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Manuka honey reduces the motility of *Pseudomonas aeruginosa* by suppression of flagella-associated genes

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Objectives: Manuka honey is a broad-spectrum antimicrobial agent that seems to affect different bacteria in many different ways. It has been shown to be bactericidal against *Pseudomonas aeruginosa* by destabilizing the cell wall, but we aimed to investigate whether there were further intracellular target sites.

Methods: In this study inhibitory effects of manuka honey on *P. aeruginosa* were investigated using hydrophobicity assays, two-dimensional electrophoresis, quantitative RT–PCR, transmission electron microscopy and motility assays.

Results: Exposure of *P. aeruginosa* to manuka honey reduced both swarming and swimming motility. Moreover, this was a consequence of de-flagellation of the bacterial cell, which was correlated with decreased expression of the major structural flagellin protein, FliC, and concurrent suppression of flagellin-associated genes, including *fliA*, *fliC*, *flhF*, *fleN*, *fleQ* and *fleR*. The differential expression of the flagellar regulon in the presence of manuka honey was mapped schematically. Flagella are integral to bacterial adhesion, the initiation of infection and biofilm formation, and swarming has been associated with increased virulence.

Conclusions: By limiting motility *in vitro*, we infer that manuka honey impacts on the virulence of *P. aeruginosa*. This deduction must now be tested *in vivo*.

Keywords: hydrophobicity, swimming, swarming, virulence, wound infection

Introduction

Pseudomonas aeruginosa is a Gram-negative opportunistic pathogen that demonstrates exceptional environmental versatility and extensive antimicrobial resistance. More recently, it has gained notoriety in nosocomial settings due to the emergence and persistence of MDR strains. Between 1993 and 2002, for example, the number of MDR strains isolated from ICUs with resistance to more than three antimicrobials quadrupled ($n=13\,999$) and this trend has continued with time. *P. aeruginosa* has been implicated in both acute and chronic infections. In wounds, *P. aeruginosa* is problematic in burns and neutropenic patients and ~12% of chronic wounds are colonized.[1–3] Venous leg ulcers are particularly susceptible to *P. aeruginosa* infection, resulting in larger wound sites with delayed healing.[4] Furthermore, chronic venous leg ulcers that are colonized with *P. aeruginosa* exhibit a marked decrease in the success rate of skin grafts.[5] An association between chronic wounds and biofilms has been established,[6] and production of rhamnolipid by *P. aeruginosa* has been postulated to contribute to the failure of these wounds to heal.[7]

Many virulence determinants of *P. aeruginosa* have been identified,[8] and this versatile bacterium responds to changing environmental influences that dictate whether a planktonic or biofilm phenotype ensues.[9] Initiation of infection and biofilm formation depend on bacterial adhesion within the wound environment, which is facilitated by altered motility and cell surface interactions. In acute infections the bacterium is usually invasive and relies on the secretion of an array of toxins and proteases with the involvement of flagella, type II and III secretion systems and type IV pili. During the initial stages of cutaneous wound infection, bacterial cells interact with subcutaneous surfaces, resulting in one of two distinct surface-associated behaviours: translocation or attachment.[10] Surface translocation usually precedes attachment and allows the spread of bacterial cells within the wound bed prior to their adherence and growth into a multicellular community. Various motility appendages and a sophisticated chemotaxis system aligns cells along concentration gradients, enabling *P. aeruginosa* to traverse various types of surface by swarming, swimming or twitching, depending on the solidity of their environment.[11–13]

Chronically infected wounds can be difficult to resolve and multidrug resistance means that many currently available antimicrobial treatments are not effective. Innovative antimicrobial interventions for wound infections that have been introduced into conventional medicine during the past decade have included medical grade honey; one example that is often used in the UK and North America is manuka honey. Bactericidal activity of manuka honey against planktonic cultures of *P. aeruginosa* isolated from wounds has been reported.[14] The use of electron microscopy showed that this honey causes structural changes in the cell surface that lead to lysis.[15] These effects were confirmed by confocal microscopy and atomic force microscopy, and decreased expression of OprF (an outer membrane protein that is involved in cell wall stability, diffusion and virulence) was implicated.[16] Additionally, manuka honey inhibits siderophore production and adhesion to human keratinocytes.[17,18] This study aimed to investigate the effects of manuka honey on the motility of *P. aeruginosa* with a view to better understanding its potential to impact on virulence.

Materials and methods

Bacterial strain and culture conditions

A reference strain of *P. aeruginosa*, ATCC 9027 (also known as NCIMB 8626), was used throughout this study and was maintained on nutrient agar (NA; Oxoid, Cambridge, UK) and incubated aerobically at 37°C unless otherwise stated.

Manuka honey

Gamma-irradiated medical grade manuka honey (Advancis Medical, Nottingham, UK) was used throughout this study. The MIC of this manuka honey for the test organism is 12% (w/v).[16]

Hydrophobicity assays

Cell surface hydrophobicity of the test organism was determined using a modified microbial adhesion to hydrocarbon (MATH) test.[19] Essentially, mid-exponential phase cells were diluted 1:1 with nutrient broth (NB; Oxoid, Cambridge, UK) and NB containing manuka honey (6%, 12% and 24% w/v final concentrations) and incubated with shaking (100 rpm) at 37°C for 5 h. At 30 min intervals, 4 mL samples were collected and centrifuged at 4700 rpm for 5 min (Heraeus Multifuge X3 Benchtop Centrifuge; Thermo Scientific, Waltham, MA, USA). The supernatant was discarded and the pellet washed twice in sterile PBS. After two washes the pellet was resuspended in 3 mL of PBS and the absorbance at λ540 nm was measured (pre-hexadecane OD). To this washed cell suspension, 0.8 mL of *n*-hexadecane was added and vortexed for 30 s. The mixture was allowed to separate into an aqueous and *n*-hexadecane phase over 20 min and the absorbance of the aqueous phase was measured at λ540 nm (post-hexadecane OD). Percentage adherence to hydrocarbons was determined using the following formula:

$$\text{Percentage adherence} = \frac{[100 \times (OD_{540}\ \text{pre-hexadecane} - OD_{540}\ \text{post-hexadecane})]}{OD_{540}\ \text{pre-hexadecane}}$$

Hydrophobicity was evaluated using the percentage cell adherence cut-off values previously described.[19] Hence, values of <25%, 25%–75% and >75% were interpreted as negative, intermediate and strong-positive hydrophobicity, respectively.

Preparation of cell-free extracts

Exponential cultures of *P. aeruginosa* were obtained by diluting an overnight culture 1:50 with NB and incubating at 37°C for 5 h. Either 50 mL of NB or 50 mL of NB containing 24% w/v manuka honey was added to the cultures, which were incubated for a further 3 h. Cells were harvested by centrifugation at 10000 **g** (Sorvall RC5B, DuPont Instruments, Cincinnati, USA) for 5 min and resuspended in 3 mL of NB. Cell samples were sonicated (VCX 500, Sonics, Newtown, USA) at 40% amplitude for 2.5 min and the cellular debris was harvested by centrifugation at 13000 **g** (MSE, London, UK) for 4 min and discarded. The supernatant containing cellular proteins was stored at −20°C for no more than 14 days and the protein concentrations were determined using the Bio-Rad Bradford kit (Bio-Rad, Hemel Hempstead, UK) according to the manufacturer's instructions.

Two-dimensional electrophoresis

Methods were derived from Bernhardt *et al.*[20] Briefly, 190 μg protein samples were diluted to 200 μL with rehydration buffer and absorbed into an 11 cm pH 3–10 ReadyStrip™ immobilized pH gradient (IPG) strip (Bio-Rad) during 60 min at room temperature as per the manufacturer's instructions. The strip was then covered with a thin layer of paraffin oil (Fisher Scientific, Loughborough, UK) to prevent evaporation, and the IPG strips were incubated at room temperature overnight. IPG strips were blotted to remove excess paraffin oil and loaded into a PROTEAN® iso-electric focusing (IEF) tray (Bio-Rad) according to the manufacturer's instructions. Paper wicks soaked with 10 μL of deionized (18 MΩ · cm) water were used to connect the IPG strips with the electrode wire. Paraffin oil (2 mL) was overlaid onto the IPG strip and the PROTEAN® IEF tray was loaded into a PROTEAN® IEF cell (Bio-Rad). First-dimension separation of proteins was achieved at 50 μA per strip for 35000 V · h at 20°C, holding at 500 V upon completion. Second-dimension gel electrophoresis was achieved by removing IPG strips from the PROTEAN® IEF tray and using wet blotting paper to remove excess paraffin oil. The IPG strip was placed in an 11 cm rehydration tray, overlaid with 3 mL of equilibration buffer 1 and incubated on an orbital shaker (10 rpm) at room temperature for 30 min. This process was repeated with equilibration buffer 2. IPG strips were dipped in MOPS running buffer to remove excess equilibration buffer and loaded into Precast 4–12% Criterion™ XT Bis-Tris polyacrylamide gel cassettes (Bio-Rad) using the manufacturer's instructions. The IPG strips were sealed in place with bromophenol blue agarose and the polyacrylamide gels were loaded into a 2DGE tank (Bio-Rad). The tank was filled with MOPS running buffer and second-dimension separation of proteins was achieved at 200 V for 45 min.

Identification of differentially expressed proteins

IPG strips were discarded and polyacrylamide gels removed from their plastic casing. Polyacrylamide gels were washed three times by adding 100 mL of 18 MΩ · cm water, microwaving for 1 min at 950 W and discarding the water. Polyacrylamide gels were then stained with 25 mL of SimplyBlue™ SafeStain (Life Technologies, Paisley, UK), microwaved for 1 min at 950 W and incubated on an orbital shaker (10 rpm) at room temperature for 10 min. SimplyBlue™ SafeStain was discarded and the gels were washed with 100 mL of 18 MΩ · cm water and incubated for a further 10 min. After incubation, 20 mL of 20% w/v sodium chloride (Fisher Scientific, Loughborough, UK) was added to each gel and incubation was continued for a further 10 min. The sodium chloride was discarded and the gels were washed with 100 mL of 18 MΩ · cm water and incubated for a further 60 min.

Stained protein spots were photographed using a BioSpectrum® Advanced Imaging System (UVP, Upland, USA) and the images were analysed using PDQuest™ Basic 2-D gel analysis software (Bio-Rad). Gels

containing protein samples from untreated and manuka honey-treated cells were compared using an untreated control. Gels were aligned to the control gel and the number of protein spots that were up-/down-regulated was calculated. Only protein spots that showed >2-fold expression differences were counted during analysis. Select protein spots that demonstrated significant differences between the two gels were analysed by MALDI-TOF MS.

MS

MALDI-TOF MS was completed using a MALDI TOF/TOF mass spectrometer (4800 MALDI TOF/TOF Analyzer, Applied Biosystems, Foster City, USA) and a solid-state laser (200 Hz) operating at 355 nm.[21–24] Peptide mass fingerprinting was conducted using Global Proteome Server Explorer software (v3.6) in conjunction with the MASCOT database search engine (v2.1) on the UniProt database (downloaded 10 December 2010).[25] Searches were restricted to the *Pseudomonas* genus and proteins identified were based on high-quality tandem MS data for at least two peptides (E value $P<0.05$ for each peptide, overall $P<0.0025$).

Quantitative PCR

To investigate the expression of flagellum-associated genes (*fliA*, *fliC*, *flhF*, *fleN*, *fleQ* and *fleR*), primers were designed (Table 1) and *rpoD* was used as a housekeeping gene. *P. aeruginosa* ATCC 9027 cells treated or not treated with 12% and 24% (w/v) manuka honey for 24 h underwent RNA extraction, cDNA conversion and quantitative PCR expression analysis as described previously.[16]

Swim and swarm plate assays

Fastidious anaerobe broth (FAB; Sigma-Aldrich, Dorset, UK) supplemented with either 0.3% or 0.5% (w/v) noble agar was used to detect swimming or swarming motility, respectively.[12] Mid-exponential phase *P. aeruginosa* culture was diluted 1:1 with either NB or NB containing manuka honey (3%, 6% and 12% w/v), and incubated aerobically at 37°C for 3 h. After 3 h of incubation, 1.5 μL of each sample was injected at an angle of ∼45° under the surface of each of the swim and swarm plates and incubated aerobically at 37°C for 24 h to allow the migration of bacteria away from the point of inoculation. Then, the diameter (mm) of each zone of bacterial migration was measured using digital callipers (Mitutoyo) and mean diameters were calculated. Triple replicates were performed on nine separate occasions.

Motility agar assay

Motility agar (Mast Laboratories Ltd, Merseyside, UK) was prepared containing manuka honey at final concentrations of 0%, 1.5%, 3%, 6% and 12% (w/v). Colourless tetrazolium salts in the agar are taken up by viable bacteria and reduced to produce formazan, which is a red insoluble compound. Bacterial growth is indicated by a deep pink colour. Overnight cultures of *P. aeruginosa* were inoculated into the agar using the stab method. Plates were incubated at 37°C for 24 h and the pink zones observed.

Transmission electron microscopy

Mid-exponential phase *P. aeruginosa* cells were diluted 1:1 with either NB or NB containing manuka honey (12% w/v final concentration) and incubated aerobically at 37°C for 3 h. Samples were transferred to 400-mesh carbon-coated nickel grids fixed in 2.5% (w/v) glutaraldehyde (Fisher Scientific, Loughborough, UK) for 10 min, washed six times for 1 min in sterile filtered deionized water and stained with 4% (w/v) aqueous uranyl acetate for 10 min. Cells were visualized using transmission electron microscopy (TEM; FEICM12, Hillsboro, USA) operating at 80 kV and images were collected using a digital camera (SIS MegaView III, Olympus, Münster, Germany). The percentage of flagellated cells in each sample was then calculated.

Statistical analysis

The statistical change in hydrophobicity using the MATH assay was determined using the one-way ANOVA test. The statistical changes in quantitative PCR products were determined using Student's *t*-test (two tailed, two sample—equal variance). Finally, significant changes in swim/swarm colony diameters were determined using ANOVA. Statistical changes in the percentage of flagellated and non-flagellated cells in untreated and manuka honey-treated samples were determined using Student's *t*-test (two tailed, two sample—unequal variance).

Results

Manuka honey resulted in consistently negative hydrophobicity

In the modified MATH assay the adherence of untreated *P. aeruginosa* cells to *n*-hexadecane gradually increased over 5 h (Figure 1), indicating that hydrophobicity changed from negative to intermediate. In comparison, adherence to *n*-hexadecane of all manuka honey-treated samples (6%, 12% and 24% w/v) was significantly reduced over time ($P<0.05$), consistently demonstrating negative hydrophobicity. Differences in the adherence of *P. aeruginosa* to hydrocarbon between subinhibitory and inhibitory concentrations of manuka honey were observed, and determined to be significantly different ($P<0.05$). A concentration response in hydrophobicity values between the two inhibitory manuka honey concentrations (12% and 24% w/v) was not observed during the 5 h treatment period, but the lower values of treated cells compared with untreated cells suggests that exposure to manuka honey disrupts non-specific hydrophobic interactions and reduces adherence efficiency.

Table 1. Primers used for quantitative PCR of flagellum-associated genes

Gene name	Gene locus tag	Forward primer	Reverse primer	Amplicon (bp)
fliA	PA1455	CTCCAATTGAGCCTCGAAGA	TTCGTTGTGACTGAGGCTGG	192
fliC	PA1092	GCTTCGACAACACCATCAAC	AGCACCTGGTTCTTGGTCAG	121
flhF	PA1453	CGAGCCTGAACGTGAAGAAT	GCCTCGTCCAGCTTAGTCA	127
fleN	PA1454	GAGCCGTATACGAGGCATTC	GTGTTGGACCAGTCGTTCG	137
fleQ	PA1097	AAGGACTACCTGGCCAACCT	CCGTACTTGCGCATCTTCTC	134
fleR	PA1099	ACAGCCGCAAGATGAACCT	TGGATGGCGTTGTCGAGTT	109

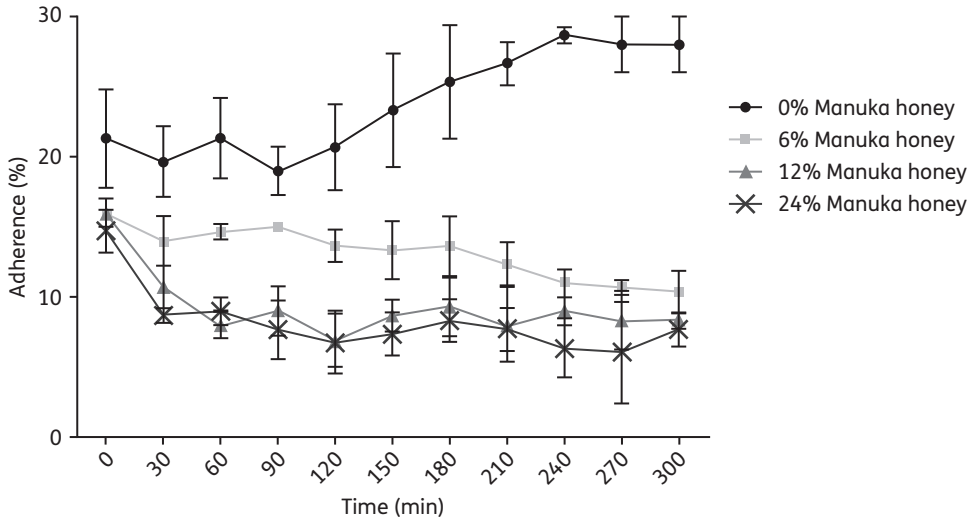


Figure 1. Adherence of *P. aeruginosa* cells to *n*-hexadecane when grown with and without manuka honey. Percentage adherence of *P. aeruginosa* cells to *n*-hexadecane in the absence and the presence of 6%, 12% and 24% (w/v) manuka honey for 5 h (mean ± SD; $n=3$ independent experiments, each with three replicates).

Manuka honey-treated P. aeruginosa exhibited differential protein expression

Following treatment with manuka honey, the expression of 142 proteins was significantly altered (100 down-regulated and 42 up-regulated) ($P<0.05$). Of these, only 111 proteins (86 down-regulated and 25 up-regulated) were differentially regulated by a factor of ≥2. Two representative images, one from untreated and one from honey-treated cells, were overlaid to find proteins with differential regulation and 10 spots (Figure 2) were selected for identification by MS. Five proteins were identified (Table 2); four were at reduced levels, of which three were identified as FliC. Dihydrolipoamide dehydrogenase (DldH2) was also down-regulated in manuka honey-treated samples and a heat shock protein (DnaK) was up-regulated in response to manuka honey.

Expression of flagellum-associated genes was suppressed following manuka honey treatment

Four (*fliA*, *fliC*, *fleN* and *fleR*) of the six flagellum-associated genes investigated showed a statistically significant reduction in gene expression following treatment with 12% (w/v) manuka honey (Table 3) and all (*fliA*, *fliC*, *flhF*, *fleN*, *fleQ* and *fleR*) were significantly reduced compared with the housekeeping gene, following treatment with 2×MIC (24% w/v) (Table 3). This suggests that manuka honey inhibits flagellum gene expression by impacting on both regulatory (*fliA*, *fleN*, *fleQ* and *fleR*) and structural genes (*fliC* and *flhF*), and supports the protein expression data.

Manuka honey resulted in reduced swimming and swarming capacity

Kearns described colonies on swim and swarm plates that typify swimming and swarming motility as colonies with a bullseye-like appearance surrounded by three or two concentric rings, respectively.[12] Such colonies were seen with untreated *P. aeruginosa* samples on swim (Figure 3a) and swarm (Figure 4a) plates. Compared with untreated samples, mean colony diameters of samples pretreated for 3 h with 3%, 6% and 12% (w/v) manuka honey (Figure 3b) on swim plates were significantly ($P<0.05$) smaller in size, by between 15% and 20%. Similarly, swarming was reduced by manuka honey and mean swarm colony diameters were also significantly smaller (17% and 20%) for samples pretreated with 3%, 6% and 12% (w/v) manuka honey (Figure 4b) ($P<0.05$). Swim (Figure 3) and swarm (Figure 4) plates did not contain honey, so the effects of manuka honey on flagellar function reflected by altered motility in these plates must have been initiated during the 3 h exposure period in broth that immediately preceded inoculation.

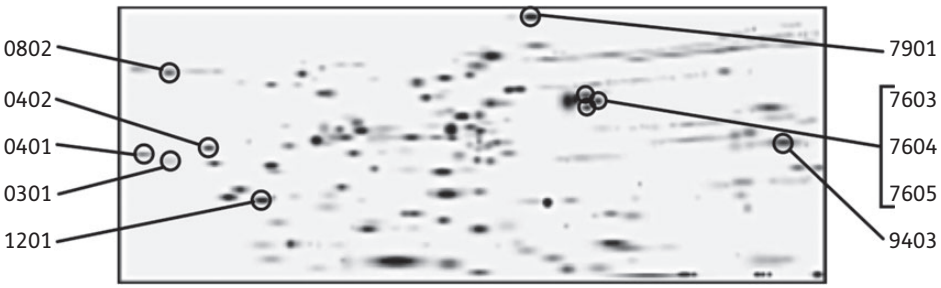
Manuka honey treatment resulted in reduced motility

Tetrazolium motility agar impregnated with manuka honey at 0%, 1.5%, 3%, 6% and 12% (w/v) clearly showed a concentration-dependent reduction in motility in response to manuka honey treatment (Figure 5). Movement through the agar was impeded as the concentration of honey increased, and at 12% (w/v) manuka honey there was no motility observed at all. In plates containing between 1.5% and 6% (w/v) manuka honey, movement was retarded compared with untreated bacteria, but not completely abrogated.

Manuka honey treatment promoted de-flagellation

Using TEM images, flagellated cells of *P. aeruginosa* were readily detected and counted (Figure 6a). Whilst the number of flagellated cells in untreated and honey-treated samples remained similar, the number of non-flagellated cells observed in the manuka honey-treated sample was twice that observed in the untreated sample (Figure 6b). The difference in cellular flagellation was statistically significant ($P<0.05$) as 79% of cells in untreated samples were flagellated, decreasing to 62% in manuka honey-treated samples (Figure 6b).

(a)



(b)

SSP ID	Relative quantity in untreated gel images	Ratio	Relative quantity in treated gel images	Ratio
0301	266*	1	1987	7.45
0401	273*	1	3903	14.27
0402	462	1	4140	8.95
0802	266*	1	4580	17.18
1201	942	1	8056	8.55
7603	2413	1	145*	0.06
7604	2341	1	142*	0.06
7605	2008	1	145*	0.07
7901	1157	1	11461	9.90
9403	10512	1	145*	0.01

Figure 2. Differential regulation of proteins from *P. aeruginosa* cells grown with and without manuka honey. Ten proteins on the master gel (a) were differentially regulated (b) in samples treated with 12% (w/v) manuka honey. *Relative quantity as determined by PDQuest 8.0 due to the absence of a corresponding protein spot.

Table 2. Differentially regulated proteins in response to manuka honey treatment identified using MS in combination with the UniProt database

SSP ID	Protein ID	Accession number	Product	Total number of peptides	Top peptide No. 1	E values No. 2	Mascot score
402	DldH2	DLDH2_PSEAE	dihydro-lipoamide dehydrogenase	4	2.9×10^{-15}	3.2×10^{-10}	473
7603	FliC	FLICA_PSEAE	flagellin	2	1.3×10^{-14}	4.9×10^{-8}	435
7604	FliC	FLICA_PSEAE	flagellin	3	2.9×10^{-8}	7.0×10^{-5}	396
7605	FliC	FLICA_PSEAE	flagellin	2	1.6×10^{-8}	1.0×10^{-3}	250
7901	DnaK	DNAK_PSEAE	heat shock protein	2	3.8×10^{-9}	1.5×10^{-8}	519

Discussion

Detailed information on the ways in which bacteria other than *Pseudomonas* are affected by manuka honey has already been obtained by proteomic and genomic investigations.[26–29] These approaches were therefore applied to *P. aeruginosa* treated with and not treated with manuka honey to explore further inhibitory effects. Following treatment with manuka honey, swarming, swimming and general motility responses by *P. aeruginosa* were impeded. Protein expression analysis identified differential expression of FliC in the presence of manuka honey. Swarming and swimming are both flagellum-dependent; FliC is the key structural component of the flagellum filament, and therefore reduced expression following manuka honey treatment could explain the observed reduction in motility.[30]

Moreover, gene expression analysis identified concentration-dependent suppression of multiple key regulatory genes within the flagellar regulon following exposure to manuka honey, including *fliC*. Transcription of *fliC* is governed by intracellular concentrations of sigma factor (FliA) and the antiactivator of the sigma factor (FlgM).[31] It is likely that the suppression of *fliC* in response to manuka honey (12% w/v) resulted from *fliA* suppression; however, doubling the concentration of manuka honey suppressed *fliC* further whilst having no further effect on *fliA*. This suggests that manuka honey affects various aspects of the flagellar regulon, resulting in the differential suppression of *fliC* in both a FliA-dependent

Table 3. Effect of manuka honey treatment on the expression of flagellum-associated genes in planktonic organisms

	Planktonic fold changes in flagellum-associated genes following manuka honey treatment	
Gene	12% (w/v) honey	24% (w/v) honey
fliA	−2.0*	−2.0*
fliC	−2.5*	−8.3*
flhF	−1.2	−2.0*
fleN	−1.6*	−3.8*
fleQ	−0.25	−2.2*
fleR	−1.8*	−2.7*

*Statistically significant change in the level of expression compared with untreated ($P<0.05$).

and -independent manner. The basal body of flagella is formed from Class III genes of the flagellar regulon, which are under the transcriptional control of FleR and FleS. Manuka honey activity was observed to significantly suppress the expression of *fleR*, and, whilst *fleS* expression was not examined, the contiguous arrangement of *fleR* and *fleS* on a single operon suggests that parallel suppression might also occur.[32]

The ability of *P. aeruginosa* cells to produce a single flagellum located at the polar cap is governed by the Class II genes *flhF* and *fleN*.[33] Suppression of both genes was observed to occur in response to manuka honey activity (12% and 24% w/v); however, *flhF* suppression was not significant at lower manuka honey concentrations (12% w/v). Insignificant changes in the expression of *fliF* correlate with the observation of flagellated cells containing a polar flagellum when viewed using TEM. MATH data also supported a loss of flagella as *P. aeruginosa* treated with manuka honey was seen to have reduced adhesive properties. The

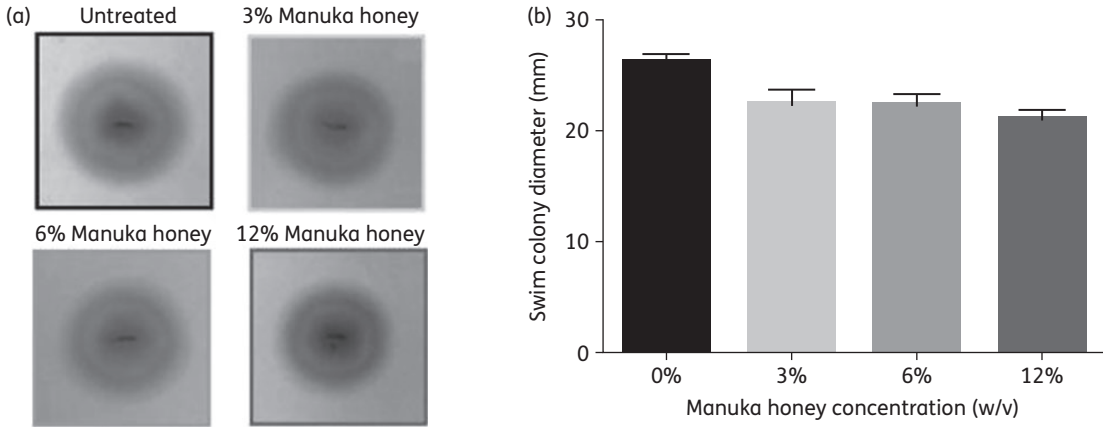


Figure 3. Swimming motility of *P. aeruginosa* cells pretreated with and without manuka honey. (a) Swim colonies of *P. aeruginosa* cells inoculated onto FAB containing 0.3% noble agar following pretreatment with or without 3%, 6% or 12% (w/v) manuka honey for 3 h. (b) Mean diameters of swim colonies (mm) (±SD) ($n=3$ independent experiments, each with three replicates). Untreated cells, black bar; cells pretreated for 3 h with 3%, 6% or 12% (w/v) manuka honey, light, mid and dark grey bars, respectively. All swim colonies produced by honey-treated cells were significantly smaller than untreated ones using the one-way ANOVA test ($P<0.05$).

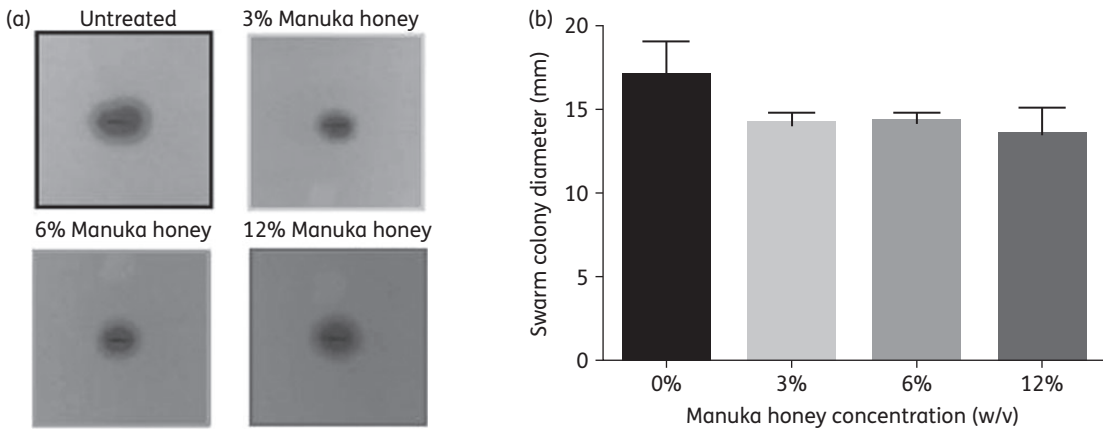


Figure 4. Swarming motility of *P. aeruginosa* cells pretreated with and without manuka honey. (a) Swarm colonies of *P. aeruginosa* cells inoculated onto FAB containing 0.5% noble agar following pretreatment with or without 3%, 6% or 12% (w/v) manuka honey for 3 h. (b) Mean diameters of swim colonies (mm) (±SD) ($n=3$ independent experiments, each with three replicates). Untreated cells, black bar; cells pretreated for 3 h with 3%, 6% or 12% (w/v) manuka honey, light, mid and dark grey bars, respectively. All swim colonies produced by honey-treated cells were significantly smaller than untreated ones using the one-way ANOVA test ($P<0.05$).

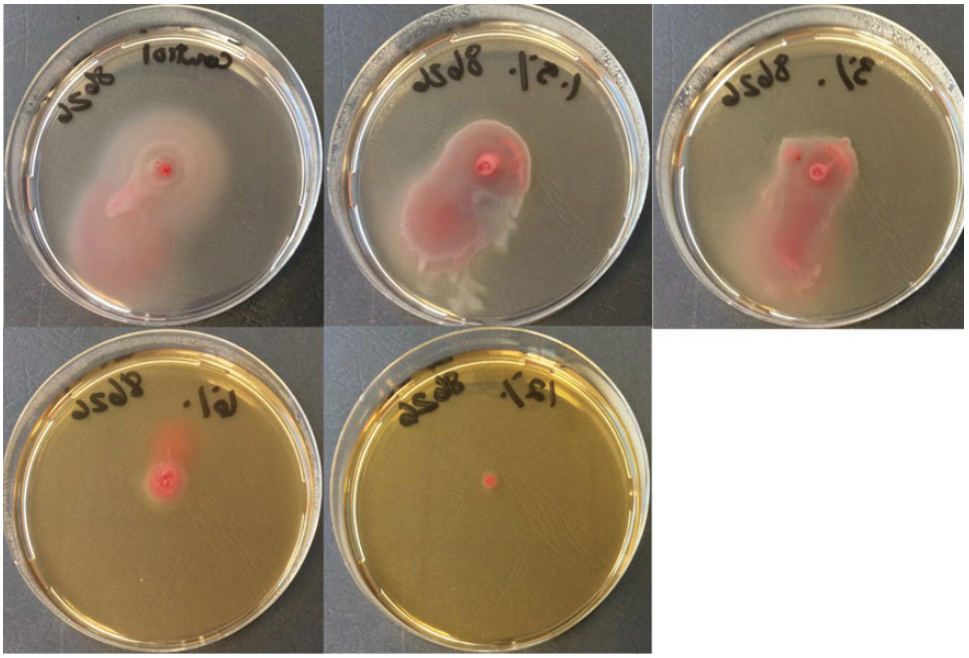


Figure 5. Concentration-dependent reduction in motility of *P. aeruginosa* in response to manuka honey treatment. Motility agar (Mast) was prepared with manuka honey at final concentrations of 0%, 1.5%, 3%, 6% and 12% (w/v), inoculated centrally by stabbing with an overnight culture of *P. aeruginosa* in NB and incubated at 37°C for 24 h. Plates from top left to right: control [0% (w/v) manuka honey]; 1.5% (w/v) manuka honey; 3% (w/v) manuka honey; 6% (w/v) manuka honey; and 12% (w/v) manuka honey (bottom right).

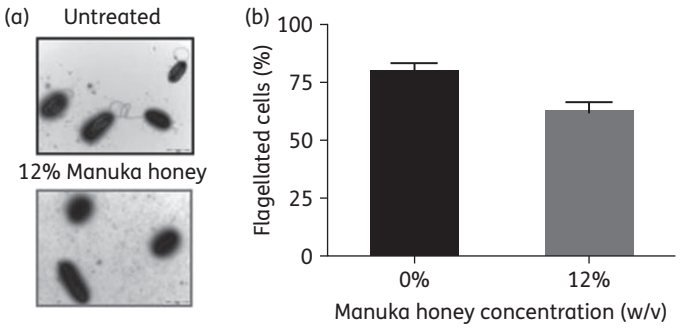


Figure 6. Flagellated state of *P. aeruginosa* cells grown with and without manuka honey. (a) TEM images ($n=102$) of *P. aeruginosa* grown with and without 12% (w/v) manuka honey for 3 h were used to calculate (b) the mean number of flagellated cells (±SD). Fewer flagellated cells were observed in honey-treated samples ($P<0.05$, using the Student's *t*-test, two sample—unequal variance).

suppression of *flhF* with higher concentrations of manuka honey (24% w/v) suggests that, should flagellated cells be observed, the flagellum may be misaligned, resulting in poor motility. However, it is most likely that cells unable to produce flagella will also have reduced expression of *flhF*.

FleN functions to negatively regulate FleQ, providing a critical feedback loop, ensuring *P. aeruginosa* only produces a single flagellum. Suppression of *fleN* following exposure to manuka honey (12% and 24% w/v) should result in the parallel suppression of FleN, increasing the relative activity of FleQ. This suggests a conflict whereby reduced *fleN* expression should concomitantly result in its own increased expression. However, manuka honey activity suppresses the expression of *fleQ*. This would cause suppression of *fleN* expression without the contradictory effect noted here, and we suggest that this occurs in manuka honey-treated samples.

The bacterial flagellum has been shown to be important in adhesion and virulence, and conditions that favour swarming in *P. aeruginosa* have been linked to the up-regulation of many virulence genes.[34,35] We therefore propose that, via the repression of flagella-associated genes, manuka honey mediates de-flagellation of *P. aeruginosa* in a concentration-dependent manner, resulting in reduced motility, adherence and virulence (Figure 7). In addition to the bactericidal effect of manuka honey against this organism,[14–16] the findings reported here reiterate the antipathogenic and antiadhesive properties of manuka honey that have been demonstrated in MRSA[29] and *Streptococcus pyogenes*,[18,36] respectively. Although the concentrations of manuka honey applied topically to wounds in licensed wound dressings exceed the MIC determined *in vitro* by factors of 8–10, it is reassuring to demonstrate that concentrations below the MIC can reduce the ability of *P. aeruginosa* to initiate infections by affecting adhesion and mobility. Antimicrobial agents that exhibit antipathogenic properties are becoming an attractive alternative to antibiotic-mediated inhibition because attenuation of virulence will result in failure of that organism to establish successful infection before it is successfully cleared by the host. This supposition must now be tested in animal models and ultimately *in vivo*, which is outside the scope of this study.

Susceptibility of *P. aeruginosa* to honeys from many countries, including Australia,[37] Chile,[38] Greece and Cyprus[39] and India,[40] has been investigated and varying MICs have been reported. However, the chemistry of honey is highly complex, with multiple components contributing to antimicrobial activity.[41] Characteristics depend on

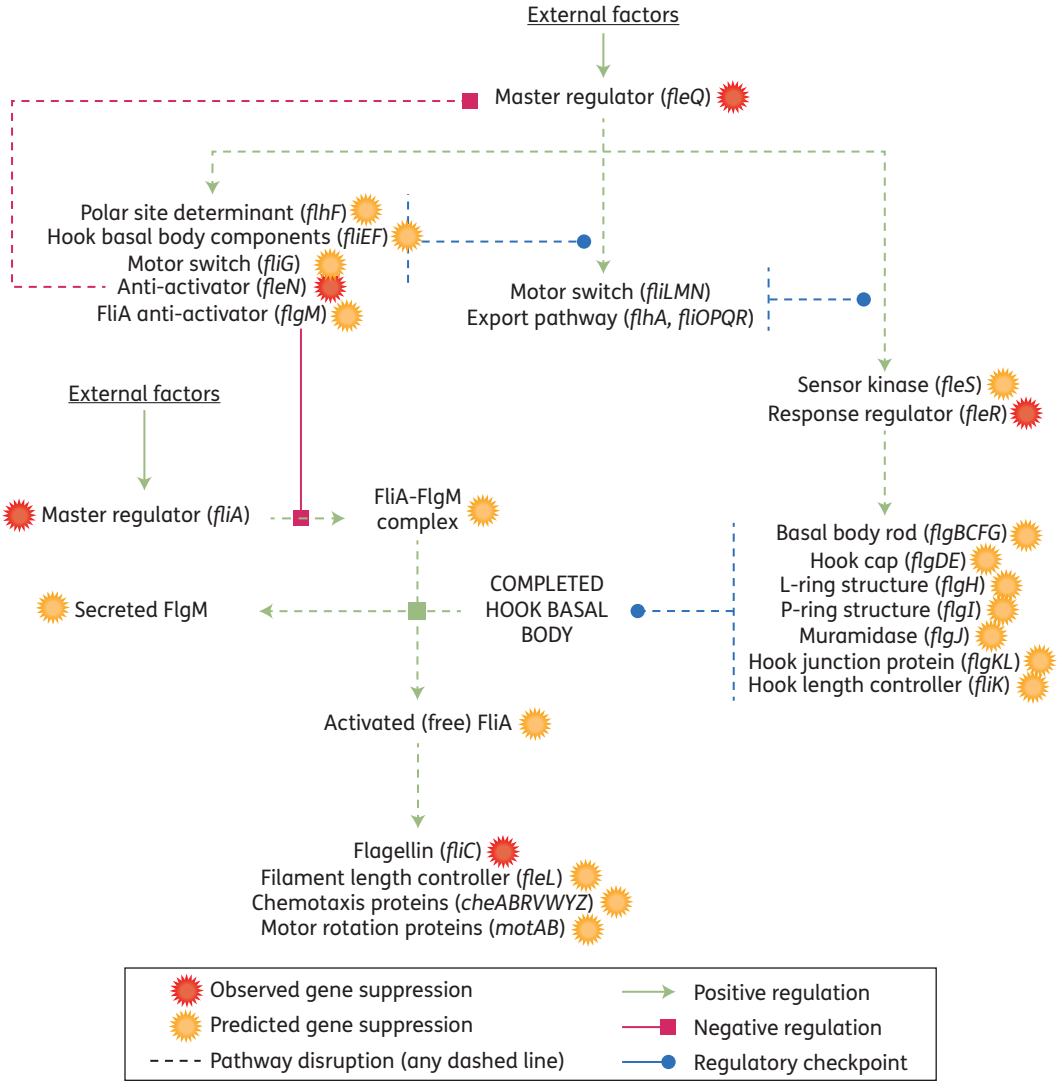


Figure 7. Differential expression of the flagellar regulon in *P. aeruginosa* cells grown with inhibitory concentrations of manuka honey. Observed (red stars) and suspected (yellow stars) suppression of flagella gene expression in *P. aeruginosa* cells treated with manuka honey for 3 h compared with untreated cells. Suspected gene expression alterations were based on regulatory check points, regulator suppression and operon structure.

floral source and bee species, but many other diverse factors have been shown to influence potency.[42,43] Here, manuka honey from New Zealand was used. It differs from other honeys in its elevated levels of methylglyoxal,[44,45] and this has recently been shown to inhibit MDR *P. aeruginosa*.[46] Whether the effects of manuka honey on flagellar structure and function can be attributed to methylglyoxal or whether other honeys elicit similar effects is unknown, but this might be evaluated in future studies.

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