RESEARCH ARTICLE

Regulation of the Arginine Deiminase System by ArgR2 Interferes with Arginine Metabolism and Fitness of *Streptococcus pneumoniae*

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ABSTRACT ***Streptococcus pneumoniae* is auxotrophic for arginine, and molecular analysis of the pneumococcal genome showed that the gene encoding an arginine-ornithine antiporter (ArcD) is organized in a cluster together with the *arcABC* genes encoding the arginine deiminase system (ADS) of pneumococci. The ADS consists of the arginine deiminase (AD), the catabolic ornithine carbamoyltransferase (cOCT), and the carbamate kinase (CK). Pneumococcal genomes contain three ArgR-type regulators (ArgR1, ArgR2, and AhrC) that are supposed to be involved in the regulation of arginine metabolism. Here, we identified ArgR2 of TIGR4 as the regulator of the ADS and ArcD. ArgR2 binds to promoter sequences of the *arc* operon, and the deficiency of ArgR2 in TIGR4 abrogates expression of the ADS, including the arginine-ornithine antiporter ArcD. Intranasal infection of mice and real-time bioimaging revealed that deletion of the *arcABCDT* genes attenuates TIGR4. However, the acute-pneumonia model and coinfection experiments indicated that the arginine-ornithine antiporter ArcD is essential to maintain fitness, while the deficiency of ADS enzymes has a minor impact on pneumococcal fitness under *in vivo* conditions. Strikingly, *argR2* mutant TIGR4 outcompeted the wild type in the respiratory tract, suggesting an increase in fitness and further regulatory functions of ArgR2. In contrast to TIGR4, other pneumococci, such as D39, lacking expression of ArgR2, constitutively express the ADS with a truncated nonfunctional AD. On the basis of these results, we propose that the arginine-ornithine antiporter is essential to maintain pneumococcal fitness and that the genes of the ADS cluster are positively regulated in a strain-specific manner by ArgR2.**

IMPORTANCE Pneumococci are the major etiologic agents of community-acquired pneumonia, causing more than 1.5 million deaths annually worldwide. These versatile pathogens are highly adapted to the nutrients provided by the host niches encountered. Physiological fitness is of major importance for colonization of the nasopharyngeal cavity and dissemination during invasive infections. This work identifies the regulator ArgR2 as the activator of the *S. pneumoniae* TIGR4 ADS and the arginine-ornithine transporter ArcD, which is needed for uptake of the essential amino acid arginine. Although ArgR2 activates ArcD expression and uptake of arginine is required to maintain pneumococcal fitness, the deficiency of ArgR2 increases TIGR4 virulence under *in vivo* conditions, suggesting that other factors regulated by ArgR2 counterbalance the reduced uptake of arginine by ArcD. Thus, this work illustrates that the physiological homeostasis of pneumococci is complex and that ArgR2 plays a key role in maintaining bacterial fitness.

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Streptococcus pneumoniae (pneumococcus), a member of the order *Lactobacillales*, is a Gram-positive bacterium residing asymptomatically as a harmless commensal in the human upper respiratory tract. However, pneumococci are also the etiologic agents of serious local and invasive infections, including otitis media, pneumonia, bacteremia, and meningitis (1–4).

During pathogenesis, pneumococci have to adapt their metabolism to different environmental conditions regarding temperature, oxygen, pH, and the availability of nutrients. Pneumococci are endowed with a large number of enzymes and transporter systems enabling them to take up and metabolize the nutrients available in their various host niches. To evade the immune defense system, they have also evolved sophisticated strategies to escape the host response and survive under harmful conditions (5).

In former studies, we demonstrated that glutamine, branched-chain amino acids, and arginine are not synthesized *de novo* by *S. pneumoniae* (6, 7). Arginine, as an essential amino acid, can be found at a concentration of about 45 μM in human plasma (8), as well as, e.g., in human cerebrospinal fluid (13 μM) (9) and muscle tissue (>1,000 μM) (8). The arginine concentration decreases during the inflammatory response (10, 11), subsequently lowering the activity of T and B cells (12). In human macrophages, arginine is important for the biosynthesis of nitric oxide, which is

toxic for invasive microbes (13). In pneumococci, arginine is also a precursor for the biosynthesis of spermine, a polyamine that acts directly as a free-radical scavenger (14–16).

During evolution, organisms have developed different mechanisms for arginine catabolism. These are the arginase pathway, the arginine transaminase pathway, the arginine decarboxylase pathway, and the arginine deiminase (AD) pathway (17–21).

Various lactic acid bacteria metabolize arginine via the AD system (ADS). The ADS includes three enzymes whose genes are commonly arranged as an operon. Many ADS operons contain a fourth and a fifth gene encoding an arginine-ornithine antiporter and a putative aminopeptidase, respectively. However, the order of the genes varies among different bacteria (22–28). Overall, the ADS yields 1 mol of ornithine and CO_2, 2 mol of ammonia, and 1 mol of ATP by substrate level phosphorylation per mol of arginine metabolized (17, 29). In *Halobacterium salinarum*, the ornithine produced by the ADS is exported in exchange for one molecule of arginine in an energy-independent manner by the membrane-bound antiporter encoded by *arcD* (30).

The ammonia produced by the ADS raises the cytoplasmic pH, thereby protecting the cell from potentially lethal effects of an acidic extracellular environment. This protective effect was described in oral bacteria like *Streptococcus sanguis*, *Streptococcus gordonii*, and *Streptococcus rattus* and partially for the pig pathogen *Streptococcus suis* (22, 24, 25, 31, 32).

Furthermore, 1 mol of ATP generated out of 1 mol of arginine allows the use of arginine as the only energy source as, e.g., in *Pseudomonas aeruginosa*. The AD pathway ArcA-ArcD is probably required for optimal anaerobic or microaerobic growth and viability of *P. aeruginosa* within cystic fibrosis airways (33). It was shown that the AD of *S. suis*, which is also present on the bacterial surface, can be induced by an increase in temperature to 42°C and a low oxygen level (34). ArcD has been characterized as an arginine transporter in several bacteria, including *S. pneumoniae* (35). It was shown that ArcD plays a role as an arginine uptake system and influences the structure of the pneumococcal capsule. This has an effect on the pathogenicity of different pneumococcal serotypes. The molecular basis of the effect of ArcD on the capsule is not clear (35).

Under anaerobic conditions, the expression of the ADS genes in *P. aeruginosa* is activated by the regulator ANR (anaerobic regulation of arginine catabolism and nitrate reduction). In the presence of arginine, the transcription of AD, induced by the ANR, is reinforced by the regulator ArgR (36). The gene encoding the ADS regulator (Crp/Fnr family), designated *argR* or *arcR*, is frequently close to the *arc* gene cluster (37–39). In *Staphylococcus aureus*, ArcR is necessary whenever arginine is the only energy source available (40). The regulation of the ADS in streptococci is highly dependent on growth conditions. Arginine induces the AD activity of *S. gordonii* and *S. sanguis*. Furthermore, AD expression is subject to carbon catabolite repression. The presence of the repressing sugar glucose lowers AD activity (37, 41). Additionally, the regulator ArgR acts as an *arcABC* operon expression activator in *S. suis* (42).

Many Gram-positive organisms, like *Bacillus subtilis* and *Lactococcus lactis*, contain a functional arginine biosynthesis pathway (43). The genome of *S. pneumoniae* D39 exhibits only two *arg* genes, namely, *argG* and *argH*, encoding argininosuccinate synthetase and argininosuccinase, respectively. These enzymes convert citrulline and aspartate to arginine (44). Remarkably, pneumococci can be positive or negative for *argGH* (16). Although pneumococci do not contain a complete set of arginine biosynthetic genes, three putative ArgR-type regulators (AhrC, ArgR1, and ArgR2) have been described (45). ArgR1 and AhrC form a heterohexameric complex that, at high arginine concentrations, represses five operons, including the *argGH* operon and the *artPQ*, *abpA*, *abpB*, and *aliB* genes, suggested to be involved in arginine uptake. In this scenario, arginine acts as an effector molecule, and under arginine limitation, the operons are derepressed (44). In the present study, we show that TIGR4, but not D39, produces the ArgR2 regulator, which activates the ADS operon in TIGR4 lacking ArgGH. In TIGR4 and other pneumococci, ArgR2 is required for optimal arginine uptake via the arginine-ornithine antiporter ArcD, and interestingly, mouse coinfections suggested that ArgR2 regulates additional genes.

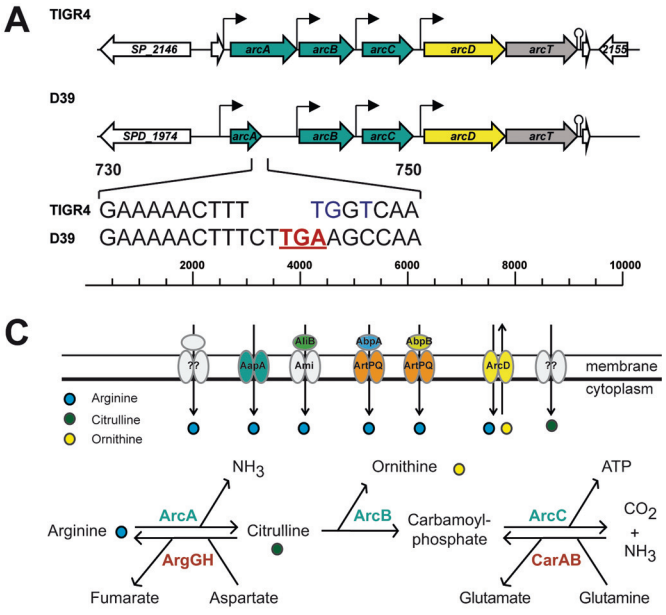
RESULTS

Bioinformatic analysis of the pneumococcal AD loci and regulatory genes in pneumococci. The proteins of the AD pathway in *S. pneumoniae* TIGR4 are encoded by the *arcABCDT* genes, which are located in a cluster. Bioinformatic analysis (Neural Network Promoter Prediction; http://www.fruitfly.org/seq_tools/promoter.html) showed putative promoter sequences upstream of the *arcA* and *arcD* genes, respectively. Further promoter structures were predicted upstream of *arcB* and *arcC*. A putative terminator sequence (TransTerm HP; http://transterm.cbcb.umd.edu/) was predicted downstream of *arcT* (Fig. 1A).

The deduced 409 amino acids (aa) of the AD (ArcA, SP_2148) of *S. pneumoniae* TIGR4 harbor conserved AD motifs, i.e., a long motif, aa 10 to 20 (SEIGKLKKVML), and three shorter conserved motifs, aa 161 to 164 (FTRD), 218 to 221 (EGGD), and 272 to 278 (MHLDTVF). All of these motifs are important for the structure and function of the AD (46). The 338 aa of the ornithine carbamoyltransferase (cOTC, ArcB, SP_2150) of TIGR4 contains the conserved carbamoylphosphate binding and catalysis (STRTR) motif (47). The amino acid sequence of the carbamate kinase (CK, ArcC, SP_2151) contains no conserved motifs (23, 48). The analysis of the arginine-ornithine antiporter (ArcD; 503 aa) shows 12 transmembrane helices (http://www.ch.embnet.org/software/TMPRED_form.html). The final gene of the ADS gene cluster, *arcT* (*SP_2153*), encodes a putative peptidase and has >60% sequence identity with ArcT of *S. sanguinis* (72%), *S. gordonii* (72%), *S. suis* (65%), and *Streptococcus pyogenes* (66%).

Comparative analyses of *arc* gene clusters, the anabolic *argGH* genes, and the genes encoding regulatory proteins ArgR1, AhrC, and ArgR2 were performed on the basis of the SYBIL database (http://strepneumo-sybil.igs.umaryland.edu/cgi-bin/current/shared/index.cgi?site=strepneumo).

Genes of the *arc* cluster (*arcA* to *arcT*) are present in the majority of pneumococcal genomes. In *S. pneumoniae* D39, R800, and R6, a stretch of 8 nucleotides (nt) starting at nt 740 differs from *arcA* sequences encoding a functionally active AD, most likely because of nucleotide insertions (CTTG) and a substitution of 3 nt (TGGT to AAGC) (Fig. 1A). This leads to a premature stop codon in the *arcA* gene, resulting in translational termination and hence in a nonfunctional AD protein. The genes *arcB* to *arcT* of R6 and D39 are identical to other pneumococcal *arcB*-to-*arcT* genes. Two other strains (*S. pneumoniae* BS292 and BS293) have neither the *arc* gene cluster nor the *argGH* operon (Fig. 1B). The regulatory genes (*argR1*, *ahrC*, and *argR2*) are annotated in all of the



B

	arcA	arcBCDT	argG	argH
S.p. TIGR4	+	+	o	-
S.p. D39	o	+	+	+
S.p. AP200	+	+	+	+
S.p. BS293	-	-	-	-
S.p. BS397	-	-	-	-
S.p. 70585	+	+	+	+
S.p. CDC1087-00	+	+	+	+
S.p. Hungary19A6	+	+	+	+
S.p. JJA	+	+	+	+
S.p. MLV-016	+	+	+	+
S.p. R6	o	+	+	+
S.p. SP11-BS70	+	+	+	+
S.p. SP195	+	+	o	-
S.p. SP9-BS68	+	+	o	-
S.p. G54	+	+	o	-
S.p. SP14-BS69	+	+	-	-
S.p. SP18-BS74	+	+	o	-
S. gordonii	+	+	+	+
S. mitis B6	-	-	+	+

FIG 1 Genomic organization of ADS gene clusters in pneumococci and distribution of *arc* genes. (A) Genomic organization of ADS operons in *S. pneumoniae* TIGR4 and D39: *arcA*, AD; *arcB*, catabolic cOCT; *arcC*, CK; *arcD*, arginine-ornithine antiporter; *arcT*, putative aminopeptidase. The AD-encoding gene (arcA) of D39 is truncated by the insertion of a stop codon (TGA). (B) Distribution of arginine metabolism genes in different strains present in the SYBIL database. *argG*, argininosuccinate synthetase; *argH*, argininosuccinase; +, present; ○, truncated; −, absent. (C) Arginine metabolism in *S. pneumoniae*. CarAB, anabolic cOCT.

strains analyzed, with the exception of *S. pneumoniae* SP14-BS69 and SP18-BS74, which lack *argR2*. In addition to the genes described here, pneumococci exhibit the anabolic carbamoyl phosphate synthetase (*carAB*), as well as the catabolic CK (*arcC*) (Fig. 1C). None of the strains sequenced has all of the eight *arg* genes of the arginine biosynthetic pathway (*argBCDEFGHJ*). Interestingly, the distribution of the anabolic genes (*argGH*) varies among pneumococci. These genes were present in only 8 of the 33 pneumococcal strains listed in the SYBIL database, among them, *S. pneumoniae* D39 and R6 but not TIGR4 (Fig. 1B).

The proteins encoded by *arc* genes form a regulatory unit. Reverse transcription (RT)-PCR analysis was performed to investigate whether the *arcABCDT* genes are cotranscribed. RNA isolated from *S. pneumoniae* TIGR4 grown in a chemically defined medium (CDM) was used to generate cDNA and PCR products representing gene-spanning sequences and intergenic regions between selected *arc* genes were amplified with specific primer pairs. The results suggest that the genes *arcABCDT* form a polycistronic operon in *S. pneumoniae* TIGR4 (Fig. 2A). Considering the putative promoter and terminator structures, a complete *arcABCDT* transcript would have a calculated length of about 6.5 kb. However, because of the predicted additional promoter and terminator structures, transcripts of 2.3 kb (*arcAB*), 3.4 kb (*arcABC*), and 3.0 kb (*arcDT*) are also possible. Northern blot analysis was conducted with digoxigenin (DIG)-labeled RNA probes homologous to the *arcA*, *arcB*, *arcC*, and *arcDT* mRNAs, respectively, to investigate the transcript lengths. All four probes detected specific transcripts of 2.6 and 1.6 kb in the wild-type TIGR4 strain, which are not consistent with the theoretical calculations (Fig. 2B). The Δ*arcA-C*, Δ*arcA-T*, and Δ*argR2* mutants lack *arcA-C*-specific transcripts. Similarly, the expression of *arcDT* was abolished in the Δ*arcA-T* and Δ*argR2* mutants (Fig. 2B), suggesting *arcABC* and *arcDT* regulation by ArgR2. The knockout of *argR1* or *ahrC* also influenced *arcABCDT* gene expression (Fig. 2B), which suggests that ArgR1 and AhrC are involved in the complex regulation of the ADS.

ArgR2 activates the ADS and arginine uptake in TIGR4. Three genes encoding ArgR-type regulatory proteins (*ArgR1*, *ArgR2*, and *AhrC*) are annotated in the TIGR4 genome (Fig. 3A), and if present, these genes are listed in pneumococcal genomes deposited in the SYBIL database (44). The amino acid sequence of the nutritional regulators is highly conserved (>98%) in streptococci and also shows high homology to *L. lactis* ArgR-type regulators. The *argR2* genes of *S. pneumoniae* TIGR4 and D39 show a GA transition at bp 94 (TIGR4 guanine; D39 adenine), resulting in an amino acid substitution at position 31 (E31K) of ArgR2 (TIGR4 glutamate, D39 lysine). ArgR1 and AhrC form a heterohexameric complex with arginine as an effector molecule to ensure optimal uptake of arginine from the surrounding milieu (44). However, the regulator of the ADS and its impact on arginine uptake by the ArcD antiporter were unknown.

Immunoblot analyses were performed to assess the role of the ArgR-type regulators in the expression of proteins encoded by the AD operon. The expression of ArcA, ArcB, ArcC, and the three regulatory proteins AhrC, ArgR1, and Arg2 in pneumococcal wild-type strains TIGR4 and D39, respectively, was compared to that in the isogenic mutants by using specific mouse polyclonal antibodies generated against ArcA, ArcB, ArcC, or the regulatory proteins (Fig. 3C). The TIGR4 wild type expressed all three enzymes of the ADS, and all of the regulator proteins were detected. The mutants deficient for ArcA to ArcC and ArcA to ArcT showed no expression of the enzymes. In contrast to the *argR1* and *ahrC* mutants, the expression of the catabolic enzymes (ArcA, ArcB, and ArcC) was diminished in the *argR2* mutant, supporting the

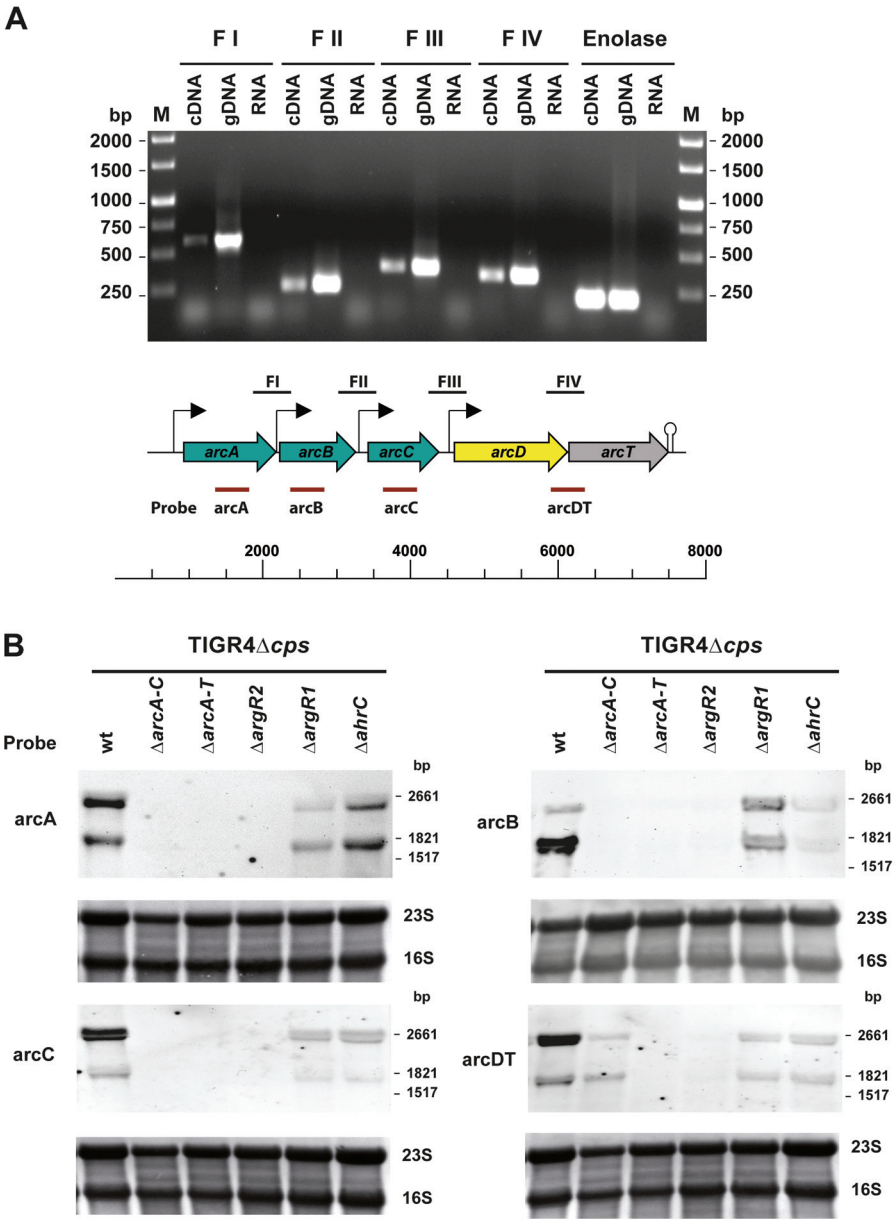


FIG 2 Analysis of the *arc* gene complex expression. (A) RT-PCR analysis of *arc* gene cluster regions. Total mRNA was isolated from *S. pneumoniae* grown in THY medium, and random primers were used to amplify cDNA. Genomic DNA (gDNA) and RNA served as positive and negative controls, respectively. The following PCR fragments representing *arc* gene and intergenic sequences were amplified after RT: FI, *arcAB* intergenic region; FII, *arcBC* intergenic region; FIII, *arcCD* intergenic region; FIV, *arcDT* intergenic region. The enolase gene was used as a positive control. Lanes M contained molecular size markers. (B) Northern blot analysis of *arcABCDT* transcripts in wild-type (wt) *S. pneumoniae* D39 and TIGR4 and in the isogenic TIGR4 Δ*arcA-C*, Δ*arcA-T* Δ*argR2*, Δ*argR1*, and Δ*ahrC* deletion mutants, respectively. Total RNA isolated from cultures grown in CDM to an OD_{600} of 0.4 was hybridized with DIG-labeled RNA probes for *arcA* (P704, P611), *arcB* (P933, P934), *arcC* (P935, P936), and *arcDT* (P705, P613). Methylene blue was used to verify the amount of RNA on hybridization membranes.

hypothesis that ArgR2 is a specific activator of the ADS and arginine uptake by ArcD.

It is noteworthy that the results of protein expression by *S. pneumoniae* D39 and isogenic mutants differed significantly from those of protein expression by TIGR4. Immunoblot analyses showed a truncated ArcA peptide due to the premature stop codon. The full-length ArcB and ArcC proteins were detected in the D39 wild type and, remarkably, also in the isogenic *argR2* mutant. Similar to TIGR4, the deficiency of ArgR1 or AhrC had no effect on the expression of the ADS enzymes. Importantly, no protein signal could be detected for the regulator ArgR2 in D39, although the upstream sequence of *argR2* is identical to the TIGR4 sequence and the only amino acid exchange (E31K) occurred at position 31. This is of particular interest because the *arc* genes are expressed in D39 although it lacks ArgR2, while *arc* gene expression in TIGR4 requires the ArgR2 regulator.

To elucidate whether the absence of ArgR2 in D39 is due to a lack of gene expression, RT-PCR analysis was performed. As depicted in Fig. 3B, a PCR fragment with intergenic *argR2* primers was amplified for *S. pneumoniae* TIGR4, while D39 showed no

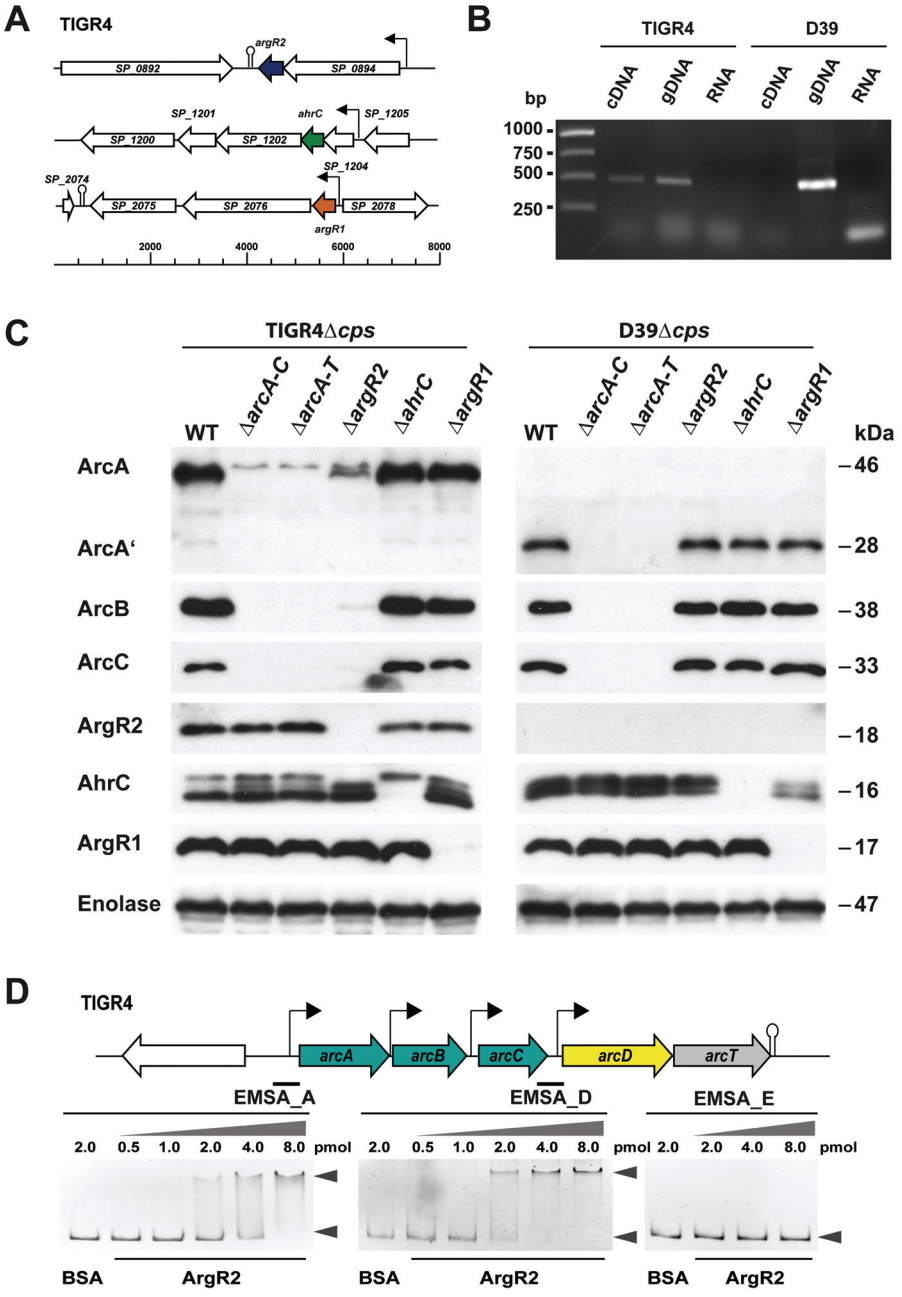


FIG 3 The AD gene cluster of *S. pneumoniae* is regulated by ArgR2. (A) Genetic regions of the three ArgR-type transcriptional regulators encoded in the TIGR4 genome. Regions: *SP_0892*, type I restriction enzyme; *SP_0893*, transcriptional regulator of arginine metabolism ArgR2; *SP_0894*, X-prolyl-dipeptidyl aminopeptidase gene *pepX*; *SP_1200*, GTP-binding protein LepA; *SP_1201*, serine/threonine protein phosphatase; *SP_1202*, DNA repair protein RecN; *SP_1203*, transcriptional regulator of arginine metabolism AhrC; *SP_1204*, 23S rRNA (cytidine1920-2′-O)/16S rRNA (cytidine1409-2′-O)-methyltransferase; *SP_1205*, geranyltranstransferase; *SP_2075*, ABC transporter ATP-binding protein/permease; *SP_2076*, pseudogene; *SP_2077*, transcriptional regulator of arginine metabolism ArgR1; *SP_2078*, arginyl-tRNA synthetase gene *argRS*. (B) RT-PCR analysis of *argR2* expression in TIGR4 and D39. Random primers were used to amplify cDNA. Genomic DNA was used as a positive control, and RNA was used as a negative control. (C) Immunoblot analysis of ArcA, ArcB, ArcC, ArgR2, ArgR1, and AhrC synthesis in nonencapsulated pneumococcal strains (TIGR4Δ*cps* and D39Δ*cps*) and isogenic regulatory mutants with bacterial whole-cell cytoplasmic protein lysates. Proteins were detected with specific mouse anti-ArcA, ArcB, ArcC, ArgR2, ArgR1, or AhrC antiserum. Pneumococcal enolase was used as a loading control. The predicted molecular masses are as follows: mature ArcA in TIGR4, 46.6 kDa; fragmented ArcA′ in D39, 28.3 kDa; ArcB, 37.9 kDa; ArcC, 33.6 kDa; ArgR2, 17.8 kDa; ArgR1, 16.2 kDa; AhrC, 17.1 kDa. WT, wild type. (D) Interaction of ArgR2 with putative promoter regions of the *arc* operon. Purified recombinant His-ArgR2 (0.5 to 8 pmol of protein) was incubated with the 437-bp *arcA* promoter fragment (EMSA_A), the 391-bp *arcD* promoter fragment (EMSA_D), or the 307-bp enolase promoter fragment (EMSA_E, negative control; DNA, 0.2 pmol).

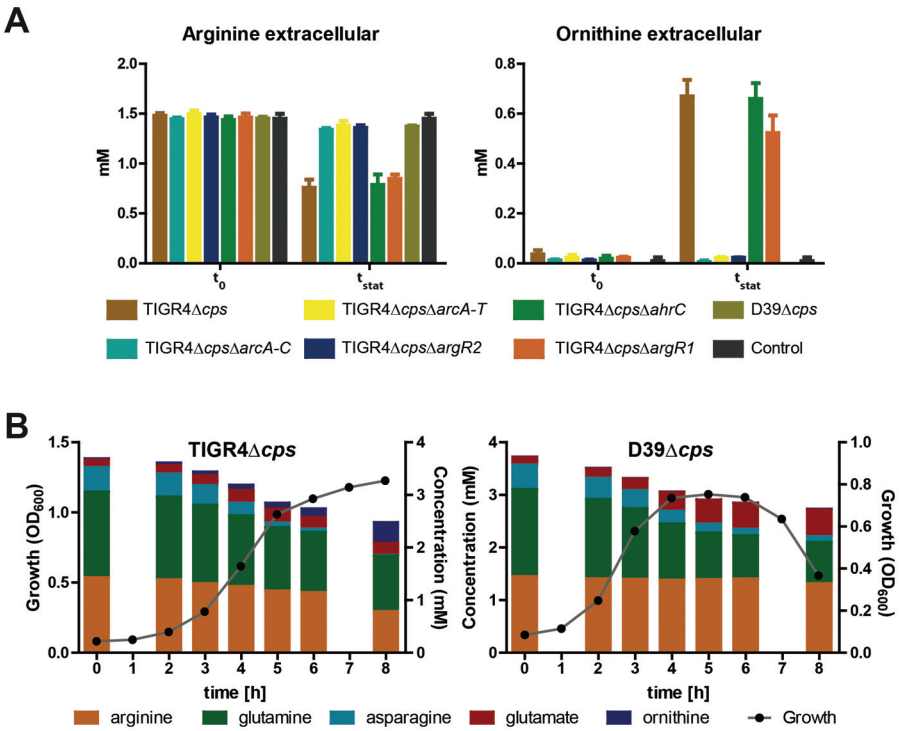


FIG 4 Impact of the pneumococcal ADS regulator ArgR2 on consumption of nitrogen-rich arginine. (A) Uptake of arginine and export of ornithine by TIGR4Δ*cps* and its isogenic mutants in CDM. The extracellular concentrations (millimolar) of arginine and ornithine were measured prior to the start of the cultivation (t_0) and at the late exponential phase (OD_{600}, 0.8; t_1). Medium without bacterial culture was used as a control. (B) Growth curves, nitrogen-rich amino acid consumption, and end products of nitrogen metabolism formed by the TIGR4Δ*cps* and D39Δ*cps* strains cultured in CDM under microaerophilic conditions at 37°C. Culture supernatant samples for substrate and end product analysis by ^{1}H-NMR were harvested at the time points indicated, and the results are represented by bars in the plots. Data from four independent experiments are shown.

PCR product, suggesting that the *argR2* gene of D39 is not transcribed.

ArgR2 binds to promoter regions of the ADS. To investigate whether ArgR2 controls the expression of the *arc* operon by binding to the predicted promoter regions of *arcA* and *arcD* (Fig. 1A), electrophoretic mobility shift assays (EMSAs) were performed with purified recombinant His_6-ArgR2 protein. DNA-binding reactions were carried out with a 437-bp *arcA* or a 391-bp *arcD* fragment. As shown in Fig. 3D, both DNA fragments were retarded by the addition of His_6-ArgR2. The amount of the DNA-protein complex increased in an ArgR2 concentration-dependent manner. These results confirm that ArgR2 acts as specific activator of the genes of the ADS.

Strain-specific expression and regulation of ADS. Arginine is essential for pneumococcal growth (6). ^{1}H nuclear magnetic resonance (NMR) analysis of the growth medium indicated that the wild-type TIGR4 strain takes up arginine and releases ornithine (Fig. 4A). The analysis of the extracellular metabolites showed that the uptake of arginine lacking functional ArcD was significantly reduced in TIGR4. Moreover, the Δ*arcA-C*, Δ*arcA-T*, and Δ*argR2* TIGR4 mutants showed no secretion of ornithine into the medium. Importantly, uptake of arginine by D39 was 20-fold lower than that by TIGR4 and correlated with the lower expression of the AD enzymes. Consequently, ornithine secretion was not detected in D39 (Fig. 4A). Pneumococci catabolize glutamine and asparagine as main nitrogen sources, while dispensable glutamate is secreted. Strains expressing a functional ADS, such as TIGR4, are additionally able to utilize arginine as a nitrogen source. This is accompanied by less glutamine uptake by TIGR4 than by D39 expressing a nonfunctional ADS (Fig. 4B).

The expression of cytoplasmic ArcA and ArgR2 in 24 different pneumococcal strains and serotypes was assessed by immunoblot analysis (Fig. 5A). The results indicated the presence of ArcA in all of the strains analyzed. However, serotypes 35A (SP37) and 2 (SP51), including D39 and strains R6 and R800, produced only a truncated ArcA protein. Interestingly, the nonfunctional ArcA protein correlated with low levels or a lack of ArgR2 expression (Fig. 5B). However, ArgR2 expression was also absent from some strains expressing a full-length and functional ArcA protein (for example, SP55) (Fig. 5A and B).

Enzymatic activity of the AD. The activity of the AD was monitored in a colorimetric enzyme assay measuring the amount of produced citrulline (Fig. 5B). The results showed no enzyme activity for D39, TIGR4Δ*arcAT*, TIGR4Δ*argR2*, and serotype 35A (SP37). In contrast, AD enzyme activity was observed for TIGR4, TIGR4Δ*argR1*, TIGR4Δ*ahrC*, SP55, SP56, and *S. gordonii*. Taken together, the immunoblot and Northern blot analyses, the enzyme activity assay, and EMSA indicate that ArgR2 is a specific positive transcriptional regulator of the pneumococcal *arcABCDT* operon for some *S. pneumoniae* strains.

Influence of the ADS, ArcD, and ArgR2 pneumococcal colonization and virulence. To assess the impact of the ADS and its regulator ArgR2 on TIGR4 colonization and virulence, the mouse acute-pneumonia model was used. Female CD-1 outbred

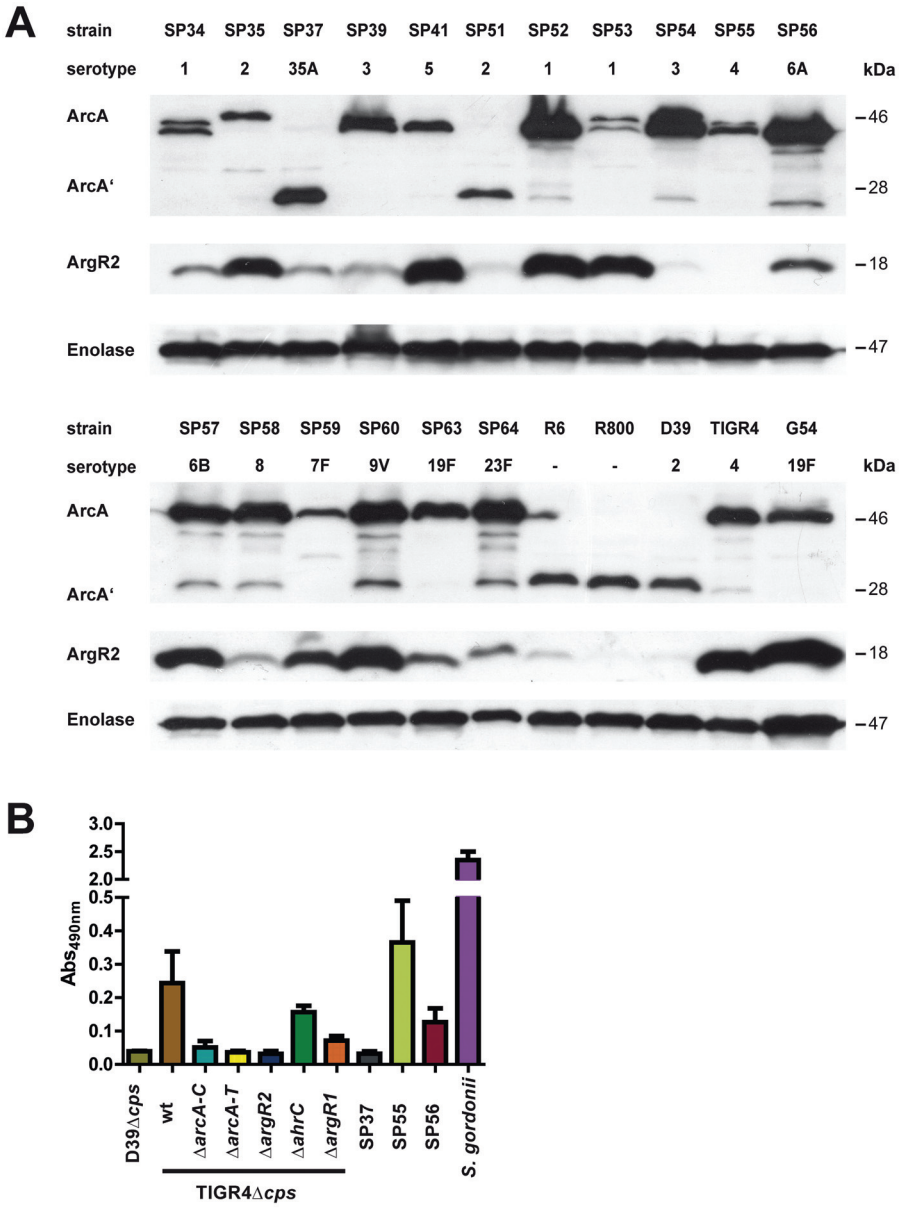


FIG 5 Strain-specific production of ArgR2 and AD activity. (A) Immunoblot analysis of ArcA and ArgR2 expression in pneumococci. Proteins were detected with specific anti-ArcA or ArgR2 antibodies. (B) AD activity was monitored in a colorimetric enzyme assay by determination of the citrulline produced. The strains were cultivated in THY medium to an OD_{600} of 1.0, and 20 μg of whole protein extract was used. wt, wild type.

mice ($n = 12$) were intranasally infected with 7.5×10^7 wild-type or isogenic TIGR4luxΔ*arcA-C*, TIGR4luxΔ*arcA-T*, and TIGR4luxΔ*argR2* pneumococci. The infection process was monitored in real time with an optical bioluminescence imaging system (IVIS Spectrum System). No significant differences were observed between the survival time of mice infected with wild-type or mutant pneumococci deficient for the proteins ArcA to ArcC or regulator ArgR2, while the deficiency of ArcD in the Δ*arcA-T* mutant attenuated pneumococci (Fig. 6A–C). To investigate the effects in more detail, coinfection experiments were conducted to decipher directly deficiencies in the *in vivo* bacterial fitness of the mutants and their abilities to colonize mice or to cause invasive infections. Mice ($n = 9$) were coinfected intranasally with equal amounts (each strain received 2.5×10^7 CFU) of the mutant strain TIGR4luxΔ*arcA-C*, TIGR4luxΔ*arcA-T*, or TIGR4luxΔ*argR2* and parental strain TIGR4lux (Fig. 6D). The ratio of wild-type to mutant bacteria was calculated 24 and 48 h postinfection by determination of the bacterial loads in the nasopharynx and airways of each individual mouse. The results of the nasopharyngeal and bronchoalveolar lavage fluid analysis showed that the Δ*arcA-C* and Δ*arcA-T* TIGR4 mutants, respectively, were outcompeted by wild-type bacteria in these host niches. In contrast, the *in vivo* survival rate of the TIGR4Δ*argR2* mutant was obviously higher in both habitats than that of the isogenic wild-type strain. Importantly, the TIGR4 and D39 mutants deficient in genes of the ADS or the regulator ArgR2 had no growth defect in complex medium

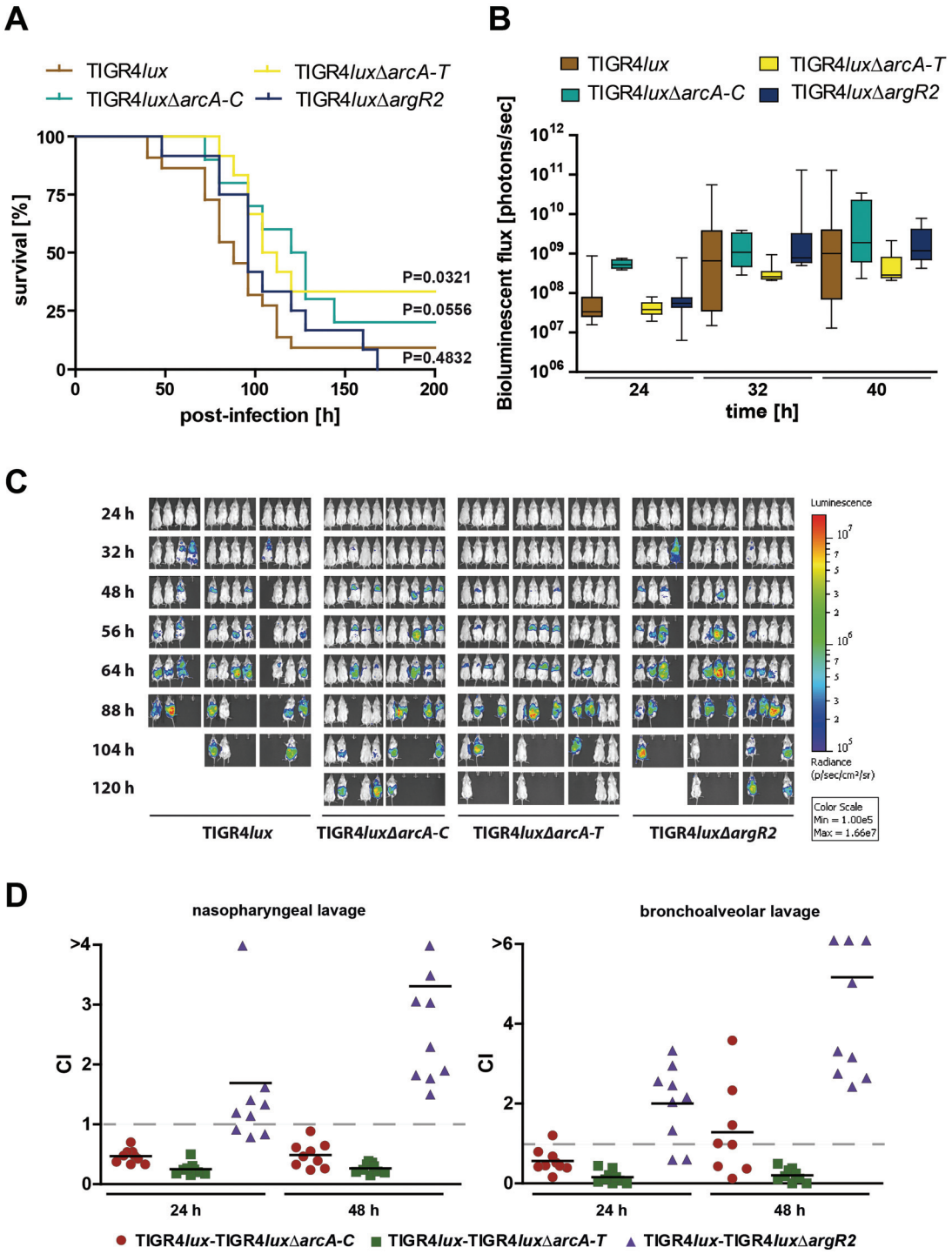


FIG 6 Impact of the ADS enzymes, ArcD antiporter, and regulator ArgR2 on pneumococcal colonization and virulence in mice. (A) Survival of CD-1 mice after intranasal infection with pneumococci. Groups of mice ($n = 12$) were intranasally infected with 7.5×10^7 CFU of wild type *S. pneumoniae* TIGR4 or the isogenic Δ*arcA-C*, Δ*arcA-T*, or Δ*argR2* mutant. (B and C) Bioluminescent optical imaging of pneumococcal dissemination after intranasal infection of CD-1 mice ($n = 12$). Dissemination of bioluminescent TIGR4*lux*, TIGR4*lux*Δ*arcA-C*, TIGR4*lux*Δ*arcA-T*, or TIGR4*lux*Δ*argR2* was analyzed at the time points indicated by determination of the luminescence intensity measured with the IVIS Spectrum System. The bioluminescent flux of grouped mice is represented in the box whisker graph (B). (D) Intranasal coinfection of mice with bioluminescent TIGR4*lux* together with TIGR4*lux*Δ*arcA-C*, TIGR4*lux*Δ*arcA-T*, or TIGR4*lux*Δ*argR2*. Groups of CD-1 mice ($n = 9$) were infected with 2.5×10^7 CFU of the wild type and one of the isogenic pneumococcal mutants. At the time points indicated, mice were sacrificed and bacterial loads in the nasopharynx (nasopharyngeal lavage) and airways (bronchoalveolar lavage) were counted after the bacteria were plated on blood agar plates. CI values lower than 1 indicate higher growth of wild-type bacteria.

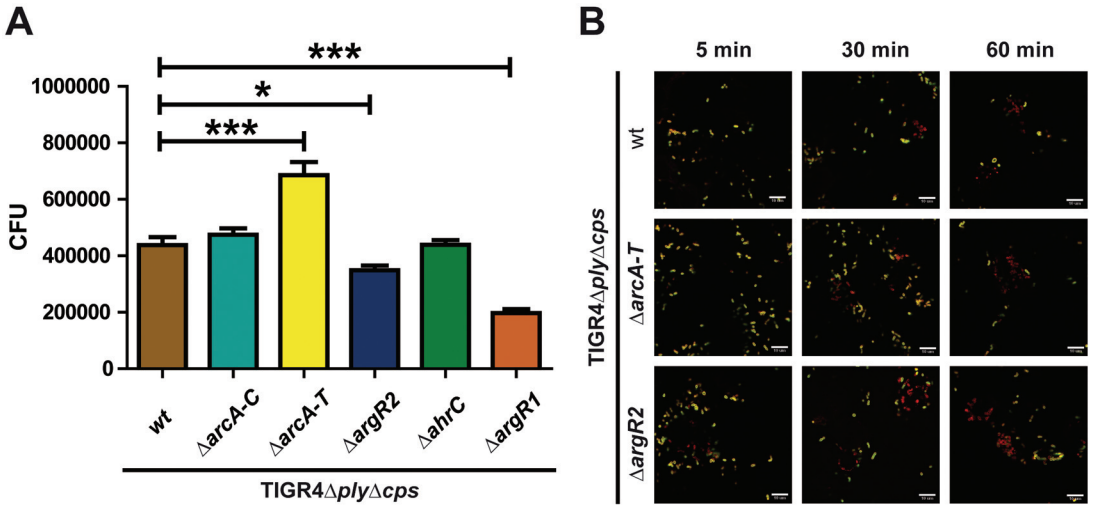


FIG 7 Deficiency of arginine uptake and arginine metabolism regulator ArgR2 interferes with pneumococcal phagocytosis. J774A.1 murine macrophages were infected with a multiplicity of infection of 50 pneumococci of wild-type (wt) strain TIGR4 or its isogenic *arcA-C*, *arcA-T*, or *argR2* mutant. (A) Viable and recovered intracellular pneumococci were determined by colony counting 30 min postinfection by applying the antibiotic protection assay. Shown are the mean values and standard deviations of three independent experiments performed in triplicate. *, $P < 0.05$; ***, $P < 0.001$. (B) Immunofluorescence microscopy of host cell-attached and intracellular pneumococci. J774A.1 murine macrophages were infected with pneumococcal strain TIGR4 or the Δ*arcA-T* or Δ*argR2* mutant for 5, 30, or 60 min. Intracellular pneumococci were stained with Alexa 568 (red), while adherent bacteria appear yellow (Alexa 488 and Alexa 568). Bars represent 10 μm.

or in CDM (data not show). Hence, the *in vivo* experiments showed that the fitness of the TIGR4 ADS-deficient mutants was reduced under infection conditions and that arginine metabolism is coupled to virulence. Remarkably, the ArgR2-deficient mutant outcompeted the isogenic TIGR4 wild type, suggesting other, as-yet-unknown, regulatory effects of ArgR2.

Influence of the ADS and regulator ArgR2 on pneumococcal phagocytosis by macrophages. To investigate whether the ADS protects pneumococci against phagocytosis by macrophages or improves intracellular survival, nonencapsulated TIGR4Δ*cps* pneumococci and the Δ*arcA-C*, Δ*arcA-T*, Δ*argR2*, Δ*argR1*, and Δ*ahrC* isogenic mutants were incubated for 30 min with J774 macrophages. After the extracellular bacteria were killed, the numbers of internalized and recovered pneumococci were determined. The phagocytosis rate of TIGR4Δ*cps* and its isogenic Δ*arcA-C* mutant was not significantly altered. However, the deletion of all of the genes of the AD operon (Δ*arcA-T* mutant) increased the internalization of pneumococci in macrophages significantly. In contrast, mutations of the regulatory proteins ArgR2 (Δ*argR2*) and AhrC (Δ*ahrC*) decreased the rate of phagocytosis significantly (Fig. 7). The latter results may explain why the ArgR2-deficient mutant outcompeted the wild type under *in vivo* conditions.

DISCUSSION

Arginine is one of the amino acids that cannot be synthesized *de novo* and have to be taken up by pneumococci (6, 44), since the complete biosynthetic pathway is missing from all of the pneumococcal genomes that have been sequenced. The only genes found in some pneumococcal genomes, like that of D39, encode the argininosuccinate synthetase (ArgG) and the argininosuccinase (ArgH) (16, 44). These enzymes catalyze the conversion of citrulline to arginine. Mouse models of meningitis and pneumonia showed that ArgG/ArgH-deficient pneumococci are attenuated in outgrowth in the lungs, blood, and cerebrospinal fluid (16). The catabolic ADS has been described in other streptococcal species, such as *S. suis*, where it covers a broad spectrum of functions. It consists of the AD, the cOCT, and the CK and catalyzes the conversion of arginine to ornithine under the release of ammonia and carbon dioxide while simultaneously generating ATP. In contrast to other prokaryotic organisms, including *P. aeruginosa* (33) and *S. aureus* (40), it seems unlikely that the main function of the ADS in group A streptococci, including *S. pneumoniae*, is to provide energy and metabolic intermediates. For several streptococci, such as oral bacteria and *S. pyogenes*, the release of ammonia plays an important role in adaptation to an acidic environment (22, 24, 31, 32, 49). Importantly, the enzymes of the ADS are still active at a very low environmental pH (50). However, pneumococci cultured under acidic conditions (pH 5.5) did not survive better than isogenic *arcA-C* mutants, which are unable to produce ammonia as the end product of the catabolism of arginine by the ADS (data not shown). Hence, the levels of ammonia produced are most likely not sufficient to alter the environmental pH or acid conditions in phagocytic compartments.

The regulation of arginine metabolism is generally performed by ArgR-type regulators, of which the pneumococcal genome encodes three. In *S. pneumoniae* D39, ArgR1 and AhrC form a heterohexameric complex that is bound to the effector molecule arginine. Under arginine-rich conditions, this complex directly represses five amino acid transport operons in a cooperative way, and it has been suggested that AbpA, AbpB, and ArtPQ, which are located in three of the five operons, form an arginine uptake unit. In addition, AliB, which is required for growth in medium with peptides (in particular, Arg-Pro-Pro and Arg-Pro-Pro-Gly-Phe) as the sole source of arginine is also controlled by ArgR1/AhrC (44). Importantly, ArgR1 and AhrC have no impact on the catabolic *arcABCDT* operon of the ADS of pneumococcal strain D39

(44) and differences in arginine uptake have not been measured. In *L. lactis*, the ArgR/AhrC complex represses in the presence of arginine the three arginine biosynthetic operons *argCJDBF*, *gltS-argE*, and *argGH* while activating the catabolic *arcABD1C1C2TD2-yvaD* operon (51–53). Also in other organisms like *Escherichia coli* (54), *P. aeruginosa* (55, 56), *B. subtilis* (57–59), *L. plantarum* (60), and *Enterococcus faecalis* (38), ArgR-type regulators are mediators of arginine metabolism and thus influence the expression of arginine biosynthetic and catabolic genes.

Here we show for the first time that, in *S. pneumoniae* TIGR4 and probably other pneumococcal strains, ArgR2 positively regulates the expression of the ADS, including the arginine-ornithine antiporter ArcD, which is shown to be essential for arginine uptake from the extracellular milieu. In addition, ArcD is essential for the full virulence of TIGR4 while the enzymes of the ADS have only a minor impact on pneumococcal fitness under *in vivo* conditions. Strikingly, a deficiency of ArgR2 has a detrimental effect despite downregulation of the ADS, suggesting that ArgR2 regulates other genes important for bacterial fitness or virulence and compensates for the ADS defect.

Immunoblot and Northern blot analyses demonstrated that ArgR2 is an activator of ArcABCDT expression in *S. pneumoniae* TIGR4 (Fig. 2B and 3C). The ArgR2-deficient TIGR4Δ*argR2* mutant showed a significant decrease in *arc* gene and protein expression. *In silico* analysis and RT-PCR suggested that the *arcABCDT* genes form an operon. However, the transcript lengths of 2.6 and 1.6 kb observed by Northern blot analysis may point to a degradation by RNase(s) or, alternatively, that the genes of the *arc* operon are not transcribed as a single transcriptional unit. The phenomenon of the inconsistent size of mRNA transcripts has previously also been observed in *S. suis* (42; Markus Fulde personal communication). To draw reliable conclusions about transcript size, further studies are required. The results of the EMSA with fragments of the *arcABC* promoter and recombinant ArgR2 reveal that ArgR2 interacts with the *arcABC* promoter under *in vitro* conditions (Fig. 3D). The ArgR-type transcriptional regulators influence gene expression by binding to so-called ARG operator sites that precede target genes. ARG operator sites consist of pairs of 18-bp palindromic sequences (called ARG boxes). For *E. coli*, a consensus sequence was characterized (5′-TNTGNATW WWWATNCANA-3′ [conserved residues are underlined, N is any nucleotide, and W is A or T]) (54). Similar sequences have been described for other microorganisms (61–63). Even though the typical ARG box could not be determined for the promoter regions of *arcA* and *arcD*, these genes are regulated by ArgR2.

Similar to pneumococci, *S. suis* also contains three ArgR-type regulators. The regulator ArgR of *S. suis*, exhibiting high sequence homology to pneumococcal ArgR2, was found by microarray and quantitative RT-PCR analyses to regulate the *S. suis arc* operon (42). In streptococcal species such as *S. rattus* (24) and *S. gordonii* (37, 64), the expression of the *arc* genes is also ArgR2 dependent, suggesting that ArgR2 or its orthologues are specific regulators of ADS operons.

Immunoblot analyses of selected pneumococcal strains showed heterogeneity of expression for ArcA and ArgR2. In TIGR4, the *arc* operon is regulated by ArgR2 while in other strains the levels of ArgR2 and ArcA do not correlate, suggesting strain-specific regulation of the ADS in pneumococci. It is also still unclear whether ArgR2 regulates or whether ArgR2 itself is regulated by unknown stimuli. By comparing pneumococcal strain TIGR4 with D39, R6, SP37, SP51, and R800, respectively, several differences in the ADS and the ArgR-type regulators have been identified in this study. Although the regulator ArgR2 is the most important activator of the ADS in TIGR4, its expression could not be detected or was extremely low in D39, R6, SP37, SP51, and R800 (Fig. 3C and 5A). Despite lacking or producing low levels of the regulatory protein ArgR2, D39 and the other strains showed constitutive expression of the enzymes of the ADS (AD, OCT, CK) (Fig. 3C). In this respect, it is important to mention that the gene encoding the AD has a premature stop codon in these strains and that these strains consequently lack a functional AD (Fig. 5B). Previous transcriptome analysis demonstrated that the D39 *arc* operon is repressed by CcpA, which can be derepressed under carbon limitation, probably allowing the use of arginine as an alternative energy source (44, 65). However, the growth of wild-type TIGR4 and that of the isogenic Δ*arcA-C* mutant cultured in CDM with a low glucose concentration and in the presence of increasing arginine concentrations did not differ significantly (data not shown).

The deficiency of enzymes of the ADS and also the deficiency of ArgR2 did not impair the full virulence of TIGR4 in the mouse acute-pneumonia model (Fig. 6A), whereas the lack of a functional ArcD antiporter attenuated TIGR4. However, coinfection experiments indicated that the absence of ADS enzymes reduced the fitness of TIGR4 because the wild type outcompeted TIGR4Δ*arcA-C* in the nasopharynx and lungs. Similar to the acute-pneumonia model, TIGR4Δ*arcA-T* is significantly attenuated, highlighting the importance of the antiporter ArcD for the proper fitness and virulence of TIGR4 and probably also other pneumococcal strains. According to the metabolome analysis performed in this study and a recent study, the antiporter ArcD is the major pneumococcal arginine uptake system and is essential in strains lacking ArgGH, like TIGR4 (35). Additionally, it has to be mentioned here that the antiporter ArcD requires ornithine for its functional activity and that the ADS is the only source of ornithine in pneumococci. The lack of the ArcD transporter affected capsule expression in *S. pneumoniae* D39, which results in significantly enhanced phagocytosis and attenuation of D39 under *in vivo* conditions (35). However, when measuring the relative amount of capsular polysaccharide (CPS) by flow cytometry with anti-serotype 2- or anti-serotype 4-specific antiserum (66), the mutant D39Δ*arcA-T* generated in this study and lacking functional ArcD showed only a slightly smaller amount of CPS than the isogenic wild-type D39 (geometric mean fluorescence intensity [GMFI] values: D39, 389; D39Δ*arcA-T*, 269; D39Δ*arcA-C*, 327; D39Δ*argR2*, 408; D39Δ*cps*, 76). Importantly, the mutant TIGR4Δ*arcA-T* produces amounts of CPS similar to those of isogenic wild-type TIGR4 (GMFI values: TIGR4, 342; TIGR4Δ*arcA-T*, 356; TIGR4Δ*arcA-C*, 346; TIGR4Δ*argR2*, 285; TIGR4Δ*cps*, 141). Hence, the effect seems to be strain dependent and if there is any reduction of CPS, it is only to a minor degree compared to the nonencapsulated phenotypes.

The analysis of extracellular metabolites of pneumococci cultured in CDM showed that the arginine uptake of TIGR4 is 20-fold higher than that of D39, suggesting that uptake of arginine is highly reduced in pneumococci lacking a functional arginine degradation pathway, like D39. Because there is no link between arginine metabolism and alterations of cell wall composition, the results of this study and that of Gupta et al. (35) may imply that

ArcD has another, not-yet-identified, function that affects the linkage of the CPS to the pneumococcal cell wall. As a secondary effect, the virulence of D39 pneumococci is impaired. The function of ArcT as an Xaa-His dipeptidase is based on *in silico* analysis and has not been experimentally proven. However, the *arcT* gene is also present in the genomes of other streptococcal species and it has been shown to be essential in a chinchilla otitis media model (35, 67).

Strikingly, ArgR2-deficient TIGR4Δ*argR2* has a higher virulence potential than the wild type, which is indicated by its ability to outcompete the wild type in the nasopharynx and lungs (Fig. 6D). This suggests that ArgR2 regulates other genes encoding proteins important for bacterial fitness and virulence, respectively, and maybe compensates for the defect of the ADS and ArcD. Phagocytosis assays (Fig. 7) indicate a higher uptake of the mutant TIGR4Δ*arcA-T* than the isogenic wild type. In contrast, the uptake of the *argR2* mutant was lower than that of TIRG4. However, accelerated or decelerated killing of intracellular pneumococci was not detected (data not shown), suggesting that the impaired colonization of the mouse lower airways by ADS-deficient pneumococci is due to their higher rates of uptake by professional phagocytes. In contrast, the *argR2* mutant may overgrow the wild type because of its reduced phagocytosis. To explore a potential effect of ArgR2 deficiency on the transcriptome of TIGR4 and to identify further genes regulated by ArgR2, microarray analysis was performed. The results confirmed the downregulation of *arcABCDT* gene expression (mutant/wild-type ratios of 0.19, 0.17, 046, 0.42, and 0.38, respectively) and the absence of an *argR2* transcript (ratio of 0.01) in the mutant TIGR4Δ*argR2*. Other downregulated genes were not identified. Importantly, only a few genes were upregulated in the *argR2* mutant, such as the genes encoding the AdhE (alcohol dehydrogenase) or SP_1282 (ABC transporter ATP-binding protein) and the *kdgA*, *kdgG*, and *gno* genes encoding the enzymes of 2-keto-3-deoxygluconate metabolism. These differentially regulated genes probably do not enhance pneumococcal virulence. Hence, further studies are needed to decipher other potential targets of ArgR2.

In conclusion, this study identified ArgR2 as a key regulator of the ADS of *S. pneumoniae* TIGR4, and it can be assumed that this also applies to other pneumococcal strains. The regulation of the pneumococcal ADS seems to be strain dependent, as indicated by the differences between TIGR4 and D39. Importantly, ArcD, encoded by the *arc* operon, has been identified as a major arginine transporter that is essential for full virulence. Finally, the increased fitness of the *argR2* mutant under *in vivo* conditions pointed to additional regulatory functions of ArgR2. The additional genes targeted by ArgR2 have to be identified in further studies to unravel the other regulatory functions of ArgR2 and understand the higher virulence potential of TIGR4Δ*argR2*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *S. pneumoniae* TIGR4 (serotype 4) and *S. pneumoniae* D39 (serotype 2; NCTC7466) and their isogenic mutants (see Table S1 in the supplemental material) were cultured to mid-log phase (OD_{600}, 0.35 to 0.4) in THY medium (Todd-Hewitt broth [Oxoid, Basingstoke, England] supplemented with 0.5% yeast extract [Roth, Karlsruhe, Germany]) or CDM or grown on Columbia blood agar plates (Oxoid) at 37°C in 5% CO_2. In this study, we established RPMI 1640 (PAA Laboratories) supplemented with 30.5 mM glucose, 0.65 mM uracil, 0.27 mM adenine, 1.1 mM glycine, 0.24 mM choline chloride, 1.7 mM $NaH_2PO_4 \cdot H_2O$, 3.8 mM Na_2HPO_4, and 27 mM $NaHCO_3$ as CDM. The *E. coli* strains used in cloning procedures (see Table S1) were cultured on Luria-Bertani (LB) agar or in LB broth. Transformation of *E. coli* and pneumococci was conducted by standard protocols described recently (68). Pneumococcal mutants were cultured in the presence of the appropriate antibiotics, i.e., chloramphenicol (4 μg/ml), erythromycin (5 μg/ml), and/or kanamycin (150 μg/ml).

DNA techniques and sequence analysis. For the primers and plasmids used in this study, see Tables S2 and S3 in the supplemental material. Bacterial chromosomal DNA isolation and purification were performed by a standard phenol-chloroform extraction method as described previously (69). PCR amplifications were carried out in 50-μl volumes, and reactions were subjected to denaturation at 95°C, 30 cycles, primer annealing for 0.5 min, and elongation at 72°C. The annealing temperatures depended on the primers used, and the extension time depended on the length of the expected PCR product. PCR products were purified with the Wizard SV Gel and PCR Cleanup System (Promega), and plasmids were extracted according to the Wizard Plus SV Minipreps DNA Purification System (Promega) protocol. Oligonucleotides were synthesized by Eurofins MWG Operon (Germany).

Construction of plasmids for mutagenesis and pneumococcal transformation. Pneumococcal mutants deficient in genes of the ADS operon or regulatory genes *argR1*, *argR2*, and *ahrC*, respectively, were generated by insertion deletion mutagenesis as previously described (6). The genes, including upstream and downstream sequences, were amplified by PCR with the primer combinations indicated (see Table S3 in the supplemental material). The DNA fragment for *arcA* was amplified by PCR with TIGR4 chromosomal DNA as the template and primers M9/M10. Similarly, *arcC* was amplified with M17/18, *arcT* was amplified with M23/24, *argR2* was amplified with P586/P587, *ahrC* was amplified with P590/591, and *argR1* was amplified with M27/M28. The PCR products were cloned into the pGEM-T Easy vector (Invitrogen), resulting in plasmids pGEM695 (*arcA*), pGEM611 (*arcC*), pGEM615 (*arcT*), pGEM699 (*argR2*), pGEM703 (*ahrC*), and pGEM708 (*argR1*), respectively. The recombinant plasmids harboring the desired DNA inserts were used as the templates for inverse PCRs with primer pairs M55/56 for *arcA*, M59/60 for *arc*, M63/64 for *arcT*, P588/P589 for *argR2*, P594/595 for *ahrC*, and M65/P595 for *argR1*. The deleted gene sequences were replaced with an *ermB* erythromycin resistance gene cassette and amplified by PCR with primers T99 and T100 and plasmid pJDC9 (70) as the template. This resulted in plasmids pGEM696 (*arcA*::*ermR*), pGEM612 (*arcC*::*ermR*), pGEM707 (*arcT*::*ermR*), pGEM700 (*argR2*::*ermR*), pGEM704 (*ahrC*::*ermR*), and pGEM709 (*argR1*::*ermR*), which were used to transform pneumococci and generate mutants (see Table S2 in the supplemental material). The integrity of the antibiotic resistance gene cassettes in pneumococcal mutants was verified by PCR (data not shown). In addition, immunoblot assays with specific antibodies generated against recombinant proteins of the ADS or regulators indicated successful mutagenesis.

RNA preparation, Northern blot analysis, and cDNA synthesis. Pneumococcal RNA was purified with the RNeasy minikit, including a DNase digestion step with the RNase-Free DNase Set (Qiagen), as previously described (6). RNA concentrations were determined by measuring the A_{280}/A_{260} ratio with the NanoDrop ND 1000 (PeqLab). Total RNA samples (3 to 5 μg per lane) were electrophoretically separated in denaturing agarose gels (1.2%) and vacuum transferred to Hybond N^+ nylon membranes (Amersham Biosciences). RNAs were cross-linked to the membranes by UV incubation, stained with methylene blue, and then hybridized with DIG-labeled RNA probes at 68°C overnight. Hybridization signals were detected with anti-DIG–alkaline phosphatase, Fab fragments, and CDP Star (Roche Applied Science). DIG-labeled RNA probes for detection of the *arcA*, *arcB*, *arcC*, and *arcDT* mRNAs were generated with the DIG RNA Labeling kit (SP6/T7) (Roche). For the oligonucleotides used to generate probes for Northern blot analysis, see Table S3 in the supplemental material. RT was performed to synthesize cDNA with 5 to 10 μg of RNA. RNA was incubated with 10 nmol of deoxynucleoside triphosphate in 20 μl of RNase-free water for 5 min at 65°C and kept on

ice for 1 min, and then 4 μl of First-Strand Buffer (5×), 1 μl of random primers [pd(N)6; GE Healthcare], 1 μl of dithiothreitol (DTT; 0.1 M), 1 μl of RNasin (Promega), and 1 μl of SuperScript III reverse transcriptase (Invitrogen) were added to amplify the cDNA. The cDNA was employed as the template in the PCRs, and control PCRs were conducted with genomic DNA and total RNA as the template.

Production and purification of recombinant proteins. Recombinant ArcA, ArcB, ArcC, ArgR1, ArgR2, and AhrC were produced as His-tagged fusion proteins in *E. coli* with the pET28TEV plasmid system (Novagen). Genes were amplified with deleted translation initiation codons by PCR from chromosomal DNA with specific oligonucleotide primers and incorporated restriction sites (see Table S3 in the supplemental material). PCR products were restricted by NheI and HindIII (NEB), and the fragments were cloned into similarly digested pET28TEV expression vectors (71) and verified by sequencing (Seqlab). The resulting plasmids, pET28*arcA*, pET28*arcB*, pET28*arcC*, pET28*argR1*, pET28*argR2*, and pET28*ahrC*, were transformed into *E. coli* strain BL21(DE3) (Novagen). For overexpression of recombinant proteins, the strains were grown in LB to an OD_{600} of 0.8 and induced with 1.0 mM IPTG (isopropyl-β-D-thiogalactopyranoside); the bacteria were then harvested after 2 h of induction. The N-terminally His_6-tagged proteins (His_6-ArcA, His_6-ArcB, His_6-ArcC, His_6-ArgR1, His_6-ArgR2, His_6-AhrC) were purified by affinity chromatography with His-Trap Ni-nitrilotriacetic acid columns (GE Healthcare) according to the manufacturer's instructions. Polyclonal mouse antisera were generated by immunization of mice with the purified protein derivative according to standard methods.

Immunoblot analysis. Pneumococci were cultured in THY or CDM to the late exponential growth phase (OD_{600}: THY, 1.0; CDM, 0.8), harvested by centrifugation, and washed once with phosphate-buffered saline (PBS). The bacteria were resuspended in PBS and lysed by ultrasonication. The lysate was centrifuged, and the supernatant was used for further investigation. The protein content was determined by the Bradford protein assay (Sigma-Aldrich). The protein extracts (20 μg) were mixed with an equal volume of SDS sample buffer, incubated at 95°C for 10 min, and separated by SDS–12% gel electrophoresis. The proteins were transferred to a nitrocellulose membrane with a semidry blotting system (Bio-Rad). The membranes were blocked with 10% skim milk (Roth) and incubated with mouse-derived polyclonal antiprotein antiserum (1:1,000). Goat anti-mouse IgG-peroxidase conjugate (Dianova; 1:5,000) was used as the secondary antibody, and binding activity was detected by using enhanced-chemiluminescence substrate (ECL; GE Healthcare). Rabbit-derived polyclonal antienolase antiserum and goat anti-rabbit IgG-peroxidase conjugate (Dianova; 1:5,000) was used as a loading control.

EMSA. DNA fragments of the putative *arcABCDT* promoter regions were amplified by PCR with specific primer pairs (see Table S3 in the supplemental material). The promoter sequences (0.2 pmol) were incubated with different concentrations of His_6-tagged ArgR2 in binding buffer (10 mM Tris/HCl [pH 7.5], 0.5 mM DTT, 5% [vol/vol] glycerol, 200 mM KCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 10 mM arginine) for 30 min at room temperature. Protein-DNA complexes were electrophoretically separated in a native 5% polyacrylamide gel and visualized by ethidium bromide staining. Bovine serum albumin (0.2 pmol) was used as a negative control.

Determination of extracellular metabolites. To analyze arginine uptake and ornithine secretion by pneumococci, bacteria cultured for 6 h on blood agar plates were diluted in CDM to a starting OD_{600} of 0.1 and incubated at 37°C. At each time point, 2 ml of medium was taken and filtered through a 0.2-μm sterile filter (Sarstaedt AG, Nürnberg, Germany) and directly frozen until analysis. ^{1}H-NMR analysis was performed as previously described (72). In brief, 400 μl of the sample was mixed with 200 μl of sodium hydrogen phosphate buffer (0.2 mol/liter, pH 7.0), including 1 mmol/liter trimethylsilyl propanoic acid made up with 50% D_2O for ^{1}H-NMR analysis. A Bruker AVANCE-II 600 NMR spectrometer operated by TOPSPIN 3.1 software was used (both from Bruker Biospin, Rheinstetten, Germany). Qualitative and quantitative data analyses were carried out by using AMIX (Bruker Biospin, Rheinstetten, Germany).

Enzymatic activity of the AD. *S. pneumoniae* was grown in THY medium at 37°C in 5% CO_2. AD enzyme activity was measured by monitoring the production of citrulline from arginine as previously described (73, 74). Briefly, 100 μg of protein extract from bacteria grown in THY medium was incubated for 2 h at 37°C in 0.1 M potassium phosphate buffer containing 10 mM arginine (500 μl). After the addition of 250 μl of a mixture of H_2SO_4 and H_3PO_4 (1:3, vol/vol) and 31.3 μl of a 3% diacetyl monoxime solution, the suspension was heated for 15 min at 99°C. The relative level of citrulline, as a readout of AD activity, was determined colorimetrically at a wavelength of 490 nm.

Ethics statement. The animal experiments conducted in this study were done in strict accordance with the guidelines of the ethics committee at The University of Greifswald, the German regulations of the Society for Laboratory Animal Science (GVSOLAS), and the European Health Law of the Federation of Laboratory Animal Science Associations (FELASA). The experiments were approved by the Landesamt für Landwirtschaft, Lebensmittelsicherheit und Fischerei Mecklenburg-Vorpommern (LALLFV M-V; Rostock, Germany) and the LALLFV M-V ethics board (LALLF M-V permits 7221.3-1.1-006/09 and 7221.3-1.1-019/11). All efforts were made to minimize suffering and ensure the highest ethical standards.

The mouse acute-pneumonia model and coinfections. Female CD-1 outbred mice (Charles River, Sulzfeld, Germany) 8 to 10 weeks of age were used in infection experiments. Pneumococci were cultured in THY supplemented with 10% fetal bovine serum (FBS) to an OD_{600} of 0.35, washed once with 0.5% PBS–1% FBS, and diluted in PBS–1% FBS to get infection doses of 7.5×10^7 CFU/10 μl. Prior to intranasal infection, mice were anesthetized by intraperitoneal injections of ketamine (Ketanest S; Pfizer Pharma, Karslruhe, Germany) and xylazine (Rompun; Provet AG, Lyssach, Germany) as previously described (71, 75). Each animal was scuffed, with the nose held upright, and a 20-μl bacterial suspension (10 μl of bacteria and 10 μl of hyaluronidase [90 U]) was administered intranasally by adding a series of small droplets into the nostrils for involuntary inhalation. The infection dose was confirmed by the determination of CFU counts on blood agar plates.

In competition infection experiments, 2.5×10^7 CFU of wild-type and mutant (TIGR4lux Δ*arcAC*, TIGR4lux Δ*arcAT*, or TIGR4lux Δ*argR2*) bacteria were mixed at a 1:1 ratio. Determination of CFU counts in the nasopharynx and bronchi was performed after intranasal infection at prechosen time intervals (24 and 48 h) postinfection and as described previously (68, 76). Briefly, mice were sacrificed and their tracheas were dissected. One milliliter of sterile PBS–0.5% FBS was passed through the nasopharynx or inserted into the lung with a tracheal cannula and collected after passage (77). The output of mutant versus wild-type bacteria was determined on selective blood agar plates (Oxoid, Basingstoke, United Kingdom) containing kanamycin and/or erythromycin. The competitive index (CI) was calculated as the ratio of mutant to wild-type output CFU counts divided by the ratio of mutant to wild-type input CFU counts. A value of 1 indicates identical output CFU counts of wild-type and mutant bacteria, while a CI value lower than 1 indicates a higher output of wild-type bacteria.

Phagocytosis experiments and antibiotic protection assays. Phagocytosis assays were conducted with J774A.1 murine macrophages (DSMZ, Braunschweig, Germany). Macrophages were incubated for 30 min with a multiplicity of infection (MOI) of 50 bacteria per cell, and the numbers of intracellular and recovered viable pneumococci were quantified by the antibiotic protection assay as described previously (7, 76). All experiments were performed at least three times in triplicate.

Cell culture adherence assays and immunofluorescence microscopy. Pneumococcal adherence to the human lung epithelial cell line A549 (ATCC CCl-185; type II pneumocytes) was tested. The eukaryotic cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% FBS at 37°C and 5% CO_2 and infected at an MOI of 25 bacteria per cell as previously described (68, 78). Postinfection, unbound bacteria

were removed and the infected host cells were fixed on glass coverslips with 3.7% paraformaldehyde. Extracellular bacteria were stained with antipneumococcal antibodies and Alexa 488-conjugated goat anti-rabbit secondary antibody, and after permeabilization with Triton X-100 (0.1%), intracellular bacteria were stained with antipneumococcal antibodies and Alexa 568- and Alexa 488-conjugated goat anti-rabbit IgG secondary antibodies, respectively, as previously described (68, 76). Immunofluorescence microscopy was performed with a fluorescence microscope (Zeiss Axio-Observer.Z1 with VisiGrid, Coolsnap HQ), and image acquisition was done with the VisiView Imaging software (Visitron Systems GmbH, Puchheim, Germany). Each bar in the images represents 10 μm. All experiments were performed at least three times with two or more replicate wells tested for each experimental setup.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01858-14/-/DCSupplemental.

Table S1, PDF file, 0.1 MB.
Table S2, PDF file, 0.1 MB.
Table S3, PDF file, 0.04 MB.

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