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Proteomic and genomic analysis of methicillin-resistant *Staphylococcus aureus* (MRSA) exposed to manuka honey *in vitro* demonstrated down-regulation of virulence markers

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Objectives: Methicillin-resistant *Staphylococcus aureus* (MRSA) is an important pathogen. Its resistance to multiple antibiotics and its prevalence in healthcare establishments make it a serious threat to human health that requires novel interventions. Manuka honey is a broad-spectrum antimicrobial agent that is gaining acceptance in the topical treatment of wounds. Because its mode of action is only partially understood, proteomic and genomic analysis was used to investigate the effects of manuka honey on MRSA at a molecular level.

Methods: Two-dimensional gel electrophoresis with dual-channel imaging was combined with matrix-assisted laser desorption ionization–time of flight mass spectrometry to determine the identities of differentially expressed proteins. The expression of the corresponding genes was investigated by quantitative PCR. Microarray analysis provided an overview of alterations in gene expression across the MRSA genome.

Results: Genes with increased expression following exposure to manuka honey were associated with glycolysis, transport and biosynthesis of amino acids, proteins and purines. Those with decreased expression were involved in the tricarboxylic acid cycle, cell division, quorum sensing and virulence. The greatest reductions were seen in genes conferring virulence (*sec3*, *fnb*, *hlgA*, *lip* and *hla*) and coincided with a down-regulation of global regulators, such as *agr*, *sae* and *sarV*. A model to illustrate these multiple effects was constructed and implicated glucose, which is one of the major sugars contained in honey.

Conclusions: A decreased expression of virulence genes in MRSA will impact on its pathogenicity and needs to be investigated *in vivo*.

Keywords: quorum sensing, biofilms, wounds, 2D-DIGE, microarrays

Introduction

The advent of antibiotics generated confidence that effective means of treating infections were available, but the emergence of antibiotic resistance has altered our perceptions. In 1961, when methicillin resistance in *Staphylococcus aureus* was discovered, its future global impact was unimaginable. Now the widespread prevalence of strains with multiple antibiotic resistance determinants has made methicillin-resistant *S. aureus* (MRSA) a serious threat to human health. The limited development of antimicrobial agents in recent times has compounded the situation[1] and increased the necessity to search for alternative remedies to complement or replace antibiotics. Natural compounds isolated from plants have historically been used as templates for successful antimicrobial therapies.[2] Before the discovery of antibiotics, honey was used for thousands of years in the topical treatment of wounds. During the past decade, it has been formulated into a range of modern wound dressings and has been reintroduced into conventional medicine.

Honey is a broad-spectrum antimicrobial agent with bactericidal activity against a number of wound pathogens.[3–7] Its high osmolarity, acidity, generation of hydrogen peroxide on dilution and insect-derived antimicrobial peptides contribute to antibacterial activity, yet not all honeys are equivalent.[8] Manuka honey is a distinctive honey that is produced in New Zealand and is used as a medical-grade honey in the manufacture of wound dressings licensed for clinical use in Australasia, Europe and North America. Whereas the antibacterial effect of many honeys on dilution is confined to the generation of hydrogen peroxide, manuka honey possesses

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additional antibacterial factors. Methylgloxal[9,10] and leptosin[11] were recently identified, and further bioactive factors may yet be discovered.

The bactericidal effects of manuka honey on wound pathogens have been investigated *in vitro*, especially in staphylococcal species. The role of manuka honey in preventing cell division in *S. aureus* was deduced from electron micrographs of honey-treated cells, where elevated numbers of cells with entire septa were found to accumulate. Autolysins (also known as murein hydrolases) were implicated in this effect.[12] Similarly an inability to complete the cell cycle was later observed in MRSA treated with manuka honey; despite an increased expression of the autolysin (*atl*) gene, murein hydrolase activity was at undetectable levels. Failure to cleave peptidoglycan was thought to contribute to the persistence of the septa and to the failure to divide and complete the cell cycle.[13] In addition, a decreased expression of universal stress protein A indicated that MRSA was unable to accommodate the stresses caused by exposure to manuka honey.[14]

Transcriptome analysis of *Escherichia coli* exposed to manuka honey revealed multiple cellular effects.[5] More recently, a proteomic study found that 12 proteins were differentially expressed in *S. aureus* following treatment with manuka honey,[15] but those effects were not investigated by transcriptome analysis. Both of these studies showed that manuka honey had a distinct mode of action that involved multiple cellular processes.

In chronic wounds, an association between the persistence of the wounds and the presence of biofilms[16] has increased the urgency to find effective antimicrobial agents that inhibit not only planktonic bacterial cells, but also those contained in antibiotic-tolerant biofilm communities. The aim of this study was therefore to investigate changes in protein and gene expression in MRSA caused by treatment with manuka honey, with a view to elucidating the mechanisms that influence pathogenicity.

Materials and methods

Bacterial strains and growth conditions

The test bacterium used in this study was EMRSA-15 NCTC 13142. This was grown at 37°C with shaking at 120 rpm in tryptone soya broth (TSB; Oxoid Cambridge, UK), with or without 10% (w/v) sterile medical-grade manuka honey (Manukacare 18+, Comvita, UK).

Two-dimensional (2D) electrophoresis

For the preparation of cell extracts, cells were grown in 50 mL of TSB with or without 10% (w/v) manuka honey. At 4 h, the culture was harvested by centrifugation at 10000 **g** for 5 min. The supernatant was discarded and the cells were resuspended in 10 mL sterile water. The cells were then disrupted at 4°C using 0.1 mm glass beads in a bead beater (BioSpec, Bartlesville, USA) using three homogenization cycles of 60 s each. The liquid phase was gently decanted from the beads and the beads were discarded. Insoluble or aggregated proteins in the retained supernatant were sedimented by a 4 min centrifugation at 13000 *g*. The supernatant was then transferred into clean tubes and stored at −80°C.

The 2D gel electrophoresis was performed using an immobilized pH gradient technique adapted from published methods.[17,18] Briefly, the 24 cm pH 3–10 strips were rehydrated in the Ettan IPGphor3 IEF system (GE Healthcare, Little Chalfont, UK). Prior to electrophoresis, 160 μg of protein from each of the control and honey-treated cells was incubated with fluorochromes Cy5 and Cy3 respectively according to manufacturer's instructions (CyDye DIGE Fluors; GE Healthcare, Little Chalfont, UK). Protein extracts were then combined and soluble proteins were loaded onto the rehydrated IPG strip before being isoelectrically focused for 60000 Vh. The IPG strip was next equilibrated in buffer containing 1% DTT for 15 min (reduction) and then in buffer containing 2.5% iodoacetamide for 15 min (alkylation).

2D gels were prepared (270×210, 1 mm 10% SDS polyacrylamide gels) and proteins were separated after embedding the IPG into gel using 1% agarose (Sigma, Dorset, UK). 2D electrophoresis was performed using an Ettan IPGPhor system (GE Healthcare, Little Chalfont, UK) and the proteins were stained with Coomassie blue.

The difference gel electrophoresis (DIGE)-labelled gels were scanned using a Typhoon Trio variable-mode imager (GE Healthcare, Little Chalfont, UK) with 580BP 30Cy3, TAMRA, Alexa Fluor 546 and 670BP 30Cy5 emission filters, and then saved as .ds files. These files were then analysed using Decyder 2D version 6.5 software (GE Healthcare, Little Chalfont, UK). Gels were checked for spot resolution, and exclusion filters were set at slope 3.2, Area 120, Volume 25000 and Peak height 100–6500. Spot-difference analysis allowed the identification of spots with a 2-fold or more up- or down-regulation compared with the control.

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. Peptides were then recovered following trypsin digestion using a modified version of that described by Shevchenko *et al*.[19] Sequencing grade modified trypsin (Promega, UK) was used at 6.25 ng/μL in 25 mM NH_4HCO_3 and incubated at 37°C for 3 h. Finally, the dried peptides were resuspended in 5 μL of 50% (v/v) acetonitrile in 0.1% (v/v) trifluoroacetic acid (TFA) for MS analysis, and an aliquot corresponding to 10% of the material (0.5 μL) was spotted onto a 384-well MS plate. The samples were allowed to dry and were overlaid with 0.5 μL of α-cyano-4-hydroxycinnamic acid [Sigma, Dorset, UK; prepared by mixing 5 mg of matrix with 1 mL of 50% (v/v) acetonitrile in 0.1% (v/v) TFA].

MS analysis

MS was performed using matrix-assisted laser desorption ionization–time of flight (MALDI TOF) MS (4800 MALDI TOF-TOF Analyzer; Applied Biosystems, Foster City, CA, USA) with a 200 Hz solid state laser operating at a wavelength of 355 nm.[20,21] 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, and 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 (the collision gas being air at a pressure of 1.6×10^{-6} Torr) and default calibration.

Combined PMF and MS/MS queries were performed using the MASCOT Database search engine v2.1 (Matrix Science Ltd, London, UK)[22] embedded in Global Proteome Server (GPS) 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 (with one missed cleavage allowed), 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. Search results were evaluated by manual inspection, and conclusive identification confirmed whether there were 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 the E value had a value of $P<0.0001$).

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Extraction of RNA for real-time PCR and microarray analysis

Cells were grown with and without manuka honey as described above. RNA was isolated using a 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. The RNA was treated with an extra DNAse treatment using Ambion DNA-free according to the manufacturer's instructions to avoid a carry-over of genomic DNA (Invitrogen, Paisley, UK). Real-time PCR was performed on all samples, using Fast SYBR Green (Applied Biosystems, Foster City, CA, USA), with the procedures suggested by the manufacturer on a CFX96 real-time PCR system (Bio-Rad). Primers for quantitative PCR (qPCR) (Table 1) were designed using NCBI Primer-BLAST to be 20–24 bases long, with a GC content of more than 50% and a melting temperature of around 60°C. All reactions were carried out in triplicate, and the expression of genes was analysed with reference to the expression of the housekeeping gene acetyl coenzyme A (*yqiL*).

For microarray analysis, RNA was isolated as above, hybridized, stained and scanned on Affymetrix arrays according to the manufacturer's instructions for prokaryotic target preparation. All experiments were carried out with three biological replicates and the mean values are presented here. The fold changes have been corrected and normalized to account for background noise. Genes showing more than a 2-fold differential regulation at a significance of $P = 0.001$ using a Bayesian *t*-test were examined.

The data discussed in this publication have been deposited in NCBI's Gene Expression Omnibus (GEO)[13] and are accessible through GEO Series accession number GSE31592 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE31592).

Table 1. Primers used in this study

Target gene	Direction	Primer sequence (5′ – 3′)
yqiL	forward	GACGTGCCAGCCTATGATTT
yqiL	reverse	ATTCGTGCTGGATTTTGTCC
pykA	forward	TGCAGCAAGTTTCGTACGTC
pykA	reverse	GGGATTTCAACACCCATGTC
clpC	forward	GTTGGTGCTCCTCCAGGATA
clpC	reverse	ACTTGAACCACCGAATCCAG
argF	forward	CCAAGCAGAATTCGAAGGA
argF	reverse	GGATGCGCACCTAAATCAAT
adh	forward	GTTGCCGTTGGTTTACCTGT
adh	reverse	TTCAGCAGCAAATTCAAACG
menB	forward	CTGGGGAAGGTGATTTAGCA
menB	reverse	ACCGCCACCTACAGCATAAC
pur7	forward	GAAGCGCATTTTCTCAACAA
pur7	reverse	CCCTTACCTGCCATTGTGTC
pdp	forward	GCAATGCGCTTGAGTTACAA
pdp	reverse	TATTGAGCTTGTGGCAAACG
fabG	forward	CCGGGACAAGCAAACTATGT
fabG	reverse	CCAAAACGTGCTAACGGAAT
glmM	forward	AGGTGTCGCAAACCAAGAAC
glmM	reverse	TCGCGACCTACAAGTACACG
argF	forward	GCCCATTCGAAGAAAACGTA
argF	reverse	ACCTAATGCTGGCGCTAATG
scdA	forward	CGAAAGCAGCGGATATTTTT
scdA	reverse	GCGAACCTGGTGTATTCGTT

Results

The mode of action of manuka honey on MRSA was investigated initially by proteomic analysis, identifying proteins with differential expression. The expression of genes encoding for those identified proteins was then determined using qPCR and whole-genome effects were explored using microarray analysis.

Proteomic analysis

Proteins extracted from MRSA treated with and without a bactericidal concentration (10% w/v) of manuka honey for 4 h were separated by 2D -DIGE and 15 proteins whose levels of expression were altered by a factor of at least two were selected for further characterization by MS. Figure 1 shows an example of a gel with a protein spot that was characterized by MS as an ATP-binding subunit of an ATP-dependent Clp protease (ClpC). Five proteins were measured at increased levels compared with untreated cells (Table 2) and 10 proteins were decreased (Table 3). Three protein spots were characterized as being pyruvate kinase (Table 2), two were characterized as phosphoglucosamine mutase (Table 3) and one was tentatively identified as transaldolase. Therefore 15 samples represented 12 different proteins, of which 11 were successfully characterized. They included proteins involved in carbohydrate metabolism, cell wall biosynthesis and the stress response. Two were pertinent to two of our previous studies: the cell wall-related protein (ScdA) where cell division was affected[13] and the Clp protease (ClpC) where inability to cope with stress was observed.[14]

qPCR analysis

Excluding transaldolase, the effect of manuka honey on the expression of the genes that coded for the 11 identified proteins was investigated quantitatively with PCR. It was found that changes in gene expression (Table 4) did not necessarily concur with the altered levels of protein expression observed using proteomic analysis (Tables 2 and 3). The expression of five corresponding proteins and genes did show the same trend (Table 4). Two genes (*fbaA* and *pdp*) had unchanged levels of expression and four genes showed increased expression (Table 4) in comparison with their respective proteins (Table 3). Hence six genes showed higher levels of expression by qPCR after honey treatment, although their proteins appeared diminished by 2D-DIGE. One explanation might be that changes in protein structure were caused directly by exposure to manuka honey or indirectly by altered post-translational modification.

Microarray analysis

Microarray analysis was undertaken to resolve the anomalies found with proteomic analysis and qPCR and to investigate wider changes in gene expression. Raw data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus and are accessible through GEO series accession number GSE31592. Genes were sorted into The Institute for Genomic Research (TIGR) categories using the information available in the J. Craig Ventnor Institute Comprehensive Microbial Resource. Only changes relevant to our study are discussed in detail here.

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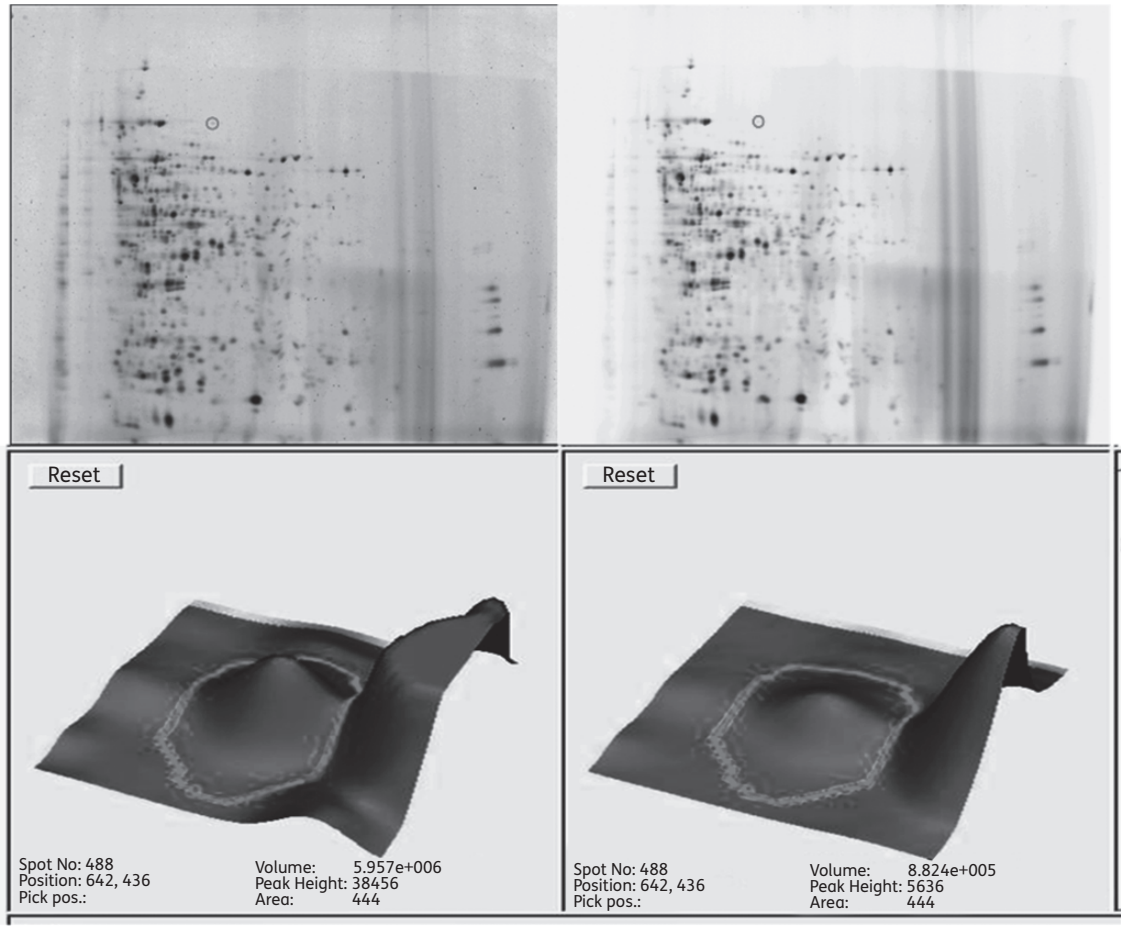


Figure 1. 2D-DIGE gel showing spots of interest with proteins isolated from untreated MRSA (Cy5; left) and proteins from honey-treated MRSA (Cy3; right). The expression of protein spot 488 (grey circle) was analysed using Decyder 2D version 6.5 software (GE Healthcare, Little Chalfont, UK) and was down-regulated 2-fold. It was picked and identified as Clp protease using the Applied Biosystems 4800 MALDI TOF/TOF Analyzer (Foster City, CA, USA).

Table 2. Proteins (determined by MS) in MRSA that increased 2-fold or more following exposure to manuka honey

Accession number	Protein name/function	Best MS sequence	Mascot score	Second best MS sequence	Mascot score	Third best MS sequence	Mascot score
C2GBD3_STAAU	possible transaldolase	EITEAVTEGVPTYVSVFAGR	1.20E-10	EIPDASISFEVFADDLETMEK	4.70E-11	LNVEVFADGADIEEMK	5.20E-07
KYPK_STAAR 551	pyruvate kinase	STDALLNNAVATAVETGR	1.9E-013	ENVDFIAASFVR	1.5E-011	IHLVGDEIANGQGIGR	1.8E-006
KYPK_STAAR	pyruvate kinase	KSTDALLNNAVATAVETGR	1.7E-018	STDALLNNAVATAVETGR	7.3E-015	ENVDFIAASFVR	5.8E-011
KYPK_STAAR	pyruvate kinase	KSTDALLNNAVATAVETGR	1.5E-015	STDALLNNAVATAVETGR	5.7E-013	IHLVGDEIANGQGIGR	7.6E-009
MENB_STAAR	naphthoate synthase	VGSFDAGYGSGYLAR	3.30E-10	GHGGYVGEDQIPR	9.60E-08		

Not all of the genes investigated by qPCR were among the 463 most highly differentially expressed genes identified by microarray analysis. This could be due in part to the fact the microarray chip was constructed from four strains of *S. aureus*—N315 (National Institute of Technology and Evaluation, Japan), Mu50 (National Institute of Technology and Evaluation, Japan), NCTC 8325 (University of Oklahoma, lab strain), and COL (TIGR)—which differed from the epidemic strain of MRSA that is prominent in the UK and that was used in this study. A specific chip for our test organism was not available. At the level of significance chosen here ($P=0.001$), analysis indicated that 290 genes had increased levels of transcription and 173 genes showed decreased levels of transcription following exposure of MRSA to manuka honey compared with untreated MRSA. Of the 11 proteins/genes that had been investigated by proteomics and

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Table 3. Proteins (determined by MS) in MRSA that were found to be decreased following exposure to manuka honey

Accession number	Protein name/function	Best MS sequence	Mascot score	Second best MS sequence	Mascot score	Third best MS sequence	Mascot score
ALF2_STAAR	fructose-bisphosphate aldolase	DVLNNDKEVYDPR	1.20E-07				
ADH_STAAR	alcohol dehydrogenase	NADFGDVTGVTLGHEGIGK	4.30E-15	LVLDGIEVVGSLVGTR	7.70E-14	KLEEINDIFEEMENGTITGR	7.20E-12
FABG_STAAR	3-oxoacyl reductase	EVVSQFGSLDVLVNNAGITR	6.40E-15	GVDSFAIQANVADADEVK	0.00033	FGQDTDIANTVAFLASDK	0.00027
PUR7_STAAR	phosphoribosylamino-imidazole-succinocarboxamide synthase	TETGQILLADEISPDTCR	5.70E-13	NNTGSLIETYQIFLNK	3.60E-07	ATNANFDKDVYR	3.00E-07
PDP_STAAR	pyrimidine-nucleoside phosphorylase	VEEGESLLTIHSNR	1.10E-06	LPQAQYQIEYK	8.90E-05		
OTCC_STAAR	ornithine carbamoyltransferase	ENFGYLEGINLTYVGDGR	6.40E-18	AEFEGLIDFAITLK	1.90E-09	AAFTVASIDLGAHPEFLGK	7.70E-09
GLMM_STAAR	phosphoglucosamine mutase	VVETESDFGLAFDGDGDR	3.70E-12				
GLMM_STAAR	phosphoglucosamine mutase	VVETESDFGLAFDGDGDR	6.10E-07				
SCDA_STAAR	cell wall-related protein	LNEVEQTNTPGSLNPK	5.80E-08	NVDLNELLQR	6.20E-07	VHGPNHPYLVELK	1.00E-06
CLPC_STAAR 488	Clp protease	DAAVHAQEFENAANLR	6.9E-009	FAGFGGSSDGQDYETIR	0.00011		

Table 4. Changes in gene expression in MRSA following treatment with bactericidal concentrations as determined by qPCR

Gene	Gene product	Function	Fold change
pykA	**pyruvate kinase**	glycolysis	+6
fbaA	fructose-bisphosphate aldolase	glycolysis	no change
adh	**alcohol dehydrogenase**	fermentation	−2
menB	**naphthoate synthetase**	anaerobic electron transport	+5
fabG	3-oxoacyl reductase	fatty acid biosynthesis	+4
purC	**phosphoribosylaminoimidazole-succinocarboxamide synthase**	purine biosynthesis	−2
pdp	pyrimidine-nucleoside phosphorylase	pyrimidine biosynthesis	no change
argF	**ornithine carbamoyltransferase**	virulence	−2
glmM	phosphoglucosamine mutase	cell wall	+4
scdA	cell wall related protein	cell wall	+2
clpC	Clp protease	stress	+16

Fold changes are shown in relation to untreated MRSA cells, and genes in bold show the same trend as proteins identified by 2D-DIGE.

qPCR, down-regulation of alcohol dehydrogenase (*adh*) and up-regulation of pyruvate kinase (*pykA*) was confirmed.

Globally, genes involved in the biosynthesis of amino acids, proteins, co-factors, prosthetic groups and carriers, purines, pyrimidines, nucleosides and nucleotides and cell envelope, as well as transport and binding proteins, were among the 290 genes found to have increased levels of expression in MRSA, while genes with decreased levels of expression mainly included those involved in energy metabolism and cellular processes (Figure 2). This suggests that growth and division was compromised in MRSA by manuka honey.

By mapping the affected genes to central pathways, a number of genes concerned with carbohydrate metabolism were found to be altered in MRSA by exposure to manuka honey (Figure 3). Essentially, glycolysis was promoted by increased transcription of *pfkA*, *tpi*, *gapA*, *pgk*, *pgm* and *pykA* (≥2-fold) and gluconeogenesis was restricted by a decreased transcription of *gapB* (33-fold). This effect was likely to be due to an increased expression of *gapR* (6.8-fold), which has been shown to regulate *gapA* and *gapB* reciprocally in response to glucose concentration, with glucose inducing *gapA* and repressing *gapB*.[23] Since glucose accounts for approximately 33% of honey by weight, this effect was not unexpected. We have previously shown, however, that the antibacterial effect of manuka honey is not attributable solely to its sugar content.[3]

All of the tricarboxylic acid (TCA) cycle genes reported by microarray assay were found to be repressed, but those involved in fermentation showed varying patterns of expression (Figure 3). Essentially, manuka honey seemed to promote glycolysis and fermentation at the expense of oxidative metabolism. This would lead to the accumulation of acidic end-products and reduce the supply of ATP available to drive active transport and biosynthetic pathways.

The most notable changes in gene expression provided by the microarray data indicated that manuka honey had a marked effect on the expression of important MRSA virulence determinants, such as haemolysins, leucocidin, lipase and fibronectin-binding protein (Table 5). The largest change was seen in the gene coding for enterotoxin type C3 (*sec*3), which was down-regulated by a factor of 109. Another important observation was that three genes within the accessory gene regulator (*agrB*, *agrC* and *agrD*) exhibited decreased levels of expression, as did the genes within the two component histidine kinase regulators (*saeS* and *saeR*). Hence genes within two global regulatory operons (*agrABCD* and *saeSR*) were found to be repressed in MRSA exposed to manuka honey. Both regulate not only virulence, but also biofilm formation in staphylococci.[24]

The elevated expression of *sarV* (Table 5), another regulator gene,[25] suggested that manuka honey might increase levels of autolysins and proteases in MRSA. Three genes that code for proteins involved in cell wall functions and division (*ftsL*, *cidB* and *scpA*) were found in transcriptome data to have decreased levels of expression, while *lrgB*, which functions as an antiholin in reducing the extracellular activity of murein hydrolase,[26] was increased 22-fold. Some markers of the stress response were also changed (Table 5).

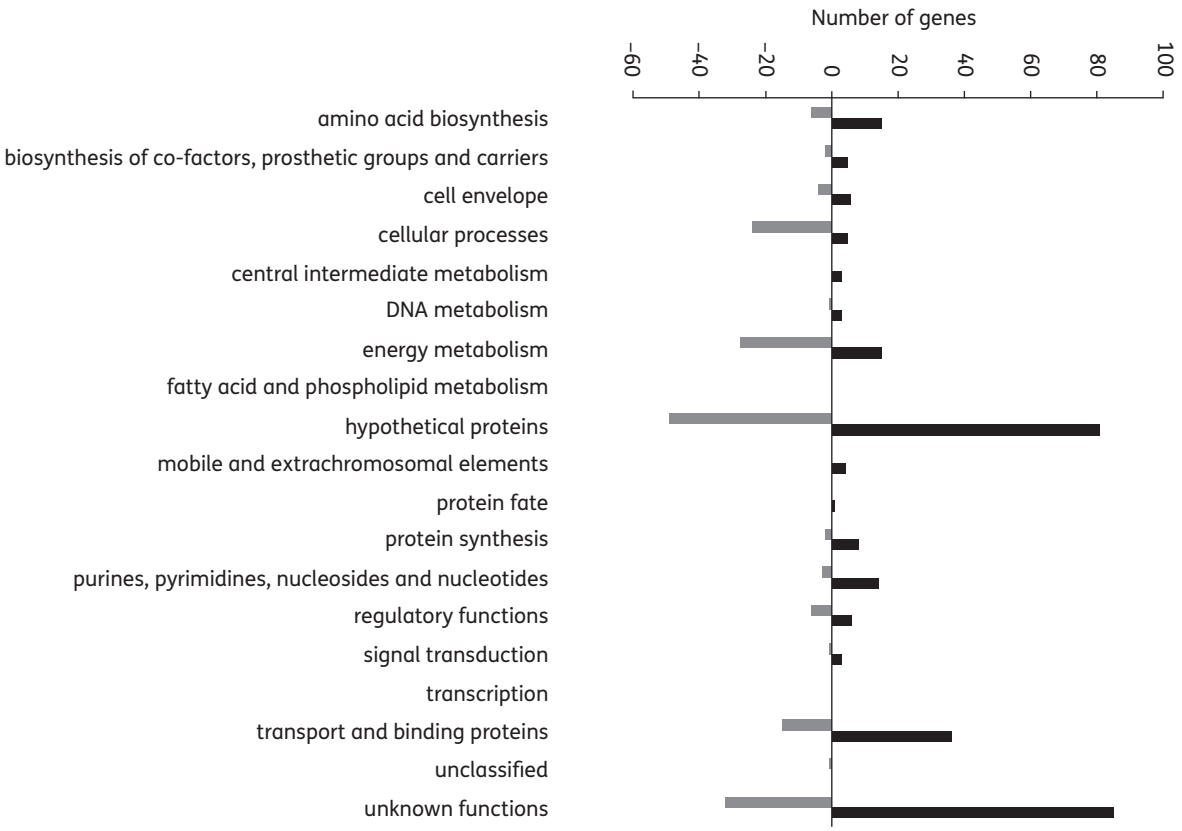


Figure 2. Changes in gene transcription (microarray data) classified by main functions. Genes with altered levels of transcription after exposure of MRSA to 10% (w/v) manuka honey for 4 h at 37°C (compared with untreated cells) were divided into categories based on main functions according to the J. Craig Ventor Institute Comprehensive Microbial Resource.

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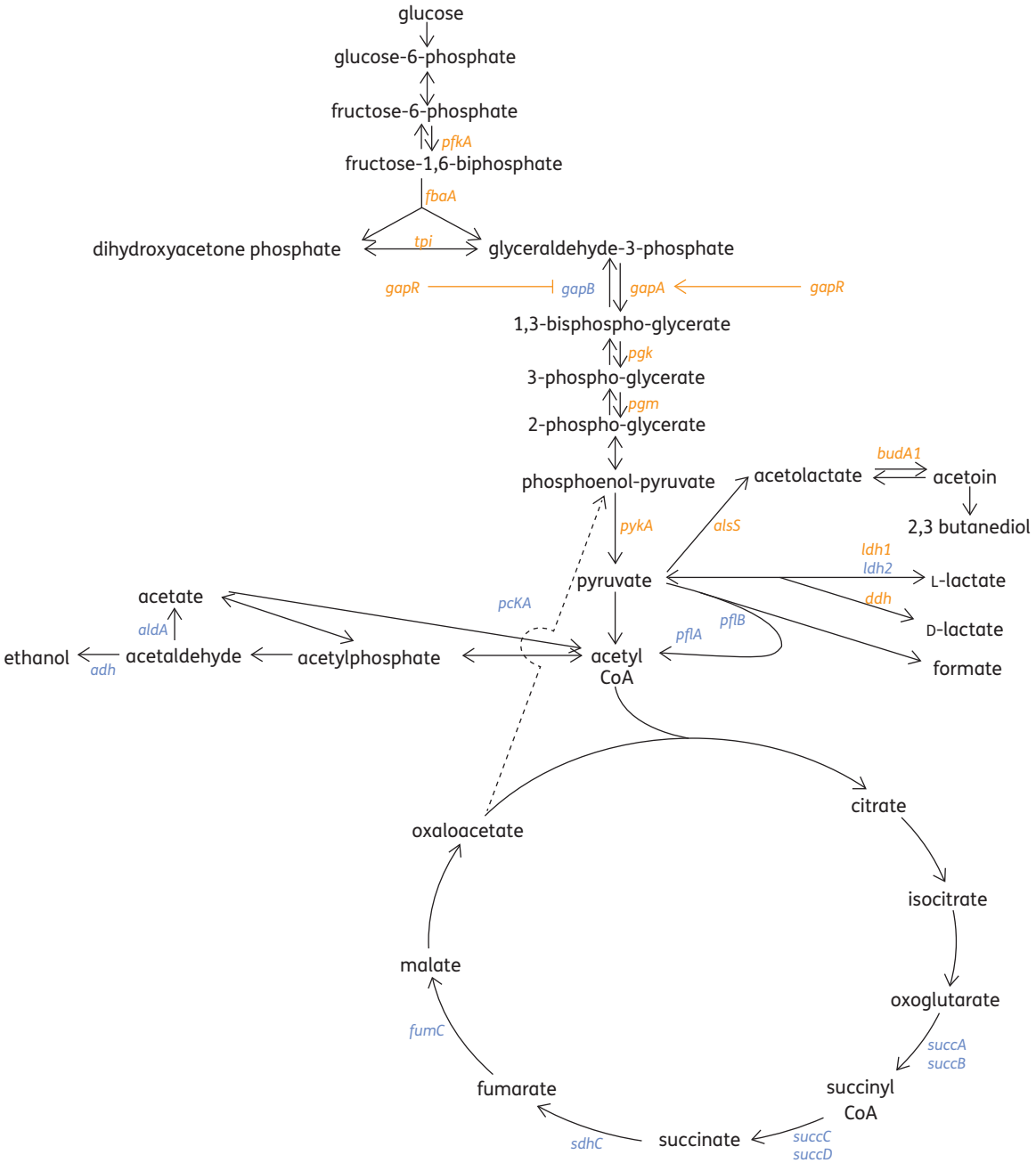


Figure 3. Effect of manuka honey on the expression of genes concerned with carbohydrate metabolism in MRSA treated with and without 10% (w/v) manuka honey for 4 h at 37°C, as determined by transcriptome analysis. Up-regulated genes are shown in orange and down-regulated genes are shown in blue.

Discussion

Investigations into the impact of manuka honey on transcription in *E. coli*[5] and on protein expression in *S. aureus*[15] have demonstrated multiple effects on bacterial function. The differential expression of the proteins and genes identified in this study largely differed from those previously reported[15] and may perhaps reflect the different strains, exposure times and honey concentrations employed in each study. Here, a bactericidal concentration of manuka honey was used [10% (w/v)], which was approximately twice the MIC and at least eight times lower than that normally used in the topical treatment of wounds. Data generated indicate multiple effects that impact on the ability of MRSA to grow and divide, as well as on its pathogenicity.

Virulence factors

The role of virulence determinants of *S. aureus* in human disease is well established.[24] Virulence genotyping of strains of *S. aureus* isolated from diabetic foot ulcers showed that *sec* was one of the

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Table 5. Genes of potential clinical significance in MRSA identified by microarray analysis following treatment with and without 10% (w/v) manuka honey

Function	Gene	Gene product	Fold change
Virulence	*sec3*	enterotoxin type C3	−109
	fnb	fibronectin binding protein	−54.5
	hlgA	gamma haemolysin component A	−54
	lip	lipase	−44.4
	hla	alpha-haemolysin	−27.5
	SA1813	leucocidin protein	−23
	sspA	serine protease	−3.8
	hlgB	gamma haemolysin component B	−2.7
	hlgC	gamma haemolysin component C	−2.3
Virulence regulators	*sarV*	HTH type regulator	+2
	agrB	accessory gene regulatory operon: membrane-associated protein	−5
	agrC	accessory gene regulatory operon: membrane-associated autoinducer peptide sensor	−2.6
	agrD	accessory gene regulatory operon: pro-autoinducer peptide	−2.4
	saeS	histidine kinase sensor protein	−11
	saeR	response regulator	−13
Cell envelope and cell division	*ftsL*	cell division protein	−2.6
	scpA	segregation and condensation protein A	−2.1
	mecR1	methicillin-resistance regulator protein	−3
	lrgB	antiholin	+22
	cidB	holin	−1.9
Stress	*sod*	superoxide dismutase	+1.6
	acpD	FMN-dependent NADH azoreductase	+4
	mscL	large-conductance mechanosensitive channel	+2.5
	asp23	alkaline shock protein 23	−1.4
	SACOL1759	putative universal stress protein	−4.4

genes significantly more frequently associated with strains isolated from infected ulcers compared with non-infected ones.[27] Reducing the transcription of *sec* in wounds by applying manuka honey can, therefore, be expected to confer a clinical benefit, but this effect will have to be investigated *in vivo*.

Reduced expression of the enterotoxin gene in MRSA in the presence of honey has considerable relevance for the food industry. Honey has been used as a food preservative for many years on the premise that it prevents microbial growth, but it may have an additional benefit in reducing staphylococcal pathogenicity, with the risk of staphylococcal food poisoning possibly being decreased by incorporating honey into perishable foodstuffs. Further investigation is needed to determine whether the repression of the enterotoxin gene is limited to manuka honey, or whether a wider variety of honeys produced for human consumption have a similar effect.

Many cell surface and extracellular proteins that contribute to virulence have been identified in *S. aureus* (Table 6); the expression of these genes is usually controlled by global virulence regulators including the accessory gene regulator (*agrABCD*) and a staphylococcal accessory regulator (*sarA*).[28,29] In this study, a decreased transcription of three genes within the *agr* operon (Table 5) was found in treated MRSA. The *agr* locus (Figure 4) is a quorum-sensing gene cluster containing five genes (*agrB*, *agrD*, *agrC*, *agrA* and *hla*),[30] four of which facilitate the production and detection of an autoinducing peptide (AIP) to regulate the expression of genes coding for virulence factors.[31,32] Although certain honeys have been shown to inhibit quorum sensing in Gram-negative bacteria,[33–35] this is the first indication that manuka honey inhibits quorum sensing in Gram-positive bacteria. Thus, the repression of some of the regulatory genes within the *agr* cluster is likely to account for the decreased expression of virulence factors under their control.

Biofilm genes

Manuka honey has been demonstrated to prevent the formation of biofilms and to disrupt established staphylococcal biofilms *in vitro*,[36,37] although the underlying mechanism has been unknown. Alpha-haemolysin (*hla*), a protein that elicits host cell lysis by disrupting host cell membranes, was found to be down-regulated 27.5-fold by manuka honey (Table 5). Mutants of *S. aureus* defective in *hla* have been shown to be unable to form biofilms due to a requirement for cell–cell interaction mediated by this toxin.[38] Although *agr* has been implicated in the regulation of *hla*, another regulator (*sae*) has also been suggested.[39] This global regulator of virulence in *S. aureus* is the two-component *saeSR* system, which is thought to be activated by *agr* and is essential for the expression of staphylococcal adhesins.[40] Here, decreased expression of the histidine kinase sensor (*saeS*) and regulator (*saeR*) genes in manuka honey-treated cells (Table 5) might explain this observation since mutation of *saeRS* has been shown to increase protease activity and restrict biofilm formation.[41] The adhesion of bacteria to host cells, wound beds or indwelling medical devices is an important

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Table 6. *S. aureus* virulence factors controlled by the global regulators *agr*, *sarA*, *sarE* and *sae*

Virulence factors (gene)	Virulence factors (gene)
Toxins	Surface proteins
alpha haemolysin (*hla*)	bone sialoprotein-binding protein
beta haemolysin (*hlb*)	clumping factor A (*clfA*)
delta haemolysin (*hld*)	clumping factor B (*clfB*)
gamma haemolysin (*hlgA-C*)	collagen-binding protein (*can*)
enterotoxin A (*sea*)	extracellular fibrinogen
enterotoxin B (*seb*)	binding protein (*efb/fib*)
enterotoxin C (*sec3*)	**fibronectin-binding protein A (*fnbA*)**
enterotoxin E-I (*entE-I*)	fibronectin-binding protein B (*fnbB*)
exfoliative toxin A (*etaA*)	lactoferrin-binding protein
exfoliative toxin B (*etaB*)	laminin-binding protein
leucocidin P-V (*lukS/F*)	lectin-like protein
toxic shock syndrome toxin-1 (*tst*)	MHC-II analogous protein (*map*)
Enzymes	plasminogen-binding protein
	protein A (*spa*)
alkaline/phosphatase	Sdr A-D (*sdrA*, *B*, *C*, *D*)
beta lactamase (*blaZ*)	thrombospondin-binding protein
coagulase (*coa*)	vitronectin-binding protein
cysteine protease (*sspB*)	
fatty acid modifying enzyme (FAME)	
glycerol ester hydrolase (*geh*)	
hyaluronate lyase (*hysA*)	
lipase/butyrylesterase (*lip*)	
metalloprotease/aureolysin (*aur*)	
thermonuclease (*nuc*)	
PI-phospholipase C (*plc*)	
staphopain/proteasell (*scp*)	
staphylokinase (*sak*)	
V8 serine protease (*sspA*)	

Those factors in bold were found to be down-regulated by treatment with honey in this study.

prerequisite to both infection and the initiation of biofilm formation, and interventions that prevent or disrupt biofilms in wounds may improve wound healing outcomes. The recent demonstration of an association between wound chronicity and the presence of biofilm[16,42] reinforces the clinical importance of anti-biofilm effects.

Reduced transcription of the gene coding for fibronectin-binding protein by MRSA after treatment with manuka honey (Table 5) is likely to impact on the ability of the bacterial cells to adhere to host fibronectin within a wound and will further reduce the opportunity to initiate infection and biofilm formation. Manuka honey has already been shown to attenuate the efficacy of binding in *Streptococcus pyogenes* by reducing the expression of two fibronectin-binding proteins, Sof and Sfbl.[6] In the case of *S. aureus*, the glycolytic enzyme glyceraldehyde 3-phosphate dehydrogenase was also demonstrated to function as a cell wall transferrin-binding protein and fibronectin-binding protein.[43] Microarray data showing decreased expression of *fnb* and *gapB* genes (Table 5) give a strong indication that manuka honey impairs the efficacy of ligand binding required for adherence that confers enhanced virulence and biofilm formation in MRSA.

Cell wall functions and cell division

The *agr* operon has also been linked to the control of β-lactam resistance via the regulation of autolysis;[44] hence the reversion of oxacillin resistance to oxacillin susceptibility owing to a reduced expression of *mecR1* caused by manuka honey[45] may have resulted from a down-regulation of *agr*. Autolysis is important in bacterial growth and cell division and is controlled by complex mechanisms. The increased expression of one pertinent transcriptional regulator (*sarV*) was seen (Table 5), which positively regulates the transcription of *scdA*, *lrgB*, *atl*, *splA* and *aur*.[25] Increased levels of ScdA (Table 3) and *scdA* were found (Table 4) and the expression of *lrgB* was increased 22-fold (Table 5). An increased transcription of *atl* was reported in MRSA in response to treatment with manuka honey, but the gene product (murein hydrolase) exhibited undetectable activity in both cell-free and extracellular extracts.[45] An inability to degrade peptidoglycan at the cell equator due to a diminished activity of murein hydrolase helps to explain why MRSA failed to execute cell division and complete its cell cycle.

Cell wall-related protein (ScdA) is a di-iron protein involved in the repair of proteins that result from conditions of oxidative stress and is also required for cell division.[46] Changes in the expression of *scdA* affect peptidoglycan cross-linking, and cells with depleted levels of ScdA have been reported to form large aggregated clumps of cells with aberrant septum placement. The diminished rate of autolysis was attributed to structural changes in peptidoglycan rather than altered murein hydrolase activity.[46] Transcription of *sarV* in *S. aureus* has been shown to be repressed by *sarA* and *mgrA*[25] and *sarA* is normally repressed by *agrA*. Altered levels of *agr A* were not discovered in this study, yet three of the genes of the *agr* locus exhibited decreased levels of expression (Table 5). If *agrA* had been repressed too, *sarV* would have been derepressed and transcription of the regulator gene would have been allowed. Increased levels of expression of *sarV*, *scdA* and *lrgB* (Table 5) support this hypothesis.

Bacterial growth and division is intimately linked to the controlled synthesis and cleavage of peptidoglycan in the cell wall by autolysins (or murein hydrolases). The *cidABC* operon works in conjunction with the *lrgAB* operon to regulate murein hydrolase, as well as antibiotic tolerance.[26,47] The export of murein hydrolases is regulated by the *cidABC* operon, which promotes murein hydrolase activity and increases sensitivity to penicillin, while the *lrgAB* operon decreases murein hydrolases activity and penicillin sensitivity.[26,47] An increased transcription of *lrgB* (22-fold) and a decreased transcription of *cidB* (1.9) (Table 5) were observed in this study. Since the products of the *cidABC* operon act as holins to facilitate the export of murein hydrolase, and the products of the *lrgAB* operon act as antiholins to restrict export, the deduction that manuka honey interferes with the cell cycle[45] is strengthened. Further evidence of an impact on cell division was provided by the diminished transcription of two other genes whose products are involved in cell division: *scpA* (for segregation) and *ftsL* (in septum formation) (Table 5).

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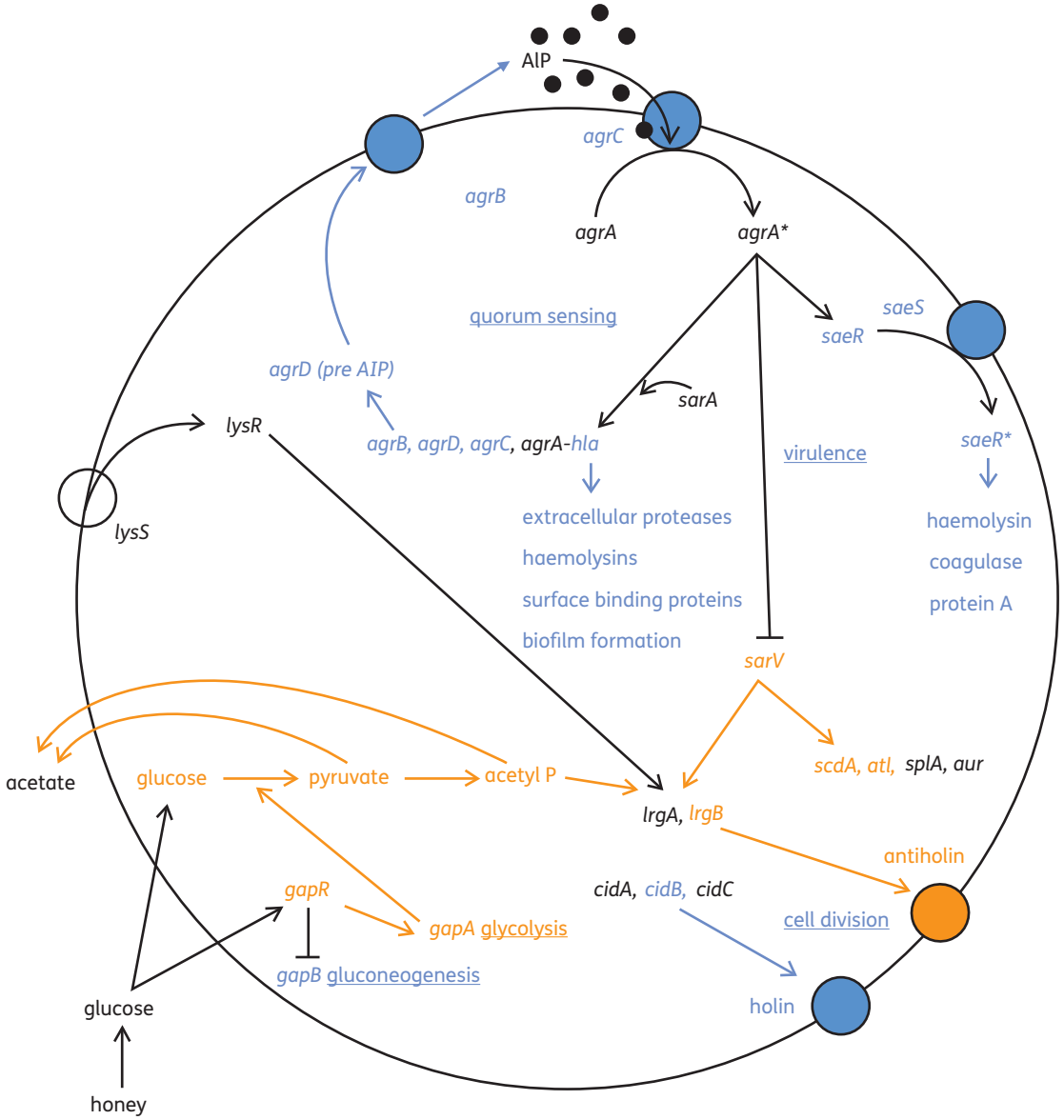


Figure 4. A model to show how manuka honey affected genes of clinical importance in MRSA. Up-regulated genes are shown in orange, down-regulated genes are shown in blue and genes with unknown levels of transcription are shown in black. Affected cellular processes are underlined.

The transcription of *cidABC* and *lrgAB* has been shown to be influenced by the metabolism of glucose when fermentation end-products such as acetic acid and lactate accumulate and acidic stress increases.[48] In this study, increased glycolysis together with a reduced expression of *pflB* and a reduced oxidation of acetyl CoA by the TCA cycle might be expected to induce such conditions.

Phosphoglucosamine mutase (GlmM) demonstrated decreased protein expression (Table 3) and increased gene expression (Table 4). GlmM converts glucosamine 6-phosphate into glucosamine 1-phosphate, which is an essential precursor of peptidoglycan, lipopolysaccharide and teichoic acids.[49] Hence perturbations in this enzyme will affect the cell wall composition, reducing both cell stability and resistance to antibiotics, and inhibition has been linked to dramatic morphological changes.[50]

Stress

Exposure of MRSA to manuka honey has already been shown to reduce levels of universal stress protein A.[14] Here, further evidence of stress was found, with an up-regulation of *sod*, *acpD* and *mscL* and a down-regulation of *asp*23 and a putative stress protein (Table 5). ATP-dependent ClpC showed reduced levels by proteomics, but the equivalent gene (*clpC*) was found to be up-regulated by qPCR. This might have been brought about by altered post-transcriptional events that may have affected its migration pattern during 2D-DIGE, perhaps causing the dissociation of the two parts of the enzyme. This protease complex comprises an ATPase specificity factor and a proteolytic domain[51] and has been demonstrated to play a role in bacterial adaptation to multiple stresses by the degradation of accumulated misfolded

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proteins. In *S. aureus*, *clpC* has been shown to regulate the TCA cycle, which is required for metabolism and growth in recovery from the stationary phase and cell death.[52] Within *S. aureus*, it is also thought to be essential for virulence, long-term survival and intracellular replication; mutants lacking *clp* showed attenuated virulence in mouse models.[53]

Summary

In this study, we compared patterns of protein expression in MRSA cells treated with and without manuka honey, examined the expression of pertinent genes by qPCR and investigated the whole-genome response by microarray analysis. In order to understand the complex effects of manuka honey on MRSA, changes in gene expression and cellular processes were mapped (Figure 4).

Essentially, the glucose in honey promoted glycolysis via *gapA* to generate pyruvate, acetate and acetyl phosphate. An accumulation of acetate is capable of activating the LytSR protein kinase system to induce the transcription of *lrgAB*, and acetyl phosphate has been postulated to activate *lytR* alone to promote *lrgAB* transcription.[54] Only an up-regulation of *lrgB* was detected here, but since both *lrgA* and *lrgB* are regulated by LytSR,[55] it is reasonable to assume that antiholin was induced and would have limited the export of murein hydrolases. The down-regulation of *cidB* indicated that the *cidABC* operon was not transcribed and that holin was in limited supply for the export of murein hydrolase. A repression of quorum sensing was deduced by the reduced expression of three of the genes in the *agr* operon and this would have resulted in diminished virulence in MRSA and a failure to initiate biofilm formation. Knock-on effects were a repression of *saeRS*, with a repression of further virulence genes and a derepression of *sarV*, which promoted the induction of *lrgB*, *scdA* and *atl*. The products of these three genes can be assumed to also limit cell wall functions and cell division (Figure 4), and this supports previous deductions that manuka honey interrupts cell division in MRSA.[13]

The differential expression of proteins and genes observed in this study provided a valuable insight into the mechanisms by which growth and pathogenicity in MRSA were inhibited and confirms the multifactorial effects of manuka honey on bacterial cells. Although the precise mode of inhibition of quorum sensing was not found, this study provides many leads for further investigation. Collectively, the observations made here support the hypothesis that honey reduces the fitness of MRSA to initiate infections or biofilms *in vitro*; whether this will be elicited in wounds colonized by MRSA in the human host must be explored. We have addressed the major responses observed, but it is important to remember that one cannot expect to see a complete correlation between gene expression and the corresponding activity level of the gene product. In addition, genes showing the highest fold change might not necessarily coincide with the most important bacterial physiological response. There remains a further opportunity to explain how manuka honey affects other genes in MRSA.

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