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Purification of High Molecular-Weight Antibacterial Proteins of Insect Pathogenic *Brevibacillus Laterosporus* Isolates

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Abstract: *Brevibacillus laterosporus* (*Bl*) is a Gram-positive and spore-forming bacterium belonging to the *Brevibacillus brevis* phylogenetic cluster. Globally, insect pathogenic strains of the bacterium have been isolated, characterised, and some activities patented. Two isolates, *Bl* 1821L and *Bl* 1951, exhibiting pathogenicity against the diamondback moth and mosquitoes, are under development as a biopesticide in New Zealand. However, due to the suspected activity of putative antibacterial proteins (ABPs), the endemic isolates often grow erratically. Various purification methods including size exclusion chromatography, sucrose density gradient centrifugation, polyethylene glycol precipitation, and ammonium sulphate precipitation employed in this study enabled the isolation of two putative antibacterial proteins of ~30 kD and ~48 kD from *Bl* 1821L and one putative antibacterial protein of ~30 kD from *Bl* 1951. Purification of the uninduced cultures of *Bl* 1821L and *Bl* 1951 also yielded the protein bands of ~30 kD and ~48 kD on SDS-PAGE which indicated their spontaneous induction. Disc diffusion assay was used to determine the antagonistic activities of the putative ABPs. Subsequent transmission electron microscope (TEM) examination of purified putative antibacterial protein-containing solution showed the presence of encapsulin (~30 kD) and polysheath (~48 kD) like structures. Although only the ~30 kD protein was purified from *Bl* 1951, both structures were seen in this strain under TEM. Furthermore, while assessing the antibacterial activity of some fractions of *Bl* 1951 against *Bl* 1821L in size exclusion chromatography method, population of *Bl* 1821L persister cells was noted. Overall, this work added a wealth of knowledge for the purification of the HMW proteins (bacteriocins) of the Gram-positive bacteria including *Bl*.

Keywords: antibacterial proteins; encapsulating protein; high molecular-weight bacteriocins; insect pathogenic bacterium; phage tail-like protein; purification methods

1. Introduction

Bacteriocins are ribosomally synthesised compounds released extracellularly by diverse lineages of bacteria [1,2] and are classified into two basic groups: low molecular-weight (LMW) and high molecular-weight (HMW) [3]. LMW bacteriocins are trypsin-sensitive, thermostable, and unsedimentable, whereas HMW bacteriocins are sedimentable, trypsin-resistant, thermolabile, and visible under an electron microscope as phage-like components [3,4]. HMW or phage tail-like bacteriocins (PTLBs), often called “tailocins” [5,6], morphologically resemble phage tails and a common ancestral relationship between tailocins and phages has been defined [7]. Two morphologically distinct types of tailocins have been distinguished: the R-type tailocins are rigid and contractile particles [8] whereas the F-type tailocins represent flexible, non-contractile structures [9]. The common feature of the two forms is how they perpetuate in nature [10]. Lysogeny is a commonly occurring phenomenon in phages and PTLBs [11,12] and both the bacterial antagonists are released upon lysis of the cell after induction [13,14]. The major components present in crude lysate

apart from phages or PTLBs may include bacterial debris (mainly membranes with bacterial proteins), nucleic acids, and ribosomes [15]. To identify and characterise the protein of interest it is vital to purify from this lysed homogenate [16,17]. A novel class of HMW complex antagonistic proteins “encapsulins” first identified in the supernatant of bacterium, *Brevibacterium linens*, also exhibited bacteriostatic activity against various strains of *Arthrobacter*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, and *Listeria* [18].

Brevibacillus laterosporus (*Bl*) is a Gram-positive and spore-forming bacterium belonging to the *Brevibacillus brevis* phylogenetic cluster [19]. *Brevibacillus* species are a rich source of antimicrobial peptides (AMPs) [20] and >30 AMPs including antibacterial, antifungal, and anti-invertebrate agents have been isolated from different species [20,21]. However, only a limited number of LMW bacteriocins have been defined [22-24]. Globally, strains of the bacterium demonstrating pathogenicity against a wide range of organisms including insect have been isolated, characterised [25], and some activities patented [26-28]. The New Zealand isolates *Bl* 1821L and *Bl* 1951 exhibit pathogenicity against the diamondback moth, *Plutella xylostella*, and larvae of the mosquitoes (*Culex pervigilans* and *Opifex fuscus*) [27,29] and are under development as a biopesticide. However, due to the suspected activity of putative antibacterial proteins on the growth of *Bl* 1821L and *Bl* 1951, the endemic strains often lose potency [29,30]. To identify the HMW antagonistic proteins (bacteriocins) belonging to the different Gram-positive bacteria, various purification methods have been used [18,31-33]. However, there is limited work about the purification of HMW proteins (bacteriocins) from the insect pathogenic isolates [13,34].

Herein, we describes the purification and identification of putative antibacterial proteins of *Bl* 1821L and *Bl* 1951.

2. Materials and Methods

2.1. Purification of putative antibacterial proteins using size exclusion chromatography (SEC)

Bacterial Strains and Growth Conditions

Isolates *Bl* 1821L and *Bl* 1951, held in the Bioprotection Research Centre Culture Collection, Lincoln University, New Zealand were used in this study. Luria-Bertani medium broth (LB, Miller, Sigma) was routinely used for growing bacteria on an orbital shaker (Conco, TU 4540, Taibei, Taiwan) at 250 rpm and 30°C overnight for further usage.

Mitomycin C induction of putative antibacterial proteins

Single colonies of bacteria were used to inoculate 5 mL of LB broth (Miller, Sigma), which was placed on an orbital shaker (Conco, TU 4540, Taibei, Taiwan) at 250 rpm and 30°C overnight. Aliquots (500 µl) of the overnight culture were independently transferred to replicated flasks of 25 mL of LB broth. The inoculated cultures were then grown at 250 rpm and 30°C on the orbital shaker for 10-12 hours. Mitomycin C (Sigma, Sydney, NSW, Australia) was added into the flasks, which were left shaking overnight at 40 rpm and ambient temperature (24°C). The flasks were monitored to view for signs of lysis (clearing of the culture or accumulation of bacterial debris). For *Bl* 1821L, 1 µg/mL [34] and for *Bl* 1951, mitomycin C at 3 µg/mL [35] was used to induce the putative antibacterial proteins with some modifications in the protocol of [36].

SEC of putative antibacterial proteins

Mitomycin C induced cultures were centrifuged at 16,000 g for 10 min and the supernatants were passed through a 0.22 µm filter. Cell free supernatants (CFS) were ultracentrifuged at 35,000 rpm (151,263 x g) in a swing bucket rotor (41Ti, Beckman, California, USA) for 70 min. The concentrated pellet was resuspended in 100-150 µl of TBS (Tris buffer saline: 25 mM Tris-HCl, 130 mM NaCl, pH 7.5). Prior to SEC, the resuspended pellet was passed through a 0.45 µm filter. For SEC, a Bio-Rad column (1.5 x 46 cm) was filled with the gel matrix (Sephacryl S-400) according to the manufacturer's instructions (GE Healthcare Life Sciences, Auckland, New Zealand). Next, 800 µl of the ultracentrifuged

sample was loaded onto the SEC column (BioLogic LP System) which had been pre-equilibrated with TBS buffer to a volume of approximately 150-200 mL at a flow rate of 1 mL/minute. The sample was run at 0.5 mL/minute and the purified/separated protein mixture was monitored using the BioLogic LP System at 280 nm.

Assay, protein quantification, and SDS-PAGE analysis of SEC fractions

Antagonistic activity of SEC derived fractions was tested against *Bl* 1821L and *Bl* 1951 as the host bacterium through the Kirby-Bauer disc diffusion assay [37,38]. A single colony of the host bacterium was inoculated into 5 mL LB broth (Miller) and shaken on an orbital shaker (Conco, TU-4540, Taiwan) at 250 rpm and 30°C for 18-20 hours. LB agar plates were inoculated by dipping a sterile swab into the culture and swabbed over the surface of the medium three times. The inoculum was left to dry for 10-15 min at room temperature (22°C). A sterile 8 mm diameter paper disc (ADVANTEC, Niigata, Japan) was placed in the middle of an LB agar plate and 80 µl of each SEC fraction was pipetted onto the paper disc. Disc diffusion assays were performed in triplicate assessing independently undiluted SEC fractions, from where SEC fractions exhibiting the inhibitory activities were pooled (*Bl* 1821L) and concentrated at 35,000 rpm (151,263 × g) in a swing bucket rotor (41Ti, Beckman) for 70 min. The concentrated SEC fractions were quantified (µg/mL) using a Qubit protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA).

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of SEC fractions after concentrating the putative antibacterial proteins was performed using the protocol of Laemmli [39]. Gels were run for 50 min at 200 volts and then rinsed four times with H₂O before staining with silver [40]. Five µl of protein ladder (BIO-RAD, Precision Plus Protein™ Standards, Auckland, New Zealand) was used.

TEM analysis of SEC fractions

A 5 µl aliquot of SEC purified and concentrated sample was applied to a freshly glow discharged plastic-coated hydrophilic 200 mesh EM grid (ProSciTech; Thuringowa, Australia) and stained with 3 µl of 0.7% uranyl acetate (UA, pH 5). The samples were examined at 18,000 to 25,000 magnification in Morgagni 268D (FEI, Hillsboro, OR, USA) TEM operated at 80 KeV. The images were photographed using the TENGRA camera. TEM analysis was performed at AgResearch, Lincoln, New Zealand.

2.2. Purification of putative antibacterial proteins using sucrose density gradient (SDG) centrifugation

CFS of mitomycin C- induced cultures were ultracentrifuged as described above and the concentrated pellet was resuspended in 100-150 µl of TBS buffer. Two groups of sucrose density gradients were used in this study. Group A comprising of 10%, 20%, 30%, 40%, and 50% gradients and group B comprising of 10%, 20%, 30%, 40%, 50%, and 60% gradients were created by applying layers of 1.25 mL of freshly prepared sucrose solution sequentially from greatest to lowest sucrose concentration. After ultracentrifugation, 200 µl of lysate (*Bl* 1821L/*Bl* 1951) was applied on top of each group of gradients and centrifuged at 35,000 rpm (151,263 × g) in a swing bucket rotor (41Ti, Beckman) for 70 min to concentrate the putative antibacterial proteins. Similarly, CFS derived from mitomycin C- induced (without ultracentrifugation) and uninduced (without mitomycin C) cultures were independently applied (200 µl) at the top of both the groups of gradients to concentrate the putative antibacterial proteins. Sucrose density layers of each gradient were carefully drawn out according to the added volume and evaluated for their antagonist activities against *Bl* 1821L and *Bl* 1951 as the host bacterium using through Kirby-Bauer disc diffusion assay. After assay test, volume of each gradient was made 7-7.5 mL with the TBS buffer and that pellet was resuspended in 100-150 µl TBS buffer that was further used in SDS-PAGE analysis.

Purified *Bl* 1821L putative antibacterial proteins of ~30 kD from 20% gradient (Group A) and ~48 kD from 60% gradient (Group B), and for *Bl* 1951 purified protein of ~30 kD from 50% gradient (Group A) were further concentrated and cleaned using an Amicon

Ultra-0.5 (10 kD) centrifugal filter (Millipore, Cork, Ireland). SDS-PAGE analysis of mitomycin C- induced (with/without ultracentrifugation) and uninduced cultures (without mitomycin C) but with ultracentrifugation was performed as outline above for SEC purified fractions. Likewise, the purified and 10 kD MWCO membrane concentrated samples were also subjected to SDS-PAGE and TEM analysis.

2.3. Purification of putative antibacterial proteins using polyethylene glycol (PEG) precipitation

Mitomycin C- induced cultures were centrifuged at 16,000 g for 10 min and the supernatants were passed through a 0.22 µm filter prior to the addition of PEG 8000 (10%) and 1M NaCl. The mixture was incubated in an ice bath for 60 min and subsequently centrifuged at 16,000 g for 30 min at 4°C. The pellet was resuspended in 1/10th the original volume of TBS buffer. PEG residues were removed by two sequential extractions with an equal volume of chloroform, which was combined with the resuspended pellet and vortexed for 10-15 sec. The mixture was centrifuged at 16,000 g for 10 min and the upper aqueous phase was transferred to a fresh micro centrifuge tube. This extraction process was repeated until no white interface between the aqueous and organic phases was visible. Next, PEG 8000 precipitated cultures were ultracentrifuged in a swing bucket rotor (41Ti, Beckman) at 35,000 rpm (151,263 x g) for 70 min. The pellet was resuspended in 100-150 µl of TBS buffer and purified using sucrose density gradient centrifugation, concentrated by ultracentrifugation, and assessed by SDS-PAGE as outlined in the preceding section.

2.4. Purification of putative antibacterial proteins using ammonium sulphate precipitation (ASP)

Mitomycin C- induced cultures were transferred into 50 mL tubes and centrifuged at 10,000 g for 10 min and 4°C to remove cell debris and the supernatants passed through a 0.22 µm filter. The supernatant were transferred into a 100 mL beaker with a magnetic stirrer placed in an ice bucket and precipitated using ammonium sulphate (AS) until 85% saturation was reached (calculated 85% quantity from <http://www.encorbio.com/protocols/AM-SO4.htm>, accessed 20 November 2019). The precipitated proteins were harvested by centrifugation at 10,000 g for 20 min, and the pellets were independently resuspended in 5 mL of phosphate buffer + 150 mM NaCl. Ammonium sulphate was removed through buffer exchange using dialysis tubing and pre chilled phosphate buffer (4°C), which was replaced after every three hours. After the third buffer change, the sample within the dialysis tube was transferred into a 15 mL tube and stored at -80°C. Subsequently, precipitates were placed for 2-3 days in a freeze dryer maintained at -80°C. The precipitated cultures were dissolved in TBS buffer and ultracentrifuged in a swing bucket rotor (41Ti, Beckman) at 35,000 rpm (151,263 x g) for 70 min. The concentrated pellet was resuspended in 100-150 µl of TBS buffer. Sucrose density gradient purification was performed and the concentrated samples were assessed by SDS-PAGE as outlined above.

3. Results

3.1. Purification of putative antibacterial proteins using SEC

Purification of *Bl* 1821L putative antibacterial protein

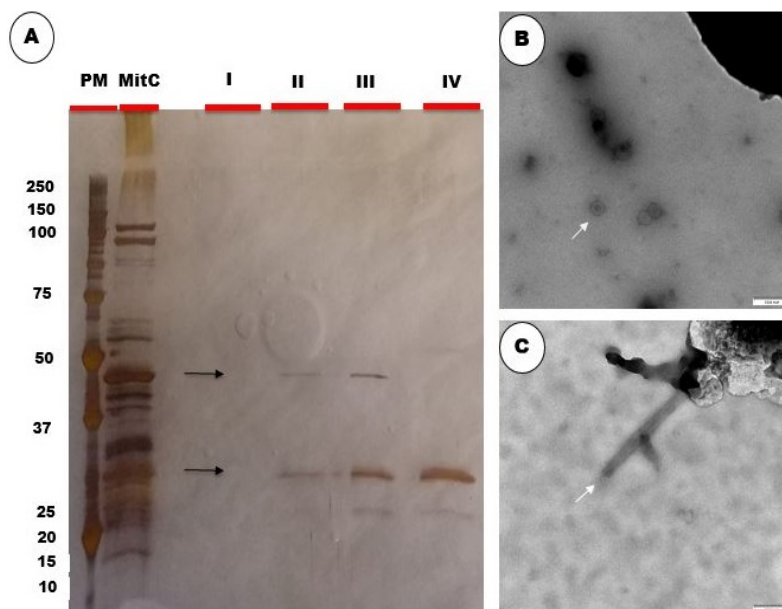
Through assessment of *Bl* 1821L derived SEC fractions for activity against *Bl* 1821L and *Bl* 1951 prominent antibacterial activity was identified in fractions 1-32 (Supplementary (S) Material, Figure S1). A disc assay test exhibited a prominent zone of inhibition with 32 of the SEC fraction against *Bl* 1821L while 27 fractions were also found active against *Bl* 1951 (Table 1). Fractions exhibiting prominent activities were pooled (Figure S1 & Table S1) and further assays test revealed strong differences in antagonistic activity among these pooled groups (Table S1). Pool I fractions (3, 4, 5) demonstrated antibacterial activity against both *Bl* 1821L and *Bl* 1951, but the pools II (11, 12, 13, 14, 15) and III (16, 17, 18, 19) had antagonistic activities only against the *Bl* 1821L. Pool IV (20, 21, 22, 23, 24)

displayed antibacterial activity only against *Bl* 1951, except fraction no. 21, which was active against both the hosts (*Bl* 1821L & *Bl* 1951) (Table S1).

Table 1. *Bl* 1821L SEC active fractions of the assay test

Host bacterium	SEC fractions	Total fractions
<i>Bl</i> 1821L	2-19, 21, 26, 27, 34, 37, 39-42, 45, 49, 52, 53, 55, 58	32
<i>Bl</i> 1951	2, 3, 4, 5, 7, 8, 10, 20-32, 41, 42, 47, 49, 52, 59, 61	27

SDS-PAGE analysis of *Bl* 1821L various SEC fractions revealed two prominent bands of ~30 kD and ~48 kD molecular mass. These bands were observed in pools II and III while the ~30 kD band only was observed in pool IV (Figure 1A). No proteins were observed by SDS-PAGE in pool I (Figure 1A), although protein was detected when measured using qubit (Table S1). Assessments of electron micrographs of SEC purified pools revealed a hollow tube-like structure in pool III (Figure 1C) and hexagonal or phage capsid-like structures of uniform sizes in the pool IV (Figure 1B).



Note: PM and MitC stand for Protein marker and Mitomycin C respectively.

Figure 1. SDS-PAGE and TEM analysis of *Bl* 1821L putative antibacterial proteins purified using SEC. SDS-PAGE showing purified ~30 kD and ~48 kD proteins (Fig. 1A, shown with dark arrows) of different pooled fractions. Electron micrographs of *Bl* 1821L SEC purified putative antibacterial proteins pool-III (Fig. 1C) and pool-IV (Fig. 1B). The arrows denote a hexagonal or phage capsid-like structure of uniform size (Fig. 1B) and a hollow tube-like structure (Fig 1C). Scale bar = 100 nm.

Purification of *Bl* 1951 putative antibacterial protein

Of the collected 61 fractions of *Bl* 1951, fractions 6-22 (Figure S2) displayed prominent antagonistic activity in the assay tests. The quantified protein contents of SEC purified and concentrated fractions are also presented in Table S2. Antagonistic activity of SEC derived 16 fractions was observed against *Bl* 1951 and 11 fractions against *Bl* 1821L as the host bacterium (Table 2). Unexpectedly, while assessing the inhibitory activity of *Bl* 1951 SEC fractions (12, 13, 14, 15, 21, 40) against *Bl* 1821L, instead of producing a prominent zone of inhibition, reduced growth of the host bacterium around the paper discs was observed (Figure 2), possibly indicating the presence of persister cells. These resistant cells

(persister) were retrieved and cultivated overnight. Subsequent assessment of mitomycin C- induced filtered supernatant of *Bl* 1951 against the cultivated lawn of *Bl* 1821L persister cells produced a prominent zone of inhibition (Figure 3), which suggests the state was not maintained.

Table 2. *Bl* 1951 SEC active fractions of the assay test

Host bacterium	SEC fractions	Total fractions
<i>Bl</i> 1951	6, 9, 16, 21, 22, 27, 28, 31, 34, 40, 47, 50, 52, 55, 58, 61	16
<i>Bl</i> 1821L	8, 10-19, 40	11

Similar to *Bl* 1821L, some of the *Bl* 1951 SEC fractions (12, 13, 14, 15, 40) were only active against *Bl* 1821L and some fractions (18, 21, 22, 27, 28, 61) only active against *Bl* 1951 (Figure S2 & Table 2). Assessment of these fractions by SDS-PAGE revealed the presence of a shared ~30 kD protein (Figures 4A & 4C). TEM examination of SEC purified fractions no. 15 and no. 27 revealed the presence of hexagonal or phage capsid-like structures of a consistent size in both fractions (Figures 4B & 4D).

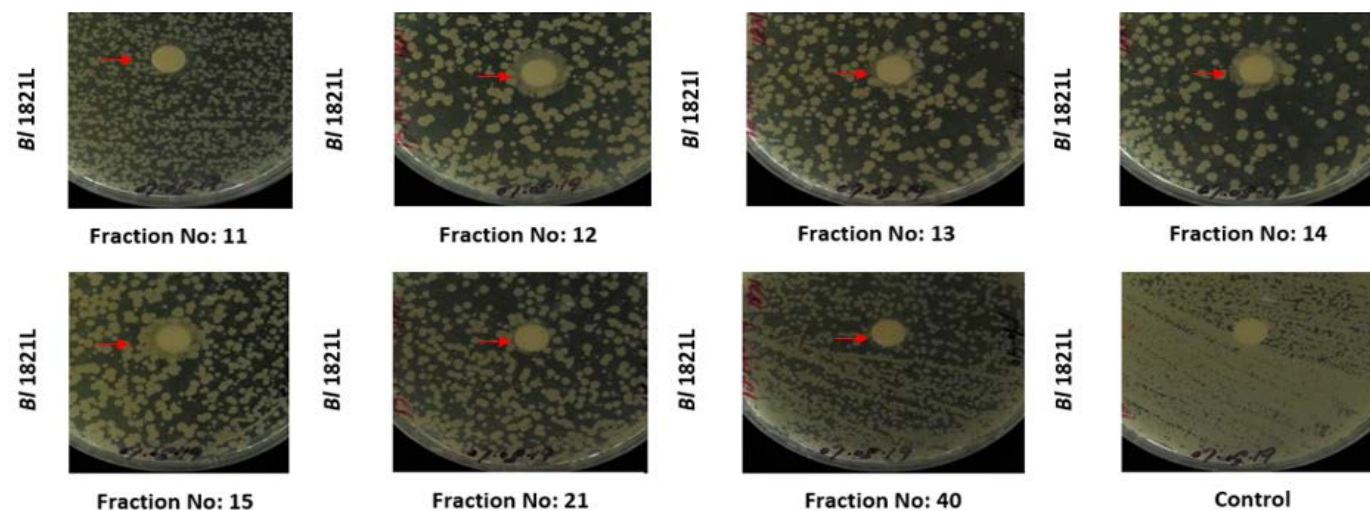


Figure 2. Disc diffusion assay test of *Bl* 1951 SEC fractions against *Bl* 1821L as the host bacterium. The red arrow denotes the formed persister cells.

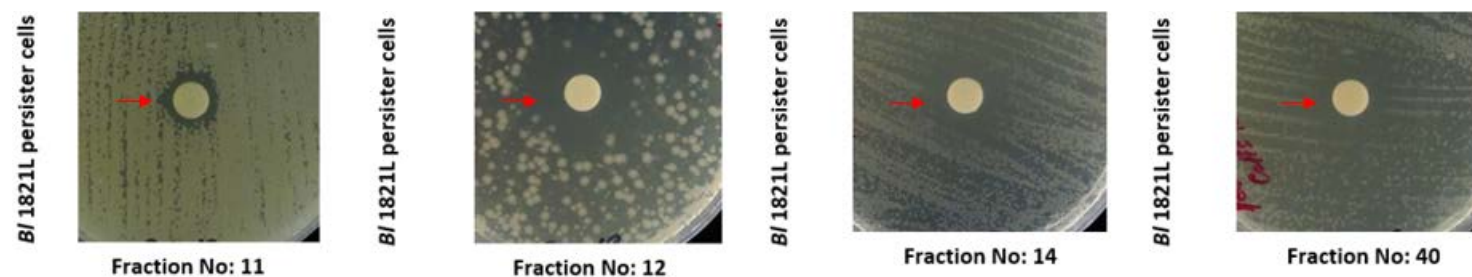


Figure 3. Disc diffusion assay test of *Bl* 1951 mitomycin C- induced CFS against *Bl* 1821L persister cells. The red arrow denotes the zone of inhibition due to the activity of mitomycin C- induced CFS against the *Bl* 1821L persister cells.

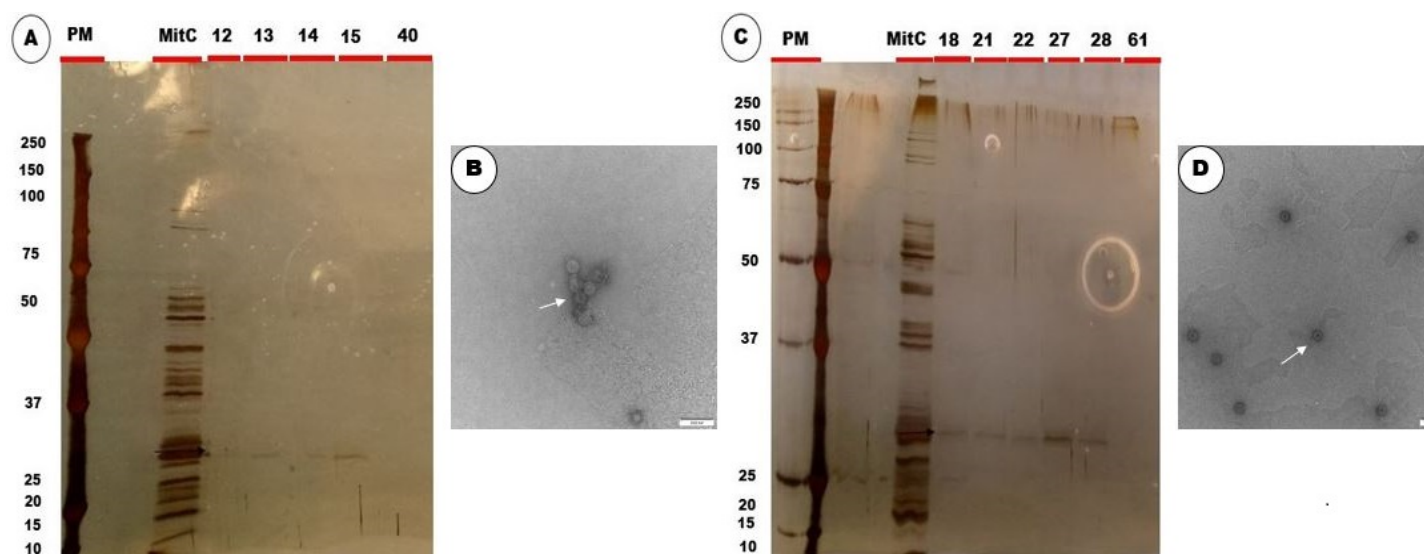


Figure 4. SDS-PAGE and TEM analysis of *Bl* 1951 putative antibacterial protein purified using SEC.

SDS-PAGEs of SEC fractions 12, 13, 14, 15, 40 (Fig. 4A) and SEC fractions 18, 21, 22, 27, 28, 61 (Fig. 4C) showing purified protein of ~30 kD (shown with dark arrow; See SEC chromatogram Fig. S2). Electron micrographs of *Bl* 1951 SEC purified putative antibacterial protein fraction no. 15 (Fig. 4B) and fraction no. 27 (Fig. 4D) showing uniform sized hexagonal or phage capsid-like structures (with white arrows). Scale bar = 100 nm.

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3.2. Purification of putative antibacterial proteins using SDG centrifugation

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Purification of *Bl* 1821L putative antibacterial proteins

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Antibacterial activity of *Bl* 1821L group A gradients indicated narrow zones of inhibition (9-10.5 mm) against *Bl* 1821L as the host bacterium and assessments of the group B gradients showed zones of inhibition that varied from 9-14 mm (Table 3). However, for *Bl* 1951 as the host bacterium using the purified sucrose A and B gradients of both groups resulted in similar halo sizes (Table 3). SDS-PAGE analysis of *Bl* 1821L sucrose density gradients revealed the presence of two protein bands of ~30 kD and ~48 kD. A purified putative antibacterial protein of ~30 kD molecular mass from the 20% and 30% gradients of group A (Figure S3A) and ~48 kD band was visualised in 20%, 40%, and 50% gradients (Figure S3A). The purified protein of ~48 kD was also prominently observed in group B gradients (40% to 60%) (Figure S3B).

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SDS-PAGE analysis of uninduced (without mitomycin C) cultures of *Bl* 1821L subjected to the same purification strategy revealed the presence of a ~30 kD protein on the gel from 20% to 50% gradients of group A (Figure S4A). From these uninduced cultures, both the proteins (~30 kD & ~48 kD) were purified and visible in group B gradients but the ~48 kD protein was observed in the gradients 40% to 60% (Figure S4B). Mitomycin C-induced CFS of *Bl* 1821L not subjected to high-speed centrifugation was also assessed directly by SDS-PAGE with both the groups of gradients and only the ~30 kD protein was visualised in 30% and 40% gradients of group A (Figure S5).

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Assessment of SDS-PAGE of group A (20%) and group B (60%) purified and 10 kD MWCO membrane concentrated solutions of *Bl* 1821L revealed prominent proteins of ~30 kD and ~48 kD molecular mass (Figure 5A). Electron micrographs of the concentrated solution containing ~30 kD protein displayed globular or phage capsid-like structures (Fig. 5D) and with ~48 kD protein long rigid polysheath-like structures were visualised (Fig. 5B-C).

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Table 3. *Bl* 1821L putative antibacterial protein assay test and quantification using group A (10%-50%) and group B (10%-60%) SDGs.

Group A SDG (%)	Protein concentration ($\mu\text{g/mL}$)	Zone of inhibition diameter (mm)		Group B SDG (%)	Protein concentration ($\mu\text{g/mL}$) (mm)	Zone of inhibition diameter (mm)	
		<i>Bl</i> 1821L as host bacterium	<i>Bl</i> 1951 as host bacterium			<i>Bl</i> 1821L as host bacterium	<i>Bl</i> 1951 as host bacterium
10	75.5	9.0	12.5	10	101.0	12.0	9.5
20	74.9	9.5	12.0	20	117.0	14.0	10.5
30	89.3	10.5	12.0	30	101.0	12.5	11.0
40	87.2	9.0	11.5	40	100.0	12.0	11.0
50	116.0	10.0	10.5	50	90.1	11.0	9.5
				60	84.1	9.00	9.0

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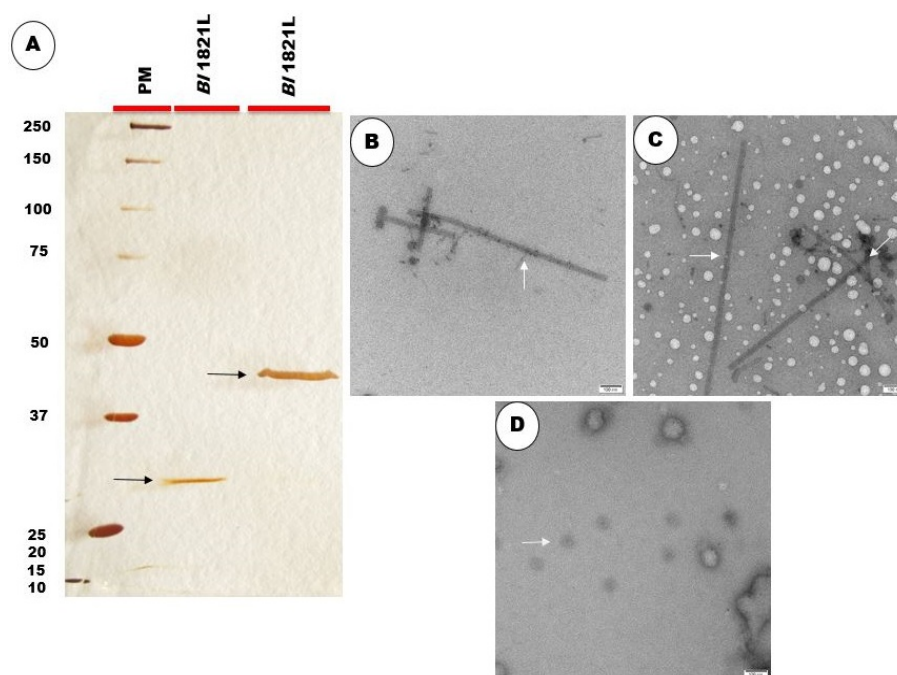


Figure 5. SDS-PAGE and TEM analysis of *Bl* 1821L purified and 10 kD MWCO membrane concentrated putative antibacterial proteins. SDS-PAGE analysis of *Bl* 1821L putative antibacterial proteins showing the ~30 kD and ~48 kD purified proteins band (Fig. 5A, shown with dark arrow). Electron micrographs of ~30 kD purified putative antibacterial protein showing globular or phage capsid-like structures (Fig. 5D). TEM images of ~48 kD purified putative antibacterial protein showing long rigid polysheath-like structures (Fig. 5B-C). Scale bar= 100 nm.

Purification of *Bl* 1951 putative antibacterial protein

Bl 1951 group A sucrose density gradients exhibited similar antagonistic activity against both the hosts (*Bl* 1951 & *Bl* 1821L) producing narrow zones of inhibition (9-11.5 mm) (Table 4). Likewise, the activity of the *Bl* 1951 derived group B gradients was similar across all the gradients but slightly differed in activity from each other against the tested bacterium (Table 4). A putative antibacterial protein of ~30 kD molecular mass was purified from the crude lysate of *Bl* 1951. Excluding the uppermost gradient (10%) of both the groups (A & B), the active putative antibacterial protein was purified from all the gradients (Figures S6 A-B), although some other co-purified proteins were also visualised (Figure S6A).

Gel electrophoresis of *Bl* 1951 culture without mitomycin C treatment after ultracentrifugation purified a protein of ~30 kD that was visualised on SDS-PAGE across all the gradients of group A and group B except for the 10% gradient (Figures 7A-B). All the gradients of group A excluding the uppermost (10%) and group B gradients (40% to 60%) showed the ~48 kD purified protein (Figure 7B). SDS-PAGE analysis of mitomycin C-induced CFS of *Bl* 1951 without ultracentrifugation with both the groups of gradients only showed the two protein bands of ~30 kD and ~48 kD in the top 10% and 20% gradients of group B (Figure S8).

Assessment of SDS-PAGE of group A (50%) purified and 10 kD MWCO membrane concentrated protein of *Bl* 1951 revealed a prominent protein of ~30 kD (Figure 6A). TEM examination of the concentrated *Bl* 1951 protein displayed globular or phage capsid-like structures and long nanotubes (polysheaths) (Figure 6B).

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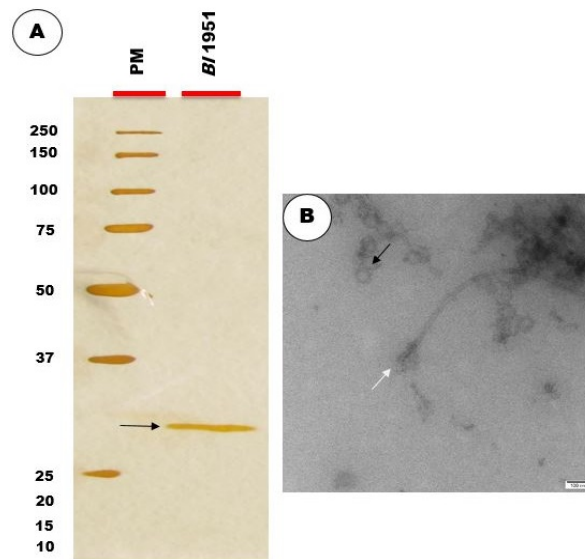


Figure 6. SDS-PAGE and TEM analysis of *Bl* 1951 purified and 10 kD MWCO membrane concentrated putative antibacterial protein. SDS-PAGE analysis of *Bl* 1951 putative antibacterial protein showing the ~30 kD purified protein band (Fig. 6A, shown with dark arrow). Electron micrographs of ~30 kD purified putative antibacterial protein of *Bl* 1951 showing globular or phage capsid-like (Fig. 6B, with dark arrow) and polysheath-like structure (Fig. 6B, with white arrow). Scale bar= 100 nm.

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Table 4. *Bl* 1951 putative antibacterial protein assay test and quantification of using group A (10%-50%) and group B (10%-60%) SDGs.

Group A SDG (%)	Protein concentration ($\mu\text{g/mL}$)	Zone of inhibition diameter (mm)		Group B SDG (%)	Protein concentration ($\mu\text{g/mL}$) (mm)	Zone of inhibition diameter (mm)	
		<i>Bl</i> 1821L as host bacterium	<i>Bl</i> 1951 as host bacterium			<i>Bl</i> 1821L as host bacterium	<i>Bl</i> 1951 as host bacterium
10	88.9	9.5	9.0	10	115.0	11.5	10.0
20	84.9	9.5	9.5	20	97.5	11.5	9.0
30	77.7	9.0	9.0	30	119.0	11.0	9.0
40	75.2	9.5	10.0	40	118.0	11.0	10.0
50	92.6	9.5	11.0	50	111.0	10.5	10.5
				60	109.0	10.5	9.0

3.3. Purification of putative antibacterial proteins of *Bl* 1821L using precipitation methods

PEG 8000 (10%) precipitated putative antibacterial proteins of *Bl* 1821L upon further purification with the group B sucrose density gradients showed protein bands of ~30 kD and ~48 kD. SDS-PAGE of PEG 8000 (10%) lysate exhibited a very minor band of ~48 kD in 40% and 60% gradients protein when compared to ~30 kD (Figure S9).

SDS PAGE of *Bl* 1821L ammonium sulphate (85%) precipitated culture with group B sucrose density gradients revealed a ~50 kD protein band from the 50% and 60% gradients (Figure S10).

4. Discussion

Protein purification is an intrinsic step to understand the nature of a targeted protein [17]. Therefore, various methods such as SEC, sucrose density gradient centrifugation, PEG precipitation, and ammonium sulphate precipitation were undertaken to purify the putative antibacterial proteins of insect pathogenic isolates *Bl* 1821L and *Bl* 1951. Two putative antibacterial proteins (~30 kD & ~48 kD) of *Bl* 1821L and one ~30 kD of *Bl* 1951 were purified. Electron micrographs of purified proteins showed different phage structural components similar to that seen in defective phages. Furthermore, SDS-PAGE of the purified products of uninduced cultures (without mitomycin C) also showed the same protein bands as mitomycin C-induced cultures. Through assessment of *Bl* 1951 SEC fractions a population of transient resistant cells (persisters) in the *Bl* 1821L isolate was noted.

Bacteria predominantly harbour prophages in their chromosomes either in true or defective lysogenic forms [11,41] and can be induced by DNA damaging agents such as UV radiation or mitomycin C [42,43]. The induction is suicidal for the cells as it results in bacterial cell lysis [44,45] which extracellularly releases numerous proteins apart from phages or PTLBs [15]. Therefore, to identify and characterise the protein of interest it is vital to purify from this lysed homogenate [16,17]. Ultracentrifugation is a preferred method due to its rapidity and low cost, but there are also reports that the structural components of viruses may be damaged due to the high speed [46,47]. Despite its limitations, density gradient ultracentrifugation is a common technique used to isolate and purify biomolecules and cell structures [31,46]. Purification of *Bl* 1821L putative antibacterial proteins using sucrose density gradient centrifugation showed two prominent bands of ~30 kD and ~48 kD molecular mass. Electron micrographs of *Bl* 1821L purified putative antibacterial proteins revealed structural differences, where phage encapsulating (capsid-like) structures were observed in ~30 kD containing gradient. Polysheath-like structures were seen in ~48 kD containing purified gradient suggesting that these structures were assembled due to the polymerisation of different units. Similar polysheath structures have been defined as aberrant assemblies of tail material in a structure identical with a contracted sheath and may be found in a "smooth" or "helical" form [48]. Polysheaths were classified as phage tail-like defective bacteriophages together with rhabdosomes and particularly bacteriocins such as R-pyocins [49]. Previously, bacteria producing the long and ordered nanotube-like structures (polysheaths) were believed to harbour a true prophage, but over time, the genetic information for the phage has decreased to such an extent that the information for the sheaths is the only structural information left [48,50]. Polysheath structures are very stable and can withstand treatments with various chemical and physical factors [51,52]. Electron micrographs of the polysheaths-like structures in the current study were in agreement with previous work in various bacteria [13,34,52-55]. A protein of ~30 kD molecular mass was also purified from the crude lysate of *Bl* 1951. TEM examination of the purified and 10 kD MWCO membrane concentrated solutions of *Bl* 1951 containing ~30 kD protein revealed the presence of both globular or phage capsids-like and polysheaths-like structures.

The ~30 kD and ~48 kD proteins were also observed in the sucrose density gradient centrifugation of non-induced (without mitomycin C) crude lysate of *Bl* 1821L and *Bl* 1951;

these might be the product of spontaneous prophage induction (SPI). SPI is the activation of bacteriophages and prophage elements, pathogenicity islands, and phage morons (an extra gene in a prophage genome without a function) from the bacterial cells in the absence of an external trigger [56,57]. This phenomenon is potentially considered a detrimental process for bacterial populations as a small percentage of cells would be lost continuously due to the lysis of the bacterial cells [56]. Earlier studies have reported the spontaneous release of free bacteriophages and phage tail-like particles in the supernatants of non-induced cultures of various lysogenic bacteria [58-60].

Size exclusion chromatography or gel filtration is a technique that is widely used to separate macromolecules based on their relative size [61,62]. Similar to sucrose density gradient centrifugation, the proteins of identical structures with equivalent molecular masses were purified. However, some differences were noted in the activity of SEC fractions. SEC pooled fractions II and III displaying both the protein bands of ~30 kD and ~48 kD on SDS-PAGE demonstrated antagonistic activity against *Bl* 1821L while the purified pooled IV fraction showing ~30 kD protein was active against *Bl* 1951. Typically, the putative antibacterial proteins (bacteriocins) are antagonistic to the closely related producer bacterial strains and species but the producer strains are immune to their lethal effects [10,14]. However, some members of a genetically identical population can kill their siblings (autocidal) [63,64]. An example is a bacteriocin, hyicin 3682, which exhibited antagonistic activity against the producer strain, *Staphylococcus hyicus* [65]. The antagonistic activity of ~30 kD encapsulating-like protein of *Bl* 1821L against *Bl* 1951 was in line with the work of various studies [18,66]. For example, Linocin M18, a putative encapsulating protein (bacteriocin) of 31 kD from *B. linens* M18 inhibited the growth of *Listeria* spp., several *Corynebacterium*, and other Gram-positive bacteria [18,66]. Notably, some of the *Bl* 1951 SEC fractions in the assay, instead of producing a prominent zone of inhibition on the lawns of *Bl* 1821L, caused the growth of cells around the paper discs which, based on the literature, are proposed to be persister cells. All the known lineages of bacterial populations are known to harbour a small fraction of transiently antibiotic-tolerant cells known as "persisters" [67]. These cells are characterised by their dormant nature and reduced metabolic activity [68,69]. The genetic basis of persister cells formation is attributed to the role of toxin-antitoxin (TA) systems in dormancy induction [70]. Several TA systems have been suggested as the basis of persister cell formation [68,71,72]. TA systems [73] typically consist of a stable toxin (always a protein) that disrupts an essential cellular process (e.g., translation via mRNA degradation) and a labile antitoxin (either RNA or a protein) that prevents toxicity [74]. Numerous environmental stimuli are also involved in the persister cells formation [75]. [76] demonstrated that DNA damage in *Escherichia coli* inducing the SOS response led to the formation of persisters by stimulating the expression of TisB toxin. The growth phase of the bacterium plays a crucial role in determining the number of persisters, with the highest percentage of persisters found at the stationary phase [77]. Persisters are typically absent in the early exponential phase of growth, but by the mid-exponential phase, persisters begin to appear in the population, and a maximum of approximately 1% is reached during the stationary phase [77-79]. Therefore, it might be possible that in the current study, *Bl* 1821L persister cells were produced in the mid/late exponential phase and a small percentage of these cells exhibited resistance against some of the *Bl* 1951 SEC fractions. However, *Bl* 1821L persister cells lost their resistance upon treatment with the mitomycin C- induced supernatant of *Bl* 1951 confirming their transient nature.

Protein purification through precipitation by using various salts like ammonium sulphate (AS) and polyethylene glycol (PEG) is also in use as a method to purify viral proteins (phages). PEG precipitation followed by sucrose density gradient centrifugation in SDS-PAGE analysis showed a low abundance of the ~48 kD protein band as compared to ~30 kD. A putative antibacterial protein of ~50 kD was purified from *Bl* 1821L strain after ammonium sulphate (85%) precipitation and subsequent sucrose density gradient centrifugation in the bottom 50% and 60% gradients. Since each purification step necessarily involves loss of some of the targeted proteins [80] it is possible that both the precipitation

methods could not purify *Bl* 1951 putative antibacterial protein due to the involvement of several purification steps *i.e.* it was lost through successive purification steps.

5. Conclusions

Using different purification methods putative antibacterial proteins of *Bl* 1821L and *Bl* 1951 were purified and appeared as phage-like capsids (encapsulin) and polysheath-like structures under an electron microscope. Based on these observation, antibacterial activity of these preparations, and the bioinformatics analysis [34,35] these antibacterial proteins are considered as the HMW bacteriocins that are involved in the antagonistic activities against the insect pathogenic isolates *Bl* 1821L and *Bl* 1951. Though, transient in nature but the appearance of persister cell in the population of *Bl* 1821L strain can be useful in future to counter the lethal effects of bacterial antagonists. Overall, this work added a wealth of knowledge for the purification of the HMW proteins (bacteriocins) of the Gram-positive bacteria including *Bl*

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: Size exclusion chromatograph of the ultracentrifuged supernatant of mitomycin C- induced culture of *Bl* 1821L. SEC fractions (pooled) showing putative antibacterial activity upon assessment in the disc diffusion assay are indicated. Figure S2: Size exclusion chromatograph of the ultracentrifuged supernatant of mitomycin C induced culture of *Bl* 1951. SEC fractions showing putative antibacterial activity upon assessment in the disc diffusion assay are indicated. Figure S3: SDS-PAGE analysis of *Bl* 1821L putative antibacterial proteins purified using sucrose density gradient (SDG) centrifugation. (A) Group A (10%-50%) and (B) group B (10%-60%) gradients. Figure S4: SDS-PAGE analysis of *Bl* 1821L putative antibacterial proteins from the culture without mitomycin C treatment purified using sucrose density gradient (SDG) centrifugation. (A) Group A (10%-50%) and (B) Group (B) (10%-60%) gradients. Figure S5: SDS-PAGE analysis of *Bl* 1821L (CFS) putative antibacterial protein purified using sucrose density gradients (SDG) centrifugation. Figure S6: SDS-PAGE analysis of *Bl* 1951 putative antibacterial proteins purified using sucrose density gradient (SDG) centrifugation. (A) Group A (10%-50%) and (B) Group B (10%-60%) gradients. Figure S7: SDS-PAGE analysis of *Bl* 1951 putative antibacterial proteins from the culture without Mitomycin C treatment purified using sucrose density gradient (SDG) centrifugation (A) Group A (10%-50%) and (B) Group B (10%-60%) gradients. Figure S8: SDS-PAGE analysis of *Bl* 1951 (CFS) putative antibacterial protein purified using sucrose density gradient (SDG) centrifugation. The red arrow denotes a faint band of ~30 kD in SDG 40%. Figure S9: SDS-PAGE analysis of *Bl* 1821L putative antibacterial proteins purified using 10% poly ethylene glycol (PEG) 8000 precipitation and sucrose density gradient (SDG) centrifugation. Figure S10: SDS-PAGE analysis of *Bl* 1821L putative antibacterial protein purified using 85% ammonium sulphate precipitation (ASP) and sucrose density gradient (SDG) centrifugation.

Table S1: *Bl* 1821L putative antibacterial proteins assay test and quantification of SEC fractions.

Table S2: *Bl* 1951 putative antibacterial proteins assay test and quantification of SEC fractions. Table

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