Role of glucose and CcpA in capsule expression and virulence of *Streptococcus suis*

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*Streptococcus suis* is one of the most important pathogens in pigs and is also an emerging zoonotic agent. After crossing the epithelial barrier, *S. suis* causes bacteraemia, resulting in meningitis, endocarditis and bronchopneumonia. Since the host environment seems to be an important regulatory component for virulence, we related expression of virulence determinants of *S. suis* to glucose availability during growth and to the sugar metabolism regulator catabolite control protein A (CcpA). We found that expression of the virulence-associated genes *arcB*, representing *arcABC* operon expression, *cps2A*, representing capsular locus expression, as well as *sly*, *ofs*, *sao* and *epf*, differed significantly between exponential and early stationary growth of a highly virulent serotype 2 strain. Deletion of *ccpA* altered the expression of the surface-associated virulence factors *arcB*, *sao* and *eno*, as well as the two currently proven virulence factors in pigs, *ofs* and *cps2A*, in early exponential growth. Global expression analysis using a cDNA expression array revealed 259 differentially expressed genes in early exponential growth, of which 141 were more highly expressed in the CcpA mutant strain 10Δ*ccpA* and 118 were expressed to a lower extent. Interestingly, among the latter genes, 18 could be related to capsule and cell wall synthesis. Correspondingly, electron microscopy characterization of strain 10Δ*ccpA* revealed a markedly reduced thickness of the capsule. This phenotype correlated with enhanced binding to porcine plasma proteins and a reduced resistance to killing by porcine neutrophils. Taken together, our data demonstrate that CcpA has a significant effect on the capsule synthesis and virulence properties of *S. suis*.

INTRODUCTION

*Streptococcus suis* is a Gram-positive, facultative anaerobic pathogen colonizing the upper respiratory tract, preferentially the tonsils of swine. It is endemic in nearly all pig-producing industries. Some strains are able to cross the epithelial barrier, causing bacteraemia and resulting in meningitis, arthritis, endocarditis and bronchopneumonia. As a zoonotic agent, *S. suis* can cause meningitis and sepsis in humans (Arends & Zanen, 1988; Chanter et al., 1993; Clifton-Hadley & Alexander, 1980; Rosenkranz et al., 2003). Among the 33 serotypes described in *S. suis*, serotype 2 strains seem to be the most virulent (Gottschalk et al., 2010). However, the mechanisms contributing to the virulence of *S. suis* in pigs and humans are only poorly understood.

The capsule of highly virulent *S. suis* serotype 2 strains has been proven to be a major virulence factor (Charland et al., 1998; Smith et al., 1999). It is proposed that during infection, *S. suis* downregulates capsule expression for increased adhesion to epithelial cells and upregulates it for protection against phagocytosis after entering the bloodstream (Gottschalk & Segura, 2000). Besides the capsular polysaccharide, the opacity factor of *S. suis* (*ofs*) is the only proven virulence factor for pig infection so far (Baums et al., 2006). Other virulence-associated factors have been...
described, as the surface-associated muramidase-released protein (mrp), the extracellular factor (epf), the secreted haemolysin suilysin (sly), the cytosolic and surface-located enzyme enolase (eno), surface antigen one (sao), and the arginine deiminase system (ADS) (Baums & Valentin-Weigand, 2009; Vecht et al., 1992; Wisselink et al., 2000).

In bacteria, the generation of energy by the uptake and consumption of environmental carbohydrates is tightly regulated. Thus, the availability of sugars which can be metabolized easily, such as glucose, activates sugar catabolism and suppresses other energy-providing mechanisms (Titgemeyer & Hillen, 2002). These phenomena are called carbon catabolite activation (CCA) and carbon catabolite repression (CCR), respectively, and are crucial during bacterial growth in culture, a phenomenon known as growth phase regulation (Seshasayee et al., 2006). The catabolite control protein A (CcpA) is the major mediator of CCR, repressing gene expression in the presence of excess sugar during growth (Kietzman & Caparon, 2010; Titgemeyer & Hillen, 2002; Zomer et al., 2007). In addition, CcpA has recently been shown to be important for the virulence of a number of bacterial species. Bacterial growth, haemolysin production, biofilm formation and capsule expression have been shown to be influenced by CcpA depletion in other streptococci (Shelburne et al., 2008; Wen & Burne, 2002). In the human pathogen Streptococcus pneumoniae, mutation in CcpA results in attenuated virulence in mouse infection models (Giammarinaro & Paton, 2002; Iyer et al., 2005). On the other hand, ccpA deficiency in Streptococcus pyogenes leads to both hypervirulence and hypovirulence, in the same set-up of experimental mouse infection (Kinkel & McIver, 2008; Wen & Burne, 2002). The mechanisms contributing to this, however, are poorly understood, and nothing is known about the contribution of CcpA to the virulence of S. suis.

During infection, S. suis has to adapt to different host environments in terms of sugar availability, pH and temperature, for example. We have previously shown that the alternative energy-providing system of S. suis, the ADS, is temperature-induced and expressed on the streptococcal surface (Winterhoff et al., 2002). Furthermore, we were able to show that the ADS is inducible by arginine and O2 tension, is subject to CCR, and is important for bacterial survival (Benga et al., 2004; Gruening et al., 2006).

In the present study we demonstrate that expression of virulence features, including capsule under conditions of high glucose availability, depends on CcpA. We further show that CcpA depletion in S. suis results in a strongly attenuated phenotype that resembles that of non-encapsulated S. suis. As a consequence, CcpA-deficient S. suis displayed poor resistance to phagocytic killing, in contrast to the parental strain. Overall, our data provide evidence that glucose-mediated regulation contributes to the virulence of S. suis and indicate that CcpA-dependent capsule expression might be a major component of carbon catabolite-regulated virulence.

**METHODS**

**Chemicals, bacterial strains and growth conditions.** If not otherwise stated, all chemicals were purchased from Sigma. The highly virulent serotype 2 strain 10, the capsule-deficient strain 10AcpsEF (Smith et al., 1999; Vecht et al., 1996), the suilysin-deficient strain 10Asly (Benga et al., 2008), the 10AcAlpA strain and its complementation c10AcAlpA were used. Bacteria were routinely grown in Todd–Hewitt broth (THB; Becton Dickinson Diagnostics). Subculturing was performed overnight at 37 °C on Columbia Blood Agar Base (Difco) containing 6% (v/v) sheep blood or horse blood supplemented with the appropriate antibiotics. To analyse bacterial growth, streptococci were grown in THB medium overnight and adjusted to OD600 0.02 on the next day. Then, OD600 was measured every hour and bacterial growth kinetics were determined in three independent experiments. For further experiments, bacteria were grown and harvested in different growth phases. As indicated in Fig. 2(a), time point P0 relates to early exponential, P1 to mid-exponential, P2 to late-exponential and P3 to early stationary bacterial growth. Escherichia coli was subcultured and maintained on Luria-Bertani (LB) agar plates. If required, antibiotics were added at the following concentrations: spectinomycin at 100 μg ml−1 (S. suis) and 50 μg ml−1 (E. coli), erythromycin at 1 μg ml−1 (S. suis) and 400 μg ml−1 (E. coli), ampicillin at 100 μg ml−1 (E. coli).

**Glucose measurement.** Determination of the glucose concentration in bacterial cultures was performed using the Glucose (GO) Assay kit (Sigma). Briefly, bacteria were grown in THB medium to the indicated time points (Fig. 2a) and then pelleted by centrifugation. The supernatants were collected and sterile-filtered (pore size 0.2 μm), and the glucose concentration (in μg ml−1) was determined.

**DNA techniques.** Chromosomal S. suis DNA was prepared according to standard procedures as described by Sambrook et al. (1989). Isolation of streptococcal plasmid DNA was done with the Promega PureYield Midiprep system according to the manufacturer’s instructions, with additional lysozyme (10 mg ml−1) digestion. Plasmid preparations of E. coli were performed with the NucleoSpin Plasmid kit (Macherey-Nagel) according to the manufacturer’s instructions. If not stated otherwise, all restriction enzymes were purchased from New England Biolabs (NEB). Southern analyses were performed with Sau3AI-cleaved and SmaI/NcoI double-digested genomic DNA according to standard protocols (Sambrook et al., 1989).

**RNA isolation and Northern blotting.** For total RNA extraction, the wild-type strain 10 and the respective mutant strain 10AcAlpA were grown to time points P0 and P3 as described above. Harvested bacteria were then resuspended in 1 ml TRIzol reagent (Invitrogen), ruptured using the FastPrep instrument (Qbiogene) three times for 45 s at intensity setting 6.5, and cooled on ice. After chloroform extraction and phenol extraction of the aqueous phase, the RNA samples were precipitated with ethanol. DNA was removed by RNAse treatment before ethanol precipitation. The RNA samples were then resuspended in 1 ml water. The isolated RNA was qualified by gel electrophoresis. A total of 1 μg RNA was separated on a 1.5% agarose gel, visualized with ethidium bromide and quantified with a gel documentation system (G:Box, Syngene). The RNA was used for Northern blot analysis.

**Production and purification of recombinant CcpA.** Recombinant CcpA was produced as a 6× His-tagged fusion protein in E. coli M15 with the pET-19a pQE plasmid system (Qiagen). The respective cpaA gene was amplified by PCR from chromosomal DNA with
the specific oligonucleotide primer pair CcpAfor_SacI and CcpArev_HindIII (Supplementary Table S1) using Pfu polymerase (Promega). The amplificate was digested with the respective enzymes and subsequently cloned into the SacI/HindIII-digested expression vector pQE30. The resulting plasmid pQE30-rCcpA was introduced into E. coli strain M15 (Qiagen). Purified plasmid DNA was verified by restriction analysis and sequencing. After IPTG induction, CcpA was overexpressed as an N-terminal hexahistidyl derivative and isolated by Ni²⁺-nitrilotriacetic acid affinity chromatography according to the manufacturer’s instructions (Macherey–Nagel, Protino Ni-TED 2000).

Polyclonal antiserum against purified recombinant CcpA was raised in a New Zealand white rabbit (Charles River Laboratories) by three consecutive immunizations with 100 μg purified protein and 50% Freund’s incomplete adjuvant. Generation of antibodies against ArcB was done as described previously (Gruening et al., 2006).

**Mutagenesis and ccpA complementation.** The ccpA gene (SSU1202) was inactivated by insertion mutagenesis of S. suis strain 10. Briefly, the gene encoding CcpA was amplified from the streptococcal genome using primer pairs CcpAfor_EcoRI and CcpArev_EcoRI (Supplementary Table S1). The PCR products were digested with HpaI and religated, resulting in a 311 bp deletion of the native ccpA gene. The ligation product was amplified using the primer pair CcpAfor_EcoRI and CcpArev_EcoRI and cloned into the vector pGEM-T Easy (Promega). The resulting plasmid pGEM-ccpA was linearized by the restriction enzyme HpaI, and the PvuII-digested erythromycin-resistance cassette from pICerm was introduced to disrupt ccpA. Electroporation of S. suis was performed as previously described (Smith et al., 1995). Putative mutants were confirmed by Southern blot and Northern blot analysis (Supplementary Fig. S1).

For the complementation of the ccpA-deficient strain, the entire ccpA gene and its putative promoter were amplified via a Pfu-based PCR using the primers CcpAfor_EcoRI and CcpArev_EcoRI (Supplementary Table S1). The PCR products were digested with EcoRI and ligated. The plasmid pGA14-CcpA was electrotransformed into competent ΔccpA bacteria, and transformants were screened by plating onto spectinomycin-containing blood media.
agar plates. Putative c10ΔccpA transformants were confirmed by Western blot analysis using polyclonal anti (α)-CcpA antiserum (Supplementary Fig. S1).

Preparation of whole-cell lysates and Western blot analysis.

Ten millilitres of bacterial cultures representing the desired growth phase were harvested and centrifuged for 15 min at 4000 g. The pellet was resuspended in 1 ml cell lysis buffer [CLB; 50 mM Tris/HCl (pH 8.0), 10 mM EDTA, 1% SDS, 1× 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride (AEBSF; Calbiochem)], and whole bacterial cell lysates were obtained by sonication (Branson, Cell Sonifier) and subsequent centrifugation. Protein concentrations were determined using the Bio-Rad DC Protein Assay. For CcpA Western blotting, proteins were separated by SDS-PAGE with a 4% stacking and a 12% separating gel. Samples were blotted onto a PVDF membrane (Serva), and membranes were blocked overnight with 3% BSA. Then, membranes were incubated with polyclonal antiserum raised against CcpA (diluted 1:1000 in 1% BSA) for 60 min. Recombinant CcpA (rCcpA) was included as a control, and the detection of ArcB protein levels represents ArcABC expression (top panel). The silver-stained gel served as a loading control (bottom panel).

Real-time quantitative RT-PCR (qRT-PCR). Wild-type strain 10 and the ccpA-deficient mutant strain 10ΔccpA were grown in THB medium to early exponential (P0) and early stationary (P3) phase, and RNA was prepared as described above. Two micrograms of RNA were reverse-transcribed with random primers (Promega) and analysed by
real-time qRT-PCR with a Stratagene Mx3005P system (primers listed in Supplementary Table S1). The reaction mixture contained 2 µL cDNA, 400 nM primers and 10 µL SYBR Green Mix (Qiagen) in a total volume of 20 µL. The PCR conditions were 95 °C for 10 min, 95 °C for 20 s, 55 °C for 30 s and 72 °C for 20 s, followed by a melting curve of the product as control. Data were normalized to a non-regulated housekeeping gene (dnaH), and the relative transcript levels were calculated by the ΔΔCt method. Statistical analyses were performed by one-way analysis of variance (ANOVA) using Dunnett’s adjustment to the non-regulated argK gene.

cDNA microarray analysis. RNA was extracted from wild-type strain 10 and the respective mutant strain 10ΔccpA that were grown in THB to OD600 0.3 (P0). Following this, cDNA microarray analysis was performed as previously described (Fulde et al., 2011).

Electron microscopy. For morphological analysis of the capsule structure, samples of early exponential (P0)-grown bacteria were fixed according to the lysine-acetate-based formaldehyde/glutaraldehyde ruthenium red-osmium (LRR) fixation procedure, as described previously (Benga et al., 2004). In addition, the capsule thickness of randomly selected bacteria was measured by the iTEM software.

Neutrophil killing assay. Neutrophil killing in the presence of 20 % (v/v) naïve porcine serum was assayed as described by Baums et al. (2009), except for the following modifications. S. suis strains 10, 10ΔcpsEF, 10ΔccpA and c10ΔccpA were grown in THB to P0. Glycerol (15 %, v/v, final volume) was added to each culture, and 500 µl aliquots were flash-frozen in liquid nitrogen. Frozen cultures were kept at −80 °C. The same batch of frozen cultures was used during the whole experiment. The bacteria were incubated with porcine neutrophils at an m.o.i. of 1 : 1, as described by Baums et al. (2009). Strain 10ΔcpsEF was included as a positive control in all killing assays. Results were expressed as survival factors, representing the ratio of c.f.u. at 1 h to c.f.u. at time 0. Finally, the survival factor was divided by the bacterial growth rate of each strain in RPMI medium without neutrophils, resulting in the relative survival factor. Statistical analysis was performed using ANOVA followed by a post-Tukey test.

Porcine plasma protein binding assay. For detecting porcine plasma binding, strains 10, 10ΔccpA, 10ΔcpsEF and c10ΔccpA were grown to the early exponential (P0) growth phase, harvested and resuspended in 2 ml PBS. Equal amounts were controlled by determination of c.f.u. ml−1. Bacterial suspensions were mixed with 5 ml porcine plasma prepared from fresh porcine heparinized blood and incubated for 2 h at room temperature on a rotator. After centrifugation for 15 min at 2316 g, the precipitated pellet was washed three times with 3 ml PBS. Bound proteins were eluted by addition of 1 ml glycine buffer (0.1 M, pH 2.0) and incubated on a rotator for 30 min at room temperature. Then, samples were centrifuged for 7 min at 8000 g, and supernatants were neutralized with 1 M Tris/HCl (pH 8.0) and subsequently concentrated by Microcon (Millipore) ultrafiltration with a cut-off of 10 kDa. The concentrated proteins were separated on an SDS gel and either stained with Coomassie brilliant blue or transferred onto a PVDF membrane for soluble fibrinogen detection using an anti-human fibrinogen antibody (Sigma, diluted 1:1000 in 1 % BSA).

Haemolytic activity assay. The haemolytic activity of streptococcal culture supernatants due to the secreted toxin slyusin was determined as described by Schaufuss et al. (2007), with some modifications. Briefly, strains 10, 10ΔccpA and 10Δsly were grown to the early exponential (P0) growth phase and pelleted by centrifugation. A 100 µl volume of serial twofold-diluted bacterial supernatants was mixed with 100 µl 2 % sheep red blood cells. The plates were incubated at 37 °C for 2 h, and non-lysed erythrocytes were removed by centrifugation at 1000 g for 5 min. A 100 µl volume of the supernatant was transferred to a flat-bottomed, 96-well microtitre plate for determination of A550 (Tecan, GENios Pro reader). Results were expressed as percentage haemolysis (haemolytic activity) compared with hypo-osmotic haemolysis by double-distilled H2O.

RESULTS

Growth-dependent expression of virulence-associated factors

Many factors have been shown to contribute to S. suis virulence, but for only a few of them has gene regulation been elucidated. One example for an environmentally highly regulated virulence-associated factor is the ADS. As an alternative energy-providing system, the ADS has been shown to be subject to CCR (Gruening et al., 2006). To analyse glucose-mediated regulation of virulence, we first related glucose availability during S. suis growth to ADS expression, to characterize more precisely the growth phases with high and low glucose. ArcB expression was used to indicate ADS expression during growth of S. suis (Fig. 1a). ArcB expression was inversely related to glucose concentration; for example, ArcB expression was highest under glucose-deprivation conditions, i.e. in the early stationary growth phase (P3). Based on these findings, we next compared the expression of eight selected well-known virulence-associated genes, including arcB, for arcABC operon expression, cps2A, representing capsule locus expression, sly, ofs, sao, eno, mrp and epf, between early exponential (P0, high glucose) and early stationary (P3, low glucose) growth. As shown in Fig. 1(b), with the exception of eno and mrp, all other genes showed growth phase-dependent differential expression. Expression of arcB and sly was highly upregulated in stationary phase-grown bacteria, whereas the relative transcript levels of cps2A and the surface-located proteins sao and ofs were lower as compared with the early exponential growth phase. These results suggested a glucose-mediated regulation of virulence-associated factors.

Effect of ccpA depletion on virulence-associated factor expression

CCR in Gram-positive bacteria is mediated by the transcriptional regulator CcpA. To analyse the role of CcpA in growth-dependent virulence gene expression, we constructed the ccpA-deficient strain 10ΔccpA (Supplementary Fig. S1). Insertion of the antibiotic-resistance cassette did not affect expression of the genes located up- and downstream of ccpA (SU1202; Supplementary Table S2). The growth of the CcpA-deficient strain was similar to that of the wild-type strain 10 (Fig. 2a). However, as expected, CCR of arcABC operon expression was abolished, indicated by the elevated ArcB expression during early exponential growth (P0) in strain 10ΔccpA (Fig. 2b). Next, we analysed virulence-associated factor regulation in the ccpA-knockout strain. For this, we related the relative transcript levels of arcB, sly, ofs, sao, eno,
**Effect of ccpA depletion on global gene expression**

To gain more insight into the role of CcpA in *S. suis* virulence and metabolism, we performed whole-genome cDNA microarray analyses with RNA extracted from strain 10ΔccpA and wild-type strain 10 during early exponential growth (P₀). The comparison of microarray data revealed 259 differentially expressed genes (13.2% of the *S. suis* genome) with expression changes of greater than twofold. The expression of 141 genes was higher, and 118 genes showed lower expression in 10ΔccpA (Supplementary Table S2). As shown by immunoblot analysis, the arcABC operon (>10-fold higher expression) belonged to the most strongly CcpA-repressed genes during early exponential growth. A Clusters of Orthologous Groups (COG) analysis of all affected genes is shown in Table 1. The majority of differentially expressed genes (56) in strain 10ΔccpA were related to carbohydrate transport. Notably, genes associated with cell wall biogenesis, including the entire capsule synthesis (*cps*) and sialic acid synthesis (*sao*) gene clusters, were strongly affected by the ccpA knockout. Similar to other Gram-positive bacteria, including *S. pyogenes* (Kinkel & McIver, 2008; Moreno et al., 2001; Shelburne et al., 2008; Zomer et al., 2007), in *S. suis*, CcpA seems to negatively regulate several operons for sugar utilization in the exponential growth phase. In detail, a maltose/maltodextrin ABC transporter and putative sugar-specific phosphotransferase system (PTS) for maltose, mannose, fructose, lactose and N-acetylglactosamine were upregulated in strain 10ΔccpA. The ccpA knockout positively and negatively influenced the mRNA level of 14 other transcriptional regulators. Interestingly, as CcpA has mainly been described as a repressor, the ccpA knockout resulted in a downregulation of about 6% of all ORFs throughout the genome. Besides the capsule synthesis cluster (*cps2ABCDGFGHI*), we found other genes encoding virulence-associated factors, such as opacity factor (*ofs*), surface-anchored SAO protein (*sao*) and enolase (*eno*), to be negatively affected by the ccpA knockout, which corresponded to our qRT-PCR shown in Fig. 2(c).

<table>
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<th>Putative function*</th>
<th>Genes with lower expression in strain 10ΔccpA</th>
<th>Genes with higher expression in strain 10ΔccpA</th>
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<td>Energy production (C)</td>
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</tr>
<tr>
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<tr>
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</tr>
<tr>
<td>Total (percentage of whole genome)</td>
<td>259 (13.2%)</td>
<td></td>
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</table>

*COG analysis was performed for differentially expressed genes and the respective one-letter code is shown in parentheses.

**Effect of ccpA depletion on capsule-associated virulence properties**

The virulence of *S. suis* is closely connected to capsule expression, at least in serotype 2 strains. As demonstrated above, the transcript levels of all genes contributing to capsule expression as well as several other genes contributing to cell wall synthesis were reduced in strain 10ΔccpA, suggesting that CcpA depletion alters capsule and cell wall structure. Therefore, we studied the capsules of wild-type strain 10, the capsular mutant 10ΔcpsEF, strain 10ΔccpA and the complemented strain c10ΔccpA during early exponential growth (P₀) by electron microscopy. As shown in Fig. 3, capsule expression was substantially reduced in strain 10ΔccpA. The wild-type phenotype could be restored after complementation of strain 10ΔccpA with plasmid-encoded CcpA. Quantification of capsule thickness revealed that compared with the wild-type strain 10, the capsule thickness of strain 10ΔccpA was significantly reduced (Fig. 4a). Thus, the CcpA-deficient phenotype closely resembled that of the capsular mutant 10ΔcpsEF. In the latter, however, capsule expression was completely abolished. The wild-type phenotype could be restored...
after complementation of strain 10ΔccpA with plasmid-encoded CcpA (c10ΔccpA).

The above data indicated that CcpA depletion affected capsule expression. Therefore, we tested the binding capacity of bacteria to porcine plasma proteins. Fig. 4(b) shows a Coomassie brilliant blue-stained SDS gel of eluted bacteria-bound plasma proteins. Strain 10ΔcpsEF possessed the highest binding capacity to porcine plasma proteins compared with the encapsulated wild-type strain 10. Strain 10ΔccpA displayed a higher binding than that of wild-type strain 10 but lower than that of strain 10ΔcpsEF. Correspondingly, using Western blot analysis, we found binding of porcine fibrinogen to strains 10ΔccpA and 10ΔcpsEF, but not to the wild-type strain 10. Notably, binding capacity was abolished by complementation of strain 10ΔccpA with plasmid-encoded CcpA (c10ΔccpA). Next, we studied the ability of the CcpA mutant strain to resist killing by porcine polymorphonuclear leukocytes (PMNs). For this, wild-type strain 10 and mutant strain 10ΔccpA were incubated with porcine PMNs in the presence of naive swine sera. As shown in Fig. 4(c), the relative survival of strain 10ΔccpA was significantly reduced (~62 vs ~100%) compared with the wild-type strain 10. The non-encapsulated strain 10ΔcpsEF, which we used as a control, was not able to resist phagocytic killing. The effects were not due to cytotoxicity, as the haemolytic activities of wild-type strain 10 and the mutant strain 10ΔccpA were similar (Fig. 4d). Complementation of the CcpA knock-out strain restored killing to the wild-type level. These results indicated that the CcpA depletion was responsible for the reduced resistance of S. suis to phagocytic killing.

**DISCUSSION**

It is well accepted that nutrient availability in different ecological niches of the host directs the expression of the virulence features of pathogenic bacteria. One prominent example of this is capsule expression during pneumococcal infection (Kadioglu et al., 2008). Thus, levels of *cps* mRNA...
are significantly different between pneumococci isolated from the blood of infected mice and those grown in vitro (Ogunniyi et al., 2002). At present, there is only indirect evidence that capsule expression in \textit{S. pneumoniae} (and some other pathogenic streptococci) is regulated by glucose availability via RegM/CcpA (Giammarinaro & Paton, 2002). In the present study, we found that glucose mediates virulence-associated gene expression via CcpA in \textit{S. suis}. Furthermore, we demonstrate for what is believed to be the first time that CcpA is necessary for capsule expression and, thereby, is important for resistance to phagocytosis in \textit{S. suis}.

CcpA has long been studied as a global bacterial sensor of glucose availability. More recently, it has become clear that such metabolic regulators can also contribute to the virulence of pathogens (Hondorp & McIver, 2007; Poncet et al., 2009). As a global regulator, CcpA has been investigated in several Gram-positive bacteria, including \textit{Bacillus subtilis}, \textit{Lactococcus lactis} and some streptococci (Deutscher, 2008; Sonenshein, 2007; Zomer et al., 2007).

Since growth phase-dependent regulation of virulence factor expression can influence pathogen fitness within...
the host (Kreikemeyer et al., 2003), we analysed the expression of virulence-associated factors during growth to reflect the adaptation of a pathogen to changing host environments (Hondorp & McIver, 2007; Mekalanos, 1992). Thus, in group A streptococci (GAS), a correlation between in vivo expression patterns and in vitro cultures of early stationary-grown bacteria has been observed (Cho & Caparon, 2005). Here, we found that the expression of major virulence-associated factors of S. suis, including arcABC, sly, ofs, sao, cps2A and epf, is dependent on the bacterial growth phase. The arcABC operon is controlled by CCR (Gruening et al., 2006; Zeng et al., 2006). As a consequence, it was strongly induced when the glucose concentration in the medium decreased. Since CcpA has been described as the main regulatory sensor of CCR, we constructed a ccpA-knockout strain. Deletion mutagenesis resulted in the abolition of the repressive glucose effect and an uncoupling of arcABC expression from growth phase regulation. Based on this observation, CCR mediated by putative orthologous pathways such as CcpB (Chauvaux et al., 1998), CcpC (Jourlin-Castelli et al., 2000), CcpN (Eckart et al., 2009) and the PTS (Deutscher et al., 2008) could be excluded for arcABC expression control. Overall, these observations fit well with results observed for other streptococci (Chaussee et al., 2003; Dong et al., 2004; Shelburne et al., 2008).

To determine the possible role of CcpA in the regulation of other virulence-associated factors we compared virulence gene expression of the wild-type strain 10 with that of strain 10ΔccpA. During exponential growth (Fig. 2c), the relative expression levels of arcB, cps2A, ofs and sao showed the same regulation pattern in exponential and stationary growth in the wild-type strain. These observations led us to presume that CcpA was the mediator of the growth phase-dependent regulation of these virulence-associated factors. Analysing the global expression differences in strain 10ΔccpA revealed 259 differentially expressed genes in early exponential growth when the glucose levels in the growth medium were still high. CcpA deficiency resulted in 118 (45.5 %) down- and 141 (54.5 %) upregulated genes, which differ in the relative distribution from what has been published for GAS in which approximately 90 % of the differentially regulated genes between the wild-type and a ccpA knockout have been found to be repressed by CcpA (Kinkel & McIver, 2008; Shelburne et al., 2008).

Four genes were confirmed by real-time qRT-PCR, indicating a significant correlation of the assays (Supplementary Fig. S2). COG analysis revealed that 56 genes (45 upregulated, 11 downregulated) of the 259 affected genes in the CcpA-knockout strain were related to carbohydrate transport and 17 (nine upregulated, eight downregulated) to amino acid transport, as well as 12 (six upregulated, six downregulated) to energy production, indicating the relevance of CcpA as a metabolic regulator in S. suis. Thus, the microarray data confirmed the repressive effect of CcpA on the arcABC operon, supporting earlier studies in other streptococci (Chaussee et al., 2003; Dong et al., 2004). CcpA depletion relieved CCR of operons for carbohydrate acquisition, such as the mannose-specific PTS (manLMN), N-acetylglactosamine PTS (agaVWD), fructose-specific PTS (fruA), cellobiose/lactose PTS (SSU1855–SSU1859), a maltose/maltodextrin-binding protein (malX) and an ABC-transport system (malCDAR), as well as the glycogen synthase pathway (gglCAB), which has been postulated to be repressed by CcpA in B. subtilis (Deutscher et al., 2006).

Among the differentially expressed genes we found 14 transcriptional regulators (seven upregulated, seven downregulated), further explaining the impact of the ccpA knockout on the whole regulatory network of S. suis transcription. Another very striking observation was that 18 genes were relieved from CCA, i.e. their expression was considerably lower in the mutant strain. The majority of these genes are responsible for the synthesis of the capsule, the most important virulence factor of S. suis. The capsule of S. suis serotype 2 strains is composed of galactose, glucose, N-acetyl-d-glucosamine, rhamnose and N-acetylneuraminic acid (sialic acid) (Elliott & Tai, 1978; Van Calsteren et al., 2010). In the microarray the entire capsule synthesis cluster cps2ABCDENFGHIJ and, additionally, the sialic acid synthase cluster (neuBCDA) were downregulated in strain 10ΔccpA. This finding has also been observed in other streptococci, including S. pneumoniae (Gianninannoto & Paton, 2002; Shelburne et al., 2008), but none of these studies analysed it more in depth.

We focused on the phenotypic characterization of strain 10ΔccpA, with emphasis on capsule-associated features under glucose-rich conditions. Electron microscopy demonstrated that the capsule thickness was considerably reduced in strain 10ΔccpA. Complementation of the mutant strain restored capsule thickness to the wild-type level, confirming the importance of CcpA for capsule expression.

Capsule expression of S. suis is of particular importance for resistance to uptake and killing by mononuclear phagocytes (Segura et al., 2004; Smith et al., 1999). It is hypothesized that during infection, S. suis first down-regulates capsule expression for increased adhesion to epithelial cells and then upregulates it for protection against phagocytosis after entering the bloodstream (Gottschalk & Segura, 2000). Accordingly, the increased plasma protein-binding capacity of strain 10ΔccpA (Fig. 4b) indicated a more adhesive phenotype, which would be necessary for adhesion (and invasion) in a low-glucose environment. However, despite the reduced thickness of the capsule in strain 10ΔccpA, biofilm formation and adherence to epithelial cells were not altered (data not shown). A possible explanation is that CcpA deficiency might affect the expression of other components necessary for biofilm formation and/or not-yet-characterized adhesins. Thus, the lower expression of the surface-located virulence-associated factors SAO, OFS and enolase may contribute to the observed phenotype, though their role in
adhesion has not yet been proven. On the other hand, the resistance of strain 10ΔccpA to neutrophil killing was markedly reduced, indicating that CcpA is necessary for capsule expression of S. suis in glucose-rich environments. This might be of relevance, for example when S. suis enters the bloodstream of the host, where glucose is the major sugar source (5.4 mM) (Baker et al., 2007), in order to facilitate resistance to killing by porcine neutrophils. Nevertheless, the lower expression of known surface-located proteins (SAO, OFS and enolase) and yet-uncharacterized proteins may also contribute to resistance to phagocytic killing.

In conclusion, our results show the relevance of the global regulator CcpA for virulence-associated factor expression in S. suis, and underline the link between metabolism and virulence in bacterial pathogens.

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