Catabolite control protein A has an important role in the metabolic regulation of *Streptococcus suis* type 2 according to iTRAQ-based quantitative proteomic analysis

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Abstract. The catabolite control protein A (ccpA) regulates the carbon metabolism in *Streptococcus suis* type 2 and has pleiotropic regulatory functions in bacterial virulence and transcription. The present study systematically investigated ccpA activity in *Streptococcus suis* type 2 using isobaric tag for relative and absolute quantification (iTRAQ) liquid chromatography-tandem mass spectrometry-based proteomics. Gene Ontology and Kyoto Encyclopedia of Genes and Genomes analyses demonstrated that ccpA is an important protein for the regulation of metabolism, virulence and immune pathways in *Streptococcus suis* type 2. The present study therefore expanded the current understanding of the effects of ccpA on virulence, metabolic regulation and transcription in *Streptococcus suis* type 2 and other important pathogens.

Introduction

Streptococcus suis type 2 (*S. suis* 2) is an important zoonotic pathogen with a global distribution. This strain can cause acute septicemia, meningitis, arthritis and endocarditis with potentially fatal outcomes. Infections can lead to severe toxic symptoms with high mortality in humans (1). Since this bacterium can be transmitted in a variety of ways, it is a serious threat to the pig industry and its workers. Therefore, this strain has aroused significant concern in this community due to economic losses in the meat industry and threats to human health (2,3).

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Carbon catabolite repression (CCR) is important for microbial catabolism, and catabolite control protein A (ccpA) is a key regulatory factor for CCR and global carbon metabolism. CcpA-mediated catabolite repression is necessary for the adaptation of Gram-positive bacteria to changes in the environment (4). It is currently thought that ccpA-mediated CCR is present in a variety of Gram-positive bacteria with low GC content. CcpA regulates gene expression in important metabolic pathways through specific functional domains and impacts bacterial virulence through the metabolic regulatory functions of ccpA (5,6).

As an important member of the *Lac*I/*Gal*R transcriptional regulator family, ccpA controls numerous metabolic processes in Gram-positive bacteria and has important roles in the biofilm formation by *S. suis* (7). Previous studies have found that the envelope of *S. suis* was affected by the deletion of ccpA, creating strains similar to those without an envelope. Furthermore, the anti-phagocyte capabilities of strains without the ccpA protein were significantly reduced as compared with those of parental strains, indicating that ccpA has an important regulatory role in cellular metabolism and structural features of *S. suis* (8). Since ccpA has pleiotropic regulatory functions, ccpA knockout affects numerous physiological processes. Therefore, it is necessary to study the association between ccpA structure and function. Investigating the effect of ccpA on protein regulation is important for understanding this protein's functional domains and active sites.

In the present study, isobaric tag for relative and absolute quantification (iTRAQ)-based proteomics technology was used to analyze the regulatory effect of the ccpA gene on the biological functions of various genes and to provide gene expression levels for further study of this gene in *S. suis* pathogenicity.

Materials and methods

Strains and culture methods. S. suis and the ccpA-mutant strains from our laboratory (9) were cultured in Todd-Hewitt broth (Oxoid Ltd., Basingstoke, UK) at 37˚C under 5% CO_2. Cells in the exponential growth phase were used.

Sample preparation. After 200 μg cells were collected by centrifugation, SDT lysis buffer [4% SDS, 100 mM Tris-HCl pH 8.0, 100 mM dithiothreitol (DTT)] purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA) was added and samples were then mixed by vortexing and placed in a boiling water bath for 10 min. The samples were then ultrasonically disrupted and heated again for 5 min in a boiling water bath. The supernatant was removed and protein quantification was performed using the bicinchoninic acid method.

Enzyme digestion and peptide labeling. From each sample, 200 μg protein was taken and DTT was added to a final concentration of 100 mM. The mixtures were heated in a boiling water bath for 3 min and then cooled to room temperature. The products were combined with 200 μl UA buffer (Bio-Rad Laboratories, Inc.; 8 M Urea and 150 mM Tris HCl, pH 8.0) and mixed. The mixtures were transferred into ultrafiltration centrifuge tubes with a 10-kDa cutoff point and centrifuged at 14,000 xg for 15 min. The precipitates were re-suspended in 200 μl UA buffer and centrifuged at 14,000 xg for 15 min. The filtrates were discarded and the precipitates were re-suspended in 100 μl Iodoacetamide (Bio-Rad Laboratories, Inc.; 50 mM in UA) in the dark for 30 min and then centrifuged at 14,000 xg for 10 min. The precipitates were re-suspended in 100 μl UA buffer and centrifuged at 14,000 xg for 10 min. This process was repeated twice. The precipitates were re-suspended in 100 μl dissolution buffer and centrifuged at 14,000 x g for 10 min, which was also repeated twice. The precipitates were re-suspended in 40 μl trypsin buffer (Promega, Madison, WI, USA; 5 μg trypsin in 40 μl dissolution buffer), agitated at 600 rpm for 1 min and incubated at 37˚C for 16-18 h. The products were centrifuged at 14,000 xg for 10 min using fresh collection tubes, the filtrates were collected, and peptide quantification was performed by measuring the optical density at 280 nm. After enzymolysis, peptide labeling was performed using an iTRAQ 8-plex Multiplex kit (Ab Sciex, Framingham, MA, USA) according to the manufacturer's instructions. For native *S. suis*, iTRAQ reagents 113, 114 and 115 were used, and for mutant strains, reagents 116, 117 and 118 were used.

Peptide fractionation with strong cation exchange (SCX) chromatography. SCX chromatography was performed using an AKTA Purifier 100 (GE Healthcare, Little Chalfont, UK) with a 4.6x100 mm polysulfoethyl column (5 μm; 200 Å) (PolyLC Inc., Columbia, MD, USA). Buffer A contained 10 mM KH_2PO_4 and 25% acetonitrile (ACN; pH 3.0; Sigma-Aldrich, St. Louis, MO, USA). Buffer B contained 10 mM KH_2PO_4 pH 3.0, 500 mM KCl and 25% ACN. Samples were collected and lyophilized prior to desalination in a C18 Cartridge (Sigma-Aldrich).

Mass spectrometry (MS). An Easy nLC system (Thermo Fisher Scientific, Waltham, MA, USA) run at a nanoliter flow rate was used for liquid separation of each sample. Buffer A was 0.1% formic acid in water and Buffer B was 0.1% formic acid in 84% ACN. Chromatographic columns were equilibrated with 95% Buffer A. Samples were loaded into a Thermo scientific EASY-Spray column (2 cmx100 μm 5 μm-C18) using an auto sampler and then separated on a Thermo scientific EASY column (75 μmx100 mm 3 μm-C18) at a flow rate of 250 nl/min. MS was performed using a Q-Ex active mass spectrometer (Thermo Fisher Scientific) with an analysis time of 120 min, a positive ion detection method, a precursor ion scanning range of 300-1,800 m/z, a first-order mass spectrum resolution of 70,000 at m/z 200, an AGC target of 3e6, a first order maximum injection time (IT) of 10 msec, one scan range and a dynamic exclusion of 40.0 sec. The mass-charge ratios of the peptides and peptide fragments were obtained as follows: Ten-fragments spectra (MS2 scan) were collected after each full scan, the MS2 Activation Type was HCD, the isolation window was 2 m/z, the second-order MS resolution was 17,500 at m/z 200 with one microscan. The second-order Maximum IT was 60 msec, the normalized collision energy was 30 eV and the under fill ratio was 0.1%.

Data analysis. The raw data of the MS analysis were derived from RAW files. Database searches and quantitative analyses were performed using the software Mascot 2.2 of Proteome Discoverer 1.4 (Thermo Fisher Scientific). For protein-abundance ratios measured using iTRAQ, a 1.2-fold change was set as the threshold and a two-tailed P-value <0.05 to identify significant changes. The database was downloaded from the National Center of Biotechnology Information (NCBI) on 2013-08-09, and the NCBI_*Streptococcus_suis*. Fasta results contained 89,409 sequences (http://www.ncbi.nlm.nih.gov/protein?term=txid1307[Organism]). Mascot 2.2 was used for the library search. The localized sequence alignment software NCBI Basic Local Alignment Search Tool (BLAST 2.2.28+-win32.ext; http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to perform sequence alignment between the identified proteins and protein sequences in the NCBInr database. Using the similarity principle, functional information from homologous proteins was used to create protein function annotation. The mapping function of BLAST2GO (https://www.blast2go.com/; version 2.7.0) was used to extract gene ontology (GO) function entries correlated with the aligned sequences for all differentially expressed proteins. The Go Slim was annotated for the function of the target protein by GO for generic.obo. The KEGG (Kyoto Encyclopedia of Genes and Genomes) Automatic Annotation Server (KAAS; http://www.genome.jp/tools/kaas/) was used to align target-protein and Streptococcal sequences in the KEGG GENES database. KEGG Orthology (KO) identifiers of homologous/similar proteins were assigned to the relevant KEGG pathway. Carbon metabolism pathway analysis was performed with the Pathway Builder Tool 2.0 (Protein Lounge, San Diego, CA, USA).

Statistical analyses. All statistical analyses were performed using the Student's *t*-test by Perseus 1.3 (http://141.61.102.17/perseus_doku/doku.php?id=start). Differences with a P-value of 0.05 or less were considered statistically significant.

Results

iTRAQ analysis results. A total of 1,167 proteins were identified from *S. suis* and ccpA mutant strains from the original mass spectrometry data, 55 of which were differentially expressed.

The mapping function of Blast2GO (Version 2.7.0) was used to extract the GO function entries correlated with the aligned sequences for all of the differentially expressed

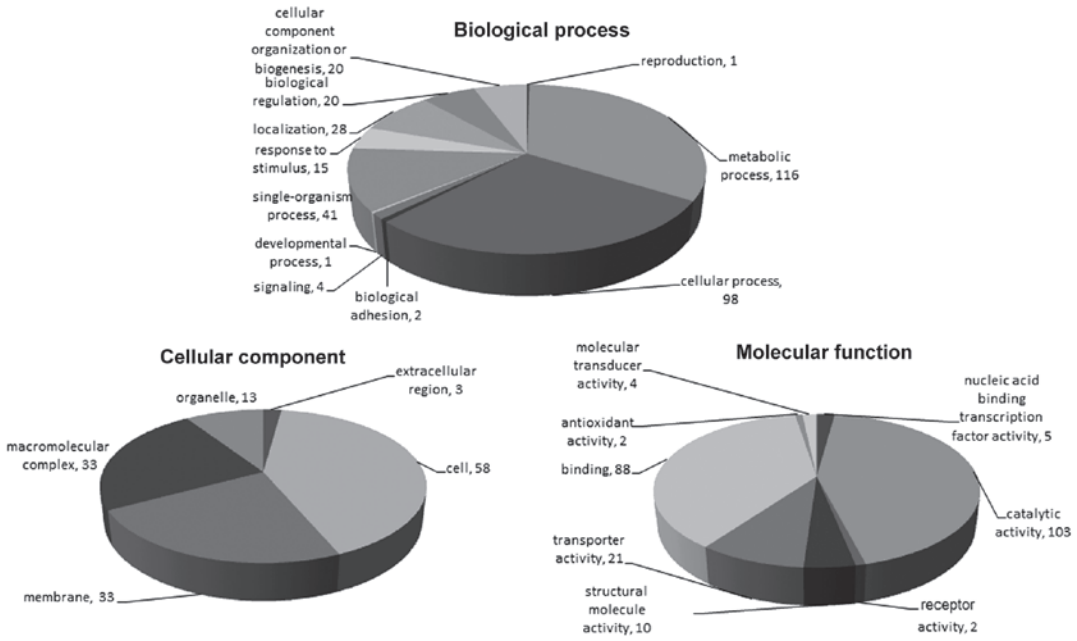

Figure 1. Classification of identified proteins according to gene ontology annotations.

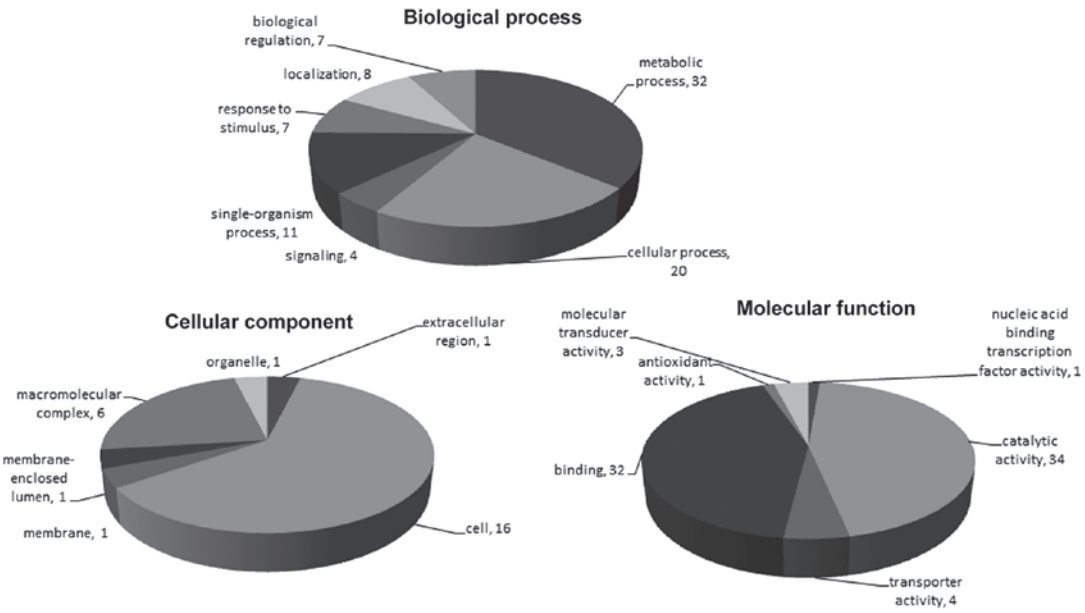

Figure 2. Classification of identified proteins according to gene ontology slim annotations.

proteins. As a result, 269 GO function entries associated with the sequences of 45 differentially expressed proteins (81.8%) were extracted. In the functional annotation process, the sequences of a total of 37 differentially expressed proteins were annotated with 87 GO function entries. The final statistical results after supplementary annotation showed that a total of 47 proteins were annotated with 188 GO function entries (Fig. 1).

GO for generic.obo was used to annotate the functions of the target proteins, and a total of 47 protein sequences were annotated by 194 GO Slim function entries (Fig. 2).

KAAS was used to align target-protein and Streptococcal sequences from the KEGG GENES database, and the KO identifiers of homologous/similar proteins were assigned to the relevant KEGG pathways. A total of 34 KEGG signal/metabolic pathways associated with the sequences of 21 differential proteins were extracted (Fig. 3).

Regulatory function of proteins or enzymes in carbon metabolic pathways. The data of the metabolic pathway analyses indicated that dihydrolipoyl transacetylase and dihydroxyacetone kinase and acetate kinase exhibited differential effects on the carbon metabolism between the native *S. suis* type 2 and ccpA mutant strains. These differential effects on the carbon metabolic pathways between the native and the ccpA mutant strain indicate that ccpA is directly or indirectly

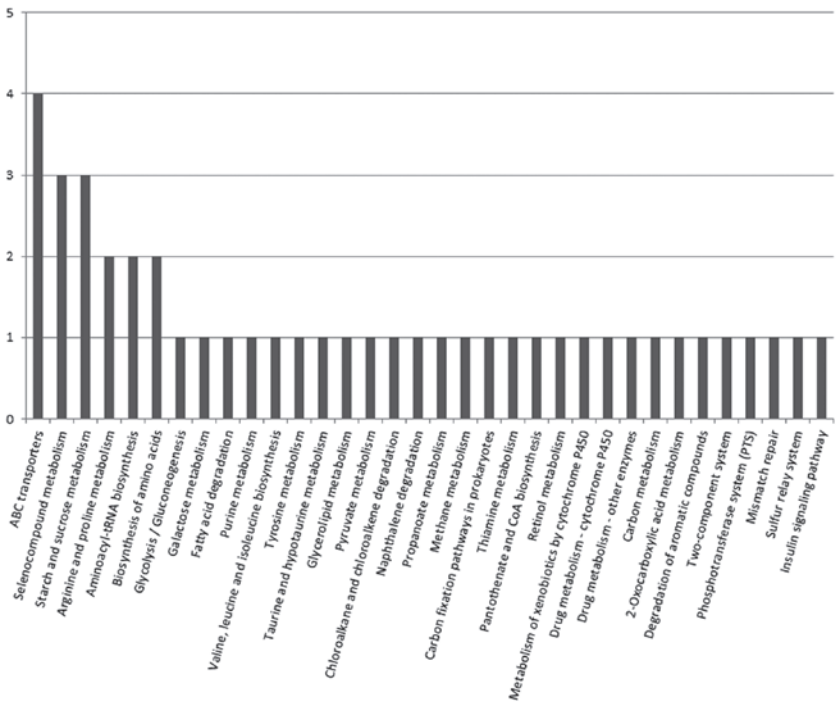

Figure 3. Pathways associated with differentially expressed proteins as determined by Kyoto Encyclopedia of Genes and Genomes analysis. ABC, adenosine triphosphate binding cassette; CoA, coenzyme A.

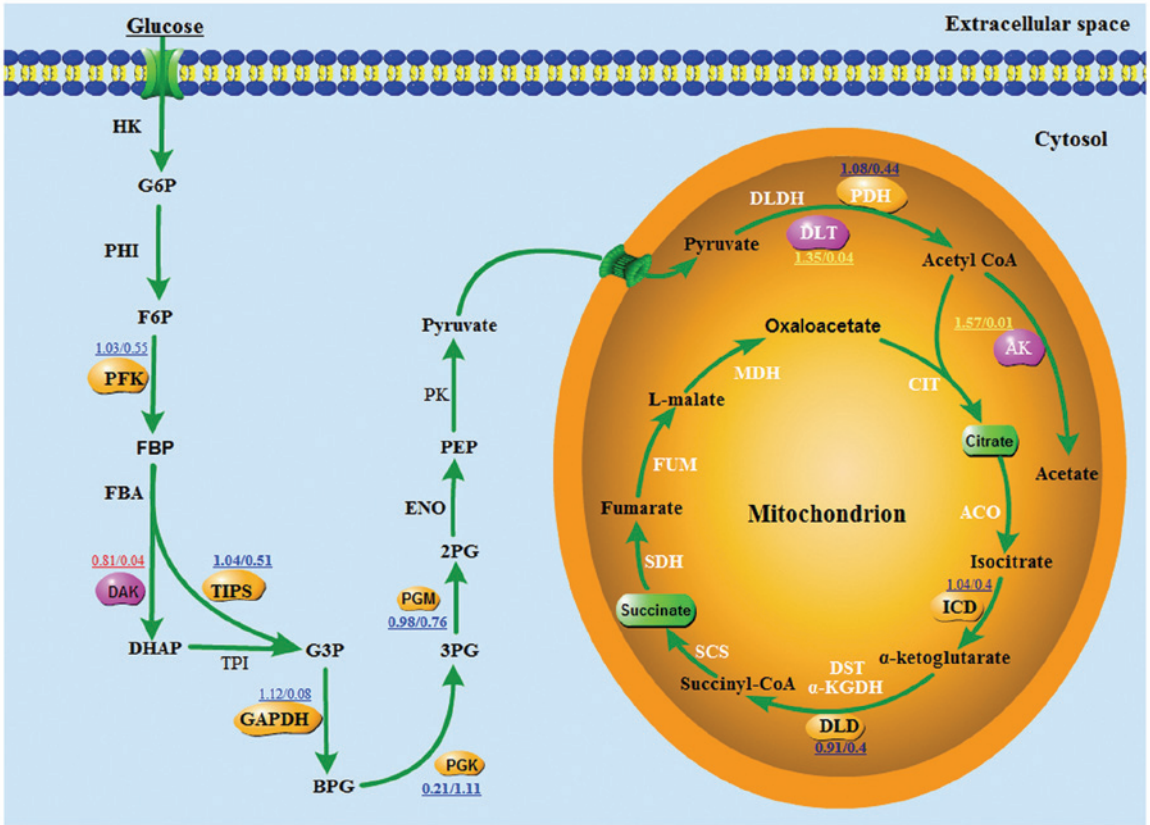

Figure 4. Regulatory function of proteins and enzymes in metabolic pathways.

involved in the carbon metabolism of bacteria. In particular, ccpA was indicated to have certain effects on the cellular metabolism of bacteria (Fig. 4).

Discussion

Two large-scale outbreaks of *S. suis* infection occurred in

Jiangsu Province and Sichuan Province of China in 1998 and 2005, respectively. These two public health events attracted worldwide attention and became the focus of domestic and international studies (10,11). Although the exact pathological mechanisms of *Streptococcus suis* has remained elusive, a number of potential virulence factors have been uncovered (12).

CcpA is not only involved in the regulation of carbon and nitrogen metabolism in bacteria, but also in specific physiological processes, including sporification, solvent production and virulence-gene expression in various microorganisms (13). In pathogens such as *Staphylococcus aureus*, ccpA directly regulates and activates the expression of virulence genes (12), while in *Clostridium perfringens*, it may regulate sporification and the expression of virulence genes, thereby affecting cellular function (14).

This gene may regulate the metabolic processes of bacteria at different levels and affect bacterial virulence, thereby exerting differential effects to cause pathological changes in infected organisms (6). CcpA deletion can reduce the activity of glycolytic enzymes, including enolase and other metabolic enzymes. In infected hosts, certain enzymes are associated with pathogenic virulence and specific metabolic requirements. Therefore, the virulence of a ccpA-deletion strain is obviously lower than that of a wild-type parent strain (15,16). However, ccpA directly activates the expression of virulence genes in certain pathogens, such as *Streptococcus pyogenes* (17,18). Thus, it is necessary to conduct in-depth studies of the structure, metabolic regulation and immunogenicity of ccpA, a regulator with multiple effects.

In the present study, differentially expressed proteins or peptides that primarily impact bacterial functions, including catalytic activity, metabolic processes, ion binding and nucleotide binding, were identified using iTRAQ technology. Bioinformatic analysis showed that ccpA markedly influenced protein metabolism in the bacterium as well as the processes of growth, metabolism and infection. Metabolism, signal transduction and pathway regulation for these processes were similar to the observations of the metabolomics analysis of the ccpA gene of *S. suis* performed in the present study. The metabolomics analysis of the present study found that ccpA mutation caused changes in metabolites, including glutamate, guanine, uridine and inosine, which are primarily involved in the metabolism of amino acids, nucleic acids, fats and certain small-molecule organic acids. These results have been verified by a further metabolomics study by our group (19). A comparison of the data from the two studies indicated that ccpA affected overall pathogenicity, metabolites and virulence through different pathways (9,19). The altered functions of various metabolic proteins, including peptidoglycan glycosyltransferase, enolase, sortase, GAPDH, 6-phosphogluconate dehydrogenase and glutamine synthetase may have affected the virulence of the bacteria, which was also reported by a previous study (20).

These results indicated that ccpA serves similar roles in *S. suis* and *Streptococcus pneumoniae*. CcpA regulates carbohydrate metabolism in bacteria by various mechanisms, thereby affecting the function and virulence of bacteria (8,21). The regulation of bacterial proteins by ccpA influences transcriptional regulation and signaling-pathway activation via different pathways, thereby impacting the phagocytosis of bacteria and bacterial virulence (22,23). The differentially expressed proteins identified in the present study included proteins associated with metabolism and molecular transport. This demonstrated that ccpA regulates the expression of intracellular products and various associated proteins by affecting metabolism, molecular transport and transcription, hence influencing the metabolic processes and virulence of bacteria. These changes were similar to regulatory processes in Gram-positive bacteria, including *Streptococcus pneumonia* and *Clostridium difficile* (24,25).

In the present study, iTRAQ-based proteomic technology has proven a useful tool for the functional study of the ccpA protein in *S. suis*. It was shown that ccpA influences important metabolic and regulatory pathways of this bacterium. These effects are mainly associated with glucose metabolism, amino acid metabolism, nucleic acid synthesis and adenosine triphosphate binding cassette transporters. The study of the specific impact of ccpA on metabolism, the mechanism of regulatory function through functional domain transformation and the transformation from multiple, complex effects to a single, simple effect are the focus of future research.

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References

1. Arends JP and Zanen HC: Meningitis caused by *Streptococcus suis* in humans. Rev Infect Dis 10: 131-137, 1988.
2. Gottschalk M, Xu J, Calzas C and Segura M: *Streptococcus suis*: A new emerging or an old neglected zoonotic pathogen? Future Microbiol 5: 371-391, 2010.
3. Zhang J, Zhu J, Ren H, *et al*: Rapid visual detection of highly pathogenic *Streptococcus suis* serotype 2 isolates by use of loop-mediated isothermal amplification. J Clin Microbiol 51: 3250-3256, 2013.
4. Kaufman GE and Yother J: CcpA-dependent and-independent control of beta-galactosidase expression in *Streptococcus pneumoniae* occurs via regulation of an upstream phosphotransferase system-encoding operon. J Bacteriol 189: 5183-5192, 2007.
5. Hondorp ER and McIver KS: The Mga virulence regulon: Infection where the grass is greener. Mol Microbiol 66: 1056-1065, 2007.
6. Poncet S, Milohanic E, Mazé A, *et al*: Correlations between carbon metabolism and virulence in bacteria. Contrib Microbiol 16: 88-102, 2009.
7. Sumby P, Barbian KD, Gardner DJ, *et al*: Extracellular deoxyribonuclease made by group A Streptococcus assists pathogenesis by enhancing evasion of the innate immune response. Proc Natl Acad Sci USA 102: 1679-1684, 2005.
8. Willenborg J, Fulde M, de Greeff A, *et al*: Role of glucose and CcpA in capsule expression and virulence of *Streptococcus suis*. Microbiology 157: 1823-1833, 2011.
9. Lang X, Wan Z, Pan Y, Wang X, Wang X, Bu Z, Qian J, Zeng H and Wang X: Investigation into the role of catabolite control protein A in the metabolic regulation of *Streptococcus suis* serotype 2 using gene expression profile analysis. Exp Ther Med 10: 127-132, 2015.
10. Tang J, Wang C, Feng Y, *et al*: Streptococcal toxic shock syndrome caused by *Streptococcus suis* serotype 2. PLoS Med 3: e151, 2006.

11. Sriskandan S and Slater JD: Invasive disease and toxic shock due to zoonotic *Streptococcus suis*: An emerging infection in the East? PLoS Med 3: e187, 2006.
12. Seidl K, Stucki M, Ruegg M, *et al*: *Staphylococcus aureus* CcpA affects virulence determinant production and antibiotic resistance. Antimicrob Agents Chemother 50: 1183-1194, 2006.
13. Warner JB and Lolkema JS: CcpA-dependent carbon catabolite repression in bacteria. Microbiol Mol Biol Rev 67: 475-490, 2003.
14. Varga J, Stirewalt VL and Melville SB: The CcpA protein is necessary for efficient sporulation and enterotoxin gene (cpe) regulation in *Clostridium perfringens*. J Bacteriol 186: 5221-5229, 2004.
15. Wang Y, Dang Y, Wang X, *et al*: Comparative proteomic analyses of *Streptococcus suis* serotype 2 cell wall-associated proteins. Curr Microbiol 62: 578-588, 2011.
16. Titgemeyer F and Hillen W: Global control of sugar metabolism: A gram-positive solution. Antonie Van Leeuwenhoek 82: 59-71, 2002.
17. Deutscher J, Herro R, Bourand A, Mijakovic I and Poncet S: P-Ser-HPr-a link between carbon metabolism and the virulence of some pathogenic bacteria. Biochim Biophys Acta 1754: 118-125, 2005.
18. Cho K and Caparon M: Patterns of virulence gene expression differ between biofilm and tissue communities of *Streptococcus pyogenes*. Mol Microbiol 57: 1545-1556, 2005.
19. Lang X, Wan Z, Bu Z, Wang X, Wang X, Zhu L, Wan J, Sun Y and Wang X: Catabolite control protein A is an important regulator of metabolism in *Streptococcus suis* type 2. Biomed Rep 2: 709-712, 2014.
20. Meijerink M, Ferrando ML, Lammers G, Taverne N, Smith HE and Wells JM: Immunomodulatory effects of *Streptococcus suis* capsule type on human dendritic cell responses, phagocytosis and intracellular survival. PLoS One 7: e35849, 2012.
21. Iyer R, Baliga NS and Camilli A: Catabolite control protein A (CcpA) contributes to virulence and regulation of sugar metabolism in *Streptococcus pneumoniae*. J Bacteriol 187: 8340-8349, 2005.
22. Seshasayee AS, Bertone P, Fraser GM and Luscombe NM: Transcriptional regulatory networks in bacteria: from input signals to output responses. Curr Opin Microbiol 9: 511-519, 2006.
23. Segura M, Gottschalk M and Olivier M: Encapsulated *Streptococcus suis* inhibits activation of signaling pathways involved in phagocytosis. Infect Immun 72: 5322-5330, 2004.
24. Kaufman GE and Yother J: CcpA-dependent and -independent control of beta-galactosidase expression in *Streptococcus pneumoniae* occurs via regulation of an upstream phosphotransferase system-encoding operon. J Bacteriol 189: 5183-5192, 2007.
25. Karlsson S, Burman LG and Åkerlund T: Induction of toxins in *Clostridium difficile* is associated with dramatic changes of its metabolism. Microbiology 154: 3430-3436, 2008.