A novel bacteriocin-like substance (BLIS) from a pathogenic strain of *Vibrio harveyi*

Sathish Prasad, Peter C. Morris, Rasmus Hansen, Philip G. Meaden and Brian Austin

School of Life Sciences, John Muir Building, Heriot-Watt University, Riccarton, Edinburgh EH14 4AS, UK

Inter-strain and inter-species inhibition mediated by a bacteriocin-like inhibitory substance (BLIS) from a pathogenic *Vibrio harveyi* strain VIB 571 was demonstrated against four isolates of the same species, and one culture each of a *Vibrio* sp., *Vibrio fischeri*, *Vibrio gazogenes* and *Vibrio parahaemolyticus*. The crude BLIS, which was obtained by ammonium-sulphate precipitation of the cell-free supernatant of a 72 h broth culture of strain VIB 571, was inactivated by lipase, proteinase K, pepsin, trypsin, pronase E, SDS and incubation at ≥60 °C for 10 min. The activity was stable between pH 2–11 for at least 5 h.

Anion-exchange chromatography, gel filtration, SDS-PAGE and two-dimensional gel electrophoresis revealed the presence of a single major peak, comprising a protein with a pl of ~5.4 and a molecular mass of ~32 kDa. The N-terminal amino acid sequence of the protein comprised Asp-Glu-Tyr-Ile-Ser-X-Asn-Lys-X-Ser-Ser-Ala-Asp-Ile (with X representing cysteine or modified amino acid residues). A similarity search based on the matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS) generated peptide masses and the N-terminal sequence did not yield any significant matches.

**INTRODUCTION**

Bacteriocins are heterogeneous antagonists of a proteinaceous nature that are produced by bacteria, and demonstrate inhibitory and/or bactericidal activity against closely related strains (Reeves, 1965, 1972; Tagg *et al.*, 1976; Klaenhammer, 1988). Several uncharacterized substances with bacteriocin-like activity have been identified and are referred to as bacteriocin-like inhibitory substances (BLIS/BLS) (Tagg, 1992; Dav & Falkiner, 1996). Bacteriocin-mediated antagonism was first reported between *Escherichia coli* V (E. coli CA7) and E. coli Ø (E. coli CA81) by Gratia (1925), for which the bactericidal factor, colicin V, was the first bacteriocin to be described (Reeves, 1972).

At present, a large number of bacteriocins have been reported from many Gram-negative and Gram-positive bacterial taxa. Most of the well-characterized bacteriocins reported from Gram-negative bacteria are from *E. coli*, and are referred to as colicins. A small number of bacteriocins have been identified in other Gram-negative organisms, for example, pyocins in *Pseudomonas* sp. (Jacob, 1954), vibriocin in ‘*Vibrio comma*’ (*Vibrio cholerae*; Himsley & Sey Fried, 1962; Jayawardene & Himsley, 1969), alveicins in *Hafnia alvei* (Hamon & Peron, 1963), klebicins or pneumocins in *Klebsiella pneumoniae* (Reeves, 1972; Chhibber & Vadehra, 1986), enterocticin in *Yersinia enterocolitica* (Strauch *et al.*, 2001), BC1 and BC2 in *Vibrio vulnificus*, IW1 in *V. cholerae* (Shehane & Sizemore, 2002), and BLS in *Aeromonas hydrophila* (Messi *et al.*, 2003). The colicins serve as the model for other Gram-negative bacteriocins. They share certain common characteristics, such as being plasmid encoded (Riley & Wertz, 2002) and their release usually involving the lysis of the producer, although there are exceptions for which the mechanism of lysis is unclear (Cursino *et al.*, 2002). Furthermore, the production of most colicins is mediated by the SOS regulon, and can be artificially induced by DNA damaging agents, such as mitomycin C and UV (Herschman & Helsinki, 1967; Zhang *et al.*, 1985; Riley & Wertz, 2002). Apart from being large in size, not all Gram-negative bacteriocins share all of the attributes of colicins, for example, the pyocins of *Pseudomonas aeruginosa* are chromosomally encoded (Riley & Wertz, 2002) and the bacteriocin from *Myxococcus coralloides* D is not inducible by mitomycin C or UV (Munoz *et al.*, 1984).

*Vibrio harveyi* is a Gram-negative organism with quorum-sensing-dependent bioluminescence (Nealson & Hastings, 1979; Miller & Bassler, 2001), which is found in tropical-marine and brackish-water environments as a member of...
the bacterioplankton, or in association with other aquatic fauna (O'Brien & Sizemore, 1979). The organism is a serious pathogen of many vertebrate and invertebrate marine animals (Lavilla-Pitogo et al., 1990; Karunasagar et al., 1994; Zhang & Austin, 2000; Alcaide et al., 2001). The first and only reference to a bacteriocin or BLIS in *V. harveyi* was by McCall & Sizemore (1979), who reported the production of a bacteriocin in a strain of *Beneckea harveyi* SY (*V. harveyi*). The bacteriocin, ‘*harveyicin SY*’, with an estimated molecular mass of 24 kDa, was lethal to two strains of *V. harveyi*, KN96 and BBP8. Furthermore, the susceptibility of *harveyicin SY* to proteolytic enzymes, and its apparent plasmid association (Hoyt & Sizemore, 1982), makes it the only definitive bacteriocin to be reported from *V. harveyi* to date.

Recently, whilst screening various *V. harveyi* isolates from our culture collection we recognized a possible BLIS production by one strain of *V. harveyi* (VIB 571). Interestingly, this strain has been demonstrated to be pathogenic to rainbow trout (*Oncorhyncus mykiss*) and Atlantic salmon (*Salmo salar*) by Zhang & Austin (2000). This present study was carried out to examine the phenomenon of BLIS production by *V. harveyi* VIB 571.

### METHODS

**Bacterial cultures.** The strains used in this study were the BLIS producing strain *V. harveyi* VIB 571, 15 other *V. harveyi* strains, 35 different *Vibrio* spp, 2 strains of methicillin-resistant *Staphylococcus aureus* (MRSA) and 2 strains of vancomycin-resistant enterococci (VRE), obtained from the culture collection of the School of Life Sciences, Heriot-Watt University. These bacterial isolates are listed in the Supplementary Table available with the online journal. All bacterial cultures were verified for authenticity according to the original descriptions. Of these cultures, the vibrios were grown on tryptone soya agar (TSA; Oxoid) supplemented with 1% (w/v) NaCl (TNA), and/or tryptone soya broth (TSB; Oxoid) supplemented with 1% (w/v) NaCl (TNB), with incubation overnight at 28°C.

**Detection of BLIS activity.** A modified double-layer plate assay (DLPA) was used (Tagg et al., 1976) in which the producer culture, *V. harveyi* VIB 571, and the indicator culture, *V. harveyi* VIB 295, were mixed in soft agar before plating. The producer and indicator strains were inoculated separately into 10 ml TNB, with incubation at 28°C overnight. Soft agar, i.e. TNA supplemented with 0.7% (w/v) agar, was sterilized at 121°C for 15 min and cooled in a water bath to 42°C. The producer culture was then diluted tenfold in 0.9% (w/v) saline, and 100 μl of each dilution was added to 100 μl of the indicator culture in sterile test tubes containing 3.8 ml soft agar. The resulting suspension was vortexed briefly, and poured rapidly over previously prepared TNA plates. Incubation was overnight at 28°C, after which the plates were examined for zones of inhibition.

A second method involved a spot-on-lawn assay (Tagg et al., 1976), which was used for determining the inhibitory spectrum of *V. harveyi* VIB 571 against a range of bacterial cultures. For this, the indicator cultures were spread evenly over separate TNA plates and incubated for 10 min at 28°C, after which 10–20 μl of *V. harveyi* VIB 571 was spotted over the lawns, with incubation for 12–24 h at 28°C. The plates were then examined for zones of inhibition around the VIB 571 producer culture.

The flip-streak method of Kekessey & Piquet (1970) was used to distinguish inhibition by BLIS from inhibition by bacteriophage induction, as suggested by De Vuyst & Vandamme (1994). An overnight culture of strain VIB 571 was streaked along the middle of a fresh TNA plate of 2–3 mm in thickness. The plates were incubated for 3 days at 28°C, after which the agar was flipped over using a flamed forceps, and an overnight TNB culture of the *V. harveyi* VIB 295 indicator strain was swabbed over the reverse side of the agar before incubation overnight. Evidence of inhibition of the indicator strain was recorded.

**Partial purification of crude BLIS.** The crude BLIS was partially purified using a modified method of Hoyt & Sizemore (1982). For this, 10 ml of an overnight TNB culture of *V. harveyi* VIB 571 (~10^8 cells ml^-1; as determined with a haemocytometer (Improved Neubauer type; Weber)) was inoculated into 990 ml TNB and incubated at 28°C for 72 h on an orbital shaker at 80 r.p.m. Then, the culture was centrifuged at 5000 r.p.m. for 30 min at 4°C, and the supernatant was pooled and filtered through a 0.45 μm nitrocellulose filter (Sartstedt). The cell-free supernatant (CFS) was precipitated using ammonium sulphate (BDH) to 60% saturation (390 g l^-1), with slow and continuous stirring on a magnetic stirrer at 4°C for 12–16 h. The precipitate was collected by centrifugation at 12000 r.p.m. for 10 min at 4°C, and dissolved in 25 ml PBS (pH 7.4). The crude extract was then dialysed against PBS in Visking dialysis tubing (Medicell International, 12000–14000 MWCO), with two changes of buffer running one day each at 4°C over a magnetic stirrer. Sterile (121°C/15 min) 10% (v/v) glycerol was added to the dialysed crude extract before transfer to sterile Petri dishes. The crude extract was frozen at −40°C and lyophilized in a freeze dryer (Edwards-Super Modulyo) at 5 mbar for 18 h. The freeze-dried sample was reconstituted with 10 ml sterile Milli Q water, and dialysed against PBS (pH 7.4). The crude extract was dialysed against PBS in Visking dialysis tubing (Medicell International, 12000–14000 MWCO), with two changes of buffer running one day each at 4°C over a magnetic stirrer. Sterile (121°C/15 min) 10% (v/v) glycerol was added to the dialysed crude extract before transfer to sterile Petri dishes. The crude extract was frozen at −40°C and lyophilized in a freeze dryer (Edwards-Super Modulyo) at 5 mbar for 18 h. The freeze-dried sample was reconstituted with 10 ml sterile Milli Q water, and dialysed against PBS (pH 7.4). The crude extract was dialysed against PBS in Visking dialysis tubing (Medicell International, 12000–14000 MWCO), with two changes of buffer running one day each at 4°C over a magnetic stirrer. Sterile (121°C/15 min) 10% (v/v) glycerol was added to the dialysed crude extract before transfer to sterile Petri dishes. The crude extract was frozen at −40°C and lyophilized in a freeze dryer (Edwards-Super Modulyo) at 5 mbar for 18 h. The freeze-dried sample was reconstituted with 10 ml sterile Milli Q water, and dialysed against PBS (pH 7.4). The crude extract was dialysed against PBS in Visking dialysis tubing (Medicell International, 12000–14000 MWCO), with two changes of buffer running one day each at 4°C over a magnetic stirrer. Sterile (121°C/15 min) 10% (v/v) glycerol was added to the dialysed crude extract before transfer to sterile Petri dishes. The crude extract was frozen at −40°C and lyophilized in a freeze dryer (Edwards-Super Modulyo) at 5 mbar for 18 h. The freeze-dried sample was reconstituted with 10 ml sterile Milli Q water, and dialysed against PBS (pH 7.4).

**Semi-quantitative and qualitative bioassay of inhibitory activity.** The inhibitory activity of the CFS and the crude BLIS was quantified using a modification of the critical-dilution method (Yamamoto et al., 2003). Briefly, 10 μl of twofold dilutions of the CFS or crude BLIS in sterile PBS (pH 7.4) were spotted onto freshly inoculated lawns of *V. harveyi* VIB 295, with incubation at 28°C for 12–24 h. The titre was defined as the reciprocal of the highest dilution showing inhibition, and the activity calculated using the formula: 1 AU ml^-1=2^n ×(1000 μl/10 μl), where AU ml^-1 is the arbitrary unit ml^-1, and n is the reciprocal of the highest dilution showing inhibition. For all qualitative screening, a rapid bioassay was used in which a loopful of the indicator culture from 24 h cultures on TNA was dispersed evenly with a sterile inoculation loop in 10 ml TNB, with vortexing to achieve a dense suspension. The indicator suspension was incubated for 30 min at 28°C on an orbital shaker at 200 r.p.m., and then swabbed over a two-day old TNA plate. This was allowed to air dry, after which 10 μl CFS or crude BLIS was spotted onto the lawn before incubation at 28°C for 5–6 h. The presence or absence of inhibition was recorded.

**Effect of physicochemical factors on the activity of BLIS.** The effect of proteolytic enzymes on the activity of the crude BLIS was determined by treating 100 μl of the crude preparation with proteinase K (Qiagen), trypsin (Sigma), pronase E (Boehringer Mannheim, from Streptomyces griseus) and pepsin (Sigma). The enzymes in their
respective buffers were added at a final concentration of 1 mg ml\(^{-1}\), and incubated at their optimum temperatures for 30 min as recommended by the manufacturer.

Lysozyme (Sigma) in 66 mM PBS (pH 6-2) was added at a final concentration of 1 mg ml\(^{-1}\) to crude BLIS (pH adjusted to 6-6-7-0). The samples were briefly vortexed, centrifuged, and then incubated at 28°C for 30 min. Lipase (Sigma) in 80 mM Tris/HCl buffer (pH 8-0) was added to the crude BLIS (pH adjusted to 8-0 with 1 M NaOH) at a final concentration of 1 mg ml\(^{-1}\), and then incubated at 40°C on a dry block for 30 min. α-Amylase (Sigma), in 66 mM potassium phosphate buffer (pH 6-2), was added to the crude BLIS (pH adjusted to 6-0 with 1 M HCl) at 1 mg ml\(^{-1}\) final concentration, and incubated at 28°C for 30 min. Two controls, one with sterile TNB and the respective enzyme (1 mg ml\(^{-1}\)), and the second with untreated crude BLIS, were also included. The residual activity after enzyme treatment was determined as described previously.

The effect of detergents on activity was determined using 100 µl of the crude BLIS with Tween 20, 40, 60 and 80, Triton X-100 (Sigma), NP-40 (BDH) at 1 % (v/v), and SDS and sodium lauryl sarcosine (SLS) at 1 % (w/v) final concentration. Incubation was at 28°C for 30 min when the residual activity was determined qualitatively.

The effect of a serine-protease and metalloprotease inhibitor on activity was determined by treating 100 µl crude BLIS with PMSF (Sigma) or EDTA (Sigma), respectively, at a final concentration of 1 % (w/v). Two controls, one containing TNB and the protease inhibitor, and the other with crude BLIS alone, were included. Incubation was at 28°C for 30 min, after which the residual activity was determined by the rapid qualitative bioassay.

The thermal stability of the crude BLIS was determined by incubating 100 µl volumes at 40, 60, 70, 80, 90 and 100°C, and the residual inhibitory activity was determined by the qualitative bioassay after incubation at these temperatures for 5, 10, 15, 20, 30, 40, 50 and 60 min.

The effect of pH 2-0, 3-0, 5-0, 9-0, 11-0 and 14-0 on stability was examined after incubation for 5 h at 28°C, after which the pH was readjusted to ~7-4 and the residual inhibitory activity determined by the qualitative bioassay.

**Effect of mitomycin C on BLIS induction.** A study of the effect of mitomycin C on the induction of BLIS was carried out as described by Hardy & Meynell (1972), with minor modifications. Briefly, 100 µl strain VIB 571 (~10\(^{10}\) cells ml\(^{-1}\)) was inoculated in two conical flasks containing 100 ml sterile TBN, and then incubated at 28°C on an orbital shaker at 80 r.p.m. for 12-16 h. After incubation, mitomycin C (Sigma) was added at a final concentration of 1 µg ml\(^{-1}\) was added to one of the flasks and incubated for a further 5 h along with the control flask (no mitomycin C). Following incubation, the cultures were centrifuged at 10 000 r.p.m. at 4°C for 20 min and the supernatants filtered separately through a 0-22 µm syringe filter (Millipore Millex GP PES, 33 mm). Ten millilitres of the filtrates were then concentrated to 1 ml using centrifugal filtration devices (Amicon Ultra-15; 10 000 MWCO) at 5000 r.p.m. for 20-25 min at 4°C. The concentrates thus obtained were precipitated with three volumes of ice-cold acetone, and incubated overnight at −20°C. The protein pellets obtained after centrifugation at 13 000 r.p.m. for 30 min at 4°C was subjected to SDS-PAGE using the system of Laemmli (1970), as described by Gallagher (1996). The gel was stained for protein (Garfin, 1990), and the molecular mass of the protein band(s) was determined using Image Master TotalLab v.2.01 software (Amersham Pharmacia Biotech).

**Two-dimensional gel electrophoresis (2-DE) and MALDI-TOF-MS analysis.** The most active fractions from 10 successive gel filtration runs were pooled and concentrated to 1 ml by ultrafiltration (Amicon Ultra-15; 10 000 MWCO), involving centrifugation at 5000 r.p.m. for 20–25 min at 4°C, and reconstitution in sterile MilliQ water. Following this, the BLIS was precipitated, as before, in acetone, the pellet dissolved in 125 µl rehydration buffer [8 M urea, 2% (w/v) CHAPS, 0-002% (w/v) bromophenol blue, 0-5% IPG (immobilized pH gradient) buffer, 30 mM DTT], and subjected to first dimension IEF on a 7 cm Immobiline DryStrip pH 3–10 NL gel (Amersham Biosciences). Electrophoresis in the second dimension was on a 10% polyacrylamide mini gel, after which staining was with colloidal Coomassie brilliant blue (Neuhoff et al., 1988). The protein spot was picked, and the gel plug was washed twice in Milli Q water and destained by 3 washes for 20 min with 100 µl of 50 mM ammonium bicarbonate in 50% (v/v) acetonitrile. The gel plugs were dehydrated in 70% (v/v) acetonitrile for 20 min, following which acetonitrile was aspirated and the gel plug dried at room temperature. Ten microlitres trypsin (20 ng µl\(^{-1}\); Trypsin Gold; Promega) in 25 mM ammonium bicarbonate was added to the gel plug, which was then incubated for 5 h at 37°C. One microlitre of cyano-4-hydroxycinnamic acid (CHCA) matrix (10 mg ml\(^{-1}\) in 0-5% (v/v) trifluoroacetic acid, 50% (v/v) acetonitrile) was mixed with 1 µl trypsin-digested sample, and spotted on to the sample slide and analysed using an Ettan MALDI-TOF-MS Pro analyser (Amersham Pharmacia Biotech).

**FPLC anion-exchange chromatography.** The active fractions obtained from 10 gel filtration runs were pooled and concentrated by ultrafiltration to 1 ml. The concentrate was reconstituted with 5 µl 20 mM PIPES (pH 6-0) buffer, and centrifuged again to change the buffer. After three such changes, 200 µl of the concentrate was applied to a FPLC-MonoQ anion-exchange column and eluted with a 0–1 M NaCl gradient in 20 mM PIPES pH 6-0, at a flow rate of 2 ml min\(^{-1}\). The elution profile was monitored at A\(_{280}\). All the fractions were subjected to the qualitative bioassay, and the active fraction(s) was desalted and the buffer exchanged by ultrafiltration prior to SDS-PAGE analysis.

**Determination of the N-terminal amino acid sequence.** Protein samples for N-terminal amino acid sequencing were prepared according to Ausubel et al. (1996). Pooled active fractions from gel filtration of the crude BLIS were concentrated by ultrafiltration, and precipitated with nine volumes of ice-cold 100% ethanol (Sigma) overnight at −20°C. The protein pellets obtained following centrifugation at 13 000 r.p.m. for 30 min were dissolved in SDS-PAGE reducing buffer to achieve a final protein concentration of 1-2 µg µl\(^{-1}\). The sample, after heat denaturation for 5 min, was subjected to SDS-PAGE on a 10% pre-electrophoresed polyacrylamide gel. Subsequent to electrophoresis, the gels were equilibrated in Western blot transfer buffer [2-21 g l\(^{-1}\) cyclohexylaminopropionic sulphonic acid (CAPS), 0-5 g l\(^{-1}\) DTT, 15% (v/v) methanol, pH 10-5] at 4°C for 10 min, and then electroblotted onto a PVDF membrane (Millipore PSQ). The blotted membranes were stained in 0-1% Coomassie blue R-250 in 50% methanol for 5 min, then destained in 10% (v/v) acetic acid in 50% (v/v) methanol. The protein band was excised, dried at room temperature, and then sequenced in a Procise 494 high-throughput gas-phase/liquid-pulse sequencer (Applied Biosystems) at the School of Biochemistry and Molecular Biology, University of Leeds.
RESULTS AND DISCUSSION

Detection of BLIS production in strain VIB 571

Plaque-like zones of clearing developed in the top soft-agar layer of the DLPA when strain VIB 571 was plated in combination with the strain VIB 295 (Fig. 1a). This indicated that strain VIB 571 produces a diffusible inhibitory substance(s), which is supported by the spot-on-lawn assay data (Fig. 1b). The flip-streak method (Fig. 1d) further confirmed that inhibition was not a result of bacteriophage induction, as bacteriophages cannot pass through the agar barrier (De Vuyst & Vandamme, 1994).

Inhibitory spectrum of *V. harveyi* VIB 571 and crude BLIS

The inhibitory spectrum of strain VIB 571 is given in Table 1. Inhibition was observed against four *V. harveyi* cultures, i.e. strains VIB 295, VIB 286, VIB 646 and VIB 651, and one culture each of *Vibrio* sp. (VIB 20), *Vibrio fischeri* (VIB 291), *Vibrio gazogenes* (VIB 294) and *Vibrio parahaemolyticus* (VIB 304). Overall, the inhibitory activity of *V. harveyi* VIB 571 was most pronounced against *V. harveyi* VIB 295 (Fig. 1b) and *V. fischeri* VIB 291 (Fig. 1c) compared to the other cultures examined in the study. The crude BLIS exhibited similar results, except inhibition was not recorded against *V. fischeri* and *V. gazogenes*. Moreover, inhibition by strain VIB 571 and its crude BLIS was not observed against MRSA 1, MRSA 2, VRE 1 and VRE 2 isolates.

Using the modified critical dilution method, the activity of the CFS and the crude BLIS was estimated to be 1600 and 25 600 AU ml⁻¹, respectively. This increase in activity indicated the precipitability of the inhibitory factor by ammonium sulphate at 60 % saturation. The use of doubling dilutions further substantiated that the inhibitory activity was not as a result of bacteriophage induction, because phage-associated inhibition results in plaque formation rather than zones of inhibition of diminishing size (De Vuyst & Vandamme, 1994). Furthermore, the retention of the active factor during dialysis (12 000–14 000 MWCO) indicated the approximate molecular size of the inhibitory factor to be >14 kDa.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Identity</th>
<th>Spot-on-lawn assay</th>
<th>Crude BLIS</th>
</tr>
</thead>
<tbody>
<tr>
<td>VIB 295</td>
<td><em>V. harveyi</em></td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>VIB 286</td>
<td><em>V. harveyi</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VIB 646</td>
<td><em>V. harveyi</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VIB 651</td>
<td><em>V. harveyi</em></td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>VIB 20</td>
<td><em>Vibrio</em> sp.</td>
<td>++</td>
<td>+</td>
</tr>
<tr>
<td>VIB 291</td>
<td><em>V. fischeri</em></td>
<td>+++++</td>
<td>+</td>
</tr>
<tr>
<td>VIB 294</td>
<td><em>V. gazogenes</em></td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>VIB 304</td>
<td><em>V. parahaemolyticus</em></td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

*Fig. 1. BLIS production by *V. harveyi* VIB 571. DLPA showing plaque-like inhibitory zones with a *V. harveyi* VIB 295 indicator strain (a), spot-on-lawn assay showing antibiotic-like inhibition against *V. harveyi* VIB 295 (b) and *V. fischeri* VIB 291 (c), and flip-streak method showing inhibition of *V. harveyi* VIB 295 (d).*
The effect of physiochemical factors on the stability of the crude BLIS

The effect of physiochemical factors on the stability of crude BLIS is summarized in Table 2. In short, the crude material was inactivated by proteolytic enzymes and SDS, indicating a proteinaceous nature for the inhibitory factor. Of interest was the inactivation by lipase, which suggests the presence of a lipid moiety in the BLIS. In contrast, lysozyme and α-amylase did not have any effect on the inhibitory activity. However, Tween 20 and 60, and SLS inhibited the indicator, therefore their effects on the stability of the crude BLIS could not be assessed. The serine-protease inhibitor PMSF and the chelating agent EDTA did not alter the activity of the crude material, thus eliminating the possibility that the BLIS was a serine protease or a metalloprotease/divalent cation-dependent enzyme, respectively. The BLIS activity remained thermally stable until reaching 60 °C for 10 min, but inactivation occurred when incubation continued for longer. BLIS activity was relatively stable between pH 2–11 for 5 h.

Effect of mitomycin C on the induction of BLIS

The residual activity of the BLIS from both the mitomycin C induced culture and the control remained the same, and was estimated to be 6400 AU ml⁻¹, indicating the absence of any effect of mitomycin C on BLIS induction.

Purification of the crude BLIS

The FPLC-gel filtration fractionation of the crude BLIS yielded 5 active fractions, i.e. 9, 10, 11, 12 and 13, and the maximum activity was observed in fraction 10. The SDS-PAGE protein profile of the active fractions showed that the predominant polypeptide was ~32 kDa (Fig. 2), the intensity of which correlated with the activity of the fraction. Calibration of the gel filtration column with proteins of known size suggests that a native molecular mass from the BLIS fractions was in the range of 30–40 kDa (data not shown).

Table 2. Effect of various physiochemical factors on the stability of the crude BLIS

<table>
<thead>
<tr>
<th>Physiochemical parameter</th>
<th>Residual activity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Proteolytic enzymes</strong></td>
<td></td>
</tr>
<tr>
<td>Proteinase K</td>
<td>–</td>
</tr>
<tr>
<td>Pepsin</td>
<td>–</td>
</tr>
<tr>
<td>Trypsin</td>
<td>–</td>
</tr>
<tr>
<td>Pronase E</td>
<td>–</td>
</tr>
<tr>
<td><strong>Other enzymes</strong></td>
<td></td>
</tr>
<tr>
<td>Lysozyme</td>
<td>+</td>
</tr>
<tr>
<td>Lipase</td>
<td>–</td>
</tr>
<tr>
<td>α-Amylase</td>
<td>+</td>
</tr>
<tr>
<td><strong>Detergents</strong></td>
<td></td>
</tr>
<tr>
<td>Tween 20</td>
<td>ND</td>
</tr>
<tr>
<td>Tween 40</td>
<td>+</td>
</tr>
<tr>
<td>Tween 60</td>
<td>ND</td>
</tr>
<tr>
<td>Tween 80</td>
<td>+</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>+</td>
</tr>
<tr>
<td>NP-40</td>
<td>+</td>
</tr>
<tr>
<td>SDS</td>
<td>–</td>
</tr>
<tr>
<td>SLS</td>
<td>ND</td>
</tr>
<tr>
<td><strong>Serine-protease inhibitor</strong></td>
<td></td>
</tr>
<tr>
<td>PMSF</td>
<td>+</td>
</tr>
<tr>
<td><strong>Metalloprotease inhibitor</strong></td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>+</td>
</tr>
<tr>
<td><strong>Temperature</strong></td>
<td></td>
</tr>
<tr>
<td>40–50 °C for 60 min</td>
<td>+</td>
</tr>
<tr>
<td>60 °C for 10 min</td>
<td>+</td>
</tr>
<tr>
<td>60 °C for &gt; 10 min</td>
<td>–</td>
</tr>
<tr>
<td><strong>pH</strong></td>
<td></td>
</tr>
<tr>
<td>pH &gt;2.0 and &lt;11 for 5 h</td>
<td>+</td>
</tr>
</tbody>
</table>

2-DE of the pooled active fraction from successive gel filtration runs yielded a major protein spot resembling a doublet, with a pI of ~5.4 (Fig. 3a). MALDI-TOF-MS analysis of multiple samples of the trypsin digested protein spot yielded a series of peaks of varying peptide masses (Fig. 3b). Similarity searches using the MASCOT (www.matrixscience.com; Perkins et al., 1999) peptide mass fingerprint program against MSDB, NCBInr and SWISSProt databases did not yield any significant matches for proteins.

Anion-exchange chromatography of the pooled active fractions succeeded in purifying the BLIS (Fig. 4a), with a single peak (A_{280}, fraction 9) eluting at 50–70 mM NaCl.

![Fig. 2. SDS-PAGE protein profile of FPLC-gel filtration fractions. Lane (a), Bio-Rad low-range protein standard; lanes (b–i), protein profile of gel filtration fractions 9–16 with the corresponding activity of each fraction shown below. The most active fraction (10) in lane (c) shows the ~32 kDa protein.](http://mic.sgmjournals.org)
at pH 6-0. Maximum and weak inhibitory activity was observed in fractions 9 and 10, respectively (Fig. 4b). SDS-PAGE analysis of the active fractions again showed the presence of an ~32 kDa protein in fraction 9 (Fig. 4c), but not in fraction 10. However, this may reflect loss during sample preparation.

**N-terminal sequencing of BLIS**

N-terminal sequencing of the ~32 kDa protein yielded the following sequence: Asp-Glu-Tyr-Ile-Ser-X-Asn-Lys-X-Ser-Ser-Ala-Asp-Ile. The ‘X’ represents probable cysteine residues or modified amino acids. A BLAST (Altschul et al., 1997) search for short, nearly exact sequences against NCBI and SWISS-PROT databases did not yield any significantly similar matches. This suggests that the protein could be unrelated to other types of BLIS.

As a development of previous work by McCall & Sizemore (1979), we demonstrated BLIS production in *V. harveyi* VIB 571. The inhibitory activity was due to the ~32 kDa protein, as confirmed by its presence in the active fractions resulting from gel- and anion-exchange filtration. The production of a possible additional inhibitory factor by strain VIB 571 with activity against *V. fischeri* (Fig. 1c) may indicate the presence of one or more specific means of inhibition, such as those brought about by microcins, which are small inhibitory polypeptides produced by Gram-negative bacteria (Jack & Jung, 2000). The loss in BLIS activity with lipase suggests a role for a lipid moiety in the activity, or maybe contamination by proteolytic enzymes. Nevertheless, bacteriocins that require non-protein moieties for their activity have been reported in Gram-positive organisms, and include lactocin 27, which is a glycoprotein from a homofermentative *Lactobacillus* sp. (Upreti & Hindsdill, 1975), and lactstrepcin, which is a lipoprotein from *Streptococcus lactis* (Kozak et al., 1977).

The ability to subculture strain VIB 571 from the spot-on-lawn assay plates demonstrating inhibitory activity, and the microscopic evidence of cell viability in the 72 h broth culture, suggest that BLIS production is not a lethal or self-destructive process for strain VIB 571, unlike for strains producing most other colicins. Enhanced bacteriocin production by DNA-damaging reagents, such as mitomycin C, is brought about by the triggering of the SOS response as a result of structural damage to the DNA, which involves activation of the RecA protease, which then degrades the LexA protein, a repressor of several DNA repair and colicin genes (Spangler et al., 1985). However, similar effects of mitomycin C on BLIS production by strain VIB 571 was not observed, as demonstrated using the semi-quantitative bioassay, implying its production was not SOS-regulon mediated. Furthermore, plasmid-purification trials (data not shown) did not show the presence of plasmids in strain VIB 571, suggesting that the BLIS may be chromosomally encoded, unlike harveyicin SY and most other colicins.

The search for similarity using the MALDI-TOF-MS generated peptide masses and the N-terminal sequence of the protein against databases did not provide significantly similar matches, and may reflect the likely uniqueness of the protein. Certainly, the narrow spectrum of activity and the proteinaceous nature are both regarded as important criteria for defining a bacteriocin. However, it is appropriate to retain the more general term of BLIS until further characterization of the protein.

**Fig. 3.** (a) The 2-DE profile of the active BLIS fraction on a 10% polyacrylamide gel showing a major protein spot with a pl ~5.4. (b) MALDI-TOF-MS chromatogram of the purified BLIS.
simulated enteric habitat against a mutant culture *V. harveyi* (*Lum*”) and the plasmid cured *V. harveyi SY* strain. From the perspective of a pathogen, such as *V. harveyi* VIB 571, it may be assumed that bacteriocinogeny may be beneficial insofar as there could be a competitive advantage over sensitive strains, facilitating access to resources within a host, and thus ensuring survival and dominance of the producer within the host. Based on the findings of other workers, Cursino *et al.* (2002) presented two observations that link colicin production and pathogenicity: firstly, the higher frequency of colicinogeny in pathogenic isolates compared with commensal isolates, and secondly, the association of the synthesis of certain virulence factors, such as aerobactin, alpha-haemolysin and P-fimbria, with some colicins. A similar association of BLIS production and the pathogenicity of strain VIB 571 remains a possibility. However, further work is required to validate this.

**ACKNOWLEDGEMENTS**

S. P. acknowledges a scholarship from the School of Life Sciences, Heriot-Watt University. We thank Dawn Austin for providing the isolates, Dr Jeff N. Keen, University of Leeds, for N-terminus sequencing, Sam de Costa for helpful suggestions, and Elise Cachat and Ross Alexander for assistance in the MALDI-TOF MS and 2-DE analyses.

**REFERENCES**


---

**Fig. 4.** FPLC anion-exchange profile of the BLIS. (a) Anion-exchange chromatograph (Photoshop enhanced) showing a single peak eluting at 50–70 mM NaCl in 20 mM PIPES pH 6.0. (b) Spot-on-lawn assay showing significant activity in fraction 9, and mild activity in fraction 10. (c) SDS-PAGE protein profile of the active fractions 9 (F9) and 10 (F10), showing a ~32 KDa protein band in fraction 9.


