Archives of Microbiology (2018) 200:783–791
https://doi.org/10.1007/s00203-018-1483-5

ORIGINAL PAPER

Polynucleotide phosphorylase is involved in the control of lipopeptide fengycin production in *Bacillus subtilis*

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Received: 24 August 2017 / Revised: 18 January 2018 / Accepted: 23 January 2018 / Published online: 8 February 2018

Abstract

Bacillus subtilis is a wealth source of lipopeptide molecules such as iturins, surfactins and fengycins or plipastatins endowed with a range of biological activities. These molecules, designated secondary metabolites, are synthesized via non-ribosomal peptides synthesis (NRPS) machinery and are most often subjected to a complex regulation with involvement of several regulatory factors. To gain novel insights on mechanism regulating fengycin production, we investigated the effect of the fascinating polynucleotide phosphorylase (PNPase), as well as the effect of lipopeptide surfactin. Compared to the wild type, the production of fengycin in the mutant strains *B. subtilis* BBG235 and BBG236 altered for PNPase has not only decreased to about 70 and 40%, respectively, but also hampered its antifungal activity towards the plant pathogen *Botrytis cinerea*. On the other hand, mutant strains BBG231 (*srfAA*$^{-}$) and BBG232 (*srfAC*$^{-}$) displayed different levels of fengycin production. BBG231 had registered an important decrease in fengycin production, comparable to that observed for BBG235 or BBG236. This study permitted to establish that the products of *pnpA* gene (PNPase), and *srfAA*$^{-}$ (surfactin synthetase) are involved in fengycin production.

Keywords Polynucleotide phosphorylase · Fengycin · Surfactin · *B. subtilis*

Introduction

Bacillus subtilis is known to produce a wealth of active compounds through its secondary metabolism machinery (Sinchaikul et al. 2002). According to Stein (2005) and Chen et al. (2007), about 4–5% of *B. subtilis* genome (8.5% for *Bacillus amyloliquefaciens*) is thought to be devoted to synthesis of antimicrobial compounds including the lipopeptides group, which are defined as non-ribosomally synthesized peptides (NRPS) (Nakano et al. 1991). Fengycin or plipastatin contains ten amino acids, which are incorporated by five NRP Synthetases (Fen1 to Fen5/Pps1-5) (Steller et al. 1999; Jacques 2011). The fengycin operon is composed of five open reading frames (ORFs) named *fenA-E* (or *ppsA-E*) (Steller et al. 1999; Tsuge et al. 1999; Jacques 2011). The expression of lipopeptide operons involves complex regulatory machinery, which is linked to a quorum sensing as reported for surfactin or mycosubtilin, a member of iturins family (Nakano et al. 1991; Duitman et al. 2007).

It has been reported that in many Bacilli, besides DNA coding for fengycin, there is at least one other lipopeptide-codifying DNA, and surfactin is most often this additional lipopeptide (Roongsawang et al. 2002, 2010; Kim et al. 2010). As recalling fact, and depicted in Fig. 1, surfactin operon contains four large ORFs coding for surfactin synthetases and designated *srfAA, srfAB, srfAC* and *srfAD* (Galli et al. 1994; Lee et al. 2007). The regulatory *comS* gene which is requested for *B. subtilis* competence, is located within the region coding for the fourth amino acid activation domain of *srfAB*, and it is co-expressed with the *srfA* operon (Liu et al. 1996; Nakano and Zuber 1991; Hamoen et al. 1995; Jacques 2011). In terms of mRNA stability, only the effect of PNPase on surfactin has been yet reported (Luttinger et al. 1996).

PNPase catalyzes the 3′-->5′ phosphorolysis of polyribonucleotides leading to nucleoside diphosphates (NDPs), and the 5′-->3′ polymerization of ribonucleoside diphosphates, releasing inorganic phosphate. PNPase catalytic activity is

Communicated by Djamel DRIDER.

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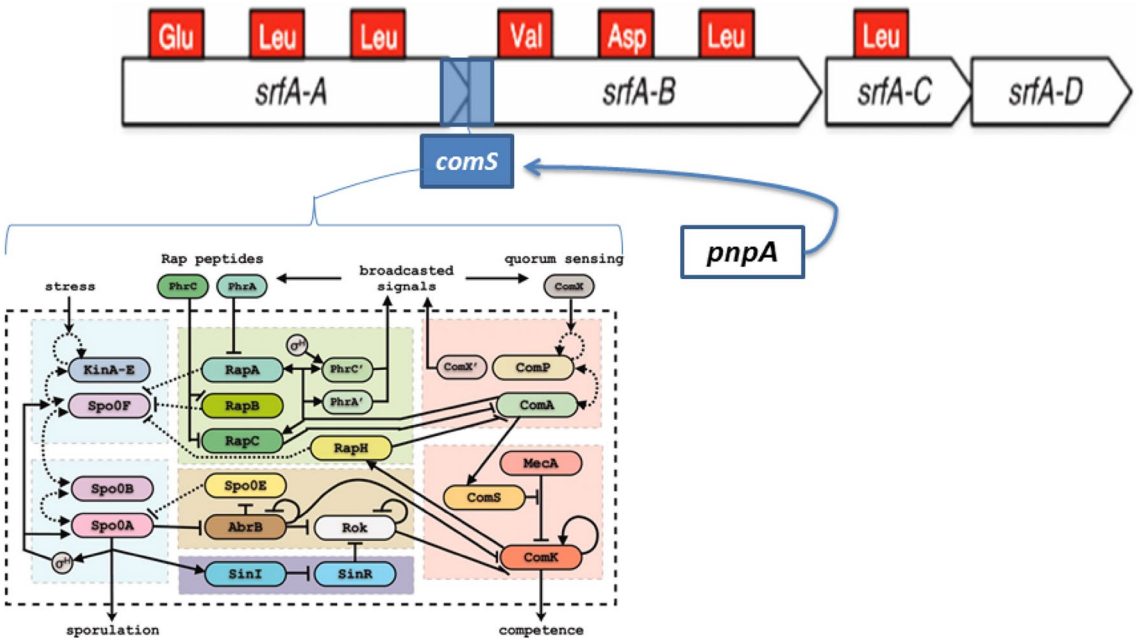

Fig. 1 Schematic regulatory interaction between the operon of surfactin including the genes *comS* and *pnpA* in *B. subtilis* [adapted from Schultz et al. 2009; Jacques 2011]

Mg^{2+}-dependent and inhibited by high Mn^{2+} concentration. According to Kakiuchi et al. (1979), a large variety of ribonucleoside diphosphatases may serve as template for the polymerization reaction.

PNPase has been studied by Severo Ochoa and Arthur Kornberg, who were awarded for Nobel Prize in 1959. After this discovery, PNPase remains still a fascinating enzyme playing a multifaceted role in bacterial cells. Previously, it was established that accumulation of mRNA decay intermediates was associated with strains lacking PNase activity, underlining the role of this major ribonuclease (Liu et al. 2014). Recent review from Briani et al. (2016) listed the allocated functions for PNPase. Briefly, these functions include the control of mRNA decay, processing of rRNA and maturation of tRNA, stability control of small noncoding RNAs, control of growth at low temperature in different Gram-negative and Gram-positive bacteria, or biofilm control.

Understanding gene regulation in bacteria is an important feature for designing the genetic circuit promoting overproduction of molecules of industrial interest such as lipopeptides, which are expected to be the next generation of bio-pesticides. Within the lipopeptides repertoire, fengycin is considered as promising biocontrol agent. Indeed, different biological functions were allocated to this lipopeptide antibiotic, including growth of filamentous fungi, control of apoptosis and necrosis (Tang et al. 2014), role in the development and progress of colon cancer via the cells apoptosis and cell cycle targeting Bax/Bcl-2 pathway (Cheng et al. 2016), coating for preventing fungal infection associated with silicone medical devices (Ceresa et al. 2016), and protection of *Caenorhabditis elegans* from Gram-positive infection (Iatsenko et al. 2014).

The present work aimed at showing the role of PNPase on fengycin production, as well as that of the operon coding for surfactin on fengycin production.

Materials and methods

Bacterial strains, plasmids and primers

Plasmids and bacteria used in this study are listed in Table 1. All primers used for genetic construction or checking of derivative DNA are depicted in Table 2.

Bacillus engineering and growth conditions

All strains derived from *B. subtilis* 168 were transformed by natural competence method (Sambrook and Russell 2001). Transformation of BBG201 was performed with a modified electroporation method requiring trehalose (Cao et al. 2011).

Bacillus strains and *Escherichia coli* JM109 were grown in Luria–Bertani medium (LB), at 37 °C under shaking, at 130 rev min^{-1}. Media were supplemented, when necessary, with apramycin, sulphate salt (100 µg ml^{-1}), erythromycin (1 µg ml^{-1}), chloramphenicol (1 µg ml^{-1}), phleomycin (4 µg ml^{-1}), or neomycin, trisulphate salt (5 µg ml^{-1}). All these

Table 1 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, Columbus, OH, 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, *Eco*RI-*Hind*III *srfAC-srfAC* amplicon from *B. subtilis* BBG21; *bla*^{R} (4630 bp)	This study
pBG333	pMUTIN-GFP+, *bla*^{R}, *erm*^{R}, *Kpn*I-*Xma*III *ribC-pnpA* amplicon from *B. subtilis* BBG21 (7,801 bp)	This study
pBG335	pOJ206, *bla*^{R}, *am*^{R}, *Eco*RI-*Hind*III *srfAC-srfAC* amplicon from *B. subtilis* BBG21	This study
pBG334	pMUTIN-GFP + *bla*^{R}, *erm*^{R}, *Kpn*I-*Xma*III *hxlR-srfAA* amplicon from *B. subtilis* BBG21, (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*+	Dhali (2016)
BBG201	*tet*^{R}, *B. subtilis* BBG21(pMMComK)	Yaseen et al. (2016)
BBG231	*erm*^{R}, *B. subtilis* BBG21Δ*srfAA*::(*srfAA*/BBG21-*gfp-erm*^{R})	This study
BBG232	*am*^{R}, *B. subtilis* BBG21Δ *srfAC::(srfAC*/BBG21) *am*^{R}	This study
BBG235	*erm*^{R}, *B. subtilis* BBG201Δ*pnpA*::(*pnpA*/BBG21-*gfp-erm*^{R})	This study
BBG236	*phl*^{R}, *B. subtilis* BBG258Δ*pnpA*::(*pnpA*/BBG258-*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.

am^R, resistance to apramycin; *bla*^{R}, resistance to ampicillin; *erm*^{R}, resistance to erythromycin; *phl*^{R}, resistance to phleomycin; and *tet*^{R}, resistance to tetracycline

antibiotics were purchased from Sigma–Aldrich (St. Louis, MO, USA). The hemolytic activity was detected upon overnight growth of *Bacillus* strains on LB blood plates (Coutte et al. 2010). Microbial growth was monitored by optical density OD_{600nm} 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 basic medium for lipopeptides production with 10% filling ratio of Erlenmeyer, at 30 °C and 160 rev min^{-1} (Hussein 2011).

DNA manipulation

Polymerase chain reaction (PCR) was performed using the PCR Master Mix (2X) (Thermo Scientific Fermentas, Villebon sur Yvette, France), as a mixture of Taq DNA polymerase. DNA extraction was performed with the Wizard® Genomic DNA Purification Kit (Promega Corp., Madison, WI, USA). PCR products were cloned into the pGEM-T Easy vector (Promega Corp.). Plasmid extraction was carried out with the GeneJET Plasmid DNA Purification Kit (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). Restriction endonucleases were supplied by Thermo Scientific Fermentas. Recovery of DNA from agarose gels was performed with GeneJET Gel Extraction kit (Thermo Scientific Fermentas). All the sequences were checked by DNA sequencing realized at Eurofins Genomics (Ebersberg, Germany).

Construction of polynucleotide phosphorylase-deficient strains

The DNA amplicon obtained from *B. subtilis* BBG21 with primers *ribC* fwd and *pnpA ylxY* rev (Table 2), was cloned into pGEM-T Easy vector. The resulting plasmid pBG330 was *Kpn*I-*Xma*III double digested and inserted between the *Kpn*I and *Xma*III sites of pMUTIN-GFP$^+$ vector, leading to the recombinant plasmid pBG333, which was successfully transferred into *B. subtilis* BBG201 by electroporation procedure (Cao et al. 2011). The resulting derivative was designated BBG235.

Two DNA fragments from *B. subtilis* BBG258, a derivative of *B. subtilis* 168, were amplified using primers *pnpA* Fw, *pnpA* delta Rv and the primers *pnpA* delta Fw, *pnpA* Rv (Table 2). Notably, primers P3 and P5 permitted to amplify the fragment k7 containing the phleomycin resistance gene.

Table 2 PCR primers used for genetic constructions

Primer	Primer sequence (5′→3′)	Amplicon length (bp)
HxlR B.s. BBG21 KpnI fwd	CACAGGGAAGTGGTACCAGT	902
srfAA B.s. BBG21 XmaIII rv	AATCGGCCGCTCAACAAG	
srfAC B.s. BBG21 EcoRI fwd	GAATTCGATGAGGAAGCCAAC	1620
srfAC B.s. BBG21 HindIII rv	CTTCAAACAAAAGCTTCACTGG	
pnpA B.s. KpnI fwd	ATATCGGTACCGCGCTTCAA	1551
pnpA B.s. XmaIII rv	CGGCCGTCCGGTCTTACTTT	
pnpA B.s. ribC fwd	CCTTAATGTGCAGCACGCAG	3409
pnpA B.s. ylxY rv	TTTTAATCAATGTGGCATGG	
pnpA fwd	TCATGGTGATAAAAGAGGGC	1069
pnpA Delta rv	CGACCTGCAGGCATGCAAGCTACCGT AGCGGATCATCACAGCACCATTTGC	
pnpA Delta fwd	GCTCGAATTCACTGGCCGTCGGCAAATG GTGCTGTGATGATCCGCTACGGTACAAGGA CGAGTGAATTTATCCCGCAAAGC	1039
pnpA rv	TCGGATGCATTAAAATCATGGC	
K7 P3	AGCTTGCATGCCTGCAGGTCG	2660
K7 P5	CGACGGCCAGTGAATTCGAGC	
PpsA q fw	GAGCCTGAGGTCAGCTTTAAT	112
PpsA q rv	GTTCTCTCTCACCCGCTATTTC	
PpsB q fw	CAGACTACACCGGGCATATT	95
PpsB q rv	CGGGCTGATCCGTTACTATTT	
PpsC q fw	AGGACATGAATGAGGCAGAAG	123
PpsC q rv	CTCTTGCTGGGTAGGTGTATTT	
PpsD q fw	GCAGCCGGAAGATGTAAAGA	103
PpsD q rv	GAGTTCGGTATGCGGACATATAG	
PpsE q fw	CCAGATCTCGCGCTTCATAATA	84
PpsE q rv	CCCGCATAGTCTCTCTTCATTT	

The three fragments were ligated using the joint PCR program NEBuilder Assembly (New England Biolabs Inc., Ipswich, MA, USA). After natural competence transformation, the BBG236 strain was obtained.

Construction of *B. subtilis* altered in surfactin operon

Fragments of *hxlR-srfAA* and *srfAC–srfAC* were amplified by PCR using primers HxlR B.S. BBG21-SrfAA B.S. BBG21 and primers SrfAC fw B.S. BBG21-SrfAC rv B.S. BBG21, respectively. These two fragments were cloned into pGEM-T Easy vector, leading to plasmids 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. Then, plasmids pOJ206 and pBG332 were *Eco*RI and *Hind*III double digested. The *srfAC–srfAC* fragment was inserted between the *Eco*RI and *Hin*dIII sites of pOJ206 to obtain plasmid pBG335. In *B. subtilis*, the surfactin operon was disrupted by targeting two *srfA* genes separately. Thus strain BBG201 was successfully transformed by the corresponding vector (pBG334) carrying the fused *hxlR-srfAA::gfp* integrated at the promoter of surfactin operon, disrupting *srfAA* and the resulting derivative was named BBG231. Using the vector pBG335, the second disruption was performed in *srfAC* (after the *srfAB*-nested *comS* gene), generating strain BBG232. The hemolytic activity was then checked (data not shown).

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). The measurement of lipopeptides concentration was performed as previously described (Coutte et al. 2010).

Antifungal assessment

An about 5 mm square-sided mycelium mat of the soil-borne fungus *Botrytis cinerea* was placed on one side of a potato dextrose agar (PDA, MB Cell, Los Angeles, CA, USA), and each bacterial isolate was streaked on the other side of the medium. The PDA plate was incubated at 28 °C for 4 days. Notably, during the cultivation, the antifungal effects of bacterial isolates were confirmed by inhibition zones formed between the bacterial and fungal isolates. The dual culture was performed at least in three replicates (Kim et al. 2010).

RNA extraction and RT-PCR experiments

BBG235, BBG236 (*pnpA* mutant strains), BBG201 and BBG258 (wild-type strains) were inoculated in 50 mL of Landy MOPS and cultured at 30 °C, 160 rev min^{-1} for 16 h, when the maximum fengycin production was observed (Yaseen et al. 2016). An equivalent volume of about 2^{10} cells was obtained from each point of the kinetics previously defined; these volumes were centrifuged (11,000 g, −9 °C, 5 min), the supernatant was discarded, and the pellet was stored in 1 ml Ambion RNA later (Thermo Fisher Scientific) at −20 °C.

Quantitative PCR

Total RNA was extracted by re-suspending a culture of about 10^9 cells according to the GeneJET RNA purification Kit (Thermo Scientific Fermentas). The quantity and quality of isolated RNA were determined for each sample by UV absorbance and analyses by agarose gel electrophoresis. Reverse transcription was performed using ReverAid H Minut first Strand cDNA synthesis kit (Thermo Fisher Scientific). Quantitative PCR was performed using StepOnePlus instrument (Applied Biosystems, Foster City, CA, USA). Six primers pairs were designed for the five genes of fengycin operon *fenA* to *fenE* and the gene *rrnA* as a reference gene (Table 2). 2 µl of 1:16 cDNA dilution of each strain mixed with 23 µl of the master mix (SYBR Green PCR Master Mix, Applied Biosystems), containing 9 µl of RNase-free water mixed with 12.5 µl SYBR mix (2X) and 0.75 µl of the forward and reverse oligos. All qPCRs were conducted at 95 °C for 10 min then 40 cycles of 95 °C-15 s + 55 °C-30 s + 72 °C-30 s and finally 95 °C for 15 s + 55 °C for 30 s and 95 °C for 15 s. The specificity of the reaction was checked by melting curve analysis. Indeed, melting curve analysis was used to monitor the specificity of the reaction (data not shown). The $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expression. Accordingly, genes with values under 0.5 are down-regulated, whilst those comprised between 0.5 and 2 are considered as unchanged, and finally genes exhibiting values higher to 2 are considered as up-regulated (Pfaffl 2004; Beltramo et al. 2006).

Results

Inactivation on the pnpA gene reduced the level of the fengycin production

To shed light on the role of PNPase on fengycin production, two mutant strains carrying deletion of the *pnpA* gene were constructed and designated BBG235 and BBG236. Briefly, mutant BBG235 was obtained from BBG201, while BBG236 was obtained from *B. subtilis* BBG258. Both mutants were characterized morphologically by a multiseptate form (Fig. 2), and a decrease in both fengycin and surfactin productions (Table 3).

Production of fengycin in BBG235 was estimated at 96.4 mg µg L^{-1}, and that of the wild type at 346 mg L^{-1}, making a difference of about 72%. Concomitantly, the mutation performed in the *pnpA* gene affected as well the production of lipopeptide surfactin in BBG235 of about 40%, compared to the parental strain BBG201. The production of surfactin in BBG235 was estimated at 390.4 mg L^{-1}, and that of the wild type at 644 mg L^{-1}. The difference in the fengycin production observed for BBG236 and its parent BBG258 was not as important as that detected for BBG235 and its parent.

A significant decrease in the antifungal activity against the plant pathogen *Botrytis cinerea* was also observed for BBG235, arguing on the role of PNPase on fengycin production (Fig. 3).

Expression of genes coding for fengycin increased alongside its decrease in production

The fengycin operon expression was investigated in both BBG235 and BBG236, as well as in the wild-type BBG201 using the qPCR technology. The expression of genes constituting the fengycin operon increased, in both PNPase null-mutants BBG235 and BBG236. Nevertheless, this expression resulted to be exerted in a gene-dependent manner. The upmost expression levels were registered for *fenB* and *fenD* genes with an enhancement of about 4.2- and 3.8-fold, respectively. In spite of this enhancement, production of fengycin decreased in both BBG235 and BBG236 (Fig. 4).

Effects of location of *srf* mutations on fengycin production

To see whether there is any interplay between surfactin and fengycin regulations, derivatives carrying mutations in the

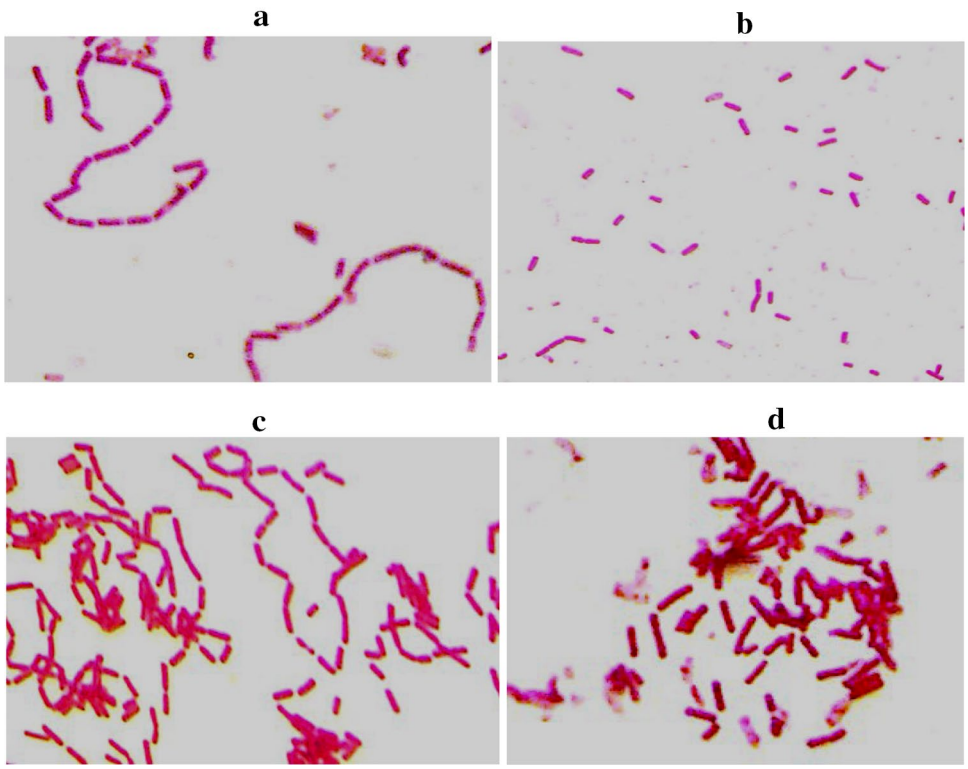

Fig. 2 The multi-septate form of the *pnpA* mutant strains versus wild-type strains: **a** BBG235, **b** BBG201, **c** BBG236, **d** BBG258

Table 3 Lipopeptide production in batch by four strains of *B. subtilis* after 48 h of growth in Landy MOPS medium, pH 7, 10% filling ratio, at 30 °C and 160 rev min^{-1}

Strain	Fengycin (mg L^{-1})	Fengycin (mg L^{-1}. OD_{600})	Surfactin (mg L^{-1})	Surfactin (mg $^{L-1}$. OD_{600})
BBG21 (wild type)	346.0 ± 12.3	37.6 ± 3.2	644.0 ± 22.2	70.7 ± 2.1
BBG235 (*pnpA*$^-$)	96.4 ± 11.4	13.2 ± 1.8	390.4 ± 23.7	54.1 ± 3.3
BBG258 (wild type)	20.34 ± 1.32	2.2 ± 0.9	746.4 ± 17.3	81.3 ± 5.3
BBG236 (*pnpA*$^-$)	7.4 ± 1.1	0.98 ± 0.3	478.0 ± 11.1	63.7 ± 4.3

surfactin operon were constructed. The fengycin and surfactin productions were quantified in the wild-type and the derived strains as depicted in Table 4. As expected, surfactin production was altered in both BBG231 and BBG232. In these strains, the amount of fengycin had decreased from 385 mg L^{-1} (wild-type BBG201) to 76.4 mg L^{-1} (mutant BBG231), but not for the mutant BBG232 disrupted in the *srfAC* gene.

Discussion

After 60 years of investigation on the fascinating 3′–5′ exoribonuclease PNPase, we are far from deciphering the final role of this unessential but important and abundant enzyme. *B. subtilis* altered for PNPase activity has diverse phenotypes as a long multi-septate form, sensitivity to low temperatures, and antibiotics resistance, deficiency for DNA competence (Luttinger 1996; Cardenas et al. 2009).

Further implications of PNPase in cell biology, specifically on the biosynthesis of lipopeptide fengycin are provided here. To this end, mutant strains altered for PNPase activity were conceived, constructed and assessed for fengycin production. It is noteworthy that *B. subtilis* BBG235, the strain carrying the *pnpA* gene disruption, displayed a decrease of about 70% in fengycin production. Attempts to link PNPase activity to this decrease in the fengycin production were performed. Expression of genes coding for fengycin, as studied by qPCR technology, revealed significant enhancement of *fenB* and *fenD* in BBG235 and BBG236, which carry a mutation in the *pnpA* gene. This data advocates the involvement of PNPase in the control of fengycin expression.

Another hypothesis to explain the decrease in fengycin production in BBG235 and BBG236 could be associated

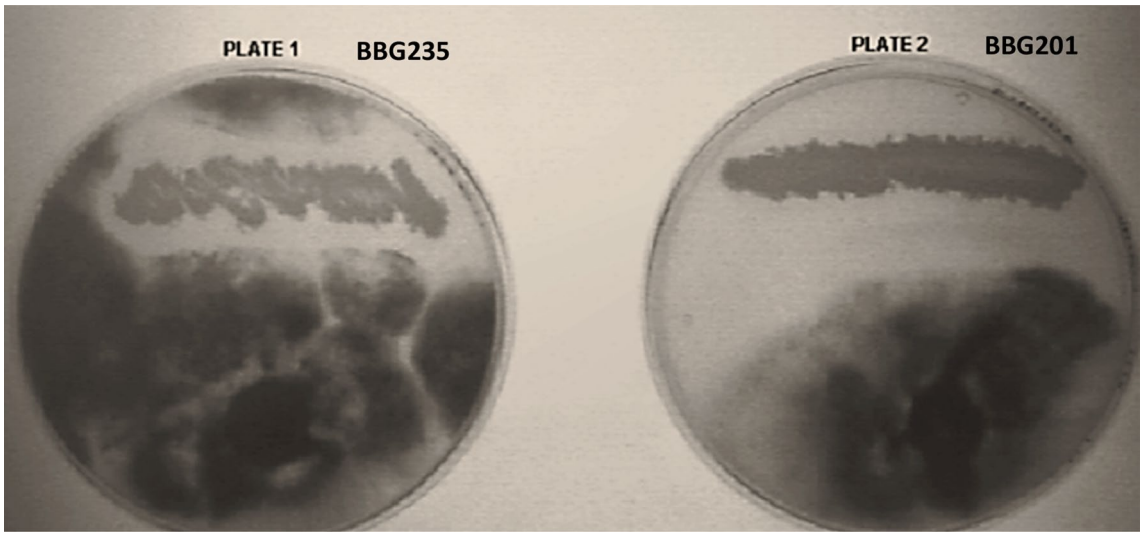

Fig. 3 In vitro growth inhibition of *Botrytis cinerea* caused by the mutant strain BBG235 (Plate 1) and the wild-type strain BBG201 (Plate 2) on potato dextrose agar (PDA) medium. The antagonism was inspected after incubation of the plates for 3 days at 25°C

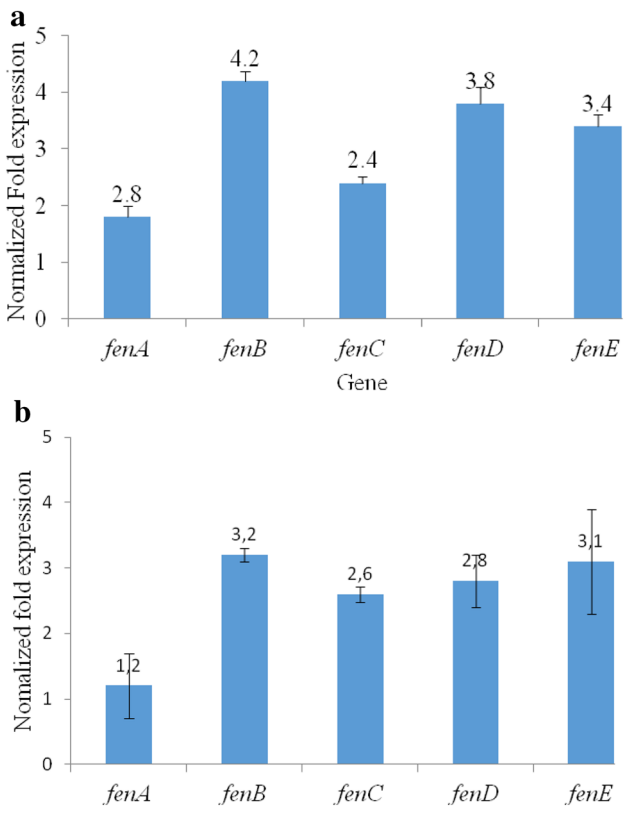

Fig. 4 Relative fengycin genes expressions from the *pnpA* mutant strains. The expression was measured using the quantitative PCR technique using the wild-strain BBG201 as control and the gene *rrnA* as reference gene. The expressions were obtained after incubation of the bacteria in 50 mL of Landy MOPS at 30 °C, 160 rev min^{-1} for 16 h. The $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expressions. **a** BBG235 and **b** BBG236

with expressions of both *comS* gene and *srfA* operon, which code for a competence factor and the lipopeptide surfactin, respectively. It should be noted that early report from Luttinger et al. (1996) unveiled already the effect of PNPase on surfactin production through the control of *comS* gene. A recent report from Gamba et al. (2015) confirmed the role of PNPase on *comS* gene expression. Taking the aforementioned studies and our data together, we can postulate on a lead of PNPase on biosynthesis of fengycin via a complex regulatory system involving *comS* gene, or a direct control of mRNAs from fengycin operon.

The influence of *pnpA* on the fengycin production may occur via different pathways. Commichau et al. (2009) and Salvo et al. (2016) have postulated that PNPase interacts with enolase and phosphofructokinase, which are essential enzymes for glycolytic pathway. Based on data gathered in this study, we hypothesize therefore that *pnpA* gene deletion may afflict the precursor synthesis for the carbon metabolism, which ultimately impacts the lipopeptide production.

Moreover, Liu et al. (2016) have recently reported that *B. subtilis* deficient in PNPase activity displayed a high expression of regulator *sigB* gene. Interestingly, the product of *sigB* gene was recently shown to exert a repressing effect on fengycin operon expression (Allenby et al. 2005).

Zhao et al. (2016) have utilized a genome shuffling approach to increase production of fengycin in *B. amyloliquefaciens* ES-2-4. Thus, after two rounds of genome shuffling, the authors noticed a rise in biosynthesis of fengycin of about 8.9-fold, and linked this rise to important expression of fengycin synthetase gene (*fenA*), which was 12.77-fold greater in the shuffling strains than in the parental strain ES-2-4.

Table 4 Lipopeptide production in Landy MOPS medium, pH 7, 10% filling ratio, at 30 °C and 160 rev min^{-1}, after 48 h by *B. subtilis* BBG201 and *sfr* mutants (BBG231 and BBG232)

Strain	Fengycin (mg L^{-1})	Fengycin (mg L^{-1}. OD_{600})	Surfactin (mg L^{-1})
BBG201 (wild-type)	385.0 ± 16.3	35.0 ± 2.9	644.0 ± 22.2
BBG231 (*sfrAA*$^-$)	76.4 ± 5.6	8.4 ± 0.9	0.0
BBG232 (*sfrAC*$^-$)	402.6 ± 21.4	37.9 ± 3.3	0.0

The data obtained in this study indicate that PNPase could interfere with fengycin production through the product of *srfAA* or *comS*, which regulate the natural competence in *Bacillus* via *comK*. Remarkably, *comK* gene was reported to negatively regulate *degQ* expression (Ogura et al. 2002), which is a regulator of fengycin operon (Tsuge et al. 1999).

On the other hand, production of different lipopeptides within a same strain might be subjected to any interplay and switch-on or switch-off regulations. Related to this, Karatas et al. (2003) reported that bacilysin production in *B. subtilis* ATCC 21332 decreased when this strain was devoid of SrfA activity. In direct line, Béchet et al. (2013) mentioned similar impact of *srfAA* mutation on mycosubtilin production in *B. subtilis* ATCC 6633. Inversely, we reported that disruption of *fenA* gene led to 30% enhancement of surfactin production in *B. subtilis* (Yaseen et al. 2016).

To examine the impact of surfactin-codifying DNA on fengycin production, we assessed production of this lipopeptide in *B. subtilis* BBG231 and BBG232. While no effect was registered for BBG232 (*srfAC* mutant), a significant decrease in fengycin production was noticed for BBG231, arguing on the role of *srfAA*/*comS* in fengycin production. These data are in good agreement with those previously reported by Ongena et al. (2007) who indicated the absence of any effect on fengycin production in *B. subtilis* 168, when harboring deletions of the *srfAB, srfAC* and *srfAD* genes. Another point that needs to be underlined is the role of PNPase on the antifungal activity. Related to this, we noticed a decrease of the antifungal activity in *B. subtilis* carrying mutations in *pnpA* and *srfAA* genes. In direct line, Zeriouh et al. (2014) reached a similar conclusion for *B. subtilis* carrying a mutation in the *sfrAB* gene.

Conclusion

This study showed the global regulatory role of PNPase on fengycin production. Indeed, the knock-out of *pnpA* gene in *B. subtilis* resulted in a decrease in fengycin production, compared to the parental strain. To the best of knowledge, this is the first report underpinning the impact of PNPase activity on fengycin production. Besides, we showed the effect of *srf* operon on fengycin production, therefore opening a new avenue aiming at understanding the interplay between genes coding for lipopeptides within a same strain.

Acknowledgements YY was a recipient of PhD scholarship awarded by Campus France through joint French-Iraqi governments program. The authors express their gratitude for "Région des Hauts-de-France" for CPER-FEDER Alibiotech project.

Compliance with ethical standards

Conflict of interest The authors declare no conflicts of interest for this article.

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