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ORIGINAL ARTICLE

Manuka honey inhibits siderophore production in *Pseudomonas aeruginosa*

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Abstract

Aims: The aim of this study was to determine whether manuka honey affected siderophore production by three strains of *Pseudomonas aeruginosa*.
Methods and results: The minimum inhibitory concentration (MIC) of manuka honey against each of the test bacteria was determined. The effect of manuka honey on siderophore production by three strains of *Ps. aeruginosa* was investigated using the Chrome azurol S assay (CAS) and CAS-agar plates. Manuka honey at ½ and ¼ of the MIC for each strain led to reduced production of siderophores (1·3–2·2-fold less) which was found to be statistically significant when compared to the untreated control.
Conclusions: Manuka honey effectively inhibited siderophore production by all three strains of *Ps. aeruginosa* used in this study. This suggests that manuka honey may impact on bacterial iron homoeostasis and identified a new target for manuka honey in *Ps. aeruginosa*.
Significance and impact of study: *Pseudomonas aeruginosa* is an opportunistic human pathogen that can cause acute, life-threatening or persistent wound infections. Part of the virulence repertoire of this micro-organism includes the ability to sequester iron from the host during infection by the synthesis and secretion of siderophores. Manuka honey may limit wound infection by *Ps. aeruginosa* by limiting its ability to capture iron. This is the first time this mechanism has been investigated.

Introduction

Infections are a considerable risk for patients with wounds and can result in increased morbidity and mortality. Gram negative bacteria cause the most severe infections, especially in burns patients, and *Pseudomonas aeruginosa* is most commonly encountered (McManus *et al.* 1985; Tredget *et al.* 2004). Systemic antibiotics represent the current treatment of choice despite problems sometimes associated with side effects and insufficient tissue penetration as a consequence of impaired blood supply to wound tissues. Increasingly, multidrug-resistant strains of *Ps. aeruginosa* are found associated with wound infections, and untreated or untreatable infections can result in impaired wound healing, bacteraemia or sepsis (Aloush *et al.* 2006; Strateva and Yordanov 2009). Topical antimicrobial agents are attractive forms of treatment that are applied directly to the wound, negating the need for an intact blood supply to the damaged tissues. Manuka honey is an example of a versatile topical antimicrobial agent that is effective against over 80 different species of bacteria, but its precise mode of antibacterial action is only just beginning to be understood. So far the bacterial 'target sites' identified include genes involved in stress response, cell division and adhesion to human proteins (Henriques *et al.* 2011; Jenkins *et al.* 2011; Maddocks *et al.* 2012; Roberts *et al.* 2012).

Bacteria colonizing the human host are subject to iron restriction with the majority of iron bound tightly to host proteins (such as transferrin, lactoferrin and ferritins), which form part of the first line of defence against pathogens. To obtain sufficient iron, bacteria must compete with the host and many rely on secreted siderophores to sequester available iron. *Pseudomonas aeruginosa* produces two extensively characterized siderophores, pyochelin and pyoverdin. Pyoverdin is known to have a high affinity for

iron, whereas pyochelin is a lower affinity siderophore (Cox and Adams 1985). Siderophores have long been regarded as virulence factors and siderophores in *Ps. aeruginosa* have been shown to function as signalling molecules (Beare *et al.* 2003). Ordinarily, siderophores are produced only under iron limitation, and therefore interfering with bacterial iron homoeostasis could be one of the ways in which manuka honey limits wound infections caused by *Ps. aeruginosa*. In this study, the universal chrome azurol S assay was used to determine whether manuka honey has the capacity to inhibit siderophore production, and whether a combination of iron limitation and manuka honey impacts on the survival of *Ps. aeruginosa.*

Materials and methods

Bacterial strains

Pseudomonas aeruginosa reference strain ATCC 9027 (NCIMB 8626) and clinical isolates 867 and LE08 were used throughout the study. The clinical isolates were from wound swabs collected from patients with chronic wounds who attended an outpatient clinic at the University Hospital of Wales, Cardiff. All strains were cultured aerobically at 37°C in nutrient broth. To attain iron restriction, 2,2-dipyridyl was added to the media to achieve a concentration of 2 mmol l^{-1}.

Manuka honey

Sterile (gamma irradiated) medical grade manuka honey (Medihoney^{TM}) was provided by Comvita in 50-g tubes and is available as a licensed, commercial medical device (Comvita, Berkshire, UK). It is supplied as a standardized, 100% pure honey derived from the *Leptospermum scoparium* plant in New Zealand.

Minimum inhibitory concentration

The minimum inhibitory concentration for manuka honey against the test bacteria was determined by serial dilution (0 – 50% w/v) in a total volume of 5-ml nutrient broth (Oxoid, Cambridge, UK) (according to British Society for Antimicrobial Chemotherapy methodology for determining minimum inhibitory concentration (MIC); Andrews 2011; Roberts *et al.* 2012). Cultures were incubated for 16 h at 37°C in aerobic conditions. Assays were carried out in triplicate on each of three separate occasions. Where combinations of manuka honey and 2,2-dipyridyl were tested, cultures were incubated with 2,2-dipyridyl at concentrations of 0, 1, 2 and 3 mmol l^{-1} in media containing manuka honey equivalent to ¼ and ½ MIC (for each strain tested). Cultures were incubated as described above. MIC readings were taken using a Spectrostar Nano spectrophotometer at a wavelength of 620 nm.

Chrome azurol S assay

The method to detect siderophore production was based on that described by Schwyn and Neilands (Schwyn and Neilands 1987), cultures were grown under conditions of iron restriction by the addition of 2,2-dipyridyl (2 mmol l^{-1} was found to be the optimum concentration for siderophore production; data not shown) as described above. Chrome azurol S assay (CAS) solution was used for quantification of siderophores in culture supernatants. Cultures were centrifuged for 10 000 ***g*** for 10 min and mixed with the CAS reagent at a ratio of 1 : 1. After reaching equilibration, absorbance readings were measured at 620 nm. CAS-agar plates were prepared as described previously (Deng *et al.* 2006). Standardized suspensions (OD 0·5 at 620 nm) of *Ps. aeruginosa* were inoculated onto separate CAS-agar plates. This procedure was performed in triplicate, and plates were incubated at 37°C for 48 h. Callipers were used to measure the diameter (mm) of observable zones.

Results

A combination of iron restriction and manuka honey treatment impairs growth of *Pseudomonas aeruginosa*

The MIC for the reference strain (ATCC 9027) was found to be 30% (w/v) for manuka honey, and 10% (w/v) for each of the clinical isolates (LE08 and 867) (data not shown). For ATCC, 9027 growth was inhibited as the concentration of 2,2-dipyridyl increased, and the reduction was statistically significant with each increment ($P < 0{\cdot}05$, using Student's t-test) (Fig 1a). With 1 mmol l^{-1} 2,2-dipyridyl, ½ MIC manuka honey completely inhibited growth; with 2 and 3 mmol l^{-1} 2,2-dipyridyl, both ¼ and ½ MIC completely inhibited growth (Fig. 1a). The same pattern of inhibition was observed for clinical isolate 867 (Fig. 1b). However, LE08 required 2 mmol l^{-1} 2,2-dipyridyl and above combined with ¼ and ½ MIC to completely inhibit growth (Fig. 1c). When supplemented with 200 μmol l^{-1} ferric citrate, MICs were restored to that equivalent to honey treatment alone (data not shown). This suggests that manuka honey treatment in combination with 2,2-dipyridyl was more deleterious for microbial growth than either condition individually.

Manuka honey at sublethal concentrations inhibits siderophore production

Each strain of *Ps. aeruginosa* was grown under iron-limited conditions and assessed for siderophore production

using the Chrome azurol S (CAS) assay in liquid culture as well as on solid media. Untreated cells were compared to honey-treated cells using either ½ or ¼ MIC of manuka honey for each of the three *Ps. aeruginosa* strains studied. Cultures were equilibrated to OD 0·5 (A620 nm) prior to assay. Quantitative CAS assay showed 1·8- and 2·2-fold reductions in siderophore production by strain ATCC 9027 treated with ¼ and ½ MIC manuka honey, respectively (Fig. 2). The clinical strains 867 and LE08 showed similar results of 1·6- and 1·3-fold (¼ MIC) and 2·2- and 1·8-fold (½ MIC) reductions in siderophore production, respectively, following honey treatment. In each case, the reduction in siderophore production compared to the untreated control, at either ¼ or ½ MIC, was found to be statistically significant ($P < 0·05$). However, there was no statistically significant reduction in siderophore production between ¼ and ½ MIC. CAS-agar plate assays supported this data, showing a marked reduction in siderophore production evident as a smaller zone of yellow colouration on the blue CAS-agar, following honey treatment at both ¼ and ½ MIC for each strain studied (Table 1). Again a statistically significant reduction in zone size was evident for both ¼ and ½ MIC as compared to the untreated control, but not between ¼ and ½ MIC.

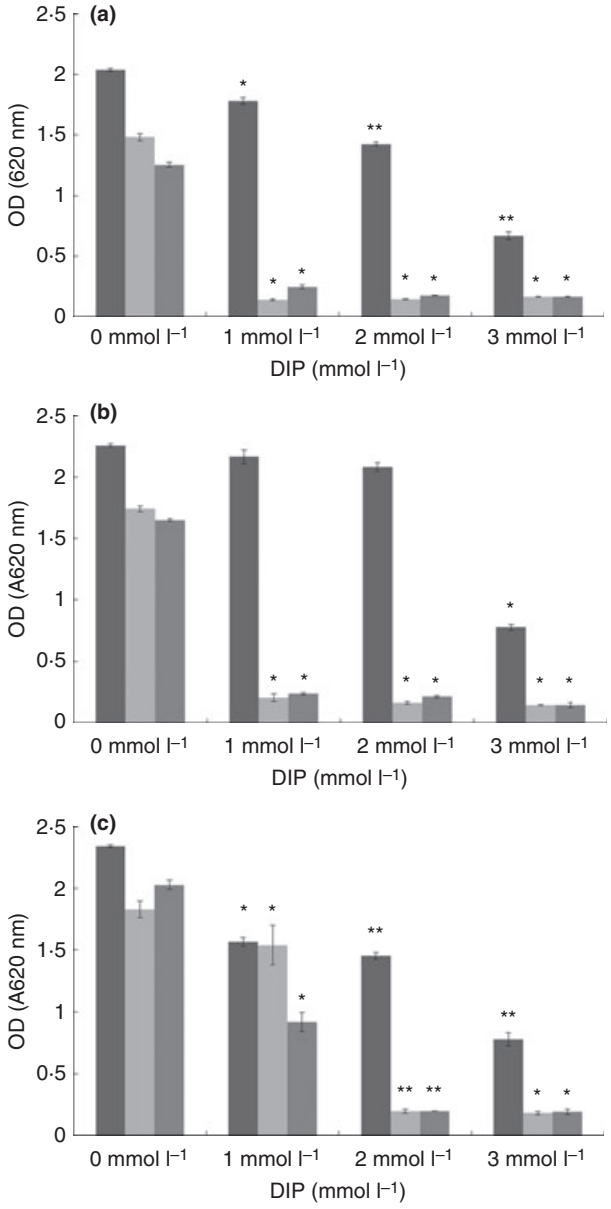

Figure 1 The combined effect of iron limitation and manuka honey on growth of *Pseudomonas aeruginosa*; MIC values are for manuka honey to which 2,2-dipyridyl has been added. (a) strain ATCC 9027; (b) strain 867; (c) strain LE08. Dark grey bars, no manuka honey; light grey bars, ¼ MIC; mid-grey bars, ½ MIC. DIP = 2,2-dipyridyl. Error bars are the standard error of triplicate biological replicates calculated using Minitab (v13). * indicates a statistical difference between the test condition and the control ($P < 0·05$); ** indicates a statistical difference between both the control and consecutive test condition ($P < 0·05$).

Discussion

During the 1960s the relationship between iron and bacterial virulence was recognized (Bullen *et al.* 1967). Due to the presence of numerous iron-containing proteins in the mammalian host, free iron is maintained at a concentration of 10^{-18} mol l^{-1} (Rogers 1973; Fischer *et al.* 1990). This is far below levels of iron necessary to sustain bacterial growth, most species require between 10^{-9} and

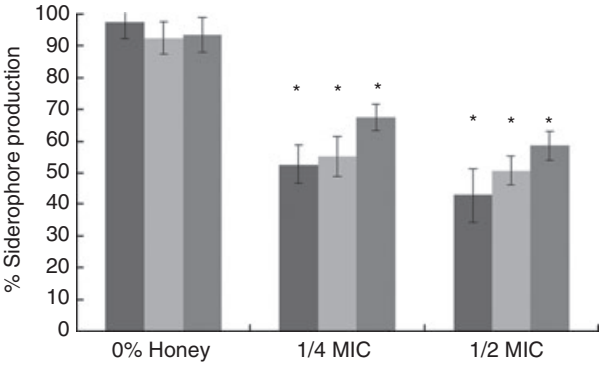

Figure 2 Chrome azurol S assay assay (in liquid) to quantify siderophore production in response to manuka honey treatment. Dark grey, strain ATCC 9027 (¼ MIC = 7·5%; ½ MIC = 15%); Light grey, strain 8626 (¼ MIC = 2·5%; ½ MIC = 5%); mid-grey, strain LE08 (¼ MIC = 2·5%; ½ MIC = 5%). Error bars are the standard error of triplicate biological replicates calculated using Minitab (v13). *indicates a statistical difference between the test condition and the control ($P < 0·05$).

Table 1 Chrome azurol S (CAS)-agar plate assay

CAS-agar plate assay: zone sizes in mm			
Honey treatment	Bacterial strains tested		
	ATCC 9027	LE08	827
0%	7·67 (SE: 0·23)	8·26 (SE: 0·1)	9·77 (SE: 0·1)
¼ MIC	4·59* (SE: 0·18)	5·56* (SE: 0·16)	5·04* (SE: 0·01)
½ MIC	4·26* (SE: 0·03)	4·58* (SE: 0·13)	4·38* (SE: 0·02)

Showing zone sizes (indicating siderophore production) in mm for *Pseudomonas aeruginosa* ATCC 9027, LE08 and 867 in response to manuka honey at the appropriate MIC, ½ MIC and ¼ MIC for each strain studied. Statistically significant ($P < 0·05$) changes as compared to the untreated control are marked with an asterisk (*). The difference between ¼ and ½ MIC was not statistically significant ($P > 0·05$).

10^{-6} mol l^{-1} (Pradel *et al.* 2000). It is the lack of available iron in the mammalian host that is believed to act as a signal to up-regulate both iron acquisition pathways and virulence components in bacteria.

It is evident from the data presented in this study that manuka honey, at sublethal doses, reduced siderophore production in both reference and clinical strains of *Ps. aeruginosa*. The loss of ability to scavenge iron would be highly detrimental to *Ps. aeruginosa* in the host environment. In *Ps. aeruginosa*, the production of pyoverdin is regulated in part by PvdQ, LasR (involved in the regulation of quorum sensing), PtxR (a LysR-type transcriptional regulator) and Fur (Ochsner *et al.*, 1995; Nadal-Jimenez *et al.* 2010; Jimenez *et al.* 2012). Despite the mechanism remaining unknown, it is possible that reduced siderophore production was the consequence of altered responses by these transcriptional regulators. Therefore potentially, honey treatment could also impact upon quorum sensing; it is known that chestnut honey can inhibit quorum sensing in several bacteria including *Yersinia enterocolitica*, *Erwinia carotovora* and *Aeromonas hydrophila* (Truchado *et al.* 2009).

In addition to the observed effect of manuka honey on siderophore production, this study has revealed that under conditions of iron limitation lower concentrations of manuka honey effectively inhibit bacterial growth and to levels equivalent to the MIC. Iron limitation is known to have an inhibitory effect on the growth of many micro-organisms and is regarded as being part of the innate immune defence mechanism to prevent colonization by pathogenic bacteria – the combined effect of manuka honey and low intracellular iron would incur a significant strain on the bacterial cell. However for some pathogens, iron limitation promotes growth and makes the population more stable, this has been noted for mucoid strains of *Ps. aeruginosa* isolated from cystic fibrosis sufferers and is often coincident with increased antimicrobial resistance (Anwar *et al.* 1989). Conversely, this study indicated that manuka honey was more efficacious under iron-limiting conditions; however, this may not necessarily be as a consequence of improved antimicrobial activity. Therefore, the arrest of growth observed in this study might be in part a consequence of the inhibition of siderophore production rather than the direct antimicrobial effects of manuka honey.

Under the conditions of iron limitation used in this study, *Ps. aeruginosa* was hypothesized to rely on siderophore production to sequester any available iron; if siderophore production was inhibited by manuka honey, fewer siderophores would be available to sequester and deliver iron to the micro-organisms, thus impeding growth. Manuka honey contains a relatively low concentration of iron, following dilution of honey in this study; the iron concentration would have been between $8·5 \times 10^{-11}$ and $2·55 \times 10^{-9}$ mol l^{-1} (Crane 1975), which is at the very threshold required for bacterial growth and would likely have been sequestered by 2,2-dipyridyl. Consequently, this was unlikely to contribute significantly to the overall iron availability, thus negating the possibility that additional iron present in the honey would have impacted on siderophore production.

During infection, bacteria are exposed to iron restriction and the application of manuka honey may further compound bacterial stress potentially leading to greater inhibition of growth as observed in this study. Siderophores are classed as virulence factors and their reduced production in response to manuka honey indicates that it acts as an 'antivirulence' therapy. The growing problem of antimicrobial resistance means that identifying new, efficacious treatments are imperative. Compounds that exhibit antivirulence properties are attractive because they don't afford the same evolutionary pressures associated with medications that result in bacterial death. Previous studies have clarified that manuka honey is indeed bactericidal, and this study has shown that it is also an antivirulence agent. The apparent multifaceted action of manuka honey therefore makes it both versatile and effective as an antimicrobial treatment which is paramount in an age where antibiotics are becoming increasingly inadequate.

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