



1 **Abstract**

2

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

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22

23 keywords: arginine repressor, arginine deiminase system, chromatin immunoprecipitation

24

## 1 **Introduction**

2

3           The regulation of arginine metabolism is mediated by the members of the ArgR/AhrC  
4 family of transcriptional regulators. These regulators and their cognate target sites are highly  
5 conserved among very diverse organisms, including Gram-positive and Gram-negative  
6 bacteria. In general, ArgR/AhrC proteins regulate their target genes by binding to operator  
7 sites, leading to repression of arginine biosynthetic genes and activation of catabolic genes in  
8 the presence of arginine (Gardan *et al.*, 1995; Grandori *et al.*, 1995; Kiupakis & Reitzer,  
9 2002; Klingel *et al.*, 1995; Lu & Abdelal, 1999; Makarova *et al.*, 2001). Furthermore, it is  
10 now clear that in *Escherichia coli* ArgR is not only involved in regulation of arginine  
11 metabolism but also regulates various genes of arginine transport (Caldara *et al.*, 2006).

12           Mechanisms for arginine catabolism differ among organisms (Blakemore & Canale-  
13 Parola, 1976; Broman *et al.*, 1978; Floderus *et al.*, 1990; Mercenier *et al.*, 1980). A widely  
14 distributed system in many bacteria, including homofermentative cocci, is the arginine  
15 deiminase system (ADS). It allows degradation of arginine into ornithine, ammonia, and  
16 carbon dioxide catalyzed by the arginine deiminase (ArcA), the ornithine carbamoyl  
17 transferase (ArcB), and the carbamate kinase (ArcC) (Barcelona-Andres *et al.*, 2002; Burne *et*  
18 *al.*, 1989; Champomier Verges *et al.*, 1999; Chaussee *et al.*, 2003; Crow & Thomas, 1982;  
19 Zuniga *et al.*, 2002b). Expression of the ADS is often closely connected to carbon metabolism  
20 via carbon catabolite repression [CCR, (Liu *et al.*, 2008; Zeng *et al.*, 2006)]. The generation  
21 of ATP by the conversion of arginine to ornithine plays an important role in supplying the  
22 bacteria with energy during nutrient starvation. Furthermore, the ADS facilitates evasion from  
23 acid stress by production of ammonium, and it supplies carbamoyl phosphate, which is  
24 essential for *de novo* synthesis of pyrimidines (van den Hoff *et al.*, 1995).

25           In many bacteria the ADS has been shown to be regulated by the members of the  
26 ArgR/AhrC family. In addition, the CCR regulator protein CcpA contributes to its regulation

1 as a repressor, whereas the members of the CRP/FNR family of transcriptional regulators  
2 have been shown to positively regulate ADS expression (Barcelona-Andres *et al.*, 2002; Dong  
3 *et al.*, 2004; Maghnouj *et al.*, 2000; Zuniga *et al.*, 2002a).

4 *Streptococcus suis* is a Gram-positive, facultative anaerobic pathogen colonizing  
5 mainly the upper respiratory tract of swine. It is endemic in nearly all pig producing industries  
6 and causes high economical losses due to meningitis, septicemia, arthritis, endocarditis, and  
7 bronchopneumonia. As a zoonotic agent *Streptococcus suis* can cause meningitis and sepsis in  
8 humans (Arends & Zanen, 1988; Chanter *et al.*, 1993; Clifton-Hadley & Alexander, 1980;  
9 Gottschalk *et al.*, 2010; Rosenkranz *et al.*, 2003).

10 Little is known about the virulence factors or protective antigens of *Streptococcus*  
11 *suis*. Previously, we have identified two proteins of the *Streptococcus suis* ADS, which were  
12 temperature induced and expressed on the streptococcal surface (Winterhoff *et al.*, 2002).  
13 Furthermore, we could show that the ADS is inducible by arginine, O<sub>2</sub> tension, subjected to  
14 CCR, and that it contributes to survival under acidic conditions (Gruening *et al.*, 2006). The  
15 arginine catabolic genes *arcA*, *arcB* and *arcC*, which are transcribed polycistronically, are  
16 clustered together with the genes for a putative arginine/ornithine antiporter (*arcD*), a putative  
17 Xaa-His dipeptidase (*arcT*), and a putative endo-beta-galactosidase C (*arcH*). The ADS gene  
18 cluster is confined by the genes of two putative transcriptional regulators, the FNR-like  
19 protein of *Streptococcus suis* (FlpS) at the 5' end of the *arcABC*-operon, and *argR*, encoding  
20 for a repressor of the ArgR/AhrC family (ArgR) at the 3' end of *arcH*.

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

## 1 **Methods**

2

### 3 **Bacterial strains and growth conditions**

4       The highly virulent serotype 2 strain 10 and its capsule deficient mutant strain  
5 10 $\Delta$ *cpsEF::spcR* were used as parental strains (Smith *et al.*, 1999). Bacteria were routinely  
6 grown in Todd Hewitt Broth (Becton Dickinson Diagnostics, Heidelberg, Germany) or, if  
7 indicated, in a tryptone-yeast (TY) minimal medium with 10 mM galactose as a non-  
8 repressive sugar. Subculturing was performed on Columbia blood agar base (Difco, Detroit,  
9 USA) containing 6% (vol/vol) sheep blood overnight at 37°C. To analyze the external pH  
10 during bacterial growth, bacteria were grown in TY medium overnight. Then, streptococci  
11 were adjusted to an optical density at 600 nm (OD<sub>600</sub>) of 0.02, and the pH was monitored at  
12 indicated time-points. *Escherichia coli* was subcultured and maintained on Luria Bertani agar  
13 plates. If required, antibiotics were added at following concentrations: spectinomycin at 100  
14  $\mu$ g/ml (*Streptococcus suis*) and 50  $\mu$ g/ml (*Escherichia coli*), erythromycin at 1  $\mu$ g/ml  
15 (*Streptococcus suis*) and 300  $\mu$ g/ml (*Escherichia coli*), ampicillin at 100  $\mu$ g/ml (*Escherichia*  
16 *coli*).

17

### 18 **DNA techniques**

19       Chromosomal *Streptococcus suis* DNA was prepared according to standard procedures  
20 (Sambrook *et al.*, 1989). Plasmid DNA was purified with the NucleoSpin<sup>®</sup> Plasmid Kit  
21 (Macherey-Nagel, Dueren, Germany) according to manufacturer's instructions. If not stated  
22 otherwise, all restriction enzymes were purchased from NEB (New England Biolabs,  
23 Frankfurt am Main, Germany).

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## 1 **5'-RACE**

2 The transcriptional start point of the *arcABC*-operon was determined by rapid  
3 amplification of cDNA ends (RACE) with the 5'RACE kit (Invitrogen, Groningen,  
4 Netherlands). Briefly, cDNA was synthesized from DNaseI (Ambion Inc., Texas, USA)  
5 treated RNA of *Streptococcus suis* strain 10 with primer GSP2 (supplementary table 1)  
6 according to the manufacturer's instructions. After tailing, PCR was performed with the  
7 primer pair ADSprimerext/AAP, followed by a nested PCR with primer pair  
8 ADSprimerext/AUAP (supplementary table 1). Sequencing was performed by SeqLab  
9 Laboratories, Goettingen, Germany.

10

## 11 **Production and purification of recombinant ArgR**

12 Recombinant ArgR was produced as a 6xHis-tagged fusion protein in *Escherichia coli*  
13 with the QIAexpress paired *pREP4-pQE* plasmid system (Qiagen, Hilden, Germany). Briefly,  
14 *argR* was amplified by PCR from chromosomal DNA with the specific oligonucleotide  
15 primer pair ArgR-*PstI* and ArgR-*SphI* (supplementary table 1) and cloned into the *PstI/SphI*  
16 digested expression vector *pQE30*. The resulting plasmid *pQE30argR* was introduced into  
17 *Escherichia coli* strain M15[*pREP4*] (Qiagen). Isopropyl- $\beta$ -D-thiogalactopyranoside (IPTG)  
18 induced recombinant ArgR was purified by Ni<sup>2+</sup>-nitrilotriacetic acid affinity chromatography  
19 according to the manufacturer's instructions (Qiagen). A polyclonal antiserum against  
20 recombinant ArgR was raised in a New Zealand White rabbit (Charles River Laboratories,  
21 Sulzfeld, Germany).

22

## 23 **Electrophoretic mobility shift assay (EMSA)**

24 To localize the ArgR binding site *in vitro*, different fragments of the promoter region  
25 of the *arcABC*-operon were generated by PCR with the forward primers 1-EMSA, 2-EMSA,  
26 3-EMSA and 4-EMSA in combination with the reverse primer EMSA-rev (supplementary

1 table 1). The DNA fragments were purified with the QIAquick<sup>TM</sup> PCR Purification Kit  
2 (Qiagen) according to the manufacturer's instructions. Then, 200 ng of DNA were incubated  
3 with or without 1 µg of recombinant ArgR and incubated in binding buffer (10 mM Tris-HCl  
4 [pH 7.5], 50 mM DTT, 5 % [v/v] glycerol, 10 mM NaCl, 1 mM MgCl<sub>2</sub>) for 1 h at room  
5 temperature (RT). Protein-DNA complexes were separated electrophoretically by a native 5%  
6 polyacrylamide gel and visualized by ethidium bromide staining.

7

## 8 **Mutagenesis**

9 The *arcABC*-operon and the ADS-associated regulator ArgR were inactivated by  
10 insertion mutagenesis in *Streptococcus suis* strain 10 and 10Δ*cpsEF*::*spcR* (10Δ*cpsEF*).  
11 Briefly, the gene encoding the putative arginine regulator ArgR was amplified from the  
12 streptococcal genome with the primers *argRK*Olin and *argRK*Orec (supplementary table 1)  
13 and subsequently introduced into the cloning vector pCR<sup>®</sup>2.1-TOPO (Invitrogen). The  
14 resulting plasmid pTOPO-*argR* was linearized by *HincII*. Then, the *PvuII* released  
15 erythromycin resistance cassette derived from vector pICerm was introduced to disrupt *argR*.  
16 Electroporation of the parental strains was performed as previously described (Gruening *et*  
17 *al.*, 2006; Smith *et al.*, 1995). For inactivation of the *arcABC*-operon the plasmid pGEMAD  
18 carrying *arcABC* was linearized with the restriction enzyme *EcoRV* and the operon was  
19 disrupted by insertion of an erythromycin resistance cassette in *arcA*. The resulting plasmid  
20 was transformed into *Streptococcus suis* strain 10 and 10Δ*cpsEF*. Mutants were controlled by  
21 PCR and immunoblot analysis.

22

## 23 **Construction of *gfp*-reporter strains**

24 For transcriptional fusion of the *arcABC* promoter to the GFP reporter protein, a 2836  
25 bp fragment containing the promoter-operator region of the *arcABC*-operon and the *arcA*  
26 gene was amplified with the primer pair IFL/IFR (supplementary table 1) from the

1 streptococcal genome and subsequently cloned into *pGEMT-Easy* (Promega, Mannheim,  
2 Germany). The resulting plasmid *pIFGEMT-Easy* was used as template for inverse PCR  
3 using the primer pair backbone1-*HindIII*/backbone2-*KpnI* (supplementary table 1), and the  
4 PCR product was digested with the respective restriction enzymes. In parallel, the  
5 promoterless *gfp* allele *gfpmut3\** was amplified from the Gram-positive shuttle vector  
6 *pDL278-gfpmut3\** with the primer pair *gfp-HindIII/gfp-KpnI* (supplementary table 1) and  
7 digested with the same enzymes inserted into the PCR amplified vector backbone. The  
8 resulting fragment containing the promoter-*gfp* fusion was subcloned into the shuttle vector  
9 *pGA14<sub>spc</sub>* (Smith *et al.*, 1995) to obtain the reporter plasmid *pGA14<sub>spc</sub>-Parc709-gfp*. Reporter  
10 plasmids carrying either the truncated *arcABC* promoter fusion (*pGA14<sub>spc</sub>-Parc187-gfp*) or  
11 no promoter fusion (*pGA14<sub>spc</sub>-gfp*) were generated in the same way using different primers  
12 (supplementary table 1) for inverse PCR. Reporter plasmids were introduced into  
13 *Streptococcus suis* strain 10 or strain 10 $\Delta$ *argR* by electro-transformation. Transformants were  
14 screened by plating onto spectinomycin-containing blood agar plates and plasmid isolation  
15 with following *EcoRI* restriction digest. For promoter studies, bacteria were grown overnight  
16 in TY medium supplemented with 10 mM galactose. Then, bacterial suspensions were  
17 adjusted to an OD<sub>600</sub> of 0.02 in the same medium and cultured at 37°C for approx. eight hours  
18 to an OD<sub>600</sub> of 0.2. One ml of bacterial culture was harvested by centrifugation, washed twice  
19 with PBS and resuspended in 1 ml PBS. One hundred  $\mu$ l of the suspension was used for *gfp*-  
20 measurements. Fluorescence was measured in a fluorescence reader (excitation: 485 nm,  
21 emission: 535 nm). Relative fluorescence values were calculated by subtracting extinction  
22 from the PBS background. Experiments were carried out in triplicates and repeated at least  
23 twice.

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25

26



## 1 **Chromatin immunoprecipitation**

2 For ChIP analysis the wildtype strain 10 and strain 10 $\Delta$ argR were grown in THB  
3 media to OD<sub>600</sub> of 0.3 and 0.9, representing the mid exponential and early stationary growth  
4 phase, respectively. The *in vivo* cross-linking of protein-bound DNA was done with 1%  
5 formaldehyde (v/v) for 5 min at room temperature and then stopped by addition of glycine in  
6 a final concentration of 0.125 M. The bacterial cells were collected by centrifugation at 4,000  
7 x g and 4°C and washed twice with ice-cold 50 mM Tris (pH 8.0). The pellet was  
8 resuspended in cell lysis buffer (50 mM Tris-HCl [pH 8.0], 1% sodium dodecyl sulfate, 10  
9 mM EDTA, 1mM AEBSF (Merck Biosciences, Darmstadt, Germany)) and 1x protease  
10 inhibitor for bacterial cell extracts (Sigma, Munich, Germany), and was ruptured using the  
11 FastPrep<sup>®</sup> instrument (Qbiogene, Eschwege, Germany) three times for 45 sec at highest  
12 intensity. Bacterial chromatin was sheared by ultrasonic disintegration. The cleared lysates  
13 were diluted 5-fold in ChIP dilution buffer (16.5 mM Tris-HCl [pH 8.0], 1.2 mM EDTA, 167  
14 mM NaCl, 1.1 % Triton X 100, 0.01 % SDS, 1 mM AEBSF). Following, 25  $\mu$ g of ChIP lysate  
15 chromatin were mixed with 30  $\mu$ l salmon sperm DNA/protein-A agarose matrix and 10  $\mu$ l of  
16 polyclonal rabbit  $\alpha$ -rArgR antiserum or preimmune serum, respectively. Immunoprecipitation  
17 was carried out on a rotator at room temperature for 90 minutes. The  
18 protein-A/antibody/DNA complexes were washed and DNA was extracted as described  
19 (Braunstein *et al.*, 1993). ChIP-DNA was analysed by quantitative real-time PCR using  
20 primer pairs (supplementary table 1) flanking the *arcABC*, *sly* and *glgC* promoter region,  
21 respectively. The amount of immunoprecipitated DNA was assayed by real-time PCR using a  
22 Stratagene (Mx3005P) instrument. The reaction mixture contained 1  $\mu$ l of ChIP- or input-  
23 DNA, 400 nM primers and 10  $\mu$ l of SYBRGreen Mix (Qiagen) in a total volume of 20  $\mu$ l. The  
24 PCR conditions were 95 °C for 10 min, 95 °C for 20 s, 55 °C for 30 s, 72 °C for 20 s followed  
25 by a melting curve of the product as control. Serial dilutions of input DNA revealed linearity  
26 from 250 pg to 8 pg and were used to calculate absolute amounts of PCR products (Hoffmann

1 *et al.*, 2008). Significance ( $P < 0.01$ ) was calculated in a two- sample *t*-test by comparison of  
2 DNA obtained after immunoprecipitation with the  $\alpha$ -rArgR antiserum or preimmune serum.

3

#### 4 **DNA microarray analysis**

5 For RNA extraction the wildtype strain and its mutant strain 10 $\Delta$ *argR* were grown in  
6 THB to an OD<sub>600</sub> of 0.3 or 0.9. The cultures were immediately cooled on ice and the bacteria  
7 were harvested by centrifugation. Bacteria were resuspended in 1 ml Trizol<sup>®</sup> reagent  
8 (Invitrogen), disrupted by FastPrep<sup>®</sup> for three times of 45 sec at intensity setting 6.5 and  
9 cooled on ice. After chloroform extraction and isopropanol precipitation the RNA was further  
10 purified using the RNeasy Mini-Kit (Qiagen) according to the manufacturer's  
11 recommendations. RNA concentration was determined spectrophotometrically, and quality  
12 and integrity were confirmed by agarose gel electrophoresis (2100 Bioanalyzer, Agilent  
13 Technologies, Santa Clara, USA). For cDNA synthesis and labelling a total of 10  $\mu$ g RNA of  
14 three independent replicated biological preparations was pooled. Ten  $\mu$ g of pooled RNA was  
15 reverse transcribed and labelled with the CyScribe<sup>™</sup> Post-Labeling Kit (GE Healthcare,  
16 RPN5660, Munich, Germany) and then purified via the GFX purification kit (GE Healthcare)  
17 according to the manufacturer's instructions. Complementary DNA from wildtype strain 10  
18 and mutant strain 10 $\Delta$ *argR* was labelled with Cy3 or Cy5, respectively, and analyzed in dye  
19 swap microarray experiments to avoid dye related effects. Equivalent amounts (25 pmol) of  
20 Cy3/Cy5 labelled cDNA were mixed together in hybridization buffer of the *In situ*  
21 hybridization kit Plus (Agilent Technologies) following instructions of the manufacturer.  
22 Hybridization was performed at 60°C for 17 h. Slides were washed for 10 min in 6 x  
23 SSC/0.05% Triton-X102 at room temperature, followed by 5 min in 0.1 x SSC/0.05% Triton  
24 X102 at 4°C. Slides were dried using pressured air and scanned in a GenePix<sup>®</sup> 4200AL  
25 (Molecular Devices, Sunnyvale, USA). Scans were analyzed using the GenePix software  
26 (Molecular Devices). A customized in house developed R-based normalization procedure was

1 performed to fit the data. Subsequently, data were analysed using SAM. After statistical  
2 normalization and evaluation of the dye swap experiments genes with more than a 2-fold ratio  
3 change and  $P$  value  $< 0.05$  were dedicated as regulated genes.

4 The microarray slides used for this study were produced by Agilent Technologies, according  
5 to a custom probe design based on the genome sequence of *Streptococcus suis* serotype 2  
6 strain P1/7. A total of 7651 unique 60-mers having a theoretical melting temperature of  
7 approximately 81°C and representing 1960 ORFs were selected as described by (Saulnier *et*  
8 *al.*, 2007). Genes were represented to 91%, 4%, 2% and 3% by 4, 3, 2 oligonucleotides or 1  
9 oligonucleotide, respectively. A total of 25 putative genes were not represented on the array  
10 because no unique 60-mer satisfying the selection criteria could be selected. Most of the  
11 putative genes not represented on the array were relatively short and encoded hypothetical  
12 proteins, transposase fragments, prophage proteins, ribosomal proteins and tRNAs.

13

#### 14 **Real-time quantitative reverse transcription PCR (qRT-PCR)**

15 Wildtype strain 10 and its mutant strain 10 $\Delta$ *argR* were grown in THB medium to  
16 early stationary (stat) phase and RNA was prepared as described above. Two  $\mu$ g of RNA were  
17 reverse transcribed (primers listed in supplementary table 1) and analysed by real-time qRT-  
18 PCR with a Stratagene Mx3005P system. QRT-PCR settings and conditions were used as  
19 described for the ChIP PCR. Data were normalized to a non regulated housekeeping gene  
20 (*dnaH*) and the relative transcript levels were calculated by the  $\Delta\Delta C_T$  method. A two- sample  
21 *t*-test was used to calculate the significance ( $P < 0.05$ ) or differential gene expression in strain  
22 10 $\Delta$ *argR* compared to the wildtype strain.

23

#### 24 **Preparation of whole cell lysates and Western blot analysis**

25 Bacteria were grown in THB to mid exponential and early stationary growth phase and  
26 lysates were prepared as described above. Protein concentrations were determined using the

1 Bio-Rad D<sub>c</sub> protein assay (BioRad, Munich, Germany). Proteins were separated by SDS-  
2 PAGE and electroblotted onto a PVDF-membrane (Serva, Heidelberg, Germany). Parallel  
3 gels were silver stained to control protein loading. Membranes were blocked for 1 h with 5 %  
4 skim milk, and then incubated for 2 h with polyclonal antisera raised against recombinant  
5 ArcB (Gruening *et al.*, 2006) or ArgR diluted 1:100 or 1:1,000, respectively, in 0.1 % skim  
6 milk. Membranes probed with anti-ArcB were developed with conjugated anti-rabbit  
7 immunoglobulin G (Amersham, Freiburg, Germany) and chemiluminescence.

8

### 9 **Determination of ammonia in the culture supernatant**

10 Ammonia production of the wildtype and the respective mutant strains was monitored  
11 with the ammonia assay kit (Sigma). For this, bacteria were grown in TY media and harvested  
12 by centrifugation 24 h after inoculation. The amount of ammonia in the supernatant was  
13 determined according to manufacturer's instructions. To calculate ammonia production  
14 (mg/ml), the ammonia content of TY medium was subtracted from the amount of the 24 h  
15 culture supernatants.

16

### 17 **Survival in cells**

18 The ability of the wildtype strain 10 and the different mutant strains to survive in HEp-  
19 2 cells was performed as described previously (Benga *et al.*, 2004) with the modification that,  
20 in addition to untreated cells HEp-2 cells, parallel assays were done with HEp-2 cells which  
21 had been pre-treated with bafilomycin (200 nM) for 1 h to inhibit endosomal acidification.  
22 The number of CFU was determined and expressed as percentage invasion of the respective  
23 inoculum used for infection.

### 24 **Statistical analysis**

25 When not stated otherwise statistical analysis was performed by ANOVA followed by  
26 a post *t*-test. A *P*-value of < 0.05 was considered significant.

## 1 **Results**

2

### 3 **ArgR is essential for *arcABC*-operon expression in *Streptococcus suis***

4 We have previously characterized the ADS of *Streptococcus suis* as a gene cluster that  
5 is confined at its 5' and 3' end by the genes *flpS* and *argR*, respectively, which are putative  
6 regulators of the *arcABC*-operon (Gruening *et al.*, 2006). In *Streptococcus gordonii*, the ADS  
7 is under control of ArcR, a member of the arginine repressor family of transcriptional  
8 regulators that is homologous to *argR*. This prompted us to elucidate the role of ArgR in  
9 *arcABC*-operon regulation of *Streptococcus suis*. For this, *argR* was deleted by insertion  
10 mutagenesis. Integrity of the mutant strain 10 $\Delta$ *argR* was controlled by immunoblot analysis  
11 of bacterial lysates with a polyclonal antiserum raised against recombinant ArgR (figure 1A).  
12 To analyse the relevance of ArgR for *arcABC*-operon expression we determined ArcB  
13 expression in strain 10 $\Delta$ *argR* and compared it to the wildtype strain 10 and strain  
14 10 $\Delta$ *arcABC* which is deficient in *arcABC*-operon expression. As shown by immunoblot  
15 analysis (figure 1B), in mid exponential growth phase ArcB expression was not detectable in  
16 both the wildtype strain 10 and the ArgR mutant strain. At early stationary growth ArcB  
17 expression was enhanced only in the wildtype strain 10, but it was not induced in strain  
18 10 $\Delta$ *argR* and was completely abolished in strain 10 $\Delta$ *arcABC*. These results indicate that  
19 ArgR is essential for ArcABC expression of *Streptococcus suis*.

20

### 21 **ArgR is important for growth and survival of *Streptococcus suis***

22 Further, we compared growth kinetics of the mutant strain 10 $\Delta$ *argR* with that of the  
23 wildtype strain and strain 10 $\Delta$ *arcABC* in TY medium supplemented with galactose. As  
24 shown in figure 2A, starting at 4 h of culture, both mutant strains were significantly reduced  
25 in their growth rate when compared to the wildtype strain. Furthermore, the growth of strain  
26 10 $\Delta$ *argR* exceeded that of strain 10 $\Delta$ *arcABC* which was most clearly seen at 6, 8, and 24 h.

1 These data indicated that *argR* and the *arcABC*-operon are necessary for optimal growth of  
2 *Streptococcus suis*. Next we monitored pH and ammonia production during growth of  
3 wildtype strain 10, strain 10 $\Delta$ *argR*, and strain 10 $\Delta$ *arcABC*. As shown in figure 2B, both  
4 mutant strains were unable to neutralize growth dependent acidification. Thus, after 24 h of  
5 growth we could determine a pH value of approx. 7.9 in the supernatant of the wildtype strain  
6 10, whereas pH values of approx. 5.6 were detected for the strains 10 $\Delta$ *argR* and 10 $\Delta$ *arcABC*,  
7 respectively. The differences in neutralising growth dependent acidification after 24 h  
8 corresponded to a loss in ammonia production (figure 2C). In contrast to wildtype strain 10  
9 (1.15 mg/ml) ammonia production of strain 10 $\Delta$ *arcABC* (0.03 mg/ml) and strain 10 $\Delta$ *argR*  
10 (0.06 mg/ml) was markedly reduced. Finally, survival of ArgR deficient *Streptococcus suis* in  
11 eukaryotic cells was investigated using the human epithelial cell line HEP-2. Since the  
12 polysaccharide capsule prevents *Streptococcus suis* uptake by epithelial cells, we generated  
13 *argR* and *arcABC* deficient strains in a non-encapsulated background (strain 10 $\Delta$ *cpsEF*). As  
14 shown in figure 2D, strain 10 $\Delta$ *cpsEF* was able to survive and multiply intracellularly at a rate  
15 of approx. 125 % of the initial inoculum. In contrast, significantly lower survival rates (60 %)  
16 were determined for both double knock-out mutant strains. To analyse whether reduced  
17 survival correlated with the inability of strains 10 $\Delta$ *cpsEF* $\Delta$ *argR* and 10 $\Delta$ *cpsEF* $\Delta$ *arcABC* to  
18 generate ammonia and prevent acidification, cells were treated with bafilomycin to inhibit  
19 endosomal acidification before infection. Compared to the infection of untreated cells, the  
20 pre-treatment of the cells with bafilomycin significantly increased the survival rates of the  
21 strains 10 $\Delta$ *cpsEF* $\Delta$ *argR* and 10 $\Delta$ *cpsEF* $\Delta$ *arcABC*. These data suggest that *Streptococcus suis*  
22 is able to resist endosomal acidification due to ArgR dependent *arcABC*-operon induction  
23 and ammonia production.

24

25

26

## 1 **ArgR deficiency specifically affects *arcABC*-operon expression**

2           The above results indicated that ArgR is an important regulator of the *arcABC*-operon  
3 in *Streptococcus suis*. The differences in the growth kinetic of 10 $\Delta$ *argR* and 10 $\Delta$ *arcABC*,  
4 however, suggested that ArgR might have additional regulatory functions. To test this, we  
5 performed whole genome cDNA microarray analyses to evaluate the relevance of ArgR in  
6 *Streptococcus suis* gene expression. RNA was extracted from strain 10 $\Delta$ *argR* and wildtype  
7 strain 10 grown to mid exponential (reflecting a similar growth of 10 $\Delta$ *argR* and  
8 10 $\Delta$ *arcABC*), and to early stationary phase when growth of 10 $\Delta$ *argR* and 10 $\Delta$ *arcABC* clearly  
9 differed from each other (figure 2 A). After reverse transcription, cDNA of strain 10 $\Delta$ *argR*  
10 and the wildtype strain 10 were analysed on a whole genome *Streptococcus suis* strain P1/7  
11 microarray. The comparison of microarray data of the bacteria grown to mid exponential  
12 growth phase revealed no significantly up- or down-regulated genes by ArgR (supplementary  
13 table 2). In early stationary growth phase, 26 genes were differentially expressed (with *P*  
14 values of <0.05) between the wildtype and the mutant strain (table 1). Interestingly,  
15 expression of only 5 genes was significantly reduced in the mutant strain. As expected, one of  
16 them was *argR* of which mRNA expression was abolished (down regulation 124-fold). The  
17 other 4 genes with strongly reduced mRNA expression in the mutant strain (down regulation  
18 of  $\leq -28$ -fold, table 1) represented the members of the *arcABC*-operon, *arcA*, SSU0581,  
19 *arcB* and *arcC*. In addition, only mRNA expression of a putative trehalose-6-phosphate  
20 hydrolase (*treA*) was negatively influenced by ArgR deficiency (-3.7-fold). The group of the  
21 20 genes with higher mRNA expression in the mutant strain comprised 7 ribosomal proteins  
22 (*rplM*, *rplT*, *rplI*, *rpsD*, *rpsL*, *rpsP*, *rpsU*), 6 hypothetical proteins (SSU0068, SSU297,  
23 SSU0810, SSU1181, SSU1391, SSU1936), 2 hydrolase family proteins (SSU1578,  
24 SSU1763), the adenylate kinase (*adk*), an exodeoxyribonuclease (*exoA*), the translation  
25 initiation factor IF-3 (*infC*), a mechanosensitive channel protein (*mscL*) and sulysin (*sly*).  
26 Their lower level of expression differences, which was at max less than 7.3-fold, compared to

1 the genes of the *arcABC*-operon suggests that these genes and *treA* might be influenced  
2 indirectly by the ArgR deficiency. QRT-PCR analyses were performed to confirm the array  
3 data. Expression levels of *arcB*, which is representative for the *arcABC*-operon, *argR*, *sly*,  
4 *treA*, *adk* and *glgC* were determined. Interestingly, significant differences in gene expression  
5 of stationary grown strain 10 and 10 $\Delta$ *argR* could be confirmed only for *arcB* and *argR*. In  
6 contrast, the expression levels of *sly*, *treA* and *adk* did not significantly differ from *glgC*  
7 which was included as control gene with similar expression in strain 10 and 10 $\Delta$ *argR*.

8 Another interesting finding was that ArgR deficiency did not alter mRNA expression  
9 of the arginine anabolic genes, arginino succinate synthase (*argG*) and arginino succinate  
10 lyase (*argH*), which were reported to be regulated by ArgR in other Gram-positive bacteria  
11 (Larsen *et al.*, 2004; Larsen *et al.*, 2005; Larsen *et al.*, 2008; Ryan *et al.*, 2009) (table 1). This  
12 was confirmed by qRT-PCR analysis (figure 3). In conclusion, the results of microarray and  
13 qRT-PCR analyses strongly suggest that in *Streptococcus suis* ArgR seems to be a specific,  
14 positive transcriptional regulator of the *arcABC*-operon.

15

### 16 **ArgR binds to and activates the *arcABC*-operon promoter**

17 To further analyse the regulatory function of ArgR in *arcABC*-operon expression we  
18 cloned a 788 bp fragment representing the promoter-operator region of the *arcABC*-operon in  
19 the *gfp*-reporter vector *pGA14<sub>spc</sub>-gfp* and transformed it into *Streptococcus suis* strains 10 and  
20 10 $\Delta$ *argR*. As a control, wildtype strain 10 was transformed with the promoterless *pGA14<sub>spc</sub>-*  
21 *gfp* vector. Bacteria were grown in galactose containing TY medium until stationary growth,  
22 and reporter gene activity was then determined by fluorescence measurement. As shown in  
23 figure 4 A, compared to the control, the wildtype strain was able to induce reporter gene  
24 activity. In contrast, nearly no *gfp* expression could be detected in strain 10 $\Delta$ *argR*, showing  
25 that *argR* is essential for reporter gene activity. In conclusion, ArgR was shown to interact  
26 with the *arcABC*-operon promoter.



1 We have previously shown that the *arcABC*-operon promoter region contains a  
2 putative far up-stream ArgR binding site (Gruening *et al.*, 2006). Therefore, we transformed  
3 wildtype strain 10 with *gfp*-reporter vector *pGA14<sub>spc</sub>-gfp* in which the putative ArgR binding  
4 site was deleted by exclusion of 522 bp of the 5' *arcABC*-operon promoter-operator region.  
5 As shown in figure 4B, similar to the full length promoter, reporter gene expression was still  
6 present after deletion of the putative ArgR binding site indicating that ArgR binding occurred  
7 further downstream. *In silico* analysis of the 5'-truncated *arcABC*-operon promoter-operator  
8 region using the virtual footprint promoter analysis program (Munch *et al.*, 2005) revealed 3  
9 AT-rich elements (ARG-boxes) with homologies to the predicted ArgR binding site in  
10 *Escherichia coli* (figure 4C). According to the positions of the ARG-boxes we generated 4  
11 PCR fragments of different length (figure 4C) which were investigated for ArgR DNA  
12 binding by EMSA. The DNA fragments were incubated with recombinant ArgR, and DNA  
13 binding to the PCR fragments was assayed after native gel electrophoresis and ethidium  
14 bromide staining. As shown in figure 4 D, recombinant ArgR was able to bind to the  
15 fragments 3 and 4 containing ARG-boxes 2 and 3 (indicated by the retarded mobility of DNA  
16 fragments after incubation with ArgR), whereas the shorter fragments 1 and 2 containing  
17 ARG-box 1 were unable to bind ArgR.

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

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

26

## 1 **ArgR interacts with the *arcABC*-operon promoter *in vivo***

2           The above results indicated the relevance of ArgR and the ArgR binding sites within  
3 the *arcABC* promoter for *arcABC*-operon expression. Next we analysed whether ArgR  
4 physically interacts with the -147-72-box of the *arcABC* promoter *in vivo* by chromatin  
5 immunoprecipitation analyses. For this, wildtype strain 10 and 10 $\Delta$ *argR* grown either to mid  
6 exponential or to early stationary phase were analysed by ChIP assays with preimmune or  $\alpha$ -  
7 ArgR antiserum. After DNA extraction, binding of ArgR to the -147-72-box was determined  
8 by real-time qRT-PCR with the oligonucleotide primer pair ChiP-for/ChiP-rev indicated in  
9 figure 4 C and calculating the amounts of amplified fragments. As controls we investigated  
10 the ArgR binding to the promoter region of the ADP-glucose pyrophosphorylase gene (*glcC*)  
11 and suilysin gene (*sly*). According to results of our real-time qRT-PCR studies these genes  
12 were not regulated by ArgR (figure 3) and thus the promoters should not be recruited by  
13 ArgR. As depicted in figure 5, approx. 0.2 ng of *arcABC* promoter DNA was precipitated in  
14 mid exponential phase with antibodies directed against ArgR using wildtype strain 10.  
15 Growth to stationary phase enhanced the yield of DNA to 0.35 ng, which indicated *in vivo*  
16 binding of ArgR to the *arcABC* promoter. In contrast, only lowest amounts of DNA were  
17 detected in experiments using the preimmune serum or in experiments using strain 10 $\Delta$ *argR*,  
18 indicating a high specificity of the  $\alpha$ -ArgR antiserum. As expected, the ChIP experiments  
19 revealed that ArgR did not bind to the *sly* and *glcC* promoters. These analyses demonstrate  
20 that ArgR binds to the *arcABC* promoter *in vivo*.

## 1 **Discussion**

2

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

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

18           Here we used the isogenic *argR* negative mutant strain 10 $\Delta$ *argR* and immunoblot analysis to  
19 demonstrate that ArgR is a positive regulator of *arcABC* expression in *Streptococcus suis*,  
20 which is essential for induction of *arcABC* expression. This result is in agreement with  
21 findings that ArgR proteins are activators of arginine catabolic genes, as it has been described  
22 for other Gram-positive bacteria such as *Bacillus licheniformis* and *Streptococcus gordonii*  
23 (Maghnouj *et al.*, 1998; Zeng *et al.*, 2006). However, the array and qRT-PCR analyses  
24 revealed that ArgR seems to solely regulate the *arcABC*-operon. This was indicated by the  
25 significantly reduced mRNA expression of the *arcABC* genes in strain 10 $\Delta$ *argR* at early  
26 stationary growth phase in both assays. A single system regulation by ArgR has not been

1 described yet and is different to many other bacteria, in which ArgR has been shown to  
2 regulate both arginine anabolic and catabolic genes (Hashim *et al.*, 2004; Hernandez-Flores *et*  
3 *al.*, 2004; Larsen *et al.*, 2004; Larsen *et al.*, 2005; Larsen *et al.*, 2008; Lu *et al.*, 2004; Park *et*  
4 *al.*, 1997). From our array analyses we could exclude any polar effects due to the insertion  
5 mutation since expression of the genes upstream (SSU0587) and downstream (SSU0589) of  
6 *argR* (SSU0588) was not significantly altered (supplementary table 2).

7 The fact that ArgR seems to be a highly specific regulator of the *arcABC*-operon in  
8 *Streptococcus suis* was underlined by several findings. First of all, our array analyses with  
9 RNA from mid exponential growth did not reveal any significantly differentially expressed  
10 genes in strain 10 $\Delta$ *argR*. Furthermore, there was no evidence of an ArgR dependent  
11 regulation of the *argGH* operon, which seems to be the only arginine anabolic system in  
12 *Streptococcus suis* as no other putative anabolic gene clusters like *argCJDBF* or *glfS-argE*  
13 could be identified in the *Streptococcus suis* genome annotation. Secondly, at early stationary  
14 growth the array analyses revealed a low number of differentially expressed genes which had  
15 a low degree of differential expression in strain 10 $\Delta$ *argR*. The qRT-PCR analyses revealed  
16 that the genes were regulated 2-fold maximally. Notably, most of them are connected to ATP  
17 generation, which seems to be plausible since ATP production by ArcABC is strongly  
18 reduced in strain 10 $\Delta$ *argR*. For example, the adenylate kinase (Adk) produces ATP and AMP  
19 from two moles of ADP (Willemoes & Kilstrup, 2005). The trehalose-6-phosphate hydrolase  
20 (TreC) catalyzes the conversion from trehalose-6-phosphate to glucose and glucose-6-  
21 phosphate (Rimmele & Boos, 1994). Thus, it is very likely that these genes are not primarily  
22 regulated by ArgR. Thirdly, our ChIP analysis demonstrated clearly that ArgR binds to the  
23 *arcABC* promoter *in vivo*. Finally, all our phenotypical tests revealed that, in comparison to  
24 the wildtype strain 10, strain 10 $\Delta$ *argR* was similarly affected in growth and biological fitness  
25 as strain 10 $\Delta$ *arcABC* (figure 2). These assays also demonstrated that enhanced *arcABC*

1 expression mediated by ArgR is essential for optimal growth and intracellular survival of  
2 *Streptococcus suis*.

3 Regulation of the ArgR family of transcriptional regulators is exerted by binding to so-called  
4 ARG operator sites preceding the relevant target genes. ARG operator sites consist of pairs of  
5 18-bp palindromic sequences (called ARG-boxes), of which the  
6 5'-TnTGnATwwwATnCANa-3' (conserved residues are capitalized, n represents any  
7 nucleotide, and w represents A or T) consensus sequence in *Escherichia coli* (Maas, 1994) is  
8 conserved with only small variations in various other organisms studied (Cherney *et al.*, 2008;  
9 Garnett *et al.*, 2008; Makarova *et al.*, 2001). Our promoter studies with the *arcABC*  
10 promoter-operator-*gfp* constructs indicated that ArgR is responsible for *arcABC* promoter  
11 activity. Furthermore, we could exclude the relevance of a putative ARG-box in the  
12 5' promoter region of the *arcABC* promoter that we identified in a previous study (Gruening  
13 *et al.*, 2006) for *arcABC* promoter activity. Further *in silico* analysis of the *arcABC*  
14 promoter-operator region with the virtual footprint promoter prediction program (Munch *et*  
15 *al.*, 2005) revealed three further putative ARG-boxes. Gel retardation assays with  
16 recombinant ArgR and truncated fragments of the *arcABC* promoter-operator region allowed  
17 us to identify a DNA binding region of 75 bp that was able to interact with recombinant  
18 ArgR. Since identification of potential A+T rich ArgR binding sites *in silico* and EMSA do  
19 not reflect the *in vivo* situation (not all binding sites may be predicted *in silico* or weak  
20 binding sites relevant *in vivo* may not be recognised by EMSA) we performed ChIP analysis  
21 which demonstrated that ArgR physically interacts with *arcABC*-promoter region *in vivo*. The  
22 context of ArgR binding to the ARG-boxes in the promoter sequence, however, awaits further  
23 studies.

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

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41

1 **Figure legends**

2

3 **Figure 1: ArgR is essential for ADS expression**

4 (A) Immunoblot analyses of whole cell lysates of *Streptococcus suis* wildtype strain 10 (wt)  
5 and strain 10 $\Delta$ argR grown to early stationary phase in THB media. As a control, recombinant  
6 ArgR (rArgR) was run in the same gel. Membranes were analysed with a polyclonal  
7 antiserum raised against recombinant ArgR. (B) Immunoblot analysis of whole cell lysates  
8 obtained from wildtype strain 10 (wt), strains 10 $\Delta$ argR and 10 $\Delta$ arcABC probed with  
9 polyclonal antisera against recombinant ArcB (top) and ArgR (mid). Bottom, silver stained  
10 gel for loading control. MW: molecular weight standard.

11

12

13 **Figure 2: Influence of ArgR deficiency on the biological fitness of *Streptococcus suis***

14 (A) *Streptococcus suis* wildtype strain 10 (wt), strain 10 $\Delta$ argR, and strain 10 $\Delta$ arcABC were  
15 cultured in tryptone-based (TY) minimal medium supplemented with 10 mM galactose. The  
16 graph shows bacterial growth including means and standard deviations of the optical density  
17 at 600 nm (OD<sub>600</sub>) from a representative experiment performed in triplicate. (B) Bacteria were  
18 grown as described in (A). The graph represents the pH value of the growth medium with  
19 means and standard deviations from a representative experiment performed in triplicate. (C)  
20 Wildtype strain 10 (wt), 10 $\Delta$ argR, and 10 $\Delta$ arcABC were cultured as described in (A).  
21 Ammonia production is given as mg ammonia per ml medium. (D) Intracellular survival of  
22 the capsule deficient strains 10 $\Delta$ cpsEF, 10 $\Delta$ cpsEF $\Delta$ argR, and 10 $\Delta$ cpsEF $\Delta$ arcABC in HEp-2  
23 cells that have been treated with bafilomycin (200 nM) for 1 hour before infection (white  
24 bars) and untreated cells (black bars). Results are given as percentage of survival in  
25 comparison to the initial inoculum. Data represent means and standard deviations of three

1 independent experiments. Results were considered statistically significant with  $P < 0.05$  as  
2 indicated by an asterisk (\*).

3

4 **Figure 3: ArgR mutation specifically affects the *arcABC*-operon expression**

5 *Streptococcus suis* wildtype strain 10 and the *argR* deficient mutant strain 10 $\Delta$ *argR* were  
6 grown in THB medium to early stationary (stat) phase. Transcript levels of the indicated  
7 genes were determined by real-time qRT-PCR. Signals were normalized to the housekeeping  
8 gene (*dnaH*) and relative gene expression was calculated by the  $\Delta\Delta C_t$  method. The relative  
9 fold-change ratio indicates the ratio of the values obtained for 10 $\Delta$ *argR* and wildtype. Data  
10 are depicted with mean standard deviation for three independent real-time qRT-PCR  
11 performed in duplicate. A significant difference ( $P < 0.05$ ) was defined as at least 2-fold  
12 change gene expression compared to the wildtype strain 10. *arcB*, ornithine carbamoyl  
13 transferase; *argG*, arginino succinate synthase; *sly*, sullysin; *treA*, putative trehalose-6-  
14 phosphate hydrolase; *glgC*, ADP-glucose pyrophosphorylase; *adk*, adenylate kinase; *argR*,  
15 arginine repressor (SSU0588).

16

17 **Figure 4: ArgR binds to and activates the *arcABC*-operon promoter.**

18 *Streptococcus suis* wildtype strain 10 and strain 10 $\Delta$ *argR* harbouring the plasmid *pGA14<sub>spc</sub>-*  
19 *Parc709-gfp* (A) or wildtype strain 10 harbouring either plasmid *pGA14<sub>spc</sub>-Parc709-gfp*  
20 representing the whole *arcABC* promoter region (*Parc709*) or the far upstream ARG-box  
21 deleted version (*Parc187*) (B) were grown for eight hours in TY medium. Bars represent the  
22 relative fluorescence units (RFU) after subtracting the absolute values of the PBS control.  
23 Wildtype strain 10 harbouring the promoterless construct *pGA14<sub>spc</sub>-gfp* served as negative  
24 control. Experiments were carried out in triplicates and repeated at least twice. (C) Schematic  
25 representation of the *Streptococcus suis* *arcABC*-operon promoter. The three putative ArgR  
26 14-bp binding sites with highest homology to *Escherichia coli* ArgR binding site are shown in

1 grey. The transcriptional start point determined by 5'-RACE is indicated (+1), the predicted  
2 amino acid sequences of the 3'-primed *flpS* gene and 5'-primed *arcA* gene are shown in  
3 italics. Primer sequences used for amplification of *arcABC* promoter segments analysed by  
4 EMSA and ChIP are indicated by underlining arrows. (D) PCR fragments were generated  
5 with the primers 1-EMSA, 2-EMSA, 3-EMSA and 4-EMSA (1-4) in combination with  
6 EMSA-rev (positions as indicated in C) and analyzed in an EMSA after a 1 h incubation with  
7 (+) or without (-) recombinant ArgR (rArgR). Gel retardation by DNA protein complexes was  
8 monitored after ethidium bromide staining.

9

#### 10 **Figure 5: ArgR binds to the *arcABC* promoter *in vivo***

11 For ChIP assays wildtype strain 10 and strain 10 $\Delta$ *argR* were grown in THB medium to  
12 exponential (exp) and stationary phase (stat). ChIP lysate chromatin was precipitated using  
13 either  $\alpha$ -ArgR or preimmune serum. ChIP-DNA was quantified by real-time qRT-PCR using  
14 primer pairs for the *arcABC* (black bars), *sly* (grey bars) and *glgC* (white bars) promoter  
15 region. The graphs represent means and standard deviations of immunoprecipitated DNA  
16 from three independent experiments. Significance ( $P < 0.01$ ) was calculated in a two-sample  
17 *t*-test by comparing wildtype strain 10 and strain 10 $\Delta$ *argR*.

**Table 1:** Comparative microarray analysis of *Streptococcus suis* wildtype strain 10 and 10Δ*argR* grown to early stationary phase (OD<sub>600</sub> 0.9). Significantly regulated genes are shown for a lowest false discovery rate of  $P < 0.05$ .

ORF in strain P 1/7	Gene name	Gene product	Fold change gene expression <sup>1</sup>
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>	exodeoxyribonuclease	+ 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 <sup>2</sup>
SSU1104	<i>rptT</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>shy</i>	suiIysin (hemolysin)	+ 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

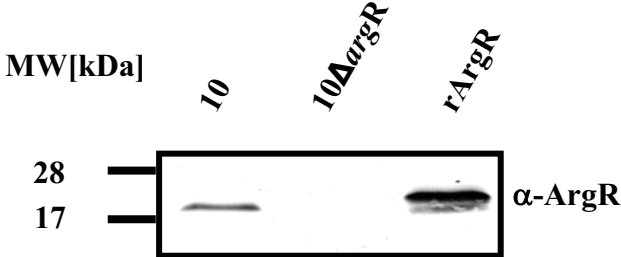
<sup>1</sup> Fold change gene expression is given as positive (+) or negative (-) value representing either upregulation or downregulation in 10Δ*argR* strain, respectively.

<sup>2</sup> NS: not significant



Fig.1

A



B

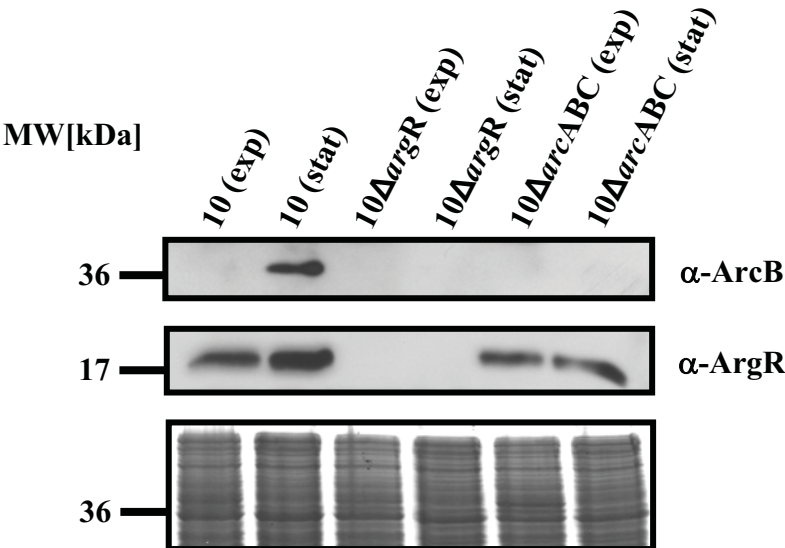
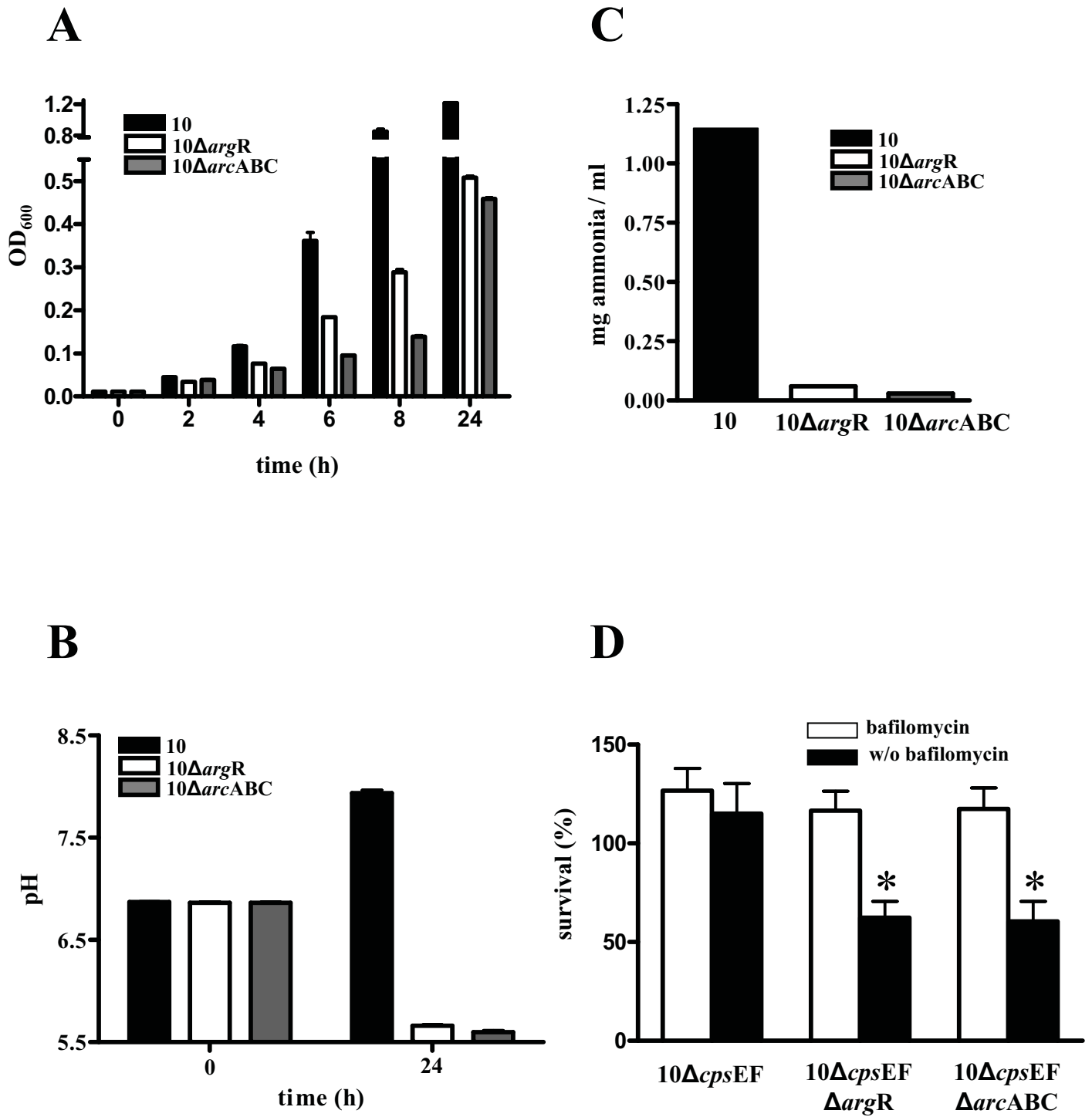
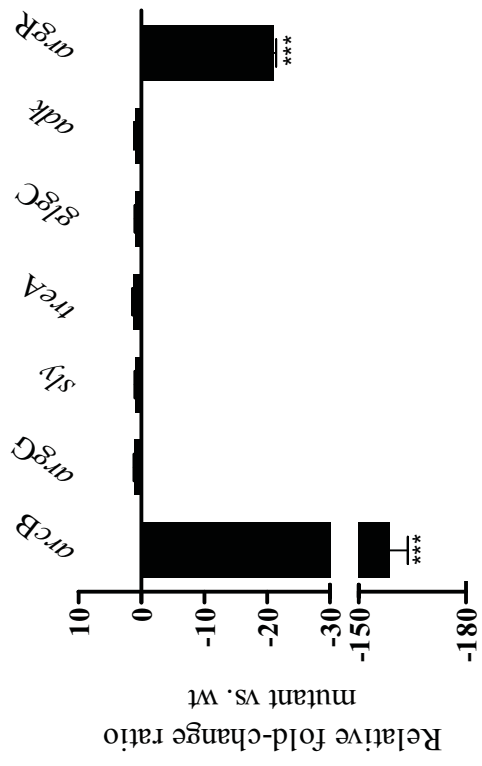




Fig.2

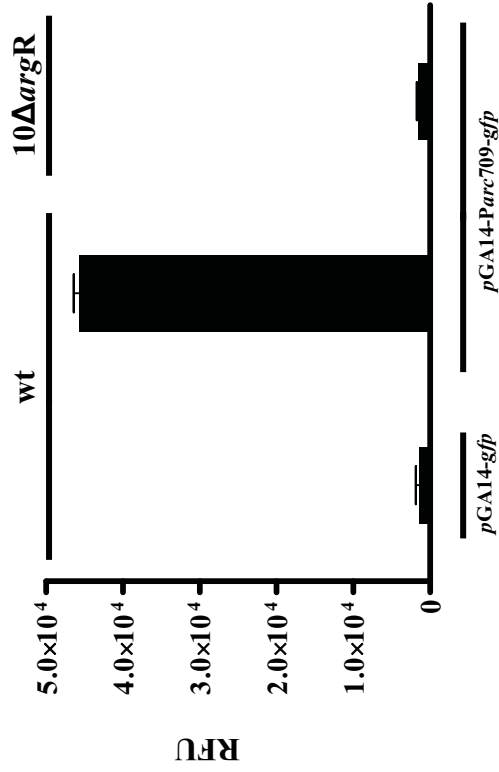




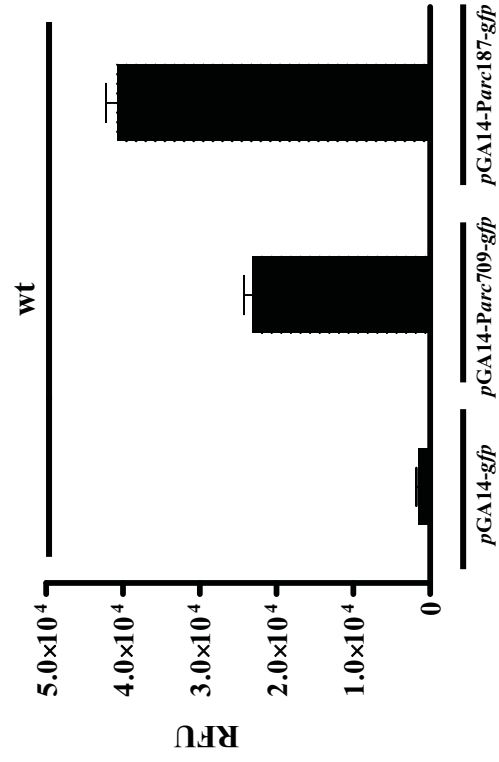
**Fig.3**

Fig.4

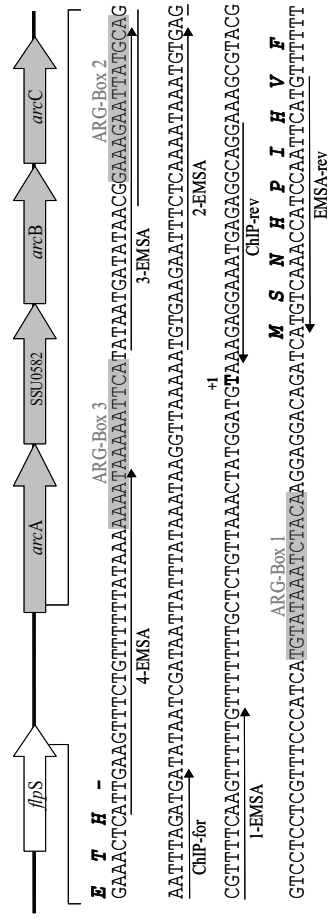
**A**



**B**



**C**



**D**

