Short Communication

MARINE BIOTECHNOLOGY

Recovery and Characterization of a 30.7-kDa Protein from *Bacillus licheniformis* Associated with Inhibitory Activity Against Methicillin-Resistant *Staphylococcus aureus*, Vancomycin-Resistant Enterococci, and *Listeria monocytogenes*

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Abstract

Of 131 bacterial isolates from seaweed, a culture of *Bacillus licheniformis* produced a novel protein with antibacterial activity against methicillin-resistant *Staphylococcus aureus*, vancomycin-resistant enterococci, and *Listeria monocytogenes*. The antibacterial activity was maximal in cultures prepared in Columbia broth containing pieces of synthetic polyurethane sponge and shaken at 210 to 230 rpm. Antibacterial activity was not found in cultures grown statically or with different speeds of rotary shaking. Reduced activity was apparent in supernatants prepared from marine 2216E broth and tryptone soya broth with or without 1% (wt/vol) sodium chloride. The antibacterial compound was sensitive to proteinase K, pronase, and trypsin, but was not affected by Tween−20, −40, −60, or −80, or α− or β-amylase. Activity was not adversely affected by heating up to 40°C or treatment at pH 5 to 14. The bioactive compound was determined to be associated with a protein of 30.7 kDa, which had homology to the YbdN protein of *B. licheniformis* ATCC 14580.

Keywords: antibacterial protein — *Bacillus licheniformis* — MRSA — VRE

Introduction

With the emergence and spread of highly antibiotic-resistant strains of bacterial pathogens, such as methicillin-resistant *Staphylococcus aureus* (MRSA) and vancomycin-resistant enterococci (VRE) which cause intractable wound infections often as a result of surgery (Richards et al., 2005), there have been extensive efforts to develop effective disease control strategies including chemotherapy involving new compounds, e.g., linezolid (Prokop et al., 2002). However, the search for new chemotherapeutic agents needs to continue in order to discover compounds with effectiveness against antibiotic-resistant strains of bacterial pathogens. In this respect, the marine environment may well be a source of new pharmaceuticals, including antibiotics (Austin, 2001). Consequently, this study focused on the recovery and characterization of compounds with effectiveness against antibiotic-resistant strains of some common bacterial pathogens. The approach was based on the growth of marine bacteria in biofilms, insofar as earlier work (Austin and Billaud, 1990; Yan et al., 2003) demonstrated that the culture of marine bacteria at an interface could induce the production of antimicrobial compounds, which are not otherwise released during planktonic growth.

Materials and Methods

Bacterial Cultures. Fronds of seaweed, *Fucus serratus*, were collected from the foreshore of the Firth of Forth, near Edinburgh, Scotland, and transported to the laboratory in plastic bags, after which 10.0-g

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quantities were shaken for 1 min in 100 ml of sterile 0.9% (wt/vol) saline. A series of 10-fold dilutions up to 10^{-3} were prepared, and 0.1-ml aliquots were spread over the surface of triplicate plates of diagnostic sensitivity test agar (DST; Oxoid), nutrient agar (Oxoid), and tryptone soya agar (TSA; Oxoid) supplemented with 1% (wt/vol) NaCl (TNA) and marine 2216E agar (Difco) and incubated at 28°C for up to 7 days. Bacterial colonies were picked randomly, and 131 were examined for inhibitory activity against MRSA 9551 and MRSA J2407, VRE 788 and VRE 1349 (supplied by Professor S.G.B. Amyes, University of Edinburgh), *Listeria monocytogenes* (NCTC [National Collection of Type Cultures, Colindale, London] 10357 and NCTC 7973), *Escherichia coli* (laboratory culture), *Pseudomonas aeruginosa* (laboratory culture), and *Salmonella enteritidis* (laboratory culture) using a previously described cross-streaking method (Robertson et al., 2000). Briefly stated, the cultures from seaweed were streaked at right angles across lines of inocula of the pathogens, with incubation at 37°C for 24 h. Interruption in the growth of the pathogens was used as evidence for the production of antimicrobial compounds. The purity of the inhibitory cultures was confirmed by colony purification on fresh medium. Pure cultures were stored at −70°C in tryptone soya broth (TSB; Oxoid) with 1% (wt/vol) NaCl (TNB) supplemented with 15% (v/v) glycerol. The identity of the microorganism present in each culture was determined by examination of key phenotypic traits (after Sneath, 1986) and by 16S rRNA gene sequencing. For this, the cultures were grown in TNB for 24 h at 28°C, and the cells harvested by centrifugation at 5000*g* for 10 min at 4°C. The bacterial cells were then lysed, and the DNA extracted using a DNeasy tissue kit (Qiagen), following the protocol for isolation of total DNA from animal tissues. Sequencing was performed via MWG-Biotech using universal primers amplifying a 1500-bp region of the 16S rRNA gene (9F: 5′-GAGTTTGATCCTGGCTCAG-3′, 1492R: 5′- GGYTACCTTGTTAACGACTT -3′). The resulting 16S rRNA gene sequences were used to search the nucleic acid sequence databases using the basic local alignment search tool (BLAST) (Altschul et al., 1990).

Determination of Inhibitory Activity. The methods described by Yan et al. (2003) were used to assess inhibitory activity. In particular, attention focused on biofilm formation in conical flasks, which contained media and pieces of synthetic polyurethane sponges, which were obtained from local supermarkets. Briefly stated, 100-ml volumes of either marine 2216E broth (Difco), TSB, a 1:1 mixture of marine 2216E broth and TSB, or Columbia broth (Oxoid) were dispensed into 250-ml capacity conical flasks to which were added 5×1 cm^3 pieces of synthetic polyurethane sponges to induce biofilm formation. The flasks were then sterilized by autoclaving at 121°C 15 min^{-1}. Inoculation was with 10-μl volumes of overnight broth cultures of the bacterial isolates. The cultures were incubated at 28°C with shaking at 0, 150, 190, 200, 210, 220, 230, 240, 250, and 260 rev min^{-1} on an Innova 2100 Platform shaker (New Brunswick) shaker for 1, 2, 3, 4, 5, 6, or 7 days. Thereafter, the cultures were centrifuged (10,000g for 15 min at 4°C), and the supernatant filtered through 0.22-μm Millipore Millex porosity filters. This was used to assess the presence of antimicrobial compounds by well diffusion (after Jack et al., 1995) and by disk diffusion using 6-mm diameter Whatman No. 1 filter paper disks, which were impregnated with 110-μl volumes of the filtrate. Inhibitory activity was assessed against the bacterial cultures listed previously.

Isolation, Purification and Characterization of the Inhibitory Compounds. Purification of the bioactive molecule(s) was achieved using Columbia broth cultures with pieces of synthetic polyurethane sponges incubated at 28°C for 7 days with shaking at 210 rpm. The culture was centrifuged (10,000*g* for 15 min at 4°C), and the supernatant filtered through 0.2-μm Millipore Millex porosity filters and concentrated by ultrafiltration (Amicon Ultra-15; 10000 MWCO) in which proteins with molecular masses of greater than 10 kDa were collected, concentrated, and taken up in phosphate-buffered saline (PBS; Oxoid; pH 7.4). Concentrated proteins were applied to a Superose 12 HR 10/30 column (Pharmacia, LKB) for gel filtration in PBS at a flow rate of 30 ml h^{-1}. One-milliliter fractions were collected and tested for antimicrobial activity, as before. Also, different solvent systems, i.e., acetic acid, methanol, butanol, and hexane, which achieved different polarities, were used before and after gel filtration and ion exchange to assay for the presence of any active peptide compounds. The procedure involved extraction using a 1:3 mixture of fraction:solvent, with rotary evaporation.

Four milliliters of pooled active fractions (Figure 1; fractions 27 to 30) from each of four gel filtration runs were applied to a FPLC-MonoQ

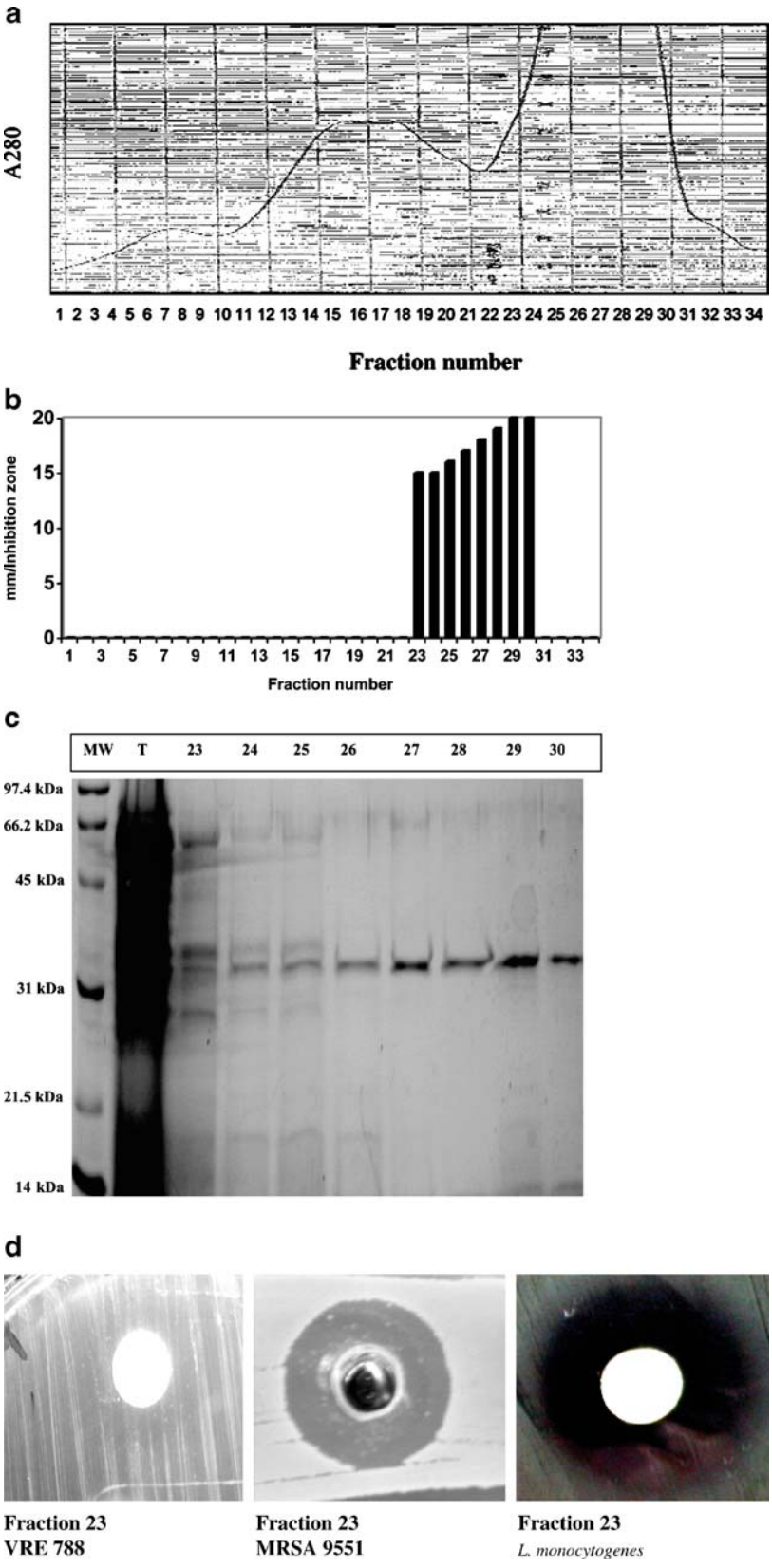

Fig. 1. (**a**) A_{280} profile of the supernatant subjected to gel filtration. (**b**) Inhibitory profile of gel filtration fraction 23 to 30. (**c**) Lanes 23 to 30, SDS-PAGE analysis of the active antibacterial fractions after gel filtration. Lane T contains the supernatant before fractionation. MW contains the molecular weight markers. (**d**) Zones of inhibition resulting from fraction 23.

anion-exchange column and eluted with a 0 to 1 M NaCl gradient in phosphate buffer (pH 7.0) at a flow rate of 1 ml min^{-1}. All the fractions were tested for inhibition against MRSA 9551, MRSA J2407, VRE 788, VRE 1347, and *L. monocytogenes*, and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). This was used to determine the molecular weight of proteins in fractions displaying inhibitory activity (after Laemmli, 1970). A 10% continuous gel was used, and 20-μl amounts of acetone-precipitated protein were loaded together with 5-μl volumes of SDS-PAGE molecular weight standard markers (Bio-Rad, Low range) (diluted 1:20 in SDS reducing sample buffer). After electrophoresis (Bio-Rad 1000/1500) at 100 mA for 30 min and 150 mA for 60 min, the gel was stained with Coomassie brilliant blue (4% wt/vol Coomassie Blue R-250 [Sigma] in 45% methanol-10% acetic acid-45% H_2O [vol/vol]).

The major protein band was excised from the SDS-PAGE gel, and the gel slice was washed twice in MilliQ water and destained by three washes for 20 min with 100 μl of 50 mM ammonium bicarbonate in 50% (vol/vol) acetonitrile. The gel slices were dehydrated in 70% (vol/vol) acetonitrile twice for 20 min, after which the acetonitrile was aspirated and the gel slice dried at room temperature. Ten microliters of 20 ng μl^{-1} trypsin (Trypsin Gold Promega) in 25 mM ammonium bicarbonate was added to the gel slice, which was then incubated for 5 h at 37°C. One microliter of cyano-4-hydroxycinnamic acid matrix (10 mg ml^{-1} CHCA in 0.5% [vol/vol] trifluoroacetic acid and 50% [v/v] acetonitrile) was mixed with 1 μl of trypsin digested sample and spotted onto the sample slide and analyzed via an Ettan MALDI-TOF-MS-PRO analyzer (Amersham Pharmacia Biotech).

The effect of temperature (0, 10, 20, 30, 40, 50, 60 70, 80, 90, and 100°C for 30 min, and 121°C for 20 min), pH 1 to 14 at 28°C for 24 h, and 1.0 mg ml^{-1} α-amylase (Sigma A3403) and β-amylase (Sigma A7005), proteinase K (Sigma P2308), pronase (Sigma P5145), trypsin (Sigma T1426), and 1% (vol/vol) Tween–20, –40, –60, and –80 for 30 min on the inhibitory activity of 100-μl volumes of supernatant and the purified inhibitory compound was examined by means of well buffer diffusion. The enzymes in their respective buffers were incubated with the supernatant/purified inhibitory compound at their optimal temperatures. Experiments with detergent (Tween–20, –40, –60, and –80) were carried out at 28°C. Also, controls involved the use of the enzymes in distilled water. The experiments were carried out in duplicate.

Results and Discussion

From an examination of 131 bacterial isolates, five cultures demonstrated antimicrobial activity against MRSA 9551, MRSA J2407, VRE 788, VRE 1349, and *L. monocytogenes* NCTC 7973, NCTC 10357 by the cross-streaking method. Of these, attention focused on one particularly inhibitory culture, SAFN031, which comprised Gram-positive endospore-forming catalase-positive rods and was identified as *Bacillus licheniformis* (100% similarity by 16S rRNA gene sequencing). Antibacterial activity was maximal in cultures prepared with Columbia broth in flasks, which contained pieces of synthetic polyurethane sponge that floated in the medium, with shaking at 210 to 230 rpm. With this approach, visible growth could be seen on the sponge. In contrast, antibacterial activity was not found in cultures grown statically. Under those conditions, there was less evidence of bacterial biomass in the sponge. Instead, the broth supported more pronounced planktonic growth and displayed increased turbidity. Reduced activity was apparent in supernatants prepared from marine 2216E broth, TSB, or TNB. Moreover, the antibacterial component of the supernatant and the purified inhibitory compound was sensitive to proteinase K, pronase, and trypsin, but was not affected by Tween–20, –40, –60, or –80, or α– or β-amylase, indicating that it was most probably a protein. Activity of the bioactive compound was not adversely affected by temperatures up to 40°C or at pH 5 to 14 (Table 1).

The bioactive protein in the supernatant, which could not be extracted with a variety of solvent systems, was purified by gel filtration and active

Table 1. Effect of Physiochemical Factors on the Activity of the Supernatant and the Purified Protein, YbdN

Physiochemical factor	*Supernatant*	*Purified protein, YbdN*
Proteinase K	–	–
Pronase	–	–
Trypsin	–	–
α-Amylase	+	+
β-Amylase	+	+
Tween-20	+	+
Tween-40	+	+
Tween-60	+	+
Tween-80	+	+
0–40°C	+	+
40–100°C	–	–
121°C	–	–
pH 1–4	–	–
pH 5–14	+	+

+, Activity present; –, no activity present.

fractions were analyzed by electrophoresis; active fractions contained a single protein band of 30.7 kDa by electrophoresis (Figure 1), which was inhibitory to the cultures of MRSA, VRE, and *L. monocytogenes* at doses down to 2 μg of protein on paper discs. When the active fraction from gel filtration was applied to ion-exchange chromatography, inhibitory activity and the protein coeluted as a single peak at 400 mM NaCl (Figure 2). By MALDI-TOF-MS analysis and comparison with the NCBI database, the tryptic digest fingerprint of this protein enabled an identification as YbdN (gi 52784125) of *B. licheniformis* ATCC (American Type Culture Collection) 14580, with a *P*-value of 0.001 and a sequence coverage of 33.5%. This protein is included in the complete genome sequence of *B. licheniformis* (Rey et al., 2004), and a homologous protein is also produced by *E. coli* O157:H7 EDL933 and *B. subtilis.* An alignment of peptide sequences revealed that YbdN of *B. licheniformis* has only 29.1% and 10.8% sequence identity to YbdN from *B. subtilis* and *E. coli*, respectively. Hitherto, YbdN has been regarded as an extracellular protein with unknown function (Tjalsma et al., 2004); however, the NCBI database entry AAU21908 suggests that *B. licheniformis* YbdN has sequence similarity to pectin lyase.

The approach of using Columbia broth, synthetic polyurethane sponge, and shaking at 210 to 230

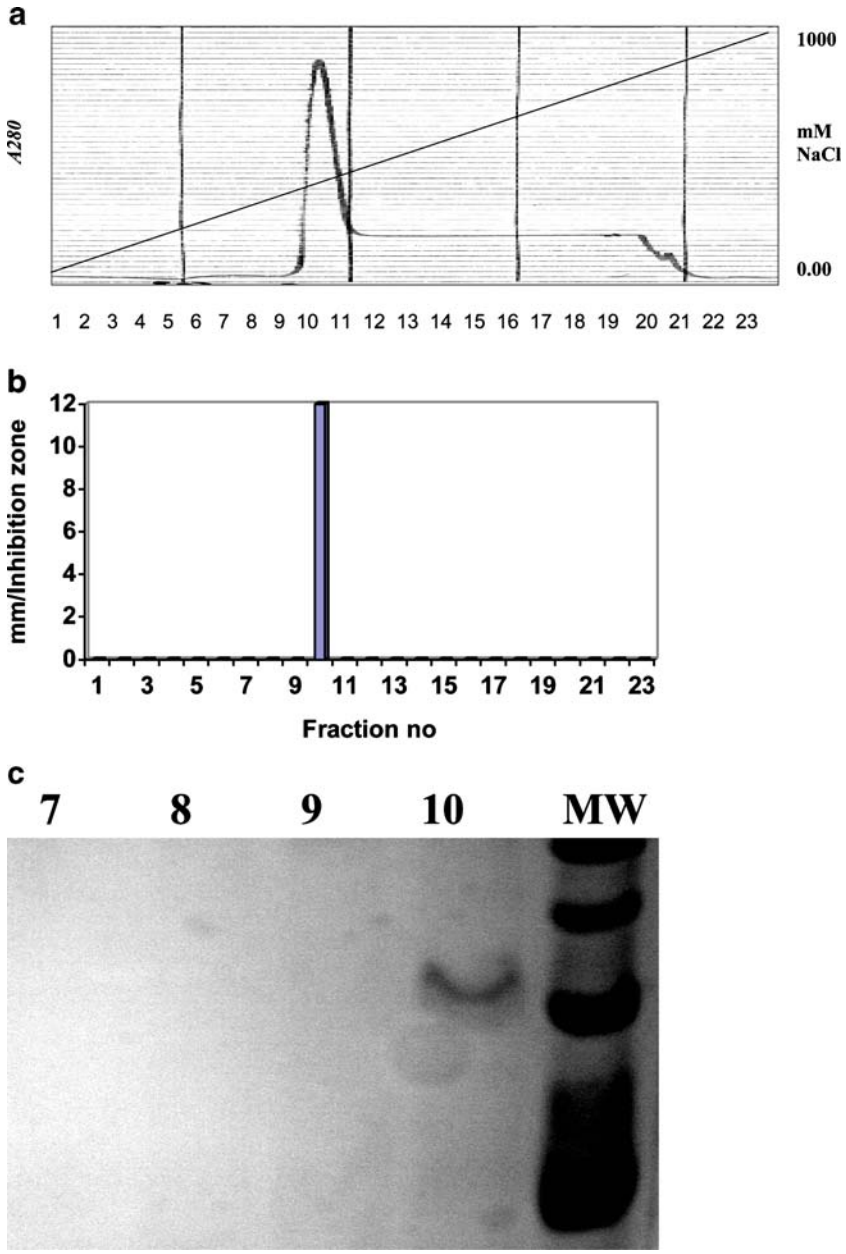

Fig. 2. (**a**) A single protein peak that eluted at 37% to 40% (wt/vol) NaCl was revealed by anion-exchange chromatography of the active gel filtration fractions (see Figure 1). (**b**) Inhibitory profile of ion-exchange fraction (**c**) s. SDS-PAGE analysis after anion exchange of fractions 7 to 10, with inhibitory activity in fraction 10. Lane MW contains the molecular weight markers.

rpm permitted the recovery of a novel protein with effectiveness against antibiotic-resistant strains of human pathogens, specifically MRSA and VRE. The intricacies of this particular method are obscure, and it is difficult to explain the benefit at shaking at 210 to 230 rpm compared to higher or lower settings. This possibly reflects a requirement for optimum oxygen levels, which may be important for biofilm formation (Brindle et al., 1998), or the ability of the sponge to enable the development of a biofilm capable of secreting antibacterial protein.

Gram-positive endospore forming bacilli have long been associated with the production of antibacterial compounds. For example, a wide range of antibacterial peptides has been recovered from *B. subtilis*, *B. brevis*, and *B. polymyxa* and include bacitracin, gramicidin, and polymyxin, respectively (Marahiel et al., 1993). Indeed, the potential value of *B. licheniformis* is attested by the recovery of bacitracin and lichenin (Marahiel et al., 1993; Cladera et al., 2004). Of relevance to this study, some antibacterial proteins have been previously recovered from bacilli, albeit not from *B. licheniformis*. Examples include thuricin (from *B. thuringiensis* and *B. cereus;* Paik et al., 1997; Cherif et al., 2001) and cerein from *B. cereus* (Oscariz et al., 1999). An antibacterial role, specifically against antibiotic-resistant strains of *S. aureus* and enterococci, from the protein recovered in this study is exciting and deserves further examination, especially with regard to cloning the gene encoding YbdN from this isolate.

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