The *Staphylococcus aureus* Response to Unsaturated Long Chain Free Fatty Acids: Survival Mechanisms and Virulence Implications

John G. Kenny¹, Deborah Ward¹, Elisabet Josefsson², Ing-Marie Jonsson², Jason Hinds³, Huw H. Rees¹, Jodi A. Lindsay³, Andrej Tarkowski², Malcolm J. Horsburgh¹

¹ School of Biological Sciences, University of Liverpool, Liverpool, United Kingdom, ² Department of Rheumatology and Inflammation Research, University of Gothenburg, Göteborg, Sweden, ³ Division of Cellular & Molecular Medicine, St George’s, University of London, London, United Kingdom

Abstract

*Staphylococcus aureus* is an important human commensal and opportunistic pathogen responsible for a wide range of infections. Long chain unsaturated free fatty acids represent a barrier to colonisation and infection by *S. aureus* and act as an antimicrobial component of the innate immune system where they are found on epithelial surfaces and in abscesses. Despite many contradictory reports, the precise anti-staphylococcal mode of action of free fatty acids remains undetermined. In this study, transcriptional (microarrays and qRT-PCR) and translational (proteomics) analyses were applied to ascertain the response of *S. aureus* to a range of free fatty acids. An increase in expression of the σ² and CtsR stress response regulators was observed. This included increased expression of genes associated with staphyloxanthin synthesis, which has been linked to membrane stabilisation. Similarly, up-regulation of genes involved in capsule formation was recorded as were significant changes in the expression of genes associated with peptidoglycan synthesis and regulation. Overall, alterations were recorded predominantly in pathways involved in cellular energetics. In addition, sensitivity to linoleic acid of a range of defined (sigB, arCA, sasF, sarA, agr, crtM) and transposon-derived mutants (vraE, SAR2632) was determined. Taken together, these data indicate a common mode of action for long chain unsaturated fatty acids that involves disruption of the cell membrane, leading to interference with energy production within the bacterial cell. Contrary to data reported for other strains, the clinically important EMRSA-16 strain MRSA252 used in this study showed an increase in expression of the important virulence regulator RNAIII following all of the treatment conditions tested. An adaptive response by *S. aureus* of reducing cell surface hydrophobicity was also observed. Two fatty acid sensitive mutants created during this study were also shown to display altered pathogenesis as assessed by a murine arthritis model. Differences in the prevalence and clinical importance of *S. aureus* strains might partly be explained by their responses to antimicrobial fatty acids.

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* E-mail: M.J.Horsburgh@Liverpool.ac.uk

Introduction

*Staphylococcus aureus* is the aetiological agent for a wide range of human infections, including abscesses, septicaemia, arthritis and endocarditis. The increased prevalence of meticillin resistant-(MRSA) and vancomycin insensitive-*S. aureus* strains, and the emergence of community-acquired MRSA make investigations into the pathogenicity of this species imperative. Inevitably, this focuses research into the development of novel antimicrobial agents, which requires a rigorous study of staphylococcal physiology. Long chain unsaturated free fatty acids (LC-uFFAs), typically ≥C16, are known to possess anti-staphylococcal activity comprising a pool of monoglycerides and free fatty acids [4–7]. Assay of staphylococcal abscess homogenates has revealed the presence of anti-staphylococcal activity comprising a pool of monoglycerides and free fatty acids [8–10]. The most abundant compound present in this active pool was identified as linoleic acid and was found at millimolar concentrations.

FFAs of various chain lengths and with different levels of unsaturation are primarily effective against Gram-positive bacteria [11–18]. Inhibition of several membrane-enveloped viruses has also been demonstrated [19–21]. Although several studies have attempted to pinpoint the specific cellular target(s) of LC-uFFAs, the actual anti-bacterial mechanism has not been unambiguously...
determined. Conflicting data have proposed that LC-uFFAs inhibit all major bacterial biosynthetic pathways within the cell, or alternatively, that they specifically inhibit FabI, which catalyses the final and rate-limiting step in fatty acid biosynthesis [12,18,22,23]. Oleic acid was proposed by Won et al. [24] to inhibit glucosyltransferases, while other proposed mechanisms for LC-uFFA-mediated growth inhibition include peptidoglycan (PG) precipitation, peroxidative stress, interference with energy metabolism and alteration of the membrane permeability or fluidity [12,16,18,22,25,26].

A diversity of mechanisms have been proposed to account for resistance to LC-uFFAs in S. aureus. Enhanced production of the carotenoid staphyloxanthin (giving S. aureus its golden title) has been proposed as a mechanism to relieve the inhibitory effects of increased membrane fluidity due to insertion of LC-uFFAs into the lipid bilayer in S. aureus [26–28]. Increased staphylococcal resistance to LC-uFFAs was positively correlated with pigmentation, although these experiments were performed using non-isogenic strains [29]. A fatty acid modifying enzyme (FAME), which catalyses the esterification of FFAs with cholesterol has also been purified from several S. aureus strains and its production correlated with increased disease severity in an abscess model [29–32]. Nonetheless the gene encoding FAME remains unidentified. Furthermore, in Neisseria gonorrhoea, FFA resistance has been linked to the presence of FFA-specific efflux pumps [33] while in S. aureus, the expression of Fur-iron-regulated staphylococcal surface-associated protein IsdA was identified as contributing to FA resistance in iron-limited environments by reducing cellular hydrophobicity [34]. Another proposed mechanism included the increased production of a ‘protective slime’ composed of precipitated PG complexed to fatty acids [25].

Previous studies demonstrated that S. aureus responds to the C12 monoester glycerol monolaurate (GML) and the component FFA lauric acid by reducing levels of expression of alpha toxin (Hla) [35–37]. Similarly, Clarke et al. [34] showed that expression of hla was reduced following exposure of S. aureus to the LC-uFFA hexadecenoic acid [C16:1 (n-6)]. More recently, GML was shown to inhibit the synthesis of toxins in several Gram-positive bacteria and also limited the effect of these toxins on eukaryotic cells [38–40].

While the biological effects of free fatty acids as antimicrobial compounds have been catalogued, there remains no unequivocal identification of the targets or mechanisms of action in relation to S. aureus. Transcriptomic and proteomic analyses have the potential to elucidate complex cellular and metabolic responses and are applied here for the first time to analyse the reaction of S. aureus to the LC-uFFAs linoleic, oleic and hexadecenoic acid. In addition, an analysis of existing well-characterised mutants and the generation of new allelic replacement mutants based on gene array data coupled to transposon screens was carried out to identify loci important for survival. Finally, a murine arthritis model of infection was used to ascertain whether two of the genes highlighted in this study have a role in pathogenesis.

Results

Comparative resistance of S. aureus strains to unsaturated C18 free fatty acids

The relative resistances of different strains of S. aureus to the unsaturated C18 free fatty acids linoleic acid [C18:2 (n-6,9)] and oleic acid [C18:1 (n-9)] were compared using a previously described agar plate assay [13]. Many strains, such as MSSA476 and N315, were unable to grow on emulsion agar plates containing 1 mM linoleic acid (Fig. 1A). In contrast MRSA252,
an epidemic ERMSA-16 strain, and the laboratory strain SH1000 displayed high levels (>60%) of survival at millimolar concentrations. Consequently, all subsequent experiments were performed using MRSA252 and SH1000 strains of S. aureus, owing to their enhanced growth in the presence of C18 LC-uFFAs.

**Growth of MRSA252 in the presence of LC-uFFAs**

To facilitate analysis of gene transcription and protein expression, a range of different concentrations of linoleic or oleic acid and the timing of their addition were examined during growth (data not shown). Upon inoculation 0.01 mM linoleic acid was determined to be the maximum concentration, which did not retard the aerobic growth of MRSA252 in BHI broth (Fig. 1B). Cells were subsequently grown in the presence of 0.01 mM linoleic or oleic acid with the FFAs being added at the start of growth (growth exposure conditions). To test the response of MRSA252 to LC-uFFAs under slightly different conditions, a higher concentration of linoleic acid (0.1 mM) was added during the late-exponential growth phase (OD_{600} = 3) where it was observed to reduce subsequent growth (challenge conditions) (Fig. 1C). These culture conditions were repeated for independent samples and cells were harvested to determine the transcriptional and translational responses of the cells to treatment with LC-uFFAs.

**The transcriptional response of S. aureus to C18 free fatty acids**

A pronounced differential transcriptional response was observed in MRSA252 cells treated with linoleic acid when it was added to a final concentration of 0.1 mM for 20 min during late-exponential growth (linoleic acid challenge) compared to unexposed control cells; 213 genes were up-regulated (Table 1) and 179 genes were down-regulated (Table 2). When transcription was analysed for cells grown in the presence of a lower concentration of linoleic acid (0.01 mM) from the time of inoculation (linoleic acid growth exposure) a correspondingly smaller subset of genes displayed differential transcription; 37 genes were up-regulated (Table 3) and 28 genes were down-regulated (Table 4). Oleic acid differs from linoleic acid in its degree of unsaturation, containing one less double bond in the chain. When cells were grown under the conditions of oleic acid growth exposure, 20 genes were up-regulated (Table 5) and 23 genes were down-regulated (Table 6).

The sudden imposition of linoleic acid during exponential growth at OD_{600} = 3 (linoleic acid challenge) resulted in large-scale transcriptional reprogramming of genes in four major discernible categories, including: virulence, energy metabolism, stress resistance and cell wall synthesis. In contrast, the presence of linoleic at 0.01 mM, a non-growth limiting concentration (linoleic acid growth exposure), resulted in changes in transcription of fewer genes in the same categories, with the exception of cell wall synthesis.

**Effect of linoleic acid on S. aureus MRSA252 transcription**

A distinctive feature of linoleic acid addition to cells of MRSA252 under both challenge and growth exposure conditions was observed to be the 10- and 2-fold up-regulation of the virulence regulator RNAIII, respectively (Table 1, 3). Previous studies have not reported changes in regulation of this locus after exposure to FFAs in S. aureus [34,35]. Moreover, after linoleic acid challenge the virulence regulator sarA was up-regulated as was cfbJ, encoding clumping factor A and genes required for capsule formation (cfaF, cfaM, cfaN), while the genes encoding the proteases staphopain and aureolysin were down-regulated (Table 1, 2). Further virulence-associated loci up-regulated in the presence of linoleic acid during growth included the eod locus encoding ESAT-6-like proteins and the genes coding for their synthesis/secretion [41] and tsaR that encodes a MarR regulator of SarS and SarF expression [42] (Table 3).

Many genes involved in sugar metabolism showed altered levels of regulation. In particular, several genes in the fructose and mannose metabolism pathways were down-regulated. These include SAR0753 [fraU] and SAR0752, involved in the importation and phosphorylation of fructose, respectively. Genes with similar functions involving the importation and phosphorylation of glucose, mannose, maltose and galactitol, namely SAR0255, SAR1777 (yjfK), SAR2720, SAR2721 (pgm) and SAR0242 and SAR0263 were also down-regulated. This could indicate an alteration of central metabolism via the action of the linoleic acid. Here, in concert with these changes, many genes in glycolysis were up-regulated, including SAR2684 (fdi), SAR0830 (pgk), SAR0831 (pgm) and SAR2596 (pgp). In addition, the SAR1014 (dmo), SAR0574 and SAR0575 genes involved in pentose and glucuronate interconversions were up-regulated, which would increase the availability of substrates for glycolysis or pentose phosphate pathways. The down-regulation of the putative UTP-glucose-1-phosphate uridylyltransferases SAR2252 and SAR2579 (gabB), which are predicted to catalyse the conversion of glucose-1-phosphate to UDP-glucose, would maintain the pool of phosphorylated glucose available for glycolysis.

In addition to increased transcription of genes encoding glycolytic enzymes, the cells exposed to a linoleic acid challenge alter metabolism to maintain levels of pyruvate. The up-regulation of SAR0204 which encodes malate dehydrogenase (converting malate to pyruvate) is predicted to increase pyruvate levels. Concomitantly, there was down-regulation of genes involved in pyruvate utilisation, including ldhA, SAR1088 (pycA), ald2 and SAR0555 converting pyruvate to lactate, oxaloacetate, alanine and cysteine, respectively. Reduced transcription of SAR2143 (ldcB) could further lower the expenditure of cellular pyruvate via amino acid synthesis, and down-regulation of SAR0522 and SAR0523 encoding predicted enzymes utilising glyceraldehyde-3-phosphate would prevent diversion of this intermediate from glycolysis. The reduced importation of substrates for glycolysis would explain increased levels of glycolytic enzymes and modulation of other pathways to increase pyruvate production. Under such potentially energy starved conditions, the pool of pyruvate would be pushed toward energy creation at the expense of less critical pathways.

There was up-regulation of many genes involved in cellular stress responses, including the CtsR regulon genes clpB, dnaJ and dnak suggesting that linoleic acid addition is perceived by S. aureus as a stressor. Moreover, the transcripts of several σ^{54}-regulated genes were up-regulated, including kspA, asp23 and clpA, and the ctsM, ctsN, ctsO, ctsP, ctsQ genes involved in staphyloxanthin biosynthesis. The mevalonate pathway generates the isopentenyl-diphosphate precursor for biosynthesis of this carotenoid, and the pathway genes mevA,K1, mevD and mevK2 were up-regulated accordingly (Table 1). Linoleic acid has been proposed to interfere with membrane function by increasing fluidity, which has the potential to perturb the electron transport chain. The production of carotenoids, which insert into the membrane has been reported to decrease fluidity and counteract the effect of LC-uFFAs [26]. In response to linoleic acid challenge the menaquinone biosynthesis pathway genes SAR1017 (menD) and SAR1018 involved in the conversion of chorismate to menaquinone (MK), and present in an operon with menB, were up-regulated indicating an increase in MK biosynthesis. This up-regulated MK synthesis could be a response to perturbation of the electron transport chain. The SAR1179, SAR1480 (menH) and SAR1481 genes synthesise heptaprenyl diphosphate for the isoprenoid moiety of MK-7, while SAR1278
| Group Functions       | MRSA252 ORF | MRSA252 Gene | MRSA252 Gene Product                                      | Fold Change Up Regulated | P-value |
|-----------------------|------------|--------------|----------------------------------------------------------|--------------------------|---------|
| Virulence Factors     | SAR0156    | capF         | capsular polysaccharide synthesis enzyme                  | 2.23                     | 4.22E-02|
| and Regulators        | SAR0163    | capM         | capsular polysaccharide synthesis enzyme                  | 2.23                     | 3.20E-02|
|                       | SAR0164    | capN         | capsular polysaccharide synthesis enzyme                  | 2.83                     | 1.79E-02|
|                       | SAR0625    | sarA         | staphylococcal accessory regulator A                      | 2.14                     | 1.50E-02|
|                       | SAR0842    | clfA         | clumping factor                                           | 4.12                     | 6.58E-03|
|                       | SAR2122    | hld          | delta-hemolysin precursor                                 | 3.28                     | 1.23E-02|
| Stress Response       | SAR2295    |              | putative exported MAP/eap domain protein                  | 3.21                     | 8.77E-04|
|                       | SAR243     | tcaR         | MarR family regulatory protein                             | 3.15                     | 1.76E-03|
| RNAIII                | SAR0777    | proP         | putative proline/betaine transporter                      | 8.31                     | 5.78E-04|
|                       | SAR0859    |              | putative organic hydroperoxide resistance protein         | 3.82                     | 1.23E-02|
|                       | SAR0938    | clpB         | putative ATPase subunit of an ATP-dependent protease      | 2.49                     | 8.15E-04|
|                       | SAR1344    | katA         | catalase                                                  | 5.71                     | 1.86E-03|
|                       | SAR1656    | dnaI         | chaperone protein                                         | 2.30                     | 4.25E-02|
|                       | SAR1657    | dnaK         | chaperone protein                                         | 2.41                     | 2.17E-03|
|                       | SAR2273    | asp23        | alkaline shock protein 23                                  | 2.06                     | 3.86E-02|
|                       | SAR2276    | opuD2        | glycine betaine transporter 2                             | 4.42                     | 6.16E-03|
|                       | SAR2561    |              | alkylhydroperoxidase, AhpD family                         | 6.63                     | 8.77E-04|
|                       | SAR2628    | clpL         | putative ATPase subunit of an ATP-dependent protease      | 4.06                     | 4.79E-03|
| Energy Metabolism     | SAR0113    | lldP1        | L-lactate permease 1                                       | 2.15                     | 1.07E-03|
|                       | SAR0188    |              | putative isochorismatase                                  | 4.91                     | 8.03E-04|
|                       | SAR0141    | drm          | putative phosphopentomutase                               | 2.45                     | 1.31E-02|
|                       | SAR0574    |              | putative hexulose-6-phosphate synthase                    | 2.36                     | 1.80E-03|
|                       | SAR0575    |              | putative 6-phospho-3-hexuliosomerase                       | 2.16                     | 5.11E-03|
|                       | SAR0775    |              | Osmoprotectant ABC transporter                             | 2.13                     | 4.80E-03|
|                       | SAR0776    |              | Osmoprotectant ABC transporter, permease protein           | 2.99                     | 3.00E-04|
|                       | SAR0824    |              | putative malolactic enzyme                                 | 2.59                     | 9.27E-03|
|                       | SAR0830    | tpiA         | triosephosphate isomerase                                 | 2.22                     | 3.39E-02|
|                       | SAR0831    | pgm          | putative phosphoglycerate mutase                          | 2.64                     | 1.39E-02|
|                       | SAR1017    | menD         | putative menaquinone biosynthesis bifunctional protein    | 2.24                     | 1.65E-03|
|                       | SAR1018    |              | putative hydrolase                                        | 2.80                     | 1.65E-03|
|                       | SAR2386    |              | putative NAD-dependent dehydrogenase                      | 3.73                     | 3.00E-04|
|                       | SAR2506    | dpgm         | putative phosphoglycerate mutase                          | 2.06                     | 7.33E-04|
|                       | SAR2684    | fda          | fructose-bisphosphase aldolase class I                     | 2.02                     | 5.85E-03|
|                       | SAR2687    |              | putative AMP-binding enzyme                                | 2.01                     | 9.65E-03|
|                       | SAR2724    |              | isochorismatase family protein                             | 3.00                     | 8.30E-04|
| DNA Repair and        | SAR0363    | ssb          | putative single-strand DNA-binding protein                 | 2.26                     | 3.29E-03|
| Replication           | SAR0744    |              | putative DNA photolase                                    | 3.46                     | 6.97E-04|
|                       | SAR0813    | uvrA         | excinuclease ABC subunit A                                 | 2.45                     | 2.06E-03|
|                       | SAR0836    | rr           | putative ribonuclease R                                   | 3.40                     | 3.55E-03|
| Protein Synthesis     | SAR0837    | smpB         | putative tmRNA-binding protein                             | 3.07                     | 3.70E-04|
|                       | SAR0364    | rpsR         | 30S ribosomal protein S18                                  | 2.40                     | 1.99E-02|
|                       | SAR0552    | fus          | translation elongation factor G                            | 2.10                     | 3.39E-02|
|                       | SAR1638    | rpoD         | RNA polymerase sigma factor                                | 2.86                     | 3.70E-04|
|                       | SAR2308    | rplQ         | 50S ribosomal protein L17                                  | 2.60                     | 1.99E-02|
|                       | SAR2309    | rpoA         | DNA-directed RNA polymerase alpha chain                    | 2.36                     | 3.35E-02|
| Group Functions | MRSA252 ORF | MRSA252 Gene | MRSA252 Gene Product | Fold Change Up Regulated | P-value |
|----------------|------------|-------------|----------------------|-------------------------|---------|
| Peptidoglycan Synthesis | SAR0878 | csdB | putative selenocysteine lyase | 2.52 | 3.07E-02 |
| | SAR1026 | atl | bifunctional autolysin precursor | 2.65 | 6.18E-03 |
| | SAR1158 | mraY | phospho-N-acetyluramoyl-pentapeptide-transferase | 2.13 | 9.56E-04 |
| | SAR1159 | murD | UDP-N-acetyluramoylanaline-D-glutamate ligase | 2.39 | 7.64E-03 |
| | SAR1160 | infA | translation initiation factor IF-1 | 2.10 | 1.31E-02 |
| | SAR1290 | murG | putative N-acetylglucosamine transferase | 5.26 | 1.86E-03 |
| Fatty Acid Metabolism | SAR1438 | conserved hypothetical protein | 2.64 | 4.94E-03 |
| | SAR2187 | fabZ | putative hydroxymyristoyl-(acyl carrier protein) dehydratase | 2.41 | 4.22E-02 |
| Carotenoid Biosynthesis | SAR0596 | mvaK1 | mevalonate kinase | 2.32 | 3.00E-04 |
| | SAR0597 | mvaD | mevalonate diphosphate decarboxylase | 3.35 | 9.23E-04 |
| | SAR0598 | mvaK2 | phosphomevalonate kinase | 3.18 | 5.09E-04 |
| | SAR2642 | crn | squalene synthase | 4.95 | 8.03E-04 |
| | SAR2643 | crnM | squalene desaturase | 7.18 | 2.38E-02 |
| | SAR2645 | crnQ | putative glycosyl transferase | 6.07 | 3.00E-03 |
| | SAR2646 | crnP | putative phytoene dehydrogenase related protein | 6.28 | 1.73E-03 |
| | SAR2647 | putative membrane protein | 4.47 | 1.73E-03 |
| Antibiotic Resistance | SAR0139 | putative tetracycline resistance protein | 4.06 | 1.59E-03 |
| | SAR1622 | metallo-beta-lactamase superfamily protein | 2.08 | 3.93E-03 |
| | SAR1785 | metallo-beta-lactamase superfamily protein | 3.05 | 1.08E-03 |
| | SAR1831 | blaZ | beta-lactamase precursor | 2.02 | 2.72E-02 |
| | SAR2505 | mdeA | putative transport system protein | 3.93 | 7.74E-03 |
| | SAR2558 | conserved hypothetical beta-lactamase-like protein | 8.72 | 3.70E-04 |
| | SAR2632 | putative MMPL efflux pump | 2.03 | 4.58E-02 |
| | SAR2655 | putative glyoxylase | 5.15 | 1.11E-03 |
| | SAR2668 | putative tetracycline resistance protein | 4.06 | 1.59E-03 |
| | SAR1738 | tnpB2 | transposase B 2 | 2.14 | 1.25E-03 |
| | SAR2725 | sasF | putative surface anchored protein | 16.80 | 4.68E-05 |
| Metabolism | SAR0108 | putative peptidase | 2.98 | 5.22E-03 |
| | SAR0109 | putative transporter protein | 2.37 | 1.52E-02 |
| | SAR0170 | putative cation efflux system protein | 2.50 | 1.77E-03 |
| | SAR0306 | ABC transporter ATP-binding protein | 6.10 | 1.68E-03 |
| | SAR0324 | putative lipoate-protein ligase A | 2.09 | 4.31E-03 |
| | SAR0325 | putative reductase | 4.80 | 8.17E-04 |
| | SAR0556 | ThiJ/Ppl family protein | 7.20 | 7.59E-04 |
| Group Functions | MRSA252 ORF | MRSA252 Gene | MRSA252 Gene Product | Fold Change Up Regulated | P-value  |
|-----------------|------------|-------------|----------------------|-------------------------|---------|
| SAR0589         | SAR0589    | putative amino acid permease | 4.19 | 3.75E-03 |
| SAR0600         | SAR0600    | pyridine nucleotide-disulphide oxidoreductase protein | 2.26 | 2.77E-04 |
| SAR0624         | SAR0624    | putative esterase | 6.49 | 7.59E-04 |
| SAR0729         | SAR0729    | putative acetyltransferase | 2.92 | 3.23E-03 |
| SAR0732         | SAR0732    | putative acetyltransferase | 2.34 | 3.00E-04 |
| SAR0756         | SAR0756    | aldo/keto reductase family protein | 2.96 | 6.16E-04 |
| SAR0757         | SAR0757    | putative glucosyl transferase | 3.49 | 7.59E-04 |
| SAR0764         | SAR0764    | putative 6-pyruvoyl tetrahydropyran synthase | 3.70 | 8.03E-04 |
| SAR0841         | SAR0841    | putative acetyltransferase | 5.22 | 3.29E-03 |
| SAR0883         | SAR0883    | putative dioxygenase | 5.40 | 1.75E-03 |
| SAR0903         | SAR0903    | putative pyridine nucleotide-disulphide oxidoreductase | 2.82 | 3.00E-04 |
| SAR0953         | SAR0953    | transport system extracellular binding lipoprotein | 2.18 | 6.05E-03 |
| SAR1076         | SAR1076    | Spermidine/putrescine-binding protein homolog. | 4.46 | 8.77E-04 |
| SAR1247         | SAR1247    | putative tRNA pseudouridine synthase B | 2.31 | 2.10E-03 |
| SAR1340         | SAR1340    | thb | homoserine kinase | 2.20 | 3.71E-02 |
| SAR1431         | SAR1431    | putative acetyltransferase | 4.58 | 1.80E-03 |
| SAR1439         | SAR1439    | dhb | dihydrofolate reductase type I | 2.13 | 6.34E-03 |
| SAR1440         | SAR1440    | thyA | thymidylate synthase | 5.32 | 1.58E-03 |
| SAR1585         | SAR1585    | malR | maltose operon transcriptional repressor | 2.21 | 2.20E-02 |
| SAR1655         | SAR1655    | putative methyltransferase | 2.25 | 6.08E-03 |
| SAR2210         | SAR2210    | moaA | putative molybdenum cofactor biosynthesis protein A | 2.07 | 1.86E-03 |
| SAR2352         | SAR2352    | putative Na+/H+ antiporter | 2.35 | 1.34E-02 |
| SAR2385         | SAR2385    | inositol monophosphatase family protein | 2.90 | 7.59E-04 |
| SAR2413         | SAR2413    | putative short chain dehydrogenase | 4.63 | 3.66E-03 |
| SAR2460         | SAR2460    | putative acetyltransferase (GNAT) family protein | 2.26 | 6.97E-04 |
| SAR2485         | SAR2485    | narH | nitrate reductase beta chain | 2.16 | 1.02E-02 |
| SAR2541         | SAR2541    | putative carboxylesterase | 2.45 | 1.79E-02 |
| SAR2544         | SAR2544    | ABC transporter ATP-binding protein | 6.01 | 1.68E-03 |
| SAR2559         | SAR2559    | putative short chain dehydrogenase | 6.85 | 6.16E-04 |
| SAR2659         | SAR2659    | putative short chain dehydrogenase | 2.65 | 1.76E-03 |
| SAR2661         | SAR2661    | putative hydrolase | 8.11 | 8.27E-04 |
| SAR2754         | SAR2754    | hisE | putative histidine biosynthesis bifunctional protein | 2.09 | 2.19E-02 |
| SAR2778         | SAR2778    | putative nickel transport protein | 2.51 | 3.59E-03 |
| SAR0111         | SAR0111    | putative myosin-crossreactive antigen | 5.96 | 2.43E-03 |
| SAR0112         | SAR0112    | putative membrane protein | 3.57 | 4.80E-04 |
| SAR0171         | SAR0171    | hypothetical protein | 2.64 | 3.01E-02 |
| SAR0269         | SAR0269    | LacI family regulatory protein | 2.57 | 3.59E-03 |
| SAR0299         | SAR0299    | hypothetical protein | 2.05 | 4.99E-02 |
| SAR0305         | SAR0305    | putative membrane protein | 3.89 | 6.02E-03 |
| SAR0390         | SAR0390    | putative lipoprotein | 3.97 | 1.68E-03 |
| SAR0392         | SAR0392    | putative membrane protein | 2.54 | 1.20E-02 |
| SAR0405         | SAR0405    | hypothetical protein | 2.76 | 1.07E-02 |
| SAR0444         | SAR0444    | putative lipoprotein | 2.31 | 2.16E-03 |
| SAR0498         | SAR0498    | yabJ | putative regulatory protein | 3.65 | 1.07E-03 |
| SAR0499         | SAR0499    | spoVG | stage V sporulation protein G | 2.83 | 1.11E-02 |
| SAR0601         | SAR0601    | putative DNA-binding protein | 2.15 | 4.48E-04 |
| SAR0670         | SAR0670    | putative sensor histidine kinase protein | 2.01 | 4.80E-04 |
| SAR0721         | SAR0721    | multicopper oxidase protein | 2.29 | 4.39E-03 |
| Group Functions | MRSA252 ORF | MRSA252 Gene | MRSA252 Gene Product | Fold Change Up Regulated | P-value |
|-----------------|------------|--------------|----------------------|-------------------------|---------|
| SAR0733         | conserved hypothetical protein | 3.04 | 1.99E-03 |
| SAR0734         | conserved hypothetical protein | 2.23 | 1.59E-03 |
| SAR0821         | conserved hypothetical protein | 3.19 | 6.54E-03 |
| SAR0825         | conserved hypothetical protein | 5.06 | 1.86E-03 |
| SAR0840         | putative membrane protein | 5.25 | 2.63E-03 |
| SAR0849         | hypothetical protein | 2.81 | 6.05E-03 |
| SAR0850         | hypothetical protein | 2.94 | 6.81E-04 |
| SAR0854         | hypothetical protein | 4.07 | 1.56E-02 |
| SAR0855         | hypothetical protein | 2.53 | 1.94E-03 |
| SAR0867         | hypothetical protein | 2.54 | 4.55E-03 |
| SAR0877         | conserved hypothetical protein | 2.34 | 3.01E-02 |
| SAR0879         | NifU-like protein | 2.06 | 3.39E-02 |
| SAR0880         | conserved hypothetical protein | 2.14 | 3.47E-03 |
| SAR0882         | putative membrane protein | 4.05 | 4.12E-03 |
| SAR0931         | putative membrane protein | 7.87 | 4.28E-04 |
| SAR1055         | hypothetical protein | 4.50 | 3.08E-03 |
| SAR1077         | putative membrane protein | 2.54 | 5.69E-03 |
| SAR1227         | conserved hypothetical protein | 2.11 | 1.71E-02 |
| SAR1258         | putative DNA-binding protein | 2.12 | 3.07E-04 |
| SAR1289         | putative exported protein | 3.49 | 1.65E-03 |
| SAR1306         | hypothetical protein | 2.20 | 5.66E-03 |
| SAR1429         | putative membrane protein | 5.74 | 1.84E-03 |
| SAR1528         | hypothetical phage protein | 6.04 | 4.99E-02 |
| SAR1623         | conserved hypothetical protein | 2.29 | 1.39E-02 |
| SAR1669         | conserved hypothetical protein | 2.01 | 1.03E-02 |
| SAR1670         | conserved hypothetical protein | 2.53 | 5.36E-03 |
| SAR1671         | probable nicotinate-nucleotide adenyllytransferase | 2.03 | 8.07E-03 |
| SAR1816         | putative membrane protein | 2.82 | 6.16E-04 |
| SAR1854         | hypothetical protein | 4.98 | 1.27E-03 |
| SAR1965         | ThiJ/Ppl family protein | 2.25 | 4.83E-02 |
| SAR1970         | conserved hypothetical protein | 2.17 | 4.68E-02 |
| SAR1972         | putative exported protein | 5.71 | 6.59E-03 |
| SAR2010         | hypothetical protein | 3.49 | 6.58E-03 |
| SAR2047         | hypothetical phage protein | 2.12 | 1.16E-02 |
| SAR2085         | hypothetical phage RecT family protein | 2.18 | 9.84E-04 |
| SAR2088         | hypothetical phage protein | 2.62 | 2.28E-02 |
| SAR2094         | hypothetical phage protein | 2.69 | 3.06E-03 |
| SAR2095         | hypothetical phage protein | 4.03 | 5.44E-03 |
| SAR2098         | hypothetical phage protein | 2.02 | 2.87E-03 |
| SAR2189         | putative membrane protein | 2.94 | 6.07E-03 |
| SAR2232         | conserved hypothetical protein | 8.26 | 2.06E-03 |
| SAR2245         | putative transcriptional antiterminator | 6.03 | 1.76E-03 |
| SAR2270         | hypothetical luxA/luxC family protein | 3.36 | 3.80E-03 |
| SAR2274         | putative membrane protein | 4.59 | 2.04E-03 |
| SAR2275         | putative membrane protein | 3.98 | 6.16E-04 |
| SAR2347         | putative membrane protein | 2.21 | 6.54E-03 |
| SAR2392         | conserved hypothetical protein | 3.03 | 5.20E-03 |
| SAR2393         | putative molydopterin dinucleotide binding domain protein | 3.26 | 2.50E-03 |
| SAR2444         | putative membrane protein | 4.38 | 2.77E-04 |
| SAR2469         | putative pyridoxamine 5’-phosphate oxidase | 4.72 | 1.11E-03 |
The MK-7 isoprenolog genes were down-regulated, which is consistent with a reduction of temperature and oxygen levels [43].

Logs, up to MK-9, and alters their ratio in response to changes in modulated in linoleic acid challenge conditions but not in the growth regulated, as was in the synthesis of the pentaglycine precursor in PG synthesis were up-regulated, as was atl, encoding the major cellular autolysin (Table 1) [44]. There was down-regulation of the two-component regulatory system lytRS, the holin-like bgA and cidA and the putative transglycosylase SAR1807, which have cell wall modulatory roles (Table 2) [45,46]. In addition to these changes, an assortment of transcriptionally modulated genes was observed, which would function to maintain the level of constituents for the PG-pentapeptide transcriptionally modulated genes was observed, which would function to maintain the level of constituents for the PG-pentapeptide precursor. SAR2109 (dagIE), which catalyses the formation of a substrate for lysine biosynthesis and the lysine-specific permease SAR1761 (lysP) were up-regulated, and this would increase the pool of L-lysine in the cell. Up-regulation of SAR2420 (huA), and down-regulation of SAR2669 encoding a putative dihydroorotate dehydrogenase, SAR0228 encoding a putative glutamine amidotransferase and SAR1732 (hemL), in concert, would maintain glutamate levels within the cell. SAR2269, a putative alanine racemase, was up-regulated thereby increasing synthesis of D-alanine by isomerising L-alanine. The microarray data also revealed increased transcription of the tagA, tagG and tagB genes concerned with teichoic acid biosynthesis.

The fatty acid biosynthesis enzyme FabI was previously reported to be inhibited by linoleic acid and was therefore proposed to be a key target for its antibacterial activity [23]. Here, within fatty acid metabolism, only fabZ was up-regulated in linoleic acid challenge conditions, whereas fabD, fabA and fad were down-regulated. fabZ is directly downstream of mraA1 within a predicted operon which may explain why fabZ alone is up-regulated amongst the fatty acid biosynthesis genes.

**Quantitative Real-Time PCR**

Confirmation of the microarray data was performed using qRT-PCR to test selected transcriptional changes of known genes from different functional subsets. To this end, the expression level of genes involved in staphyloxanthin synthesis (crtM), PG biosynthesis (murG, cidA and lytR), stress responses (katA and ctpR), virulence (RNAIII, sapA, arca, hla and spa) and fatty acid metabolism (fabZ, fabI, fadD and fadA) were analysed. In addition, the saIF gene was analysed to confirm the particularly high levels of transcript that were observed under the challenge experimental conditions. Most genes tested showed the same pattern of up- or down-regulation (Table 7) that was predicted by microarray analysis under any given set of conditions. The only exceptions were the fatty acid degradation pathway genes fadD and fadA. While fadD was 2.15 fold down-regulated after linoleic acid challenge when analysed by microarray, this was identified as a 3.16 fold up-regulation when tested by qRT-PCR. The fabA gene lies within a predicted operon with fadD and would thus be co-regulated. A 3.1 fold up-regulation of fabA was similarly measured by qRT-PCR when the cells were challenged with linoleic acid, which supports the reproducibility of the qRT-PCR analysis of fadD and its likely operon arrangement with fabA. Therefore, with the exception of the fab operon, the microarray data was shown to be consistent when tested by qRT-PCR.

The transcription of a subset of genes was examined by qRT-PCR during mid-exponential growth phase and late exponential-phase (OD₆₀₀ = 8) (Table 7), to examine the potential effect of the increased

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**Table 1.**

| Group Functions | MRSA252 ORF | MRSA252 Gene Product | Fold Change Up Regulated | P-value |
|-----------------|-------------|-----------------------|--------------------------|---------|
| SAR2496         | putative solute binding lipoprotein | 2.60 | 3.66E-03 |
| SAR2525         | hypothetical protein | 5.28 | 2.33E-05 |
| SAR2532         | CapD domain protein | 2.48 | 4.16E-03 |
| SAR2542         | putative transport protein | 2.01 | 5.64E-04 |
| SAR2543         | putative membrane protein | 6.37 | 6.16E-04 |
| SAR2568         | hypothetical protein | 4.66 | 1.65E-03 |
| SAR2656         | conserved hypothetical protein | 3.35 | 6.16E-04 |
| SAR2657         | hypothetical protein | 2.40 | 8.80E-04 |
| SAR2658         | TetR family regulatory protein | 2.22 | 4.39E-04 |
| SAR2660         | conserved hypothetical protein | 7.26 | 6.16E-04 |
| SAR2665         | conserved hypothetical protein | 2.19 | 4.08E-03 |
| SAR2666         | hypothetical protein | 2.75 | 1.87E-03 |
| SAR2667         | hypothetical protein | 2.19 | 1.37E-02 |
| SAR2686         | hypothetical protein | 7.55 | 3.70E-04 |
| SAR2689         | hypothetical protein | 2.53 | 1.84E-02 |
| SAR2726         | conserved hypothetical protein | 5.07 | 1.80E-03 |
| SAR2727         | glycosyl transferase, group 1 family protein | 4.11 | 6.70E-03 |
| SAR2739         | conserved hypothetical protein | 4.21 | 2.06E-03 |
| SAR2740         | conserved hypothetical protein | 2.05 | 3.09E-02 |
| SAR2777         | putative DNA-binding protein | 2.40 | 1.90E-03 |
| SAR2780         | putative membrane protein | 7.38 | 4.80E-04 |

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Table 2. MRSA252 genes down-regulated following the addition of linoleic acid (0.1 mM) to exponentially growing cells (linoleic acid challenge).

| Group Functions and Regulators | MRSA 252 ORF | MRSA252 Gene | MRSA252 Gene Product | Fold Change | Down Regulated | P-value |
|-------------------------------|--------------|--------------|----------------------|-------------|----------------|---------|
| Virulence Factors and Regulators | SAR0105 | plc | 1-phosphatidylinositol phosphodiesterase | 3.85 | 4.39E-04 | |
| | SAR1574 | fur | iron uptake regulatory protein | 2.08 | 2.46E-02 | |
| | SAR1984 | ferritin | 2.56 | 4.39E-03 | |
| | SAR2001 | staphopain protease | 2.44 | 3.00E-04 | |
| | SAR2474 | sarZ | MarR family virulence regulator | 2.22 | 2.18E-02 | |
| | SAR2155 | nsbU | putative sigma factor sigB regulation protein | 2.56 | 1.58E-03 | |
| | SAR2715 | argR | arginine repressor family protein | 2.27 | 4.27E-03 | |
| | SAR2716 | aur | zinc metalloproteinase aureolysin precursor | 2.17 | 1.76E-03 | |
| Energy Metabolism | SAR0234 | ldh1 | L-lactate dehydrogenase 1 | 2.94 | 2.99E-05 | |
| | SAR0235 | | putative PTS system, IIBC component | 2.22 | 1.30E-03 | |
| | SAR0242 | | putative galactitol PTS component | 2.13 | 1.76E-02 | |
| | SAR0263 | | putative PTS system protein | 2.50 | 3.44E-02 | |
| | SAR0355 | | Cys/Met metabolism PLP-dependent enzyme | 2.04 | 2.74E-02 | |
| | SAR0522 | | putative pyridoxal 5-phosphate biosynthesis protein | 3.03 | 3.00E-04 | |
| | SAR0523 | | SNO glutamine amidotransferase family protein | 2.70 | 3.70E-04 | |
| | SAR0752 | | putative phosphofructokinase | 2.38 | 3.27E-02 | |
| | SAR0753 | fruA | fructose-specific PTS system component | 2.50 | 3.78E-02 | |
| | SAR0766 | | glutamine amidotransferase class-I protein | 2.04 | 6.16E-04 | |
| | SAR1088 | | putative pyruvate carboxylase | 2.5 | 8.78E-04 | |
| | SAR1450 | tdcB | putative threonine dehydratase | 2.22 | 5.47E-03 | |
| | SAR1451 | ald2 | alanine dehydrogenase 2 | 3.03 | 1.15E-03 | |
| | SAR1777 | pfkA | 6-phosphofructokinase | 2.86 | 2.87E-03 | |
| | SAR1789 | ackA | acetate kinase | 2.33 | 9.23E-03 | |
| | SAR2143 | ilvC | ketol-acid reductoisomerase | 2.22 | 2.80E-02 | |
| | SAR2213 | fba | putative tagatose-bisphosphate aldolase | 3.13 | 6.59E-03 | |
| | SAR2262 | | putative uridylyltransferase | 2.27 | 8.35E-03 | |
| | SAR2579 | gtaB | putative uridylyltransferase | 2.63 | 6.84E-03 | |
| | SAR2720 | | putative PTS system component | 4.17 | 3.54E-03 | |
| | SAR2721 | pmi | mannos-6-phosphate isomerase | 3.57 | 2.76E-03 | |
| Cell Wall Synthesis | SAR0228 | | putative glutamine amidotransferase class-I | 2.13 | 2.08E-03 | |
| | SAR0257 | lrtK | autolysin sensor kinase protein | 3.33 | 3.00E-03 | |
| | SAR0258 | lrtR | autolysin response regulator protein | 3.57 | 9.15E-03 | |
| | SAR0259 | lrgA | holin-like protein | 2.22 | 1.20E-03 | |
| | SAR0646 | tagA | teichoic acid biosynthesis protein | 2.78 | 6.80E-03 | |
| | SAR0648 | tagG | teichoic acid ABC transporter permease protein | 2.38 | 1.32E-02 | |
| | SAR0649 | tagB | teichoic acid biosynthesis protein | 2.50 | 4.14E-04 | |
| | SAR1143 | | putative carbamate kinase | 2.27 | 6.79E-03 | |
| | SAR1752 | hemA | glutamyl-IRNA reductase | 2.27 | 3.93E-02 | |
| | SAR1807 | | putative transglycosylase | 2.04 | 8.78E-04 | |
| | SAR2472 | gilT | putative proton/sodium-glutamate symport protein | 2.04 | 2.97E-02 | |
| | SAR2621 | cidA | holin-like protein | 2.27 | 1.18E-02 | |
| | SAR2669 | | putative dihydroorotate dehydrogenase | 2.86 | 3.75E-03 | |
| Fatty Acid Metabolism | SAR0225 | fadD | putative acyl-CoA dehydrogenase | 2.17 | 3.27E-02 | |
| | SAR0227 | fadK | putative acetyl-CoA transferase | 2.13 | 4.95E-02 | |
| | SAR0803 | | conserved hypothetical protein | 3.23 | 1.02E-02 |
| Group Functions                          | MRSA 252 ORF | MRSA252 Gene | MRSA252 Gene Product                             | Fold Change Down Regulated | P-value      |
|-----------------------------------------|--------------|--------------|-------------------------------------------------|---------------------------|-------------|
| Carotenoid Biosynthesis                 | SAR1278      | miaA         | putative isopentenylpyrophosphate transferase   | 2.00                      | 9.23E-03    |
|                                         | SAR1479      | menH         | heptaprenylnaphthoquinone methyltransferase     | 2.78                      | 1.62E-02    |
|                                         | SAR1481      | hplB         | putative hexaprenyl diphosphate synthase        | 3.13                      | 1.79E-02    |
| DNA Repair and Replication              | SAR0001      | dnaA         | chromosomal replication initiator protein DnaA  | 2.04                      | 2.17E-03    |
|                                         | SAR0004      | recF         | DNA replication and repair protein RecF         | 2.08                      | 8.51E-03    |
|                                         | SAR0028      | repB         | replication protein (pseudogene)               | 4.35                      | 1.48E-02    |
|                                         | SAR0485      | holB         | putative DNA polymerase III, delta' subunit     | 3.03                      | 1.26E-02    |
|                                         | SAR0711      |              | putative replication initiation protein         | 2.50                      | 3.43E-02    |
|                                         | SAR3429      |              | putative 3-methylpurine glycosylase            | 2.22                      | 1.87E-03    |
| Metabolism                              | SAR0246      | ispD         | conserved hypothetical protein                  | 2.00                      | 2.27E-03    |
|                                         | SAR0261      |              | putative nitric oxide reductase                | 2.22                      | 6.16E-04    |
|                                         | SAR0302      |              | putative formate/nitrite transporter            | 2.38                      | 8.03E-03    |
|                                         | SAR0524      | nupC         | nucleoside permease                            | 2.94                      | 3.96E-03    |
|                                         | SAR0562      |              | putative deoxyadenosine kinase protein          | 2.17                      | 2.64E-02    |
|                                         | SAR0563      |              | putative deaminase                             | 2.50                      | 3.75E-03    |
|                                         | SAR0569      |              | putative glycosyl transferase                  | 2.13                      | 4.40E-03    |
|                                         | SAR0642      |              | ABC transporter permease protein                | 2.56                      | 9.65E-03    |
|                                         | SAR0643      |              | ABC transporter ATP-binding protein             | 3.70                      | 9.25E-03    |
|                                         | SAR0655      |              | putative Na+ dependent nucleoside transporter   | 2.17                      | 2.25E-03    |
|                                         | SAR0743      |              | putative sodium:sulfate symporter protein       | 2.22                      | 4.39E-04    |
|                                         | SAR0847      | nuc          | thermonuclease precursor                        | 3.33                      | 3.70E-04    |
|                                         | SAR0916      |              | putative peptidyl-prolyl cis-trans isomerase    | 2.13                      | 6.54E-03    |
|                                         | SAR1008      |              | putative glycosyl transferases                 | 4.00                      | 1.65E-03    |
|                                         | SAR1014      |              | acetyltransferase (GNAT) family protein         | 2.27                      | 6.54E-03    |
|                                         | SAR1090      | ctaB         | putative protopheme IX farnesyltransferase      | 2.04                      | 2.66E-02    |
|                                         | SAR1185      |              | putative guanylate kinase                      | 2.78                      | 6.16E-03    |
|                                         | SAR1449      |              | amino acid permease                            | 2.50                      | 2.65E-03    |
|                                         | SAR1478      | ndk          | putative nucleoside diphosphate kinase          | 2.38                      | 3.03E-02    |
|                                         | SAR1598      |              | arginine repressor                             | 2.50                      | 3.23E-03    |
|                                         | SAR1627      |              | S-formyltetrahydrofolate cyclo-ligase family protein | 2.78                   | 2.27E-03    |
|                                         | SAR1707      |              | putative ATPase                                | 2.13                      | 1.36E-02    |
|                                         | SAR1714      | relA         | GTP pyrophosphokinase                           | 2.27                      | 3.96E-03    |
|                                         | SAR1717      | secF         | putative protein-export membrane protein        | 2.27                      | 6.05E-03    |
|                                         | SAR1804      |              | putative aconitase                             | 2.44                      | 2.99E-02    |
|                                         | SAR2129      | scrR         | sucrose operon repressor                        | 2.56                      | 1.38E-02    |
|                                         | SAR2130      |              | ammonium transporter family protein             | 2.04                      | 1.65E-03    |
|                                         | SAR2340      |              | acetyltransferase (GNAT) family protein         | 3.03                      | 8.77E-04    |
|                                         | SAR2363      | modA         | putative molybdate-binding lipoprotein precursor | 2.08                      | 2.97E-02    |
|                                         | SAR2432      |              | CoR-like Mg2+ transporter protein               | 2.44                      | 5.61E-03    |
|                                         | SAR2493      |              | putative formate/nitrite transporter            | 2.22                      | 8.71E-03    |
|                                         | SAR2594      |              | ABC transporter ATP-binding protein             | 2.38                      | 1.65E-03    |
|                                         | SAR2789      |              | putative subtilase family protease             | 2.04                      | 2.27E-03    |
| Hypothetical Genes                      | SAR0013      |              | putative membrane protein                       | 2.17                      | 1.81E-02    |
|                                         | SAR0024      |              | conserved hypothetical protein                  | 3.03                      | 2.27E-03    |
|                                         | SAR0030      |              | hypothetical protein                            | 2.38                      | 6.16E-03    |
|                                         | SAR0048      |              | putative membrane protein                       | 2.08                      | 1.08E-02    |
|                                         | SAR0061      |              | putative membrane protein                       | 2.08                      | 4.25E-02    |
| Group Functions | MRSA 252 ORF | MRSA252 Gene | MRSA252 Gene Product | Fold Change | Down Regulated | P-value |
|-----------------|-------------|-------------|---------------------|-------------|----------------|---------|
| SAR0063         | hypothetical protein | 2.56 | 1.02E-02 |
| SAR0075         | hypothetical protein | 2.04 | 6.16E-04 |
| SAR0078         | hypothetical protein | 2.08 | 9.65E-03 |
| SAR0097         | putative DNA-binding protein | 2.17 | 2.99E-03 |
| SAR0145         | putative lipoprotein | 2.13 | 1.56E-02 |
| SAR0197         | hypothetical protein | 286 | 2.14E-02 |
| SAR0216         | putative lipoprotein | 2.04 | 6.16E-04 |
| SAR0338         | putative membrane protein | 2.86 | 2.40E-03 |
| SAR0383         | abortive infection bacteriophage resistance related | 4.76 | 1.99E-02 |
| SAR0618         | putative iron compound-binding protein | 2.27 | 4.08E-02 |
| SAR0673         | conserved hypothetical protein | 2.70 | 4.55E-03 |
| SAR0694         | putative bacteriocin | 2.38 | 3.75E-03 |
| SAR0695         | putative bacteriocin-immunity membrane protein | 2.22 | 2.08E-03 |
| SAR0718         | putative membrane protein | 3.33 | 9.93E-04 |
| SAR0761         | putative lipoprotein | 2.86 | 3.00E-04 |
| SAR0793         | hypothetical protein | 2.56 | 1.58E-02 |
| SAR0846         | secreted von Willebrand factor-binding homolog | 2.17 | 1.94E-02 |
| SAR0890         | conserved hypothetical protein | 2.56 | 8.06E-04 |
| SAR0893         | putative membrane protein | 2.13 | 4.74E-02 |
| SAR0898         | conserved hypothetical protein | 2.70 | 1.59E-02 |
| SAR0899         | conserved hypothetical protein | 2.33 | 4.94E-03 |
| SAR0915         | kinase-associated protein B | 2.44 | 8.06E-04 |
| SAR0970         | protozoan/cyanobacterial globin family protein | 2.38 | 1.11E-02 |
| SAR0971         | conserved hypothetical protein | 2.78 | 1.87E-03 |
| SAR0979         | putative membrane protein | 2.50 | 2.25E-03 |
| SAR0981         | putative esterase | 2.44 | 1.55E-03 |
| SAR0982         | putative restriction-modification system protein | 2.44 | 1.37E-03 |
| SAR0983         | putative restriction-modification system protein | 2.56 | 2.42E-03 |
| SAR0985         | putative 2'-5' RNA ligase family | 2.13 | 2.32E-02 |
| SAR0987         | putative monogalactosyldiacylglycerol synthase | 2.56 | 6.54E-03 |
| SAR1066         | putative lipoprotein | 2.50 | 4.74E-02 |
| SAR1085         | conserved hypothetical protein | 2.33 | 3.44E-02 |
| SAR1086         | conserved hypothetical protein | 3.45 | 6.54E-03 |
| SAR1095         | conserved hypothetical protein | 2.86 | 2.16E-02 |
| SAR1114         | putative cell division protein ZapA | 2.38 | 3.96E-03 |
| SAR1148         | putative DNA-binding protein | 2.38 | 2.66E-02 |
| SAR1154         | MraZ protein | 2.50 | 3.00E-03 |
| SAR1312         | hypothetical protein | 3.85 | 3.27E-02 |
| SAR1315         | hypothetical protein | 2.38 | 2.99E-03 |
| SAR1316         | hypothetical protein | 2.27 | 1.79E-02 |
| SAR1320         | hypothetical protein | 4.00 | 1.46E-02 |
| SAR1335         | putative exported protein | 2.27 | 7.38E-03 |
| SAR1389         | conserved hypothetical protein (pseudogene) | 2.33 | 5.63E-03 |
| SAR1448         | major facilitator superfamily transporter protein | 2.04 | 4.57E-03 |
| SAR1556         | putative phage regulatory protein | 2.08 | 5.64E-03 |
| SAR1558         | putative phage lipoprotein | 2.44 | 1.24E-03 |
| SAR1559         | hypothetical phage protein | 2.33 | 4.80E-04 |
| SAR1560         | hypothetical phage protein | 2.04 | 1.72E-02 |
| SAR1561         | putative phage membrane protein | 2.13 | 1.10E-03 |
| SAR1581         | conserved hypothetical protein | 2.86 | 1.81E-02 |
levels of the density-signalling effector RNAIII on transcription of regulated genes (e.g. spa, hla and sarA). qRT-PCR analysis was performed on MRSA252 genes under linoleic and oleic acid growth exposure conditions. The RNAIII and clpB transcripts were consistently up- or down-regulated, respectively, at all of the points tested during growth; at OD$_{600} = 8$ RNAIII was massively up-regulated (150-fold) in the presence of either linoleic or oleic acid. The transcription of sarA was up-regulated 1.5- to 2-fold in post-exponential phase in these conditions. Post-exponential transcription of hla was 6-fold higher after growth with either linoleic or oleic acid in comparison with the untreated control. Interestingly, this increase was moderate compared to that observed for RNAIII of the agr locus, which is known to up-regulate expression of hla. This reflects the complex regulation of hla and may be due to the increase in sarA levels.

Several genes showed fluctuations in relative transcript levels during the growth cycle. For example, arcA transcription varied over the different sample points, with gene up-regulation at OD$_{600} = 3.0$ for the linoleic growth experiment as per the microarray results. However, arcA was down-regulated in post-exponential phase in the presence of linoleic acid.

The observation of increased expression of RNAIII, hla and spa in MRSA252 in response to LC-uFFAs is significantly different to previously published experiments for these transcripts in alternative strains [34,35]. The expression of a large subset of genes, confirmed by qRT-PCR to be altered following exposure of MRSA252 to linoleic acid (Table 7), were subsequently examined in SH1000 to determine whether they were similarly regulated (Table 8). This revealed that in SH1000 the up- or down-regulation of several genes was in direct contrast to the pattern

### Table 2. Cont.

| Group Functions | MRSA 252 ORF | MRSA252 Gene | MRSA252 Gene Product | Fold Change | Down Regulated | P-value |
|-----------------|-------------|--------------|----------------------|-------------|----------------|---------|
| SAR1592         | conserved hypothetical protein | 2.27 | 1.16e-02 |
| SAR1699         | conserved hypothetical protein | 2.00 | 3.92e-03 |
| SAR1706         | putative transcriptional regulator | 3.45 | 2.16e-02 |
| SAR1708         | conserved hypothetical protein | 2.04 | 3.14e-03 |
| SAR1770         | putative membrane protein | 2.13 | 2.99e-03 |
| SAR1834         | putative leucyl-tRNA synthetase | 2.17 | 3.65e-02 |
| SAR1885         | hypothetical protein | 2.63 | 6.97e-04 |
| SAR1897         | hypothetical protein | 3.03 | 2.86e-02 |
| SAR1935         | probable phosphoesterase | 2.78 | 2.37e-03 |
| SAR1938         | putative DNA-binding protein | 2.38 | 2.80e-02 |
| SAR2020         | putative membrane protein | 2.44 | 6.39e-03 |
| SAR2035         | putative exported protein | 2.86 | 1.61e-02 |
| SAR2113         | hypothetical protein | 2.86 | 4.64e-02 |
| SAR2114         | hypothetical protein | 2.56 | 3.43e-02 |
| SAR2115         | hypothetical protein | 2.86 | 4.22e-02 |
| SAR2118         | putative membrane protein | 2.00 | 3.28e-03 |
| SAR2119         | membrane anchored protein | 2.44 | 1.08e-03 |
| SAR2156         | pemK-like protein | 3.03 | 5.61e-03 |
| SAR2219         | hypothetical protein | 2.78 | 6.21e-03 |
| SAR2261         | putative membrane protein | 2.08 | 6.02e-04 |
| SAR2263         | putative membrane protein | 2.17 | 2.89e-03 |
| SAR2299         | hypothetical protein | 2.04 | 3.47e-03 |
| SAR2369         | putative acyl-CoA dehydrogenase | 2.86 | 1.94e-03 |
| SAR2425         | putative membrane protein | 2.13 | 5.63e-03 |
| SAR2428         | putative membrane protein | 2.00 | 3.23e-03 |
| SAR2435         | putative acyl hydrolase | 2.50 | 3.02e-02 |
| SAR2439         | tetR family regulatory protein | 2.22 | 3.23e-03 |
| SAR2473         | putative exported protein | 3.85 | 3.97e-03 |
| SAR2500         | putative lipoprotein | 2.86 | 8.48e-04 |
| SAR2546         | putative lipoprotein | 3.13 | 2.76e-02 |
| SAR2595         | putative membrane protein | 2.78 | 1.65e-03 |
| SAR2718         | putative exported protein | 2.04 | 4.80e-04 |
| SAR2719         | transcriptional regulator | 3.13 | 3.07e-04 |
| SAR2792         | putative membrane protein | 3.85 | 2.78e-03 |
| SAR2793         | putative membrane protein | 3.70 | 8.17e-04 |

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observed in MRSA252. For example, where both microarray data and qRT-PCR data showed that there was a large up-regulation of RNAIII after challenge or growth exposure in MRSA252, pronounced down-regulation was observed in SH1000 by qRT-PCR. Contrasts in regulation between MRSA252 and SH1000 were also observed for sarA, spa and sasF. However, several genes not predicted to be RNAIII-regulated, including lytR, clpB, fabI, murG, and arcA exhibited similar patterns of regulation in both strains under the conditions tested.

### Proteomic analysis

The proteome of MRSA252 was analysed by 2D-PAGE to identify protein expression changes in exponentially growing cells that were exposed to linoleic acid under the challenge conditions used for the microarray experiments. This analysis was performed to determine whether the large-scale transcriptional modulation described above was translated into a correspondingly large-scale proteomic shift. Under these conditions, 58 proteins were significantly (P<0.05) up-regulated ≥2-fold and 15 proteins were

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**Table 3.** MRSA252 genes up-regulated during growth in the presence of linoleic acid (0.01mM) (linoleic acid growth exposure).

| Group Functions | MRSA252 ORF | MRSA252 Gene | MRSA252 Gene Product | Fold Change Up Regulated | P-value |
|-----------------|-------------|--------------|-----------------------|--------------------------|---------|
| Virulence Factors and Regulators | SAR0279 | exsA | virulence factor exsA | 5.93 | 7.03E-08 |
| | SAR0280 | exsA | putative membrane protein | 4.32 | 4.53E-06 |
| | SAR0281 | exsA | putative membrane protein | 2.70 | 1.66E-03 |
| | SAR0282 | exsB | conserved hypothetical protein | 2.65 | 3.35E-03 |
| | SAR0284 | exsC | putative membrane protein | 2.56 | 1.25E-03 |
| | SAR0284v | exsC | putative membrane protein | 2.49 | 4.23E-03 |
| | SAR2123 | agrB | putative autoinducer processing protein | 9.36 | 1.74E-05 |
| | SAR2125 | agrC | autoinducer sensor protein | 5.39 | 4.33E-05 |
| | SAR2126 | agrA | autoinducer sensor protein regulator protein | 2.25 | 1.41E-03 |
| | agrIII | agrIII | Class III accessory gene regulator (agr) locus | 8.71 | 4.16E-06 |
| | RNAIII | RNAIII | RNAIII accessory gene regulator (agr) locus | 10.20 | 1.21E-05 |
| Metabolism | SAR0150 | adhE | putative aldehyde-alcohol dehydrogenase | 2.25 | 1.67E-02 |
| | SAR0190 | gicA | glucose-specific PTS transporter protein, IIABC component | 2.05 | 3.76E-02 |
| | SAR0829 | pgk | phosphoglycerate kinase | 2.76 | 2.16E-03 |
| | SAR0830 | tpiA | triosephosphate isomerase | 2.75 | 1.69E-03 |
| | SAR0831 | pgm | putative phosphoglycerate mutase | 2.83 | 2.22E-03 |
| | SAR0832 | eno | putative enolase | 2.15 | 5.88E-03 |
| | SAR2296 | alsD | putative acetolactate decarboxylase | 2.43 | 3.32E-03 |
| | SAR2297 | alsS | putative acetolactate synthase | 2.17 | 1.41E-03 |
| | SAR2618 | glcB | PTS system, glucose-specific IIABC component | 2.78 | 1.41E-02 |
| | SAR2711 | arcC | carbamate kinase | 2.40 | 3.41E-02 |
| | SAR2712 | arcD | arginine/ornithine antiporter | 2.21 | 1.88E-02 |
| | SAR2713 | arcB | putative ornithine carbamoyltransferase | 2.31 | 1.88E-02 |
| | SAR2714 | arcA | arginine deiminase | 2.89 | 1.41E-02 |
| Hypothetical Genes | SAR0111 | putative myosin-crossreactive antigen | 2.44 | 6.52E-05 |
| | SAR0277 | putative exported protein | 3.76 | 4.22E-05 |
| | SAR0278 | putative CHAP domain protein | 2.69 | 1.22E-04 |
| | SAR0299 | possible pseudogene | 2.95 | 3.30E-03 |
| | SAR0301 | putative membrane protein | 3.44 | 1.93E-03 |
| | SAR0385 | similar to putative pathogenicity island gene orf3 | 4.09 | 1.93E-03 |
| | SAR0839 | putative lipoprotein | 3.36 | 5.41E-05 |
| | SAR1564 | hypothetical protein | 2.09 | 5.32E-04 |
| | SAR1565 | putative lipoprotein | 2.38 | 3.02E-03 |
| | SAR2426 | putative membrane protein | 2.09 | 2.11E-03 |
| | SAR2427 | ABC transporter ATP-binding protein | 2.14 | 4.82E-03 |
| | SAR2428 | putative membrane protein | 3.73 | 1.21E-05 |
| | SAR2569 | hypothetical protein | 6.01 | 4.75E-02 |

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microarray and proteomics data. The ald2 experiment. A few contradictions were observed between the eno all glycolytic genes except metabolism pathways were up-regulated (e.g. Gap1, Pgi), whereas microarrays. Several proteins within the glycolysis and pyruvate proteomic data were often complementary to those from the proteins and those linked to pyruvate metabolism. Moreover, the linoleic acid resulted in up-regulation of glycolysis pathway regulated over 3-fold. From the proteomic data, challenge with the CapA protein involved in capsule biosynthesis was up-
biosynthesis were modulated in response to linoleic acid. Similarly,
Proteins associated with stress responses and PG and MK effects of the fatty acid upon the cell were therefore corroborated.
In terms of the assigned metabolic pathways, the interpreted functions of the genes modulated in the microarray experiments. expression due to linoleic acid challenge exposure and the encoded proteins were unambiguously determined (Table 9 and 10). There significantly (P ≤ 0.05) down-regulated ≥ 2-fold. MALDI-MS was used to identify the most intense protein spots on the gel corresponding to proteins that were modulated by linoleic acid, and the identities of 38 up-regulated and 5 down-regulated proteins were unambiguously determined (Table 9 and 10). There was strong agreement between the observed changes in protein expression due to linoleic acid challenge exposure and the encoded functions of the genes modulated in the microarray experiments. In terms of the assigned metabolic pathways, the interpreted effects of the fatty acid upon the cell were therefore corroborated. Proteins associated with stress responses and PG and MK biosynthesis were modulated in response to linoleic acid. Similarly, the CapA protein involved in capsule biosynthesis was up-regulated over 3-fold. From the proteomic data, challenge with linoleic acid resulted in up-regulation of glycolysis pathway proteins and those linked to pyruvate metabolism. Moreover, the proteomic data were often complementary to those from the microarrays. Several proteins within the glycolysis and pyruvate metabolism pathways were up-regulated (e.g. Gap1, Pgi), whereas all glycolytic genes except eno were up-regulated in the microarray experiment. A few contradictions were observed between the microarray and proteomics data. The ald2, ackA, 3phD, SAR0985 and SAR2369 proteins were determined by proteomics to be up-regulated but were down-regulated according to microarray analysis. In addition to linoleic acid, the effect of the skin-associated LC-uFFA hexadecenoic acid [C16:1 (n-6)] on the cellular proteome was studied to determine whether there was a common response to LC-uFFAs on S. aureus MRSA252. Analysis of 2D-SDS-PAGE gels revealed strong spot conservation for proteins exhibiting modulated expression in response to hexadecenoic acid compared to linoleic acid. Under challenge conditions with 0.1 mM hexadecenoic acid, 95 proteins were significantly (P ≤ 0.05) up-regulated ≥ 2-fold and 7 proteins were significantly (P ≤ 0.05) down-regulated ≥ 2-fold. MALDI-MS was used to identify 63 of the most intense protein spots on the gel corresponding to proteins that were modulated by linoleic acid and the identities of 56 up-regulated and 5 down-regulated proteins were unambiguously determined (Table 9 and 12). Many of the same proteins, or different proteins within the same metabolic pathways e.g. glycolysis and pyruvate metabolism, were identified after exposure to hexadecenoic acid and linoleic acid. This indicates that there is commonality in the metabolic response to the cellular perturbations caused by exposure to these LC-uFFAs, which differ in chain length, and the number and position of double bonds.

Table 4. MRSA252 genes down-regulated during growth in the presence of linoleic acid (0.01mM) (linoleic acid growth exposure).

| Group Functions | MRSA252 ORF | MRSA252 Gene | MRSA252 Gene Product | Fold Change Down Regulated | P-value |
|-----------------|-------------|--------------|-----------------------|---------------------------|---------|
| Strep Response  | SAR0525     | ctsR         | stress regulatory protein | 3.57 | 6.52E-05 |
|                 | SAR0526     | uvrB/uvrC domain protein | 4.35 | 4.16E-06 |
|                 | SAR0528     | clpC         | putative stress response-related Clp ATPase | 4.17 | 5.47E-05 |
|                 | SAR0823     | clpP         | putative ATP-dependent Clp protease proteolytic subunit | 2.04 | 3.82E-04 |
|                 | SAR0938     | clpB         | putative ATPase subunit of an ATP-dependent protease | 9.09 | 5.72E-06 |
|                 | SAR1657     | dnaK         | chaperone protein | 2.94 | 5.41E-05 |
|                 | SAR1658     | grpE         | GrpE protein | 3.57 | 7.03E-08 |
|                 | SAR2116     | groEL        | 60 kDa chaperonin | 2.44 | 1.05E-03 |
|                 | SAR2117     | groES        | 10 kDa chaperonin | 2.78 | 1.92E-04 |
| Metabolism      | SAR0189     | putative thiamine pyrophosphate enzyme | 2.94 | 1.51E-04 |
|                 | SAR0208     | putative sugar transport system permease | 2.94 | 2.68E-02 |
|                 | SAR0209     | putative oxidoreductase | 4.75 | 1.21E-02 |
|                 | SAR0210     | putative oxidoreductase | 9.09 | 3.75E-03 |
|                 | SAR0527     | putative phosphotransferase | 4.55 | 7.03E-08 |
|                 | SAR0752     | putative phosphofructokinase | 2.44 | 3.20E-02 |
|                 | SAR0753     | fruA         | fructose-specific PTS system component | 3.45 | 1.21E-02 |
|                 | SAR1274     | gllF         | putative glycerol uptake facilitator protein | 3.70 | 3.36E-03 |
|                 | SAR1275     | g1pK         | glycerol kinase | 4.17 | 6.42E-04 |
|                 | SAR1276     | gldK         | glycerol-3-phosphate dehydrogenase | 7.69 | 4.83E-06 |
|                 | SAR2244     | mtdA         | mannitol-specific PTS system component | 2.08 | 4.75E-02 |
|                 | SAR2445     | hrtA         | Heme-regulated transporter ATPase | 2.94 | 3.11E-04 |
|                 | SAR2594     | ABC transporter ATP-binding protein | 2.33 | 3.32E-03 |
| Hypothetical Genes | SAR0100    | putative membrane protein | 2.56 | 2.28E-02 |
|                 | SAR0211     | conserved hypothetical protein | 11.11 | 3.02E-03 |
|                 | SAR0584     | vraX         | predicted role in inipenime resistance | 2.27 | 3.15E-02 |
|                 | SAR0750     | conserved hypothetical protein | 2.22 | 1.32E-02 |
|                 | SAR0939     | LysR family regulatory protein | 2.94 | 5.81E-05 |
|                 | SAR2595     | putative membrane protein | 2.04 | 7.18E-03 |

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Identification of survival mutants

Allelic replacement mutants were constructed in the genes sarF and arcA, which displayed altered transcription in response to linoleic acid and a further mutant was constructed in vraS, encoding a cell wall synthesis regulator. The contribution of these genes to survival in the presence of LC-uFFAs was tested on agar plates containing linoleic acid. Additionally, existing mutants of genes identified by microarray analysis to display altered transcription in response to linoleic acid, or regulators of these genes, were tested. Furthermore, a 5,000 clone Tn917 mutant library was also screened to identify survival mutants. Analysis of the mutant clones was performed in SH1000 since MRSA252 is resistant to most antibiotics used for gene inactivation studies. Importantly, many of the mutant strains tested exhibited increased sensitivity to LC-uFFAs when compared to the wild-type, including those harbouring mutations in the genes: sarF (Liv694), sarM (Liv681), arcA (Liv692), sigB (Liv130), agr (Liv038) and sarA (Liv039) (Fig. 2A, 2B). In contrast clfB (Liv442), vraS (Liv718), katD (Liv750), tfosR (Liv101) and clpC (Liv671) did not contribute to survival under the conditions tested in a SH1000 background. Screening of the Tn917 transposon library identified two further clones with defective survival. Sequencing upstream and downstream of the transposon in these mutants revealed insertion of Tn917 in the Sar2632 (Liv766) and vraE (Liv753) genes. Complementation of the fatty acid sensitivity of the sarF, arcA, vraS and SAR2632 mutants was achieved by individually cloning each gene into the low copy number shuttle vector pSK5630 [75] and transforming each mutant with the relevant plasmid. Complementation restored survival of each mutant in LC-uFFA resistance assays (data not shown).

Autolysis assays

Cells grown in the presence of linoleic acid under constant growth conditions displayed reduced expression of the CtsR regulon, which is known to impact on cell autolysis [47]. Consequently we addressed the impact of the presence of LC-uFFAs upon autolysis of treated and control cells of MRSA252 and SH1000. A significantly increased rate of autolysis was observed in linoleic acid treated cells of each strain (Fig. 3A, 3B). This increase is in accordance with the reduced expression of the CtsR regulon in treated cells.

Cell hydrophobicity

IsdA reduces cell surface hydrophobicity and acts to increase staphylococcal resistance to LC-uFFAs [34] while a GML resistant mutant of Enterococcus faecalis was found to be less hydrophobic than the wild type parent strain [48]. Partitioning of cells in the non-polar solvent hexadecane was measured to determine whether modulating cell hydrophobicity was a S. aureus response to growth in the presence of fatty acids. Growth in the presence of 0.1 mM linoleic acid resulted in both strains exhibiting decreased partitioning indicating a decrease in cell surface hydrophobicity (Fig. 3C). The change in cell hydrophobicity was particularly dramatic for MRSA252 with partitioning reduced from over 90% to less than 20% of cells upon growth in the presence of linoleic acid. The adaptive decrease in cell hydrophobicity makes conditions less favourable for interactions between the cell and the amphipathic fatty acid. Alterations to cell surface charge via the dlt and mprF loci have also been linked to S. aureus evasion of a number of innate immune system components including cationic antimicrobial

| Table 5. MRSA252 genes up-regulated during growth in the presence of oleic acid (0.01 mM) (oleic acid growth exposure). |
|-----------------|-----------------|-----------------|-----------------|
| **Group**       | **MRSA252 ORF** | **MRSA252 Gene** | **MRSA252 Gene Product** | **Fold Change Up Regulated** | **P-value** |
| Virulence Factors and Regulators | SAR0279 | esaA | virulence factor EsxA | 3.20 | 1.51E-05 |
| SAR0280 | esaA | putative membrane protein | 2.67 | 1.07E-04 |
| SAR2122 | hld | delta-hemolysin precursor | 6.02 | 5.33E-04 |
| SAR2123 | agrB | putative autoinducer processing protein | 6.54 | 3.55E-07 |
| SAR2125 | agrC | autoinducer sensor protein | 3.77 | 9.89E-05 |
| SAR2126 | agrA | autoinducer sensor protein response regulator protein | 2.01 | 2.21E-04 |
| SAR2127 | agrl | agrl | Class III accessory gene regulator (agr) locus | 6.30 | 1.12E-07 |
| SAR2296 | alaD | conserved hypothetical protein | 2.05 | 4.08E-03 |
| SAR2297 | alaS | putative acetolactate synthase | 2.49 | 1.19E-03 |
| SAR2711 | arcC | carbamate kinase | 4.09 | 6.41E-03 |
| SAR2712 | arcD | arginine/ornithine antipporter | 3.55 | 7.34E-04 |
| SAR2713 | arcB | putative ornithine carbamoyltransferase | 3.41 | 1.97E-03 |
| SAR2714 | arcA | arginine deiminase | 4.03 | 3.28E-03 |
| SAR2277 | putative exported protein | 2.00 | 7.31E-03 |
| SAR0301 | putative membrane protein | 2.15 | 2.54E-02 |
| SAR0385 | putative membrane protein | 2.88 | 8.02E-03 |
| SAR0839 | putative lipoprotein | 2.07 | 1.97E-03 |
| SAR1448 | major facilitator superfamily | 2.03 | 1.32E-02 |
| SAR2710 | putative regulatory protein | 2.62 | 6.93E-05 |

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### Table 6. MRSA252 genes down-regulated during growth in the presence of oleic acid (0.01 mM) (oleic acid growth exposure).

| Group          | Functions       | MRSA252 ORF | MRSA252 Gene | MRSA252 Gene Product                                      | Fold Change Down Regulated | P-value          |
|----------------|-----------------|-------------|--------------|----------------------------------------------------------|----------------------------|------------------|
| Stress Response|                 | SAR0525     | ctsR         | stress regulatory protein                                  | 3.23                       | 3.26E-05         |
|                |                 | SAR0526     | uvrB/uvrC domain protein |                                 | 4.17                       | 3.26E-05         |
|                |                 | SAR0527     | putative phosphotransferase   |                                 | 3.85                       | 2.20E-07         |
|                |                 | SAR0528     | clpC         | putative stress response-related Clp ATPase              | 3.23                       | 1.01E-04         |
|                |                 | SAR0938     | clpB         | putative ATPase subunit of an ATP-dependent protease      | 8.33                       | 1.30E-06         |
|                |                 | SAR1119     | uvrC         | putative excinuclease ABC subunit C                       | 3.70                       | 7.00E-03         |
|                |                 | SAR1657     | dnaK         | chaperone protein                                         | 2.63                       | 1.01E-04         |
|                |                 | SAR1658     | grpE         | GrpE protein                                              | 2.86                       | 4.78E-06         |
|                |                 | SAR2116     | groEL        | 60 kDa chaperonin                                         | 2.38                       | 1.82E-03         |
|                |                 | SAR2117     | groES        | 10 kDa chaperonin                                         | 2.44                       | 2.21E-04         |
| Metabolism     |                 | SAR0120     | putative ornithine cyclodeaminase |                         | 2.38                       | 4.46E-02         |
|                |                 | SAR0354     | putative homocysteine S-methyltransferase |                             | 2.13                       | 1.60E-02         |
|                |                 | SAR0452     | putative NADH-Ubiquinone protein |                               | 2.00                       | 1.32E-02         |
|                |                 | SAR1274     | glpF         | putative glycerol uptake facilitator protein              | 4.35                       | 9.16E-03         |
|                |                 | SAR1275     | glpK         | glycerol kinase                                           | 3.57                       | 1.59E-02         |
|                |                 | SAR1276     | glpD         | aerobic glycerol-3-phosphate dehydrogenase                | 4.76                       | 3.26E-05         |
|                |                 | SAR1849     | proline dehydrogenase |                                               | 3.23                       | 8.02E-03         |
|                |                 | SAR2445     | hra          | Heme-regulated transporter ATPase                         | 2.94                       | 1.01E-04         |
|                |                 | SAR2446     | hrb          | Heme-regulated transporter permease                       | 2.22                       | 3.98E-02         |
|                |                 | SAR2582     | gntP         | putative gluconate permease                               | 5.88                       | 4.53E-03         |
|                |                 | SAR2583     | gntK         | putative gluconokinase                                    | 4.55                       | 3.60E-02         |
| Hypothetical   |                 | SAR0939     | LysR family regulatory protein |                        | 2.86                       | 2.76E-04         |
| Genes          |                 | SAR2581     | hypothetical protein |                                            | 4.55                       | 3.99E-02         |

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### Table 7. qRT-PCR analysis of gene expression in MRSA252.

| ORF     | Gene          | Linoleic Challenge | Linoleic Growth OD<sub>600 = 3</sub> | Linoleic Growth OD<sub>600 = 8</sub> | Oleic Growth OD<sub>600 = 3</sub> | Oleic Growth OD<sub>600 = 8</sub> | Linoleic Growth OD<sub>600 = 3</sub> | Linoleic Growth OD<sub>600 = 8</sub> | Oleic Growth OD<sub>600 = 3</sub> | Oleic Growth OD<sub>600 = 8</sub> |
|---------|---------------|--------------------|--------------------------------------|-------------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|--------------------------------|
| SAR0114 | spa           | 1.46 (0.37)        | 1.01 (0.07)                          | −1.08 (0.11)                        | −3.03 (0.14)                  | −2.92 (0.24)                  |
| SAR0223 | ladA          | 3.10 (0.08)        | nd                                   | nd                                  | nd                            | nd                            |
| SAR0225 | ladD          | 3.16 (0.61)        | −1.34 (0.03)                         | −1.88 (0.02)                        | −1.06 (0.03)                  | −1.01 (0.02)                  |
| SAR0258 | lyrR          | −5.03 (0.01)       | nd                                   | nd                                  | nd                            | nd                            |
| SAR0625 | sarA          | 3.84 (0.81)        | 1.10 (0.11)                          | −1.12 (0.07)                        | 1.52 (0.55)                  | 2.05 (1.12)                  |
| SAR2621 | cidA          | −1.93 (0.02)       | 1.02 (0.33)                          | 1.41 (0.03)                         | −2.75 (0.01)                  | −1.39 (0.02)                  |
| SAR0938 | clpB          | 3.90 (0.08)        | −8.55 (0.01)                         | −10.31 (0.01)                      | −3.16 (0.01)                  | −3.00 (0.01)                  |
| SAR0978 | fabi          | 1.25 (0.02)        | nd                                   | nd                                  | nd                            | nd                            |
| SAR1136 | hla           | −1.60 (0.20)       | 1.19 (0.19)                          | −1.71 (0.09)                        | 6.38 (2.91)                  | 7.88 (5.16)                  |
| SAR1344 | katA          | 7.27 (0.18)        | nd                                   | nd                                  | nd                            | nd                            |
| SAR1430 | murG          | 7.59 (0.22)        | nd                                   | nd                                  | nd                            | nd                            |
| SAR2187 | fabZ          | 3.37 (0.64)        | −1.10 (0.02)                         | 1.40 (0.02)                         | 1.10 (0.04)                  | 1.20 (0.06)                  |
| SAR2643 | cmeM          | 3.72 (0.08)        | nd                                   | nd                                  | nd                            | nd                            |
| SAR2714 | arcA          | 1.61 (0.03)        | 1.86 (0.04)                          | 2.17 (0.04)                         | −2.19 (0.02)                  | −1.50 (0.01)                  |
| SAR2725 | satF          | 31.86 (0.69)       | nd                                   | nd                                  | nd                            | nd                            |
| RNAIII   |               | 7.86 (0.15)        | 56.14 (1.40)                         | 34.28 (0.74)                        | 156.12 (6.95)                | 153.30 (2.81)                |

The values correspond to the fold change for each gene tested under the relevant fatty acid treatment conditions when compared to the untreated control. The standard deviation for each measurement is in parentheses. nd, not determined. ORF indicates the gene locus in MRSA252 (http://www.genedb.org/genedb/saureusMRSA/).

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peptides [49–51]. The SH1000 mutants were exposed to linoleic acid [C16:1 (n-6)] and oleic acid [C18:1 (n-6)] to test the LC-uFFA survival genes identified in this study as having decreased survival upon exposure to LC-uFFAs. The results showed that SasF and VraE might make contributions to the pathogenesis of S. aureus, which esterifies LC-FFAs with cholesterol, thereby reducing toxicity [32]. However, this activity was demonstrated to be agr-regulated [29,33], producing the anomaly that in strains with SH1000-like regulation, expression of the detoxifying enzyme would be down-regulated upon exposure to its substrate. MRSA252 is a successful epidemic strain of S. aureus and the ability to persist in an environment containing LC-uFFAs such as on the skin surface (hexadecenoic acid) or in skin infections (linoleic and oleic acid) would aid the transmission of the organism. In this scenario, the specific up-regulation of agr in response to LC-uFFAs observed in MRSA252 [EMRSA-16] may contribute towards its success as an epidemic strain. Superior skin colonisation was previously suggested as a reason for the epidemic nature of the EMRSA-15 and -16 strains, which together are responsible for over 95% of MRSA from cases of nosocomial bacteraemia in the UK [54,55].

Microarray analysis revealed further virulence factors exhibiting increased transcription, including the exs locus, which encodes a specific secretion system and the ESAT-6-like proteins that have been confirmed as having a role in the pathogenesis of murine abscesses [41]. Increased transcription of the exs locus was only observed after growth exposure to linoleic or oleic acid and not in response to linoleic acid challenge conditions. Increased transcription of the arcABDC operon, encoding the arginine deiminase (ADI) pathway enzymes, was observed under the same conditions where the exs locus is up-regulated. The ADI pathway enables the utilisation of arginine as an energy source under anaerobic conditions of growth. Concomitant with the expression of the ADI pathway, there was an up-regulation of many glycolytic enzymes, suggesting that a net effect of growth exposure to linoleic acid was metabolic alterations leading toward anaerobic growth. To test the importance of the ADI pathway under these conditions, an arcA allelic replacement mutant of SH1000 was generated (arcA) was transcriptionally up-regulated in both SH1000 and MRSA252. The arcA strain was found to display a reduction in growth on agar plates containing 1 mM linoleic acid, with a 25-fold lower survival than the parental strain. The alteration in metabolism via up-regulation of the ADI pathway is therefore important for survival under these conditions. The ADI pathway may also contribute to virulence since some ST38-SCCmecIVa (USA300) MRSA clones carry the arginine catabolism mobile element (ACME), which contains an extra copy of the arc operon [56]. This leads to the hypothesis that the arcABDC operon facilitates pathogenicity by increasing survival of S. aureus in the presence of LC-uFFAs.

The sarF gene showed the largest change in expression of any gene in response to linoleic acid challenge (>16-fold and >30-fold up-regulation in MRSA252 by microarray and qRT-PCR, respectively). Expression of SasF, an LPXAG motif cell wall-anchored surface protein, is repressed by TcaR, the teicoplanin-associated locus of S. aureus Response to LC-uFFAs

### Table 8. qRT-PCR analysis of gene expression in SH1000.

| ORF     | Gene | Linoleic Challenge | Linoleic Growth | Oleic Growth |
|---------|------|--------------------|-----------------|--------------|
| SAR0114 | spa  | 2.19 (0.09)        | −1.94 (0.02)    | 1.66 (0.04)  |
| SAR0258 | lytF | −2.31 (0.02)       | nd              | nd           |
| SAR0625 | sarA | 1.26 (0.05)        | −3.79 (0.02)    | −3.45 (0.03) |
| SAR0938 | clpB | 1.95 (0.08)        | −2.11 (0.02)    | −2.58 (0.03) |
| SAR0978 | fabI | −1.20 (0.04)       | nd              | nd           |
| SAR1136 | hla  | −3.60 (0.01)       | −2.11 (0.02)    | −2.58 (0.03) |
| SAR1430 | mnrG | 1.84 (0.11)        | nd              | nd           |
| SAR2643 | crfM | 1.32 (0.05)        | nd              | nd           |
| SAR2714 | arcA | nd                 | 2.19 (0.10)     | 4.39 (0.18)  |
| SAR2725 | sarF | 1.49 (0.06)        | nd              | nd           |
|         | RNAIII | −1.79 (0.03)   | −3.29 (0.01)    | −1.95 (0.01) |

The values correspond to the fold change for each gene tested under the relevant fatty acid treatment conditions when compared to the untreated control. The standard deviation for each measurement is in parentheses. nd, not determined. ORF indicates the gene locus in MRSA252 (http://www.genedb.org/genedb/saureusMRSA/) that was tested in SH1000.

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The tcaR gene was found by microarray analysis to be up-regulated (3-fold) in MRSA252 under the linoleic acid challenge conditions (Table 1). However, the SH1000 strain harbours a truncated copy of tcaR and synthesises a non-functional protein. This could explain why the sasF gene was only slightly up-regulated in SH1000 since its transcription may already be very high as its expression is reduced as part of the TcaR regulon. Many of the differences observed in the transcriptional responses of SH1000 and MRSA252 to the presence of fatty acids (Table 7, 8) are thus likely to be due to differential responses modulating RNAIII production, altered sarA transcription and differences between the strains in respect of the functionality of TcaR. The importance of sasF transcription for adaptation and survival of S. aureus to linoleic acid was tested by constructing an allelic replacement mutant in SH1000.

**Table 9.** MRSA252 proteins up-regulated following the addition of linoleic acid (0.1 mM) to exponentially growing cells (linoleic acid challenge).

| Group Functions and Regulators | MRSA252 ORF | MRSA252 Gene | MRSA252 Gene Product | Fold Change Up Regulated | P-value |
|--------------------------------|-------------|-------------|----------------------|-------------------------|---------|
| Virulence Factors and Regulators | SAR2745 | capA | Capsular polysaccharide biosynthesis protein | 3.36 | 2.27E-04 |
| Energy Metabolism | SAR0140 | deoC1 | deoxyribose-phosphate aldolase | 4.34 | 2.49E-03 |
| | SAR0217 | formate acetyltransferase | | 2.30 | 1.50E-03 |
| | SAR0394 | phosphoglycerate mutase family protein | | 2.94 | 1.67E-02 |
| | SAR0828 | gap1 | glyceraldehyde 3-phosphate dehydrogenase 1 | 2.07 | 3.53E-03 |
| | SAR0924 | pgII | glucose-6-phosphate isomerase | 4.18 | 7.43E-03 |
| | SAR1451 | alld | alanine dehydrogenase 2 | 2.13 | 1.32E-03 |
| | SAR1789 | ackA | acetate kinase | 2.81 | 6.97E-04 |
| | SAR2506 | dpgm | putative phosphoglycerate mutase | 2.15 | 3.52E-02 |
| | SAR2685 | mqa2 | malatequinone oxidoreductase | 3.39 | 8.08E-03 |
| DNA Repair and Replication | SAR1639 | dnaG | DNA primase | 2.66 | 2.61E-03 |
| | SAR1966 | lig | DNA Ligase | 2.09 | 6.11E-04 |
| Protein Synthesis | SAR0552 | fus | elongation factor G | 4.47 | 2.51E-04 |
| | SAR0552 | fus | elongation factor G | 2.10 | 2.09E-02 |
| Peptidoglycan Synthesis | SAR1216 | trmA | putative tRNA (guanine-7-)methyltransferase | 2.33 | 4.84E-04 |
| Carotenoid Biosynthesis | SAR1720 | queA | S-adenosylmethionine-tRNA ribosyltransferase-isomerase | 2.11 | 1.68E-02 |
| SAR2309 | rpoA | RNA polymerase alpha subunit | 2.61 | 2.40E-03 |
| Miscellaneous | SAR0218 | putative pyruvate formate-lyase activating enzyme | | 2.60 | 3.46E-02 |
| | SAR0403 | putative DNA binding protein | | 2.72 | 3.48E-02 |
| | SAR0207 | putative oxygenase/mitric oxide synthase | | 2.04 | 7.13E-03 |
| | SAR0207 | putative nitric oxide synthase | | 2.66 | 2.38E-02 |
| Metabolism | SAR0150 | adhE | putative aldehyde-alcohol dehydrogenase | 3.48 | 5.07E-03 |
| | SAR0246 | ispD | 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase | 2.43 | 4.84E-04 |
| | SAR0246 | ispD | 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase | 2.21 | 8.75E-03 |
| | SAR0564 | pdhD | putative halocid dehalogenase-like hydrolase | 2.22 | 1.01E-02 |
| | SAR1070 | pdhD | dihydrodihydroxy acid dehydrogenase | 2.22 | 2.62E-02 |
| | SAR2353 | mobA | molybdopterin-guanine dinucleotide biosynthesis protein | 3.12 | 8.32E-04 |
| | SAR2513 | 6-carboxyhexanoate-CoA ligase | | 4.84 | 4.13E-05 |
| | SAR2641 | putative aminotransferase | | 2.07 | 1.54E-02 |
| Hypothetical Proteins | SAR0985 | putative RNA ligase protein | | 2.31 | 2.12E-02 |
| | SAR2064 | hypothetical phage protein | | 2.06 | 1.41E-03 |
| | SAR2369 | Acyl-CoA dehydrogenase-related protein | | 3.36 | 4.69E-03 |
The sarF mutant showed much reduced survival on agar plates containing 1 mM linoleic acid, exhibiting a 6-fold lower level of survival than the parental strain (Fig. 2A). The expression of this cell wall-anchored protein is therefore important for survival under these conditions. SasF may also contribute to virulence since in a murine arthritis model of infection a sarF allelic replacement mutant of SH1000 caused significantly less weight loss of the animals compared to control cells (Fig. 4A). Reduced numbers of bacteria were harvested from the kidney in mice infected with the sarF mutant strain compared to the control but the difference was not significant (Fig. 4B). SasF did not significantly affect development or severity of arthritis.

A screen for additional mutants of SH1000 that were defective for survival in the presence of linoleic acid identified several Tn917 transposants from a 5,000 clone library of mutants. Sequencing located the transposons within genes encoding VraE (ABC transporter permease) and SAR2632 (MMPL domain, putative efflux pump). The mutants Liv753 (vraE) and Liv766 (SAR2632) had reduced survival using this agar plate-based assay exhibiting 150-fold and 4-fold reductions in viability, respectively, at 1 mM linoleic acid (Fig. 2A). Each of these genes encodes transporter proteins of unknown function. The gene vraE is located downstream of vraD in a bicistronic operon and is a member of the GraSR regulon proposed to regulate traffic of cell wall substrates [59,60]. Two studies have shown that S. aureus vraE mutants display decreased resistance to meticillin and increased susceptibility to human β-defensin 3. [61,62]. VraE may also contribute to virulence, since in a murine arthritis model of infection a vraE allelic replacement mutant of SH1000 resulted in significantly less weight loss of the animals compared to control cells (Fig. 4A). Reduced numbers of bacteria were harvested from the kidney in mice infected with the vraE mutant strain compared to control but the difference was not significant (Fig. 4B). VraE did not significantly affect the development or severity of arthritis (data not shown). SAR2632 is a predicted transporter protein of the MMPL domain family proposed to be involved in lipid transport [63].

The identification of cell envelope mutants correlated with the gene expression and proteomic data, in which altered levels of cell wall synthesis and regulation components was observed (Fig. 5A). An increase in autolysis was observed under growth exposure conditions, although whether this is due to changes in expression of PG synthesis genes or down-regulation of the CtsR regulon remains unelucidated. The overall up-regulation of many cell wall synthesis genes could have two possible explanations. Firstly, the increased synthesis may be required to maintain the integrity of the cell wall, damaged due to loss of material through the precipitation of PG by LC-uFFAs as described by Campbell et al. [25]. The binding of LC-uFFAs to PG would not be unexpected given that chitosan, which has a very similar structure to PG, has been shown to bind lipids [64]. Secondly, an increase in cell wall and capsule synthesis could act as a defense mechanism since an increase in ionically charged material surrounding the cell would mitigate against access of the non-polar carbon chain of LC-uFFAs to the cell membrane. Reduced cell surface hydrophobicity and increased thickness of the cell wall have been suggested as Gram-positive defense mechanisms to limit interactions with lipids [34,48]. Therefore, the reduced cell surface hydrophobicity of both the MRSA252 and SH1000 strains, observed here following overnight growth in the presence of linoleic acid, represents a pathogen countermeasure to this component of the innate immune system.

Previous studies have used gene expression profiling to determine the cellular pathway targeted by antimicrobial agents [65,66]. In this study, there was no change in expression of fatty acid biosynthesis genes, other than fabZ, which is located on the same operon as the PG synthesis gene murA. This suggests that the anti-staphylococcal toxicities of the LC-uFFAs used in this study are not a consequence of inhibiting fatty acid biosynthesis. Prior studies on the action of LC-uFFAs upon cells of S. aureus demonstrated membrane perturbations [12,18,22,26]. This supports the finding of Chamberlain et al. that increased fluidity of S. aureus membranes resulted from exposure to LC-uFFAs [26]. Furthermore, these authors demonstrated that carotenoid-dependent pigmentation in non-isogenic clinical isolates positively correlated with increased survival from LC-uFFAs and showed that the carotenoid staphyloxanthin acted to decrease membrane fluidity and reduce its damaging effects. Interestingly, genes involved in carotenoid biosynthesis were up-regulated in response to LC-uFFAs (Fig. 5B), and Liv681 (crtM), which cannot produce staphyloxanthin, was shown here to have a >5-fold reduced survival to 1 mM linoleic acid. Staphyloxanthin production is regulated via σB, which also regulates many stress response components observed to be up-regulated in the array data. Consequently, the general stress response, including the production of staphyloxanthin serves as an important component of defence against LC-uFFAs, given the discovery that Liv130 (sigB) exhibited a >75-fold reduction in survival to 1 mM linoleic acid. σB has previously been shown to contribute to S. aureus resistance to antimicrobials [67]. The CtsR regulon was strongly up-regulated after linoleic acid challenge, but was down-regulated after growth exposure and may therefore also participate in the adaptation to this environment. A consequence of LC-uFFAs inserting in the cell membrane could be the disruption of the electron transport chain. This would explain the numerous changes in expression of genes associated

Table 10. MRSA252 proteins down-regulated following the addition of linoleic acid (0.1 mM) to exponentially growing cells (linoleic acid challenge).

| Group                  | Functions          | MRSA252 ORF | MRSA252 Gene       | MRSA252 Gene Product | Fold Change Down Regulated | P-value |
|------------------------|--------------------|-------------|--------------------|----------------------|---------------------------|---------|
| Protein Synthesis      | SAR0927            | spsB        | signal peptidase lb| 5.88                 | 9.88E-04                  |         |
|                        | SAR1755            | tig         | trigger factor     | 2.56                 | 1.93E-02                  |         |
|                        | SAR2179            |            | putative membrane protein | 2.17                 | 2.05E-02                  |         |
| Peptidoglycan Synthesis| SAR1284            | glnA        | glutamine synthetase| 2.33                 | 5.00E-02                  |         |
| Metabolism             | SAR0814            | hprK        | kinase/phosphorylase| 3.03                 | 6.66E-03                  |         |

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References:
[25] Campbell et al.
[26] Liu et al.
[27] Chamberlain et al.
### Table 11. MRSA252 proteins up-regulated following the addition of hexadecenoic acid (0.1 mM) to exponentially growing cells (hexadecenoic acid challenge).

| Group Functions                     | MRSA252 ORF | MRSA252 Gene | MRSA252 Gene Product               | Fold Change Up regulated | P-value   |
|-------------------------------------|-------------|--------------|------------------------------------|--------------------------|-----------|
| **Stress Response**                 |             |              |                                    |                          |           |
| Stress Response SAR2116             | groEL       | chaperonin   | 2.03                               | 3.49E-02                 |           |
| SAR2273                             | asp23       | alkaline shock protein 23 | 2.89 | 8.90E-03 |           |
| SAR2461                             | pyridine nucleotide-disulphide oxidoreductase family protein | 2.05 | 3.99E-02 |           |
| SAR2461                             | pyridine nucleotide-disulphide oxidoreductase family protein | 2.00 | 2.13E-03 |           |
| SAR2461                             | pyridine nucleotide-disulphide oxidoreductase family protein | 2.59 | 2.49E-02 |           |
| **Energy Metabolism**               |             |              |                                    |                          |           |
| Energy Metabolism SAR0140           | deoC1       | deoxyribose-phosphate aldolase | 4.21 | 4.66E-03 |           |
| SAR0394                             | phosphoglycerate mutase family protein | 4.79 | 1.06E-03 |           |
| SAR0828                             | gap1        | glyceraldehyde 3-phosphate dehydrogenase 1 | 2.86 | 2.51E-03 |           |
| SAR0830                             | tpiA        | triosephosphate isomerase | 3.40 | 4.42E-03 |           |
| SAR0832                             | eno         | enolase      | 3.20 | 1.83E-02 |           |
| SAR0832                             | eno         | enolase      | 3.09 | 4.60E-03 |           |
| SAR0832                             | eno         | enolase      | 2.07 | 5.15E-03 |           |
| SAR0924                             | pgp         | glucose-6-phosphate isomerase | 2.31 | 2.73E-02 |           |
| SAR1068                             | pdhB        | putative pyruvate dehydrogenase E1 component | 2.72 | 2.29E-04 |           |
| SAR1121                             | sdhA        | putative succinate dehydrogenase flavoprotein | 2.24 | 1.36E-02 |           |
| SAR1451                             | ald2        | glyceraldehyde 3-phosphate dehydrogenase 2 | 2.21 | 2.30E-03 |           |
| SAR2605                             | ddh         | D-lactate dehydrogenase | 3.11 | 8.87E-03 |           |
| SAR2685                             | mgo2        | malate:quinone oxidoreductase | 4.79 | 1.06E-03 |           |
| SAR2685                             | mgo2        | malate:quinone oxidoreductase | 3.13 | 2.18E-03 |           |
| **DNA Repair and Replication**      |             |              |                                    |                          |           |
| DNA Repair and Replication SAR1997  | pcrA        | ATP-dependent DNA helicase | 2.23 | 3.49E-02 |           |
| **Protein Synthesis**               |             |              |                                    |                          |           |
| Protein Synthesis SAR0552           | fus         | translation elongation factor G | 3.56 | 3.84E-02 |           |
| SAR0553                             | tuf         | translation elongation factor Tu | 2.65 | 3.41E-02 |           |
| SAR0553                             | tuf         | elongation factor Tu | 4.13 | 7.56E-03 |           |
| SAR1216                             | trmD        | putative tRNA (guanine-7-)-methyltransferase | 2.80 | 1.69E-03 |           |
| SAR1485                             | rpsA        | putative 30S ribosomal protein S1 | 2.91 | 4.91E-02 |           |
| SAR1719                             | tgt         | queuine tRNA-ribosyltransferase | 2.01 | 4.96E-02 |           |
| SAR1720                             | queA        | S-adenosylmethionine:tRNA ribosyltransferase-isomerase | 2.49 | 1.44E-02 |           |
| SAR2309                             | rpoA        | DNA-directed RNA polymerase subunit alpha | 2.44 | 9.73E-03 |           |
| **Peptidoglycan Synthesis**         |             |              |                                    |                          |           |
| Peptidoglycan Synthesis SAR1048     | purD        | putative phosphoribosylamine–glycine ligase | 3.98 | 2.08E-02 |           |
| SAR1762                             | thrS        | theonine-tRNA synthetase | 2.51 | 1.51E-02 |           |
| SAR2212                             | murA2       | UDP-N-acetylglucosamine 1-carboxyvinyltransferase | 2.13 | 1.36E-02 |           |
| **Cell Division**                   |             |              |                                    |                          |           |
| Cell Division SAR1795               | ezrA        | putative septation ring formation regulator | 3.11 | 3.71E-03 |           |
| SAR1795                             | ezrA        | putative septation ring formation regulator | 2.56 | 5.16E-03 |           |
| **Miscellaneous**                   |             |              |                                    |                          |           |
| Miscellaneous SAR0218               |             | putative pyruvate formate-lyase activating enzyme | 2.39 | 4.03E-03 |           |
| SAR0403                             |             | putative DNA-binding protein | 2.84 | 8.32E-03 |           |
| SAR2007                             |             | putative oxygenase | 2.85 | 2.92E-02 |           |
| **Metabolism**                      |             |              |                                    |                          |           |
| Metabolism SAR0351                  | thl         | acetyl-CoA acetyltransferase | 2.85 | 2.59E-04 |           |
| SAR0351                             | thl         | acetyl-CoA acetyltransferase | 2.77 | 9.07E-04 |           |
| SAR0514                             |             | putative O-acetylserine (thiol)-lyase | 2.41 | 4.43E-03 |           |
| SAR1142                             |             | ornithine carbamoyltransferase | 2.03 | 5.43E-03 |           |
| SAR2352                             |             | putative molybdenum cofactor biosynthesis protein A | 3.73 | 6.34E-03 |           |
| SAR2352                             |             | putative molybdenum cofactor biosynthesis protein A | 2.25 | 9.64E-03 |           |
| SAR2460                             |             | putative acetyltransferase (GNAT) family protein | 5.37 | 6.22E-05 |           |
| SAR2460                             |             | putative acetyltransferase (GNAT) family protein | 5.20 | 2.03E-02 |           |
| SAR2641                             |             | putative aminotransferase | 2.12 | 8.47E-03 |           |
| SAR2694                             |             | putative anaerobic ribonucleotide reductase activating protein | 3.38 | 4.66E-04 |           |
with energy creation within the cell (Fig. 5C, 5D) and appears to constitute the main cellular response to LC-uFFAs. The overall trend is one of increasing levels of pyruvate and alterations in menaquinone synthesis. Moreover, the ADI pathway for anaerobic utilisation of arginine was up-regulated under growth exposure conditions. The genes affected by LC-uFFAs include those involved in the glycolytic and fermentative pathways, cell wall synthesis, cell division, and capsule synthesis. These pathways have also been shown to be modulated in a S. aureus small colony variant, which has a mutation in the hemB gene of the electron transport chain [68]. This supports a mode of action for LC-uFFAs of disturbing cell energetics via membrane disruption. Finally, the overall similarities in responses to the LC-uFFAs employed in this study appear to indicate a common mode of action amongst the linoleic, oleic and hexadecenoic acids. This corroborated dataset on the transcriptional and translational responses of S. aureus should provide a useful resource for further studies on this pathogens response to the host environment.

Materials and Methods

Bacterial strains, plasmid and growth conditions

Strains and plasmids used in this study are listed in Table 13. Unless indicated otherwise, bacteria were grown in brain heart infusion broth (BHI)(Merck) at 37°C with shaking at 125 rpm. When included, antibiotics were added at the following concentrations: erythromycin, 5 μg ml⁻¹; lincomycin, 25 μg ml⁻¹; tetracycline, 5 μg ml⁻¹; chloramphenicol 10 μg ml⁻¹.

Transposon mutagenesis and screening for sensitivity to LC-uFFAs

Transposon mutagenesis was performed on the SH1000 strain of S. aureus using the Tn917 containing plasmid pLTV1, as described previously [69]. Single colonies from a transposon library grown on BHI agar containing erythromycin and lincomycin were inoculated into 96-well plates containing 200 μl of BHI. From this library 5,000 clones were cultured and stored at −80°C in 10% glycerol. After repeat growth of clones overnight at 37°C, without shaking, cultures were diluted 100-fold before 5 μl was spotted onto BHI agar with or without 0.5 mM linoleic acid. After overnight incubation, strains with decreased resistance to linoleic acid, when compared to wild type (WT) SH1000, were selected. The transposon-mediated mutations in these strains were transduced into the WT SH1000 using ø11 as described previously [70]. The linoleic acid sensitivity of these mutants was reconfirmed, proving the phenotype was transposon-linked, by repeat assay using serial dilutions of the mutant strains onto BHI agar containing millimolar concentrations of linoleic acid. The locations of the Tn917 insertions within the genome of mutants were determined using arbitrary primed nested PCR and DNA sequencing of regions upstream and downstream of the transposon [71].

Construction of sasF, arcA and vraS insertional mutants and complementation plasmids

Construction of sasF, arcA and vraS mutants was performed as described by Horsburgh et al. [72] using the oligonucleotides described in Table 14. Briefly this was as follows: the sasF, arcA or vraS genes were amplified as upstream and downstream fragments using

| Table 11. Cont. |

| Group Functions | MRSA252 ORF | MRSA252 Gene | MRSA252 Gene Product | Fold Change | P-value |
|-----------------|-------------|--------------|----------------------|-------------|---------|
| Hypothetical Proteins | SAR2694 | nrdG | putative anaerobic ribonucleotide reductase activating protein | 2.48 | 3.43E-02 |
| SAR0246 | ispD | 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase | 3.37 | 2.20E-04 |
| SAR0246 | ispD | 2-C-methyl-D-erythritol 4-phosphate cytidylyltransferase | 2.90 | 1.69E-03 |
| SAR0985 | lyrA | putative RNA ligase protein | 2.11 | 1.68E-02 |
| SAR1105 | lyrA | hypothetical protein | 2.25 | 3.60E-03 |
| SAR2206 | hypothetical phage protein | 2.06 | 1.41E-03 |
| SAR2369 | hypothetical phage protein | 2.61 | 2.43E-04 |
| SAR2545 | hypothetical phage protein | 2.08 | 1.72E-02 |
| SAR2674 | hypothetical phage protein | 2.45 | 5.46E-04 |

| Table 12. MRSA252 proteins down-regulated following the addition of hexadecenoic acid (0.1 mM) to exponentially growing cells (hexadecenoic acid challenge). |

| Group Functions | MRSA252 ORF | MRSA252 Gene | MRSA252 Gene Product | Fold Change | P-value |
|-----------------|-------------|--------------|----------------------|-------------|---------|
| Protein Synthesis | SAR0927 | spsB | signal peptidase Ib | 4.55 | 8.44E-03 |
| Peptidoglycan Synthesis | SAR0920 | NAD-specific glutamate dehydrogenase | 2.17 | 8.21E-03 |
| Miscellaneous | SAR2262 | lyrA | family regulatory protein | 2.13 | 1.21E-02 |
| Metabolism | SAR0483 | tmk | putative thymidylate kinase | 2.27 | 1.04E-03 |
| SAR1399 | pstB | ABC transporter ATP-binding protein | 3.23 | 1.80E-03 |
primer pairs sasF_BamI/sasF_NotI and sasF_KpnI/sasF_EcoRI, or arcA_BamI/arcA_NotI and arcA_KpnI/arcA_EcoRI or vraS_BamI/vraS_NotI and vraS_KpnI/vraS_EcoRI, respectively. The tetracycline resistance gene from pDG1513 [73] was amplified by using the primer pair Tet_NotI/Tet_KpnI. The upstream, downstream and tet gene fragments were digested with BamHI and NotI, or KpnI and EcoRI, or NotI and KpnI, respectively, and simultaneously ligated into pAZ106, which had been previously digested with BamHI and EcoRI. The resulting constructs were confirmed by restriction digest and then used to transform electrocompetent S. aureus RN4220 by the method of Schenk and Ladagga [74]. Strains of RN4220 containing the Campbell integration of the plasmid were resolved in SH1000 by transductional outcross using ø11. Clones of SH1000, which had now lost the plasmid and contained an allelic replacement with the tetracycline resistance gene, were confirmed as mutants by PCR amplification. Correct allelic replacement was confirmed in each case.

Complementation of the sasF, arcA, vraE and SAR2632 mutants was performed by amplifying each gene with sufficient upstream and downstream DNA using the primer pairs listed in Table 14. The fragments were ligated into pSK5630 [75] following digestion with BamHI/SalI, and the resulting constructs and the control plasmid were transformed into E. coli DH5α, with selection on agar plates containing ampicillin. The resulting constructs were confirmed by restriction digest and then used to transform electrocompetent S. aureus RN4220. The plasmids were then

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**Figure 2. Plate based survival assay.**

A Graph showing the percentage survival of WT and mutant variants of SH1000 when serial dilutions of the strains were plated on BHI agar containing 1 mM linoleic acid. Survival is expressed as a percentage of viable cell counts obtained for control plates lacking linoleic acid. Values are the mean of multiple independent experiments. Error bars indicate standard errors of the mean. p<0.005 for each mutant by Student’s t-test. B Plates showing the relative survival of SH1000 and the sasF (Liv694) and vraE (Liv753) mutants on BHI agar containing 0 or 1 mM linoleic acid. The 10⁻¹ to 10⁻⁶ dilution series of cultures are as indicated. doi:10.1371/journal.pone.0004344.g002
Figure 3. Physiological effects of linoleic acid on *S. aureus*. The result of growth of MRSA252 and SH1000 in the absence (closed triangle) or presence (open box) of 0.01mM linoleic acid on autolysis is shown in A and B, respectively. Survival is expressed as a percentage of OD₆₀₀ at T=0. Values are from three independent experiments. Error bars indicate standard errors of the mean. **p<0.01, *p<0.05 by Student’s t test. C Relative hydrophobicity of the MRSA252 and SH1000 strains following overnight growth in BHI +/- 0.1 mM linoleic acid. Values are from three independent experiments. Error bars indicate standard errors of the mean. **p<0.01, *p<0.05 by Student’s t test.

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WT SH1000 (open box) and mutations of SR1 (diagonal hatched box) on percentage change in weight of infected mice. *p<0.05, **p<0.01 by Dunn’s test.

To ascertain the transcriptional responses of MRSA252 to fatty acids, overnight cultures (18 h) of MRSA252 were used to inoculate 100 ml of BHI (Merck) with or without 10 μM of oleic or linoleic acid in 250 ml conical flasks. These 100 ml cultures were placed in a shaking water bath at 37°C in 250 ml conical flasks. These 100 ml cultures were placed in an Agilent Technologies 2100 Bioanalyzer by using the RNA 6000 Nano LabChip Kit. The RNA was converted to cDNA and labelled by incorporation of Cy5 dCTP during reverse transcription of RNA using the enzyme Superscript II (Amersham). DNA used in the microarray hybridisations was extracted from 5 ml of an overnight culture (18 h) of MRSA252 using the Edge Biosystems Bacterial Genomic DNA purification kit according to manufacturer’s instructions. The DNA was labeled by the incorporation of Cy3 dCTP using Klenow (Invitrogen). cDNA derived from RNA and genomic DNA were pooled and hybridized on whole-genome microarrays supplied by the Bacterial Microarray Group at St. George’s Hospital (Bugs@G [http://bugs.scul.ac.uk]) before washing and scanning [76]. Microarrays were scanned using an Affymetrix 428 scanner and image data extracted using ImAge 5.2 (BioDiscovery). Fully annotated microarray data have been deposited in Bugs@G (accession number E-BUGS-68; http://bugs.scul.ac.uk/E-BUGS-68) and also ArrayExpress (accession number E-BUGS-68). Two independent labelling reactions and hybridisations were carried out for each RNA sample. Image data was analysed using the GeneSpring 7.3.1 software (Silicon Genetics).

Briefly, data were normalized relative to the corresponding untreated controls. Signals below 0.01 were taken as 0.01. Genes were then filtered on expression level to remove non-changing genes, with only those genes that changed by at least two-fold considered biologically significant. Changing genes were then filtered on confidence applying the Benjamini and Hochberg false discovery rate algorithm with a maximum significance cut-off at 0.05 to eliminate the chance of false-positives [77].

Quantitative Real-Time PCR

To confirm the validity of microarray data gene specific mRNAs were quantified from treated and untreated cultures by quantitative real-time PCR (qRT-PCR). Cells were grown in biological triplicate exactly as described above for the microarray experiments and bacterial RNA was isolated using the Pro-Blue Fast RNA kit (MP Biomedicals). DNA was removed from the samples by DNase I treatment (Ambion) according to manufacturer’s instructions. The purified RNA was quantified using the nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific) and the integrity assessed by electrophoresis. 0.5 μg of RNA was reverse transcribed with 100 U of Bioscript Reverse Transcriptase (Bioline) using 0.2 μg of random hexamer primers (Promega) according to manufacturer’s instructions. qRT-PCR was performed using the 7500 Fast System (Applied Biosystems) and the Quantifast SYBR Green PCR kit (Qigen) according to manufacturer’s instructions. The relative levels of gene expression in fatty acid treated cells and the non-treated controls were calculated by relative quantification using gyrB as the endogenous reference gene. The choice of gyrB as a single reference gene was based on its consistent levels in microarray in all conditions and at all timepoints that were analysed. The oligonucleotides used for qRT-PCR are listed in Table 15. All samples were amplified in triplicate and the data analysis was carried out using the 7500 Fast System Software (Applied Biosystems).

Sample preparation for 2D-PAGE

Cultures of MRSA252 (100 ml) were grown to late exponential phase (OD600 = 3.0) and exposed to 0.1 mM linoleic acid or 0.1 mM hexadecenoic acid as described above. Cells were harvested by centrifugation at 5000 g for 10 min at 4°C. After two washes in PBS the cells were resuspended in 2 ml of lysis buffer (PBS, 1 mg/ml DNase I, 100 μM benzamidine, 100 μM PMSF, 1 mg/ml RNase, 2 mg/ml lysozyme) and incubated at 37°C for 20 min before chilling on ice. Cell debris and insoluble material was pelleted by centrifugation at 4°C for 20,000 g for 20 min. The supernatant was stored at −20°C. Protein samples were purified from RN4220 and transformed into the corresponding mutant strains.

Microarray analysis

To ascertain the transcriptional responses of MRSA252 to fatty acids, overnight cultures (18 h) of MRSA252 were used to inoculate 100 ml of BHI (Merck) with or without 10 mM of oleic or linoleic acid in 250 ml conical flasks. These 100 ml cultures were placed in a shaking water bath at 37°C at 250 rpm and 10 ml samples were taken from the flask when the cultures reached late exponential phase (OD600 = 3). Identical inoculations were performed to 100 ml of BHI lacking additional fatty acids. 100 μM of linoleic acid in ethanol or an equal volume of the ethanol used to dilute the fatty acid was added to these cultures at an OD600 = 3.0 and the RNA extracted from treated and untreated cells 20 min post-treatment. Each treatment and control culture was performed in biological triplicate. The concentrations of fatty acids used in these experiments did not alter the pH of the media. RNA was extracted from 10 ml samples of culture taken at the indicated time intervals and stabilised by the addition of 20 ml of RNA Bacteria Protect (Qagen). The cells were subsequently harvested by centrifugation at 5000 rpm for 10 min and cell pellets resuspended in lysis buffer (10 mM Tris, pH8.0) containing 200 U ml−1 of lysozyme in 400 U ml−1 of mutanolysin, and incubated for 90 min at 37°C with gentle shaking every 10–15 min. The RNA was subsequently extracted using the RNeasy Midi kit (Qagen) and DNase treated whilst on the purification column using the RNase-Free DNase Set according to manufacturer’s instructions (Qagen). The quantity and quality of the RNA was assessed on an Agilent 2100 bioanalyzer by using the RNA 6000 Nano LabChip Kit. The RNA was subsequently extracted using the RNeasy Midi kit (Qagen) and DNase treated whilst on the purification column using the RNase-Free DNase Set according to manufacturer’s instructions (Qagen).
were quantified using the BioRad Protein assay. The protein samples were desalted using Slide-A-Lyzer Mini Dialysis Units with a 3.5 kDa MWCO (Thermo Scientific).

**2D-PAGE**

Soluble protein (120 μg) was brought up to 320 μl with rehydration buffer (8 M urea, 2M thiourea, 4% (w/v) CHAPS, 20 mM DTT, 1% (v/v) ASB 14 detergent and 0.5% (v/v) carrier ampholytes (Bio-lyte 3/10, Bio-Rad)). Samples were incubated for an hour at room temperature with gentle shaking, before centrifugation at 8,000 g for 5 min. Samples were in-gel rehydrated and focused on 17 cm, pH 4–7 IPG strips (Bio-Rad) for a total of 40000 V h (150V for 1 h, 300V for 1 h, 600V for 1 h, 1200V for 1 h, 1200–8000V over 1 h (linear gradient), 8000 V to 40000 V (steady state)), using a Protean IEF Cell (Bio-Rad). After focusing, strips were equilibrated in 50 mM Tris (pH 6.8), 6 M urea, 2% (w/v) SDS, 30% (w/v) glycerol, and bromophenol blue, containing 20 mM DTT in the reduction step (15 min) and 25 mM iodoacetamide in the alkylation step (15 min). IPG strips were run in the second dimension on 20x20cm 12.5% SDS-PAGE gels using a Protean II xi 2D Cell (Bio-Rad). Gels were run in triplicate, silver-stained [78] and scanned (GS-710 Densitometer, Bio-Rad) as gray-scale tiff files at 16 bit and 300 dpi and uploaded into the Progenesis ‘SameSpots’ (Non Linear Dynamics) gel image analysis Software. Quantitative analysis was based on average gels created from three gel replicates. Spots in the treated

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**Figure 5. Schematic representation of cellular pathways displaying changes in gene transcription in response to linoleic acid challenge conditions.** Sections A, B, C and D highlight the various genes involved in peptidoglycan, carotenoid, menaquinone and energy metabolism respectively. Genes in red and blue boxes are up- and down-regulated, respectively. See text for details. doi:10.1371/journal.pone.0004344.g005

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samples with a p≤0.05 and ≥ two-fold difference from the control sample were considered statistically significant. For protein identification by mass spectrometry 2 gels containing 800 mg each of soluble protein (a pool from each growth condition) were prepared as above and stained with Colloidal Coomassie Brilliant Blue [79]. The scanned images were uploaded into Progenesis ‘SameSpots’ and matched to the analytical gels.

| Strain or Plasmid | Comment | Reference or Source |
|------------------|---------|---------------------|
| Strains: E. coli: | a80 [lacZ]M15 (argF-lacU169 endA1 recA1 hsdR17 rK2 mK+deoR thi-1 supE44 gyrA96 relA1 | [86] |
| DH5α | Functional rsbU derivative of B325-4 rsbU | [87] |
| MIRSA252 | Wild-type clinical isolate | [52] |
| RN4220 | Restriction-deficient transformation recipient mutant of B325-4 | [88] |
| N315 | Wild-type pharyngeal smear clinical isolate | [99] |
| MSSA476 | Wild-type clinical isolate | [52] |
| Liv033 (katA) | B325-4 katA::Tn917 | [87] |
| Liv038 (agr) | SH1000 agr::tet | [87] |
| Liv039 (sarA) | SH1000 sarA::kan | [87] |
| Liv101 (lytS) | SH1000 lytS::pER1 | [45] |
| Liv130 (sigB) | SH1000 sigB::tet | [87] |
| Liv142 (att) | SH1000 att::lacZ pAZ106 | [44] |
| Liv405 (clfA) | B325-4 clfA::lacZ pAZ106 | T. Foster |
| Liv442 (clfA) | SH1000 clfA transduced from Liv405 | This Study |
| Liv671 (clpC) | SH1000 clpC::erm | [90] |
| Liv673 (crtM) | Newman crtM::cat | [91] |
| Liv684 (sasF) | RN4220 sasF::tet | This Study |
| Liv686 (arcA) | RN4220 arcA::tet | This Study |
| Liv692 (arcA) | SH1000 arcA::tet transduced from Liv686 | This Study |
| Liv694 (sasF) | SH1000 sasF::tet transduced from Liv684 | This Study |
| Liv718 (vraS) | SH1000 vraS::tet transduced from Liv723 | This Study |
| Liv723 (vraS) | RN4220 vraS::tet | This Study |
| Liv750 (katA) | SH1000 katA::Tn917 transduced from Liv033 | This Study |
| Liv753 (vraE) | SH1000 SAR2632::Tn917 | This Study |
| Liv766 (SAR2632) | SH1000 SAR2632::Tn917 | This Study |
| Liv994 | RN4220 pSKS630::sasF | This Study |
| Liv995 | RN4220 pSKS630::arcA | This Study |
| Liv996 | RN4220 pSKS630::vraE | This Study |
| Liv997 | RN4220 pSKS630::SAR2632 | This Study |
| Liv1000 (sasF) | Liv694 complemented with pSKS630::sasF | This Study |
| Liv1001 (arcA) | Liv692 complemented with pSKS630::arcA | This Study |
| Liv1002 (vraE) | Liv753 complemented with pSKS630::vraE | This Study |
| Liv1003 (SAR2632) | Liv766 complemented with pSKS630::SAR2632 | This Study |
| Plasmids: | | |
| pLT1 | Temperature-sensitive plasmid harbouring Tn917 | [69] |
| pAZ106 | Promoterless lacZ erm insertion vector | [92] |
| pDG1513 | pMTL22 derivative [tet'] | [73] |
| pSKS630 | Low copy number E. coli-S. aureus shuttle vector [cm'] | [75] |

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Trypsin digestion and mass spectrometric identification of proteins

Spots for identification were excised and digested in-gel with trypsin. Gel plugs were destained in 50% (v/v) acetonitrile:50% (v/v) 50 mM ammonium bicarbonate (37°C), dehydrated in 100% acetonitrile (37°C), and rehydrated overnight (37°C) in 10 μl of 50mM ammonium bicarbonate containing trypsin (1 μl of 100 ng
trypsin stock reconstituted in 50 mM acetic acid (Promega). Supernatants containing the extracted peptides were removed and analyzed by MALDI-TOF.

Peptide Mass Fingerprinting (PMF) was conducted on a reflectron MALDI-TOF instrument (M@LDE, Waters-Micromass, UK). Samples were mixed in a 1:1 ratio with a saturated solution of uis using Savitzky Golay methods et al. The acquired spectra were analyzed using MassLynx v 4.0 (Waters-Micromass, UK) and were all externally calibrated with a mixture of proteins. For each sample, all acquired spectra were combined and processed as follows using MassLynx v 4.0: smoothing, 2 channels and 2 ″ smooth using a Savitzky Golay method set at +/-3 channels and background subtraction using a polynomial of order 1 and 40% below the curve in order to reduce background noise. To get accurate mono isotopic peak data all processed spectra were centred using the top 80% of each peak. Lists were generated using ProteinLynx, part of MassLynx v 4.0. Monoisotopic peptide masses in the mass range of 800–4000 Da were used in the database search. The resulting peptide mass lists were used to interrogate S. aureus MRSA252 sequences to generate statistically significant candidate identifications using the Mascot search engine (Matrix Science).

Table 14. Oligonucleotides used for the construction of mutants.

| Oligonucleotides | Sequence (5’ to 3’) |
|------------------|---------------------|
| sasF_BamHI       | CCAAGGATCCCGTATGATGTTTTG |
| sasF_NotI        | ATAACTGGCGGCCGTTTAAACGGTTCCTCG |
| sasF_KpnI        | CCGTACGTTATACAGCAGAATAGAG |
| sasF_EcoRI       | ACATGAAATCAGAAGAGGTTGCG |
| arcA_BamHI       | CCAAGGATCCGACAGATGATGTTTTG |
| arcA_NotI        | ATAACTGGCGGCCGTTTAAACGGTTCCTCG |
| arcA_KpnI        | CCGTACGTTATACAGCAGAATAGAG |
| arcA_EcoRI       | ACATGAAATCAGAAGAGGTTGCG |
| vraS_BamHI       | CCAAGGATCCCGTATGATGTTTTG |
| vraS_NotI        | ATAACTGGCGGCCGTTTAAACGGTTCCTCG |
| vraS_KpnI        | CCGTACGTTATACAGCAGAATAGAG |
| vraS_EcoRI       | ACATGAAATCAGAAGAGGTTGCG |
| Tet_NotI         | ATAACTGGCGGCCGTTTAAACGGTTCCTCG |
| Tet_KpnI         | CCGTACGTTATACAGCAGAATAGAG |
| Complementation  |                     |
| Sar2725_SasF_For | ACAGGTACGTTATATGATGTTTTG |
| Sar2725_SasF_Rev | ACAGGTACGTTATATGATGTTTTG |
| arcA_For         | ACAGGTACGTTATATGATGTTTTG |
| arcA_Rev         | ACAGGTACGTTATATGATGTTTTG |
| Sar2632_For      | ACAGGTACGTTATATGATGTTTTG |
| Sar2632_revE_For | ACAGGTACGTTATATGATGTTTTG |
| Sar2782_vraE_For | ACAGGTACGTTATATGATGTTTTG |
| Sar2782_vraE_Rev | ACAGGTACGTTATATGATGTTTTG |

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Cell surface hydrophobicity assays

Cell surface hydrophobicity of S. aureus strains was measured as described previously [81]. Briefly, stationary-phase cells (10-h cultures) grown in the presence or absence of 0.1 mM linoleic acid were harvested, washed three times and resuspended to an OD440 of 0.5 in PBS. 3 ml aliquots of each of these bacterial suspensions were vortexed for 1 min with 200 µl n-hexadecane (Sigma). After 15 min incubation to enable partitioning, 1 ml was removed from the aqueous layer and the OD440 recorded. Cell surface hydrophobicity was calculated as the percentage decrease in OD as a result of cells partitioning into the hexadecane.

Cell Autolysis Assay

Cell autolysis rates were determined on cells exposed to linoleic acid using an assay modified from that described by Blackman et al. [82]. Briefly, cells were grown in 100 ml volumes of BHI to an OD440 of 0.5.

Table 15. Oligonucleotides used for qRT-PCR analysis.

| Oligonucleotide | SAR Number | Sequence (5’ to 3’) |
|-----------------|------------|---------------------|
| glyb_For        | SAR0005    | ATCGACTTCAAGAGAGTTTG |
| glyb_Rev        | SAR0005    | CCGTATTCGGTTTCAATGCA |
| spa_For         | SAR0114    | GAAGCAACACCACAACAGTC |
| spa_Rev         | SAR0114    | ACCTGCAACTTAAACGCAGT |
| fadA_For        | SAR0223    | GAAGATGCTAATGTTGCTAGC |
| fadA_Rev        | SAR0223    | TGTACATCGGATGACAGTAC |
| fadD_For        | SAR0225    | TTACATGCAAGAATAGACTAG |
| fadD_Rev        | SAR0225    | TCGGTTTGAGAGATCCCTTG |
| lyrR_For        | SAR0258    | TTGTTCGAATCGCAGACGCAA |
| lyrR_Rev        | SAR0258    | TATCATTCGTTTCGTTATGTC |
| sarA_For        | SAR0625    | TAAACTCAAAACACCAAAAGT |
| sarA_Rev        | SAR0625    | TTCGATTTGTTACGTTGTCG |
| clpB_For        | SAR0938    | GAAGCGAAGAATATGAGTCAG |
| clpB_Rev        | SAR0938    | GCCTTTGATCTACAGGAGTTG |
| fabl_For        | SAR0978    | GTGATGGGGTGTGCTATAAGG |
| fabl_Rev        | SAR0978    | AACCACCCACACCTTTGCAC |
| hla_For         | SAR1136    | GTGCAATAACGTGAAATGAG |
| hla_Rev         | SAR1136    | CCAATTTTTCCGAACTTAC |
| katA_For        | SAR1344    | AATAGTGTAAGACAGGAGCTCA |
| katA_Rev        | SAR1344    | AACATCCGCGCAAGCAGAC |
| murG_For        | SAR1430    | ATCCCGAGGGCAGAAATGAA |
| murG_Rev        | SAR1430    | AATCCGCGCAAGCAGAC |
| fabZ_For        | SAR2186    | AATAGTGAAGAATGCTAAAG |
| fabZ_Rev        | SAR2186    | ACCGCCCTTGGTGAACTG |
| cidA_For        | SAR2621    | GCCGGAGTATTTGTGCTCA |
| cidA_Rev        | SAR2621    | TTAATACCTAACAATCAGGACATGA |
| cfrM_For        | SAR2643    | TGATCACTGATAGATGTTTTAG |
| cfrM_Rev        | SAR2643    | ACATCGTGAAGGGCCATCATG |
| arcA_For        | SAR2714    | TCGAGGATCTGAAAGAGA |
| arcA_Rev        | SAR2714    | GTGCCATCTGAGGCTTGG |
| sais-For        | SAR2725    | CACAAATCGGAGAATAGCTCAG |
| sais_Rev        | SAR2725    | GCCTTTGATCTACAGGAGTTG |
| RNAIII_For      | RNAIII     | ACATGGTTATTAAGGTTGAGT |
| RNAIII_Rev      | RNAIII     | TAAATGAGTTATACAGCAGA |

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OD₆₀₀ of 0.8–1.0 in the presence or absence of 0.01 mM linoleic acid. Following harvesting of the cells by centrifugation, the cells were washed in PBS and resuspended to an OD₆₀₀ = 0.6 in 0.5% (v/v) Triton X-100. The cells were incubated with shaking at 37 °C and the OD₆₀₀ was monitored over time.

Experimental septic arthritis

A well described mouse model of septic arthritis was used to test the in vivo role of genes implicated in resistance to fatty acids in the strains [83–85]. Seven week old female NMRI mice were obtained from Charles River Laboratories (Sulzfeld, Germany) and maintained in the animal facility of the Department of Rheumatology and Inflammation Research, University of Göteborg, Sweden. All mice were maintained according to the local ethical board animal husbandry standards. The mice were housed 10 to a cage under standard conditions of temperature and light and were fed standard laboratory chow and water ad libitum.

Bacteria were grown on blood agar plates for 24 h, harvested and stored frozen at −20°C in PBS containing 5% bovine serum albumin and 10% dimethyl sulfoxide. Before injection into animals, the bacterial suspensions were thawed, washed in PBS, and adjusted to appropriate cell concentrations. Mice were inoculated in the tail vein with 0.2 ml of bacterial suspension.

The number of viable bacteria was measured in conjunction with each challenge by counting colonies following culture at 37 °C for 24 hours on blood agar plates. Ten mice were infected with each strain of S. aureus by i.v. injection in the tails of 3.2–3.5 × 10⁶ CFU of bacteria for induction of septic arthritis. The mice were weighed regularly and examined for arthritis until death by cervical dislocation 14 days after challenge. The kidneys were aseptically dissected and kept on ice, homogenized, diluted in PBS and inoculated on blood agar plates. Data were presented as CFU per kidney pair.

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Author Contributions

Conceived and designed the experiments: JGK DW EJ MJH. Performed the experiments: JGK DW EJ MJH. Analyzed the data: JGK DW EJ MJH. Contributed reagents/materials/analysis tools: JL. Wrote the paper: JGK DW HR MJH.
53. Chamberlain NR, Brueggemann SA (1997) Characterisation and expression of

54. Wydro P, Krajewska B, Hac-Wydro K (2007) Chitosan as a lipid binder: a

55. Johnson AP, Aucken HM, Cavendish S, Ganner M, Wale MC, et al. (2001)

56. Sass V, Pag U, Bierbaum G, Sahl HG (2008) Mode of action of human

57. Roche FM, Massey R, Peacock SJ, Day NP, Visai L, et al. (2003)

58. Weidenmaier C, Peschel A, Kempf VA, Lucindo N, Yeaman MR, et al. (2005)

59. Qoronfleh MW, Gustafson JE, Wilkinson BJ (1998) Conditions that induce

60. McCallum N, Bischoff M, Maki H, Wada A, Berger-Bachi B (2004) TcaR, a

61. De Lencastre H, Wu SW, Pinho MG, Ludovice AM, Filipe S, et al. (1999)

62. Sass V, Pag U, Bierbaum G, Sahl HG (2008) Mode of action of human

63. Foster SJ (1995) Molecular characterization and functional analysis of the major

64. Wydro P, Krajewska B, Hac-Wydro K (2007) Chitosan as a lipid binder: a

65. Freiberg C, Fischer HP, Brunner NA (2005) Discovering the mechanism of

66. Hutter B, Schaaf C, Albrecht S, Borgmann M, Brunner NA, et al. (2004)

67. Bierbaum G, Sahl HG (2008) Mode of action of human

68. Bierbaum G, Sahl HG (2008) Mode of action of human

69. Li S, Huang X, Zhu X, Yang F, Qiu Z, et al. (2008) Targeting of s-bm3 antibiotic

70. Linares M, Linares M, Legrand E, Buhot S, Brodin E, et al. (2008) Targeting of s-bm3 antibiotic

71. Foster SJ (1995) Molecular characterization and functional analysis of the major

72. Foster SJ (1995) Molecular characterization and functional analysis of the major

73. Foster SJ (1995) Molecular characterization and functional analysis of the major

74. Schenk S, Ladagga RA (1992) Improved methods for electroporation of

75. Schenk S, Ladagga RA (1992) Improved methods for electroporation of

76. Schenk S, Ladagga RA (1992) Improved methods for electroporation of

77. Schenk S, Ladagga RA (1992) Improved methods for electroporation of

78. Yan J, Wait R, Berkelman T, Harry R, Westbrook J, et al. (2000) A modified

79. Pappin DJ, Hojrup P, Bleasby AJ (1993) Rapid identification of proteins by

80. Pappin DJ, Hojrup P, Bleasby AJ (1993) Rapid identification of proteins by

81. Pappin DJ, Hojrup P, Bleasby AJ (1993) Rapid identification of proteins by

82. Blackman SA, Smith TJ, Foster SJ (1998) The role of autolysins during

83. Blackman SA, Smith TJ, Foster SJ (1998) The role of autolysins during

84. Blackman SA, Smith TJ, Foster SJ (1998) The role of autolysins during

85. Blackman SA, Smith TJ, Foster SJ (1998) The role of autolysins during

86. Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory

87. de Lencastre H, Nyholm H, Boetsch G, Pag U, V１lles S, et al. (2003) Mode of action of human

88. de Lencastre H, Nyholm H, Boetsch G, Pag U, V１lles S, et al. (2003) Mode of action of human

89. Kuroda M, Ohta T, Uchiyama I, Baba T, Yuzawa H, et al. (2001) Whole

90. Altwegg R, Huebner K, Schaefer S, Heesemann J, Wieser H, et al. (2005) Identification of

91. Altwegg R, Huebner K, Schaefer S, Heesemann J, Wieser H, et al. (2005) Identification of

92. Altwegg R, Huebner K, Schaefer S, Heesemann J, Wieser H, et al. (2005) Identification of

93. Altwegg R, Huebner K, Schaefer S, Heesemann J, Wieser H, et al. (2005) Identification of

94. Foster SJ (1995) Molecular characterization and functional analysis of the major

95. Foster SJ (1995) Molecular characterization and functional analysis of the major

96. Foster SJ (1995) Molecular characterization and functional analysis of the major

97. Foster SJ (1995) Molecular characterization and functional analysis of the major