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Staphylococcus aureus Redirects Central Metabolism to Increase Iron Availability

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Staphylococcus aureus pathogenesis is significantly influenced by the iron status of the host. However, the regulatory impact of host iron sources on S. aureus gene expression remains unknown. In this study, we combine multivariable difference gel electrophoresis and mass spectrometry with multivariate statistical analyses to systematically cluster cellular protein response across distinct iron-exposure conditions. Quadruplicate samples were simultaneously analyzed for alterations in protein abundance and/or post-translational modification state in response to environmental (iron chelation, hemin treatment) or genetic (Δfur) alterations in bacterial iron exposure. We identified 120 proteins representing several coordinated biochemical pathways that are affected by changes in iron-exposure status. Highlighted in these experiments is the identification of the heme-regulated transport system (HrtAB), a novel transport system which plays a critical role in staphylococcal heme metabolism. Further, we show that regulated overproduction of acidic end-products brought on by iron starvation decreases local pH resulting in the release of iron from the host iron-sequestering protein transferrin. These findings reveal novel strategies used by S. aureus to acquire scarce nutrients in the hostile host environment and begin to define the iron and heme-dependent regulons of S. aureus.

Introduction

Staphylococcus aureus requires iron to successfully colonize the host [1]. To ensure efficient uptake and metabolism of host iron sources, bacterial pathogens regulate a variety of genes in response to the levels of available iron. The canonical bacterial repressor responsible for this iron-dependent regulation is the ferric uptake regulator (Fur) [2]. S. aureus has a functional Fur which has been implicated in the iron-dependent repression of a subset of genes [3–5]. An S. aureus Δfur mutant has a significant defect in virulence in a mouse model of infection, underscoring the importance of iron metabolism to staphylococcal pathogenicity [6]. The consensus sequence to which the S. aureus Fur binds has been predicted using in silico techniques [7]; however, a global analysis of Fur and iron-affected proteins in this important human pathogen has not been reported. The demonstrated role for iron and Fur in staphylococcal pathogenesis emphasizes the importance of identifying the iron-dependent Fur regulon of S. aureus.

Heme is the preferred iron source of S. aureus, and heme acquisition contributes to staphylococcal infection [8]. We have proposed a model for heme-Fe acquisition that involves the hemolysin-dependent lysis of host erythrocytes followed by hemoglobin recognition, heme removal, and transport into the bacterial cytoplasm [9]. Once inside the bacterium, heme can either be degraded by staphylococcal heme monooxygenases [10,11] or segregated to the bacterial membrane, where it is likely incorporated intact into bacterial heme-binding proteins [8]. It is possible that the different potential fates for intracellular heme are dependent on the level of iron and/or heme exposure experienced by the bacterium in distinct host environments. If correct, this model suggests that bacterial pathogens monitor the level of intracellular heme and alter protein expression in response to changes in heme status.

Based on the demonstrated role for iron, Fur, and heme in staphylococcal pathogenesis, we sought to evaluate changes in global protein status in response to alterations in bacterial iron status using two-dimensional (2D) difference gel electrophoresis (DIGE). DIGE enables quantitative differential-display analysis with statistical confidence and is based on 2D gel separations whereby thousands of protein features can be resolved based on isoelectric point and by apparent molecular mass. It uses spectrally resolvable fluorescent dyes (Cy2/3/5) to prelabel samples that are then multiplexed onto the same gel, allowing for direct quantification of each resolved protein feature between the three dye channels without analytical (gel-to-gel) variation. Multiple samples from a complex experiment can be analyzed across several DIGE gels, whereby an internal standard comprised of every sample present in the experiment is included in each multiplexed gel [12–15]. Within each gel, quantitative measurements are made for each resolved protein feature relative to the cognate signal from the internal standard, which is then used to normalize the intragel ratios between gels in a coordinated experiment. Thus, DIGE enables...
Synopsis

Virtually all bacterial pathogens require iron to successfully infect their human hosts. This presents a problem to invading bacteria because the majority of iron in humans is tightly bound by iron-binding proteins. To counteract this host defense, bacterial pathogens have developed elaborate mechanisms to acquire nutrient iron during infection. To gain insight into how the amount of available iron impacts the human pathogen *Staphylococcus aureus*, the authors identified proteins that increase or decrease abundance upon alterations in iron status. The authors found that under conditions of iron starvation, the Fur regulatory protein of *S. aureus* coordinates a redirection of the central metabolic pathways causing the bacteria to produce large amounts of acidic end-products. The accumulation of these acidic end-products facilitates the release of iron from host iron-binding proteins, in effect increasing the availability of this precious nutrient source. These findings provide a mechanistic explanation for how *S. aureus* alters its local microenvironment during infection to increase the availability of nutrient iron. Based on the well-established role for bacterial iron acquisition during pathogenesis, systems involved in iron acquisition represent excellent potential therapeutic targets against bacterial infection.

Results

Changes in Iron Status Alter Staphylococcal Protein Expression Patterns

To identify proteins that are affected by alterations in host iron sources, we performed differential expression analyses on *S. aureus* cultures grown under various conditions of iron exposure. Cytoplasmic proteins were prepared from wild-type and Δfur mutant cells grown under either iron-replete conditions, after iron starvation elucidated by treatment with 2,2’-dipyridyl (DIP), or after exposure to hemin. Protein extracts from each of the four conditions were independently isolated in quadruplicate to control for nonbiological variation, and the resulting 16 extracts were simultaneously coresolved across eight DIGE gels that were coordinated by a Cy2-labeled 16-mix pooled-sample internal standard as described in Materials and Methods (Figure 1). PCA was used to group the 16 individual Cy3- or Cy5-labeled proteome maps based on the overall expression pattern from the more than 1,000 resolved protein forms under survey. PCA allowed for independent confirmation of distinct expression patterns from the four groups and demonstrated high reproducibility between the replicate samples. The four groups (control, iron-starved, Δfur, and hemin) all clustered into separate quadrants with only one proteome map from the hemin group clustering equidistant from the hemin group and the control group (Figure 1B). These assignments were reiterated in an unsupervised hierarchical clustering of the independent proteome maps presented as a heat map, where expression patterns of the individual proteins can also be compared (Figure 1D).

DIGE analysis combined with subsequent PCA and hierarchical clustering allowed for the grouping of protein expression changes between these four conditions into the following five classes: (I) proteins that are affected by hemin independent of Fur or iron (Table 1), proteins that are more abundant as shown in Figure 1C), (II) proteins that are negatively affected by iron and Fur (Table 2 and Figure 1C), (III) proteins that are positively affected by iron and Fur (Table 3 and Figure 1C), (IV) proteins that are affected by iron independent of Fur (Table 4), and (V) proteins that are affected by Fur independent of iron (Table 5). We identified 29 resolved protein features (representing 20 distinct proteins including isoforms) under iron-dependent negative control by Fur, 30 distinct features (25 proteins including isoforms) under iron-dependent positive Fur-mediated control, and 21 distinct proteins that are exclusively affected by hemin.

Using the PCA and hierarchical clustering approach, we were able to further group the proteome expression maps into two primary clusters, one containing all eight samples prepared from bacteria grown in TSB or TSB containing hemin and a second containing all eight samples prepared from Δfur bacteria or bacteria starved for iron. This comprised the first principle component, which accounted for 62.3% of the variance in the system. These multivariate analyses of protein expression changes allow for a global representation of the similar patterns in protein expression that occur upon inactivation of fur versus those that occur upon iron starvation. Furthermore, this analysis reveals that exposure of *S. aureus* to hemin results in a vastly different, and less severe, change in cellular protein expression as compared to altering the iron status of the bacterium (Figure 1D).

*S. aureus* Proteins Regulated by Hemin Independently of Iron or Fur (Class I)

The reactive nature of heme presents a unique problem to bacterial pathogens, as they must maintain an intracellular...
heme homeostasis that prevents toxicity while internalizing enough heme for nutrient iron needs [8]. This raises the possibility that bacterial pathogens undergo a coordinated change in protein expression in response to exogenous heme. To identify proteins that respond to heme, we grew S. aureus in the presence of 10 μM hemin and identified proteins that change expression upon hemin exposure but not upon inactivation of fur or iron starvation. Twenty-one proteins were identified that responded exclusively to excess hemin with statistical confidence (0.04 > p > 0.0000037), 16 of which were down-regulated between 1.25-fold and 3.6-fold (Table 1). These proteins represent a variety of predicted biochemical functions without clear overrepresentation of any one physiological pathway. Only five proteins increased expression exclusively in response to hemin, comprising a hemin-activated regulon (Figure 1C). These proteins are involved in lactate metabolism (Ddh, 5.26-fold, p = 0.000067), gene regulation (SaeR, 1.34-fold, p = 0.02), and stress response (YaaD, 3.78-fold, p = 0.000037, ClpL, 1.58-fold, p = 0.033). The fifth protein, a putative conserved ABC transporter (SAV2359), exhibited a 45-fold increase (p = 0.0000037) upon exposure to hemin, and is predicted to encode for a conserved transporter with no demonstrated function. These experiments describe the first global analysis of the heme-regulon of a bacterial pathogen and identify a putative transport system that is highly up-regulated exclusively upon exposure to hemin.

The Hrt System Is Required for S. aureus Growth in Hemin

The protein demonstrating the most significant increase upon hemin exposure (45-fold increase) is an ATP-binding component (SAV2359) of a previously uncharacterized ABC-type transport system. The gene encoding for SAV2359 is located immediately adjacent to a predicted permease component (SAV2360) of the same transport system. Based on our proteomic observations, we have named these proteins the heme-regulated transporter ATPase (HrtA) and permease (HrtB). Importantly, genomic analyses demonstrate that this transport system is conserved across many patho-
genic bacteria, including *Bacillus anthracis*, *Listeria monocytogenes*, and *Enterococcus faecalis* (unpublished data).

To explore the contribution of the Hrt system to heme transport, we investigated whether strains inactivated for *hrtA* or *hrtB* can grow when hemin is the sole available iron source. There was no detectable difference in the ability of wild-type, *hrtA* or *hrtB* mutants to grow in medium where the sole iron source was FeSO₄ (Figure 2). In contrast, compared to wild-type, the *hrtA* and *hrtB* mutant strains are severely impaired in their ability to grow when hemin is the sole iron source (Figure 2). To further confirm that the heme-dependent growth defect exhibited by strains inactivated for *hrtA* and *hrtB* is dependent on the insertional mutations, and as an attempt to rule out the possibility that the observed growth defects were due to secondary mutations, we transduced the *hrtA* and *hrtB* mutations into a clean wild-type background as previously described [10]. Successful transductants exhibited identical phenotypes as the original *hrtA* or the *hrtB* mutants, suggesting that inactivation of the Hrt system is responsible for the observed inability to grow in the presence of high heme concentrations (Figure 2). Taken together, these observations identify the HrtAB as a novel staphylococcal heme transport system that is critically important to proper heme metabolism.

### S. aureus Proteins Negatively Regulated by Iron and Fur (Class II)

Proteins that increase abundance upon iron starvation or inactivation of *fur* (via release from repression) represent proteins negatively regulated by Fur in an iron-dependent manner, and hence comprise the canonical Fur regulon of the bacterium. We identified 29 distinct protein features comprising 20 unique cytoplasmic proteins that are increased from 1.3-fold to over 9-fold in the absence of iron or Fur (0.04 > p > 0.0000012, Table 2). These results demonstrate a strong correlation between expression changes upon iron chelation versus the absence of Fur. As expected, iron acquisition systems previously shown to be iron-regulated via Fur are up-regulated under these conditions including proteins involved in siderophore synthesis (ShnE isoforms exhibiting over 3-fold increases in minus iron or *Afur*, 0.013 > p > 0.000013 [16] and transport (FhuA, over 8-fold increases, p < 0.0000016) [17].

Five of 21 proteins that were classified as having Class II expression patterns with mostly moderate increases (approximately 1.5-fold) are enzymes of the glycolytic pathway including fructose 1-P kinase (FruB), fructose bisphosphate aldolase (FbaA), triosephosphate isomerase (Tpi), glyceraldehyde 3-phosphate dehydrogenase (Gap), and transketolase (Tkt) (Table 2). This observation is consistent with a systemic up-regulation of glycolysis upon iron starvation, which would lead to a commensurate increase in pyruvate for subsequent use in the tricarboxylic acid (TCA) cycle or as a substrate for fermentative metabolism (Figure 3).

### S. aureus Proteins Positively Affected by Iron and Fur (Class III)

Fur is traditionally considered a repressor of iron-regulated gene transcription. However, recent work has highlighted a role for Fur in the direct and/or indirect activation of a small subset of genes in *Helicobacter pylori* [18], *Vibrio cholerae* [19], *Neisseria meningitidis* [20], *Escherichia coli* [21,22], and *Bacillus subtilis* [23]. We identified 30 distinct protein features representing 25 unique proteins that were...
positively affected by Fur in an iron-dependent manner as measured by decreased detection in the absence of Fur or upon iron starvation (Table 3).

Numerous regulatory factors were positively affected by Fur in an iron-dependent manner including RbsU. RbsU, down 2.12-fold ($p = 0.0014$) and 1.48-fold ($p = 0.016$) in $Afur$ and iron-deplete conditions, respectively, controls the expression of a variety of virulence genes and regulatory systems. Based on this pleiotropy, small changes in RbsU expression may have profound affects on cellular metabolism. In particular, RbsU activates acetate catabolism; therefore, the Fur-dependent activation of RbsU is consistent with a down-regulation of the TCA cycle upon iron starvation or fur inactivation [24].

The value of iron to the bacterium is underscored by the Fur-mediated iron dependent increase of four separate proteins that are predicted to contain iron-sulfur clusters. Three of these proteins are the TCA cycle enzymes succinate dehydrogenase (SdhA, decreased 6.42-fold in $Afur$, $p = 0.0015$ and 3.8-fold in iron-depleted, $p = 0.00053$), aconitate hydratase (CitB, decreased over 4-fold, $p < 0.0002$ in both conditions), and fumarate hydratase (CitG, decreased over 2-fold in both conditions, $p < 0.00025$). A fourth TCA cycle enzyme, phosphoenolpyruvate carboxykinase (PckA), which converts oxaloacetate to phosphoenolpyruvate during gluconeogenesis, was decreased approximately 1.7-fold upon inactivation of fur ($p = 0.0023$) or iron depletion ($p = 0.037$).

Two additional proteins associated with central metabolism and demonstrating group III expression patterns are α-fructose-6-phosphate amidotransferase (GlmS) exhibiting 2.29-fold ($p = 0.00063$) and 1.8-fold ($p = 0.0038$) decreases in $Afur$ and iron-depleted conditions, respectively, and glyceraldehyde 3-P dehydrogenase (GapB), exhibiting 14.5-fold ($p = 0.0023$) or iron depletion ($p = 0.037$). GlmS converts fructose 6-P to glucosamine-6-P, and hence depletes substrate for phosphofructokinase in effect antagonizing glycolysis. GapB is a second glyceraldehyde 3-P dehydrogenase (GapB), exhibiting 14.5-fold ($p = 0.00052$) and 6.1-fold ($p = 0.00088$) decreases in $Afur$ and iron-deplete conditions, respectively. GlmS converts fructose 6-P to glucosamine-6-P, and hence depletes substrate for phosphofructokinase in effect antagonizing glycolysis. GapB is a second glyceraldehyde 3-P dehydrogenase of $S. aureus$ and based on its function in Bacillus subtilis, is predicted to possess an enzymatic GAPDH activity involved in gluconeogenesis [25]. These results support the Fur-mediated up-regulation of glycolysis upon iron starvation and suggest a commensurate systemic and regulated inhibition of the TCA cycle. Together, these findings support a model whereby in iron-starved $S. aureus$, excess pyruvate produced as a result of an up-

Table 2. Proteins Negatively Affected by Fur and Iron

| Protein IDa | SAV Locusb | Gene Namec | Functiond | Fold Change (−Fe) | $p$-Valuee | Fold Change (↑fur) | $p$-Valuee |
|------------|------------|-----------|-----------|------------------|------------|------------------|------------|
| 1888 0116  | NA         | Cysteine synthetase | 9.41 | 0.0012 | 9.98 | 0.00054 |
| 1902 0116  | NA         | Cysteine synthetase | 3.00 | 0.00018 | 2.64 | 0.00056 |
| 1740 0117  | NA         | Ornithine cyclodeaminase | 3.12 | 0.0038 | 3.83 | 0.00022 |
| 1742 0117  | NA         | Ornithine cyclodeaminase | 14.13 | 0.0022 | 17.88 | 0.00086 |
| 1002 0120  | sbrE       | Siderophore synthesis | 3.22 | 0.00044 | 4.13 | 0.00013 |
| 1003 0120  | sbrE       | Siderophore synthesis | 4.47 | 0.0025 | 5.63 | 0.0011 |
| 1004 0120  | sbrE       | Siderophore synthesis | 4.07 | 0.013 | 5.22 | 0.0067 |
| 2790 0123  | sodM       | Superoxide dismutase | 1.57 | 0.033 | 2.13 | 0.0022 |
| 2793 0123  | sodM       | Superoxide dismutase | 2.37 | 0.00032 | 2.07 | 0.0021 |
| 2548 0255  | ispD       | 2-C-methyl-4-erythritol-4-phosphate cytidylyltransferase | 1.73 | 0.0013 | 1.32 | 0.039 |
| 2037 0513  | cysK       | D-Acetylserine (thiol)-lyase | 1.36 | 0.013 | 1.70 | 0.00095 |
| 2139 0551  | hchA       | Chaperone | 1.99 | 0.00015 | 1.42 | 0.027 |
| 2410 0647  | fhuA       | Ferrichrome transport | 8.19 | 0.0000012 | 8.34 | 0.0000016 |
| 2066 0699  | frbA       | Fructose 1-phosphate kinase | 1.59 | 0.0097 | 1.43 | 0.031 |
| 1700 0772  | gap        | Glyceroldehyde 3-phosphate dehydrogenase | 1.69 | 0.0054 | 2.27 | 0.0048 |
| 1723 0772  | gap        | Glyceroldehyde 3-phosphate dehydrogenase | 1.53 | 0.014 | 1.66 | 0.0011 |
| 1726 0772  | gap        | Glyceroldehyde 3-phosphate dehydrogenase | 1.51 | 0.036 | 1.44 | 0.022 |
| 2492 0774  | tpi        | Triosephosphatase isomerase | 1.46 | 0.0068 | 1.52 | 0.0085 |
| 2188 0968  | NA         | Decarboxylase | 1.57 | 0.0036 | 1.22 | 0.046 |
| 1432 0984  | NA         | 3-Oxoacyl synthase | 1.73 | 0.0038 | 1.30 | 0.021 |
| 2217 1011  | fabI       | Enoyl-[acyl carrier protein] reductase [NADH] | 2.07 | 0.000046 | 1.42 | 0.026 |
| 2725 1259  | tfr        | Ribosome recycling factor | 1.49 | 0.00081 | 1.27 | 0.03 |
| 0859 1342  | tkt        | Transketolase | 2.37 | 0.0000066 | 1.51 | 0.0013 |
| 0866 1342  | tkt        | Transketolase | 2.59 | 0.000041 | 1.78 | 0.00078 |
| 0956 1630  | aspS       | Aspartyl-RNA-synthetase | 1.26 | 0.0034 | 1.31 | 0.015 |
| 2168 2125  | fbsA       | Fructose bisphosphate aldolase | 1.53 | 0.00061 | 1.49 | 0.00016 |
| 2169 2125  | fbsA       | Fructose bisphosphate aldolase | 1.40 | 0.029 | 1.46 | 0.002 |
| 1994 2125  | fbsA       | Fructose bisphosphate aldolase | 5.19 | 0.0000098 | 5.93 | 0.0000051 |
| 2005 2125  | fbsA       | Fructose bisphosphate aldolase | 2.66 | 0.0001 | 4.63 | 0.000005 |
| 1786 2302  | NA         | α-Octopine dehydrogenase | 1.93 | 0.0028 | 1.33 | 0.042 |
| 1750 2455  | NA         | Endo-1,4β-glucanase | 1.94 | 0.0015 | 1.52 | 0.016 |

aProtein ID corresponds to Master Number in the Master Table S1.
bSAV number corresponds to position in the annotated S. aureus Mu50 genome.
cGene name corresponds to name listed in annotation of Mu50 genome. NA signifies no gene name listed.
dGene name and function were determined based on closest hit in a BLAST search with an e value of less than 10−10.
e$p$-Values were calculated using the Student’s t-test.

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$S. aureus$ Iron–Dependent Gene Regulation

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regulators Zur [26], PerR [27], and MntR [28]. The presence of

### Table 3. Proteins Positively Affected by Fur and Iron

| Protein ID | SAV Locus | Gene Name | Function | p-Value | Fold Change | p-Value |
|------------|-----------|-----------|----------|----------|-------------|---------|
| 2302       | 0018      | vicR      | Response regulator | 0.03 | -1.39 | 0.01 |
| 0825       | 0226      | pfbB      | Formate acetyltransferase | 0.0022 | -7.04 | 0.012 |
| 0846       | 0226      | pfbB      | Formate acetyltransferase | 0.0043 | -7.30 | 0.0094 |
| 0845       | 0226      | pfbB      | Formate acetyltransferase | 0.0033 | -6.67 | 0.0054 |
| 1964       | 0519      | NA        | Pyridoxine biosynthesis | 0.078 | -2.23 | 0.0025 |
| 2748       | 0520      | NA        | SNO glutamine amidotransferase | 0.0083 | -1.67 | 0.0044 |
| 1979       | 0548      | tuF       | Translation elongation TU | 0.036 | -4.07 | 0.035 |
| 1855       | 0605      | adh1      | Alcohol dehydrogenase | 0.0046 | -1.73 | 0.037 |
| 1867       | 0605      | adh1      | Alcohol dehydrogenase | 0.0074 | -1.63 | 0.05 |
| 1543       | 0938      | NA        | Pyridine nucleotide disulfide oxidoreductase | 0.000003 | -2.61 | 0.000053 |
| 0567       | 1139      | pheT      | Phe-tRNA synthetase | 0.043 | -1.19 | 0.0039 |
| 1066       | 1148      | sdiA      | Succinate dehydrogenase | 0.0053 | -3.80 | 0.0015 |
| 3073       | 1178      | NA        | Hypothetical protein | 0.0073 | -1.61 | 0.041 |
| 2428       | 1255      | codY      | Transcriptional repressor | 0.052 | -1.49 | 0.0031 |
| 0487       | 1350      | citB      | Aconitate hydratase | 0.0013 | -4.15 | 0.000087 |
| 2235       | 1492      | srrA      | Staphylococcal respiratory regulator | 0.0011 | -1.87 | 0.00012 |
| 1679       | 1512      | NA        | Tripeptidase | 0.0022 | -1.22 | 0.0014 |
| 1595       | 1687      | gapB      | Glyceraldehyde 3-phosphate dehydrogenase 2 | 0.000088 | -6.10 | 0.000052 |
| 1554       | 1729      | tyrS      | Tyrosyl RNA synthetase | 0.017 | -1.24 | 0.033 |
| 1133       | 1791      | pckA      | Phosphoenolpyruvate carboxykinase | 0.037 | -1.77 | 0.0023 |
| 1382       | 1851      | citG      | Fumarate hydratase | 0.0025 | -2.47 | 0.00001 |
| 1943       | 2067      | thrU      | Sigma B | 0.016 | -1.48 | 0.0004 |
| 1526       | 2136      | pnp       | Pyridine-nucleoside phosphorylase | 0.0028 | -1.77 | 0.00034 |
| 0970       | 2154      | glmS      | α-Fructose-6-phosphate amidotransferase | 0.024 | -1.53 | 0.006 |
| 0968       | 2154      | glmS      | α-Fructose-6-phosphate amidotransferase | 0.007 | -1.42 | 0.00001 |
| 0969       | 2154      | glmS      | α-Fructose-6-phosphate amidotransferase | 0.0038 | -1.80 | 0.00063 |
| 1765       | 2165      | NA        | MRP-like ATP-binding protein | 0.0052 | -1.59 | 0.0026 |
| 2216       | 2204      | NA        | Hypothetical protein | 0.0044 | -1.39 | 0.0001 |
| 1694       | 2285      | NA        | Butyryl-CoA dehydrogenase | 0.00057 | -2.15 | 0.00002 |
| 1782       | 2305      | NA        | Glycerate dehydrogenase | 0.0073 | -1.67 | 0.00067 |

*a-Protein ID corresponds to Master Number in the Master Table S1.
*b-SAV number corresponds to position in the annotated S. aureus Mu50 genome.
*c-Gene name corresponds to name listed in annotation of Mu50 genome. NA signifies no gene name listed.
*d-Function name corresponds to functional term listed in the Master Table S1.

In keeping with the above model, Class III proteins associated with fermentative metabolism were represented by alcohol dehydrogenase (Adh1), butyryl-CoA dehydrogenase (between 1.5- to 7-fold decreases for the two conditions, 0.05 > p > 0.00002), and three distinct isoforms of formate acetyltransferase (PfB) (between 3.6 to 7.3-fold decreases for the two conditions across isoforms). These three enzymes are involved in the conversion of pyruvate to distinct end-products of fermentative metabolism: formate, ethanol, or butyrate, respectively. These decreases upon iron chelation suggest that iron-starved *S. aureus* metabolize pyruvate through fermentation to metabolic end-products other than formate, ethanol, or butyrate. These results suggest that excess pyruvate produced as a result of increased glycolysis is converted to other predicted products of staphylococcal fermentative metabolism, such as 2,3-butanediol and/or lactate (Figure 3).

*S. aureus* Proteins Regulated by Iron Independently of Fur (Class IV)

In addition to Fur, *S. aureus* possesses the metal-dependent regulators Zur [26], PerR [27], and MntR [28]. The presence of multiple metal-specific regulatory factors raises the possibility that additional as-yet-unidentified factors other than Fur respond to changes in cellular iron content. We identified 22 unique proteins that were positively or negatively affected by iron starvation, but whose expression was not affected by inactivation of fur (Table 4). Four of these proteins are associated with cellular respiration including formate dehydrogenase (Fdh) which was up-regulated 22.32-fold in the absence of iron (fur) and Hpr kinase (HprK), NAD synthase (NadE), and a single isoform of formate acetyltransferase (PfB). Thus, we have identified a significant pool of proteins that are affected by iron independently of Fur, raising the possibility that an additional transcriptional regulator exists in *S. aureus* to monitor intracellular iron status. It should be pointed out that DIP binds divalent cations other than iron which might be responsible for some of the Class IV expression changes that were observed.

*S. aureus* Proteins Regulated by Fur Independently of Iron (Class V)

We also identified 24 unique proteins that changed expression upon inactivation of fur without any significant changes in iron availability status (Class V, Table 5). One Class V protein, GapR, is an activator of Gap expression and is
### Table 4. Proteins Affected by Iron Independently of Fur

| Protein IDa | SAV Locusb | Gene Namec | Functiond | Fold Change (−Fe) | p-Valuee | Fold Change (−fur) | p-Valuef |
|-------------|------------|------------|-----------|-------------------|----------|-------------------|----------|
| 1757        | 0177       | fhf        | Formate dehydrogenase | 22.32 | 0.015 | 1.77 | 0.24 |
| 0811        | 0226       | pfB        | Formate acetyltransferase | 2.03 | 0.045 | −2.66 | 0.035 |
| 1177        | 0380       | ahpF       | Alkyl hydroperoxide reductase subunit F | −1.7 | 0.0036 | 2.05 | 0.00023 |
| 2771        | 0381       | ahpC       | Alkyl hydroperoxide reductase subunit C | −1.73 | 0.00096 | 2.24 | 0.0000025 |
| 2162        | 0551       | hchA       | Chaperone | 1.93 | 0.00099 | 1.32 | 0.06 |
| 2259        | 0566       | NA         | Hypothetical | 1.55 | 0.0046 | −1.09 | 0.5 |
| 2508        | 0587       | NA         | Hypothetical | 1.27 | 0.0062 | 1.07 | 0.4 |
| 1933        | 0760       | hprK       | Hpr kinase | 1.14 | 0.05 | −1.22 | 0.061 |
| 1392        | 0844       | NA         | Aminotransferase NitF homologue | 1.22 | 0.05 | 1.11 | 0.15 |
| 2197        | 0968       | NA         | Decarboxylase | 1.4 | 0.037 | 1.0 | 0.87 |
| 2435        | 1088       | NA         | Potassium transport | 1.36 | 0.032 | 1.22 | 0.066 |
| 2285        | 1533       | NA         | Lipoate protein ligase | 1.66 | 0.00071 | 1.05 | 0.62 |
| 2040        | 1557       | NA         | Endonuclease IV | 1.53 | 0.0056 | −1.08 | 0.61 |
| 0848        | 1683       | thrS       | Threonyl-tRNA synthetase | −2.61 | 0.0023 | −1.61 | 0.069 |
| 2803        | 1854       | NA         | Hypothetical | 1.28 | 0.05 | −1.15 | 0.35 |
| 1872        | 1912       | nadE       | NAD synthetase | −1.39 | 0.022 | −1.2 | 0.19 |
| 2831        | 1929       | NA         | Hypothetical | 1.26 | 0.019 | 1.0 | 0.93 |
| 3469        | 2030       | groES      | GroES | 1.75 | 0.0061 | 1.38 | 0.054 |
| 1476        | 2124       | muZ        | UDP-N-acetylglucosamine carboxyvinyl transferase | 1.56 | 0.0072 | 1.16 | 0.059 |
| 2552        | 2229       | adK        | Adenylate kinase | 1.55 | 0.0013 | 1.26 | 0.069 |
| 1753        | 2455       | NA         | Endo-1,4β-glucanase | 1.33 | 0.036 | −1.71 | 0.00022 |
| 2315        | 2699       | NA         | N-Hydroxyarylamine O-acetyltransferase | 2.06 | 0.0016 | 1.14 | 0.47 |

aProtein ID corresponds to Master Number in the Master Table S1.
bSAV number corresponds to position in the annotated S. aureus Mu50 genome.
cGene name corresponds to name listed in annotation of Mu50 genome. NA signifies no gene name listed.
dFunction corresponds to Master Number in the Master Table S1.
ep-Value was calculated using the Student’s t-test.
fp-Value was calculated using the Student’s t-test.

### Table 5. Proteins Affected by Fur Independently of Iron

| Protein IDa | SAV Locusb | Gene Namec | Functiond | Fold Change (−Fe) | p-Valuee | Fold Change (−fur) | p-Valuef |
|-------------|------------|------------|-----------|-------------------|----------|-------------------|----------|
| 2456        | 0126       | butA       | Acetoin reductase | −1.27 | 0.15 | −1.55 | 0.024 |
| 2776        | 0133       | sodM       | Superoxide dismutase | −1.46 | 0.062 | −2.33 | 0.0024 |
| 1456        | 0139       | dsm        | Phosphopentomutase | 1.2 | 0.096 | −1.47 | 0.0087 |
| 0807        | 0226       | pfB        | Formate acetyltransferase | 1.75 | 0.065 | −3.5 | 0.0053 |
| 0811        | 0226       | pfB        | Formate acetyltransferase | 2.03 | 0.045 | −2.66 | 0.035 |
| 1177        | 0380       | ahpF       | Alkyl hydroperoxide reductase subunit F | −1.7 | 0.0036 | 2.05 | 0.00023 |
| 2771        | 0381       | ahpC       | Alkyl hydroperoxide reductase subunit C | −1.73 | 0.00096 | 2.24 | 0.0000025 |
| 2312        | 0491       | NA         | TatD-related DNaese | 1.01 | 0.83 | −1.76 | 0.033 |
| 2042        | 0513       | cysK       | O-Acetylserine (thiol)-lyase | 1.3 | 0.093 | 1.42 | 0.025 |
| 2465        | 0531       | NA         | Arabinose repressase | 1.13 | 0.26 | 1.51 | 0.00085 |
| 1771        | 0771       | gapR       | Glycolytic operon regulator | 1.16 | 0.44 | 1.24 | 0.05 |
| 2503        | 0842       | NA         | ABC transporter | −1.22 | 0.061 | −1.4 | 0.0067 |
| 1712        | 0957       | rocD       | Ornithine-oxo-acid transaminase | −1.51 | 0.067 | −3.14 | 0.0012 |
| 1434        | 0958       | gudB       | NAD-glutamate dehydrogenase | −1.04 | 0.7 | −1.42 | 0.013 |
| 0595        | 0975       | cliB       | CIPB chaperone | 1.29 | 0.09 | 2.06 | 0.0055 |
| 2560        | 1339       | lexA       | Transcriptional repressor | 1.02 | 0.84 | −1.59 | 0.026 |
| 1916        | 1425       | NA         | Hypothetical | −1.1 | 0.3 | −1.55 | 0.000079 |
| 1580        | 1694       | citC       | Isocitrate dehydrogenase | −1.12 | 0.7 | −2.8 | 0.018 |
| 1582        | 1694       | citC       | Isocitrate dehydrogenase | −1.4 | 0.12 | −3.85 | 0.000048 |
| 1572        | 1737       | NA         | 3-Deoxy-7-phosphoheptulonate synthase | −1.02 | 0.76 | −1.53 | 0.0053 |
| 1142        | 1791       | pckA       | Phosphoenolpyruvate carboxykinase | −1.35 | 0.12 | −2.85 | 0.00039 |
| 2397        | 2416       | psgA       | Phosphoglycerate mutase | −1.06 | 0.96 | −1.94 | 0.24 |
| 2398        | 2416       | psgA       | Phosphoglycerate mutase | 1.27 | 0.093 | −2.02 | 0.000075 |
| 1753        | 2453       | NA         | Endo-1,4β-glucanase | 1.33 | 0.036 | −1.71 | 0.00022 |

aProtein ID corresponds to Master Number in the Master Table S1.
bSAV number corresponds to position in the annotated S. aureus Mu50 genome.
cGene name corresponds to name listed in annotation of Mu50 genome. NA signifies no gene name listed.
dFunction corresponds to Master Number in the Master Table S1.
ep-Value was calculated using the Student’s t-test.
fp-Value was calculated using the Student’s t-test.

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increased in the absence of Fur (1.24-fold, $p = 0.05$). This moderate increase in expression may contribute to the increase in Gap expression observed upon inactivation of fur (as much as 2.27-fold, $p = 0.0048$; Table 2). Inactivation of fur leads to down-regulation of PckA (2.85-fold, $p = 0.00039$) and isocitrate dehydrogenase (CitC; 3.85-fold, $p = 0.0018$), consistent with our previous observation of Fur-mediated activation of the TCA cycle. Furthermore, acetoin reductase (ButA) is decreased by inactivation of fur (1.55-fold, $p = 0.024$) implying a commensurate decrease in the production of 2,3-butanediol upon iron starvation. Taken together with results described above, this suggests that a major metabolic end-product of carbohydrate metabolism produced by iron-starved S. aureus is lactate (Figure 3).

Iron-Starved S. aureus Produce Excess Lactate Leading to a Decrease in Local pH

Iron-starved S. aureus are known to restrict oxidative capacity by oxidizing glucose with the accumulation of much lactate and minor amounts of pyruvate, acetate, and acetoin [29]. The coordinated expression changes of the staphylococcal central metabolic pathways identified using DIGE/MS are summarized in Figure 3 and provide a mechanistic explanation for this observation. Our results suggest that iron-starved S. aureus undergo a Fur-mediated redirection of central metabolic pathways leading to the production of lactate as a primary end-product of fermentative metabolism.

To test this hypothesis, we measured the amount of lactate produced by S. aureus after growth in either iron-replete conditions, upon iron starvation elicited by DIP, or upon fur inactivation. S. aureus grown under iron-starved conditions produced approximately 3-fold more lactate than S. aureus grown in the presence of iron. Similarly, inactivation of fur increased lactate production by approximately 2-fold in iron-replete medium (Figure 4A). To test whether this increase in lactate production contributes to a commensurate decrease in pH, we measured the pH of medium from cultures of iron-replete, iron-deplete, $Afu$, and $Afu$ containing a full-length copy of fur provided in trans ($Afu$ + fur). These experiments demonstrated a drop in the pH of the iron-starved culture from 7.2 to 5.2 upon either iron starvation or fur inactivation (Figure 4B). Providing fur in trans complemented the pH decrease of the $Afu$ strain linking the observed decrease in pH to an absence of fur. When subjected to identical growth conditions, iron-replete cultures increased pH to close to 8.0 (Figure 4B). From these data, we conclude that S. aureus elaborates a Fur-mediated redirection of central metabolism under iron starvation to increase lactate production and decrease pH.

Transferrin-iron represents a viable iron source to invading bacterial pathogens. In order to utilize transferrin-iron as a nutrient iron source, the iron must be dissociated from transferrin and transported into the bacterium. Free Fe$^{3+}$ is more readily utilized as a nutrient source than transferrin-bound iron, and iron is known to dissociate from transferrin upon changes in pH [30]. We hypothesized that the Fur-dependent redirection of central metabolism by iron-starved S. aureus facilitates the release of iron from transferrin through a decrease in local pH. To test this hypothesis, we measured iron release from transferrin mediated by spent medium from either iron-replete, iron-deplete, $Afu$, or $Afu$ + fur staphylococcal cultures. We found that medium from iron-starved or $Afu$ staphylococcal cultures significantly increased the rate of iron release from transferrin and this phenotype was partially complemented by providing fur in trans (Figure 4C). A similar pattern of iron release was observed upon incubation of transferrin in the presence of lactate (Figure 4D). Taken together, these results demonstrate that the Fur-mediated production of lactate by iron-starved S. aureus facilitates the release of iron from host iron-sequestering proteins.

Discussion

In this study, we have analyzed changes in the cytoplasmic protein profile of S. aureus upon genetic ($Afu$) and biochemical (iron chelation, hemin treatment) alterations in iron exposure. Using large-format, high-resolution DIGE with mixed-sample internal standards, we simultaneously surveyed the S. aureus proteome in response to these manipulations versus control in quadruplicate to provide for statistical confidence. Overall, 156 protein features of interest, specifying approximately 120 individual proteins (including changes in post-translational modification) were identified by mass spectrometry and placed into functional groups defining Fur-dependent and independent iron regulation as well as hemin-affected proteins.

The hemin-affected proteins were particularly of note because this class of proteins has not previously been characterized in bacterial pathogens, despite the identification of heme-regulated proteins in other bacterial pathogens such as Corynebacterium diphtheria [31] and Bordetella sp. [32,33]. The majority of the 21 proteins in this class were decreased upon hemin exposure. However, a few notable exceptions were identified, including a dramatic 45-fold increase of a single protein component (SAV2359) of a putative transporter system which we have named the heme-regulated
transporter (HrtAB). This dramatic increase in abundance in the presence of hemin suggests a role for the HrtAB system in heme transport. As a preliminary test of this hypothesis, we individually inactivated \textit{hrtA} and \textit{hrtB} and monitored the ability of these strains to grow in the presence of hemin as a sole iron source. These experiments demonstrated a severe growth restriction on hemin upon inactivation of \textit{hrtAB} and identify the HrtAB system as a critical component of staphylococcal heme metabolism. The Hrt system joins the heme transport system (HtsABC) and iron-regulated surface determinant system, as a third membrane-associated heme transporter [4,8]. The presence of three separate membrane-associated transport systems with roles in heme transit underscores the value of heme metabolism to \textit{S. aureus}.

The response regulator SaeR was also increased upon exposure to hemin, whereby the modest 1.34-fold increase (\( p = 0.02 \)) may well have profound affects on gene transcription of target proteins. SaeR together with SaeS activates the transcription of several exoproteins including \( \alpha \)-hemolysin and \( \beta \)-hemolysin [34], two proteins with potent erythrocyte lysis activity. It is tempting to speculate that the recognition of host heme up-regulates SaeR expression, in turn activating \( \alpha \)- and \( \beta \)-hemolysins, which would lead to an increase in local erythrocyte hemolysis and free hemoglobin concentrations. This might represent a positive regulatory circuit used by \textit{S. aureus} to increase local heme concentrations, and hence nutrient iron availability.

Another noteworthy class of proteins identified in our study was decreased upon inactivation of \textit{fur} in an iron-dependent manner, suggesting a Fur-mediated increase in abundance of these proteins. In gram-negative bacteria, the mechanism for Fur-mediated positive regulation of proteins has been elucidated by elegant studies beginning with the work of Masse et al. [35]. These and other investigations have identified the small regulatory RNA (sRNA) RhyB as being responsible for Fur-dependent protein activation in \textit{E. coli} [35], \textit{Pseudomonas aeruginosa} [36], \textit{V. cholera} [37], and \textit{Shigella flexneri} [38]. The targets of RhyB include some of the same
genes identified in our study as being positively regulated by Fur, including the TCA cycle enzymes aconitase (acnA), fumarase (fumA), and succinate dehydrogenase (sdhCDAB) [21]. This observation suggests that a similar mechanism of iron-dependent gene regulation is occurring in S. aureus, however we were unable to identify any potential homologues to RyhB in any Gram positive bacterial genome using traditional BLAST analyses (unpublished data).

S. aureus has been reported to express at least 12 sRNAs with predicted roles in translational regulation through message stability [39]. It is likely that an as-yet-undiscovered sRNA-mediated regulatory system exists in S. aureus responsible for iron homeostasis through targeted mRNA degradation.

Our data indicate that iron starvation leads to the reversible inactivation (or down-regulation) of TCA cycle enzymes including aconitase, the down-regulation of which has been implicated as a survival response to oxidative stress induced during the host-pathogen interaction [40]. In S. aureus, downregulation of the TCA cycle through aconitase inactivation prevents maximal expression of the virulence factors lipase, staphylococcal enterotoxin C, and α- and β-hemolytic toxins and therefore alters the interaction between S. aureus and the host [40]. Additionally, inactivation of the TCA cycle or growth in iron-deplete conditions leads to a decrease in production of formylated delta-toxin, a potent neutrophil attractant [41]. Combined, these two factors have led to the suggestion that down-regulation of the TCA cycle may protect against host immune-mediated recognition of infecting S. aureus [41].

We propose a model whereby upon iron starvation, such as would be encountered inside the host, S. aureus up-regulates glycolysis through the release of Fur-mediated repression of glycolytic enzymes. Based on the simultaneous Fur-mediated down-regulation of TCA cycle enzymes, pyruvate does not enter the TCA cycle but instead is acted on by fermentative pathways. We have demonstrated here that four separate branches of fermentative metabolism are down-regulated under iron starvation, which we predict funnels excess pyruvate into acidic fermentative end-products including lactate (Figure 3).

The production of the acidic end-product lactate contributes to a decrease in the local pH of the microenvironment surrounding infecting staphylococci, a hypothesis supported by the observation that the pH of the spent medium from iron-starved or Δfur staphylococci is significantly more acidic than that of spent medium from iron-replete cultures (Figure 4B).
This overproduction of lactate and subsequent decrease in pH dissociates iron from host iron-sequestering molecules (Figure 4C and 4D). Further, the decrease in the local pH combined with a commensurate decrease in Eh (oxidation reduction potential) would be expected to change the oxidation state of host iron atoms converting the insoluble ferric iron to the more bioavailable ferrous iron. An increase in local ferrous iron concentrations would significantly relieve the iron stress placed on the bacterium and provide a growth advantage to invading staphylococci. The Fur-mediated redirection of central metabolic pathways to increase iron availability is supported by published results showing that the uptake of iron (presented as $\text{FeSO}_4$) by S. aureus is twice as great at pH 4.7 as it is at a pH 7.4 [42]. It is possible that additional acidic end-products of fermentative metabolism contribute to the acidification of the culture medium upon growth of iron-starved S. aureus. For instance, the production of formate as a fermentative end-product would contribute to a decrease in the pH of the microenvironment surrounding iron-starved S. aureus. Acetyl-CoA is converted to formate by formate acetyltransferase (PBf), an enzyme that was identified in our proteomic analyses by three separate isoforms exhibiting decreased abundance upon iron starvation or fur inactivation (Table 3). These results suggest that formate does not significantly contribute to the acidification of spent medium from iron-starved staphylococcal cultures. However, we did identify a single and separate isoform of PBf that increased expression upon iron starvation (2.03-fold; Table 4). Formate dehydrogenase, which subsequently converts formate to NADH, H$^+$, and CO$_2$, also exhibited an increase in abundance upon iron starvation (22.32-fold), consistent with the possibility that appreciable amounts of formate are formed by iron-starved staphylococci. We were unable to detect a significant increase in formate accumulation in the medium of iron-starved staphylococcal cultures (unpublished data), suggesting that if formate is being accumulated as a result of iron starvation, it is a transient increase due to catabolism by Fdh.

Although the experiments described here were performed in vitro, the severe iron restriction encountered by S. aureus once inside the host strongly supports an in vivo relevance for these findings. These fundamental changes in metabolic function potentially provide a survival advantage to S. aureus by preventing maximal activation of the immune system while the bacteria struggle to alter its microenvironment to access host iron.

**Materials and Methods**

**Bacterial strains and growth conditions.** S. aureus clinical isolate Newman was used in all experiments. Prior to cytoplasmic extraction, bacteria were grown in TSB for 15 h at 37°C with shaking at 180 rpm. Iron starvation was achieved by addition of 1 mM DIP to the growth cultures prior to inoculation. Hemin treatment was achieved by addition of 10 μM hemin to the growth cultures prior to inoculation. All cultures were incubated in the dark to maintain the integrity of the hemin. To avoid differential gene expression due to growth phase, the cultures were harvested at comparable optical densities during early stationary phase. Newman Δfur was created through transduction of the previously created Δfur allele from RN4220 [43] to strain Newman with the transducing phage Φ85 as previously described [10].

S. aureus strain Newman Δfur was complemented by providing a full-length copy of fur (SAV1498) under the control of its native promoter in the context of a promoterless pOS1-derived vector. fur was PCR amplified from S. aureus Newman genomic DNA using a 5′ primer containing an EcoRI site and a 3′ primer containing a BamHI site. The PCR product was cloned into pCR2.1 (Invitrogen, Carlsbad, California, United States) and excised by digestion with EcoRI and BamHI (New England Biolabs, Beverly, Massachusetts, United States). pOS1 was digested with EcoRI and BamHI and fur was inserted, yielding pOS1/fur, where fur is under the control of its native promoter. pOS1/fur and pOS1 are electroporation-deficient primary recipient RN4220, after which they were electroporated into appropriate electroporation-deficient secondary recipient strains (Newman and Newman Δfur for pOS1, Newman Δfur for pOS1 fur). S. aureus strains harboring plasmids were selected on and grown in either tryptic soy agar or tryptic soy broth containing 10 μg/ml chloramphenicol.

ΔtruA and ΔhrtB mutants were obtained from the Phoenix (N) library, clones PHINE03177 (SAV2359) and PHINE01762 (SAV2360) [44]. The Phoenix mutant isolates are derivatives of the clinical S. aureus isolate Newman that has been subjected to two rounds of mutagenesis using the bursa aurealis transposon. The exact site of transposon insertions have been determined by DNA sequencing and inactivated genes annotated using the S. aureus Mu50 genome [44]. The bursa aurealis insertions in ΔtruA and ΔhrtB were transduced into wild-type S. aureus Newman with the transducing phage Φ85 as previously described [10].

**Preparation of cytoplasmic fractions.** Cytoplasmic extracts were prepared upon completion of 15 h of bacterial growth. Cells were pelleted by centrifugation at 6,000 g for 15 min. Pellets were resuspended in a lysis buffer (100 mM Tris pH 7.5, 100 mM sucrose, 100 mM MgCl$_2$) and incubated at 37°C for 45 min in the presence of 1 mg of lysostaphin. Following cell wall digestion, protoplasts were isolated by centrifugation at 13,700 g and washed once and resuspended in 20 ml of buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 100 μM phenylmethylsulfonyl fluoride [PMSF]). To lyse the protoplasts, samples were subjected to two rounds of osmotic mediated lysis at 20,000 psi. Insoluble material was removed by ultracentrifugation at 100,000 g for 45 min. The collected supernatant, representing the cytoplasmic fraction, was used in subsequent analyses. Successful fractionation was confirmed by immunoblot using monoclonal antibodies which recognize the cytoplasmic (α-IsdG), membrane (α-IsdB), and cell wall (α-IsdIB) of S. aureus [10].

**DIGEMS.** Quadruplicate samples from the four conditions were independently prepared as described above. For each sample, 0.25 mg of protein was separately precipitated with methanol and chloroform [45] and resuspended in 30 μl of lysis buffer (7 M urea, 2 M thioura, 4% CHAPS, 30 mM Tris, 5 mM magnesium acetate). The NHS-ester dyes Cy2/3/5 were used for the minimal labeling protocol using an internal standard [12,13,46]. Briefly, one-third of each sample (10 μl, 83 μg) was removed and combined into a single tube to comprise the pooled-sample internal standard (1,350 μg total). The remaining two-thirds of each individual sample (20 μl, 167 μg) was individually labeled with 200 pmol of either Cy3 or Cy5, while the pooled-sample was labeled en masse with 1,600 pmol of Cy2. The samples were quenched with 10 mM lissamine (2 μl for each 290 pmol) for 10 min on ice. Finally, the Cy3-labeled sample was equal volume 2× Cy3 buffer (7 M urea, 2 M thioura, 4% CHAPS, 4 mg/ml DTT). Pairs of Cy3/Cy5-labeled samples were mixed with an equal aliquot of the Cy2-labeled internal standard according to the schema in Figure 1. Tripartite samples were brought up to 450 μl with 1× rehydration buffer (same as 2× buffer but with 2 mg/ml DTT and 0.5% IPI buffer 4–7) and passively rehydrated into 24 cm-4 7-iminoimidized pH gradient (IPI) strips for 24 h (total of 500 μg per gel). All 2D DIGE-associated instrumentation was manufactured by GE Healthcare/Amersham Biosciences (Piscataway, New Jersey). First-dimensional separations were performed on a modified first-dimension-equipped IPGphor first-dimension isoelectric focusing unit, and second-dimensional 12% SDS-PAGE was carried out using hand-cast gels that had one plate presilanized (to ensure subsequent accurate robotic protein excision) using an Etan DALT 12 unit, both according to the manufacturer’s protocols. Cy2/3/5-specific 16-bit data files were acquired at 100 μm resolution separately by dye-specific excitation and emission wavelengths using a Typhoon 9400 Variable Mode Imager, and the gels were stained for total protein content with SyproRuby (Molecular Probes/Invitrogen) per the manufacturer’s instructions.

The DeCyder v6.5 suite of software tools (Amersham Biosciences/GE Healthcare) was used for DIGE analysis. The normalized volume ratio of each individual protein spot-feature from a Cy3- or Cy5-labeled sample was directly quantified relative to the Cy2-signal from the corresponding pool sample in the same channel. This is performed for all resolved features in a single gel where no gel-to-gel variation exists between the three co-resolved content with SyproRuby (Molecular Probes/Invitrogen) per the manufacturer’s instructions.
to normalize and compare Cy3/Cy2 and Cy5/Cy2 abundance ratios across the eight-gel set, enabling statistical confidence to be associated with each change in abundance or charge-altering post-translational modification using Student’s t-test and ANOVA analyses (Table S1). Unsupervised PCA and hierarchical clustering was performed using the DeCyder Extended Data Analysis (EDA) module. Proteins were robotically excised from gels, digested in gel with modified porcine trypsin protease (Trypsin Gold; Promega, Madison, Wisconsin, United States) and peptides applied to a stainless steel target using an integrated Spot Handling Workstation per the manufacturer’s recommendations. Matrix-assisted laser desorption/ionization, time-of-flight mass spectrometry (MALDI-TOF MS) and data-dependent TOF/TOF tandem MS/MS was performed on a Voyager 4700 (Applied Biosystems, Framingham, Massachusetts, United States). The resulting peptide mass maps and the associated fragmentation spectra were collectively used to interrogate S. aureus Mu50 sequences to generate statistically significant candidate identifications using GPS Explorer software (Applied Biosystems, Foster City, California, United States) running the MASCOT search algorithm (http://www.matrixscience.com). Searches were performed allowing for complete carbamidomethylation of cysteine, partial oxidation of methionine residues, and one missed cleavage. Molecular Weight Search (MOWSE) scores, number of matched ions, number of matching ions with independent MS/MS matches, percent protein sequence coverage, and correlation of gel region to predicted protein were collectively considered for each protein identification (all data are presented in Table S1).

**Growth curve assays.** S. aureus cultures were grown overnight under low-iron conditions by inoculating strains in RPMI supplemented with 1% casamino acids plus 200 μM DIP. Overnight cultures were then subcultured in NRPMI (Chelex- treated RPMI) containing 500 μM DIP, and inoculated into NRPMI (NRPMI containing 25 μM ZnCl2, 25 μM MnCl2, 1 mM MgCl2, 100 μM CaCl2) supplemented with 500 μM DIP, and 20 μM iron sulfate or 10 μM hemin as indicated. Cultures were grown at 37°C with aeration in a round-bottom 96-well plate and bacterial growth was monitored by increase of absorbance at 630 nm.

**Measuring pH, transferrin-Fe release, and lactate.** Bacteria were grown in 10 ml of tryptic soy broth (TSB) in a 50-ml flask for 15 h at 37°C with 180 rpm shaking. All strains containing derivatives of pOS1 were grown in the presence of 10 μg/ml chloramphenicol to ensure successful maintenance of the plasmid and to normalize growth conditions. Iron was chelated from the media by adding DIP to a final concentration of 1 mM. After 15 h, the cultures were centrifuged and the supernatants were collected. All pH values were measured using a S29 SevenEasy pH meter (Mettler Toledo).

Measuring the release of iron from transferrin was performed as previously described [47]. Iron-bound transferrin exhibits an absorption peak at 470 nm. As iron dissociates from transferrin the intensity of the peak at 470 nm absorption decreases. Absorption at 470 nm was measured every 30 s for 15 min upon introduction of the samples. Transferrin stock solutions of 400 μM were prepared by suspending hemin-containing transferrin (Sigma, St. Louis, Missouri, United States) in distilled water. Transferrin stock solutions were added at a final concentration of 40 μM to all samples. All absorption readings were measured using a Cary 100 UV-Vis spectrophotometer (Varian).

To measure lactate concentrations, bacteria were grown in 5 ml TSB for 15 h at 37°C with 180 rpm shaking. Lactate levels were measured using a Lactate Assay Kit according to manufacturer’s recommendations (Promega, Madison, Wisconsin, United States) running the MASCOT search algorithm (http://www.matrixscience.com). Searches were performed allowing for complete carbamidomethylation of cysteine, partial oxidation of methionine residues, and one missed cleavage. Molecular Weight Search (MOWSE) scores, number of matched ions, number of matching ions with independent MS/MS matches, percent protein sequence coverage, and correlation of gel region to predicted protein were collectively considered for each protein identification (all data are presented in Table S1).

**Supporting Information**

Table S1. Master Table of All DIGE Profiling and MS Database Search Results

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**Author contributions**. DBF, DLS, GP, VJT, and EPS conceived and designed the experiments. DBF, DLS, GP, CWV, VJT, and EPS performed the experiments. DBF, DLS, CWV, VJT, and EPS analyzed the data. DBF, DLS, and EPS wrote the paper.

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**Competing interests.** The authors have declared that no competing interests exist.

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