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Short Communication

An isolate of *Haemophilus haemolyticus* produces a bacteriocin-like substance that inhibits the growth of nontypeable *Haemophilus influenzae*

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ABSTRACT

Nontypeable *Haemophilus influenzae* (NTHi) frequently colonises the upper respiratory tract and is an important cause of respiratory infections. Resistance to antibiotics is an emerging trend in NTHi and alternative prevention or treatment strategies are required. *Haemophilus haemolyticus* is a common commensal occupying the same niche as NTHi and, if able to produce substances that inhibit NTHi growth, may have a role as a probiotic. In this study, ammonium sulphate extracts from broth culture of 100 *H. haemolyticus* isolates were tested for the presence of substances inhibitory to NTHi using a well diffusion assay. One isolate produced a substance that consistently inhibited the growth of NTHi. The substance was inactivated by protease enzymes and had a molecular size of ca. 30 kDa as determined by size exclusion chromatography. When the substance was tested against bacteria from eight Gram-negative and three Gram-positive genera, only *Haemophilus* spp. were inhibited. Quantitative PCR testing showed the substance to be different to 'haemocin', the previously described bacteriocin of *H. influenzae* type b. These molecular characteristics, together with narrow-spectrum activity, suggest the substance may be a novel bacteriocin, and there is potential for this *H. haemolyticus* isolate to function as a probiotic for reduction of colonisation and subsequent infection with NTHi.

1. Introduction

Nontypeable *Haemophilus influenzae* (NTHi) is an important cause of lower respiratory tract infections, particularly for the elderly, smokers and aboriginal populations [1]. Options for managing these infections are becoming limited due to the emergence of antibiotic resistance [2] and the inefficacy of candidate vaccines [3,4]. To address this problem, the development of a non-antibiotic therapeutic approach should be considered.

Prevention of *Streptococcus pyogenes* respiratory infections by a probiotic strain of *Streptococcus salivarius* has been demonstrated [5], and a similar strategy may prove successful against NTHi infection. *Haemophilus haemolyticus* is a common respiratory tract commensal that occupies the same niche as NTHi and, as such, may have potential as a probiotic. This potential was investigated in a recent in vitro study in which *H. haemolyticus* reduced the attachment to and invasion of pharyngeal cells by NTHi [6].

To further investigate the probiotic potential of *H. haemolyticus*, in this study a collection of *H. haemolyticus* isolates was screened for secretion of substances inhibitory to NTHi. A bacteriocin-like substance (BLS) from one isolate was characterised by determining its spectrum of activity, stability to heat and pH extremes, resistance to enzymatic degradation and molecular size. Quantitative PCR (qPCR) was used to investigate whether the BLS was haemocin, the bacteriocin produced by *H. influenzae* type b (Hib) [7].

2. Materials and methods

2.1. Bacterial collection

Haemophilus haemolyticus ($n = 100$) and NTHi ($n = 100$) isolates were obtained from respiratory tract specimens of patients at five Australian hospitals and were identified by 16S rRNA sequencing and qPCR for species-specific markers as previously described [8]. Hib isolates ($n = 20$) were kindly provided by Dr Ben Howden (Doherty Institute for Infection & Immunity, Melbourne, VIC, Australia). Details of all strains and clinical isolates in this study are given in Supplementary Table S1.

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2.2. Bacterial culture

Bacteria were stored at –80 °C and were cultured initially on chocolate agar for 12–16 h at 35 °C in a humidified atmosphere with 5% CO_2. Broth culture for BLS production was in sBHI, which consisted of brain–heart infusion (Oxoid Ltd., Basingstoke, UK) supplemented with 2% (v/v) Vitox® (Oxoid Ltd.) and 15 mg/L of both NAD and haematin (Oxoid Ltd.). Broth was inoculated to ca. 5×10^7 CFU/mL and was incubated at 37 °C with shaking at 200 rpm for 24 h, except as shown in Fig. 1. The population density was quantified by serial dilution plating on chocolate agar and measurement of the culture broth optical density at 600 nm (OD_{600}).

2.3. Enrichment of bacteriocin-like substance (BLS) in culture broth

The procedure for enrichment of BLS in culture broth was the same as that described for haemocin [9], with the following modifications. For initial screening of *H. haemolyticus* for secretion of substances inhibitory to NTHi, ammonium sulphate was added to 55% saturation. Subsequent testing showed that 70% saturation was optimal for the recovery of a BLS, and this concentration was used for all other enrichments. The ammonium sulphate precipitates were dissolved in a volume of Dulbecco's phosphate-buffered saline equal to 1/20th of the culture broth volume and were then heated at 90 °C for 3 min (except when testing for resistance to inactivation by heating). Enriched solutions were dialysed into 50 mM Tris–HCl (pH 7.5) using a 3500 Da molecular weight cut-off membrane (Snakeskin Dialysis Tubing; Thermo Fisher, Waltham, MA) and were concentrated using a 10 kDa molecular weight cut-off centrifugal filter unit (Merck, Darmstadt, Germany), and finally passed through a 0.2 μm filter prior to chromatography and inactivation studies.

2.4. Well diffusion assay

The presence and activity of BLS were determined by a well diffusion assay as described previously [10], with the modifications described below. The medium consisted of half-strength sBHI solidified with 7.5 g/L Bacteriological Agar (Oxoid Ltd.). Petri dishes containing 10 mL of solidified medium were overlaid with ca. 10^5 CFU of indicator bacteria suspended in 5 mL of the same medium. NTHi strains NCTC 4560 and NCTC 11315 as well as Hib strain ATCC 43163 were used as indicator strains when screening for production of a BLS. A volume of 25 μL of BLS-enriched culture broth was fully absorbed into 5-mm diameter wells in the overlaid plate medium and was incubated at 35 °C in a humidified atmosphere with 5% CO_2 for 18–24 h. Annular radii of clearing zones were then measured.

2.5. Quantitative PCR for haemocin

DNA was extracted using a DNeasy® Blood and Tissue Kit (QIAGEN, Hilden, Germany) and was tested for the presence of the haemocin gene *hmcA* by SYBR Green qPCR at an annealing temperature of 60 °C using the primers 2533F (5′-CAGCTTCGCTAGCAAGT AGTAATG-3′) and 2683R (5′-TGTTTTGCTCCGCATATTGA-3′) designed from the Hib haemocin locus (GenBank accession no. **U68399**).

2.6. Spectrum of activity

The spectrum of activity of a BLS was determined using well diffusion assays as described above, but with the overlaid indicator bacteria variably composed of 102 NTHi, 21 Hib and 30 *H. haemolyticus* as well as strains from the clinically relevant bacteria *Acinetobacter baumannii* ($n = 1$), *Burkholderia cepacia* ($n = 1$), *Enterococcus* spp. ($n = 4$), *Escherichia coli* ($n = 3$), *Haemophilus parainfluenzae* ($n = 2$), *H. influenzae* type c ($n = 1$), *Moraxella catarrhalis* ($n = 1$), *Neisseria lactamica* ($n = 1$), *Proteus vulgaris* ($n = 1$), *Pseudomonas aeruginosa* ($n = 1$), *Staphylococcus* spp. ($n = 4$) and *Streptococcus* spp. ($n = 4$).

2.7. Resistance to inactivation by heat, pH extremes and enzymes

Resistance to inactivation by heat and pH extremes was determined using BLS-enriched culture broths kept at temperatures of 40–90 °C for 8 min, 110 °C for 15 min and pH 1.6–9.7 for 1 h at room temperature. Resistance to enzymatic degradation was tested in 1 mg/mL proteinase K (Bioline, London, UK), 1 mg/mL trypsin (Sigma-Aldrich, St Louis, MO) and 100 U/mL DNase I (Invitrogen, Waltham, MA) with incubation at 37 °C for 20 h and then heating at 90 °C for 3 min.

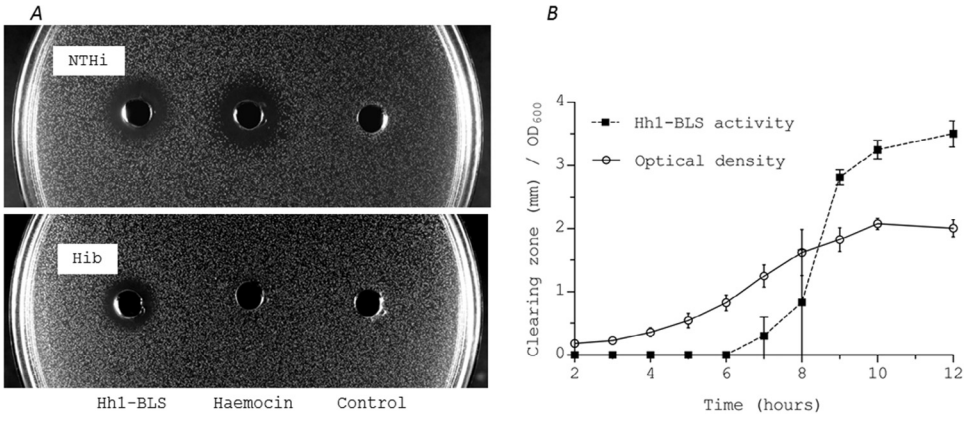

Fig. 1. Activity and production of a bacteriocin-like substance (BLS) by *Haemophilus haemolyticus*. (A) Well diffusion assays demonstrate the inhibitory activity of a BLS produced by *H. haemolyticus* against both nontypeable *Haemophilus influenzae* (NTHi) and *H. influenzae* type b (Hib), whereas haemocin, the bacteriocin produced by Hib, inhibits NTHi only. Wells contain ammonium sulphate extracts from culture broth of *H. haemolyticus* Hh1 (Hh1-BLS), Hib ATCC 43163 (haemocin) and NTHi NCTC 4560 (control, non-producer of BLS or haemocin). (B) Production of Hh1-BLS in broth culture, with time and population density. Activity was first measurable at an OD_{600} of 1.5 at 7 h of incubation and continued to increase until after stationary phase was attained. The medium consisted of sBHI broth at 37 °C with 200 rpm agitation. Annular radii of clearing zones in well diffusion assays were measured. Mean ± standard error of the mean of five independent experiments. OD_{600}, optical density at 600 nm; sBHI, brain–heart infusion supplemented with 2% (v/v) Vitox® and 15 mg/L of both NAD and haematin.