RESEARCH LETTER

Isolation and characterization of a protective bacterial culture isolated from honey active against American Foulbrood disease

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Abstract

Bacterial strains isolated from US domestic honey were screened for antibacterial activity against *Paenibacillus larvae* ssp. *larvae*, the causative agent of American Foulbrood (AFB) in apiaries. A bacterial isolate (TH13) showing a high level of antimicrobial activity against *P. larvae* ssp. *larvae* ATCC 9545 was selected and identified as *Paenibacillus polymyxa* by 16S rRNA gene sequencing. The antimicrobial compound was purified by 80% saturated ammonium sulfate precipitation followed by CM-sepharose chromatography and reverse-phase HPLC. The molecular mass of the compound was determined to be 1168.78 Da by ESI-qTOF MS, matching that of polymyxin E1. The producer strain showed a broad range of antibacterial activity against Gram-positive and -negative bacteria including *P. larvae* ssp. *larvae* ATCC 25747 and foodborne pathogens such as *Bacillus cereus* F4552 and *Escherichia coli* O157:H7 ATCC 43895. The selection of antibiotic-producing bacterial strains indigenous to honey as protective cultures against AFB may lessen the use of antibiotics in apiaries.

Introduction

A spore-forming bacterium, *Paenibacillus larvae* ssp. *larvae*, is established to cause American Foulbrood (AFB), which results in the annihilation of honeybee (*Apis mellifera* L.) larvae and pupae, consequently devastating infected apiaries (Shimanuki, 1990). AFB-infected apiaries suffer from severe economic losses, resulting from significant decreases in honeybee populations and honey production. In addition, *P. larvae*-contaminated honey represents a risk for further spread of this devastating disease (Antúnez *et al.*, 2004). Current control methods include burning contaminated honeybee colonies or treatment of colonies with antibiotics (Shimanuki, 1990). However, the use of antibiotics such as tetracycline and its derivatives to treat AFB can result in the contamination of honey with residual antibiotics (Khong *et al.*, 2005; Martel *et al.*, 2006). Chloramphenicol has been detected in honey and other apiary products in numerous countries (Nakajima *et al.*, 1997; Bogusz *et al.*, 2004; Bogdanov, 2006; Sheridan *et al.*, 2008). Consequently, the residues of antibiotics in bee products may cause potentially lethal allergenic reactions or serious side effects in hypersensitive consumers. An additional concern with prolonged use of antibiotics in apiaries is the emergence of antibiotic-resistant *P. larvae* strains. It has been reported that *P. larvae* ssp. *larvae* isolates from AFB-infected sources exhibited resistance to tetracyclines, the primary antibiotics used to prevent and control AFB (Alippi *et al.*, 2007). Recently, resistance to tetracyclines was elucidated to be due to the existence of a cryptic plasmid in *P. larvae*, and the repetitive use of tetracyclines *in vitro* could induce resistant *P. larvae* strains (Alippi *et al.*, 2007).

Honeybee-associated sources have been tested for their antagonist activity against *P. larvae* ssp. *larvae*. Propolis was tested *in vivo* to cure AFB (Lindenfelser, 1968) and the activities of Brazilian and US propolis against *P. larvae* ssp. *larvae* were investigated by comparison with the conventional antibiotics (Bastos *et al.*, 2008). It was also reported that mid-gut extracts of adult honeybees were assayed to determine their antibacterial activity against wild *P. larvae* ssp. *larvae* (Riessberger-Gallé *et al.*, 2001). Recently, bacterial isolates from honeybee larvae were identified and evaluated to determine their antibacterial activity against AFB disease (Evans & Armstrong, 2006). In addition, aerobic spore-forming bacteria from honeys and apiarian sources were investigated to evaluate their antagonistic activity

against *P. larvae* ssp. *larvae* (Alippi & Reynaldi, 2006). Most of the isolates were identified as *Bacillus* spp. and exhibited very strong activity against the honeybee pathogen. Evans & Lopez (2004) fed nonpathogenic bacteria to honeybee larvae as probiotic cultures to induce an immune response through the upregulated expression of two antibacterial peptides.

In a previous study, we isolated bacterial strains from eight different varieties of honey active against common food spoilage and pathogenic microorganisms (Lee *et al.*, 2008a). Subsequently, characterization of antifungal compounds produced by a bacterial isolate from honey has been conducted (Lee *et al.*, 2008b). In this study, indigenous microbiota from US domestic honey samples were screened for the production of the antimicrobial compounds active against *P. larvae* ssp. *larvae* in an attempt to identify potential protective cultures against AFB. As a potential AFB control method, protective bacterial cultures isolated from healthy honeybee colonies could be used to provide 'natural' protection against *P. larvae* ssp. *larvae*. *Paenibacillus polymyxa* TH13, a bacterial isolate from honey, was found to exhibit a high level of inhibitory activity against *P. larvae* ssp. *larvae* and the antibacterial compound was purified and characterized.

Materials and methods

Screening of honey bacterial stains

Bacterial strains were isolated from a US domestic honey and screened for the production of antimicrobial activity against *P. larvae* ssp. *larvae*. Briefly, honey was harvested from healthy hives without AFB in New York State. One gram of the honey sample was added to 1 mL of sterile deionized water to make a 50% honey solution. One hundred microliters of the solution was spread onto tryptic soy agar (TSA; Hardy Diagnostics, Santa Maria, CA) plates and incubated at 30 °C for 24 h. The isolates were replica plated onto TSA plates and preserved at 4 °C for further study. All of the isolates were stored in 15% (v/v) glycerol at − 80 °C.

Antibacterial activity assay of bacterial isolates

Antibacterial activity of the honey bacterial isolates was tested by deferred inhibition assay (Harding & Shaw, 1990). The isolates were replica plated onto TSA plates and incubated at 30 °C for 48 h. As an indicator bacterium, *P. larvae* ssp. *larvae* ATCC 9545 was grown in American Type Culture Collection (ATCC) medium 540 [brain heart infusion (BHI; BD, Sparks, MD) supplemented with 1 mg of thiamine hydrochloride (Acros Organics, Morris Plains, NJ) per liter of BHI] at 30 °C for 24 h. Molten soft ATCC medium 540 [0.75% (w/v) agar] was mixed with the culture [1% (v/v) inoculum] of the indicator and overlaid onto the plates containing the bacterial isolates. The solidified plates were incubated at 30 °C until the zones of inhibition were discernible. The inhibition zones were then measured and recorded for comparison of activities.

Identification of the producer strains

A bacterial isolate (TH13) displaying a high level of antimicrobial activity was selected for further characterization of the antimicrobial compound produced by this strain. Gram staining and 16S rRNA gene sequencing was conducted to identify the producer strain. Chromosomal DNA was prepared based on the method described by Mengaud *et al.* (1991). The chromosomal DNA of the isolate was used as a template for PCR, with primers designed from the conserved sequence in 16S rRNA genes of bacteria. A set of primers, 16S-FOR (5′-AGAGTTTGATCCTGGCTCAG-3′) and 16S-REV (5′-AAGGAGGTGATCCAGCCGCA-3′), were used to amplify the region of interest. The PCR mixture was heated at 94 °C for 3 min for one cycle and subjected to 30 cycles of 1 min of denaturation at 94 °C, 2 min of annealing at 37 °C, and 1.5 min of polymerization at 72 °C, followed by one cycle of 72 °C for 8 min. The PCR product was run onto 0.7% (w/v) agarose gel and the amplified corresponding band of the conserved sequence was extracted using QIAquick Gel Extraction Kit (Qiagen Inc., Valencia, CA) as per the manufacturer's instructions. Sequencing of the fragment was conducted on the Applied Biosystems Automated 3730 DNA analyzer (Foster City, CA) at the Biotechnology Resource Center, Cornell University (Ithaca, NY). The DNA sequences were analyzed using the NCBI BLAST homology search to identify the genus and species of the isolate.

Purification of the antibacterial compound

The producer strain (TH13) was inoculated into 200 mL of tryptic soy broth (TSB; BD) and grown at 30 °C with agitation (250 r.p.m.) for 36 h. The culture supernatant was recovered by centrifugation at 15 000 ***g*** for 10 min at 4 °C. The antibacterial compound was precipitated by the addition of ammonium sulfate (80% saturation) to the culture supernatant. The precipitate was resuspended in sterile deionized water and loaded onto CM-Sepharose Fast Flow (GE Healthcare, Piscataway, NJ), and equilibrated with buffer A [50 mM Tris-Cl (pH 8.0)]. The cation-exchange chromatography was eluted with buffer A, 0.5 M NaCl in buffer A, and 1.0 M NaCl in buffer A sequentially. The pooled active fractions from the cation exchanger were injected onto an HPLC system (Agilent 1100 series, Agilent Technologies, Wilmington, DE) equipped with a C18 reverse-phase column [Discovery Bio Wide pore C18 (5 μm, 4.6 × 150 cm), Supelco, Bellefonte, PA]. The antimicrobial compound was eluted with a linear gradient of deionized water and 100% acetonitrile (0–100%) containing 0.05%

trifluoroacetic acid (Fisher Scientific, Hampton, NH). The signal intensity was monitored at 214 nm. As external standards, polymyxin B sulfate (Sigma, St. Louis, MO) and colistin sulfate salt (polymyxin E; Sigma) solutions were prepared in sterile deionized water (1000 U mL^{-1}) and injected onto the same reverse-phase (RP)-HPLC system under the same conditions as described above.

Electrospray ionization (ESI)-MS

The RP-HPLC-purified active compound from *P. polymyxa* TH13 was collected and lyophilized to perform further chemical characterization of the antimicrobial compound. MS was conducted to determine the molecular mass of the purified compound at Proteomics and Mass Spectrometry, Donald Danforth Plant Science Center (St. Louis, MO). The compound was analyzed on an ABI QSTAR XL (Applied Biosystems/MDS Sciex) hybrid quadrupole time-of-flight (qTOF) MS system with a nanoelectrospray source.

Activity assay of partially purified antibacterial compound

The 80% saturated ammonium sulfate precipitant was assayed for its antibacterial activity using the well-diffusion method (Ahn & Stiles, 1990). One hundred microliters of the precipitant was loaded into a well (8 mm in diameter) of a TSA plate and allowed to absorb into the agar. Five milliliters of soft ATCC medium 540 [0.75% (w/v) agar] inoculated with 50 μL of *P. larvae* ssp. *larvae* ATCC 9545 overnight culture was overlaid onto the TSA plate containing the precipitant and incubated at 30 °C until the zone of inhibition was observed.

Inhibition spectrum of the producer strain

The producer bacterium (TH13) was replica plated onto TSA plates and incubated at 30 °C for 48 h. The indicator strains (Table 1), including food spoilage and pathogenic bacteria, were inoculated in TSB and incubated at 30 or 37 °C according to their optimal growth temperature. Fifty microliters of overnight culture of the bacterial indicator strains was mixed with TSA soft agar [0.75% (w/v) agar] and the mixture was overlaid on the prepared plates to determine the antibacterial activity of the producer strain. As for *P. larvae* ssp. *larvae* ATCC 9545 and 25747, the activity assays were performed as described in the previous section. The overlaid plates were incubated at 30 or 37 °C according to the optimal growth temperatures of the individual indicators until the indicators had grown completely. Antimicrobial activity was measured by designating + to +++++ for a range of the lowest to the highest activity, with the results of the arbitrary estimation of the inhibition zone size (diameter in mm) formed.

Table 1. Antibacterial spectrum of *Paenibacillus polymyxa* TH13

Indicator strains	Producer strain *P. polymyxa* TH13*
Bacillus cereus F4552	++++
Bacillus cereus ATCC 14579	+++
Bacillus subtilis ATCC 23857	++++
Bacillus subtilis CU1065	+++
Bacillus subtilis CU1065 (sig neg)	++++
Bacillus subtilis JH642 (1A96)	+++
Bacillus subtilis 168 (BGSC no1A1)	−
Bacillus licheniformis ATCC 14580	−
Paenibacillus larvae ssp. *larvae* ATCC 25747	+++++
Paenibacillus larvae ssp. *larvae* ATCC 9545	+++++
Listeria monocytogenes 104035	+++++
Listeria monocytogenes F2 582 1053	+++++
Listeria innocua	−
Staphylococcus aureus ATCC 9144	++
Staphylococcus aureus ATCC 25923	−
Bordetella bronchiseptica ATCC 4617	+++++
E. coli BF2	+++++
E. coli ATCC 25922	++++
E. coli O157:H7 ATCC 33150	+
E. coli O157:H7 933	++
E. coli O157:H7 ATCC 43889	+++++
E. coli O157:H7 ATCC 43895	++
Salmonella Typhimurium ATCC 14028	++++
Salmonella Hartford H0778	++++
Salmonella Gaminara H0662	−
Salmonella Rubislaw F2883	−
Yersinia enterocolitica	−
Klebsiella pneumoniae TR154	++++
Shigella sonnei	++++
Erwinia amylovora SW-2	−
Pseudomonas putida FSL D3375	−
Pseudomonas aeruginosa ATCC 10145	++
Pseudomonas syringae pv. *syringae* B301D	+++
Pseudomonas fluorescens ATCC 11150	++

**Paenibacillus polymyxa* TH13 was spotted onto TSA medium and was assayed to determine the antibacterial activity by deferred inhibition assay. Antibacterial activity was graded from + (lowest) to +++++ (highest activity).

Results

Isolation and identification of the producers

Among the collection of bacterial isolates from US domestic honey samples, one bacterium (TH13) exhibited the highest level of antibacterial activity against *P. larvae* ssp. *larvae* ATCC 9545 and was selected for further characterization of the antibacterial compound. The TH13 strain was a short rod-shaped, Gram-positive bacterium and was determined to be *P. polymyxa* by the NCBI BLAST homology search with the results of 16S rRNA gene sequencing. The producer strain was named *P. polymyxa* TH13.

Antibacterial activity and spectrum of *P. polymyxa* TH13

The antimicrobial production of *P. polymyxa* TH13 was determined by two methods: deferred inhibition and well-diffusion methods. *Paenibacillus polymyxa* TH13 (Fig. 1a) and 80% ammonium sulfate precipitate of the culture supernatant (Fig. 1b) exhibited high levels of antibacterial activity against *P. larvae* ssp. *larvae* ATCC 9545.

Paenibacillus polymyxa TH13 exhibited the highest activity against most *Bacillus* spp. and two *P. larvae* ssp. *larvae* strains (Table 1). *Paenibacillus polymyxa* TH13 also exhibited a broad range of inhibition to Gram-negative human pathogenic bacteria including *Bordetella bronchiseptica* ATCC 4617, several *Escherichia coli* O157:H7 strains, *Pseudomonas* spp. and *Klebsiella pneumoniae* TR154. In addition, the growth of Gram-positive bacteria including food spoilage and pathogenic bacteria, such as *Bacillus cereus* strains and two *Listeria monocytogenes* strains, were strongly inhibited. However, staphylococci were only slightly inhibited or resistant to *P. polymyxa* TH13.

Purification of the antibacterial compounds

The antibacterial activity was detected in the cell-free culture supernatant after 24-h incubation in TSB. The centrifuged culture broth was precipitated by ammonium sulfate, which yielded $>$ 90% recovery of the activity. The compound from *P. polymyxa* TH13 was purified by two steps of column chromatography. Using CM-sepharose, $>$ 90% of the activity from the previous step was eluted with 0.5 M NaCl in buffer A. The fractionated active compound from the cation exchange chromatography was injected onto the RP-HPLC system to obtain the purified compound. The chromatogram of RP-HPLC resulted in a single peak retaining the antibacterial activity (Fig. 2b) and the activity of the purified antimicrobial compound was confirmed by well-diffusion assay (data not shown).

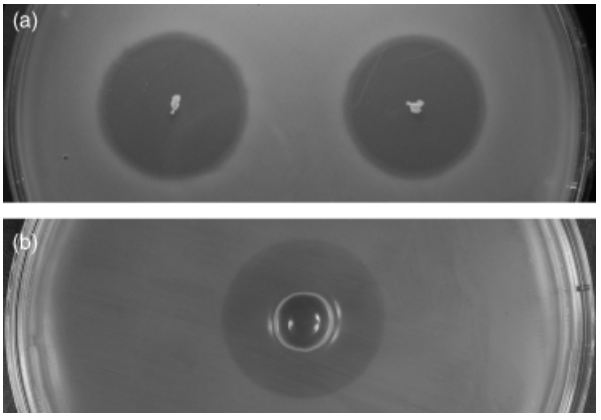

Fig. 1. Deferred inhibition assay of *Paenibacillus polymyxa* TH13 strain (a) and well-diffusion assay of 80% ammonium sulfate precipitate of *P. polymyxa* TH13 culture supernatant (b) against *Paenibacillus larvae* ssp. *larvae* ATCC 9545.

Molecular mass determination

The HPLC-purified antibacterial compound produced by *P. polymyxa* TH13 was characterized by ESI-qTOF MS and the molecular mass of the peptide was calculated to be 1168.78 Da (Fig. 3a). Comparison with all known antimicrobial compounds produced by *P. polymyxa* and other *Bacillus* spp. revealed that the molecular mass was identical to that of polymyxin E1 (Fig. 3b; 1168.77 Da).

Discussion

AFB is a catastrophic disease, resulting in considerable economic loss to the honey industry. In this study, we attempted to screen bacterial strains isolated from honey for the production of antibacterial compounds targeting *P. larvae* ssp. *larvae* as potential protective cultures against

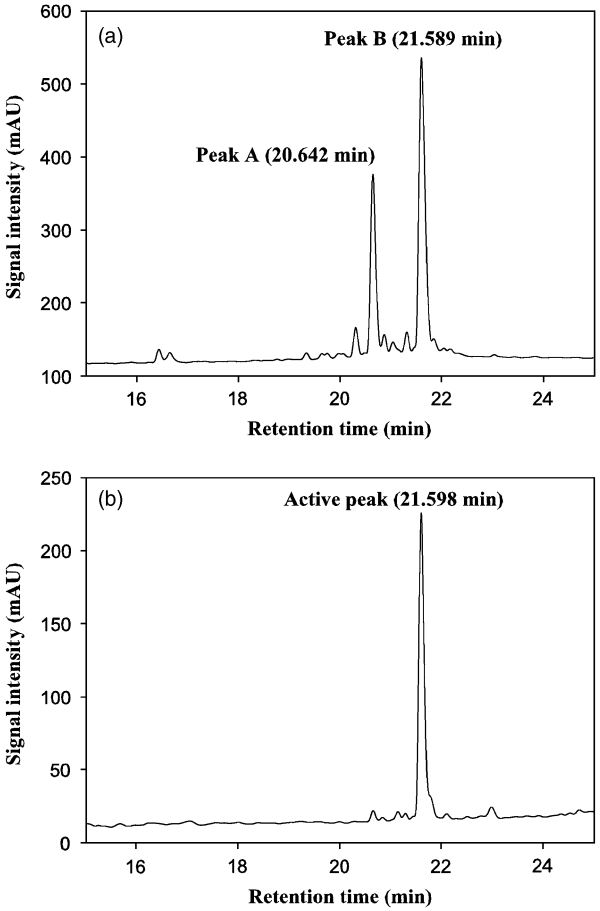

Fig. 2. Chromatogram of colistin sulfate salt (polymyxin E) (a) and CM-sepharose Fast Flow active fraction on RP-HPLC (b) with a C18 column by the gradient of deionized water and acetonitrile as the mobile phase.

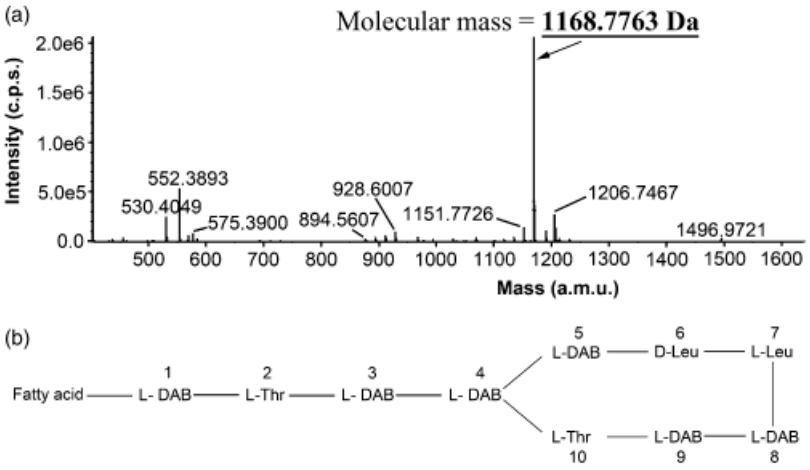

Fig. 3. ESI-qTOF mass spectrum of the purified antibacterial peptide from *Paenibacillus polymyxa* TH13 (a), and the chemical structure of polymyxin E (b). In (b), DAB is α,γ-diaminobutyric acid and fatty acid represents 6-methyloctanoic acid (polymyxin E1) or 6-methylheptanoic acid (polymyxin E2).

AFB. For the characterization of the antimicrobial compounds produced by *P. polymyxa* TH13, polymyxin B sulfate and colistin sulfate were analyzed as external standards on RP-HPLC to compare the retention time of their major peaks with that of the purified antimicrobial compound. The chromatogram of polymyxin B sulfate did not show any peak eluting at the same retention time as that of the purified compound from *P. polymyxa* TH13 (data not shown). However, one of the major peaks from the colistin sulfate solution injection (Fig. 2a; peak B) was eluted at 21.6 min, the same retention time as that of the purified compound (Fig. 2b). The HPLC results were confirmed by the result of MS; the molecular mass of the compound exactly matched with that of polymyxin E1, 1168.77 Da (Li *et al.*, 2001).

Identification of select bacterial strains from the screening showed a high incidence of *P. polymyxa* that exhibited strong antimicrobial activity against *P. larvae* ssp. *larvae*. Paenibacilli have been known to produce polymyxins with broad spectrums of activity that includes Gram-negative as well as Gram-positive pathogenic bacteria (Storm *et al.*, 1977). More than 15 polymyxins, including polymyxins B, E, M, and their derivatives, have been identified. Polymyxins are classified as peptide antibiotics synthesized by multienzyme complexes, assembling each amino acid and fatty acid moiety step-wise into the chemical structure. Polymyxins are known to bind to lipid A of the bacterial cell membrane, resulting in membrane disruption and causing cell death (Martin *et al.*, 2003). Polymyxins have been reported to exhibit nephrotoxicity and neurotoxicity, which limits the direct application of the purified compounds to apiaries due to possible residues being carried over into the honey. However, polymyxins have been recently reevaluated as good candidates to treat multidrug-resistant pathogens, including *Pseudomonas aeruginosa*, and were described to be less toxic than had been reported previously (Li *et al.*, 2005; Falagas & Kasiakou, 2006).

The population of bacterial strains in finished honey is influenced by the environment during nectar collection and the resident bacterial microbiota in hives as well as honeybee sacs, where honey is matured through dehydration. During honey maturation, the microbiota of honey are subjected to different types of stresses, including osmotic pressure, acidity, hydrogen peroxide and phytochemicals, which selects for microorganisms that are able to survive these stresses. *Paenibacillus polymyxa* TH13 is a spore-forming bacterium, capable of surviving the harsh conditions. Therefore, *P. polymyxa* TH13 may be a promising candidate as a protective strain for honeybee larvae and pupae against AFB. In this study, the honey microbiota was shown to inhibit the growth of the causative agent of AFB. The use of protective cultures isolated from healthy hives could have potential application in apiaries that are prone to AFB, to prevent or control the incidence of AFB. Consequently, balanced populations of microbiota producing antimicrobial compounds active against *P. larvae* ssp. *larvae* in hives as a barrier against AFB may reduce the need for antibiotic treatments.

To our knowledge, this is the first report that identifies an antimicrobial compound produced by a bacterial strain from honey that is capable of inhibiting *P. larvae* ssp. *larvae*. The identification of indigenous bacterial strains that exhibit high antimicrobial activity against *P. larvae* ssp. *larvae* is the first step towards the ultimate goal of providing protection against AFB and potentially lessening the incidence of extraneous antibiotics found in honey.

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