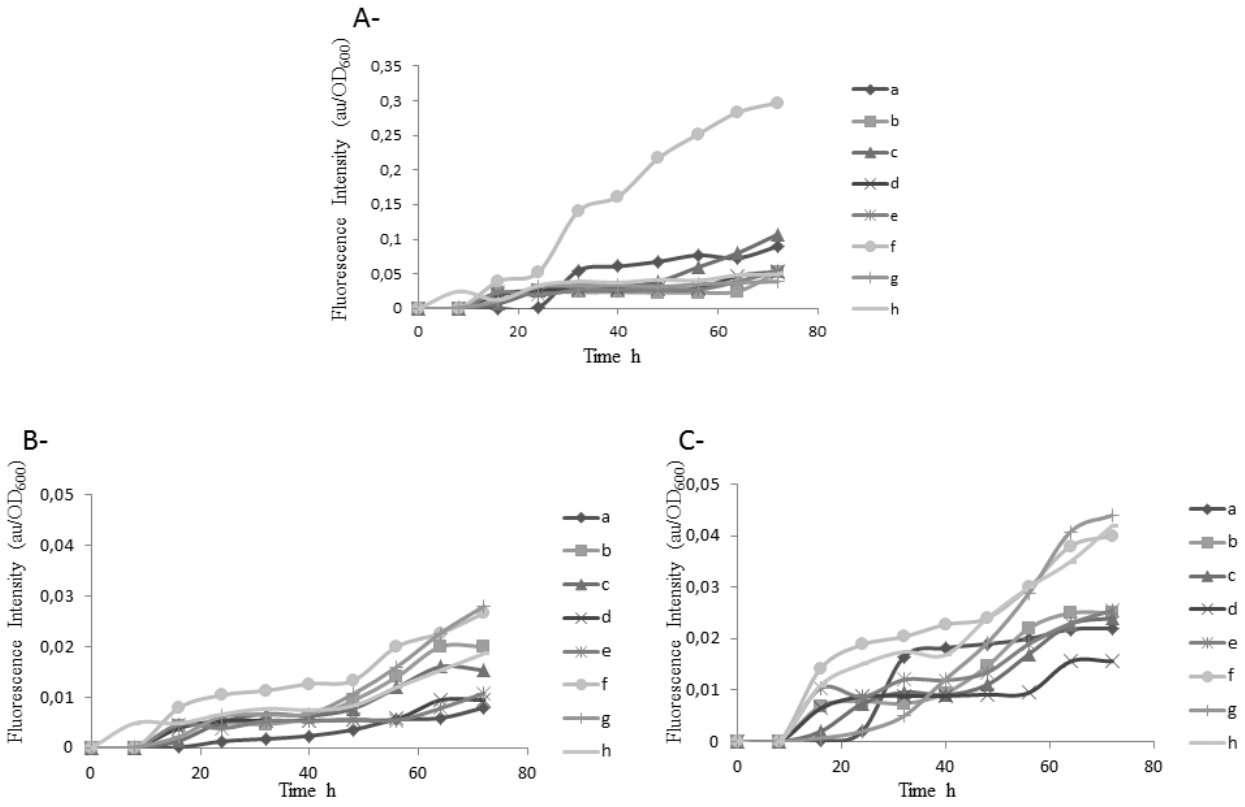

Fig.1. **Fluorescence intensity (au/OD_{600}) in strain BBG205 (1-A), BBG206 (1-B) and BBG207 (1-C) grown in Landy medium under eight conditions.** (a) Landy medium; (b) Landy MOPS tyrosine; (c) Landy MOPS urea+NH_4Cl; (d) Landy MOPS threonine; (e) Landy MOPS valine; (f) Landy MOPS mannitol; (g) Landy MOPS, 1.2 mL volume; and (h) Landy MOPS (control)**.** Fluorescence was measured by BioLector**.**

3.3. Comparative studies of promoter strength in different environmental conditions

The influence, on promoter strength, of several parameters known to be important for the synthesis of fengycin was then tested in these three strains. Based on literature information, eight experiments were designed to test the influence of the pH, the oxygenation rate, the carbon (glucose or mannitol) and nitrogen sources (tyrosine, threonine, valine or urea + NH_4Cl). The results obtained for the three strains are given in Fig.1.

According to these results, the strength of the promoters from BBG21 is between two to ten times (depending on the medium condition) better than that of the two other promoters whatever the environmental tested conditions. These results also highlight the different

conditions which are favorable for the best expression of the different promoters. For BBG205, the highest fluorescence intensity was obtained when glucose was replaced by mannitol (0.3 au/OD_{600}). There was no noticeable effect of both decrease in the oxygen rate and the absence of MOPS buffer on GFP expression. Addition of three amino acid precursors of fengycin peptidic moiety (tyrosine, threonine and valine) allowed obtaining about 0.05 au/OD_{600}; this addition did not increase GFP expression compared to Landy MOPS as control. For strains BBG206 and BBG207, there was no significantly difference in fluorescence under the different conditions.

Fig.2. shows the fluorescence intensity (au and au/OD_{600}) and the biomass obtained for the three mutants incubated in Landy medium complemented with mannitol or glucose as carbon sources. The fluorescence intensity of BBG205 with mannitol was about 10-fold more compared to the two other mutants. It was about 2 to 4-fold more with glucose, while the differences in biomass between the three strains were not significant under each condition. Biomass was significantly lower in Landy MOPS mannitol culture medium than in Landy glucose one. The results demonstrated also that the promoter expression started after 24 h of culture corresponding to the end of the log phase and the beginning of the stationary phase.

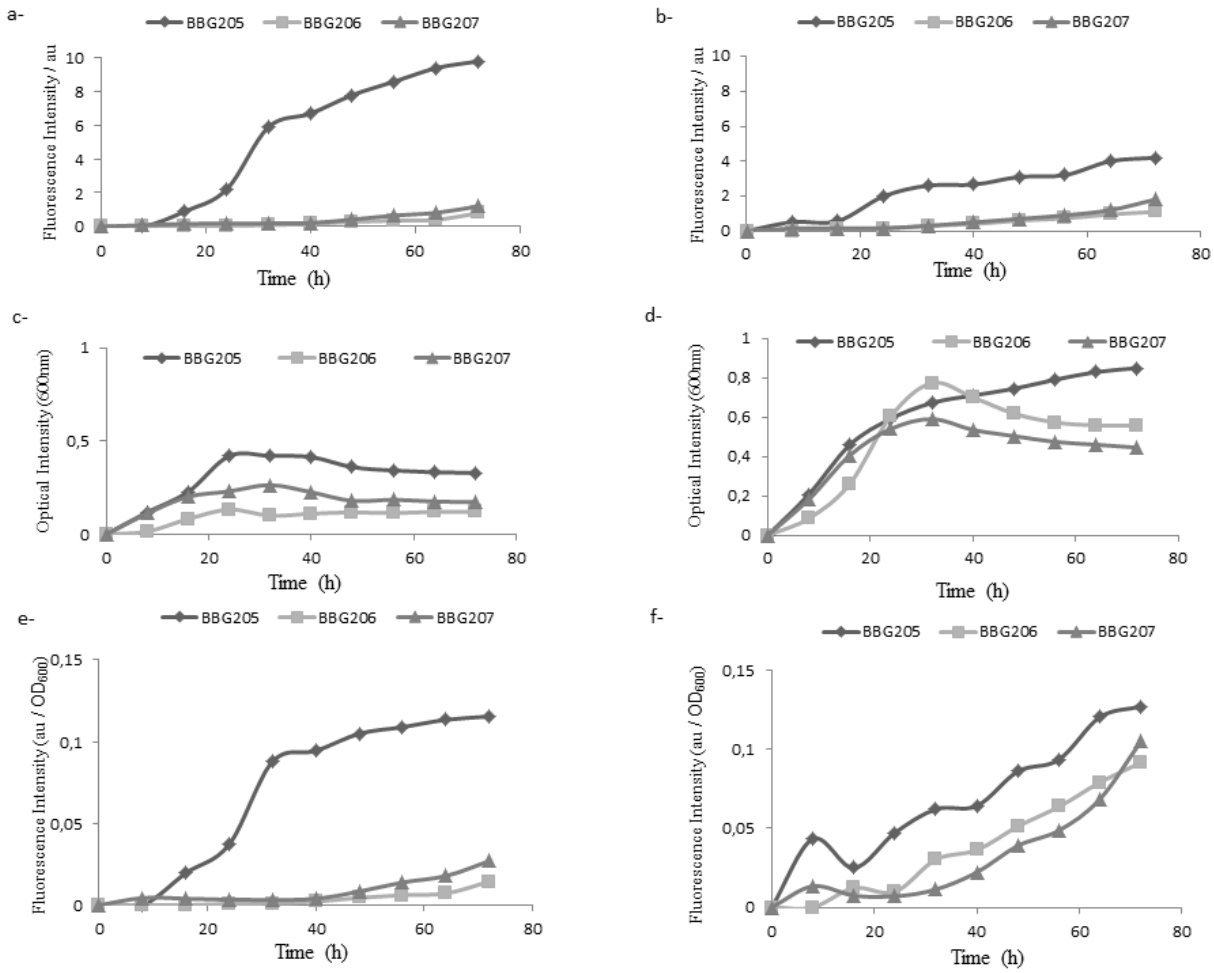

Fig.2. **Fluorescence intensity (au and au/OD_{600}) and the optical density at (600 nm) measured by BioLector for three *Bacillus* mutant strains (BBG206, BBG207 and BBG208) grown in Landy medium with mannitol and glucose.**

- ***a***- *Fluorescence intensity (au) in Landy MOPS-mannitol.*
- ***b***- *Fluorescence intensity (au) in Landy MOPS-glucose.*
- ***c***- *Optical density in Landy MOPS-mannitol.*
- **d**- *Optical density in Landy MOPS-glucose*.
- **e**- *Fluorescence intensity (au/OD_{600}) in Landy MOPS-mannitol*
- **f**- *Fluorescence intensity (au/OD_{600}) in Landy MOPS-glucose.*

3.4. Simultaneous analysis of fengycin operon expression and fengycin production

To complete these experiments, a second group of mutants were constructed, allowing the simultaneous production of fengycin and GFP protein under the control of P*pps*/P*fen* promoters. These strains were constructed using pBF01 plasmid. The integrative plasmids (pBG314 and pBG315) containing P*fen*/P*pps-gfp* flanked by two *amyE* border cassettes were used to recombine at the amylose degradation *amyE* locus by double crossover in *B. subtilis* BBG201 and *B. amyloliquefaciens* FZB42. The resulting erythromycin resistant strains were named BBG208 (from BBG201) and BBG209 (from FZB42). Experimental conditions previously used were tested with the mutant strains *B. subtilis* BBG208 and BBG209. The results permitted to establish that there were no significant differences between the GFP expression from these strains and *B. subtilis* BBG205 and BBG207.

In order to corroborate the results obtained from *B. subtilis* BBG205, production of lipopeptides from BBG208 was investigated after 48 h of incubation under three different conditions: Landy culture medium, Landy culture medium with mannitol instead of glucose and Landy culture medium with urea and NH_4Cl instead of glutamic acid. Fig.3. shows that the highest fengycin yield (445 mg/L) was obtained with mannitol used as carbon source. This quantity was twice higher than those obtained under the other two conditions. Surfactin production was also affected by changing the carbon and nitrogen sources. The use of urea and NH_4Cl as sources of nitrogen enhanced the production of surfactin which reached 803 mg/L, while it was close to the fengycin level with Landy + mannitol (480 mg/L). The maximum fengycin production for the other two strains BBG111 and BBG209 under the same conditions were 22.43 and 68.8 mg/L respectively (data not shown).

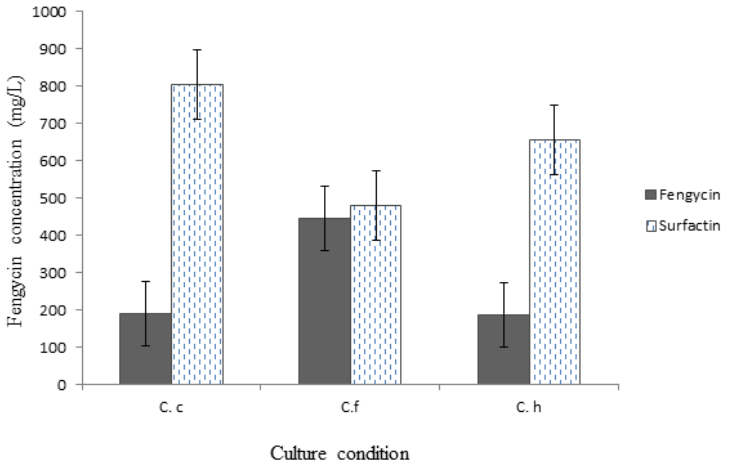

Fig.3. **Lipopeptide production by BBG208 after 48 h of growth under three conditions**. (c): Landy MOPS urea +NH4Cl; (f) Landy MOPS mannitol; and (h) Control Landy MOPS glucose.

*3.5. P*pps *replacement mutant*

In order to confirm the role played by the promoter of BBG21 in its fengycin overproducing phenotype, the P*pps* promoter from *B. subtilis* BBG111 was successfully replaced by the promoter P*fen* from BBG21, generating a novel strain named *B. subtilis* BBG203. This mutant was characterized by growth in LB agar containing 100 µg/mL spectinomycin, and verified by PCR (data not shown). As shown in Table.4, fengycin specific production in BBG203 was increased 8-11.8 fold depending on the culture conditions (0.49 to 5.8 and 1.4 to 11.2 mg/OD_{600} under the conditions (a) and (b) respectively) as well in the presence of ammonium sulphate with either 10 or 33% filling volume. On the other hand, no increase in fengycin production was observed in the mutant strain BBG216 which contains the fengycin promoter of ATCC 21332 comparing to its mother strain BBG111 under the two tested conditions (Table 4).

This result confirms that the differences observed between the promoters of BBG21 and BBG111 influences fengycin production. Nevertheless, this production in BBG203 remained low compared to that of *B. subtilis* BBG21, under both conditions.

Table.3. Lipopeptide production in batch by four strains of *Bacillus spp.*

Strain	Time (h)	Condition a		Condition b	
		Fengycin	Surfactin	Fengycin	Surfactin
BBG21	48	19.1± 1.2	125.8 ± 12.4	50 ± 1.6	44.6 ± 3.5
	72	23.9 ± 1.6	105.2 ± 9.8	52.3 ± 2.5	64.4 ± 10.9
ATCC 21332	48	3.7 ± 0.17	123 ± 5.3	6.4 ± 0.9	83.9 ± 2.6
	72	4.1 ± 0.37	183.2 ± 12.4	7.9 ± 0.8	110.3 ± 1.3
BBG111	48	0.76 ± 0.3	57.7 ± 0.8	1.85 ± 0.36	43.1 ± 7.2
	72	0.8 ± 0.17	53.3 ± 7.7	1.56 ± 0.5	52.3 ± 5.2
FZB42	48	7.9 ± 0.7	41.1 ± 1.5	7.2 ± 0.54	32.4 ± 3.8
	72	8.7 ± 0.9	49.9 ± 14.7	8.9 ± 0.6	40.8 ± 3.7

(Expressed in specific production)

Specific fengycin production was calculated as the ratio of fengycin (mg/L) over OD_{600} unit, both of them were determined at the same time point.
*Condition **a**- Landy MOPS medium, pH 7, 10% filling ratio, 30°C and 160 rpm.*
*Condition **b**- Landy MOPS medium pH 7, (contains 2.2 g/L ammonium sulphate with 2.5g/L glutamic acid] with 33% filling ratio, 30°C and 160 rpm.*

Table 4. Fengycin production from BBG203, BBG216 and their mother strain BBG111 after 48 h of growth under two different conditions.

Strain	Condition a	Condition b
	*Fengycin mg/OD_{600}	Fengycin mg/OD_{600}
BBG111	0.49 ± 0.14	1.4 ± 0.5
BBG203 (BBG111 $P_{fen\ BBGG21}$)	5.8 ± 0.75	11.5 ± 1.46
BBG216 (BBG111 $P_{fen\ ATCC\ 21332}$)	0.76 ± 0.36	1.54 ± 0.48

* *Specific fengycin production was calculated as the ratio of fengycin (mg/L) over OD_{600} unit, both of them were determined at the same time point.*
*Condition **a**- Landy MOPS medium, pH 7, 10% filling ratio, 30°C and 160 rpm.*
*Condition **b**- Landy MOPS medium pH 7, (contains 2.2 g/L ammonium sulphate with 2.5g/L glutamic acid) with 33% filling ratio, 30°C and 160 rpm.*

3.6. Sequence analysis of fengycin operon promoters

Sequences of P_{fen}/P_{pps} promoters were analyzed in order to check differences that could explain the overproduction of fengycin in *B. subtilis* BBG21 (Fig. 4). The sequences of *B. subtilis* BBG21 and *B. subtilis* ATCC 21332 promoters were nearly identical (> 99% identity) with only one nucleotide different in the UP element sequence. To confirm this little difference, the sequence was performed three times. Two supplementary differences were observed between *B. subtilis* 168 and *B. subtilis* BBG21 (identity percentage was 97%): one A was added seven nucleotides before the UP element, as well as a small sequence of 10 nucleotides between the -10 TATA box and the ribosome binding site (RBS). The identity percentage between BBG21 and *B. amyloliquefaciens* FZB42 was only 77%.

Fig.4. **Alignment of the *Pfen* promoter of *B. subtilis* BBG21 with the other strains ones.** (a) Alignment of the *Pfen* promoter of *B. subtilis* BBG21 with its mother strain *B. subtilis* ATCC 21332. (b) Alignment of the *Pfen* promoter of *B. subtilis* BBG21 with *B. subtilis* 168. (c) Alignment of the *Pfen* promoter of *B. subtilis* ATCC 21332 with *B. subtilis* 168.

4. Discussion

B. subtilis BBG21 is a spontaneous mutant strain from *B. subtilis* ATCC 21332, known to produce two types of lipopeptides, i.e., fengycin and surfactin. Fengycin production reached up to 500 mg/L when BBG21 was grown under optimal conditions, while only 40 mg/L were produced in the case of *B. subtilis* ATCC 21332 [28].

To highlight on the particularly elevated fengycin productivity of *B. subtilis* BBG21, comparison between the lipopeptide yield of this strain and those of *B. subtilis* ATTC 21332, *B. subtilis* BBG111 and *B. amyloliquefaciens* FZB42, was realized in batch culture under two conditions. The results confirmed the high productivity of fengycin for *B. subtilis* BBG21 compared to the three other strains, especially when low oxygen rate is provided and when ammonium sulphate is used as nitrogen source. Previous works in our laboratory have clearly established the effect of oxygen rate on both surfactin and fengycin productions from *B. subtilis* ATCC 21332 and the results were comparable to those found in this study [3,25]. Another work performed on *B. subtilis* BBG21 demonstrated that fengycin production is influenced by the oxygen transfer limitation and increased at low culture temperature (30°C), whereas surfactin production shows a high correlation with the enhancement of oxygen transfer rate [28]. The efficiency of the BBG21 promoter was then confirmed in different environmental conditions, using the green fluorescent protein. Using GFP expression system as a reliable and quantitative reporter of underlying varieties of the promoter activity is a performant biological tool [36,37]. This sensor can provide high accuracy description of the promoter action in reply to each stimulation factor [38,39]. Under different medium compositions known to influence fengycin production [5,17], the expression was monitored by fluorescence spectroscopy using a BioLector device. Different fluorescence intensities were recorded with the three promoters under the same conditions. Under all tested conditions, the strain BBG205 which contains the fengycin promoter of BBG21 showed the highest expression in comparison to the other strains. This expression was significantly influenced by the medium composition. Nicolas *et al.* [40] studied the effect of 104 nutritional and environmental conditions on the promoter expression in *B. subtilis* 168 using 269 RNA samples and, in the best of our knowledge, this is the only study on the direct impact of the environmental factors on the fengycin promoter activity. The *ppsA* gene expression responded positively with the oxygen stress during the growth and from many carbon sources the highest expression obtained was in M9 medium with fructose [40].

In our experiments, the use of mannitol as carbon source or urea and NH_4Cl as nitrogen source enhanced the fengycin promoter expression. The mannitol was already used as a sole

carbon source in the optimal medium for the fengycin production for the strain F29-3 and it was the best carbon source for lipopeptide production from different *Bacillus* strains [10,24,41].

Our results also showed that replacement of glutamic acid in Landy medium by different amino acids (tyrosine, threonine and valine) did not have any effect on *gfp* expression. Previous study showed that supplying the culture with the amino acids precursors changes the fengycin type; however, the study did not refer to the effect of those amino acids on fengycin production [42]. The effect of amino acids that are structural components of iturinA on the production was variable with each amino acid [21].

To explain the different rate of fengycin synthesis, promoter sequences from the four strains have been investigated. Promoter sequence of *B. subtilis* BBG21 appeared rather identical to the promoter sequence of its "mother" strain as only one nucleotide was different. In a previous study, it was established that the sequences in the promoter upstream from the *fenC gene* of *B. subtilis* F29-3 and the P*pps of B. subtilis* 168 promoters differed by one nucleotide in the -10 region[17]. In this work, we show that the difference is located neither in the -10 nor in -35 regions, but in UP element sequence. This sequence is very critical for the promoter activity and the mutation in this sequence can reduce the production of fengycin until 85% in *B. subtilis* F29-3 [17]. In addition Meyer *et al.* [43] reported that the change in the UP element sequence located upstream of the -35 boxes enhances promoter activity *in vivo* as well as *in vitro* in three *B. subtilis* 168 promoters. In a previous study, the promoter of BBG111 was replaced by promoter P*amyQ*, the production of fengycin increased about 10-fold in the mutant strain (Bs2508) grown in the optimal medium [44]. A similar result was obtained by replacing the native promoter of *B. subtilis* BBG111 by the P*fen* of *B. subtilis* BBG21, leading to a significant increase of fengycin production, while no increase in the fengycin yield was observed by replacing the native promoter of *B. subtilis* BBG111 by the P*fen* of *B. subtilis* ATCC 21332. The results obtained with the two mutants confirm that the point mutation in the UP element is responsible for the increase in fengycin yield in BBG21 strain. Moreover, another similar result was obtained when the *degQ* regulator was introduced into the strain 168, suggesting that this is a major factor in plipastatin operon regulation [15].

5. Conclusion

We can conclude from the sequence analysis and promoter expression data, that a point mutation in UP element can significantly influence the expression of fengycin operon and the fengycin yield. However, the promoter is not the sole factor involved in this complex regulation system. To our knowledge, this is also the first study that used the *gfp* expression as indicator of fengycin promoter activity. This system provides huge and accurate information that can be used as a database to optimize the lipopeptide production or to study lipopeptide biosynthesis in natural conditions (rhizosphere ecosystem, for example). Many conditions as well as many promoters could be investigated in a short time, allowing attaching the role of the promoters in the operon regulation without passing through the long procedure of lipopeptide production, extraction and estimation.

Conflict of interest

Authors declare that they have no conflict of interest

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References

[1] Ongena M, Jacques P, *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. Trends Microbiol 2008;16:115–25.

[2] Jacques P, Surfactin and other lipopeptides from *Bacillus* spp.*,* in Soberon-Chavez G (Ed) Biosurfactants, Microbiology Monographs, Springer-Verlag, Berlin Heidelberg,, 2011, vol. 20, pp 57-91.

[3] Hathout YA, Ho YP, Ryzhov V, Demirev P, Fenselau C, Kurstakins: a new class of lipopeptides isolated from *Bacillus thuringiensis*. J Nat Prod. 2000; 63:1492-6.

[4] Abderrahmani A, Tapi A, Nateche F, Chollet M, Leclère V, Wathelet B, et al. Bioinformatics and molecular approaches to detect NRPS genes involved in the biosynthesis of kurstakin from *Bacillus thuringiensis*. Appl Microbiol Biotechnol. 2011;92:571-81.

[5] Jacques P, Hbid C, Destain J, Razafindralambo H, Paquot M, De Pauw E, et al. Optimization of biosurfactant lipopeptide production from *Bacillus subtilis* S499 by Plackett-Burman design. Appl Biochem Biotechnol 1999;77-79:223–33.

[6] Chen XH, Koumoutsi A, Scholz R, Schneider K, Vater J, Süssmuth R, et al. Genome analysis of *Bacillus amyloliquefaciens* FZB42 reveals its potential for biocontrol of plant pathogens. J Biotechnol 2009;140:27–37.

[7] Huang QX, Lu ZX, Zhao HZ, Bie XM, Lu FX, Yang SJ, Antiviral activity of antimicrobial lipopeptide from *Bacillus subtilis* fmbj against Pseudorabies Virus, Porcine Parvovirus, Newcastle Disease Virus and Infectious Bursal Disease Virus in vitro. Int J Pept Res Therap 2006;12:373-7.

[8] Fracchia L, Cavallo M, Giovanna M, Banat IM, Biosurfactants and bioemulsifiers biomedical and related applications, Present Status and Future Potent, CC BY 3.0 license (2012).

[9] Yin H, Guo C, Wang Y, Liu D, Lv Y, Lv F , et al. Fengycin inhibits the growth of the human lung cancer cell line 95D through reactive oxygen species production and mitochondria-dependent apoptosis. Anticancer Drugs, 2013;24:587-98.

[10] Wei YH, Wang LC, Chen WC, Chen SY, Production and characterization of fengycin by indigenous *Bacillus subtilis* F29-3 originating from a potato farm. Int J Mol Sci, 2010;11:4526–38.

[11] Ramarathnam R, Bo S, Chen Y, Fernando WGD, Xuewen G, de Kievit T, Molecular and biochemical detection of fengycin- and bacillomycin D-producing *Bacillus* spp., antagonistic to fungal pathogens of canola and wheat. Can J Microbiol 2007;53:901–11.

[12] Steller S, Vollenbroich D, Leenders F, Stein T, Conrad B, Hofemeister J, et al. Structural and functional organization of the fengycin synthetase multienzyme system from *Bacillus subtilis* b213 and A1/3. Chem. Biol 1999;6:31–41.

[13] Tosato V, Albertini AM, Zotti M, Sonda S, Bruschi CV, Sequence completion, identification and definition of the fengycin operon in *Bacillus subtilis* 168. Microbiology 1997;143:3443–50.

[14] Wu CY, Chen CL, Lee YH, Cheng YC, Wu YC, Shu HY, et al. Nonribosomal synthesis of fengycin on an enzyme complex formed by fengycin synthetases. J Biol Chem 2007;282:5608–16.

[15] Tsuge K, Ano T, Hirai M, Nakamura Y, Shoda M, The genes *degQ*, *pps*, and *lpa-8* (*sfp*) are responsible for conversion of *Bacillus subtilis* 168 to plipastatin production. Antimicrob Agents Chemother, 1999;43:2183–92.

[16] Tsuge K, Matsui K, Itaya M, Production of the non-ribosomal peptide plipastatin in *Bacillus subtilis* regulated by three relevant gene blocks assembled in a single movable DNA segment. J Biotechnol. 2007;129(4):592-603.

[17] Ke XJ, Chang BY, Lin TP, Liu ST, Activation of the promoter of the fengycin synthetase operon by the UP element. J Bacteriol 2009;191:4615–23.

[18] Singh AK, Rautela R, Cameotra SS, Substrate dependent in vitro antifungal activity of *Bacillus* sp. strain AR2. Microb Cell Fact, 2014;13:67.

[19] Volpon L, Besson F, Lancelina JM, NMR structure of antibiotics plipastatins A and B from *Bacillus subtilis* inhibitors of phospholipase A2. FEBS Lett 2000;485:76-80.

[20] Islam R, Jeong T, Lee YS, Song SH, Isolation and Identification of antifungal compounds from *Bacillus subtilis* C9 inhibiting the growth of plant pathogenic fungi. Mycobiology 2012;40:59-66.

[21] Besson F, Chevanet C, Michel G, Influence of the culture medium on the production of iturin A by *Bacillus subtilis.* J Gen Microbiol 1987;3:767–72.

[22] Cameotra SS, Makkar RS, Kaur J, Mehta SK, Synthesis of biosurfactants and their advantages to microorganisms and mankind. Adv Exp Med Biol 2010;672:261–80.

[23] Chen XH, Koumoutsi A, Scholz R, Eisenreich A, Schneider K, Heinemeyer I, et al. Comparative analysis of the complete genome sequence of the plant growth–promoting bacterium *Bacillus amyloliquefaciens* FZB42. Nat Biotechnol 2007;25:1007–14.

[24] Koumoutsi A, Chen XH, Henne A, Liesegang H, Hitzeroth G, Franke P, Structural and functional characterization of gene clusters directing nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. J Bacteriol 2004;186:1084–96.

[25] Coutte F, Leclère V, Béchet M, Guez JS, Lecouturier D, Chollet-Imbert M, Effect of *pps* disruption and constitutive expression of *srfA* on surfactin productivity, spreading and antagonistic properties of *Bacillus subtilis* 168 derivatives. J Appl Microbiol 2010;109:480–91.

[26] Gancel F, Montastruc L, Liu T, Zhao L, Nikov I, Lipopeptide overproduction by cell immobilization on iron-enriched light polymer particles. Process Biochem, 2009;44:975–78.

[27] Chtioui O, Dimitrov K, Gancel F, Dhulster P, Nikov I, Selective fengycin production in a modified rotating discs bioreactor. Bioprocess Biosyst Eng, 2014;37:107-14.

[28] Fahim S, Dimitrov K, Gancel F, Vauchel P, Jacques P, Nikov I, Impact of energy supply and oxygen transfer on selective lipopeptide production by *Bacillus subtilis* BBG21. Bioresour Technol 2012;126:1-6.

[29] Sambrook J, Russell DW, Molecular Cloning: A Laboratory Manual, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 2001.

[30] Romero D, Perez-Garcia A, Veening JW, de Vicente A, Kuiper OP, Transformation of undomesticated strains of *Bacillus subtilis* by protoplast electroporation. J Microbiol Meth 2006;66:556-9.

[31] Cao G, Zhang X, Zhong L, Lu Z. A modified electro-transformation method for *Bacillus subtilis* and its application in the production of antimicrobial lipopeptides. Biotechnol. Lett 2011;33:1047-51.

[32] Mathew CD, Rathnayake S, Isolation and characterization of alpha amylase isolated from a hot water spring in Sri Lanka. Int Res J. Microbiol. 2014;5:50-61.

[33] Landy M, Warren GH, Rosenman SB, Colio LG. Bacillomycin an antibiotic from *Bacillus subtilis* active against pathogenic fungi. Proc Soc Exp Biol Med, 1948;67:530–41.

[34] HusseinW, Study on the regulation and biosynthesis of fengycin and plipastatin produced by *Bacillus subtilis.* PhD. Thesis, Lille1 University, France, 2011.

[35] Coutte F, Lecouturier D, Yahia SA, Leclère V, Béchet M, Jacques P, et al. Production of surfactin and fengycin by *Bacillus subtilis* in a bubbleless membrane bioreactor. Appl Microbiol Biotechnol. 2010;87:499–507.

[36] Fan B, Plant colonization by GFP-labeled *Bacillus amyloliquefaciens* FZB42 and transcriptomic profiling of its response to plant root exudates. PhD Thesis, Humboldt-Universität zu Berlin, Germany, 2011.

[37] Lewis PJ, Thaker SD, Errington J. Compartmentalization of transcription and translation in *Bacillus subtilis.* EMBO J 2000;15:710–8.

[38] Botella E, Sebastian H, Hokamp K, Hansen A, Bisicchia P. Cell envelope gene expression in phosphate-limited *Bacillus subtilis* cells. Microbiology 2011;157:2470–84.

[39] Soboleski MR, Oaks J, Halford WP, Green fluorescent protein is a quantitative reporter of gene expression in individual eukaryotic cells. FASEB J 2005;19:440-2.

[40] Nicolas P, Mäder U, Dervyn E, Rochat T, Leduc A, Pigeonneau N, et. al. Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis.* Science 2012;2:1103-6.

[41] Makkar RS, I.M Banat SS. Advances in utilization of renewable substrates for biosurfactant production. AMB Express 2011;1:5.

[42] Akpa E, Jacques P, Wathelet B, Paquot M, Fuchs R, Budzikiewicz H, et al. Influence of culture conditions on lipopeptide production by *Bacillus subtilis.* Appl Biochem Biotechnol 2001;93:551–61.

[43] Meijer WJ, Salas M. Relevance of UP elements for three strong *Bacillus subtilis* phage phi29 promoters. Nucleic Acids Res. 2004;32(3):1166-76.

[44] Ongena M, Jourdan E, Adam A, Paquot M, Brans A, Joris B. Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. Environ Microbiol, 2007:9:1084–90.

Chapter 4

Study of the correlation between fengycin operon expression and fengycin production under different culture conditions

General introduction

In the previous chapter, we verified the promoter activity by constructing several mutants integrating the GFP marker under the control of fengycin promoter in several strains. GFP marker showed a high sensitivity reading according to the different strains and conditions which influenced the promoter expression. The clear effect of the conditions previously analyzed on the promotor expression and lipopeptide production lead us to test the influence of the carbon and nitrogen sources in the culture medium.

Study of the correlation between fengycin operon expression and fengycin production by *Bacillus subtilis* under different culture conditions

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Abstract

Aims: To establish the correlation between fengycin operon expression and fengycin production under different culture conditions and to optimise fengycin production taking into account the study of this correlation.

Material and results: The strain *Bacillus subtilis* BBG208 (derived from a fengycin overproducing strain called BBG21, and harbouring the GFP protein under the control of fengycin promoter) was used to study the influence of different carbon and nitrogen sources on lipopeptide (surfactin and fengycin) production as well as the fengycin promoter expression. Significant promoter expression and fengycin as well as surfactin productions were obtained using starch or mannitol as carbon source and urea or a mix of urea and ammonium carbonate as nitrogen source. Some conditions (maltose, galactose and sorbitol) showed disaccording results between the production levels of surfactin and fengycin. Temperature, pH and oxygenation influenced the lipopeptide biosynthesis differently according to the provided substrates. Optimisation of the production medium enabled the fengycin production to be increased more than twice, to reach until 768 mg L^{-1}, the highest production reported from this strain.

Conclusion: This study permits to demonstrate that the nutrient conditions play an important role on the fengycin biosynthesis, either by their influence on the operon expression or at another step of this biosynthesis.

Significance and Impact of the Study: This study provides insightful information regarding fengycin production. This data will serve to scale-up production of lipopeptides and likely help to decrease the production costs.

Keywords: Fengycin, surfactin, promoter expression, carbon and nitrogen sources, culture conditions

Introduction

Gram positive bacteria belonging to the *Bacillus* genus are known to produce more than twenty different secondary metabolites with antagonistic activities (Sinchaikul *et al.* 2002; Chen *et al.* 2007). The non-ribosomally synthesized lipopeptides are the most important ones. These substances are cyclic peptides linked to a fatty acid chain. The lipopeptides group includes five families: surfactins/lichenysins/pumilacidins, fengycins/plipastatins, iturins (bacillomycins/iturins A/mycosubtilins), kurstakins and locillomycins (Hathout *et al.* 2000; Ongena and Jacques 2008; Chen *et al.* 2009; Luo *et al.* 2015). Fengycins are cyclic lipodecapeptides containing a β-hydroxy fatty acid chain of 16 to 19 carbon atoms. This family includes fengycins A and B, also named plipastatins, (Jacques *et al.* 1999; Ramarathnam *et al.* 2007; Raaijmakers *et al.* 2010; Wei *et al.* 2010). Non-Ribosomal Peptide Synthetases responsible for fengycins/plipastatins biosynthesis are encoded by five genes named *fenA* to *fenE* or *ppsA* to *ppsE* (Tosato *et al.* 1997; Steller *et al.* 1999; Wu *et al.* 2007). Fengycins have been characterized for their antibacterial, antiviral and antifungal properties (Tosato *et al.* 1997; Huang *et al.* 2006; Fracchia *et al.* 2012; Cawoy *et al.* 2015). Notably, fengycins are also endowed with anti-cancer activity by inhibiting the growth of human lung cancer cells (Yin *et al.* 2013; Ditmer *et al.* 2014). Environmental conditions appeared to influence the type and yield of the lipopeptides produced (Volpon *et al.* 2000; Islam *et al.* 2012). Regarding fengycin specifically, four cultivation parameters have been highlighted for their influence on its production: source of nutriments, oxygen transfer rate, temperature and pH (Pryor *et al.* 2007b, Jacques 2011; Fahim *et al.* 2012, Varadavenkatesan and Murty 2013, Zhu *et al.* 2014). The source of nitrogen was shown to strongly influence the biosurfactant synthesis, suggesting that fengycin synthesis was correlated with the nitrogen metabolism (Makkar and Cameotra 1998; Steller *et al.* 1999; Jacques 2011). A few studies were realized in order to establish the effect of these factors on the amount of fengycin produced. To our knowledge, there is no study correlating the fengycin promoter activity with the fengycin produced under different biotic or abiotic conditions. Recently, we cloned, the gene coding for the green fluorescent (GFP) protein under the control of the fengycin promoter strain in *B. subtilis* BBG21, a spontaneous mutant of *B. subtilis* ATCC 21332, overproducing fengycin. The resulting mutant strain was called BBG208 (Yaseen *et al.* 2016). According to its fluorescence stability and absence of adverse effect, the GFP protein was used as a promoter marker activity in a myriad of studies. In this work, we used GFP expression to study the effects of biotic factors (carbon, hydrocarbon and nitrogen sources) or abiotic factors (pH,

temperature and oxygen transfer rate) on fengycin biosynthesis regulation, by correlating the promoter expression and the fengycin production under every condition. In the last experiments, the fengycin biosynthesis was optimised.

Materials and methods

Media and strains used in this work

The Landy medium (Landy *et al.* 1948) containing 100 mM of 3-(N-morpholino) propanesulphonic acid (MOPS) was used as the basic medium for lipopeptide production. *B. subtilis* BBG208 was the strain used in all the experiments. This BBG21 derivative produces surfactin and fengycin, and carries the GFP ORF integrated within the *amyE* gene under the control of fengycin promoter as described in a previous work (Yaseen *et al.* 2016).

Assessment of lipopeptide production and promoter activity by using BioLector

The promoter expression was tested via the high-throughput system of fermentation BioLector (Mp2-labs GmbH, Baesweiler, Germany). Pre-cultures were effected at 30°C in 500 mL Erlenmeyer flasks containing 50 mL of Landy medium (10% filling volume). Cultures were carried out in 48 wells flower plate designed for the BioLector, containing pH, dissolved oxygen, fluorescence and biomass optodes in 1 mL of Landy medium at 30°C, 1100 rpm and initial pH of 7.0 during 72 h. All experiments were performed in triplicate wells for each condition and repeated at least twice and the standard deviation was less than 1% for all the means. Eleven carbon sources (at 20 g L^{-1}) were used to replace glucose in Landy medium, i.e., galactose, lactose, maltose, mannitol, sucrose, starch, fructose, arabinose, glycerol, mannose and sorbitol. Different nitrogen sources (at 5 g L^{-1}) were used to replace glutamic acid in Landy medium: urea, beef extract, peptone, casein hydrolysate, tryptone, seven different amino acids (alanine, proline, threonine, ornithine, valine, glycine and isoleucine) and several inorganic nitrogen sources including NH_4NO_3, NH_4HCO_3, $NaNO_3$, KNO_3 and NH_4HCO_3 mixed with urea. Vegetable and hydrocarbon oils (including sunflower oil, olive oil, paraffin oil and silicon oil), benzene and diesel were tested individually by adding them at 1% (v/v) into Landy medium. Biomass and optical density measurements for BioLector calibration were carried out as described previously (Yaseen *et al.* 2016).

Influence of physical and chemical parameters on fengycin production

An overnight culture of *B. subtilis* BBG208 grown in Landy MOPS medium was inoculated in Landy glucose (20 g L^{-1}) + urea (5 g L^{-1}) medium or Landy glutamic acid (5 g L^{-1}) + mannitol (20 g L^{-1}) medium with an initial OD_{600} of 0.05. Cultures were performed at 160 rpm, 10 % filling volume at 30°C. After 16 h of growth in Landy urea medium, the cultures were centrifuged at 2300 g, 20 °C for 10 min and the supernatants were discarded. Cells were inoculated in seven different media named C1 to C7 as described in Table 1, to quantify the lipopeptide production. In the same manner, after 15 h of growth in Landy mannitol medium, cultures were centrifuged and cells were inoculated in seven different media named C'1 to C'7 as described in Table 1. For all the cultures, the pH, OD_{600} and fengycin concentration were recorded at 24 and 48 h.

Influence of nitrogen and carbon sources on fengycin production

As previously described, the *B. subtilis* BBG208 was grown during 16 h in Landy MOPS glucose + urea to test nitrogen source or during 15 h in Landy MOPS glutamic acid + mannitol to test carbon source. Cultures were centrifuged at 2300 g, 20 °C for 10 min and the supernatants were discarded. According to the preculture (influence or carbon or nitrogen sources) cells were then replaced in fresh Landy MOPS media complemented with glutamic acid and one carbon source (glucose or maltose or sucrose) at 160 rpm, 10 % filling volume at 30°C or in fresh Landy MOPS media complemented with glucose and one nitrogen source (glutamic acid or a mix of glutamic acid or ammonium nitrate).

Influence of nitrogen or carbon source concentration on lipopeptide production

The influence of nitrogen concentration was tested using Landy medium complemented with glucose (20 g L^{-1}) and 4, 5, 6, 8 or 10 g L^{-1} of urea. The influence of carbon concentration was tested by using Landy medium complemented with glutamic acid (5 g L^{-1}) and 15, 20, 25, 35 or 45 g L^{-1} of mannitol.

Lipopeptide purification and quantification

1 mL of supernatant was extracted using C18 cartridges (Extract-clean SPE 500 mg, Grace Davison-Alltech, Deerfield, IL, USA). Lipopeptide productions were quantified by HPLC (Waters Corporation, Milford, MA, USA) using a C18 column (5 µm, 250 × 4.6 mm, VYDAC 218 TP, Hesperia, CA, USA). Analyses of fengycin were performed as previously described (Coutte *et al.* 2010).

Table 1. Experimental conditions for lipopeptide production by *B. subtilis* BBG208

Conditions	Nitrogen source ($5\ g\ L^{-1}$)	Carbon source ($20\ g\ L^{-1}$)	Filling Volume (%)	Initial pH	Temperature
U1	Urea	Glucose	10	7.0	25°C
M1	Glutamic Acid	Mannitol	10	7.0	25°C
U2	Urea	Glucose	10	7.0	30°C
M2	Glutamic Acid	Mannitol	10	7.0	30°C
U3	Urea	Glucose	10	7.0	37°C
M3	Glutamic Acid	Mannitol	10	7.0	37°C
U4	Urea	Glucose	30	7.0	30°C
M4	Glutamic Acid	Mannitol	30	7.0	30°C
U5	Urea	Glucose	50	7.0	30°C
M5	Glutamic Acid	Mannitol	50	7.0	30°C
U6	Urea	Glucose	10	7.5	30°C
M6	Glutamic Acid	Mannitol	10	7.5	30°C
U7	Urea	Glucose	10	6.5	30°C
M7	Glutamic Acid	Mannitol	10	6.5	30°C

Results

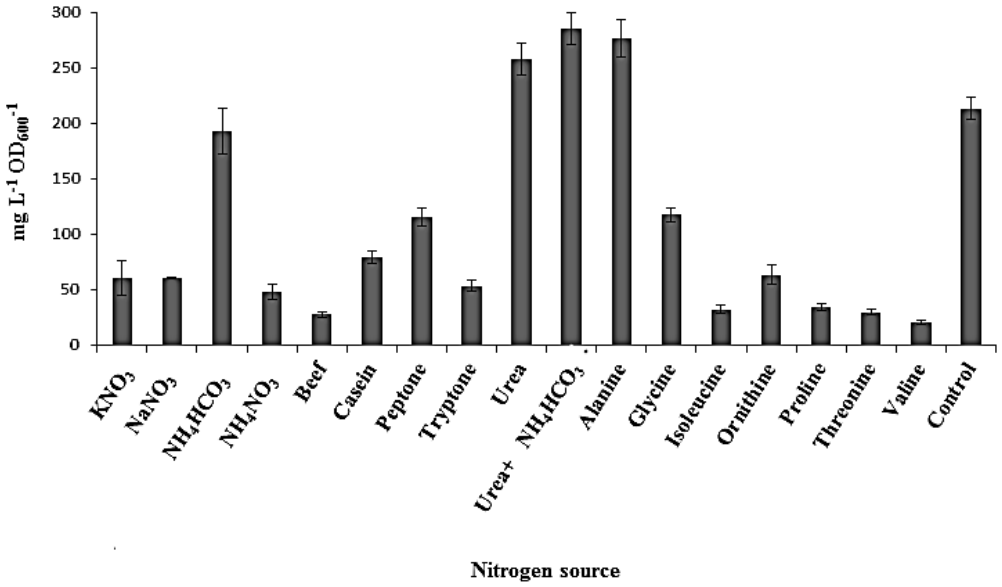
High-throughput screening of the influence of culture conditions on lipopeptide production

In order to study the effect of several substrates on surfactin and fengycin productions and on fengycin operon promoter expression by *B. subtilis* BBG208, 18 nitrogen sources, 12 carbon sources and six hydrocarbon and vegetable sources were tested in Landy MOPS medium. The seven amino acids residues tested as nitrogen sources are precursors of the fengycin peptide chain. Most of the other substrates have been mentioned in previous studies to have a positive effect on the lipopeptide production.

Surfactin production

The strain BBG208 is a co-producer of surfactin and fengycin. First, the specific production of surfactin was measured under the 36 conditions after 72 h of culture. The results are shown in Fig 1. There was no significant difference in the growth rate under the different conditions (data not shown). When the effect of nitrogen source in the medium was evaluated, the surfactin specific production was ranging from 20.34 to 285 mg L^{-1} OD_{600}^{-1}. The conditions showing the highest specific productions were urea, urea with ammonium carbonate and alanine, i.e., 257.8 mg L^{-1} OD_{600}^{-1} (surfactin production: 2.1 g L^{-1}), 285 mg L^{-1} OD_{600}^{-1} (surfactin production: 2.43 g L^{-1}) and 276 mg L^{-1} OD_{600}^{-1} (surfactin production: 2 g L^{-1}), respectively. A second set of interesting nitrogen sources were glutamic acid (213 mg L^{-1} OD_{600}^{-1}), usually recognized as a central point in the nitrogen metabolism and present in the Landy medium and ammonium carbonate (192 mg L^{-1} OD_{600}^{-1}). Peptone, casein and glycine gave intermediary results while the nitrate salts, the other amino acid residues, the beef extract and the tryptone led to low specific production of surfactin (Fig. 1.a). Most of the carbon sources led to a specific production of 100 to 224 mg L^{-1} OD_{600}^{-1}. However, when galactose or arabinose was used, production dropped to 30 mg L^{-1} OD_{600}^{-1}. Lactose gave logically average production of those obtained with glucose and galactose. Interestingly, maltose was less efficient than glucose but also than starch (Fig. 1.b). In the media supplemented with 1% vegetable and hydrocarbon sources, surfactin production did not increase as compared to reference Landy medium (Fig. 1.c).

a.



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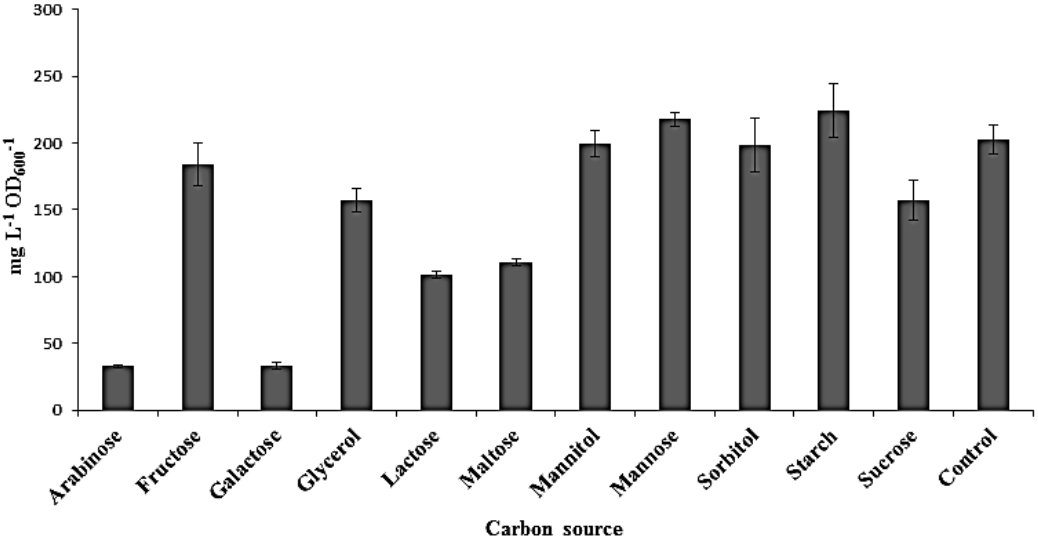


c.

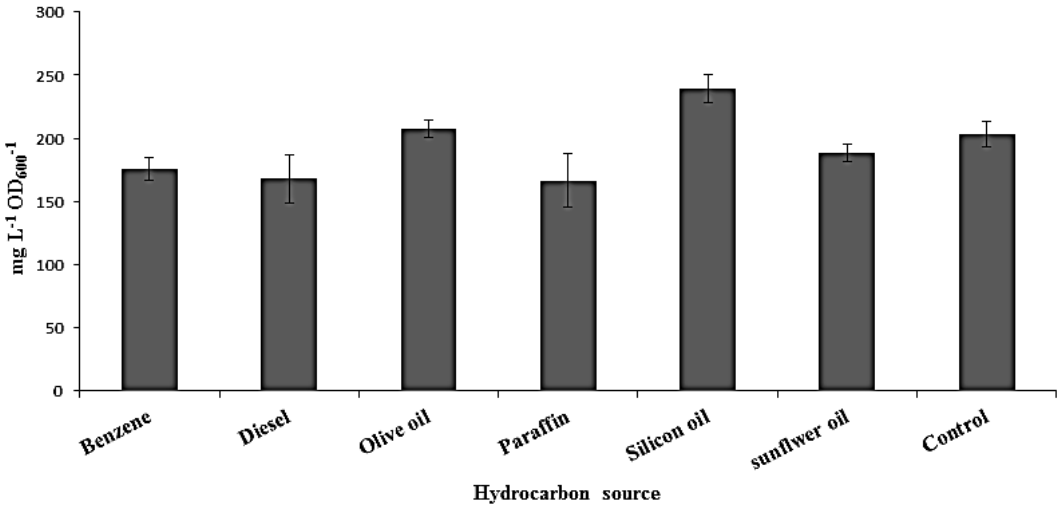


Fig. 1. Surfactin production by *B. subtilis* BBG208 after 48 h of growth in the presence of different substrates

a. Nitrogen sources: Landy medium (control) was modified by replacing the glutamic acid with 5 g of 17 different nitrogen sources: KNO_3, $NaNO_3$, NH_4HCO_3, NH_4NO_3, Beef extract, Casein, Peptone, Tryptone, Urea, Urea (2.5g)+ NH_4HCO_3 (2.5 g), Alanine, Glycine, Isoleucine, Ornithine, Proline, Threonine, Valine.

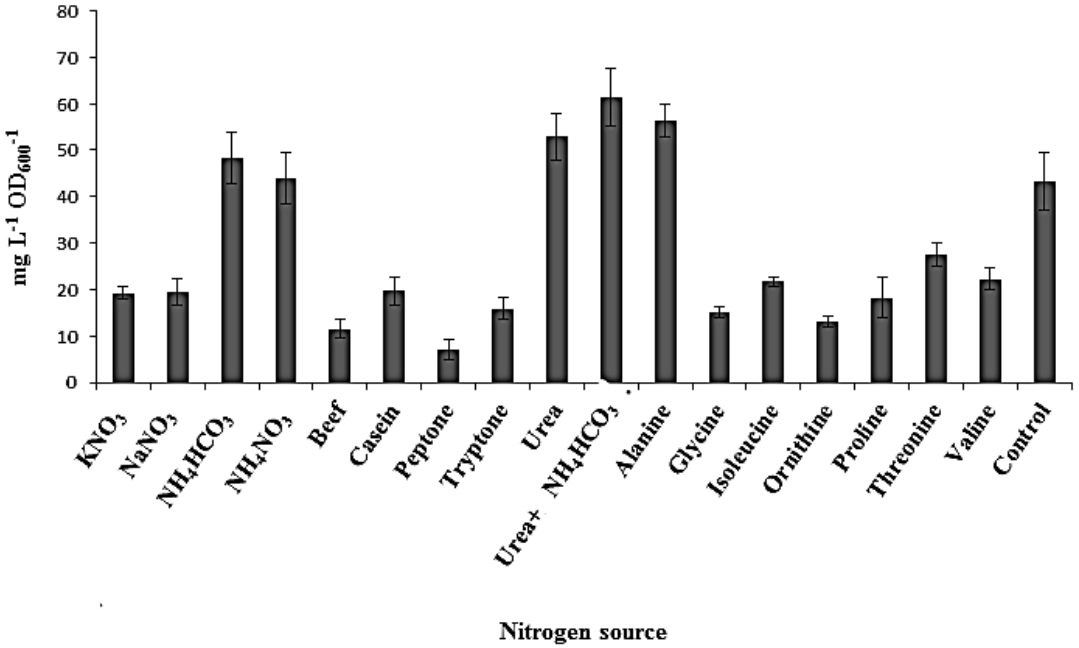
b. Carbon sources: Landy medium (control) was modified by replacing the glucose with 20 g of 11 different carbon sources: Arabinose, Fructose, Galactose, Glycerol, Maltose, Mannitol Mannose, Lactose, Sorbitol, Starch, and Sucrose.

c. Addition of hydrocarbon sources: Landy medium (control) was modified by supplying the medium with 1% of 6 different hydrocarbon sources: Benzene, Diesel, Olive oil, Paraffin, Silicon oil, Sunflower oil.

Fengycin production

The synthesis of fengycin was also measured under the conditions described above. When the effect of nitrogen source in the medium was evaluated, fengycin specific production was ranging from 7 to 61.4 mg L^{-1} OD_{600}^{-1}. As for surfactin, the highest productions were obtained with urea, urea + NH_4CO_3, NH_4CO_3, alanine and glutamic acid (52, 61.46, 48.39, 56.38 and 43.3 mg L^{-1} OD_{600}^{-1}, respectively). Contrary to surfactin, NH_4NO_3 also led to high specific production of fengycin (43.8 mg L^{1} OD_{600}^{-1}). Low productions were observed for casein, tryptone, beef extract and peptone, while less than 28 mg L^{-1} OD_{600}^{-1} were produced using the other amino acids than alanine and glutamic acid (Fig. 2.a). Among the 12 different carbon sources, mannitol significantly enhanced the production (77.7 mg L^{-1} OD_{600}^{-1}) with about 17-fold more than the lowest production level obtained with mannose (5.7 mg L^{-1} OD_{600}^{-1}). The production was 47.7 mg L^{-1} OD_{600}^{-1} when using starch as carbon source. The production with the disaccharides (maltose, sucrose and lactose) was between 37 and 44 mg L^{-1} OD_{600}^{-1}. With arabinose or sorbitol, production was less than 20 mg L^{-1} OD_{600}^{-1}. (Fig. 2.b). When unusual carbon sources (diesel, benzene, sunflower oil, silicon oil, olive oil, and paraffin) were added (1%) to the medium, a slight increase was observed with sunflower oil (53.6 mg L^{-1} OD_{600}^{-1}) compared to the control. Equivalent production to the control was obtained with diesel and olive oil. Using the media containing paraffin, silicon oil benzene or olive oil, the productions were under 30 mg L^{-1} OD_{600}^{-1} (Fig. 2.c).

a.



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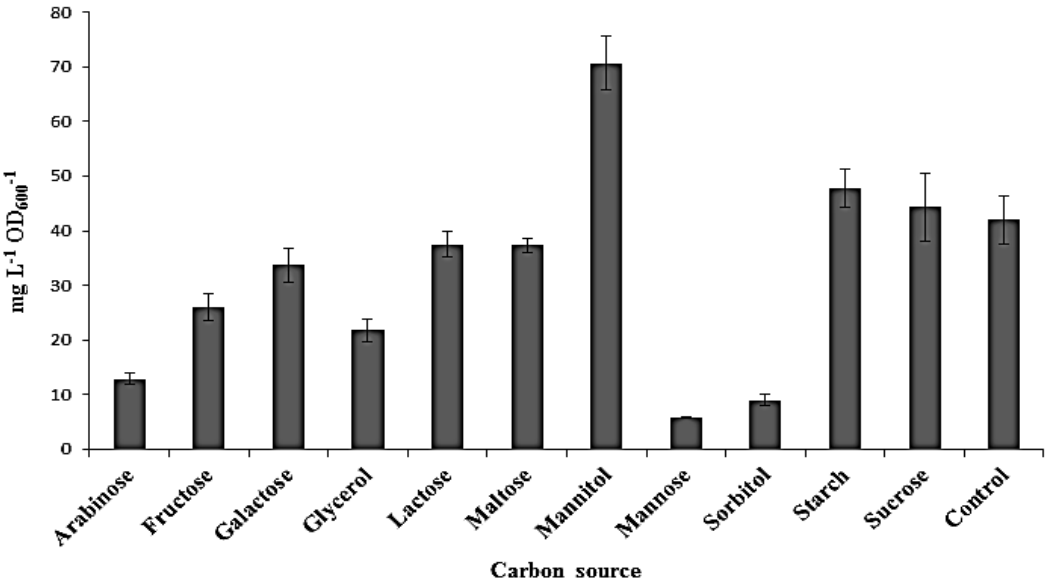

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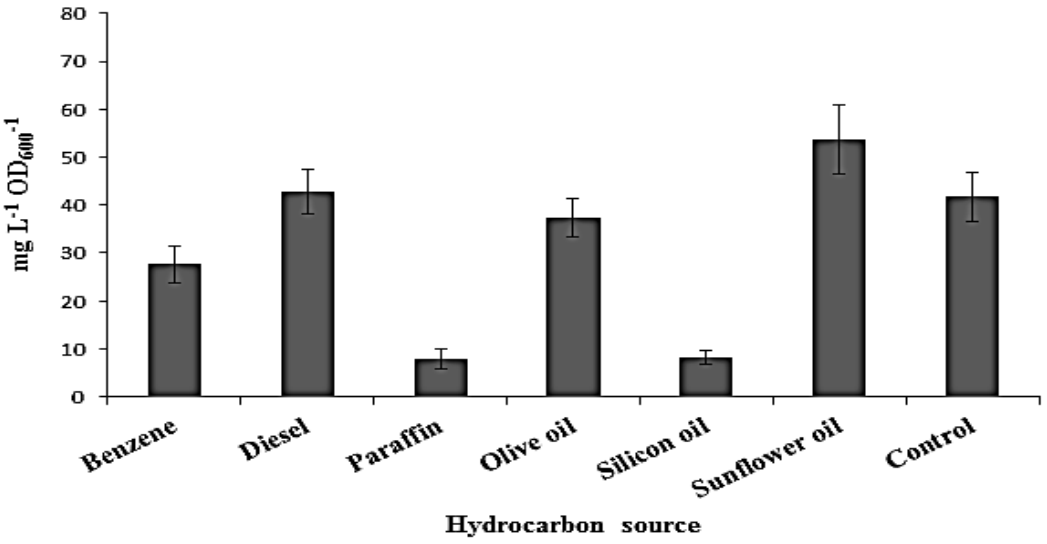


Fig. 2. Fengycin production by *B. subtilis* BBG208 after 48 h of growth in the presence of different substrates

a. Nitrogen sources: Landy medium (control) was modified by replacing the glutamic acid with 5 g of 17 different nitrogen sources: KNO_3, $NaNO_3$, NH_4HCO_3, NH_4NO_3, Beef extract, Casein, Peptone, Tryptone, Urea, Urea (2.5g)+ NH_4HCO_3 (2.5 g), Alanine, Glycine, Isoleucine, Ornithine, Proline, Threonine, Valine.

b. Carbon sources: Landy medium (control) was modified by replacing the glucose with 20 g of 11 different carbon sources: Arabinose, Fructose, Galactose, Glycerol, Lactose, Maltose, Mannitol Mannose, Sorbitol, Starch, Sucrose.

c. Addition of hydrocarbon sources: Landy medium (control) was modified by supplying the medium with 1% of 6 different hydrocarbon sources: Benzene, Diesel, Olive oil, Paraffin, Silicon oil, Sunflower oil.

Fengycin operon promoter activity

Surfactin regulation has been well studied while only little information is available concerning the fengycin biosynthesis regulation. This fact encouraged us to focus on the production of fengycin by comparing it with the fengycin operon promoter expression under the same tested conditions. In BBG208, the fused P*fen::gfp* cassette was integrated at the *amyE* gene. Using the BioLector device, it is possible to follow on-line the fluorescence emitted by GFP and thus the expression of the operon in the presence of the different nitrogen, carbon or hydrocarbon and vegetable sources tested in order to compare the fengycin production with the fengycin operon expression. Interestingly, analysis of the production and promoter activity showed that a high expression level does not always correspond to a high fengycin production. Some conditions permitted to stimulate the promoter although the production was not significantly important or on the contrary, a good production was obtained in spite of a moderate gene expression.

Nitrogen sources

The promoter expression under different nitrogen sources is shown in Fig. 3. The highest fluorescence intensity was registered with urea (1.9 au OD_{600}^{-1}), urea + NH_4HCO_3 (1.48 au OD_{600}^{-1}) and alanine (1.24 au OD_{600}^{-1}). Under these conditions, the promoter expression was correlated to high levels of fengycin production. On the contrary, low expression (0.3 to 0.66 au OD_{600}^{-1}) was obtained with glutamic acid, NH_4NO_3, and NH_4HCO_3, even though the fengycin production under the same conditions reached 43 to 48 mg L^{-1} OD_{600}^{-1}. Under other conditions, there was no significant expression and synthesis (Fig. 3.a). Measuring the fengycin promoter activity enabled us to conclude that the expression started after 10 h of cultivation in Landy urea medium, and, in most of the tested conditions, stopped (0.6 au $OD_{600}^{-1}\Delta t^{-1}$) upon 18 and 20 h. Under most of the conditions, the pH level decreased after 14 h of incubation to the minimum value after 30 h before it raised up again to achieve a maximum level in the end of the culture incubation. The highest level was recorded with urea (pH 8.1) after 36 h and the lowest level was also obtained after 36 h with NH_4HCO_3 (pH 5.8). Under the other conditions, the pH level was between 6.2 to 7.2 (data not shown).

Carbon sources

The highest promoter expression was obtained with starch (1.2 au OD_{600}^{-1}), whereas the production under this condition was only 61% of that obtained with mannitol. Differences in *fen* genes expression and fengycin production were observed with mannitol, lactose, glucose

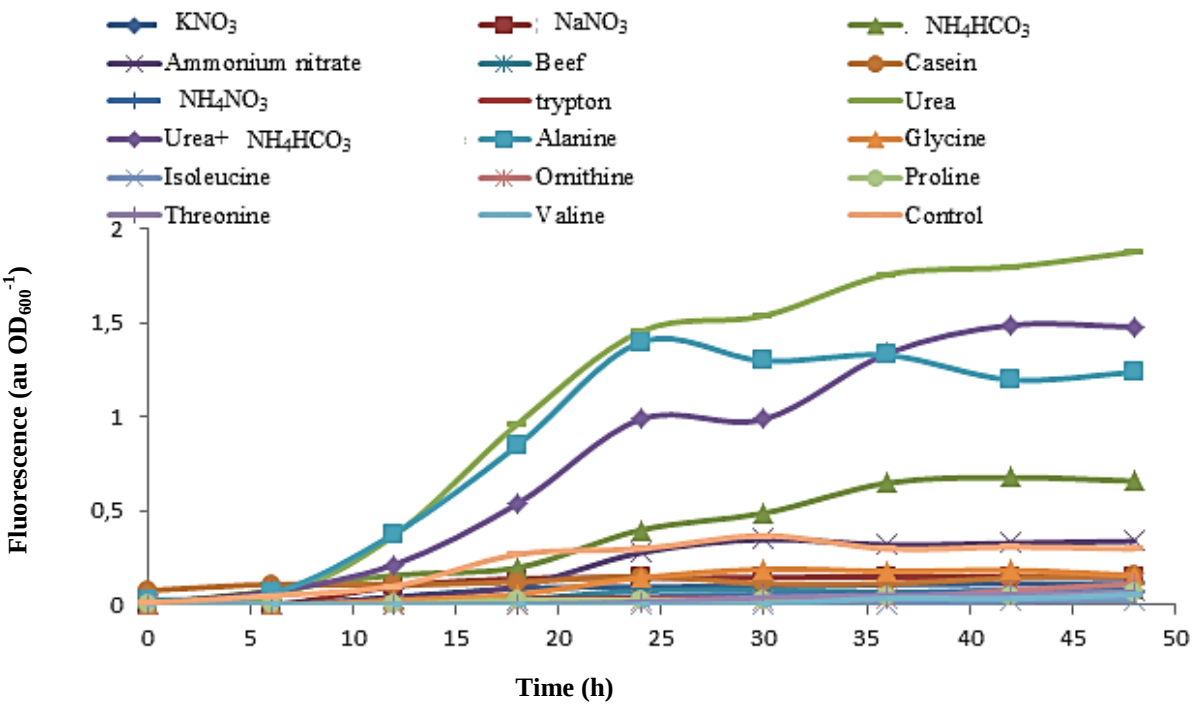
and sucrose. Indeed, with mannitol which showed the highest fengycin specific production, promoter expression reached only 0.63 au OD_{600}^{-1}, unlike the expression with lactose (0.8 au OD_{600}^{-1}) which was correlated with a low fengycin production (37 mg L^{-1} OD_{600}^{-1}). Expression with sucrose was nearly half of those obtained with glucose but productions remained identical (40 mg L^{-1} OD_{600}^{-1}) (Fig. 3.b).

For most of the conditions, the expression stopped at 18 h. The highest promoter activity was recorded in Landy starch at 22h while it was between 16 and 18 h in Landy mannitol. During the fermentation, the changes in the pH were not important, the pH remaining under all the conditions between 6.7 and 7.2 (data not shown).

Influence of the addition of different hydrocarbon sources

The effect of adding different hydrocarbon sources (1%) on the promoter expression was also analyzed (Fig. 3.c). The best promoter expression was obtained in Landy complemented with sunflower oil (0.78 au OD_{600}^{-1}). The expression was about twice more important than that obtained in the control assay (Landy medium), while the specific fengycin production was about 1.28-fold more important than that in the control medium. No significant difference in the expression level under the other conditions could be observed. The changes in the pH were not important, the pH remaining under all the conditions between 6.7 and 7.2 (data not shown).

a.



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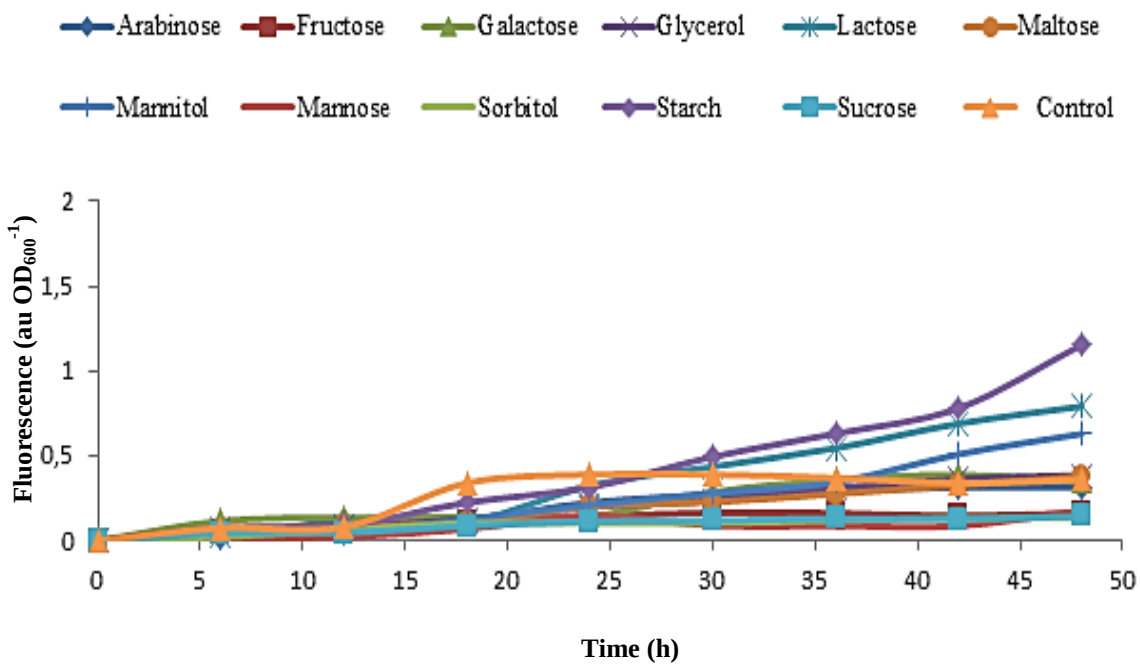

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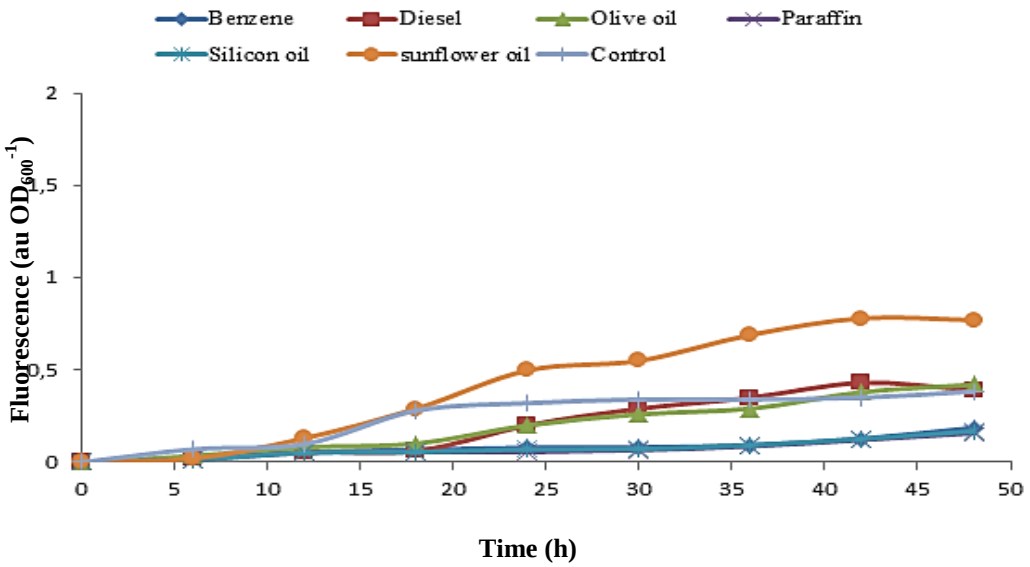


Fig. 3. Fengycin promoter expression by *B. subtilis* BBG208 after 48 h of growth in the presence of different substrates (All experiments were performed in triplicate wells for each condition and the values of standard deviation were less than 1%):

a. Nitrogen sources: Landy medium (control) was modified by replacing the glutamic acid with 5 g of 17 different nitrogen sources: KNO_3, $NaNO_3$, NH_4HCO_3, NH_4NO_3, Beef extract, Casein, Peptone, Tryptone, Urea, Urea (2.5g)+ NH_4HCO_3 (2.5 g), Alanine, Glycine, Isoleucine, Ornithine, Proline, Threonine, Valine.

b. Carbon sources: Landy medium (control) was modified by replacing the glucose with 20 g of 11 different carbon sources: Arabinose, Fructose, Galactose, Glycerol, Lactose, Maltose, Mannitol, Mannose, Sorbitol, Starch, Sucrose.

c. Addition of hydrocarbon sources: Landy medium (control) was modified by supplying the medium with 1% of 6 different hydrocarbon sources: Benzene, Diesel, Olive oil, Paraffin, Silicon oil, Sunflower oil.

Fengycin production optimisation

Effects of temperature, pH and aeration rate

As the highest expression was obtained with urea, this nitrogen source was used with glucose in order to observe the influence of different physiological and chemical conditions on the fengycin production. Separately, mannitol was used also as carbon source with glutamic acid as nitrogen source. After 16h of cultivation, the cells were centrifuged and transferred in fresh media under different aeration rates, initial pHs or temperatures as shown in Table 1. In the presence of urea, the highest fengycin production was obtained after 48 h at 30°C (550 mg L^{-1}), with a filling volume of 10% at pH7.0 (C2) (Table 2). When the temperature was 25 or 37°C, the production was lower. The reduction in the oxygen rate and the modification of initial pH of the medium influenced also the production. Interestingly with mannitol, the best production was obtained after 48 h at 25 °C (C'1) with 588 mg L^{-1} (Table 3). At 30°C, a high aeration rate and an initial pH of 7.0 were the best conditions but the influence of the initial pH was less important than in the presence of urea.

Table 2. Influence of temperature, pH and aeration rate on growth and fengycin production by *B. subtilis* BBG208 in the presence of urea

Culture conditions*	Fengycin (mg L^{-1})	Biomass (OD_{600})	Relative fengycin yield (mg L^{-1} OD_{600}^{-1})	Final pH
U1	368 ± 22	9.6 ± 0.6	38.3	8.0
U2	550 ± 15	10.5 ± 0.6	52.2	8.1
U3	380 ± 14	10.5 ± 1.1	35.5	7.7
U4	332 ± 13	10.1 ± 0.9	32.8	8.5
U5	328 ± 15	10.4 ± 0.7	31.5	8.3
U6	187 ± 11	9.2 ± 1.2	20.3	8.7
U7	345 ± 12	9.0 ± 1.2	38.3	8.3

**Culture conditions are described in table 1.*

Table 3. Influence of temperature, pH and aeration rate on growth and fengycin production by *B. subtilis* BBG208 in the presence of mannitol.

Culture conditions*	Fengycin (mg L^{-1})	Biomass (OD_{600})	Relative fengycin yield (mg L^{-1} OD_{600}^{-1})	Final pH
M1	588 ± 31	10.2 ± 0.3	57.6	7.3
M2	532 ± 25	10.2 ± 1.0	52.2	6.6
M3	240 ± 14	9.0 ± 1.1	26.6	6.4
M4	488 ± 17	10.7 ± 0.7	45.6	7.1
M5	328 ± 15	9.4 ± 0.8	34.9	7.3
M6	510 ± 11	9.8 ± 0.8	52.1	7.1
M7	398 ± 12	11.0 ± 1.2	36.1	6.1

**Culture conditions are described in table 1.*

Influence of urea and mannitol concentrations

To optimise the fengycin production we took into account the previous results, and two sets of experiments were performed. In the first set, mannitol was added (15, 20, 25, 35 or 45 g L^{-1}) in Landy media with glutamic acid (5 g L^{-1}). In the second set urea was used (4, 5, 6, 8 or 10 g L^{-1}) in Landy media with glucose (20 g L^{-1}). The best specific fengycin production was obtained by adding 20 or 25 g L^{-1} of mannitol (60.5 mg L^{-1} OD_{600}^{-1}), whereas 5 or 6 g L^{-1} of urea in the culture was enough to produce the highest fengycin concentration with 43.8 mg L^{-1} OD_{600}^{-1} (Fig. 4). Moreover, 78.5 mg L^{-1} OD_{600}^{-1} (648 mg L^{-1}) of fengycin was obtained using Landy medium with 6 g L^{-1} of urea and 25 g L^{-1} of glucose as optimal culture condition (data not shown).

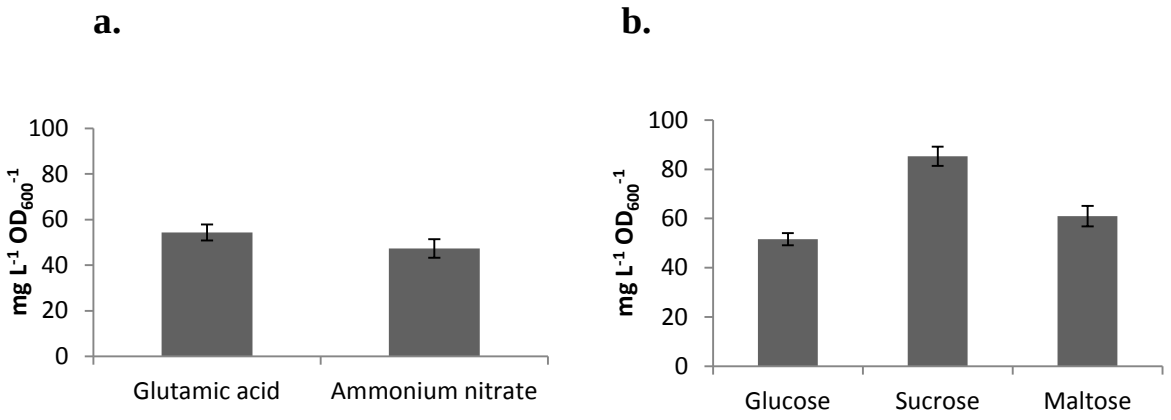


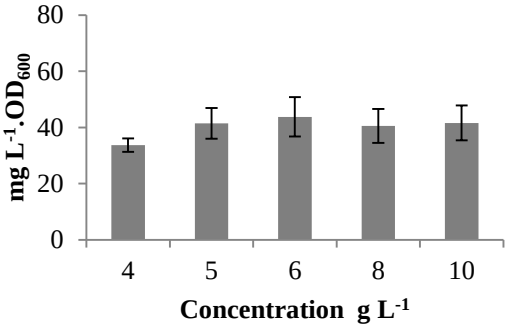
Fig. 5. Effect of successive cultivations in the presence of two different nitrogen or carbon sources on specific fengycin production by *B. subtilis* BBG208: **a.** Nitrogen sources and **b.** Carbon sources.

B. subtilis BBG208 was grown during 16 h in Landy glucose + urea to test nitrogen sources **(a)** or during 15 h in Landy glutamic acid + mannitol to test carbon sources **(b)**. Cultures were centrifuged at 2300 g for 10 min and the supernatants were discarded. According to the preculture (influence or carbon or nitrogen sources), the cells were then replaced in fresh Landy medium (control) or in a Landy medium in which glucose is replaced by maltose or sucrose, and incubated at 160 rpm, with 10 % filling volume at 30°C or in fresh Landy medium or in a Landy medium in which glutamic acid was replaced by ammonium nitrate.

Cultures with two successive different nitrogen or carbon sources

After 16 h or 15 h of growth in Landy urea or Landy mannitol culture medium, the cells were centrifuged and resuspended in five fresh different Landy media with two other nitrogen sources or three other carbon sources, respectively. These sources are selected for their ability to favour fengycin production but not fengycin operon expression. Optimal temperature (30°C), initial pH (7.0) and filling volume (10 %) were selected as described in Materials and Methods. The successive use of urea and glutamic acid or ammonium nitrate, or mannitol and glucose or maltose did not significantly increase the specific fengycin production (Fig. 5). But the successive use of mannitol and sucrose increase the specific fengycin production of 20,7 % (85.3 mg L^{-1} OD_{600}^{-1}) compared to the use of mannitol only (70.7 mg L^{-1} OD_{600}^{-1}). In these conditions, the fengycin production is also the highest obtained (768 mg L^{-1}).

a.



b.

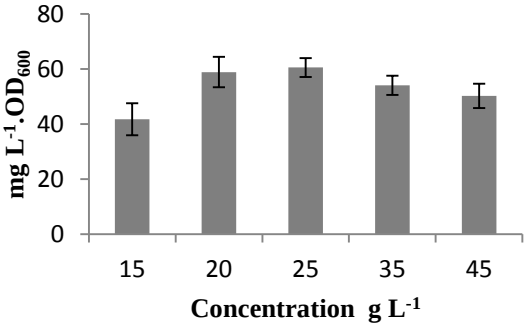


Fig. 4. Specific fengycin production by *B. subtilis* BBG208 after 48 h of growth in the presence of five different concentrations of: a. Urea and b. Mannitol.

Discussion

Production of secondary metabolites such as lipopeptides is governed by complex cascades of mechanisms involving the different steps of their biosynthesis. Five main steps can be easily identified: the transcription of the operon, the translation of the mRNA in the NonRibosomal Peptide Synthetases, the post-translation modification of NRPS from apo-protein in holo-protein by the phosphotransferase encoded by the *sfp* gene, the biosynthesis of the lipopeptide by the multienzyme assembly line machinery, and the excretion of the compound by a transporter. Most of the previous works dedicated to the optimisation of the lipopeptide production completely ignore these different steps, considering the all process as a black box. In this work, we undertook, using GFP reporter genes and BioLector device, to screen the influence of a high set of 38 substrates on both operon expression and lipopeptide production. The choice of different conditions was inspired from several publications in literature showing in different strains, the influence of these substrates on the production of different lipopeptides. We also decided to check the feeding of fengycin precursors, such as the amino acid residues of the peptide chain. This strategy should lead to distinguish between parameters influencing the first step of the synthesis from others playing a role in the following steps especially the supply of precursors to the synthetases. This work should also lead defining new strategy of culture for overproducing these compounds. We decided to focus our work on fengycin, a lipopeptide displaying several interesting biological activities (antifungal, immunomodulatory, anticancer compounds and inducers of systemic resistance in plants), and whose regulation of its production was not well described in literature. The strain we used was a derivative of BBG21, a spontaneous mutant of ATCC 21332, able to produce increased amounts of fengycin. This strain is a co-producer of surfactin and fengycin and thus the opportunity of this high-throughput analysis of 36 conditions was taken to compare their effect on both lipopeptide production. The ratio of specific production of surfactin/fengycin varies between 1 and 36 depending on the substrate tested. The biggest difference was observed with mannose which clearly favors surfactin production and with valine which leads to a more equivalent production between both lipopeptides but with low concentrations. Singh *et al.* (2014) also showed that some environmental conditions can orient the production of a specific lipopeptide at the expense of the other one. Recently, we also observed a significant enhancement of surfactin production in a mutant disrupted in fengycin operon (Yaseen *et al.* 2016). Previous studies on *B. subtilis* ATCC 21332 stated that surfactin regulation depends on the nutrient sources provided in the medium, and was

strongly influenced by the conditions of nitrogen metabolism (Davis *et al.* 1999). Further, Abushady *et al.* (2005) reported that ammonium salts and urea are among the preferred nitrogen sources for surfactin production; the positive influence of urea and ammonium carbonate was confirmed in the present study for both lipopeptides. From an industrial point of view, these nitrogen sources are less expensive than glutamic acid which is currently used for lipopeptide production. Surfactin and fengycin differ in their amino acid composition: surfactin contains 1 Asp, 1 Glu, 1 Val/Ala/Leu/Ile, 2 Leu and 2 Leu/Ile/Val, while fengycin is composed of 1 Orn, 1 aThr, 1 Pro, 1 Ile, 1 Gln, 2 Tyr, 2 Glu and 1 Ala/Val. Except for proline, the positive effect of which could be linked to the presence of proline in fengycin, the influences of the other amino acid residues are more complex to be analysed. For example, the role of the different branched amino acid residues such as valine, leucine and isoleucine which are substrates of both lipopeptide synthetases but which are also known for their role in the modulation of the *codY* regulator which represses the surfactin operon directly and indirectly via the *Ilv-Leu* operon (Handke *et al.* 2008; Belitsky, 2015). Ammonium nitrate is more favourable to fengycin production. This mineral source of nitrogen was shown to be favourable to surfactin production (Cooper *et al.* 1981) but with a surfactin monoproducing strain. It could play a role in oxygen limitation conditions to modify the metabolism of the cells (Kim *et al.*, 1997). This result has to be correlated with those obtained by Fahim *et al.* (2012) with the strain BBG21, who have shown that oxygen transfer rate can modulate the ratio of surfactin/fengycin. A moderate oxygen transfer rate is favourable to fengycin biosynthesis. To end with this influence of substrates on surfactin/fengycin ratio, we have to highlight that mannose, sorbitol, silicon oil and paraffin are more favourable to surfactin production. Abushady *et al.* (2005) found that using glucose, sucrose and mannose as carbon source in the medium enhanced the production of high yield of surfactin from *B. subtilis* BBK1. When starch was used as a carbon source to optimise the surfactin production from *B. subtilis* NEL-01, the synthesis achieved 1,879 mg L^{-1}(Zheng *et al.* 2013). Abdel-Mawgoud *et al.* (2008) showed that using galactose and lactose increased surfactin production, which was not the case here, and confirms that the influence of environmental factors on lipopeptide production is strain-dependent.

The second part of this study aimed to correlate the influence of environmental factors on both fengycin operon expression and fengycin production. Thus, the influences on the fengycin promoter activity (via the GFP expression) of the previous conditions were tested. Regarding the nitrogen source used, four (urea, urea + ammonium carbonate, alanine and ammonium carbonate) of the six best substrates for fengycin production also showed the best

positive effect on the fengycin operon expression. Interestingly the fifth one, glutamic acid, frequently used for lipopeptide production, did not lead to a high expression. The last one, ammonium nitrate also exerts its positive influence on fengycin production at another level than transcription. Globally, the main significant expression was obtained with urea, urea with ammonium carbonate or alanine as the sole nitrogen source in medium. Indeed, a 20-fold higher expression was obtained with Landy urea than Landy beef extract, while no significant difference in biomass was observed between the two conditions. Mols and Abee (2008) reported that *B. subtilis* catalyses the conversion of urea into ammonia. NH_4 was found to be the main nitrogen source absorbed by *B. subtilis* ATCC 21332 and the use of NH_4 increased *B. subtilis* cell growth (Makkar and Cameotra 1997; Huang *et al.* 2015). This ammonia release could also slightly increase the intracellular pH. The alkaline pH was shown to positively influence surfactin expression (Cosby *et al.* 1998). According to Mukherjee and Das (2005), the use of ammonium as nitrogen source could allow the best biosurfactant production in *B. subtilis* DM-03 and the same result was obtained with *B. subtilis* ATCC 21332, which preferred this nitrogen source due to the short assimilation pathway of ammonium (Huang *et al.* 2015). But urea may influence the fengycin promoter expression in other ways. The use of urea as sole nitrogen source may lead to the activation of a switching signal to the secondary metabolism that promoted the synthesis of fengycin.

It is known that the *degQ* gene is one of the fengycin synthesis regulatory factors (Wang *et al.* 2015). According to an early study by Msadek *et al.* (1991), two sets of growth limiting conditions, amino acid deprivation and poor carbon sources, increased strongly *degQ* gene expression.

This gene is also one of the targets of *codY* which can repress about 200 other genes, and also interact with the *sfp* gene activity which is essential for fengycin production. The decrease in *codY* repressive activity occurs under certain culture conditions, thereby activating genes such as *sfp* and *degQ*. (Wray *et al.* 1997 ; Molle *et al.* 2003; Vargas-Bautista *et al.* 2014). That could also explain the low fengycin concentration obtained using valine and isoleucine, the branched chain amino acids which activate *codY* (Belitsky 2015).

Compared to the other amino acid tested in this study, alanine was the best nitrogen source to enhance the production. This could result from an efficient alanine dehydrogenase which catalyzes the reversible conversion of alanine to its keto acid pyruvate, with reduction of NAD^+ and release of ammonia (Allaway *et al.* 2000).

Among the 12 carbon sources tested in Landy medium, starch, lactose, mannitol and glucose improved the promoter expression. The highest fengycin production was obtained with

mannitol (14-fold more than the lowest one and 1.6-fold more than with glucose). Mannitol was stated in several studies to be a preferable carbon source for fengycin production (Besson *et al.* 1998; Wei *et al.* 2010; Islam *et al.* 2012). In the presence of glucose, the reference carbon source for *Bacillus* growth, the level of fengycin operon expression reach a maximum value after 18h which is faster than with the other sources. It should be pointed out that starch is a good promoter activator but is not the best carbon source for the production. For several *B. subtilis* strains, starch was reported to be the best source for the lipopeptide production (Zheng *et al.* 2013). As reported similarly in *B. subtilis* ATCC 21332, starch is difficult to metabolise according to its low aqueous solubility even with the presence of amylase gene (Thompson *et al.* 2001). Conversely, strains are stimulated to produce higher amount of lipopeptides, which might help to adapt to the low aqueous solubility of the substrate. The addition of crude lipopeptides in the culture increases the growth rate and the biosurfactants production in *B. subtilis* ATCC 21332 (Mukherjee and Das 2005).

Recently, it was reported that the fengycin operon is regulated by the PhoP regulon which represses or activate the expression of phosphate-regulated genes (Guo *et al.* 2010; Dong *et al.* 2014). This regulon was indirectly influenced by the carbon source available in the medium because PhoP represses CcpA, which functions as a DNA binding protein, either activating or repressing many genes in the presence of particular carbon source (Puri-Taneja *et al.* 2006). The complex metabolism of the carbon sources in *B. subtilis* and the expected regulation interacting with the lipopeptide syntheses can give many hypotheses to explain the effect of each carbon source on the fengycin production.

The use of sunflower oil was reported as improving biosurfactant production from *Bacillus* sp. (Ghribi and Ellouze-Chaabouni 2011; Rajendran *et al.* 2014). In the present study, sunflower oil as a supplementary carbon source in the medium was the only vegetable source able to enhance both promoter activity and fengycin synthesis.

In a third step of this work, we focused on the optimisation of fengycin production taking into account the previous results. Thus, we first studied the effects of temperature, pH and aeration in two different nutrient conditions allowing a good promoter expression level: with urea as nitrogen source and with mannitol as carbon source. The results showed that conditions such as temperature level for optimal production of fengycin can differ in function of the substrate used. Indeed the best specific fengycin production was obtained at 30°C in the presence of urea but at 25°C in the presence of mannitol. The optimum initial pH was 7.0 for both conditions. Influence of the final pH level during production seems to be very

important for the fengycin production. Indeed, minimal production was recorded for a final pH of 8.9 (urea with initial pH 7.5), while a significant decrease in production was observed with mannitol for a final pH of 6.1 (mannitol with an initial pH of 6.5). According to Varadavenkatesan and Murty (2013), the optimum pH for fengycin production was between 6.5 and 7.5. In our work, a pH ranging from 6.3 and 8.1 still allowed an important production. Decreasing the oxygen supplied to the culture led to a decrease in fengycin production under both conditions. On the contrary, a maximal fengycin production (about 0.3 g L^{-1}) was obtained by Chtioui *et al.* (2014) at moderate oxygen supply with kLa = 0.01 s^{-1}. Generally, the aeration has a strong positive effect on lipopeptide production.

We also checked if a culture using substrate recognized for its positive influence on fengycin operon expression in a first step, followed by its replacement by a substrate leading to a good fengycin biosynthesis in a second step, may lead to a better production. Here, we found that the successive use of mannitol and sucrose enhanced the specific fengycin production of 97% compared to the control, 93% compared to the production obtained in Landy sucrose and 20 % compared to the production obtained in Landy mannitol.

In conclusion, the use of different nitrogen and carbon sources strongly influences the ratio of specific production surfactin/fengycin (varying from 1 to 36) and fengycin promoter expression, which can either or not reflect directly on fengycin production. Activation of promoter by mannitol followed by feeding the culture with sucrose leads to the highest specific production. This strategy enabled us to increase the fengycin production more than twice with many sources to reach 768 mg L^{-1}, as the highest production never reported from this strain. Maximum surfactin production (2.33 g L^{-1}) was observed when using a mix of urea and ammonium carbonate as nitrogen source, which was also a favorable source for fengycin production as many other sources, leading to conclude that both lipopeptides (fengycin and surfactin) produced from this strain probably shared several direct or indirect regulation factors.

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References

Abdel-Mawgoud, M., Aboulwafa, M. and Hassouna, H. (2008) Optimization of surfactin production by *Bacillus subtilis* isolate BS5. *Appl Biochem Biotechnol* **150**, 305-325.

Abushady, M., Bashandy, S., Aziz, H. and Ibrahim, M. (2005) Molecular characterization of *Bacillus subtilis* surfactin producing strain and the factors affecting its production. *Int J Agric Biol* **3**, 337-344.

Allaway, D., Lodwig, M., Crompton, A., Wood, M., Parsons, R., Wheeler, R. and Poole, S. (2000) Identification of alanine dehydrogenase and its role in mixed secretion of ammonium and alanine by pea bacteroids. *Mol Microbiol* **36**, 508-515.

Belitsky, B.R. (2015) Role of branched-chain amino acid transport in *Bacillus subtilis* CodY activity. *J Bacteriol* **197**, 1330-1338.

Besson, F., Chevanet, C. and Michel, G. (1987) Influence of the culture medium on the production of iturin A by *Bacillus subtilis. J Gen Microbiol* **3**, 767–772.

Cawoy, H., Debois, D., Franzil, L., De Pauw, E., Thonart, P. and Ongena, M. (2015) Lipopeptides as main ingredients for inhibition of fungal phytopathogens by *Bacillus subtilis/amyloliquefaciens*. *Microb Biotechnol* **8**, 281-295.

Chen, X.H., Koumoutsi, A., Scholz, R., Eisenreich, A., Schneider, K., Heinemeyer, I. *et al.* (2007) Comparative analysis of the complete genome sequence of the plant growth–promoting bacterium *Bacillus amyloliquefaciens* FZB42. *Nat Biotechnol* **25**,1007–1014.

Chen, X.H., Koumoutsi, A., Scholz, R., Schneider, K., Vater, J., Süssmuth, R. *et al.* (2009) Genome analysis of *Bacillus amyloliquefaciens* FZB42 reveals its potential for biocontrol of plant pathogens. *J Biotechnol* **140**, 27–37.

Chtioui, O., Dimitrov, K., Gancel, F., Dhulster, P. and Nikov, I. (2014) Selective fengycin production in a modified rotating discs bioreactor. *Bioprocess Biosyst Eng* **37**, 107-114.

Cooper, D.G., Macdonald, C.R., Duff, S.J. and Kosaric, N. (1981) Enhanced production of surfactin from *Bacillus subtilis* by continuous product removal and metal cation additions. *Appl Environ Microbiol* 42, 408-412.

Cosby, M., Vollenbroich, D., Lee, H. and Zuber, P. (1998). Altered *srf* expression in *Bacillus subtilis* resulting from changes in culture pH is dependent on the Spo0K oligopeptide permease and the ComQX system of extracellular control. *J Bacteriol* **180**, 1438-1445.

Coutte, F., Lecouturier, D., Yahia, S.A., Leclère, V., Béchet, M., Jacques, P. *et al.* (2010) Production of surfactin and fengycin by *Bacillus subtilis* in a bubbleless membrane bioreactor. *Appl Microbiol Biotechnol* **87**, 499–507.

Davis, A., Lynch, C. and Varley, J. (1999) The production of surfactin in batch culture by *Bacillus subtilis* ATCC 21332 is strongly influenced by the conditions of nitrogen metabolism. *Enz Microb Technol* **25**, 322-329.

Ditmer, E. M. (2014) Evaluation of the anti-proliverative effect of the lipopeptides iturin A and fengycin of *Bacillus* spp. and the viral fusion protein PTD4-VP3 on human cancer and normal cells. Masters Dissertation, Federal University of Rio Grande do Sul, Brazil, 115 p.

Dong, W.X., Li, S.Z., Lu, X.Y., Zhang, X.Y., Wang, P.P, Ma, P. and Guo, Q.G. (2014) **Regulation of fengycin biosynthase by regulator PhoP in the *Bacillus subtilis* strain NCD-2.** *Acta Phytopathol Sinica* **44**, 180-187.

Fahim, S., Dimitrov, K., Gancel, F., Vauche,l P., Jacques, P. and Nikov, I. (2012). Impact of energy supply and oxygen transfer on selective lipopeptide production by *Bacillus subtilis* BBG21. *Bioresour Technol* **126**,1-6.

Fracchia, L., Cavallo, M., Giovanna, M. and Banat, I.M. (2012). Biosurfactants and bioemulsifiers biomedical and related applications, Present Status and Future Potent, CC BY 3.0 license.

Ghribi, D. and Ellouze-Chaabouni, S. (2011). Enhancement of *Bacillus subtilis* lipopeptide biosurfactants production through optimization of medium composition and adequate control of aeration. *Biotechnol Res Int* doi: 10.4061/2011/653654. Epub 2011 Sep 27.

Guo, Q., Li, S., Lu, X., Li, B. and Ma, P. (2010). PhoR/PhoP two component regulatory system affects biocontrol capability of *Bacillus subtilis* NCD-2. *Genet Mol Biol* **33**, 333-340.

Handke, L., Shivers, P. and Sonenshein, L. (2008). Interaction of *Bacillus subtilis* CodY with GTP. *J Bacteriol* **190**, 798-806.

Hathout, Y.A., Ho, Y.P., Ryzhov, V., Demirev, P. and Fenselau, C. (2000). Kurstakins: a new class of lipopeptides isolated from *Bacillus thuringiensis*. *J Nat Prod* **63**, 1492-1496.

Huang, Q.X., Lu, Z.X., Zhao, H.Z., Bie, X.M., Lu, F.X. and Yang, S.J., (2006). Antiviral activity of antimicrobial lipopeptide from *Bacillus subtilis* fmbj against Pseudorabies Virus, Porcine Parvovirus, Newcastle Disease Virus and Infectious Bursal Disease Virus *in vitro*. *Int J Pept Res Therap* **12**, 373-377.

Huang, X., Liu, J. N., Wang, Y., Liu, J. and Lu, L. (2015). The positive effects of Mn2+ on nitrogen use and surfactin production by *Bacillus subtilis* ATCC 21332. *Biotechnol Biotechnol Equip **29***, 381-389.

Islam, M., Jeong, T., Lee, S. and Song, H. (2012). Isolation and identification of antifungal compounds from *Bacillus subtilis* C9 inhibiting the growth of plant pathogenic fungi. *Mycobiology* **40**, 59-66.

Jacques, P., Hbid, C., Destain, J., Razafindralambo, H., Paquot, M., De Pauw, E. *et al.* (1999). Optimization of biosurfactant lipopeptide production from *Bacillus subtilis* S499 by Plackett-Burman design. *Appl Biochem Biotechnol* **77-79**, 223–233.

Jacques, P. (2011). Surfactin and other lipopeptides from *Bacillus* spp., in Soberon-Chavez G (Ed) Biosurfactants, Microbiology Monographs, Springer-Verlag, Berlin Heidelberg, vol. 20, pp 57-91.

Kim, H.-S., Yoon, B.-D., Lee, C.-H., Suh, H.-H., Ho, H.-M., Katsuragi, T. and Tani, Y. (1997) Production and properties of a lipopeptide biosurfactant from *Bacillus subtilis* C9. *J Ferment Bioeng* **84**, 41-46.

Landy, M., Warren, G.H., Rosenman, S.B. and Colio, L.G. (1948). Bacillomycin an antibiotic from *Bacillus subtilis* active against pathogenic fungi. *Proc Soc Exp Biol Med* **67**, 530-541.

Luo, C., Liu, X., Zhou, X., Guo, J., Truong, J., Wang, X., Zhou, H., Li, X. and Chen, Z. (2015) Unusual biosynthesis and structure of locillomycins from *Bacillus subtilis* 916. *Appl Environ Microbiol* **81**, 6601-6609.

Makkar, S. and Cameotra, S. (1997). Biosurfactant production by a thermophilic *Bacillus subtilis* strain. *J Ind Microbiol Biotechnol* **18**, 37-42.

Molle, V., Nakaura, Y., Shivers, R. P., Yamaguchi, H., Losick, R., Fujita, Y. and Sonenshein, A. L. (2003). Additional targets of the *Bacillus subtilis* global regulator CodY identified by chromatin immunoprecipitation and genome-wide transcript analysis. *J Bacteriol* **185**, 1911-1922.

Mols, M. and Abee, T. (2008). Role of ureolytic activity in *Bacillus cereus* nitrogen metabolism and acid survival. *Appl Environ Microbiol* **74**, 2370-2378.

Msadek, T., Kunst, F., Klier, A. and Rapoport, G. (1991). DegS-DegU and ComP-ComA modulator-effector pairs control expression of the *Bacillus subtilis* pleiotropic regulatory gene *degQ*. *J Bacteriol* **173**, 2366-2377.

Mukherjee, A. K. and Das, K. (2005). Correlation between diverse cyclic lipopeptides production and regulation of growth and substrate utilization by *Bacillus subtilis* strains in a particular habitat. *FEMS Microbiol Ecol* **54**, 479-489.

Ongena, M. and Jacques, P. (2008). *Bacillus* lipopeptides: versatile weapons for plant disease biocontrol. *Trends Microbiol* **16**, 115-125.

Pryor, S.W., Siebert, K.J., Gibson, D.M., Gossett, J.M. and Walker, L.P. (2007b) Modeling production of antifungal compounds and their role in biocontrol product inhibitory activity. *J Agric Food Chem* **55**, 9530-9536.

Puri-Taneja, A., Paul, S., Chen, Y. and Hulett, F. M. (2006). CcpA causes repression of the *phoPR* promoter through a novel transcription start site, PA6. *J Bacteriol* **188**, 1266-1278.

Raaijmakers, M., De Bruijn, I., Nybroe, O. and Ongena, M. (2010). Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. *FEMS Microbiol Rev* **34**, 1037-1062.

Rajendran, D., Venkatachalam, P. and Ramakrishnan, J. (2014). Response surface methodology: optimisation of antifungal bioemulsifier from novel *Bacillus thuringiensis*. *Scient World J* doi: 10.1155/2014/423289. Epub 2014 Oct 14.

Ramarathnam, R., Bo, S., Chen, Y., Fernando, W.G.D., Xuewen, G. and de Kievit, T. (2007). Molecular and biochemical detection of fengycin- and bacillomycin D-producing *Bacillus* spp., antagonistic to fungal pathogens of canola and wheat. Can J Microbiol **53**, 901–911.

Sinchaikul, S., Sookkheo, B., Topanuruk, S., Juan, H.-F., Phutrakul, S. And Chen, S.T. (2002) Bioinformatics, functional genomics, and proteomics study of *Bacillus* sp. *J Chromatog* B **771**, 261-287.

Singh, K., Rautela, R. and Cameotra, S. (2014). Substrate dependent *in vitro* antifungal activity of *Bacillus* sp. strain AR2. *Microb Cell Fact* **13**, 67-77.

Steller, S., Vollenbroich, D., Leenders, F., Stein, T., Conrad, B., Hofemeister, J. *et al.* (1999). Structural and functional organization of the fengycin synthetase multienzyme system from *Bacillus subtilis* b213 and A1/3. *Chem Biol* **6**, 31-41.

Thompson, N., Fox, L. and Bala, A. (2001). The effect of pretreatments on surfactin production from potato process effluent by *Bacillus subtilis*. In Twenty-Second Symposium on Biotechnology for Fuels and Chemicals ed. Davison, B.H., McMillan, J.D. and Finkelstein, M. pp.487-501. Humana Press, New York: Springer.

Tosato, V., Albertini, A.M., Zotti, M., Sonda, S. and Bruschi, C.V. (1997). Sequence completion, identification and definition of the fengycin operon in *Bacillus subtilis* 168. *Microbiology* **143**, 3443–3450.

Varadavenkatesan, T. and Murty, R. (2013). Production of a lipopeptide biosurfactant by a novel *Bacillus* sp. and its applicability to enhanced oil recovery. *ISRN Microbiol* doi: 10.1155/2013/621519. eCollection 2013.

Vargas-Bautista, C., Rahlwes, K. and Straight, P. (2014). Bacterial competition reveals differential regulation of the *pks* genes by *Bacillus subtilis*. *J Bacteriol* **196**, 717-728.

Volpon, L., Besson, F. and Lancelina, J.M. (2000). NMR structure of antibiotics plipastatins A and B from *Bacillus subtilis* inhibitors of phospholipase A2. *FEBS Lett* **485**, 76-80.

Wang, P., Guo, Q., Ma, Y., Li, S., Lu, X., Zhang, X. and Ma, P. (2015). DegQ regulates the production of fengycins and biofilm formation of the biocontrol agent *Bacillus subtilis* NCD-2. *Microbiol Res* **178**, 42-50.

Wei, Y.H., Wang, L.C., Chen, W.C. and Chen, S.Y. (2010). Production and characterization of fengycin by indigenous *Bacillus subtilis* F29-3 originating from a potato farm. *Int J Mol Sci* **11**, 4526-4538.

Wray, V., Ferson, E. and Fisher, H. (1997). Expression of the *Bacillus subtilis ureABC* operon is controlled by multiple regulatory factors including CodY, GlnR, TnrA, and Spo0H. *J Bacteriol* **179**, 5494-5501.

Wu, C.Y., Chen, C.L., Lee, Y.H., Cheng, Y.C., Wu, Y.C., Shu, H.Y. *et al.* (2007). Nonribosomal synthesis of fengycin on an enzyme complex formed by fengycin synthetases. *J Biol Chem* **282**, 5608–5616.

Yaseen, Y., Gancel, F., Drider, D., Béchet, M. and Jacques, P. (2016) Influence of promoters on the production of fengycin in *Bacillus* spp. *Res Microbiol* **167**, 272-281.

Yin, H., Guo, C., Wang, Y., Liu, D., Lv, Y., Lv, F. *et al.* (2013). Fengycin inhibits the growth of the human lung cancer cell line 95D through reactive oxygen species production and mitochondria-dependent apoptosis. *Anticancer Drugs* **24**, 587-598.

Zheng, M., Wang, P. and Wu, Q. (2013). Optimization of culture conditions for biosynthesis of lipopeptide by *Bacillus subtilis* with starch. In *Applied Mechanics and Materials*, Vol. 291, pp. 225-229.

Zhu, Z., Sun, L., Huang, X., Ran, W. and Shen, Q. (2014) Comparison of the kinetics of lipopeptide production by *Bacillus amyloliquefaciens* XZ-173 in solid-state fermentation under isothermal and non-isothermal conditions. *World J Microbiol Biotechnol* **30**, 1615-1623.

Chapter 5

Impacts of *srfA* and *pnpA* pleitropic genes on the fengycin production in *Bacillus subtilis*

General introduction

As shown in the previous chapter the use of different carbon and nitrogen sources in culture medium allowed different levels of expression of fengycin promoter and lipopeptide production. In all experiments, surfactin and fengycin productions have been analyzed. Some conditions proved that there is a significant link between of surfactin and fengycin productions, while others showed different results. These results raised the following query: how fengycin and surfactin productions are they correlated?

In order to answer to this question, two surfactin negative mutants were constructed using BBG201 strain as mother strain. The first mutant was obtained by disrupting the *srfAA* gene and the second by the *srfAC* gene disruption. In both mutants fengycin production was verified and compared to wild strain.

A second interesting results generated by this previous chapter is, in some culture conditions, the absence of a correlation between the level of fengycin operon expression and fengycin production. Indeed in the same medium, high fengycin promoter expression could be obtained while low fengycin is produced. One of the hypotheses to explain this result could be a low stability of mRNA. Indeed fengycin operon is quite big and long mRNA are well known foe their high sensitivity to RNAse. We thus decided to check the role of the mRNA degradation enzymes such as PnpA. So, we carried out *pnpA* deletions in BBG 21 and 168 derivatives. The effects of knock out were analysed on fengycin and surfactin productions and, antifungal activities of the mutant strains.

Abstract

Bacillus subtilis produces a large variety of lipopeptides trough a non-ribosomal mechanism, i.e., iturins, surfactins and fengycins or plipastatins. These molecules show a broad range of biological properties. Their biosynthesis is most often subject to complex regulation. Thus several regulatory factors could play a role on the production of these secondary metabolites. This work focuses on the regulatory effects of surfactin operon on the fengycin production as well as on the role of the mRNA stability on lipopeptide expression. Several mutant strains were obtained via the knock-out of two genes of the surfactin operon (srfA), one gene of the fengycin operon (pps) and the gene *pnpA* which encodes the Polynucleotide phosphorylase 3'-5' RNA-degrading enzyme. The results showed a clear regulatory interaction between the fengycin and the surfactin operons. In *srfAA* mutant a significant decrease in fengycin production was obtained while the production was not influenced in the *srfAC* mutant. In contrast, the fengycin disrupted mutant BBG205 increased the surfactin production up to 30%. The deletion of *pnpA* showed also a significant decrease on the production of the two lipopeptides: 95% and 70% for fengycin and surfactin respectively.

Introduction

Polynucleotide Phosphorylase (PNPase) is a multifunctional enzyme responsible for $Mg2^{+}$- and inorganic phosphate dependent 3′ → 5′ possessive exoribonuclease activity [Luttinger *et al.*, 1996]. In *B. subtilis* PNPase is the major RNA-degrading enzyme as well as it is involved in various nucleic acid metabolic pathways [Cardenas, 2009]. It has been reported that PNPase is the major mRNA decay enzyme and its lack resulted in the accumulation of decay intermediates for several small, monocistronic mRNAs [Liu *et al.*, 2014]. Besides the role of PNPase in quality control of RNA precursors, this enzyme is involved in DNA repairing via its important role in the degradation of ssDNA [Liu *et al.* 1996; Oussenko *et al.* 2005]. PNPase has been shown to influence many genes such as *sinR, abrB, mecA, comS* [Cardenas *et al.* 2009]. Liu *et al.* [2014] reported that among 2276 genes tested 66% showed significant deferential expression in the wild type comparing to the *pnpA* mutant strain in *B subtilis.*

A *B.subtilis pnpA* mutant strain showed a number of phenotypes including cold sensitivity or competence deficiency which is controlled by the regulator comS (within the surfactin operon), tetracycline sensitivity and long multi-septate growth resulting of the effect of the pnpA on the regulatory factors of degD. All of these phenotypes are presumably linked to mRNA turnover and/or recycling of NDPs [Luttinger, 1996; Wang and Bechhofer, 1996; Cardenas, 2009].

Most of *Bacillus* strains which produce fengycins contain at least one other operon responsible for production of other NRP products. Surfactin is most of the time synthetized with fengycin [Roongsawang *et al.*, 2002; Roongsawang *et al.*, 2010; Kim *et al.*, 2010]. Organization and regulation of surfactin operon are well known. Four large ORFs coding for surfactin synthetases are designated *srfAA, srfAB, srfAC* and *srfAD* (Fig. 1) [Galli *et al*., 1994; Lee *et al*., 2007].

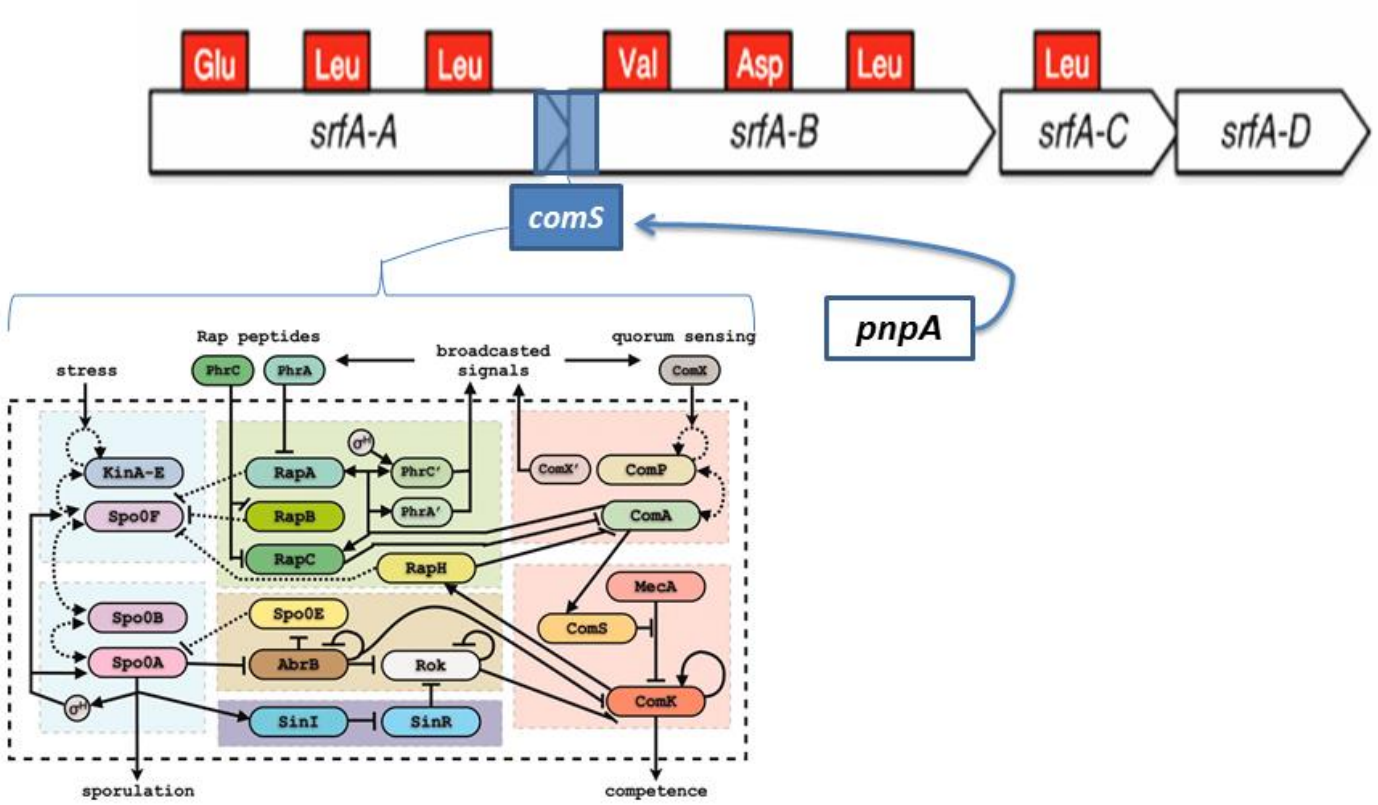


Figure 1. schematic regulatory interaction between the operon of surfactin including the gene comS and pnpA gene in *B.subtilis* [this figure is adapting from Schultz *et al.*, 2009; Jaccques, 2001].

The small *comS* gene, is located within the coding region of the fourth amino acid-activation domain of *srfAB* and thus co-expressed with the *srfA* operon. The ComS protein which contains 46 amino acid residues, is required for competence development in *B. subtilis* [Nakano and Zuber 1991; Hamoen *et al*., 1995; Jacques 2011].

Surfactin-deficient mutants were found to influence production of the other non-ribosomally synthesized lipopeptides. Indeed several studies showed that surfactin mutant influence directly or indirectly on the production of fengycin, mycosubtilin and bacilysin [Karata *et al.*, 2003; Zeriouh *et al.*, 2014; Luo *et al.*, 2015].

This work aimed at understanding the influence of the *pnpA* gene on the fengycin expression in both *B. subtilis* BBG21 and *B. subtilis* 168. Furthermore, the work highlighted the regulation role of *srfA* operon on the fengycin synthesis thanks to two surfactin mutant strains with *srfAA* and *srfAC* in order to better understand the relation between the two lipopeptides produced by the same strain.

Material and methods

Bacterial strains, plasmids and primers

Plasmids and strains used in this study are listed in Table 1. All the primers used for genetic construction or inspection are shown in Table 2.

Bacillus transformation

All the strains derived from *B. subtilis* 168 were transformed by natural competence method [Sambrook and Russell 2001]. A modified electroporation method using trehalose was used for transformation of *B. subtilis* BBG201 [Cao *et al.*, 2011].

Culture conditions

For transformation experiments, *B.* strains and *Escherichia coli* JM109 were grown at 37°C under shaking at 130 rev min^{-1} in Luria–Bertani medium (LB) [Sambrook and Russell 2001]. Media were supplemented with antibiotics (Sigma-Aldrich, St. Louis, MO, USA): apramycin (100 µg/mL), erythromycin (1 µg/mL), chloramphenicol (1 µg/mL), phleomycin (4 µg/mL) and neomycin (5 µg/mL) when required. The haemolysis activity was detected by growing *Bacillus* colonies overnight on LB blood plates [Coutte *et al.*, 2010]. Microbial growth was monitored by optical density (OD) at 600 nm with an Uvikon 940 spectrophotometer (Kontron Instruments, Plaisir, France). Landy medium [Landy *et al.*, 1948] containing 100 mM 3-(N-morpholino) propanesulfonic acid (MOPS) was used as the basic medium for lipopeptide production with 10% filling ratio of Erlenmeyer, 30°C and 160 rpm [Hussein 2011].

DNA manipulation

Polymerase chain reaction (PCR) was done using the PCR Master Mix (2X) (Thermo Scientific Fermentas, Villebon sur Yvette, France) as a mixture of Taq DNA polymerase. DNA extraction was performed using the Wizard® Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA). Ligation of PCR products was done into pGEM-T Easy vector (Promega Corp.). Plasmid extraction was carried out using GeneJET Plasmid DNA Purification Kit (Thermo Scientific Fermentas). Restriction endonucleases were supplied by Thermo Scientific Fermentas. Ligation of inserts to different vectors was effected using the DNA Ligation Kit <Mighty Mix> from Takara (Ozyme, Saint Quentin en Yvelines, France). Recovery of DNA from agarose gels was performed with GeneJET Gel Extraction kit

(Thermo Scientific Fermentas). In all cases, the instructions of the suppliers were followed. All the construction sequences were verified by DNA sequencing (Eurofins Genomics, Ebersberg, Germany).

Surfactin mutant construction

Fragments of *hxlR-srfAA* and *srfAC-srfAC* were amplified by PCR using respectively, the primers HxlR B.S. BBG21-SrfAA B.S. BBG21 and primers SrfAC fw B.S. BBG21-SrfAC rv B.S. BBG21. The two fragments were cloned in pGEM-T Easy vector. The resulting plasmids were named pBG331 and pBG332 respectively. Plasmids pMUTIN-GFP+ and pBG331 were *Kpn*I and *Xma*III double digested. The *hxlR-srfAA* fragment was inserted between the *Kpn*I and *Xma*III sites of pMUTIN-GFP+ to obtain pBG334. While the plasmids pOJ206 and pBG332 were *EcoRI* and *HindIII* double digested. The *srfAC-srfAC* was inserted between *EcoRI* and *HindIII* of pOJ206 to obtain pBG335.

PNPase mutant derivative strains

The expected DNA fragments were amplified by PCR using the primers *ribC* fwd and *pnpA* rev from BBG21 (Table 1), and then cloned in pGEM-T Easy. The resulting plasmids pBG330 was *KpnI-XmaIII* double digested. The fragment then was inserted between *KpnI* and *XmaIII* sites, in pMUTIN-GFP+ generating the plasmid pBG333. The latter were successfully transferred into the strain BBG201 by electroporation transformation [Cao *et al.*, 2011], leading to the recombinant strain BBG230.

Using the primers *pnpA* Fw, *pnpA* delta Rv and the primers *pnpA* delta Fw, *pnpA* Rv from the strain *B. subtilis* BBG258 and *B. subtilis* BSB1 (derivatives of the strain *B.subtilis* 168); four fragments were amplified by PCR. The primers P3, P5 were used to amplify the fragment k7 contain the phleomycin resistant gene. The three fragments were ligated using the joint PCR program (NEBuilder Assembly, Ipswich, USA). Natural competence transformation was carried out resulted the strain BBG236 and BBG237.

Lipopeptide purification and quantification

1 mL of supernatant was extracted using C18 cartridges (Extract-clean SPE 500 mg, Grace Davison-Alltech, Deerfield, IL, USA). Lipopeptide production was quantified by HPLC (Waters Corporation, Milford, MA, USA) using a C18 column (5 µm, 250 × 4.6 mm, VYDAC 218 TP, Hesperia, CA, USA). Analyses of lipopeptides were performed as previously described [Coutte *et al.*, 2010].

Antifungal test

5µL of culture (O.D 600 nm = 0.2) were spotted in the center of a PDA Petri dish. A small square of *Botrytis cinerea* was spotted on one side of the dish and the bacteria on the other side. Plates were incubated at 25 ºC for 4 days to measure the inhibition zone.

Table 1. PCR primers used for genetic constructions.

PRIMER	PRIMER SEQUENCE 5'—3'	Fragment length bp
HxlR B.s. 186 KpnI fwd srfAA B.s. 186 XmaIII rv	CACAGGGAAGTGGTACCAGT AATCGGCCGCTCAACAAG	902
srfAC B.s. EcoRI fwd srfAC B.s. HindIII rv.	GAATTCGATGAGGAAGCCAAC CTTCAAACAAAAGCTTCACTGG	1620
pnpA B.s. KpnI fwd pnpA B.s. XmaIII rv.	ATATCGGTACCGCGCTTCAA CGGCCGTCCGGTCTTACTTT	1551
pnpA B.s. ribC fwd pnpA B.s. ylxY rv.	CCTTAATGTGCAGCACGCAG TTTTAATCAATGTGGCATGG	3409
pnpA fwd pnpA Delta rv	TCATGGTGATAAAAGAGGGC CGACCTGCAGGCATGCAAGCTACCGT AGCGGATCATCACAGCACCATTTGC	1069
pnpA Delta fwd pnpA rv	GCTCGAATTCACTGGCCGTCGGCAAATG GTGCTGTGATGATCCGCTACGGTACAAG GACGAGTGAATTTATCCCGCAAAGC TCGGATGCATTAAAATCATGGC	1039
K7 P3 K7 P5	AGCTTGCATGCCTGCAGGTCG CGACGGCCAGTGAATTCGAGC	2660

Table.2. Strains and plasmids used in this study

Plasmid or bacterial strain	**plasmid description or Genotype (Phenotype)**	**Reference**
pGEM-T Easy	*bla*^{R}	Promega Corp.
pMUTIN-GFP+	*bla*^{R}, *erm*^{R} , *gfp*, (6192 bp)	Bacillus genetic stock center, USA
pBG330	pGEM-T Easy, *Kpn*I-*Xma*III *ribC-pnpA* amplicon from *B. subtilis* BBG21; *bla*^{R}, (4625 bp)	This study
pBG331	pGEM-T Easy, *Kpn*I-*Xma*III *hxlR -srfAA* amplicon from *B. subtilis* BBG21; *bla*^{R}, (3903 bp)	This study
pBG332	pGEM-T Easy , *EcoRI-HindIII srfAC-srfAC* amplicon from *B. subtilis* BBG21; *bla*^{R} (4630 bp)	This study
pBG333	*bla*^{R}, *erm*^{R}, *Kpn*I-*Xma*III *ribC-pnpA* amplicon from *B. subtilis* BBG21, pMUTIN-gfp+, (7801 bp)	This study
pBG335	*bla*^{R}, *am*^{R}, *EcoRI-HindIII srfAC-srfAC* amplicon from *B. subtilis* BBG21, pOJ206	This study
pBG334	*bla*^{R}, *erm*^{R}, *Kpn*I-*Xma*III *hxlR -srfAA* amplicon from *B. subtilis* BBG21, pMUTIN-gfp+, (7078 bp)	This study
B. subtilis BBG21	Spontaneous mutant of *B. subtilis* ATCC 21332, *fen*+, s*rf*+	Fahim *et al.* (2012)
B.subtilis BBG258	*cat*^{R}, *B. subtilis* 168 *sfp*+, *pps*+, *srf*+	Dellhi *et al.* (2016)
B.subtilis BSB1	*nem*^{R}, *B. subtilis* 168 *sfp*-,	
BBG201	*tet* ^{R}, *B. subtilis* BBG21 PMM ϖ *comK*+	This study
BBG231	*erm* ^{R}, *B. subtilis* BBG21 Δ*srfAA*::(*srfAA* BBG21 –*gfp-erm*^{R})	This study
BBG232	*am*^{R}, *B. subtilis* BBG21 Δ *srfAC*:: *srfAC* / BBG21 *am*^{R}	This study
BBG235	*erm* ^{R}, *B. subtilis* BBG21 Δ*pnpA*::(*pnpA* BBG21 –*gfp-erm*^{R})	This study
BBG236	*phl* ^{R}, *B. subtilis* BBG258 Δ*pnpA*::(*pnpA* BBG258 –*phl*^{R}-*pnpA*)	This study
BBG237	*phl* ^{R}, *B. subtilis* BSB1 Δ*pnpA*::(*pnpA* BSB1 –*phl*^{R}-*pnpA*)	This study
Escherichia coli JM109	*recA1, endA1,gyrA96, thi, hsdR17, supE44* Δ(*lac-proAB*)/F'[*traD36, proAB+, lacIq, lacZ*ΔM15]	Promega Corp.

Ap^R*, resistance to ampicillin; Spc*^{R}*, resistance to spectinomycin ; Erm*^{R}*, resistance to erthromycin; Cm*^{R}*, resistance to chloramphenicol; Tc*^{R}*, resistance to tetracycline and phl*^{R}*, resistant to phleomycin.*

Results and discussion

Surfactin mutants

The surfactin operon was disrupted in the strain BBG201 targeting two *srf* genes. The first mutant was obtained by disrupting *srfAA* and the resultant strain was named BBG231. The second disruption was done in *srfAC* and the resultant strain was named BBG232. The haemolytic activity was verified in strains (Figure 2). The fengycin and surfactin were quantified using HPLC method in the wild type and mutant strains as shown in table 3.

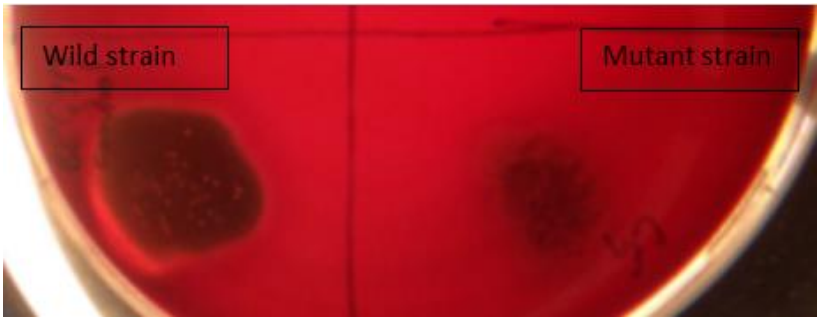


Figure 2. Haemolysis activity test on LB blood agar medium for the wild strain BBG201 and mutant strain BBG231

Table.3. Lipopeptide production in Landy MOPS medium, pH 7, 10% filling ratio, 30°C and 160 rpm, after 48 hours by *B. subtilis* BBG201 and *sfr* mutants

Strain	**Fengycin mg/L**	**Fengycin mg/L.OD$_{600}$**	**Surfactin mg/L**
BBG201 (wild type)	385 ± 16.3	35 ± 2.9	644 ± 22.2
BBG231 (*sfrAA*$^-$)	76.4 ± 5.6	8.4 ± 0.9	00
BBG232 (*sfrAC*$^-$)	402.6 ± 21.4	37.9 ± 3.3	00

As expected the surfactin production was zero for *srfAA* mutant (strain BBG231) and BBG232 (*srfAC* mutant strain). Surprisely, in BBG231 the fengycin production showed also an important decrease (80%) when compared to the mother strain (385 mg/L versus 76.4 mg/L in the mutant strain). In another hand, the disruption of *srfAC* in BBG232 did not influence the fengycin production as 402.6 mg/L were reached.

The significant decrease in the fengycin production from the mutant strain BBG231 indicates that the gene *srfAA* play a role on the fengycin production. This gene also includes *comS* which regulates the natural competence in *Bacillus* via *comK*. The latter gene (*comK*) negatively regulates *degQ* expression [Ogura *et al.,* 2002], which is wellknown regulator of the fengycin operon [Tsuge *et al.,* 1999]. This hypothesis could explain the positive effect of *srfAA* on fengycin production. Althought, previous studies showed that the surfactin operon influenced the other lipopeptides produced from the same strain. For example, the production of bacilysin from *B.subtilis* ATCC 21332 was reduced to 12.5% in a *SrfA*$^-$ mutant [Karatas *et al*., 2003]. In contrast, the deletions of *srfAB, srfAC* and *srfAD* had no effect on the fengycin yield in *B. subtilis* 168 [Ongena *et al.,* 2007]. Previous work in our laboratory showed that the *srfAA* mutation in the surfactin operon influences negatively mycosubtilin production by *B. subtilis* ATCC 6633 [Béchet *et al.,* 2013]. Zeriouh *et al.,* [2014] showed also that the antifungal activity of the fengycin was significantly reduced in *B. subtilis sfrAB* mutant.

Interestingly, the role of fengycin on the surfactin production has been shown in our previous work. Disruption of *fenA* has been carried out in order to measure the fengycin promoter activity by introducing *gfp* in front of fengycin promoter (the strain BBG205). Surfactin production increased in the *fenA* mutant (30%). The enhanced of the surfactin production could be simply due to the availability of the precursors when no fengycin is produced.

Whatever the interaction between the lipopeptides and the regulation (direct or indirect influences) of the genes involved in synthesis of these lipopeptides, remain unclear and need more study.

PnpAse mutants

In order to study the influence of the pnpAse on the fengycin regulation and production, three *pnpA* disrupted mutants were constructed as described in materials and methods. The *B. subtilis* strain BBG201 was successfully transformed by electroporation method producing the strain BBG235. On the other hand, two strains derived from *B. subtilis* 168; BBG258 (*sfp*+) and BSB1 (*sfp*-) were also successfully transformed by natural competence transformation generating two strains named BBG236 and BBG237 respectively. All the mutant strains were shown as the multi-septate form resulting from the *pnpA* deletion (Fig. 3)

For BBG235 and BBG236, the fengycin and surfactin production were measured and compared in the wild type and mutant strains (table 4).

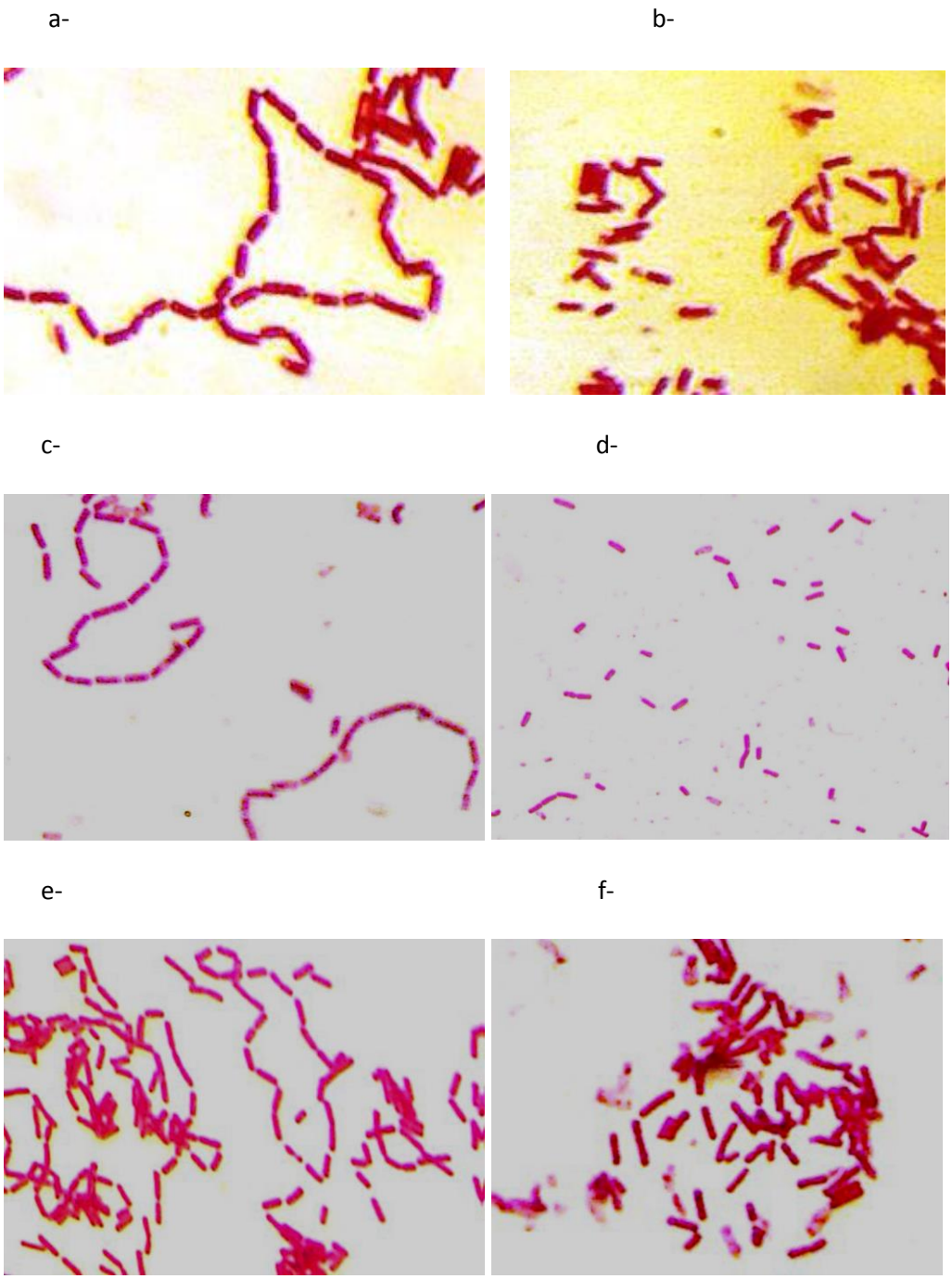


Figure 3. The multi-septate form of the *pnpA* mutant strains versus wild type strains:

BBG235, b- BBG201, c- BBG236, d- BBG258, e- BBG237, f- BSB1

Table.4. Lipopeptide production in batch by four strains of *B. subtilis* after 48 hours of growth in Landy MOPS medium, pH 7, 10% filling ratio, 30°C and 160 rpm.

Strain	**Fengycin mg/L**	**Fengycin mg/L.OD$_{600}$**	**Surfactin mg/L**	**Surfactin mg/L.OD$_{600}$**
BBG21 (wild type)	346 ± 12.3	37.6 ± 3.2	644 ± 22.2	70 ± 2.1
BBG235 (*pnpA*$^-$)	18.4 ± 1.4	2.96 ± 0.8	190.4 ± 13.7	30.7 ± 3.3
BBG258 (wild type)	15.34 ± 1.32	1.9 ± 0.9	746.4 ± 17.3	81.3 ± 5.3
BBG236 (*pnpA*$^-$)	4.4 ± 1.1	0.4 ± 0.7	388 ± 11.1	42 ± 4.3

The fengycin production showed a significant decreased (95%) in the mutant strain BBG 235 with only 18.4 mg/L compared to 346 mg/L in the wild strain. In this strain, production of surfactin was also influenced negatively with a decrease of 70 % in the total production in the mutant strain compared the wild strain with 190.4 mg/L and 644 mg/L respectively. The reduction in the lipopeptide production in the mutant strain BBG236 compared to the wild type BBG258 was less than that observed with BBG201 strain but still significant.

A significant decrease in the antifungal activity against the plant pathogen fungi *Botrytis cinerea* was observed with the mutant strain BBG235 comparing to the wild strain, confirming the negative influence on the fengycin production by disrupting the *pnpA* gene in mutant strain (Fig. 4).

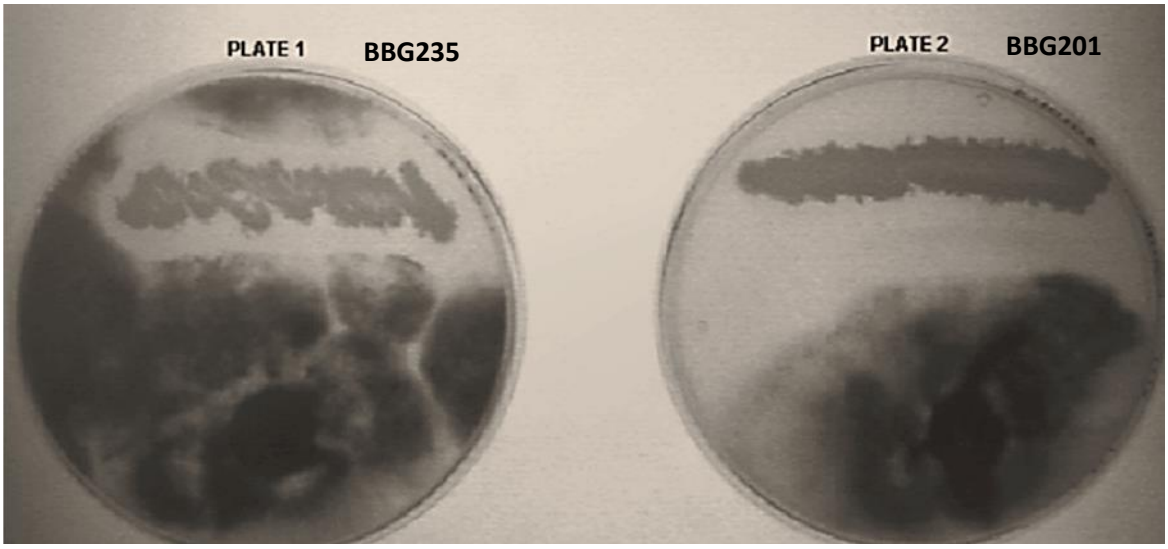


Figure 4. *In vitro* growth inhibition of *Botrytis cinerea* caused by the mutant strain BBG235 (plate 1) and the wild strain BBG201 (plate 2) on potato dextrose agar (PDA) medium. The antagonism was scored after incubation of the plates for 3 days at 25°C.

The results of the lipopeptides production as well as the antifungal activity test showed an important role of *pnpA* on the fengycin and surfactin productions. The positive influence of this gene may occur via different pathways. The first pathway proposed is the role of *pnpA* on the *comS* and *srfA* regulation. Actualy, the effect of *pnpA* mutant on the surfactin production has been already carried out. The promoter activity of the surfactin operon showed slightly decreased (as well as the expression of *comS* as it is controlled by the same promoter) while, significant negative effect have been noticed on *srfAB, srfAC, srfAD*. The post transcription effect, as well as the expression of *comS* (the gene controlled by *srfAA*) was also decreased [Luttinger *et al.*, 1996]. Gamba *et al.*, (2015) indicated that *pnpA* is required for the expression of *comS* in *B. subtilis*. Moreover, the *pnpA* mutant strain showed twofold higher in the expression of *sigB* [Liu *et al.*, 2016]. Knowing that the regulator *sigB* has been mentioned to has a repressing effect on fengycin expression [Allenby *et al.*, 2016].

The second pathway PnpA interacts with the essential genes in the glycolytic pathway (*pfkA* and *eno*) [Commichau *et al.*, 2009]. We hypothesize that deletion of this gene might have an effect on the precursor synthesis from the cental carbon metabolism which ultimately have a significant effect on the production of the lipopeptides.

A direct effect of the lack of PNPase was observed for about 10% of expressed genes, for which there was a significant increase in the level of 5′-proximal reads relative to the level of 3′-proximal reads while most of the changes in gene expression in the Δ*pnpA* strain are likely due to indirect effects [Liu *et al.*, 2016]. In gram negative bacteria *pnpA* plays a role in expression of virulence functions and growth resumption after cold shock [Song *et al.*, 2013]. The fact that effective decay of an mRNA figures obviously in the regulation of a large number of genes supports the conception that models of gene expression networks must consider not only transcriptional and translational control, but also control at the level of mRNA decay [Liu *et al.*, 2016].

Conclusion

The positive role of *srfAA* or *comS* which is controlled by *srfAA* on the fengycin regulation has been highlighted, while, there is no post-transcriptional effect of the surfactin operon on fengycin production. Conversely, mutant in the fengycin operon stopped the biosynthesis of this lipopeptide as well as increased the surfactin production. The influence of *pnpA* on the lipopeptide biosynthesis was significant based on the decline on the productivity between the mutant and the wild type strains. To strengthen this original data, more experiments are requested especialy to confirm the role of *comS* which influenced by the *srfAA* and *pnpA* mutants, resulting, a significant reduction in the fengycin production.

References

Allenby, N. E., O'Connor, N., Prágai, Z., Ward, A. C., Wipat, A., & Harwood, C. R. (2005). Genome-wide transcriptional analysis of the phosphate starvation stimulon of *Bacillus subtilis*. *Journal of Bacteriology*, *187*(23), 8063-8080.

Béchet, M., Castéra-Guy, J., Guez, J. S., Chihib, N. E., Coucheney, F., Coutte, F., ... & Jacques, P. (2013). Production of a novel mixture of mycosubtilins by mutants of *Bacillus subtilis*. *Bioresource technology*, *145*, 264-270.

Cao G, Zhang X, Zhong L, Lu Z. A modified electro-transformation method for *Bacillus subtilis* and its application in the production of antimicrobial lipopeptides. Biotechnol. Lett 2011;33:1047-51.

Cardenas, P., Carrasco, B., Sanchez, H., Deikus, G., Bechhofer, H., & Alonso, J. C. (2009). *Bacillus subtilis* polynucleotide phosphorylase 3′-to-5′ DNase activity is involved in DNA repair. *Nucleic acids research*, *37*(12), 4157-4169.

Commichau F. M., et al. 2009. Novel activities of glycolytic enzymes in *Bacillus subtilis*: interactions with essential proteins involved in mRNA processing. *Mol. Cell. Proteomics* 8:1350–1360

Coutte F, Lecouturier D, Yahia SA, Leclère V, Béchet M, Jacques P, et al. Production of surfactin and fengycin by *Bacillus subtilis* in a bubbleless membrane bioreactor. Appl Microbiol Biotechnol. 2010;87:499–507.

Galli, G., Rodriguez, F., Cosmina, P., Pratesi, C., Nogarotto, R., de Ferra, F., & Grandi, G. (1994). Characterization of the surfactin synthetase multi-enzyme complex. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology*, *1205*(1), 19-28.

Gamba, P., Jonker, M. J., & Hamoen, L. W. (2015). A novel feedback loop that controls bimodal expression of genetic competence. *PLoS Genet*, *11*(6), e1005047.

Hamoen, W., Eshuis, H., Jongbloed, J., Venema, G., & Sinderen, D. (1995). A small gene, designated comS, located within the coding region of the fourth amino acid-activation domain of srfA, is required for competence development in *Bacillus subtilis*. *Molecular microbiology*, *15*(1), 55-63.

Hussein W, Study on the regulation and biosynthesis of fengycin and plipastatin produced by *Bacillus subtilis.* PhD. Thesis, Lille1 University, France, 2011.

Jacques P. (2011) Surfactin and other lipopeptides from *Bacillus* spp. Chapter 3: 58-83

Karatas, Y., Çetin, S., & Özcengiz, G. (2003). The effects of insertional mutations in comQ, comP, srfA, spo0H, spo0A and abrB genes on bacilysin biosynthesis in *Bacillus subtilis*. *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression*, *1626*(1), 51-56.

Kim, I., Ryu, J., Kim, H., & ChI, T. (2010). Production of biosurfactant lipopeptides Iturin A, fengycin and surfactin A from *Bacillus subtilis* CMB32 for control of Colletotrichum gloeosporioides. *J Microbiol Biotechnol*, *20*(1), 138-145.

Landy M, Warren GH, Rosenman SB, Colio LG. Bacillomycin an antibiotic from *Bacillus subtilis* active against pathogenic fungi. Proc Soc Exp Biol Med, 1948;67:530–41.

Lee, K., Yoon, D., Yoon, H., Lee, G., Song, J., Kim, G., & Kim, S. (2007). Cloning of srfA operon from *Bacillus subtilis* C9 and its expression in E. coli. *Applied microbiology and biotechnology*, *75*(3), 567-572.

Liu, B., Deikus, G., Bree, A., Durand, S., Kearns, B., & Bechhofer, H. (2014). Global analysis of mRNA decay intermediates in *Bacillus subtilis* wild-type and polynucleotide phosphorylase-deletion strains. *Molecular microbiology*, *94*(1), 41-55.

Liu, B., Kearns, B., & Bechhofer, H. (2016). Expression of multiple *Bacillus subtilis* genes is controlled by decay of slrA mRNA from Rho-dependent 3′ ends. *Nucleic acids research*, gkw069.

Liu, L., Nakano, M., Lee, H., & Zuber, P. (1996). Plasmid-amplified comS enhances genetic competence and suppresses sinR in *Bacillus subtilis*. *Journal of bacteriology*, *178*(17), 5144-5152.

Luo, C., Liu, X., Zhou, H., Wang, X., & Chen, Z. (2015). Nonribosomal Peptide Synthase Gene Clusters for Lipopeptide Biosynthesis in *Bacillus subtilis* 916 and Their Phenotypic Functions. *Applied and environmental microbiology*, *81*(1), 422-431.

Luttinger, A., Hahn, J., & Dubnau, D. (1996). Polynucleotide phosphorylase is necessary for competence development in *Bacillus subtilis*. *Molecular microbiology*, *19*(2), 343-356.

Nakano M., Zuber P. (1991). The primary role of ComA in stablishment of the competent state in *Bacillus subtilis* is to activate expression of srfA. J Bacteriol 173:7269-7274.

Ogura, M., Liu, L., LaCelle, M., Nakano, M., & Zuber, P. (1999). Mutational analysis of ComS: evidence for the interaction of ComS and MecA in the regulation of competence development in *Bacillus subtilis*. *Molecular microbiology*, *32*(4), 799-812.

Ogura, M., Yamaguchi, H., Kobayashi, K., Ogasawara, N., Fujita, Y., & Tanaka, T. (2002). Whole-genome analysis of genes regulated by the *Bacillus subtilis* competence transcription factor ComK. *Journal of bacteriology*, *184*(9), 2344-2351.

Ongena M., Jourdan E., Adam A., Paquot M., Brans A., Joris B., Arpigny L., Thonart P. (2007). Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. Environm Microbiol 9:1084-1090.

Oussenko, A., Abe, T., Ujiie, H., Muto, A., & Bechhofer, H. (2005). Participation of 3′-to-5′ exoribonucleases in the turnover of *Bacillus subtilis* mRNA. *Journal of bacteriology*, *187*(8), 2758-2767.

Roongsawang, N., Thaniyavarn, J., Thaniyavarn, S., Kameyama, T., Haruki, M., Imanaka, T., & Kanaya, S. (2002). Isolation and characterization of a halotolerant *Bacillus subtilis* BBK-1 which produces three kinds of lipopeptides: bacillomycin L, plipastatin, and surfactin. *Extremophiles*, *6*(6), 499-506.

Roongsawang, N., Washio, K., & Morikawa, M. (2010). Diversity of nonribosomal peptide synthetases involved in the biosynthesis of lipopeptide biosurfactants. *International journal of molecular sciences*, *12*(1), 141-172.

Salvo, E., Alabi, S., Liu, B., Schlessinger, A., & Bechhofer, D. H. (2016). Interaction of *Bacillus subtilis* Polynucleotide Phosphorylase and RNase Y: Structural Mapping and Effect on mRNA Turnover. *Journal of Biological Chemistry*, jbc-M115.

Sambrook J, Russell DW, Molecular Cloning: A Laboratory Manual, 3rd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 2001.

Schultz, D., Wolynes, G., Jacob, B., & Onuchic, N. (2009). Deciding fate in adverse times: sporulation and competence in *Bacillus subtilis*. *Proceedings of the National Academy of Sciences*, *106*(50), 21027-21034.

Song, Y., Rubio, A., Jayaswal, R. K., Silverman, J. A., & Wilkinson, B. J. (2013). Additional routes to *Staphylococcus aureus* daptomycin resistance as revealed by comparative genome sequencing, transcriptional profiling, and phenotypic studies. *PloS one*, *8*(3), e58469.

Tsuge K, Ano T, Hirai M, Nakamura Y, Shoda M, The genes *degQ*, *pps*, and *lpa-8* (*sfp*) are responsible for conversion of *Bacillus subtilis* 168 to plipastatin production. Antimicrob Agents Chemother, 1999;43:2183–92.

Wang, W., & Bechhofer, H. (1996). Properties of a *Bacillus subtilis* polynucleotide phosphorylase deletion strain. *Journal of Bacteriology*, *178*(8), 2375-2382.

Zeriouh, H., Vicente, A., Pérez-García, A., & Romero, D. (2014). Surfactin triggers biofilm formation of *Bacillus subtilis* in melon phylloplane and contributes to the biocontrol activity. *Environmental microbiology*, *16*(7), 2196-2211.

Chapter 6
General discussion and perspectives

Part 1: General discussion

During the past decades, lipopeptides have gained attention and interest for developing environmentally friendly approaches allowing to reduce the use of chemicals. These lipopeptides are especialy studied for their potential uses in several domains such as agriculture with natural pesticides **[**Ongena, 2007**]**, cosmetics industry **[**Meena and Kanwar, 2015**],** pharmaceutical uses [Moryl *et al.,* 2014], bioremediation of polluted soils **[**Sang-Cheol *et al.,* 2007**]** and much else. Fengycins are important molecules synthesized by *Bacillu*s, however, little information is available concerning their biosynthesis regulation. These facts encouraged us to study this family of lipopeptides regarding two general aspects: molecular regulation and production (physiology) of fengycins in *Bacillus spp*.

The bibliographic study **(chapter 1)** shows that many quorum sensing compounds or regulators could play a role as activator or suppressor to regulate the fengycin operon either directly or indirectly. Among these factors PhO-PhR, ComA-ComP, DegQ-DegR, AbrB, SinR, CodY can be mentioned. Most of these regulators are directly or indirectly stimulated by the nutrient and stress conditions **[**Msadek *et al.,* 1990; Allenby *et al.,* 2005; Chumsakul *et al.* 2010; Guo *et al.,* 2014; İrigül-Sönmez *et al*., 2014**]**.

In chapter 3 *B. subtilis* BBG21 strain (a spontaneous mutant of *B. subtilis* ATCC 21332 overproducing fengycins) was used to study the fengycin regulation and production. Whole genome sequence for this strain was previously carried out in our laboratory.

The analysis by the AntiSmash software indicated that this strain contains the two known NRPs clusters of surfactin and fengycin, as well as three secondary NRPS clusters (bacillaene, bacillibactin and bacilysin). Comparison of the genome sequence of this strain with sequence of *B. subtilis* 168 showed 99% of identity, while only 95% of identity was obtained between the fengycin clusters in the two strains. These differences in fengycin clusters lead us to study the 5% of dissimilarity in order to check their influence on fengycin biosynthesis. We first highlight that *B. subtilis* BBG21 produce more than 10-fold of fengycin than *B subtilis* BBG111 (*B. subtilis* 168 strain modified with *sfp*). By comparing the promoter sequence of both strains we showed that there one nucleotide differs and 10 nucleotides are lacking. The different nucleotide is situated in the UP element zone is the sole difference between the promoter of BBG21 and its mother strain ATCC 21332. Meijer and Salas (2004) still refer to the critical role of the UP element sequence and its effect on the

promoter stability and activity in *B. subtilis* and according to Tsuge *et al*. (2007) a significant decreased of the fengycin production were observed after a mutation in this sequence in the strain *B. subtilis* F29-3. In order to confirm that the differences in the production background between the strains are a result from the differences in promoter sequences, we integrated the GFP protein in front of the promoter of three fengycin producer strains (BBG21, BBG111 and *B. amyloliquefaciens* FZB42). The results of the promoter expression showed that the promoter of BBG21 strain is more active than other promoters under all the tested conditions. The study of Nicolas *et al.,* [2012] still clarified the effect of several conditions on the fengycin promoter expression, but under our knowledge this work showed for the first time the effect of the different medium conditions on the fengycin operon expression as well as the fengycin production.

Therefore, **in chapter 4** the influences on the promoter expression of different nutrient conditions as carbon and nitrogen sources in the medium have been studied. Influences were tested directly using GFP mutants and indirectly by measuring the fengycin produced under these conditions.

Most of the conditions showed related effect on both promoter activity and fengycin production as shown below in table 1. The effects of urea as nitrogen source were positive allowing to increase the promotor expression as well as the production. Actually, the expression with urea showed 6-fold higher than that obtained with glutamic, and the production was 20% higher.

Positive effects were also observed with mannitol as carbon source when expression was 3-fold higher and production about 2-fold compared to that one obtained with glucose. Negative effects with valine, peptone, galactose, and sorbitol were observed for both expression and production.

Actually, the influence of urea and mannitol on the fengycin expression and production have been well discussed in chapter four as a hypotheses to explain their effect [Koumoutsi *et al., 2004;* Makkar *et al., 2011 ;* Huang *et al. 2015]*.

Using starch or lactose**,** production of fengycin was not significant comparing to the high expression obtained. On the contrary, significant productions were obtained with glucose and sucrose while moderate promoter expression was shown. Sucrose has been mentioned as a favorable carbon source to produce the fengycin from Bacillus sp. [Liu et al. 2013]. In our experiments replacing mannitol by sucrose in the medium after 16 hours of growth increased

the production of fengycin 30% to reach 768 mg/L. This production is the highest production reported from the strain BBG21.

Table 1. Tested conditions that positively influenced either the expression or the production of fengycin.

Conditions	Promoter expression	Fengycin synthesis
Urea	++	
Glutamic acid		+
Alanine	++	
Ammonium		+
Glucose		+
Mannitol	++	+
Starch	++	
Galactose		+
Maltose		+
Sucrose		++
Lactose	+	

The use of different nitrogen and carbon sources strongly influences the fengycin promoter expression which can either reflect directly on fengycin production or the gene translation scale-down under certain conditions. According to Nicolas *et al.*, 2012, the *ppsA* gene expression responded positively with many carbon sources and the highest expression obtained was in M9 medium with fructose.

Few studies have compared the influence of nutrient conditions on two families of lipopeptides produced from one strain [Zeriouh *et al.*, 2014; Luo *et al.*, 2015]. In this work surfactin was also analyzed under numerous conditions and the results showed that the production varied in the same way as fengycin under most of the conditions. The highest surfactin productions obtained were with the urea and ammonium carbonate as nitrogen sources and starch or mannitol as carbon sources. Previous studies showed the positive influence using the urea or ammonium as nitrogen source as well as mannitol or starch as

carbon sources **[**Abushady *et al.* 2005; Abdel-Mawgoud *et al.* 2008; Zheng *et al.* 2013**]**. Few carbon substrate conditions (mannose, maltose, and galactose) showed disaccording results between the productions level of surfactin and fengycin. These observations indicated that expression of NRPS genes could act as a response to the environmental conditions, while some nutrient sources could play a role as lipopeptide precursors. We can hypothesize from these results that there might be an interaction among the regulatory factors of the two operons. However in other works, some culture conditions direct the production toward a specific lipopeptide at the expense of the other one [Singh *et al* 2014].

To confirm this regulatory relation, surfactin or fengycin negative mutants were constructed **(Chapter 5)**. The fengycin production was slightly increased in *srfAC*$^{-}$ mutant (10%) but surprisingly, the fengycin concentration was significantly decreased in *srfAA*$^{-}$ mutant. Published results indicate that *srfAA* is probably not involved directly in the regulation of fengycin but may act indirectly through *comS* competence gene which is situated at the end of the *srfAA* gene and controlled ComA-ComP and *degQ* [Molle *et al.,* 2003; Ogura and Tsukahara, 2010]. On the other hand, the surfactin production increased for 30% in the *fenA*$^{-}$ mutant this could result from a competition for precursors between both synthetases.

The *comS* expression requires *pnpA* (gene controlling mRNA degradation enzyme PnpAse) [Gamba *et al., 2015;* Liu *et al.*, 2016]. In the last part of work, effect of deletion of *pnpA* in *B. subtilis* BBG21 and *B. subtilis* 168 strains were studied. The mutation significantly reduced the productions of both fengycin (70-80%) and surfactin (40-50%) compared with wild strains. All the molecular results were concluded in figure 1.

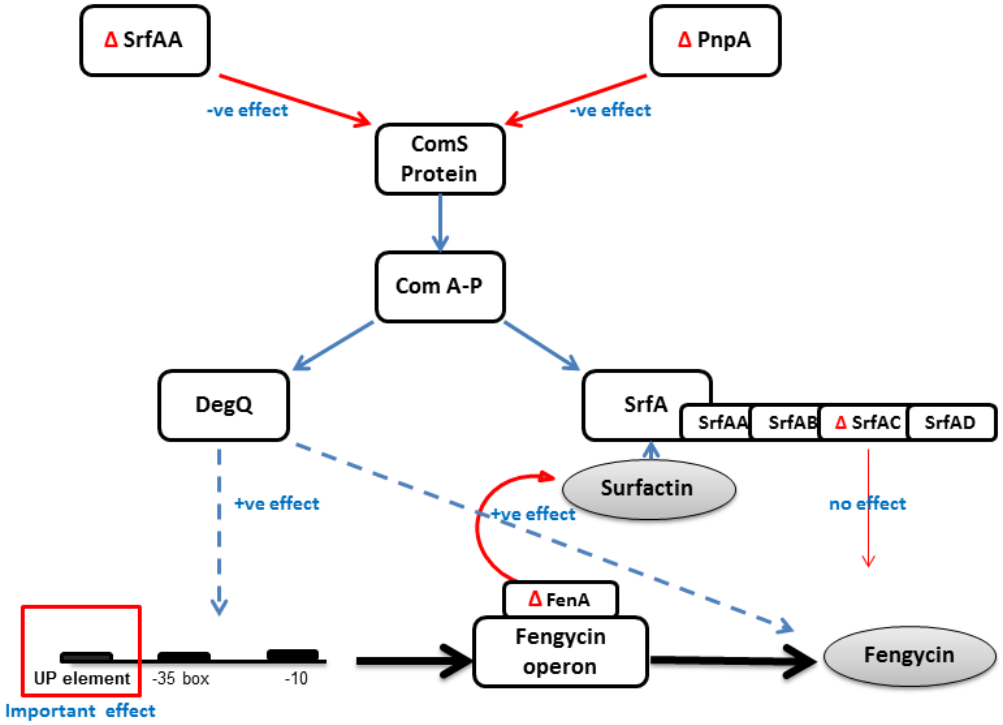


Figure 1. Summary of the results obtained in this thesis about the molecular mechanism involved in the regulation of fengycin operon expression.

Δ: knockout of the gene. Red arrows represent the work realized in this study.

-ve effect: negative effect; +ve effect: positive effect.

Part 2: Future prospects

Regarding the results obtained in this study, the following perspectives are proposed:

1- All the physiological parameters tested in this study showing positive results regarding the fengycin production could be tested in continuous culture to allow the optimization of the production.

2- The interactions between the fengycin and surfactin biosynthesis which were obvious in the results of *srfAA* and *fenA* mutants, as well as the role of *comS* need to be confirmed. The study of the overlaps and the ability of binding for these genes in the fengycin promoter region can be also verified using DNase I fingerprinting as well as the potential competition for common substrates for both synthetases.

3- Transcriptomic of the fengycin producer strains could be tested under several conditions such as urea or mannitol using microarray technology. This technique help to highlight expected regulator factors which could play a role in the activating or repressing the fengycin operon.

4- We could specify the roles of several regulators which may have an influence on the fengycin regulation (AbrB, CodY, ComP, and SinR). By knock-outing the genes of interest we can verify their role in the fengycin biosynthesis either directly by measuring the fengycin operon expression or indirectly by observing the fengycin production.

5- We could intend to characterize the structure and the functional for the fengycin isoforms produced under different nitrogen or carbon sources.

6- We could intend to study *the post-translated* factors which may lead to degrade the fengycin produced (protease).

References

Abdel-Mawgoud, M., Aboulwafa, M., & Hassouna, H. (2008). Optimization of surfactin production by *Bacillus subtilis* isolate BS5. *Applied biochemistry and biotechnology*, *150*(3), 305-325.

Abushady, M., Bashandy, S., Aziz, H., & Ibrahim, M. (2005). Molecular characterization of *Bacillus subtilis* surfactin producing strain and the factors affecting its production. *International Journal of Agriculture and Biology*, *3*, 337-344.

Allenby, N. E., O'Connor, N., Prágai, Z., Ward, A. C., Wipat, A., & Harwood, C. R. (2005). Genome-wide transcriptional analysis of the phosphate starvation stimulon of *Bacillus subtilis*. *Journal of Bacteriology*, *187*(23), 8063-8080.

Chumsakul, O., Nakamura, K., Kurata, T., Sakamoto, T., Hobman, L., Ogasawara, N., & Ishikawa, S. (2013). High-resolution mapping of in vivo genomic transcription factor binding sites using in situ DNase I footprinting and ChIP-seq. *DNA research*, dst013.

Chumsakul, O., Takahashi, H., Oshima, T., Hishimoto, T., Kanaya, S., Ogasawara, N., & Ishikawa, S. (2010). Genome-wide binding profiles of the *Bacillus subtilis* transition state regulator AbrB and its homolog Abh reveals their interactive role in transcriptional regulation. *Nucleic acids research*, gkq780.

Gamba, P., Jonker, M. J., & Hamoen, L. W. (2015). A novel feedback loop that controls bimodal expression of genetic competence. *PLoS Genet*, *11*(6), e1005047.

Guo, Q., Dong, W., Li, S., Lu, X., Wang, P., Zhang, X., ... & Ma, P. (2014a). Fengycin produced by Bacillus subtilis NCD-2 plays a major role in biocontrol of cotton seedling damping-off disease. *Microbiological research*, *169*(7), 533-540.

Huang, X., Liu, J. N., Wang, Y., Liu, J., & Lu, L. (2015). The positive effects of Mn2+ on nitrogen use and surfactin production by *Bacillus subtilis* ATCC 21332. *Biotechnology & Biotechnological Equipment*, *29*(2), 381-389.

İrigül-Sönmez, Ö., Köroğlu, E., Öztürk, B., Kovács, Á. T., Kuipers, O. P., & Yazgan-Karataş, A. (2014). In *Bacillus subtilis* LutR is part of the global complex regulatory network governing the adaptation to the transition from exponential growth to stationary phase. *Microbiology*, *160*(Pt 2), 243-260.

Koumoutsi A, Chen X, Henne A, Liesegang H, Hitzeroth G, Franke P, Vater J, Borriss R. (2004), Structural and functional characterization of gene clusters directing

nonribosomal synthesis of bioactive cyclic lipopeptides in *Bacillus amyloliquefaciens* strain FZB42. J Bacteriol 186:1084–1096.

Liu, B., Kearns, B., & Bechhofer, H. (2016). Expression of multiple *Bacillus subtilis* genes is controlled by decay of slrA mRNA from Rho-dependent 3′ ends. *Nucleic acids research*, gkw069.

Liu, X., Ren, B., Gao, H., Liu, M., Dai, H., Song, F., & Zhang, L. (2012). Optimization for the production of surfactin with a new synergistic antifungal activity. *PloS one*, *7*(5), e34430.

Luo, C., Liu, X., Zhou, H., Wang, X., & Chen, Z. (2015). Nonribosomal Peptide Synthase Gene Clusters for Lipopeptide Biosynthesis in *Bacillus subtilis* 916 and Their Phenotypic Functions. *Applied and environmental microbiology*, *81*(1), 422-431.

Makkar S, Banat S., (2011). Advances in utilization of renewable substrates for biosurfactant production. AMB Express; 1:5.

Meena, R., & Kanwar, S. (2015). Lipopeptides as the Antifungal and Antibacterial Agents: Applications in Food Safety and Therapeutics. *BioMed research international*, *2015*.

Meijer, W. J., & Salas, M. (2004). Relevance of UP elements for three strong *Bacillus subtilis* phage ϕ29 promoters. *Nucleic acids research*, *32*(3), 1166-1176.

Molle, V., Nakaura, Y., Shivers, R. P., Yamaguchi, H., Losick, R., Fujita, Y., & Sonenshein, A. L. (2003). Additional targets of the *Bacillus subtilis* global regulator CodY identified by chromatin immunoprecipitation and genome-wide transcript analysis. *Journal of bacteriology*, *185*(6), 1911-1922.

Moryl, M., Spętana, M., Dziubek, K., Paraszkiewicz, K., Różalska, S., Płaza, G. A., & Różalski, A. (2014). Antimicrobial, antiadhesive and antibiofilm potential of lipopeptides synthesised by *Bacillus subtilis*, on uropathogenic bacteria. *Acta biochimica Polonica*, *62*(4), 725-732.

Msadek, T., Kunst, F., Henner, D., Klier, A., Rapoport, G., & Dedonder, R. (1990). Signal transduction pathway controlling synthesis of a class of degradative enzymes in *Bacillus subtilis*: expression of the regulatory genes and analysis of mutations in degS and degU. *Journal of bacteriology*, *172*(2), 824-834.

Nicolas, P., Mäder, U., Dervyn, E., Rochat, T., Leduc, A., Pigeonneau, N., & Bessières, P. (2012). Condition-dependent transcriptome reveals high-level regulatory architecture in *Bacillus subtilis*. *Science*, *335*(6072), 1103-1106.

Ogura, M., & Tsukahara, K. (2010). Autoregulation of the *Bacillus subtilis* response regulator gene degU is coupled with the proteolysis of DegU-P by ClpCP. *Molecular microbiology*, *75*(5), 1244-1259.

Ongena M, Jourdan E, Adam A, Paquot M, Brans A, Joris B, Arpigny JL, Thonart P (2007) Surfactin and fengycin lipopeptides of *Bacillus subtilis* as elicitors of induced systemic resistance in plants. *Environm Microbiol* 9:1084-1090.

Sang-Cheol, L., Kim, H., Park, H., Chung, Y., Chandra, S., & Yong-Lark, C. (2010). Isolation, purification, and characterization of novel fengycin S from *Bacillus amyloliquefaciens* LSC04 degrading-crude oil. *Biotechnology and Bioprocess Engineering*, *15*(2), 246-253.

Singh, K., Rautela, R., & Cameotra, S. (2014). Substrate dependent in vitro antifungal activity of Bacillus sp. strain AR2. *Microb Cell Fact, 13*, 67-77.

Tsuge K, Matsui K, Itaya M, (2007). Production of the non-ribosomal peptide plipastatin in *Bacillus subtilis* regulated by three relevant gene blocks assembled in a single movable DNA segment. J Biotechnol.;129(4):592-603.

Zeriouh, H., Vicente, A., Pérez-García, A., & Romero, D. (2014). Surfactin triggers biofilm formation of *Bacillus subtilis* in melon phylloplane and contributes to the biocontrol activity. *Environmental microbiology*, *16*(7), 2196-2211.

Zheng, M., Wang, P., & Wu, Q. (2013). Optimization of culture conditions for biosynthesis of lipopeptide by *Bacillus subtilis* with starch. In *Applied Mechanics and Materials* (Vol. 291, pp. 225-229).

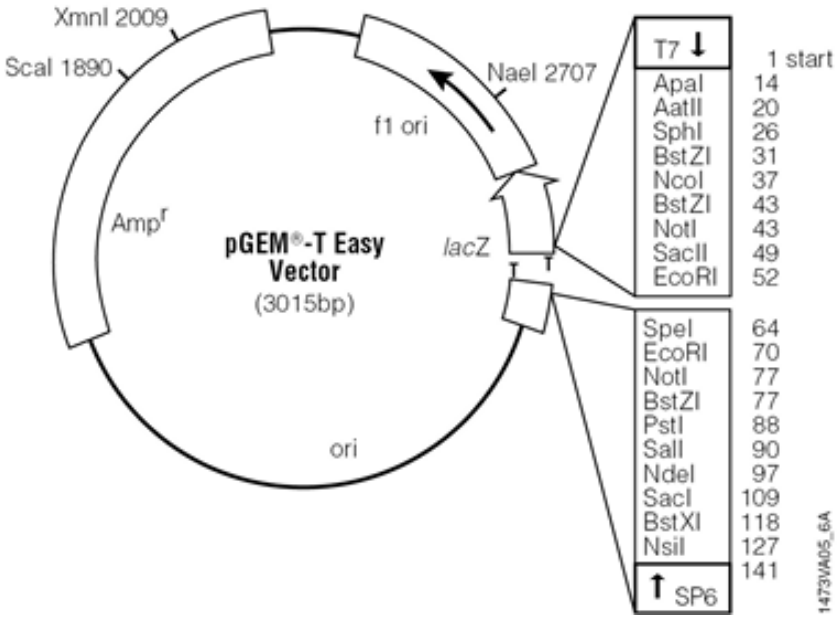
Chapter 7
Appendix

Part 1: supplementary data chapter 3

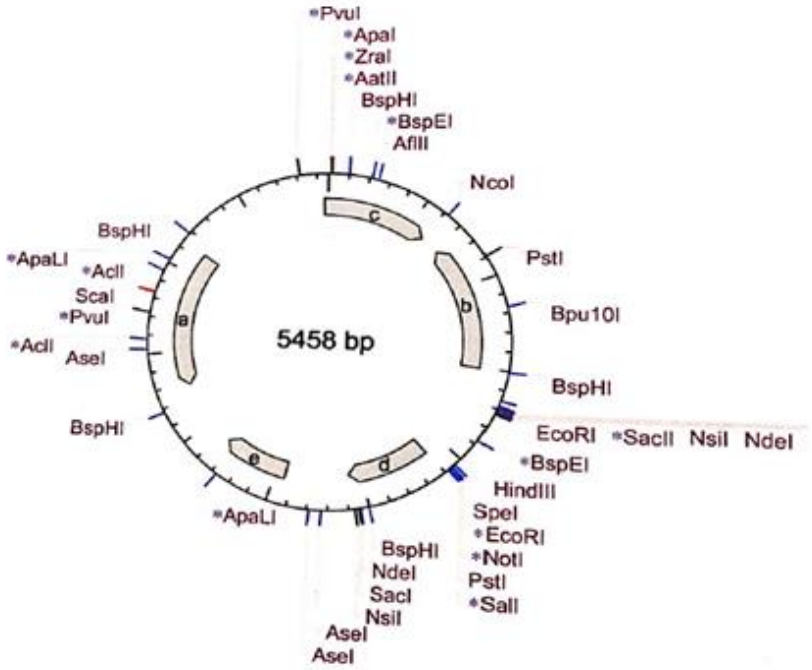
Supplementary 1: Victors used in this study:

Al the victors used have been described in table (1)

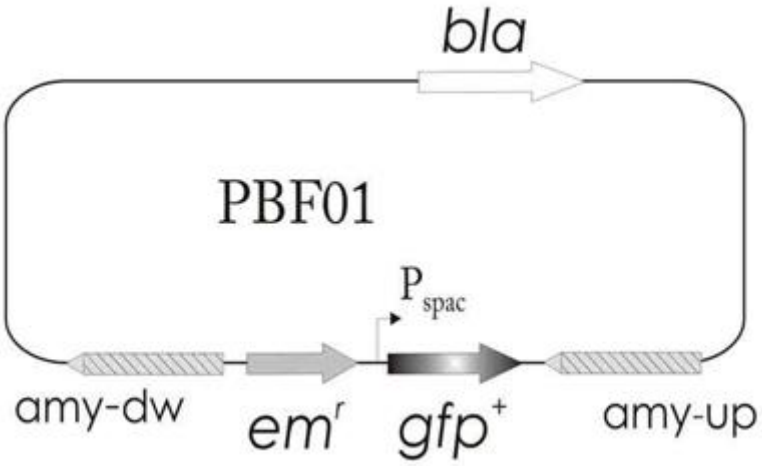
a- pGEM-T Easy



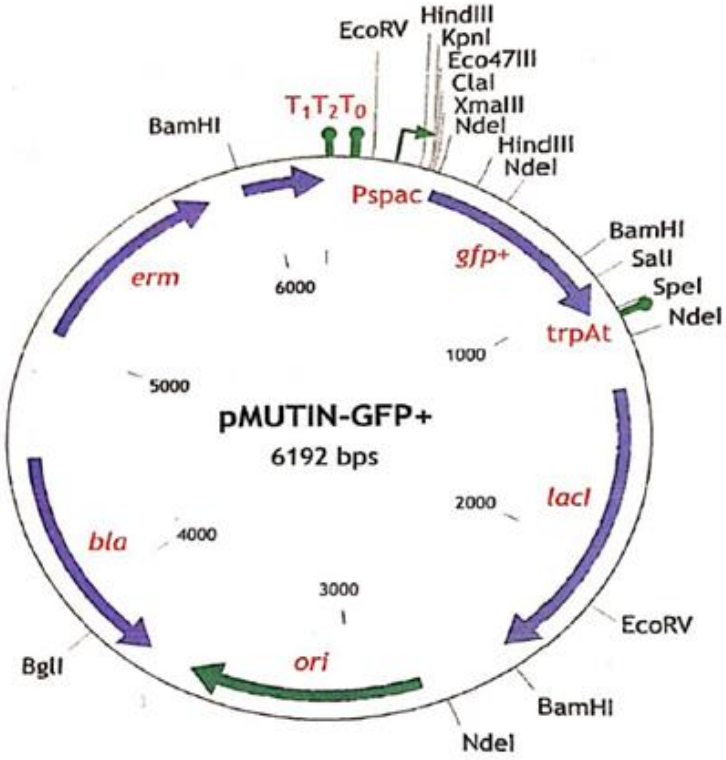
b- **pBG193**



c- pFB01



d- pMUTIN-GFP+

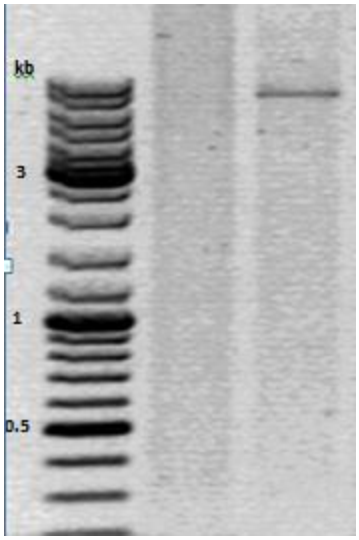


Supplementary 2: Insert *ComK* (the natural competence gene) in BBG21

After many unsuccessful trials to insert the GFP gene in the strain BBG21 by electroporation method, we choose another strategy. We first transfer the natural competence gene *comK* in the wild strain BBG 21 then we try to insert the GFP gene in the strain. Between many protocols the protoplast electroporation was the only method that gave the positive result for *ComK* to produce the strain BBG201;

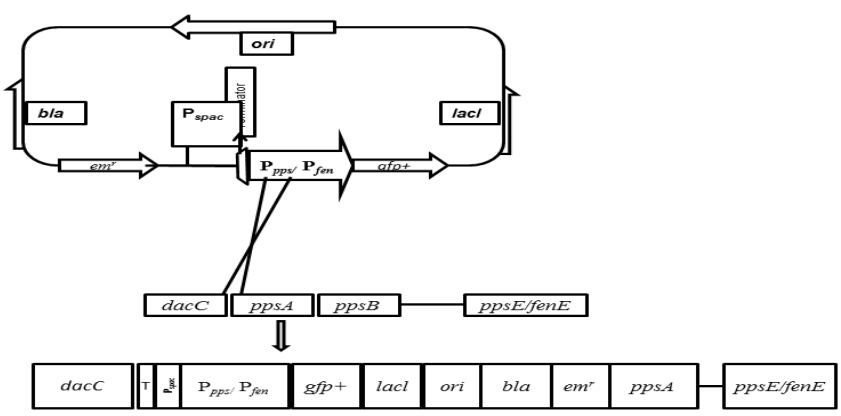
Supplementary 2.a. Protoplast electroporation

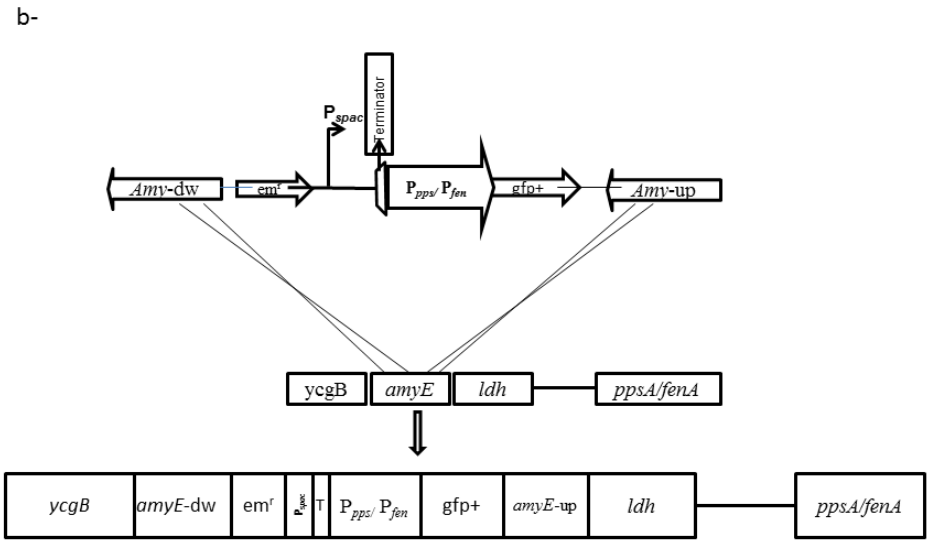
Cells were overnight grown in 10ml of X1 PAB (Penassy broth) at 37°C, the culture diluted 1/10 with 10ml X1 PAB, incubated 2 hours at 37°C until (OD_{450} = 1.5-2). Subsequently cells were collected by centrifugation, suspended in 5-10ml SMPP medium, and protoplasts were obtained after incubation at 37°C on a rotary shaker at 100 rpm for 30 min in presence of lysozyme (10mg/ml). The presence of protoplasts was verified by phase contrast microscopy. Protoplasts were then carefully harvested by centrifugation 5000 tim/min for 5 min, washing two times with SMM buffer and suspended in 2ml of SMMP medium. For the electroporation trials, volumes of 150µl of protoplast suspensions were mixed with of 0.5-1µg DNA and kept on ice for at least 2min.The mixture was then transferred to a pre-chilled electroporation cuvette (0.2 cm electrode gap) and exposed to a single electrical pulse in a Gene Pulser Xcell System (Bio-Rad Laboratories, USA) set at 25 µF, 400 Ω and0.7 kV. Immediately after the pulse delivery, 1 ml of recovering medium (SMMP) was added to the cuvette, and the mixture was transferred to a 2 ml tube and incubated at 37 °C and 100 rpm for 2h. Then, the cells are spread on LB plates with the selective antibiotic for 24 h at 37 ºC.



Supplementary 2.b: verification of pMMcomK insertion in the strain BBG21 producin the strain BBG201, lane 1 represent the mother strain, lane 2 represent the mutant strain.

a-





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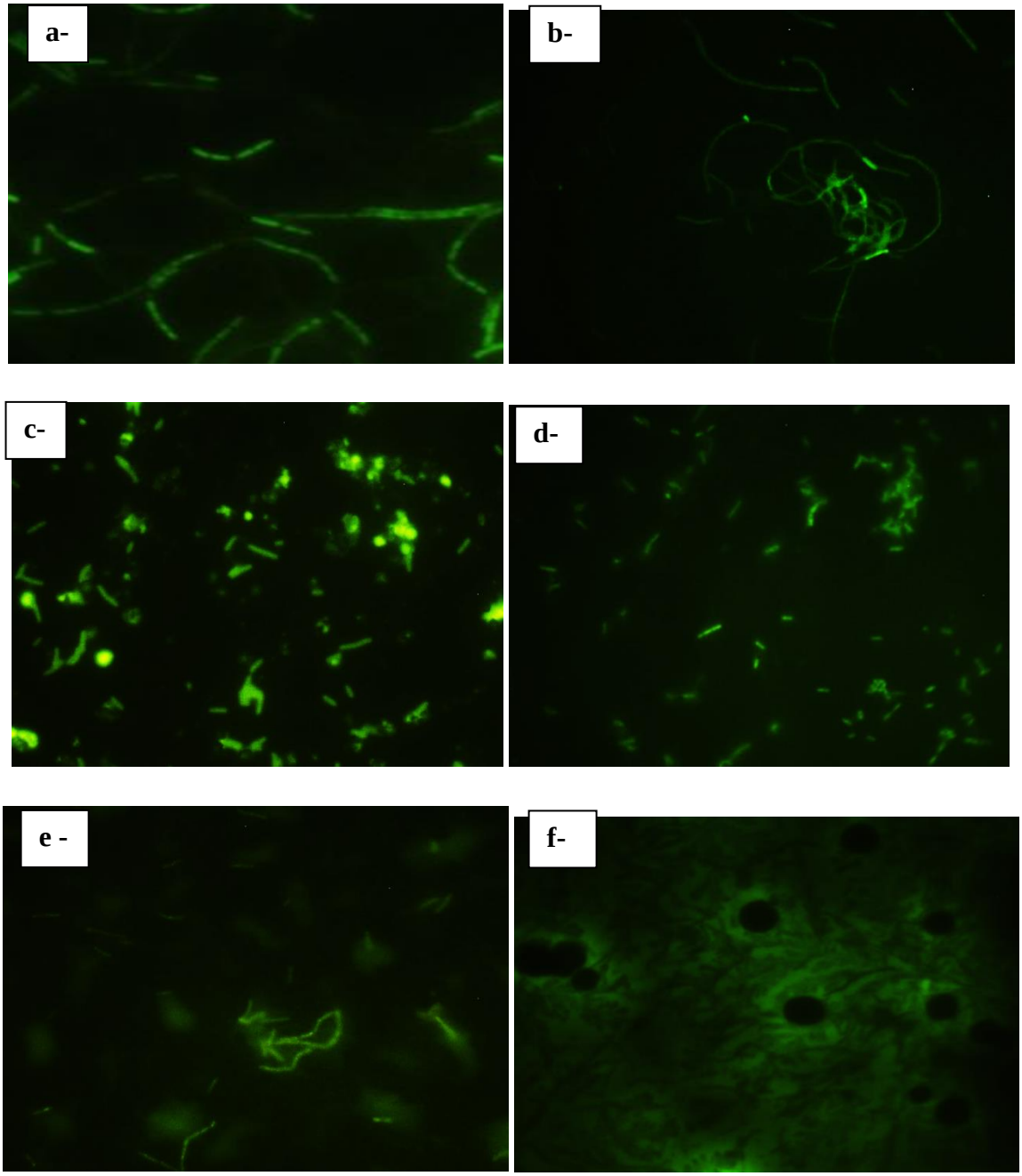
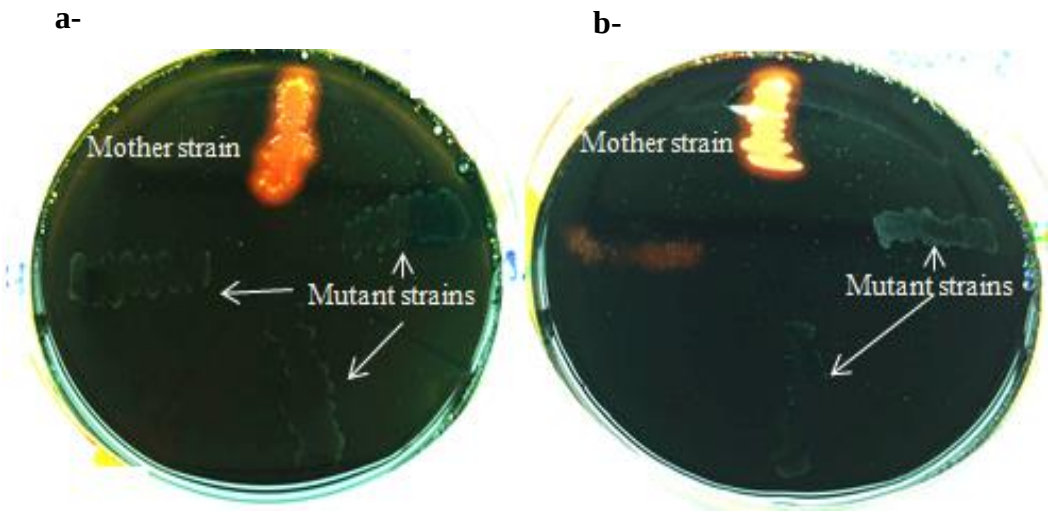
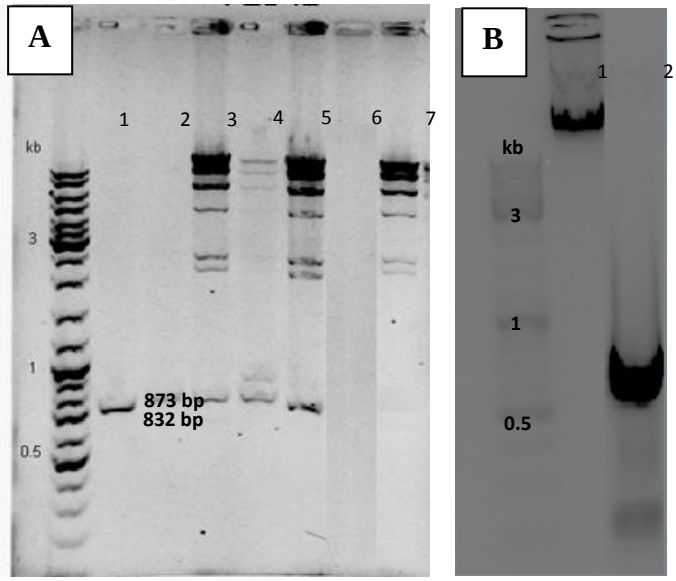
Supplementary 2: Schematic representation of *gfp* insertion using the two vectors pMUTIN-GFP+ and pFB01. (a) Transformation of *B. subtilis* BBG201, BBG111 and *B. amyloliquefaciens* FZB42 with pMUTIN-GFP+ by homologous recombination through a single crossing-over between the end of the gene *dacC* and the start beginning of the gene *fenA*/*ppsA*. (b) Transformation of *B. subtilis* BBG201 and *B. amyloliquefaciens* FZB42 with pFB01 by homologous recombination through a double crossing-over up and down of the gene *amyE*.



Supplementary 3: Fluorescence microscopic observations for the Bacillus modified strain takes using Nikon fluorescence microscope under 490 nm excitation at 100X magnification. Images are: a- *B. subtilis* BBG21/pMUTIN. b- *B. subtilis* BBG21/pFB01 c- *B. amyloliquefacienes*FZB42/pMUTIN. d- - *B. amyloliquefacienes*FZB42/pFB01 d- *B. subtilis* BBG111/pMUTIN. f- *B. subtilis* 168 (control).



Supplementary 4: Starch analysis test for the mutant cells: a- *B. subtilis* BBG21/pFB01 and b- *B. amyloliquefacienes*FZB42/pFB01.



Supplementary 6: Verification of the insertion of *gfp* gene in the different strains:

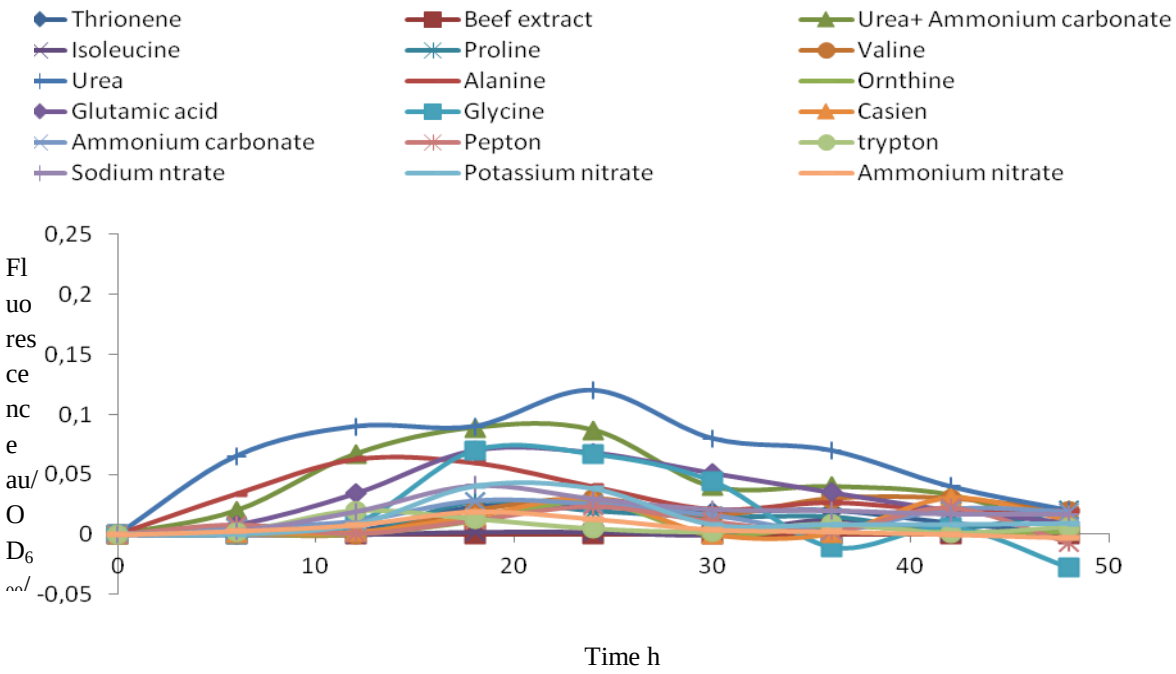
A-*Verification of gfp insertion in fengycin operon under the control of its promoters using PCR of dacC upstream-gfp dwonstream and amyE upstream-gfp dwonstream ampliconts in the muatnt and the mother strains: 1- BBG205 transferd by pMUTIN-GFP+ carring Pfen BBG21. 2- BBG208 transferd by pFB01 carring Pfen BBG21. 3- BBG209 transferd by pFB01 carring Pfen FZB42. 4- BBG209 (another colony) transferd by pFB01 carring Pfen FZB42. 5- BBG207 transferd by pMUTIN-GFP+ carring Pfen FZB42. 6- BBG21 mother strain. 7- FZB42 mother strain.*

B- *Verification of gfp insertion in the strain BBG111: lane1 represent the mutant strain BBG206, lane 2 represent the mother strain.*

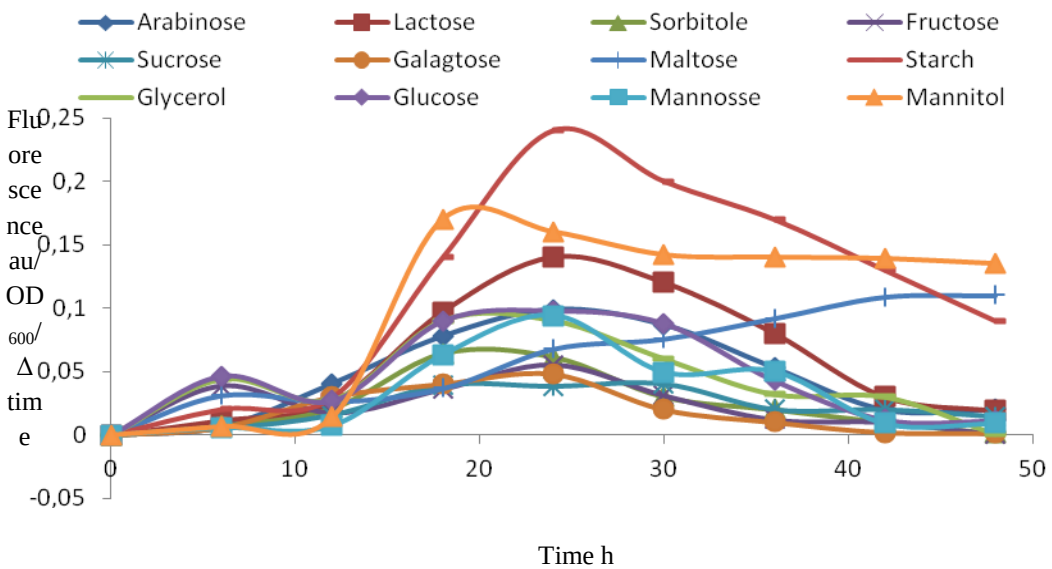
Part 2: supplementary data chapter 4

Supplementary data 1:

a-



b-



c-

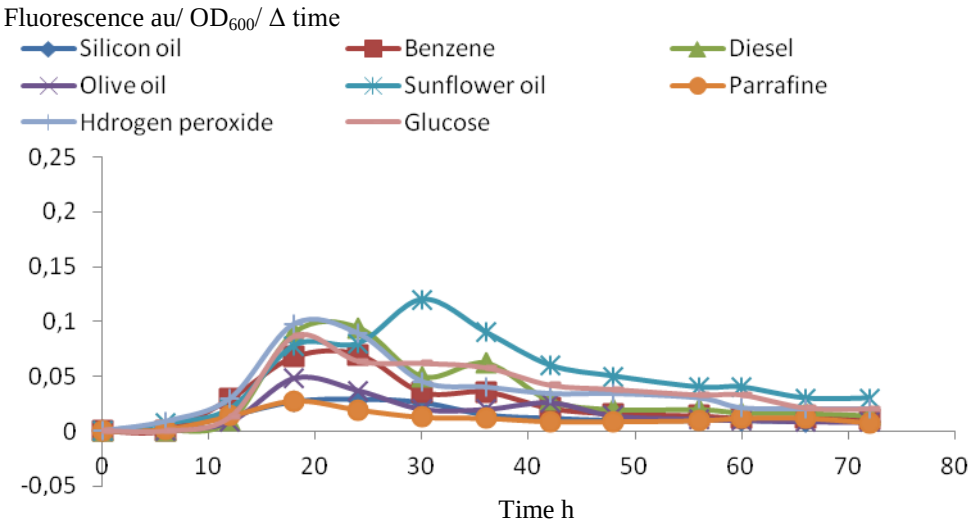


Figure 1. The fengycin promoter activity (**Fluorescence intensity/ OD_{600}/ Δtime (when time = time 2- time1)** by BBG208 after 48 h of growth under Landy medium modified with different substrate conditions:

a. Nitrogen sources **Landy medium was modified by replacing the glutamic acid in Landy medium with 5 g of 18 different nitrogen sources: Landy Urea, Landy Urea- NH_4HCO_3, Landy NH_4HCO_3, Landy NH_4NO_3, Landy Tryptone, Landy Peptone, Landy Beef extract, Landy Casein, Landy Valine, Landy Proline, Landy Isoleucine, Landy Ornithine, Landy Threonine, Landy Glycine, Landy Alanine, Landy KNO_3, Landy $NaNO_3$ and Landy Glutamic acid.*

b. Carbon sources **Landy medium was modified by replacing the glucose in Landy medium with 5 g of 13 different carbon sources: Landy Arabinose, Landy Lactose, Landy Fructose, Landy Sucrose, Landy Mannose, Landy Sorbitol, Landy Maltose, Landy Starch, Landy Galactose, Landy Glycerol, Landy Mannitol, and Landy Glucose.*

c. Hydrocarbon sources **Landy medium was modified by supplied the medium with 1% of 6 different hydrocarbon sources: Landy diesel, Landy benzene, Landy sunflower oil, Landy silicon oil, Landy paraffin, Landy olive oil, Landy H_2O_2 (oxidative stress condition), where Landy medium without any addition used as control.*

Supplementary data 2

Table 1: Effect of the use the Urea as nitrogen source with different culture conditions on surfactin production by Bacillus subtilis BBG208 strain.

Condition*	Surfactin mg/L	∗Relative surfactin yield $mg/L.DO_{600}$
C.1	480 ± 17	43.6
C.2	483 ± 16.4	63.5
C.3	510 ± 23.2	55.4
C.4	435 ± 11.8	47.8
C.5	327 ± 9.7	43.6
C. 6	400 ± 31.1	48.7
C.7	450 ± 13	69.2

** Condition 1: Landy Urea 25°C, Condition 2: Landy Urea 30°C, Condition 3: Landy Urea 37 °C, Condition 4: Landy Urea 30% filing volume, Condition 5: Landy Urea 50% filing volume, Condition 6: Landy Urea pH 7.5, Condition 7: Landy Urea pH 6.5.*

Table 6: Effect of the use the Mannitol as carbon source with different culture conditions on surfactin production by *Bacillus subtilis* BBG208 strain.

Condition	**surfactin mg/L**	**∗Relative surfactin yield mg/ L.DO$_{600}$**
C.1	649 ± 28.2	63.7
C.2	525 ± 8.8	59.6
C.3	529 ± 12.6	58.8
C.4	600 ± 41.1	56.1
C.5	476 ± 9.2	50.5
C.6	625 ± 7.7	63.7
C.7	751 ± 22.2	68.1

**Condition 1: Landy Mannitol 25°C, Condition 2: Landy Mannitol 30°C, Condition 3: Landy Mannitol 37 °C, Condition 4: Landy Mannitol 30% filing volume, Condition 5: Landy Mannitol 50% filing volume, Condition 6: Landy Mannitol pH 7.5, Condition 7: Landy Mannitol pH 6.5.*

Part 3: chapter 5 supplementary data

Supplementary chapter 5

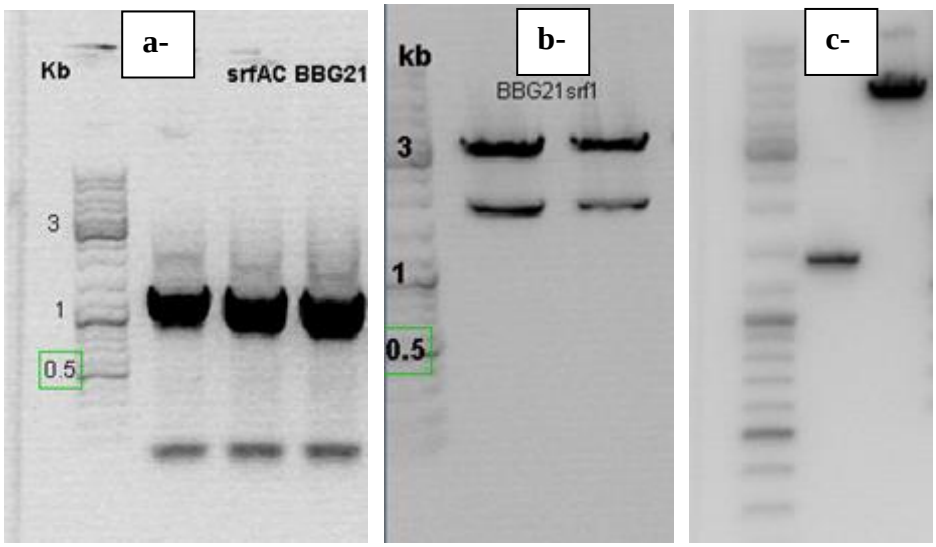


Figure 1. Agarose gel represents:

a- PCR amplicon for the fragment *srfAC-srfAC* (1620 bp).
b- pOJ260 double digested by EcoRI-HindIII.
c- PCR for verified the disruption of *srfAC* using the primer *srfAC-srfAC.*

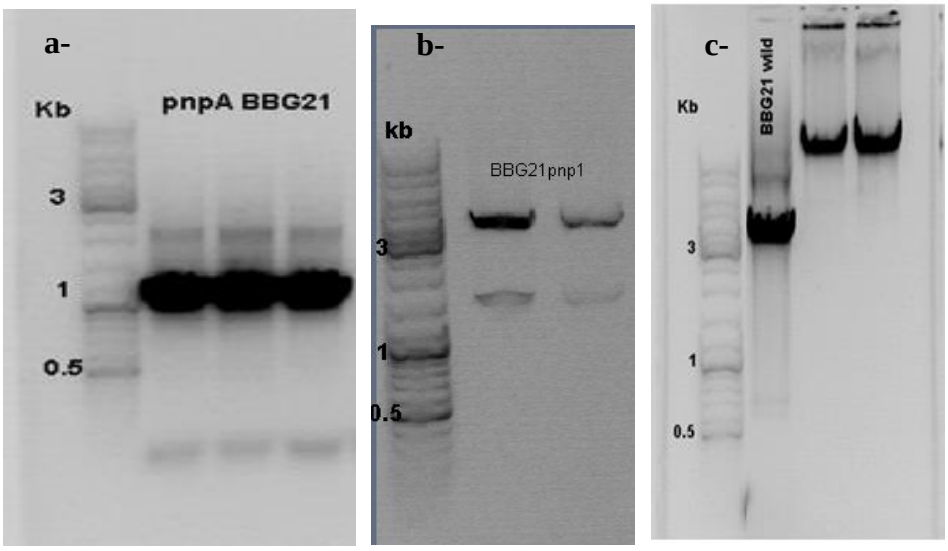


Figure 2. Agarose gel represents:

a- PCR amplicon for the fragment *ribC-pnpA* (1551 bp).

b- pMUTIN double digested by KpnI-XmaII.

c- PCR mutant verified by the primer *ribCfwd –ylxYrv*.

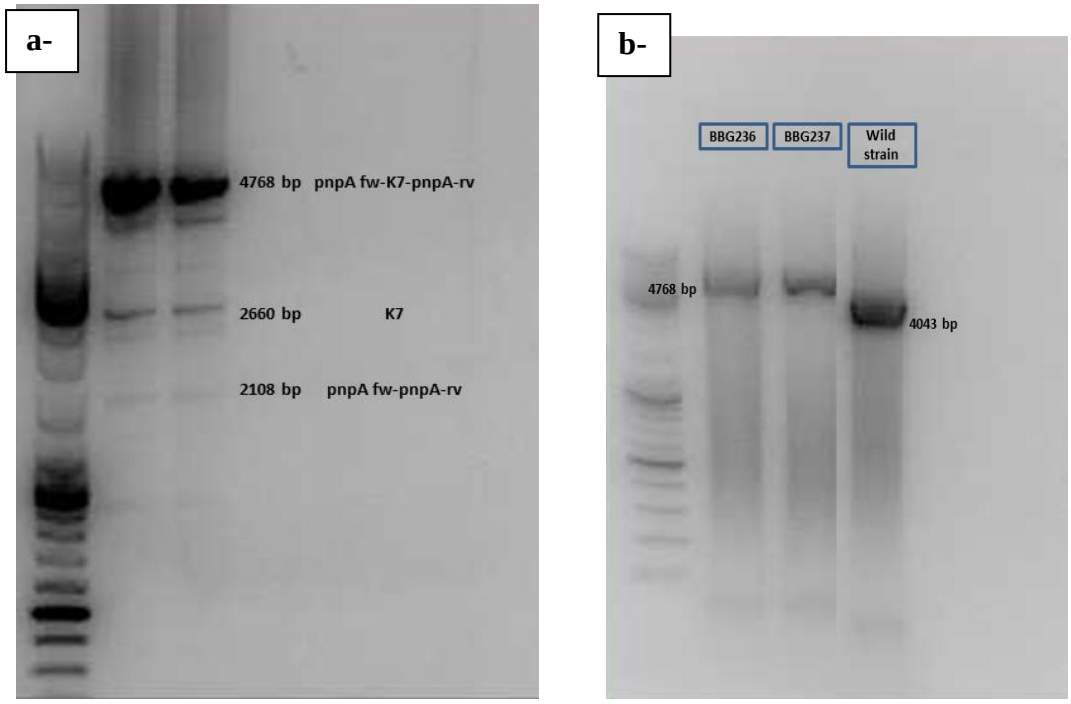


Figure 3. Agarose gel represents:

a- The joint PCR amplicon whine the three fragments are ligated (pnpA fw,pnpA delta rv – K7 p3,K7 p5 - pnpA delta fw, pnpA rv) generated a liner fragment with 4768 bp.

b- Verification of the disruption mutant using the primers pnpA fw – pnpA rv.