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Shortomics

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SHORTOMICS

Proteomic analyses of iron-responsive, Clp-dependent changes in *Staphylococcus aureus*

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One sentence summary: Proteomic analyses reveal Clp protease targets under varying nutrient iron conditions in the human pathogen *Staphylococcus aureus*.

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ABSTRACT

Staphylococcus aureus is a frequent human pathogen that is capable of causing a wide range of life-threatening infections. A promising antibacterial target is the Clp proteolytic system, which performs the vital function of maintaining protein turnover within the cell. This system primarily impacts the bacterial response to various stresses by degrading specific proteins but can also regulate a number of physiological processes through protein degradation. A critical stress to which *S. aureus* must adapt during infection of a vertebrate host is nutrient iron limitation. We have previously shown that the Clp system impacts expression of genes required for heme-iron acquisition during iron limitation and is required for staphylococcal infection. Based on these data, we sought to further define the Clp-dependent impact on *S. aureus* during iron limitation by characterizing the proteomic profiles of mutants inactivated for components of the Clp protease, including ClpP, ClpC and ClpX, in high- and low-iron conditions. Our results reveal numerous proteins altered in abundance in the *clp* mutants and provide new insights into the staphylococcal proteolytic network during nutrient iron limitation.

Key words: protein; degradation; 2D-DIGE

Staphylococcus aureus is an important human pathogen and significant public health concern due to widespread antibiotic resistance and frequent community- and hospital-associated infections. Characterization of factors that allow *S. aureus* to evade clearance in the host will aid in designing new anti-staphylococcal therapeutics. During infection, *S. aureus* experiences a number of stresses ranging from immune system attack to nutrient limitation. The major protease in *S. aureus* is the Clp protease, which relieves stress by degrading accumulated and misfolded proteins (Katayama-Fujimura, Gottesman and Maurizi 1987; Wickner, Maurizi and Gottesman 1999). The Clp protease also regulates numerous physiological processes, including metabolism, virulence and antibiotic resistance (Hecker, Schumann and Völker 1996; Conlon *et al.*, 2013). This proteolytic system is critical to staphylococcal pathogenesis as inactivation of *clp* genes significantly impairs virulence (Mei *et al.*, 1997; Frees *et al.*, 2003; Farrand *et al.*, 2013). The Clp protease has been successfully targeted by acyldepsipeptides, which activate ClpP and

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cause uncontrolled proteolysis resulting in bacterial cell death (Brotz-Oesterhelt *et al.*, 2005). Acyldepsipeptides eliminate *S. aureus* biofilms and cure persistent infections in animals, further supporting the intriguing possibility of Clp proteolytic dysregulation as a viable treatment strategy (Brotz-Oesterhelt *et al.*, 2005; Conlon *et al.*, 2013). Furthermore, targeting the Clp protease represents a potential therapeutic for several important Gram-positive pathogens, including *Streptococcus pneumoniae*, *S. pyogenes* and *Enterococcus faecalis* (Brotz-Oesterhelt *et al.*, 2005).

Nutrient limitation is a critical challenge that *S. aureus* must overcome during infection. This is particularly true in the case of iron, an important bacterial nutrient that is sequestered by host proteins to protect against infection. One strategy employed by some Gram-positive bacteria to circumvent iron sequestration is the iron-regulated surface determinant (Isd) system, which extracts and imports iron-containing heme from host hemoglobin (Mazmanian *et al.*, 2003; Pishchany, Dickey and Skaar 2009). We recently demonstrated that the staphylococcal Clp genes impact heme acquisition by altering expression of Isd genes, resulting in impaired heme import, reduced growth in low iron and decreased pathogenesis in a systemic infection model (Farrand *et al.*, 2013). We hypothesize that alteration of *isd* expression occurs through Clp-dependent proteolytic regulation of an as-yet-unidentified transcriptional regulator. To better understand the role of the Clp protease during iron limitation, we performed 2D difference in gel (2D-DIGE) analysis on cytoplasmic fractions isolated from *S. aureus* mutants inactivated for the Clp protease, ClpP or the Hsp100/Clp ATPases ClpC and ClpX, which interact with ClpP and initiate proteolysis (Wawrzynow 1995; Kim *et al.*, 2000; Singh *et al.*, 2000).

Clp mutants were generated in the *S. aureus* Newman background (Frees *et al.*, 2011; Farrand *et al.*, 2013). Three biological replicates of wild type, Δ*clpP*, Δ*clpC* or Δ*clpX*, were grown to stationary phase in tryptic soy broth with or without the iron chelator 2,2-dipyridyl overnight (DIP; 1 mM final concentration). Bacteria were harvested, lysostaphin treated to digest cell wall and lysed in the presence of phenylmethylsulfonyl fluoride using an Emulsiflex-C3 high pressure homogenizer (Avestin). Cellular debris was removed with ultracentrifugation (100 000 × g) and cytoplasmic fractions were isolated.

2D-DIGE using the mixed-sample internal standard method was performed essentially as described previously (Friedman *et al.*, 2006) using GE Healthcare Ettan DIGE instrumentation and software (Piscataway, New Jersey) per manufacturer's protocols. Twenty four individual samples, each containing 1 mg total protein, were coresolved using a 12-gel matrix where each gel contained a mixture of a pre-labeled Cy3/Cy5 pair of any two samples (167 μg protein each) plus a 167 μg aliquot of the Cy2-labeled pooled internal standard, for a total of 500 μg per gel. Proteins were resolved using 24 cm pH 4–7 IPG strips followed by 20 cm 12.5% polyacrylamide gel electrophoresis. 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 analyzed for significant protein abundance changes (Student's *t*-test and ANOVA) using the DeCyder v6.5 suite of software tools. The gels were stained for total protein content with SyproRuby (Molecular Probes/Invitrogen) for robotic spot picking and digestion with porcine trypsin protease (Trypsin Gold; Promega, Madison, WI, USA) using a GE Healthcare Spot Handling Workstation.

Peptide hydrolysates were analyzed by a 45 min data-dependent LC-MS/MS analysis. Peptides were resolved using an Eksigent 1D+ ultraHPLC equipped with an AS1 autosampler on an 18 cm Jupiter (3 μm, 300A) 100 μm internal diameter, self-packed analytical column coupled directly to an LTQ-orbitrap (Thermo Fisher) via a nano-electrospray source. A full scan mass spectrum followed by five data-dependent tandem mass spectra (MS/MS) was collected throughout the run using dynamic exclusion to minimize acquisition of redundant spectra. MS/MS spectra were searched against the *S. aureus* Newman protein database and human protein database (UniprotKB v155) using SEQUEST (www.ncbi.nlm.nih.gov/pubmed/7741214) and results filtered and collated using Scaffold (www.proteomesoftware.com). MS data are available at https://medschool.vanderbilt.edu/skaar-lab/our-projects.

Proteomic analyses of *S. aureus* Clp mutants revealed many proteins that changed in abundance in varied iron levels (Table 1). These proteins impact numerous cellular processes, including metabolism, stress response, transcriptional regulation, protein synthesis and electron transport (Fig. 1). Interestingly, many proteins altered in Δ*clpP* were similarly changed in Δ*clpX*, suggesting that the ClpXP protease targets these proteins.

We identified proteins that have previously been shown to change in an iron-dependent manner (Friedman *et al.*, 2006; Hempel *et al.*, 2011). Some proteins (PflB, RocD2, decarboxylase NWMN_0839, fumarylacetoacetate hydrolase-like protein NWMN_0839) likely change upon iron starvation and are not impacted by the Clps, as levels changed equivalently in low iron regardless of *clp* expression. Other proteins known to be affected by iron starvation changed in the absence of *clp* expression, including metabolic proteins FruB and Tkt, which were upregulated in low iron in wild-type *S. aureus* (Friedman *et al.*, 2006) but were reduced in Δ*clpX* or Δ*clpP*. Conversely, the ATP-binding Mrp/Nbp35 family protein (NWMN_2067) and peptidase M20/M25/M40 (NWMN_1418) were decreased in low iron in wild type (Friedman *et al.*, 2006) but were more abundant in Δ*clpP* regardless of iron status. A hydrolase (NWMN_0521) was increased upon iron starvation in wild type (Hempel *et al.*, 2011) but was downregulated in Δ*clpP* in both conditions. The aldehyde dehydrogenase AldA was decreased in low iron in wild type (Hempel *et al.*, 2011) but increased in Δ*clpX*. These results suggest the Clp protease or ATPases could be important for degrading or stabilizing these proteins under iron restriction.

These results are further supported by Clp-trap experiments in which mutant forms of ClpP or ClpC that bind but do not degrade proteins were used to identify Clp targets (Feng *et al.*, 2012; Graham, Lei and Lee 2013). Proteins altered in the *clpP* mutant and pulled down by $\text{ClpP}^{\text{trap}}$ included CodY, HslO, GuaB and SufD (Feng *et al.*, 2012).CodY and HslO, a transcriptional regulator and a heat shock protein, respectively, were more abundant in Δ*clpP* in iron-rich conditions, consistent with these proteins being Clp targets. Interestingly, GuaB and SufD, proteins involved in purine metabolism or iron–sulfur cluster assembly, were reduced in Δ*clpP* in low iron, perhaps suggesting that ClpP is required to maintain protein levels during iron limitation. Several proteins bound by $\text{ClpC}^{\text{trap}}$ were impacted by the loss of *clpC*, such as CodY, ClpB, ClpP, NWMN_0976 and an ATP-binding Mrp/Nbp35 family protein (Graham, Lei and Lee 2013), all of which were enhanced in this mutant.

These data revealed many proteins involved in iron homeostasis and transport that were impacted by the Clps in iron-rich conditions. The iron storage molecule ferritin (NWMN_1831), which is normally downregulated in low iron (Stenz *et al.*, 2011), was decreased in all three mutants in iron-replete conditions. This is consistent with lower reported cell-associated iron levels in Δ*clpC* compared to wild type during exponential

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Table 1. Compilation of proteins identified in 2D-DIGE analyses of *S. aureus clp* mutants compared to wild-type cells in iron-replete and -deplete (+ DIP) conditions. # = spot number. Protein = protein name or *S. aureus* Newman gene identifier. Acc number = NCBI accession number. % Cov = percent sequence coverage. # Pep = number of peptides identified. Fold = fold change relative to wild-type cells. P = *p* value according to Student's *t*-test. * = No reliable signal. *Staphylococcus aureus* proteins not included in this list were not changed in *clp* mutants relative to wild-type cells in iron-replete or -deplete conditions. Empty boxes reflect no observed change in the abundance of the protein compared to wild type in the specified condition.

#	Protein	Acc number	MWpI	% Cov	# Pep	Δ*clpP* Fold	P	Δ*clpP* ± DIP Fold	P	Δ*clpC* Fold	*p*	Δ*clpC* ± DIP Fold	*p*	Δ*clpX* Fold	*p*	Δ*clpX* ± DIP Fold	*p*
1	ClpB	150373857	98 4.96	35	32			2.91	0.006			3.82	0.004			−2.31	0.041
2	ClpB	150373857	98 4.96	45	45			3.83	0.014			6.46	0.003				
2	ValS	150374570	102 4.99	17	18			3.83	0.014			6.46	0.003				
3	SecA	150373734	96 5.11	33	33			4.33	0.018								
4	FusA	151220721	77 4.81	28	23	6.31	0.012	1.68	0.059	6.48	0.046			6.23	0.006		
4	ClpC	150373499	91 5.51	13	9	6.31	0.012	1.68	0.059	6.48	0.046			6.23	0.006		
4	PurL	150373949	80 4.77	13	9	6.31	0.012	1.68	0.059	6.48	0.046			6.23	0.006		
5	ClpC	151220699	91 5.51	35	34	15.2	0.068	12.31	.0004			−3.95	0.02			−6.54	0.018
6	ClpC	151220699	91 5.51	32	29	5.93	0.0002	11.21	0.001					−2.94	0.001		
7	ClpB	150373857	98 4.96	46	56	26.65	0.01	25.97	0.004	8.58	0.06	30.55	0.004				
7	TypA	150373986	69 4.93	18	12	26.65	0.01	25.97	0.004	8.58	0.06	30.55	0.004				
8	PflB	150373174	85 5.31	31	28											−16.6	0.054
9*																	
10	ClpL	150375460	78 4.88	46	49	2.09	0.043			3.09	0.004			1.63	0.007	−3.95	0.046
10	PykA	150374604	63 5.23	28	15	2.09	0.043			3.09	0.004			1.63	0.007	−3.95	0.046
11	Tkt	150374266	72 5.00	61	38	−1.9	0.003	−1.53	0.03					−1.33	0.035		
12	GuaA	150373393	58 5.02	26	18			−2.08	0.005							−2.06	0.042
12	SufB	15373801	53 5.08	30	14			−2.08	0.005							−2.06	0.042
12	Tkt	150374266	72 5.00	26	14			−2.08	0.005							−2.06	0.042
12	_0050	150373062	68 5.00	18	14			−2.08	0.005							−2.06	0.042
12	Zwf	150374424	57 5.31	17	10			−2.08	0.005							−2.06	0.042
13	SufD	150373798	49 5.28	22	12			−1.63	0.007							−1.72	0.01
13	GltX	150373502	56 5.21	35	22			−1.63	0.007							−1.72	0.01
14	SufD	150373502	49 5.28	32	16			−1.72	0.026							−2.02	0.048
14	GltX	150373798	56 5.21	43	29			−1.72	0.026							−2.02	0.048
15	_2026	150375038	52 5.04	45	33									2.08	0.004	2.77	0.069
15	AldA	150373125	54 5.12	40	21									2.08	0.004	2.77	0.069
15	GatB	150374849	54 5.04	41	22									2.08	0.004	2.77	0.069
16	GuaB	150373392	53 5.61	55	35			−2.53	0.008							−3.1	0.028
16	Mqo2	150375516	56 6.12	19	9			−2.53	0.008							−3.1	0.028
16	_2506	150375518	61 5.56	14%	9			−2.53	0.008							-3.1	0.028
17	AtpA	150375021	55 4.86	42	25	−3.62	0.001					−1.51	0.017	−2.6	0.004		
18	AtpD	150375019	51 4.68	47	23	−3.77	0.005	−1.88	0.011					−3.17	0.032		
18	TufA	150373522	43 4.74	36	13	−3.77	0.005	−1.88	0.011					−3.17	0.032		
19	Pgi	150373845	50 4.83	42	30	−1.71	0.005	−1.32	0.076								
19	TufA	150373522	43 4.74	47	16	−1.71	0.005	−1.32	0.076								
20	GlyA	150375029	45 5.75	45	27	1.63	0.004							1.94	0.026	1.76	0.093
21	_1317	150374329	44 5.76	54	26	3.18	0.009			3.52	0.04			5.47	0.003		
22	_0175	150373187	43 5.29	35	16	3.07	0.023	5.28	0.035	2.92	0.047			2.84	0.052		
22	_0512	150373524	43 5.20	22	10	3.07	0.023	5.28	0.035	2.92	0.047			2.84	0.052		
22	AckA	150374617	44 5.65	23	8	3.07	0.023	5.28	0.035	2.92	0.047			2.84	0.052		
23	CitC	150374599	46 4.84	32	18	2.2	0.022	5.23	6E-05					3.08	0.028	5.08	0.03
23	_2072	150375084	45 4.86	27	9	2.2	0.022	5.23	6E-05					3.08	0.028	5.08	0.03
23	PepS	150374829	47 4.84	16	8	2.2	0.022	5.23	6E-05					3.08	0.028	5.08	0.03
24	_1418	150374430	40 5.04	31	16	1.47	0.003	1.89	0.048								
25	_0826	150373838	42 5.24	26%	14	3.4	0.007			2.08	0.02			3.72	0.003	-1.57	0.039
25	RocD2	150373839	43 5.21	23	12	3.4	0.007			2.08	0.02			3.72	0.003	−1.57	0.039
26	_1434	150374446	39 5.23	32	15			4.1	0.005	1.96	.0001			1.6	0.008	2.4	0.03
26	_2067	150375079	38 5.29	31	13			4.1	0.005	1.96	.0001			1.6	0.008	2.4	0.03
27	Pta	150373563	35 4.72	34	19	−2.2	0.003	−1.63	0.026								
27	PpaC	150374869	34 4.69	30	11	−2.2	0.003	−1.63	0.026								
28	PhdB	150373972	35 4.65	47	17			−1.57	0.028							−1.44	0.04
28	PpaC	150374869	34 4.69	28	9			−1.57	0.028							−1.44	0.04
29	Tsf	150374179	32 5.09	85	39												
29	Ldh1	151220388	35 4.95	42	16												
30	CcpA	150374641	36 5.58	32	16	5.4	0.004	2.11	0.031					2.91	0.025		
31	SucD	150374168	32 5.47	54	23	2.18	0.058							2.83	0.019		
32	_2422	150375434	35 5.06	48	20	8.13	0.001	11.71	0.007	3.41	0.077			4.97	0.004		
33	Fda	150375515	33 4.92	72	31	2.35	.0001	−1.69	0.002	1.97	0.052			1.93	0.011		
34	Fda	150375515	33 4.92	89	43	5.16	0.005			3.31	0.092			4.33	0.009		
35	_2480	150375492	31 4.67	50	19			−3.09	0.003			−1.71	0.004			−1.98	0.015
36	Nfo	150374472	33 5.63	40	23	−1.37	0.072	−1.64	0.004								
37	RpsB	151221378	29 5.42	41	11	−2.49	0.005									−1.72	0.05
37	FruB	150373680	33 4.64	17	9	−2.49	0.005									−1.72	0.05

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Table 1 *continued.*

#	Protein	Acc number	MWpI	% Cov	# Pep	Δ*clpP* Fold	P	Δ*clpP* ± DIP Fold	P	Δ*clpC* Fold	*p*	Δ*clpC* ± DIP Fold	*p*	Δ*clpX* Fold	*p*	Δ*clpX* ± DIP Fold	*p*
38	MemB	150373927	30 5.41	42	16			−1.73	0.008							−1.86	0.094
39	HchA	150373525	32 4.90	35	14	−3.06	0.011	−2.31	0.012								
39	_0521	150373533	32 4.91	45	12	−3.06	0.011	−2.31	0.012								
40	Tuf	150373522	43 4.74	31	11			4.48	0.006								
40	HchA	150373525	32 4.90	23	8			4.48	0.006								
41	_0839	150373851	33 4.84	61	21	2.03	0.07	2.05	0.019	1.98	0.09	1.78	0.06	4.03	0.001		
42	GrpE	150374496	24 4.42	58	17			2.69	0.005					−1.34	0.089		
43	_0881	150373893	28 5.64	44	17	2.76	0.032	4.29	0.008								
44	HslO	150373486	32 4.96	44	15	1.49	0.03							2.05	0.007		
45	_0035	150373047	33 5.71	15	5	4.35	0.007							2.4	0.026		
46	NagB	150373544	28 5.43	37	14	2.93	0.003	2.59	0.027	1.99	0.046			2.89	0.001		
47	_2596	150375608	30 5.99	34	11									1.77	0.010		
48	_2596	150375608	30 5.99	30	8					−1.24	0.067			1.48	.0002		
49	GpmA	150375327	27 5.23	40	15	−5.39	0.004	−1.79	0.009	−2.75	0.049			−2.65	0.044		
49	_1921	150374933	27 5.24	36	9	−5.39	0.004	−1.79	0.0087	−2.75	0.049			−2.65	0.044		
50	Pfs/ MtnN	150374513	25 4.83	30	9	−1.3	0.034	−2.71	0.008					1.28	0.006		
51	PanB	150375508	29 5.61	45	16	−1.92	0.027							−1.83	0.013		
52	CodY	150374177	29 5.87	33	14	5.16	0.004	1.77	0.087	2.84	0.014			4.35	0.004		
53	_1102	150374114	24 4.91	38	14												
53	_0071	150373083	27 5.04	25	8												
54	IspD1	150373197	27 5.69	48	13	−1.67	0.005							−1.68	0.005		
55	Adk	150375143	24 4.80	61	22	−1.78	0.001										
56	DeoC	150375053	23 4.71	40	12			1.39	0.025					1.91	0.007	2.13	0.099
56	_1672	150374684	26 4.72	44	11			1.39	0.025					1.91	0.007	2.13	0.099
56	Adk	150375143	24 4.80	47%	16			1.39	0.025					1.91	0.007	2.13	0.099
57	_2421	150375433	25 5.52	35	12	3.83	0.013	1.59	0.09	3.25	0.052			3.42	0.002		
58	Ppi	150373836	22 4.53	17	3			−1.93	0.063							2.11	0.048
59	Ppi	150373836	22 4.53	29	9			−1.5	0.003					1.7	0.077		
59	_0533	150373545	22 4.57	23	5			−1.5	0.003					1.7	0.077		
60	_1941	150374953	24 5.27	39	14	−3.93	0.001	−2.2	0.055					−2.18	0.01	−3.89	0.001
61	VraR	151222034	24 5.46	47	10	3.36	0.013	1.64	0.016					3.55	0.009		
62	FabG	150374153	26 5.33	34	13	5.51	0.003	2.57	0.015	4.26	0.042	1.8	0.085	6.77	0.005		
63	Frr	150374181	20 5.04	55	22	−1.8	.0006										
64	ClpP	150373748	22 5.13	28	7	−6.54	0.002	−10.18	.0004			4.46	.0005				
65	MsrA	150374347	21 6.15	22	7	3.73	8E-05	2.36	0.001	2.5	0.025	2.46	0.011	5.41	0.002		
66	_1767	150374779	19 4.59	18	5			−2.44	0.001			−1.37	0.02				
67	_1856	150374868	215.66	33	6					3.59	0.021	2.77	0.001				
68	_0115	150373127	19 4.68	38	14	−3.44	.0004	−2.52	0.003			−2.22	0.015	−1.88	0.001		
69	_0885	150373897	19 5.04	29	5												
70	AtpH	150375022	20 6.15	47	11	3.76	.0004			2.48	0.081			2.56	0.003	−2.11	0.079
71*																	
72	_1213	150374225	18 6.31	54	13	3.19	0.006			2.18	0.004			2.97	0.05		
73	_1604	150374616	18 5.60	33	8					4.48	0.002	1.68	0.078	3.15	0.016		
74	_2086	150375098	19 5.13	38	9	−5.14	0.003	−2.93	0.031	−2.82	0.042	−2.3	0.059	−3.34	0.002		
74	Apt	150374549	19 4.74	20	5	−5.14	0.003	−2.93	0.031	−2.82	0.042	−2.3	0.059	−3.34	0.002		
75	GreA	150374523	18 4.53	51	12	2.51	0.011							3.15	0.017		
76	_1831	150374843	20 4.64	42	10	−16.0	.0002			−5.52	0.001			−1.69	0.077	−1.76	0.082
77	Tpx	150374619	18 4.56	18	4												
78	_1755	150374767	17 5.48	63	19	7.92	0.002	1.96	0.089					5.57	0.004		
79	_0976	150373988	19 5.16	30	5	3.76	0.003			2	0.062			4.24	0.002		
80	_0533	150373545	22 4.57	37	10	−2.65	.0004	−2.71	0.003	−1.74	0.061			−1.55	0.019		
81	Adh1	150373589	36 5.34	55	30	−7.09	0.010	−3.9	0.004					−4.51	0.012	−6.03	.0008

and early stationary growth (Chatterjee *et al.*, 2009). Two proteins involved in iron–sulfur cluster formation, SufB and SufD (NWMN_0789 and NWMN_0786), were decreased in Δ*clpP* and Δ*clpX* upon iron starvation. Flavohemoprotein (NWMN_0175), a component of the nitrosative stress response that binds heme, iron and other metals, was increased in all *clp* mutants in iron-rich conditions. Alteration of proteins involved in iron homeostasis in *clp* mutants may reflect the importance of this protease to withstanding stresses including oxidative stress, which would be enhanced in high iron.

The indirect impact of Clp protease activity on protein abundance under varying iron conditions is likely far reaching due to the effect of the Clps on transcriptional regulators. CodY, a global virulence repressor (Stenz *et al.*, 2011), was increased in all *clp* mutants. VraR controls expression of cell wall stress response and antimicrobial resistance genes (Kuroda *et al.*, 2003; Gardete *et al.*, 2006), and was more abundant in Δ*clpP* and Δ*clpX*. CcpA, a regulator of carbon metabolism and virulence (Seidl *et al.*, 2006), was also increased in Δ*clpP* and Δ*clpX*. These data suggest that Clp proteolytic regulation likely plays an important role in controlling levels of these regulators, thus indirectly influencing genes under their control.

Proteomic analyses of cytoplasmic fractions isolated from *S. aureus clp* mutants revealed many proteins altered in abundance by the Clps in an iron-dependent manner. Some proteins altered in the Clp mutants are confirmed in other studies (Friedman *et al.*, 2006; Michel *et al.*, 2006; Chatterjee *et al.*, 2009). However, many proteins revealed in these analyses were not previously known to be impacted by the Clp protease. Thus, these results represent a broader view of the cellular processes influenced by this system and highlight the role of the Clps under nutrient limitation similar to that experienced

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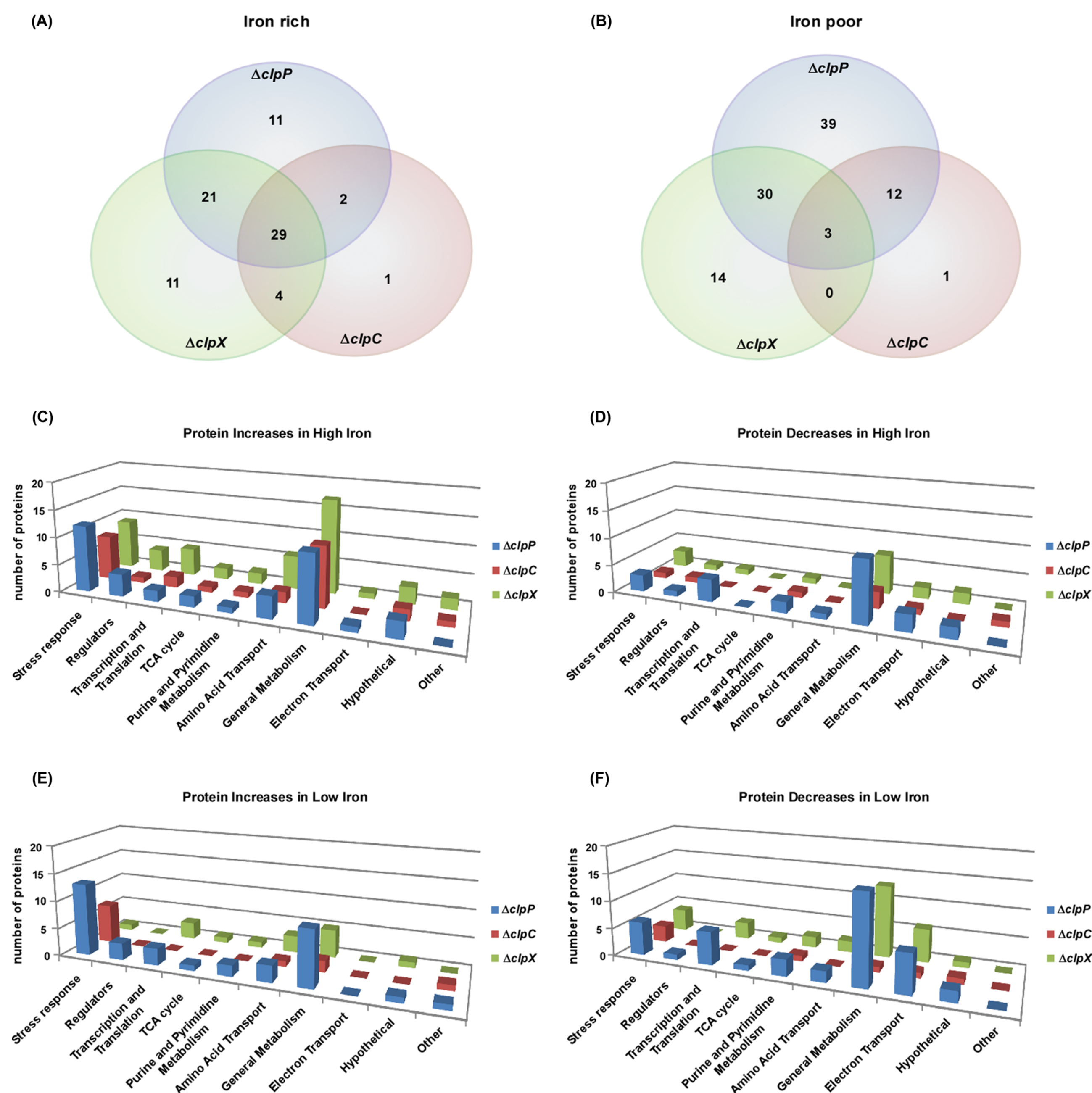

Figure 1: (**A** and **B**) Venn diagrams displaying the number of proteins altered in abundance in the *clp* knockout strains compared to wild-type *S. aureus* in iron-rich and iron-poor conditions. (**C–F**) Pathways impacted by proteins affected in *clp* knockout strains compared to wild-type *S. aureus* in iron-rich and iron-poor conditions.

during infection. Moreover, these data represent the first proteomic analyses of a *S. aureus* Δ*clpX* in iron-replete and -deplete conditions. These studies reveal new insights into the *S. aureus* proteolytic network and underscore the importance of the Clp protease for withstanding stresses such as nutrient iron limitation.

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