ArgR is an essential local transcriptional regulator of the *arcABC* operon in *Streptococcus suis* and is crucial for biological fitness in an acidic environment

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Streptococcus suis is one of the most important pathogens in pigs and can also cause severe infections in humans. Despite its clinical relevance, very little is known about the factors that contribute to its virulence. Recently, we identified a new putative virulence factor in *S. suis*, the arginine deiminase system (ADS), an arginine catabolic enzyme system encoded by the *arcABC* operon, which enables *S. suis* to survive in an acidic environment. In this study, we focused on ArgR, an ADS-associated regulator belonging to the ArgR/AhrC arginine repressor family. Using an *argR* knockout strain we were able to show that ArgR is essential for *arcABC* operon expression and necessary for the biological fitness of *S. suis*. By cDNA expression microarray analyses and quantitative real-time RT-PCR we found that the *arcABC* operon is the only gene cluster regulated by ArgR, which is in contrast to the situation in many other bacteria. Reporter gene analysis with *gfp* under the control of the *arcABC* promoter demonstrated that ArgR is able to activate the *arcABC* promoter. Electrophoretic mobility shift assays with fragments of the *arcABC* promoter and recombinant ArgR, and chromatin immunoprecipitation with antibodies directed against ArgR, revealed that ArgR interacts with the *arcABC* promoter *in vitro* and *in vivo* by binding to a region from –147 to –72 bp upstream of the transcriptional start point. Overall, our results show that in *S. suis*, ArgR is an essential, system-specific transcriptional regulator of the ADS that interacts directly with the *arcABC* promoter *in vivo*.

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INTRODUCTION

The regulation of arginine metabolism is mediated by the members of the ArgR/AhrC family of transcriptional regulators. These regulators and their cognate target sites are highly conserved among very diverse organisms, including Gram-positive and Gram-negative bacteria. In general, ArgR/AhrC proteins regulate their target genes by binding to operator sites, leading to repression of arginine biosynthetic genes and activation of catabolic genes in the presence of arginine (Gardan *et al.*, 1995; Grandori *et al.*,

1995; Kiupakis & Reitzer, 2002; Klingel *et al.*, 1995; Lu & Abdelal, 1999; Makarova *et al.*, 2001). Furthermore, it is now clear that in *Escherichia coli*, ArgR not only is involved in regulation of arginine metabolism but also regulates various genes of arginine transport (Caldara *et al.*, 2006).

Mechanisms for arginine catabolism differ among organisms (Blakemore & Canale-Parola, 1976; Broman *et al.*, 1978; Floderus *et al.*, 1990; Mercenier *et al.*, 1980). A widely distributed system in many bacteria, including homo-fermentative cocci, is the arginine deiminase system (ADS). It allows degradation of arginine into ornithine, ammonia and carbon dioxide catalysed by arginine deiminase (ArcA), ornithine carbamoyltransferase (ArcB) and carbamate kinase (ArcC) (Barcelona-Andrés *et al.*, 2002; Burne *et al.*, 1989; Champomier Vergès *et al.*, 1999; Chaussee *et al.*, 2003; Crow & Thomas, 1982; Zuñiga *et al.*, 2002b). Expression of the ADS is often closely connected to carbon metabolism via carbon catabolite repression (CCR) (Liu *et al.*, 2008; Zeng *et al.*, 2006). The generation of ATP by the conversion of arginine to ornithine plays an

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Abbreviations: ADS, arginine deiminase system; CCR, carbon catabolite repression; EMSA, electrophoretic mobility shift assay; qRT-PCR, quantitative RT-PCR; 5'-RACE, rapid amplification of 5' cDNA ends.

The microarray data discussed in this paper are available from ArrayExpress under accession number E-MEXP-2945.

Two supplementary tables, showing the primers used in this study and genes up- or downregulated by ArgR, are available with the online version of this paper.

important role in supplying the bacteria with energy during nutrient starvation. Furthermore, the ADS facilitates the evasion of acid stress by production of ammonium, and it supplies carbamoyl phosphate, which is essential for *de novo* synthesis of pyrimidines (van den Hoff *et al.*, 1995).

In many bacteria, the ADS has been shown to be regulated by members of the ArgR/AhrC family. In addition, the CCR regulator protein CcpA contributes to its regulation as a repressor, whereas the members of the CRP/FNR family of transcriptional regulators have been shown to positively regulate ADS expression (Barcelona-Andrés *et al.*, 2002; Dong *et al.*, 2004; Maghnouj *et al.*, 2000; Zuñiga *et al.*, 2002a).

Streptococcus suis is a Gram-positive, facultatively anaerobic pathogen that colonizes mainly the upper respiratory tract of swine. It is endemic in nearly all pig-producing industries and causes high economic losses due to meningitis, septicaemia, 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; Gottschalk *et al.*, 2010; Rosenkranz *et al.*, 2003).

Little is known about the virulence factors or protective antigens of *S. suis*. Previously, we identified two proteins of the *S. suis* ADS which were 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 contributes to survival under acidic conditions (Gruening *et al.*, 2006). The arginine catabolic genes *arcA*, *arcB* and *arcC*, which are transcribed polycistronically, are clustered together with the genes for a putative arginine/ornithine antiporter (*arcD*), a putative Xaa-His dipeptidase (*arcT*), and a putative endo- β -galactosidase C (*arcH*). The ADS gene cluster is bounded by the genes of two putative transcriptional regulators, the FNR-like protein of *S. suis* (FlpS) at the 5' end of the *arcABC* operon, and *argR*, encoding a repressor of the ArgR/AhrC family (ArgR) at the 3' end of *arcH*.

In the present study we found that in *S. suis*, ArgR is essential for *arcABC* operon expression activity and specifically regulates the *arcABC* operon by directly interacting with its promoter. Furthermore, we provide evidence that ArgR is essential for the biological fitness of *S. suis*.

METHODS

Bacterial strains and growth conditions. The highly virulent serotype 2 strain 10 and its capsule-deficient mutant strain 10 Δ cpsEF::spcR were used as parental strains (Smith *et al.*, 1999). Bacteria were routinely grown in Todd-Hewitt Broth (Becton Dickinson Diagnostics) or, if indicated, in a tryptone-yeast (TY) minimal medium with 10 mM galactose as a non-repressive sugar. Subculturing was performed on Columbia blood agar base (Difco) containing 6% (v/v) sheep blood overnight at 37 °C. To analyse the

external pH during bacterial growth, bacteria were grown in TY medium overnight. Then, streptococci were adjusted to OD₆₀₀ 0.02, and the pH was monitored at the indicated time points. *E. coli* was subcultured and maintained on Luria-Bertani agar plates. If required, antibiotics were added at the following concentrations: spectinomycin at 100 μ g ml⁻¹ (*S. suis*) and 50 μ g ml⁻¹ (*E. coli*), erythromycin at 1 μ g ml⁻¹ (*S. suis*) and 300 μ g ml⁻¹ (*E. coli*), and ampicillin at 100 μ g ml⁻¹ (*E. coli*).

DNA techniques. Chromosomal *S. suis* DNA was prepared according to standard procedures (Sambrook *et al.*, 1989). Plasmid DNA was purified 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.

Rapid amplification of 5' cDNA ends (5'-RACE). The transcriptional start point of the *arcABC* operon was determined by RACE with a 5' RACE kit (Invitrogen). Briefly, cDNA was synthesized from DNase I-treated (Ambion) RNA of *S. suis* strain 10 with primer GSP2 (Supplementary Table S1) according to the manufacturer's instructions. After tailing, PCR was performed with the primer pair ADSprimerext/AAP, followed by a nested PCR with primer pair ADSprimerext/AUAP (Supplementary Table S1). Sequencing was performed by SeqLab Laboratories, Göttingen, Germany.

Production and purification of recombinant ArgR. Recombinant ArgR was produced as a 6 $\times$ His-tagged fusion protein in *E. coli* with the QIAexpress paired pREP4-pQE plasmid system (Qiagen). Briefly, *argR* was amplified by PCR from chromosomal DNA with the specific oligonucleotide primer pair ArgR-PstI/ArgR-SphI (Supplementary Table S1) and cloned into the PstI/SphI-digested expression vector pQE30. The resulting plasmid, pQE30*argR*, was introduced into *E. coli* strain M15[pREP4] (Qiagen). IPTG-induced recombinant ArgR was purified by Ni²⁺-nitrilotriacetic acid affinity chromatography according to the manufacturer's instructions (Qiagen). A polyclonal antiserum against recombinant ArgR was raised in a New Zealand white rabbit (Charles River Laboratories).

Electrophoretic mobility shift assay (EMSA). To localize the ArgR-binding site *in vitro*, different fragments of the promoter region of the *arcABC* operon were generated by PCR with the forward primers 1-EMSA, 2-EMSA, 3-EMSA and 4-EMSA in combination with the reverse primer EMSA-rev (Supplementary Table S1). The DNA fragments were purified with the QIAquick PCR Purification kit (Qiagen) according to the manufacturer's instructions. Then, 200 ng DNA was incubated with or without 1 μ g recombinant ArgR and incubated in binding buffer (10 mM Tris/HCl, pH 7.5, 50 mM DTT, 5%, v/v, glycerol, 10 mM NaCl, 1 mM MgCl₂) for 1 h at room temperature. Protein-DNA complexes were separated electrophoretically in a native 5% polyacrylamide gel and visualized by ethidium bromide staining.

Mutagenesis. The *arcABC* operon and the ADS-associated regulator ArgR were inactivated by insertion mutagenesis in *S. suis* strains 10 and 10 Δ cpsEF::spcR (10 Δ cpsEF). Briefly, the gene encoding the putative arginine regulator ArgR was amplified from the streptococcal genome with the primers argRKolin and argRKOrec (Supplementary Table S1) and subsequently introduced into the cloning vector pCR2.1-TOPO (Invitrogen). The resulting plasmid, pTOPO-*argR*, was linearized by HincII. Then, the PvuII-released erythromycin-resistance cassette derived from vector pICerm was introduced to disrupt *argR*. Electroporation of the parental strains was performed as previously described (Gruening *et al.*, 2006; Smith *et al.*, 1995). For inactivation of the *arcABC* operon, the plasmid pGEMAD carrying *arcABC* was linearized with the restriction enzyme EcoRV and the operon was disrupted by insertion of an erythromycin-resistance cassette in *arcA*. The resulting plasmid was transformed into *S. suis*

strains 10 and 10Δ*cps*EF. Mutants were controlled by PCR and immunoblot analysis.

Construction of *gfp* reporter strains. For transcriptional fusion of the *arcABC* promoter to the GFP reporter protein, a 2836 bp fragment containing the promoter–operator region of the *arcABC* operon and the *arcA* gene was amplified with the primer pair IFL/IFR (Supplementary Table S1) from the streptococcal genome and subsequently cloned into *pGEMT*-Easy (Promega). The resulting plasmid, *pIFGEMT*-Easy, was used as template for inverse PCR using the primer pair Backbone1-*Hind*III/Backbone2-*Kpn*I (Supplementary Table S1), and the PCR product was digested with the respective restriction enzymes. In parallel, the promoterless *gfp* allele *gfpmut3** was amplified from the Gram-positive shuttle vector *pDL278-gfpmut3** with the primer pair *gfp-Hind*III/*gfp-Kpn*I (Supplementary Table S1) and digested with the same enzymes inserted into the PCR-amplified vector backbone. The resulting fragment containing the promoter-*gfp* fusion was subcloned into the shuttle vector *pGA14_{spc}* (Smith *et al.*, 1995) to obtain the reporter plasmid *pGA14_{spc}-Parc709-gfp*. Reporter plasmids carrying either the truncated *arcABC* promoter fusion (*pGA14_{spc}-Parc187-gfp*) or no promoter fusion (*pGA14_{spc}-gfp*) were generated in the same way using different primers (Supplementary Table S1) for inverse PCR. Reporter plasmids were introduced into *S. suis* strain 10 or strain 10Δ*argR* by electro-transformation. Transformants were screened by plating onto spectinomycin-containing blood agar plates and plasmid isolation with subsequent *Eco*RI restriction digestion. For promoter studies, bacteria were grown overnight in TY medium supplemented with 10 mM galactose. Then, bacterial suspensions were adjusted to OD₆₀₀ 0.02 in the same medium and cultured at 37 °C for ~8 h to OD₆₀₀ 0.2. One millilitre of bacterial culture was harvested by centrifugation, washed twice with PBS and resuspended in 1 ml PBS. One hundred microlitres of the suspension was used for *gfp* measurements. Fluorescence was measured in a fluorescence reader (excitation, 485 nm; emission, 535 nm). Relative fluorescence values were calculated by subtracting extinction from the PBS background. Experiments were carried out in triplicate and repeated at least twice.

Chromatin immunoprecipitation. For ChIP analysis the wild-type strain 10 and strain 10Δ*argR* were grown in THB medium to OD₆₀₀ 0.3 and 0.9, representing the mid-exponential and early stationary growth phases, respectively. The *in vivo* cross-linking of protein-bound DNA was done with 1% formaldehyde (v/v) for 5 min at room temperature and then stopped by addition of glycine in a final concentration of 0.125 M. The bacterial cells were collected by centrifugation at 4000 g and 4 °C, and washed twice with ice-cold 50 mM Tris (pH 8.0). The pellet was resuspended in cell lysis buffer [50 mM Tris/HCl, pH 8.0, 1% SDS, 10 mM EDTA, 1 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride (AEBSF) (Merck Biosciences)] and 1× protease inhibitor for bacterial cell extracts (Sigma), and was ruptured using the FastPrep instrument (Qbiogene) three times for 45 s at highest intensity. Bacterial chromatin was sheared by ultrasonic disintegration. The cleared lysates were diluted fivefold in ChIP dilution buffer (16.5 mM Tris/HCl, pH 8.0, 1.2 mM EDTA, 167 mM NaCl, 1.1% Triton X-100, 0.01% SDS, 1 mM AEBSF). Then, 25 µg ChIP lysate chromatin was mixed with 30 µl salmon sperm DNA/protein-A agarose matrix and 10 µl polyclonal rabbit anti-rArgR (α-rArgR) antiserum or preimmune serum. Immunoprecipitation was carried out on a rotator at room temperature for 90 min. The protein-A-antibody-DNA complexes were washed and DNA was extracted as described by Braunstein *et al.* (1993). ChIP-DNA was analysed by quantitative real-time PCR using primer pairs (Supplementary Table S1) flanking the *arcABC*, *sly* and *glgC* promoter regions. The amount of immunoprecipitated DNA was assayed by real-time PCR using a Stratagene (Mx3005P) instrument. The reaction mixture contained 1 µl ChIP or input DNA, 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. Serial dilutions of input DNA revealed linearity from 250 to 8 pg, and were used to calculate absolute amounts of PCR products (Hoffmann *et al.*, 2008). Significance ($P < 0.01$) was calculated in a two-sample *t* test by comparison of DNA obtained after immunoprecipitation with the α-rArgR antiserum or preimmune serum.

DNA microarray analysis. For RNA extraction, the wild-type strain and its mutant strain 10Δ*argR* were grown in THB to OD₆₀₀ 0.3 or 0.9. The cultures were immediately cooled on ice and the bacteria were harvested by centrifugation. Bacteria were resuspended in 1 ml TRIzol reagent (Invitrogen), disrupted by FastPrep three times for 45 s at intensity setting 6.5, and cooled on ice. After chloroform extraction and 2-propanol precipitation, the RNA was further purified using the RNeasy Mini kit (Qiagen) according to the manufacturer's recommendations. RNA concentration was determined spectrophotometrically, and quality and integrity were confirmed by agarose gel electrophoresis (2100 Bioanalyser, Agilent Technologies). For cDNA synthesis and labelling, a total of 10 µg RNA from three independent replicate biological preparations was pooled. Ten micrograms of pooled RNA was reverse-transcribed and labelled with the CyScribe Post-Labeling kit (GE Healthcare, RPN5660) and then purified with the GFX purification kit (GE Healthcare) according to the manufacturer's instructions. Complementary DNA from wild-type strain 10 and mutant strain 10Δ*argR* was labelled with Cy3 or Cy5, respectively, and analysed in dye-swap microarray experiments to avoid dye-related effects. Equivalent amounts (25 pmol) of Cy3/Cy5-labelled cDNA were mixed together in hybridization buffer of the *in situ* Hybridization Kit Plus (Agilent Technologies) following the instructions of the manufacturer. Hybridization was performed at 60 °C for 17 h. Slides were washed for 10 min in 6× SSC/0.05% Triton X-102 at room temperature, followed by 5 min in 0.1× SSC/0.05% Triton X-102 at 4 °C. Slides were dried using compressed air and scanned in a GenePix 4200AL microarray scanner (Molecular Devices). Scans were analysed using GenePix software (Molecular Devices). A customized in-house-developed *R*-based normalization procedure was performed to fit the data. Subsequently, data were analysed using significance analysis of microarrays (SAM). After statistical normalization and evaluation of the dye-swap experiments, genes with more than a twofold ratio change and a *P* value < 0.05 were regarded as regulated genes.

The microarray slides used for this study were produced by Agilent Technologies, according to a custom probe design based on the genome sequence of *S. suis* serotype 2 strain P1/7. A total of 7651 unique 60-mers having a theoretical melting temperature of approximately 81 °C and representing 1960 ORFs were selected as described by Saulnier *et al.* (2007). Genes were represented at 91, 4, 2 and 3% by four, three, two oligonucleotides and one oligonucleotide, respectively. A total of 25 putative genes were not represented on the array because no unique 60-mer satisfying the selection criteria could be selected. Most of the putative genes not represented on the array were relatively short and encoded hypothetical proteins, transposase fragments, prophage proteins, ribosomal proteins and tRNAs.

Real-time quantitative RT-PCR (qRT-PCR). Wild-type strain 10 and its mutant strain 10Δ*argR* were grown in THB medium to early stationary phase and RNA was prepared as described above. Two micrograms of RNA were reverse-transcribed (primers listed in Supplementary Table S1) and analysed by real-time qRT-PCR with a Stratagene Mx3005P system. qRT-PCR settings and conditions were used as described for the ChIP PCR. Data were normalized to a non-regulated housekeeping gene (*dnaH*) and the relative transcript levels were calculated by the $\Delta\Delta C_t$ method. A two-sample *t* test was used to calculate the significance ($P < 0.05$) or differential gene expression in strain 10Δ*argR* compared with the wild-type strain.

Preparation of whole-cell lysates and Western blot analysis.

Bacteria were grown in THB to the mid-exponential and early stationary growth phases, and lysates were prepared as described above. Protein concentrations were determined using the Bio-Rad D_c protein assay. Proteins were separated by SDS-PAGE and electro-blotted onto a PVDF membrane (Serva). Parallel gels were silver-stained to control protein loading. Membranes were blocked for 1 h with 5 % skimmed milk, and then incubated for 2 h with polyclonal antisera raised against recombinant ArcB (Gruening *et al.*, 2006) or ArgR diluted 1:100 or 1:1000, respectively, in 0.1 % skimmed milk. Membranes probed with anti-ArcB were developed with conjugated anti-rabbit immunoglobulin G (Amersham) and chemiluminescence.

Determination of ammonia in the culture supernatant.

Ammonia production of the wild-type and the respective mutant strains was monitored with an Ammonia Assay kit (Sigma). For this, bacteria were grown in TY medium and harvested by centrifugation 24 h after inoculation. The amount of ammonia in the supernatant was determined according to the manufacturer's instructions. To calculate ammonia production (mg ml^{-1}), the ammonia content of TY medium was subtracted from the value for the 24 h culture supernatants.

Survival in cells. The ability of the wild-type strain 10 and the different mutant strains to survive in HEP-2 cells was determined as described previously (Benga *et al.*, 2004), with the modification that, in addition to untreated HEP-2 cells, parallel assays were done with HEP-2 cells that had been pre-treated with bafilomycin (200 nM) for 1 h to inhibit endosomal acidification. The c.f.u. value was determined and expressed as percentage invasion of the respective inoculum used for infection.

Statistical analysis. If not stated otherwise, statistical analysis was performed by ANOVA followed by a post *t* test. A *P* value of <0.05 was considered significant.

RESULTS

ArgR is essential for *arcABC* operon expression in *S. suis*

We have previously characterized the ADS of *S. suis* as a gene cluster that is bounded at its 5' and 3' ends by the genes *flpS* and *argR*, respectively, which are putative regulators of the *arcABC* operon (Gruening *et al.*, 2006). In *Streptococcus gordonii*, the ADS is under the control of ArcR, a member of the arginine repressor family of transcriptional regulators that is homologous to *argR*. This prompted us to elucidate the role of ArgR in *arcABC* operon regulation in *S. suis*. For this, *argR* was deleted by insertion mutagenesis. The integrity of the mutant strain 10 Δ *argR* was controlled by immunoblot analysis of bacterial lysates with a polyclonal antiserum raised against recombinant ArgR (Fig. 1a). To analyse the relevance of ArgR to *arcABC* operon expression, we determined ArcB expression in strain 10 Δ *argR* and compared it with that of the wild-type strain 10 and strain 10 Δ *arcABC*, which is deficient in *arcABC* operon expression. As shown by immunoblot analysis (Fig. 1b), in the mid-exponential growth phase, ArcB expression was not detectable in either the wild-type strain 10 or the ArgR mutant strain. In early stationary growth, ArcB expression was enhanced in the wild-type strain 10 alone, while it was not induced in strain

Fig. 1. ArgR is essential for ADS expression. (a) Immunoblot analyses of whole-cell lysates of *S. suis* wild-type strain 10 and strain 10 Δ *argR* grown to early stationary phase in THB medium. As a control, recombinant ArgR (rArgR) was run in the same gel. Membranes were analysed with a polyclonal antiserum raised against rArgR. (b) Immunoblot analysis of whole-cell lysates obtained from wild-type strain 10, and strains 10 Δ *argR* and 10 Δ *arcABC* probed with polyclonal antisera against recombinant ArcB (top) and ArgR (centre). Bottom, silver-stained gel as a loading control. MW, molecular weight.

10 Δ *argR* and was completely abolished in strain 10 Δ *arcABC*. These results indicate that ArgR is essential for *ArcABC* expression of *S. suis*.

ArgR is important for growth and survival of *S. suis*

Further, we compared growth kinetics of the mutant strain 10 Δ *argR* with those of the wild-type strain and strain 10 Δ *arcABC* in TY medium supplemented with galactose. As shown in Fig. 2(a), starting at 4 h of culture, both mutant strains were significantly reduced in their growth rate when compared with the wild-type strain. Furthermore, the growth of strain 10 Δ *argR* exceeded that of strain 10 Δ *arcABC*, and this was most clearly seen at 6, 8 and 24 h. These data indicated that *argR* and the *arcABC* operon are necessary for optimal growth of *S. suis*. Next we monitored pH and ammonia production during growth of wild-type strain 10, strain 10 Δ *argR* and strain 10 Δ *arcABC*. As shown in Fig. 2(b), both mutant strains were unable to neutralize growth-dependent acidification. Thus, after 24 h of growth, we could determine a pH value of about 7.9 in the supernatant of the wild-type strain 10, whereas pH values of about 5.6 were detected for strains 10 Δ *argR* and 10 Δ *arcABC*, respectively. The differences in neutralizing

Fig. 2. Influence of ArgR deficiency on the biological fitness of *S. suis*. (a) *S. suis* wild-type strain 10, strain 10ΔargR and strain 10ΔarcABC were cultured in tryptone-based (TY) minimal medium supplemented with 10 mM galactose. The graph shows bacterial growth, including means and SDs, of the OD₆₀₀ from a representative experiment performed in triplicate. (b) Bacteria were grown as described in (a). The graph represents the pH value of the growth medium with means and SDs from a representative experiment performed in triplicate. (c) Wild-type strain 10, strain 10ΔargR and strain 10ΔarcABC were cultured as described in (a). Ammonia production is given as mg ammonia (ml medium)⁻¹. (d) Intracellular survival of the capsule-deficient strains 10ΔcpsEF, 10ΔcpsEFΔargR and 10ΔcpsEFΔarcABC in HEp-2 cells that were treated with bafilomycin (200 nM) for 1 h before infection (white bars) and in untreated cells (black bars). Results are given as percentage survival in comparison with the initial inoculum. Data represent means and SDs of three independent experiments. Results were considered statistically significant with $P < 0.05$, as indicated by asterisks.

growth-dependent acidification after 24 h corresponded to a loss in ammonia production (Fig. 2c). In contrast to wild-type strain 10 (1.15 mg ml⁻¹), the ammonia production of strains 10ΔarcABC (0.03 mg ml⁻¹) and 10ΔargR (0.06 mg ml⁻¹) was markedly reduced. Finally, the survival of ArgR-deficient *S. suis* in eukaryotic cells was investigated using the human epithelial cell line HEp-2. Since the polysaccharide capsule prevents *S. suis* uptake by epithelial cells, we generated *argR*- and *arcABC*-deficient strains in a non-encapsulated background (strain 10ΔcpsEF). As shown in Fig. 2(d), strain 10ΔcpsEF was able to survive and multiply intracellularly at a rate that was about 125% of the initial inoculum. In contrast, significantly lower survival rates (60%) were determined for both double-knockout mutant strains. To analyse whether reduced survival correlated with the inability of strains

10ΔcpsEFΔargR and 10ΔcpsEFΔarcABC to generate ammonia and prevent acidification, cells were treated with bafilomycin to inhibit endosomal acidification before infection. Compared with the infection of untreated cells, the pretreatment of the cells with bafilomycin significantly increased the survival rates of strains 10ΔcpsEFΔargR and 10ΔcpsEFΔarcABC. These data suggest that *S. suis* is able to resist endosomal acidification by ArgR-dependent *arcABC* operon induction and ammonia production.

ArgR deficiency specifically affects *arcABC* operon expression

The above results indicated that ArgR is an important regulator of the *arcABC* operon in *S. suis*. The differences in the growth kinetics of strains 10ΔargR and 10ΔarcABC,

however, suggested that ArgR might have additional regulatory functions. To test this, we performed whole-genome cDNA microarray analyses to evaluate the relevance of ArgR to *S. suis* gene expression. RNA was extracted from strain 10 Δ argR and wild-type strain 10 grown to the mid-exponential (reflecting a similar growth of 10 Δ argR and 10 Δ arcABC) and early stationary phases, when the growth of 10 Δ argR clearly differed from that of 10 Δ arcABC (Fig. 2a). After reverse transcription, cDNA of strain 10 Δ argR and the wild-type strain 10 was analysed on a whole-genome *S. suis* strain P1/7 microarray. A comparison of the microarray data of the bacteria grown to the mid-exponential growth phase revealed no genes significantly up- or downregulated by ArgR (Supplementary Table S2). In the early stationary growth phase, 26 genes were differentially expressed (with *P* values of <0.05) between the wild-type and the mutant strain (Table 1). Interestingly, expression of only five genes was significantly reduced in the mutant strain. As expected, one of them was *argR*, for which mRNA expression was abolished (downregulation 124-fold). The other four genes with strongly reduced mRNA expression in the mutant strain (downregulation of $\leq$ 28-fold, Table 1) represented the members of the *arcABC* operon, *arcA*, SSU0581, *arcB* and *arcC*. In addition, only mRNA expression of a putative trehalose-6-phosphate hydrolase (*treA*) was negatively influenced by ArgR deficiency (−3.7-fold). The 20 genes with higher mRNA expression in the mutant strain comprised seven ribosomal proteins (*rplM*, *rplT*, *rpmI*, *rpsD*, *rpsL*, *rpsP* and *rpsU*), six hypothetical proteins (SSU0068, SSU297, SSU0810, SSU1181, SSU1391 and SSU1936), two hydrolase family proteins (SSU1578 and SSU1763), the adenylate kinase (*adk*), an exoDNase (*exoA*), the translation initiation factor IF-3 (*infC*), a mechanosensitive channel protein (*mscL*) and sullysin (*sly*). Their lower level of expression differences, which was at maximum less than 7.3-fold, compared with the genes of the *arcABC* operon, suggests that these genes and *treA* might be influenced indirectly by the ArgR deficiency. qRT-PCR analyses were performed to confirm the array data. Expression levels of *arcB*, which is representative of the *arcABC* operon, *argR*, *sly*, *treA*, *adk* and *glgC* were determined. Interestingly, significant differences in the gene expression of stationary-grown strains 10 and 10 Δ argR could be confirmed only for *arcB* and *argR*. In contrast, the expression levels of *sly*, *treA* and *adk* did not differ significantly from that of *glgC*, which was included as a control gene with similar expression in strains 10 and 10 Δ argR.

Another interesting finding was that ArgR deficiency did not alter mRNA expression of the arginine anabolic genes argininosuccinate synthase (*argG*) and argininosuccinate lyase (*argH*), which have been reported to be regulated by ArgR in other Gram-positive bacteria (Larsen *et al.*, 2004, 2005, 2008; Ryan *et al.*, 2009) (Table 1). This was confirmed by qRT-PCR analysis (Fig. 3). In conclusion, the results of microarray and qRT-PCR analyses strongly suggest that in *S. suis*, ArgR is a specific, positive transcriptional regulator of the *arcABC* operon.

ArgR binds to and activates the *arcABC* operon promoter

To further analyse the regulatory function of ArgR in *arcABC* operon expression, we cloned a 788 bp fragment representing the promoter–operator region of the *arcABC* operon into the *gfp* reporter vector pGA14_{spc}-*gfp* and transformed it into *S. suis* strains 10 and 10 Δ argR. As a control, wild-type strain 10 was transformed with the promoterless pGA14_{spc}-*gfp* vector. Bacteria were grown in galactose-containing TY medium to the stationary growth phase, and reporter gene activity was then determined by fluorescence measurement. As shown in Fig. 4(a), compared with the control, the wild-type strain was able to induce reporter gene activity. In contrast, nearly no *gfp* expression could be detected in strain 10 Δ argR, showing that *argR* is essential for reporter gene activity. In conclusion, ArgR was shown to interact with the *arcABC* operon promoter.

We have previously shown that the *arcABC* operon promoter region contains a putative far-upstream ArgR-binding site (Gruening *et al.*, 2006). Therefore, we transformed wild-type strain 10 with the *gfp* reporter vector pGA14_{spc}-*gfp*, in which the putative ArgR-binding site was deleted by excision of 522 bp of the 5' *arcABC* operon promoter–operator region. As shown in Fig. 4(b), similar to the full-length promoter, reporter gene expression was still present after deletion of the putative ArgR-binding site, indicating that ArgR binding occurred further downstream. *In silico* analysis of the 5'-truncated *arcABC* operon promoter–operator region using the virtual footprint promoter analysis program (Münch *et al.*, 2005) revealed three AT-rich elements (ARG-boxes) with homologies to the predicted ArgR-binding site in *E. coli* (Fig. 4c). According to the positions of the ARG-boxes, we generated four PCR fragments of different lengths (Fig. 4c), which were investigated for ArgR DNA binding by EMSA. The DNA fragments were incubated with recombinant ArgR, and DNA binding to the PCR fragments was assayed after native gel electrophoresis and ethidium bromide staining. As shown in Fig. 4(d), recombinant ArgR was able to bind to fragments 3 and 4 containing ARG-boxes 2 and 3 (indicated by the retarded mobility of DNA fragments after incubation with ArgR), whereas the shorter fragments 1 and 2 containing ARG-box 1 were unable to bind ArgR.

Interestingly, the ArgR-binding region was located within the predicted operator region of the *arcABC* operon, which is contradictory to its role as a positive regulator. This prompted us to redefine the transcriptional start point and to revise the *arcABC* promoter structure. Repeated 5'-RACE analyses identified a tyrosine 79 bp upstream of the ArcA ATG as the transcriptional start (data not shown and Fig. 4c).

These data revealed that, based on the corrected transcriptional start point, a region between −147 and −72 bp within the *arcABC* promoter sequence (−147-72-box) seems to be necessary for ArgR binding *in vitro*.

Table 1. Comparative microarray analysis of *S. suis* wild-type strain 10 and strain 10 Δ argR grown to early stationary phase (OD₆₀₀ 0.9)Significantly regulated genes are shown for a lowest false-discovery rate of $P < 0.05$. NS, Not significant.

ORF in strain P 1/7	Gene	Gene product	Fold change gene expression*
SSU0068	<i>comX2</i>	Putative competence-specific global transcription modulator (fragment)	+ 3.11
SSU0094	<i>adk</i>	Adenylate kinase	+ 3.08
SSU0148	<i>rpsL</i>	30S ribosomal protein S12	+ 2.84
SSU0216	<i>treA, treC</i>	Putative trehalose-6-phosphate hydrolase	− 3.68
SSU0297		Putative transcription regulation protein	+ 3.61
SSU0580	<i>arcA</i>	Arginine deiminase	− 28.15
SSU0581		Acetyltransferase (GNAT) family protein	− 36.66
SSU0582	<i>arcB</i>	Ornithine carbamoyltransferase	− 29.96
SSU0583	<i>arcC</i>	Carbamate kinase	− 28.49
SSU0588	<i>argR</i>	Arginine repressor	− 124.67
SSU0627	<i>exoA</i>	ExoDNase	+ 3.63
SSU0742	<i>rpsP</i>	30S ribosomal protein S16	+ 3.16
SSU0810		Conserved hypothetical protein	+ 6.18
SSU0870	<i>glgC</i>	ADP-glucose pyrophosphorylase	NS
SSU1104	<i>rplT</i>	50S ribosomal protein L20	+ 2.88
SSU1105	<i>rpmI</i>	50S ribosomal protein L35	+ 3.29
SSU1106	<i>infC</i>	Translation initiation factor IF-3	+ 3.03
SSU1181		Putative membrane protein	+ 3.01
SSU1231	<i>sly</i>	Suilysin (haemolysin)	+ 3.23
SSU1257	<i>mscL</i>	Large-conductance mechanosensitive channel	+ 4.34
SSU1259	<i>rpsU</i>	30S ribosomal protein S21	+ 4.01
SSU1391		Putative membrane protein	+ 3.98
SSU1463	<i>argR</i>	Putative arginine repressor	NS
SSU1578		Gamma-glutamyl hydrolase	+ 4.22
SSU1692	<i>rplM</i>	50S ribosomal protein L13	+ 2.77
SSU1763		MutT/NUDIX hydrolase family protein	+ 2.92
SSU1806	<i>argG</i>	Argininosuccinate synthase (citrulline-aspartate ligase)	NS
SSU1807	<i>argH</i>	Argininosuccinate lyase	NS
SSU1909	<i>argR</i>	Putative arginine repressor	NS
SSU1935	<i>rpsD</i>	30S ribosomal protein S4	+ 3.51
SSU1936		Hypothetical protein	+ 7.84

*Fold change gene expression is given as a positive (+) or negative (−) value representing upregulation or downregulation in strain 10 Δ argR, respectively.

ArgR interacts with the *arcABC* operon promoter *in vivo*

The above results indicated the relevance of ArgR and the ArgR-binding sites within the *arcABC* promoter to *arcABC* operon expression. Next we analysed whether ArgR physically interacts with the −147-72-box of the *arcABC* promoter *in vivo* by chromatin immunoprecipitation analyses. For this, wild-type strain 10 and strain 10 Δ argR grown either to mid-exponential or to early stationary phase were analysed by ChIP assays with preimmune or α -ArgR antiserum. After DNA extraction, binding of ArgR to the −147-72-box was determined by real-time qRT-PCR with the oligonucleotide primer pair ChIP-for/ChIP-rev indicated in Fig. 4(c) and calculating the amounts of amplified fragments. As controls, we investigated ArgR binding to the promoter region of the ADP-glucose

pyrophosphorylase gene (*glgC*) and the suilysin gene (*sly*). According to the results of our real-time qRT-PCR studies, these genes were not regulated by ArgR (Fig. 3), and thus the promoters should not be recruited by ArgR. As depicted in Fig. 5, ~0.2 ng of *arcABC* promoter DNA was precipitated in the mid-exponential phase with antibodies directed against ArgR, using wild-type strain 10. Growth to stationary phase enhanced the yield of DNA to 0.35 ng, which indicated *in vivo* binding of ArgR to the *arcABC* promoter. In contrast, only low amounts of DNA were detected in experiments using the preimmune serum or in experiments using strain 10 Δ argR, indicating a high specificity of the α -ArgR antiserum. As expected, the ChIP experiments revealed that ArgR did not bind to the *sly* and *glgC* promoters. These analyses demonstrate that ArgR binds to the *arcABC* promoter *in vivo*.

Fig. 3. ArgR mutation specifically affects *arcABC* operon expression. *S. suis* wild-type strain 10 and the *argR*-deficient mutant strain 10 Δ *argR* were grown in THB medium to early stationary phase. Transcript levels of the indicated genes were determined by real-time qRT-PCR. Signals were normalized to a housekeeping gene (*dnaH*) and relative gene expression was calculated by the $\Delta\Delta C_t$ method. The relative fold-change ratio indicates the ratio of the values obtained for strain 10 Δ *argR* and the wild-type. Data are shown with mean and SD for three independent real-time qRT-PCR analyses performed in duplicate. A significant difference ($P < 0.05$) was defined as at least a twofold change in gene expression compared with wild-type strain 10. *arcB*, ornithine carbamoyltransferase; *argG*, argininosuccinate synthase; *sly*, suilysin; *treA*, putative trehalose-6-phosphate hydrolase; *glgC*, ADP-glucose pyrophosphorylase; *adk*, adenylate kinase; *argR*, arginine repressor (SSU0588).

DISCUSSION

This study showed that ArgR is essential for *arcABC* operon expression of the ADS gene cluster in *S. suis*. Furthermore, we could demonstrate that, in contrast to other bacteria, ArgR is a local regulator that specifically interacts with the *arcABC* operon promoter in *S. suis*. In our previous work we found that in *S. suis*, the genes of the ADS are located on a gene cluster consisting of the *arcABC* operon, the genes for a putative arginine/ornithine antiporter (*arcD*), a putative Xaa-His dipeptidase (*arcT*), a putative endo- β -galactosidase (*arcH*), and the genes of two regulatory factors, FlpS and ArgR, at the 5' and 3' ends, respectively (Gruening *et al.*, 2006).

The members of the ArgR/AhrC family of transcriptional regulators are mediators of arginine metabolism regulation. ArgR proteins and their cognate target sites are highly conserved among very diverse organisms, including Gram-positive and Gram-negative bacteria. In general, ArgR proteins regulate their target genes by binding to operator sites, leading to repression of arginine biosynthetic genes and activation of catabolic genes (Gardan *et al.*, 1995; Grandori *et al.*, 1995; Kiupakis & Reitzer, 2002; Klingel *et al.*, 1995; Lu & Abdelal, 1999; Makarova *et al.*, 2001).

Here we used the isogenic *argR*-negative mutant strain 10 Δ *argR* and immunoblot analysis to demonstrate that ArgR is a positive regulator of *arcABC* expression in *S. suis*

which is essential for induction of *arcABC* expression. This result is in agreement with findings that ArgR proteins are activators of arginine catabolic genes, as has been described for other Gram-positive bacteria such as *Bacillus licheniformis* and *S. gordonii* (Maghnouj *et al.*, 1998; Zeng *et al.*, 2006). However, the array and qRT-PCR analyses revealed that ArgR seems solely to regulate the *arcABC* operon. This was indicated by the significantly reduced mRNA expression of the *arcABC* genes in strain 10 Δ *argR* in the early stationary growth phase in both assays. Regulation of a single system by ArgR has not yet been described and is different to that in many other bacteria, in which ArgR has been shown to regulate both arginine anabolic and arginine catabolic genes (Hashim *et al.*, 2004; Hernández-Flores *et al.*, 2004; Larsen *et al.*, 2004, 2005, 2008; Lu *et al.*, 2004; Park *et al.*, 1997). From our array analyses we could exclude any polar effects due to the insertion mutation, since expression of the genes upstream (SSU0587) and downstream (SSU0589) of *argR* (SSU0588) was not significantly altered (Supplementary Table S2).

The fact that ArgR seems to be a highly specific regulator of the *arcABC* operon in *S. suis* was underlined by several findings. First of all, our array analyses with RNA from mid-exponential growth did not reveal any significantly differentially expressed genes in strain 10 Δ *argR*. Furthermore, there was no evidence of an ArgR-dependent regulation of the *argGH* operon, which seems to be the only arginine anabolic system in *S. suis*, as no other putative anabolic gene clusters such as *argCJDBF* or *gltS-argE* can be identified in the *S. suis* genome annotation. Second, in early stationary growth, the array analyses revealed a small number of genes with a low degree of differential expression in strain 10 Δ *argR*. The qRT-PCR analyses revealed that the genes were regulated twofold maximally. Notably, most of them are connected to ATP generation, which is plausible, as ATP production by ArcABC is strongly reduced in strain 10 Δ *argR*. For example, the adenylate kinase (Adk) produces ATP and AMP from two moles of ADP (Willemoës & Kilstrup, 2005). The trehalose-6-phosphate hydrolase (TreC) catalyses the conversion of trehalose 6-phosphate to glucose and glucose 6-phosphate (Rimmele & Boos, 1994). Thus, it is very likely that these genes are not primarily regulated by ArgR. Third, our ChIP analysis demonstrated clearly that ArgR binds to the *arcABC* promoter *in vivo*. Finally, all our phenotypical tests revealed that, in comparison with the wild-type strain 10, strain 10 Δ *argR* was affected in growth and biological fitness to an extent similar to that of strain 10 Δ *arcABC* (Fig. 2). These assays also demonstrated that enhanced *arcABC* expression mediated by ArgR is essential for optimal growth and intracellular survival of *S. suis*.

Regulation of the ArgR family of transcriptional regulators is exerted by binding to so-called ARG operator sites that precede the relevant target genes. ARG operator sites consist of pairs of 18 bp palindromic sequences (called ARG-boxes), of which the 5'-TnTgNATwwwATnCANa-3' (conserved residues in upper-case type; n, any nucleotide;

Fig. 4. ArgR binds to and activates the *arcABC* operon promoter. *S. suis* wild-type strain 10, and strain 10Δ*argR* harbouring the plasmid pGA14_{spc}-*Parc709-gfp* (a) or wild-type strain 10 harbouring either plasmid pGA14_{spc}-*Parc709-gfp* representing the whole *arcABC* promoter region (*Parc709*) or the far upstream ARG-box-deleted version (*Parc187*) (b), were grown for 8 h in TY medium. Bars represent the relative fluorescence units (RFU) after subtracting the absolute values for the PBS control. Wild-type strain 10 harbouring the promoterless construct pGA14_{spc}-*gfp* served as negative control. Experiments were carried out in triplicate and repeated at least twice. (c) Schematic representation of the *S. suis* *arcABC* operon promoter. The three putative ArgR 14 bp binding sites with highest homology to the *E. coli* ArgR-binding site are shown in grey. The transcriptional start point determined by 5'-RACE is indicated (+1); the predicted amino acid sequences of the 3'-primed *flpS* gene and 5'-primed *arcA* gene are shown in italic type. Primer sequences used for amplification of *arcABC* promoter segments analysed by EMSA and ChIP are indicated by underlining arrows. (d) PCR fragments were generated with the primers 1-EMSA, 2-EMSA, 3-EMSA and 4-EMSA (1–4) in combination with EMSA-rev [positions as indicated in (c)] and analysed in an EMSA after a 1 h incubation with (+) or without (–) recombinant ArgR (rArgR). Gel retardation by DNA–protein complexes was monitored after ethidium bromide staining.

Fig. 5. ArgR binds to the *arcABC* promoter *in vivo*. For ChIP assays, wild-type strain 10 and strain 10Δ*argR* were grown in THB medium to the exponential (exp) and stationary (stat) phases. ChIP lysate chromatin was precipitated using either α-ArgR or preimmune serum. ChIP DNA was quantified by real-time qRT-PCR using primer pairs for the *arcABC* (black bars), *sly* (grey bars) and *glgC* (white bars) promoter regions. The graphs represent means and SDs of immunoprecipitated DNA from three independent experiments. Significance ($P < 0.01$) was calculated in a two-sample *t* test by comparing wild-type strain 10 and strain 10Δ*argR*.

w, A or T) consensus sequence in *E. coli* (Maas, 1994) is conserved with only small variations in various other organisms studied (Cherney *et al.*, 2008; Garnett *et al.*, 2008; Makarova *et al.*, 2001). Our promoter studies with the *arcABC* promoter–operator–*gfp* constructs indicated that ArgR is responsible for *arcABC* promoter activity. Furthermore, we could exclude the relevance to *arcABC* promoter activity of a putative ARG-box in the 5′ promoter region of the *arcABC* promoter that we identified in a previous study (Gruening *et al.*, 2006). Further *in silico* analysis of the *arcABC* promoter–operator region with the virtual footprint promoter prediction program (Münch *et al.*, 2005) revealed three further putative ARG-boxes. Gel retardation assays with recombinant ArgR and truncated fragments of the *arcABC* promoter–operator region allowed us to identify a DNA-binding region of 75 bp that was able to interact with recombinant ArgR. Since the identification of potential A+T-rich ArgR-binding sites *in silico* and by EMSA does not reflect the *in vivo* situation (not all binding sites may be predicted *in silico* or weak binding sites relevant *in vivo* may not be recognized by EMSA), we performed ChIP analysis, which demonstrated that ArgR physically interacts with the *arcABC* promoter region *in vivo*. The context of ArgR binding to the ARG-boxes in the promoter sequence, however, awaits further studies.

In conclusion, our data indicate that ArgR is an essential, local transcriptional regulator of *arcABC* operon expression in *S. suis*. Its specificity for the ADS makes it highly relevant to the biological fitness of *S. suis*.

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