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

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Abstract

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 contributing to its virulence. Recently, we identified a new putative virulence factor in *Streptococcus suis*, the arginine deiminase system (ADS), an arginine catabolic enzyme system encoded by the *arc*ABC-operon, which enables *Streptococcus suis* to survive in acidic environment. In this study, we focused on ArgR, an ADS associated regulator belonging to the ArgR/AhrC arginine repressor family. Using an *arg*R knock-out strain we could show that ArgR is essential for *arc*ABC-operon expression and necessary for the biological fitness of *Streptococcus suis*. By cDNA expression microarray analyses and quantitative real time RT-PCR we found that the *arc*ABC-operon is the only gene cluster regulated by ArgR, which is in contrast to many other bacteria. Reporter gene analysis with *gfp* under the control of the *arc*ABC promoter demonstrated that ArgR is able to activate the *arc*ABC promoter. Electrophoretic mobility shift assays with fragments of the *arc*ABC promoter and recombinant ArgR, and chromatin immunoprecipitation with antibodies directed against ArgR revealed that ArgR interacts with the *arc*ABC 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 *Streptococcus suis* ArgR is an essential, system specific transcriptional regulator of the ADS directly interacting with the *arc*ABC promoter *in vivo*.

keywords: arginine repressor, arginine deiminase system, chromatin immunoprecipitation

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 is not only 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 homofermentative cocci, is the arginine deiminase system (ADS). It allows degradation of arginine into ornithine, ammonia, and carbon dioxide catalyzed by the arginine deiminase (ArcA), the ornithine carbamoyl transferase (ArcB), and the carbamate kinase (ArcC) (Barcelona-Andres *et al.*, 2002; Burne *et al.*, 1989; Champomier Verges *et al.*, 1999; Chaussee *et al.*, 2003; Crow & Thomas, 1982; Zuniga *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 important role in supplying the bacteria with energy during nutrient starvation. Furthermore, the ADS facilitates evasion from 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 the 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-Andres *et al.*, 2002; Dong *et al.*, 2004; Maghnouj *et al.*, 2000; Zuniga *et al.*, 2002a).

Streptococcus suis is a Gram-positive, facultative anaerobic pathogen colonizing mainly the upper respiratory tract of swine. It is endemic in nearly all pig producing industries and causes high economical losses due to meningitis, septicemia, arthritis, endocarditis, and bronchopneumonia. As a zoonotic agent *Streptococcus 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 *Streptococcus suis*. Previously, we have identified two proteins of the *Streptococcus suis* ADS, which were temperature induced and expressed on the streptococcal surface (Winterhoff *et al.*, 2002). Furthermore, we could show that the ADS is inducible by arginine, O_2 tension, subjected to CCR, and that it contributes to survival under acidic conditions (Gruening *et al.*, 2006). The arginine catabolic genes *arc*A, *arc*B and *arc*C, which are transcribed polycistronically, are clustered together with the genes for a putative arginine/ornithine antiporter (*arc*D), a putative Xaa-His dipeptidase (*arc*T), and a putative endo-beta-galactosidase C (*arc*H). The ADS gene cluster is confined by the genes of two putative transcriptional regulators, the FNR-like protein of *Streptococcus suis* (FlpS) at the 5` end of the *arc*ABC-operon, and *arg*R, encoding for a repressor of the ArgR/AhrC familiy (ArgR) at the 3´end of *arc*H.

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

Methods

Bacterial strains and growth conditions

The highly virulent serotype 2 strain 10 and its capsule deficient mutant strain 10Δ*cps*EF::*spc*R were used as parental strains (Smith *et al.*, 1999). Bacteria were routinely grown in Todd Hewitt Broth (Becton Dickinson Diagnostics, Heidelberg, Germany) 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, Detroit, USA) containing 6% (vol/vol) sheep blood overnight at 37°C. To analyze the external pH during bacterial growth, bacteria were grown in TY medium overnight. Then, streptococci were adjusted to an optical density at 600 nm (OD_{600}) of 0.02, and the pH was monitored at indicated time-points. *Escherichia coli* was subcultured and maintained on Luria Bertani agar plates. If required, antibiotics were added at following concentrations: spectinomycin at 100 µg/ml (*Streptococcus suis*) and 50 µg/ml (*Escherichia coli*), erythromycin at 1 µg/ml (*Streptococcus suis*) and 300 µg/ml (*Escherichia coli*), ampicillin at 100 µg/ml (*Escherichia coli*).

DNA techniques

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

5´-RACE

The transcriptional start point of the *arc*ABC-operon was determined by rapid amplification of cDNA ends (RACE) with the 5´RACE kit (Invitrogen, Groningen, Netherlands). Briefly, cDNA was synthesized from DNaseI (Ambion Inc., Texas, USA) treated RNA of *Streptococcus suis* strain 10 with primer GSP2 (supplementary table 1) 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 1). Sequencing was performed by Seqlab Laboratories, Goettingen, Germany.

Production and purification of recombinant ArgR

Recombinant ArgR was produced as a 6xHis-tagged fusion protein in *Escherichia coli* with the QIAexpress paired *p*REP4-*p*QE plasmid system (Qiagen, Hilden, Germany). Briefly, *arg*R was amplified by PCR from chromosomal DNA with the specific oligonucleotide primer pair ArgR-*Pst*I and ArgR-*Sph*I (supplementary table 1) and cloned into the *Pst*I/*Sph*I digested expression vector *p*QE30. The resulting plasmid *p*QE30*arg*R was introduced into *Escherichia coli* strain M15[*p*REP4] (Qiagen). Isopropyl-β-D-thiogalactopyranoside (IPTG) induced recombinant ArgR was purified by Ni^{2+}-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, Sulzfeld, Germany).

Electrophoretic mobility shift assay (EMSA)

To localize the ArgR binding site *in vitro*, different fragments of the promoter region of the *arc*ABC-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 1). The DNA fragments were purified with the QIAqick™ PCR Purification Kit (Qiagen) according to the manufacturer´s instructions. Then, 200 ng of DNA were incubated with or without 1 µg of 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 $MgCl_2$) for 1 h at room temperature (RT). Protein-DNA complexes were separated electrophoretically by a native 5% polyacrylamide gel and visualized by ethidium bromide staining.

Mutagenesis

The *arc*ABC-operon and the ADS-associated regulator ArgR were inactivated by insertion mutagenesis in *Streptococcus suis* strain 10 and 10Δ*cps*EF::*spc*R (10Δ*cps*EF). Briefly, the gene encoding the putative arginine regulator ArgR was amplified from the streptococcal genome with the primers *arg*RKOlin and *arg*RKOrec (supplementary table 1) and subsequently introduced into the cloning vector *p*CR®2.1-TOPO (Invitrogen). The resulting plasmid *p*TOPO-*arg*R was linearized by *Hinc*II. Then, the *Pvu*II released erythromycin resistance cassette derived from vector *p*IC*erm* was introduced to disrupt *arg*R. Electroporation of the parental strains was performed as previously described (Gruening *et al.*, 2006; Smith *et al.*, 1995). For inactivation of the *arc*ABC-operon the plasmid *p*GEMAD carrying *arc*ABC was linearized with the restriction enzyme *Eco*RV and the operon was disrupted by insertion of an erythromycin resistance cassette in *arc*A. The resulting plasmid was transformed into *Streptococcus suis* strain 10 and 10Δ*cps*EF. Mutants were controlled by PCR and immunoblot analysis.

Construction of *gfp*-reporter strains

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

streptococcal genome and subsequently cloned into *p*GEMT-Easy (Promega, Mannheim, Germany). The resulting plasmid *p*IFGEMT-Easy was used as template for inverse PCR using the primer pair backbone1-*Hind*III/backbone2-*Kpn*I (supplementary table 1), and the PCR product was digested with the respective restriction enzymes. In parallel, the promoterless *gfp* allele *gfp*mut3* was amplified from the Gram-positive shuttle vector *p*DL278-*gfp*mut3* with the primer pair *gfp*-*Hind*III/gfp-*Kpn*I (supplementary table 1) 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 *p*GA14$_{spc}$ (Smith *et al.*, 1995) to obtain the reporter plasmid *p*GA14$_{spc}$-P*arc*709-*gfp*. Reporter plasmids carrying either the truncated *arc*ABC promoter fusion (*p*GA14$_{spc}$-P*arc*187-*gfp*) or no promoter fusion (*p*GA14$_{spc}$-*gfp*) were generated in the same way using different primers (supplementary table 1) for inverse PCR. Reporter plasmids were introduced into *Streptococcus suis* strain 10 or strain 10Δ*arg*R by electro-transformation. Transformants were screened by plating onto spectinomycin-containing blood agar plates and plasmid isolation with following *Eco*RI restriction digest. For promoter studies, bacteria were grown overnight in TY medium supplemented with 10 mM galactose. Then, bacterial suspensions were adjusted to an OD_{600} of 0.02 in the same medium and cultured at 37°C for approx. eight hours to an OD_{600} of 0.2. One ml of bacterial culture was harvested by centrifugation, washed twice with PBS and resuspended in 1 ml PBS. One hundred µl 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 triplicates and repeated at least twice.

Chromatin immunoprecipitation

For ChIP analysis the wildtype strain 10 and strain 10Δ*arg*R were grown in THB media to OD_{600} of 0.3 and 0.9, representing the mid exponential and early stationary growth phase, 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 4,000 x *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% sodium dodecyl sulfate, 10 mM EDTA, 1mM AEBSF (Merck Biosciences, Darmstadt, Germany)) and 1x protease inhibitor for bacterial cell extracts (Sigma, Munich, Germany), and was ruptured using the FastPrep® instrument (Qbiogene, Eschwege, Germany) three times for 45 sec at highest intensity. Bacterial chromatin was sheared by ultrasonic disintegration. The cleared lysates were diluted 5-fold 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). Following, 25 µg of ChIP lysate chromatin were mixed with 30 µl salmon sperm DNA/protein-A agarose matrix and 10 µl of polyclonal rabbit α-rArgR antiserum or preimmune serum, respectively. Immunoprecipitation was carried out on a rotator at room temperature for 90 minutes. The protein-A/antibody/DNA complexes were washed and DNA was extracted as described (Braunstein *et al.*, 1993). ChIP-DNA was analysed by quantitative real-time PCR using primer pairs (supplementary table 1) flanking the *arc*ABC, *sly* and *glg*C promoter region, respectively. The amount of immunoprecipitated DNA was assayed by real-time PCR using a Stratagene (Mx3005P) instrument. The reaction mixture contained 1 µl of ChIP- or input-DNA, 400 nM primers and 10 µl of SYBRGreen 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, 72 °C for 20 s followed by a melting curve of the product as control. Serial dilutions of input DNA revealed linearity from 250 pg 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 wildtype strain and its mutant strain 10Δ*arg*R were grown in THB to an OD_{600} of 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® for three times of 45 sec at intensity setting 6.5 and cooled on ice. After chloroform extraction and isopropanol 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 Bioanalyzer, Agilent Technologies, Santa Clara, USA). For cDNA synthesis and labelling a total of 10 µg RNA of three independent replicated biological preparations was pooled. Ten µg of pooled RNA was reverse transcribed and labelled with the CyScribe™ Post-Labelling Kit (GE Healthcare, RPN5660, Munich, Germany) and then purified via the GFX purification kit (GE Healthcare) according to the manufacturer's instructions. Complementary DNA from wildtype strain 10 and mutant strain 10Δ*arg*R was labelled with Cy3 or Cy5, respectively, and analyzed 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 instructions of the manufacturer. Hybridization was performed at 60°C for 17 h. Slides were washed for 10 min in 6 x SSC/0.05% Triton-X102 at room temperature, followed by 5 min in 0.1 x SSC/0.05% Triton X102 at 4°C. Slides were dried using pressured air and scanned in a GenePix® 4200AL (Molecular Devices, Sunnyvale, USA). Scans were analyzed using the GenePix software (Molecular Devices). A customized in house developed R-based normalization procedure was

performed to fit the data. Subsequently, data were analysed using SAM. After statistical normalization and evaluation of the dye swap experiments genes with more than a 2-fold ratio change and *P* value < 0.05 were dedicated 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 *Streptococcus 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 to 91%, 4%, 2% and 3% by 4, 3, 2 oligonucleotides or 1 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 reverse transcription PCR (qRT-PCR)

Wildtype strain 10 and its mutant strain 10Δ*arg*R were grown in THB medium to early stationary (stat) phase and RNA was prepared as described above. Two µg of RNA were reverse transcribed (primers listed in supplementary table 1) 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 (*dna*H) 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*arg*R compared to the wildtype strain.

Preparation of whole cell lysates and Western blot analysis

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

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

Determination of ammonia in the culture supernatant

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

Survival in cells

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

Statistical analysis

When 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 *arc*ABC-operon expression in *Streptococcus suis*

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

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

Further, we compared growth kinetics of the mutant strain 10Δ*arg*R with that of the wildtype strain and strain 10Δ*arc*ABC in TY medium supplemented with galactose. As shown in figure 2A, starting at 4 h of culture, both mutant strains were significantly reduced in their growth rate when compared to the wildtype strain. Furthermore, the growth of strain 10Δ*arg*R exceeded that of strain 10Δ*arc*ABC which was most clearly seen at 6, 8, and 24 h.

These data indicated that *arg*R and the *arc*ABC-operon are necessary for optimal growth of *Streptococcus suis.* Next we monitored pH and ammonia production during growth of wildtype strain 10, strain 10Δ*arg*R, and strain 10Δ*arc*ABC. As shown in figure 2B, both mutant strains were unable to neutralize growth dependent acidification. Thus, after 24 h of growth we could determine a pH value of approx. 7.9 in the supernatant of the wildtype strain 10, whereas pH values of approx. 5.6 were detected for the strains 10Δ*arg*R and 10Δ*arc*ABC, respectively. The differences in neutralising growth dependent acidification after 24 h corresponded to a loss in ammonia production (figure 2C). In contrast to wildtype strain 10 (1.15 mg/ml) ammonia production of strain 10Δ*arc*ABC (0.03 mg/ml) and strain 10Δ*arg*R (0.06 mg/ml) was markedly reduced. Finally, survival of ArgR deficient *Streptococcus suis* in eukaryotic cells was investigated using the human epithelial cell line HEp-2. Since the polysaccharide capsule prevents *Streptococcus suis* uptake by epithelial cells, we generated *arg*R and *arc*ABC deficient strains in a non-encapsulated background (strain 10Δ*cps*EF). As shown in figure 2D, strain 10Δ*cps*EF was able to survive and multiply intracellularly at a rate of approx. 125 % of the initial inoculum. In contrast, significantly lower survival rates (60 %) were determined for both double knock-out mutant strains. To analyse whether reduced survival correlated with the inability of strains 10Δ*cps*EFΔ*arg*R and 10Δ*cps*EFΔ*arc*ABC to generate ammonia and prevent acidification, cells were treated with bafilomycin to inhibit endosomal acidification before infection. Compared to the infection of untreated cells, the pre-treatment of the cells with bafilomycin significantly increased the survival rates of the strains 10Δ*cps*EFΔ*arg*R and 10Δ*cps*EFΔ*arc*ABC. These data suggest that *Streptococcus suis* is able to resist endosomal acidification due to ArgR dependent *arc*ABC-operon induction and ammonia production.

ArgR deficiency specifically affects *arc*ABC-operon expression

The above results indicated that ArgR is an important regulator of the *arc*ABC-operon in *Streptococcus suis.* The differences in the growth kinetic of 10Δ*arg*R and 10Δ*arc*ABC, 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 in *Streptococcus suis* gene expression. RNA was extracted from strain 10Δ*arg*R and wildtype strain 10 grown to mid exponential (reflecting a similar growth of 10Δ*arg*R and 10Δ*arc*ABC), and to early stationary phase when growth of 10Δ*arg*R and 10Δ*arc*ABC clearly differed from each other (figure 2 A). After reverse transcription, cDNA of strain 10Δ*arg*R and the wildtype strain 10 were analysed on a whole genome *Streptococcus suis* strain P1/7 microarray The comparison of microarray data of the bacteria grown to mid exponential growth phase revealed no significantly up- or down-regulated genes by ArgR (supplementary table 2). In early stationary growth phase, 26 genes were differentially expressed (with *P* values of <0.05) between the wildtype and the mutant strain (table 1). Interestingly, expression of only 5 genes was significantly reduced in the mutant strain. As expected, one of them was *arg*R of which mRNA expression was abolished (down regulation 124-fold). The other 4 genes with strongly reduced mRNA expression in the mutant strain (down regulation of ≤ –28-fold, table 1) represented the members of the *arc*ABC-operon, *arc*A, SSU0581, *arc*B and *arc*C. In addition, only mRNA expression of a putative trehalose-6-phosphate hydrolase (*tre*A) was negatively influenced by ArgR deficiency (-3.7-fold). The group of the 20 genes with higher mRNA expression in the mutant strain comprised 7 ribosomal proteins (*rpl*M, *rpl*T, *rpm*I, *rps*D, *rps*L, *rps*P, *rps*U), 6 hypothetical proteins (SSU0068, SSU297, SSU0810, SSU1181, SSU1391, SSU1936), 2 hydrolase family proteins (SSU1578, SSU1763), the adenylate kinase (*adk*), an exodeoxyribonuclease (*exo*A), the translation initiation factor IF-3 (*inf*C), a mechanosensitive channel protein (*msc*L) and suilysin (*sly*). Their lower level of expression differences, which was at max less than 7.3-fold, compared to

the genes of the *arc*ABC-operon suggests that these genes and *tre*A might be influenced indirectly by the ArgR deficiency. QRT-PCR analyses were performed to confirm the array data. Expression levels of *arc*B, which is representative for the *arc*ABC-operon, *arg*R, *sly*, *tre*A, *adk* and *glg*C were determined. Interestingly, significant differences in gene expression of stationary grown strain 10 and 10Δ*arg*R could be confirmed only for *arc*B and *arg*R. In contrast, the expression levels of *sly*, *tre*A and *adk* did not significantly differ from *glg*C which was included as control gene with similar expression in strain 10 and 10Δ*arg*R.

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

ArgR binds to and activates the *arc*ABC-operon promoter

To further analyse the regulatory function of ArgR in *arc*ABC-operon expression we cloned a 788 bp fragment representing the promoter-operator region of the *arc*ABC-operon in the *gfp*-reporter vector *p*GA14$_{spc}$-*gfp* and transformed it into *Streptococcus suis* strains 10 and 10Δ*arg*R. As a control, wildtype strain 10 was transformed with the promoterless *p*GA14$_{spc}$-*gfp* vector. Bacteria were grown in galactose containing TY medium until stationary growth, and reporter gene activity was then determined by fluorescence measurement. As shown in figure 4 A, compared to the control, the wildtype strain was able to induce reporter gene activity. In contrast, nearly no *gfp* expression could be detected in strain 10Δ*arg*R, showing that *arg*R is essential for reporter gene activity. In conclusion, ArgR was shown to interact with the *arc*ABC-operon promoter.

We have previously shown that the *arc*ABC-operon promoter region contains a putative far up-stream ArgR binding site (Gruening *et al.*, 2006). Therefore, we transformed wildtype strain 10 with *gfp*-reporter vector *p*GA14$_{spc}$-*gfp* in which the putative ArgR binding site was deleted by exclusion of 522 bp of the 5' *arc*ABC-operon promoter-operator region. As shown in figure 4B, 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 *arc*ABC-operon promoter-operator region using the virtual footprint promoter analysis program (Munch *et al.*, 2005) revealed 3 AT-rich elements (ARG-boxes) with homologies to the predicted ArgR binding site in *Escherichia coli* (figure 4C). According to the positions of the ARG-boxes we generated 4 PCR fragments of different length (figure 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 figure 4 D, recombinant ArgR was able to bind to the 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 *arc*ABC-operon, which is contradictory to its role as a positive regulator. This prompted us to redefine the transcriptional start point and to revise the *arc*ABC promoter structure. Repeated 5´ RACE analyses identified a tyrosin 79 bp upstream of the ArcA ATG as transcriptional start (data not shown and figure 4 C).

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

ArgR interacts with the *arc*ABC-operon promoter *in vivo*

The above results indicated the relevance of ArgR and the ArgR binding sites within the *arc*ABC promoter for *arc*ABC-operon expression. Next we analysed whether ArgR physically interacts with the -147-72-box of the *arc*ABC promoter *in vivo* by chromatin immunoprecipitation analyses. For this, wildtype strain 10 and 10Δ*arg*R 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 figure 4 C and calculating the amounts of amplified fragments. As controls we investigated the ArgR binding to the promoter region of the ADP-glucose pyrophosphorylase gene (*glc*C) and suilysin gene (*sly*). According to results of our real-time qRT-PCR studies these genes were not regulated by ArgR (figure 3) and thus the promoters should not be recruited by ArgR. As depicted in figure 5, approx. 0.2 ng of *arc*ABC promoter DNA was precipitated in mid exponential phase with antibodies directed against ArgR using wildtype strain 10. Growth to stationary phase enhanced the yield of DNA to 0.35 ng, which indicated *in vivo* binding of ArgR to the *arc*ABC promoter. In contrast, only lowest amounts of DNA were detected in experiments using the preimmune serum or in experiments using strain 10Δ*arg*R, indicating a high specificity of the α-ArgR antiserum. As expected, the ChIP experiments revealed that ArgR did not bind to the *sly* and *glg*C promoters. These analyses demonstrate that ArgR binds to the *arc*ABC promoter *in vivo*.

Discussion

This study showed that ArgR is essential for *arc*ABC-operon expression of the ADS gene cluster in *Streptococcus suis*. Furthermore we could demonstrate that, in contrast to other bacteria, ArgR is a local regulator that specifically interacts with the *arc*ABC-operon promoter in *Streptococcus suis*. In our previous work we found that in *Streptococcus suis* the genes of the ADS are located on a gene cluster consisting of the *arc*ABC-operon, the genes for a putative arginine/ornithine antiporter (*arc*D), a putative Xaa-His dipeptidase (*arc*T), a putative endo-beta-galactosidase (*arc*H), and the genes of two regulatory factors FlpS and ArgR at the 5' and 3' end, 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 *arg*R negative mutant strain 10Δ*arg*R and immunoblot analysis to demonstrate that ArgR is a positive regulator of *arc*ABC expression in *Streptococcus suis*, which is essential for induction of *arc*ABC expression. This result is in agreement with findings that ArgR proteins are activators of arginine catabolic genes, as it has been described for other Gram-positive bacteria such as *Bacillus licheniformis* and *Streptococcus gordonii* (Maghnouj *et al.*, 1998;Zeng *et al.*, 2006). However, the array and qRT-PCR analyses revealed that ArgR seems to solely regulate the *arc*ABC-operon. This was indicated by the significantly reduced mRNA expression of the *arc*ABC genes in strain 10Δ*arg*R at early stationary growth phase in both assays. A single system regulation by ArgR has not been

described yet and is different to many other bacteria, in which ArgR has been shown to regulate both arginine anabolic and catabolic genes (Hashim *et al.*, 2004; Hernandez-Flores *et al.*, 2004; Larsen *et al.*, 2004; Larsen *et al.*, 2005; Larsen *et al.*, 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 *arg*R (SSU0588) was not significantly altered (supplementary table 2).

The fact that ArgR seems to be a highly specific regulator of the *arc*ABC-operon in *Streptococcus 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 10Δ*arg*R. Furthermore, there was no evidence of an ArgR dependent regulation of the *arg*GH operon, which seems to be the only arginine anabolic system in *Streptococcus suis* as no other putative anabolic gene clusters like *arg*CJDBF or *glt*S-*arg*E could be identified in the *Streptococcus suis* genome annotation. Secondly, at early stationary growth the array analyses revealed a low number of differentially expressed genes which had a low degree of differential expression in strain 10Δ*arg*R. The qRT-PCR analyses revealed that the genes were regulated 2-fold maximally. Notably, most of them are connected to ATP generation, which seems to be plausible since ATP production by ArcABC is strongly reduced in strain 10Δ*arg*R. For example, the adenylate kinase (Adk) produces ATP and AMP from two moles of ADP (Willemoes & Kilstrup, 2005). The trehalose-6-phosphate hydrolase (TreC) catalyzes the conversion from 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. Thirdly, our ChIP analysis demonstrated clearly that ArgR binds to the *arc*ABC promoter *in vivo.* Finally, all our phenotypical tests revealed that, in comparison to the wildtype strain 10, strain 10Δ*arg*R was similarly affected in growth and biological fitness as strain 10Δ*arc*ABC (figure 2). These assays also demonstrated that enhanced *arc*ABC

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

Regulation of the ArgR family of transcriptional regulators is exerted by binding to so-called ARG operator sites preceding the relevant target genes. ARG operator sites consist of pairs of 18-bp palindromic sequences (called ARG-boxes), of which the 5'-TnTGnATwwwwATnCAnA-3' (conserved residues are capitalized, n represents any nucleotide, and w represents A or T) consensus sequence in *Escherichia 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 *arc*ABC promoter-operator-*gfp* constructs indicated that ArgR is responsible for *arc*ABC promoter activity. Furthermore, we could exclude the relevance of a putative ARG-box in the 5' promoter region of the *arc*ABC promoter that we identified in a previous study (Gruening *et al.*, 2006) for *arc*ABC promoter activity. Further *in silico* analysis of the *arc*ABC promoter-operator region with the virtual footprint promoter prediction program (Munch *et al.*, 2005) revealed three further putative ARG-boxes. Gel retardation assays with recombinant ArgR and truncated fragments of the *arc*ABC promoter-operator region allowed us to identify a DNA binding region of 75 bp that was able to interact with recombinant ArgR. Since identification of potential A+T rich ArgR binding sites *in silico* and EMSA do 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 recognised by EMSA) we performed ChIP analysis which demonstrated that ArgR physically interacts with *arc*ABC-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 the *arc*ABC-operon expression in *Streptococcus suis.* Its specificity for the ADS makes it highly relevant for biological fitness of *Streptococcus suis.*

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Figure legends

Figure 1: ArgR is essential for ADS expression

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

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

(A) *Streptococcus suis* wildtype strain 10 (wt), strain 10Δ*arg*R, and strain 10Δ*arc*ABC were cultured in tryptone-based (TY) minimal medium supplemented with 10 mM galactose. The graph shows bacterial growth including means and standard deviations of the optical density at 600 nm (OD_{600}) 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 standard deviations from a representative experiment performed in triplicate. (C) Wildtype strain 10 (wt), 10Δ*arg*R, and 10Δ*arc*ABC were cultured as described in (A). Ammonia production is given as mg ammonia per ml medium. (D) Intracellular survival of the capsule deficient strains 10Δ*cps*EF, 10Δ*cps*EFΔ*arg*R, and 10Δ*cps*EFΔ*arc*ABC in HEp-2 cells that have been treated with bafilomycin (200 nM) for 1 hour before infection (white bars) and untreated cells (black bars). Results are given as percentage of survival in comparison to the initial inoculum. Data represent means and standard deviations of three

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

Figure 3: ArgR mutation specifically affects the *arc*ABC-operon expression

Streptococcus suis wildtype strain 10 and the *arg*R deficient mutant strain 10Δ*arg*R were grown in THB medium to early stationary (stat) phase. Transcript levels of the indicated genes were determined by real-time qRT-PCR. Signals were normalized to the housekeeping gene (*dna*H) 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 10Δ*arg*R and wildtype. Data are depicted with mean standard deviation for three independent real-time qRT-PCR performed in duplicate. A significant difference ($P < 0.05$) was defined as at least 2-fold change gene expression compared to the wildtype strain 10. *arc*B, ornithine carbamoyl transferase; *arg*G, arginino succinate synthase; *sly*, suilysin; *tre*A, putative trehalose-6-phosphate hydrolase; *glg*C, ADP-glucose pyrophosphorylase; *adk*, adenylate kinase; *arg*R, arginine repressor (SSU0588).

Figure 4: ArgR binds to and activates the *arc*ABC-operon promoter.

Streptococcus suis wildtype strain 10 and strain 10Δ*arg*R harbouring the plasmid *p*GA14$_{spc}$-P*arc*709-*gfp* (A) or wildtype strain 10 harbouring either plasmid *p*GA14$_{spc}$-P*arc*709-*gfp* representing the whole *arc*ABC promoter region (P*arc*709) or the far upstream ARG-box deleted version (P*arc*187) (B) were grown for eight hours in TY medium. Bars represent the relative fluorescence units (RFU) after subtracting the absolute values of the PBS control. Wildtype strain 10 harbouring the promoterless construct *p*GA14$_{spc}$-*gfp* served as negative control. Experiments were carried out in triplicates and repeated at least twice. (C) Schematic representation of the *Streptococcus suis arc*ABC-operon promoter. The three putative ArgR 14-bp binding sites with highest homology to *Escherichia 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 *flp*S gene and 5′-primed *arc*A gene are shown in italics. Primer sequences used for amplification of *arc*ABC 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 analyzed 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.

Figure 5: ArgR binds to the *arc*ABC promoter *in vivo*

For ChIP assays wildtype strain 10 and strain 10Δ*arg*R were grown in THB medium to exponential (exp) and stationary phase (stat). 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 *arc*ABC (black bars), *sly* (grey bars) and *glg*C (white bars) promoter region. The graphs represent means and standard deviations of immunoprecipitated DNA from three independent experiments. Significance ($P < 0.01$) was calculated in a two-sample *t*-test by comparing wildtype strain 10 and strain 10Δ*arg*R.

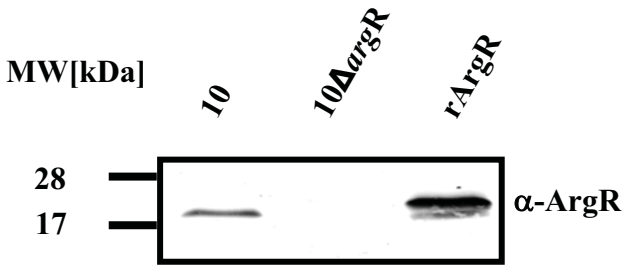
Table 1: Comparative microarray analysis of *Streptococcus suis* wildtype strain 10 and 10Δ*arg*R grown to early stationary phase (OD_{600} 0.9). Significantly regulated genes are shown for a lowest false discovery rate of $P < 0.05$.

ORF in strain P 1/7	Gene name	Gene product	Fold change gene expression[1]
SSU0068	*com*X2	putative competence-specific global transcription modulator (fragment)	+ 3.11
SSU0094	*adk*	adenylate kinase	+ 3.08
SSU0148	*rps*L	30S ribosomal protein S12	+ 2.84
SSU0216	*tre*A, *tre*C	putative trehalose-6-phosphate hydrolase	- 3.68
SSU0297		putative transcription regulation protein	+ 3.61
SSU0580	*arc*A	arginine deiminase	- 28.15
SSU0581		acetyltransferase (GNAT) family protein	- 36.66
SSU0582	*arc*B	ornithine carbamoyltransferase	- 29.96
SSU0583	*arc*C	carbamate kinase	- 28.49
SSU0588	*arg*R	arginine repressor	- 124.67
SSU0627	*exo*A	exodeoxyribonuclease	+ 3.63
SSU0742	*rps*P	30S ribosomal protein S16	+ 3.16
SSU0810		conserved hypothetical protein	+ 6.18
SSU0870	*glg*C	ADP-glucose pyrophosphorylase	NS[2]
SSU1104	*rpl*T	50S ribosomal protein L20	+ 2.88
SSU1105	*rpm*I	50S ribosomal protein L35	+ 3.29
SSU1106	*inf*C	translation initiation factor IF-3	+ 3.03
SSU1181		putative membrane protein	+ 3.01
SSU1231	*sly*	suilysin (hemolysin)	+ 3.23
SSU1257	*msc*L	large-conductance mechanosensitive channel	+ 4.34
SSU1259	*rps*U	30S ribosomal protein S21	+ 4.01
SSU1391		putative membrane protein	+ 3.98
SSU1463	*arg*R	putative arginine repressor	NS
SSU1578		gamma-glutamyl hydrolase	+ 4.22
SSU1692	*rpl*M	50S ribosomal protein L13	+ 2.77
SSU1763		MutT/NUDIX hydrolase family protein	+ 2.92
SSU1806	*arg*G	argininosuccinate synthase (citrulline-asparate ligase)	NS
SSU1807	*arg*H	argininosuccinate lyase	NS
SSU1909	*arg*R	putative arginine repressor	NS
SSU1935	*rps*D	30S ribosomal protein S4	+ 3.51
SSU1936		hypothetical protein	+ 7.84

[1] Fold change gene expression is given as positive (+) or negative (-) value representing either upregulation or downregulation in 10Δ*arg*R strain, respectively.
[2] NS: not significant

Fig.1

A


MW[kDa]
10
10ΔargR
rArgR
28
17
α-ArgR

B

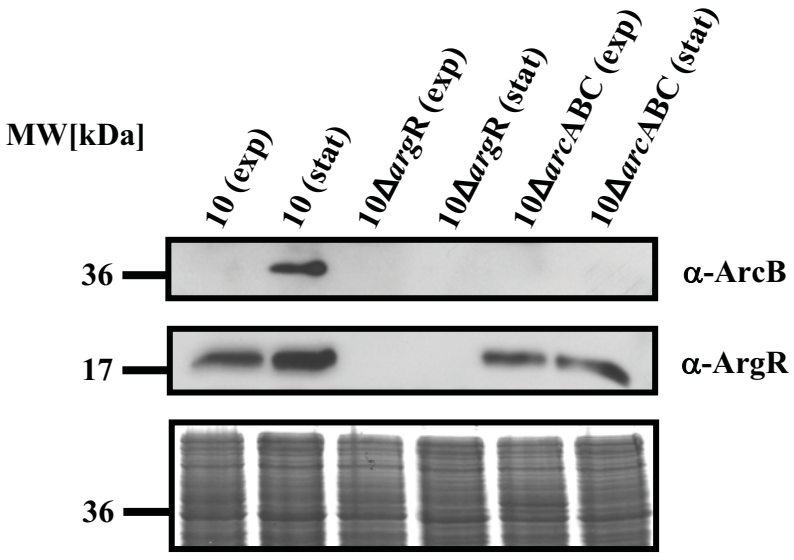

MW[kDa]
10 (exp)
10 (stat)
10ΔargR (exp)
10ΔargR (stat)
10ΔarcABC (exp)
10ΔarcABC (stat)
36
α-ArcB
17
α-ArgR
36

Fig.2

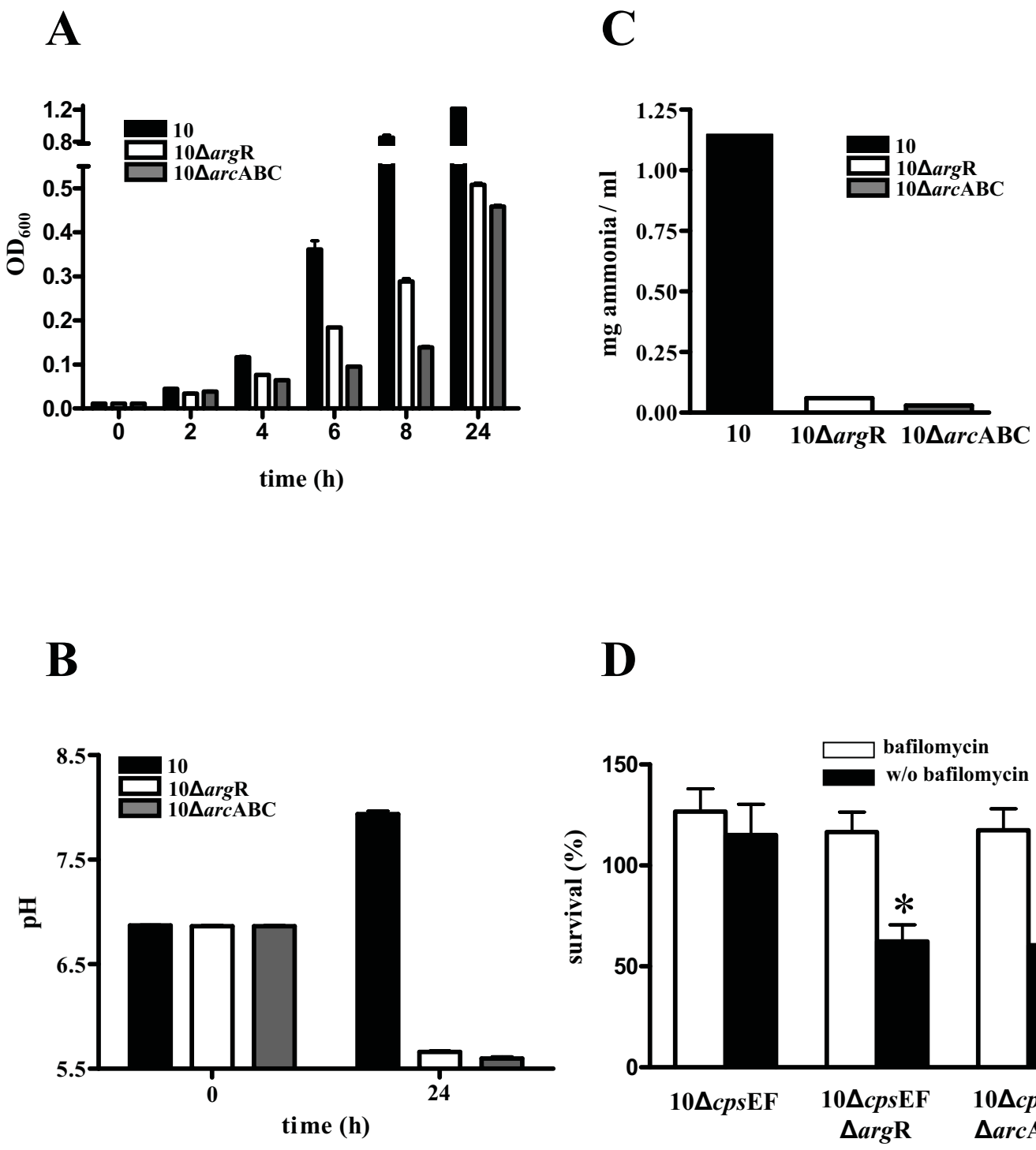
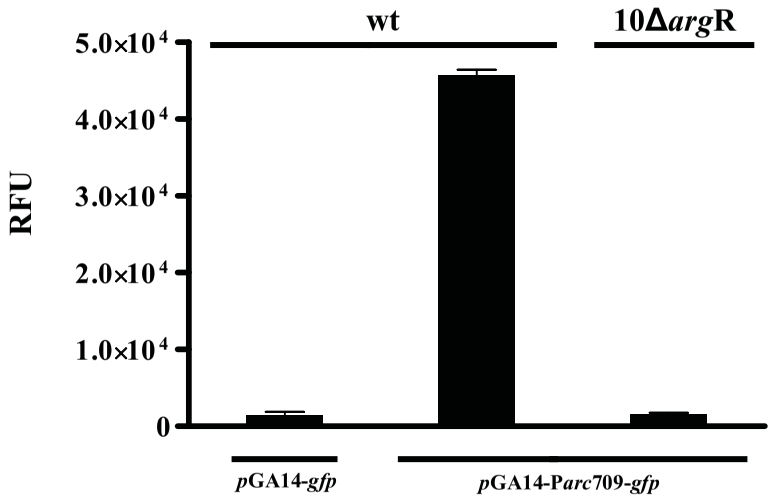

A
1.2
0.8
0.5
0.4
0.3
0.2
0.1
0.0
OD600
10
10ΔargR
10ΔarcABC
0
2
4
6
8
24
time (h)
C
1.25
1.00
0.75
0.50
0.25
0.00
mg ammonia / ml
10
10ΔargR
10ΔarcABC
10
10ΔargR
10ΔarcABC
B
8.5
7.5
6.5
5.5
pH
10
10ΔargR
10ΔarcABC
0
24
time (h)
D
150
100
50
0
survival (%)
bafilomycin
w/o bafilomycin
*
*
10ΔcpsEF
10ΔcpsEF ΔargR
10ΔcpsEF ΔarcABC

Fig.3

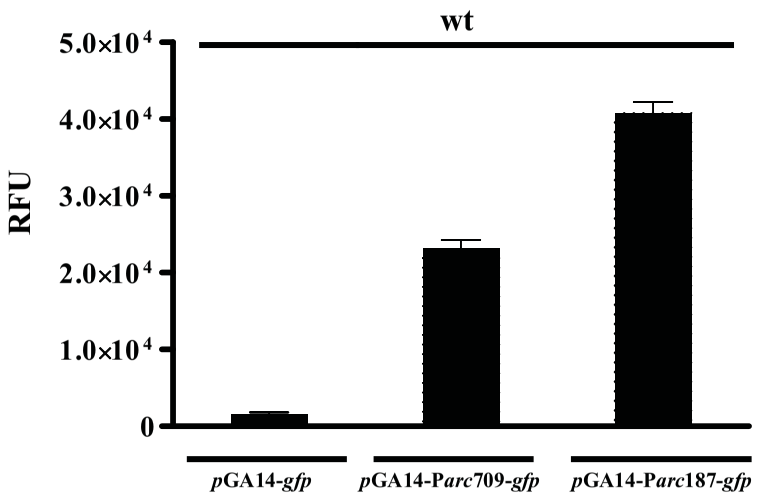
Relative fold-change ratio
mutant vs. wt
10
0
-10
-20
-30
-150
-180
***
***
argR
cadR
glgC
treA
sly
argG
argCB

Fig.4

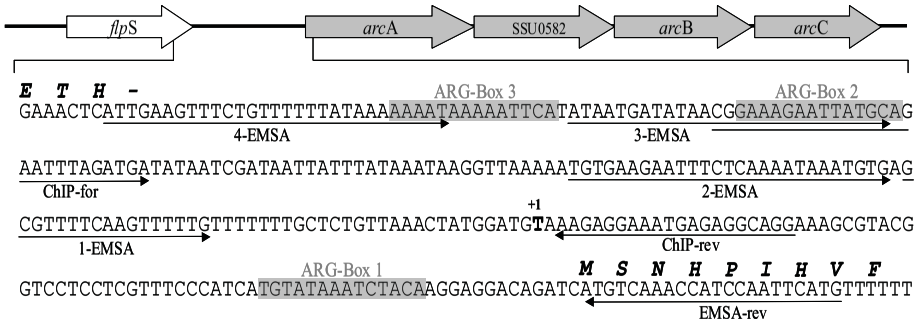
A


wt
10ΔargR
5.0×10⁴
4.0×10⁴
3.0×10⁴
2.0×10⁴
1.0×10⁴
0
RFU
pGA14-gfp
pGA14-Parc709-gfp

B


wt
5.0×10⁴
4.0×10⁴
3.0×10⁴
2.0×10⁴
1.0×10⁴
0
RFU
pGA14-gfp
pGA14-Parc709-gfp
pGA14-Parc187-gfp

C


flpS
arcA
SSU0582
arcB
arcC
E T H -
ARG-Box 3
ARG-Box 2
GAAACTCATTGAAGTTTCTGTTTTTTATAAAAAAATAAAAATTCATATAATGATATAACGGAAAGAATTATGCAG
4-EMSA
3-EMSA
AATTTAGATGATATAATCGATAATTATTTATAAATAAGGTTAAAAATGTGAAGAATTTCTCAAAATAAATGTGAG
ChIP-for
2-EMSA
+1
CGTTTTCAAGTTTTTGTTTTTTTTGCTCTGTTAAACTATGGATGTTAAAGAGGAAATGAGAGGCAGGAAAGCGTACG
1-EMSA
ChIP-rev
ARG-Box 1
M S N H P I H V F
GTCCTCCTCGTTTCCCATCATGTATAAATCTACAAGGAGGACAGATCATGTCAAACCATCCAATTCATGTTTTTT
EMSA-rev

D

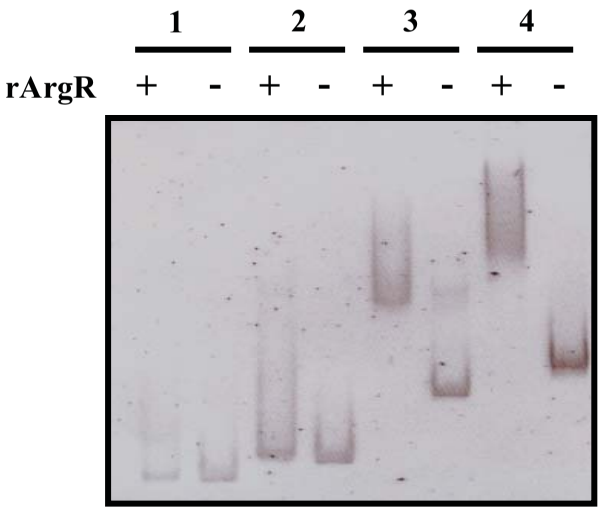
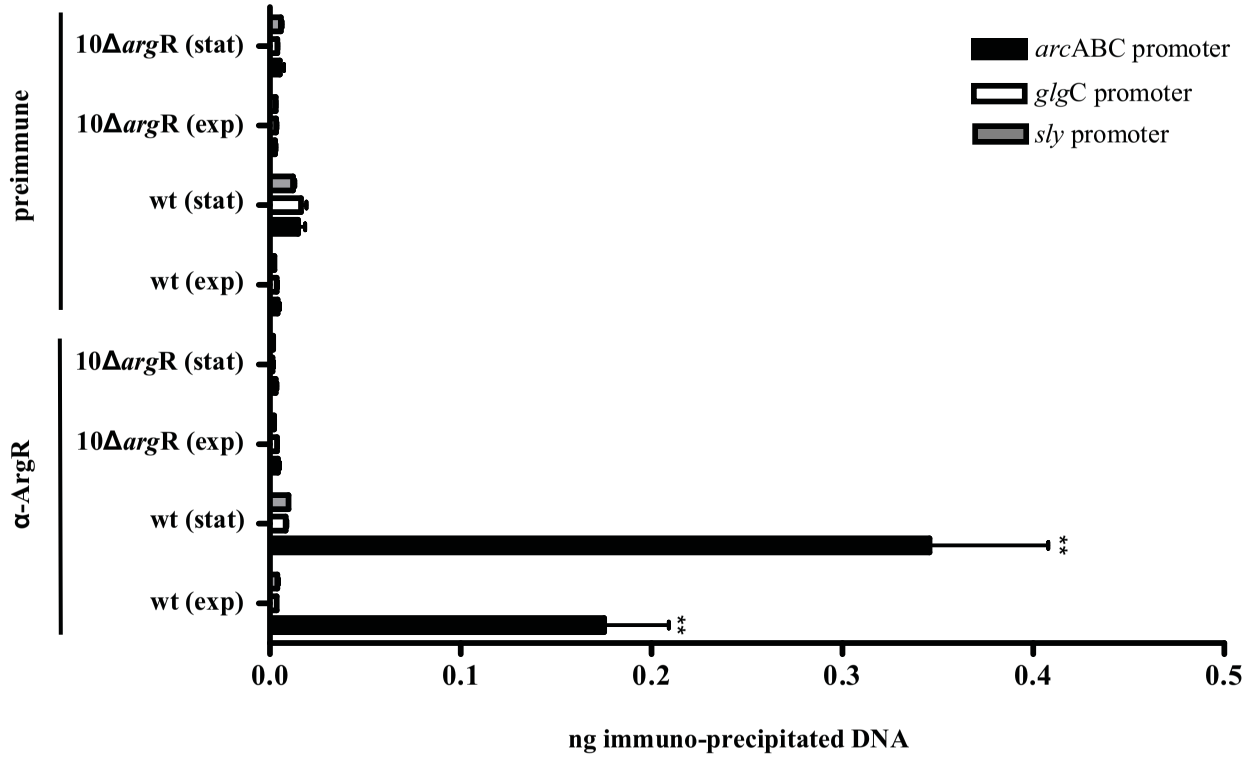

1
2
3
4
rArgR
+ - + - + - + -

Fig.5


preimmune
α-ArgR
10ΔargR (stat)
10ΔargR (exp)
wt (stat)
wt (exp)
10ΔargR (stat)
10ΔargR (exp)
wt (stat)
wt (exp)
arcABC promoter
glgC promoter
sly promoter
**
**
0.0
0.1
0.2
0.3
0.4
0.5
ng immuno-precipitated DNA