Acid-shock responses in *Staphylococcus aureus* investigated by global gene expression analysis

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A general overview is presented of the changes in the genetic expression along a time curve through the first 20 min after acidification to pH 4.5 of exponentially growing cultures of the food pathogenic strain *Staphylococcus aureus* 50583. A newly developed method for statistical significance testing was used to detect significant gene expression responses. Most responses showed an increase or decrease from time zero to 10 min after acidification, and then generally a stabilization in expression level from 10 to 20 min. Increased urease activity appeared to be an important factor in the acid defence, along with proton excretion by NADH dehydrogenase and macromolecule repair mechanisms. Oxidative-stress responses, such as increased expression of thioredoxin genes and upregulation of pentose phosphate pathway genes to generate more reducing power, were also induced. A general reduction in the expression of genes encoding ribosomal proteins and genes involved in nucleotide synthesis, as well as fatty acid and lipoprotein metabolism, reflected the lowered growth rate after acidification. The pH shock did not appear to trigger major virulence responses or biofilm formation. Metal ion regulation and transport were affected by the acid shock, and production of several cofactors such as molybdopterin was increased. Many of the presented observations could be explained, while some represent still-unknown mechanisms. The patterns of regulation were confirmed by quantitative reverse transcriptase PCR (QRT-PCR). Together, these results showed the main responses of *S. aureus* and will be a good starting point for future, more specific, in-depth studies of specific gene responses that occur in conjunction with the acid-stress defence of *S. aureus*.

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INTRODUCTION

Staphylococcus aureus is a major human pathogen that causes a wide spectrum of infections, ranging from superficial wound infections to life-threatening septicaemia and toxic-shock syndrome (Lowy, 1998). It is also a major food poisoning bacterium posing a great risk to consumer health, mainly through its production of heat-stable enterotoxins. *S. aureus* is considered to be a poor competitor in complex microbial populations. Therefore, the greatest risk of staphylococcal food poisoning is associated with food

contaminated with *S. aureus* after the normal microflora has been destroyed. Although *S. aureus* is found in many environments, the source of contamination is often human, since a large part of the healthy human population carry the bacteria as part of the normal microflora associated with the nose, throat and skin.

This work constitutes a part of our effort to study different stress responses of *S. aureus* in food. Acidification is one of the major ways to reduce growth of bacteria in food processing, and *S. aureus* is quite acid tolerant. In general, bacteria possess a multitude of defence mechanisms to cope with a sudden drop in pH (for a review for Gram-positive bacteria, see Cotter & Hill, 2003). The most direct approach is the use of proton pumps, which literally pump protons out of the cell to keep the internal pH at an acceptable level. Another approach is to increase the concentration of alkaline compounds within the cell to counteract the acidification of the cytoplasm. Repair mechanisms are needed due to an increased rate of damage to macromolecules. Biofilm formation can also affect the acid resistance of the cells. Energy metabolism and

Abbreviations: CAI, codon adaptation index; 6-FAM, 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein; FDR, false-discovery rate; PPP, pentose phosphate pathway; QRT-PCR, quantitative reverse transcriptase PCR; TAMRA, tetramethyl-6-carboxyrhodamine; TCA cycle, tricarboxylic acid cycle.

The array data discussed in this publication have been deposited in the NCBI Gene Expression Omnibus (GEO; <http://www.ncbi.nlm.nih.gov/geo/>) and are accessible through GEO series accession number GSE7273.

A complete table showing genes up- and down-regulated in *S. aureus* after acid shock is available with the online version of this paper.

metabolic pathways are altered by the shift of energy usage from mainly growth to defence mechanisms.

In contrast to the situation for *Escherichia coli*, much less is known about the general stress responses in *S. aureus*. Although some studies have shown the involvement of specific genes or genetic systems, few overall studies have elucidated the general mechanisms employed by *S. aureus* to cope with acidic environments. Most published work on how *S. aureus*, or similar bacteria such as *Streptococcus*, responds to low pH have chosen a steady-state approach, studying growth at low pH in parallel with growth under neutral-pH conditions (Len *et al.*, 2004; Weinrick *et al.*, 2004; Wilkins *et al.*, 2002). In this work, we have chosen to focus on the genetic expression of a food poisoning *S. aureus* strain along a time curve through the first 20 min after a sudden acid stress to pH 4.5 of exponentially growing cultures. By using a microarray approach in combination with a novel improved statistical analysis, we aspire to give a general overview of how this important pathogen responds to cope with this sudden change of environmental conditions.

METHODS

Strain and growth conditions. *S. aureus* 50583 used for this work was a gift from the Norwegian Veterinary Institute, and was isolated from a foodborne outbreak in which the source of infection was traced to a pizza. The strain identity was confirmed as *S. aureus* by 16S rDNA sequencing. The active toxin was classified as toxin B. Bacteria were stored at -80°C in TSB medium supplemented with 15% glycerol. Precultures of *S. aureus* 50583 were prepared by inoculation of frozen glycerol culture to 1 ml Tryptic Soy Broth (TSB; Oxoid) and incubation overnight at 200 r.p.m. and 37°C . The precultures were transferred to 99 ml TSB in 500 ml baffled shake flasks and incubated at 200 r.p.m. and 37°C . At an OD_{600} of 1.0, corresponding to 10^8 c.f.u. ml^{-1} , control samples were withdrawn immediately prior to the addition of 450–500 μl 6.5 M HCl, which directly brought the pH down to 4.5 ± 0.1 . Samples were withdrawn 2, 5, 10 and 20 min after addition of acid. All samples were directly transferred to RNeasy Protect Bacteria Reagent (Qiagen).

This procedure was repeated on three different days for a total of three sample sets (in parallel). Separate array experiments were performed on all three parallel sample sets.

RNA extraction. Total RNA was extracted by using the RNeasy Protect Bacteria Mini Prep kit (Qiagen). Briefly, 1.5 ml bacterial culture was added directly to 3 ml RNeasy Protect Bacteria Reagent, vortexed for 5 s, incubated at room temperature for 5 min and then centrifuged at 10 000 g for 10 min at room temperature. The supernatant was discarded and the pellet resuspended in 40 μl Tris/EDTA buffer followed by addition of 50 μl lysozyme stock solution (40 mg ml^{-1}) and 10 μl lysostaphin stock solution (1 mg ml^{-1}). The samples were incubated at 37°C with shaking for 10 min. The manufacturer's protocol, including use of kit buffers, was followed from this point. RNase-free water was added to the membrane of the RNeasy Mini Spin column (Qiagen), and after 1 min incubation at room temperature the RNA was eluted by centrifugation of the column at 10 000 g for 1 min. The concentration and purity of the total RNA were analysed with an Ultrospec 3000 spectrophotometer (Pharmacia Biotech) and the RNA 600 Nano LabChip system (Agilent Technologies).

Labelling of total RNA and microarray hybridization. *S. aureus* genome microarrays (version 1) were kindly provided by The Institute for Genomic Research (TIGR). The slides contained PCR products of 2576 ORFs from *S. aureus* COL. In addition the arrays contained 117 unique ORFs from strains Mu50 (60), MW2 (51) and N315 (6). Total RNA was reverse-transcribed by using random hexamers in the presence of aminoallyl-dUTP, followed by labelling with Cy3 and Cy5 monoreactive dyes (TIGR protocol, standard operating procedure no. M007; <http://pfgrc.tigr.org/protocols/M007.pdf>). Briefly, 2 μg total RNA and 6 μg random hexamers (Invitrogen) in a reaction volume of 18.5 μl were denatured at 70°C for 10 min, snap-cooled on ice for 30 s and centrifuged briefly to bring down condensation. Then 6 μl First Strand buffer (Invitrogen), 3 μl 0.1 M DTT, 1.2 μl 12.5 mM deoxynucleoside triphosphate-aminoallyl-dUTP labelling mix (Invitrogen) and 400 U Superscript II reverse transcriptase (Invitrogen) were added. The labelling reaction mixture was incubated at 42°C overnight. The first-strand synthesis reaction was stopped by adding 10 μl 0.5 M EDTA and 10 μl 1 M NaOH. The reaction mixture was incubated at 65°C for 15 min, and then 25 μl 1 M Tris/HCl (pH 7.0) was added. The removal of unincorporated aminoallyl-dUTP and free amines was performed according to the TIGR protocol with the Qiagen QIAquick PCR purification kit. After being dried in a Speed Vac (Savant), the samples were stored at -80°C . Coupling of aminoallyl-labelled cDNA to CyDye Ester (Amersham Biosciences) was done according to the TIGR protocol. The eluted Cy3- and Cy5-labelled samples were mixed and dried in a Speed Vac. The hybridization of the labelled cDNA probes was performed according to a protocol from TIGR (standard operating procedure no. M008; <http://pfgrc.tigr.org/protocols/M008.pdf>). Briefly, slides were incubated in a preheated prehybridization buffer [$5 \times \text{SSC}$ ($1 \times \text{SSC}$ is 0.15 M NaCl, 0.015 M sodium citrate), 0.1% SDS, 1% BSA], at 42°C for at least 1 h. After prehybridization, the slides were washed in distilled water (five times 2 min in 50 ml tubes) and then in 50 ml isopropanol. The slides were dried by centrifugation at 90 g for 12 min at room temperature. The dried Cy3- and Cy5-labelled cDNA was resuspended in 60 μl hybridization buffer (50% formamide, $5 \times \text{SSC}$, 0.1% SDS, 300 μg salmon sperm DNA), denatured at 95°C for 5 min, vortexed at low speed and heated again at 95°C for 5 min. The sample was centrifuged briefly and applied to the prehybridized microarray under a coverslip (LifterSlip, Erie Scientific). The slides were placed in a waterproof hybridization chamber for hybridization in a 42°C water bath overnight. After hybridization, the slides were washed in $2 \times \text{SSC}$ buffer with 0.1% SDS (55°C , 2 min), then washed in $0.1 \times \text{SSC}$ with 0.1% SDS buffer (room temperature, 2 min), followed by two washes in $0.1 \times \text{SSC}$ buffer (room temperature, 2 min). The slides were dried by centrifugation at 90 g at room temperature for 12 min.

Reverse transcription and quantitative reverse transcriptase PCR (QRT-PCR). Total RNA was reverse-transcribed by using random primers. The reaction mixture (20 μl), containing 300 ng total RNA, 100 ng random primers (Invitrogen) and 1 μl 10 mM dNTP mix in a reaction volume of 13 μl , was denatured at 65°C for 5 min, snap-cooled on ice and centrifuged briefly. Then 4 μl $5 \times$ First Strand buffer (Invitrogen) and 2 μl 0.1 M DTT were added and the reaction was incubated at 42°C for 2 min before 1 μl (200 U μl^{-1}) SuperScript II reverse transcriptase (Invitrogen) was added. The reaction was incubated at 25°C for 10 min and 42°C for 50 min. The reaction was centrifuged briefly and incubated at 70°C for 15 min before being centrifuged briefly again. cDNA was synthesized from two biological parallel samples grown on different days. Three reverse-transcriptase reactions were made for each biological replicate sample of RNA in addition to one negative control (without enzyme). After the reverse-transcriptase reaction, the cDNA was added in 30 μl H_2O , and 1.5 μl was used as template in the QRT-PCR analyses. Primers and probes (Table 1) were designed using Primer Express

Table 1. Primers and probes used in QRT-PCR expression studies

TIGR locus	Gene*	Name	Sequence (5'–3')†
SA2280	<i>ureA</i>	UreaF	TTGTAGTGGCGGCGGAAG
		UreaR	TGCCTCAGGATGATTTAGTTTCAA
		UreApr	TGCACGTCGTCGTAAAGCACGTGG
SA2282	<i>ureC</i>	UrecF	GTGGTACTGGTGCTTCAGAAGGT
		UrecR	CGGCAGCTTCTAACATTCTATGAAT
		UrecPr	AGCAACAACCTGTAACCTCCAGGTCCATGGC
SA2284	<i>ureF</i>	UreFF	ACACACTTAAGATTATTTTCAGTTCTGTGATT
		UreFR	TTCGTTGAATATATGTTTCAAGACCAA
		UreFpr	ACAGTTTCCAACAGGTGCTTTCAGTCATTCA
SA2654	<i>arcC2</i>	ArcC2 F	CGCCGGCAATGCCA
		ArcC2R	CAGTTTCCAACCAATAGCCTATCA
		ArcC2pr	TGGTACTTGTGGTGCAATGTCACAGGG
SA0494	<i>nuoF</i>	NuoFF	GATTGAATCTGTAACDGCACCA
		NuoFR	ACCACCTGCATTACAAATTCC
		NuoFpr	CGCCAGTATCGGCAATTATGCATGC
SA2097	<i>atpA</i>	AtpAF	GACGTATGGCTGGTGAGCTAGT
		AtpAR	CACACCCACGTTTGACTCTTCA
		AtpApr	AATTCCATAACGGCGTACTTGGTTTAGCCC
SA1549	<i>zwf</i>	Zwff	CAAACACATCCCWTGTTTAATCACA
		Zwfr	TGGAATATTGATGGAAACAACCTTACG
		Zwfpr	TCTTTGGTGCAACTGGTGACTTAAGCC
Sa2178	<i>Sa2178</i>	SA2178 F	GGTCTGCTTGATATTGGACAACC
		SA2178R	ACTGAACCTACAGCACCAGATGC
		SA2178pr	AAAGCCGGTGATACTGTTGTGGTTTCAGC
SA1368	<i>katA</i>	KatAF	AACCGAAATGTTTGCCCGT
		KatAR	ATGTCACGCTCCGCATCA
		KatApr	TCTCTACTGTAGCAGGAGAACGTGGTGCTG
SACOL_Sa16SA‡	<i>16S</i>	16SF2	CCAGCAGCCGCGGTAAT
		16SR2	CGCGCTTTACGCCCAATA
		16SFpr	CGTAGGTGGCAAGCGTTATCCGGA
SA0005‡§	<i>gyrB</i>	GyrBF2	AGTAACGGATAACGGACGTGGTA
		GyrBR2	CCAACACCATGTAAACCACCAGAT
		GyrBpr	CCGCCACCGCCGAATTTACCACCA

*Gene sequences from *S. aureus* COL accession no. NC002951.

†Primers were consensus primers for the known *S. aureus* sequences. All probes were labelled with 6-FAM at the 5' end and TAMRA at the 3' end.

‡Used as reference genes.

§Design from Beenken *et al.* (2004).

(version 1.0) ABI PRISM (Applied Biosystems) software. The probes contained 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (6-FAM) linked to the 5' end, and tetramethyl-6-carboxyrhodamine (TAMRA) at the 3' end. Amplification efficiency was analysed by performing PCR on dilution series of DNA. The amplification reaction mixture (25 µl) contained: 1.5 µl template cDNA (1000-fold diluted for the 16S PCR); 1 × TaqMan buffer A; 5 mM MgCl₂; 200 µM each of dATP, dCTP and dGTP, and 400 µM dUTP; 80 µM AmpErase uracil N-glycosylase; 0.2 µM forward and reverse primer; 0.1 µM MGB probe; and 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems). Before amplification, the reaction mixture was heated to 50 °C for 2 min and then denatured at 95 °C for 10 min. The amplification profile was as follows: 40 cycles of 95 °C for 15 s and then 60 °C for 1 min. Reactions were performed in the ABI PRISM 7900HT Sequence Detection system and data analysed using the SDS 2.2 software (Applied Biosystems). $\Delta\Delta C_T$ was calculated as the C_T value from the unstressed control cells minus the C_T value from the stressed cells and corrected for differences in signals from the 16S or *gyrB* controls.

Data acquisition and analysis. Slides were scanned with the ScanArray Express 1.0 scanner (Packard BioScience), following the manufacturer's guidelines. The intensities of the fluorescent spots were quantified with ImaGene 5 software (Biodiscovery). Background subtraction and normalization were performed in GeneSpring 6.1 (Silicon Genetics). All genes were spotted in triplicate on the slides to increase the accuracy of the measured intensities.

The data were evaluated statistically according to a recently developed approach (Langsrud, 2005) for analysing experiments designed with multiple responses and the calculations were made using the Matlab program at www.matforsk.no/ola/program.htm.

To detect significant differences, all four time points need to be modelled simultaneously. Our analysis was based on the second-order model:

$$\log(\text{ratio}) = A + B_i + C \cdot \log(\text{time}) + D \cdot (\log(\text{time}))^2$$

where B_i ($i=1, 2, 3$) represents a block effect of biological replicates. The time (min) is log-transformed to make the trends with time

closer to linear. The second-order term is included to take into account possible deviations from linearity. If there is no effect of acid shock, all parameters in the above model are zero.

The constant term, A , represents the mean difference between the control and the four time points. An ordinary significance test of A would, however, give misleading results. The reason is that we have a common (not replicated) control at the four time points and therefore the SD of A is underestimated. It can be shown that a conservative estimate can be obtained by scaling the SD by a factor of $\sqrt{5}$. We applied this property to perform significance testing of A in a conservative way. The significance tests of C (linear trend) and D (deviation from linearity) were conducted as usual.

However, with several multiple responses, ordinary significance testing leads to a large number of falsely significant results (5% of the total number of genes). Therefore, to draw reliable significance conclusions, the P value of each gene was adjusted using a newly developed rotation-testing procedure; see Appendix in Moen *et al.* (2005). This method combines general linear modelling with P value adjustments according to false-discovery rates (FDRs). When using adjusted P values according to the FDR, one accepts that 5% of the genes reported as significantly differentially expressed at the 5% level are false results (31 of 622 genes). Compared to other FDR variants, an advantage of the rotation-testing method is that any kind of dependence between the genes is allowed.

RESULTS AND DISCUSSION

DNA microarray measurements and statistics

Growth of *S. aureus* after addition of HCl to pH=4.5 is shown in Fig. 1. Control samples for microarray analyses were withdrawn immediately prior to the acid shock that lowered pH of the culture to 4.5, and this was defined as

Fig. 1. Growth of *S. aureus* after addition of HCl to pH=4.5. Cells were grown to $OD_{600}=1.0$ before addition of acid (indicated by the arrow). Samples were then withdrawn and plated on TSA agar. ■, Acidified cells; ▲, control without acid addition. The results are the means of four experiments; error bars represent SD.

time zero. The other samples for microarray analysis were withdrawn 2, 5, 10 and 20 min after the acid shock. In the microarray analyses, a total of 622 genes showed a statistically significant (at 5% FDR) change in expression level over the four time intervals measured. This result was due to significant mean differences and/or significant linear trends (log scale). No log ratios of gene expression showed significant deviation from linearity in the statistical model. Of the significant gene responses, 311 genes were upregulated and 311 genes down-regulated (Table 3). In general, most of the changes occurred within the first 10 min, and for most of the responses the expression levelled out between 10 and 20 min after the acid shock (Fig. 2). The acid shock reduced the growth rate of the culture and the genetic expression profiles showed this clearly through the down-regulation of the biosynthesis of many macromolecules (Table 3, and see below). Of the genes showing a statistically significant change in expression, 59.6% showed a maximum change of expression larger than twofold, 23.3% were larger than threefold, 10.5% were larger than fourfold and 5.6% were larger than fivefold. Relatively few genes showed an aberrant pattern, a very fast upregulation or a very fast down-regulation, and none of these is commonly reported to be involved in acid-stress responses. We cannot address all responses in this paper, but an outline of the major responses observed is given. It is important to bear in mind that one cannot expect to see full correlation between gene expression levels and the corresponding activity of the gene product. Likewise, genes showing the highest fold-change might not coincide with the most important genetic response. Generally, one might expect an accumulation of many of the proteins for which gene expression was unchanged in the cell after acid shock, since growth was significantly reduced. No experiments to

Fig. 2. Expression ratio (ER) profile for genes that showed statistically significant changes after acid-shocking. The scale is semi-logarithmic. $ER=1$ refers to the level of expression of the control, while $ER=2$ and $ER=0.5$ represent an increase or decrease, respectively, in expression by a factor of two.

determine the extent to which this occurred were carried out, however. The literature on stress mechanisms in *S. aureus* is relatively sparse. Our results (Table 3) closely resembled responses described in the literature on acid stress in *S. aureus* or other bacteria. We also observed a number of additional responses.

Array data are often reported to underestimate the fold-change. To test the validity of the microarray data, cells were grown to OD₆₀₀=1 before acid was added. After 10 min acid stress, cells were harvested. We then carried out QRT-PCR analysis on nine genes reacting to the acid stress with a range of fold-changes using 16S and *gyrB* expression as the reference genes (Table 2). The two reference genes gave similar results. The QRT-PCR analysis agreed well with the microarray data and provided independent verification of changes in the transcript levels of the genes that we discuss in this paper.

Direct acid-defence systems

Neutralizing components. The urease genes encoding urease subunits A, B and C (*ureA*, *ureB* and *ureC*), and *ureF* encoding the urease accessory protein UreF, were all strongly upregulated to a maximum ratio of 3.6–5.6 (Table 3, section F3). This result was similar to other findings where *S. aureus* was grown at pH 5.5 or the bacterium was exposed to 1 mM peracetic acid (Weinrick *et al.*, 2004; Chang *et al.*, 2006, respectively). Urease is a multimeric, nickel-containing enzyme that consists of six

UreA and six UreB subunits. Urease activity is known to be the major acid-resistance mechanism in *Helicobacter pylori*, which has adapted itself to live in the strongly acidic environment of the human stomach. In *H. pylori*, urease is expressed at very high levels, and the main role of the enzyme is thought to be neutralization of acidic micro-environments by production of NH₃ and CO₂. The NH₃ produced can act as a buffer leaving the bacterial cytoplasm and entering the bacterial periplasm, by the formation of NH₄⁺ (Valenzuela *et al.*, 2003). Urease is expressed by a wide variety of bacteria, although not at the high levels seen in *H. pylori*. In other bacteria, the urease activity is regulated in response to environmental changes such as changes in pH, urea and nitrogen availability, and growth phase (Burne & Chen, 2000; Chen *et al.*, 1998). In being a nickel metalloenzyme, urease requires active uptake of nickel ions from the environment to maintain its activity. The *nixA* gene, encoding a nickel transporter necessary for full urease activity (Bauerfeind *et al.*, 1996; Davis & Mobley, 2005; Mobley *et al.*, 1995; van Vliet *et al.*, 2001), was found to be upregulated in our study at a similar level of expression as the urease genes (maximum 5.8-fold after 10 min). Enhanced urease activity therefore seemed to be an important acid-shock mechanism for *S. aureus* 50583 to counteract the acidic environment.

The arginine deiminase (ADI) pathway generates alkaline compounds to help raise the cellular pH as a response to acid shock in many bacteria (Cotter & Hill, 2003). Of the three main enzymes in the ADI pathway, only *arcC2*

Table 2. QRT-PCR expression studies

TIGR locus	Gene	Gene function	Category*	ΔΔC _T † (16S)	Fold-change (16S) PCR, 10 min	ΔΔC _T ‡ (<i>gyrB</i>)	Fold-change (<i>gyrB</i>) PCR, 10 min	Fold-change§	
								Arrays, 10 min	Arrays, 20 min
SA2280	<i>ureA</i>	Urease, gamma subunit	Central intermediary metabolism	5.57 ± 0.97	47.34	4.90 ± 0.33	29.75	2.76	3.59
SA2282	<i>ureC</i>	Urease, alpha subunit	Central intermediary metabolism	4.67 ± 0.83	25.37	4.00 ± 1.46	15.94	3.89	5.60
SA2284	<i>ureF</i>	Urease accessory protein	Central intermediary metabolism	3.33 ± 2.34	10.02	2.66 ± 2.98	6.30	2.66	4.48
SA2654	<i>arcC2</i>	Carbamate kinase	Energy metabolism	−0.37 ± 0.13	0.77	−1.04 ± 0.76	0.49	0.64	0.61
SA0494	<i>nuoF</i>	NADH dehydrogenase, putative	Energy metabolism	2.66 ± 0.11	6.30	1.99 ± 0.74	3.96	4.76	3.81
SA2097	<i>atpA</i>	ATP synthase F ₁ , α subunit	ATP–proton motive force interconversion	−1.01 ± 0.37	0.50	−1.68 ± 1.01	0.31	0.51	0.44
SA1549	<i>zwf</i>	Glucose-6-phosphate 1-dehydrogenase	PPP	1.62 ± 0.00	3.07	0.95 ± 0.64	1.93	3.12	2.58
SA2178	SA2178	Alcohol dehydrogenase, zinc-containing	Fermentation	2.03 ± 0.49	4.07	1.36 ± 1.12	2.56	3.41	2.96
SA1368	<i>kata</i>	Catalase	Detoxification	1.86 ± 0.60	3.62	1.19 ± 0.04	2.27	4.53	3.17

*According to TIGR database.

†ΔΔC_T values are shown with SDs. 16S rRNA expression was used as reference.

‡ΔΔC_T values are shown with SDs. *gyrB* expression was used as reference.

§Data extracted from Table 3.

Table 3. Genes up- and down-regulated in *S. aureus* after acid-shocking

The table includes only genes commented on in the text; a more complete version is available as Supplementary Table S1. Statistically significant changes in expression are given as ratio of sample versus control (fold-change) for samples withdrawn 2, 5, 10 and 20 min after pH 4.5 acid shock. The data are grouped according to the categories given by the TIGR database (www.tigr.org). Major groups are numbered A1, A2, B1, etc. Highest and lowest ratios for each time curve are given in bold type.

Group	Locus	Gene	Common name	Ratio				
				2 min	5 min	10 min	20 min	
A, purines, pyrimidines, nucleosides and nucleotides								
A1, purine ribonucleotide biosynthesis down-regulated	SA0018	<i>purA</i>	Adenylosuccinate synthase	0.77	0.59	0.46	0.42	
	SA0544	<i>prsA</i>	Ribose-phosphate pyrophosphokinase	0.86	0.74	0.67	0.69	
	SA1073	<i>purE</i>	Phosphoribosylaminoimidazole carboxylase, catalytic subunit	0.56	0.27	0.23	0.24	
	SA1074	<i>purK</i>	Phosphoribosylaminoimidazole carboxylase, ATPase subunit	0.68	0.29	0.21	0.21	
	SA1075	<i>purC</i>	Phosphoribosylaminoimidazole-succinocarboxamide synthase	0.77	0.37	0.25	0.24	
	SA1076	<i>purS</i>	Conserved hypothetical protein TIGR00302	0.88	0.47	0.23	0.26	
	SA1077	<i>purQ</i>	Phosphoribosylformylglycinamide synthetase I	0.96	0.44	0.27	0.25	
	SA1078	<i>purL</i>	Phosphoribosylformylglycinamide synthase II	1.05	0.61	0.39	0.31	
	SA1079	<i>purF</i>	Amidophosphoribosyltransferase	1.39*	0.82	0.45	0.35*	
	SA1080	<i>purM</i>	Phosphoribosylformylglycinamide cyclo-ligase	1.45	1.03	0.67	0.40	
	SA1081	<i>purN</i>	Phosphoribosylglycinamide formyltransferase	1.28	0.92	0.37	0.33	
	SA1082	<i>purH</i>	Phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase	1.38	1.03	0.72	0.46	
	SA1083	<i>purD</i>	Phosphoribosylamine-glycine ligase	1.18	1.45	0.86	0.44	
	SA1969	<i>purB</i>	Adenylosuccinate lyase	0.65	0.54	0.41	0.42	
	A2, pyrimidine ribonucleotide biosynthesis down-regulated	SA1212	<i>pyrB</i>	Aspartate carbamoyltransferase	0.55	0.53	0.28	0.29
		SA1214	<i>carA</i>	Carbamoyl-phosphate synthase, small subunit	0.58	0.33	0.28	0.21
		SA1215	<i>carB</i>	Carbamoyl-phosphate synthase, large subunit	0.62	0.38	0.28	0.17
		SA1216	<i>pyrF</i>	Orotidine 5'-phosphate decarboxylase	0.55	0.36	0.20	0.15
		SA1217	<i>pyrE</i>	Orotate phosphoribosyltransferase	0.76	0.53	0.30	0.26
SA2119		<i>pyrG</i>	CTP synthase	0.58	0.52	0.39	0.31	
SA2606		<i>pyrD</i>	Dihydroorotate dehydrogenase	0.39	0.30	0.21	0.27	
A3, nucleotide and nucleoside interconversions down-regulated		SA0603		Deoxynucleoside kinase family protein	0.83	0.69	0.59	0.66
	SA0604		Deoxynucleoside kinase family protein	1.01	0.63	0.58	0.65	
	SA1277	<i>pyrH</i>	Uridylate kinase	0.85	0.71	0.60	0.61	
	SA1371	<i>guaC</i>	GMP reductase	0.95	0.70	0.47	0.32	
	SA1509		Nucleoside diphosphate kinase	0.93	0.70	0.71	0.83	
	SA2111	<i>tdk</i>	Thymidine kinase	0.62	0.47	0.35	0.44	
	SA2218	<i>adk</i>	Adenylate kinase	1.12	0.83	0.63	0.44	
	A6, DNA metabolism: DNA replication, recombination and repair upregulated	SA0970	<i>rexB</i>	Exonuclease RxB	1.28	1.86	2.24	1.70
SA0971		<i>rexA</i>	Exonuclease RxA	1.02	1.26	2.27	1.95	
SA1157		<i>uvrC</i>	Excinuclease ABC, C subunit	1.06	1.35	1.94	2.01	
SA1224		<i>priA</i>	Primosomal protein N'	1.16	1.49	1.97	1.46	
SA1492		<i>nth</i>	Endonuclease III	1.30	1.98	1.81	1.19	
SA1737		<i>polA</i>	DNA polymerase I	1.17	1.38	1.87	1.54	
SA1955		<i>dinP</i>	DNA-damage-inducible protein P	2.16	3.91	3.63	2.86	
Down-regulated		SA1381	<i>sbcD</i>	Exonuclease SbcD	0.95	0.86	0.72	0.64
B, transporters								
B2, transport and binding proteins: (anions) ² , cations and iron-carrying compounds upregulated	SA0687		Na ⁺ /H ⁺ antiporter	1.23	1.39	2.34	2.67	
	SA2572		Copper-translocating P-type ATPase	1.62	2.15	3.16	2.38	
	SA2721	<i>nixA</i>	High-affinity nickel transporter	1.72	3.43	5.82	5.14	

Table 3. cont.

Group	Locus	Gene	Common name	Ratio				
				2 min	5 min	10 min	20 min	
C, resistance, toxins, pathogenicity and detoxification								
C1, adaptation to atypical conditions upregulated	SA2131		Dps family protein	1.98	3.53	2.46	1.77	
C2, toxin production and resistance upregulated	SA2347		Drug resistance transporter, EmrB/QacA subfamily	6.12	10.35	15.45	12.09	
	SA2348		Drug transporter, putative	6.44	12.12	11.98	8.72	
	SA0754	norA	Multidrug resistance protein (NorA)	0.59	0.32	0.35	0.34	
	SA2437	bcr	Bicyclomycin resistance protein	0.64	0.45	0.46	0.56	
Down-regulated	SA2460		Drug transporter, putative	0.80	0.57	0.47	0.56	
C3, pathogenicity + (toxin production and resistance) ³ upregulated	SA0762		Haemolysin, putative	1.08	1.81	2.27	1.82	
	SA0856	clfA	Clumping factor A	0.83	0.82	0.96	2.22	
	SA0900		Pathogenicity island protein	1.22	1.38	1.69	1.37	
	SA0902		Pathogenicity island protein	1.22	2.22	2.20	1.68	
Down-regulated	SA1411		FemB protein (cell envelope)	0.96	1.11	1.15	1.25	
	SA2570	lacA ³	Galactoside O-acetyltransferase (cell envelope)	1.14	1.77	2.85	2.60	
	SA0478	³	Exotoxin 3	0.46	0.33	0.15	0.13	
	SA0536	ksgA ³	Dimethyladenosine transferase	0.86	0.79	0.67	0.68	
	SA0857		Staphylocoagulase precursor, putative	0.74	0.83	0.55	0.44	
	SA1173	hly ³	Alpha-haemolysin precursor	0.64	0.69	0.47	0.36	
	SA2004	³	Leukocidin precursor, F subunit, putative	0.99	0.73	0.77	0.57	
	SA2006	³	Leukotoxin LukM	0.90	0.76	0.56	0.48	
	SA2151	glmM ³	Phosphoglucosamine mutase GlmM (cell envelope)	0.76	0.63	0.51	0.67	
	SA2291		Staphyloxanthin biosynthesis protein	0.29	0.13	0.09	0.08	
	SA2352	tcaA ³	TcaA protein	0.66	0.66	0.59	0.72	
	SA2418		IgG-binding protein SBI	0.44	0.29	0.16	0.10	
	SA2511		Fibronectin-binding protein A (cell envelope)	0.95	0.63	0.52	0.55	
	SA2581		Staphyloxanthin biosynthesis protein	0.58	0.54	0.41	0.37	
	C4, detoxification upregulated	SA0451	ahpF	Alkyl hydroperoxide reductase, subunit F	1.26	1.97	2.09	1.56
		SA0452	ahpC	Alkyl hydroperoxide reductase, subunit C	1.46	2.31	1.95	1.35
SA1368		kataA	Catalase	1.32	2.41	4.53	3.17	
SA1610		sodA2	Superoxide dismutase	0.95	1.78	2.09	2.58	
D, energy metabolism								
D1, electron transport (anaerobic) ⁴ upregulated	SA0494	nuoF	NADH dehydrogenase, putative	3.17	3.45	4.76	3.81	
	SA0829	trxB	Thioredoxin reductase	0.96	1.73	1.83	2.01	
	SA1155	trxA	Thioredoxin	1.13	1.82	2.08	1.58	
	SA1794		Thioredoxin, putative	1.13	1.52	1.80	1.98	
D2, ATP–proton motive force interconversion down-regulated	SA2094	atpC	ATP synthase F ₁ , ε subunit	1.20	0.94	0.71	0.50	
	SA2095	atpD	ATP synthase F ₁ , β subunit	1.08	0.91	0.58	0.41	
	SA2096	atpG	ATP synthase F ₁ , γ subunit	1.15	1.03	0.74	0.53	
	SA2097	atpA	ATP synthase F ₁ , α subunit	1.06	0.78	0.51	0.44	
	SA2098	atpH	ATP synthase F ₁ , δ subunit	1.11	0.67	0.38	0.37	
	SA2099	atpF	ATP synthase F ₀ , b subunit	0.97	0.66	0.39	0.40	
	SA2100	atpE	ATP synthase F ₀ , c subunit	1.02	0.68	0.54	0.46	
D3, TCA cycle upregulated	SA2101	atpB	ATP synthase F ₀ , a subunit	1.02	0.68	0.55	0.49	
	SA1448	sucB	2-Oxoglutarate dehydrogenase, E2 component, dihydrolipoamide succinyltransferase, authentic frameshift	1.01	1.06	1.41	1.64	
	SA1449	sucA	2-Oxoglutarate dehydrogenase, E1 component	1.13	1.20	1.85	2.08	
	SA1741	icd	Isocitrate dehydrogenase, NADP-dependent	1.03	1.69	2.29	2.51	
	SA1742	gltA	Citrate synthase	1.21	1.53	2.07	2.28	
	SA2362	mgo1	Malate:quinone oxidoreductase	1.07	1.35	1.61	1.70	

Table 3. cont.

Group	Locus	Gene	Common name	Ratio			
				2 min	5 min	10 min	20 min
D4, glycolysis/gluconeogenesis upregulated	SA0842	<i>eno</i>	Enolase	1.13	1.14	1.42	1.66
	SA0966	<i>pgi</i>	Glucose-6-phosphate isomerase	0.75	1.26	1.25	1.67
	SA2415	<i>gpm</i>	Phosphoglycerate mutase	9.37	28.53	24.11	24.06
	SA2622	<i>fdaB</i>	Fructose-diphosphate aldolase, class I	1.20	2.83	4.09	7.10
D5, PPP upregulated	SA1377	<i>tkt</i>	Transketolase	1.31	1.31	1.79	2.11
	SA1549	<i>zwf</i>	Glucose-6-phosphate 1-dehydrogenase	1.50	2.34	3.12	2.58
	SA1554	<i>gnd</i>	6-Phosphogluconate dehydrogenase, decarboxylating	1.04	1.58	2.09	1.55
D7, fermentation upregulated	SA0154	<i>aldA1</i>	Aldehyde dehydrogenase	0.76	1.07	1.38	1.76
	SA1984	<i>aldA2</i>	Aldehyde dehydrogenase	0.84	0.84	1.33	1.56
	SA2198	<i>aldC/ budA1</i>	Alpha-acetolactate decarboxylase, putative	1.75	4.51	5.73	5.30
	SA2199	<i>budB</i>	Acetolactate synthase, catabolic	2.52	4.45	4.26	4.82
	SA2624	⁶	Acetyl-CoA synthetase, putative	1.68	2.95	4.06	4.22
D8, amino acids and amines + (other energy metabolism) ⁶ upregulated							
Down-regulated	SA2654	<i>arcC2</i>	Carbamate kinase	0.80	0.69	0.64	0.61
E, protein synthesis							
E3, protein synthesis (translation factors, tRNA and rRNA base modification, tRNA aminoacylation) upregulated	SA0576	<i>cysS</i>	Cysteinyl-tRNA synthetase	1.20	1.31	1.34	1.71
	SA1808	<i>leuS</i>	Leucyl-tRNA synthetase	1.27	1.74	2.08	2.23
Down-regulated	SA0562	<i>lysS</i>	Lysyl-tRNA synthetase	0.98	0.82	0.80	0.73
	SA0574	<i>gltX</i>	Glutamyl-tRNA synthetase	0.88	0.70	0.55	0.58
	SA0663	<i>argS</i>	Arginyl-tRNA synthetase	0.92	0.82	0.67	0.64
	SA1148	<i>pheS</i>	Phenylalanyl-tRNA synthetase, alpha subunit	1.01	0.75	0.72	0.82
	SA1778	<i>tyrS</i>	Tyrosyl-tRNA synthetase	0.80	0.73	0.57	0.73
E5, protein fate: degradation of proteins, peptides, and glycopeptides upregulated	SA0085		Peptidase, M20/M25/M40 family	3.61	4.58	4.01	2.36
	SA0570	<i>clpC</i>	ATP-dependent Clp protease, ATP-binding subunit ClpC, authentic frameshift	1.04	1.81	3.57	2.46
	SA0595		Peptidase, M20/M25/M40 family	1.23	2.07	2.69	2.41
	SA0979	<i>clpB</i>	ATP-dependent Clp protease, ATP-binding subunit ClpB	1.53	4.34	8.23	6.56
	SA1555		Peptidase, M20/M25/M40 family	1.60	3.10	4.36	3.77
	SA1588		Proline dipeptidase	0.90	1.50	1.62	1.61
	SA1756	<i>pepQ</i>	Proline dipeptidase	1.00	1.43	1.73	2.22
	SA1777		Serine protease HtrA	0.86	1.13	1.27	1.50
	SA1795	<i>pepA1</i>	Glutamyl aminopeptidase	1.19	1.53	2.03	1.93
	SA2007		Peptidase, M20/M25/M40 family, authentic frameshift	0.91	1.15	1.52	2.39
	SA2463	<i>pepA2</i>	Glutamyl aminopeptidase	1.29	2.49	3.35	3.28
	SA2563		ATP-dependent Clp protease, putative	0.95	0.80	1.28	3.33
F, metabolism							
F3, central intermediary metabolism; (amino sugars) ⁸ , (nitrogen) ⁹ , (sulfur) ¹⁰ , (one-carbon) ¹¹ , other upregulated	SA2280	<i>ureA</i> ⁹	Urease, gamma subunit	1.18	1.58	2.76	3.59
	SA2281	<i>ureB</i> ⁹	Urease, beta subunit	1.51	2.14	2.84	5.39
	SA2282	<i>ureC</i> ⁹	Urease, alpha subunit	1.51	2.47	3.89	5.60
	SA2284	<i>ureF</i> ⁹	Urease accessory protein UreF	1.17	1.87	2.66	4.48
G, regulatory functions							
G1, two-component system + protein interactions + (pathogenicity) ¹² , (DNA interactions) ¹³ , (toxin production and resistance) ¹⁴ upregulated	SA1740	<i>phoP</i> ¹³	Alkaline phosphatase synthesis transcriptional regulatory protein PhoP	1.32	1.69	1.88	1.85
	SA1943	<i>vraS</i> ¹⁴	Sensor histidine kinase	0.88	1.14	1.62	1.57
	SA2070	<i>kdpD</i>	Sensor histidine kinase	1.25	1.75	2.51	2.49

Table 3. cont.

Group	Locus	Gene	Common name	Ratio			
				2 min	5 min	10 min	20 min
Down-regulated	SA0765	<i>saeS</i> ¹²	Sensor histidine kinase SaeS	1.06	0.86	0.66	0.48
	SA0766	<i>saeR</i> ¹²	Two-component signal transduction protein SaeR	0.89	0.60	0.39	0.35
G2, protein interactions down-regulated	SA2057	<i>rsbU</i>	σ^B regulator protein	0.98	0.83	0.80	0.73
G3, RNA interactions down-regulated	SA1210	<i>pyrR</i>	Pyrimidine operon regulatory protein	0.41	0.47	0.20	0.21
G4, DNA interactions upregulated	SA0567	<i>ctsR</i>	Transcriptional regulator CtsR	1.55	3.14	3.42	2.31
	SA1541		Transcriptional regulator, Fur family	1.71	2.11	1.92	0.98
	SA2193		Transcriptional regulator, MerR family	1.62	1.72	2.38	1.96
	SA2517		Transcriptional regulator, MerR family	1.03	1.38	1.91	2.32
H, cell envelope							
H1, biosynthesis and degradation of surface polysaccharides and lipopolysaccharides down-regulated	SA2689	<i>icaA</i>	IcaA protein; intercellular adhesion protein A	0.83	0.38	0.21	0.15
	SA2690	<i>icaD</i>	IcaD protein; intercellular adhesion protein D	0.96	0.40	0.20	0.13
	SA2691	<i>icaB</i>	IcaB protein; intercellular adhesion protein B	0.86	0.34	0.17	0.12
	SA2692	<i>icaC</i>	IcaC protein; intercellular adhesion protein D; authentic frameshift	0.92	0.45	0.21	0.14
H3, other (cell envelope) down-regulated	SA2509	<i>fnbB</i>	Fibronectin-binding protein B	0.94	0.65	0.47	0.34

*When there was a clear upregulation at one time point and a clear down-regulation at an earlier or later time point, both values are given in bold type.

²⁻¹⁴Footnote numbers refer to subgroups of genes in groups for which the heading includes several types of gene. A full set of footnotes is given in Supplementary Table S1.

encoding carbamate kinase showed any significant change in our data, and this gene was down-regulated (minimum 0.61), suggesting that the ADI pathway is not a major acid-shock response in the *S. aureus* 50583 strain under the conditions given. A similar down-regulation of *arcC2* has been observed in peracetic acid-treated cells (Chang *et al.*, 2006).

Proton efflux. The gene *nuoF* encoding an NADH dehydrogenase was found to be upregulated (maximum ratio of 4.76 after 10 min). This dehydrogenase moves $2H^+$ across the membrane and out of the cell, converting one NADH to NAD^+ , and thereby helps to increase the internal pH of the cell. Somewhat surprisingly, we found that the F_0F_1 ATPase was down-regulated at pH 4.5. All genes encoding subunits for both F_1 (α , β , γ , δ and ϵ) and F_0 (a, b and c) were down-regulated to minimum ratios of the order of 0.37–0.53 (Table 3, section D2). In the literature, F_0F_1 ATPases are often mentioned as one of the primary mechanisms for extruding protons in many Gram-positive bacteria (Foster, 2004). Len *et al.* (2004) have shown increased levels of F_0F_1 ATPase at pH 5.0 in *Streptococcus mutans*. On the other hand, microarray analysis of *S. aureus* does not show any significant change in expression of the F_0F_1 ATPase genes when cells are grown at pH 5.5 or subjected to 1 mM peracetic acid (Weinrick *et al.*, 2004; Chang *et al.*, 2006). In addition, recent work by Maurer *et al.* (2005) has shown that in *E. coli*, the ATPase

genes are induced at high pH rather than at low pH. The genetic expression was clearly reduced to about 50 %, but the ATPase could still play an important role by pumping H^+ out of the cells, since regulation of ATPase activity apparently occurs at the assembly stage (Arikado *et al.*, 1999). The gene SACOL0687, encoding a Na^+/H^+ antiporter, was upregulated.

Reducing power. As the acid shock leads to increased consumption of reducing power in the form of NADPH and NADH, an urgent need to generate more reducing power arises in bacterial cells. For NADPH, this need can be met by increased flow through the pentose phosphate pathway (PPP). Several genes encoding enzymes in the PPP and in the overlap between the PPP and glycolysis were upregulated after the acid shock (*zwf*, *gnd*, *tkt*, *pgi*, *fdaB*). None of these genes has been reported to show changes in expression when cells are grown at pH 5.5 (Weinrick *et al.*, 2004). The gene *zwf*, encoding the glucose-6-phosphate dehydrogenase, is the first irreversible enzyme leading into the PPP. Together with *gnd* (6-phosphogluconate dehydrogenase) it controls the carbon flow into the PPP. Both these genes were found to be upregulated in our study (*zwf* 3.1-fold and *gnd* 2.1-fold after 10 min). Upregulation of *zwf* as a response to oxidative stress and recovery from heat stress has been demonstrated for Gram-negative bacteria (Cohen *et al.*, 1993; Greenberg *et al.*, 1990; Kobayashi *et al.*, 2005; Ma *et al.*, 1998).

Several genes encoding enzymes in the tricarboxylic acid (TCA) cycle were also upregulated after the acid shock (*sucA*, *sucB*, *icd*, *gltA*, *mgo1*). The *sucA* and *sucB* genes in the TCA cycle encode 2-oxoglutarate dehydrogenase. It has been proposed that this complex may provide succinyl-CoA for phosphorylation of GDP and ADP under conditions of restricted NAD⁺ availability, which could be essential upon accumulation of NADH and exhaustion of the pyridine nucleotide pool (Bunik, 2003).

Several genes involved in conversion of acetate to ethanol were upregulated after the acid shock. Among these were four zinc-containing alcohol dehydrogenases, two aldehyde dehydrogenases (AldA1, AldA2), and α -acetolactate decarboxylase (BudA1) and acetolactate synthase (BudB). Increased activity of these dehydrogenases would accelerate the regeneration of NAD⁺, and one might also speculate that this could raise the intracellular pH by the removal of acetate. Len *et al.* (2004) have reported a 4.6-fold higher level of ethanol produced by *Strep. mutans* grown at pH 5.0 compared with pH 7.0.

Among other energy-metabolism genes showing a significant increase in expression was an acetyl-CoA synthetase (SACOL2624), which could generate acetyl-CoA from acetate for use in the TCA cycle. The two genes *budA1* and *budB* (maximum upregulation 5.7- and 4.8-fold, respectively) are also part of the C₅-branched dibasic acid metabolism pathway and encode enzymes that produce acetoin. It is known that conversion of pyruvate to acetoin contributes to maintenance of pH homeostasis in *Lactobacillus plantarum* (Tsau *et al.*, 1992).

Regulators. Several regulatory genes play a role in the exponential-phase acid-tolerance response. Among these, *rsbU* was found to be down-regulated, while *phoP* and SACOL1541 (transcriptional regulator of the iron regulator Fur family) were found to be upregulated (Table 3, section G). Also, many other transcriptional regulators showed a significant change of expression in our data (Table 3, section G). The *rsbU* gene encodes the σ^B regulator RsbU, which is thought to be the major activator of the σ^B response to acid stress in *S. aureus* (Palma & Cheung, 2001). The down-regulation of this gene is in agreement with the lack of any significant increase for σ^B . σ^B partially controls another well-studied signal-transduction regulatory component, *sarA* (Deora *et al.*, 1997; Manna *et al.*, 1998; Miyazaki *et al.*, 1999), the expression of which remained unchanged after the acid shock. In *Salmonella enterica*, σ^S , PhoP and Fur are known to be induced by acid shock to pH 4.5, and *rpoS*, *phoP* and *fur* mutants are known to be acid sensitive (Foster, 2004). Besides *phoP*, two genes involved in different two-component systems were upregulated (*kdpD* and *vraS*), while two were down-regulated (*saeS* and *saeR*). The latter two genes are known to be important for virulence (Novick & Jiang, 2003), while KdpD is involved in sensing K⁺ levels (Epstein, 2003; Wood, 1999) and VraS is involved in the cell wall stimulon response (Kuroda *et al.*, 2003).

Indirect responses

Oxidative stress response. The ability to defend against oxidative stress is of vital importance to *S. aureus* when attacked by host phagocytes at the site of bacterial infection. Oxidative-defence mechanisms are also generally important for bacterial survival in the environment, and the enzymes involved may provide the bacteria with a defence against oxidative threats such as the disinfectants employed in the food industry and in hospitals. Our results showed upregulation of the superoxide dismutase (SOD) gene *sodA* and the catalase gene *katA*, thus connecting the acid response to the oxidative-stress response. The SodA protein can reduce the rate of neutrophil-mediated killing and destroys highly reactive O₂⁻ (Clements & Foster, 1999). Adaptation of *S. aureus* to acid leads to induction of *sodA*, and *sodA* mutants are known to be more sensitive to acid (Clements *et al.*, 1999). KatA is the only major catalase in *S. aureus* and accumulates extracellularly, converting H₂O₂ to water. When cells are grown at pH 5.5, no increase in *katA* and *sodA* expression has been reported (Weinrick *et al.*, 2004). Somewhat surprisingly, this is also the case when cells are subjected to 1 mM peracetic acid (Chang *et al.*, 2006).

Our results also showed an upregulation of the genes *ahpC* and *ahpF* that encode the alkyl hydroperoxide reductase subunits C and F, respectively. Alkyl hydroperoxide reductase (AhpC) protects cells against OONO⁻, which is also generated within neutrophils and macrophages. The gene *ahpC* has been shown to be induced by osmotic stress but not by oxidative stress in *S. aureus* (Armstrong-Buisseret *et al.*, 1995). Regulation of *katA* and *ahpC* via *perR* has been described previously (Horsburgh *et al.*, 2001).

Three thioredoxin genes were shown to be upregulated in our data (*trxA*, *trxB* and SACOL1794). Thiol-specific redox systems are vital for the protection of cells against toxic oxygen species. They also play a major role in maintaining the intracellular thiol-disulfide balance and in providing reducing power to the key reductive enzymes, such as ribonucleotide reductases. In many prokaryotes, thioredoxin and glutaredoxin execute these tasks. *S. aureus* lacks glutathione and must therefore employ thioredoxin and alternative thioredoxin-based systems, such as alkyl hydroperoxide reductases, to carry out these cellular processes (Hofmann *et al.*, 2002). Thioredoxins are small ubiquitous proteins participating in numerous dithiol-disulfide interchange reactions. One of the established functions of thioredoxins is to reduce disulfide bonds in target proteins. The reducing power for this is provided by thioredoxin reductase, which in turn is reduced by NADPH. Thioredoxin is also essential for growth (Uziel *et al.*, 2004). Linkage between acid and oxidative responses has also been suggested by others, for both *S. aureus* and *E. coli* (Clements & Foster, 1999; Clements *et al.*, 1999; Maurer *et al.*, 2005).

Metal transporters. In general, the expression of many genes that encode transporters was significantly changed after the acid shock, and that of ABC transporters in

particular. The gene SACOL2572, encoding a copper-translocating P-type ATPase, was upregulated. The copper-translocating P-type ATPase can pump excess Cu^{2+} ions out of the cell at the expense of ATP. Low pH can increase metal ion toxicity in bacteria, and an excess of metal ions causes oxidative damage. The sensing and regulation of metal ion levels are therefore essential for the cell in a stressed situation. The *merR* regulators are known to be involved in the regulation of heavy metal ion concentrations in many bacteria (for a review see Brown *et al.*, 2003), and two of these *merR* regulator genes (SACOL2193 and SACOL2517) were upregulated in the acid-shocked cells.

Genes encoding transporters of metal ions, such as nickel, iron and manganese, as well as genes encoding ABC transporter systems for the transport of molybdenum and zinc into cells, were upregulated after the acid shock (Table 3, sections B2 and B5). One might speculate that this is due to an increased need for these essential metal ions in enzyme systems (Kisker *et al.*, 1997). As stated above, several zinc-containing alcohol dehydrogenase genes and the *nixA* gene encoding a nickel transporter were found to be upregulated. Likewise, eight genes involved in molybdopterin cofactor synthesis were upregulated. In the Gram-positive bacterium *Streptomyces coelicolor*, others have linked molybdopterin biosynthesis to disulphide stress caused by the thioredoxin activity (see above) which follows from oxidative stress (Paget *et al.*, 2001).

Other transporters. Several genes encoding transporters of osmoprotectants were found to be upregulated. Down-regulated transporter genes included those encoding transporters for formate and lactate, spermidine/putrescine, sugars and amino acids. The reduction in expression of transporters for spermidine and putrescine is interesting, since these are alkaline compounds that might contribute to keeping a higher pH inside the cell.

DNA-repair systems. It is well known that low pH causes damage to DNA. One would therefore expect expression changes in genes involved in DNA replication and repair.

DNA-damage-inducible protein P (*dinP*) showed an early increase in expression. Also, several genes involved in DNA replication and repair, such as *polA*, encoding DNA polymerase I, and *rexA* and *rexB*, encoding the exonucleases RexA and RexB, respectively, were found to be upregulated. In Gram-positive bacteria, the exonuclease/helicase RexAB is a major component of the homologous recombination process and is essential for double-strand DNA break repair (Chedin & Kowalczykowski, 2002; Quiberoni *et al.*, 2001). On the other hand, other genes involved in DNA repair and replication were found to be down-regulated, e.g. DNA-repair protein RecN and exonuclease SbcD. The gene *priA*, encoding a helicase (Lasken & Kornberg, 1988), was found to be upregulated. Others have shown that inactivation of this gene induces the SOS response in *E. coli* (Nurse *et al.*, 1991).

Changes related to metabolism and growth. The lower growth rate of the bacterium after the acid shock was reflected in the down-regulation of a high number of genes involved in protein synthesis, as well as in fatty acid and phospholipid metabolism. A total of 42 ribosomal protein genes showed significant down-regulation after the pH drop (Table 3, section E2). Only two were upregulated (*rpmG* and *rpsA*). In addition, *frr*, encoding ribosome recycling factor, was down-regulated. The level of down-regulation was in the range 0.25–0.63 with an average of 0.46, with a general tendency for a gradual decrease from time zero to 20 min. We also found several other genes involved in protein synthesis to be down-regulated, including translation factors, tRNA and rRNA base modification genes, and genes linked to tRNA aminoacylation. Among these, five tRNA synthetase genes (*tyrS*, *pheS*, *argS*, *gltX* and *lysS*) were shown to be down-regulated, while cysteinyl- and leucyl-tRNA synthetases (CysS and LeuS) were upregulated.

Likewise, the lower growth rate resulted in down-regulation of seven and 14 genes, respectively, of the pyrimidine and purine ribonucleotide biosynthesis pathways (Table 3, sections A1–A2). The same was also the case for seven genes involved in nucleotide and nucleoside interconversions (Table 3, section A3). While most of the PPP was upregulated to generate reducing power, the gene *prsA* (ribose-phosphate pyrophosphokinase), catalysing the first step from D-ribose-5-phosphate from the PPP into the pyrimidine and purine synthesis pathways, was found to be down-regulated. Also the regulator gene *pyrR*, encoding pyrimidine operon regulatory protein, showed a major down-regulation in expression level after the acid shock.

In contrast to the major down-regulation of genes involved in protein and nucleotide synthesis, we found that a number of genes involved in the degradation of proteins, peptides and glycopeptides were upregulated at relatively high ratios (Table 3, section E5). Four genes involved in protein modification and repair were also upregulated. The upregulation of these types of genes could be a result of both an increased need for the degradation of proteins subjected to acid denaturation and inactivation, and a need for amino acids for the increased synthesis of proteins involved in the acid response. Three peptidases were also found to be down-regulated (M16 family and U32 family).

Our results indicated that both *clpB* and *clpC*, encoding subunits of an ATP-dependent Clp protease, are part of an acid-shock response in *S. aureus* 50583, as we saw a major upregulation with maximum expression 10 min after the acid shock (8.2-fold for *clpB* and 3.6-fold for *clpC*), followed by a slight decrease in expression from 10 to 20 min. This decrease is consistent with the increase in expression of the *ctsR* gene, encoding a negative heat-shock regulator known to control expression of *clpB* and *clpC* (Frees *et al.*, 2004). Those authors have shown that ClpC is required for the repressor activity of CtsR, and suggest that

ClpC is a stress sensor for *S. aureus* during environmental stress and infection (Frees *et al.*, 2004). The gene SACOL2563, encoding a putative ATP-dependent Clp protease, was also found to be upregulated after the acid shock. Others have recently shown that *S. aureus* ClpC is required for resistance to stresses such as oxidative and heat stress, aconitase activity, growth recovery and death (Chatterjee *et al.*, 2005; Frees *et al.*, 2004), as well as biofilm formation and thermotolerance, in which ClpB also plays an important role (Frees *et al.*, 2004). In *Listeria monocytogenes*, *clpB* has been linked to virulence and shown not to be involved in stress tolerance (Chastanet *et al.*, 2004). Upregulation of *clpB* in *S. aureus* exposed to acid has also been reported by Weinrick *et al.* (2004) at steady state after 6 h growth. The gene SACOL1777, encoding HtrA serine protease, was also found to be upregulated. In *E. coli*, the HtrA protease is known to be heat-shock induced, and its main role is to degrade misfolded proteins in the periplasm (Pallen & Wren, 1997).

Genes encoding enzymes in the major energy metabolic pathway, glycolysis, generally showed only minor changes in expression after the acid shock. The exception was the gene *fdxB*, encoding fructose-diphosphate aldolase, which showed an increase in expression of up to 7.1-fold after 20 min. The genes *pgi* (glucose-6-phosphate isomerase) and *eno* (enolase) showed a small but statistically significant increase (~1.6-fold). Both glucose-6-phosphate isomerase and fructose-diphosphate aldolase are enzymes that are active in the junction between PPP and glycolysis, and this could explain the upregulation of the genes encoding these enzymes.

S. aureus contains several genes that encode phosphoglycerate mutase. One of these genes, *gpm*, was highly upregulated (28.5-fold). Phosphoglycerate mutase is one of the enzymes in the glycolysis pathway. Given the high level of upregulation of this gene, a codon adaptation index (CAI) analysis was carried out (<http://www.evolvingcode.net/codon/CalculateCAIs.php>; University of Maryland, Baltimore County, MD) to check if *gpm* was a glycolytic enzyme. The CAI analysis showed that this enzyme is probably not part of the glycolysis pathway (results not shown). This was also supported by the fact that the *gpm* gene was not a member of the gap operon (*gapA1-pgk-tpiA-pgm-eno*). Further work would be needed to elucidate the significance of this upregulation.

The expression of nine genes linked to electron transport was significantly changed by the acid shock. Among these were the three thioredoxin genes (*trxAB* and SACOL1794) discussed above and the NADH dehydrogenase gene (*nuoF*), and also two upregulated NAD(P)H oxidoreductase genes (*frp* and SACOL0453).

Effects of acid shock on virulence and persistence factors. The acid shock did not seem to influence the induction of toxins, since most genes encoding toxins were down-regulated (Table 3, section C3). This is in accordance

with their expected initiation at post-exponential growth. The active toxin in *S. aureus* 50583 is toxin B. SarA has been shown to upregulate both toxic shock syndrome toxin 1 gene (*tst*) expression and staphylococcal enterotoxin B production (Chan & Foster, 1998). Neither the *sarA* gene nor the toxin B gene showed a significant change of expression level after the acid shock. The two-component system genes *saeR* and *saeS*, which are parts of the *sae* signalling locus involved in the regulation of many exoproteins (Novick & Jiang, 2003), were both down-regulated (*saeR* 0.35-fold and *saeS* 0.48-fold after 20 min). These results are consistent with those of Weinrick *et al.* (2004), which show that mild acid (3 h at pH 5.5) reduces expression of *sae* genes 2.1-fold. For other regulators, the results were different, e.g. in the case of the *rot* (repressor of toxins) locus, for which we did not observe a down-regulation of expression.

Besides the enterotoxins, a large number of other secreted proteins (haemolysins, proteases, lipases and coagulases) as well as cell-surface-associated proteins (protein A, and fibrinogen-, fibronectin- and collagen-binding proteins) are essential for *S. aureus* pathogenicity. With the exceptions of a putative haemolysin gene (SACOL0762) and two pathogenicity island genes (SACOL0900 and SACOL0902), most virulence genes were down-regulated or showed no statistically significant change in expression ratio.

Of the genes directly related to drug resistance and detoxification of the cell, the genes encoding a Dps family protein (SACOL2131), alkaline-shock protein 23 and three drug-transporter genes were all upregulated. The *asp23* gene, encoding the alkaline shock protein 23, showed a somewhat unusual response in that it was first down-regulated and then upregulated only after 20 min. The pattern of expression remains unclear, since *asp23* is reported to be under the control of σ^B (Giachino *et al.*, 2001), which itself showed no change in expression. The upregulation of the putative drug-resistance transporter *EmrB/QacA* (SACOL2347 and SACOL2348) was at an especially high level, with factors of 12.1 and 15.5, respectively (Table 3, section C2). The multidrug-resistance gene *norA*, the drug-transporter gene SACOL2460 and the bicyclomycin-resistance gene *bcr* were all down-regulated.

For the extracellular enzymes, our results showed down-regulation of staphylocoagulase precursor (SA0857), which is involved in clumping bacteria together as an antiphagocytic defence. Catalase, which protects the cells from being killed by polymorphonuclear leukocytes (PMNs), was (as mentioned above) clearly upregulated after acidification. Higher levels of catalase activity in virulent strains than in non-virulent strains of *S. aureus* have been found (Kanafani & Martin, 1985). Nucleases are important for virulence in that they permit the reuse of bacterial and host nucleic acids. Several nucleases were found to be upregulated (*uvrC*, *nth*, *rexA*, *rexB*), while some were down-regulated (*sbcD*, *recJ*, *nfo*).

The results indicated lack of adhesion and initiation of biofilm formation in the first 20 min following the acid

shock. None of the major biofilm regulators *sigB*, *agr* or *sarA* showed a statistically significant change in expression after the acid shock. The *ica* genes (*icaADBC*), known to be central to intercellular adhesion and biofilm formation, were all strongly down-regulated to a minimum ratio of 0.12–0.15. This was in accordance with the result obtained when cells were subjected to peracetic acid (Chang *et al.*, 2006), but in contrast to work showing upregulation of the *icaA* and *icaD* genes when cells are grown at pH 5.5 (Weinrick *et al.*, 2004). Two other adherence-associated genes, *fmbA* (SACOL2511) and *fmbB*, encoding fibronectin-binding proteins A and B, respectively, were down-regulated. The *clfA* gene, encoding clumping factor A, mediates adherence to polyethylene and polyvinylchloride (Vaudaux *et al.*, 1995), and showed a clear increase in expression from 10 to 20 min after the acid shock. The increase seen for clumping factor A could be an indication of adhesion after 20 min, but further work is necessary to confirm this. It should be added that *S. aureus* 50583 produces relatively little biofilm compared to other *S. aureus* strains at 37 °C (Rode *et al.*, 2007).

Genes of unknown function. Among the 622 genes that showed a statistically significant change of expression level, 29 (4.7 %) encode enzymes of unknown specificity, 38 (6.1 %) encode proteins of unknown function and 133 (21.4 %) encode hypothetical proteins. Of the genes encoding hypothetical proteins, 59 (9.5 % of 622) were up- or down-regulated more than twofold and were included in Table 3 (section J3). Further work will be required to elucidate their role in the acid response.

Conclusions

Most responses showed an increase or decrease from time zero to 10 min after acidification and then generally a stabilization in expression level from 10 to 20 min. Increased urease activity appears to be an important factor in the acid defence of *S. aureus* 50583, along with proton excretion by NADH dehydrogenase and macromolecule-repair mechanisms. Oxidative-stress responses, such as increased expression of thioredoxin genes and upregulation of PPP genes to generate more reducing power, were observed. A general reduction in the expression of genes encoding ribosomal proteins and genes involved in nucleotide synthesis, as well as fatty acid and lipoprotein metabolism, was typical, and reflected the lowered growth rate after acidification. Repair mechanisms and degradation of damaged proteins were induced. The acid shock did not initiate a virulence response. Many of the presented observations can be explained; some are unknown or cannot easily be interpreted. Nevertheless, together, these results show the main responses of *S. aureus* and will be a good starting point for future, more specific, in-depth studies of gene responses associated with the acid-stress defence of *S. aureus*.

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