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ORIGINAL ARTICLE

Inhibitory potential of biosurfactants from *Bacillus amyloliquefaciens* derived from mangrove soil against *Vibrio parahaemolyticus*

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Abstract *Vibrio parahaemolyticus* is a halophilic bacterium that causes seafood-borne gastroenteritis which can occur through direct or cross-contamination. In this study, biosurfactant-producing marine bacteria were isolated from 28 soil samples collected from mangrove and coastal regions. Using the cross streak technique, 26 isolates were found to inhibit the growth of *V. parahaemolyticus*. Biosurfactant lipopeptides were obtained by acid precipitation and their antimicrobial potentials were assessed by the agar well diffusion technique. The extract of a bacterial isolate SM11 derived from mangrove soil showed the strongest inhibitory activity against *V. parahaemolyticus*. The inhibition zones against *V. parahaemolyticus* of the extract obtained by chloroform and methanol at concentrations of 900, 1800 and 2600 μg/mL were 16.9 ± 0.2, 18.4 ± 0.5 and 25.0 ± 0.1 mm, respectively. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract were 256 to 512 and 512 to 1024 μg/mL, respectively. The extract of biosurfactants showed more than 60 % reduction in *V. parahaemolyticus* adhesion. 16S rRNA gene sequencing of SM11 revealed that this isolate is *Bacillus amyloliquefaciens*. The active fractions obtained from anion exchange chromatography and HPLC as analysed by ESI-Q-TOF mass spectrometry indicated that those biosurfactants were mycosubtilin, surfactin and iturin A. Besides highlighting the merits of biosurfactants as antagonistic agents, this study suggests the possibility of using them to decrease cross-contamination of *V. parahaemolyticus* on cooking or food processing surfaces.

Keywords Biosurfactants · *Vibrio parahaemolyticus* · *Bacillus amyloliquefaciens* · Mycosubtilin · Surfactin Iturin A

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Introduction

Vibrio parahaemolyticus is a Gram-negative marine bacterium which has been isolated from seawater, shellfish and fish worldwide (Su and Liu 2007). Human pathogenic strains produce major virulence factors, a thermostable direct hemolysin (TDH) and a TDH-related hemolysin (TRH), which are encoded by *tdh* and *trh* genes, respectively. In humans, *V. parahaemolyticus* commonly causes acute gastroenteritis, with diarrhoea, headache, vomiting, nausea and abdominal cramps, after consuming contaminated seafood (Yeung and Boor 2004). *Vibrio parahaemolyticus* isolates carrying *tdh* or *trh* or both genes have been frequently isolated from clinical samples and are rarely detected in marine environments (Vuddhakul et al. 2000). In 1996, the pandemic O3:K6 clone of *V. parahaemolyticus* appeared in Bangladesh. The bacteria from this original clone have spread worldwide, including the United States (Okuda et al. 1997),

Europe and many countries in Asia (Matsumoto et al. 2000). Cross-contamination during food preparation has also been reported to be a cause for *V. parahaemolyticus* infections. An investigation of the outbreaks of *V. parahaemolyticus* in Maryland, USA, has shown that steamed crabs were contaminated with *V. parahaemolyticus* during transportation and storage with live crabs (Dadisman et al. 1972). In Chinese markets, the investigation of six species of crustaceans demonstrated *V. parahaemolyticus* in 22 out of 45 samples. Interestingly, this bacterium was also detected in mitten crabs, which are supposed to be produced in freshwater ponds. Confirmation by examination of six mitten crabs obtained from two freshwater ponds revealed that none of the isolates was *V. parahaemolyticus*, suggesting that cross-contamination occurred among crustaceans sold at the markets (Yano et al. 2006). In Thailand, acute gastroenteritis was reported in a group of nursing students with watery diarrhoea (90.8 %) and abdominal cramps (71.3 %). Around 50 % of the isolates obtained from the rectal swabs indicated *V. parahaemolyticus* with serotype O4:K55, and all of them possessed the *tdh* gene. Retrospective analysis revealed that the implicated food risk factor for the gastroenteritis was boiled eggs and cross-contamination could occur through utensils used for shelling (Jatapai et al. 2010).

Though various techniques such as high pressure, thermal and depuration processing have been demonstrated to reduce *V. parahaemolyticus* contamination in seafood (Shen et al. 2009; Su et al. 2010; Ma and Su 2011), their utility to large surfaces such as work places, benches etc. in food processing industries and household utensils is limited. Hence, an antimicrobial agent with potential action against *V. parahaemolyticus* will be of considerable use in such cases. Biosurfactants are surface-active compounds that are usually extracellularly released from bacteria, yeast or fungi. They are capable of reducing surface and interfacial tension and have a wide range of industrial and environmental applications (Mukherjee and Das 2010). The present study intends to identify biosurfactants effective against *V. parahaemolyticus* to control microbial colonisation over inanimate surfaces.

Materials and methods

Bacterial strains used in this study

Three clinical and five environmental isolates of *V. parahaemolyticus* were obtained from the bacterial stock culture in the Department of Microbiology, Faculty of Science, Prince of Songkla University, Hat Yai, Thailand. A clinical isolate of *V. parahaemolyticus* PSU 4118 was used as a tested strain throughout the study, unless other conditions were indicated.

Bacterial isolation and screening for antimicrobial activity against *V. parahaemolyticus*

A total of 28 soil samples were collected from various mangrove and other coastal regions in southern Thailand. They were vigorously mixed and diluted in normal saline solution before spreading on trypticase soy agar (TSA) supplemented with 1 % NaCl. After overnight incubation at room temperature, bacteria were selected according to their colonial characteristics. Their inhibitory activity against a clinical *V. parahaemolyticus* isolate (PSU 4118) was evaluated by the cross streak technique using TSA containing 1 % NaCl (Lertcanawanichakul and Sawangnop 2011). After incubating the plates at room temperature for 24 h, the isolates able to inhibit *V. parahaemolyticus* were selected and stored at −80 °C.

Extraction and purification of lipopeptide biosurfactants

Lipopeptide biosurfactants were extracted by the acid precipitation technique with slight modification (Rivardo et al. 2009). Briefly, to support the growth of bacteria as well as to promote biosurfactants production, a 2-mL volume (containing 10^8 cfu/mL) of bacterial isolate was cultured in 100 mL of modified synthetic seawater medium (MSSW, prepared by adding casein peptone, yeast extract and palm oil) (Amano et al. 1982; Button et al. 1993). The culture was incubated at room temperature for 48 h and the lipopeptides were obtained by precipitation of the supernatant with 6 N HCl at 4 °C for 6 h. The crude extract was further precipitated by mixing it with 2:1 (v/v) chloroform and methanol, and this step was repeated thrice, followed by drying in a rotary vacuum evaporator R-124 (BÜCHI, Bern, Switzerland) (Kim et al. 2010). The yellow-brown sediment was dissolved in sterile distilled water with pH 8 and filtered through a 0.45-μM filter (Corning, NY, USA). The filtrate obtained was loaded into the DEAE-cellulose anion exchange column (Sigma Chemical Co., St. Louis, MO, USA) saturated with 20 mM Tris HCl at pH 8 and eluted stepwise with the same buffer supplemented with NaCl from 200 mM to 1000 mM. The flow rate was kept constant at 0.7 mL/min at room temperature. Each fraction was pooled and analysed using an UV-Vis spectrophotometer at 210 nm (Das et al. 2008). In addition, it was evaluated for inhibitory activity against *V. parahaemolyticus* using the agar well diffusion assay.

The active fractions were further purified by reverse-phase HPLC (Supelcosil™ LC-18, Sigma) using 0.1 % trifluoroacetic acid (A) and acetonitrile (B) as mobile phase at a flow rate of 0.5 mL/min. The gradient elution (A/B) was

done as follows: 90:10 for 0–5 min, 40:60 for 6–15 min, 0:100 for 16–45 min and final elution at 40:60 for 46–50 min. Each fraction was collected at different retention times and assayed for inhibitory activity against *V. parahaemolyticus*. The active fraction was evaporated and subjected to electrospray ionisation quadrupole time-of-flight mass spectrometry (ESI-Q-TOF MS) to detect the molecular mass of the compounds (Bruker Daltonics, Billerica, MA, USA).

Agar well diffusion assay

The extract obtained from acid precipitation was tested for inhibitory activity against *V. parahaemolyticus* by the agar well diffusion technique (Vuddhakul et al. 2007). The crude extract was dissolved in distilled water and its pH was adjusted to 7.5 before assay.

Surface tension measurement

The acid precipitation extract was dissolved in distilled water and neutralised with NaOH. The surface tension was determined via the ring method using a du Noüy tensiometer (K6; Kruss, Hamburg, Germany) (Cooper and Goldenberg 1987).

Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

In order to determine the lowest concentration able to inhibit the growth of *V. parahaemolyticus* (MIC) or to kill it (MBC), biosurfactants obtained from chloroform:methanol extraction were subjected to MIC and MBC evaluation with three clinical and five environmental *V. parahaemolyticus* isolates. Briefly, the biosurfactants were diluted two-fold in Mueller Hinton broth supplemented with 1 % NaCl, followed by inoculation with *V. parahaemolyticus* (2.5×10^5 cfu/mL). The MIC was determined after incubation at 37 °C for 24 h. The highest dilution tube that showed no growth was regarded as the MIC and the highest dilution tube that exhibited no growth after culturing on Mueller Hinton agar supplemented with 1 % NaCl was considered as the MBC.

Anti-adhesive potential of biosurfactants against *V. parahaemolyticus*

The inhibition of *V. parahaemolyticus* adhesion to inanimate surface was tested in a 96-well flat bottom plate (Janek et al. 2012). Briefly, the biosurfactants were dissolved in PBS pH 7.4 and filtered through a 0.45-μM membrane filter. Then, the wells were coated with a 200-μL volume of the biosurfactants at concentrations ranging from 500 to 1000 μg/mL and the plate was incubated at room temperature for 4–20 h. After removing the residual biosurfactants, *V. parahaemolyticus* (1.0×10^7 cfu/mL in PBS) was added to each well and the plate was placed on a rotary shaker at 300 rpm for 2 h at room temperature. Non-adherent cells were removed by washing thrice with PBS and the wells were stained with 0.1 % crystal-violet for 30 min and washed thrice with PBS to remove excess stain. The wells were then destained with 150 μL of isopropanol-0.04 N HCl and 50 μL of 0.25 % SDS. The optical density of each well was determined with a spectrophotometer at 590 nm. All experiments were done in triplicate. The adhesion inhibition was calculated as:

$$\text{Adhesion inhibition } (\%) = [1 - (\text{ODT}/\text{ODC})] \times 100$$

where:

ODT Optical density of well with *V. parahaemolyticus* and biosurfactants

ODC Optical density of well with *V. parahaemolyticus* and PBS

Identification of biosurfactant-producing bacterium

The biosurfactant-producing bacterium was identified by the standard method described in Bergey's Manual of Determinative Bacteriology (Holt et al. 1994) and was confirmed by the 16S rRNA gene sequencing. The bacterium was cultured in Luria-Bertani broth (Difco) and genomic DNA was extracted using the Gentra Puregene extraction kit (QIAGEN, Germany). The 16S rRNA gene was amplified using the universal primers 27F and 1492R (Lane 1991) in a thermal cycler (Perkin Elmer GeneAmp 2400). The amplified products were sequenced in an ABI 377 genetic analyser (Applied Biosystems) and the sequences obtained were searched against the public database using BLAST. A homology of >99 % identity was the criterion used to identify the bacterium to the species level.

Statistical analysis

One-way ANOVA (Duncan's multiple-range test) using SPSS software was performed for the statistical analysis.

Results and discussion

Twelve and 16 soil samples were collected from mangrove and coastal areas, respectively. A total of 462 bacterial isolates were obtained from TSA. Using the cross streak technique, 11 and 15 isolates (5.6 % of the total isolates) from mangrove and coastal soils, respectively, exhibited antimicrobial activity against *V. parahaemolyticus*.

Crude extracts derived from acid precipitation of seven bacterial isolates affected *V. parahaemolyticus* with inhibition

Table 1 Inhibitory activity against *Vibrio parahaemolyticus* PSU 4118 and surface tension of crude extracts obtained from soil bacteria

Crude extracts from strain	Sources	Inhibition zone (mm)	Surface tension (mN/m)*
S15	Coastal area	12.4 ± 0.2	38.3 ± 0.1
YS24	Mangrove	11.8 ± 0.1	38.0 ± 0.0
KS43	Coastal area	12.4 ± 0.1	36.1 ± 0.1
KS21	Mangrove	13.3 ± 0.1	35.6 ± 0.1
SM6	Mangrove	12.1 ± 0.1	34.6 ± 0.0
KS33	Coastal area	14.3 ± 0.1	34.3 ± 0.1
SM11	Mangrove	13.2 ± 0.1	33.2 ± 0.0**

The agar well diffusion assay was performed with 50 μL of the crude extracts. Each experiment was done in triplicate

*Initial surface tension of medium = 45.2 ± 0.2 mN/m

**Mean ± SD significantly different from other values ($p \leq 0.05$)

zones between 11.8 and 14.3 mm (Table 1). Potential biosurfactants have surface tension properties ranging from 27 to 35 mN/m (Lee et al. 2002). The surface tension of the crude extract obtained from those isolates was between 33.2 and 38.3 mN/m (Table 1). The crude extract of an SM11 bacterial isolate exhibited significantly the lowest surface tension property and this bacterium was selected for further studies. The SM11 bacterium was obtained from mangrove soil, which has been reported as a rich microorganism source, including a potential source of biosurfactant-producing bacteria (Saimmai et al. 2012; Thatoi et al. 2013). The chloroform:methanol extract of SM11 at concentrations of 900, 1800 and 2600 μg/mL produced inhibition zones against *V. parahaemolyticus* with diameters of 16.9 ± 0.2, 18.4 ± 0.5 and 25.0 ± 0.1 mm, respectively. The MIC and MBC were evaluated against three clinical and five environmental *V. parahaemolyticus* strains with *Escherichia coli* ATCC 25922 as a control (Table 2). The MIC of the extract was between 256 and 512 μg/mL, whereas the MBC was between 512 and 1024 μg/mL. No significant differences were observed in the MIC and MBC between the clinical and environmental isolates.

In order to determine the effectiveness of biosurfactants to decrease *V. parahaemolyticus* on surface areas, the anti-adhesive property of the extract was evaluated. The wells coated with biosurfactants at a concentration of 500 μg/mL inhibited more than 60 % of *V. parahaemolyticus* adhesion after 4 h of incubation (Fig. 1), whereas incubation for 20 h showed no significant difference (data not shown). This might be due to the interaction between bacterium and biosurfactants achieving equilibrium in 4 h and no further activity being observed at longer incubation. In addition, there was no significant difference in the anti-adhesive property even after increasing the concentrations of biosurfactants to 750 and 1000 μg/mL.

The SM11 isolate was identified as *Bacillus* sp. by biochemical tests. Further confirmation by 16S rRNA gene sequencing revealed its identity as *Bacillus amyloliquefaciens*. *Bacillus* spp. have been reported to produce many kinds of biosurfactants (Vater et al. 2002; Kim et al. 2010) for

Table 2 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the chloroform and methanol extract of the SM11 isolate against *V. parahaemolyticus*

V. parahaemolyticus	Sources	Serotypes	Virulence factors		MIC (μg/mL)	MBC (μg/mL)
			tdh	*trh*		
PSU 4118	C	O1:K25	+	−	256	512
PSU 4211	C	O3:K6	+	−	256	512
PSU 2598	C	O4:K68	+	−	256	512
PSU 5313	E	O1:KUT	−	−	256	512
PSU 4413	E	O1:KUT	+	+	512	1024
PSU 5321	E	O2:KUT	−	−	256	512
PSU 4886	E	O3:K6	+	−	512	1024
PSU 5310	E	O8:KUT	−	−	256	512

C clinical strain; *E* environmental strain

For the control, the MIC and MBC to tetracycline of *E. coli* ATCC 25922 were 32 μg/mL and 128 μg/mL, respectively

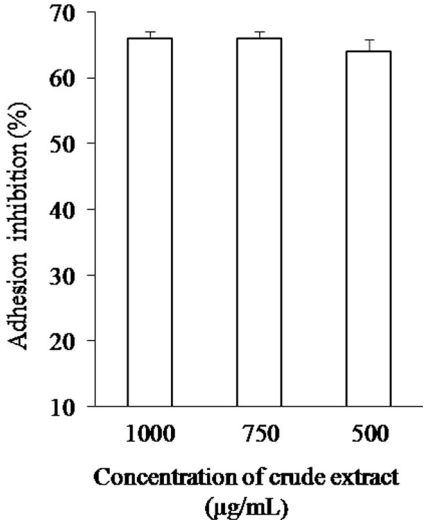

Fig. 1 Anti-adhesive properties of biosurfactants from *Bacillus amyloliquefaciens* against *Vibrio parahaemolyticus*

increasing of the surface area of water-insoluble substrates, heavy metal binding, bacterial pathogenesis and biofilm formation (Mulligan 2005; Ron and Rosenberg 2001). Those are important for their development and survival in natural environments.

Microbial surfactants have been characterised as glycolipids, lipopeptides, fatty acids, neutral lipids, phospholipids, polymeric and particulate biosurfactants (Vater et al. 2002; Padmavathi and Pandian 2014). To characterise biosurfactants obtained in this work, the extract collected from chloroform:methanol precipitation was purified by DEAE-cellulose ion exchange chromatography. After elution, six different fractions were obtained, in which one fraction exhibited strong antimicrobial activity against *V. parahaemolyticus*. The active fraction was subjected to further purification by reverse-phase HPLC, in which five fractions (designated as PSM1–PSM5) were obtained (Fig. 2). Though all the five

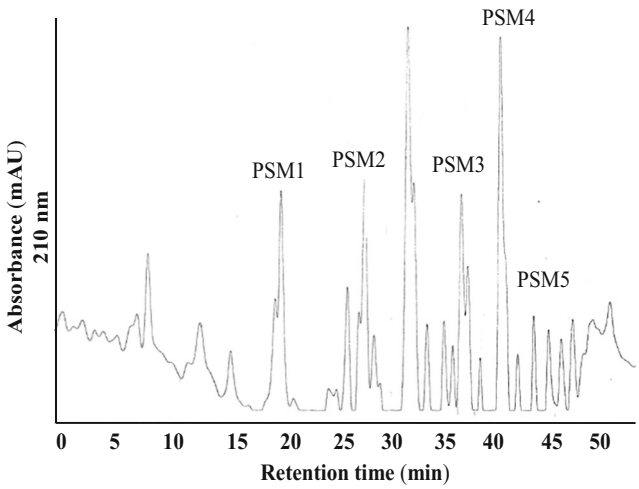

Fig. 2 Active fractions (PSM1–PSM5) of biosurfactants detected by reverse-phase HPLC

fractions exhibited inhibitory activity against *V. parahaemolyticus* with diameters of clear zones between 20.1 and 32.9 mm, the PSM4 fraction exhibited the maximum activity (Table 3). ESI-Q-TOF MS analysis of PSM1 and PSM2 showed a cluster of molecular mass (*m*/*z*) between 1079.5 and 1107.5. Based on the available literature and mass spectral database analysis, the peaks were identified as mycosubtilin lipopeptides (Leclère et al. 2005). PSM3 showed a cluster of molecular mass between 1002.5 and 1088.5, whereas PSM4 showed an *m*/*z* of 1044.6. The mass spectral analysis indicated that these peaks corresponded to lipopeptides and belonged to surfactins (Vater et al. 2002; Hofemeister et al. 2004; Pathak and Keharia 2013). PSM5 showed major peaks of surfactin and iturin A, with molecular masses of 1102.6 and 1120.6, respectively (Hofemeister et al. 2004; Koumoutsi et al. 2004).

Mycosubtilin and iturin A are biosurfactants in the iturin family, which is well known for strong antifungal activity (Maget-Dana and Peypoux 1994; Leclère et al. 2005). However, the antimicrobial activity of the iturin family against bacteria is limited (Ongena and Jacques 2008). The effect of iturin A on *B. subtilis* surface hydrophobicity has been documented (Ahimou et al. 2000). In addition, iturin A efficiently lyses the protoplasts of *Micrococcus luteus* and forms channels in artificial lipid membranes (Maget-Dana and Peypoux 1994). In this study, the antimicrobial activity of iturin A and surfactins (PSM5) against *V. parahaemolyticus* was higher than those observed from mycosubtilins (PSM1 and PSM2) (Table 3).

PSM3 contained short chain and long chain of six isoforms of surfactins (C11–C16) and the intensity of the peaks was lower than the PSM4 surfactin (C14). This correlated with its inhibitory activity against *V. parahaemolyticus* because PSM3 showed an inhibition zone of 24.9 ± 0.1 mm, whereas the inhibitory activity of PSM4 was 32.9 ± 0.1 mm (Table 3). The surfactin isoforms which comprise both short and long carbon chains may influence antimicrobial activity, as it has been demonstrated that long carbon chain surfactin analogs increase antimicrobial activity more than short chain analogs

Table 3 Antimicrobial activity against *V. parahaemolyticus* of fractions PSM1–PSM5 obtained from reversed-phase HPLC

Fractions	Retention time (min)	Inhibition zone (mm)
PSM1	19.9	24.7 ± 0.1*
PSM2	27.1	20.1 ± 0.3**
PSM3	37.3	24.9 ± 0.1*
PSM4	42.2	32.9 ± 0.1***
PSM5	44.1	29.9 ± 0.3

Different symbols show values that are significantly different ($p < 0.05$)

(Vollenbroich et al. 1997a; Dufour et al. 2005). In addition, the C14–C15 carbon atoms of surfactins show higher antiviral activity than the C13 isoform. Therefore, the hydrophobic character of the long chain carbon atom may be the key to its antimicrobial activity.

Surfactin is a cyclic lipo-heptapeptide which contains a hydroxyl fatty acid. It decreases surface tension and increases surface area for anti-adhesion activity to prevent biofilm formation of human bacterial pathogens (Rivardo et al. 2009). Surfactin can also induce the formation of ion channels in a lipid bilayer membrane and it has been reported to inactivate both herpes and retroviruses (Vollenbroich et al. 1997b; Deleu et al. 2003). In addition, it has been demonstrated to prevent viruses and mycoplasma in biotechnological products (Vollenbroich et al. 1997a, b).

In this study, the crude extract of biosurfactants at a concentration of 500 μg/mL could significantly inhibit the adhesion of *V. parahaemolyticus* by more than 60 % (Fig. 1). Thus, the crude extract may be more useful than the purified form in treating inanimate surfaces. A previous study has reported that most biosurfactants are not toxic to plants and animals and do not irritate human skin (Cameotra and Makkar 2004), suggesting that biosurfactants obtained in this study may be applied in food processing industries, such as cooking surfaces, packing benches and surfaces of food containers, to reduce cross-contamination of *V. parahaemolyticus*. In addition, they may be applied to aquaculture industries to decrease *V. parahaemolyticus* contamination in seafood.

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