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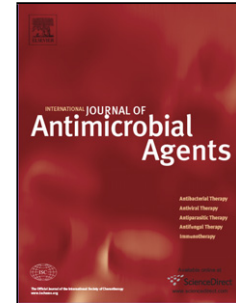
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Effect of manuka honey on the expression of universal stress protein A in meticillin-resistant *Staphylococcus aureus*

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ABSTRACT

Staphylococcus aureus is an important pathogen that can cause many problems, from impetigo to endocarditis. With its continued resistance to multiple antibiotics, *S. aureus* remains a serious health threat. Honey has been used to eradicate methicillin-resistant *S. aureus* (MRSA) strains from wounds, but its mode of action is not yet understood. Proteomics provides a potent group of techniques that can be used to analyse differences in protein expression between untreated bacterial cells and those treated with inhibitory concentrations of manuka honey. In this study, two-dimensional (2D) electrophoresis was combined with matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) to determine the identities of proteins whose levels of expression were changed at least two-fold following treatment with manuka honey. Protein extracts were obtained from cells grown in tryptone soy broth (with or without manuka honey) by mechanical disruption and were separated on 2D polyacrylamide gels. A protein was isolated in gels prepared from untreated cell extract that was absent from gels made using honey-treated cell extract. Using MALDI-TOF MS, the protein was identified as universal stress protein A (UspA). Downregulation of this protein was confirmed by real-time polymerase chain reaction (PCR), which showed a 16-fold downregulation in honey-treated cells compared with untreated samples. This protein is involved in the stress response and its downregulation could help to explain the inhibition of MRSA by manuka honey.

1. Introduction

The prevalence of *Staphylococcus aureus* strains with resistance determinants to multiple antibiotics, both in healthcare settings and in the community, represents a serious health threat and has impacted on clinical outcomes [1]. Currently, β -lactams are ineffective against methicillin-resistant *S. aureus* (MRSA), and multiresistance to antimicrobials including fluoroquinolones and glycopeptides has also been observed [2]. The consequence of increasing antimicrobial resistance is increased patient mortality and morbidity, which is compounded by the relative lack of new antimicrobials in development [3]. To help combat antibiotic resistance, development of new therapies directed at novel targets as potential alternatives to antibiotics as well as evaluation of former remedies is worthwhile.

Honey has been acknowledged since ancient times as a substance of medicinal importance that was used to clear infections and to promote wound healing. Inhibition and eradication of wound pathogens by manuka honey has been demonstrated, with antibiotic-sensitive and -resistant strains proving to be equally susceptible to topical applications of manuka honey [4,5]. It has recently been re-introduced into modern medicine and various formulations are available as regulated wound care products. However, until its mechanism of action is determined, its use may be limited.

Manuka honey is made from nectar that is collected by honey bees (*Apis mellifera*) from a shrub that is indigenous to New Zealand, known as manuka or *Leptospermum scoparium*. Its antibacterial activity was shown to be derived from

components in the honey rather than the sugar content or hydrogen peroxide activity [4], and methylglyoxal (or unique manuka factor) has been identified as the active antibacterial component [6]. Preliminary experiments in our laboratory indicated that exposure of *S. aureus* to 10% (w/v) manuka honey for 4 h caused an interruption to the cell cycle at the stage of cytokinesis [7]. Investigation of the proteins expressed in *S. aureus* during its life cycle and while exposed to honey could potentially help to elucidate mechanisms of action and the response of MRSA to this particular stress.

2. Material and methods

2.1. Bacterial strains, growth conditions and protein extraction

The test organism used in this study was epidemic MRSA type 15 (EMRSA-15) (NCTC 13142). The strain was grown at 37 °C with shaking at 120 rpm in tryptic soy broth (TSB) (Oxoid Ltd., Cambridge, UK) with or without 10% (w/v) manuka honey.

For preparation of cell extracts, cells were grown in 50 mL of TSB with or without 10% (w/v) manuka honey. After 4 h the culture was harvested by centrifugation at $10\,000 \times g$ for 5 min. The supernatant was discarded and the cells were re-suspended in 10 mL of sterile deionised water. Cells were then disrupted at 4 °C using 0.1-mm glass beads in a bead beater (BioSpec, Bartlesville, OK) using three homogenisation cycles of 60 s each. The liquid phase was cleared from beads and insoluble or aggregated proteins by 4 min centrifugation at $13\,000 \times g$ and was then transferred into clean tubes and stored at -80 °C .

2.2. Two-dimensional (2D) electrophoresis

2D electrophoresis methods were adapted from Bernhardt et al. [8] and Kohler et al. [9]. Briefly, 150 μg of protein was added to rehydration buffer giving a final sample volume of 200 μL . An 11-cm pH 3–10 IPG strip (Bio-Rad, Hemel Hempstead, UK) was laid gel-side down onto the sample and was incubated at room temperature for 1 h before covering with mineral oil and incubating at room temperature overnight. The IPG strip was transferred into a focusing cell (Bio-Rad PROTEAN[®] IEF Cell) and was treated at 50 μA for 50 000 volt-hours at 20 °C. IPG strips were removed from the focusing tray and were blotted gently. Strips were placed in the rehydration tray for 30 min gel-side up in 3 mL of equilibrium buffer 1 [6 M urea, 0.375 M HCl, 2% sodium dodecyl sulphate (SDS), 20% glycerol, 2.5% dithiothreitol (DTT)] and shaken in an orbital incubator at room temperature for 30 min. Equilibrium buffer 1 was removed and 3 mL of equilibrium buffer 2 (6 M urea, 0.375 M HCl, 2% SDS, 20% glycerol, 2.5% iodoacetamide) was added and incubated at room temperature, shaken gently for 30 min. Strips were transferred into a pre-cast Criterion[™] Gel Cassette (Criterion[™] XT Precast Cell, 4–12% Bis–Tris; Bio-Rad) sealed with molten agarose and run at 200 V for 55 min in a Bio-Rad Criterion[™] Cell.

Gels were stained using SimplyBlue[™] SafeStain (Invitrogen, Paisley, UK) following the manufacturer's instructions.

Gel images were captured using the UVP AutoChemi[™] gel documentation system and were analysed using PDQuest[™] Basic software v8.0 (Bio-Rad). Gels were run in triplicate.

2.3. Sample preparation of spots for mass spectrometry (MS) analysis

Gel plugs (1.5 mm diameter) of spots of interest were manually excised and placed in a 96-well plate and peptides were recovered following trypsin digestion using a modified version of the method of Shevchenko et al. [10]. Sequencing-grade modified trypsin (Promega, Southampton, UK) was used at 6.25 ng/ μ L in 25 mM NH_4HCO_3 and was incubated at 37 °C for 3 h. Dried peptides were re-suspended in 50% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA) (5 μ L) for MS analysis, and an aliquot corresponding to 10% of the material (0.5 μ L) was spotted onto a 384-well MS plate. Samples were allowed to dry and were overlaid with α -cyano-4-hydroxycinnamic acid (Sigma, Dorset, UK) [0.5 μ L prepared by mixing 5 mg matrix with 1 mL of 50% (v/v) acetonitrile in 0.1% (v/v) TFA].

2.4. Mass spectrometry analysis

MS was performed using a matrix-assisted laser desorption/ionisation time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometer (4800 MALDI TOF/TOF™ Analyzer; Applied Biosystems, Foster City, CA) with a 200 Hz solid-state laser operating at a wavelength of 355 nm [11]. MALDI mass spectra and subsequent MS/MS spectra of the eight most abundant MALDI peaks were obtained following routine calibration.

Common trypsin autolysis peaks and matrix ion signals and precursors within 300 resolutions of each other were excluded from the selection; the peaks were analysed

with the strongest peak first. For positive-ion reflector mode spectra, 800 laser shots were averaged (mass range 700–4000 Da, focus mass 2000). In MS/MS positive ion mode, 4000 spectra were averaged with 1 kV collision energy (collision gas was air at a pressure of 1.6×10^{-6} Torr) and default calibration.

Combined peptide mass fingerprinting and MS/MS queries were performed using the MASCOT Database search engine v2.1 (Matrix Science Ltd., London, UK) embedded in GPS (Global Proteome Server) Explorer™ software v3.6 (Applied Biosystems) on the Swiss Prot database (download date 16 December 2009). Searches were restricted to bacterial taxonomy with trypsin specificity (one missed cleavage allowed), with the tolerances set for peptide identification searches at 50 ppm for MS and 0.3 Da for MS/MS. Cysteine modification by iodoacetamide was employed as a fixed modification, with methionine oxidation as a variable modification.

Results were evaluated by manual inspection and conclusive identification was confirmed if there was high-quality tandem MS (good *y*-ion) data for two or more peptides (*E*-value $P < 0.05$ for each peptide; overall $P < 0.0025$) or one peptide (only if *E*-value was $P < 0.0001$).

2.5. Extraction of RNA and real-time polymerase chain reaction (PCR)

Cells were grown as above. RNA was isolated using Promega SV Total RNA Isolation Kit and cDNA was prepared using an Applied Biosystems High-Capacity cDNA Reverse Transcription Kit, both according to the manufacturer's instructions.

Real-time PCR was performed on all samples using Fast SYBR Green (Applied Biosystems), with the procedures suggested by the manufacturer, on a CFX96 Real Time PCR System (Bio-Rad). Primers (Table 1) were designed using the National Center for Biotechnology Information's Primer-BLAST to be 20–24 bases long, with a GC content of >50%, a melting temperature of ca. 60 °C and to amplify PCR products of 137 bp (Yqil) and 380 bp (UspA). All reactions were carried out in triplicate and expression of UspA was analysed with reference to the expression of the housekeeping gene acetyl coenzyme A.

3. Results and discussion

Analysis of the 2D gel prepared from manuka honey-treated cell extract showed that one spot was absent compared with the control cell extract gel (Fig. 1). After analysis by MALDI-TOF, the protein was identified as universal stress protein A (UspA). The protein was Y1788_STAAR, putative universal stress protein SAR1788 OS=*Staphylococcus aureus* (strain MRSA252) Category 1 ID. The total number of peptides with tandem MS data was 6. Percentage sequence coverage 60%. The three best sequence and the *E*-values to go with those were:

LAHEINADLIMSGTSGLNAVER Expect: 9e-015

TYSSYEVYDAQFTEK Expect: 1.4e-010

FIVGSVSESIVR Expect: 1.1e-009

The UspA superfamily is found in many microorganisms, including bacteria, Archaea and fungi as well as some higher organisms [12]. They are normally induced in response to stress conditions such as temperature shock, starvation and the presence of agents that arrest cell growth. Mutant cells lacking UspA have been shown to be less fit by growing more slowly and dying prematurely during growth arrest.

In this study, a decrease in UspA was confirmed by quantitative PCR, which showed a 16-fold decrease in the expression of UspA in honey-treated cells compared with control cells. This was an unexpected response as stress conditions usually cause increased expression of UspA, which is an autophosphorylating serine and threonine phosphoprotein [12]. Only treatment of cells with tetracycline and extreme temperatures has previously indicated a drop in UspA expression [12]. Cells treated with manuka honey have been shown to become unable to divide, dying in stasis [7]. This kind of premature death has also been reported in *Escherichia coli* UspA mutants [13] and supports our inference that manuka honey lethally affects the cell cycle. As UspA is responsible for a general stress endurance activity, its removal would make the cells more vulnerable to environmental perturbations. Deletion of UspA results in increased sensitivity of *E. coli* cells to stress agents and DNA-damaging agents [14,15]. It raises the question of whether, in combination with antibiotics, honey could make MRSA more susceptible to antibiotics by removing the general stress endurance response.

MRSA is able to colonise and survive in numerous environments and causes a range of illnesses. It can be difficult to control as its adaptability allows it to overcome

many stresses. This new insight into the mode of action of manuka honey may help to encourage its acceptance as a first-line treatment. It is thought that functioning UspA is a key element required for full pathogenicity [14]. Removal of a part of the general stress response of MRSA may explain how honey is able to eradicate bacteria from colonised wounds and limit wound infections. This is the first time that an alteration in the expression of a specific protein following exposure to manuka honey has been demonstrated. Further investigation into the other proteins upregulated and downregulated by treatment with manuka honey is in progress.

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Competing interests

RC has received grants from the British Society for Antimicrobial Chemotherapy (BSAC), the Society for General Microbiology (SGM), the European Wound Management Association, the University of Waikato (in collaboration with the National Honey Board) and the Waterloo Foundation. Sponsorship to attend scientific meetings has been received from Capilano and Derma Sciences Inc.; consultancy has been undertaken for Aspen Medical, Brightwake Ltd., Comvita UK, Derma Sciences Inc., Medlock Medical and Medihoney; remuneration for

presentations has been received from the Tissue Viability Society, the American Professional Wound Care Association, Derma Sciences Inc., Comvita UK, World Union of Wound Healing Societies and numerous beekeeping organisations. RJ and NB declare no competing interests.

Ethical approval

Not required.

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Fig. 1. Two-dimensional gels of proteins from epidemic meticillin-resistant *Staphylococcus aureus* type 15 (EMRSA-15) strain (NCTC 13142): (a) control cell extract gel with mass spectrometry-identified universal stress protein A (UspA); and (b) honey-treated cell extract gel with UspA spot absent. Sample load was 150 μg of protein.

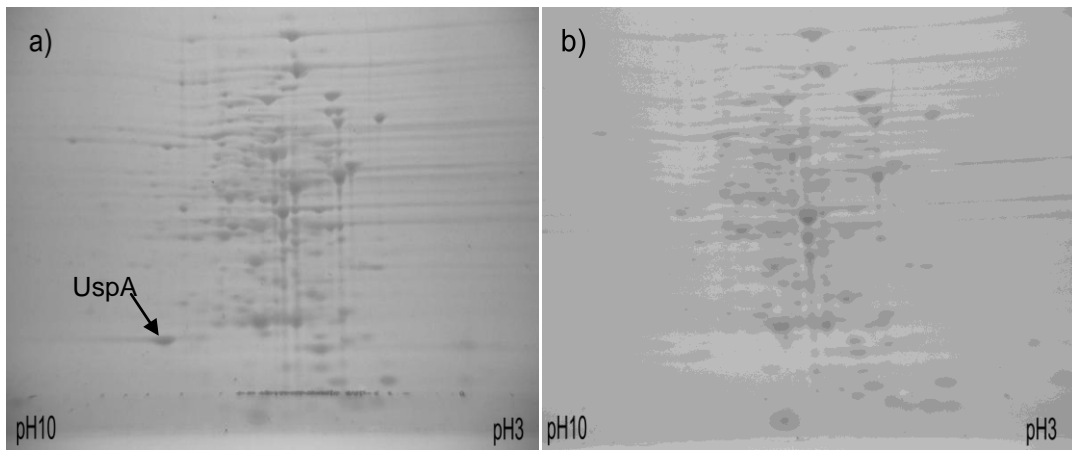
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Table 1

Primers used in this study

Target	Direction	Primer sequence (5'→3')
Yqil	Forward	GACGTGCCAGCCTATGATTT
Yqil	Reverse	ATTCGTGCTGGATTTTGTCC
UspA	Forward	GGTTCACATGAAGCGGAATGGGCA
UspA	Reverse	ACGTCACATGGAGCATGACGAACG

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