Characterization of the two nonidentical ArgR regulators of *Tetragenococcus halophilus* and their regulatory effects on arginine metabolism

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Running Title: Roles of the duplicated ArgR regulators of *T. halophilus*

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ABSTRACT

The halophilic lactic acid bacterium *Tetragenococcus halophilus* has been widely used in high-salinity fermentation processes of food. Previous studies have indicated that the catabolism of arginine may contribute to the osmotic stress adaptation of *T. halophilus*. Unusually, in the chromosome of *T. halophilus*, preceding the arginine deiminase (ADI) operon, locates two co-transcribed genes, both encoding an ArgR regulator; similar structure was rarely found and the roles of the regulators haven't been demonstrated. In the current study, regulatory roles of these two nonidentical ArgR regulators on the arginine metabolism of *T. halophilus* were investigated. The results show that these two regulators play different roles in arginine metabolism, ArgR1 acts as a negative regulator of the ADI pathway by binding to the promoter sequences and repressing the transcription of genes, and the addition of arginine or hyper-osmotic stress conditions can abolish the ArgR1 repression; whereas ArgR2 negatively regulates the genes involved in arginine biosynthesis. Our study found that despite the commonly known roles of the ArgR regulators as the activator of arginine catabolism and the repressor of arginine biosynthesis, which are found in most studied bacteria possessed one ArgR regulator, the two non-identical ArgR regulators of *T. halophilus* both act as repressors, and the repression by which is regulated when sensing changes of environments. By reveling the regulation of arginine metabolism, the current study provides molecular insights and potential tools for future applications of halophiles in biotechnology.

Key points:

The expression of the ADI pathway of *T. halophilus* is regulated by carbon sources and osmotic stress.

The arginine metabolism process of *T. halophilus* is fine-tuned by the two ArgR regulators.

The ADI pathway may contribute to the osmotic stress adaptation by generating more energy and

accumulating citrulline which acts as compatible solute.

Keywords: *Tetragenococcus halophilus*, ADI pathway, ArgR regulator, osmotic stress.

Introduction

The arginine deiminase pathway (ADI) is one of the main arginine catabolic pathways in Gram-positive bacteria. The ADI pathway has been identified in many Gram-positive bacteria (Zúñiga et al. 2002b) although many Gram-positive bacteria including *Streptomyces* and *Bacillus subtilis* do not have an active ADI pathway (Botas et al. 2018; Xiong et al. 2016). The ADI pathway converts arginine via citrulline and carbamoyl phosphate to ornithine, ammonia and ATP. The ADI pathway thus provides ATP, acts as a source of carbamoyl phosphate for pyrimidine biosynthesis, and contributes to pH homeostasis and acid resistance (Abdelal 1979; Casiano-Colón and Marquis 1988; De Angelis et al. 2002). Arginine conversion to citrulline is catalyzed by arginine deiminase (ADI; EC 3.5.3.6), ornithine transcarbamylase (OTC; EC 2.1.3.3) phosphorolyzes citrulline to ornithine and carbamoyl phosphate, and carbamate kinase (CK; EC 2.7.2.2) further catalyzes phosphotransfer and generates ATP, CO_2 and NH_3 (Abdelal 1979; Barcelona-Andrés et al. 2002). The genes *arcA*, *arcB*, *arcC* encoding the enzymes respectively are generally clustered and form an operon (Barcelona-Andrés et al. 2002). Other genes including *arcD*, encoding an arginine-ornithine antiporter, or *arcT*, a transaminase-encoding gene, are sometimes part of the same operon (Maghnouj et al. 2000a; Ohtani et al. 1997; Verhoogt et al. 1992).

Arginine metabolism is modulated by various environmental conditions. In most bacteria studied, the ADI pathway is induced by arginine and subjected to carbon catabolite repression (Budin-Verneuil et al. 2006; Leisner et al. 1994; Liu and Pilone 1998), some studies also showed that anaerobic conditions activate the expression of the ADI pathway (Hall and Ji 2013). The ADI pathway also protects bacterial cells from stress conditions. Catabolism of arginine protects *Streptococcus* species and other lactic acid bacteria against acid stress by producing ammonia and raising the cytoplasmic pH (Cusumano and Caparon 2015; Griswold et al.

2004; Gruening et al. 2006). In *Staphylococcus aureus*, the activation of the ADI pathway also conveyed resistance to vancomycin (Tan et al. 2017). In addition, studies have indicated altered expression of genes of the ADI pathway under salt stress and varied temperature (Vrancken et al. 2009; Xiong et al. 2015). Thus, the ADI pathway may play a significant role in the survival of bacteria under diverse stress conditions.

The regulation of the ADI pathway in Gram-positive bacteria is variable and complex to allow fine-tuning of arginine biosynthesis and arginine catabolism in response to the substrate supply and environmental conditions. The repressor of arginine biosynthesis ArgR was first identified in *Escherichia coli*, where it acts as a repressor of arginine biosynthesis and an activator of arginine catabolism (Van Duynef et al. 1996). Arginine biosynthesis in *Bacillus subtilis* is regulated by the ArgR homologue AhrC (Garnett et al. 2008); *Lactococcus lactis* regulates arginine metabolism by ArgR and AhrC. Although both regulators can repress the biosynthesis of arginine, only AhrC can activate the ADI pathway (Larsen et al. 2004). Beside regulating arginine metabolism, recent studies employing genome-scale analysis have revealed that ArgR acts as a global transcriptional regulator in *E. coli* (Cho et al. 2012; Cho et al. 2015). *Streptomyces* lacks an active ADI pathway, however, a transcriptomic study found that *Streptomyces coelicolor* ArgR is a pleiotropic regulator which affects various genes involving in different aspects of bacterial life (Botas et al. 2018; Pérez-Redondo et al. 2012), indicating that the role of ArgR in bacterial life varies and needs further investigations.

Tetragenococcus halophilus is a halophilic lactic acid bacterium which has been widely used in high salinity fermentation processes. *T. halophilus* grows optimally at a 1 M NaCl and tolerates saturated NaCl solutions. The ability to grow at high salinity is a prerequisite for the use of *T. halophilus* in traditional food fermentations and in industrial fermentation applications. Osmotolerance of *T. halophilus* relates to the uptake of compatible solutes including glycine betaine, and the overexpression of molecular chaperons such as ClpB

and DnaK (Robert et al. 2000; Sugimoto et al. 2003; Sugimoto et al. 2006). A proteomic study additionally indicated that the expression of the ADI pathway may also play a significant role in the *T. halophilus* adaptation to osmotic stress conditions (Lin et al. 2017). Both genome sequences and proteomic study demonstrated that the ADI pathway is the only arginine catabolism pathway of *T. halophilus*. A recent pan-genome analysis also showed that citrulline, a by-product of the ADI pathway, may protect *T. halophilus* against high salinity-induced osmotic stress (Chun et al. 2019). The regulation of the ADI pathway during adaptation of *T. halophilus* to osmotic stress, however, remains unknown.

In the halotolerant *Enterococcus faecalis,* genes involved in ADI pathway form an operon but their organization differs from other lactic acid bacteria (Barcelona-Andrés et al. 2002). In *E. faecalis*, the operon containing genes involved in the ADI pathway is comprised of *arcA* (*EF_RS00460*, with NCBI reference sequence NC_004668.1), *arcB* (*EF_RS00465*), *arcC* (*EF_RS00470*), the regulator *arcR* (*EF_RS00475*), and *arcD* (*EF_RS00480*), *argR1* (*EF_RS00455*), and *argR2* (*EF_RS00450*) locating on the antisense strand of the chromosome. Although ADI pathway is generally reported to be regulated either by ArcR or ArgR, it is rare to find an ArcR and two ArgR regulators closely clustered in the bacterial genome (Barcelona-Andrés et al. 2002). Analysis of the genome sequence of *T. halophilus* (Genbank accession number NC_016052.1) indicates a similar organization of the ADI pathway and putative regulators of the pathway, however, the roles of these regulators in this species remain uncharacterized. The low amino acid identity of ArgR1 and ArgR2 in *E. faecalis* (Barcelona-Andrés et al. 2002) and *T. halophilus* (NC_016052.1) indicates that these two regulators have different roles. Thus, identification of the roles of *T. halophilus* ArgR regulators can extend our current understanding of the arginine metabolism and its contribution to salt tolerance in *T. halophilus*. The current study aims to identify the regulation of arginine metabolism in *T. halophilus* and to

reveal its possible roles in stress adaptation. The gene organization of the *T. halophilus* ADI pathway was investigated by identifying the co-transcription structures and promoter sequences and *in vivo* and *in vitro* experiments were conducted to study the regulatory effects of ArgR1 and ArgR2 on arginine metabolism.

Materials and Methods

Bioinformatics Analysis

The reference genomic sequence of *T. halophilus* was obtained from NCBI with the reference number NC_016052.1. The amino acid sequences of the ADI pathway of *T. halophilus* and their homologs were obtained from the Universal Protein Resource (UniProt) database. Multiple sequences alignments of amino acid sequences were performed with CLUSTAL W(Thompson et al. 1994) program using MEGA 6.0. Phylogenetic analysis was conducted using Maximum Likelihood method and the number of bootstrap replications was 1000. Promoters of ADI pathway of *T. halophilus* were predicted using the BPROM module on the SoftBerry website, and transcription terminator was predicted using the FindTerm module on the SoftBerry website (http://www.softberry.com/berry.phtml) (Solovyev and Salamov 2011).

Bacterial strains and growth conditions

T. halophilus CICC 10469 was grown in De Man, Rogosa, and Sharpe (MRS) mediums at 30°C with an optimum NaCl concentration of 1 M (De Man et al. 1960; Liu et al. 2015). *E. coli* strains were grown at 37°C in Luria broth (LB) mediums; and the mediums were supplemented with ampicillin (100 μg/ml), or isopropyl β-D-1-thiogalactopyranoside (IPTG) whenever appropriate.

Construction of enhanced green fluorescent protein (eGFP) expression plasmids

The activities of the putative promoter fragments were examined by eGFP expression. Promoter-reporter plasmids were constructed using the Gibson assembly method. Briefly, plasmid pUC19 was digested with

*Nde*I and *Pvu*II to remove the original promoter *Plac*, resulting in a backbone fragment called pUC in this study; gene sequences were amplified using primers listed in Table S3 to introduce homologous arms. Promoter-reporter plasmids were constructed by fusing the promoter fragments and the eGFP coding sequence with the pUC fragment using ClonExpress MultiS One Step Cloning Kit (Vazyme Biotech Co.,Ltd). The control plasmid pUC::eGFP was constructed by introducing restriction sites for *Nde* I and *Pvu* II using primers listed in Table S3 into the coding sequence of eGFP and fusing the resulted fragment with pUC, the resulted promoter-less plasmid was used as control.

To investigate the regulatory effects of ArgR regulators, promoter-regulator-reporter plasmids were constructed with primers listed in Table S4. Briefly, coding sequences of promoters, regulators and reporters were amplified to introduce homologous arms of adjacent sequences, specially, the ribosomal binding site of the respective promoters were introduced between the regulator sequence and the reporter sequence to ensure the recognition of ribosome; resulted fragments were fused using Gibson assembly method to generate promoter-regulator-reporter plasmids. To evaluate the regulatory effects of the ArgR regulators on the promoters, corresponding plasmids containing mutated regulators which lost the binding ability were constructed by site-directed mutation of the promoter-regulator-reporter plasmids; primers used in site-directed mutation are listed in Table S5.

All plasmids were verified by DNA sequencing before being transferred into *E. coli* strain BL21(DE3) for further studies. The strains containing the recombinant plasmids were grow in LB mediums till mid-logarithmic phase, and fluorescence was measured using an excitation wavelength of 488nm and emission wavelength of 597nm, OD_{600} was measured at the same time.

RNA isolation, reverse transcription, quantitative reverse-transcriptase PCR (qRT-PCR) and co-

transcript structure analysis using RT-PCR

T. halophilus cells were grown in MRS medium supplied with 1 M of NaCl until exponential phase of growth and harvested by centrifugation afterwards. For salt stress experiments, the cells were incubated in MRS mediums with a final NaCl concentration of 0 M, 1 M, and 3.5 M respectively for 3 h; for arginine addition and carbon catabolite repression study, cells were incubated in MRS mediums with or without the addition of 5 mM arginine or 2% glucose. After incubation, total RNA was extracted with Qiagen RNeasy Mini Kit and treated with RQ1 RNase-Free DNase (Promega) to remove residual DNA according to the manufacturer's instruction. Then, 1 μg of total RNA was converted to cDNA with random primers using the High-Capacity cDNA Reverse Transcription Kit (Thermofisher). RNA samples without the reverse transcription step were used as templates for the negative control of RT-PCR experiments.

qRT-PCR was performed using the 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with the primers listed in Table S1. The following procedures were applied: 95°C for 30 s, followed by 40 cycles of incubation at 94°C for 5 s and at 60°C for 34 s. Three biological replicates for each sample and technical triplicate for all genes were performed. We selected the 16S rRNA gene to normalize the data, and qRT-PCR data were calculated using the $2^{-\Delta\Delta Ct}$ method (Pfaffl 2001). Primers used in qRT-PCR are listed in Table S1.

RT-PCR experiments were conducted to investigate the transcription structure of the ADI pathway of *T. halophilus*. Primers amplifying different lengths of fragments are listed in Table S2. The cDNA products were used as template. The chromosomal DNA of *T. halophilus* was used as the template for positive control and RNA samples without the reverse transcription step were used as the template for negative control.

Overexpression and purification of recombinant His_6-tagged regulators

The gene sequences encoding ArgR1 and ArgR2 were amplified from the chromosomal DNA of *T. halophilus* using primers listed in Table S6, resulted fragments were cloned into the *Pst* I and *EcoRV* restriction sites of the expression plasmid pETDuet, which introduced a N-terminal His_6 tag to the recombinant regulators. After verification by DNA sequencing, plasmids were transferred into *E. coli* strain BL21(DE3) for protein overexpression. Briefly, recombinant strains were grown at 37°C until OD_{600} reached 0.5, followed by induction with 1 mM IPTG for 4 h at 20°C. Protein extractions were conducted using Bacterial Protein Extraction Kit (Sangon Biotech, Shanghai) according to the manufacturer's instructions. Briefly, cells were collected by centrifugation at 5000 × *g* for 10 mins, 4°C. After washing with PBS buffer, cell pellets were resuspended in cell lysis buffer. PMSF (Phenylmethanesulfonyl fluoride) and lysozyme were then added, and after incubation at 37°C for 30 mins, the resultant mixtures were vortexed in a rocking platform for 10 min, followed by the addition of DNase I/RNase and further incubation at 37°C for 10 mins. The resulting crude protein extracts were purified using Ni-NTA column, and recombinant proteins were eluted with imidazole and dialyzed into storage buffer, glycerol was added to a final concentration of 20% to preserve proteins. Protein concentrations were determined with Bradford reagents and purities were assessed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

DNA binding assays

EMSAs were conducted to study the DNA binding characteristic of ArgR1 and ArgR2. Upstream sequences of *arcA*, *arcC*, *arcR*, *argR1* and *argR2* containing putative promoter sequences (listed in Table S7) were synthesized and labeled with biotin by Sangon Biotech (Shanghai) Co., Ltd. The resulting products were annealed prior to EMSAs according to the manufacturer's protocol and used as probes for EMSAs.

EMSAs experiments were conducted using the LightShift Chemiluminescent EMSA Kit (Pierce

Biotechnology, Rockford, IL) according to the manufacturer's instructions. Bindings were performed in a 20 μl reaction volume containing 50 ng μl-1 of poly (deoxyinosinic-deoxycytidylic) acid [poly (dI•dC)], 2.5% glycerol, 5 mM $MgCl_2$, 0.05% NP-40 and 10 fmol biotin-labeled probes, reaction mixtures were incubated at room temperature for 20 min and loaded onto a native 6% polyacrylamide gel, and electrophoresed at 4°C, 100V for 35 mins. For each promoter, 2 pmol of unlabeled sequences were used to conduct control reactions to verify specific bindings. Results were detected by chemiluminescence immunoassay according to the manufacturer's instructions.

Overexpression and purification of regulator mutants

Site directed mutations were conducted on plasmids pETDUET::ArgR1 and pETDUET::ArgR2 using primers listed in Table S5 to mutate the predicted DNA binding sites. Briefly, after PCR amplification, the parental plasmid strands were digested with Dpn1 and the mutated plasmids were chemically transformed into *E. coli* strain DH5α and selected on LB plates containing 100μg ml^{-1} ampicillin. Successful mutagenesis was confirmed by DNA sequencing. Mutated regulators were overexpressed and purified as described for His_6-tagged regulators.

Crosslinking analysis

To verify the interference of arginine on the polymer status of ArgR1, purified ArgR1 was crosslinked using 0.1% glutaraldehyde in 50 mM HEPES buffer containing 150 mM KCl with the addition of 0, 5, 10 and 20mM L-arginine. The reaction mixtures were incubated at room temperature for 2 hours and analyzed by 10% SDS-PAGE and stained with Coomassie Brilliant Blue.

Results

Genetic organization of the ADI pathway in *T. halophilus*

The organization of *T. halophilus* ADI pathway is shown in Fig. 1. Genes *arcA*, *arcB* and *arcC*, encoding arginine deiminase, ornithine transcarbamylase and carbamate kinase respectively, are located on the complementary strand of the *T. halophilus* genome and arranged in order of the enzymatic steps in the pathway (Fig. 1). As shown in Fig. S1, the signature ADI sequences of ArcA, the carbamoyl phosphate and ornithine binding sites of ArcB are highly conserved (Barcelona-Andrés et al. 2002; Knodler et al. 1998; Kraus et al. 1985). Downstream of *arcC* gene lies a putative *arcR* gene encoding a Crp/Fnr family transcriptional regulator with an identity of 51.97% with the ArcR of *E. coli*, and a putative *arcD* gene which encodes an arginine-ornithine antiporter. Two genes encoding ArgR family transcriptional regulators, termed *argR1* and *argR2*, are preceding *arcA* on the sense strand.

Sequences upstream of the start codons of the 7 genes presented in the pathway (*arcA*, *arcB*, *arcC*, *arcD*, *arcR*, *argR1* and *argR2*) were examined for putative promoter activity with BPROM. A putative promotor was identified upstream of each of the seven genes. To confirm promotor activity of the 7 predicted promotors, each putative promoter sequence was fused with the reporter gene eGFP to qualitatively assess the promoter activity. Promoter activities were also confirmed by measuring the fold change of fluorescence/OD_{600} ratios of the promoter-reporter plasmids compared to the promotorless control. As shown in Fig. 1B, the upstream sequences of *arcA*, *arcB*, *arcC*, *arcR*, *argR1* and *argR2* initiated expression of eGFP, while *ParcD* did not enhance eGFP expression relative to the promotorless control. Confocal microscopy observations confirmed the results of fluorescence measurements. As shown in Fig. 1C, strong green fluorescence was observed in strains harboring plasmid pUC-ParcA-eGFP, pUC-ParcR-eGFP, pUC-PargR1-GFP and pUC-PargR2-eGFP, only weak fluorescence was observed in strains harboring pUC-ParcB-eGFP and pUC-ParcC-eGFP, while control and pUC-ParcD-eGFP showed no fluorescence. Although a promotor was identified upstream of *arcB*,

results of RT-PCR (Fig. S2) indicated the co-transcription of *arcB* with *arcA*, so only *ParcA*, *ParcC*, *ParcR*, *PargR1* and *PargR2* were investigated for regulatory effects in the following studies.

ADI pathway was induced by arginine and subject to carbon catabolite repression

qRT-PCR was conducted to study the regulatory effects of arginine and glucose on *T. halophilus* ADI pathway. The transcription of *arcA*, *arcB*, *arcC*, *arcD* and *arcR* was strongly repressed in the presence of glucose, and induced in absence of glucose and presence of arginine (Fig. 2). The regulatory effects of arginine and glucose on ArgR regulators were different from their effect on the *arc* operon. The transcriptional levels of both ArgR regulators were higher in the presence of glucose than arginine (Fig. 2).

ArgR1 acts as a negative regulator of the ADI pathway

To understand the role of ArgR1 in regulation of the ADI pathway, EMSAs were performed to study the binding effects of ArgR1 on *ParcA*, *ParcC*, *ParcR*, *PargR1* and *PargR2*. Sequences labeling with biotin were used as probes, 50ng/μL poly (dI•dC) was added to each reaction and sequences without label were used to confirmed specific bindings. As shown in Fig. 3A, when incubating with elevated concentration of ArgR1, bands corresponding to every sequence shifted, indicating that ArgR1 can regulate the expression of both the *arc* operon and ArgR regulators. Interestingly, when incubating ArgR1 with *ParcA*, a band shift to a lower molecular mass occurred, possibly resulting from the binding of trimeric ArgR1 instead of hexamer.

Sequence alignments showed that the DNA binding domain of ArgR regulator, Ser42-Arg43, is conserved in ArgR1 (Fig. S3A). Site-directed mutation was conducted to confirm the roles of these two residues. As shown in Fig S3B, the mutant ArgR1 S42D R43D showed no binding to promoter sequences, indicating the mutation of Ser42-Arg43 abolished the binding ability.

To investigate the regulatory roles of ArgR1 on the ADI pathway, promoter-regulator-reporter plasmids were

constructed as Fig. 4A. In the plasmids, the coding sequences of ArgR1 and eGFP were linked by a ribosome binding site and transcribed from the promotors *ParcA*, *ParcC*, *ParcR*, *PargR1* or *PargR2*, respectively. To clarify the effects of ArgR1 binding on these promoters, corresponding plasmids containing the mutated ArgR1 (S42D R43D) which lost the binding ability were constructed, the plasmids containing wild type regulator pUC-promoter-ArgR1-eGFP and the plasmids containing mutated plasmid were transferred to *E. coli*, respectively, and the florescence of both series of strains was measured. By comparing the ratio of fluorescence/OD_{600} of the strain containing pUC::promoter-ArgR1-eGFP and pUC::promoter-ArgR1mut-eGFP, the effect of the regulator on each promoter can be estimated. As shown in Fig. 4B, mutation on ArgR1 significantly enhanced the fluorescence in all the five strains containing mutant ArgR1, indicating that by binding to the promoter sequences, ArgR1 repressed the transcription of the *arc* operon and *argR1* itself.

ArgR2 acts as a negative regulator of *arcA*

Since the amino acid sequence identity of ArgR1 and ArgR2 is only 29.49%, the regulatory roles of ArgR2 may be different from ArgR1. Testing the binding ability of ArgR2 to the promoters of ADI pathway using EMSA showed that clear band shifts only occurred when ArgR2 was incubated with *ParcA* (Fig. 3B). Although ArgR2 showed a weak binding with its own promoter *PargR2* at high concentration, results of RT-PCR (Fig. S2) showed that argR1 and argR2 are co-transcribed, so only the regulatory effect of binding to *ParcA* was studied using eGFP fusion.

Promoter-regulator-reporter plasmids were constructed to study the regulatory effect of ArgR2 on *ParcA* as described above. As shown in Fig. 4C, mutation of the DNA binding sites of ArgR2 led to higher expression of eGFP, indicating that by binding to the promoter sequence, ArgR2 repressed the transcription of *arcA*.

The regulatory effect of ArgR1 and ArgR2 on the arginine biosynthesis pathway.

The existence of genes encoding argininosuccinate synthase (ArgG, *TEH_12490*) and argininosuccinate lyase (ArgH, *TEH_12480*) were identified by protein Blast in the *T. halophilus* genome using the protein sequences of the ArgG (UniProtKB accession number P0A6E4) and ArgH (UniProtKB accession number P11447) of *E. coli*. ArgG and ArgH are involved in the biosynthesis of arginine, their coding sequences are adjacent, forming the *argGH* operon. Genome sequencing and proteomic study indicated that *argG* and *argH* are the only arginine biosynthesis genes in *T. halophilus*. These two genes are located on the complementary strand of the genome, the *argH* coding sequence is preceded by *argG*, with a 3bp intermediate sequence. Analyzing the sequences found a promoter upstream of *argG* and no predicted transcription terminator was found upstream of *argH*, indicated that these two genes are possibly co-transcribed by the promoter of *argG*, termed *PargG* in the current study. *PargG* was used in further studies about the regulation of arginine biosynthesis pathway in *T. halophilus*. Since previous studies have reported regulatory effects of ArgR regulators on arginine biosynthesis, EMSAs were conducted to investigate the roles of ArgR1 and ArgR2 on *argGH* operon. Results showed that both regulators bound to *PargG* and clear band shifts were observed (Fig. 5A). To clarify the effects of the regulators on the arginine biosynthesis, promoter-regulator-reporter plasmids containing either wild type or mutant regulators were constructed as described above, and the fluorescence and OD_{600} of the strains containing the respective plasmids were measured; the regulatory effects of regulators were evaluated by comparing the fluorescence/OD_{600} ratio of recombinant strains containing wild-type regulator with that of strains containing mutated regulator. As shown in Fig. 5B, both regulators exhibited negative regulatory effects on *PargG*.

The effect of arginine on the DNA binding of the regulators

Arginine plays an important role in the activity of ArgR regulators and qRT-PCR demonstrated that arginine

induced the *arc* operon in *T. halophilus*. To better illustrate the regulatory mechanism of arginine metabolism of *T. halophilus*, the effects of arginine addition on the regulatory effects of ArgR1 and ArgR2 were further investigated.

EMSAs were conducted to study the impact of arginine on the binding of both regulators. As shown in Fig. S4A, the higher-molecular mass bands that were observed when incubating ArgR1 with *ParcA* and *PargG*, were no longer visible after addition of arginine, and arginine also decreased the binding of ArgR1 to the promoter sequences of *arcB, arcC, arcD*; which will result in the de-repression of ArgR1 and the expression of the ADI pathway. The effects of arginine on the binding of ArgR2 to *ParcA* were weaker but increasing addition of arginine still resulted in a faded band shift and an increase of free probe. The addition of arginine hardly affected the binding of ArgR2 to *PargG* (Fig. S4B). The results indicated that in the presence of arginine, both ArgR1 and ArgR2 were released from the promoter sequences of the *arc* operon and the transcription of the ADI pathway was induced; while ArgR2 still bound to the promoter sequence and repressed the transcription of arginine biosynthesis pathway in the presence of arginine.

The regulatory effects of osmotic stress on arginine metabolism

T. halophilus grows over a broad range of salinity conditions, which requires the ability to regulate specific genes to adapt to changing environments. qRT-PCR experiments were conducted to examine how arginine metabolism is regulated in response to osmotic stresses. As shown in Fig. S5, the transcription of the ADI pathway and the arginine biosynthesis pathway were expressed during growth at high salinity but not differentially expressed at different salt concentrations. Likewise, *argG* and *argH* were not differentially expressed in hypo-osmotic or hyper-osmotic condition.

DISCUSSION

The current study aimed to identify the ADI pathway and the regulation of arginine metabolism of *T. halophilus*. The ADI pathway generates metabolic energy through formation of ATP and consumption of intracellular protons; in addition, the products of the ADI pathway thicken the cell walls of bacteria and protect cells from stress conditions (Cheng et al. 2017; Cusumano and Caparon 2015; Tan et al. 2017; Vrancken et al. 2009). *T. halophilus* is a halophilic lactic acid bacterium that is widely used in food fermentation processes with high salinity but also occurs as spoilage organism during extraction of sucrose from sugar beets (Justé et al. 2008; Lin et al. 2017). *T. halophilus* possesses an ADI pathway encoded in an operon. The expression of the ADI pathway was increased under higher salt concentration and the intracellular concentration of citrulline under hyper-osmotic condition was higher than that under optimal growth conditions (Chun et al. 2019; He et al. 2017; Lin et al. 2017), indicating that the ADI pathway contributes to the salt tolerant ability of *T. halophilus*. This study demonstrated that expression of the ADI is fine-tuned by two regulatory proteins, ArgR1 and ArgR2.

The arrangement of genes of coding for the ADI pathway and the presence of regulatory proteins is diverse even in closely related microorganism (Barcelona-Andrés et al. 2002; Zúñiga et al. 2002b). The *T. halophilus arc* operon is composed of 5 genes *arcA*, *arcB*, *arcC*, *arcR*, and *arcD* located in the complementary strand; 359 bp upstream of *arcA* are two homologous ArgR/AhrC-type regulators ArgR1 and ArgR2. The amino acid sequences of ArgR1 and ArgR2 are only 29% identical to each other, indicating a different function of the two regulatory proteins. The organization of the *T. halophilus* ADI pathway is similar with that of *E. faecalis* (Barcelona-Andrés et al. 2002). The presence of multiple regulators indicates complex regulations of the pathway. Of the promotors controlling expression of the *arc* operon, *ParcA, ParcB, ParcC, ParcR* showed promoter activities. Although the upstream sequence of *arcB* also possessed promoter activity, results of RT-

335 PCR showed that it was co-transcribed with *arcA*. RT-PCR also verified the co-transcription of ArgR1 and
336 ArgR2. Because both regulators present an active promoter, and due to the low sequence identity of the two
337 regulators, we suspected that these two regulators act differently and both promoter sequences were used in
338 further studies.
339 This study assessed the regulation of arginine metabolism of *T. halophilus* by quantification of mRNA, and
340 determination of the effect of arginine to binding of ArgR1 and ArgR2 to promotors of the *arc* operon. Results
341 of qRT-PCR showed that the ADI pathway was subject to carbon catabolite repression, which is consistent
342 with the regulation in *Lactococcus lactis* and *Latilactobacillus sakei* (previously known as *Lactobacillus*
343 *sakei*) (Budin-Verneuil et al. 2006; Larsen et al. 2004; Zheng et al. 2020; Zúñiga et al. 2002a). EMSAs
344 confirmed the direct binding of ArgR1 to the promoters of the *arc* operon, *argR1*, *argR2*, and the *argGH*
345 operon, indicated the broad regulatory effects of ArgR1 on the arginine deiminase pathway, ArgR regulators
346 and arginine biosynthesis pathway. ArgR2 bound only to *ParcA* and *PargG*, indicating that the regulatory
347 effects of ArgR1 and ArgR2 of *T. halophilus* are different.
348 In *E. coli* and *B. subtilis*, the N-terminal of ArgR contains the DNA-binding domain, and the conserved
349 residues Ser and Arg are essential to the DNA binding (Barcelona-Andrés et al. 2002; Garnett et al. 2008;
350 Sunnerhagen et al. 1997). The “SR” domain lies in the third helix of the ArgR regulator, and mutation of this
351 domain alleviated the regulatory effects (Sunnerhagen et al. 1997). This “SR” domain (Ser42-Arg43) is
352 conserved in ArgR1 and ArgR2 (Fig. S3A). Site directed mutation on Ser42 and Arg43 residues of ArgR1
353 and ArgR2 abolished binding of the proteins to promotor sequences (Fig. S3B, C), indicating that the “SR”
354 domain of both regulators acts as DNA binding domain. Due to the lack of gene manipulation methods in *T.*
355 *halophilus*, the regulatory effects of ArgR binding were evaluated by comparing the activity of promoters

356 regulated by either wild-type ArgR or the S42D-R43D mutant which lost the binding ability. Surprisingly,
357 results of *in vivo* experiments using reporter gene eGFP showed that ArgR1 and ArgR2 act as negative
358 regulators to the ADI pathway and the arginine biosynthesis pathway. This is uncommon because generally
359 ArgR regulators act as a positive regulator of arginine catabolism pathway and negative regulator of arginine
360 biosynthesis pathway. ArgR was first identified in *E. coli* as a repressor of the arginine biosynthesis pathway
361 and an activator of arginine catabolism though the arginine succinyltransferase (AST) pathway (Cho et al.
362 2012; Cho et al. 2015; Kiupakis and Reitzer 2002; Maas and Clark 1964). In *Corynebacterium glutamicum*,
363 ArgR represses the expression of arginine biosynthesis genes *argCJBDFRGH* (Yim et al. 2011); similarly in
364 *S. coelicolor*, ArgR represses the expression of arginine biosynthesis genes *argCJBD* (Botas et al. 2018). The
365 ArgR homologue in *B. subtilis*, AhrC, acts as a repressor of arginine biosynthesis genes and an activator of
366 the arginase pathway encoded by *roc* operons at the same time (Garnett et al. 2008). Similarly, the ArgR of
367 *Bacillus licheniformis* acts as the repressor of arginine biosynthesis genes and the activator of the ADI
368 pathway in presence of arginine (Maghnouj et al. 1998) (Maghnouj et al. 2000a). In *L. monocytogenes*, ArgR
369 represses the arginine biosynthesis with the addition of arginine and represses the ADI pathway under acidic
370 condition and in the absence of arginine (Cheng et al. 2017). *Streptococcus pneumonia* contains three ArgR-
371 type regulator; ArgR1 and AhrC repress the arginine biosynthesis genes *argGH* (Kloosterman and Kuipers
372 2011), and ArgR2 activates the expression of arginine deiminase system (Schulz et al. 2014). In *Lactococcus*
373 *lactis*, ArgR cooperates with AhrC in order to repress the expression of arginine biosynthesis operon, while
374 the ADI pathway is activated solely by AhrC instead of ArgR (Larsen et al. 2004).
375 To assess patterns of ArgR1 and ArgR2 mediated regulation of gene expression in *T. halophilus*, phylogenetic
376 analysis examined their evolutionary relationship to well-studied ArgR regulators of Gram-positive bacteria

(Fig. 6A). The regulatory effects of each regulator on the arginine biosynthesis and arginine catabolism including the ADI and arginase pathways encoded by *roc* genes were also indicated. As shown in Fig. 6B, the ArgR regulators of *T. halophilus*, *E. faecalis* and *L. monocytogenes* are closely related. While the role of ArgR regulators has not been studied in *E. faecalis*, the regulatory effects of ArgR on arginine metabolism of *T. halophilus* and *L. monocytogenes* are similar. Our results indicated that besides the commonly known regulatory roles of ArgR regulators, different regulation pattern are observed in some bacteria.

Arginine functioned as a corepressor for the regulatory effects of AhrC in *B. subtilis* and ArgR in some *Chlamydia* strains (Garnett et al. 2008; Schaumburg and Tan 2006). In the current study, we found that the addition of arginine released the binding of both regulators to the ADI promoter but not of ArgR2 and thus de-repressed the ADI pathway but not the ArgGH pathway. Similarly in *Streptomyces*, the ArgR regulator represses the arginine biosynthesis pathway and the repression cannot be released by arginine supplement (Pérez-Redondo et al. 2012). The regulation of the arginine metabolism by ArgR1 and ArgR2 in *T. halophilus* is presented in Fig. 6A. In *T. halophilus*, ArgR1 acts as the major regulator of the ADI pathway as it bound to most of the promoter sequences. Its regulation was affected by the addition of arginine. ArgR2 bound only to the promoter sequence of *arcA* and repressed expression of *argGH* in presence or absence of arginine. When arginine is available, *T halophilus* thus activates arginine utilization but not arginine synthesis. The derepression of ArgR by arginine has also been observed in *L. monocytogenes*, where presence of arginine abolished the repression of ArgR on the ADI pathway (Cheng et al. 2017). To study the effects of arginine on ArgR1, cross-linking experiments showed that the addition of arginine did not affect the multimeric state of ArgR1 (Fig. S6), thus the allosteric mechanism of the derepression of arginine binding of ArgR1 remains to be studied. Differences between the effects of arginine addition on ArgR1 and ArgR2 binding abilities also

398 demonstrated that these two regulators act differently. The C-terminal domain of ArgR is essential to its
399 multimerization and arginine binding abilities (Garnett et al. 2008; Karaivanova et al. 1999; Tian and Maas
400 1994). The "SR" domain involved in DNA binding is conserved in ArgR1 and ArgR2 but the "GDDT"
401 domain, which is involved in effector binding (Van Duynef et al. 1996), is only conserved in ArgR1 (Fig.
402 S3A). In ArgR2, the residue Asp128 is replaced by a phenylalanine, which may result in the alteration of
403 effector binding of ArgR2 and the lack of derepression of ArgR2 to arginine biosynthesis under hyper-osmotic
404 condition or in the presence of arginine. The alteration of Asp128 was also found in the ArgR1 of *E. faecalis*
405 (Barcelona-Andrés et al. 2002), indicating that the regulation pattern on arginine metabolism of this species
406 may be similar to that of *T. halophilus*, and that the two ArgR regulators are non-redundant and contribute to
407 different regulation in both species.
408 The ADI pathway in *T. halophilus* is subjected to carbon catabolic repression but glucose induces the
409 expression of arginine biosynthesis. In the absence of glucose, the ADI pathway is induced significantly,
410 generating metabolic energy in forms of ATP and proton motive force. The quantification of mRNA revealed
411 that induction of the ADI pathway by arginine is below the threshold of 2 fold overexpression that is detected
412 by RT-qPCR (this study), however, the EMSAs results obtained in this study indicated induction of the
413 pathway by arginine through derepressing regulator repression. Taken together, the ADI pathway of *T.*
414 *halophilus* is subjected to carbon catabolic repression and is induced by arginine.
415 The ADI pathway is up-regulated by osmotic stresses in *Limosilactobacillus fermentum* (previously known
416 as *Lactobacillus fermentum*) (Vrancken et al. 2009; Zheng et al. 2020). The fold changes of gene expression
417 under different salinity concentrations was too small to be detected by RT-qPCR. However, proteome analysis
418 revealed that *T. halophilus* up-regulated the ADI gene expression under higher salinity condition (Chun et al.

2019; Lin et al. 2017; Liu et al. 2015). The proteomic studies also found that ArgG is down-regulated under hyper-osmotic conditions and *T. halophilus* cells accumulated higher amounts of citrulline in the cytoplasm in response to salt stress (He et al. 2017). Citrulline acts as a compatible solutes against osmotic stress (Held and Sadowski 2016), and ADI pathway has been shown to protect cells against stress conditions such as acidic stress, salt and temperature stress and ethanol stress (Cusumano and Caparon 2015; Díez et al. 2017; Vrancken et al. 2009). The ADI pathway in *T. halophilus* thus serves a dual role in hyper-osmotic stress conditions, first, to provide metabolic energy and second to generate citrulline as a compatible solute.

A predicted Crp/Fnr-type regulator ArcR was also found in the ADI operon of *T. halophilus* and in *E. faecalis.* ArcR acts as an activator and is essential to the expression of the ADI pathway in *L. sakei* and *S. aureus* (Makhlin et al. 2007; Zúñiga et al. 2002a). In *B. licheniformis*, ArcR acts as an activator of the ADI operon and is essential for the anaerobic expression of the ADI pathway (Maghnouj et al. 2000b). In *T. halophilus* grown in presence of different carbon sources, *arcR* exhibit similar expression pattern with other genes of *arc* operon compared with *argR* genes (Fig. 2), indicating that ArcR unlikely to act as a repressor. The elucidation of its role in arginine metabolism processes of *T. halophilus* remains subject to future studies.

In conclusion, the current study indicated that despite the commonly known roles of ArgR regulator as an activator of arginine catabolism and a repressor of arginine biosynthesis at the same time, ArgR regulators negatively regulate arginine biosynthesis and catabolism pathway in some bacteria possessed two homologous ArgR, and the repression can be modulated when sensing environmental changes, which can possibly minimize energy cost and contribute to bacterial survivals in stress conditions.

Author's contributions

JL and LL conceived and designed the study, JL and XL conducted experiments, JL wrote the manuscript,

MG and LL revised the manuscript.

Compliance with Ethical Standards

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Reference

Abdelal AT (1979) Arginine catabolism by microorganisms. Annu Rev Microbiol 33(1):139-168

Barcelona-Andrés B, Marina A, Rubio V (2002) Gene Structure, Organization, Expression, and Potential Regulatory Mechanisms of Arginine Catabolism in *Enterococcus faecalis*. J Bacteriol 184(22):6289-6300 doi:10.1128/JB.184.22.6289-6300.2002

Botas A, Pérez-Redondo R, Rodríguez-García A, Álvarez-Álvarez R, Yagüe P, Manteca A, Liras P (2018) ArgR of *Streptomyces coelicolor* Is a pleiotropic transcriptional regulator: effect on the transcriptome, antibiotic production, and differentiation in liquid cultures. Front Microbiol 9:361 doi:10.3389/fmicb.2018.00361

Budin-Verneuil A, Maguin E, Yanick Auffray, Ehrlich DS, Pichereau V (2006) Genetic structure and transcriptional analysis of the arginine deiminase (ADI) cluster in *Lactococcus lactis* MG1363. Can J Microbiol 52(7):617-622 doi:10.1139/w06-009

Casiano-Colón A, Marquis RE (1988) Role of the arginine deiminase system in protecting oral bacteria and an enzymatic basis for acid tolerance. Appl Environ Microbiol 54(6):1318-1324

Cheng C, Dong Z, Han X, Sun J, Wang H, Jiang L, Yang Y, Ma T, Chen Z, Yu J, Fang W, Song H (2017) *Listeria monocytogenes* 10403S arginine repressor ArgR finely tunes arginine metabolism regulation under acidic conditions. Front Microbiol 8:145 doi:10.3389/fmicb.2017.00145

Cho B-K, Federowicz S, Park Y-s, Zengler K, Palsson BØ (2012) Deciphering the transcriptional regulatory logic of amino acid metabolism. Nat Chem Biol 8(1):65-71 doi:10.1038/NCHEMBIO.710

Cho S, Cho Y-B, Kang TJ, Kim SC, Palsson B, Cho B-K (2015) The architecture of ArgR-DNA complexes at the genome-scale in *Escherichia coli*. Nucleic Acids Res 43(6):3079-3088 doi:10.1093/nar/gkv150

Chun BH, Han DM, Kim KH, Jeong SE, Park D, Jeon CO (2019) Genomic and metabolic features of *Tetragenococcus halophilus* as revealed by pan-genome and transcriptome analyses. Food Microbiol 83:36-47 doi:10.1016/j.fm.2019.04.009

Cusumano ZT, Caparon MG (2015) Citrulline Protects *Streptococcus pyogenes* from Acid Stress Using the Arginine Deiminase Pathway and the F_1F_0-ATPase. J Bacteriol 197(7):1288-1296

473 doi:10.1128/JB.02517-14
474 De Angelis M, Mariotti L, Rossi J, Servili M, Fox PF, Rollan G, Gobbetti M (2002) Arginine catabolism by
475 sourdough lactic acid bacteria: purification and characterization of the arginine deiminase pathway
476 enzymes from *Lactobacillus sanfranciscensis* CB1. Appl Environ Microbiol 68(12):6193-6201
477 doi:10.1128/AEM.68.12.6193-6201.2002
478 De Man JC, Rogosa M, Sharpe ME (1960) A medium for the cultivation of *Lactobacilli*. J Appl Bac 23(1):130-
479 135
480 Díez L, Solopova A, Fernández-Pérez R, González M, Tenorio C, Kuipers OP, Ruiz-Larrea F (2017)
481 Transcriptome analysis shows activation of the arginine deiminase pathway in *Lactococcus lactis* as
482 a response to ethanol stress. Int J Food Microbiol 257:41-48 doi:10.1016/j.ijfoodmicro.2017.05.017
483 Garnett JA, Marincs F, Baumberg S, Stockley PG, Phillips SEV (2008) Structure and function of the arginine
484 repressor-operator complex from *Bacillus subtilis*. J Mol Biol 379(2):284-298
485 doi:10.1016/j.jmb.2008.03.007
486 Griswold A, Chen Y-YM, Snyder JA, Burne RA (2004) Characterization of the arginine deiminase operon of
487 *Streptococcus rattus* FA-1. Appl Environ Microbiol 70(3):1321-1327 doi:10.1128/AEM.70.3.1321-
488 1327.2004
489 Gruening P, Fulde M, Valentin-Weigand P, Goethe R (2006) Structure, regulation, and putative function of
490 the arginine deiminase system of *Streptococcus suis*. J Bacteriol 188(2):361-369
491 doi:10.1128/JB.188.2.361-369.2006
492 Hall JW, Ji Y (2013) Sensing and adapting to anaerobic conditions by *Staphylococcus aureus* Adv Appl
493 Microbiol. vol 84. Academic Press, pp 1-25
494 He G, Wu C, Huang J, Zhou R (2017) Metabolic response of *Tetragenococcus halophilus* under salt stress.
495 Biotechnol Bioprocess Eng 22:366-375 doi:10.1007/s12257-017-0015-5
496 Held C, Sadowski G (2016) Compatible solutes: Thermodynamic properties relevant for effective protection
497 against osmotic stress Fluid Phase Equilib 407:224-235 doi:10.1016/j.fluid.2015.07.004
498 Justé A, Lievens B, Frans I, Marsh TL, Klingeberg M, Michiels CW, Willems KA (2008) Genetic and physiological
499 diversity of *Tetragenococcus halophilus* strains isolated from sugar- and salt-rich environments.
500 Microbiology 154(9):2600-2610 doi:10.1099/mic.0.2008/018168-0
501 Karaivanova IM, Weigel P, Takahashi M, Fort C, Versavaud A, Van Duyne G, Charlier D, Hallet J-N, Glansdorff
502 N, Sakanyan V (1999) Mutational analysis of the thermostable arginine repressor from *Bacillus*
503 *stearothermophilus*: dissecting residues involved in DNA binding properties. J Mol Biol 291(4):843-
504 855 doi:10.1006/jmbi.1999.3016
505 Kiupakis AK, Reitzer L (2002) ArgR-independent induction and ArgR-dependent superinduction of the
506 *astCADBE* operon in *Escherichia coli*. J Bacteriol 184(11):2940-2950 doi:10.1128/JB.184.11.2940-
507 2950.2002
508 Kloosterman TG, Kuipers OP (2011) Regulation of arginine acquisition and virulence gene expression in the
509 human pathogen *Streptococcus pneumoniae* by transcription regulators ArgR1 and AhrC. J Biol
510 Chem 286(52):44594-44605 doi:10.1074/jbc.M111.295832
511 Knodler LA, Sekyere EO, Stewart TS, Schofield PJ, Edwards MR (1998) Cloning and expression of a prokaryotic
512 enzyme, arginine deiminase, from a primitive eukaryote *Giardia intestinalis*. J Biol Chem
513 273(8):4470-4477 doi:10.1074/jbc.273.8.4470

514 Kraus JP, Hodges PE, Williamson CL, Horwich AL, Kalousek F, Williams KR, Rosenberg LE (1985) A cDNA clone
515 for the precursor of rat mitochondrial ornithine transcarbamylase: comparison of rat and human
516 leader sequences and conservation of catalytic sites. Nucleic Acids Res 13(3):943-952
517 doi:10.1093/nar/13.3.943
518 Larsen R, Buist G, Kuipers OP, Kok J (2004) ArgR and AhrC are both required for regulation of arginine
519 metabolism in *Lactococcus lactis*. J Bacteriol 186(4):1147-1157 doi:10.1128/JB.186.4.1147-
520 1157.2004
521 Leisner JJ, Tidemand J, Larsen LM (1994) Catabolism of arginine by *Carnobacterium* spp. isolated from
522 vacuum-packed sugar-salted fish. Curr Microbiol 29:95-99
523 Lin J, Liang H, Yan J, Luo L (2017) The molecular mechanism and post-transcriptional regulation characteristic
524 of *Tetragenococcus halophilus* acclimation to osmotic stress revealed by quantitative proteomics. J
525 Proteomics 168(25):1-14 doi:10.1016/j.jprot.2017.08.014
526 Liu L, Si L, Meng X, Luo L (2015) Comparative transcriptomic analysis reveals novel genes and regulatory
527 mechanisms of *Tetragenococcus halophilus* in response to salt stress. J Ind Microbiol Biotechnol
528 42:601-616 doi:10.1007/s10295-014-1579-0
529 Liu SQ, Pilone GJ (1998) A review: arginine metabolism in wine lactic acid bacteria and its practical
530 significance. J Appl Microbiol 84(3):315-327 doi:10.1046/j.1365-2672.1998.00350.x
531 Maas WK, Clark AJ (1964) Studies on the mechanism of repression of arginine biosynthesis in *Escherichia*
532 *coli*: II. Dominance of repressibility in diploids. J Mol Biol 8(3):365-370 doi:10.1016/S0022-
533 2836(64)80200-X
534 Maghnouj A, Abu-Bakr AAW, Baumberg S, Stalon V, Vander Wauven C (2000a) Regulation of anaerobic
535 arginine catabolism in *Bacillus licheniformis* by a protein of the Crp/Fnr family. FEMS Microbiol Lett
536 191(2):227-234 doi:10.1111/j.1574-6968.2000.tb09344.x
537 Maghnouj A, Abu-Bakr AAW, Baumberg S, Stalon V, Wauven CV (2000b) Regulation of anaerobic arginine
538 catabolism in *Bacillus licheniformis* by a protein of the Crp/Fnr family. FEMS Microbiol Lett
539 191(2):227-234 doi:10.1111/j.1574-6968.2000.tb09344.x
540 Maghnouj A, de Sousa Cabral TF, Stalon V, Wauven CV (1998) The *arcABDC* gene cluster, encoding the
541 arginine deiminase pathway of *Bacillus licheniformis*, and its activation by the arginine repressor
542 ArgR. J Bacteriol 180(24):6468-6475 doi:10.1128/JB.180.24.6468-6475.1998
543 Makhlin J, Kofman T, Borovok I, Kohler C, Engelmann S, Cohen G, Aharonowitz Y (2007) *Staphylococcus*
544 *aureus* ArcR controls expression of the arginine deiminase operon. J Bacteriol 189(16):5976–5986
545 doi:10.1128/JB.00592-07
546 Ohtani K, Bando M, Swe T, Banu S, Oe M, Hayashi H, Shimizu T (1997) Collagenase gene (*colA*) is located in
547 the 3′ -flanking region of the perfringolysin O (*pfoA*) locus in *Clostridium perfringens*. FEMS
548 Microbiol Lett 146(1):155-159 doi:10.1111/j.1574-6968.1997.tb10186.x
549 Pérez-Redondo R, Rodríguez-García A, Botas A, Santamarta I, Martín JF, Liras P (2012) ArgR of *Streptomyces*
550 *coelicolor* is a versatile regulator. PLoS One 7(3):e32697 doi:10.1371/journal.pone.0032697
551 Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT–PCR. Nucleic Acids
552 Res 29(9):e45 doi:10.1093/nar/29.9.e45
553 Robert H, Le Marrec C, Blanco C, Jebbar M (2000) Glycine betaine, carnitine, and choline enhance salinity
554 tolerance and prevent the accumulation of sodium to a level inhibiting growth of *Tetragenococcus*

halophila. Appl Environ Microbiol 66(2):509-517 doi:10.1128/AEM.66.2.509-517.2000

Schaumburg CS, Tan M (2006) Arginine-dependent gene regulation via the ArgR repressor is species specific in *Chlamydia*. J Bacteriol 188(3):919-927 doi:10.1128/JB.188.3.919-927.2006

Schulz C, Gierok P, Petruschka L, Lalk M, Mäder U, Hammerschmidt S (2014) Regulation of the arginine deiminase system by ArgR2 interferes with arginine metabolism and fitness of *Streptococcus pneumoniae*. Mbio 5(6):e01858-14 doi:10.1128/mBio.01858-14

Solovyev V, Salamov A (2011) Automatic annotation of microbial genomes and metagenomic sequences. Paper presented at the Metagenomics and its applications in agriculture,

Sugimoto S, Nakayama J, Fukuda D, Sonezaki S, Watanabe M, Tosukhowong A, Sonomoto K (2003) Effect of heterologous expression of molecular chaperone DnaK from *Tetragenococcus halophilus* on salinity adaptation of *Escherichia coli*. J Biosci Bioeng 96(2):129-133 doi:10.1016/S1389-1723(03)90114-9

Sugimoto S, Yoshida H, Mizunoe Y, Tsuruno K, Nakayama J, Sonomoto K (2006) Structural and functional conversion of molecular chaperone ClpB from the gram-positive halophilic lactic acid bacterium *Tetragenococcus halophilus* mediated by ATP and stress. J Bacteriol 188(23):8070-8078 doi:10.1128/JB.00404-06

Sunnerhagen M, Nilges M, Otting G, Carey J (1997) Solution structure of the DNA-binding domain and model for the complex of multifunctiona hexameric arginine represser with DNA. Nat Struct Biol 4(10):819-826

Tan X-E, Neoh H-m, Looi M-L, Chin SF, Cui L, Hiramatsu K, Hussin S, Jamal R (2017) Activated ADI pathway: the initiator of intermediate vancomycin resistance in *Staphylococcus aureus*. Can J Microbiol 63(3):260-264 doi:10.1139/cjm-2016-0439

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22(22):4673–4680. doi:10.1093/nar/22.22.4673

Tian G, Maas WK (1994) Mutational analysis of the arginine repressor of *Escherichia coli*. Mol Microbiol 13(4):599-608 doi:10.1111/j.1365-2958.1994.tb00454.x

Van Duynef GD, Ghosh G, Maas WK, Sigler PB (1996) Structure of the oligomerization and L-arginine binding domain of the arginine repressor of *Escherichia coli*. J Mol Biol 256(2):377-391 doi:10.1006/jmbi.1996.0093

Verhoogt HJ, Smit H, Abee T, Gamper M, Driessen AJ, Haas D, Konings WN (1992) *arcD*, the first gene of the *arc* operon for anaerobic arginine catabolism in *Pseudomonas aeruginosa*, encodes an arginine-ornithine exchanger. J Bacteriol 174(5):1568-1573 doi:10.1128/jb.174.5.1568-1573.1992

Vrancken G, Rimaux T, Wouters D, Leroy F, De Vuyst L (2009) The arginine deiminase pathway of *Lactobacillus fermentum* IMDO 130101 responds to growth under stress conditions of both temperature and salt. Food Microbiol 26(7):720-727 doi:10.1016/j.fm.2009.07.006

Xiong L, Teng JLL, Botelho MG, Lo RC, Lau SKP, Woo aPCY (2016) Arginine metabolism in bacterial pathogenesis and cancer therapy. Int J Mol Sci 17(3):363 doi:10.3390/ijms17030363

Xiong L, Teng JLL, Watt RM, Liu C, Lau SKP, Woo PCY (2015) Molecular characterization of arginine deiminase pathway in *Laribacter hongkongensis* and unique regulation of arginine catabolism and anabolism by multiple environmental stresses. Environ Microbiol 17(11):4469-4483 doi:10.1111/1462-2920.12897

596 Yim S-H, Jung S, Lee S-k, Cheon C-I, Song E, Lee S-S, Shin J, Lee M-S (2011) Purification and characterization
597 of an arginine regulatory protein, ArgR, in *Corynebacterium glutamicum*. J Ind Microbiol Biotechnol
598 38(12):1911-1920 doi:10.1007/s10295-011-0977-9
599 Zheng J, Wittouck S, Salvetti E, Franz CMAP, Harris HMB, Mattarelli P, O'Toole PW, Pot B, Vandamme P, Walter
600 J, Watanabe K, Wuyts S, Felis GE, Gänzle MG, Lebeer S (2020) A taxonomic note on the genus
601 *Lactobacillus*: Description of 23 novel genera, emended description of the genus *Lactobacillus*
602 *Beijerinck* 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. Int J Syst Evol Microbiol
603 70(4):2782–2858 doi:10.1099/ijsem.0.004107
604 Zúñiga M, del Carmen Miralles M, Pérez-Martínez G (2002a) The Product of *arcR*, the sixth gene of the *arc*
605 operon of *Lactobacillus sakei*, is essential for expression of the arginine deiminase pathway. Appl
606 Environ Microbiol 68(12):6051-6058 doi:10.1128/AEM.68.12.6051-6058.2002
607 Zúñiga M, Pérez G, González-Candelas F (2002b) Evolution of arginine deiminase (ADI) pathway genes. Mol
608 Phylogenet Evol 25(3):429-444 doi:10.1016/S1055-7903(02)00277-4

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Figure legends

Figure 1. Schematic organization of *T. halophilus* ADI pathway of *T. halophilus* and the identification of putative promoter sequences. (A) The genes encoding *arc* operon and ArgR regulators were closely clustered in *T. halophilus* genome. The *arc* operon is composed of *arcA*, *arcB*, *arcC*, *arcR* and *arcD* and located on the complementary strand, directly upstream of *argR1* and *argR2*. The identified promoters were shown by arrows. The activities of the putative promoter sequences of the ADI pathway were verified by (B) evaluating the transcription of reporter gene induced by the promoter; briefly, *E. coli* strains harboring pUC-ParcA-eGFP, pUC-ParcB-eGFP, pUC-ParcC-eGFP, pUC-ParcD-eGFP, pUC-ParcR-eGFP, pUC-PargR1-eGFP, pUC-PargR2-eGFP and control plasmid pUC-eGFP were cultured until the exponential phase of growth, then the numbers of fluorescence and OD600 were measured using microplate reader, the measurements were conducted in 3 replicates. The fluorescence/OD_{600} value of each sample represents the activity of each promoter. Fold change was generated by dividing the value of each sampled by the value of the control; and (C) observing the fluorescence emission of the strains using confocal microscopy with the same parameters; the studied promoters were illustrated above or below each microscopy picture.

Figure 2. The transcription levels of genes involved in the ADI pathway in the presence or absence of arginine or glucose. 0G0A indicates no glucose or arginine was present, 0G5A indicates 5 mM arginine was added but glucose was not present, 2G0A indicates 2% glucose was added but arginine was not present, which is the normal recipe of MRS, 2G5A indicates that 2% glucose and 5 mM arginine were added to the medium. 16S rRNA was used as endogenous control and gene expression was calculated relative to 2G0A. Gene expression data under different conditions obtained from 3 biological replicates were subjected to one-way analysis of variance (one-way ANOVA), comparisons were made between the mean of each column and that of every

632 other column, expression of the same gene under different conditions is significantly different (P<0.05) if the
633 bars do not share a common superscript. 2G0A condition was chosen as control since it's the optimal growth
634 condition of *T. halophilus*.

635 Figure 3. Evaluating the binding abilities of (A) ArgR1 and (B) ArgR2 to the promoter sequences of the ADI
636 pathway. EMSAs were conducted using increasing concentration of the proteins and promoter sequences
637 labeled with biotin. "N" indicated that no protein was added in the reaction mixtures; "C" indicated 200-fold
638 molar excess of unlabeled promoter sequences were added, which was used as positive control to confirm
639 the specific binding. The number below each lane represent the amount of protein added. The amount of
640 ArgR1 in each lane was increased from 100ng to 800ng for each promoter sequence. The amount of ArgR2
641 added from lane 2 to lane 4 is 100ng, 200ng, 400ng, respectively.

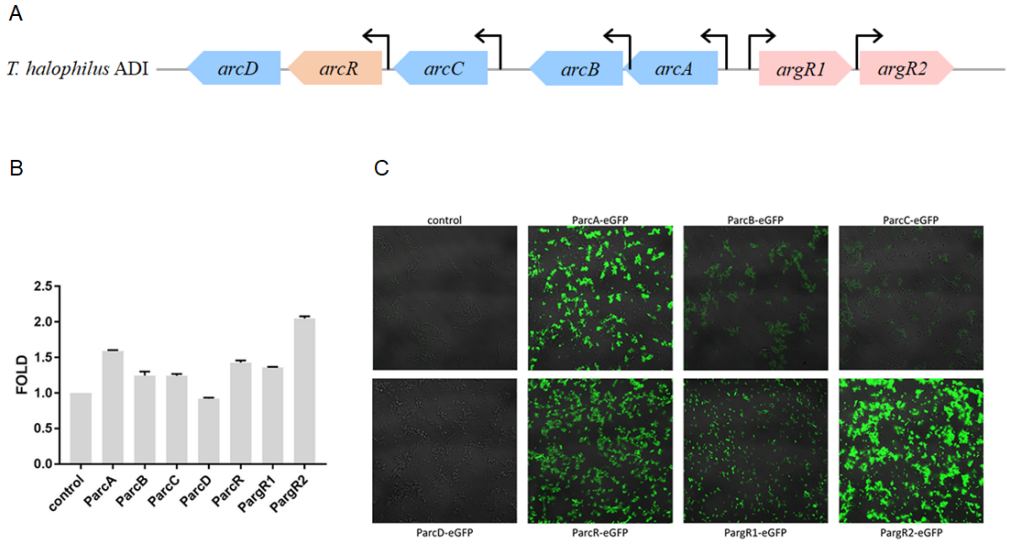
642 Figure 4. Investigating the regulatory effects of the regulators on the ADI promoters. (A) Experimental design:
643 the regulator and the reporter gene were co-transcribed by the promoter studied, by measuring the florescence
644 changes after mutating the DNA binding sites of the regulator, the effect of the binding of the regulator to
645 promoters can be evaluated. The fluorescence/OD600 values of strains containing wild-type or mutated (B)
646 ArgR1 or (C) ArgR2 were measured; the fluorescence/OD600 values of strains containing the mutated
647 regulators were illustrated by orange, the values of the wild-type were illustrated by blue.

648 Figure 5. The binding and regulatory effects of ArgR1 and ArgR2 on the promoter sequence of the arginine
649 biosynthesis pathway. (A) EMSAs were conducted with increasing concentration of ArgR1 (200ng, 400ng,
650 800ng from lane 2 to lane 4) and ArgR2 (200ng, 400ng, 600ng from lane 2 to lane 4), the promoter sequence
651 *PargG* labeled with biotin was used as probe; "N" indicated that no protein was added in the reaction
652 mixtures; "C" indicated 200-fold molar excess of unlabeled promoter sequences were added, which was used

as positive control to confirm the specific binding. (B) similarly, to investigate the regulatory effects of the regulators, promoter-regulator-reporter plasmids were constructed, and the fluorescence changes after mutating the DNA binding sites of regulator were measured.

Figure 6. (A) The regulatory pattern of arginine metabolism of *T. halophilus* revealed by current study. AB indicated the arginine biosynthesis pathway. The ADI pathway and AB pathway are repressed by ArgR regulators, higher salinity concentration and the presence of arginine can induce the expression of the ADI pathway by abolishing the binding of ArgR1; while ArgR2 still binds to the promoter sequence of the AB pathway, thus arginine biosynthesis is still repressed with the addition of arginine. (B) Phylogenetic analysis of previously studied ArgR regulators, the regulatory effects of these regulators on 3 arginine metabolism pathway: arginine biosynthesis (AB), arginine deiminase (ADI) and arginase pathway (*roc*) were illustrated; red boxes indicate negative and green indicate positive regulatory effects of the regulators, minus signs indicate the absence of the pathway, multiplication signs indicate that the regulators do not regulate the pathway and blanks indicate unknown regulatory effects.

598 Figure 1



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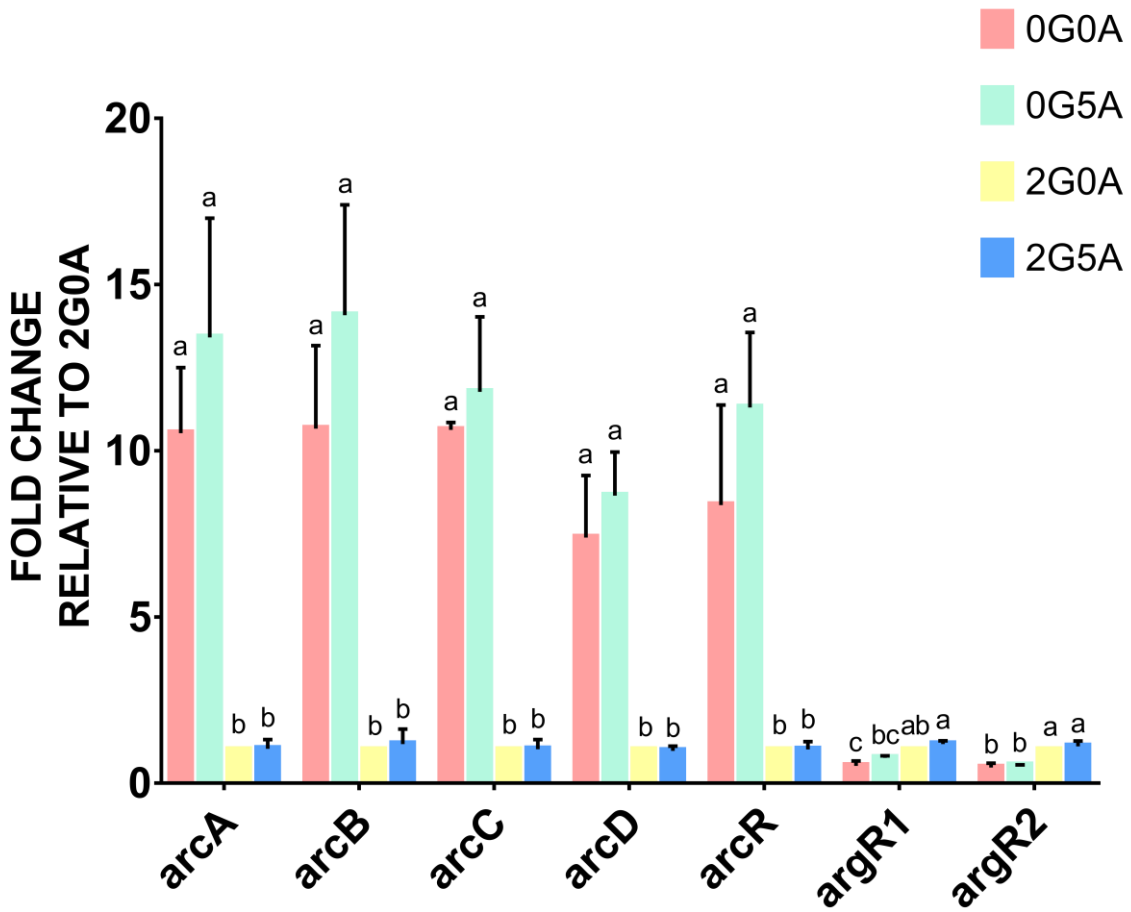
601 Figure 2



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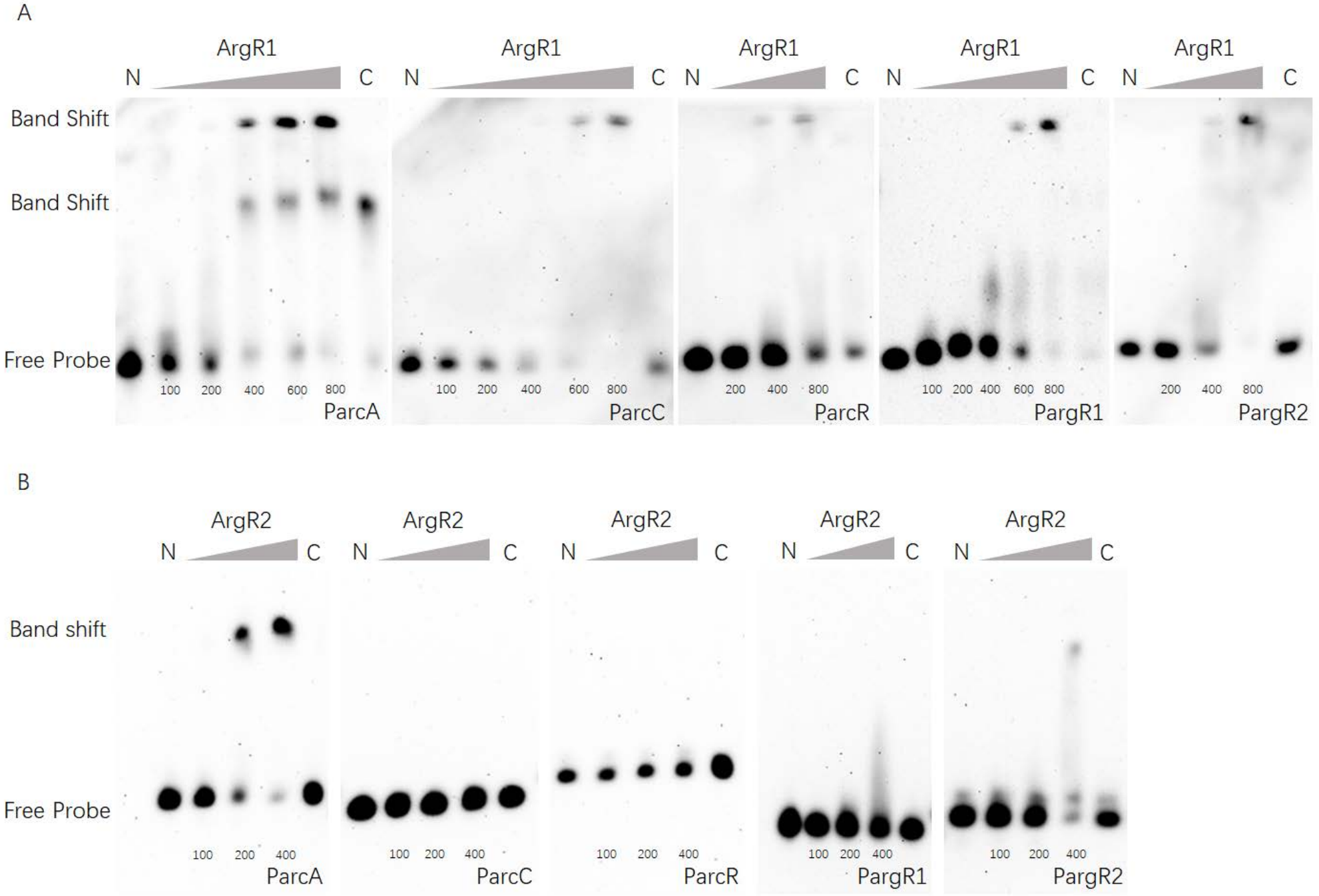
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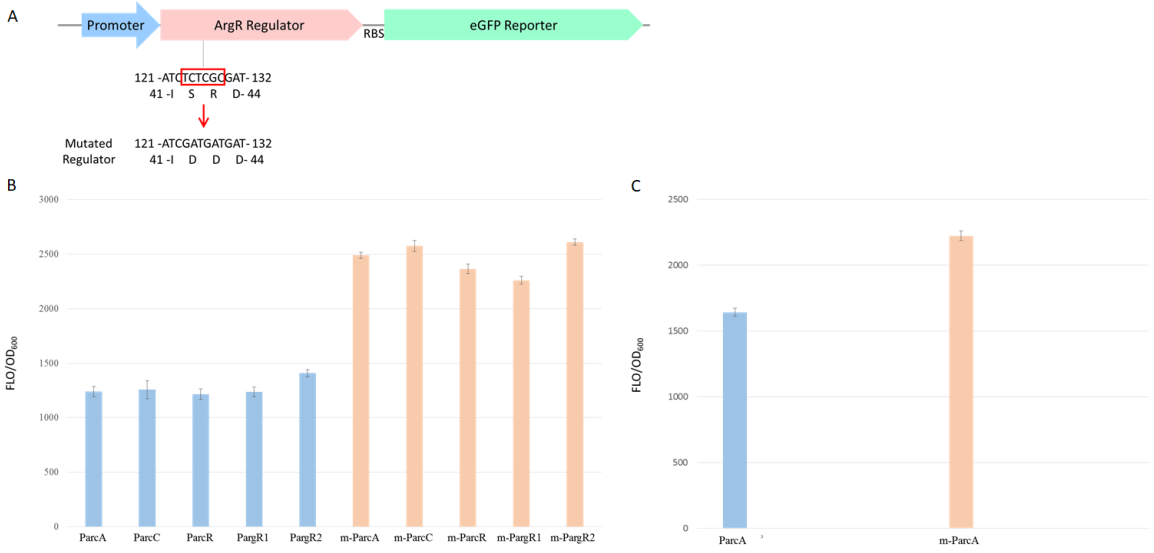
ArgR1
N
C
Band Shift
Band Shift
Free Probe
100 200 400 600 800
ParcA
ParcC
200 400 800
ParcR
PargR1
PargR2
B
ArgR2
Band shift
100 200 400
ParcA
ParcC
ParcR
PargR1
PargR2

606 Figure 4



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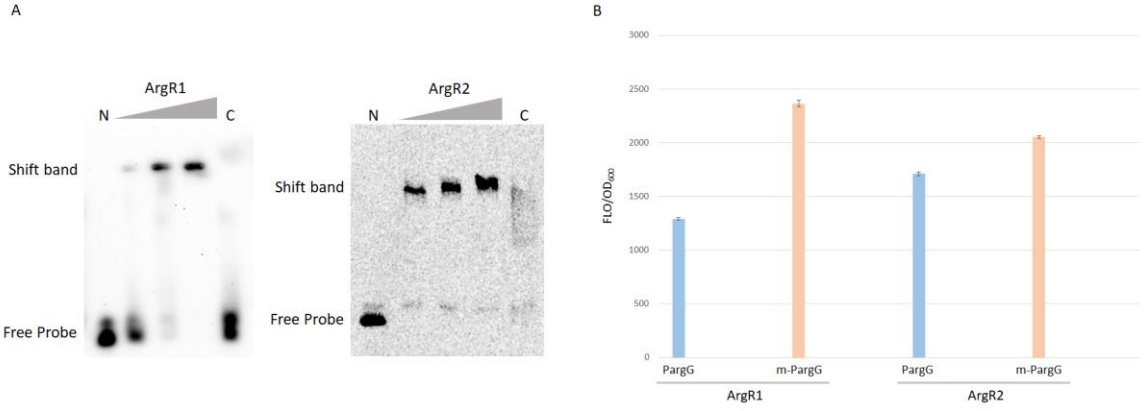
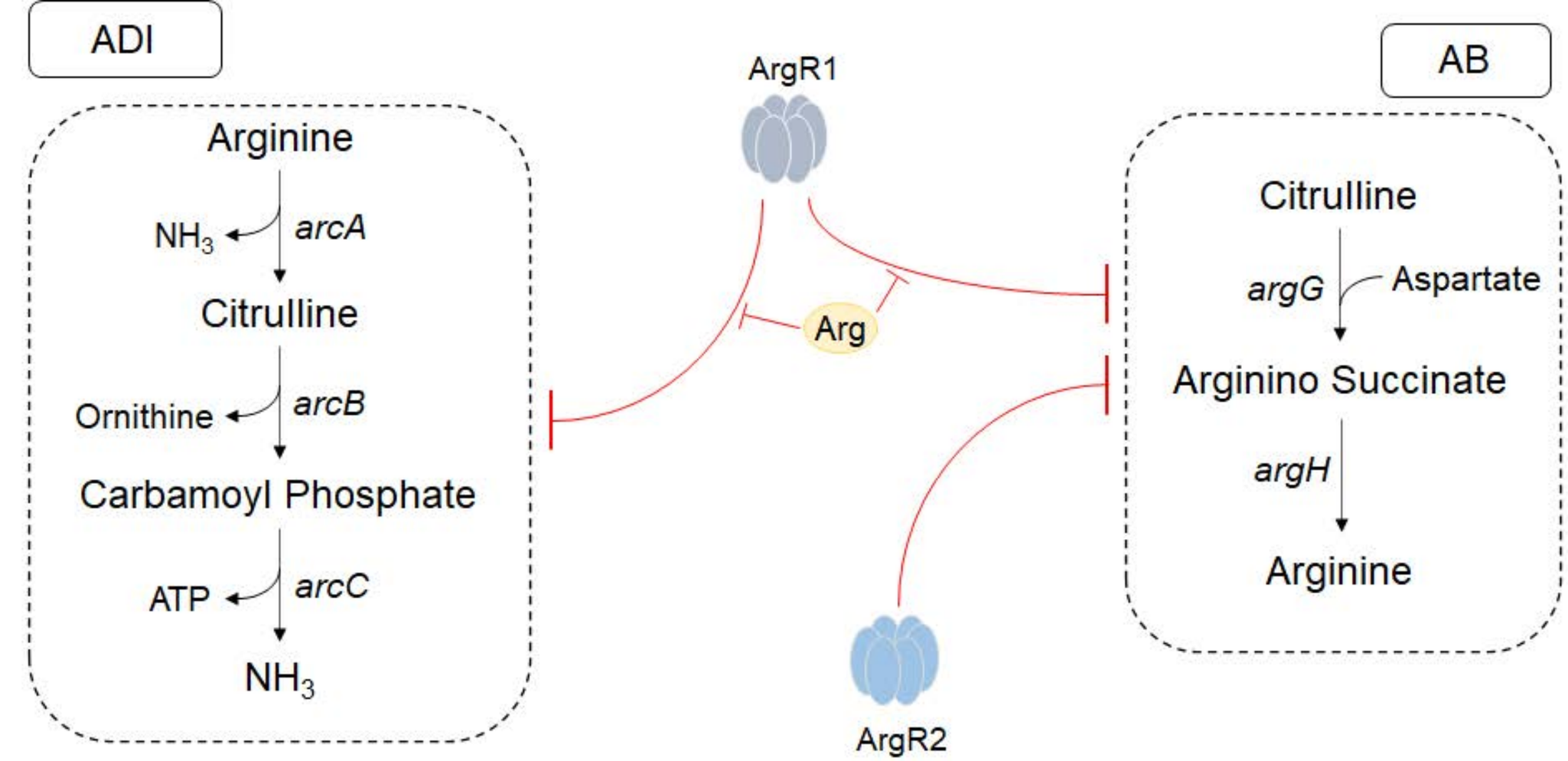
609 Figure 5



610

A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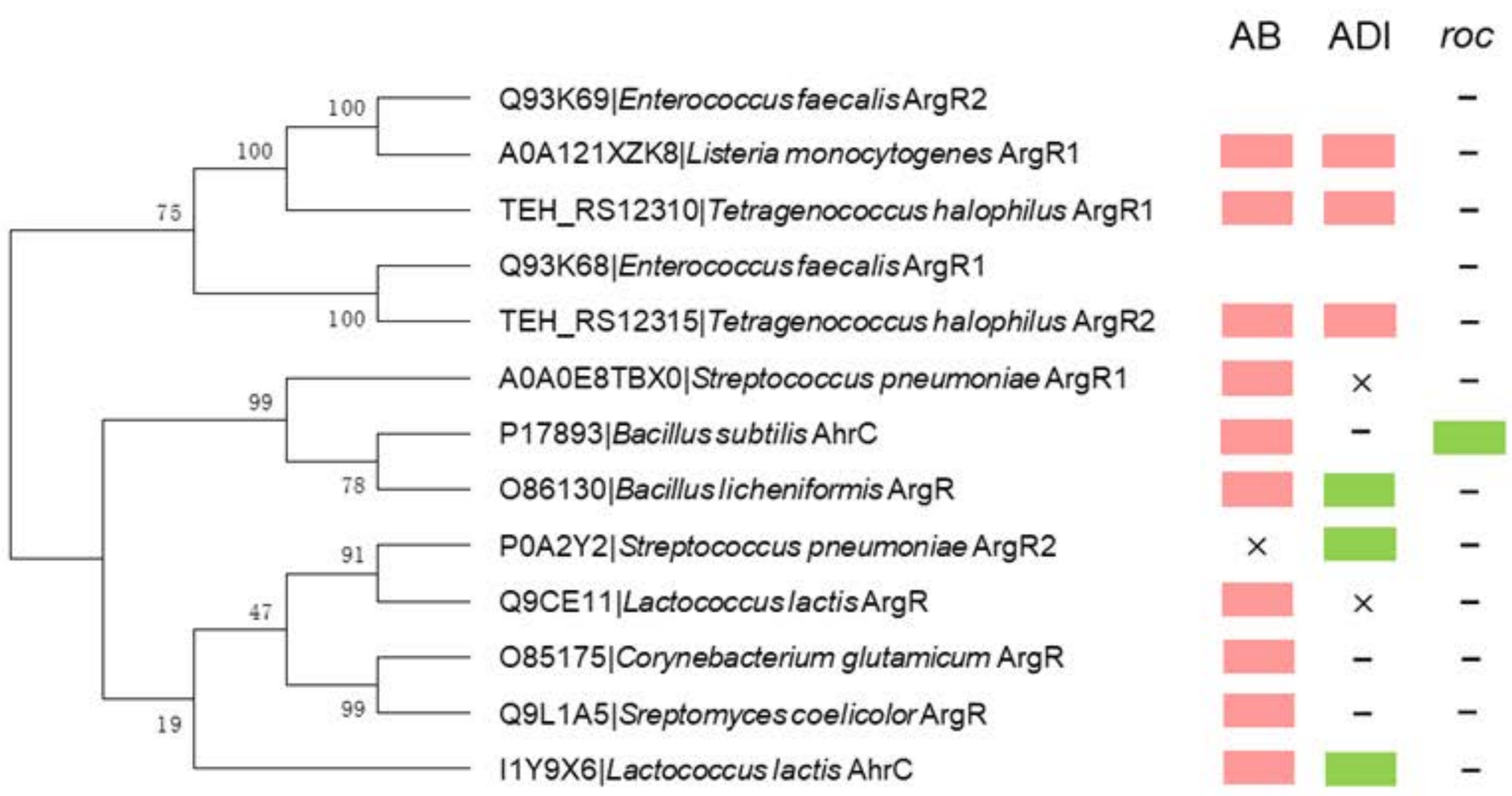
NH3
arcA
Citrulline
Ornithine
arcB
Carbamoyl Phosphate
ATP
arcC
NH3
ArgR1
Arg
ArgR2
AB
Citrulline
argG
Aspartate
Arginino Succinate
argH
Arginine

B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ADI
roc
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A0A121XZK8|Listeria monocytogenes ArgR1
TEH_RS12310|Tetragenococcus halophilus ArgR1
Q93K68|Enterococcus faecalis ArgR1
TEH_RS12315|Tetragenococcus halophilus ArgR2
A0A0E8TBX0|Streptococcus pneumoniae ArgR1
P17893|Bacillus subtilis AhrC
O86130|Bacillus licheniformis ArgR
P0A2Y2|Streptococcus pneumoniae ArgR2
Q9CE11|Lactococcus lactis ArgR
O85175|Corynebacterium glutamicum ArgR
Q9L1A5|Sreptomyces coelicolor ArgR
I1Y9X6|Lactococcus lactis AhrC
100
100
75
100
99
78
91
47
99
19

Applied Microbiology and Biotechnology

Supplemental Material of **Characterization of the two nonidentical ArgR regulators of *Tetragenococcus halophilus* and their regulatory effects on arginine metabolism**

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Table S1 Primers used in qRT-PCR

primer name	sequences	fragment amplified
qRT-arcA-F	TGCTGCTGCAAGAGAGCAATGG	arcA
qRT-arcA-R	CCACGTCCACGCACTAGTTCAC	
qRT-arcB-F	AACGTGGCGTACCGCATCATT	arcB
qRT-arcB-R	CCACACGGGAACACCAGAATGT	
qRT-arcC-F	AAGAAGATGCAGGCCGAGGTTG	arcC
qRT-arcC-R	GATGCCACCACCACCACATGAA	
qRT-arcD-F	AATCGGGCGCACTTGAATCTGG	arcD
qRT-arcD-R	GCATCCATTGCTACGCCTGTTG	
qRT-arcR-F	TCAGCAATCGCGCAAACGGATA	arcR
qRT-arcR-R	ATCGTCACCGGAGAGGCAAGTT	
qRT-argR1-F	AACAGTCCTTCCTGGCAATGGT	argR1
qRT-argR1-R	CCATGCGAGCGTCCTCTTCATT	
qRT-argR2-F	TCCTAACTATTGTCCACACACTTCCT	argR2
qRT-argR2-R	TCTCCATCTCTATAGCACGAGTTTCA	
qRT-argG-F	CAAGCCAGTCTTATGCCATTGATGC	argG
qRT-argG-R	CCATGAGCGATTGTTGTTGCGTTAG	
qRT-argH-F	GCAGCACGCTCAACCTATCTCTT	argH
qRT-argH-R	TCTCGATCACTAACTGCATCCAAGC	

Table S2 Primers used in RT-PCR

primer name	sequences	fragment amplified
RT-C-F	TTACACACGTGGGATAAATAAATTACCTA	arcB-arcA
RT-C-R	ATGAGTAAGCCAATTAATGTTTTTTCAGAAAT	
RT-D-F	TTATTTTGTAAGGTCTTCACGATATAAAGGC	arcA-argR1
RT-D-R	TTAAGAAACGTGAAATAAATATTGTTGAAAGTAT	
RT-E-F	ATGAACCGTACACAACGTCAAG	argR1-argR2
RT-E-R	TTAGTTTCCTATTTGTTCTCTTGTTTGT	

Table S3 Primer used in the construction of promoter-regulator plasmids

primer name	sequences	plasmid construced
p-ParcA-e F	TACTGAGAGTGCACCATATGTATAAAATGAAAAATTAATGAT	pUC-ParcA-eGFP
p-ParcA-e R	TCCTCGCCCTTGCTCACCATAACTTAAGTCCTCCTCTTT	
PA-eGFP-p F	TAAAGAGGAGGACTTAAGTTATGGTGAGCAAGGGCG	
PA-eGFP-p R	GCCGATTCATTAATGCAGCTGTTACTTGTACAGCTCGTCC	
p-ParcB-e F	TACTGAGAGTGCACCATATGCAAACACTTTAACAATTGCT	pUC-ParcB-eGFP
p-ParcB-e R	TCCTCGCCCTTGCTCACCATTATAAACTCTCCTGTTCTTTATT	
PB-eGFP-p F	AAAGAACAGGAGAGTTTATAATGGTGAGCAAGGGCG	
PB-eGFP-p R	GCCGATTCATTAATGCAGCTGTTACTTGTACAGCTCGTCC	
p-ParcC-e F	TACTGAGAGTGCACCATATGTGCATTCAATTAAAGCTATC	pUC-ParcC-eGFP
p-ParcC-e R	TCCTCGCCCTTGCTCACCATAATTATTATGTCCTTCCTTTCC	
PC-eGFP-p F	AAAGGAAGGACATAATAATTATGGTGAGCAAGGGCG	
PC-eGFP-p R	GCCGATTCATTAATGCAGCTGTTACTTGTACAGCTCGTCC	
p-ParcD-e F	TACTGAGAGTGCACCATATGGGTGACGATTACGGATATTT	pUC-ParcD-eGFP
p-ParcD-e R	TCCTCGCCCTTGCTCACCATTTTATTTTTCACAGTTAGAGACTG	
PD-eGFP-p F	CTCTAACTGTGAAAAATAAAATGGTGAGCAAGGGCG	
PD-eGFP-p R	GCCGATTCATTAATGCAGCTGTTACTTGTACAGCTCGTCC	
p-ParcR-e F	TACTGAGAGTGCACCATATGCAGAATTAGAAGAATATAAAG	pUC-ParcR-eGFP
p-ParcR-e R	TCCTCGCCCTTGCTCACCATTTTAACTCTCCTAAAAAGC	
PR-eGFP-p F	CTTTTTAGGAGAGTTAAATTACTTGTACAGCTCGTCC	
PR-eGFP-p R	GCCGATTCATTAATGCAGCTGTTACTTGTACAGCTCGTCC	
p-PargR1-e F	TACTGAGAGTGCACCATATGCAAATTTTTGCTGTATATTT	pUC-PargR1-eGFP
p-PargR1-e R	TCCTCGCCCTTGCTCACCATTTGTTCACCACCTTCAC	
PR1-eGFP-p F	TAAGTGAAGGTGGTGAACAAATGGTGAGCAAGGGCG	
PR1-eGFP-p R	GCCGATTCATTAATGCAGCTGTTACTTGTACAGCTCGTCC	
p-PargR2-e F	TACTGAGAGTGCACCATATGAATAGCAAAATTGTTGGCTG	pUC-PargR2-eGFP
p-PargR2-e R	TCCTCGCCCTTGCTCACCATTTTTTTCACCTCTTAATGTATC	
PR2-eGFP-p F	TACATTAAGAGGTGAAAAAAATGGTGAGCAAGGGCG	
PR2-eGFP-p R	GCCGATTCATTAATGCAGCTGTTACTTGTACAGCTCGTCC	
NdeI-eGFP-PvuII F	ATTCATATGATGGTGAGCAAGGGC	pUC-eGFP*
NdeI-eGFP-PvuII R	GCGCAGCTGTTACTTGTACAGCTC	

*Restriction sites were underlined in the primer sequences

Table S4 Primers used in the construction of the promoter-regulator-reporter plasmids

primer name	sequences	plasmid construced
p-PA-R1 F	TACTGAGAGTGCACCATATGTATAAAATGAAAAATTAATGAT	pUC-ParcA-ArgR1-RBSa-eGFP
P-PA-R1 R	TGACGTTGTGTACGGTTCATAACTTAAGTCCTCCTCTTT	
PA-R1-RBSa F	ATGAACCGTACACAACGT	
PA-R1-RBSa R	AACTTAAGTCCTCCTCTTTATTAAGAAACGTGAAATAAATATTGTTG	
RBSa-eGFP-p F	TAAAGAGGAGGACTTAAGTTATGGTGAGCAAGGGCG	
RBSa-eGFP-p R	GCCGATTCATTAATGCAGCTGTTACTTGTACAGCTCGTCC	
p-PC-R1 F	TACTGAGAGTGCACCATATGTGCATTCAATTAAAGCTATC	pUC-ParcC-ArgR1-RBSceGFP
p-PC-R1 R	TGACGTTGTGTACGGTTCATAATTATTATGTCCTTCCTTTCC	
PC-R1-RBSc F	ATGAACCGTACACAACGT	
PC-R1-RBSc R	AATTATTATGTCCTTCCTTTCCTTTTTAAGAAACGTGAAATAAATATTGTTG	
RBSc-eGFP-p F	AAAGGAAAGGAAGGACATAATAATTATGGTGAGCAAGGGCG	
RBSc-eGFP-p R	GCCGATTCATTAATGCAGCTGTTACTTGTACAGCTCGTCC	
p-PR-R1 F	TACTGAGAGTGCACCATATGCAGAATTAGAAGAATATAAAGAACAAGG	pUC-ParcR-ArgR1-RBSr-eGFP
p-PR-R1 R	TGACGTTGTGTACGGTTCATTTTAACTCTCCTAAAAAGCTTTTAT	
PR-R1-RBSr F	ATGAACCGTACACAACGT	
PR-R1-RBSr R	TTTAACTCTCCTAAAAAGCTTTAAGAAACGTGAAATAAATATTGTTG	
RBSr-eGFP-p F	AGCTTTTTAGGAGAGTTAAAATGGTGAGCAAGGGCG	
RBSr-eGFP-p R	GCCGATTCATTAATGCAGCTGTTACTTGTACAGCTCGTCC	
p-PR1-R1 F	TACTGAGAGTGCACCATATGCAAATTTTTGCTGTATATTTATCATT	pUC-PargR1-ArgR1-RBSr1-eGFP
p-PR1-R1 R	TGACGTTGTGTACGGTTCATTTGTTCACCACCTTCAC	
PR1-R1-RBSr1 F	ATGAACCGTACACAACGT	
PR1-R1-RBSr1 R	TTGTTCACCACCTTCACTTATTAAGAAACGTGAAATAAATATTGTTG	
RBSr1-eGFP-p F	TAAGTGAAGGTGGTGAACAAATGGTGAGCAAGGGCG	
RBSr1-eGFP-p R	GCCGATTCATTAATGCAGCTGTTACTTGTACAGCTCGTCC	
p-PR2-R1 F	TACTGAGAGTGCACCATATGAATAGCAAAATTGTTGGCT	pUC-PargR2-ArgR1-RBSr2-eGFP
p-PR2-R1 R	TGACGTTGTGTACGGTTCATTTTTTTCACCTCTTAATGTATCT	
PR2-R1-RBSr2 F	ATGAACCGTACACAACGT	
PR2-R1-RBSr2 R	TTTTTTCACCTCTTAATGTATTAAGAAACGTGAAATAAATATTGTTG	
RBSr2-eGFP-p F	TACATTAAGAGGTGAAAAAAATGGTGAGCAAGGGCG	
RBSr2-eGFP-p R	GCCGATTCATTAATGCAGCTGTTACTTGTACAGCTCGTCC	
p-PG-R1 F	TACTGAGAGTGCACCATATGTATAATTTGCTCCTTTGTTTAAAAATAAGTGAAA	pUC-PargG-ArgR1-RBSg-eGFP
p-PG-R1 R	TACGGTTCATAATTATGCATCCCCTAAATTGAATTTAAAC	
PG-R1-RBSg F	ATGCATAATTATGAACCGTACACAACGTCA	
PG-R1-RBSg R	AATTATGCATCCCTAAATTGTTAAGAAACGTGAAATAAATATTGTTGAAAGTATT	
RBSg-eGFP F	CAATTTAGGGATGCATAATTATGGTGAGCAAGGGCG	
RBSg-eGFP R	GCCGATTCATTAATGCAGCTGTTACTTGTACAGCTCGTCCATG	
p-PA-R2 F	TACTGAGAGTGCACCATATGTATAAAATGAAAAATTAATGAT	pUC-ParcA-ArgR2-RBSa-eGFP
p-PA-R2 R	TGCCGTTCATTTTTATGCAAAACTTAAGTCCTCCTCTTT	

PA-R2-RBSa F	TTGCATAAAAATGAACGGCAA	
PA-R2-RBSa R	AACTTAAGTCCTCCTCTTTATTAGTTTCCTATTTGTTCTCTTGT	
RBSa-eGFP F	TAAAGAGGAGGACTTAAGTTATGGTGAGCAAGGGCG	
RBSa-eGFP R	GCCGATTCATTAATGCAGCTGTTACTTGTACAGCTCGTCC	
p-PG-R2 F	TACTGAGAGTGCACCATATGTATAATTTGCTCCTTTGTTTAAAAATAAGTGAAA	pUC-PargG-ArgR2-RBSg-eGFP
p-PG-R2 R	TTTTATGCAAAATTATGCATCCCCTAAATTGAATTTAAAC	
PG-R2-RBSg F	ATGCATAATTTTGCATAAAAATGAACGGCAAAG	
PG-R2-RBSg R	AATTATGCATCCCCTAAATTGTTAGTTTCCTATTTGTTCTCTTGTTTGTT	
RBSg-eGFP F	CAATTTAGGGATGCATAATTATGGTGAGCAAGGGCG	
RBSg-eGFP R	GCCGATTCATTAATGCAGCTGTTACTTGTACAGCTCGTCCATG	

Table S5 Primers used in site direct mutation

primer name	sequences	fragment construced
R1 S42D F	CTACACAAGCAACCATCGATCGCGATATAC	ArgR1mutS42D
R1 S42D R	ATCGATGGTTGCTTGTGTAGTAAGAGCTTT	
R1 42D R43D F	CACAAGCAACCATCGATGATGATATACGGG	ArgR1mutS42D R43D
R1 42D R43D R	ATCATCGATGGTTGCTTGTGTAGTAAGAGC	
R2 S42D F	GCACAGGCAACTATTGATAGAGATATACGT	ArgR2mutS42D
R2 S42D R	ATCAATAGTTGCCTGTGCAATAGTTATATC	
R2 42D R43D F	AACTATTGCACAGGCAACTATTGATGAT	ArgR2mutS42D R43D
R2 42D R43D R	CACGTATATCATCATCAATAGTTGCCTG	

Table S6 Primers used in the overexpression of ArgR1 and ArgR2

primer name	sequences	gene amplified
P-argR1-E-F	ATGCTGCAGATGAACCGTACACA	argR1
P-argR1-E-R	GCGCGATATCTTAAGAAACGTGAAAT	
P-argR2-E-F	ATGCTGCAGTTGCATAAAAATGAAC	argR2
P-argR2-E-R	GCGCGATATCTTAGTTTCCTATTTGT	

*Restriction sites were underlined in the primer sequences

Table S7 Sequence of the probes used in EMSA assays

promoter	sequences of probes
ParcA	ATAATTGAATAACGTTGTATAATTTCCTTTTTATTTGCTGAATATTTCACAATTGGTGATTGGATAATTTATTATCCTTA
ParcC	TTATTATATAGTTTAATTAATGTTAACTGCAGACTATTTTTTACTAATAATTTTTATTTATTAAAGG
ParcR	GATAATTACATCTTTAGAAAATTTAGGTAAAATGGAACAAAATGAGACTATCGGTACCGTTATAACTAAATAAAAGCTTTTTAG
PargR1	CATTTTATACATAATTTTTTTTATTCTTTAATTTGAAACGCTTGTTCACTTTAATACAATCGTTT
PargR2	TTTATTTCACGTTTCTTAATAAAAATATTATTAAGTAGTCCTTTAGTTTTATTTTTGATATAATTAATATGAATTAAGATACATT
PargG	TGCATAAATACAACCTGTATTAAAAAATAATGAATATTTATTCGCTGATCTATTGATTAAATAAAAGTAATTTGCTAACATATAGTTTAAATTCAAT

Figure S1

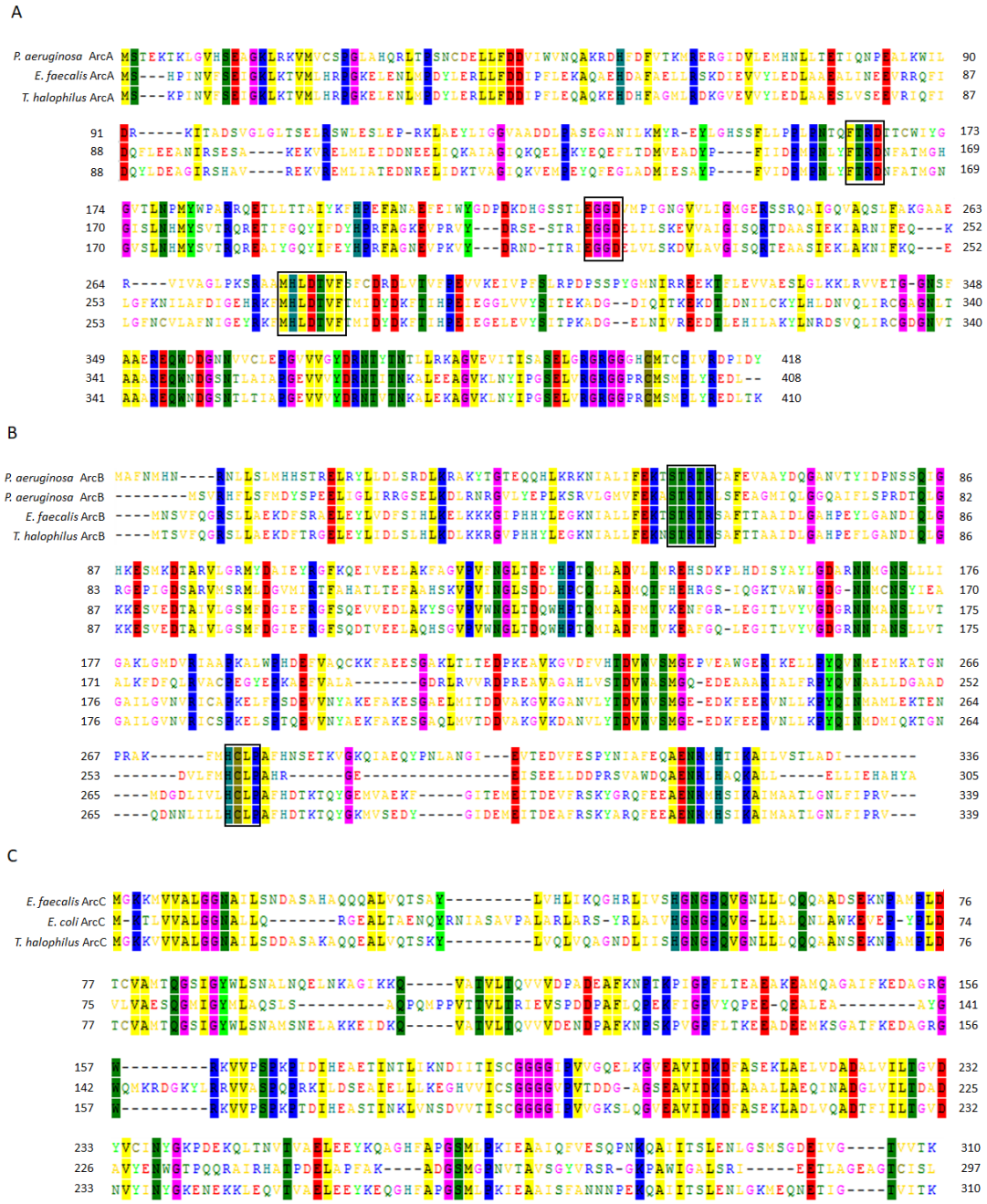

Figure S1 Amino acid sequence alignments of the ADI enzymes of *Tetragenococcus halophilus*

(A) *T. halophilus* ArcA was aligned with ArcA from *Pseudomonas aeruginosa* (P13981) and *Enterococcus faecalis* (Q93K67). The signature ADI sequences were highlighted with boxes.

(B) *T. halophilus* ArcB was aligned with catabolic (P08308) and anabolic (P11724) ornithine carbamoyltransferase from *P. aeruginosa* and from *E. faecalis* (Q839Q5). Conserved carbamoyl phosphate and ornithine binding sites were highlighted with boxes.

(C) *T. halophilus* ArcC was aligned with carbamate kinase from *E. faecalis* (P0A2X7) and *Escherichia coli* (P37306).

Figure S2

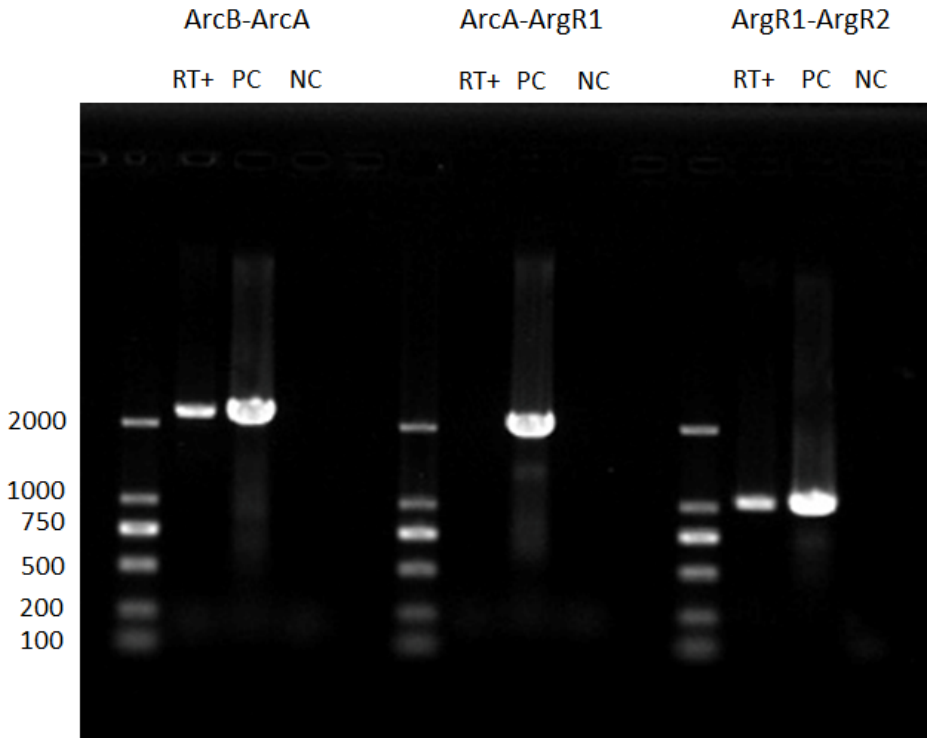

Figure S2 Results of RT-PCR performed to confirmed the co-transcription with primers designed to amplify sequences between specific genes; RT+ indicated that RT-PCR was performed using cDNA resulted from reverse transcription of RNA samples as template, PC indicated positive control, that is, using genome DNA as template, and NC indicated negative control, which is performed using the RNA samples without reverse transcript.

Figure S3

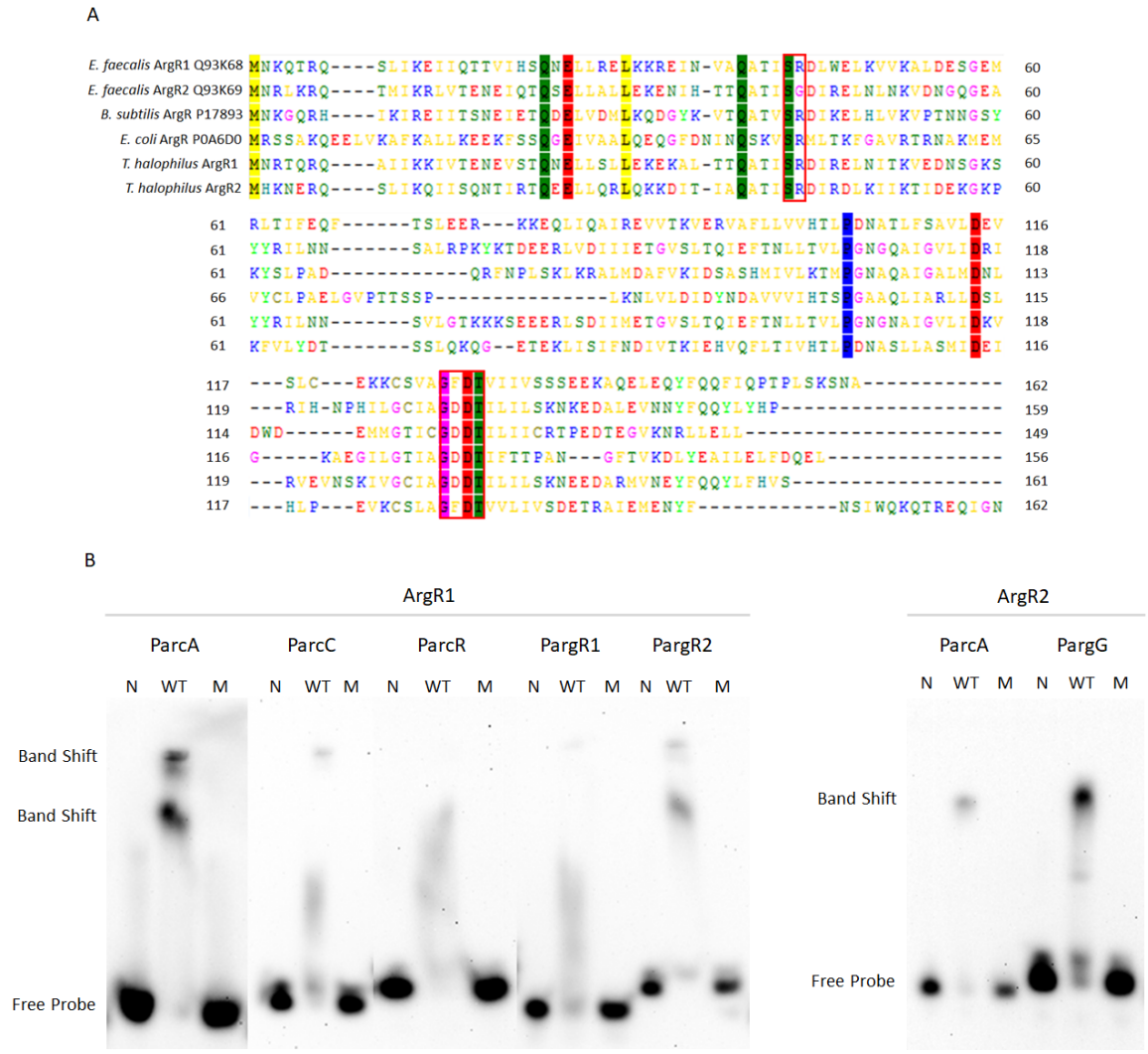

Figure S3 (A) Multiple sequence alignments indicated the conservation of the DNA binding motif "S42R43" of ArgR1 and ArgR2. (B) EMSA assays using wild type and mutated ArgR1 or ArgR2. N indicated no protein was added, WT indicated wild type protein was added, while M indicated the assays were performed using mutated regulators.

Figure S4

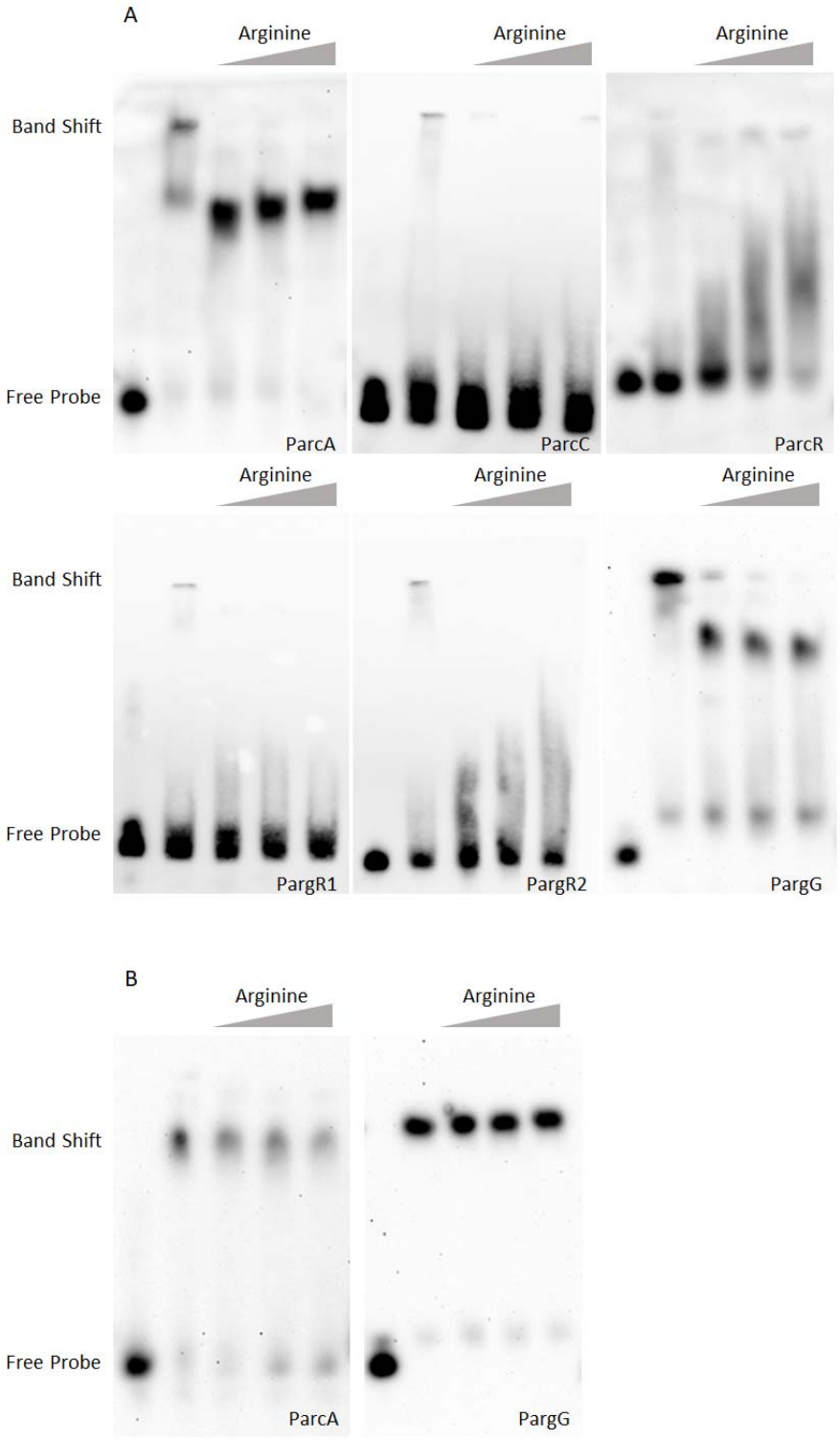

Figure S4 Effect of increasing amount of arginine on the binding ability of ArgR1 (A) and ArgR2 (B). ArgR1

bound to *ParcA* as both trimers and hexamers, resulted in two shifted bands with different molecular mass; the addition of arginine abolished the binding of hexamer ArgR1 to *ParcA* and *PargG*, since ArgR regulators act as hexamers, this will result in the release of ArgR1 repression of both promoters. For each experiment, no protein was added to lane 1. The amounts of arginine added were 0 mM, 5 mM, 10 mM, 20 mM, respectively, from lane 2 to lane 5 in each experiment; for each probe, 600 ng protein was added in each sample.

Figure S5

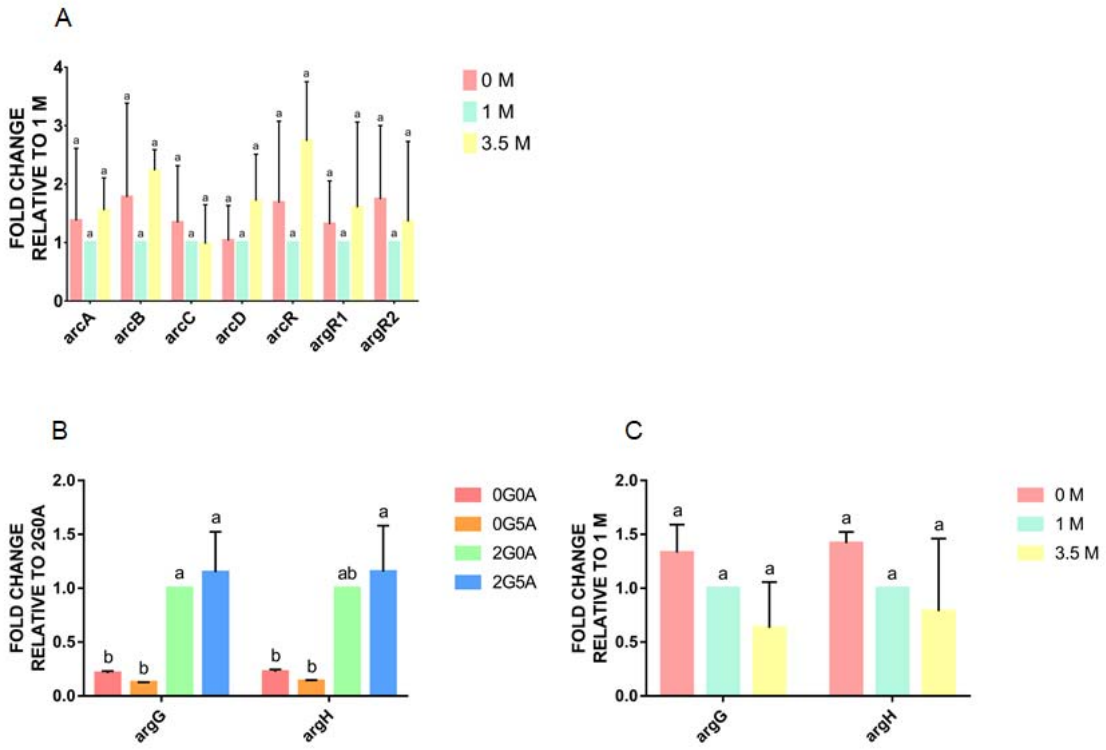

Figure S5 (A) The transcription levels of ADI genes when occurring to 0 M, 1 M, 3.5 M NaCl concentrations (16s rRNA was used as endogenous control and 1 M was used as reference condition). (B) The transcription levels of *argG* and *argH* in the presence or absence of arginine or glucose (0G0A indicating no glucose or arginine was present, 0G5A indicating 5 mM arginine was added, 2G0A indicating 2% glucose was added, 2G5A indicating that 2% glucose and 5 mM were added to the medium; 16s rRNA was used as endogenous control and 2G0A was used as reference condition).(C) The transcription levels of *argG* and *argH* under 0 M, 1 M, 3.5 M NaCl concentration (16s rRNA was used as endogenous control and 1 M was used as reference conditions). Gene expression data under different conditions obtained from 3 biological replicates were subjected to one-way analysis of variance (one-way ANOVA), comparisons were made between the mean of each column and that of every other column, expression of the same gene under different conditions is significantly different ($P<0.05$) if the bars do not share a common superscript

Figure S6

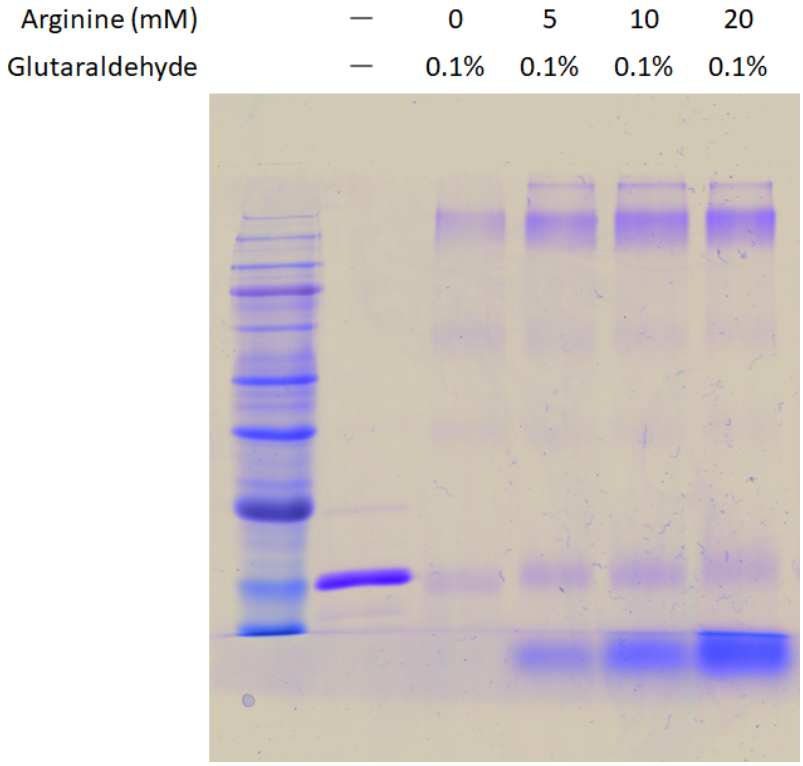

Figure S6 Results of cross-linking assays of ArgR1 with increasing amount of arginine.