



# Quantitative proteomic analysis reveals that serine/threonine kinase is involved in *Streptococcus suis* virulence and adaption to stress conditions

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## Abstract

The eukaryotic-type serine/threonine kinase of *Streptococcus suis* serotype 2 (SS2) performs critical roles in bacterial pathogenesis. In this study, isobaric tags for relative and absolute quantification (iTRAQ) MS/MS were used to analyze the protein profiles of wild type strain SS2-1 and its isogenic STK deletion mutant ( $\Delta$ stk). A total of 281 significant differential proteins, including 147 up-regulated and 134 down-regulated proteins, were found in  $\Delta$ stk. Moreover, 69 virulence factors (VFs) among these 281 proteins were predicted by the Virulence Factor Database (VFDB), including 38 downregulated and 31 up-regulated proteins in  $\Delta$ stk, among which 15 down regulated VFs were known VFs of SS2. Among the down-regulated proteins, high temperature requirement A (HtrA), glutamine synthase (GlnA), ferrichrome ABC transporter substrate-binding protein FepB, and Zinc-binding protein AdcA are known to be involved in bacterial survival and/or nutrient and energy acquisition under adverse host conditions. Overall, our results indicate that STK regulates the expression of proteins involved in virulence of SS2 and its adaption to stress environments.

**Keywords** *Streptococcus suis* · Serine/threonine kinase · Quantitative proteomic · Virulence · Stress

## Introduction

*Streptococcus suis* (*S. suis*) is a major swine pathogen that causes a wide range of diseases (Lun et al. 2007). In addition, *S. suis* is an important zoonotic agent responsible for severe human infections, including meningitis, endocarditis,

and septic shock (Tang et al. 2006; Lun et al. 2007). *Streptococcus suis* serotype 2 (SS2), which is the serotype that is most virulent and most frequently isolated from diseased pigs, is most commonly involved in human infections (Tang et al. 2006). During the past few decades, more than 100 virulence factors of *S. suis* had been identified. These factors were classified into the following subgroups: surface/secreted elements, enzymes/proteases, transcription factors/regulatory systems and others (Fittipaldi et al. 2012). However, the mechanisms underlying the pathogenesis of the different virulence factors on SS2 has still not been entirely explained.

Bacteria use regulatory systems to sense and respond to environmental signals via regulation of specific gene expression. Two-component systems (TCSs), stand-alone regulators (SARs), regulator RNAs and unknown regulators constitute bacterial regulatory networks (Fittipaldi et al. 2012; Wu et al. 2014; Segura et al. 2017; Zheng et al. 2018a). Two-component systems such as SalK/SalR, VirR/VirS and VraSR, as well as the orphan response regulators RevS and CovR have been reported to contribute to bacterial adaptation to various environments and the expression of virulence

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factors of *S. suis* (Zheng et al. 2018a). In addition, several SARs have been reported to modulate *S. suis* virulence, including Rgg (Zheng et al. 2011), CcpA (Willenborg et al. 2011), CodY (Feng et al. 2016) and Rex (Zhu et al. 2018). Small RNAs also play a pivotal role in the pathogenicity of *S. suis* (Xiao et al. 2017).

Several recent studies have shown that eukaryote-type serine/threonine kinases (ESTKs) and phosphatases (ESTPs) also play essential roles in sensing of external stimuli (Wright and Ulijasz 2014). Even though signaling systems composed of ESTKs/ESTPs do not have dedicated transcription factors, they are capable of affecting the expression of genes involved in cell growth and division, adherence to host cell, stress response, biofilm formation, and various metabolic, developmental and virulence processes (Burnside and Rajagopal 2011; Zhu et al. 2011, 2014; Wright and Ulijasz 2014; Manuse et al. 2016; Fang et al. 2017; Zhang et al. 2017).

The homologues of ESTK and ESTP in *S. suis*, which have been designated SsSTK and SsSTP, respectively, were found to contribute to bacterial adherence to host cells, survival in stress environments and virulence (Zhu et al. 2011, 2014; Fang et al. 2017; Zhang et al. 2017). Comparative proteome analysis is a powerful method for elucidating gene expression patterns in microorganisms (Chen et al. 2011; Pian et al. 2015; Yu et al. 2018). Proteome and transcriptome profiles are not exactly the same because of differences in the post-transcriptional regulation that modulates the translation rate and half-lives of specific proteins or mRNAs, as well as their intracellular location and molecular association with other proteins (Chen et al. 2011; Shen et al. 2013). A previous comparative transcriptomic analysis have shown that SsSTK affects the transcription of a set of genes encoding functions involving in cell growth and division, glycolysis, carbohydrate metabolism, membrane transport and translation (Zhang et al. 2017). In the present study, the isobaric tag for relative and absolute quantitation (iTRAQ) and liquid chromatography tandem mass spectrometry (LC–MS/MS) were used to analyze the different protein expression profiles, especially those concerning the bacterial pathogenicity, between the wild-type (WT) strain SS2-1 and its SsSTK mutant strain with the aim of revealing proteins involved in adaption to stress environments and virulence of *S. suis*.

## Materials and methods

### Bacterial strains and culture conditions

SS2 strain SS2-1, which was isolated from a diseased pig with septicemia in Jiangsu Province in 1998, has been confirmed as a highly virulent strain (Zhu et al. 2014). The SsSTK mutant of SS2-1 ( $\Delta stk$ ) was constructed in a previous study (Zhu et al. 2014). For this study, SS2 strains were

grown in Todd-Hewitt broth (THB, Becton, Dickinson and Company, USA) or plated on agar medium containing 10% fetal bovine serum at 37 °C.

### Protein digestion and iTRAQ labelin

Protein samples were performed with a few modifications as described by previous descriptions (Shen et al. 2013; Yu et al. 2018). Briefly, the WT strain SS2-1 and the mutant strain  $\Delta stk$  were each cultured in THB in triplicate (three SS2-1 and three  $\Delta stk$ ) and collected during the log-growth phase ( $OD_{600}=0.7$ ) (Shen et al. 2013; Yu et al. 2018). Cells were then centrifuged at  $10,000\times g$  for 5 min at 4 °C, after which the pellets were washed twice with PBS (Shen et al. 2013). Comparative proteome analysis was subsequently performed at Wuhan GeneCreate Biological Engineering Co., Ltd. (Wuhan, GeneCreate, China). Protein digestion was performed as previously described (Jing et al. 2008), with some modifications. Briefly, SS2 cell pellets were ground to powder in liquid nitrogen and then incubated in dissolution buffer (8 M urea/100 mM triethylammonium hydrogen carbonate buffer (TEAB), pH 8.0) containing 1 mM PMSF and 2 mM EDTA (final concentration) for 5 min, after which 10 mM DTT (final concentration) was added to the sample. Next, the suspension was sonicated for 15 min and then centrifuged at 4 °C at  $14,000\times g$  for 20 min. The supernatant was subsequently mixed with four volumes of precooled acetone at  $-20$  °C overnight. After another centrifugation, the resulting protein pellets were air-dried and resuspended in 8 M urea/100 mM TEAB (pH 8.0). Protein samples were then reduced with 10 mM DTT at 56 °C for 30 min and alkylated with 50 mM iodoacetamide (IAM) for 30 min in the dark. Next, the protein concentration was measured using a Bradford Protein Assay Kit (Beyotime, Shanghai, China). After being diluted 5 $\times$  with 100 mM TEAB, equal amounts of proteins from each sample were used for tryptic digestion. Trypsin was added at an enzyme protein ratio of 1:50 (w/w), after which samples were digested at 37 °C for 12–16 h. Following digestion, peptides were desalted using C18 columns and the resulting desalted peptides were dried under vacuum. The dried peptide powder was later re-dissolved with 0.5 M TEAB and processed according to the manufacturer's instructions for the iTRAQ Reagent-8 plex Multiplex Kit (AB Sciex U.K. Limited). Three biological replicates of SS2-1 (1A, 1B, and 1C) were labeled with iTRAQ tags 113, 114, and 115, respectively, and three biological replicates of  $\Delta stk$  (20A, 20B, and 20C) were labeled with tags 116, 117, and 118, respectively. The peptide samples were then fractionated using a Durashell C18 column (5  $\mu$ m, 100 Å, 4.6 $\times$ 250 mm) on an Ultimate 3000 HPLC system (Thermo DINOEX, USA) operating at 1 ml/min. Peptides were separated by increasing acetonitrile (ACN) concentrations under high pH (pH 10) conditions and fractions were collected at 1 ml intervals and pooled into 12 fractions. Each fraction was then dried under vacuum.

## LC–ESI–MS/MS analysis

Peptide samples were dissolved in 2% acetonitrile/0.1% formic acid and then analyzed using a Triple TOF 5600<sup>+</sup> mass spectrometer coupled with the Eksigent nanoLC System (SCIEX, USA) as previously described (Lin et al. 2015). The raw files collected from the Triple TOF 5600 were interpreted using ProteinPilot version 4.5 (July 2012, Applied Biosystems; Foster City, CA, USA). MS/MS spectra were searched against the Uniprot *S. suis* database (80,299 items, updated Jan 2017). For analysis, the instrument was set as TripleTOF 5600 plus with cysteine carbamidomethylation and 8 multiplex iTRAQ labeling was set as a fixed modification. In addition, methionine oxidation was used as a variable modification, and digestion by trypsin allowing for no more than one missed cleavage. The ratio of  $\Delta$ stk to SS2-1 represents the expression of proteins with a 1% false discovery rate for the protein identification confidence (Unwin et al. 2010). The differences in abundance were considered significant when whose criteria were met a ratio-fold change  $\geq 1.5$  or  $\leq 0.67$  (Yu et al. 2018).

## Bioinformatics analysis

Gene Ontology (GO) analysis was conducted to classify differently expressed proteins (DEPs) in three categories (cell component, molecular function, and biological process) using the UniPort-GOA database (<http://www.ebi.ac.uk/GOA/>), InterProScan (<http://www.ebi.ac.uk/interpro/>) and GO annotation (<http://geneontology.org/>). In addition, the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway (<http://www.genome.jp/kegg/>) of DEPs were further categorized utilizing the same resource. Furthermore, the protein–protein interactions for these DEPs predicted by the Virulence Factor Database (VFDB) (Chen et al. 2005) in this study and the known VFs reported by others were analyzed using the Cytoscape software to construct a virulence network of *S. suis* and screen the novel DEPs for those that are connected with the known VFs. The protein–protein interaction network was obtained from the STRING database ([http://string-db.org/newstring.cgi/show\\_input\\_page.pl](http://string-db.org/newstring.cgi/show_input_page.pl)), which defined a ‘confidence score’ to evaluate the interaction confidence. We obtained all interactions with a confidence score of at least 0.4 (Yu et al. 2018).

## Western blot validation of comparative proteomic analysis

SS2-1 and  $\Delta$ stk were prepared for western blot analysis. Two proteins, OppA (putative oligopeptide-binding protein) and DnaJ (chaperone protein DnaJ), were chosen for validation of the comparative proteomic data. In the  $\Delta$ stk/SS2-1 comparison, OppA is a lower abundance protein and DnaJ is a

higher abundance protein. EF-Tu was used as the loading control because its abundance is relatively constant. Equal amounts (30  $\mu$ g for each lane) of whole cell proteins from the WT and mutant strains were separated on a 12% SDS-PAGE gel, then transferred onto polyvinylidene fluoride (PVDF) membranes (BioRad). The membranes were then incubated with a 1:500 dilution of the primary antibodies for OppA and EF-Tu (kindly provided by Prof. Wei Zhang, Nanjing Agricultural University) and DnaJ (kindly provided by Prof. Weihuan Fang, Zhejiang University), after which they were incubated with horseradish peroxidase (HRP)-conjugated secondary antibody at a 1:10,000 dilution. Signals were detected using enhanced chemiluminescence (ECL) substrate (ThermoFisher Scientific).

## Statistical analysis

The means of groups were compared using the Student’s *t* test (unpaired, two-tailed) in GraphPad Prism 5 (San Diego, USA), with a  $P < 0.05$  considered to be statistically significant.

## Results

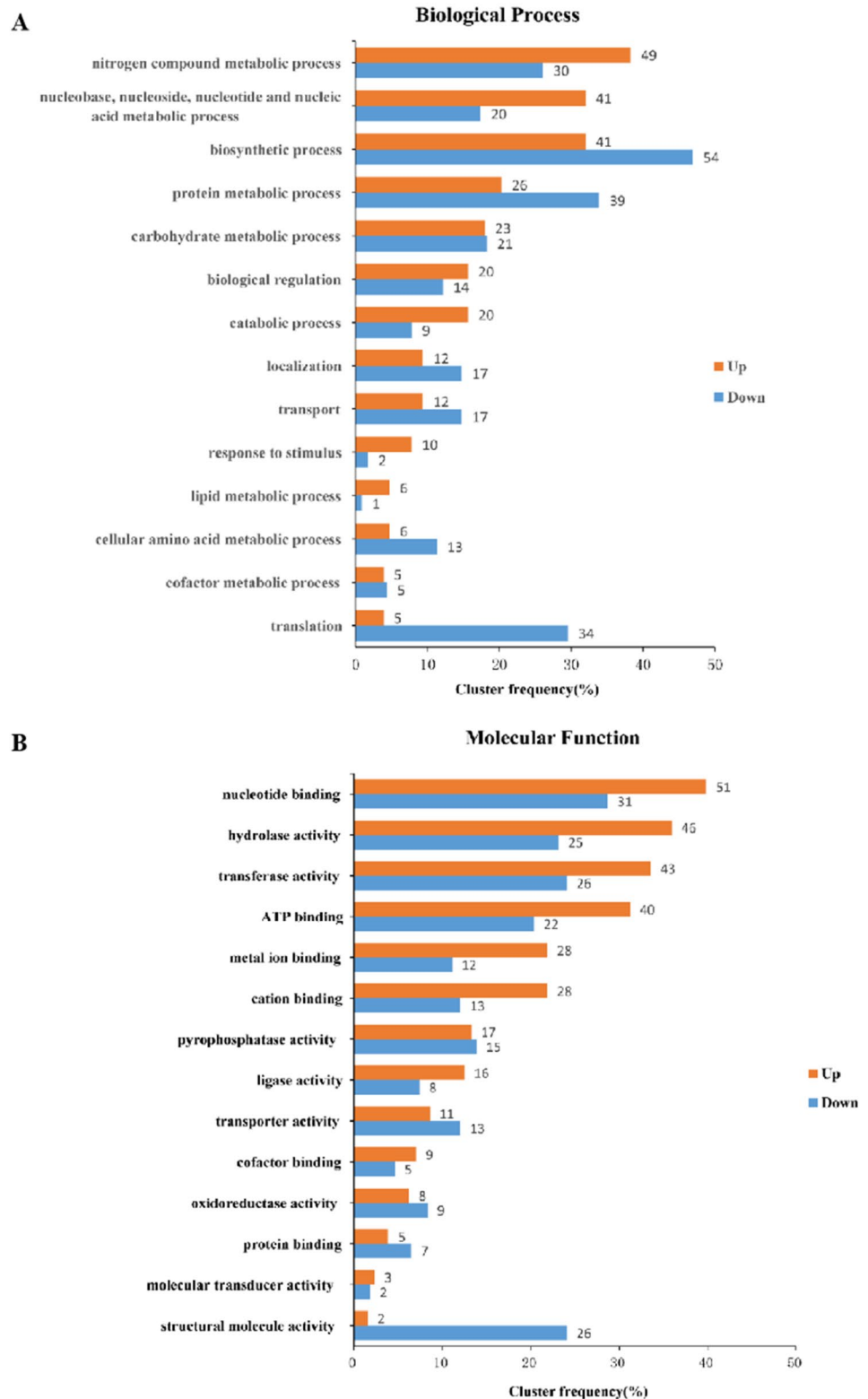
### Comparative proteome analysis of SsSTK mutant strain and WT strain

iTRAQ coupled mass spectrometry identified a total of 1120 proteins from WT strain SS2-1 and its mutant strain  $\Delta$ stk. Among these, the expression levels of 281 proteins differed significantly ( $> 1.5$ -fold change or  $< 0.67$ -fold change,  $P$ -value  $< 0.05$ ) in  $\Delta$ stk compared with its WT strain SS2-1, with 134 (47.7%) down-regulated and 147 (52.3%) up-regulated (Table S1).

### Functional classification annotation analysis of DEPs

To gain insight into the functional categories of the 281 differentially expressed proteins (DEPs), GO analysis was performed to generate classification clusters based on biological process and molecular function. In the biological processes classification cluster, the five most enriched GO terms were biosynthetic processes (41 proteins [32.03%] upregulated; 54 proteins [46.96%] downregulated), nitrogen compound metabolic processes (49 proteins [38.28%] upregulated; 30 proteins [26.09%] downregulated), protein metabolic processes (26 proteins [20.31%] upregulated; 39 proteins [33.91%] downregulated), nucleotide and nucleic acid metabolic processes (41 proteins [32.03%] upregulated; 14 proteins [28.1%] downregulated), and carbohydrate metabolic processes (23 proteins [17.97%] upregulated; 21 proteins [18.26%] downregulated) (Fig. 1a). In the molecular

**Fig. 1** GO enrichment analysis of differently expressed proteins in *Δstk*. Results are grouped by biological process (a) and molecular function (b)



function classification, the most enriched GO terms were nucleotide binding (51 proteins [39.84%] upregulated; 31 proteins [28.70%] downregulated), hydrolase activity (46

proteins [35.94%] upregulated; 25 proteins [23.15%] downregulated), transferase activity (43 proteins [31.25%] upregulated; 26 proteins [24.07%] downregulated), ATP binding

(46 proteins [39.84%] upregulated; 25 proteins [20.37%] downregulated), and cation binding (28 proteins [21.88%] upregulated; 13 proteins [12.04%] downregulated) (Fig. 1b).

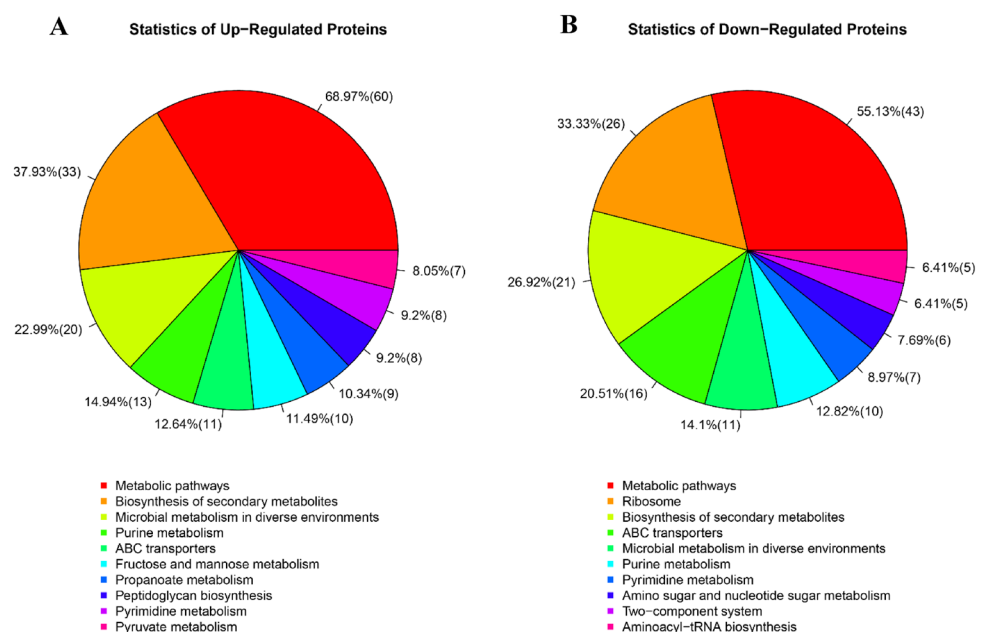
### KEGG pathway analysis of DEPs

To reveal the roles of SsSTK in SS2, KEGG pathway analysis was performed (Fig. 2). The DEPs were mainly involved in metabolic pathways (60 proteins [68.97%] up-regulated; 43 proteins [55.13%] down-regulated), biosynthesis of secondary metabolites (33 proteins [37.93%] up-regulated; 21 proteins [26.92%] down-regulated) and microbial metabolism in diverse environments (20 proteins [22.92%] up-regulated; 11 proteins, [14.1%] down-regulated, Fig. 2a). The up-regulated proteins included those associated with purine metabolism (13 proteins, 14.94%), ABC transporters (11 proteins, 12.64%), fructose and mannose (10 proteins, 11.49%), propanoate (9 proteins, 10.34%), peptidoglycan biosynthesis (8 proteins, 9.2%), pyrimidine metabolism (8 proteins, 9.2%) and pyruvate metabolism (7 proteins, 8.97%). The down-regulated pathways were associated with ribosomes (26 proteins, 33.3%), ABC transporters (16 proteins, 20.51%), purine metabolism (10 proteins, 12.82%), pyrimidine metabolism (7 proteins, 8.97%), amino sugar and nucleotide sugar metabolism (6 proteins, 7.69%), the two-component system (5 proteins, 6.41%) and aminoacyl-tRNA biosynthesis (5 proteins, 6.41%) (Fig. 2b). In general, most of these DEPs are involved in key metabolic and pathways, which may contribute to the pathogenicity of SS2.

### SsSTK regulates known virulence factors

The SsSTK deletion significantly reduced SS2 virulence. Among the 281 DEPs, there were 69 virulence factors (VFs) predicted by VFDB, including 38 down-regulated proteins (Table 1) and 31 up-regulated proteins (Table 2) in the  $\Delta stk$ , of which 26 were known VFs of SS2 (Fittipaldi et al. 2012). In addition, the following 16 VFs were down-regulated in  $\Delta stk$ : capsular polysaccharide biosynthesis locus genes CPS2A (regulation), CPS2B (chain length determination) and CPS2H (glycosyltransferase), sialic acid synthase (NeuB), UDP-N-acetylglucosamine 2-epimerase (NeuC), putative oligopeptide-binding protein (OppA), high-affinity zinc uptake system protein (ZnuA/TroA) and IgM protease (IdeS<sub>Suis</sub>) (Fittipaldi et al. 2012; Rungelrath et al. 2018). Additionally, the putative IgA-specific zinc metalloproteinase (ZmpC or IgA1) (Zhang et al. 2011; Dumesnil et al. 2018), translation initiation factor 2 (HP0272 or SadP) (Ferrando et al. 2017), chaperone protein DnaJ (Zhang et al. 2015), and sensor histidine kinase TCS VarS (Zheng et al. 2018a; Zhong et al. 2018) were significantly up-regulated in the mutant strain. Another 43 VFs that have been reported in other pathogens were identified as DEPs in the  $\Delta stk$ , including putative 5'-nucleotidase (5NuC) (Zheng et al. 2015; Ma et al. 2017), trypsin-like serine protease (HtrA) (Backert et al. 2018) and metalloendopeptidase (PepO) (Agarwal et al. 2013, 2014). These VFs were mainly assigned into eight classes: (i) amino acid transport and metabolism (12 proteins); (ii) posttranslational modification, protein turnover, chaperones (10 proteins); (iii) nucleotide transport and metabolism (9 proteins); (iv) transcription (8 proteins);

**Fig. 2** KEGG pathway enrichments in DEPs in  $\Delta stk$ . **a** Up-regulated and **b** down-regulated proteins



**Table 1** Down-regulated virulence associated factors identified by iTRAQ in  $\Delta$ stk

Accession	Gene locus	Gene name	Description/Function	Fold change Mutant/WT	P value
<i>Amino acid transport and metabolism</i>					
A0A116MPN5	ssu05-1868	OppA	Putative oligopeptide-binding protein OppA	0.260903997	0
G7SK61	ssu05-1027	GlnP	Putative glutamine ABC transporter, glutamine-binding protein/permease protein	0.412407484	2.96E-05
A4VSY1	ssu05_0252	GDH	Glutamate dehydrogenase	0.360913896	4.91E-08
A4VSP1	ssu05-0160	GlnA	Glutamine synthetase	0.318288584	1.10E-05
A4VXL3	ssu05_1886	IlvC	Ketol-acid reductoisomerase	0.598600521	6.38E-05
A4VY44	ssu05_2067	GlnQ	Putative amino acid ABC transporter	0.651608966	0.0284189
A0A0K2E4M4	ssu05-1548	LivK	Branched-chain amino acid ABC transporter substrate-binding protein LivK	0.626362412	0.00362246
A0A0Z8K531	ssu05_0718	ASD	Aspartate-semialdehyde dehydrogenase	0.597290304	0.00686381
<i>Nucleotide transport and metabolism</i>					
A0A0H3MXU4	ssu05_2118	NrdD	Anaerobic ribonucleoside-triphosphate reductase	0.262453737	5.79E-10
A0A0Z8I535	ssu05_0873	GuaA	GMP synthase	0.572778731	0.00943322
A4VSH4	ssu05_0091	ADK	Adenylate kinase	0.433358178	9.74E-05
A4VWL5	ssu05-1538	5NuC	Putative 5'-nucleotidase	0.34182614	1.38E-06
A4VU67	ssu05_0690	Fhs	Formate-tetrahydrofolate ligase	0.538793986	0.00305715
A0A0Z8Y8J2	ssu05-0033	PurD	Phosphoribosylamine-glycine ligase purD	0.441863004	0.03455731
<i>Cell wall/membrane/envelope biogenesis</i>					
A4VTS6	ssu05_0549	GlmS	Glutamine-fructose-6-phosphate aminotransferase	0.237614116	8.34E-08
A4V VX1	ssu05_1294	AltA	N-acetylmuramoyl-L-alanine amidase	0.500737006	0.00053377
A4VTV5	ssu05_0578	NeuB	Sialic acid synthase	0.363283669	0.00013004
A4VTV6	ssu05_0579	NeuC	UDP-N-acetylglucosamine 2-epimerase	0.561224277	0.04520204
A4VTU2	ssu05_0565	Cps2B	CPS biosynthesis ( chain length determination)	0.519610055	0.0295358
<i>Inorganic ion transport and metabolism</i>					
U5UKP6	ssu05-1771	MetQ	Lipoprotein	0.467159782	3.22E-05
A4VSJ3	ssu05-0112	AdcA	Zinc-binding protein AdcA	0.312008388	2.30E-06
A4VU25	ssu05-0649	FepB/FhuD	Ferrichrome ABC transporter substrate-binding protein	0.371811659	7.41E-05
A0A0M9FIU4	ssu05-0309	ZosA	Cation transport ATPase ZosA	0.211173974	0.00041522
D5AIQ2	ssu05-1418	MgtA	Magnesium-transporting ATPase	0.567922803	0.00017229
<i>Transcription</i>					
A4VTU1	ssu05_0564	Cps2A	CPS biosynthesis (regulation)	0.486937335	0.03900053
D5AFJ8	ssu05-0159	GlnR	Transcriptional regulator, glutamine synthetase repressor	0.289088662	0.02195271
A4VV72	ssu05_1045	GlpR	Transcriptional regulator of sugar metabolism	0.573794895	0.04839645
A0A123TNJ0	ssu05-0402	YebC	Probable transcriptional regulatory protein yebC	0.322587925	0.00155311
<i>Function unknown</i>					
A0A0H3MYM8	ssu05_1267	InIA	Streptococcal histidine triad-family protein	0.585290914	0.00207815
A4VTU8	ssu05_0571	Cps2H	CPS biosynthesis (glycosyltransferase)	0.59434387	0.00572005
A0A0H3MW10	ssu05-545	Ide.Ssuis	IgM protease	0.389281421	2.94E-05
B3GFZ7	ssu05-0177	EF	Extracellular factor protein	0.298326271	0.00592074
A0A123TR75	ssu05-0141	Impdh	Inosine-5'-monophosphate dehydrogenase	0.611950148	0.03139697
U5UEH8	ssu05-1257	PBP1	ABC transporter permease protein	0.202037287	0.00087703
<i>Energy production and conversion</i>					
A0A0H3MXX6	ssu05-2154	FrdA	Putative fumarate reductase flavoprotein subunit	0.228390668	0.00000546
<i>Signal transduction mechanisms</i>					
A4VTF6	ssu05-0428	STK	Serine/threonine protein kinase	0.042032747	1.71E-10
<i>Posttranslational modification, protein turnover, chaperones</i>					
A4VXJ8	ssu05-1871	SufB	FeS assembly protein SufB	0.495251882	0.00073167
A4VYG9	ssu05-2192	HtrA	Trypsin-like serine protease HtrA	0.611081572	0.0303447

**Table 2** Up-regulated virulence associated factors identified by iTRAQ in  $\Delta$ stk

Accession	Gene locus	Gene name	Description/function	Fold change mutant/WT	P value
<i>Posttranslational modification, protein turnover, chaperones</i>					
A4VSA0	ssu05-0015	FtsH	ATP-dependent zinc metalloprotease FtsH	2.666489219	8.88E-10
A4VY59	ssu05-2082	PepgO	Predicted metalloendopeptidase	1.592715723	5.40E-05
A4VTN2	ssu05_0505	Collagenase	Collagenase and related protease	2.58065221	0.00085438
A4VT31	ssu05_0302	DnaJ	Chaperone protein DnaJ	5.099121717	1.38E-10
A0A116NL00	ssu05-1737	TrxB	Thioredoxin reductase	1.587025457	0.00379886
A4VTN3	ssu05_0506	HP0506	Collagenase and related protease	1.512746775	0.01979603
G7SNC3	ssu05-0240	MrsA	Peptide methionine sulfoxide reductase	1.798334701	0.02091889
A4VT28	ssu05_0298	GrpE	Protein GrpE	1.639950423	0.00096526
<i>Amino acid transport and metabolism</i>					
A4VVS0	ssu05_1243	PepF	Oligoendopeptidase F	2.335395064	6.57E-08
A4VXS8	ssu05-1951	AmpT	Leucyl aminopeptidase	1.68826166	0.00091845
A0A0Z8CXK2	ssu05_0728	PepF1-2	Oligoendopeptidase F	2.102440266	0.00069887
A4VU01	ssu05_0624	ArcA	Arginine deiminase	2.592388155	1.04E-06
<i>Transcription</i>					
A4VUG1	ssu05-0784	CpsY	CpsY	1.665254688	0.00096483
A0A168YWM4	ssu05-1012	LysR	LysR family transcriptional regulator	1.597409841	0.02397035
A0A0Z8IRK2	ssu05_1700	ScpB	Segregation and condensation protein B	1.51173895	0.02841734
A4VT27	ssu05_0299	HrcA	Heat-inducible transcription repressor HrcA	3.027403522	0.04096617
<i>Nucleotide transport and metabolism</i>					
A4VVU8	ssu05_1271	Cmk	Cytidylate kinase	1.650953089	0.02280986
A4VUL1	ssu05_0834	ThyA	Thymidylate synthase	1.540254675	0.02311404
D5AK71	ssu05_1966	PurA	Adenylosuccinate synthetase	1.677929485	0.00077763
<i>Signal transduction mechanisms</i>					
A4VTF8	ssu05_0430	VarS	Sensor histidine kinase VarS	1.645401188	0.00250477
A4VWT3	ssu05_1606	HprK	HPr kinase/phosphorylase	1.53464737	0.00154572
<i>General function prediction only</i>					
A4VSS6	ssu05-0197	PepX	Xaa-Pro dipeptidyl-peptidase	1.836436817	0.00597575
D5AIW8	ssu05-1495	TPR	Tetratricopeptide repeat family protein	1.560591535	0.0141768
<i>Function unknown</i>					
A0A0H3MV01	ssu05_1022	ZmpC	Putative IgA-specific zinc metalloproteinase	2.251180198	1.52E-13
A4VSS5	ssu05-0196	Fhbp	Uncharacterized protein	5.347251049	8.88E-16
<i>Inorganic ion transport and metabolism</i>					
A0A0Z8XZK6	ssu05-0658	TelA	Tellurite resistance protein	1.581032077	0.00013611
<i>Replication, recombination and repair</i>					
A4VVA2	ssu05_1075	GyrA	DNA gyrase subunit A	1.663559748	0.00072441
<i>Intracellular trafficking, secretion, and vesicular transport</i>					
A4VUQ3	ssu05_0876	Ffh	Signal recognition particle protein	1.822934548	0.00080988
<i>Carbohydrate transport and metabolism</i>					
A4VU63	ssu05_0686	PMM	Phosphomannomutase	1.584042233	0.00014063
<i>Lipid transport and metabolism</i>					
G7SMB7	ssu05_1807	FabH	3-oxoacyl-[acyl-carrier-protein] synthase 3	1.534641502	0.00468633
<i>Cell wall/membrane/envelope biogenesis</i>					
A4VT01	ssu05-0272	SadP/IF-2	Translation initiation factor 2 (IF-2 GTPase)	5.605539481	2.22E-16

(v) inorganic ion transport and metabolism (6 proteins); (vi) cell wall/membrane/envelope biogenesis (6 proteins); (vii) general function prediction (3 proteins); and (viii) other proteins of unknown function (7 proteins) (Tables 1 and 2).

### Interaction network analysis confirmed the roles of the newly identified DEPs in the known virulence factor system

For further insight into the roles of these newly identified DEPs in virulence, we visualized the network formed by the known VFs of SS2 and the novel identified VFs predicted by VFDB using the Cytoscape software. The protein–protein interaction network demonstrated 241 direct physical interactions among the 80 nodes (Table S2). Sixty-six of the interactions had a score higher than 0.70 (high confidence). The 36 newly identified DEPs (yellow nodes) were implicated in and complemented the virulence interaction network, with some playing an important role of bridging to link the known VFs (green nodes and red nodes) and forming important hub proteins. Overall, the results indicated that 37 of the 69 novel DEPs are involved in the known virulence network and may play a role in virulence (Fig. 3).

### Confirmation of the proteomics results by western blot analysis

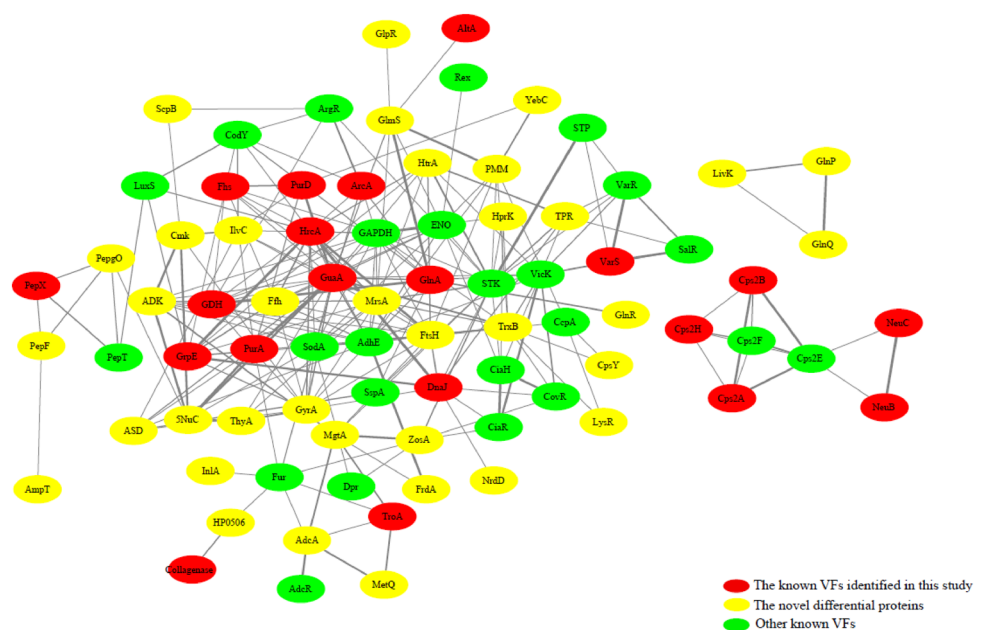
The up-regulated VF DnaJ (41kD) and down-regulated VF OppA (66kD) were selected for confirmation of the comparative proteomics analysis. The EF-Tu protein was used as an internal reference because its abundance is relatively

constant in both groups. The western blot results support those of the proteomic analysis, as the levels of OppA was decreased and the levels of DnaJ were increased in  $\Delta stk$ , which indicating that the proteomics data and western blot results agreed (Fig. 4).

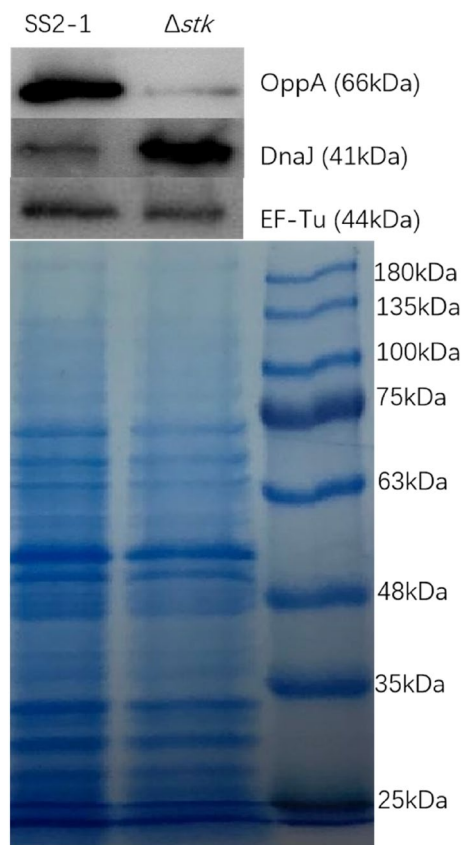
## Discussion

Bacterial ESTKs have emerged as important regulation elements that are indispensable for pathogenesis (Burnside and Rajagopal 2011; Wright and Ulijasz 2014; Manuse et al. 2016). In *Mycobacterium tuberculosis*, two-dimensional gel electrophoresis was used to investigate the effects of the serine/threonine protein kinase (pknE) on the bacterial protein expression under nitric oxide stress conditions. In response to NO stress,  $\Delta pknE$  had increased number of proteins involved in intermediary and lipid metabolism (Parandhaman et al. 2014). In *S. pneumoniae*, a mass-spectrometry based label-free quantitative (LFQ) approach was used to characterize and determine the impact of StkP on the protein expression profiles. Notable changes in the proteome of the kinase mutant  $\Delta stkP$  in comparison to the WT strain have been observed especially in the cluster of amino acid metabolism, energy metabolism, regulatory function and transcription (Hirschfeld et al. 2019). In the present study, comparative proteomics approaches revealed that SsSTK can regulate the expression of proteins involved in bacterial central metabolism, stress response and virulence. These findings provide further support for the previous data that showed attenuated growth in vivo, reduced survival rate in

**Fig. 3** Interaction networks of differentially expressed proteins predicted by the VFDB created using Cytoscape. Protein–protein interactions of differentially expressed proteins of *S. suis* that had a confidence score  $\geq 0.4$  were visualized. The lines represent the interactions that exist between proteins







**Fig. 4** Western blot analysis of comparative proteomics data. Equal amounts (30  $\mu$ g for each lane) of total bacterial cell proteins were separated on a 12% SDS-PAGE gel, then subjected to western blotting. From left to right the lanes were loaded with SS2-1 and  $\Delta$ *stk* samples. Differentially expressed OppA (66 kDa, the first line), DnaJ (41 kDa, the second line) and EF-Tu (44 kDa, the last line) proteins were analyzed using their respective antibodies. The CBB-R250-stained gel was used as a loading control. Protein bands were visualized using the ECL substrate

various stress environments and virulence of  $\Delta$ *stk* (Zhu et al. 2014).

As previously reported, the deletion of *stk* in strain SS2 resulted in alteration of bacterial pathogenicity (Zhu et al. 2014; Zhang et al. 2017). This attenuation may result from the impaired growth of  $\Delta$ *stk* in vivo and because of direct effects on the expression of VFs. The transcriptomic profiles showed that 32 VFs were down-regulated in *stk*-deletion strain of SC-19, including 9 known VFs of SS2, such as subtilisin-like protease (*SspA*), DNA nuclease (*SsnA*), mannose-specific PTS (*ManN*), adenylosuccinate synthase (*PurA*) and phosphoribosylamine-glycine ligase (*PurD*) (Wilson et al. 2007; Fittipaldi et al. 2012; Zhang et al. 2017). Our iTRAQ analysis showed 38 VFs, including 26 known VFs and 12 novel identified VFs that were predicted by VFDB, with repressed expression in  $\Delta$ *stk* such as *AtIA*, *IdeSsuis*, *OppA*, *HtrA*, *HtpsB*, *PurD* and *5NuC*

(Fittipaldi et al. 2012). In SS2 virulent strain HA9801, the *AtIA* mutant strain ( $\Delta$ *atIA*) exhibited a significant reduction in adherence to epithelial cells, biofilm formation and virulence (Ju et al. 2012). In SS2 virulent strain 10, IgM cleaving activity of *IdeSsuis* is important for bacterial survival in porcine blood and evasion of the classical complement pathway (Rungelrath et al. 2018). In *Streptococci*, *HtrA* involved in tissue invasion, chronic airway infections and secretion of VFs, such as in *S. pyogenes* (Lyon and Caparon 2004) and *S. pneumoniae* (Ibrahim et al. 2004). The histidine triad-family protein *Htps B*, a homologue of internalin A (*InIA*) of *Listeria monocytogenes*, has been reported to be important in surface invasions of bacteria and to facilitate both bacterial attachment and internalization in cells that express its receptor of E-cadherin (Bergmann et al. 2002). In SS2 virulent strains, *OppA* is involved in cell growth, binding to host proteins and virulence (Zhang et al. 2014; Zheng et al. 2018b). In SS2 strain S735, *purD* insertion mutant strain obtained by the signature-tagged mutagenesis system was showed apathogenic in both mouse and caesarian-derived, colostrum-deprived (CDCD) pig models (Wilson et al. 2007). Recently transcriptome profiles and our proteome analysis both showed that *PurD* were down-regulated in *stk* deletion mutant strain (Zhang et al. 2017). The extracellular nucleases and or nucleotidase play important roles in degradation of the DNA backbone of neutrophil extracellular traps (NETs) and allow bacteria to evade the host immune system. In *S. equi subsp. zooepidemicus* ATCC35246, 5'-Nucleotidase (*5NuC*) was found to directly degrade the NET DNA backbone to deoxyadenosine, negatively influencing macrophage phagocytic activity, while the mutant strain  $\Delta$ 5nuc exhibited lower virulence and a weaker ability to spread from blood to organs than the WT strain (Ma et al. 2017). Similar findings were reported in *S. pyogenes* for 5'-nucleotidase A (*S5nA*) (Zheng et al. 2015). Therefore, our results are in agreement those of previous studies showing *SsSTK* mutant strains displayed reduced ability to adhere to epithelial cells, increased immune evasion and increased sensitivity to phagocytosis (Zhu et al. 2014; Zhang et al. 2017).

During infection, bacteria must often cope with stress in the form of oxidative conditions, excessive temperature, extreme osmolarity, low pH, and nutrient limitations. Our previous study showed that  $\Delta$ *stk* displayed defects in the ability to adapt to various environmental conditions (Zhu et al. 2014). These may be because of down-regulation of the following series of DEPs that are necessary for bacterial survival and/or nutrient and energy acquisition under adverse host conditions. (i) **The general stress response protein** (e.g., *HtrA*). The virulence features of bacterial *HtrA*, an important stress response regulation protein, have primarily been attributed to increased fitness of pathogens because of resistance against stress conditions during infection (Backert et al. 2018). (ii) **Amino acid transport and**

**metabolism** (e.g., GlnA, IlvC, LivK and ASD). Glutamine synthase (GlnA), which converts glutamate and ammonia to glutamine, plays a central role in regulating the carbon/nitrogen balance in the metabolism and the pathogenicity of bacteria, such as in *Salmonella enterica* and SS2 (Si et al. 2009; Aurass et al. 2018). The ketol-acid reductoisomerase IlvC and branched-chain amino acid ABC transporter substrate-binding protein LivK are involved in the branched-chain amino acids (BCAAs; isoleucine, leucine, valine) biosynthesis pathway, which contributes to the virulence of pathogens (Ribardo and Hendrixson 2011; Kim et al. 2017). In *S. pneumoniae* strain D39, an *ilvC* deletion mutant ( $\Delta ilvC$ ) diminished Ply and LytA virulence factor expression and showed a higher survival rate and lower bacterial burden in a mice infection model relative to the WT strain (Kim et al. 2017). Aspartate-semialdehyde dehydrogenase (ASD) is an essential enzyme for the biosynthesis of lysine, methionine, and threonine from aspartate. In *Burkholderia pseudomallei*, a deletion mutant strain  $\Delta asd$  exhibited attenuated intracellular infectivity and the mutation showed protection against acute inhalation melioidosis in mice (Norris et al. 2011). (iii) **Inorganic ion transport and metabolism** (e.g., AdcA, FepB and MgtA). Apart from the two DEPs mentioned above, these proteins can help pathogens overcome the hostile environments created by ion starvation. The Adc protein contributes zinc uptake and streptococcal virulence. In *S. agalactiae*, Adc and Lmb are involved in zinc acquisition and contribute to bacterial growth and survival (Moulin et al. 2016). In *S. pneumoniae*, AdcA enables zinc acquisition during growth in vitro and systemic virulence in vivo (Bayle et al. 2011; Plumptre et al. 2014). In *Salmonella enterica* serovar Typhimurium wild-type strain SL1344, deletion of *fepB* attenuated *Salmonella* replication and colonization within macrophages and mice (Nagy et al. 2013). Moreover, the transcriptional level of *mgtA* in  $\Delta stk$  was found to be decreased in previous studies (Zhang et al. 2017). Down-regulation of these VFs may hinder the acquisition of nutrients by bacteria and therefore decrease the adaptation of SS2 to various stress environments.

## Conclusion

In summary, our comparative proteome analysis identified 38 down-regulated VFs in the mutant strain  $\Delta stk$  that were involved in adherence to host cells and adaption to and survival in the host environments during SS2 infection. Consistently, phenotypic assays in previous studies have confirmed that the  $\Delta stk$  mutant strain displayed deficient growth in various stress environments in vitro and in vivo and attenuated pathogenicity. Therefore, STK is important to cell growth, stress response, and virulence of SS2.

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**Author contributions** Conceived and designed the experiments: HZ, YN and KH. Performed the experiments: HZ, JZ and DW. Analyzed the data: HZ and JZ. Contributed reagents/materials/analysis tools: ZY, BL, YN, and KH. Wrote the manuscript: HZ, YN and KH. All authors read, advised, and approved the final manuscript.

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**Data availability** All data during the study appear in the submitted article and the supplementary materials.

## Declarations

**Conflict of interest** The authors declare no conflict of interest.

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