Manuka honey is bactericidal against
*Pseudomonas aeruginosa* and results in differential expression of oprF and algD

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INTRODUCTION

Burns are one of the most prevalent skin traumas in modern society, and subsequent infection is common. *Pseudomonas aeruginosa* has been associated with burn infections for decades (McManus et al., 1985; Shankowsky et al., 1994; Tredget et al., 2004), and despite progress in treatment, infection remains the main cause of death amongst burn patients. Multidrug-resistant strains of *P. aeruginosa* have become commonplace due to their intrinsic resistance to antimicrobial agents (Strateva & Yordanov, 2009) and ability to acquire new antibiotic resistance mechanisms (Aloush et al., 2006). In addition to key immune defence mechanisms (Nikaido, 1994), *P. aeruginosa* possesses numerous virulence factors that also facilitate infection (Tredget et al., 2004). Once *P. aeruginosa* infections in burn wounds have established, they often become chronic, a state that is associated with the presence of bacterial biofilms (Bjarnsholt et al., 2008; James et al., 2008). Many currently used antimicrobials are ineffective against *P. aeruginosa*; therefore, novel therapeutic agents are sought for the successful management of wounds in burn patients.

Honey is a natural product and for many centuries was held in high regard due to its antibacterial properties (Crane, 2001). Such effects have been observed against more than 80 bacterial species, including both Gram-positive and Gram-negative bacteria, and multidrug-resistant pathogens. It is for this reason that the use of honey has seen a resurgence in modern wound care management (Molan, 1992a) (for an in-depth review of this topic see Cooper et al., 2009). The inherent antibacterial properties of honey are partly conferred by sugars, which account for 80% of its weight, resulting in a high osmolarity and low water activity (Molan, 1992a). Hydrogen peroxide has been identified as the main antimicrobial component of many honeys (Allen et al., 1991), but in manuka honey, this activity is limited (Kwakman et al., 2011). Instead, methylglyoxal has been recognized as the major antimicrobial constituent (Adams et al., 2008). Furthermore, some 200–600 additional components have so far been identified, including leptosin, and some are likely to contribute to the observed antimicrobial properties of manuka honey (Bogdanov et al., 2008; Kato et al., 2012).

The method by which manuka honey inhibits bacterial cells is beginning to be understood, and so far a diverse mechanism of activity has been shown. For example, in *Staphylococcus aureus*, manuka honey interrupts the cell division process following septa formation (Jenkins et al., 2011). Conversely, electron microscopy has shown that manuka honey does not induce these same effects in *P. aeruginosa* but instead results in complete cell lysis (Henriques et al., 2011). For *Streptococcus pyogenes*, manuka honey appears to act as an anti-adhesive, preventing biofilm formation and bacterial aggregation (Maddocks et al., 2012). Therefore, data accumulated so far have suggested that there is no single mechanism of antimicrobial action and that a combination of factors appears to result in diverse modes of action.
bacterial inhibition and killing. Here we continue to build on this current body of evidence using the commercially available BacLight Live/Dead cellular viability kit, confocal microscopy, atomic force microscopy (AFM) and quantitative real-time PCR to develop a clearer understanding of the way in which manuka honey interacts with P. aeruginosa at the cellular and molecular level.

**METHODS**

**Bacterial strains and culture conditions.** *Pseudomonas aeruginosa* reference strain ATCC 9027 (NCIMB 8626) was used throughout the study. All cultures were grown aerobically in nutrient broth (NB; Oxoid) in a shaking water bath at 100 r.p.m. for 24 h at 37 °C to promote planktonic growth.

**Manuka honey.** Sterile (gamma-irradiated) medical grade manuka honey, with a peroxide antimicrobial activity equivalent to 14 % (w/v) phenol (measured as in Allen et al., 1991; data not shown).

**Minimum inhibitory and bactericidal concentrations.** MIC for manuka honey against *P. aeruginosa* ATCC 9027 was determined using a broth micro-dilution method. Stationary-phase *P. aeruginosa* cells were equilibrated to OD_{500} of 0.5 and further diluted 40-fold into NB containing a range of manuka honey concentrations (1–20 %, w/v, in increments of 2 %) in the wells of a 96-well microtitre plate. Cultures were incubated aerobically for 24 h at 37 °C. The MIC was determined as the lowest concentration with no visible growth. To establish MBC, 100 μl samples from wells with no visible growth was spread onto nutrient agar (NA; Oxoid) and incubated aerobically for 24 h at 37 °C. The MBC was determined as the concentration at which no growth was observed on NA plates. Three biological samples were analysed on separate occasions.

**Effect of manuka honey on the growth and viability of *P. aeruginosa*.** To determine the effects of manuka honey on the growth of *P. aeruginosa* ATCC 9027, microtitre plates were prepared as described above and the OD_{500} was monitored (Infinite M200; Tecan) at hourly intervals for 24 h. Unincubated control wells were used as a blank for each honey concentration, and the change in optical density was calculated over time. The effect of manuka honey on viability of *P. aeruginosa* was also monitored by estimating population sizes using total viable counts (TVCs). Stationary-phase *P. aeruginosa* cells (5 × 10^6 cells ml^{-1}) were cultured in 20 ml NB containing a range of manuka honey concentrations (3, 6, 12 and 24 %, w/v) in 100 ml Erlenmeyer flasks. Cultures were incubated for 8 h at 100 r.p.m. and 37 °C. At 60 min intervals, samples were collected and diluted via a modified Miles and Misra method (Miles et al., 1938) using 0.25 % Ringer’s solution (Oxoid) and NA plates. NA plates were incubated at 37 °C for 24 h, then the number of bacterial cells surviving treatment was estimated. Three biological samples were analysed on separate occasions and the standard error was calculated.

**BacLight Live/Dead staining.** To determine the effects of manuka honey on the viability of *P. aeruginosa* ATCC 9027, cells were stained using the BacLight Live/Dead cellular viability kit (Invitrogen) and visualized using confocal microscopy (Leica DM6000 B Upright Confocal Microscope). Stationary-phase *P. aeruginosa* cells were diluted to OD_{500} of 0.5 and further diluted 1 in 20 ml NB with and without manuka honey (20 %, w/v) in 100 ml Erlenmeyer flasks. Cultures were incubated aerobically for 2 h at 100 r.p.m. and 37 °C. At 10–30 min intervals, 1 ml samples were collected, washed twice with PBS (Oxoid) and stained with the BacLight viability kit according to the manufacturer’s instructions. Stained samples were viewed using confocal microscopy (Cardiff University, UK). The percentage of cells stained with propidium iodide (PPI; non-viable cells) and SYTO9 (viable cells) in ten random fields of view per sample was estimated using the image analysis program ImageJ.

**AFM.** Cultures were grown in NB, NB + 6 % (w/v) manuka honey or NB + 12 % (w/v) manuka honey for 24 h at 37 °C. Sterile, 12 mm, round, glass coverslips were placed into the culture for 30 min, and visualized using a Veeco Multimode V scanning probe microscope in tapping mode operating on nanoscope version 7.30 software (School of Chemistry, University of Bristol, UK). Tips were Budget Sensor Tap 300s with a nominal operating frequency of ~270 kHz in air.

**RNA extraction and cDNA conversion.** To perform gene expression analysis using quantitative real-time PCR, total RNA was extracted and converted to cDNA. *P. aeruginosa* cells were grown in NB under aerobic conditions for 5 h at 100 r.p.m. and 37 °C to achieve mid-exponential-phase cultures. Cells were then transferred into NB containing manuka honey (12 %, w/v) and incubated for 3 h at 100 r.p.m. and 37 °C. After 3 h, samples were washed twice with PBS, resuspended in 0.4 mg lysozyme ml^{-1}, and incubated at 25 °C for 3 min to lyse cells. RNA extraction was carried out using the SV Total RNA Isolation System (Promega) in accordance with the manufacturer’s instructions. RNA quantification and purity were analysed using a SpectroStar Nano LV is plate at 260 nm and 280 nm (BMG Labtech). Samples were adjusted to 100 ng μl^{-1} and converted to cDNA using the cDNA conversion kit (Applied Biosystems) as per the manufacturer’s instructions.

**Real-time PCR of aggregation genes.** Following initial observations using the BacLight Live/Dead cellular viability kit, real-time PCR primers were designed to amplify fragments of genes associated with aggregation and microcolony formation (Sriramulu et al., 2005). Primers were designed using Oligo Perfect Primer Designer (Sigma–Aldrich) and *P. aeruginosa* PAO1 sequence data from the *Pseudomonas* genome database (www.pseudomonas.com) as ATCC 9027 sequence data were unavailable. Gene targets and primer sequences were as follows: alfG (Fwd: GCGGCGAGATGATCAAGTAC; Rev: AGGTGAGGTTGTTGCTCT), oprF (Fwd: CTGGAGCCCATGTCCT; Rev: CTGTTGCATTTGATGTTGCT), lasR (Fwd: CGGTGTTCCTGGCGTGCA; Rev: TCAGTTGCTGCTGCTGCTGCTT) and rpoD (Fwd: GCCAGGTATTCAGCTAC; Rev: GCAAGGAGAAATGTGTCGAG). Real-time PCR was performed using the CFX–96 Touch real-time PCR detection system (Bio-Rad) and SsoFast EvaGreen Supermix (Bio-Rad). PCR reactions were performed in 10 μl with 5 ng template cDNA and 500 nM oligonucleotides (600 nM for the *rpoD* housekeeping gene). PCR parameters were as follows: 30 s at 95 °C, followed by 40 cycles of 3 s at 95 °C and 3 s at 60 °C. Primer dimers and secondary structure formation were evaluated using melt curve analysis. Results were analysed using the ΔΔC_t method and changes in copy number (normalized against the housekeeping gene *rpoD*) were calculated.

**RESULTS**

**Inhibition of planktonic *P. aeruginosa* by manuka honey and growth analysis**

The MIC and MBC of manuka honey against *P. aeruginosa* ATCC 9027 were determined to be 12 % (w/v) and 16 % (w/v), respectively. Growth curves with half-MIC concentrations resulted in a reduced growth rate and reduction in...
overall cell number (Fig. 1) compared to untreated cells. Growth curves for cells incubated with quarter-MIC concentration exhibited increased growth compared to untreated cultures at 12 h; however, this was not sustained, and by the onset of death phase, there was less growth overall, compared to the untreated cultures. Additionally, manuka honey at MIC values prevented the growth of \textit{P. aeruginosa} ATCC 9027. TVC data with identical concentrations of manuka honey showed a sixfold decrease in the total number of bacterial cells (Fig. 2) over an 8 h treatment period with 12\% (w/v) manuka honey. Lower concentrations of manuka honey (3 and 6\%, w/v) resulted in less than onefold reductions throughout the 8 h treatment period, with consistent TVCs below those of the untreated samples. Samples treated with double MIC concentrations (24\%, w/v) had an additional log fold reduction in c.f.u. ml\(^{-1}\) at each time point when compared to MIC values.

**Reduced viability and increased cell lysis in response to honey treatment**

Live–dead viability staining of untreated cells grown over 2 h showed an increase in the percentage of viable, SYTO9 stained cells over time (Fig. 3a) that was not observed for honey-treated cells (20\%, w/v, manuka honey) (Fig. 3b). In addition to this, there was an increase in the amount of non-viable cells from 10\% to 13\% after 60 min, which further increased to 20\% after 120 min. However, only the latter was statistically significant ($P<0.05$), and it is possible that the changes observed at earlier time points were due to experimental error.

Analysis of confocal images showed extensive cell lysis in samples treated with 20\% (w/v) manuka honey for 2 h. At higher magnification, single cell lysis and membrane disruption were observed (Fig. 4a); at lower magnification, extensive bacterial lysis throughout the entire sample was evident, with large amounts of extracellular material visible (Fig. 4b). Spatial differences between the untreated (NB) and treated (NB containing 20\%, w/v, manuka honey) \textit{P. aeruginosa} cells were also observed. The ability of untreated cells to aggregate and form microcolonies (Fig. 5a) was noted after 60 min, but aggregation between cells treated with manuka honey was not observed at any point during the treatment period (Fig. 5b). Samples treated with manuka honey were consistently seen to be stained with both PPI and SYTO9 in such a way that cells appeared two-tone (Fig. 6a). The localization of each fluorescent dye was mapped by identifying fluorescence intensity (Fig. 6b). PPI intensity was highest in the centre of the cell, with SYTO9 intensity remaining constant over the entire cell. This indicated that the PPI was able to permeate the cell despite it apparently remaining viable.

AFM images of control cells showed typical rod-shaped bacteria with single polar flagella (Fig. 7a). Cells treated with 6\% (w/v) manuka honey (half MIC) showed
numerous surface irregularities, suggesting that the cell wall or membrane had been disrupted by the treatment (Fig. 7b). At 12 % (w/v) (MIC), no entire bacterial cells were observed, only cellular debris, suggesting that cell lysis had occurred (Fig. 7c).

Genes involved in cellular aggregation and microcolony formation are differentially expressed following exposure to manuka honey

Quantitative, real-time PCR was used to determine whether the inability of *P. aeruginosa* cells to form microcolonies following honey treatment (12 %, w/v) was the result of differential expression of genes involved in bacterial adhesion or aggregation, and hence microcolony formation. Target genes used in this part of the study were *algD* (GDP-mannose dehydrogenase), *lasR* (transcriptional activator of virulence genes, including those involved in quorum sensing and biofilm development) and *oprF* (outer-membrane porin) (Sriramulu et al., 2005). C<sub>T</sub> values between biological replicas were standardized against the housekeeping gene *rpoD*, and changes in copy number relative to untreated control cells were analysed. The results showed that *algD* expression increased approximately 16-fold (*P* < 0.05) (Fig. 8), whilst *oprF* expression decreased by approximately 10-fold (*P* < 0.05) following exposure to 12 % (w/v) manuka honey. No change was observed for *lasR* (0.8-fold; *P* > 0.05).

DISCUSSION

*P. aeruginosa* is one of the most prevalent colonizers of burns and leg ulcers, leading to significant morbidity and mortality; novel therapeutic strategies are required to combat this multidrug-resistant organism. Manuka honey has previously been suggested as a topical treatment option for burns patients infected with *P. aeruginosa* (Cooper et al., 2002). This study has shown that manuka honey is
inhibitory against \textit{P. aeruginosa} with an MIC and MBC of 12\% (w/v) and 16\% (w/v), respectively. These values were found to meet the criteria for a bactericidal mode of action, confirming previous studies (Cooper \textit{et al.}, 2002; Henriques \textit{et al.}, 2011).

Manuka honey is primarily composed of various mono- and oligosaccharides (Doner, 1977; Siddiqui, 1971), which make up 95\% of its dry weight (Bogdanov \textit{et al.}, 2008). Growth studies with subinhibitory and sublethal doses of manuka honey showed that, at these concentrations (3\%, w/v), growth of \textit{P. aeruginosa} was faster than that of the untreated control. It is likely that the diluted sugars provide a nutrient source and were not yet at inhibitory levels. However, when the cultures were treated with 6\% (w/v) manuka honey, there was a notable reduction in growth over 24 h. It was supposed that, at this concentration, the higher sugar concentrations did not compensate for both the increase in osmolarity and increase in concentration of other inhibitory components of manuka honey; however, further studies to determine the effect of osmolarity alone are required to support this hypothesis (Molan, 1992b).

Growth studies using the MIC (12\%, w/v) resulted in no growth of the cultures, and verified the earlier finding that this concentration was indeed inhibitory. The growth data were corroborated by TVCs and it is clear that the antimicrobial capacity of manuka honey is dose-dependent. This is of significance since, in the wound environment, any exudate would have the potential to dilute topically applied honey. However most medical preparations ensure that manuka honey is applied undiluted, and it is unlikely that it would be diluted below the MIC of 12\% (w/v).

The bactericidal activity of manuka honey was verified using confocal microscopy, and large numbers of non-viable cells were observed following honey treatment, compared to the untreated control. Intracellular material...
was observed surrounding the bacterial cells, suggesting that extensive lysis had occurred in a relatively short period of time (2 h). AFM images verified these data, with abnormal cells being observed following treatment with manuka honey at half the MIC, and no intact cells present in samples treated with the MIC. The dual staining observed by confocal microscopy following honey treatment indicated that the PPI had permeated bacterial cells, but SYTO9 staining suggested that the cells were still viable. It is possible that these images captured the cells in the early stages of lysis, or that manuka honey at this concentration increased the permeability of the cell without killing it, thus allowing PPI to permeate. If this is the case, it might be possible that manuka honey could be used in conjunction with other antimicrobial treatments that are usually removed from the cell by efflux mechanisms. To date, no additional evidence exists to support this hypothesis; however, antibiotic activity has been shown to be improved when used in conjunction with manuka honey in vitro against wound pathogens (Jenkins & Cooper, 2012).

It was also noted that when *P. aeruginosa* was exposed to sublethal doses of manuka honey, the characteristic cell

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**Fig. 7.** AFM showing (a) control, untreated cells, (b) cells treated with 6 % (w/v) manuka honey and (c) cells treated with 12 % (w/v) manuka honey. In each case, the left-hand image shows height, and the right-hand image shows phase.
aggregates observed by microscopy for the control cells were dissipated. Studies using S. pyogenes have shown that sublethal doses of manuka honey inhibit cellular aggregation, which occurs during the early stages of biofilm formation, when microcolonies begin to establish (Maddocks et al., 2012). It is likely that this is also true for P. aeruginosa and that a sublethal dose of manuka honey inhibits cell-to-cell adhesion. It is possible that this occurs sterically, by physical disruption of cell-to-cell binding or that surface structures involved in aggregation and cellular adhesion are differentially expressed, as has been seen for S. pyogenes. Studies using a non-manuka honey and royal jelly have shown that binding and biofilm growth in P. aeruginosa are inhibited, by a process that is thought to competitively block mannose binding lectin, suggesting a steric mechanism of inhibition in which honey components mimic lectin receptor glycoconjugates (Lerer et al., 2007). Given the evidence presented here, it is not unreasonable to hypothesize that the process involves both physical blocking and differential adhesion expression, but further work is required to clarify this.

Three genes (lasR, oprF and algD) involved in microcolony formation were identified from extensive searches of the current literature as possible targets, and differential regulation in response to manuka honey treatment was determined by Q-PCR (Sriramulu et al., 2005). A 10-fold reduction in expression of oprF was observed following honey treatment. The oprF gene encodes a major outer-membrane protein, which has been extensively characterized and is regarded as a major virulence factor (Fito-Boncompte et al., 2011). It has a role in facilitating the diffusion of low molecular mass ionic species and polar nutrients below 1519 Da across the outer membrane (Nestorovich et al., 2006). The structure and positioning of OprF in the outer membrane provides structural stability, forming an anchor point between the outer membrane and peptidoglycan layer (Rawling et al., 1998). The reduced expression of this gene not only could result in the disruption of diffusion, but may also compromise cellular integrity; the latter supports the confocal microscopy and AFM data, which showed a loss of intracellular components and abnormal cell structure. Similar membrane disruption and surface furrows have been observed in previous studies (Henriques et al., 2011); however, such effects were not seen in cultures treated with an artificial honey containing only sugars. It is possible also that the dual staining observed by confocal microscopy resulted from structural surface disruption in the absence of OprF that increased cell permeability, without causing immediate cell death. As well as providing structural stability for the cell envelope, the protrusion of OprF into the external environment has been implicated in adhesion to biotic surfaces and cell binding (Aghzami et al., 2002). Furthermore, OprF has also been shown to play a role in the anaerobic growth of P. aeruginosa (Yoon et al., 2002) and subsequent biofilm formation, concurrent with chronic wound infection (Bjarnsholt et al., 2008; James et al., 2008). The observed reduction of oprF in P. aeruginosa following treatment with manuka honey may therefore, in part, account for the observed inability of honey-treated P. aeruginosa to form microcolonies.

The mucoid phenotype of P. aeruginosa is caused by excessive alginate production and has long been associated with prolonged infection of the pulmonary cavity, particularly in those suffering from cystic fibrosis. Recent studies (Wood & Ohman, 2012) have shown that alginate genes are under the regulation of sigma factor (σ73) and are upregulated as part of an extracytoplasmic stress response to cell wall stress. Of the many genes regulated, 11 are directly involved in cell envelope homeostasis. One of these encodes GDP-mannose dehydrogenase (AlgD), which is essential for the production of alginate, the protection of cells from macrophage mediated killing (Leid et al., 2005) and as a virulence factor (May et al., 1991). It is therefore possible that the 16-fold increase in algD observed in response to honey treatment may be the result of extensive cell wall stress, which itself may be mediated in part by the reduction in expression of oprF. It can therefore be postulated that the observed decrease in oprF causes instability of the cell envelope, making P. aeruginosa susceptible to the osmotic action of manuka honey, which results in the increased expression of algD as the extracytoplasmic stress response system is activated. These findings suggest that it would be inadvisable to employ sublethal honey solutions for the control of P. aeruginosa in treating lung infections of cystic fibrosis patients since any increase in algD expression is likely to promote a mucoid phenotype, which is associated with prolonged infection (Pedersen, 1992).

LasR is known to be involved in the regulation of quorum sensing in P. aeruginosa and studies have shown that in ΔlasR mutants microcolony formation is increased (Passador et al., 1993; Jimenez et al., 2012). Given the inhibition of microcolony formation observed here, it might be expected that the expression of lasR should be inhibited.
increased in response to honey treatment. However, manuka honey had no effect on the expression of lasR. Interestingly, studies have shown that chestnut honey is an effective inhibitor or regulator of quorum sensing in Erwinia carotovora, Yersinia enterocolitica and Aeromonas hydrophila (Truchado et al., 2009), therefore it is possible that other transcriptional regulators governing the expression of surface proteins involved in cellular aggregation are differentially expressed in response to manuka honey, and further work is required to investigate this phenomenon.

It is evident that manuka honey is effective at inhibiting the growth of P. aeruginosa, causing abnormal cell phenotypes by reducing structural integrity to the point of cell lysis. At the honey concentrations used in this study, these effects are unlikely to be caused by the sugars present in the honey, but when undiluted honey is used in treating wounds, its osmolarity is likely to exaggerate the observed effects (Cooper et al., 2002). The data presented here support previous findings and describe for what we believe to be the first time the effects of manuka honey on P. aeruginosa at a genetic level. The study provides insight into the cellular response to manuka honey and describes a paradigm in which bactericidal effects are facilitated by disruption of the cell envelope caused by the reduced expression of oprF thereby resulting in significant cell wall stress. These effects may be compounded by the high osmolarity of manuka honey. Whether these are the only targets remains to be determined, and the global effect of manuka honey on P. aeruginosa will be the subject of future research.

ACKNOWLEDGEMENTS

The authors would like to thank Dr Tony Hayes and Mark Isaacs at Cardiff University for their technical assistance during confocal microscopy, Dr Andy Collins at the University of Bristol for AFM and Advancis Medical for donating manuka honey to this study.

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Edited by: W. J. Quax