The Contribution of ArsB to Arsenic Resistance in Campylobacter jejuni

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Abstract

Arsenic, a toxic metalloid, exists in the natural environment and its organic form is approved for use as a feed additive for animal production. As a major foodborne pathogen of animal origin, Campylobacter is exposed to arsenic selection pressure in the food animal production environments. Previous studies showed that Campylobacter isolates from poultry were highly resistant to arsenic compounds and a 4-gene operon (containing arsP, arsR, arsC, and acr3) was associated with arsenic resistance in Campylobacter. However, this 4-gene operon is only present in some Campylobacter isolates and other arsenic resistance mechanisms in C. jejuni have not been characterized. In this study, we determined the role of several putative arsenic resistance genes including arsB, arsC2, and arsR3 in arsenic resistance in C. jejuni and found that arsB, but not the other two genes, contributes to the resistance to arsenite and arsenate. Inactivation of arsB in C. jejuni resulted in 8- and 4-fold reduction in the MICs of arsenite and arsenate, respectively, and complementation of the arsB mutant restored the MIC of arsenite. Additionally, overexpression of arsB in C. jejuni 11168 resulted in a 16-fold increase in the MIC of arsenite. PCR analysis of C. jejuni isolates from different animals hosts indicated that arsB and acr3 (the 4-gene operon) are widely distributed in various C. jejuni strains, suggesting that Campylobacter requires at least one of the two genes for adaptation to arsenic-containing environments. These results identify ArsB as an alternative mechanism for arsenic resistance in C. jejuni and provide new insights into the adaptive mechanisms of Campylobacter in animal food production environments.

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Introduction

Arsenic is a wildly distributed toxic metalloid in water, soil, and air from natural and anthropogenic sources, and exists in both inorganic and organic forms [1–3]. The most prevalent inorganic forms of arsenic include trivalent arsenite [AS(III)] and pentavalent arsenate [AS(V)]. The trivalent form is more toxic than the pentavalent form [1,3]. AS(III) impairs the functions of many proteins by reacting with their sulfhydryl groups, while AS(V) is a molecular analog of phosphate, which inhibits oxidative phosphorylation and harms the main energy-generation system [2,4]. In order to survive arsenic toxicity, microorganisms have developed different mechanisms for arsenic detoxification, including reduction of AS(V) to AS(III) by arsenate reductases and methylolation or extrusion of AS(III) by efflux transporters [5–7].

The genes encoding arsenic detoxification systems are found on both plasmids and chromosomes. Usually, the ars genes are organized as operons, such as arsRBC, arsRABC, and arsRDABC, but some ars genes exist singly [6,8–14]. ArsC is a small-molecular mass arsenate reductase, which converts AS(V) to AS(III) in the cytoplasm [5,15]. ArsIII is extruded by AS(III)-specific transporters, such as ArsB and Acr3 [5,6]. The activity of ArsB can be ATP-independent or requires the help of ArsA, an ATPase [16,17]. A recent study identified a new arsenic detoxification mechanism mediated by ArsM, an AS(III) S-adenosylmethionine methyltransferase, which methylates AS(III) to volatile trimethylarsine [7]. In addition, there are other Ars proteins involved in arsenic resistance. ArsR, a transcription regulator, modulates the expression of arsenic resistance genes [6,18–21]. ArsD, an arsenical metallochaperone, transfers As(III) to ArsA and increases the rate of arsenic extrusion [7,22–24]. ArsH, an NADPH-flavin mononucleotide oxidoreductase, also contributes to arsenic resistance, and its detoxification mechanism is probably through oxidation of arsenite to the less toxic arsenenate or reduction of trivalent arsenicals to volatile arsines that escape from cells [25,26].

Campylobacter is a leading cause of food-borne bacterial diseases in the United States and other developed countries [27]. Campylobacter infections account for 400 to 500 million cases of diarrhea each year worldwide [28]. According to a recent CDC report, campylobacteriosis is estimated to affect over 840,000 people every year in the U. S. [27]. As a zoonotic pathogen, Campylobacter is highly prevalent in food producing animals, including both livestock and poultry [29], and is frequently exposed to antimicrobials used in animal agriculture. Roxarsone (4-hydroxy-3-nitro-phenylarsonic acid), an organoarsonic compound, is frequently used as a feed additive to improve weight gain, feed utilization and pigmentation, and control of coccidiosis in the poultry industry [30]. Organic roxarsone is excreted through feces and can also be converted into inorganic AS(V) and AS(III) in the broiler digestive system, and the total arsenic
concentration in the litter can reach up to 39 mg/kg [31,32]. Due to the concern with food safety, the manufacturer of roxarsone voluntarily suspended sale of this product in the U.S. in 2011 (http://www.fda.gov/AnimalVeterinary/SafetyHealth/ProductSafetyInformation/ucm258313.htm). Given that Campylobacter is prevalent and well adapted in poultry digestive system, this organism must have the ability to deal with the toxicity of arsenic compounds used for poultry production.

Recently, Wang et al. identified a 4-gene ars operon, which is associated with high-level arsenic resistance in Campylobacter [6]. This operon encodes a putative membrane permease (ArsP), a transcriptional repressor (ArsR), an arsenate reductase (ArsC), and an efflux protein (Acr3). The expression of the whole operon is directly regulated by ArsR and is inducible by AS(III) and As(V) and an efflux protein (Acr3). The expression of the whole operon is directly regulated by ArsR and is inducible by AS(III) and As(V) [6]. According to the published whole genome sequences of Campylobacter, this ars operon is not present in all Campylobacter strains and how those strains without this ars operon adapt to arsenic selection is unknown. The first sequenced C. jejuni strain NCTC 11168 (http://www.lshrm.ac.uk/pmbn/erf/Cj_updated. art) lacks the previously characterized ars operon [6,33], but three putative ars genes are present on the chromosome. These include cj0258 (an arsR homolog and named arsR3 in this study), q0217 (an arsC homolog and named arsC2 in this study), and q1178c (an arsB homolog and named arsB in this study), and their functions remain unknown. In this study, we determine the roles of these putative ars genes in arsenic resistance and find that q1178c (arsB in this study) contributes to the resistance to AS(III). In addition, we investigated the presence of the arsB and arsC (present in the 4-gene ars operon) genes in various Campylobacter isolates. The results suggest that Campylobacter requires at least one of the two genes for adaptation to arsenic-containing environment.

Materials and Methods

Bacterial Strains and Growth Conditions

The key bacterial strains and plasmids used in this study are listed in Table 1. Escherichia coli DH5α used for genetic manipulation was grown in Luria-Bertani (LB) broth or on Mueller-Hinton (MH) agar. When required for cloning of plasmids with different selection markers, kanamycin (30 µg/ml), chloramphenicol (10 µg/ml), or ampicillin (100 µg/ml) was added to the culture media. C. jejuni strains were cultured on MH agar or in MH broth at 42°C microaerobically (5% O2, 10% CO2, and 85% N2). Kanamycin (30 µg/ml) or chloramphenicol (4 µg/ml) was supplemented to the media when needed for culturing the mutant strains that contained a selection marker.

Chemical Compounds and Antibiotics

The chemicals and antibiotics used in this study were purchased from Sigma-Aldrich Co. LLC (arsenate, arsenate, chloramphenicol, kanamycin, ampicillin, copper sulfate, erythromycin, tetracycline, ethidium bromide, azithromycin, ciprofloxacin, florfenicol, and clindamycin), Thermo Fisher Scientific Inc. (roxarsone, mercury bichloride, and telithromycin), and Alfa Aesar (anticatinoic).

Antimicrobial Susceptibility Tests

The MICs of various arsenic compounds against C. jejuni strains were determined using the agar dilution antimicrobial susceptibility testing method according to the protocol from CLSI [34]. The concentrations of arsenic compounds tested in this study ranged from 0.25 to 256 µg/ml for arsenite, 2 to 2048 µg/ml for arsenate, and 1 to 32 µg/ml for roxarsone. Bricy, Campylobacter strains grown on blood agar plates for 24 h were inoculated into Mueller-Hinton broth and then adjusted to a turbidity equivalent to a 0.5 McFarland standard by a colorimeter. A multipoint inoculants (a Cathra replicator system) with 1-mm pins (Oxoid, Inc., Ogdenburg, NY) was used to inoculate approximately 10^3 CFU of C. jejuni onto Mueller-Hinton agar containing a twofold dilution series of arsenic compounds and supplemented with 5% defibrinated sheep blood. The inoculated plates were incubated at 42°C microaerobically (5% O2, 10% CO2, and 85% N2). The MIC was defined as the lowest concentration that completely inhibited the visible growth on the plates. The MICs of various antibiotics against C. jejuni strains were determined using the broth microdilution method as described previously [35]. Each MIC test was repeated at least three times.

PCR

All primers used for PCR are listed in Table 2. PCR was performed in a volume of 50 µl containing 0.2 µM of primers, 250 µM of deoxynucleoside triphosphates, and 1.25 U of TaKaRa Ex Taq polymerase or Phusion High-Fidelity DNA Polymerase. The annealing temperature varied from 50°C to 58°C (Table 2) and the elongation time depended on the expected size of the products (1 kb/min).

Insertional Mutation of arsB

Primers arsB1929F and arsB1929R (Table 2) were used to amplify a 1929 bp arsB fragment with the ScaI and XhoI restriction sites in the middle region of the fragment. The PCR fragment was cloned into the pUC19 between the EcoRI and SaI sites, resulting in the construction of pArsB. Primers arsBCat-F and arsBCat-R (Table 2) were used to amplify the chloramphenicol resistance cat gene from pUA18 using the Phusion High-Fidelity DNA Polymerase (NEB). After the XhoI digestion, the cat cassette was ligated to the ScaI and XhoI digested pARSB to obtain plasmid pArsBcat, which was then transformed into E. coli DH5α. Suicide vector pArsBcat was introduced into C. jejuni NCTC 11168 using an electroporator (Gene Pulser Xcell System; Bio-Rad Laboratories). Transformants were selected on MH agar containing chloramphenicol at 4 µg/ml. The insertion of cat cassette into the arsB gene of C. jejuni 11168 was confirmed by PCR analysis using primers arsB1929F and arsB1929R.

Insertional Mutagenesis of arsC2 (cj0717)

Primers arsC2M1-F and arsC2M1-R were used to amplify the 5’ part of arsC2 and its upstream region (arsC2M1), while Primers arsC2M2-F and arsC2M2-R were used to amplify the 3’ part of arsC2 and its downstream region (arsC2M2). After EcoRI and KpnI digestion, the arsC2M1 PCR product was cloned into the EcoRI and KpnI digested pUC19, resulting in the construction of pC2M1. The digested arsC2M2 PCR product was cloned into the XhoI and PstI digested pC2M1, resulting in the construction of pC2M2. Primers gidAKanF and gidAKanR (Table 2) were used to amplify the aphA3 gene encoding kanamycin resistance from pMW10 using the Phusion High-Fidelity DNA Polymerase (NEB). After the KpnI and XhoI digestion, the Kan’ cassette was ligated to the KpnI and XhoI digested pC2M1M2 to obtain plasmid construct pC2M1M2Kan, which was then transformed into E. coli DH5α. Suicide vector pC2M1M2Kan was then electroporated into C. jejuni NCTC 11168. Transformants were selected on MH agar plates containing 30 µg/ml of kanamycin. The insertion of the aphA3 gene into arsC2 in the transformants was confirmed by PCR using primers arsC2M1-F and arsC2M2-R.
Insertional Mutagenesis of \( \text{arsR}3 \) (cj0258)

Primers \( \text{arsR3M1-F} \) and \( \text{arsR3M1-R} \) were used to amplify the 5’ part of \( \text{arsR3} \) and its upstream region (\( \text{arsR3M1} \)), while primers \( \text{arsR3M2-F} \) and \( \text{arsR3M2-R} \) were used to amplify the 3’ part of \( \text{arsR3} \) and its downstream region (\( \text{arsR3M2} \)). After EcoRI and \( \text{KpnI} \) digestion, the \( \text{arsR3M1} \) PCR product was cloned into the EcoRI and \( \text{KpnI} \) digested \( \text{pUC19} \), resulting in the construction of \( \text{pR3M1} \). The digested \( \text{arsR3M2} \) PCR product was cloned into the \( \text{XbaI} \) and \( \text{PstI} \) digested \( \text{pR3M1} \), resulting in the construction of \( \text{pR3M1M2} \).

As mentioned above, primers \( \text{gidAKanF} \) and \( \text{gidAKanR} \) (Table 2) were used to amplify the \( \text{aphA3} \) gene encoding kanamycin resistance from \( \text{pMW10} \) using the Phusion High-Fidelity DNA Polymerase (NEB). After the \( \text{KpnI} \) and \( \text{XbaI} \) digestion, the \( \text{Kat} \) cassette was ligated to the \( \text{KpnI} \) and \( \text{XbaI} \) digested \( \text{pR3M1M2Kan} \), which was then transformed into \( \text{E. coli} \) DH5α. Suicide vector \( \text{pR3M1M2Kan} \) was then electroporated into \( \text{C. jejuni NCTC} \) 11168. Transformants were selected on MH agar plates containing 30 \( \mu \text{g/ml} \) of kanamycin. The insertion of the \( \text{aphA3} \) gene into \( \text{arsR3} \) in the transformants was confirmed by PCR using primers \( \text{arsR3M1-F} \) and \( \text{arsR3M2-R} \).

Complementation of the \( \Delta \text{arsB::Cm} \) Mutant

The \( \Delta \text{arsB::Cm} \) mutant was complemented by inserting a wild-type copy of \( \text{arsB} \) between the 16S and 23S rRNAs as described by Muraoka and Zhang [36]. Briefly, primers \( \text{comarsB-F} \) and \( \text{comarsB-R} \) were used to amplify the intact \( \text{arsB} \) gene including its ribosome binding site. The amplicon was digested with \( \text{XhoI} \) and cloned into the p\( \text{RRK} \) plasmid, which contains an \( \text{aphA3} \) cassette in the opposite orientation to the ribosomal genes, to obtain plasmid construct p\( \text{RRKarsB} \). The direction of the insertion was confirmed by primers 16s\( \text{arsB-F} \) and 16s\( \text{arsB-R} \). The construct with \( \text{arsB} \) in the same transcriptional direction as the ribosomal genes was selected and used as the suicide vector to insert the \( \text{arsB} \) gene into the chromosome of the \( \text{arsB} \) mutant. The complemented strains were selected on MH agar containing 50 \( \mu \text{g/ml} \) of kanamycin and were confirmed by PCR using primers 16s\( \text{arsB-F} \) and 16s\( \text{arsB-R} \).

Overexpression of \( \text{arsB} \) in \( \text{C. jejuni} \) NCTC 11168

The suicide plasmid p\( \text{RRKarsB} \) constructed for complementation was electroporated into wild-type \( \text{C. jejuni} \) NCTC 11168 wild type strain, resulting in the insertion of an extra copy of \( \text{arsB} \) in the chromosome. Transformants were selected on MH agar plates containing 30 \( \mu \text{g/ml} \) of kanamycin.

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Table 1. Bacterial strains and plasmids used in this study.

| Bacterial strain or plasmid | Description or relevant genotype | Source or reference |
|----------------------------|---------------------------------|---------------------|
| **Plasmids**               |                                 |                     |
| pUOA18                     | E. coli C. jejuni shuttle vector | [48]                |
| pUC19                      | Cloning vector                  | [49]                |
| pMW10                      | Promoterless lacZ plasmid       | [50]                |
| pArsB                      | pUC19+arsB                      | This study          |
| pArscat                    | pUC19+ars8:cat                  | This study          |
| pRRK                       | pRRK::aphA3                     | [36]                |
| pRRKarsB                   | pRRK+arsB                       | This study          |
| pC2M1                      | pUC19+arsC2M1                   | This study          |
| pC2M1M2                    | pUC19+arsC2M1+arsC2M2           | This study          |
| pC2M1M2Kan                 | pUC19+arsC2M1+aphA3+arsC2M2     | This study          |
| pR2M1                      | pUC19+arsR3M1                   | This study          |
| pR2M1M2                    | pUC19+arsR3M1+arsR3M2           | This study          |
| pR2M1M2Kan                 | pUC19+arsR3M1+aphA3+arsR3M2     | This study          |
| **strains**                |                                 |                     |
| DH5α                       | Plasmid propagation E.coli strain| Invitrogen          |
| NCTC 11168                 | Wild-type C. jejuni             | [33]                |
| 11168&arsB                 | NCTC 11168 derivative, \( \Delta \text{arsB::Cm} \') | This study          |
| 11168&arsC2                | NCTC 11168 derivative, \( \Delta \text{arsC2::aphA3} \) | This study          |
| 11168&arsR3                | NCTC 11168 derivative, \( \Delta \text{arsR3::aphA3} \) | This study          |
| 11168&arsB&arsC2           | NCTC 11168 derivative, \( \Delta \text{arsB::Cm} \)', \( \Delta \text{arsC::aphA3} \) | This study          |
| 11168&arsB                 | NCTC 11168 derivative, \( \text{mcarsB} \) | This study          |
| 11168&arsB&arsB            | NCTC 11168 derivative, \( \text{mcarsB} \) | This study          |
| ATCC 33560                 | Wild-type C. jejuni             | ATCC                |
| 33560&arsB                 | ATCC 33560 derivative, \( \Delta \text{arsB::Cm} \') | This study          |
| CB5-28                     | Wild-type C. jejuni             | [6]                 |
| CB5-28&arsB                | CB5-28 derivative, \( \Delta \text{arsB::Cm} \') | This study          |
| CB5-28&arsC                | CB5-28 derivative, \( \Delta \text{arsC::aphA3} \) | [6]                 |
| CB5-28&arsC&arsB           | CB5-28 derivative, \( \Delta \text{arsB::Cm} \', \Delta \text{arsC::aphA3} \) | This study          |

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Arsenic Resistance in \( C. jejuni \)
containing 30 μg/ml of kanamycin and confirmed by PCR using primers 16sars-F and 16sars-R.

Real-time qRT-PCR
To determine if the \( \text{arsB} \) gene is inducible by arsenic compounds, \( \text{C. jejuni} \) NCTC 11168 was cultured in MH broth with or without added arsenite and arsenate for 20 h. The final concentrations of arsenite and arsenate in the culture were 0.125, 0.25, and 0.5 times of their corresponding MIC in NCTC 11168. Total RNA was extracted from three biological replicate cultures using the RNeasy mini kit (Qiagen) according to the protocol supplied with the product and further treated with the Turbo DNA-free kit (Ambion) to eliminate DNA contamination in each preparation. For real-time quantitative reverse transcription-PCR (qRT-PCR), primers \( \text{arsB-F}1 \) and \( \text{arsB-R}1 \) (Table 2) specific for \( \text{arsB} \) were designed using the Primer3 online interface (http://frodo.wi.mit.edu/). Real-time qRT-PCR analyses were conducted using the iScript one-step RT-PCR kit with SYBR green (Bio-Rad) along with the MyiQ iCycler real-time PCR detection system (Bio-Rad, Hercules, CA), and the 16S rRNA gene was used for normalization as described in a previous publication [37]. Briefly, for each RNA template, to generate the standard curve for quantification of the target transcript, a 10-fold dilution series between 25 ng/μl and 0.0025 ng/μl were made and used for RT-PCR. Triplicate reactions in a volume of 15 μl were performed for each dilution of the RNA template. Thermal cycling conditions were as follows: 10 min at 50°C, 5 min at 60°C followed by 5 min at 95°C, and then 40 cycles of 10 s at 95°C and 30 s at 58°C. Melt-curve analysis was performed immediately after the amplification. Each specific amplicon was verified both by the presence of a single melting temperature peak and by the presence of a single band of expected size on agarose gel after electrophoresis. Cycle threshold values were determined with the MyiQ software (BioRad). The relative changes (n-fold) of transcription in \( \text{arsB} \) between the induced and noninduced samples were calculated using the \( 2^{-\Delta\Delta C_T} \) method as described by Livak and Schmittgen [38].

Analysis of \( \text{ars} \) Gene Distribution by PCR
To determine the distribution of the \( \text{arsB} \) and \( \text{acr3} \) genes in various \( \text{C. jejuni} \) isolates, \( \text{arsB} \)-specific primers (arsB-F and arsB-R) and \( \text{acr3} \)-specific primers (cje1733F and cje1733R) [6] were designed from the genomic sequence of \( \text{C. jejuni} \) NCTC 11168 and RM1221, respectively, and used in PCR analyses with the genomic templates of different \( \text{C. jejuni} \) strains and the Ex Taq polymerase (TaKaRa Bio Inc., Japan). These \( \text{C. jejuni} \) isolates were derived from human, chicken, and turkey.

Results

Genetic Features of \( \text{arsB}, \text{arsC2}, \) and \( \text{arsR3} \)

The \( \text{arsB} \) gene encodes a putative arsenic efflux membrane protein (426 amino acids) and shows amino acid sequence

| Table 2. PCR primers used in this study. |
|------------------------------------------|
| **Primers** | **Sequence (5′→3′)** | **Annealing temperature (°C)** |
| arsB1929F | ACAAAGAATTCATGCTGATGTTAAGGC | 56 |
| arsB1929R | ATCACTAGCCTACCAAGGCCAATGCCTTCGG | 56 |
| arsBCat-F | CCGTIGTACGAGGAGTAGTTAATGCTGCTTTCCTTCTTT | 58 |
| arsBCat-R | GTGCTAGCTTAGAAGACAAAGACAAAGACATGATAGAAT | 58 |
| comarsB-F | GCGGCTAGCAAGAGATTTAATGCTGCTTTCCTTCTTT | 52 |
| comarsB-R | GTGCTAGCTTAGAAGACAAAGACAAAGACATGATAGAAT | 52 |
| gidAKanF | TATGTACCCGCCTACATATATATAGATTGAAAT | 50 |
| gidAKanR | AGCCTAGAAATGCTGCTTACGACAATCCTAAA | 50 |

| arsBqidAF | CACTATAAACCTCTTCAACCATT | 58 |
| arsBqidAR | AAGAATCTCTCTCIAAACCAACAG | 58 |
| arsR3M1-F | TGGGTAGTGGAGCGCTTAAATCACAACCTTA | 52 |
| arsR3M2-R | TAAGGTACCTCCCTTATCAAGGCTTTCATCATCATAT | 52 |
| arsR3M2-F | CGATCTAGATGGAATAGCGGATGAAA | 52 |
| arsR3M2-R | ATTGTCCAGACCATGACCTTTAGAAG | 52 |
| arsC2M1-F | TGGGTAGTGGAGCGCTTAAATCACAACCTTA | 52 |
| arsC2M1-R | GCTGTGATAGAATGCTTACGACTCTTATCATCATAT | 52 |
| arsC2M2-R | TGTGCTAGCCACAAGTTGTTATTAAAGGCTTCT | 52 |
| arsC2M2-R | AATCTGCAGCCCATGCTGCTATCATCATATCAT | 52 |
| 16arsB-F | ATGTAGGGATCACCATCAGAAT | 54 |
| 16arsB-R | GATAATCAACCCAACCAAG | 54 |
| arsB-F1 | AGTATAATCAACCCAACCAAG | 58 |
| arsB-R1 | CGTCCATGGAATTTAATCATTAG | 58 |
| arsB56F | GGAATTTACCTTATTTTGGAT | 50 |
| arsB1185R | ATATTTGCTTGGTTCTTGACC | 50 |
| cje1733F | ATGTAGGTGGTTATCGATAGAT | 50 |
| cje1733R | TCACTGAGCTGTGATTTTG | 50 |

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homology to ArsB in *Shewanella* sp. ANA-3 (32% identity; E = 8e−55) [39], *Staphylococcus aureus* (33% identity; E = 2e−65) [40], *Escherichia coli* (32% identity; E = 3e−54) [41–43], and *Acidithiobacillus caldus* (33% identity; E = 1e−57) [44]. ArsB contains eleven probable transmembrane helices predicted by TMHMM2.0 (Fig. 1). Analysis of several published genome sequences of *C. jejuni* strains showed that the *arsB* gene is conserved and immediately downstream of the *gidA* gene (Fig. 2A), which encodes a putative tRNA uridine 5-carboxymethylaminomethyl modification enzyme [45]. RT-PCR (using primers *arsBgidAF* and *arsBgidAR*) amplified a transcript spanning both *gidA* and *arsB*, suggesting that these two genes form an operon and are co-transcribed. *gj0717* encodes a small protein (109 aa), which is predicted to belong to the arsenate reductase (ArsC) family and the YlfB subfamily. YlfB is an uncharacterized bacterial protein encoded by the *yffb* gene, marginally similar to the amino-acid sequences of classical arsenate reductases (ArsC) (Fig. 2B). *gj0258* encodes an ORF of 81 aa, which is predicted to contain a helix-turn-helix motif at aa 35–56 and belongs to the *arsR* family [33,45]. To differentiate *gj0258* from the *arsR* genes and *gj0717* from the *arsC* gene previously identified in *C. jejuni* [6], we named them as *arsR3* and *arsC2* in this study, respectively (Fig. 2B and C).

**Role of *arsB*, *arsC2*, and *arsR3* in Arsenic Resistance**

To define the role of *arsB*, *arsC2*, and *arsR3* in arsenic resistance in *Campylobacter*, their insertionally mutated were compared with the wild-type strain NCTC 11168 for susceptibility to arsenic compounds. According to the MIC results from the agar dilution method, inactivation of *arsB* resulted in 8- and 4-fold reduction in the MICs of arsenite and arsenate, respectively, while mutation of *arsC2* or *arsR3* did not affect the MICs of arsenite and arsenate (Table 3). All three mutants showed no changes in the MIC of roxarsone. Chromosomal complementation of *arsB* restored the MIC of arsenite to wild type, and over-expression of *arsB* showed 16-fold increase in the MIC of arsenite compared to the wild-type strain. Interestingly, chromosomal complementation could not restore the MIC of arsenite to the wild-type level and over-expression of *arsB* showed no change in the MIC of arsenate compared to the wild-type strain. Furthermore, we transferred the *arsB* mutation to two additional *Campylobacter* strains (ATCC 33560 and CB5-28) by natural transformation. Inactivation of *arsB* in ATCC 33560 resulted in 9-fold reduction in the MICs of arsenite and had no affect on the MIC of arsenate and roxarsone (Table 3). Inactivation of *arsB* in CB5-28, which harbors the 4-gene *ars* operon as described in a previous study [6], did not affect the MICs of arsenite, arsenate, and roxarsone, suggesting the function of *arsB* in CB5-28 is masked by the fully functional *ars* operon.

**Mutation of the *arsB* did not Affect the Susceptibility to the Other Antibiotics**

To examine if *arsB*, *arsC2*, and *arsR3* are associated with resistance to other heavy metals and antibiotics, we compared the susceptibilities of the *arsB*, *arsC2*, and *arsR3* mutants with the wild-type strain to antimonate, copper sulfate, mercury bichloride, erythromycin, tetracycline, ethidium bromide, azithromycin, ciprofloxacin, florfenicol, telithromycin, and clindamycin using the broth microdilution method. The results showed no differences between the wild type and mutants in the susceptibilities to these compounds (data not shown), indicating that these genes do not confer resistance to other heavy metals and antibiotics.

**The *arsB* is Inducible by Arsenite and Arsenate**

To determine if the expression of the *arsB* is inducible by arsenic compounds, strain NCTC11168 was cultured in MH broth with different concentrations of arsenite and arsenate. The transcription levels of *arsB* in these cultures were compared with those grown in MH broth without arsenic compounds using real time qRT-PCR. As shown in Figure 3, the expression of *arsB* was induced in a dose-dependent manner. At 0.5 times of MIC, both arsenite and arsenate produced approximately 16-fold induction in the expression of *arsB*. This result clearly indicates that the *arsB* gene in *Campylobacter* is inducible by both arsenite and arsenate.

![Figure 1. The membrane topologies of ArsB predicted by TMHMM.](https://doi.org/10.1371/journal.pone.0058894.g001)

- The transmembrane domains are shaded in red. The blue line indicates loops facing inside (cytoplasm), while the pink line depicts loops facing outside (periplasmic space). The numbers at the bottom indicate the amino acid numbers in ArsB.

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**Table 3. MICs of arsenite, arsenate, and roxarsone.**

|                | Wild Type | *arsB* | *arsC2* | *arsR3* |
|----------------|-----------|--------|---------|---------|
| MIC of Arsenite | 0.066     | 0.008  | 0.008   | 0.008   |
| MIC of Arsenate | 0.133     | 0.033  | 0.033   | 0.033   |
| MIC of Roxarsone| 0.006     | 0.006  | 0.006   | 0.006   |

**Figure 2**. The membrane topologies of ArsB predicted by TMHMM. The transmembrane domains are shaded in red. The blue line indicates loops facing inside (cytoplasm), while the pink line depicts loops facing outside (periplasmic space). The numbers at the bottom indicate the amino acid numbers in ArsB.

**Figure 3**. The expression of *arsB* is inducible by arsenic compounds in C. jejuni. As shown in Figure 3, the expression of *arsB* was induced in a dose-dependent manner. At 0.5 times of MIC, both arsenite and arsenate produced approximately 16-fold induction in the expression of *arsB*. This result clearly indicates that the *arsB* gene in *Campylobacter* is inducible by both arsenite and arsenate.

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**Figure 3**. The expression of *arsB* is inducible by arsenic compounds in C. jejuni. As shown in Figure 3, the expression of *arsB* was induced in a dose-dependent manner. At 0.5 times of MIC, both arsenite and arsenate produced approximately 16-fold induction in the expression of *arsB*. This result clearly indicates that the *arsB* gene in *Campylobacter* is inducible by both arsenite and arsenate.
Distribution of \textit{arsB} and \textit{acr3} Genes in \textit{Campylobacter} Isolates

Data described above indicated that ArsB contributes to arsenic resistance in \textit{C. jejuni}. Additionally, Acr3 is associated with high-level of arsenic resistance in certain \textit{Campylobacter} strains [6]. We determined the distribution of the \textit{arsB} and \textit{acr3} genes in various \textit{Campylobacter} isolates of different animal origins. As shown in Table 4, \textit{arsB} was present in 76 of the 98 isolates examined in this study, while \textit{acr3} were present in 58 of the 98 isolates. Interestingly, all the tested strain contains at least one of the two genes. Furthermore, \textit{arsB} is more prevalent in the chicken (97.1%) and human (92.0%) isolates than in the turkey isolates (50.0%) \((p<0.001\) and \(p<0.005\)), while the prevalence of \textit{acr3} is higher in the turkey isolates (84.2%) than in the chicken (45.7%) \((p<0.005\) and human (40.0%) \((p<0.005\)) isolates.

Discussion

The results from this study identified ArsB involved in arsenic resistance in \textit{C. jejuni}. This conclusion is based on the following findings: first, inactivation of \textit{arsB} resulted in reduced resistance to both arsenite and arsenate; second, complementation of the \textit{arsB} mutant restored the MIC of arsenite (but not arsenate) to that of the wild-type strain; and third, overexpression of \textit{arsB} in \textit{C. jejuni} 11168 increased the MIC of arsenite by 16-fold, but did not affect the MIC of arsenate. These results suggest that ArsB in \textit{C. jejuni} contributes resistance to arsenite, but not for arsenate. However, arsenate can be converted to arsenite by ArsC in bacteria including \textit{C. jejuni}, where arsenite can be subsequently extruded by ArsB and Acr3 [6]. Thus, ArsB contributes to the resistance to arsenate in an indirect manner. These results are consistent with the \textit{arsB} findings in other bacterial species.

The ArsB in \textit{C. jejuni} shares homology with the other members of the ArsB family. ArsB is employed by many bacteria as an arsenic detoxification method and is proposed to have 12 membrane-spanning regions [46]. ArsB appears to be an unipporter which extrudes As(III) at a moderate rate using membrane potential. In some cases, with the help from ArsA (ATPase), ArsB can extrude As(III) more efficiently [5]. Several previous studies also showed that Sb(III) is a substrate for certain ArsB transpoters [47]. In this study, we found that the ArsB in \textit{C. jejuni} does not play a role in the resistance to other heavy metals and antibiotics. The inability of \textit{C. jejuni} ArsB to extrude Sb(III) is different from the result reported in other bacteria [47] and suggests that the ArsB in \textit{C. jejuni} is more or less unique. Indeed, the predicted transmembrane topology of the ArsB in \textit{C. jejuni} contains 11 transmembrane domains, instead of 12 of typical ArsB proteins, which might explain the difference in substrate specificities.

The contributions of \textit{arsB} to arsenic resistance vary in different \textit{Campylobacter} strains. The role of ArsB in mediating arsenic

\begin{table}
\centering
\caption{The MICs of roxarsone, arsenate and arsenite in various \textit{C. jejuni} strains as determined by the agar dilution method.}
\begin{tabular}{|c|c|c|c|}
\hline
\textbf{Strains} & \textbf{Arsenite} & \textbf{Arsenate} & \textbf{Roxarsone} \\
\hline
NCTC 11168 & 8 & 512 & 8 \\
11168\textperiodcentered\textit{arsB} & 1\(\downarrow\) 8 & 128\(\downarrow\) 4 & 8 \\
11168\textperiodcentered\textit{acr3} & 8 & 512 & 8 \\
11168\textperiodcentered\textit{arsB\textperiodcentered\textit{arsC}} & 1\(\downarrow\) 8 & 128\(\downarrow\) 4 & 8 \\
11168\textperiodcentered\textit{arsB\textperiodcentered\textit{arsR3}} & 128 & 128 & 8 \\
11168\textperiodcentered\textit{arsB\textperiodcentered\textit{arsC}} & 128\(\uparrow\) 16 & 512 & 8 \\
ATCC 33560 & 8 & 32 & 8 \\
33560\textperiodcentered\textit{arsB} & 1\(\downarrow\) 8 & 32 & 8 \\
CB5\textperiodcentered28 & 64 & 1024 & 64 \\
CB5\textperiodcentered28\textperiodcentered\textit{arsB} & 64 & 1024 & 64 \\
CB5\textperiodcentered28\textperiodcentered\textit{arsC} & 8 & 64 & 64 \\
CB5\textperiodcentered28\textperiodcentered\textit{arsB\textperiodcentered\textit{arsC}} & 4\(\downarrow\) 2 & 64 & 64 \\
\hline
\end{tabular}
\end{table}

*the numbers in parentheses indicate fold-changes, either increase (\(\uparrow\)) or decrease (\(\downarrow\)).
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Figure 2. Diagrams showing the genomic localizations and mutant generation of various \textit{ars} genes. (A) Genomic organization of \textit{arsB} and inactivation of \textit{arsB} by insertion of a choramphenicol resistance cassette. (B) Genomic localization of \textit{arsC2} and inactivation of this gene by insertion of a kanamycin resistance cassette. (C) \textit{arsB} and its flanking gene. Inactivation of \textit{arsR3} was accomplished by insertion of a kanamycin resistance cassette. (D) Complementation of the \textit{arsB} mutant by insertion of an extra copy of the \textit{arsB} gene downstream of 16S rRNA. (E) The \textit{ars} operon identified in \textit{C. jejuni} CBS-28 and inactivation of \textit{arsC} by insertion of a kanamycin resistance cassette.
In addition, the expression level of affecting its contribution to arsenic resistance. As shown in Table 3, Campylobacter findings suggest that ArsB mediated arsenic resistance level in a drastic increase in the resistance to arsenite, to a level that is C. jejuni ArsA (ATPase) [5,16]. However, analysis of the whole genomes of indicated that ArsB functions more efficiently when facilitated by explained for two reasons. The published data in other bacteria that in the wild-type strain [6]. Thus, the residual expression of acr3 mutation did not totally inactivate the function of acr3 genes (Table 3). To test if the function of ArsB is masked by the presence of the ars operon, we constructed an arsB and arsC double knockout strain (CB5-28ΔarsBΔarsC) in the CB5-28ΔarsC background [6]. Compared to CB5-28ΔarsC, CB5-28ΔarsBΔarsC showed 2-fold reduction in the MIC of arsenite, but not 8-fold reduction as observed in NCTC 11168 (Table 3). This could be explained by the fact that the polar effect caused by the arsC mutation did not totally inactivate the function of acr3 and residual expression of acr3 still existed in the C. jejuni mutant compared with that in the wild-type strain [6]. Thus, the residual expression of acr3 could still play a role in arsenite resistance. These results suggest that the function of arsB is most likely masked in those C. jejuni strains harboring a fully functional ars operon.

The level of arsenic resistance mediated by ArsB in C. jejuni is not as high as that mediated by the ars operon. This could be explained for two reasons. The published data in other bacteria indicated that ArsB functions more efficiently when facilitated by ArsA (ATPase) [5,16]. However, analysis of the whole genomes of C. jejuni did not identify an arsA homology in the organism. Thus, the lack of arsA in C. jejuni might reduce the efflux ability of ArsB. In addition, the expression level of arsB might be another factor affecting its contribution to arsenic resistance. As shown in Table 3, artificial overexpression of arsB in C. jejuni NCTC 11168 resulted in a drastic increase in the resistance to arsenite, to a level that is even higher than the resistance conferred by the ars operon. These findings suggest that ArsB mediated arsenic resistance level in Campylobacter is mainly dependent on the expression level of arsB.

The putative arsR (arsR3) gene did not contribute to arsenic resistance in C. jejuni NCTC 11168. As a transcriptional repressor, ArsR modulates the expression of ars genes through interaction with the arsenite substrate [6]. In this study, the induction experiment revealed that addition of arsenite or arsenate in culture media induced the expression of arsB, and the induction was dose-dependent (Fig. 3). Thus, we speculated that the expression of arsB is modulated by an ArsR like regulator. However, inactivation of arsR3, which is separated from the arsB gene on chromosome, did not affect the expression of arsB in C. jejuni NCTC 11168, suggesting that the expression of arsB is not modulated by arsR3 and is likely regulated by an unknown mechanism.

The putative arsC (arsC2) gene did not contribute to arsenic resistance C. jejuni NCTC 11168. Conversion of AS(V) to AS(III) by arsenate reductase and then extrusion by arsenite transporters is an important detoxification mechanism used by many bacterial organisms [6,15]. The previously characterized ars operon in C. jejuni contains an arsC, which mediates arsenic resistance in Campylobacter [6]. Inactivation of arsC2 in C. jejuni NCTC 11168 did not change the susceptibility to arsenic compounds. Additionally, we inactivated arsC2 in the arsB mutant background of C. jejuni NCTC 11168, and the the arsB and arsC double knockout did not further alter the resistance to arsenate compared to the arsB mutant (data not shown), further suggesting that arsC2 is not involved in arsenic resistance in Campylobacter.

As mentioned in the introduction, organic arsenic compounds (roxarsone and p-arsanilic acid) are extensively used as feed additives in the poultry industry and Campylobacter is exposed to the selection pressure. ArsB is a putative efflux transporter for inorganic arsenic and does not seem to directly contribute to the resistance to roxarsone (Table 3). However, roxarsone is converted into inorganic species such as AS(V) and AS(III) in poultry litter [32]. Thus, ArsB is expected to facilitate Campylobacter adaptation to the toxic effect of roxarsone in an indirect manner. To date, the identified mechanisms of arsenic resistance in bacteria are all related to detoxification of inorganic arsenic, and the efflux transporters that directly extrude organic arsenic compounds have not been reported.

Interestingly, the distribution of both arsB and acr3 genes in human isolates is similar to those in chicken isolates, but differ from those in turkey isolates (Table 4). According to a report from American Meat Institute on April 2009, per capita consumption of chicken was five times more than that of turkey in 2007. In addition, poultry is the main reservoir for human C. jejuni infections. Thus, the big portion of Campylobacter infections is probably caused by consumption of chicken. This might explain that the presence of ars genes in human isolates is similar to those in chicken isolates. Furthermore, the results revealed a broad distribution of arsB and acr3 genes (ars operon) in C. jejuni isolates of different animal origins (Table 4) and suggest that at least one of the two genes is required for the adaptation of Campylobacter in arsenic-rich niches. These

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**Table 4. Distribution of arsB and acr3 in C. jejuni isolates of different origins.**

| Source of isolates | Total number | arsB-positive | acr3-positive | Positive with both arsB and acr3 | Positive with either arsB or acr3 |
|-------------------|--------------|---------------|---------------|---------------------------------|---------------------------------|
| Chicken           | 35           | 34 (97.1%)    | 16 (45.7%)    | 15 (42.9%)                      | 35 (100.0%)                    |
| Turkey            | 38           | 19 (50.0%)    | 32 (84.2%)    | 13 (34.2%)                      | 38 (100.0%)                    |
| Human             | 25           | 23 (92.0%)    | 10 (40.0%)    | 8 (32.0%)                       | 25 (100.0%)                    |
| total             | 98           | 76 (77.6%)    | 58 (59.2%)    | 36 (36.7%)                      | 98 (100.0%)                    |

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findings provide new insights into the adaptive mechanisms of Campylobacter in the poultry production system.

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Author Contributions

Conceived and designed the experiments: ZS QZ. Performed the experiments: ZS JH YW OS. Analyzed the data: ZS JH YW OS QZ. Contributed reagents/materials/analysis tools: ZS JH YW OS QZ. Wrote the paper: ZS QZ.

Arsenic Resistance in C. jejuni

Conceived and designed the experiments: ZS QZ. Performed the experiments: ZS JH YW OS. Analyzed the data: ZS JH YW OS QZ. Contributed reagents/materials/analysis tools: ZS JH YW OS QZ. Wrote the paper: ZS QZ.