J Ind Microbiol Biotechnol (2015) 42:93–103
DOI 10.1007/s10295-014-1527-z

FERMENTATION, CELL CULTURE AND BIOENGINEERING

SIMB
Society for Industrial Microbiology and Biotechnology

Overexpression of specific proton motive force-dependent transporters facilitate the export of surfactin in *Bacillus subtilis*

Xu Li · Huan Yang · Donglai Zhang · Xue Li · Huimin Yu · Zhongyao Shen

Received: 25 July 2014 / Accepted: 17 October 2014 / Published online: 4 November 2014

Abstract A novel surfactin producer, *Bacillus subtilis* THY-7, was isolated from soil. Using liposomes and transmembrane transport inhibitors, the surfactin efflux in THY-7 was determined to be mainly dependent on proton motive force (PMF), not ATP hydrolysis. YcxA, KrsE and YerP, three putative lipopeptide transporters with PMF as energy source, were then highlighted in this work. A mutant YcxA named as YcxAmt, with 2 transmembrane helices deletion due to a code-shift mutation of the encoding gene, was identified in THY-7. This truncated YcxAmt was confirmed unable to transfer surfactin; on the contrary, overexpression of the natural full-lengthYcxA enhanced the secretion of surfactin by 89 %. KrsE, a putative kurstakin transporter, was found also capable of transporting surfactin. Overexpression of KrsE increased the production of surfactin by 52 %. In the culture of YerP-overexpressing strain at 24 h, surfactin titer reached 1.58 g L^{-1}, which was 145 % higher than that of the control. This indicated that YerP acted as the major surfactin exporter in *B. subtilis* THY-7.

Keywords *Bacillus subtilis* · Surfactin · Proton motive force · Overexpression · Lipopeptide transporters

Electronic supplementary material The online version of this article (doi:10.1007/s10295-014-1527-z) contains supplementary material, which is available to authorized users.

X. Li · H. Yang · D. Zhang · X. Li · H. Yu (✉) · Z. Shen
Key Laboratory for Industrial Biocatalysis,
Ministry of Education, Department of Chemical Engineering,
Tsinghua University, 100084 Beijing, China
e-mail: yuhm@mail.tsinghua.edu.cn; yuhm@tsinghua.edu.cn

Introduction

Surfactants have a major impact on both our daily lives and industries, from laundry products to oil recovery enhancement [23]. Since the industrial sustainability and environmental safety became a concern of the whole society, biosurfactants including glycolipids, lipopeptides and phospholipids have attracted great interest as environmentally friendly alternatives of chemical surfactants [3, 11]. Although some biosurfactants showed excellent efficacy in small-scale experiments and trials, their adoption in large-scale use may eventually face the cost and production issues [3]. At present, glycolipids can be produced with yields >10 g L^{-1}, with the example of sophorolipid as high as 100 g L^{-1}. On the contrary, lipopeptides ususally can only be produced at, or even lower than, 1 g L^{-1} [23].

Bacillus species were reported to produce a variety of bioactive secondary metabolites, especially lipopeptides with antimicrobial activity and surface activity [6, 33, 38]. Screening and characterization of strains producing novel metabolites or with high productivity have attracted people's attention for a long time due to the application potential of these strains and molecules [2]. Lipopeptides produced by micro-organisms can be identified by matrix-assisted laser desorption/ionization time-of-flight mass spectrum (MALDI-TOF–MS), which gave information on molecular weights of different lipopeptides [32, 45]. According to their structure, *Bacillus* lipopeptide can be divided into several families, namely surfactin family (consisting of surfactin, lichenysin, esperin and pumilacidin), fengycin family (consisting of fengycin and plipastatin), iturin family (consisting of bacillomycin, iturin and mycosubtilin) and kurstakin family [16].

Surfactin, a representative lipopeptide discovered decades ago, is considered as one of the most powerful

biosurfactants [16, 29]. A solution of 20 μM surfactin can lower the surface tension of water from 72 to 27 mM m^{-1} [42]. Surfactin is synthesized by SrfA, a non-ribosomal peptide synthase (NRPS) with seven modules, and exported to the outside of cell [35, 43]. Although the biosynthesis of surfactin has been well documented [8, 12, 26], how surfactin is transported through the cell membrane is not clear yet. Whether transmembrane efflux is an obstacle to higher surfactin production becomes an urgent concern of surfactin production improvement.

Permeabilization, destabilization and dissociation of biological membrane induced by lipopeptides, especially surfactin, have been well described through both experimental and computational approaches [9, 10]. At low concentration (below or near critical micelle concentration, CMC), surfactin monomer can insert into phospholipid layers in biomimetic membrane systems, inducing mild content leakage. When surfactin concentration is high, membrane solubilization and vesicle destruction were observed [10]. Given its amphiphilic character, transmembrane efflux of surfactin, therefore, may simply be the result of membrane insertion and penetration of surfactin monomers or oligomers. However, Tsuge et al. [44] reported that a null mutation in *yerP*, a gene encoding a protein with homology to RND (resistance, nodulation and cell division) family efflux pump, will significantly reduce production of surfactin and resistance to it in *B. subtilis* host. These findings suggested that there may be a mechanism of surfactin resistance or, to be more specific, transportation mediated by protein transporters, and YerP may play a role in the efflux of surfactin, although there has been no further proof yet. Other putative transmembrane protein genes, such as *ycxA* and *krsE*, have been identified together with NRPS genes responsible for the synthesis of lipopeptides, but the roles of these potential transporters in lipopeptide efflux were largely unknown [1, 7].

In this work, the main driving force for surfactin's active transmembrane efflux in THY-7, a surfactin-producing *B. subtilis* strain, was determined first by both liposomes and transmembrane transport inhibitors. The effect of two putative lipopeptide transporters (YcxA and KrsE) and the previously reported YerP on production of surfactin in THY-7 was also investigated by cloning and overexpression of the three transporters.

Materials and methods

Isolation and identification of the biosurfactant-producing strain

Soil samples collected from multiple locations were dissolved in autoclaved water and plated on blood agar plate. A biosurfactant-producing bacterium, designated as THY-7 was isolated from an oil-polluted soil sample and deposited in China General Microbiological Culture Collection Center (CGMCC No. 8906). Production of biosurfactants by THY-7 was verified by oil spreading assay [47]. Partial 16S rDNA gene of THY-7 was amplified using primers 16SF and 16SR. The amplicon was purified and sequenced, and alignment analysis of THY-7 16S rDNA sequence was performed using BLAST (http://blast.ncbi.nlm.nih.gov).

Bacterial strains, plasmids and growth conditions

Bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* TOP10 was used for cloning, and Luria–Bertani (LB) medium was used for *E. coli* cell-growth. For surfactin fermentation, *B. subtilis* colonies on LB agar plate were inoculated in LB medium and incubated at 37 °C and 200 rpm for 16 h. Aliquots of this culture were inoculated into 500 mL baffled flasks containing 100 mL fermentation broth (sucrose 70 g L^{-1}, yeast extract 1 g L^{-1}, $NaNO_3$ 25 g L^{-1}, KH_2PO_4 0.333 g L^{-1}, $Na_2HPO_4{\cdot}12H_2O$ 1 g L^{-1}, $MgSO_4{\cdot}7H_2O$ 0.15 g L^{-1}, $CaCl_2$ 7.5 mg L^{-1}, $MnSO_4{\cdot}H_2O$ 6 mg L^{-1}, $FeSO_4{\cdot}7H_2O$ 6 mg L^{-1}) to reach $OD_{600} = 0.2$ and incubated at 200 rpm and 37 °C.

Purification, identification and quantification of lipopeptide

Cells in fermentation culture of THY-7 were removed by centrifugation at 4 °C and 12,000×*g* for 20 min. Supernatant was adjusted to pH 2.0 by HCl and left at 4 °C for 12 h. Precipitation was collected by centrifugation (4 °C, 12,000×*g*, 20 min), washed by 0.01 M HCl (pH = 2.0) and air dried. The crude biosurfactants were extracted by methanol, and the extraction was air dried. Components of purified biosurfactants produced by THY-7 were determined by MALDI-TOF-MS. MS Analysis was performed by Center of Biomedical Analysis of Tsinghua University, on SCIEX 4800 Analyzer (Applied Biosystem, Foster City, CA, USA) with 337 nm nitrogen laser. An equal volume of 0.1 % α-cyano-4-hydroxycinnamic acid (CHCA) was used as the matrix solution. Peaks in *m*/*z* range of 600–1,300 were displayed.

Surfactin concentration in the *B. subtilis* fermentation broth or solution of the purified biosurfactants was determined using high-performance liquid chromatography (HPLC). LC-20A HPLC system (Shimadzu, Kyoto, Japan) equipped with an ODS-SP column (250 × 4.6 mm, 5 μm, GL Sciences, Kyoto, Japan) and a DA detector (Shimadzu, Kyoto, Japan) was used for analysis. Fermentation culture samples were centrifuged at 12,000×*g* for 3 min. Supernatant was diluted tenfold by 1 g L^{-1} $NaHCO_3$, filtrated through 0.45 μm PES filter, and injected (20 μL) into the HPLC for analysis. A mixture of acetonitrile:

Table 1 Bacterial strains and plasmids used in this study

Strains/plasmids	Descriptions	Sources/references
B. subtilis strains		
THY-7	Wildtype (CGMCC No. 8906); *ycxAmt*, *yerP*	This group
TS589	THY-7 carrying pTS532 (P_{grac}-*ycxA*)	This work
TS593	THY-7 carrying pTS549 (P_{grac}-*krsE*)	This work
TS658	THY-7 carrying pTS638 (P_{grac}-*ycxAmt*)	This work
TS662	THY-7 carrying pTS609 (P_{grac}-*yerP*)	This work
TS762	THY-7 carrying pHT08	This work
1012	Derivated from *B. subtilis* strain 168; *ycxA*	MoBiTec/[37]
B.thuringiensis strains		
HD73	ATCC35866; *krsE*	[4]
E. coli strains		
TOP10	Cloning host strain	Lab stock
Plasmids		
pHT08	*E. coli-B. subtilis* shuttle vector; *bla*, *cat*, P_{grac}, 8×His-tag	[25]
pTS532	*bla*, *cat*, P_{grac} -*ycxAmt*	This work
pTS549	*bla*, *cat*, P_{grac} -*krsE*	This work
pTS609	*bla*, *cat*, P_{grac} -*yerP*	This work
pTS638	*bla*, *cat*, P_{grac} -*ycxA*	This work

H_2O: trifluoroacetic acid = 85:15:0.1 was used as the mobile phase, and the flow rate was 0.8 mL min^{-1}. Surfactin was detected under the wavelength of 205 nm. Surfactin concentration was calculated according to the surfactin standard purchased from Sigma (St. Louis, MO, USA). Cell dry weight (CDW) was determined as follows: 20 mL fermentation broth was centrifuged at 4 °C and 12,000×*g* for 20 min. Cell pellets were dried to constant weight at 110 °C after washing with 20 mL distilled water for one time. Experiments were performed with three replicates.

Intracellular content of surfactin in *B. subtilis* cells was also determined: cells were collected by centrifugation at 4 °C and 12,000×*g* for 20 min. Cell pellets were washed with distilled water for three times, resuspended in 1 g L^{-1} $NaHCO_3$, and disrupted by sonification (250 W for 10 min, 5 s/5 s intervals). Cell debris was removed by centrifugation, and surfactin released was quantified by HPLC.

Preparation of liposome and treatment by surfactin

Phosphatidylcholine mixed with vitamin E (8:1 mass ratio) was dissolved in chloroform, and the solvent was evaporated under vacuum. The lipid film was hydrated with 10 mM Tris buffer (pH 7.0), and the resulting liposome suspension were sonicated at 200 W for 10 min (10 s/10 s intervals) to form the small unilamellar vesicles (SUV). The suspension of liposomes was used for transmission electron microscope (TEM) observation directly or after treated by 10 or 500 mg L^{-1} surfactin.

Investigation on effects of transmembrane transporter inhibitors

Four transmembrane transporter inhibitors, sodium orthovanadate (SOV), glybenclamide (GLB), Phe-Arg-β-naphtylamide (PAβN) and carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), were selected to measure their effects on THY-7 surfactin production. Each inhibitor with appropriate concentration was added when cell growth reached the mid-logarithmic phase (OD_{600}~10). Surfactin production of THY-7 under working concentrations of four different inhibitors was monitored by HPLC. Experiments were performed with three replicates.

Gene cloning and plasmid construction

Chromosomal DNA of *B. subtilis* 1012, THY-7, and *Bacillus thuringiensis* HD73 was isolated using E.Z.N.A Bacterial DNA Kit (OMEGA Bio-Tek, Norcross, GA, USA) and used as PCR templates. PCR primers were designed according to published genomic sequence of *B. subtilis* strain 168 (GenBank accession No. AL009126) and *B. thuringiensis* strain HD73 (GenBank accession No. CP004069). Primers yerPFB/yerPRS, ycxAFB/ycxARS and krsEFB/krsERS were used to amplify the coding regions of *yerP*, *ycxA* and *krsE,* respectively. Sequences of primers were listed in Table 2. DNA polymerase used for gene cloning was *Pyrobest* DNA polymerase, purchased from TaKaRa (Dalian, China). PCR amplification products were purified by agarose electrophoresis, digested by

Table 2 PCR primers used in this work

Name	Sequence (5′–3′)[a]	Restriction site
yerPFB	GCTCTAGAATGACCAGTCAGTCAATAA AAAATG	*Xba* I
yerPRS	TCCCCCGGGTTACTCTTCTTCCGTTCC CG	*Sma* I
ycxAFB	GCTCTAGAATGCACACGTCTCCCAGGAT	*Xba* I
ycxARS	TCCCCCGGGTTATATTGATTGGTGGGTT TCTTTT	*Sma* I
krsEFB	GCTCTAGAATGAAAACTAC-CGATTCAAAAGG	*Xba* I
krsERS	TCCCCCGGGTCAAAAATTATTTGCTCT TATAGGTTC	*Sma* I

[a] Restriction sites are indicated by underlines

Xba I and *Sma* I and ligated to *E. coli-B. subtilis* shuttle vector pHT08 digested by same enzymes. Ligation mixture was transformed into *E. coli* TOP10 competent cells. Ampicillin-resistant colonies were picked and verified by PCR (using cloning primers yerPFB/yerPRS, ycxAFB/ycxARS or krsEFB/krsERS, respectively) and sequencing.

Recombinant plasmids were then introduced into *B. subtilis* THY-7 cells by electroporation. Preparation of *B. subtilis* competent cells and transformation condition were according to Xue et al. [48] with electrical pulse at 12.5 kV/cm on Micropulser (Bio-rad, Richmond, CA, USA). Transformed cells were plated on LB-agar plates containing 5 μg mL^{-1} chloramphenicol. Resistant clones were picked and verified by PCR.

Expression of transmembrane transporters in recombinant *B. subtilis*

Fermentation of *B. subtilis* TS589, TS593, TS658 and TS662 was performed. Overnight culture of *B. subtilis* strains was inoculated into 100 mL fermentation broth with OD_{600} = 0.2 (0 h). IPTG (final concentration 1 mM) was added into fermentation culture at 4 h. Both cell density and surfactin concentration were recorded at 12, 24 and 36 h, respectively. THY-7 transformed with empty pHT08 vector (TS762) was used as control. Experiments were performed with three replicates.

Accession numbers

The nucleotide sequences of 16S rRNA gene of THY-7, *yerP*, *ycxAmt*, *ycxA* and *krsE* have been deposited in the GenBank database under accession numbers KJ777688–KJ777692, respectively.

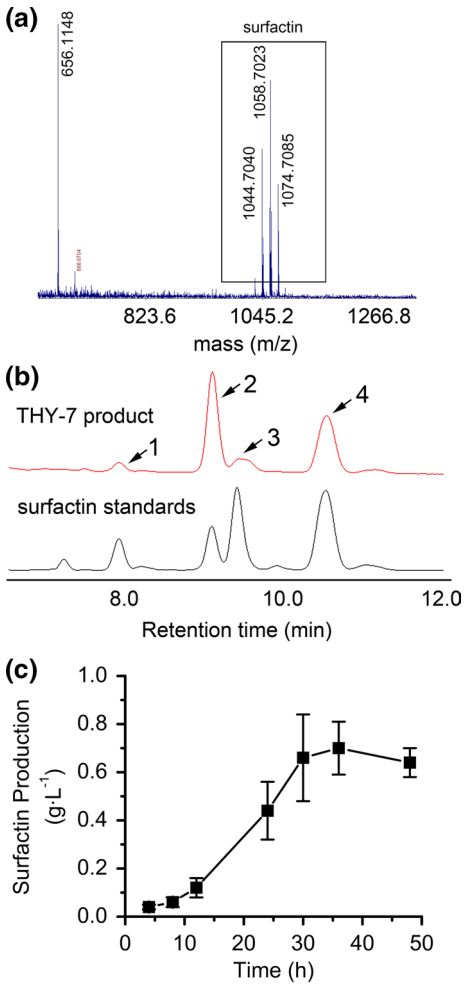

Fig. 1 Identification of lipopepetides produced by THY-7. **a** MALDI-TOF mass spectrum of biosurfactants produced by THY-7. Peaks between *m/z* 1,044.7 and 1,074.7 belonged to surfactin family. **b** HPLC analysis of surfactin in THY-7 fermentation culture (*red*) and surfactin standards (*blue*), between which the surfactin component ratios were shown to be different. **c** surfactin titer changes during the flask culture of THY-7 (color figure online)

Results

Isolation and identification of the lipopeptide-producing strain THY-7

From a cooking-oil-polluted soil sample in Tsinghua University, Beijng, China, a strain, designated as THY-7, which formed halo on blood agar plate was isolated. Cells of THY-7 were rod-shaped, with spore-forming ability (Fig. S1). According to the result of 16S rDNA sequencing and blast, THY-7 was closely related to *Bacillus subtilis*.

Components of the purified biosurfactants from the fermentation culture of THY-7 were determined by MALDI-TOF–MS, as shown in Fig. 1a. The results revealed that the

biosurfactants produced by THY-7 were mainly surfactin (*m/z* 1,044.7, 1,058.7, 1,074.7, etc.), according to the mass data of lipopeptides reported previously [32]. This surfactin product was further inspected by HPLC. Four main components matched with the surfactin standards but in different relative ratio (Fig. 1b). The four components corresponding to peak 1, 2, 3 and 4 in Fig. 1b were collected and analyzed by MS individually, and their features detailed in Table 3. These four components differed in the amino acid residues at position 2, 4 and 7 of the circular peptide (leucine, isoleucine or valine) and the length of the carbon chain (C_{13} to C_{15}). After 48 h of culture, the cell density (OD_{600}) of THY-7 was ~17.0 and the surfactin titer reached ~660 mg L^{-1} (Fig. 1c). Surfactin production of THY-7 was relatively high among other reported surfactin producer [21, 28, 40]. Therefore, this strain was used for further investigation.

Active export of surfactin in THY-7 mainly depended on transmembrane proton gradient

Using liposome as the simulated cell membrane, the effect of surfactin on the integrity of the liposome was observed. Results showed that as low as 10 mg L^{-1} surfactin, which is close to the CMC (9.4×10^{-6} mol L^{-1}, [15]), began to affect the integrity of the liposome. When we increased the surfactin concentration to 500 mg L^{-1}, which is close to the fermentation titer of THY-7, the spheral structure of the liposome was thoroughly destroyed (Fig. 2). This indicated that the intracellular concentration of surfactin in the living microbial cells could not be too high, or the cell membrane might be destroyed. Combining with the characteristics of the micelle formation of surface-active material, we paid more attention to the active efflux of surfactin in this work rather than the passive diffusion which was driven by transmembrane concentration gradient and in which the intracellular surfactin concentration should be higher than that of extracellular.

In order to identify the mechanism of the active surfactin efflux, sub-lethal working concentrations of four active transport inhibitors (CCCP, PAβN, SOV and GLB) were determined as 2.5 μM, 20 μM, 1 mM and 10 μM, respectively, according to their effects on THY-7 cell growth (Fig. S2). After addition of 2.5 μM CCCP, a PMF inhibitor, into the culture broth of THY-7, surfactin production decreased obviously compared with control. The inhibitory effect became statistically significant at 16 h and declined along with time (Fig. 3a). Adding of 20 μM PAβN (a competitive inhibitor of some PMF-driven transporters) showed slight effect on surfactin secretion at 14 h, though without statistical significance (Fig. 3b). Adding of ATP-dependent transportation inhibitor, 1 mM SOV or 10 μM GLB, did not consistently affect the surfactin production of THY-7 (Fig. 3c, d).

Table 3 Assignments of the structures of surfactin isoforms in THY-7 bio-surfactant product

lipopeptide	Peak	Mass (*m/z*)	Assignment	Amino acids at position 2,4 and 7
Surfactin	1	1,030.7	C_{13}, $[M + Na]^+$	2-Leu, 4-Val, 7-Leu
	2	1,044.7	C_{14}, $[M + Na]^+$	2-Leu, 4-Val, 7-Leu
	3	1,030.6	C_{14}, $[M + Na]^+$	2-Val, 4-Leu, 7-Val
	4	1,058.7	C_{15}, $[M + Na]^+$	2-Leu, 4-Val, 7-Leu

Transporter YcxA in THY-7 was a truncated protein without export function of surfactin

Synthase gene cluster of lipopeptides usually contains at least one deduced transmembrane protein. The close genetic relationship between these transmembrane proteins and lipopeptide NRPS indicated that they might play a role in lipopeptide export. The gene *ycxA*, locating in the surfactin synthase gene cluster just downstream of *srfA* operon (Fig. 4a), encodes a protein homologous to members of major facilitator superfamily (MFS) [36]. The length of coding region of *ycxA* is 1,227 bp (Fig.

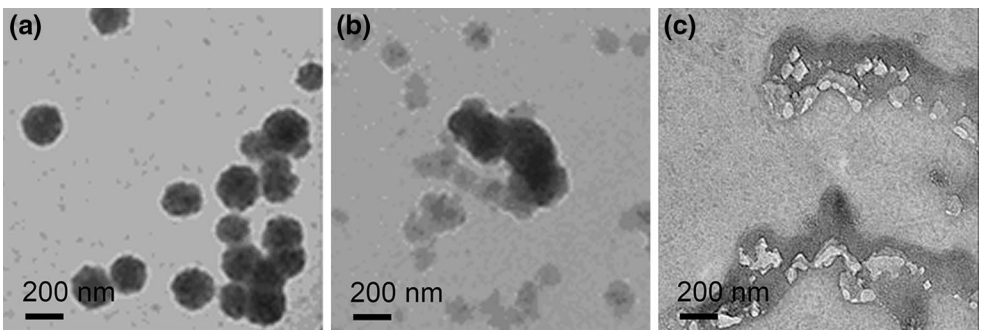

Fig. 2 Morphology of liposomes in buffer with 0 (**a**), 10 mg L^{-1} (**b**) and 500 mg L^{-1} (**c**) biosurfactant produced by THY-7. Phospholipid bilayer was unstable at surfactin concentration as low as 10 mg L^{-1}, and was totally destroyed by 500 mg L^{-1} surfactin

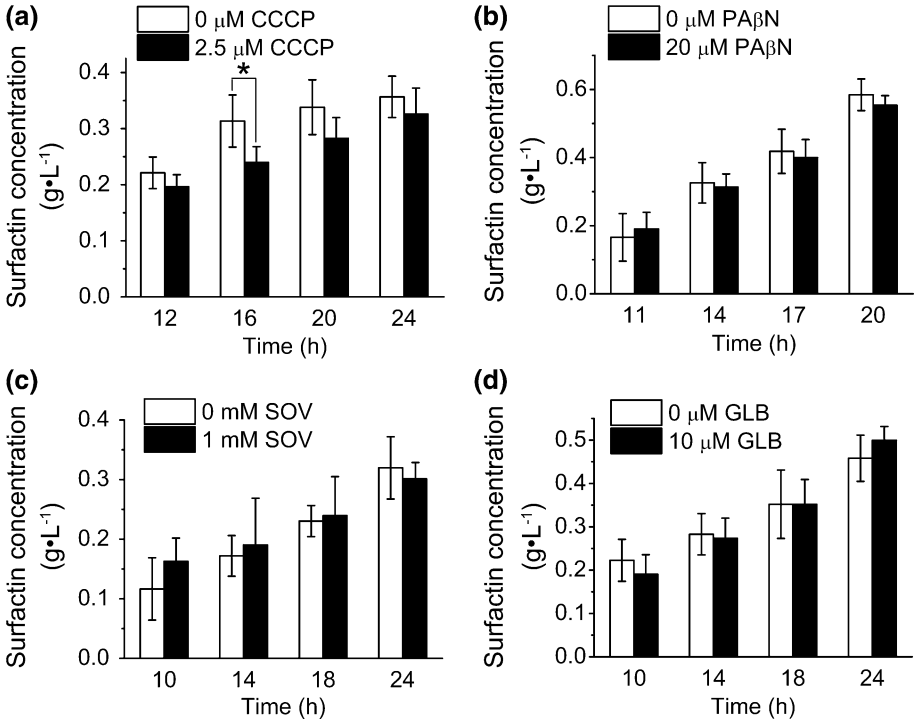

Fig. 3 Effect of transporter inhibitors on surfactin production. Inhibitors (**a** CCCP; **b** PAβN; **c** SOV; **d** GLB) were added to THY-7 fermentation culture at OD_{600}~10, and surfactin production was monitored afterwards. Data were presented as mean ± SD ($n = 3$). *$P < 0.05$ by Student's test

S3), which encoded a deduced protein of 408 aa (mass of 44.9 kDa). When using genomic DNA of THY-7 as PCR template, a mutant of *ycxA* gene with a 2-base deletion (C_{986} and G_{987}) was isolated (Fig. 4b). The mutation of 2 bp deletion caused a coding shift mutation, and the 1,225-bp mutant *ycxA* (*ycxAmt*) encoded a truncated YcxA of 366 aa (mass of 40.0 kDa) (Fig. 4c). In order to get full-length *ycxA* gene, a reference strain *Bacillus subtilis* 1012 was used as PCR template, in addition to THY-7. Protein hydrophobicity analysis demonstrated that YcxAmt may form only first 10 transmembrane helices (TMs), comparing with YcxA forming 12 TMs, and 3-D structure modeling confirmed the absence of the last 2 TMs in YcxAmt (Fig. S4).

Both *ycxA* and *ycxAmt* were overexpressed in THY-7, to reveal their function of surfactin export. For cell growth, no statistical difference between transporter-expressing group and control group was observed after 1 mM IPTG induction (Fig. S5); but for surfactin efflux, the expression results of YcxA with the intact 12 TMs and YcxAmt with 2 deleted TMs in THY-7 showed significant difference. Production of surfactin in TS589 (expressing the wild-type *ycxA*) was higher than TS762 control after IPTG induction, and a significant enhancement of about 0.86-fold was observed at 24 h (1.15 g L^{-1} for TS589 and 0.61 g L^{-1} for THY-7, $P < 0.05$, Fig. 5). Overexpression of *ycxAmt*, on the contrary, did not show any significant effect on surfactin production, at both 12 and 24 h (Fig. 5).

KrsE of *B. thuringiensis* also served as a surfactin transporter in THY-7

Except *ycxA*, a 1,254-bp *krsE* gene was also cloned from the genome of *B. thuringiensis* that can produce a newly discovered lipopeptide kurstakin (Fig. S3). The gene of *krsE* encoded a deduced protein of 417 aa (mass of 45.7 kDa) which is also homologous to the MFS members. Therefore, the KrsE protein was presumed to be an efflux transporter of kurstakin [1]. When we overexpressed the *krsE* in the engineered TS593 (THY-7 expressing *krsE* under IPTG induction), the surfactin yield was 0.93 g L^{-1} (85.2 mg g^{-1} CDW) at 24 h, displaying a 52 % increase with respect to the control (Fig. 6a). The similar molecular structure of surfactin and kurstakin (both circular heptapeptide with fatty acid chain, Fig. 6b) could explain why KrsE can also play the role of surfactin transporter.

Overexpression of YerP significantly promoted the export of surfactin in THY-7

The gene of *yerP* was cloned from the genomic DNA of *B. subtilis* THY-7 (Fig. S3) and then overexpressed in THY-7 cells with a plasmid vector, forming a recombinant strain TS662. Through 1 mM IPTG induction, the surfactin production of TS662 increased remarkably after 12 h and reached 1.58 g L^{-1} (157.8 mg g^{-1} CDW) at 24 h, 1.45-fold higher than that of control (0.64 g L^{-1}, 55.8 mg g^{-1} CDW) (Fig. 7). The highest surfactin production occurred at 36 h

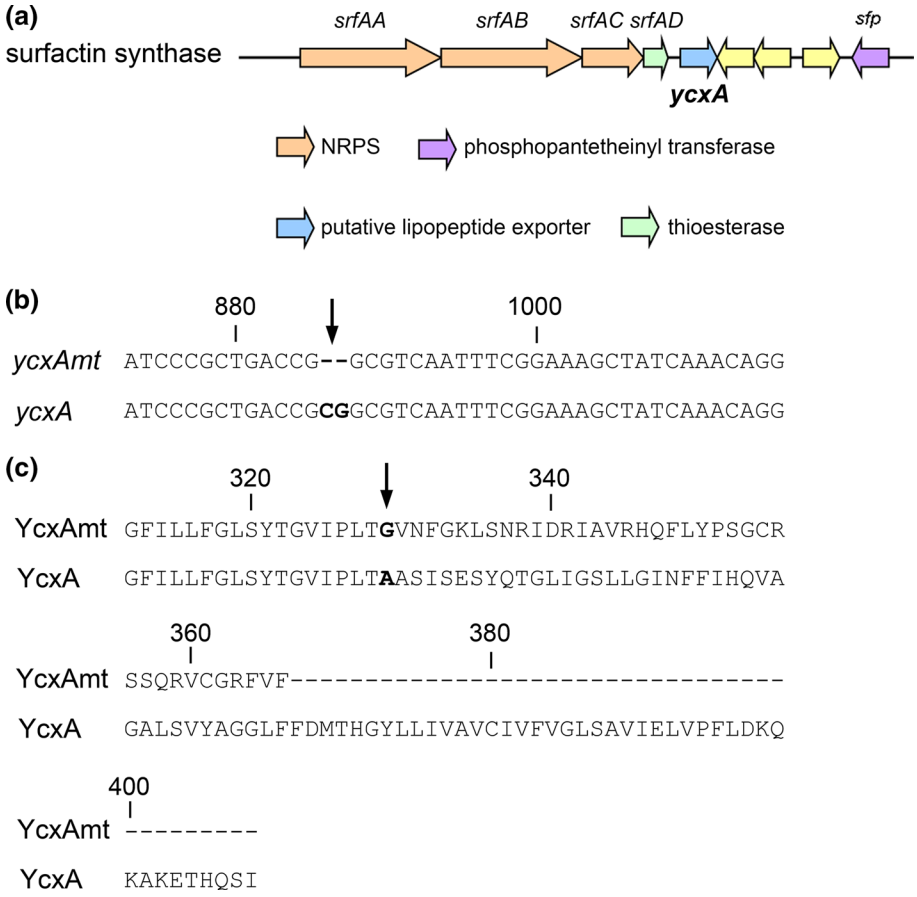

Fig. 4 Analysis of YcxA and YcxAmt. **a** Genetic organization of the synthase gene cluster responsible for synthesis of surfactin. Surfactin synthase *srfA* operon was followed by *ycxA*, encoding a deduced MFS family transporter. **b** Nucleotide sequence alignment between *ycxAmt* and *ycxA*. The *arrow* indicated the deletion of C_{986} and G_{987} in *ycxAmt*. **c** Amino acid sequence alignment between YcxAmt and YcxA. The *arrow* indicated the beginning of the code-shift mutation caused by 2-bp deletion in *ycxAmt*

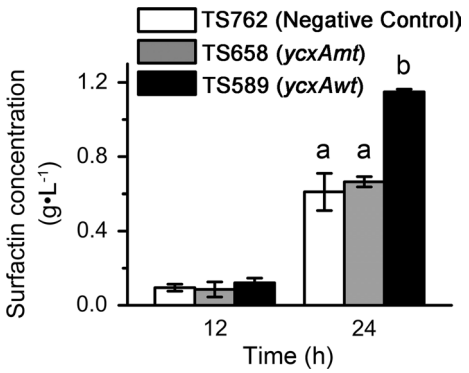

Fig. 5 Effect of *ycxA* and *ycxAmt* overexpression on THY-7 surfactin production. Surfactin production was promoted significantly by overexpression of *ycxA* (TS589), comparing with empty pHT08 control (TS762). Surfactin concentration of TS658 (THY-7 overexpressing YcxAmt) was similar to control. Statistical significance was indicated by *lowercase letters*, $P < 0.05$. Data were presented as mean ± SD ($n = 3$)

for both TS662 (1.67 g L^{-1}, 219.3 mg g^{-1} CDW) and TS762 (0.75 g L^{-1}, 99.7 mg g^{-1} CDW), the control strain containing the blank plasmid. Furthermore, cell growth of TS662 was not influenced by the overexpression of YerP (Fig. S5). Therefore, the promotional effect on surfactin efflux of YerP was greater than either YcxA (0.89-fold) or KrsE (0.52-fold) (as shown in Fig. S6). Using HPLC, we further compared the surfactin isoforms produced by different engineers with elevated producing yield, as shown in Fig. S7. Results indicated that overexpression of the three putative transporters (i.e. YcxA, KrsE and YerP) in THY-7 did not show selective effect towards specific surfactin isoforms.

Discussion

The mechanism for transmembrane efflux of surfactin is of great importance for its high titer production. Since surfactin is a big molecule with a long fatty acid chain and a circular oligopeptide head, both passive diffusion driven by transmembrane concentration gradient and active transport assisted by transmembrane protein transporters are probable export ways. The interaction between surfactin and phospholipid bilayers is highly dependent on surfactin concentration and lipid components [9, 13, 14]. Previous studies reported that DOPC (1, 2-dioleoyl-*sn*-glycero-3-phosphocholine) and DPPC (1, 2-dipalmitoyl -*sn*-glycero-3-phosphocholine) bilayers

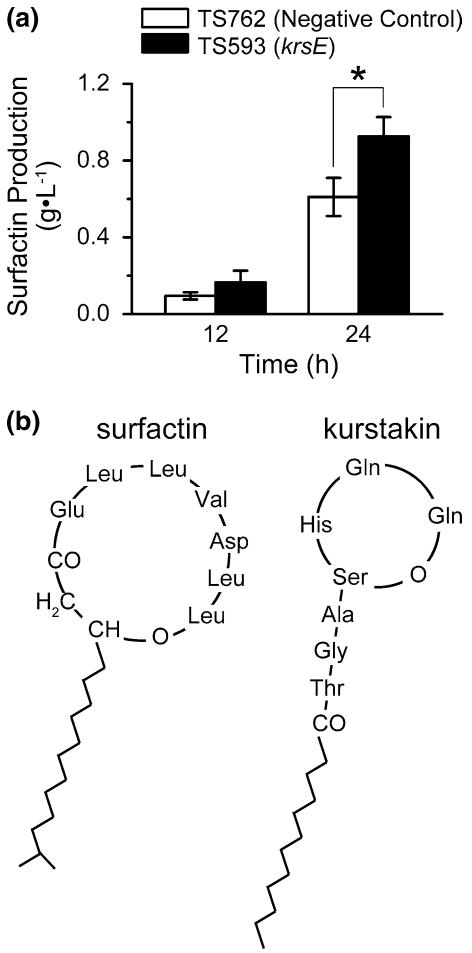

Fig. 6 Effects of KrsE expression on surfactin efflux and the structure of kurstakin. **a** Comparison of the surfactin production. TS593 (THY-7 expressing KrsE) produced significantly more surfactin than control strain TS762 at 24 h. **b** Molecular structure of surfactin and kurstakin

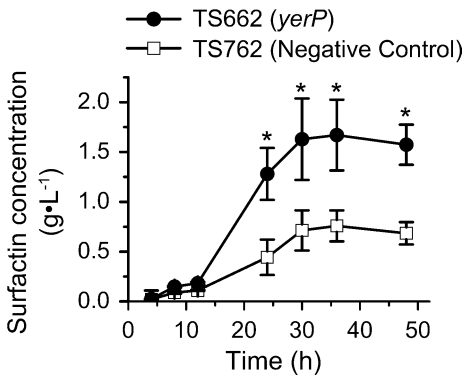

Fig. 7 Effect of YerP overexpression on surfactin production. Difference of surfactin production between TS662 (THY-7 expressing YerP) and TS762 (negative control) became significant since 12 h. Statistical significance is indicated by *asterisk*, $P < 0.05$. Data are presented as mean ± SD ($n = 3$)

disintegrated quickly at 100 μM surfactin (~0.1 g L^{-1}), which is lower than the titer in fermentation broth (usually >0.5~1.0 g L^{-1}) [5]. However, according to the intracellular surfactin content (Fig. S8) and the volume of THY-7 cells (2~3 μm × 0.8 μm, Fig. S1), the intracellular surfactin concentration (~1 g L^{-1} at 12 h and ~3 g L^{-1} at 24 h) was higher than CMC of surfactin (~10–20 mg L^{-1}). The intra- and extra-cellular concentration of free surfactin, therefore, should be both CMC. The same concentration of surfactin monomer across the cell membrane hampered the passive diffusion of surfactin. Comparison of the intra- and extra-cellular production in THY-7 (ca. 1 mg g^{-1} CDW and 60 mg g^{-1} CDW at 24 h, respectively, Fig. S8) revealed that almost all synthesized surfactin was efficiently transported outside of the cells. The obvious inhibition of surfactin even low as 10 mg L^{-1} on cell growth of the *yerP*-deficient *B. subtilis* [44] also indicated that the efflux of surfactin might be mainly facilitated by transmembrane transporters.

Bacterial transmembrane transporters can be briefly divided into two categories according to the energy source they used, ATP or proton motive force (PMF, i.e. transmembrane proton concentration gradient) [22]. Efflux of secondary metabolites in Gram-positive bacteria tends to be mediated mainly by major facilitator superfamily (MFS, depending on PMF) and ATP-binding cassette (ABC, depending on ATP) family exporters [19, 34]. Carbonyl cyanide m-chlorophenylhydrazone (CCCP), as a PMF uncoupler, can destroy proton gradient across plasma membrane and inhibit metabolites efflux driven by PMF accordingly [41]. Phe-Arg-β-naphtylamide (PAβN) was shown to be a competitive inhibitor of some transporters driven by PMF [22]. Sodium orthovanadate (SOV) interacts with nucleotide binding domain of bacterial ABC transporters and inhibits their ATPase activity [30]. Glybenclamide (GLB) has also been shown to inhibit the activities of various ABC transporters [39]. Since different energy sources were utilized by two sorts of transporters, it is possible to identify the major exporter family for certain metabolites by inhibitors of different exporters, especially via inhibition of energy utilization. Martins et al. [24], for instance, revealed the mechanism of ethidium bromide extrusion in *E. coli* using PMF inhibitor CCCP and ATP-dependent transportation inhibitor Verapamil. Similarly, arthrofactin exportation by ABC-transporters in *Pseudomonas* was confirmed using ATP-dependent transportation inhibitor SOV and GLB [20]. Norfloxacin efflux mediated by BC4707, a MFS transporter in *Bacillus cereus*, was determined to be PMF-dependent by adding CCCP [41]. The mechanism of surfactin efflux in THY-7, accordingly, may also be demonstrated by transportation inhibitors. The differences after addition of sub-lethal concentration of PMF inhibitor (CCCP and PAβN) and ATP-dependent transportation inhibitor (SOV and GLB) showed that surfactin efflux in

B. subtilis THY-7 was greatly affected by the elimination of transmembrane proton gradient, but not ATP hydrolysis. Therefore, it could be concluded that surfactin efflux in *B. subtilis* THY-7 was facilitated by PMF-dependent exporters, for example, members of MFS.

There is a 12-TM (transmembrane helices) rule in MFS family. Almost all MFS proteins possess a uniform topology of 12 transmembrane helices, with few exceptions of 14-, 24- and 6-TM proteins [19, 34]. The coding region of *ycxAmt* from THY-7 stopped after TM10, producing a truncated YcxA protein with only 10 transmembrane helices (Fig. S4). There is little evidence that a 10-TM MFS protein can be functional in Gram-positive bacteria. Not surprisingly, overexpression of *ycxAmt* did not show any positive effect on surfactin production. Surfactin production of TS589 (expressing full-length *ycxA*), however, was significantly higher than that of control (Fig. 5). Our results indicated (1) that the truncated YcxA with 10 TMs was non-functional, and (2) that the expression of full-length YcxA could further promote surfactin transportation, which together revealed that for YcxA itself, the integrity of 12 TMs was fundamental for its own transporting function. Surfactin production of THY-7 (with a non-functional *ycxAmt*), nevertheless, was not abolished, which demonstrated that YcxA was not essential for surfactin efflux, and surfactin could be secreted by other functional transporters, probably YerP. These results had shed some light on the relationship between structure and function of MFS family transporters, especially YcxA.

It is interesting that KrsE could play the role of surfactin transporter. Relatively low substrate specificity of MFS family exporters [19] may explain why KrsE, a putative permase in kurstakin synthase gene cluster, facilitated surfactin efflux, and partially why enhancement of surfactin production of *krsE* over-expression strain was not as high as *ycxA* over-expression strain. It is believed that only a few amino acid residues at the substrate-binding site of MFS transporters determine the specificity of substrate [19]. The polar amino acid moiety of both lipopeptides may react with certain amino acid residues at the substrate-binding site of KrsE, and the lipid moiety may facilitate the membrane binding and penetration through hydrophobic channels formed by KrsE, resulting in efflux of lipopeptides, such as surfactin and kurstakin. However, these descriptions could only be general and hypothetical, due to the lack of specific structural and functional information about lipopeptide transporters in Gram-positive bacteria.

Besides YcxA and KrsE, YerP is another probable lipopeptide transporter with transmembrane proton gradient as driving force. YerP is homologous with the resistance, nodulation and cell division (RND) family PMF-dependent efflux pumps and has been shown to be essential for surfactin resistance in *B. subtilis* [44]. Since *yerP-deleted* mutant *B. subtilis* cells have shown lower surfactin production and higher susceptibility to surfactin than wild-type strain, YerP might be a satisfying candidate for surfactin transmembrane transporter. To further confirm the function of YerP toward surfactin transport, *yerP* was cloned and introduced back into THY-7. Promotion effect on surfactin production of YerP was most significant among three transporters investigated, which demonstrated the major role of *yerP* on surfactin efflux. Extrusion of certain molecules was a common mechanism for toxin resistance in bacteria [31]. For instance, BC 4707, a multidrug transporter, mediated the resistance to norfloxacin in *Bacillus cereus* [41]. AcrAB transportation system was also shown to be responsible for the resistance of several antibiotics (such as tetracycline and chloramphenicol) in *E. coli* K12 [27, 46]. It could be inferred from the surfactin production enhancing effect of YerP (Fig. 7) and results of *yerP*-deficient strain reported by Tsuge et al. [44] that surfactin resistance mediated by YerP is intrinsically the transmembrane transportation of surfactin by YerP. The capacity of surface motility in *B. subtilis*, so called swarming, depended on the existence of extracellular surfactin [17]. A *B. subtilis* strain was unable to swarm after invalidation of *swrC* (synonymous with *yerP*), and swarming ability was regained after the addition of extracellular surfactin [18].These reports indicated that SwrC (YerP) contributed to the extracellular accumulation of surfactin. Considering the reported sequential/structural homology of YerP and our results, YerP might act as the major surfactin exporter, responsible for both surfactin efflux and resistance in *B. subtilis*.

In this study, a *B. subtilis* strain THY-7 producing surfactin was isolated. Active efflux of surfactin in THY-7 was determined to be dependent on transmembrane proton motive force, rather than ATP hydrolysis. Overexpression of YerP, YcxA and KrsE were all beneficial to surfactin production, among which surfactin efflux elevated most greatly in the YerP-overexpressing strain, indicating that YerP acted as the major surfactin exporter in *B. subtilis* THY-7. Overexpression of a truncated YcxA with 2 transmembrane helices deletion could not promote the surfactin export. On the contrary, KrsE, the putative kurstakin transporter, could also play the role of surfactin transporter. To our knowledge, this is the first report with direct evidence that transmembrane exporters dependent on PMF could facilitate the surfactin efflux in *B. subtilis*. Using PMF as energy source, export of surfactin may also be significantly affected by medium pH, which might guide further optimization of surfactin fermentation conditions. Further studies are needed to clarify the function of efflux proteins in surfactin transmembrane transportation. A deeper understanding on mechanisms of surfactin efflux would undoubtedly contribute to the solution of the cost issue on lipopeptide production.

Acknowledgments This work is supported by National Key Basic Research Project 973 (2013CB733600) and Tsinghua University Initiative Scientific Research Program (No. 20111081120).

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Béchet M, Caradec T, Hussein W, Abderrahmani A, Chollet M, Leclère V, Dubois T, Lereclus D, Pupin M, Jacques P (2012) Structure, biosynthesis, and properties of kurstakins, nonribosomal lipopeptides from *Bacillus* spp. Appl Microbiol Biotechnol 95(3):593–600. doi:10.1007/s00253-012-4181-2
2. Baindara P, Mandal SM, Chawla N, Singh PK, Pinnaka AK, Korpole S (2013) Characterization of two antimicrobial peptides produced by a halotolerant *Bacillus subtilis* strain SK.DU.4 isolated from a rhizosphere soil sample. AMB Express 3(1):2. doi:10.1186/2191-0855-3-2
3. Banat I, Franzetti A, Gandolfi I, Bestetti G, Martinotti M, Fracchia L, Smyth T, Marchant R (2010) Microbial biosurfactants production, applications and future potential. Appl Microbiol Biotechnol 87(2):427–444. doi:10.1007/s00253-010-2589-0
4. Baum JA, Coyle DM, Gilbert MP, Jany CS, Gawron-Burke C (1990) Novel cloning vectors for *Bacillus thuringiensis*. Appl Environ Microbiol 56(11):3420–3428
5. Carrillo C, Teruel JA, Aranda FJ, Ortiz A (2003) Molecular mechanism of membrane permeabilization by the peptide antibiotic surfactin. Biochim Biophys Acta (BBA)-Biomembr 1611(1):91–97
6. Cheng F, Tang C, Yang H, Yu H, Chen Y, Shen Z (2013) Characterization of a blend-biosurfactant of glycolipid and lipopeptide produced by *Bacillus subtilis* TU2 isolated from underground oil-extraction wastewater. J Microbiol Biotechnol 23(3):390–396
7. Cosmina P, Rodriguez F, Ferra F, Grandi G, Perego M, Venema G, Sinderen D (1993) Sequence and analysis of the genetic locus responsible for surfactin synthesis in *Bacillus subtilis*. Mol Microbiol 8(5):821–831
8. Das P, Mukherjee S, Sen R (2008) Genetic regulations of the biosynthesis of microbial surfactants: an overview. Biotechnol Genet Eng Rev 25(1):165–185
9. Deleu M, Bouffioux O, Razafindralambo H, Paquot M, Hbid C, Thonart P, Jacques P, Brasseur R (2003) Interaction of surfactin with membranes: a computational approach. Langmuir 19(8):3377–3385. doi:10.1021/la026543z
10. Deleu M, Lorent J, Lins L, Brasseur R, Braun N, El Kirat K, Nylander T, Dufrêne YF, Mingeot-Leclercq M-P (2013) Effects of surfactin on membrane models displaying lipid phase separation. Biochim Biophys Acta (BBA)-Biomembr 1828(2):801–815. doi:10.1016/j.bbamem.2012.11.007
11. Deravel J, Lemière S, Coutte F, Krier F, Hese N, Béchet M, Sourdeau N, Höfte M, Leprêtre A, Jacques P (2014) Mycosubtilin and surfactin are efficient, low ecotoxicity molecules for the biocontrol of lettuce downy mildew. Appl Microbiol Biotechnol 98(14):6255–6264. doi:10.1007/s00253-014-5663-1
12. Hayashi K, Ohsawa T, Kobayashi K, Ogasawara N, Ogura M (2005) The H_2O_2 stress-responsive regulator PerR positively regulates *srfA* expression in *Bacillus subtilis*. J Bacteriol 187(19):6659–6667. doi:10.1128/jb.187.19.6659-6667.2005
13. Heerklotz H, Seelig J (2007) Leakage and lysis of lipid membranes induced by the lipopeptide surfactin. Eur Biophys J 36(4–5):305–314
14. Henry G, Deleu M, Jourdan E, Thonart P, Ongena M (2011) The bacterial lipopeptide surfactin targets the lipid fraction of the plant plasma membrane to trigger immune-related defence responses. Cell Microbiol 13(11):1824–1837
15. Ishigami Y, Osman M, Nakahara H, Sano Y, Ishiguro R, Matsumoto M (1995) Significance of β-sheet formation for micellization and surface adsorption of surfactin. Colloids Surf B Biointerf 4(6):341–348. doi:10.1016/0927-7765(94)01183-6
16. Jacques P (2011) Surfactin and other lipopeptides from *Bacillus* spp. In: Soberón-Chávez G (ed) Biosurfactants, vol 20. Microbiology monographs. Springer, Berlin/Heidelberg, pp 57–91. doi:10.1007/978-3-642-14490-5_3
17. Kearns DB (2010) A field guide to bacterial swarming motility. Nat Rev Microbiol 8(9):634–644
18. Kearns DB, Chu F, Rudner R, Losick R (2004) Genes governing swarming in *Bacillus subtilis* and evidence for a phase variation mechanism controlling surface motility. Mol Microbiol 52(2):357–369
19. Law CJ, Maloney PC, Wang DN (2008) Ins and outs of major facilitator superfamily antiporters. Annu Rev Microbiol 62:289–305
20. Lim SP, Roongsawang N, Washio K, Morikawa M (2009) Flexible exportation mechanisms of arthrofactin in *Pseudomonas* sp. MIS38. J Appl Microbiol 107(1):157–166. doi:10.1111/j.1365-2672.2009.04189.x
21. Lin SC, Jiang HJ (1997) Recovery and purification of the lipopeptide biosurfactant of *Bacillus subtilis* by ultrafiltration. Biotechnol Tech 11(6):413–416
22. Lomovskaya O, Zgurskaya HI, Totrov M, Watkins WJ (2007) Waltzing transporters and 'the dance macabre' between humans and bacteria. Nat Rev Drug Discov 6(1):56–65
23. Marchant R, Banat IM (2012) Microbial biosurfactants: challenges and opportunities for future exploitation. Trends Biotechnol 30(11):558–565. doi:10.1016/j.tibtech.2012.07.003
24. Martins A, Spengler G, Rodrigues L, Viveiros M, Ramos J, Martins M, Couto I, Fanning S, Pagès JM, Bolla JM (2009) pH modulation of efflux pump activity of multi-drug resistant *Escherichia coli*: protection during its passage and eventual colonization of the colon. PLoS ONE 4(8):e6656
25. Nguyen H, Phan T, Schumann W (2007) Expression vectors for the rapid purification of recombinant proteins in *Bacillus subtilis*. Curr Microbiol 55(2):89–93. doi:10.1007/s00284-006-0419-5
26. Ohsawa T, Tsukahara K, Sato T, Ogura M (2006) Superoxide stress decreases expression of *srfA* through inhibition of transcription of the comQXP quorum-sensing locus in *Bacillus subtilis*. J Biochem 139(2):203–211. doi:10.1093/jb/mvj023
27. Okusu H, Ma D, Nikaido H (1996) AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J Bacteriol 178(1):306–308
28. 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. Environ Microbiol 9(4):1084–1090
29. Peypoux F, Bonmatin J, Wallach J (1999) Recent trends in the biochemistry of surfactin. Appl Microbiol Biotechnol 51(5):553–563
30. Pezza RJ, Villarreal MA, Montich GG, Argaraña CE (2002) Vanadate inhibits the ATPase activity and DNA binding capability of bacterial MutS. A structural model for the vanadate–MutS interaction at the Walker A motif. Nucleic Acids Res 30(21):4700–4708
31. Piddock LJV (2006) Multidrug-resistance efflux pumps? Not just for resistance. Nat Rev Microbiol 4(8):629–636
32. Price NPJ, Rooney AP, Swezey JL, Perry E, Cohan FM (2007) Mass spectrometric analysis of lipopeptides from *Bacillus* strains isolated from diverse geographical locations. FEMS Microbiol Lett 271(1):83–89. doi:10.1111/j.1574-6968.2007.00702.x

33. Raaijmakers JM, De Bruijn I, Nybroe O, Ongena M (2010) Natural functions of lipopeptides from *Bacillus* and *Pseudomonas*: more than surfactants and antibiotics. FEMS Microbiol Rev 34(6):1037–1062. doi:10.1111/j.1574-6976.2010.00221.x
34. Reddy VS, Shlykov MA, Castillo R, Sun EI, Saier MH Jr (2012) The major facilitator superfamily (MFS) revisited. FEBS J 279(11):2022–2035
35. Roongsawang N, Washio K, Morikawa M (2011) Diversity of nonribosomal peptide synthetases involved in the biosynthesis of lipopeptide biosurfactants. Int J Mol Sci 12(1):141–172
36. Saier MH Jr, Goldman SR, Maile RR, Moreno MS, Weyler W, Yang N, Paulsen IT (2002) Transport capabilities encoded within the *Bacillus subtilis* genome. J Mol Microbiol Biotechnol 4(1):37–67
37. Saito H, Shibata T, Ando T (1979) Mapping of genes determining nonpermissiveness and host-specific restriction to bacteriophages in *Bacillus subtilis* Marburg. Mol Gen Genet 170(2):117–122
38. Sansinenea E, Ortiz A (2011) Secondary metabolites of soil *Bacillus* spp. Biotechnol Lett 33(8):1523–1538. doi:10.1007/s10529-011-0617-5
39. Serrano-Martín X, Payares G, Mendoza-León A (2006) Glibenclamide, a blocker of K^+ ATP channels, shows antileishmanial activity in experimental murine cutaneous leishmaniasis. Antimicrob Agents Chemother 50(12):4214–4216
40. Shaligram NS, Singhal RS (2010) Surfactin- A review on biosynthesis, fermentation, purification and applications. Food Technol Biotechnol 48(2):119–134
41. Simm R, Vörös A, Ekman JV, Sødring M, Nes I, Kroeger JK, Saidijam M, Bettaney KE, Henderson PJF, Salkinoja-Salonen M, Kolstø A-B (2012) BC4707 is a major facilitator superfamily multidrug resistance transport protein from *Bacillus cereus* implicated in fluoroquinolone tolerance. PLoS ONE 7(5):e36720. doi:10.1371/journal.pone.0036720
42. Stein T (2005) *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol Microbiol 56(4):845–857. doi:10.1111/j.1365-2958.2005.04587.x
43. 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. Appl Microbiol Biotechnol 85(5):1521–1531. doi:10.1007/s00253-009-2176-4
44. Tsuge K, Ohata Y, Shoda M (2001) Gene *yerP*, involved in surfactin self-resistance in *Bacillus subtilis*. Antimicrob Agents Chemother 45(12):3566–3573. doi:10.1128/aac.45.12.3566-3573.2001
45. Vater J, Kablitz B, Wilde C, Franke P, Mehta N, Cameotra SS (2002) Matrix-assisted laser desorption ionization-time of flight mass spectrometry of lipopeptide biosurfactants in whole cells and culture filtrates of *Bacillus subtilis* C-1 isolated from petroleum sludge. Appl Environ Microbiol 68(12):6210–6219. doi:10.1128/aem.68.12.6210-6219.2002
46. Viveiros M, Jesus A, Brito M, Leandro C, Martins M, Ordway D, Molnar AM, Molnar J, Amaral L (2005) Inducement and reversal of tetracycline resistance in *Escherichia coli* K-12 and expression of proton gradient-dependent multidrug efflux pump genes. Antimicrob Agents Chemother 49(8):3578–3582. doi:10.1128/aac.49.8.3578-3582.2005
47. Walter V, Syldatk C, Hausmann R (2010) Screening concepts for the isolation of biosurfactant producing microorganisms. In: Sen R (ed) Biosurfactants. Springer, New York, pp 1–13
48. Xue GP, Johnson JS, Dalrymple BP (1999) High osmolarity improves the electro-transformation efficiency of the gram-positive bacteria *Bacillus subtilis* and *Bacillus licheniformis*. J Microbiol Methods 34(3):183–191