The *srhSR* Gene Pair from *Staphylococcus aureus*: Genomic and Proteomic Approaches to the Identification and Characterization of Gene Function†

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ABSTRACT: Systematic analysis of the entire two-component signal transduction system (TCSTS) gene complement of *Staphylococcus aureus* revealed the presence of a putative TCSTS (designated SrhSR) which shares considerable homology with the ResDE His-Asp phospho-relay pair of *Bacillus subtilis*. Disruption of the *srhSR* gene pair resulted in a dramatic reduction in growth of the *srhSR* mutant, when cultured under anaerobic conditions, and a 3-log attenuation in growth when analyzed in the murine pyelonephritis model. To further understand the role of SrhSR, differential display two-dimensional gel electrophoresis was used to analyze the cell-free extracts derived from the *srhSR* mutant and the corresponding wild type. Proteins shown to be differentially regulated were identified by mass spectrometry in combination with protein database searching. An *srhSR* deletion led to changes in the expression of proteins involved in energy metabolism and other metabolic processes including arginine catabolism, xanthine catabolism, and cell morphology. The impaired growth of the mutant under anaerobic conditions and the dramatic changes in proteins involved in energy metabolism shed light on the mechanisms used by *S. aureus* to grow anaerobically and indicate that the staphylococcal SrhSR system plays an important role in the regulation of energy transduction in response to changes in oxygen availability. The combination of proteomics, bio-informatics, and microbial genetics employed here represents a powerful set of techniques which can be applied to the study of bacterial gene function.

Staphylococcus aureus has gained notoriety as an opportunistic pathogen capable of causing a wide variety of diseases ranging from skin and soft tissue surface lesions to more serious ailments including endocarditis, septic arthritis, and toxic shock syndrome (*1*). The ability of *S. aureus* to cause such varied diseases relies in part upon the controlled elaboration of a complex series of virulence factors which include surface adhesins, toxins, extracellular enzymes, and extracellular polysaccharide (*2*). To this end the organism has developed numerous regulatory effectors, such as Sar, Sae, and Agr (*3*−*5*), which are used to sense the immediate surroundings and modulate virulence gene expression accordingly. In this manner, *S. aureus* is able to rapidly adapt to changing environmental parameters and deploy only those factors required for growth and survival within a particular niche.

Within surface lesions, oxygen is freely available, and *S. aureus* is therefore able to respire normally; however, with deeper more persistent infections, oxygen quickly becomes limiting, and the invading bacteria must switch to an anaerobic form of energy generation (*6*, *7*). Thus, for *Staphylococcus* to cause disease, it must sense the immediate oxygen tension and switch to the appropriate form of energy metabolism. Although the molecular mechanisms which *S. aureus* employs to sense and respond to changes in oxygen tension have not been identified, the ability of *S. aureus* to adapt to oxygen-limiting environments has been well documented (*8*). *S. aureus* is a facultative anaerobe capable of growing in the absence of oxygen either by anaerobic respiration with nitrate as the terminal electron acceptor (*9*) or by carbohydrate fermentation (*10*). Under these conditions, ATP, derived from substrate level phosphorylation, is hydrolyzed by the F0/F1 ATPase complex in order to maintain membrane potential, and pyruvate is converted to a series of fermentation end-products which include diacetyl, ethanol, acetate, and formate (*11*).

In the related Gram-positive bacterium *Bacillus subtilis*, respiration (*12*) and anaerobic fermentation (*13*) are regulated by the *resD-resE* TCSTS.[1] ResDE positively regulate genes which are induced under anaerobic conditions, including the anaerobic regulator Fnr, the nitrate reductase genes *nasDEF*, and the flavohemoglobin gene *hmp*. A comparative pro-

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[1] Abbreviations: 2DE, two-dimensional gel electrophoresis; DTT, dithiothreitol; ES/MS/MS, electrospray tandem mass spectrometry; IPG, immobilized polyacrylamide gradient; LB, Luria broth; LC/MS/MS, liquid chromatography tandem mass spectrometry; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; SDS, sodium dodecyl sulfate; TCA, tricarboxylic acid; TCSTS, two-component signal transduction system; TSA, tryptone soy agar; TSB, tryptone soy broth.

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teomics approach recently has been used to evaluate the effects of a *resDE* mutation in *B. subtilis* (*14*). The authors found that a number of proteins including lactate dehydrogenase (LctE), the flavohemoglobin Hmp, and YwfI, a protein of unknown function, were induced under anaerobic conditions and dependent upon *resDE* expression. The pattern of regulation exhibited by the *resDE* mutant, when taken together with results from the analysis of an *fnr* mutant, suggests that the effects of a *resDE* mutation may be mediated through Fnr.

Through large-scale sequencing of the *S. aureus* genome, 16 gene pairs predicted to encode homologues of the TCSTS protein families were identified. Of these, one locus, termed *srhSR*, displayed remarkably high homology with the ResDE signaling pair of *B. subtilis*. Here we report the preliminary characterization of the *srhSR* gene pair, and the phenotypic effects of a *srhSR* deletion. We have employed a proteomics approach utilizing differential display 2D gel electrophoresis combined with mass spectrometry analysis to determine some of the putative members of the SrhSR regulon.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. S. aureus strains WCUH29 (NCIMB 40771; *15*) and RN4220 (*16*) were cultured in tryptone soy broth (TSB; Difco), agar-based TSB plates (TSA), or Luria broth (LB; Difco) at 37 °C. Aerobic cultures of *S. aureus*, grown in either TSB or LB, were inoculated with an overnight culture of *S. aureus* and incubated in conical flasks with shaking at 250 rpm. Unless otherwise specified, the culture medium did not exceed 15% of the flask volume. Anaerobic cultures of *S. aureus* were incubated in a Bactron anaerobic incubator (Sheldon Manufacturing Inc.) at 37 °C with periodic shaking. Prior to inoculation, oxyrase (Oxyrase Inc.) was added to remove any residual oxygen from the medium. Bacterial growth was monitored by measuring the optical density of the cell suspension at 600 nm. Plasmid DNA isolation, restriction analysis, ligation, PCR, and Southern blot analysis were performed as previously described (*17*).

Construction of S. aureus srhSR Deletion Mutant. The construction of a *S. aureus srhSR* deletion mutant has previously been described (*18*). Briefly, a 650 bp region corresponding to nt 1−665 of *srhR* and a 716 bp fragment corresponding to nt 162−878 of *srhS* were generated using 321HRA2/321HRB2R and 321HRC2R/321HRD2 primer pairs, respectively. The two fragments were joined by crossover PCR (*19*), and an additional in-frame termination codon was included at the junction between the truncated *srhR* gene and *srhS*. Recombination of this construct into *S. aureus* WCUH29 resulted in a deletion of 201 bp and represented a loss of the C-terminal region of the response-regulator component and a small 5′ section of the histidine kinase component effectively destroying translational coupling between these two open reading frames.

Sample Preparation for 2D SDS−PAGE. One liter of *S. aureus* cultures grown to mid-exponential phase was harvested by centrifugation at 5000*g* for 15 min. Cells were washed twice with 250 mL of ice-cold phosphate-buffered saline before being resuspended in 3 mL of lysis buffer (8 M urea, 4% CHAPS, 2% IPG buffer, pH 4−7, 50 mM DTT). An equal volume of glass beads (150−212 μm diameter) was added to the cell suspension, and the samples were then subjected to homogenization in a Biospec bead-beater for 6 × 30 s, samples being chilled on ice for 1−2 min between each cycle. The suspension was clarified by centrifugation at 12000*g* for 5 min and the supernatant stored at −80 °C for further analysis.

Analytical 2D Gel Electrophoresis and Protein Identification. Prior to electrophoresis, the protein concentration was determined using the Bradford assay (*20*). Aliquots containing approximately 100 μg of protein extract were diluted in 2D-PAGE rehydration buffer (8 M urea, 2% CHAPS, 0.5% IPG buffer, pH 4−7, 20 mM DTT) to a final volume of 350 μL and centrifuged at 100000*g* to remove any insoluble material. For the first dimension, pH 4−7 IPG strips (Amersham Pharmacia) were used. Strips were allowed to reswell in rehydration buffer containing the sample for 12 h at 30 V; focusing was then carried out for 80 000 Vh. After the first dimension, strips were incubated in equilibration buffer (6 M urea, 2% SDS, 0.1 M Tris-HCl, pH 6.8, 30% glycerol) containing 70 mM DTT for 15 min before being washed for a further 15 min in equilibration buffer containing 70 mM iodoacetamide. The second dimension separation was performed on 12% polyacrylamide gels (18 × 20 × 0.1 cm) run at 50 mA/gel in a Protean II apparatus (BioRad). Gels were stained using a modification of the Heukeshoven and Dernick protocol in which the use of glutaraldehyde was omitted for subsequent mass spectral analysis (*21*). Every sample was run at least in triplicate. Differences in the expression profile were assessed by visual inspection, and protein spots were quantified using the Image Master Elite software (Amersham Pharmacia).

Mass Spectral Analysis and Protein Identification. Differentially expressed protein spots were excised from a series of silver-stained gels (2−6 according to intensity) and in-gel-digested with trypsin (*22*). Digests were analyzed either by nano-electrospray tandem mass spectrometry (nano-ES/MS/MS) or by LC/MS/MS. Samples were desalted and concentrated (*22*) and then eluted in 2 μL of 60% methanol, 5% formic acid directly in the nanospray needle. Spectra were acquired on a QTOF hybrid instrument equipped with a Z-spray nanosource. MS/MS analysis was performed on selected ions (up to 12 per sample); the collision energy was manually adjusted to achieve optimized fragmentation.

For LC/MS/MS analysis, samples were loaded onto an LC Packing (PepMap) C18 trap cartridge, which was washed with 0.1% TFA. Peptides were backflushed off the cartridge onto a column [LC Packing, C18 (300 μm × 15 cm, 3 μm)] at 4 μL/min with a 5−50% acetonitrile−water gradient containing 0.1% TFA. The column outlet was connected to a Micromass micro-ionspray. Flow was split in the micro-ionspray source, directing only 0.5 μL/min to the 20 μm ES tip. Spectra were acquired on a QTOF hybrid instrument with automated MS to MS/MS switching for data acquisition. MS scans were acquired over the range *m*/*z* 400−1400 at 1.5 s/scan. MS/MS data were acquired for 12 s using three different preset collision energies to optimize fragmentation. Partial sequence data generated either by nano-ES/MS/MS or by micro-LC/MS/MS were matched against a *S. aureus* genome sequence database using the PepSea (Protana) search engine.

Pyelonephritis Infection Model. Overnight cultures of *S. aureus* WCUH29 were inoculated from single colonies in 5

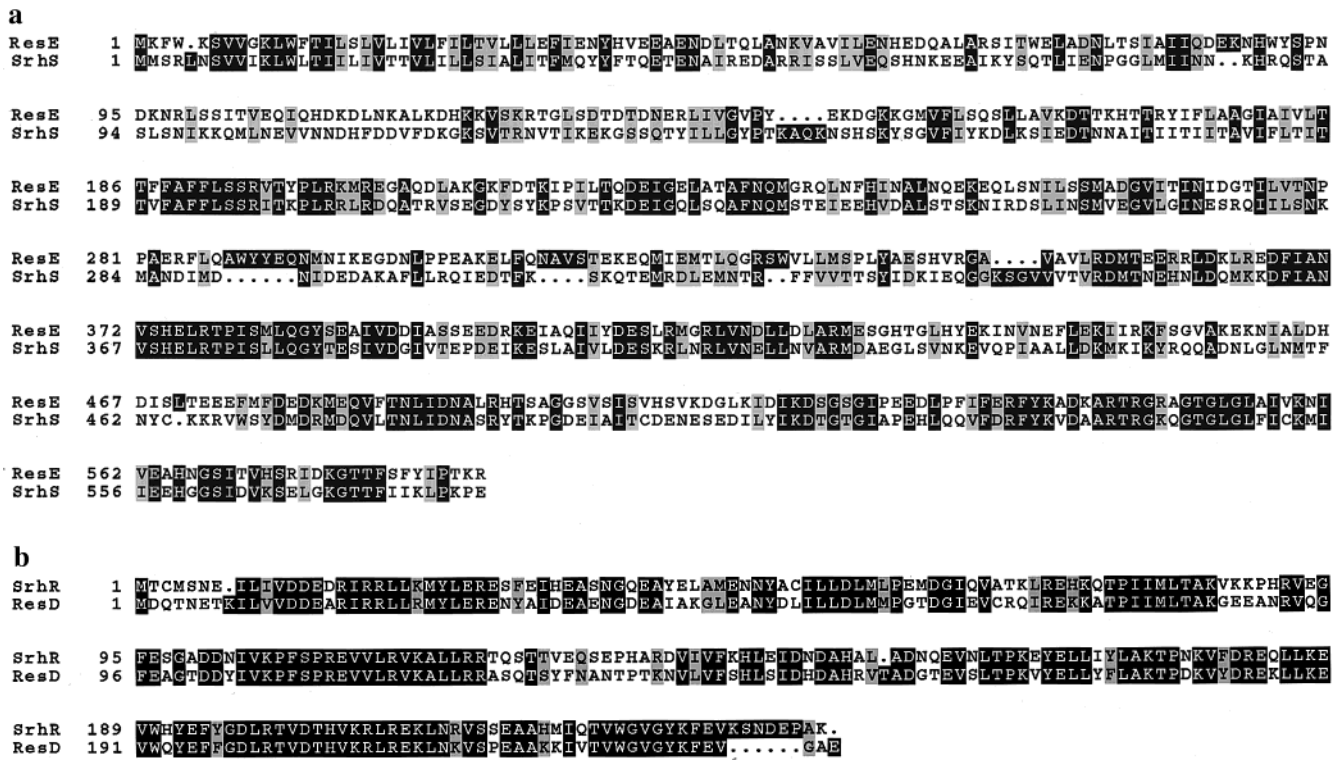

FIGURE 1: Comparison of the deduced ResE (a) and ResD (b) homologues from *B. subtilis* and *S. aureus*. The deduced amino acid sequences were aligned using the ClustalV multiple sequence alignment software. Residues that are identical or conserved are shaded in black or gray, respectively.

mL of tryptic soy broth (TSB) and grown at 37 °C with shaking. The cultures were then washed twice in sterile PBS and diluted to an A_{600} of 0.2. Female CD-1 mice (18−20 g) were inoculated with 0.2 mL of this suspension (containing approximately 10^7 bacteria) by tail vein injection.

Animals were euthanized via carbon dioxide overdose at 5 days post-inoculation. Both kidneys were removed using aseptic technique before being homogenized in 1 mL of PBS and the homogenates diluted in PBS. Viable bacteria were enumerated after plating on TSA plates.

RESULTS

Identification and Sequence Analysis of SrhSR. Analysis of the entire TCSTS gene complement of *S. aureus* identified eight loci orthologous to TCSTS gene pairs previously identified in *B. subtilis* (Throup et al., manuscript in preparation). Of these, one TCSTS displayed extensive identity with the ResDE pair of *B. subtilis* (*15*). We therefore named the *S. aureus* TCSTS, SrhSR (for **s**taphylococcal ***r****esDE* **h**omologues). Unlike the *resDE* gene pair, which along with *resABC* forms a five gene operon in *B. subtilis*, sequence analysis indicates that the *srhSR* genes are transcribed as a single gene pair and do not form part of a larger operon since the locus is apparently separated from neighboring genes by over 100 nucleotides.

The *srhS* gene of *S. aureus* encodes a predicted protein of 584 amino acids with a calculated molecular mass of 66.1 kDa which shares 33.7% identity with the ResE histidine kinase (Figure 1a). In common with its closest homologue, ResE, SrhS displays all of the characteristics typical of the histidine kinase protein family, including a conserved histidine residue (H 369), the predicted site of phosphorylation, and the H, N, D, F, and G boxes predicted to form a nucleotide binding surface within the active site of the kinase. Hydropathy plots of ResE and SrhS revealed that the N-terminal regions of both proteins contain two predicted transmembrane domains separated by an extracellular linker region of approximately 140 amino acids stretching from Y38 to T174. The N-terminal region of histidine kinases is generally considered to be the most variable domain, since it is thought to form the sensory domain responsible for binding or detecting a particular ligand or stimulus. Even within this domain (residues 1−364) both ResE and SrhS share 27.4% identity and 54% similarity, suggesting that both kinases bind and respond to the same stimuli. The *srhR* gene encodes a protein of 244 aa with a calculated molecular mass of 29.3 kDa; the deduced protein shares over 67.8% identity with ResD (Figure 1b), its closest homologue, both proteins belong to the OmpR family of response regulators and contain a winged helix−turn−helix motif.

Construction of a S. aureus srhSR Mutant. Initial attempts to disrupt the *srhSR* gene pair through plasmid insertion revealed that the mutation was unstable in the absence of selection (data not shown). We therefore adopted the "hit & run" mutagenesis approach (*18*) to generate a stable unmarked deletion within the histidine kinase and response regulator. Recombination of the construct resulted in a 201 bp deletion comprised of the C-terminal response regulator and a small 5′ section of the histidine kinase and the addition of an in-frame TAA codon to ensure translational termination of the truncated response regulator. One strain, Δ30321, was identified which contained the expected deletion. The successful construction of this mutant resulted in a deletion which would render the genes encoding the truncated histidine kinase and response regulator inactive and destroy translational coupling between *srhR* and *srhS*. Our studies

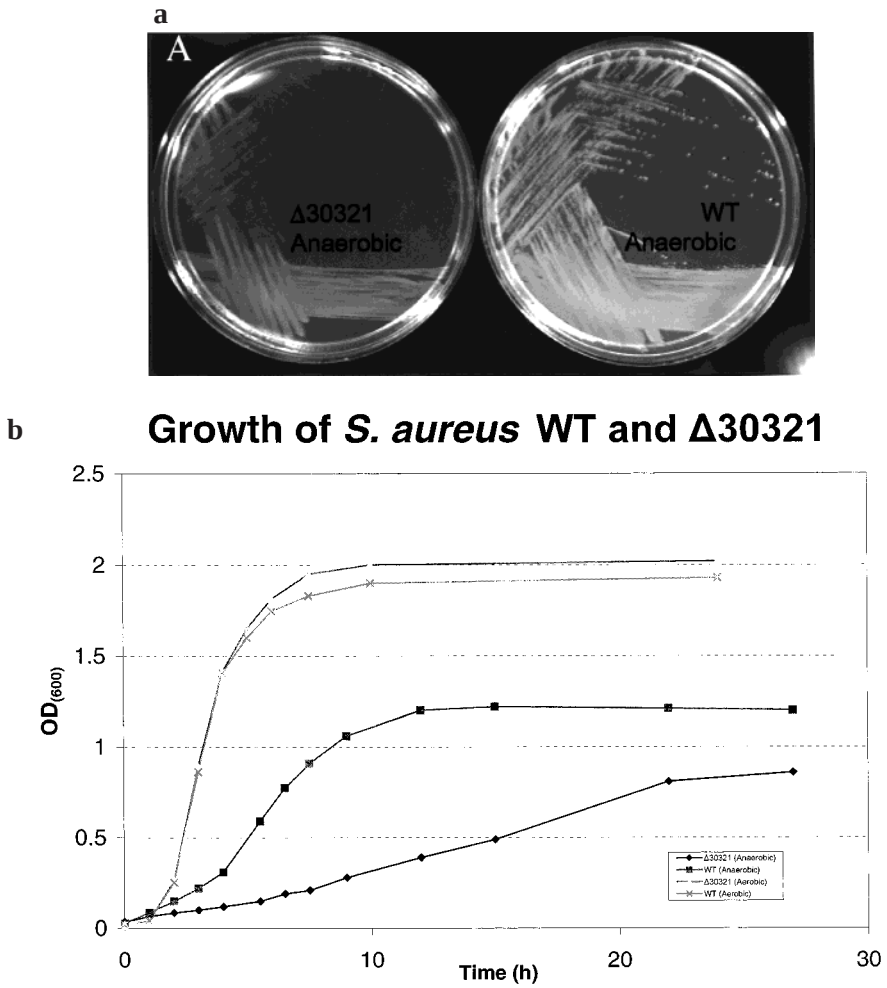

FIGURE 2: Growth of the *srhSR* mutant and *S. aureus* parental strain cultured in TSB under both aerobic and anaerobic conditions.

confirmed that the *srhSR* locus was not essential for *S. aureus* growth under aerobic conditions.

Effect of Oxygen on the Growth of a S. aureus srhSR Mutant in Vitro. Since previous studies have demonstrated that the *resDE* gene pair of *Bacillus* is important for bacterial growth under anaerobic conditions, we analyzed the growth of the *srhSR* mutant and the corresponding wild type under aerobic and anaerobic conditions. When streaked onto TSA plates and incubated under anaerobic conditions, the *srhSR* mutant displayed a marked reduction in growth when compared with the parental strain (Figure 2a); in contrast, no differences in colony size or growth rate could be detected when strains were incubated under aerobic conditions. To further quantify these differences, both the *srhSR* mutant and the wild type were cultured in TSB under aerobic and anaerobic conditions, and the growth rate of each culture was assessed by monitoring changes in optical density at 600 nm (Figure 2b). Under aerobic conditions, the growth of the mutant was indistinguishable from that of the wild type; both cultures entered stationary phase at an OD_{600} of 1.8 after approximately 5−7 h of incubation. Under anaerobic conditions, the wild type grew slowly and to a lower OD_{600} of 1.2 after 12 h, reflecting the switch from respiration to fermentation, which, in energetic terms, proves far less lucrative. Under these conditions, growth of the *srhSR* mutant was severely repressed; when compared with the wild type, the mutant only reached an OD_{600} of 0.8 after 22 h, and the doubling time at mid-exponential phase increased from 2.8 h for the parental strain to 11 h for the mutant.

Two-Dimensional Gel Electrophoresis of S. aureus and srhSR Mutant Cell Lysates. To assess the effects of the *srhSR* mutation and to determine the regulatory function of the SrhSR TCSTS pair, extracts from the mutant and the parental strain (WCUH29) grown to mid-exponential phase under aerobic or anaerobic conditions were subjected to two-dimensional gel electrophoresis (2DE). 2DE experiments were repeated at least 3 times with each sample to ensure accuracy, and gels were silver stained using a protocol compatible with subsequent mass spectrometric analysis. Whole cell preparations of *S. aureus* wild type and *srhSR* mutant resulted in well-defined 2D profiles containing approximately 400 distinct protein spots; representative profiles of the *S. aureus* samples examined are shown in Figures 3 and 4. Differences in the protein profiles of samples drawn from each strain grown under aerobic (Figure 3) or anaerobic (Figure 4) conditions were identified by visual inspection of the silver-stained gels. For a more comprehensive quantitative analysis, those spots found to be differentially expressed were further characterized using image analysis software (Tables 1 and 2). Only differences found to be conserved in all three 2DE gels and only those whose synthesis was induced or repressed by a factor of 2-fold or more in the parental strain when compared with the mutant grown under comparable conditions were con-

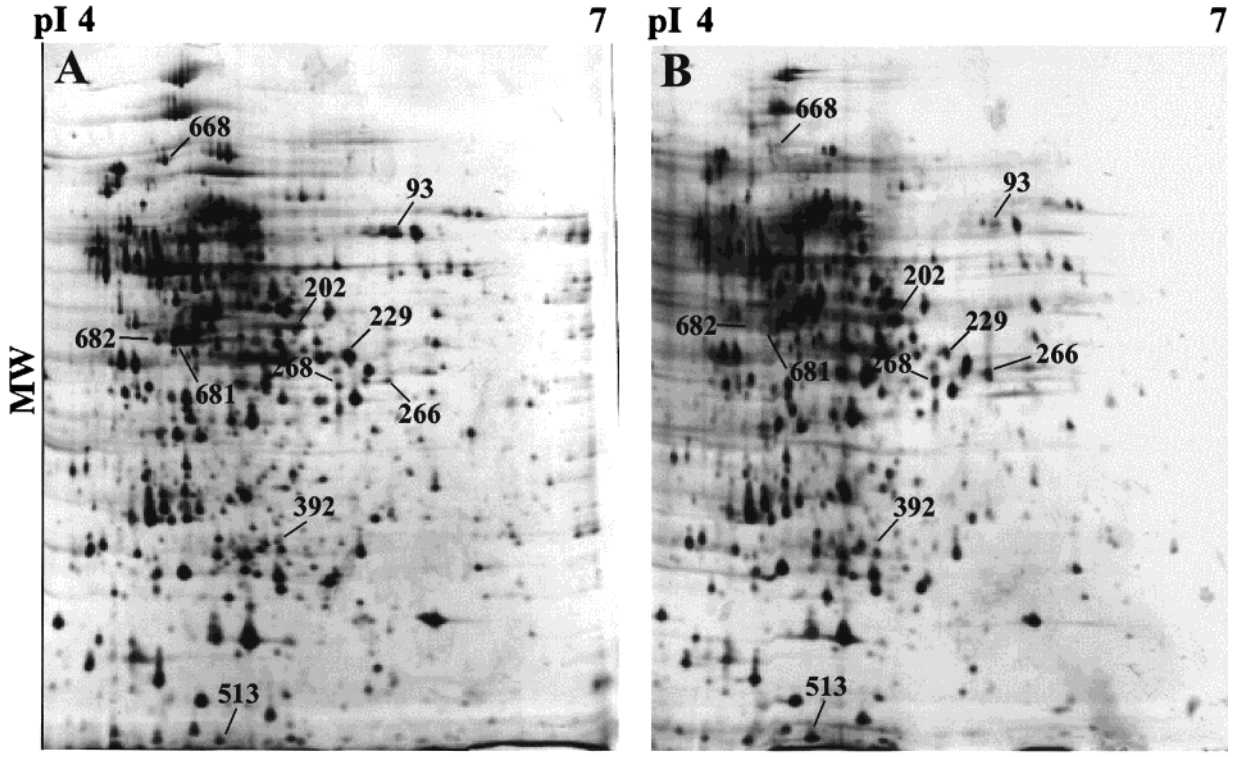

FIGURE 3: Expression profiles of cytoplasmic proteins from *S. aureus* (A) and the *srhSR* mutant DL321 (B), grown under aerobic conditions. Proteins exhibiting at least a 2-fold difference in expression in the *srhSR* mutant when compared to the parental strain grown under comparable conditions are labeled and numbered according to an *S. aureus* master gel (unpublished data). These spots were excised, and digested with trypsin. Peptides from the unfractionated tryptic digests were sequenced by mass spectrometry.

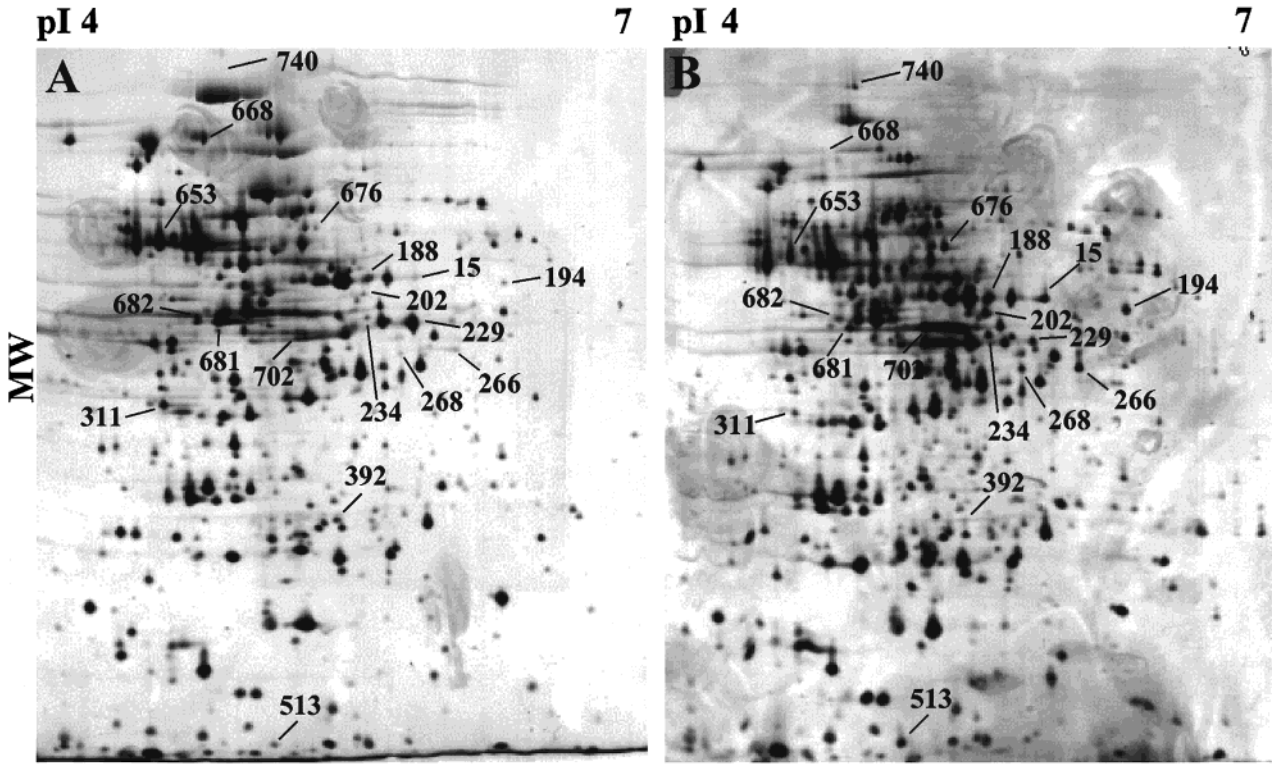

FIGURE 4: Expression profiles of cytoplasmic proteins from *S. aureus* (A) and the *srhSR* mutant DL321 (B), grown under anaerobic conditions. See Figure 3 for details.

sidered further. Although the profiles of the *srhSR* mutant and wild type were broadly similar, when cultured under comparable conditions, several differences in the protein profile of the two strains were identifiable. Under aerobic conditions, 10 protein spots were found to be differentially expressed in the *srhSR* mutant (Table 1). Of these, the expression of at least four proteins was induced by a factor of 2-fold or greater in the *srhSR* mutant; conversely, the expression of six protein spots appeared to be reduced in the mutant. In anaerobic conditions, the expression of 19

Table 1: Quantitation and Identification of Proteins Differentially Expressed in the *srhSR* Mutant, When Compared with the Wild Type, Grown under Aerobic Conditions[a]

(A) Proteins Induced

spot no.	spot volumes WT	spot volumes *srhSR*	induction (*x*-fold increase)	closest homologue	% identity	function
266	14	48	3.4	P80865	81.4	succinyl-CoA synthetase α
202	34	86	2.5	P38021	74.4	ornithine amino transferase
268	11	42	3.8	CAA70780	100.0	arginase
513	25	52	2.1	CAB11363	47.0	unknown (YlbP)

(B) Proteins Repressed

spot no.	spot volumes WT	spot volumes *srhSR*	repression (*x*-fold decrease)	closest homologue	% identity	function
392	8	<1	>8	AAB81287	100	cell shape and morphology
229	86	29	3.0	AAA27682	68.1	alcohol dehydrogenase
681	101	32	3.2	AAA22563	54.4	lactate dehydrogenase
682	34	<1	>34	AAA22563	54.4	lactate dehydrogenase
668	26	3	8.7	CAA44207	54.5	ATPase
92	53	22	2.4	S66039	77.0	inositol monophosphate dehydrogenase

[a] Spot volumes were measured and normalized as described under Materials and Methods and represent arbitrary units of expression. For this experiment, the limit of detection for each protein spot was determined to be 1.0 unit. *S. aureus* ORFs were identified by mass spectrometry-based peptide sequencing and database searching; the BLAST version 2.0.4 (*39*) software was then used to identify homologues in the nonredundant database and thereby assign a putative function.

Table 2: Quantitation and Identification of Proteins Differentially Expressed in the *srhSR* Mutant, When Compared with the Wild Type, Grown under Anaerobic Conditions[a]

(A) Proteins Induced

spot no.	spot volumes WT	spot volumes *srhSR*	induction (*x*-fold increase)	closest homologue	% identity	function
266	8	45	5.6	P80865	81.4	succinyl-CoA synthetase α
688	5	20	4.0	P80886	77.7	succinyl-CoA Synthetase *β*
202	4	40	10.0	P38021	74.4	ornithine amino transferase
268	2	19	9.5	CAA70780	100.0	arginase
702	60	138	2.3	BAA85589	67.1	lactate dehydrogenase
513	11	31	2.8	CAB11363	47.0	unknown (YlbP)
194	4	33	8.3	JE0388	65.8	alanine dehydrogenase
15	4	45	11.3	CAB07953	52.7	NADH dehydrogenase
740	1	30	30.0	P09339	72.0	aconitase hydratase
676	4	36	9.0	P07343	66.7	fumarase
234	6	23	3.8	not identified		
188	22	59	2.7	not identified		

(B) Proteins Repressed

spot no.	spot volumes WT	spot volumes *srhSR*	repression (*x*-fold decrease)	closest homologue	% identity	function
392	19	<1	>19	AAB81287	100	ScdA
229	104	31	3.4	AAA27682	68.1	alcohol dehydrogenase
681	146	41	4.6	AAA22563	54.4	lactate dehydrogenase
682	51	12	4.3	AAA22563	54.4	lactate dehydrogenase
653	107	51	2.1	CAA82256	82.7	ATPase *β*
668	39	<1	>39	CAA44207	54.5	ATPase
311	52	15	3.4	BAA75269	86.9	EF-Tu

[a] See Table 1 for details.

proteins spots was altered in the *srhSR* mutant (Table 2); 7 proteins were down-regulated, and 12 proteins were apparently up-regulated in the mutant.

Twenty-one spots which appeared to be differentially expressed in the *srhSR* mutant under either aerobic or anaerobic conditions were excised from selected silver-stained gels and in-gel-digested with trypsin. Peptides from the unfractionated tryptic digests of each individual spot were sequenced by mass spectrometry using either nano-ES/MS/MS or on-line LC/MS/MS (for example, see the nano-ES/MS/MS spectrum in Figure 5). To identify the proteins, we searched a translated *S. aureus* genomic sequence database with peptide sequence tags (*23*) derived from the MS/MS data. We chose to make the protein identifications based upon MS-derived sequence data rather than the more commonly employed peptide mass mapping (*24*) because in our experience the success rate for identifications is higher when sequence data are used for the database search (unpublished data).

MS analysis of the 21 spots yielded the identities of all but 2 of the differentially regulated proteins. BLAST searches of these proteins against the nonredundant database were

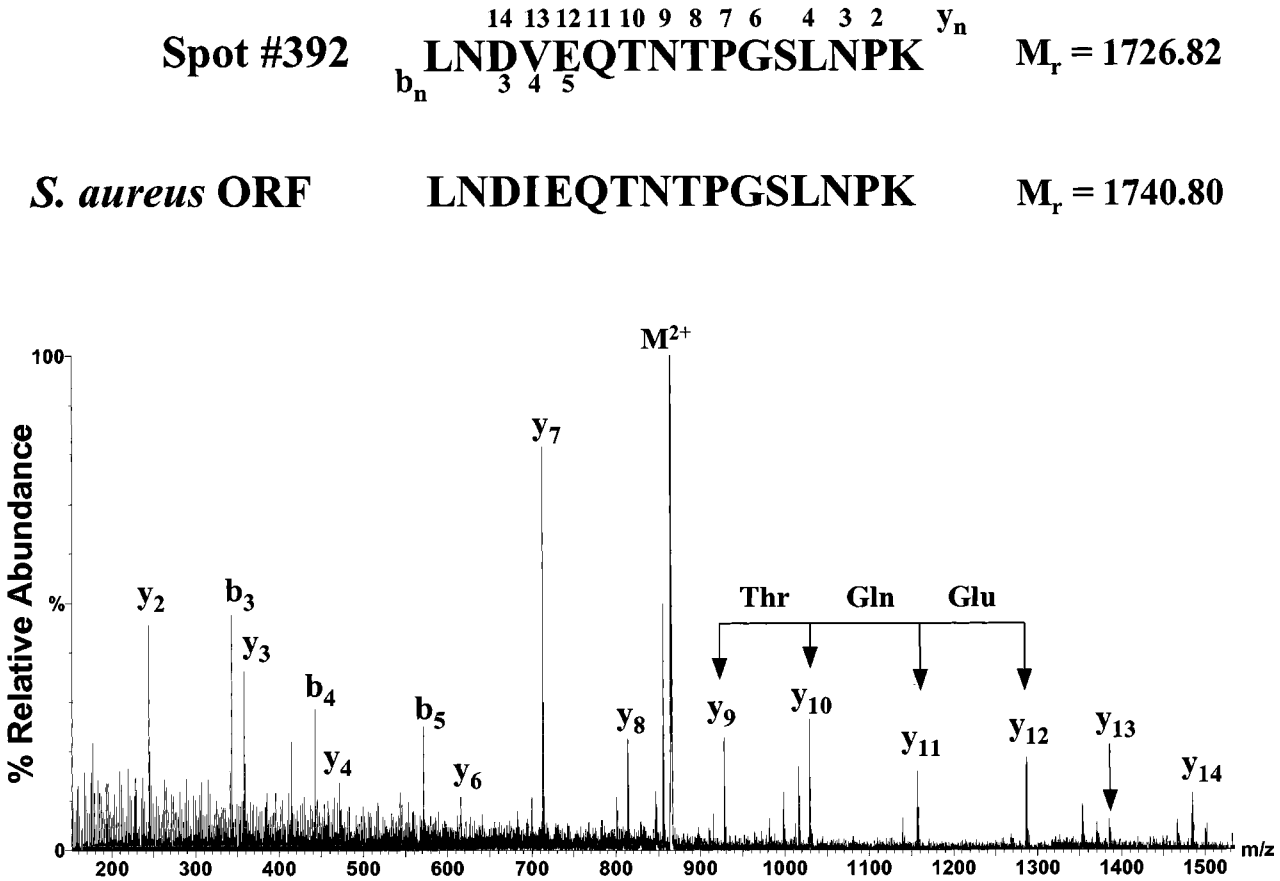

FIGURE 5: Identification of spot 392 by MS-based sequence tag database search. MS/MS product ion mass spectrum of the M^{2+} ion *m*/*z* 864.26 (M_r = 1726.52). The sequence tag (1286.9)EQT(927.8) was extracted from the spectrum and used to search a translated *S. aureus* genomic sequence database in an error-tolerant manner. A single match was found to the *S. aureus* sequence LNDIEQTNTPGSLNPK. Correlation of the mass spectrum with the matched sequence confirmed the identity of the peptide and pinpointed a valine to isoleucine substitution at position 4. Sequence coverage for the peptide derived from spot 392 is shown.

used to identify homologues of each protein and, where possible, assign a putative function; these findings are summarized in Tables 1 and 2. One protein (spot 392) corresponded to the staphylococcal ScdA protein which had previously been submitted to the GenBank database (*25*); 17 proteins had their functions inferred by homology; 1 protein (YlbP) was homologous to proteins of unknown function.

A high proportion (10/18) of proteins identified appear to participate in aspects of energy generation which include glycolysis, the TCA cycle, fermentation, and ATP synthesis. Four proteins (α/β subunits of succinyl-CoA synthetase, fumarase, and aconitase) catalyze reactions in the TCA cycle, each of which was up-regulated in the *srhSR* mutant when cultured under anaerobic conditions. Two fermentation enzymes, alcohol dehydrogenase (spot 229) and L-lactate dehydrogenase (spots 681 and 682), were down-regulated in the *srhSR* mutant in both aerobic and anaerobic conditions. In contrast, a second L-lactate dehydrogenase species (LDH II) was up-regulated in the mutant (spot 702). Under anaerobic conditions, two putative dehydrogenase enzymes [alanine dehydrogenase (spot 194) and NADH dehydrogenase (spot 15)] exhibited an approximately 10-fold increase in expression upon the deletion of *srhSR*. The deletion of *srhSR* also resulted in a greater than 2-fold reduction in expression of ATPase subunit B (spot 653) under anaerobic conditions.

In Vivo Assessment of a S. aureus srhSR Deletion Mutant. To determine whether a *srhSR* deletion had any impact on staphylococcal pathogenicity, a murine model of hematogenous pyelonephritis was employed to assess the ability of the *srhSR* mutant to establish an infection when compared with the parental strain; 0.2 mL of a suspension containing 10^7 cfu of either the *srhSR* mutant or the WCUH29 bacteria was inoculated into the tail vein of female CD1 mice. Five days post-inoculation, both kidneys were removed, and the bacterial load was determined. Figure 6 reveals that the disruption of the *srhSR* gene pair led to a 3-log reduction in kidney colonization when compared with the wild type.

DISCUSSION

Through a systematic evaluation of the genes identified within the genome sequence of *S. aureus*, we have identified one gene pair (termed *srhSR*) which shares considerable homology with the ResDE regulatory pair of *B. subtilis*. Previous studies have revealed that the *resDE* locus encodes a probable TCSTS which acts as a global regulator of aerobic and anaerobic metabolism in *Bacillus subtilis* (*26*). The high homology these two gene pairs share further indicates that they are functionally analogous in that they elicit responses to similar stimuli through a His-Asp phospho-relay cascade.

To evaluate the role of the *S. aureus srhSR* locus, we used hit and run mutagenesis (*18*) to disrupt the *srhSR* gene pair. Such an approach afforded minimal disturbance of the *srhSR*

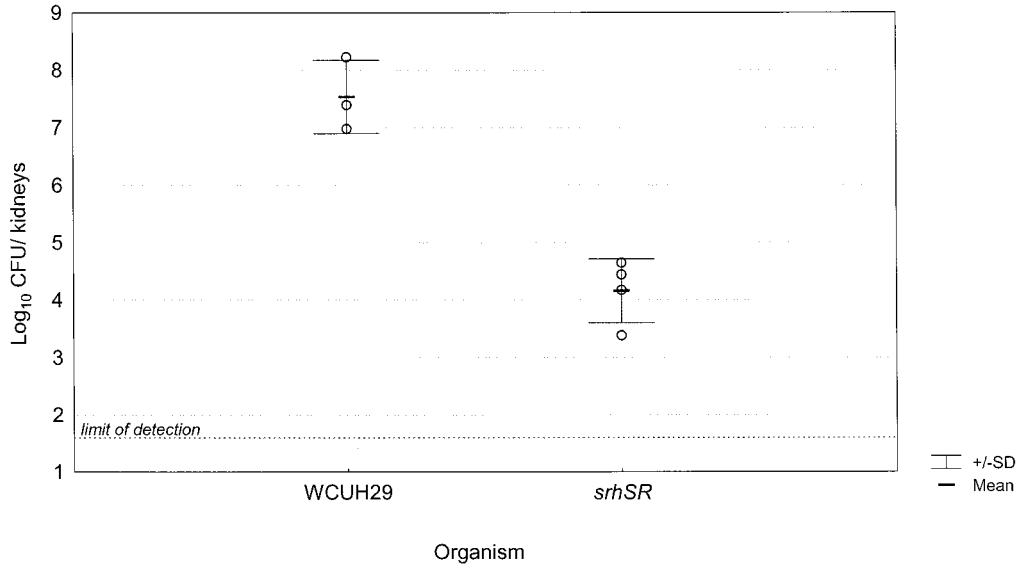

FIGURE 6: Attenuation of the *S. aureus srhSR* mutant in a mouse pyelonephritis infection model. The degree of attenuation was assessed as the reduction in mean bacterial counts recovered from animals inoculated with the mutant when compared with tissue sampled from animals infected with the parent strain.

genetic architecture and avoided the insertion of a selectable marker. Although the *srhSR* deletion did not result in any appreciable growth defects under aerobic conditions, our studies indicated that the *srhSR* gene pair is required for the fermentative growth of *S. aureus*, since the growth of a strain carrying the *srhSR* deletion was severely impaired when cultured under anaerobic conditions in the absence of an alternative electron acceptor. Previous studies using the *resDE* mutant of *B. subtilis* indicated that while *resD* is required for the regulation of aerobic and anaerobic respiration (*26*) mutations in this system resulted in a moderate reduction in fermentative growth (*13*). However, the reasons for this growth defect were not investigated. In an attempt to elucidate the function of this locus in *S. aureus*, cell lysates prepared from both the *srhSR* mutant and the parental strain were subjected to two-dimensional protein electrophoresis. The impaired growth of the mutant under anaerobic conditions and the dramatic changes in the expression of proteins involved in energy metabolism suggested that, in common with the *Bacillus subtilis resDE* gene pair, the staphylococcal system plays an important role in the regulation of energy transduction in relation to oxygen availability. 2DE studies of a *Bacillus resDE* mutant indicated that many of the regulatory effects exerted by this locus were mediated through Fnr (*14*). In contrast to *B. subtilis*, analysis of three incomplete *S. aureus* genome sequence databases,[2] each with a theoretical coverage of over 98%, has failed to identify an Fnr homologue (data not shown), indicating that *S. aureus* lacks this key regulator. Thus, it would seem likely that significant differences exist between *resDE*-mediated gene regulation in *B. subtilis* and *srhSR*-mediated gene regulation in *S. aureus*.

The media and growth conditions we employed lack sufficient alternative electron acceptors to maintain respiratory activity in the absence of oxygen. Bacteria grown under such conditions must therefore utilize fermentation to generate sufficient energy for growth (*27*). Thus, chemical energy must be derived principally from glycolysis, membrane energization is rendered dependent on ATP hydrolysis, and the role of the TCA cycle is reduced to the minimum level required to supply biosynthetic precursors (*28*). Under these conditions, NADH generated from substrate level phosphorylation must be reoxidized to allow glycolysis to continue; fermentation then acts to recycle NAD through the reduction of pyruvate and other biochemical intermediates (*28*). We found that the *srhSR* deletion led to marked changes in the expression of a number of proteins involved in energy metabolism. Among these, most striking was the down-regulation of key fermentative enzymes such as alcohol dehydrogenase and lactate dehydrogenase under both aerobic and anaerobic conditions. Both enzymes play an important role during anaerobic fermentation since each catalyzes the formation of ethanol or lactate concomitant with the reoxidation of NADH. Previous studies reported that of the two enzymes, lactate dehydrogenase is particularly important for the anaerobic growth of *S. aureus* since lactate forms the bulk (80%) of the fermentation end products (*29*, *30*). The cell seems capable of (partially) compensating for this loss through the induction of a second, less abundant lactate dehydrogenase enzyme and alanine dehydrogenase, which may support the maintenance of the NAD pool.

In the presence of a suitable electron acceptor, reducing equivalents generated throughout glycolysis and the TCA cycle are used to generate ATP through respiratory electron transport and oxidative phosphorylation. During anaerobic fermentation, however, the respiratory electron-transport chain is halted, and the process of oxidative phosphorylation is effectively reversed (resulting in the hydrolysis of ATP) to generate the electrochemical proton gradient necessary to maintain membrane function (*28*, *31*). In bacterial systems, the F0/F1 ATPase complex is responsible for both oxidative phosphorylation and ATP hydrolysis; thus, the expression of this complex remains constant irrespective of the oxygen tension (*32*, *33*). Our studies indicate that the expression of the ATPase β subunit is down-regulated in the *srhSR* mutant when cultured under anaerobic conditions. In contrast, the

[2] *S. aureus* sequence databases: http://www.tigr.org/; http://www.sanger.ac.uk; http://www.genome.ou.edu.

srhSR mutant fails to repress the biosynthesis of NADH dehydrogenase, which forms the first component of the respiratory electron-transport chain. In the presence of a suitable electron acceptor, inhibition of the ATPase complex would simply result in reduction of the growth rate. Under fermentative conditions, however, a reduction in ATPase expression brought on by a *srhSR* deletion, coupled with the failure to minimize expression of NADH dehydrogenase, would deplete the cell's ability to generate a proton gradient across the cytoplasmic membrane, leading to the abortive initiation of the respiratory electron-transport chain. Together these factors would be expected to lead to the retardation or cessation of growth under anaerobic conditions.

In addition to changes in the level of fermentative enzymes and respiratory enzymes, a series of additional enzymes appear preferentially up-regulated in the *srhSR* mutant when grown in an anaerobic environment. Aconitase, fumarase, and the succinyl-CoA synthetase α and β subunits, each of which participates in discrete steps of the TCA cycle, are up-regulated in the *srhSR* mutant when grown anaerobically. Ordinarily under such conditions the role of the TCA cycle is reduced to the minimum level required to supply biosynthetic precursors, and these enzymes are down-regulated (*28*, *34*). Failure to curtail the TCA cycle in the absence of oxidative phosphorylation allows the production of NADH to continue, which must be reoxidized through the action of fermentative enzymes such as lactate dehydrogenase—the down-regulation of lactate dehydrognase serves to further exasperate this imbalance. The up-regulation of both ornithine amino transferase and arginase in the *shrSR* mutant indicates that *srhSR* appears to repress arginine catabolism, irrespective of oxygen tension. Results from several studies indicate that *S. aureus* may utilize the arginine dihydrolase pathway as a fermentative source of ATP (*35*); moreover, gene homologues encoding the arginine dihydrolase pathway enzymes (*arcABCD*) have been identified in the genome sequence of *S. aureus* (data not shown). When grown under anaerobic conditions, the repression of the arginine catabolism pathway via *srhSR* may be required to provide substrate for this additional fermentation pathway.

Thus, while each individual change in the expression of these factors may not be sufficient to prevent the growth of *S. aureus* when grown under anaerobic fermentative conditions, in combination the deleterious effects may be enough to prevent growth completely or at least slow growth dramatically.

The biological significance of the remaining changes identified in our study remains unclear at present. ScdA has previously been implicated in the control of cell morphology (*25*), while the *ylbP* gene remains uncharacterized. Identification of the ScdA and YlbP proteins emphasizes that the influence of *srhSR* is not restricted to the regulation of energy metabolism. Interestingly, the *B. subtilis resDE* gene pair has also been implicated in the control of attributes (e.g., phosphate regulation) in addition to respiration and energy metabolism, indicating that both the staphylococcal and *Bacillus* loci act as pleiotropic regulators of bacterial gene expression. Analysis of the *srhSR* mutant grown under anaerobic conditions indicated that expression of the elongation factor EF-Tu was down-regulated 3.4-fold when compared with the parental strain. Although no differences in the expression of the protein could be detected between the wild type and *srhSR* mutant when cultured under aerobic conditions, analysis of the parental strain revealed that EF-Tu is apparently up-regulated 16-fold upon anaerobiosis. It is possible that the differences observed here may stem from changes in the modification of EF-Tu as opposed to changes in protein expression per se, since we have previously detected several discrete spots which correspond to EF-Tu (data not shown). Previous studies have demonstrated that changes in culture conditions and environmental insults can result in changes in the modifications of EF-Tu which would be expected to affect the electrophoretic mobility of a particular protein species (*36*). Thus, to conclusively determine whether the SrhSR pair acts to regulate the expression of the proteins identified through this study, further studies will be required.

Our studies indicated that the SrhSR locus is important for the growth and proliferation of *S. aureus in vivo* since a deletion of this gene pair resulted in a dramatic reduction in bacterial counts in a murine pyelonephritis model when compared with the corresponding wild-type strain. Oxygen tension is known to vary between different tissues within the host (*7*). Thus, at some stages of infection, *S. aureus* may be forced to grow anaerobically either by anaerobic respiration or by fermentation. Previous studies have demonstrated that a series of staphylococcal virulence determinants including enterotoxin B, haemolysin A, and capsular polysaccharide are regulated in response to oxygen tension (*37*, *38*, *11*). Although the precise means of this regulation have not been ascertained, it is possible that *S. aureus* has adapted the SrhSR system as a means to monitor the external environment and regulate pathogenicity.

The 2D gel-based approach employed here does not provide a truly comprehensive view of the proteome. Protein expression in most cells ranges over 5−8 orders of magnitude. Given the limited loading capacity of a standard wide p*I* range gel (typically 50−500 μg), it is unlikely that low copy number proteins (<1000 copies/cell) will be observed. In addition, hydrophobic proteins, basic proteins (in our case p*I* > 7), and very large proteins (>100 kDa) are not solubilized and brought into focus using the 2D gel methodology employed here. Thus, whereas *S. aureus* has been predicted to code for >2000 ORFs, we observe only approximately 400 spots, and at least some of these will be protein products from the same gene (for example, elongation factor EF-Tu discussed earlier). Nevertheless, the combination of proteomics, bio-informatics, and directed gene deletion employed here represents a powerful set of techniques which can be applied to the study of bacterial gene function in the post-genomic era.

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