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Catabolite Control Protein A of *Streptococcus suis* Type 2 Contributes to Sugar Metabolism and Virulence

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Catabolite control protein A (CcpA) is the major transcriptional regulator in carbon catabolite repression in several Gram-positive bacteria. We attempted to characterize the role of a CcpA homologue of *Streptococcus suis* type 2 in sugar metabolism and virulence. Addition of glucose or sucrose to the defined medium significantly reduced the activity of raffinose-inducible α-galactosidase, cellobiose-inducible β-glucosidase, and maltose-inducible α-glucosidase of the wild-type strain by about 9, 4, and 2-3 fold, respectively. Deletion of *ccpA* substantially derepressed the effects of repressing sugars on α-galactosidase or β-glucosidase activity. The *ccpA* deletion mutant showed reduced expression of virulence genes *sly* and *eno* ($P<0.05$), decreased adhesion to and invasion into endothelial cells ($P<0.05$), and attenuated virulence to mice with significant reduction of death rate and bacterial burden in organs, as compared to the wild-type strain. Both the *in vitro* and *in vivo* defect phenotypes were reversible by *ccpA* complementation. Thus, this study shows that CcpA of *S. suis* type 2 plays an important role in carbon catabolite repression and virulence.

Keywords: *Streptoccoccus suis* type 2, catabolite control protein A, carbon catabolite repression, virulence

Introduction

Streptococcus suis type 2 is a zoonotic swine pathogen that can be transmitted to humans by direct contact with infected pigs or pork-derived products, causing meningoencephalitis, arthritis, septicemia and even sudden death (Wertheim *et al.*, 2009; Tang *et al.*, 2011). The bacterium causes sporadic human cases. However, there were two human outbreaks of streptococcal toxic shock syndrome (STSS) in China in 1998 and 2005 with mortality of 62.7% and 81.3%, respectively (Tang *et al.*, 2006). In addition to known virulence factors such as suilysin (Jacobs *et al.*, 1994), peptidoglycan N-acetylglucosamine pgdA (Fittipaldi *et al.*, 2008), capsular polysaccharide (Smith *et al.*, 1999), and sortase A (Wang *et al.*, 2009), recent studies have found that some transcriptional regulators of *S. suis* type 2 are involved in regulation of virulence, including Salk-SalR (Li *et al.*, 2008), Rgg (Zheng *et al.*, 2011), and CcpA (catabolite control protein A) (Willenborg *et al.*, 2011).

The pathogenesis of *S. suis* type 2 infections is not well understood. It is believed that the bacterium enters through the respiratory route to colonize tonsils, subsequently escapes from immune surveillance and reaches the blood stream (Dominguez-Punaro Mde *et al.*, 2008). Some regulatory genes might be able to respond to environmental signals and regulate expression of genes important for survival. CcpA, as a global regulatory protein, is involved in regulation of virulence factors in Gram-positive pathogens (Iyer *et al.*, 2005; Abranches *et al.*, 2008). Willenborg *et al.* (2011) demonstrated that CcpA of *S. suis* type 2 altered expression of virulence factors and reduced resistance against porcine neutrophils. CcpA is involved in carbon catabolite repression (CCR) that has been extensively analyzed in a number of *Streptococcus* spp. (Asanuma *et al.*, 2004; Iyer *et al.*, 2005). When bacteria are exposed to two or more carbon sources, one of them is preferentially utilized (Deutscher, 2008). CCR could be important during bacterial infection of the host compartments where multiple sugars are available (Iyer *et al.*, 2005). In the present study, we characterized the roles of CcpA from *S. suis* type 2 in virulence to mice as well as in carbon catabolite repression.

Materials and Methods

Bacterial strains and culture conditions

Bacterial strains and plasmids used in the present study are listed in Table 1. The *S. suis* type 2 strain ZJJX081101 was isolated from the lung of a diseased pig (Tang *et al.*, 2011). Unless otherwise indicated, *S. suis* type 2 strains were grown in Brain Heart Infusion medium (BHI; Oxoid, UK) at 37°C. *E. coli* strains were grown in Luria-Bertani broth or agar at 37°C. Antibiotics (Sigma, USA) were added, where necessary, to the culture media at the following concentrations: spectinomycin (Spc) at 100 μg/ml and chloramphenicol (Cm) at 4 μg/ml for *S. suis* type 2, and Spc at 50 μg/ml, Cm at 8 μg/ml and ampicillin at 100 μg/ml for *E. coli*.

Construction of the *ccpA* deletion mutant

To construct the *ccpA* deletion mutant from *S. suis* type 2 strain ZJJX081101, the 5′ and 3′ flanking regions of the *ccpA*

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Table 1. Table 1. Strains and plasmids used in this study

Strains/plasmids	Characteristics/function	Source/reference
Bacterial strains		
S. suis type 2 ZJJX081101	High virulent strain of SS2 isolated from a dead pig	This study
E. coli DH5α	Cloning host for maintaining the recombinant plasmids	Promega
ΔccpA	The deletion mutant of ccpA with background of ZJJX081101, Cm^R	This study
CΔccpA	Complemented strain of ΔccpA; Spc^R; Cm^R	This study
Plasmids		
pMD-18T	Cloning vector; Amp^R	Takara
pSET2s	E.coli-S.suis shuttle vector	Takamatsu *et al.* (2001a)
pSET4s	Thermosensitive vector Cm^R	Takamatsu *et al.* (2001b)
pSET5s	Thermosensitive vector Spc^R	Takamatsu *et al.* (2001b)
pSET4s::ΔccpA	pSET4s carrying the construct for *ccpA* allelic replacement	This study
pSET2s::ccpA	pSET2s inserted with the intact *ccpA* genes and the upstream promoter	This study
pET30-ccpA	pET30a inserted with the intact *ccpA* gene for expression in *E. coli*	This study

ORF were PCR-amplified with the primer pairs ccpA-1/ccpA-2 and ccpA-3/ccpA-4 (Table 2). The PCR products were digested with *Bam*HI and *EcoT22*I or *EcoT22*I and *Sph*I, fused at the *EcoT22*I site, and cloned into the *Bam*HI and *Sph*I sites of pSET4s. The unique *EcoT22*I site was then used to introduce a chloramphenicol acetyltransferase gene (*cat*) amplified from pSET6s with the primers 6s-F and 6s-R (Table 2). The resulting insert was subcloned between the *Bam*HI and *Sph*I sites of pSET4s, generating the knockout vector pSET4s-ΔccpA.

Mutants were constructed by allelic exchange via double crossover as described previously (Osaki *et al.*, 2002). Briefly, the *S. suis* type 2 strain was electro-transformed with pSET4s-ΔccpA, and the transformants were grown to mid-logarithmic growth phase at 28°C in the presence of Cm and Spc. The cells were then transferred to fresh medium containing Cm and grown at 28°C to early logarithmic phase. The cultures were shifted to 37°C and incubated for 4 h, followed by spreading on BHI agar plates containing Cm and incubated at 37°C for 18 h. Temperature-resistant and Cm-resistant colonies were screened for loss of Spc resistance. This identified allelic exchange mutants leaving the *cat* gene in the genome as a result of homologous recombination. The mutant was designated as ΔccpA.

Complementation of the ΔCcpA mutant

A DNA fragment containing *ccpA* and its upstream promoter was amplified from *S. suis* type 2 chromosomal DNA with the primer sets CccpA-F and CccpA-R. The PCR product was digested with *Bam*HI and *Eco*RI (Table 2). The resulting DNA fragment was cloned into the shuttle vector pSET2s to generate the recombinant plasmid pSET2s::ccpA, which was then electro-transformed into the ΔccpA mutant to screen for the complemented strain on BHI agar containing Spc and Cm, and the mutant strain carrying the recombinant vector was designated as CΔccpA.

Table 2. Primers used for PCR amplification and detection

Primers	Sequences of primers	Location or description
ccpA-1F	ACTTGGATCCTGGTAGCGGGTTAAATTC	5′ flanking region of *ccpA* with *Bam*HI site
ccpA-1R	CATATTTAATGCATACGTTATGTACTCCCTTT	5′ region of *ccpA* with *EcoT22*I site
ccpA-2F	CATAACGTATGCATTAAATATGGGAGTAGCCG	5′ region of *ccpA* with *EcoT22*I site
ccpA-2R	ACGCCTCGAGAACATTTAAAACCGAAAC	5′ flanking region of *ccpA* with *Sph*I site
6s-F	TAGTATGCATTAATTCGATGGGTTCCGAGG	5′ end of cat with *EcoT22*I site
6s-R	TCACATGCATCACCGAACTAGAGCTTGATG	3′ end of cat with *EcoT22*I site
ccpA-F	TGGGGACGGGGGCATTAT	Segment of *ccp*A gene
ccpA-R	TAGCGGCAGCGTAGTCAA	Segment of *ccpA* gene
CccpA-F	GGCCGAATTCTTTCTAGCATGTAGTGG	upstream of *ccpA* gene with *Eco*RI site
CccpA-R	AATTGGATCCAGTCGAAGCAAGTTTGC	downstream of *ccpA* gene with *Bam*HI site
rccpA-F	ATGCGGATCCATGTTAAACACTGACGAT	5′ region of *ccpA* gene with *Eco*RI site
rccpA-R	CTGACTCGAGCTTAGTTGATTTACGTAC	3′ region of *ccpA* gene with *Eco*RI site
qccpA-F	CGGTGTCAGTGATATGGG	*ccpA* gene for qRT-PCR
qccpA-R	GTCAGGTTTGGACGGGTA	*ccpA* gene for qRT-PCR
q16s-F	GTAGTCCACGCCGTAAAC	16S rRNA for qRT-PCR
q16s-R	TAAACCACATGCTCCACC	16S rRNA for qRT-PCR
qsly-F	TAATCCGCCAGCAACAAC	*sly* gene for qRT-PCR
qsly-R	TCAGCACGCAATAAAGCAC	*sly* gene for qRT-PCR
qeno-F	AATCCTCGGTGTTTCTATC	*eno* gene for qRT-PCR
qeno-R	TGGAGTTGGCAATACTTT	*eno* gene for qRT-PCR
qcps2a-F	GATGTGGCGTAAGAAAG	*cps2a* gene for qRT-PCR
qcps2a-R	GACGAACGTCCGTAAT	*cps2a* gene for qRT-PCR

Cloning and expression of *S. suis ccpA* and Western blot analysis

A pair of specific primers (rccpA-F/rccpA-R) was designed for amplification of the *ccpA* gene (Table 2). The amplified product was digested with respective enzymes and subsequently cloned into the expression vector pET30a (Novagen, USA). Insertion was verified by restriction analysis and sequencing. *E. coli* BL21 transformed with pET30-ccpA was induced at 37°C for 4 h with the addition of 1.0 mmol/L isopropyl-beta-D-thiogalactopyranoside (IPTG). Bacterial pellets were sonicated and centrifuged to remove the insoluble debris. The His-tagged recombinant protein was purified by affinity chromatography on a nickel-nitrilotriacetic acid column according to the manufacturer's instructions (Wei's Bohui Chromatography Sci & Tech Co. Ltd., China). The target protein was separated by SDS-PAGE and the concentration determined by the Bradford method. Polyclonal antiserum against CcpA was raised in a New Zealand white rabbit by three immunizations with 50 μg purified protein and 50% Freund's incomplete adjuvant for Western blotting.

Quantitative PCR

To examine if *ccpA* is transcribed in the complemented mutant strain and if the ccpA deletion affects transcription of virulence factors (*sly*, *cps2a*, and *eno*), total RNA was extracted from the logarithmic phase culture (OD_{600nm}≈0.4) from ΔccpA, CΔccpA and its parent strain ZJJX081101 using the RNA simple Total RNA kit (Sangon Inc. Ltd., China) and treated with DNase I (TaKaRa, China) at 37°C for 30 min. The RNA quality was measured on the NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Only intact RNA samples with 260 nm:280 nm ratio between 2.0 and 2.2 were used for cDNA synthesis.

First-strand cDNA was synthesized from total RNA using an M-MLV First-Strand cDNA synthesis kit (Promega, USA). Quantitative PCR was conducted to detect relative ccpA mRNA expression levels with 16S rRNA serving as control using the SYBR® Green Real-time PCR Master Mix kit (ToYoBo Co. Ltd., Japan) according to the manufacturer's instructions. The 10-μl PCR mixtures included 7.5 μl of PCR master mix, 0.4 μl of 10 μM primers (Table 1), 1 μl of cDNA and 1.1 μl of Milli-Q water, and subjected to 95°C pre-denaturation for 30 sec, followed by 40 cycles at 95°C for 10 sec, 55°C for 10 sec, and 72°C for 15 sec in the iQ5™ real-time multicolor PCR detector (Bio-Rad, USA). Fold changes in gene expression were calculated as $2^{-\Delta\Delta Ct}$. All reactions were performed in quadruplicate and mRNA expression in mutant strains ΔccpA and CΔccpA was shown as percent relative to the parent strain ZJJX081101 (mean % ±SD).

Bacterial growth in sugar defined media

For the sugar hydrolysis assays, *S. suis* type 2 mutant and wild-type strains were grown in a tryptone-vitamin base (TV) medium (Burne *et al.*, 1999) containing primary carbon sources for growth. Inducing sugar (maltose, cellobiose or raffinose) was used alone or in combination with a repressing sugar (glucose or sucrose) to create catabolite repressing conditions. Sugars were used at final concentrations of 10 mM in all experiments. Bacterial growth in TV medium without any sugars was used as control.

Enzyme assays

The overnight bacterial cultures in BHI were reinoculated into defined media containing single sugars or combinations of sugars and incubated at 37°C for 6–8 h. The cultures were then pelleted at 8,000×g for 2 min and adjusted to 0.2 at $OD_{600\ nm}$ in phosphate buffered saline (PBS, 10 mM, pH 7.0). One milliliter of the bacterial suspensions was pelleted again and resuspended in 100 μl of PBS. One microliter of a 10% Triton X-100 stock was added to the bacterial suspension and incubated at 37°C for 5 min. The α-glucosidase, β-glucosidase, and α-galactosidase activities were measured as previously described (Iyer *et al.*, 2005).

Adhesion and invasion assays

The bEnd.3 endothelial cells were grown in 24-well tissue culture plates using RPMI 1640 culture medium (Invitrogen, USA). *S. suis* type 2 strains were cultured in BHI for 6 h at 37°C, harvested by centrifugation, washed twice in PBS, and resuspended in fresh RPMI 1640 medium. Confluent monolayers of bEnd.3 at 10^5 cells/well were infected with 0.4 ml aliquots of bacterial suspensions at 10^7 CFU/ml to obtain a multiplicity of infection (MOI) of 1:100. For adhesion, the plates were centrifuged at 800×g for 10 min and incubated for 1 h at 37°C and 5% CO_2. Monolayers were then washed 4 times with PBS, trypsinized and disrupted by mild pipetting. Viable bacterial cells were determined by plating suitable dilutions of the lysates onto BHI agar. For the invasion assay, the infected monolayers were incubated for 1 h at 37°C and 5% CO_2 and then subjected to antibiotic (100 μg/ml of gentamicin and 5 μg/ml of penicillin G) killing of extracellular bacteria for 1 h at 37°C. Viable intracellular bacteria were counted. Invasion and adhesion rates were expressed using the following formula: adhesion rate ‰=(CFU_{Adh}/CFU_{Total})×1000, and invasion rate %=(CFU_{Inv}/$CFU_{Adh+Inv}$)×100, where CFU_{Adh} is bacterial numbers that adhered to cells; CFU_{Total} is the total number of bacteria added to the cell monolayers; CFU_{Inv} is the numbers that invaded cells; and $CFU_{Adh+Inv}$ is the total number of bacteria adhered to and invaded into cells.

Mouse infection

A total of 70 female BALB/c mice (3 weeks old) were used in the experiment. Animals were divided into 7 groups, 10 mice per group. Groups I and II were inoculated intraperitoneally with a 0.3 ml suspension of the wild-type strain at two different inoculum levels, while groups III and IV as well as groups V and VI received similar doses of mutants ΔccpA and CΔccpA, respectively. The inoculum sizes ranged from 3.3–3.6×10^8 CFU/mouse for the lower dose and 7.0–7.3×10^8 CFU/mouse for the higher dose. Mice in control group VII were injected with 0.3 ml PBS. Mortality was recorded twice a day post-inoculation (p.i.). Surviving animals were euthanized at the 6th day p.i. In a separate experiment, the bacterial burden was examined for four mouse tissues (brain, kidney, liver and spleen) (6 mice/strain) at 30 h.p.i.

Table 3. Comparison of *S. suis* type 2 CcpA with similar gene products from other *Streptococcus* species and *B. subtilis*

Bacterium	No. Amino acids	Amino acid identity (%)	Amino acid identity of DNA binding site (%)[a]	Reference
Streptococcus suis	334			This study (TIGR)
Streptococcus agalactiae	334	82	100	Tettelin *et al.* (2002)
Streptococcus mutans	333	81	100	Simpson and Russell (1998)
Streptococcus thermophilus	333	79	100	Van den Bogaard *et al.* (2000)
Streptococcus pneumoniae	336	81	100	Giammarinaro and paton (2002)
bacillus subtilis	334	56	88	Faires *et al.* (1999)

[a] Sequences of the DNA binding sites were shown in Fig. 1.

for mice that survived after a challenge with 3.7–4.0×10^8 of the ΔccpA mutant and its parent strain. The tissues were homogenized in PBS containing 20% glycerol. The homogenates were diluted 10-fold and spread on BHI agar plates for enumeration. The animal experiments were approved by the Zhejiang University Committee for Laboratory Animal Management.

Statistics

Unless otherwise specified, all data were expressed as mean ±SD, and analyzed by two-tailed student's *t*-test. *P* values <0.05 or <0.01 were considered as statistically significant.

Results

Structural characteristics of CcpA in *S. suis* type 2

A putative *ccpA* gene was identified from the genome of *S. suis* type 2 based on the *ccpA* sequences of other streptococcal species. BLASTP analysis indicated that the putative CcpA showed significant similarity (79–82%) to members of the LacI/GalR family in other *Streptococcus* spp. (Table 3). Analysis of the promoter region of *ccpA* revealed the presence of a putative catabolite responsive element (cre) (Weickert and Chambliss, 1990) upstream of the initiation codon (Fig. 1). The DNA-binding domain of CcpA is highly conserved among the five *Streptococcus* species examined, and differs from that of *B. subtilis* by only 6 amino acids.

CcpA is involved in expression of virulence-related genes

Quantitative PCR showed that *ccpA* transcription in the complemented mutant (CΔccpA) was 1.3-fold higher than the parent strain, and the CΔccpA strain also expressed CcpA as shown on the Western blot (Fig. 2A), indicating that complementation worked. As a global regulator (Willenborg *et al.*, 2011), *ccpA* deletion could have effects on expression of virulence-related genes. Thus, we analyzed the expression of virulence genes *sly*, *cps2a*, and *eno* in the wild type, ΔccpA and CΔccpA strains. The transcription levels of *sly*, *cps2a* and *eno* in logarithmic cultures were significantly lower in the ΔccpA mutant than its parent (Fig. 2B). The transcripts of the complemented strain were partly restored to that of the

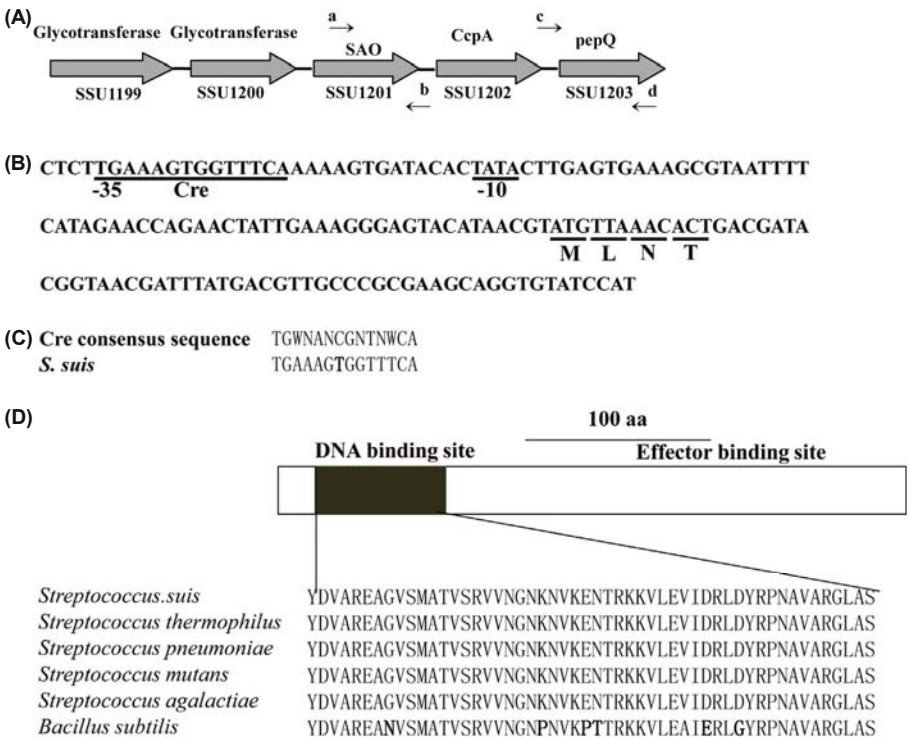

Fig. 1. Structural characteristics of the CcpA region in *S. suis* type 2. (A) Relative positions of the primers (a, b, c and d) used to create the knockout mutant are indicated by arrows. (B) The promoter region of *ccpA*: The -35 and -10 promoter elements are indicated and the 14-bp sequence with homology to the cre consensus sequence is underlined. (C) Comparison of the cre-like sequence in the *ccpA* promoter region with the consensus cre sequence that differs by only one nucleotide (indicated in bold) defined by Weickert and Chambliss (Weickert and Chambliss, 1990). (D) Comparison of the DNA binding domain of CcpA in different *Streptococcus* species and *B. subtilis*.

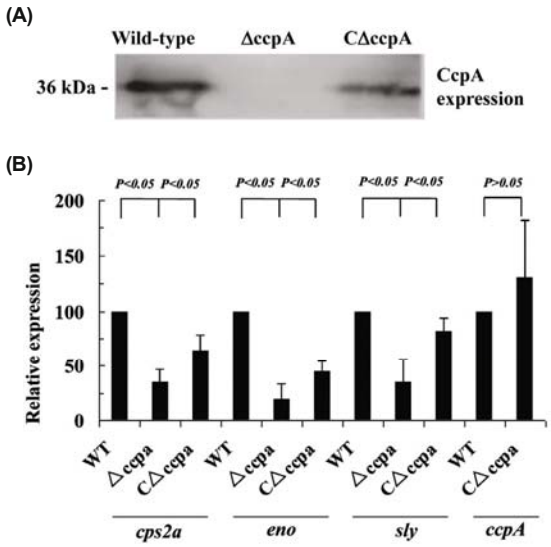

Fig. 2. Analysis of CcpA expression and virulence-related gene transcription in *S. suis* type 2 wild-type (WT), *ccpA* deletion (ΔccpA), and *ccpA* complemented (CΔccpA) strains. (A) Expression of CcpA in the wild type and CΔccpA by Western blotting. (B) Effects of *ccpA* deletion on expression of virulence-related genes *sly*, *eno*, and *cps2a* as measured by quantitative PCR.

parent strain in CΔccpA (Fig. 2B). These data indicate that CcpA might affect virulence by influencing the expression of virulence-related genes.

CcpA is involved in growth of *S. suis* type 2 in two-sugar media

Given the putative role of CcpA as a catabolite repressor, we investigated the growth rate of wild-type and mutant strains in TV media containing one of the sugars as the sole carbon source. In the single sugar medium containing cellobiose, raffinose or mannitol, there was no significant difference of growth between the wild-type and mutant strains ($P>0.05$) (Fig. 3A). However, the mutant strain exhibited significantly slower growth than the parent strain in the TV medium containing sucrose or glucose alone ($P<0.05$) (Fig. 3A). Growth was clearly slower in the two-sugar media containing sucrose or glucose together with cellobiose, raffinose or mannitol ($P<0.01$) (Fig. 3B). The combination of sucrose and glucose appeared to reduce growth of the *ccpA* deletion mutant as compared with the wild-type strain, although there was no statistical difference ($P>0.05$) (Fig. 3C).

CcpA is involved in carbon catabolite repression of α-galactosidase and β-glucosidase activities

S. suis type 2 can utilize different kinds of sugars, including maltose, cellobiose and raffinose, for its growth when provided as the sole carbon source (Fig. 3). Three enzymes α-glucosidase, β-glucosidase, and α-galactosidase were tested to examine the role of CcpA in CCR of sugar metabolism in *S. suis* type 2 strains. Presence of either glucose or sucrose in the TV medium significantly repressed raffinose-inducible α-galactosidase and cellobiose-inducible β-glucosidase activities in wild-type *S. suis* type 2, about 9-fold and 4-fold (expressed as A_{400nm}) respectively, as compared to single sugar

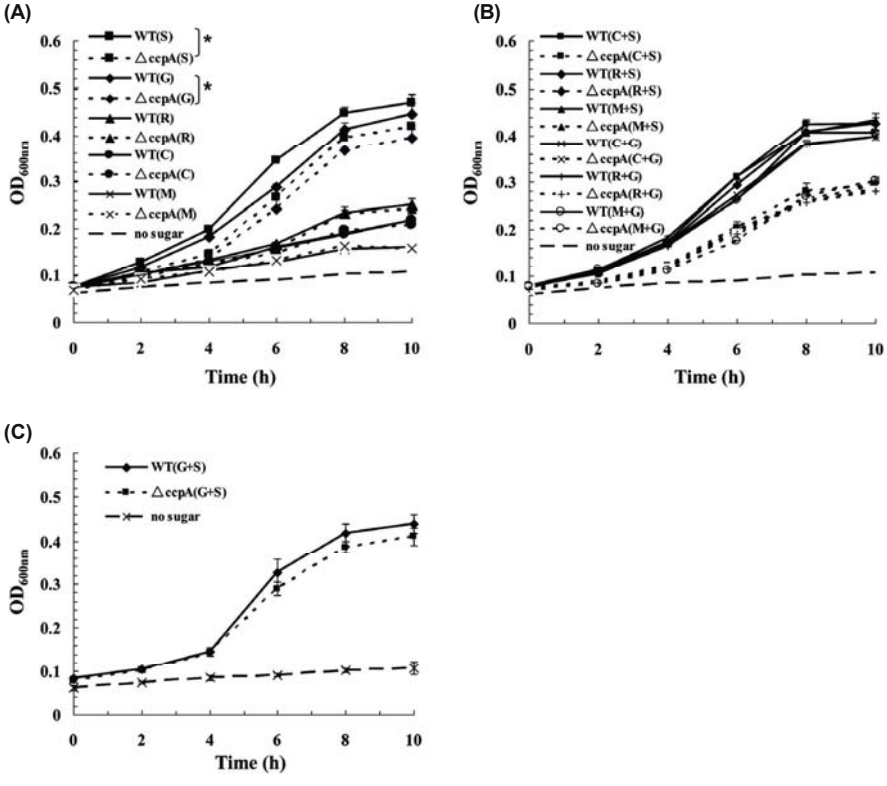

Fig. 3. Growth of *S. suis* type 2 wild-type (WT), *ccpA* deletion (ΔccpA), and *ccpA* complemented (CΔccpA) strains in TV medium with different sugars alone (A) or in combination (B and C). Abbreviations for figure labels: G, glucose; S, sucrose; R, raffinose; C, cellobiose and M, maltose. *denotes statistical difference ($P<0.05$) between *ccpA* deletion mutant and wild-type strain.

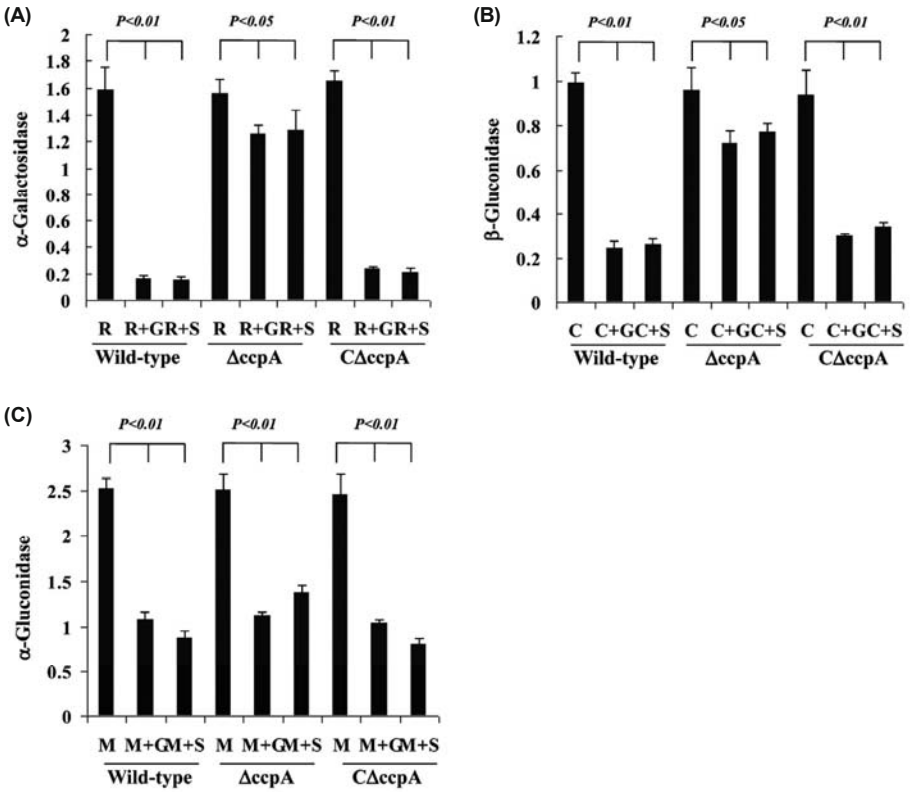

Fig. 4. Carbon catabolite repression of sugar-degrading enzymes in *S. suis* type 2 wild-type, *ccpA* deletion (ΔccpA) and *ccpA* complemented (CΔccpA) strains. (A) α-galactosidase; (B) β-glucosidase; and (C) α-glucosidase. The inducing sugars include raffinose, 'R'; cellobiose, 'C'; and maltose, 'M' either alone or in combination with the repressing sugar glucose, 'G' or sucrose, 'S'. Data are expressed as mean±SD of three independent experiments, each in duplicate wells.

controls (Figs. 4A and 4B) (P<0.01). The inhibition was also statistically significant (P<0.01), but at a lesser degree (2–3 fold) with maltose-utilizing α-glucosidase activity in presence of repressing sugars (Fig. 4C). Deletion of *ccpA* did not affect utilization of the single sugars by α-galactosidase and β-glucosidase, but did cause reduced repression of their activities, about 86% and 75% respectively, with significant differences between single and double sugars (P<0.05) (Figs. 4A and 4B). Complementation of *ccpA* restored the repression close to the wild type levels for α-galactosidase and β-glucosidase. However, *ccpA* deletion did not have a significant effect on the repression of α-glucosidase activity mediated by glucose or sucrose (Fig. 4C). These results indicate that CcpA mediates at least part of the CCR of α-galactosidase and β-glucosidase activities in the presence of glucose and sucrose.

Deletion of *ccpA* leads to reduced adhesion and invasion in endothelial cells and decreased virulence to mice

The ΔccpA mutant had significantly lower levels of adherence to and invasion into bEnd.3 endothelial cells as compared to its parent strain (3.9‰ vs 6.3‰ with P<0.05 for adhesion, and 11.2% vs 19.8% with P<0.05 for invasion) (Figs. 5A and 5B), indicating that CcpA might regulate some factors that contribute to cell adhesion and invasion.

CcpA has been shown to contribute to virulence of some Gram-positive bacteria in the murine model (Iyer *et al.*, 2005). Thus, we investigated the role of CcpA in virulence to mice. At the inoculum doses of 3.3–3.6×10^8 CFU, only one out of 10 mice challenged with the ΔccpA mutant died by day 5 p.i., whereas the death rate was 60% for mice challenged with the parent strain. Doubling of the inoculum level led to 100% death (10/10) of mice receiving the parent strain, but only 20% (2/10) death with the ΔccpA mutant by day 5 p.i. (Figs. 5C and 5D). Moreover, *ccpA* complementation restored the virulence close to its parent strain. To further confirm virulence attenuation by the *ccpA* deletion, live bacterial cells of the ΔccpA mutant and its parent strain were examined in organs of mice infected with sublethal doses of around 10^8 CFU at 30 h p.i. The numbers of wild-type strain were about 6.1 log CFU/g (range 5.0–7.2 log CFU) from brain, kidney, liver and spleen, as compared to the average of 2.5 log CFU/g (range 2.4–5.1 log CFU) for ΔccpA mutant (Fig. 5E). The numbers in the *ccpA* complemented strain rose to about 5.1 log CFU/g (range 3.3–6.8 log CFU).

Discussion

Substantial progress has been made in the search for virulence factors as well as virulence-associated factors of *S. suis* type 2 for better understanding of its pathogenesis (Feng *et al.*, 2010). In addition to known factors like extracellular protein factor, muramidase-released protein, suilysin, and fibronectin-binding protein, there are novel virulence factors such as serum opacity factor, orphan response regulator, IgA1 protease and Rgg-like regulator (Feng *et al.*, 2010; Zhang *et al.*, 2011; Zheng *et al.*, 2011). The CcpA regulator is found to have pleiotropic effects in a number of bacterial species,

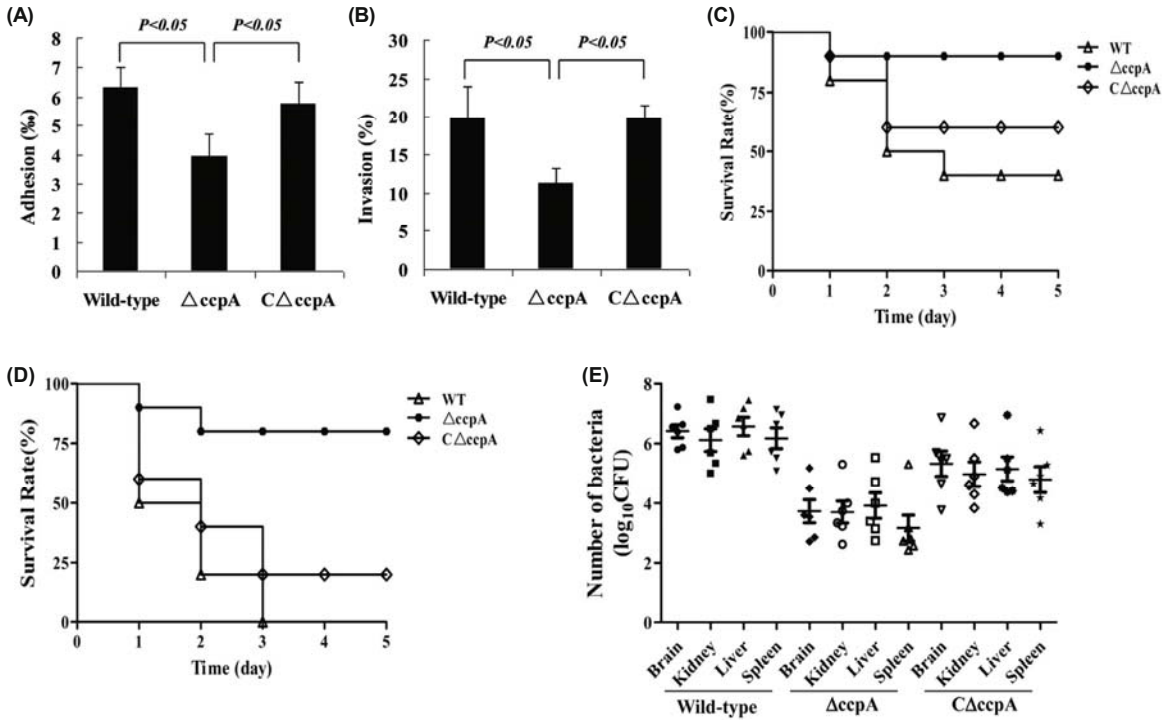

Fig. 5. Changes of virulence attributes in cultured bEnd.3 endothelial cell monolayers and in mice due to *ccpA* deletion. Adherence (A) to and invasion (B) into bEnd.3 cells by *S. suis* type 2 wild-type, *ccpA* deletion (ΔccpA), and *ccpA* complemented (CΔccpA) strains. Results are expressed as mean±SD of three independent experiments, each in duplicate wells for (A and B). Survival of mice intraperitoneally inoculated with 3.3–3.6×10^8 CFU (C) and 7.0–7.3×10^8 CFU (D) of the same sets of strains as in (A and B). (E) Bacterial burden in the brain, kidney liver and spleen of mice inoculated with 3.7–4.0×10^8 of the same sets of strains as in (A and B). Six mice in each group that survived the challenge at hour 30 post-inoculation were examined.

including alteration of growth in semi-synthetic media supplemented with two or more different sugar sources, induction of sugar metabolism enzymes, and loss of virulence (Iyer *et al.*, 2005; Antunes *et al.*, 2011; Chiang *et al.*, 2011). This study clearly indicates that CcpA in *S. suis* type 2 plays a role not only in mediating CCR but also in virulence to cultured epithelial cells and mice.

Deletion of *ccpA* resulted in decreased adhesion and invasion of *S. suis* type 2 to the endothelial cells and attenuated its virulence to mice as well. We also saw decreased expression of the *sly*, *cps2a*, and *eno* genes in the *ccpA* deletion mutant. These findings are in agreement with the recent report by (Willenborg *et al.*, 2011) that CcpA may be related to expression of several virulence genes and contribute to virulence in *S. suis* type 2. These results suggest that CcpA in *S. suis* type 2 might serve as a global regulator, acting on expression of virulence-related genes independent of carbohydrate utilization. This has been seen in other streptococcal species. CcpA could regulate some surface proteins that mediate interactions of *S. pneumoniae* with its host in colonization and multiplication (Iyer *et al.*, 2005). In *S. pyogenes*, CcpA was found to affect production of several key virulence factors not influenced during growth in nutrient-rich medium, and purified recombinant CcpA bound to the promoter region of the gene encoding streptolysin S (Shelburne *et al.*, 2008). CcpA activates the expression of *mga*, the master regulator of virulence. Mga controls the expression of *S. pyogenes* genes involved not only in sugar metabolism but also in specific virulence functions, such as adhesion and internalization in host cells (Görke and Stülke, 2008). This could be due to direct binding of CcpA to the putative cre element in the promoter region of *mga* (Almengor *et al.*, 2007).

We found that loss of CcpA affected catabolite repression of α-galactosidase and β-glucosidase activities, indicating its role in regulation of sugar metabolism. However, CcpA in *S. suis* type 2 was not involved in glucose/sucrose-mediated repression of the α-glucosidase activity. This suggests that other regulators might also contribute to CCR in *S. suis* type 2. For example, CcpB (Chauvaux *et al.*, 1998) and CcpC (Jourlin-Castelli *et al.*, 2000) of *B. subtilis* are involved in CCR of gluconate and xylose operons as well as aconitase and citrate synthase genes, respectively. In addition, (Rosenow *et al.*, 1999) reported that catabolite repression of α-galactosidase is not mediated by CcpA in *S. pneumoniae*, but rather, directly or indirectly, by a sucrose-specific phosphotransferase system. Analysis of the genomes of *S. suis* type 2 strains P1/7 and 05ZYH33 reveals that there are probably three potential phosphotransferase systems, including the loci from SSU0403 to SSU0406, SSU1055 to SSU1059, and SSU1583 to SSU1585. However, their roles in control of sugar metabolism remain unknown.

It appears that there was discrepancy between repressed galactosidase or glucosidase activity and rapid growth with the wild-type strain. This could be due to the fact that one of the sugars in the two-sugar media was glucose or sucrose,

which could be utilized preferentially for effective bacterial growth. Catabolite repression allows the wild-type bacteria to adapt quickly to a preferred (rapidly metabolisable) carbon and energy source first. This is usually achieved through inhibition of synthesis of enzymes involved in catabolism of carbon sources other than the preferred one (Deutscher, 2008). Another discrepancy is that the mutant strain showed significant growth retardation in two-sugar media containing either glucose or sucrose, as compared with the wild-type strain. One possibility could be metabolic linkage with other nutrients such as amino acids as a result of *ccpA* deletion. In *B. subtilis*, loss of *ccpA* causes a general growth defect that arises from amino acid auxotrophy, such as Glu, Met, Ile, Leu, and Val (Ludwig *et al.*, 2002).

In summary, we have demonstrated that CcpA of *S. suis* type 2 is involved not only in regulation of carbon catabolite repression of raffinose-inducible α-galactosidase and cellobiose-inducible β-glucosidase activity but also in virulence to cultured cells and mice. Further research is needed to examine if CcpA in *S. suis* type 2 acts *in trans* on virulence genes.

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