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ORIGINAL PAPER

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Isolation and characterization of a halotolerant *Bacillus subtilis* BBK-1 which produces three kinds of lipopeptides: bacillomycin L, plipastatin, and surfactin

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Abstract Twenty-three halotolerant and biosurfactant producing strains were collected from salty conditions in central Thailand. One of the strains designated BBK-1 produced the biosurfactants with the highest activity. BBK-1 was isolated from fermented foods and was identified as *B. subtilis* based on its physiological characteristics and 16S rRNA gene sequence. We show that the strain grows in media containing NaCl up to 16% (w/v) and produces biosurfactants in NaCl up to 8%. We found that *B. subtilis* BBK-1 produces three kinds of surface-active lipopeptides simultaneously. By their respective molecular weights and amino acid compositions, it is indicated that these lipopeptides are bacillomycin L, plipastatin, and surfactin. In order to analyze the production mechanism of lipopeptides further in the strain, a generally important biosynthetic gene encoding 4′-phosphopantetheinyl transferase was cloned and sequenced. The gene existed in a single copy in the genome and the deduced amino acid sequence was almost identical to that of Lpa-14 from *B. subtilis* strain RB14, which co-produces iturin A and surfactin.

Key words Bacillomycin L · *Bacillus subtilis* · Biosurfactants · Halotolerant · Lipopeptides · Plipastatin · Surfactin

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Introduction

Biosurfactants are biological surface-active compounds produced by bacteria and fungi that have a broad range of industrial applications as well as several advantages over chemical surfactants (Fiechter 1992). They are usually amphipathic and complex lipids such as glycolipids, lipopeptides, phospholipids, or fatty acids (Cooper and Zajic 1980). The genus *Bacillus* is a bacterial group most often studied for the production mechanisms of lipopeptides including biosurfactants (LPBS) and antibiotics (LPAB) (see Table 1). Both LPBS and LPAB share similar amphipathic structures containing both a hydrophilic peptide portion and a hydrophobic fatty acid portion. Most of them have cyclic structure mediated by either β-hydroxy fatty acid (β-hydroxy type) or β-amino fatty acid (β-amino type).

Surfactin is the most common LPBS produced by several bacilli whose biosynthetic mechanisms have been well studied both genetically and biochemically (Quadri et al. 1998; Peypoux et al. 1999). Three genetic loci, *srfA* operon, *comA* (previously called *srf*B), and *sfp* are essential for surfactin production in *B. subtilis*. The *srfA* operon consists of four open reading frames that encode three proteins with peptidyl carrier protein (PCP) and/or racemase domain, and a thioesterase like protein. These proteins form a nonribosomal peptide synthetase complex (surfactin synthetase). The operon encompasses 28 kb (Cosmina et al. 1993). On the other hand, *comA* and *sfp* encode a transcriptional activator of the *srfA* gene (Roggiani and Dubnau 1993) and a 4′-phosphopantetheinyl transferase (Sfp/PPTase, an activating enzyme of SrfA multienzyme complex), respectively (Nakano et al. 1992; Lambalot et al. 1996). Sfp(PPTase) converts the inactive apo-forms of the seven PCP domains of surfactin synthetase to the active holo-forms. Recently, heterologous posttranslational modification by Sfp of the polyketide 6-deoxyerythronolide B and 6-methylsalicylic synthases in *E. coli* and *S. cerevisiae* have been reported (Kealey et al. 1998; Pfeifer et al. 2001). Sfp apparently has broad substrate preferences that allow posttranslational modification of both PCP in the nonribosomal peptide synthetases and the acyl carrier protein

Table 1. Structures of LPBS/LPAB produced by *Bacillus* strains

LPBS/LPAB MW (Da.)	Producer	Structure	References
Surfactin MW = 1,007–1,035	*B. subtilis* ATCC 21332, OKB105	C_{13}-C_{15} β-hydroxy fatty acid-L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu	Kakinuma et al. (1969)
	B. pumilus A1		Morikawa et al. (1992)
Lichenysin A MW = 992–1,062	*B. licheniformis* BAS50	C_{12}-C_{17} β-hydroxy fatty acid-L-Gln-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Ile	Yakimov et al. (1999)
Lichenysin B MW = 1,035	*B. licheniformis* JF-2	C_{15} β-hydroxy fatty acid-L-Glu-L-Leu-D-Leu-L-Val-L-Asp-D-Leu-L-Leu	Lin et al. (1994)
Lichenysin C MW = 1,021–1,035	*B. licheniformis*	C_{14}-C_{15} β-hydroxy fatty acid-Glu-Leu-Leu-Val-Asp-Leu-Ile	Jenny et al. (1991)
Surfactant BL86 MW = 992–1,062	*B. licheniformis* 86	C_{12}-C_{17} β-hydroxy fatty acid -L-Glx-L-Leu-D-Leu-L-Val-L-Asx-D-Leu-L-Ile (60%)/L-Val (40%)	Horowitz and Griffin (1991)
Plipastatin MW = 1,462–1,504	*B. cereus* BMG302-fF67	C_{16}-C_{17} β-hydroxy fatty acid -L-Glu-D-Orn-L-Tyr-D-allo-Thr-L-Glu-D-[X]-L-Pro-L-Gln-D-Tyr-L-Ile X = Ala or Val	Nishikiori et al. (1986)
Iturin A MW = 1,028–1,070	*B. subtilis*	C_{13}-C_{16} β-amino fatty acid -L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Asn-L-Ser	Isogai et al. (1982)
Bacillomycin L MW = 1,020–1,048	*B. subtilis* NCIB 8872	C_{14}-C_{16} β-amino fatty acid-L-Asp-D-Tyr-D-Asn-L-Ser-L-Gln-D-Ser-L-Thr	Peypoux et al. (1986)
Mycosubtilin MW = 1,042–1,084	*B. subtilis* ATCC6633	C_{14}-C_{17} β-amino fatty acid-L-Asn-D-Tyr-D-Asn-L-Gln-L-Pro-D-Ser-L-Asn	Duitman et al. (1999)

These LPBS/LPAB are usually a mixture of compounds with different lengths and types (*normal*, *iso-* and *anteiso-*) of fatty acid chain. The β-hydroxy or β amino group of fatty acid forms an ester or peptide bond with the carboxyl group of a C-terminal amino acid residue. Plipastatin is expected to form a lactone structure between hydroxyl group of L-tyrosine and carboxyl group of C-terminal L-isoleucine

(ACP) in polyketide synthases. Thus, PPTase is a generally important and valuable enzyme for the production of LPBS and LPAB, and polyketide compounds.

Despite their great potential in industrial applications, including environmental remediation of oil spills, biosurfactants have been isolated from bacteria and fungi only under moderate conditions. There is little information available about biosurfactant production in extreme environments (Yakimov et al. 1995). In order to expand the industrial application of biosurfactant-producing bacteria, we decided to collect halotolerant bacteria that naturally produce large amounts of biosurfactants. We chose to isolate halophilic bacteria because there are many high-salt areas in Thailand. In this report, we describe the isolation and characterization of a halotolerant *B. subtilis* strain BBK-1. We demonstrate that BBK-1 co-produces bacillomycin L, plipastatin, and surfactin. To further advance the study of biosurfactant production in the halotolerant strain we take a molecular biological approach to clone the homologue of *sfp* which encodes PPTase.

Materials and methods

Isolation of halotolerant biosurfactant-producing bacteria

Samples from various sources including salty soils, sands, seawater, and fermented foods were collected in the central part of Thailand. These samples were grown on oil–LBGS agar plates. The oil–LBGS agar plates contained (w/v) 1% Bacto-tryptone, 0.5% yeast extract, 1% glucose, 5% NaCl, 1.5% agar, pH 7.5 with NaOH and were overlaid with 30 µl of crude oil. The plates were incubated at 30°C overnight. Colonies surrounded by oil-displaced halos, indicating biosurfactant production, were taken from the plates (Morikawa et al. 1992). They were then cultivated aerobically in LBGS liquid medium at 30°C for 24 h with agitation at 200 rpm. The culture supernatants were used to test for biosurfactant production.

Determination of biosurfactant production

The surface tensions of the culture supernatants were measured using a ring tensiometer (K6; Kruss, Germany). The critical micelle concentration (CMC) is determined as the concentration at which the surface tension begins to increase. The dilution required to attain the CMC is defined as CMC^{-1} and was considered proportional to the amount of biosurfactants in the original sample (Sheppard and Mulligan 1987). The bacterial strain whose culture broth was determined to have a surface tension lower than 35 mN/m and gave the highest CMC^{-1} value was selected for further experiments.

Bacterial strains and plasmids

B. subtilis BBK-1 is described in this study. Surfactin non-producing *B. subtilis* MI113 (*arg-15, trpC2, hsrM, hsmM,*

sfp[0]) (Morikawa et al. 1992), which is a derivative of Marburg strain 168, and *E. coli* DH5α [F⁻, ø80, *lac*ZΔM15, *recA*1, *end*A1, *gyrA*96, *thi*–1, *hsdR*17, r_k^-, m_k^+, *SupE*44, *relA*1, *deoR*, Δ(*lacZYA-argF*)U169, λ⁻] were used as host strains for cloning procedures. Plasmids pUC19 and pCR2.1 (Invitrogen, San Diego, CA), and pTB523 (Tc^r; Morikawa et al. 1992) were used to transform *E. coli* DH5aand *B. subtilis*, respectively.

General DNA manipulations

Genomic DNA of *B. sub*tilis MI113 and BBK-1 was extracted by the Sarkosyl method and separated by CsCl–ethidium bromide equilibrium density gradient ultracentrifugation (Morikawa et al. 1992). All DNA manipulations were performed by standard protocols (Sambrook et al. 1989). Competent cells of *B. subtilis* MI113 were prepared as described by Anagnostopoulos and Spizizen (1961) and used for transformation. DNA fragments were recovered from an agarose gel using GeneClean Kit (Bio101, La Jolla, CA). Plasmid DNA preparation on a large scale was performed using a Qiagen plasmid maxi kit (Qiagen, Hilden, Germany). PCR was performed in 30 cycles using a thermal cycler, GeneAmp PCR System 2400 (Perkin-Elmer, Foster City, CA). Oligodeoxyribonucleotides for PCR primers were synthesized at Sawady Technology (Tokyo, Japan) or Life Technologies (Tokyo, Japan). Nucleotide sequence of the gene fragments was determined by the dideoxy-chain termination method (Sanger et al. 1977) using an autosequencer ABI Prism 310 (Perkin-Elmer). DNA sequencing data were analyzed using DNASIS software (Hitachi Software, Tokyo, Japan) and BLAST program (NCBI, Bethesda, MD).

Identification of strain BBK-1

BBK-1 was physiologically tested according to *Bergey's Manual of Systematic Bacteriology* (Sneath 1986). In order to determine the 16S rRNA gene sequence, genomic DNA of strain BBK-1 was extracted using an InstaGene Matrix (BIO-RAD, Hercules, CA). The DNA region encoding 16S rRNA was amplified by standard PCR method using a set of universal primers, forward: 5′-AAGAGTTTGATCATGG CTCAG-3′ and reverse: 5′-AGGAGGTGATCCAACCG CA-3′. The amplified gene fragment was purified from agarose gel and cloned into the TA-cloning site of pCR2.1. The nucleotide sequence of 16S rRNA gene was determined as described above.

Isolation and structural analyses of LPBS and LPAB

Strain BBK-1 was grown in 250-ml Erlenmeyer flasks containing 50 ml of modified medium at 30°C for 24 h on a platform shaker at 200 rpm. The modified medium contained (w/v) 0.5% sucrose, 0.2% NH_4NO_3, 0.5% yeast extract, and 3% NaCl, pH 7.5. Lipopeptides were recovered using the following method. Bacterial cells were removed from 3 1 of surfactant-containing culture medium by centrifugation (20,000 g for 15 min at 4°C). The supernatant was subjected to acid precipitation by adding concentrated HCl to achieve a final pH of 2.0 and allowing the precipitate to form at 4°C overnight. The pellet was collected by centrifugation and washed three times with diluted HCl (pH 2.0). The surfactants were extracted three times with methanol and subjected to further purification by chromatographic procedures as described below. When necessary, the solvent was removed using a rotary evaporator under vacuum conditions.

Reverse-phase HPLC was carried out on an octadecyl silica gel column (Cosmosil $5C_{18}AR$ 4.6 × 150 mm; Nacalai, Kyoto, Japan) attached to a system HP1100 (Hewlett Packard, Palo Alto, CA). The mobile phase was changed linearly from eluent A (10% acetonitrile and 0.1% trifluoroacetic acid) to eluent B (acetonitrile and 0.05% trifluoroacetic acid) at a flow rate of 0.5 ml/min. Peaks eluting from the column were monitored by their absorbance at 220 nm. The fractions were tested for surfactant activity by the oil displacement test (Morikawa et al. 1993). The molecular weight of each component was determined by an API mass spectrometer LCQ (Thermo Finnigan, San Jose, CA). Amino acid compositions were determined by Hitachi L-8500 amino acid analyzer (Tokyo, Japan) at the Institute for Protein Research, Osaka University.

Cloning of *sfp* homologue, encoding PPTase from BBK-1

A putative *sfp*[0] gene in *B. subtilis* MI113 was amplified by PCR using the following primers, 5′-CTAGAATTCAGAT TTACGGAATTTATATG-3′ and 5′-GGGGAATTCAGG GTGTGCGGCGCATAC-3′, derived from the *sfp* gene in *B. subtilis* OKB105 (DDBJ/EMBL/GenBank, X63158). The resulting 642-bp PCR fragment was determined for its sequence, labeled by AlkPhos direct system (Amersham Pharmacia, Buckinghamshire, UK), and used to probe BBK-1 genomic DNA by Southern blot and to isolate a positive colony from the BBK-1 genomic library in *E. coli* (Sambrook et al. 1989).

Nucleotide sequence accession number

The nucleotide sequences of *B. subtilis* BBK-1 16S rRNA and *lpbk1* genes have been deposited in the DDBJ/EMBL/GenBank databases under accession numbers AB053351 and AB062550, respectively. The nucleotide sequence of *B. subtilis* MI113 *sfp*[0] has been deposited under AB061356.

Results

Isolation of halotolerant biosurfactant producers

Twenty-three positive colonies surrounded by oil-displaced halos were isolated from the central Thailand samples. Nine

Table 2. The surface tensions and the surface activities of culture supernatants of halotolerant bacterial strains

Strains	Surface tension (mN/m)	CMC^{-1} (fold)
BST1–2	30.0	6
BBW1–2	30.7	7
BBW2–2	31.2	16
BBK-1	28.0	28
BBT-2	28.0	24
BBS1–10	28.5	3
BCW3–2	33.5	2
BKS2–2	31.5	3
BBS-1	29.0	2
No cell	50.0	–

CMC^{-1} = Relative biosurfactant concentration

of these isolates showed the ability to reduce surface tension of their culture supernatant to below 35 mN/m (Table 2). These strains were considered to produce biosurfactants effectively. One strain, designated BBK-1, with the largest oil-displaced halo, exhibited the highest CMC^{-1} value (28-fold in LBGS), and had the lowest surface tension (28 mN/m) in the culture supernatant. This strain was isolated from traditional fermented foods.

Identification of strain BBK-1

Microscopic observations of BBK-1 showed a rod-shaped, spore-forming, Gram-positive, motile bacterium. The strain tested positive for catalase activity and positive for acid production when grown on a variety of carbon sources including glucose, fructose, mannitol, xylose, and arabinose. BBK-1 utilized citrate as sole carbon source. BBK-1 hydrolyzed gelatin, casein, and starch, which indicate protease and amylase production. On the other hand, both indole test and VP test showed negative for the strain. The strain did not grow under anaerobic conditions. BBK-1 was able to grow in media containing NaCl at a range of 0%–16% (w/v) and at temperatures between 25° and 50°C. Optimal growth temperature was determined as 45°C.

We sequenced 1,450 bases of the 16S rRNA gene. Comparative sequence analysis suggests that the strain BBK-1 belongs to the *Bacillus* group, with the highest identity score (99.0%) to *B. subtilis* ATCC21331 (AB18487). All of the results from physiological tests with exception of the VP test were consistent with those of *B. subtilis*. *B. subtilis* is positive for the VP test in most cases but BBK-1 was not.

Effect of NaCl on biosurfactant production

Biosurfactant production by *B. subtilis* BBK-1 was tested with various concentrations of NaCl in LBGS medium. Production was repressed by NaCl at concentrations higher than 3% whereas cell growth was not seriously affected by NaCl up to 5%. No biosurfactant was produced when the medium contained 10% NaCl. These characteristics indicate that BBK-1 is moderately halotolerant.

Structural analyses of biosurfactants

When the methanol fraction containing surfactant activity was analyzed by HPLC, it was revealed that *B. subtilis* BBK-1 produced three kinds of biosurfactants simultaneously. These were tentatively named by the order of elution time as BS-A (18–22.5 min), BS-B (23–24 min), and BS-C (29–32 min) (Fig. 1). The mass spectra of BS-A showed a series of ions at $[M + H]^+/z$ = 993, 1,007, 1,021, 1,035, 1,049, and 1,063, those of BS-B were found at $[M + H]^+/z$ = 1,463, 1,477, and 1,505, and those of BS-C at $[M + H]^+/z$ = 994, 1,008, 1,022, 1,036, and 1,050. A set of mass peaks with an interval of 14 is often observed for lipopeptides with different numbers of methylene groups (-CH_2-) in fatty acyl chains (Morikawa et al. 2000). The amino acid composition was analyzed for each compound: BS-A contained Thr, Tyr, Glu, Asp, and Ser in a ratio of 1 : 1 : 1 : 2 : 2, respectively, BS-B comprised Orn, Thr, Ala, Pro, Ile, Tyr, and Glu in a ratio of 1 : 1 : 1 : 1 : 1 : 2 : 3, and BS-C contained Glu, Asp, Val, and Leu in a ratio of 1 : 1 : 1 : 4. Glutamine and asparagine could not be differentiated from glutamic acid and aspartic acid, respectively, under these conditions, because the hydrolytic reaction gave rise to the deamination of these amino acids. The above results strongly indicate that BS-A, BS-B, and BS-C are the lipopeptides bacillomycin L, plipastatin, and surfactin, respectively (Table 1). Carbon numbers in the fatty acid portion of each were deduced as C_{12}–C_{17}, C_{16}–C_{19}, and C_{12}–C_{16}, respectively. The production ratio of BS-A, BS-B, and BS-C was approximately 5 : 1 : 15 in the modified medium (Fig. 1). Total production yield of the lipopeptides was about 480 mg/l at 30°C for 24 h.

Comparison of surfactant activity of LPBS and LPAB produced by *B. subtilis* BBK-1

The three lipopeptides produced by *B. subtilis* BBK-1 were purified by reverse-phase HPLC and tested for their surfactant activities using oil displacement assay. All three lipopeptides – bacillomycin L, plipastatin and surfactin – exhibited significant surfactant activities. Oil-displacement activity of each substance was 207.5 cm^2/mg (bacillomycin L), 659.5 cm^2/mg (plipastatin), and 1,840 cm^2/mg (surfactin).

Effect of carbon sources on the production of biosurfactants

After optimizing culture conditions for biosurfactant production, it was found that BBK-1 produced the largest quantity of biosurfactants in LBGS containing 3% NaCl (CMC^{-1} = 40-fold). Next, glucose was replaced by several carbon sources to examine the effect of carbon sources on the production. Of the carbon sources tested, glucose was found to be the best carbon source for production of all these lipopeptides (Fig. 2A). Meanwhile, sucrose and especially paraffin oil decreased the production of plipastatin and surfactin, leaving bacillomycin L almost unaffected, and glycerol dramatically reduced the production of all the three lipopeptides (Fig. 2B, C, D).

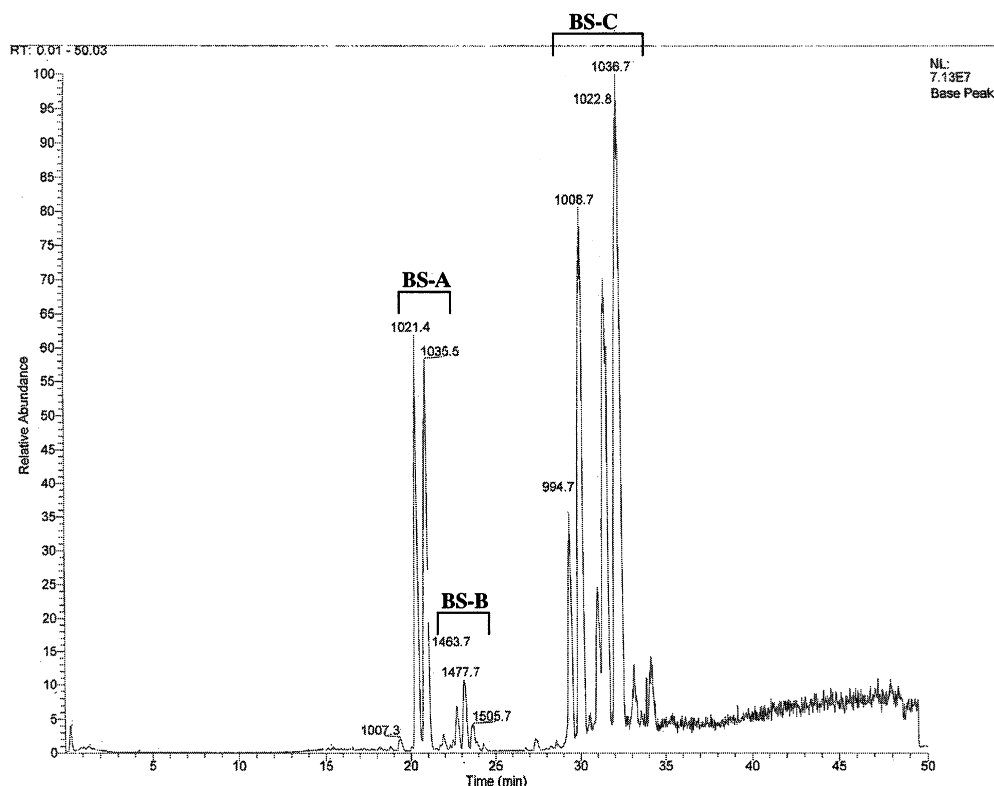

Fig. 1. HPLC/MS analysis of methanol extracts containing biosurfactant activity. The molecular weight of each component is shown *above the peak*

Cloning of the *sfp* homologue

The *sfp* gene in *B. subtilis* OKB105 has been shown to encode 4′-phosphopantheinyl transferase (PPTase), which is an essential modification enzyme of lipopeptide synthetase complex. A gene homologue of *sfp* in *B. subtilis* RB14 (*lpa-14*) has been reported to be responsible for the production of both surfactin (LPBS) and iturin A (LPAB) (Huang et al. 1993). Moreover, Sfp (PPTase) was shown to modify even acyl carrier proteins in the polyketide synthase complex (Pfeifer et al. 2001). The above information prompted us to isolate and analyze the sequence of a homologue of *sfp* in BBK-1. The nucleotide sequence analysis of *sfp*^{0} from surfactin-nonproducing *B. subtilis* MI113 differed from *sfp* gene by five base substitutions and one base insertion. The insertion results in a frame shift and a 165-amino-acid inactive protein Sfp^0. These results are consistent with the report by Nakano et al. (1992). Southern blot analysis with *sfp*^{0} of MI113 as a probe on *Eco*RI-digested BBK-1 genomic DNA revealed a 4-kb *Eco*RI fragment. We sequenced this 4-kb fragment. As expected, it contained a putative gene, which we designate as *lpbk1*, highly homologous to *sfp*. The presence of a single band on the Southern blot suggests that the BBK-1 genome contains only one *sfp*-like gene in spite of the strain being capable of producing three lipopeptides. The 4-kb *Eco*RI fragment was then subcloned into pTB523 and transferred into *B. subtilis* MI113 (surfactin nonproducer) to complement the preexisting mutations in *sfp*^{0}. All the transformants of *B. subtilis* MI113 with the recombinant plasmid produced surfactin. Moreover, introduction of a frame shift mutation into a *Sac*I site in *lpbk1* impaired the complementation ability of the gene. This clearly shows that *lpbk1* and its product PPTase are fully functional in vivo.

Comparison of *lpbk1* with other gene homologues

The *lpbk1* gene encoded PPTase with 224 amino acids. This gene is preceded by a sequence homologous to the consensus ribosome-binding site of *B. subtilis* genes ACGGAG GATC at five bases upstream of initiation ATG codon. Sequence alignment clearly indicates two gene groups (Fig. 3). The PPTase from BBK-1 shares 98% of its amino acid sequence identity with Lpa-14 of *B. subtilis* RB14 (Huang et al. 1993), while it shares only 66% identities with both Lpa-8 of *B. subtilis* YB8 (Tsuge et al. 1996) and Sfp of *B. subtilis* OKB105 (Nakano et al. 1992). The amino acid residues Ala22, Gly65, Gly69, and Glu216 in Lpa-14 are substituted by Thr, Ala, Ser, and Ala, respectively, in BBK-1 PPTase (Fig. 3). The crystal structure has already been determined for Sfp (PPTase) and a catalytic mechanism has been proposed and tested (Lambalot et al. 1996; Reuter et al. 1999). Sfp catalyzes the transfer of a phosphopantetheinyl moiety from coenzyme A to a consensus Ser residue in all seven PCP domains in SrfA with similar efficiency. BBK-1 PPTase conserves all the functionally required amino acid residues in Sfp, which are His90, Gly105, Asp107, Glu109, Trp147, Lys150, Glu151, and Lys155. This supports the experimental result that *lpbk1* complements the *sfp*^{0} mutation in *B. subtilis* MI113.

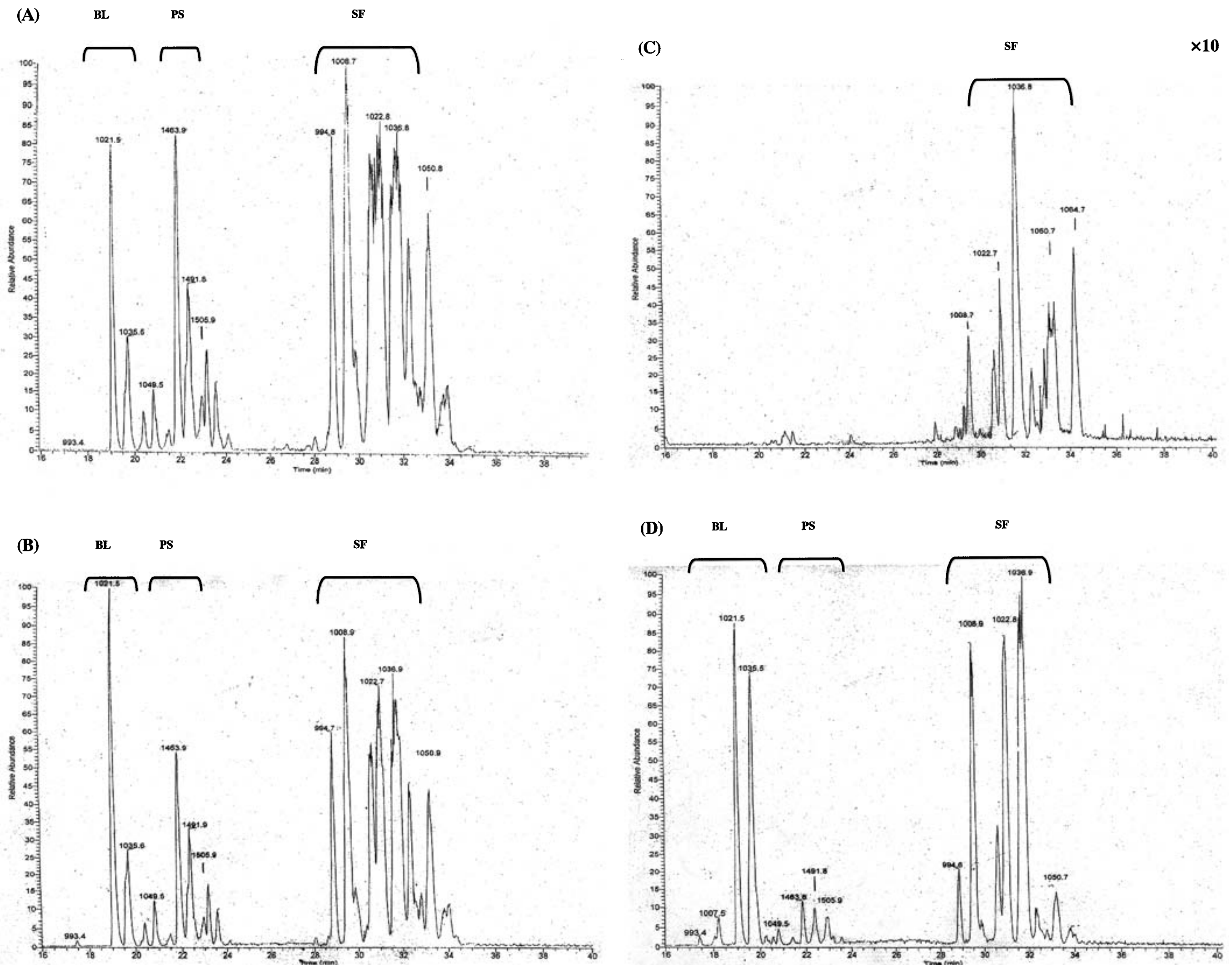

Fig. 2. HPLC/MS analysis of LPBS/LPAB produced by *B. subtilis* BBK-1 in LBGS, 3% NaCl medium with different carbon sources (1%, w/v): **A** glucose, **B** sucrose, **C** glycerol (magnified ×10), and **D** paraffin oil. Peaks corresponding to bacillomycin L (*BL*), plipastatin (*PS*), and surfactin (*SF*) are indicated

Discussion

B. subtilis RB14, YB8, and ATCC6633 have been reported to co-produce surfactin and iturin A, surfactin and plipastatin B1, and surfactin and mycosubtilin, respectively (Huang et al. 1993; Tsuge et al. 1996; Duitman et al. 1999). A halotolerant *B. licheniformis* strain BAS50 has been reported to produce lichenysin A, a derivative of surfactin (Yakimov et al. 1995). In this study, we isolated a halotolerant *B. subtilis* BBK-1 which simultaneously produces three kinds of surface-active compounds: bacillomycin L, plipastatin, and surfactin.

Bacillomycin L, plipastatin, and surfactin were purified from BBK-1 and their surfactant activities were confirmed. Surfactin is the most powerful biosurfactant of the three. Amidation and methylation of surfactin were used to demonstrate that the more hydrophobic compounds showed the higher surfactant activity (Morikawa et al. 2000). Loss of the negative charge at Asp and Glu resulted in an increase in surfactant activity by 1.2-fold. It has also been reported that lichenysin A exhibits a higher surface activity than surfactin because a glutamic acid in surfactin is replaced by glutamine in lichenysin A (Yakimov et al. 1999). Hopp and Woods (1983) estimated the hydrophilic scores of glutamic acid and glutamine to be 3.0 and 0.2, respectively. The hydrophobic nature of surfactin is demonstrated by the following facts. First is late elution time in reverse-phase HPLC (29–32 min, Fig. 1) and second is by the amino acid composition. Surfactin is composed of five hydrophobic (4 Leu and Val) and two hydrophilic (Glu and Asp) amino acids in its peptide portion. On the other hand, plipastatin comprises five hydrophobic (Ala, Ile, Pro, 2 Tyr) and five hydrophilic (Gln, 2 Glu, Orn, Thr) amino acids; and bacillomycin L contains only one polar hydrophobic residue (Tyr) out of seven. Contents of hydrophobic amino acids in

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                  10         20         30         40
BBK-1     MKIYGVYMDR PLSAGEEDRM MTAVSAEKRE KCRRFYHKED
Lpa-14    MKIYGVYMDR PLSAGEEDRM MAAVSAEKRE KCRRFYHKED
Lpa-8     MKIYGIYMDR PLSQEENERF MSFISPEKRE KCRRFYHKED
Sfp       MKIYGIYMDR PLSQEENERF MTFISPEKRE KCRRFYHKED
                                       *   *
                  50         60         70         80
BBK-1     AHRTLIGDML IRTAAAKAYG LDPAAISFSV QEYGKPYIPA
Lpa-14    AHRTLIGDML IRTAAAKAYG LDPAGISFGV QEYGKPYIPA
Lpa-8     AHRTLLGDVL VRSVISRQYQ LDKSDIRFST QEYGKPCIPD
Sfp       AHRTLLGDVL VRSVISRQYQ LDKSDIRFST QEYGKPCIPD
             *                               ****
                  90        100        110        120
BBK-1     LPDMHFNISH SGRWIVCAVD SKPIGIDIEK MKPGTIDIAK
Lpa-14    LPDMHFNISH SGRWIVCAVD SKPIGIDIEK MKPGTIDIAK
Lpa-8     LPDAHFNISH SGRWVICAFD SQPIGIDIEK TKPISLEIAK
Sfp       LPDAHFNISH SGRWVIGAFD SQPIGIDIEK TKPISLEIAK
                  **                * * *
                 130        140        150        160
BBK-1     RFFSPTEYSD LQAKHPDQQT DYFYHLWSMK ESFIKQAGKG
Lpa-14    RFFSPTEYSD LQAKHPDQQT DYFYHLWSMK ESFIKQAGKG
Lpa-8     RFFSKTEYSD LLAKDKDEQT DYFYHLWSMK ESFIKQEGKG
Sfp       RFFSKTEYSD LLAKDKDEQT DYFYHLWSMK ESFIKQEGKG
                                           *   *
                 170        180        190        200
BBK-1     LSLPLDSFSV RLKDDGHVSI ELPDGHEPCF IRTYDADEEY
Lpa-14    LSLPLDSFSV RLKDDGHVSI ELPDGHEPCF IRTYDADEEY
Lpa-8     LSLPLDSFSV RLHQDGQVSI ELPDSHSPCY IKTYEVDPGY
Sfp       LSLPLDSFSV RLHQDGQVSI ELPDSHSPCY IKTYEVDPGY

                 210        220        230        240
BBK-1     KLAVCAAHPD FCDGIAMKTY EELL
Lpa-14    KLAVCAAHPD FCDGIEMKTY EELL
Lpa-8     KMAVCAAHPD FPEDITMVSY EELL
Sfp       KMAVCAAHPD FPEDITMVSY EELL
```

Fig. 3. Homology between BBK-1 PPTase of *B. subtilis* BBK-1, with Lpa-14 of iturin A and surfactin co-producing *B. subtilis* RB14, Lpa-8 of plipastatin B1 and surfactin co-producing *B. subtilis* YB8 and Sfp of surfactin producing *B. subtilis* OKB105. *Asterisks* indicate catalytically important and highly conserved amino acid residues

each lipopeptide are consistent with the order of surfactant activity and the elution time in HPLC.

Sandrin et al. (1990) examined the production conditions of surfactin and iturin A in *B. subtilis* S499 and showed that glucose and fructose were effective substrates for the production of both lipopeptides, but sucrose was effective only for surfactin production. In our experiment, glucose was confirmed to be an effective carbon source for bacillomycin L, plipastatin, and surfactin production, suggesting a common regulation system for biosurfactant gene expression. Unlike glucose, sucrose and paraffin were good substrates for the production of bacillomycin L rather than surfactin or plipastatin. This might reflect a different synthetic mechanism of the structural group on bacillomycin L (β-amino type) compared with surfactin and plipastatin (β-hydroxy type). As recently shown, glucose and fructose can stimulate the activation of *comA* and concomitantly increase the expression level of the surfactin synthetase gene, *srfA* (Nakano and Zuber 1989; Kim et al. 2001). Unlike the upstream region of *srfA* and *licA* promoter regions, no ComA box sequence (T/GCGG-N_4-CCGCA; Roggiani and Dubnau 1993) was found in the upstream region of either the mycosubtilin or iturin synthetase gene (Duitman et al. 1999; Tsuge et al. 2001). Therefore, ComA may not be functioning as an activator for lipopeptide synthetase genes of β-amino type.

Sfp (PPTase), encoded by *sfp*, converts the inactive apo-forms of the seven PCP domains of surfactin synthetase complex to the active holo-forms. Huang et al. (1993) have shown that *lpa-14*, a homologue of *sfp* in RB14, is responsible for the production of both surfactin (β-hydroxy type) and iturin (β-amino type). Southern blot analysis in the present work showed that BBK-1 contains only one *sfp* homologue, *lpbk1*, in its genome. Although a gene disruption experiment of *lpbk1* in BBK-1 has not yet been successful, BBK-1 PPTase, which is encoded by *lpbk1*, may function to activate all three lipopeptide synthetase complexes. In fact, the amino acid sequences of BBK-1 PPTase and Lpa-14 share a significantly high similarity, 98% (Fig. 3). Strains BBK-1 and RB14 co-produce β-hydroxy type LPBS/LPAB (surfactin/plipastatin) and β-amino type LPAB (bacillomycin L or iturin A), while OKB105 and YB8 only produce β-hydroxy type LPBS/LPAB (surfactin or surfactin/plipastatin). Structural differences between BBK-1 PPTase or Lpa-14 and Sfp or Lpa-8 should reflect the different structures of β-amino type and β-hydroxy type lipopeptide synthetase complexes.

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