Catabolite control protein A is an important regulator of metabolism in *Streptococcus suis* type 2

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Abstract. *Streptococcus suis* (*S. suis*) type 2 is an extremely important Gram-positive bacterial pathogen that can cause human or swine endocarditis, meningitis, bronchopneumonia, arthritis and sepsis. Catabolite control protein A (CcpA) is a major transcriptional regulator in *S. suis* type 2 that functions in catabolite control, specifically during growth on glucose or galactose. The regulation of central metabolism can affect the virulence of bacteria. In the present study, a metabolomics approach was used along with principal components analysis (PCA) and partial least-squares-discriminant analysis (PLS-DA) models and 37 metabolites were found that differed substantially between native *S. suis* and a mutant lacking CcpA. These results showed that CcpA is an important protein in *S. suis* type 2 for studying bacterial protein function.

Introduction

Streptococcus suis (*S. suis*) type 2 is an important Gram-positive bacterial pathogen of pigs that causes large economic losses in the meat industry, has a high rate of incidence and induces mortality worldwide (1,2).

Carbon catabolite repression (CCR) has a significant function in *S. suis* type 2 as it regulates the utilization of different carbon sources. CCR also affects the expression of numerous virulence factors that are dependent on the quantity and type of carbohydrates present (3,4). Catabolite control protein A (CcpA) is the major regulator of CCR in Gram-positive bacteria and is also involved in the regulation of virulence factors (5). Transcriptomic studies that compare native and mutant strains are a powerful approach for studying the regulation of carbohydrate metabolism and virulence (6). A previous study has shown that the mutation of CcpA leads to altered biofilm phenotypes in *Streptococcus mutans* (*S. mutans*) and other oral streptococci (7). CcpA was suggested to function in the regulation of virulence in numerous Gram-positive pathogens, particularly streptococci (8,9).

Our previous study systematically analyzed the virulence in *S. suis* type 2 using proteomics and found that CcpA is an important protein in this organism (10). In the present study, a metabolomics experiments was performed to identify the proteins and metabolites that differed in *S. suis* type 2 CcpA mutant strains. These results confirmed the importance of this protein in the organism.

Materials and methods

Bacterial strains and culture conditions. Native *S. suis* type 2 and CcpA mutant strains were cultured in Todd-Hewitt broth (Oxoid Ltd., Basingstoke, Hampshire, UK) with 5% CO_2 at 37˚C. This procedure was conducted to prepare cultures in the exponential growth phase for the subsequent experiments.

Metabolomics methodology and analysis. Quenching of the *S. suis* culture for the metabolomics experiments was performed by adding 60% MeOH and 0.85% (w/v) ammonium carbonate (pH 5.5). Centrifugation was performed at 10,000 x g for 2 min. The supernatants were then frozen in liquid nitrogen, freeze-dried and stored at -80˚C until they were required for subsequent analysis. The regulation of amino acid metabolism by CcpA was detected using liquid chromatography-mass spectrometry (LC-MS)-based metabolomic methods. The results were analyzed using MassHunter software (version B.03.01) from Agilent Technologies, Inc. (Santa Clara, CA, USA). The principal components analysis (PCA) and partial least squares-discriminant analysis (PLS-DA) were performed with the SIMCA-P software package (Umetrics, Inc., Umea, Sweden). The differentially expressed compounds were distinguished by $P<0.05$, which was considered to indicate a statistically significant difference (11,12). The metabolomics methods and analysis used in the study were carried out by Shanghai Sensichip Infotech Co., Ltd. (Shanghai, China).

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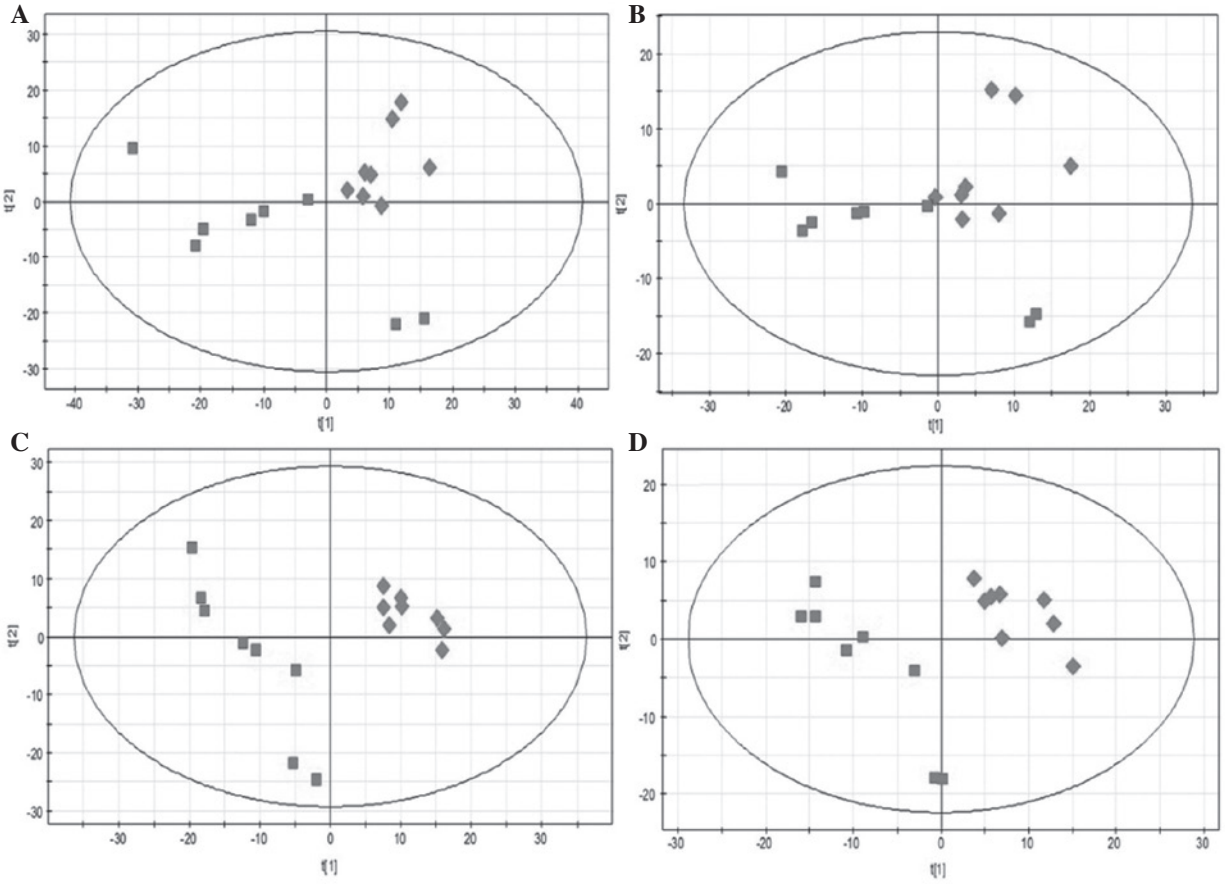

Figure 1. (A and B) Principal components analysis (PCA) and (C and D) partial least squares-discriminant analysis (PLS-DA) score plots derived from liquid chromatography-mass spectrometry results. (A and C) Electrospray ionization (ESI)+ model and (B and D) ESI- model. Rhombuses, native *Streptococcus suis* type 2; squares, catabolite control protein A mutant strain.

Results

Differences in metabolites between the bacterial strains. PCA and PLS-DA were used to analyze the metabolomics data. There was little overlap with the PCA model, suggesting this was appropriate for use in the present study. However, it was determined that PLS-DA should be used as the main model for analyzing the metabolomics samples as previously described (13). Based on the PLS-DA models, the native *S. suis* type 2 and CcpA mutant strains were successfully discriminated, with an R2X value of 0.439 and a Q2 value of 0.924 in the electrospray ionization (ESI)+ model using variable importance in the projection (VIP) of the first and second principal components (t[1] and t[2], respectively). An R2X value of 0.452 and a Q2 value of 0.878 were obtained with the ESI- model (Fig. 1). Using the T-test on the PLS-DA model, 37 differential metabolites were identified with a VIP score >1 (Table I). The model analysis suggested that the main differences were for the metabolites that were involved in amino acid, nucleic acid and fat metabolism, which all occur in the liver.

Discussion

S. suis type 2 is persistent in a wide variety of environmental conditions and this contributes to the serious economic losses endured by the swine industry when disease outbreaks occur. Another major concern to public health is the elusive mechanisms of invasion and infection, which remain to be clarified (14,15). CcpA-independent CCR appears to be important for the coordination of carbohydrate catabolism in this organism. The ability to integrate information regarding the metabolic status of the cells with a knowledge of the transcriptional responses would be advantageous. The mechanisms of catabolite control by CcpA have been studied in model bacteria previously (6). Transcriptomics and microarray studies have shown that this protein regulates carbohydrate metabolism in *S. mutans* (6). The deletion of CcpA substantially reduced the effects of repressing sugars on α- or β-glucosidase activity. CcpA regulates several proteins that are involved in metabolism, a number of which are important for bacterial colonization, survival or replication *in vivo* (16). CcpA is part of a conserved pathway in Gram-positive bacteria that controls sugar utilization, and targeting this protein or pathway is an attractive mechanism for diminishing virulence (17).

CcpA also affects sugar metabolism and virulence in *S. suis* type 2 and mutant strains lacking this protein exhibit a reduced expression of certain virulence genes, including sly and eno (1). CcpA is considered to regulate virulence factors through the expression of genes in the capsule locus (18). This is reminiscent of a functional study in *Streptococcus pneumoniae*, which showed that CcpA is a master regulator of catabolism and virulence (19). A notable impact on the capsule synthesis and virulence properties of *S. suis* has also been shown (20).

Table I. Metabolite differences between native *Streptococcus suis* type 2 and CcpA mutant strains.

LC-MS	Retention time, min	Molecular mass	Metabolite	VIP score[a]	Fold change[b]
ESI+	0.66	103.0631	γ-aminobutryic acid	1.98	0.58
	0.68	165.0456	L-methionine S-oxide	1.55	0.29
	0.68	147.0527	L-glutamate	1.98	0.42
	0.74	159.1260	DL-2-aminooctanoic acid	1.28	-0.45
	0.74	453.2959	Glycerophospho-N-palmitoyl ethanolamine	1.83	-2.38
	0.82	149.0509	L-methionine	1.84	1.63
	1.02	244.0703	Uridine	1.91	-1.56
	1.08	267.0971	Deoxyguanosine	2.29	3.20
	1.16	573.1530	Biotinyl-5'-AMP	1.74	-2.28
	1.17	268.0795	Inosine	1.67	-0.98
	3.72	441.2594	Leukotriene E3	1.28	-0.61
	10.14	255.2567	Palmitic amide	1.73	-0.63
	10.65	329.2575	4,8-Dimethylnonanoyl-carnitine	2.24	0.95
	13.44	281.2723	Oleamide	1.88	0.54
	13.93	160.0887	Homoglutamine	1.77	0.35
	15.26	384.2865	17,20-Dimethyl prostaglandin F1α	1.84	0.76
	16.07	148.0376	2-Hydroxyglutarate	2.21	0.88
ESI-	0.67	133.0378	L-aspartic acid	1.76	0.32
	0.68	147.0535	L-glutamate	1.63	0.44
	0.70	183.0656	Phosphocholine	1.82	1.19
	0.74	151.0504	Guanine	1.77	1.42
	0.75	324.0371	Uridine monophosphate	1.95	1.89
	1.00	168.0285	Uric acid	1.36	-0.97
	1.00	152.0336	Xanthine	1.46	0.36
	1.02	244.0707	Uridine	1.56	-0.58
	1.05	192.0272	Citric acid	1.64	-2.85
	1.16	268.0820	Inosine	1.67	-1.86
	1.17	118.0266	Succinic acid	1.52	-0.15
	1.23	131.0948	L-leucine	1.47	-0.82
	3.80	204.0905	L-tryptophan	1.53	-0.25
	3.93	427.2449	20-Hydroxy N-arachidonoyl taurine	1.33	-0.22
	4.80	643.3191	Gsh-prostaglandin A1	1.86	-0.32
	4.93	723.3088	Trypanothione	1.52	-0.71
	5.62	187.1212	8-Amino-7-oxononanoic acid	1.35	0.68
	5.83	193.0742	Phenylacetylglycine	1.61	-1.14
	11.72	450.1830	Geranylgeranyl PP	1.54	0.73
	14.44	166.0484	7-Methylxanthine	1.53	1.53

[a]VIP, variable importance in the projection ($P<0.05$). [b]Fold change was calculated as binary logarithm of average mass response ratio between native *Streptococcus suis* type 2 and CcpA mutant strain, where the positive value means that the native *Streptococcus suis* type 2 of the metabolite is larger than the CcpA mutant strain. CcpA, catabolite control protein A; LC-MS, liquid chromatography-mass spectrometry; ESI, electrospray ionization.

The LC-MS-based metabolomics experiments in the present study suggested that CcpA is involved in the regulation of amino acid metabolism, which may affect the metabolic fitness and is in accordance with the findings of other studies (21). The PLS-DA model showed that CcpA acts as a regulator of catabolite control, and has a particular influence on the metabolism of sugars, fats and amino acids.

The experiments also showed that CcpA is an important regulator of metabolism in *S. suis* type 2. Additionally, the analysis of the metabolomics results identified numerous metabolites, including ABC transporters, amino sugars, carbon metabolism, glycolysis and galactose metabolism. This result indicates that CcpA may be involved in mediating metabolic networks and coordinating the regulation of catabolism and anabolism to ensure optimum growth and propagation under particular growth conditions. Notably, *Bacillus subtilis* mutants lacking CcpA have a slower growth rate than native bacteria in minimal media containing glucose and ammonium

as carbon and nitrogen sources (22,23). This result suggests that CcpA may regulate carbon and nitrogen metabolic pathways and function in connecting catabolism and anabolism.

From the data of the metabolomics analyses, it was found that glutamate, glutamine and other deviants and enzymes have different effects between the native *S. suis* type 2 and CcpA mutant strains. These, and other genes, were found to have extremely important effects for the methionine acquisition and synthesis of *Streptococcus pneumoniae* in the growth and virulence when using gene deletions methods (24). By contrast, the methionine aminopeptidase is a dinuclear metalloprotease, which is conserved in all forms of life in bacteria (25). Others, including the glutamine, glutamate and uridine biosynthesis and transport, are stringently controlled during bacteria metabolism (26-28). Thus, these results may provide a clear insight into the regulation of glutamine and glutamate metabolism or another mechanism of *S. suis* type 2 mediated by CcpA.

In conclusion, the metabolomics analyses performed in the present study proved useful for studying bacterial protein function. CcpA was found to affect numerous metabolic pathways in *S. suis*. The apparent associations with the metabolism of sugars, amino acids, amino sugars, nucleotides and ABC transporters are potentially noteworthy. However, more studies are required to confirm and understand these phenomena.

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