Journal of Applied Microbiology

Journal of Applied Microbiology ISSN 1364-5072

ORIGINAL ARTICLE

Molecular characterization of piezotolerant and stress-resistant mutants of *Staphylococcus aureus*

K.A.G. Karatzas[1], N.A. Lemmens-den Toom[2], C.C. Tassou[3], W. vanLeeuwen[4] and A. vanBelkum[5]

1 Department of Food and Nutritional Sciences, University of Reading, Reading, UK
2 Department of Medical Microbiology and Infectious Diseases, Erasmus Medical Center, GD Rotterdam, The Netherlands
3 Hellenic Agricultural Organisation 'DEMETER', Institute of Technology of Agricultural Products, Attikis, Greece
4 Leiden Centre for Applied Bioscience, University of Applied Science Leiden, Leiden, The Netherlands
5 BioMérieux, Open Innovation & Partnerships, La Balme Les Grottes, France

Keywords
food preservation, food processing, food safety, Staphylococci, toxins.

Correspondence
Kimon Andreas G. Karatzas, Department of Food & Nutritional Sciences, University of Reading, Whiteknights, PO Box 226, Room 3-38, Reading RG6 6AD UK.
E-mail: k.karatzas@reading.ac.uk

2020/0582: received 30 March 2020, revised 8 July 2020 and accepted 4 August 2020

doi:10.1111/jam.14832

Abstract

Aims: In the previous work, following a pressure treatment with wild-type *Staphylococcus aureus*, we obtained piezotolerant isolates showing altered phenotypic characteristics. This work focuses on understanding the genetic background of their altered phenotype.
Methods and Results: AK23, a representative piezotolerant isolate was subjected to DNA microarrays, corroborated by PCR product sequencing and revealed 10-gene deletion. All other piezotolerant isolates possessed the mutation encompassing the region from SAR0665 to SAR0674 genes (9351 bp) which was most likely the result of recombination between two homologous loci (ATTGCGGGTG) present in both genes. RNA microarray transcriptomic analysis showed that due to partial deletion of the low-affinity phosphate transporter *pitA*, the high-affinity PhoU-PstABCS operon was upregulated in AK23 which could be the reason for piezotolerance. Furthermore, AK23 showed low levels of the virulence gene regulator *rnaIII* resulting in the downregulation of several *agr* system genes explaining the impaired virulence characteristics of the mutant.
Conclusions: Naturally occurring mutations can result in piezotolerance which can be of a concern for high hydrostatic pressure-treated foods.
Significance and Impact of the Study: A locus has been identified in piezotolerant *S. aureus* mutants providing insight into possible mechanisms associated with phenotypic characteristics of *S. aureus*. Further work should study each individual gene of the locus.

Introduction

Staphylococcus aureus is a Gram-positive, facultatively anaerobic, cluster-forming, non-motile coccus which is the leading cause of nosocomial infections (Tong *et al.* 2015). Normally these possibly lethal infections occur upon a transition from colonization to infection where the bacterium can gain access to the blood stream and translocate to different parts of the body, where it can grow and cause further damage. There is also a variety of human diseases caused by *S. aureus* (Tong *et al.* 2015). One of these is a food-borne disease occurring through growth in foods where it can produce enterotoxins (Tassou *et al.* 2008). Food-borne *S. aureus* disease occurs normally through the contamination of food by a food handler as a large proportion of humans are *S. aureus* carriers (Zeaki *et al.* 2019). If this food is kept at normal or higher temperatures, the micro-organism can grow and produce toxins which are consumed together with the food and cause a food-borne illness characterized by diarrhoea, vomiting, nausea and abdominal cramps (Guidi *et al.* 2018).

One of the novel methods for the inactivation of *S. aureus* and other bacteria in ready-to-eat foods is high hydrostatic pressure (HHP; Karatzas *et al.* 2007). Following an initial slow adoption by the food industry, the last

decade has seen a major increase in the number of food products processed by HHP entering the market and many companies adopting the method. Despite the increasing usage of HHP there is still concern about piezotolerant strains that have been described previously and can resist significant levels of pressure. Study of these strains can provide us with insights on how micro-organisms resist such pressure levels and it will detail the mode of action of HHP (Karatzas *et al.* 2003, 2005). This knowledge could be used to minimize HHP treatment intensity which can translate in lower costs or it could resolve problems with tailing effects where such strains persist HHP treatment and remain in the food product, posing a threat to the customers.

Previously, we identified piezotolerant *S. aureus* variants that derived from a clonal population and could survive high levels of HHP (Karatzas *et al.* 2007). These variants comprised 9 out of 21 isolated survivors (52% of the isolates), following a HHP treatment of 400 MPa for 30 min. They all showed a small colony phenotype, increased thermotolerance, impaired growth and reduced antibiotic resistance compared to the wild type (WT). They also showed weaker agglutination reactions, they were defective in the production of the typical *S. aureus* golden colour and showed lower invasion to intestinal epithelial cells than the WT. In an attempt to identify the mechanism causing this significant change in phenotype and based on our previous experience with piezotolerant mutants in *Listeria monocytogenes* having similar phenotypic changes that were attributed to mutations in *ctsR* gene, we analysed their *ctsR* and *hrcA* genes, but we found no mutations (Karatzas *et al.* 2007).

In the current work, we aimed to identify the mechanism behind the piezotolerance of these mutants. We carried out further analysis of one of these piezotolerant mutants (AK23) isolated following an HHP treatment of a culture of the parent strain and performed a transcriptomic and genetic analysis. Additional genetic analysis of a specific locus was also carried out for the previously isolated piezotolerant isolates.

Materials and Methods

Bacterial strains

Previously, a WT methicillin-susceptible strain of *S. aureus* isolated from ham (National Agricultural Research Foundation, Lycovrissi, Greece) was used as WT for HHP experiments. Following an HHP treatment of the WT, 21 survivors were isolated randomly, analysed and described previously (Karatzas *et al.* 2007) were used in the current study. One additional representative piezotolerant isolate (AK23) that was isolated together with the latter ones, but was not analysed previously was also used. All isolates were derived from the same parent WT strain. Due technical problems with the recovery of all other isolate stocks from the freezer, work proceeded with AK23 which, however, needed to be verified for its phenotypic characteristics. Subsequently, through detailed thorough work all other isolates were recovered but after all molecular work was completed with AK23.

The WT methicillin-susceptible *S. aureus* strain was resistant to ampicillin, amoxicillin, penicillin, nalidixic acid and sulfonamides. Stock cultures were kept at −80°C in 15% (vol/vol) glycerol or microbank tubes (Pro-Lab Diagnostics, Neston, Wirral, UK), transferred into sterile brain heart infusion (BHI) broth (Oxoid, Hampshire, UK) with or without 5% Tween 80 (ICI Surfactants, Wilmington, DE) and incubated twice at 37°C overnight (0·3% (vol./vol.) inoculum). Cultures were grown with shaking (160 rev min^{-1}), and Tween 80 was added only to those that would be subjected to HHP or heat treatment to alleviate the clumping of bacterial cells.

Colony morphology

Stationary-phase cultures of the WT and AK23 were grown as described above and passaged on Baird Parker agar, BHI agar, Columbia blood agar (COLBA) and plate count agar plates (Oxoid). Plates were incubated at 37°C for 24 h, and colonies were examined for their size, colour and haemolysis characteristics on COLBA plates.

Analysis of growth kinetics

Growth characteristics of WT and AK23 were assessed at 37°C with shaking (160 rev min^{-1}) or at 20°C under static conditions. Five microlitres from each culture was inoculated into 200 ml fresh BHI. Samples were placed onto a Sero-Well microtitre plate (Sterilin, Staffordshire, UK), and bacterial growth was assessed by measuring the optical density at 600 nm (OD_{600nm}) of the samples with a Bio-Rad model 680 microplate reader (Bio-Rad, Hercules, CA). Growth curves were constructed in triplicate. Doubling times were calculated based on values obtained from the exponential phase of growth.

Agglutination test

An agglutination test was performed using the Staphylo Monotec kit (Fluka, Buchs, Switzerland) to confirm the identity of AK23 isolate as *S. aureus*. The test reagent comprises monodispersed particles coated with fibrinogen and immunoglobulin G, which bind to the cell-associated coagulase and cell wall protein A respectively. The times

needed for agglutination for the AK23 isolate and the WT were recorded.

treatment

Cultures were placed in sterile plastic stomacher bags (Seward, London, UK) that were sealed while avoiding an excess of air bubbles. Pouches were submerged in glycol (Resato, Roden, The Netherlands), which was the fluid pressure medium, and subjected to 450 MPa for 15 min in a HHP unit (Resato) at 20°C. The viability of *S. aureus* cells was determined before and after the pressure treatment. Decimal dilutions of samples in saline solution (Oxoid) were prepared, followed by plating in triplicate onto BHI agar (1% (wt./vol.) agar). Plates were incubated at 37°C for 5 days.

Analysis of thermotolerance

BHI broth (100 ml) was inoculated (0·1%, vol./vol.) with overnight cultures of the strains incubated with shaking (160 rev min^{-1}) at 37°C. Cultures were grown until mid-exponential phase (OD_{600nm} between 0·4 and 0·6), and samples were placed in 7-ml plastic tubes (Sterilin, Staffordshire, UK) and incubated in a water bath at 58°C for 20 min. Samples were taken before and after treatment and decimal dilutions in saline solution were prepared using saline tablets (Oxoid), and viability was determined.

Gentamicin protection assay

The gentamicin protection assay was performed for the WT strain and AK23 as described previously (Elsinghorst 1994). In brief, Caco-2 human colon adenocarcinoma cells (European Collection of Cell Cultures number 86010202) were maintained in Dulbecco's modified Eagle's medium containing 2 mM glutamine, 1% non-essential amino acids and 10% foetal bovine serum (DMEM; Sigma-Aldrich Poole, UK). Penicillin or streptomycin (Invitrogen, Paisley, UK) was used at a concentration of 100 U ml^{-1} in DMEM until 3 days before cells were used for infection studies. The culture medium was changed every 2–3 days. Just before experiments, Caco-2 cells were washed thrice with sterile phosphate-buffered saline (PBS), and subsequently, 2 ml of antibiotic-free DMEM was added to each well. The OD_{600nm} of stationary-phase cultures of *S. aureus* AK23 and WT were measured, and all cultures were adjusted to similar OD_{600nm}. We previously confirmed that there was a good correlation between OD_{600nm} measurements and cell numbers for both strains, as assessed by comparing CFU and OD_{600nm} values. Fifty microlitres of the adjusted bacterial suspensions were added to the wells, resulting in 10^6 CFU per well and yielding an estimated ratio of 2·5: 1 of bacteria to cells (multiplicity of infection). Cells were incubated for 1 h at 37°C and subsequently washed twice with PBS and suspended in 2 ml of PBS containing 100 g ml^{-1} gentamicin. After 2 h at 37°C, cells from cultures exposed to gentamicin were rinsed twice with sterile PBS and lysed with 2 ml 1% Triton X-100 (vol/vol) in PBS. Following a brief incubation for 5 min at 37°C, cell lysates were serially diluted and plated onto COLBA to quantify the number of intracellular bacteria.

Antibiotic disk diffusion test

The antibiotic disk diffusion test was conducted according to the recommendations of the British Society for Antimicrobial Chemotherapy (Andrews 2005). In brief, cells from four morphologically similar colonies of each strain grown overnight on Iso-Sensitest agar plates (ISA; Oxoid) were transferred with a sterile loop into Iso-Sensitest broth (ISB; Oxoid) and incubated with shaking at 37°C until the turbidity was equal to the 0.5 McFarland standard. Subsequently, cells from the suspension were transferred with a sterile cotton-wool swab onto ISA spread evenly over the entire surface of the plate by swabbing in three directions. Immediately, after swabbing, antibiotic disks (Oxoid) were applied on the plate and following incubation at 37°C for 18 h, and zones of inhibition were measured.

Determination of MIC

The MICs of a range of antibiotics were determined using the broth doubling dilution method according to the recommendations of the British Society for Antimicrobial Chemotherapy (Andrews 2001). Overnight cultures of strains were prepared in ISB at 37°C and diluted to 0.5 McFarland. Kanamycin, gentamicin and nalidixic acid were purchased from Sigma-Aldrich (Poole, UK).

Stability of phenotype

AK23 isolate was assessed for the stability of its phenotypes. This isolate was subcultured for 10 consecutive days using 0·3% (vol./vol.) inocula in fresh BHI medium. On day 10, the culture was inoculated using a 0·3% (vol./vol.) inoculum in 100 ml of BHI broth, incubated at 37°C with shaking (160 rev min^{-1}), and tested for its growth characteristics, colony morphology and piezotolerance as described above.

Sequencing of deleted region

In previous work we attempted to investigate the source of the altered phenotype of the piezotolerant isolates by

looking at possible mutations in the *ctsR* and *hrcA*, based on previous accounts of the involvement of these genes in piezotolerance and stress resistance (Karatzas *et al.* 2003, 2005) but no mutations were found (Karatzas *et al.* 2007). In this work, we have investigated the possibility that isolates could possess a major mutation which was indicated by genomic indexing and microarray transcriptomics as both techniques showed no hybridization with all genes from SAR0666–SAR0673. Therefore, we designed primers DelFor (TATCGGGCGAATTGCTTATC) and DelRev (GGGAAATGTAGCGATGATGC) that could amplify this specific region. We were sure that these primers would work since we designed them in areas that showed hybridization with the genomic DNA of the isolate. DelFor was designed in the intragenic region between SAR0664 and SAR0665 and DelRev within the SAR0674 gene.

Chromosomal DNA from WT and all piezotolerant isolates described previously (Karatzas *et al.* 2007) and AK23 were isolated as described by Pospiech and Neumann (1995) and used as PCR templates. All primers were provided by Eurofins Genomics (Ebersberg, Germany), and amplification and sequence analysis were performed in duplicate by Eurofins Genomics.

isolation for genomic indexing

Since the parent strain and AK23 used in these experiments were not previously typed, we performed genomic indexing with the use of genomic DNA from the parent strain and the microarray SAM-62. This procedure was essential to assess the level of hybridization between the genes present in the parent strain and on the microarray chip. To isolate DNA, cells were grown overnight (18 h) at 37°C and subsequently, 7 ml from this culture was taken and centrifuged at 3500 ***g*** for 5 min at 4°C. The pellet was washed twice in 1 ml PBS and finally it was resuspended in 0·5 ml PBS and kept on ice. Subsequently, cells were transferred in SETS II tube system (Roche Diagnostics Ltd, Burgess Hill, UK), followed by shaking for 30 s at 6000 rev min^{-1} in a MagNA Lyser device (Roche Products Ltd, Welwyn Garden City, UK). The lysate was centrifuged for 2 min at 14 000 rev min^{-1} and 400–500 μl of the supernatant was transferred to a clean Eppendorf tube followed by 200 μl of ethanol (96%). This cell lysate was then used following the instructions of Qiagen DNA Minikit (Manchester, UK) from step 4. Subsequently, the DNA was eluted in 80 μl DNA/RNA-free water, its concentration was measured in a NanoDrop™ 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE) and subjected to hybridization with the microarray hybridization protocol.

isolation and slide hybridization

Overnight cultures were diluted 100 times in fresh prewarmed brain–heart infusion (BHI) broth and grown at 37°C in 5% CO_2 until an OD_{590nm} of 0·5 was reached.

A volume of 30°ml of the culture was pelleted and suspended in 1°ml RNAPro Solution (Qbiogene Inc. Heidelberg, Germany).

Staphylococcus aureus RNA was isolated using the FastRNA Pro Blue Kit according to the manufacturer's instructions (Qbiogene Inc. Heidelberg, Germany) using the Fastprep FP120 instrument (two cycles of 45 s at a speed setting of 6·0; Qbiogene). After isolation, the RNA was treated with 6 U TURBO DNase (Ambion, Austin, TX) according to the manufacturer's instructions.

Hybridization probes were generated from 2 μg total RNA of each strain according to the protocol of the Bacterial Microarray Group (BuG@s; St. George's Hospital Medical School, London, UK). RNA was mixed with 3 μg random primers (Invitrogen, Breda, The Netherlands), heat denatured and snap-cooled on ice. The RNA was reverse transcribed to cDNA to incorporate the Cy5 dCTP (GE Healthcare, Diegem, Belgium) or Cy3 dCTP (GE Healthcare).

Labelled cDNA samples were pooled and hybridized overnight to a *S. aureus* microarray with PCR amplicons printed on Ultragaps (Corning, NY) glass slides (BuG@S; Witney *et al.* 2005). Microarray slides were prehybridized in 3·5 × SSC (1 × SSC is 0·15 mol l^{-1} NaCl plus 0·015 mol l^{-1} sodium citrate)–0·1% sodium dodecyl sulfate (SDS)–10 mg ml^{-1} bovine serum albumin at 65°C for 20 min before a 1-min wash in distilled water and a subsequent 1-min wash in isopropanol. Each Cy3-labelled test strain cDNA was mixed with an equal amount of Cy5-labelled reference strain cDNA, purified using a MiniElute kit (Qiagen, Manchester, UK), denatured and mixed to achieve a final 45 μl hybridization solution of 4 × SSC–0·3% SDS. Using two 22 × 22 mm LifterSlips (Erie Scientific, Portshmouth, NH), the microarray was sealed in a humidified hybridization chamber (Telechem International, Sunnyvale, CA), hybridized overnight by immersion in a water bath at 65°C for 16–20 h. Slides were washed once in 400 ml 1 × SSC–0·06% SDS at 65°C for 2 min and twice in 400 ml 0·06 × SSC for 2 min. The array design is available in BmG@Sbase (accession no. A-BUGS-17; http://bugs.sgul.ac.uk/A-BUGS-17) and also ArrayExpress (accession no. A-BUGS-17).

Transcriptomics analysis

Microarray slides were scanned using the ScanArray Express HT scanner (Perkin Elmer, Groningen, The Netherlands) following manufacturer's instructions. The

spots were quantified using Imagene 6.0 software (BioDiscovery, Marina Del Ray, CA).

GeneSpring GX version 7.3 Software (Agilent Technologies, Santa Clara, CA) was used for normalization and further data analysis. Expression levels were quantified as the log-ratio of the signal derived from RNA isolated from the mutant divided by the signal derived from the WT strain RNA. Expression levels were averaged for the duplicate experiments.

Statistical analysis

The *t*-test for three samples, assuming equal variances, was used to determine statistical differences between the means of log reductions in CFU of each isolate and of the wild type. *P* values of less than 0·05 were considered to indicate statistical significance.

Results

Growth characteristics and colony morphology

Growth of WT and AK23 was assessed in liquid and solid media. AK23 demonstrated the typical colony morphology of *S. aureus* on Plate Count and Baird Parker agar, but it formed significantly smaller colonies than the WT on all solid media (Fig. 1). This phenotype was observed previously by (Karatzas *et al.* 2007) in piezotolerant *S. aureus* isolates. Furthermore, AK23 demonstrated a reduced ability to produce the typical golden colour of WT *S. aureus* (Fig. 1a). The small colony phenotype of AK23 indicated impaired growth which was also confirmed in liquid media when cells were grown under static conditions at 20°C (Fig. 2) and the doubling time was 0·77 and 1·46°h for WT and AK23 respectively. However, there was no difference in growth when cells were incubated at 37°C with shaking (data not shown). The above-mentioned phenotype for AK23 was stable for the 10 days of continuous cultivation.

Agglutination test

Further to identification through colony morphology on Baird Parker, AK23 was also identified as *S. aureus* by a successful agglutination test. However, the agglutination reaction was significantly weaker for AK23 compared to the WT as it took 23 s for the first and 10 s for the latter (Fig. 3). Furthermore, the coagulation reaction for AK23 was not very strong and produced smaller clumps.

Stress resistance of AK23

The AK23 isolate showed significant piezotolerance compared to its WT parent strain (Fig. 4). At 450 MPa for 15 min, AK23 showed only 3 log reduction of CFU per ml, while the WT a significant inactivation of 5·25 log reduction of CFU per ml. Furthermore, we assessed the thermotolerance of AK23 and we found that it was more resistant than the WT with statistical significance. The AK23 showed a 4·15 log reduction of CFU ml^{-1} while the WT a 6·05 log reduction of CFU per ml (Fig. 5). The piezotolerant phenotype observed for AK23 was stable for the 10 days of continuous cultivation.

Gentamicin protection assay

We found that AK23 invaded much less efficiently in Caco-2 intestinal epithelial cells, with statistical significance ($P < 0·05$). We documented 8·56 log CFU per ml for the WT and 5·10 log CFU per ml for AK23 (Fig. 6).

Antimicrobial resistance (inhibition zones and minimum inhibitory concentrations)

AK23 was significantly more susceptible than the WT to antibiotics amikacin, kanamycin, oxacillin, neomycin and gentamicin. Zones of inhibition to the above antibiotics were significantly larger than the WT (Table 1). Analysis of the MICs also showed that AK23 was more susceptible to antibiotics although they did not change the status of the AK23 to sensitive (Table 1).

Deletion present in AK23 and other piezotolerant isolates

While ultimately aiming to perform microarray transcriptomic analysis with the WT and the AK23, we first performed a genomic indexing where DNA from the WT was hybridized with the DNA on the microarray slides. Along with WT we took AK23 for these experiments and although all genes on the slide showed hybridization for the WT, genes encompassing SAR0665 until SAR0673 showed no hybridization for AK23. This suggested that a major deletion was present in AK23. Therefore, we designed primers DelFor and DelRev in the intragenic region between SAR0664 and SAR0665 and within the SAR0674 gene respectively. These primers were expected to give short amplicon when used in PCR with DNA from AK23 mutant but not for the WT. The distance between these primers is more than 10 000 bp and it was impossible to give an amplicon with the setup used for this specific PCR reaction. Furthermore, we repeated this PCR with all isolates analysed in the previous work (Karatzas *et al.* 2007) and we found that all piezotolerant isolates (1, 3, 4, 6, 7, 8, 10, 15, 21) and AK23 gave a positive PCR while all other isolates and the WT, produced no detectable PCR product. Subsequently, we

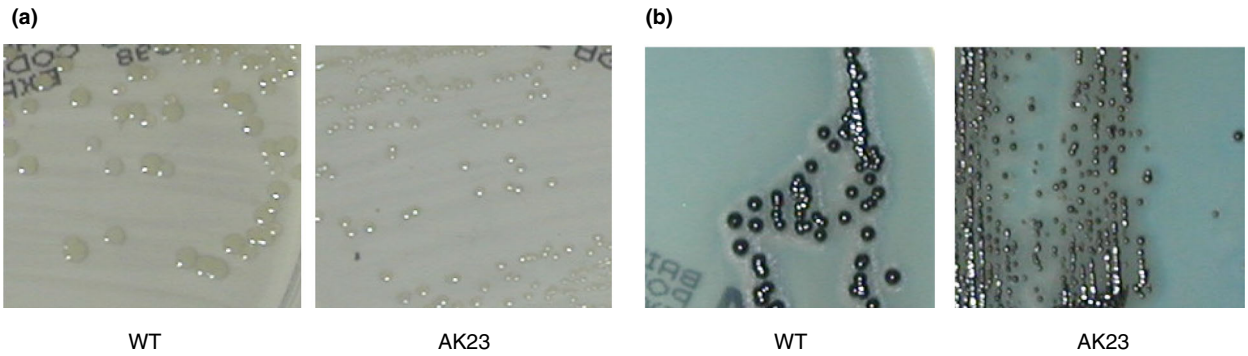

Figure 1 Colony morphology of AK23 and WT grown on plate count (a) and Baird Parker (b) agar. Plates were incubated at 37°C overnight

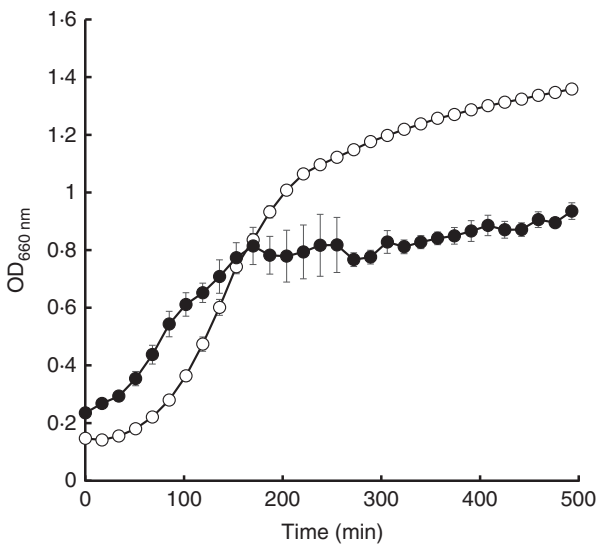

Figure 2 Growth of AK23 (black circles) and WT (white circles) grown at 20°C under static conditions

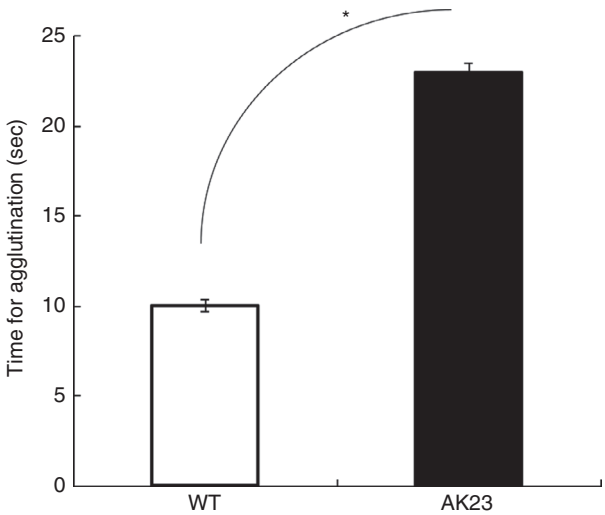

Figure 3 Time (s) taken for AK23 and WT to show a positive agglutination reaction. Asterisk denotes statistically significant difference ($P < 0{\cdot}05$) between AK23 and WT. Bars indicate average of three independent experiments and error bars denote the standard deviation

proceeded with sequencing the amplicon obtained from AK23 which showed a major deletion of 9351 bps affecting 10 genes (Table 2). The deletion most probably occurred through the homologous recombination between two relatively small homologous regions of 10 bps (ATTGCGGGTG) which are present in both genes SAR0665 and SAR0674 (Fig. 7). The deletion encompassed the *yts* operon (SAR0669–SAR0672), *pitA* (SAR0674) encoding a putative low-affinity inorganic phosphate transporter and *lipA* encoding for a putative lipase (SAR0669).

Transcriptomic analysis of AK23

We performed transcriptomic microarray analysis of AK23 in comparison with its isogenic WT. We found that all genes upregulated in AK23 were related to phosphate uptake (Table 3). The genes SAR1398–SAR1402 comprise the Pst operon in *S. aureus* which plays a role in phosphate uptake. Furthermore, the PhoB gene and SAR0110 encoding a putative Na/Pi cotransporter and the hypothetical protein SAR0584, were all upregulated.

There were also genes that were downregulated in AK23 of which the majority such as *hld*, *spa*, *rnaIII*, *plc*, *nuc*, *splB*, *hysA2*, SAR0694 and SAR0304 are part of the *agr* system (Table 4). Furthermore, genes *pyrAA* and *pyrAB* played a role in pyrimidine and arginine biosynthesis, while *nrdD* and *narJ* contributing in anaerobic respiration were also downregulated. *dltA* that contributes in the teichoic acid alanylation affecting membrane

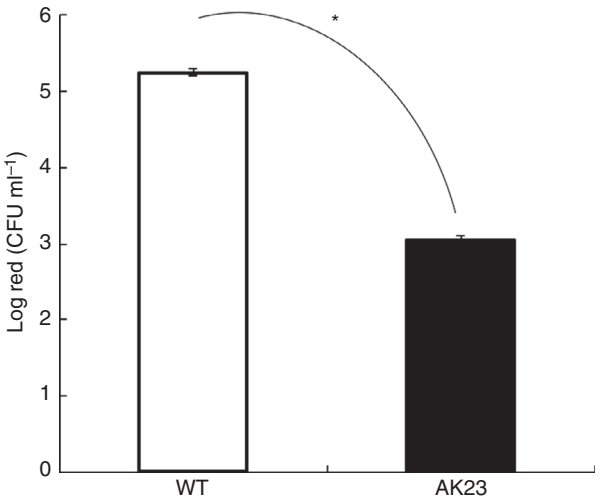

Figure 4 Piezotolerance of AK23 and WT presented as log reduction of CFU per ml. Cells were grown in BHI broth supplemented with Tween 80 (0·3% vol./vol.) at 37°C with shaking (160 rev min^{-1}) overnight and subsequently challenged at 450 MPa for 15 min. Asterisk denotes statistically significant difference ($P < 0·05$) between AK23 and WT. Bars indicate average of three independent experiments and error bars denote the standard deviation.

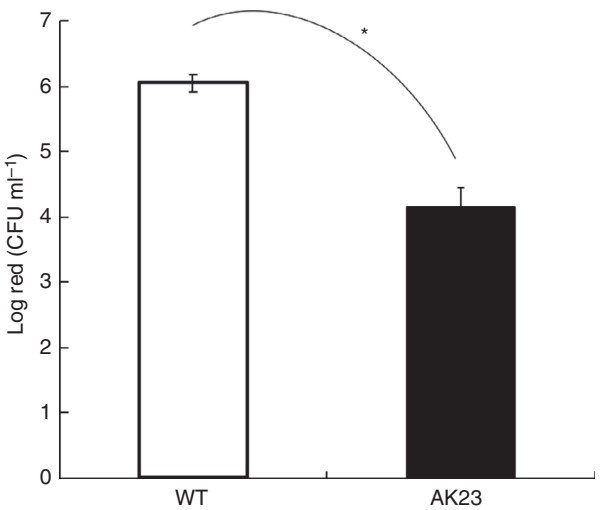

Figure 5 Thermotolerance of AK23 and WT presented as log reduction of CFU per ml. Cells were grown in BHI broth supplemented with Tween 80 (0·3% vol./vol.) at 37°C with shaking (160 rev min^{-1}) until mid-exponential phase (0·4–0·6 optical density at 600 nm) and subsequently challenged at 58°C for 20 min. Asterisk denotes statistically significant difference ($P < 0·05$) between AK23 and WT. Bars indicate average of three independent experiments and error bars denote the standard deviation

integrity and *mprF* encoding a phosphatidylglycerol lysyltransferase playing a role in antibiotic resistance were also downregulated.

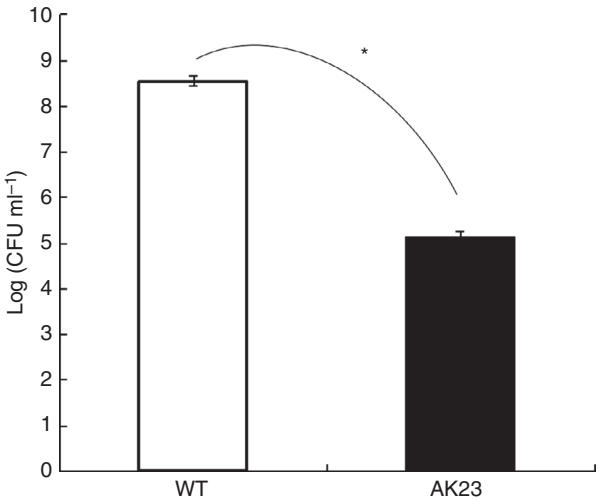

Figure 6 Invasiveness of AK23 and WT *Staphylococcus aureus* following that gentamicin protection assay in Caco-2 intestinal epithelial cells for 2 h at 37°C. Asterisk denotes statistically significant differences between the two strains ($P < 0·05$). Bars indicate average of three independent experiments and error bars denote the standard deviation

Discussion

In the previous work we have shown that clonal populations of *S. aureus* are able to give rise to stable piezotolerant variants (Karatzas *et al.* 2007), a phenomenon we also observed and investigated previously in *L. monocytogenes* (Karatzas *et al.* 2003, 2005). *Listeria monocytogenes* possesses a hypermutable region located on the *ctsR* stress gene regulator, giving rise to stress-resistant piezotolerant mutants at a rate of 1 per 10 000 cells in a WT culture. This subpopulation of *ctsR* mutants is always present in the culture and can survive HHP treatments. Such strains are a major concern since many food products nowadays are pressure treated, and their study is essential to ensure safety of such food products as their design should consider the worst-case scenario. Furthermore, such work could provide us with a broader understanding of stress resistance in bacteria impacting on various scientific areas.

During the above work, we have found that the piezotolerant *S. aureus* variants mentioned above, comprised 52% of the surviving WT population following a challenge of the WT at 400 MPa for 30 min. All these piezotolerant variants showed a small colony phenotype, increased thermotolerance, impaired growth, weaker agglutination reactions, lower invasion to intestinal epithelial cells, defective golden colour production and reduced antibiotic resistance compared to the WT. An initial attempt to identify the genetic basis of this phenotype has been unsuccessful as we did not find any

Table 1 Diameters of zones of inhibition in disk diffusion tests (upper part of table with normal fonts) and MICs (lower part of table with bold fonts) of strains with antibiotics

Strain	Amikacin (30)	Kanamycin (30)	Oxacillin (1)	Neomycin (10)	Gentamicin (10)	Nalidixic acid
WT	15 (R)	15–16 (I)	16 (R)	13–14 (R)	17 (R)	
AK23	22	22	21	19	21	
WT		50–100 (R)			50 (R)	400
AK23		25 (R)			1·5–6·2 (R)	50–100

(R) resistant; (I) intermediate. Numbers in parentheses next to the antibiotics are the μg of antibiotic contained on the disc. Zones of inhibition are expressed in mm while MICs in μg ml^{-1}.

Table 2 Genes deleted in the AK23 mutant

Gene	Functional annotation
SAR0665	Putative esterase, similar to lipase *lipA* (partly deleted)
SAR0666	Hypothetical protein
SAR0667	Putative acetyltransferase
SAR0668	Hypothetical protein
SAR0669	Hypothetical protein, similar to two-component response regulator (*ytsA*)
SAR0670	Putative membrane protein, putative sensor histidine kinase (*ytsB*)
SAR0671	Putative ABC transporter protein (*ytsC*)
SAR0672	Putative ABC transporter permease (*ytsD*)
SAR0673	Conserved hypothetical protein
SAR0674	Putative phosphate transport protein, similar to low-affinity inorganic phosphate transporter (*pitA*; partly deleted)

mutations in their *ctsR* and *hrcA* genes (Karatzas *et al.* 2007). The selection of *ctsR* was based on a similar phenotype previously observed in piezotolerant mutants of *L. monocytogenes* (Karatzas *et al.* 2003, 2005) while *hrcA* is another transcriptional regulator of stress genes that could have been involved in this phenotype.

During the above work additional isolates were obtained. In the present work, to identify the molecular basis of the piezotolerance, we used one of these additional isolates namely AK23. This was done due to initial problems faced when recovering the original isolates from the freezer which were subsequently resolved. As we decided to proceed with AK23, its phenotype had to be verified. We found that this isolate, similarly to all piezotolerant isolates obtained previously, showed the defective golden colour production (Fig. 1a), small colony phenotype (Fig. 1a,b), impaired growth (Fig. 2), delayed agglutination reaction (Fig. 3), increased piezotolerance (Fig. 4) and thermotolerance (Fig. 5) and reduced invasiveness in Caco-2 intestinal epithelial cells (Fig. 6).

Once we established that AK23 shared the same phenotype with the other piezotolerant isolates, we proceeded to identify the genetic basis of this phenotype. We decided to look at its transcriptome through microarray analysis. Prior to transcriptomics, we performed genomic indexing to assess the level of hybridization between each of the gene amplicons on the microarray slides with the genome of the WT and the AK23. During this process, we observed no hybridization with all genes encompassing the genomic region between SAR0666 and SAR0673 which were present in the WT. This suggested the presence of a major deletion in AK23 and we designed primers that could amplify the wider region where the deletion was located. Once we succeeded in recovering all isolates from our previous study, we performed the above PCR which was successful only with the piezotolerant isolates but unsuccessful with the WT and all other remaining isolates. Following sequencing of the PCR amplicons obtained from AK23 and the piezotolerant SCVs we showed that they possessed a major deletion of 9351 bps affecting 10 genes (Table 2). This explains why no amplicons were obtained from the WT and all other nonpiezotolerant isolates, as the replication of such a long amplicon would require different PCR conditions which were not used. We suggest that the deletion was the result of a homologous recombination event between two relatively small homologous regions of 10 bps (ATTGCGGGTG) which are present in both SAR0665 and SAR0674 genes (Fig. 7). In contrast to the *L. monocytogenes* mutants we identified previously (Karatzas *et al.* 2003, 2005), these *S. aureus* piezotolerant mutants could not be subjected to phase variation as the loss of this major genetic region cannot be recovered.

The transcriptomic analysis presented here revealed various differences between the AK23 and the WT. A significant percentage of genes upregulated in AK23 were related to phosphate uptake (Table 3). This is probably due to the partial deletion of *pitA* (Table 2). Overall, *S. aureus* encodes three distinct P_i transporters, PstSCAB, PitA and NptA (Kelliher *et al.* 2018). PitA is the low affinity P_i transporter which is constitutively expressed and its partial deletion in AK23 could result in lower levels of P_i in the cell. This in turn could lead to upregulation of the high affinity system for the uptake of inorganic phosphate (Pst operon) and a putative Na/P_i

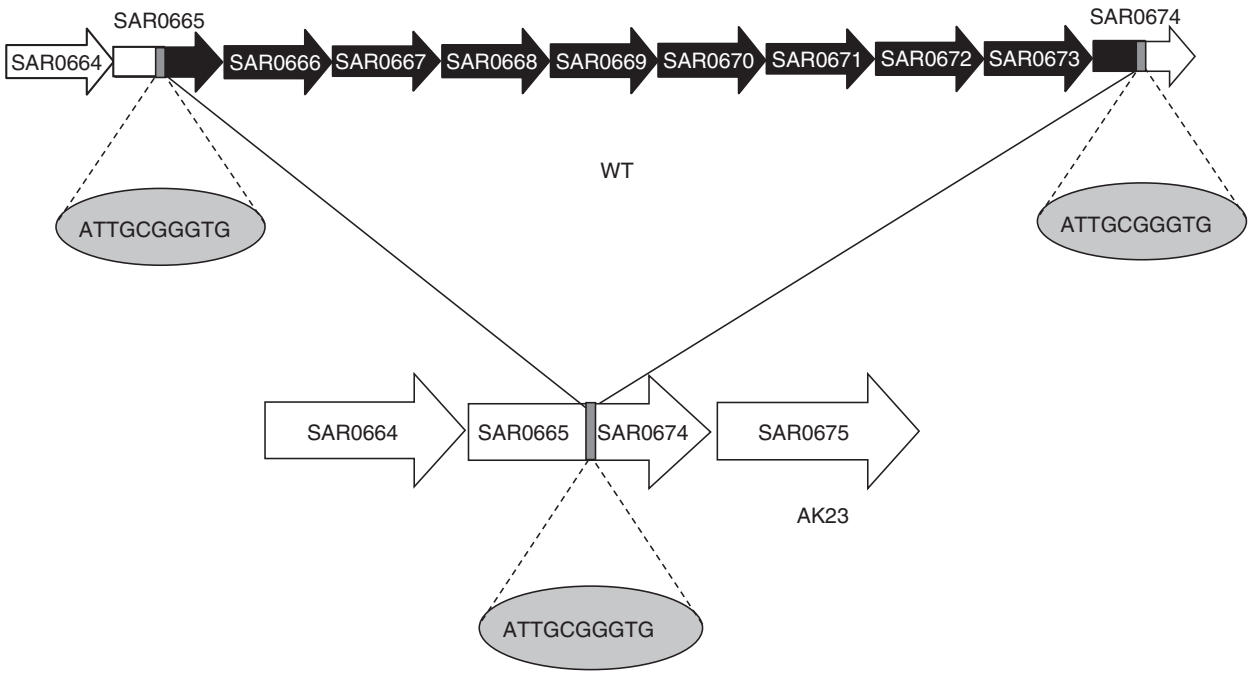

Figure 7 Schematic representation of the deletion in the mutant AK23. A whole region covering 9351 bps has been deleted affecting 10 genes. The mutation occurred through homologous recombination between two short homologous regions of 10 bps (ATTGCGGGTG)

Table 3 Genes upregulated in AK23 mutant compared to WT

Gene	Functional annotation	Percentage increase
PhoB	Alkaline phosphatase III pseudogene precursor	417
SAR0110	Putative Na/Pi cotransporter protein	571
SAR0584	Hypothetical protein	862
SAR1398	Putative phosphate transporter similar to negative regulator of phosphate (PhoU)	726
SAR1399	ABC transporter ATP-binding protein (PstB)	902
SAR1400	ABC transporter permease (PstA)	1082
SAR1401	ABC transporter permease (PstC)	1217
SAR1402	Phosphate binding lipoprotein (thioredoxine reductase; PstS)	1194

Genes SAR1398–SAR1402 belong to the *pst* operon.

Table 4 Genes downregulated in AK23 mutant compared to WT

Gene	Functional annotation	Percentage change
hld	Delta-hemolysin precursor (virulence factor)	14
spa	Immunoglobulin G binding protein A precursor (virulence factor)	10
rnaIII	rnaIII accessory gene regulator (agr) locus, elta lysin (virulence factor)	10
plc	1-phosphatidylinositol phosphodiesterase precursor (virulence factor)	30
nuc	Thermonuclease precursor	21
splB	Serine protease SplB	22
hysA2	Hyaluronate lyase precursor 2 (virulence factor)	44
SAR0694	Putative exported protein	36
SAR0304	Putative exported protein	22
nrdD	Anaerobic ribonucleoside-triphosphate reductase	21
narJ	Respiratory nitrate reductase delta chain	35
pyrAA	Putative carbamoyl-phosphate synthase, pyrimidine-specific, small chain	18
pyrAB	Putative carbamoyl-phosphate synthase, pyrimidine-specific, large chain	11
dltA	D-alanine-D-alanyl carrier protein ligase	17
mprF	Phosphatidylglycerol lysyltransferase	48

cotransporter protein (SAR0110). This partial *pitA* deletion might be responsible for the SCV and the slow growth phenotype in AK23, as this has been demonstrated previously in a *pitA S. aureus* deletion mutant (Kelliher *et al.* 2018). *phoU*, which was upregulated in AK23, is also another gene playing a role in phosphate metabolism as its negative regulator in *Escherichia coli* (Li and Zhang, 2007; Overton *et al.* 2011). However, the role of *phoU* in phosphate metabolism in *S. aureus* is not clear although it is widely accepted that it plays an important role which, however, is different from *E. coli* or *Bacillus subtilis* (Kelliher *et al.* 2018). Also the presence of *phoU* at the *pst* locus and the common upregulation might be

of significant importance for Pi metabolism. *phoU* might be also involved in the increased susceptibility of AK23 to antibiotics as its mutation has been shown to lead to increased susceptibility to antibiotics in *E. coli* (Li and Zhang, 2007). However, seeing the upregulation in AK23, it should have had the opposite effect (increased resistance). Nevertheless, as stated above, the function of PhoU is not well-understood in *S. aureus*.

Furthermore, the *pitA* mutation resulting in Pst operon upregulation, could possibly explain the piezotolerance of AK23. In the previous work on *B. subtilis*, it has been shown that *pstA* is upregulated during recovery following an HHP treatment, suggesting that it plays a role in this process (Nguyen *et al.* 2019). The authors have suggested that HHP treatment may cause phosphate limitation which can lead to upregulation of *pstA*. Therefore, the AK23 which has a *pstA* constitutively expressed, due to the loss of *pitA,* is able to recover much better than the WT after the HHP treatment. Furthermore, *phoU* which is upregulated in AK23 has been previously implicated in heat resistance in *E. coli* (Li and Zhang, 2007). The authors have shown that loss of *phoU* resulted in reduced thermotolerance. We could speculate that the increased expression of *phoU* could bring about the opposite effect, and increased thermotolerance which we observed in AK23. It is also important to note that in the present work, we did not find any alteration in the expression of toxin genes in AK23 compared to the WT. Based on that, we should assume that toxin, and since we look at piezotolerance to food HHP applications, enterotoxin production per cell is normal and similar to the WT. On the other hand, post-transcriptional effects or, altered toxin production in other stages of growth could not be excluded. Based on these findings, if AK23 has similar per cell toxin production to the WT, it is expected a lower toxin production through time, as a result of the lower growth rate of AK23.

There are also other possible reasons for the increased susceptibility of AK23 to antibiotics. The downregulation of *mprF* in AK23 observed here could be linked to the above phenotype. MprF is a multiple peptide resistance factor in *S. aureus* encoding a phosphatidylglycerol lysyltransferase that plays an important role in resistance and susceptibility to various antibiotics such as monomycin and vancomycin, methicillin, oxacillin, bacitracin, gentamicin and beta-lactams (Ruzin *et al.* 2003; Oku *et al.* 2004; Staubitz *et al.* 2004). In addition, it plays a role in resistance to synthetic peptides, human defensins (HNP1-3), evasion of oxygen-independent neutrophils and susceptibility to (hBD3, CAP18) and other cationic antimicrobial peptides (Ruzin *et al.* 2003; Oku *et al.* 2004; Staubitz *et al.* 2004). AK23 also showed a partial deletion of SAR0669 which is similar to the *ytS* gene of *B. subtilis* that could also be involved in the antibiotic sensitivity of AK23. As it has been shown previously, a Δ*ytS* mutant in *B. subtilis* was bacitracin sensitive (Bernard *et al.* 2003). Furthermore, *dltA* which is significantly downregulated in AK23 encodes a D-alanine-D-alanyl carrier protein ligase. This gene is involved in the metabolism of teichoic acids affecting membrane integrity which is also involved in antimicrobial susceptibility mainly to cationic antimicrobial peptides (Weidenmaier *et al.* 2005). All these features might be responsible for the increased susceptibility of AK23 to some of the antimicrobials.

Interestingly, the majority of genes downregulated in AK23 (*hld, spa, rnaIII, plc, nuc, splB, hysA2*, SAR0694 and SAR0304) play a role in virulence and are part of the *agr* system (Table 4; Reed *et al.* 2001; Huntzinger *et al.* 2005; White *et al.* 2014). The *agr* system has been previously shown to play a role in invasion to Caco-2 cells (Chessa *et al.* 2016) which explains the lower invasiveness of AK23 and the other isolates to Caco-2 cells. It is clear that the lower transcription levels of these *agr* system genes occur due to the downregulation of that accessory gene regulator *rnaIII* which is its effector gene (Gupta *et al.* 2015). However, we were as yet unable to identify the reason why *rnaIII* is downregulated in AK23.

Our work links a significant mutation in *S. aureus* with a range of phenotypic characteristics. However, since the mutation involves a significant number of genes, further work should focus on deleting each one of the genes individually to link them with specific phenotypic characteristics. Given that this mutation affects significant phenotypic characteristics of *S. aureus* involved in virulence, antibiotic resistance and food safety, such work could have major impact on various areas of science encompassing medical microbiology, antimicrobial chemotherapy and food safety.

Acknowledgements

We would like to thank all colleagues at the Microbiology research team, Food and Nutritional Sciences, University of Reading. The help and support of our deceased colleague Dr. Bernard Mackey has been invaluable. This work was partly supported by a Marie Curie European Reintegration Grant (grant ERG 265154) awarded to K.A.G. Karatzas, and funds from the University of Reading.

References

Andrews, J.M. (2005) BSAC standardized disc susceptibility testing method (version 4). *J Antimicrob Chemother* **56**, 60–76. https://doi.org/10.1093/jac/dki124.

Andrews, J.M. (2001) Determination of minimum inhibitory concentrations. *J Antimicrob Chemother* **48**(Suppl 1), 5–16. https://doi.org/10.1093/jac/48.suppl_1.5.

Bernard, R., Joseph, P., Guiseppi, A., Chippaux, M. and Denizot, F. (2003) YtsCD and YwoA, two independent systems that confer bacitracin resistance to *Bacillus subtilis*. *FEMS Microbiol Lett* **228**, 93–97. https://doi.org/10.1016/S0378-1097(03)00738-9.

Chessa, D., Ganau, G., Spiga, L., Bulla, A., Mazzarello, V., Campus, G.V. and Rubino, S. (2016) *Staphylococcus aureus* and *Staphylococcus epidermidis* virulence strains as causative agents of persistent infections in breast implants. *PLoS One* **11**, e0146668. https://doi.org/10.1371/journal.pone.0146668.

Elsinghorst, E.A. (1994) Measurement of invasion by gentamicin resistance. *Methods Enzym* **236**, 405–420.

Guidi, F., Duranti, A., Gallina, S., Nia, Y., Petruzzelli, A., Romano, A., Travaglini, V., Olivastri, A. *et al.* (2018) Characterization of a staphylococcal food poisoning outbreak in a workplace canteen during the post-earthquake reconstruction of Central Italy. *Toxins (Basel)* **10**, 523. https://doi.org/10.3390/toxins10120523.

Gupta, R.K., Luong, T.T. and Lee, C.Y. (2015) RNAIII of the *Staphylococcus aureus agr* system activates global regulator MgrA by stabilizing mRNA. *Proc Natl Acad Sci USA* **112**, 14036–14041. https://doi.org/10.1073/pnas.1509251112.

Huntzinger, E., Boisset, S., Saveanu, C., Benito, Y., Geissmann, T., Namane, A., Lina, G., Etienne, J. *et al.* (2005) *Staphylococcus aureus* RNAIII and the endoribonuclease III coordinately regulate spa gene expression. *EMBO J* **24**, 824–835. https://doi.org/10.1038/sj.emboj.7600572.

Karatzas, K.A.G., Valdramidis, V.P. and Wells-Bennik, M.H.J. (2005) Contingency locus in *ctsR* of *Listeria monocytogenes* Scott A: a strategy for occurrence of abundant piezotolerant isolates within clonal populations. *Appl Environ Microbiol* **71**, 8390–8396. https://doi.org/10.1128/aem.71.12.8390-8396.2005.

Karatzas, K.A.G., Wouters, J.A., Gahan, C.G.M., Hill, C., Abee, T. and Bennik, M.H.J. (2003) The CtsR regulator of *Listeria monocytogenes* contains a variant glycine repeat region that affects piezotolerance, stress resistance, motility and virulence. *Mol Microbiol* **49**, 1227–1238. https://doi.org/10.1046/j.1365-2958.2003.03636.x.

Karatzas, K.A.G., Zervos, A., Tassou, C.C., Mallidis, C.G. and Humphrey, T.J. (2007) Piezotolerant small-colony variants with increased thermotolerance, antibiotic susceptibility, and low invasiveness in a clonal *Staphylococcus aureus* population. *Appl Environ Microbiol* **73**, 1873–1881. https://doi.org/10.1128/aem.01801-06.

Kelliher, J.L., Radin, J.N., Grim, K.P., Párraga Solórzano, P.K., Degnan, P.H. and Kehl-Fie, T.E. (2018) Acquisition of the phosphate transporter NptA enhances pathogenesis by improving phosphate uptake in divergent environments. *Infect Immun* **86**, e00631–17. https://doi.org/10.1128/IAI.00631-17.

Li, Y. and Zhang, Y. (2007) PhoU is a persistence switch involved in persister formation and tolerance to multiple antibiotics and stresses in *Escherichia coli*. *Antimicrob Agents Chemother* **51**, 2092–2099. https://doi.org/10.1128/AAC.00052-07.

Nguyen, H.T.M., Akanuma, G., Tu, H.T.M., Nakai, Y., Kimura, K., Yamamoto, K. and Inaoka, T. (2019) Ribosome reconstruction during recovery from high hydrostatic pressure-induced injury in *Bacillus subtilis*. *Appl Environ Microbiol*, **86**, e01640-19. https://doi.org/10.1128/AEM.01640-19.

Oku, Y., Kurokawa, K., Ichihashi, N. and Sekimizu, K. (2004) Characterization of the *Staphylococcus aureus mprF* gene, involved in lysinylation of phosphatidylglycerol. *Microbiology* **150**, 45–51. https://doi.org/10.1099/mic.0.26706-0.

Overton, I.M., Graham, S., Gould, K.A., Hinds, J., Botting, C.H., Shirran, S., Barton, G.J. and Coote, P.J. (2011) Global network analysis of drug tolerance, mode of action and virulence in methicillin-resistant *S. aureus*. *BMC Syst Biol* **5**, 68. https://doi.org/10.1186/1752-0509-5-68.

Pospiech, A. and Neumann, B. (1995) A versatile quick-prep of genomic DNA from Gram-positive bacteria. *Trends Genet* **11**, 217–218. https://doi.org/10.1016/s0168-9525(00)89052-6.

Reed, S.B., Wesson, C.A., Liou, L.E., Trumble, W.R., Schlievert, P.M., Bohach, G.A. and Bayles, K.W. (2001) Molecular characterization of a novel *Staphylococcus aureus* serine protease operon. *Infect Immun* **69**, 1521–1527. https://doi.org/10.1128/IAI.69.3.1521-1527.2001.

Ruzin, A., Severin, A., Moghazeh, S.L., Etienne, J., Bradford, P.A., Projan, S.J. and Shlaes, D.M. (2003) Inactivation of *mprF* affects vancomycin susceptibility in *Staphylococcus aureus*. *Biochim Biophys Acta* **1621**, 117–121. https://doi.org/10.1016/s0304-4165(03)00028-x.

Staubitz, P., Neumann, H., Schneider, T., Wiedemann, I. and Peschel, A. (2004) MprF-mediated biosynthesis of lysylphosphatidylglycerol, an important determinant in staphylococcal defensin resistance. *FEMS Microbiol Lett* **231**, 67–71. https://doi.org/10.1016/S0378-1097(03)00921-2.

Tassou, C.C., Panagou, E.Z., Samaras, F.J., Galiatsatou, P. and Mallidis, C.G. (2008) Temperature-assisted high hydrostatic pressure inactivation of *Staphylococcus aureus* in a ham model system: evaluation in selective and nonselective medium. *J Appl Microbiol* **104**, 1764–1773. https://doi.org/10.1111/j.1365-2672.2007.03698.x.Ep

Tong, S.Y.C., Davis, J.S., Eichenberger, E., Holland, T.L. and Fowler, V.G. Jr (2015) *Staphylococcus aureus* infections: epidemiology, pathophysiology, clinical manifestations, and management. *Clin Microbiol Rev* **28**, 603–661. https://doi.org/10.1128/CMR.00134-14.

Weidenmaier, C., Peschel, A., Kempf, V.A.J., Lucindo, N., Yeaman, M.R. and Bayer, A.S. (2005) DltABCD- and MprF-mediated cell envelope modifications of *Staphylococcus aureus* confer resistance to platelet microbicidal proteins and contribute to virulence in a

rabbit endocarditis model. *Infect Immun* **73**, 8033–8038. https://doi.org/10.1128/IAI.73.12.8033-8038.2005.

White, M.J., Boyd, J.M., Horswill, A.R. and Nauseef, W.M. (2014) Phosphatidylinositol-specific phospholipase C contributes to survival of *Staphylococcus aureus* in human blood and neutrophils. *Infect Immun* **82**, 1559–1571. doi: 10.1128/IAI.01168-13.

Witney, A.A., Marsden, G.L., Holden, M.T.G., Stabler, R.A., Husain, S.E., Vass, J.K., Butcher, P.D., Hinds, J. *et al.* (2005) Design, validation, and application of a seven-strain *Staphylococcus aureus* PCR product microarray for comparative genomics. *Appl Environ Microbiol* **71**, 7504–7514. https://doi.org/10.1128/AEM.71.11.7504-7514.2005.

Zeaki, N., Johler, S., Skandamis, P.N. and Schelin, J. (2019) The role of regulatory mechanisms and environmental parameters in staphylococcal food poisoning and resulting challenges to risk assessment. *Front Microbiol* **10**, 1307. https://doi.org/10.3389/fmicb.2019.01307.