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Staphylococcus aureus adaptation to aerobic low redox potential environments: implications for an intracellular lifestyle

Benjamin A. F. Christmas[1], Matthew D. Rolfe[1], Matthew Rose[1] and Jeffrey Green[1,*]

Author affiliations: [1]The Florey Institute, Department of Molecular Biology and Biotechnology, University of Sheffield, Sheffield S10 2TN, United Kingdom

***Correspondence:** Jeffrey Green, jeff.green@sheffield.ac.uk

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Abbreviations: CFUs, colony forming units; $\Delta\Psi$, membrane electrical potential; DTNB, 5, 5´ dithiobis(2-nitrobenzoate); DTT, dithiothreitol; E_h, redox potential; GSH, glutathione; OD_{600}, optical density at 600 nm; PDHC, pyruvate dehydrogenase; SCVs, small colony variants

Five supplementary figures are available with the online version of this article.

Abstract

Methicillin-resistant *Staphylococcus aureus* is a 'superbug', responsible for extensive death and morbidity. Chronic *S. aureus* infections are associated with the presence of intracellular bacteria and the host cytosol is an aerobic low redox potential (E_h) environment. How *S. aureus* adapts to aerobic low E_h environments is understudied. A low external E_h, imposed by the non-metabolizable reductant dithiothreitol, resulted in transcriptional reprogramming mediated by the redox-responsive transcription factors AgrA, Rex and SrrBA resulting in a shift towards fermentative metabolism. Accordingly, in the presence of the host cytoplasmic reductant glutathione *S. aureus* aerobic respiration was impaired, the intracellular NADH:NAD$^+$ ratio increased, lactate dehydrogenase was induced, resistance to the aminoglycoside antibiotic gentamicin was enhanced and greater numbers of small colony variants (SCVs) were detected. These observations suggest that entry of *S. aureus* into the aerobic low E_h environment of the host cytosol could result in adaptive responses that promote the formation of SCVs.

INTRODUCTION

Methicillin-resistant *Staphylococcus aureus*, such as *S. aureus* USA300 JE2, is a 'superbug' that is resistant to a range of clinical antibiotics and a major healthcare problem. It is an adaptable bacterium with a formidable armoury of virulence factors leading to a range of clinical syndromes, including bacteremia, toxic shock syndrome, meningitis and pneumonia [1]. Chronic, recurrent infections are often characterized by the presence of slow growing, antibiotic-resistant small colony variants (SCVs) that have been associated with invasion of, and survival within, non-professional phagocytes [2-10]. Stable (genotypic) SCVs are often characterized by mutations in genes required for menaquinone or haem biosynthesis and consequently exhibit impaired respiratory metabolism, resulting in phenotypes resembling those observed in hypoxic environments [11]. Secondary mutations that constitutively activate the redox-sensing two-component system SrrBA, which plays a major role in adaption to hypoxic environments, permits stable SCVs to retain drug-resistance whilst regaining the capacity for rapid growth [11]. In contrast, phenotypic SCVs are unstable and revert to normal growth rates with loss of antibiotic resistance, suggesting a reversible mechanism that avoids the permanent fitness costs of genotypic resistance [12]. Adoption of the SCV phenotype within the confines of a host cell and reversion to wild-type virulence traits within 24 h of liberation is thought to be a mainstream component of the process leading to chronic infections [9,10].

The redox potential (E_h) of host cell cytoplasm is low (-220 to -285 mV) due the presence of high (30:1 to 100:1) ratios of reduced glutathione (GSH) to oxidized gluthathione (GSSG) [13]. It is clear that *S. aureus* can survive and replicate in the host intracellular milieu [9,10,14]. Therefore, the work reported here was undertaken to begin to improve understanding of how *S. aureus* adapts to an aerobic low E_h environment, such as might be encountered in the host cytosol. It is suggested that low E_h prompts *S. aureus* transcriptional reprogramming mediated by redox-responsive transcription regulators, including Agr, Rex and SrrA, resulting in induction of fermentative metabolism, lower growth rates, resistance to aminoglycoside antibiotics and enhanced formation of SCVs. Hence adaptation to the low E_h of the host cytosol could contribute to establishing and maintaining persistent infections.

METHODS

General culture conditions

The bacterial strains used were: *S. aureus* USA300 JE2 and the *S. aureus* USA300 JE2 *rex* mutant from the Nebraska Tn library [15]; *S. aureus* RN4220 was the host for the *ldh1-gfp* plasmid pGS2625 (see below) [16]. Standard culture conditions and growth media were as described in Sambrook and Russell [17]. Appropriate antibiotics were added from sterile stock solutions. Overnight liquid cultures were prepared from single colonies transferred into 5 ml of liquid medium (Brain Heart Infusion broth, Tryptic Soy broth or Luria-Bertani broth) and incubated at 37^{o}C with shaking (250 rpm). To estimate bacterial growth, optical density was measured at 600 nm (OD_{600}) using an ATI Unicam spectrophotometer. To measure viable bacterial cells, suspensions were serially diluted 1:10 in phosphate-buffered saline in triplicate and then 10 µl of each dilution was spotted onto solid medium. The dried agar plates were incubated at 37^{o}C overnight and the number of colony forming units (CFUs) was counted.

Growth in liquid media was also monitored using a Sunrise™ microplate reader (TECAN). An overnight starter culture was diluted 1:50 into 50 ml fresh medium, as indicated for each experiment, and after 3 h at 37^{o}C with shaking (250 rpm) the culture was diluted to OD_{600} 0.0125 in 200 µl fresh medium (supplemented as indicated in the text) in the wells of a 96 well plate (Thermo Scientific). The plates were incubated at 37^{o}C in the microplate reader with shaking, where OD_{600} was recorded every 15 min by the Magellan 7.1 software (TECAN).

Continuous cultures

A single colony of *S. aureus* USA300 JE2 was used to inoculate 5 ml Brain Heart Infusion broth for incubation overnight at 37^{o}C with shaking (250 rpm). This culture was then injected into a 2 l Labfors chemostat vessel (Infors-HT, Switzerland) containing 1 l of chemically defined medium with 15 mM glucose as the sole source of carbon and 0.0001% (v/v) Antifoam Y-30 Emulsion (Sigma-Aldrich) [18]. The culture was grown under batch conditions for 5−20 h before being switched to continuous culture with a dilution rate of 0.2 h^{-1}. Positive dissolved oxygen concentrations were maintained by constant addition of filtered air (1 l min^{-1}) and agitation (400 rpm). Temperature (37^{o}C) and pH (7.0) were maintained throughout the experiments. Redox potential (E_h) was measured using an ORP FermProbe (Broadly James) according to the manufacturer's instructions. An E_h of 86 mV was set by using a pH 7.0 Quinhydrone-buffered solution and an E_h of 263 mV was set by using a pH 4.0 Quinhydrone-buffered solution.

The aerobic steady-state cultures were perturbed by addition of 9.7 mM (final concentration) dithiothreitol (DTT) directly to the culture and the feed supply. Samples, for transcript profiling, enzyme assays, optical density measurements (OD_{600}) and ^{1}H-NMR measurements of exo-metabolites, were taken at 0, 2, 5, 10, 15, 30, 60 and 1440 min following the addition of DTT.

Samples for RNA isolation were taken by collection directly into 2 volumes of RNAprotect Bacteria Reagent (QIAgen). These were immediately mixed (vortexed for 5 s) and then incubated at room temperature for 5 min before centrifugation (4°C) for 10 min at 3,380 x*g*. The supernatant was then poured off and the pellet suspended in the residual solution. The suspended sample was then transferred to a 1.5 ml micro-centrifuge tube and centrifuged for 10 min at 20,000 x*g* before removing the supernatant and storing the pellet at −80°C.

Samples (5 ml) for culture medium analysis were centrifuged (4°C) for 10 min at 3,380 x*g*. The supernatant was passed through a 0.22 µm syringe filter and two 1.5 ml samples were stored in micro-centrifuge tubes at −80°C.

Samples for enzyme assays were collected at 0, 60 and 1440 min following the addition of DTT. Two 50 ml culture samples were collected in two 50 ml tubes and centrifuged in a cooled centrifuge (4°C) for 10 min at 3,380 x*g*. The supernatants were poured off and the samples stored at −80°C.

Transcript profiling

A custom Agilent microarray was designed using eArray (www.earray.chem.agilent.com). It contained probes for *S. aureus* USA300_FPR3757 and *S. aureus* N8325 (Agilent Design ID: 066358). RNA labelling was achieved using 8 µg of purified RNA in 6.15 µl molecular biology grade water and 2.5 µg of random primers (Invitrogen). This mixture was incubated for 10 min at 72°C and then placed on ice for 10 min. Addition of 6.25 µl reverse transcription mix (5x First strand buffer, 3.0 µl; 0.1 M DTT, 1.5 µl; 50x dNTP mix, 0.3 µl (dNTP mix contained 25 mM dATP, dTTP, dGTP and 10 mM dCTP) and molecular biology grade water, 1.45 µl) followed by 1 µl 1 mM Cy5-dCTP (or Cy3-dCTP for the time 0 min reference samples) (GE Healthcare) and 0.75 µl SuperScript III reverse transcriptase (200 U μl^{-1}, Invitrogen). The samples were incubated for 5 min at 25°C then overnight at 50°C before addition of 7.5 µl 0.1 M NaOH and incubation for 10 min at 72°C, followed by addition of 7.5 µl of 0.1 M HCl. The samples were purified using PCR purification kit (Qiagen) and the concentration of cDNA and labelling efficiency was measured using a Nanodrop™ (ND-1000 Spectrophotometer, Peqlab Ltd). Hybridization and washing were carried out according to the manufacturer's instructions (Agilent Two-Color Microarray-Based Prokaryotic Analysis (Fairplay III Labeling) Protocol). Cy5-labelled cDNA (300 ng) from the test sample was hybridized in tandem with 300 ng of Cy3-labelled cDNA from the 0 min (initial steady-state) sample. The array slides were incubated at 65°C for 17 h in a Hybridisation Oven (Agilent). Slides were scanned in an Agilent Microarray Scanner and data were extracted from .tif files using Agilent Feature Extraction 11.5. Data were analyzed using Agilent GeneSpring 7.3.1. Signal intensities for each array were divided by the signal obtained from the control channel (Cy3-labelled time point 0 min) with a median shift being applied across all samples included in each comparison. A Student's t-test (2 tailed, equal variance) with Benjamini-Hochberg correction was applied to the data. The complete gene expression data are available in ArrayExpress accession number E-MTAB-6942.

The TFinfer software was used to infer changes in the activities of 11 *S. aureus* transcription factors (AirR, AgrA, CstR, CymR, MgrA, NreABC, Rex, Rot, SarA, SarZ and SrrA) [19]. TFInfer is a state space model representing a simplified version of transcription based on a linear approximation (in log space) to the dynamics of transcription and treats noise in a principled way such that the estimated TF activities are associated with confidence limits [19]. The model was implemented using a connectivity matrix linking the 11 *S. aureus* transcription factors listed above to genes present in their regulons as identified by transcript profiles of parent and mutant strains and the time-resolved gene expression data for all the genes present in the connectivity matrix.

Detection of small colony variants (SCVs)

A single colony of *S. aureus* USA300 JE2 or the isogenic *rex* mutant from the Nebraska Tn mutant library [15] (confirmed by PCR) was used to inoculate 5 ml of Tryptic Soy broth and then incubated overnight at 37°C with shaking (250 rpm). This culture was used to inoculate 50 ml fresh medium to OD_{600} 0.05. After 3 h incubation at 37°C with shaking (250 rpm) GSH (10 mM) was added and the culture was incubated for a further hour. Then the culture was serially diluted 1:10 in triplicate and 10 µl spotted onto plates (Tryptic Soy agar) with and without GSH (10 mM) and with and without gentamicin (2 µg ml^{-1}). After 48 h the colony forming units were counted and the SCVs were assigned based on appearance: small (~10th size of wild-type colonies), light-coloured (white or pale yellow as compared to orange wild-type colonies) and gentamicin-resistant.

Lactate dehydrogenase assay

Aliquots of cell-free extracts (~20 ng protein) were added to 35 mM Tris-HCl, pH 7.9 containing 1 mM $MnSO_4$ and 0.15 mM NADH and absorbance at 340 nm was monitored using a spectrophotometer at 37°C. After establishing a base rate, pyruvate (5 mM) was added and the oxidation of NADH was monitored at 340 nm. The specific activity of lactate dehydrogenase was calculated in µmol NADH oxidized mg^{-1} h^{-1} using a molar extinction coefficient for NADH of 6,220 M^{-1} cm^{-1}.

Construction and assay of a *ldh1-gfp* promoter fusion plasmid

The intergenic region between *ldh1* and the divergent *SAUSA300_0234* gene was amplified from genomic DNA using PCR primers containing *Kpn*I and *Hin*dIII restriction enzyme sites. The PCR product and the reporter plasmid, pCM20, which contains the *S. aureus nuc* promoter fused to superfolder *gfp*, were digested with *Kpn*I and *Hin*dIII to release the *nuc* promoter from pCM20 and prepare the *ldh1* promoter region for ligation upstream of *gfp* [20]. After gel purification (Qiagen Gel Extraction kit), the *ldh1* promoter and the pCM20 plasmid backbone were ligated. The ligation mixes were used to transform competent *E. coli* cells and the transformants were selected by plating on agar plates containing ampicillin (100 µg ml^{-1}). The authenticity of the construct (pGS2625) was confirmed by DNA sequencing before transferring the plasmid to *S. aureus* RN2440 by selecting for growth on erythromycin (4 µg ml^{-1}).

Pyruvate dehydrogenase assay

Pyruvate dehydrogenase complex activity was estimated by measuring the rate of depletion of CoASH in a discontinuous assay. Cell extracts of bacteria grown as Brain Heart Infusion broth batch cultures were prepared by two passages through a French pressure cell. A reaction mixture containing buffer (31 mM Tris-HCl, pH 8.5), thiamine pyrophosphate (0.33 mM), cell-free extract, CoASH (0.08 mM), NAD^+ (3 mM) and NADH (0-3 mM) was prepared. The reaction was initiated with the addition of pyruvate (11.5 mM) and stopped after 10 min using 10% SDS before adding 5, 5′ dithiobis(2-nitrobenzoate) (DTNB) (0.04%). The amount of CoASH remaining was calculated by measuring absorbance at 412 nm on an

ATI Unicam UV/VIS spectrophotometer using the molar extinction coefficient for 2-nitrothiobenzoate (13,700 M^{-1} cm^{-1}).

Respiration rate measurements

Oxygen uptake rates at 37°C of washed cell suspensions were measured using a Clark-type oxygen electrode (Rank Bros Ltd). Overnight cultures were diluted 1:50 in 50 ml fresh medium incubated for 3 h at 37°C with shaking (250 rpm) to produce log phase cultures. These bacteria were collected by centrifugation (4°C) for 10 min at 3,380 x*g*. The cell pellets were suspended in pre-cooled 0.02 M phosphate-buffered saline and centrifuged in a cooled centrifuge (4°C) for 10 min at 3,380 x*g* twice before the resulting washed cell pellets were suspended in pre-cooled 0.02 M phosphate-buffered saline to a final optical density (OD_{600}) of 50. Aliquots (50 µl) of the cell suspension were added to phosphate-buffered saline (1950 µl) and after the baseline rate of oxygen uptake was established, glucose (2.5 mM final concentration) was injected. After 1 min either a control of 50 µl 0.02 M phosphate-buffered saline was added or 50 µl KCN (final concentration 25 mM) or GSH (final concentration 10 mM). Respiration rates (nmol O_2 min^{-1} OD_{600}^{-1}) were then calculated.

Growth inhibition zone assay

An overnight culture was diluted 1:100 in 20 ml of molten (50°C) soft agar (0.7% agar) Tryptic Soy medium. This was then poured onto 20 ml solid Tryptic Soy medium and left to cool before a sterile 6 mm filter paper disc was placed on top. The disc was loaded with 20 µl of gentamicin (50 mg ml^{-1}) and the plates were incubated at 37°C overnight under aerobic or anaerobic (anaerobic jars which contained an Oxoid AnaeroGen 2.5 L sachet) conditions before measurement of the growth inhibition zone. In other experiments Tryptic Soy medium was replaced by chemically defined medium with and without nitrate (10 mM) [18].

Measurement of NADH:NAD$^+$ ratios

A single colony from solid medium was used to inoculate a 5 ml chemically defined medium supplemented with 10% Luria-Bertani broth overnight at 37°C with shaking (250 rpm). This was used to inoculate the same medium (50 ml), which was incubated (37°C, 250 rpm shaking) to OD_{600} ~1.0. Two samples (5 ml) were removed from the culture and GSH (10 mM) was added to the culture, which was incubated for a further hour before taking further 5 ml samples. All samples taken were immediately placed within pre-cooled (-80°C) 50 ml tubes. The cells were collected by centrifugation and washed in M9 salts before storing at -20°C. For NAD$^+$ extraction, pellets were suspended in 0.2 M HCl and sonicated for two 30 s bursts. The sample was then centrifuged, the pellet was discarded and the supernatant was neutralized with 0.2 M NaOH. The same method was employed for NADH extraction, except that 0.2 M NaOH was initially added to suspend the cell pellets before sonication and 0.2 M HCl was used to neutralize the supernatant after centrifugation. The cell extracts obtained were then used in the coupled assay of Bernofsky and Swan [21]. Reactions contained: 0.144 M glycylglycine, pH 7.0; 0.035% ethanol; 2.4 mM phenazine ethosulfate; 0.6 mM methylthiazolyldiphenyl-tetrazolium bromide; 500 μl cell-extract; and H_2O to 995 μl. Reaction mixtures were left to equilibrate in the dark for 20 min before being started with the addition of yeast alcohol dehydrogenase (5 μl; 9-18 units μl; Roche). The reactions were followed spectrophotometrically at 570 nm.

RESULTS AND DISCUSSION

An aerobic low E_h environment induces fermentative metabolism in cultures of *S. aureus* USA300 JE2

In the first phase of this work the non-metabolizable reducing agent dithiothreitol (DTT; E_h -330 mV at pH 7.0) was used to isolate the effects of low external E_h from other potentially confounding factors that might arise from using a metabolically active physiological reductant. Aerobic steady-state chemostat cultures of *S. aureus* USA300 JE2 (triplicate independent cultures; pH 7.0, dilution rate 0.2 h^{-1}, dissolved O_2 in the medium ≥10%) were established. After baseline sampling, the steady-states were perturbed by introducing DTT (9.7 mM) to mimic the low external E_h in the aerobic but reducing environment of the host cell cytoplasm (the maximum growth rate of *S. aureus* USA300 JE2 in batch cultures containing 9.7 mM DTT was greater than the growth rate set by the dilution rate of the chemostat; Fig. S1). The external E_h of the initial steady-state cultures was 60 ±14 mV, similar to previous reports (~50 mV) [22], but after addition of DTT rapidly decreased to a final value of ~-275 ±40 mV (Fig. 1a). The dissolved O_2 concentration in the culture increased rapidly after DTT addition, before returning to the pre-perturbation value at 30 min consistent with a transient decrease in respiration rate (Fig. 1b). After perturbation the concentration of excreted lactate increased ~6-fold from ~1 mM to 6.2 ±0.1 mM in the new low E_h steady-state, indicating that ~20% of glucose carbon supplied was converted to lactate under these new conditions (Fig. 1c). Accordingly, the lactate dehydrogenase activity of cell-free extracts increased ~6-fold after 60 min and ~14-fold 1440 min after exposure to DTT (Fig. 1d). These data suggest that when *S. aureus* enters a low E_h aerobic environment, aerobic respiration is transiently inhibited (manifest as a decrease in O_2 consumption) and aerobic respiratory metabolism and fermentation co-exist (respiro-fermentative growth).

An aerobic low E_h environment triggers *S. aureus* USA300 JE2 transcriptional reprogramming consistent with a shift to respiro-fermentative metabolism

The dynamics of *S. aureus* USA300 JE2 gene expression in response to perturbation of the external redox state were measured by time-resolved transcript profiling (Table 1). TFInfer is a state space model that reveals the changes in transcription factor activities that drive transcriptional reprogramming [19]. A connectivity matrix was assembled for 11 *S. aureus* transcription factors (AirR, AgrA, CstR, CymR, MgrA, NreABC, Rex, Rot, SarA, SarZ and SrrA) that have previously been associated with redox-sensing and/or persistence [23-32]. Inference of the activities of these 11 transcription factors predicted that Rex and SrrA exhibited the greatest changes (≥7-fold, ≥90% confidence; Fig. 1e). DNA-binding by Rex is impaired when the NADH:NAD$^+$ ratio is high, resulting in derepression of genes associated with anaerobic metabolism. A reduced quinone pool activates the SrrBA two-component system resulting in activation of genes involved in anaerobic metabolism, cytochrome and heam biosynthesis and down-regulation of *agr*-RNAIII. This is consistent with the strong induction of transcripts associated with anaerobic metabolism: *pflBA, ldh1, adh, narG*, and *ddh* (Table 1). Previous work has shown enhanced abundance of fermentative enzymes pyruvate formate lyase (PflB), alcohol dehydrogenase (Adh), D- and L-lactate dehydrogenases (Ddh, Ldh1, Ldh2; the latter being maximally up-regulated 9.1-fold in response to DTT and hence just below the 10-fold cut-off applied to genes listed in Table 1) [33]. Moreover, *S. aureus* Newman strain *ldh1* and *ldh1-ldh2* double mutants are significantly attenuated in a mouse model of infection [34]. Taken together these analyses suggest that redox-sensing and induction of fermentative metabolism are important factors for intracellular survival of *S. aureus*.

The TFInfer analysis also suggested that AgrA activity decreased under low E_h conditions, manifest as severe down-regulation of the *agr* locus and lower expression of *psmB1*, *psmB2* and *hld* (Fig. 1e; Table 1). Down-regulation of the Agr-activated *psmB1* and *psmB2*, coding for the phenol soluble modulins PSMB1 and PSMB2, is a major factor in *S.*

aureus persister formation, and the δ-toxin (*hld*, PSMγ) is implicated in phagosome escape [35,36].

The host cytoplasmic reductant glutathione increases the frequency of *S. aureus* USA300 JE2 SCVs and enhances resistance to gentamicin

The experiments using DTT as an extracellular reductant indicated that *S. aureus* USA300 JE2 responded to an aerobic low E_h environment by adopting respiro-fermentative metabolism. The host cytosol is an aerobic low E_h environment maintained by the reductant GSH. The experiments using DTT suggested that exposing *S. aureus* USA300 JE2 to GSH (10 mM) would result in impaired respiration and this was found to be the case (Fig. 2a). Impairment of respiration was found to be associated with higher NADH:NAD$^+$ ratios (Fig. S2). Higher NADH:NAD$^+$ ratios are known to inhibit pyruvate dehydrogenase (PDHC) activity in several bacterial species and measurement of *S. aureus* USA300 JE2 PDHC activity showed that it was also inhibited at elevated NADH:NAD$^+$ ratios (Fig. S3) [37]. Consistent with these observations and the enhanced expression of *ldh1* after perturbation of the chemostat cultures by DTT (Table 1), expression of Green fluorescent protein from a plasmid borne (pGS2625) *ldh1-gfp* fusion in *S. aureus* RN4220 was increased in the presence of GSH (Fig. 2b). This is interpreted as indicating sensing of increased NADH:NAD$^+$ ratio by the transcription factor Rex leading to derepression of *ldh1* and consequent reduction of pyruvate to lactate.

A *S. aureus* membrane electrical potential (ΔΨ) of -75 to -95 mV is required for uptake of the aminoglycoside antibiotic gentamicin [38]. Bacteria adopting respiro-fermentative metabolism are likely to have a lower ΔΨ compared to those utilizing aerobic respiration and therefore the resistance of *S. aureus* USA300 JE2 to gentamicin was tested in the presence of GSH. Addition of GSH to the culture medium allowed *S. aureus* USA300 JE2 to grow in the presence of gentamicin (Fig. 2c). The ionophore, nigericin, is capable of restoring $\Delta\Psi$ at the expense of the transmembrane proton gradient. Addition of nigericin to low E_h cultures of *S. aureus* USA300 JE2 partially restored sensitivity to gentamicin (Fig. 2d). The increased resistance to gentamicin in the presence of GSH is therefore likely caused by a decrease in $\Delta\Psi$. Accordingly, gentamicin tolerance correlated with the E_h of the electron acceptor being used to support growth, i.e. *S. aureus* USA300 JE2 growing by aerobic respiration was less tolerant to gentamicin than when growing by anaerobic (nitrate) respiration or by anaerobic fermentation and this aerobic sensitivity was much diminished in the presence of GSH (Fig. 2e and f).

As noted above, *S. aureus* SCVs are induced by the host intracellular environment in which the bacteria are exposed to the cytoplasmic reductant GSH. Plating *S. aureus* USA300 JE2 under aerobic conditions in the presence of GSH resulted in a significant increase in the number of SCVs, suggesting that the adaptations initiated by perception of the low E_h signal contribute establishing the SCV phenotype (Fig. 2g; closed bars). One plausible explanation for the increased frequency of *S. aureus* USA300 JE2 SCVs in the presence of GSH was that mutation rates were greater under reducing conditions. However, measurements indicated that there was no significant difference (2-tailed T-test $p= 0.39$) in the frequencies of rifampicin (100 $\mu g\ ml^{-1}$) resistant mutants in the absence ($1.3 \times 10^{-7} \pm 0.7 \times 10^{-7}$; n=3) or presence ($6.8 \times 10^{-8} \pm 3.8 \times 10^{-8}$; n=3) of GSH (10 mM).

The transcriptional profiling and associated prediction of transcription factor activities indicated major changes in the regulation of the Rex regulon under low E_h conditions (Table 1). Therefore, SCV formation by the *S. aureus* USA300 JE2 *rex* mutant, from the Nebraska Tn library, was measured in the presence and absence of GSH. The frequency of *rex* mutant SCVs from cultures pre-exposed to GSH (10 mM) was similar to that of unexposed cultures when plated on Tryptic Soy agar containing gentamicin (2 $\mu g\ ml^{-1}$) (Fig. 2f, open bars) and greater than that observed for the parent strain. When the *rex* mutant was spread on plates containing gentamicin (2 μg ml^{-1}) and GSH (10 mM) bacterial lawns were observed for both pre- and un-treated cultures, suggesting that in the presence of GSH the *rex* mutant exhibits increased resistance to gentamicin. Accordingly, the frequency of *rex* mutant SCVs in the presence of GSH (10 mM) and a higher concentration of gentamicin (10 μg ml^{-1}) was greater than that observed when the plates contained the standard concentration of gentamicin (2 μg ml^{-1}) and no GSH (Fig. 2f, open bars). It was concluded that derepression of anaerobic metabolism by deletion of *rex* enhances SCV formation and that the *rex* mutant exhibits enhanced tolerance to gentamicin in the presence of GSH compared to the parent strain under these conditions.

These data suggested that GSH increased the frequency of *S. aureus* USA300 JE2 SCVs without significantly increasing the frequency of general mutation. Therefore, phenotypic stability of *S. aureus* USA300 JE2 SCVs in the absence and presence of GSH (10 mM) was determined. Small colony variants were first generated by plating aliquots of overnight Tryptic Soy broth cultures on Tryptic Soy agar supplemented with gentamicin (2 μg ml^{-1}) and incubating at 37°C for 48 h [39]. Small colony variants were picked and sequentially patched onto Tryptic Soy agar supplemented with GSH (10 mM) and unamended Tryptic Soy agar. After incubation at 37°C for 3 days the patches were scored as unstable, mixed or stable as defined by Edwards [39] (Fig. S4). Examination of 210 SCVs indicated that 66 of these acquired a mixed phenotype when plated on Tryptic Soy agar but retained a stable SCV phenotype on Tryptic Soy agar containing GSH (Table 2). Thirteen SCVs with an unstable phenotype on Tryptic Soy agar exhibited either a stable or mixed phenotype in the presence of GSH (Table 2). Thus, as well as increasing the frequency of SCVs, GSH appears to support the retention of the SCV phenotype.

Concluding remarks

Investigation of *S. aureus* adaptation to aerobic low E_h environments revealed a remodelling of central metabolism linked to changes in the activities of the redox-responsive transcription factors AgrA, Rex and SrrA (Fig. 3). The data reported here suggest that aerobic low E_h environments, such as the host cytosol, switch off AgrA, probably via leakage of electrons from reduced flavins and quinones to O_2, creating reactive oxygen species that promote formation of an intramolecular disulfide bond in the DNA-binding domain of AgrA [40]. A more reduced electron transport chain would activate SrrA, which allows stable SCVs to retain drug-resistance whilst regaining the capacity for rapid growth [11]. The increased NADH:NAD$^+$ ratios found under aerobic low E_h conditions impair DNA-binding by Rex, causing derepression of genes associated with fermentative metabolism. Reducing environments, that mimic the redox potential of the host cytoplasm, activate the virulence program of the intracellular pathogen *Listeria monocytogenes* and it was suggested that this might be explained, at least in part, by alteration in NADH:NAD$^+$ balance leading to Rex-mediated transcriptional reprogramming [41]. The evidence presented here indicates that redox-sensing and de-repression of Rex-regulated genes signalled by increased NADH:NAD$^+$ ratios is a major component of the adaptive response of *S. aureus* to an aerobic low E_h environment (Fig. 3). Hence Rex-mediated transcriptional reprogramming might be a common feature promoting the survival of Gram-positive pathogens in the host cytosol. The increased frequency of SCVs in the presence of GSH implies that intracellular *S. aureus* residing in the aerobic low E_h environment of the host cytoplasm is one possible source of phenotypic SCVs associated with difficult to treat infections. It is anticipated that these observations will prompt further work to better understand the role of the low E_h of the host cytosol in establishing and maintaining persistent infections by facultative and obligate intracellular pathogens.

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Author contributions

J.G. conceived the study and supervised the work. B.A.F.C., M.D.R. and M.R. carried out the research; all authors contributed to analyzing the data and writing the manuscript.

Conflict of interest

The authors declare that there are no conflicts of interest.

Figure legends

Fig. 1. *Staphylococcus aureus* USA300 JE2 adapts to an aerobic low redox potential (E_h) environment imposed by dithiothreitol (DTT) by adopting respiro-fermentative metabolism. (a-e) Steady-state cultures (1 l) of *S. aureus* USA300 JE2 were maintained in chemically defined medium at 37°C and pH 7.0 with constant aeration at 1 l min^{-1} and a dilution rate of 0.2 l h^{-1} before perturbation (at time zero) with 9.7 mM DTT. (a) Changes in redox potential. (b) Changes in dissolved oxygen concentration. (c) Proton-NMR measurements of lactate concentration in culture supernatants. (d) Lactate dehydrogenase activity of cell-free extracts. All data shown in these panels are mean values ± standard deviation from three independent experiments. (e) Changes in transcription factor activities in response to lowering the redox potential of *S. aureus* cultures were predicted using the TFinfer model [19]. The input data were the fold changes in expression relative to the initial steady-state for all the genes present in a binary connectivity matrix that linked 11 transcription factors (see text) to directly- or indirectly-regulated genes. The output of the model is a time-resolved series of transcription factor activities that could account for the observed changes in gene expression. The activity of SrrA (circles) was predicted to increase after perturbation, whereas the activities of AgrA (squares) and Rex (diamonds) were predicted to decrease.

Fig. 2. The host cytoplasmic reductant glutathione (GSH) inhibits *S. aureus* USA300 JE2 aerobic respiration, enhances tolerance to gentamicin and increases the frequency of small colony variant (SCV) formation. (a) Respiratory activity of suspensions of *S. aureus* in phosphate-buffered saline (PBS) in a chamber fitted with Clark-type oxygen electrode was stimulated by the addition of glucose (25 mM; arrow g). Oxygen consumption was followed and then either potassium cyanide (KCN, 25 mM; light blue trace) or GSH (10 mM; red trace) were added and the inhibition of respiration relative to suspensions to which phosphate-buffered saline was added (black trace) was calculated. Treatment with GSH (10 mM) inhibited the rate of oxygen uptake by 32±8%. The data shown are representative of

three independent experiments. (b) Induction of *ldh1* in the presence of GSH. *Staphylococcus aureus* RN4220 was transformed with pGS2625, which carries the superfolder *gfp* gene under the control of the *S. aureus* USA300 JE2 *ldh1* promoter. Aerobic batch cultures of the reporter strain were grown in Tryptic Soy broth supplemented with 0.5% glucose for 3 h at 37°C before addition of GSH (50 mM); control cultures were left untreated. After overnight incubation, bacteria were washed and suspended to constant OD_{600} and GFP was detected by scanning fluorescence spectroscopy. Typical spectra (GFP emission at 510 nm; excitation wavelength 485 nm) from triplicate experiments of suspension buffer (blue trace), untreated cell suspensions (red trace) and GSH-treated cell suspensions (green trace) are shown. (c) Growth of *S. aureus* USA300 JE2 in Luria-Bertani broth (blue trace), Luria-Bertani broth supplemented with GSH (10 mM; grey trace), Luria-Bertani broth supplemented with gentamicin (32 μg ml^{-1}; brown trace) and Luria-Bertani broth supplemented with both (black trace). (d) Restoration of membrane electrical potential antagonizes GSH-conferred gentamicin tolerance. Batch cultures of *S. aureus* USA300 JE2 were grown in triplicate in chemically defined medium containing gentamicin (2 μg ml^{-1}) in shaking 96-well plates at 37°C. The medium was supplemented with GSH (10 mM; blue trace), nigericin (2 nM; orange trace), or both (black trace). The data shown are mean optical densities (OD_{595}) ± standard deviation for three cultures. (d and e) *Staphylococcus aureus* USA300 JE2 metabolic mode affects tolerance to gentamicin. Chemically defined medium agar plates with glucose (15 mM) as the carbon source with and without nitrate (10 mM) were prepared and soft agar of matching medium seeded with *S. aureus* were overlaid on the plates. Gentamicin (1 mg) was loaded on a central disc and the plates were incubated at 37°C for 24 h before measuring the zones of inhibition (arrows). The images shown are representative of three experiments. (e) Plates incubated under: aerobic conditions – aerobic respiratory growth (left); anaerobic conditions plus nitrate – anaerobic respiratory growth (middle); anaerobic conditions – fermentative growth (right). (f) Plates prepared and incubated as for (e) but supplemented with GSH (10 mM). (g) Increased frequency of SCVs in the presence of GSH and by deletion of the transcription factor, Rex. Six cultures of *S. aureus* USA300 JE2 or the corresponding *rex* mutant were grown in Tryptic Soy broth (TSB) for 3 h at 37°C. Glutathione (GSH; 10 mM) was then added to three cultures and all cultures were incubated for a further hour. Serial dilutions were plated on Tryptic Soy agar (TSA) containing gentamicin or gentamicin and GSH and the plates were incubated at 37°C for 48 h. The concentration of gentamicin in the plates was 2 μg ml^{-1} (G2) and the concentration of GSH was 10 mM, but the *rex* mutant (open bars) exhibited enhanced resistance to gentamicin when GSH was added to the plates and the concentration of gentamicin was raised to 10 μg ml^{-1} (G10). The chart shows the frequency of SCVs per 10^8 cells for cultures unexposed (Broth: TSB; Plate: TSB + G2) and pre-exposed to GSH (Broth: TSB + GSH; Plate: TSB + G2; or Broth: TSB + GSH; Plate: TSB + G2 or G10 + GSH). The error bars show the standard deviation from the mean.

Fig. 3. Working model to account for the adaptation of *S. aureus* USA300 JE2 to low redox potential environments. In the cytosol *S. aureus* encounters an aerobic low E_h environment imposed by the presence of the reductant glutathione (GSH). Under these aerobic reducing conditions bacterial aerobic respiration is impaired and electrons leak from the reduced respiratory chain creating reactive oxygen species (ROS) that inhbit the activity of AgrA (dashed lines) [40]. The potential DNA damage caused by ROS could be a source of genotypic SCVs (dashed lines). Impaired electron transport leads to accumulation of reduced electron carriers, including menoquinol (MQH_2) and NADH. Menaquinol (MQH_2) activates the SrrBA two-component system and elevated NADH:NAD$^+$ ratios inactivate Rex, permitting expression of lactate dehydrogenase (Ldh1) by activation (→) and derepression (⊥), respectively, as well as up-regulation of terminal oxidase (*qox* and *cyd*) operons to partially restore aerobic respiration (Fig. S5). Elevated NADH:NAD$^+$ ratios also inhibit pyruvate dehydrogenase complex (PDHC) activity, leading to reduction of pyruvate to lactate, with concomitant oxidation of NADH, through the action of the newly synthesized

Ldh1 to ensure that redox balanced growth is achieved. This new mixed metabolic mode is associated with a lower membrane electrical potential ($\Delta\Psi$), which impairs uptake of aminoglycoside antibiotics and hence enhances resistance to gentamicin. These transcriptional and physiological adaptations are consistent with the characteristics of phenotypic small colony variants (SCVs).

Table 1. Transcripts that were altered in abundance by ≥10-fold in response to perturbation of aerobic steady-state cultures of *S. aureus* USA300 JE2 by addition of dithiothreitol

Gene	**Fold-change in transcript abundance at indicated time relative to the initial aerobic steady state**							**Redox regulator**	**Function**
	2 min	**5 min**	**10 min**	**15 min**	**30 min**	**60 min**	**1440 min**		
Amino acids and derivatives									
SAUSA300_0008 (*hutH*)	12.75	13.09	8.33	5.71	4.49	5.22	3.09	AgrA, AirR	histidine ammonia-lyase
SAUSA300_0434 (*metB*)	0.13	0.12	0.11	0.13	0.16	0.12	0.09	CymR	cystathionine beta-lyase
SAUSA300_1227 (*thrC*)	13.45	9.07	3.46	1.63	0.68	1.37	2.57	Rot	threonine synthase
SAUSA300_1228 (*thrB*)	15.60	10.05	3.75	1.92	0.69	1.34	2.50	Rot	homoserine kinase
SAUSA300_1330 (*ilvA*)	1.19	1.31	1.81	1.67	2.57	5.02	28.84	AgrA, AirR	threonine ammonia-lyase
SAUSA300_1711 (*putA*)	9.23	7.79	5.69	6.04	7.37	7.84	13.37	SrrBA	proline dehydrogenase
SAUSA300_2277 (*hutI*)	21.33	21.40	9.49	5.11	3.81	8.22	5.86	AgrA	imidazolonepropionase
SAUSA300_2278 (*hutU*)	43.22	44.14	12.78	6.86	6.04	15.44	12.85	AgrA	uroconate hydratase
SAUSA300_2491 (*rocA*)	7.34	8.47	9.28	8.48	8.17	7.14	12.42	AgrA, AirR, MgrA, Rot	1-pyrroline-5-carboxylate dehydrogenase
Biosynthesis of cofactors, prosthetic groups, and carriers									
SAUSA300_0228 (*fadE*)	13.34	7.88	4.60	3.32	3.89	6.12	6.23	AirR, SarZ	acylCoA synthetase
Cell envelope									

SAUSA300_0315 (*nanA*)	9.93	12.74	9.75	10.42	10.70	12.55	32.68	MgrA	N-acetylneuraminate lyase
Anaerobic metabolism									
SAUSA300_0220 (*pflB*)	22.95	55.96	81.10	75.58	59.37	63.09	337.35	Rex, SarZ, SrrBA	pyruvate formate-lyase
SAUSA300_0221 (*pflA*)	11.44	31.64	54.22	52.71	37.10	37.80	189.68	Rex, SarZ, SrrBA	pyruvate formate-lyase 1-activating enzyme
SAUSA300_0235 (*ldh1*)	13.84	18.55	18.88	15.21	21.59	25.29	123.45	Rex, SrrBA	L-lactate dehydrogenase
SAUSA300_0536 (*hchA*)	0.23	0.18	0.10	0.09	0.08	0.08	0.08	AirR, CymR	D-lactate dehydratase/chaperone
SAUSA300_0594 (*adh*)	2.3	4.3	4.2	3.8	3.4	6.7	16.7	Rex SrrBA	alcohol dehydrogenase
SAUSA300_2343 (*narG*)	6.47	6.39	3.01	2.26	2.02	4.95	18.38	AirR, NreABC, Rex, Rot, SrrBA	nitrate reductase subunit
SAUSA300_2463 (*ddh*)	9.21	14.22	19.04	26.19	26.46	31.19	69.12	AgrA, AirR, MgrA, Rot	D-lactate dehydrogenase
Carbohydrates – central metabolism									
SAUSA300_1679 (*acsA*)	21.43	19.54	12.23	10.48	10.53	13.27	24.26	SarZ, SrrBA	acetylCoA synthase
SAUSA300_1331 (*ald*)	1.53	1.56	1.55	1.48	2.70	5.96	27.50	AgrA, AirR	alanine dehydrogenase
SAUSA300_1680 (*acuA*)	9.06	6.70	5.16	5.15	5.18	5.80	14.15	AirR	acetoin dehydrogenase
SAUSA300_1681	8.54	7.45	5.76	5.69	5.25	5.41	13.58	AirR	acetylCoA synthetase deacetylase
SAUSA300_1731 (*pckA*)	7.74	8.19	6.79	7.10	9.25	10.47	8.66	AgrA, AirR, SarZ	phosphoenolpyruvate carboxykinase
Regulatory functions									
SAUSA300_1989 (*agrB*)	0.23	0.19	0.16	0.14	0.12	0.11	0.08	AgrA, SarA	accessory gene regulator protein B
SAUSA300_1990	0.19	0.16	0.13	0.13	0.11	0.10	0.08	AgrA, SarA	accessory gene

(*agrD*)									regulator protein D
SAUSA300_1991 (*agrC*)	0.22	0.22	0.17	0.16	0.13	0.13	0.08	AgrA, SarA	accessory gene regulator protein C
SAUSA300_1992 (*agrA*)	0.21	0.26	0.21	0.17	0.15	0.16	0.08	AgrA, SarA	accessory gene regulator protein A
SAUSA300_2347 (*nirR*)	7.43	6.15	4.49	4.08	4.48	7.52	23.37	AirR, NreABC, Rex	nitrate reductase regulator
Transport and binding proteins									
SAUSA300_0174 (*ssuB*)	0.11	0.09	0.09	0.08	0.10	0.06	0.06	CymR, Rot, SarZ	ABC transporter, ATP-binding protein
SAUSA300_0333	10.50	10.65	7.04	5.71	2.87	3.03	4.96	AirR	PTS system, fructose subfamily, IIA component
SAUSA300_0448 (*treP*)	6.11	12.08	10.77	9.11	5.60	5.65	6.63	AgrA, CymR, SarZ	PTS system, trehalose-specific IIBC component
SAUSA300_0887 (*oppB*)	14.09	4.82	1.48	1.15	1.13	2.37	2.44		oligopeptide ABC transporter, permease subunit
SAUSA300_0888 (*oppC*)	14.64	4.87	1.62	1.22	1.11	2.14	2.46		oligopeptide ABC transporter, permease subunit
SAUSA300_0889 (*oppD*)	14.01	6.77	2.22	1.43	0.99	2.19	2.18		oligopeptide import ATP-binding protein
SAUSA300_0890 (*oppF*)	12.58	6.20	2.25	1.38	0.91	1.76	2.00		oligopeptide import ATP-binding protein
SAUSA300_0891 (*oppA*)	11.72	9.97	3.58	1.76	1.10	2.47	1.70		oligopeptide ABC transporter, periplasmic binding protein
SAUSA300_0914	12.24	5.26	1.84	1.39	1.23	1.96	1.97	MgrA	sodium:alanine symporter family protein
SAUSA300_1329	1.23	1.68	1.67	1.69	2.03	4.35	23.15	AgrA, AirR	amino acid permease
SAUSA300_2313	9.17	13.88	10.11	10.33	13.44	16.08	35.16	Rex	L-lactate permease

(*lctP2*)									
SAUSA300_2383	12.77	13.52	12.27	13.69	10.63	8.73	20.50		amino acid permease
SAUSA300_2449	8.36	11.03	6.57	4.60	4.82	5.10	5.39	AgrA	putative MFS transporter
Virulence factors									
SAUSA300_1067 (*psmB1*)	0.04	0.04	0.02	0.02	0.01	0.01	0.00	AgrA, AirR, CymR	phenol soluble modulin β1
SAUSA300_1068 (*psmB2*)	0.04	0.05	0.04	0.02	0.02	0.01	0.00	CymR	phenol soluble modulin β2
SAUSA300_1739	17.86	14.37	17.20	17.94	19.07	17.24	47.23	MgrA	putative immunoglobulin-blocking virulence protein
SAUSA300_1988 (*hld*)	0.09	0.08	0.05	0.03	0.02	0.01	0.00	AgrA, AirR, SarA	RNA-III/delta-hemolysin
Hypothetical proteins and others									
SAUSA300_0108	2.04	3.45	3.46	3.13	2.79	4.70	10.22	AgrA, AirR, MgrA	carbon-oxygen lyase (myosin cross-reactive antigen)
SAUSA300_0173	0.19	0.19	0.15	0.13	0.11	0.11	0.07	CymR, Rot, SarZ	
SAUSA300_0227 (*fadD*)	26.45	23.40	12.99	12.14	5.89	10.16	9.83	AirR, SarZ	cyclohexanecarboxyl-CoA dehydrogenase
SAUSA300_0228 (*fadE*)	13.34	7.88	4.60	3.32	3.89	6.12	6.23	AirR, SarZ	acylCoA synthetase
SAUSA300_0229 (*fadX*)	10.78	9.51	5.16	3.81	3.22	3.79	5.35	AirR, SarZ	putative acylCoA transferase
SAUSA300_0311	5.27	4.95	3.92	6.99	5.35	11.06	27.96		ribokinase
SAUSA300_0312 (*psuG*)	4.41	5.01	5.40	6.90	6.30	10.11	27.60		hypothetical protein
SAUSA300_0862 (*glpQ*)	8.27	10.39	10.75	9.97	9.26	9.98	35.01		glycerophosphoryl diester phosphodiesterase

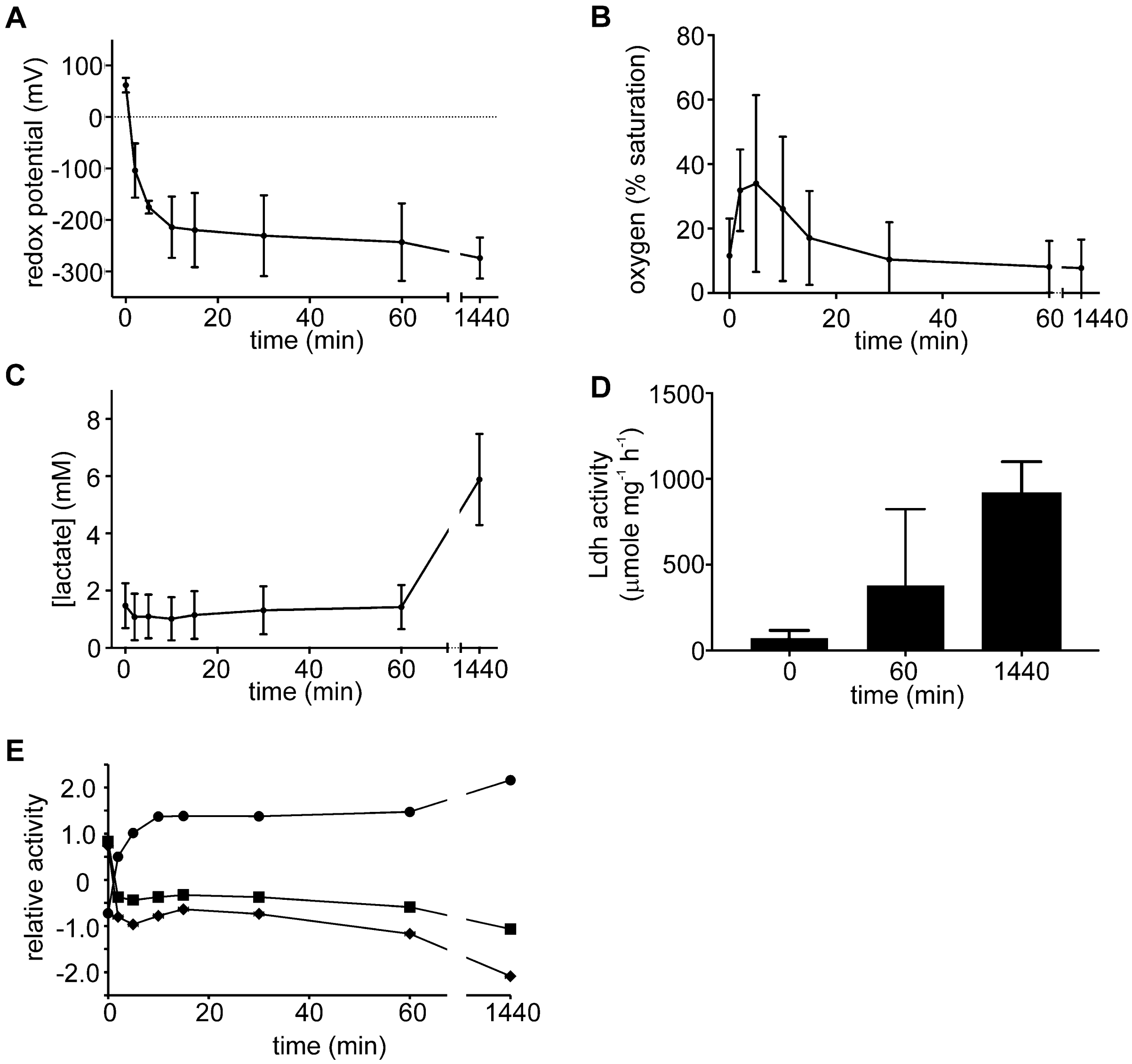
SAUSA300_1224	5.14	7.06	5.83	5.64	6.93	8.16	13.88	AirR	hypothetical protein
SAUSA300_2132	10.70	19.23	21.67	13.73	13.65	14.09	13.77		hypothetical protein
SAUSA300_2164	0.27	0.26	0.18	0.15	0.12	0.10	0.08	AgrA, AirR, CymR, SarA	hypothetical protein
SAUSA300_2423	18.18	19.72	25.19	26.99	30.41	33.81	143.61	Rot	hypothetical protein

[a] Redox responsive regulators are assigned on the basis of gene expression data [23-32].

Table 2. Glutathione increases retention of the SCV phenotype.

		Patches with the indicated phenotype[a]		
		Tryptic Soy agar		
		Unstable	**Mixed**	**Stable**
Tryptic Soy agar plus GSH	**Unstable**	20	1	0
	Mixed	6	35	7
	Stable	7	66	68

[a] Two hundred and ten *S. aureus* JE2 USA300 SCVs were picked from Tryptic Soy agar plates containing gentamicin (2 µg ml^{-1}). Each SCV was sequentially patched on to Tryptic Soy agar containing 10 mM GSH and Tryptic Soy agar alone. The plates were incubated for 3 days at 37°C and then the phenotypes were recorded: Unstable, reversion to normal phenotype; Mixed, patches were a mixture of normal and SCV phenotypes; Stable, SCV phenotype maintained. A representative pair of plates is shown in Fig. S4.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0
-100
-200
-300
0
20
40
60
1440
time (min)
B
oxygen (% saturation)
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60
40
20
0
0
20
40
60
1440
time (min)
C
[lactate] (mM)
8
6
4
2
0
0
20
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1440
time (min)
D
Ldh activity
(µmole mg-1 h-1)
1500
1000
500
0
0
60
1440
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relative activity
2.0
1.0
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-1.0
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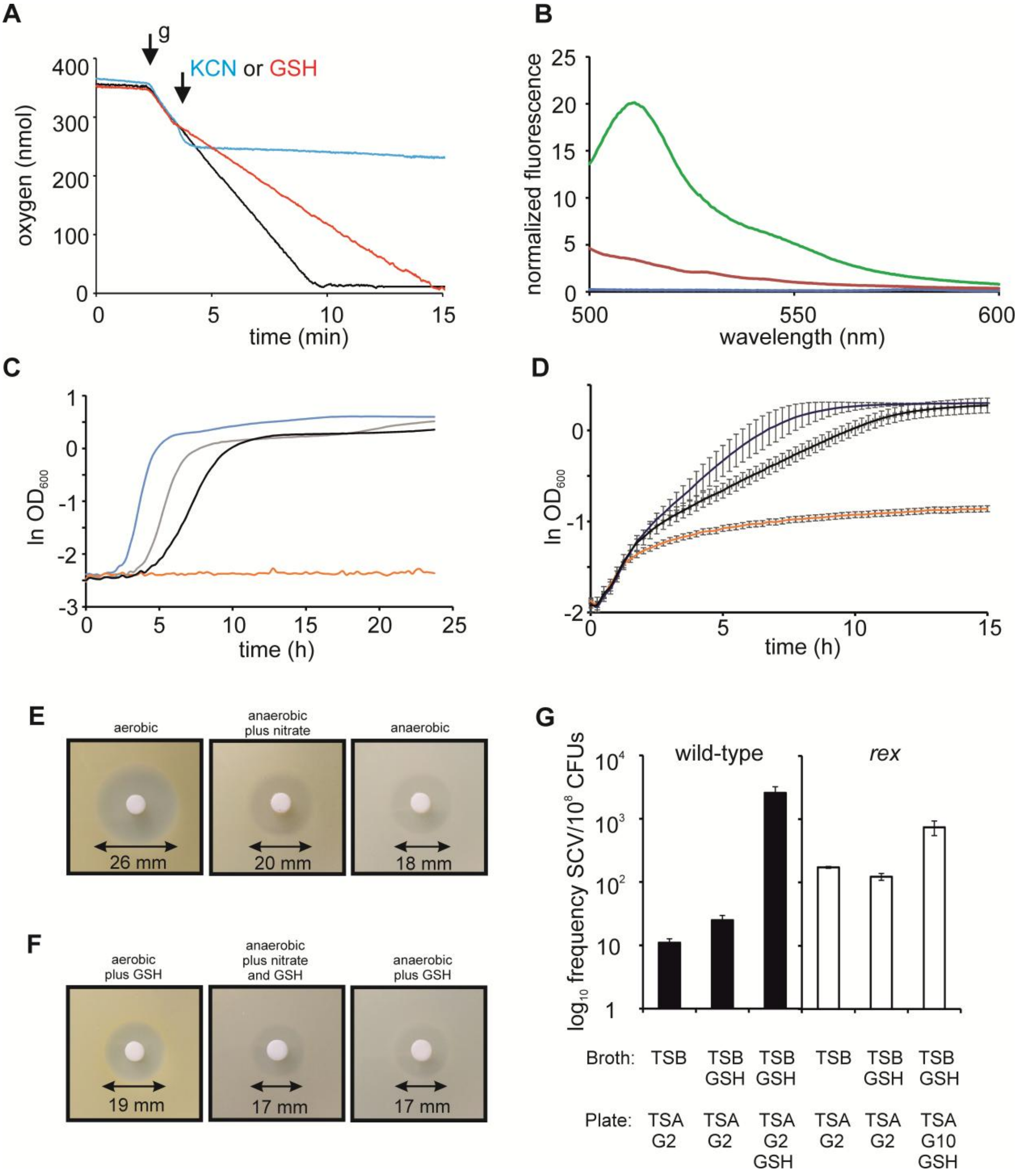
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KCN or GSH
oxygen (nmol)
400
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200
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time (min)
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normalized fluorescence
25
20
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wavelength (nm)
C
ln OD600
1
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time (h)
D
ln OD600
0
-1
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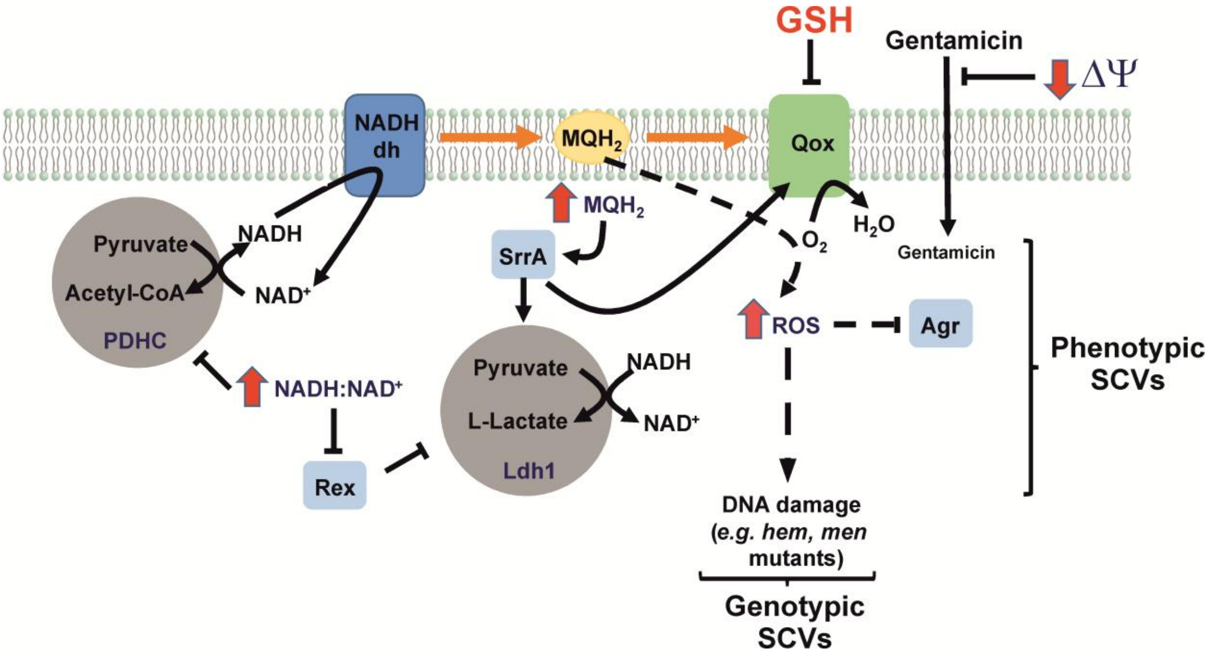
15
time (h)
E
aerobic
anaerobic plus nitrate
anaerobic
26 mm
20 mm
18 mm
F
aerobic plus GSH
anaerobic plus nitrate and GSH
anaerobic plus GSH
19 mm
17 mm
17 mm
G
log10 frequency SCV/10^8 CFUs
10^4
10^3
10^2
10
1
wild-type
rex
Broth:
TSB
TSB GSH
TSB GSH
TSB
TSB GSH
TSB GSH
Plate:
TSA G2
TSA G2
TSA G2 GSH
TSA G2
TSA G2
TSA G10 GSH


GSH
Gentamicin
ΔΨ
NADH dh
MQH2
Qox
MQH2
SrrA
O2
H2O
Gentamicin
Pyruvate
NADH
Acetyl-CoA
NAD+
PDHC
ROS
Agr
Phenotypic SCVs
NADH:NAD+
Pyruvate
NADH
L-Lactate
NAD+
Ldh1
Rex
DNA damage (e.g. hem, men mutants)
Genotypic SCVs