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Campylobacter jejuni dsb gene expression is regulated by iron in a Fur-dependent manner and by a translational coupling mechanism

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Abstract

Background: Many bacterial extracytoplasmic proteins are stabilized by intramolecular disulfide bridges that are formed post-translationally between their cysteine residues. This protein modification plays an important role in bacterial pathogenesis, and is facilitated by the Dsb (disulfide bond) family of the redox proteins. These proteins function in two parallel pathways in the periplasmic space: an oxidation pathway and an isomerization pathway. The Dsb oxidative pathway in Campylobacter jejuni is more complex than the one in the laboratory E. coli K-12 strain.

Results: In the C. jejuni 81-176 genome, the dsb genes of the oxidative pathway are arranged in three transcriptional units: dsbA2-dsbB-astA, dsbA1 and dba-dsbI. Their transcription responds to an environmental stimulus - iron availability - and is regulated in a Fur-dependent manner. Fur involvement in dsb gene regulation was proven by a reporter gene study in a C. jejuni wild type strain and its isogenic fur mutant. An electrophoretic mobility shift assay (EMSA) confirmed that analyzed genes are members of the Fur regulon but each of them is regulated by a disparate mechanism, and both the iron-free and the iron-complexed Fur are able to bind in vitro to the C. jejuni promoter regions. This study led to identification of a new iron- and Fur-regulated promoter that drives dsbA1 gene expression in an indirect way. Moreover, the present work documents that synthesis of DsbI oxidoreductase is controlled by the mechanism of translational coupling. The importance of a secondary dba-dsbI mRNA structure for dsbI mRNA translation was verified by estimating individual dsbI gene expression from its own promoter.

Conclusions: The present work shows that iron concentration is a significant factor in dsb gene transcription. These results support the concept that iron concentration - also through its influence on dsb gene expression - might control the abundance of extracytoplasmic proteins during different stages of infection. Our work further shows that synthesis of the DsbI membrane oxidoreductase is controlled by a translational coupling mechanism. The dba expression is not only essential for the translation of the downstream dsbI gene, but also Dba protein that is produced might regulate the activity and/or stability of DsbI.

Background

Campylobacter jejuni is a human pathogen and the leading cause of acute bacterial gastroenteritis. As a commensal organism for many warm-blooded animals, especially in the gastrointestinal tract of poultry, C jejuni is also isolated from a wide variety of watery environmental sources [1,2]. Thus, the ability of C. jejuni to sense and respond to diverse environmental stimuli and to adapt gene expression to changes in external conditions is crucial for its pathogenesis, commensalism and survival outside the host organism.

Recent experiments have revealed many changes in the C. jejuni transcriptome and proteome that are driven by environmental stimuli. These include temperature, oxygen tension, iron concentration, sodium deoxycholate concentration and pH of the culture medium [3-7].
C. jejuni’s phase of life - planktonic vs biofilm - also shows a great difference in the microorganism’s protein profile [8,9]. *Campylobacter* gene expression is coupled to environmental cues mostly by two-component signal transduction systems (TCSTS) [10-14]. The activity and the amount of a specific protein can also be affected by posttranslational modifications such as glycosylation, proteolysis and disulfide bond formation. That latter protein modification, which very often influences the tertiary and quaternary structure of virulence determinants, plays an important role in bacterial pathogenesis [15,16]. In Gram-negative bacteria disulfide bond formation is facilitated by the Dsb (disulfide bond) family of redox proteins, which function in the periplasmic space under oxidizing conditions. In *E. coli* the disulfide bridge formation system operates in two partially coinciding metabolic pathways: the oxidation (DsbA and DsbB) pathway and the isomerization/reduction (DsbC and DsbD) pathway. The oxidation pathway is responsible for the formation of disulfide bonds in newly synthesized proteins, just after they cross the cytoplasmic membrane. This process occurs in a rather non-selective way. The isomerization/reduction pathway rearranges improperly introduced disulfides [15,16].

The sequencing of more and more bacterial genomes has revealed that the process of disulfide bond formation in bacteria is extremely diverse, and it has become obvious that *E. coli* Dsb system cannot be considered a paradigm for Dsb activity [16,17]. The Dsb oxidative pathway of *C. jejuni* is much more complex than the oxidative pathway of the laboratory *E. coli* K-12. Depending on the strain, it is catalyzed by three or four enzymes - two localized in the inner membrane (DsbA and DsbI) and one or two in the periplasm (DsbA1 and DsbA2). DsbA1 and DsbA2 possess classic signal sequences, which potentially ensure their transport through the cytoplasmic membrane into the periplasm. They are both directly responsible for disulfide bond formation. DsbB and DsbI, orthologues of *E. coli* DsbB, are potentially involved in DsbA1/DsbA2 re-oxidation [18]. *C. jejuni* genes of the Dsb oxidation pathway are organized in two clusters located at different chromosomal loci: *dsbA2-dsbB-asta-dsbA1* and *dba-dsbI*. AstA (arylsulfatase), encoded by the gene located in the first cluster, transfers arylsulfate groups between aromatic substrates in an adenosine 3’-phosphate-5’phosphosulfate (PAPS)-independent manner, at least in an *E. coli* strain [19-21], and is a substrate for the Dsb oxidative pathway. Based on specificity toward the donor aromatic substrate, arylsulfatases are classified as PAPS-dependent or PAPS-independent enzymes. The mode of *C. jejuni* AstA action remains uncharacterized. The *dba* gene encodes a potential protein of unknown function. Except for *dsbA2*, *C. jejuni dsb* genes are highly conserved within the species. Only *dsbA2* is variable among strains [15].

An active Dsb system is required for intestinal colonization by *Campylobacter*, as shown in a chicken infection model. Additionally, *C. jejuni* strain 81-176 with a mutated *dsbB* or *dsbI* gene showed reduced invasion/intracellular survival ability in T84 cells. These data indicate that some targets of the Dsb system are involved in crucial processes of *Campylobacter* pathogenicity and commensalism [22].

The goal of this work was to analyze *C. jejuni* *dsb* oxidative gene expression by characterizing its transcriptional units, and identify control mechanisms and environmental regulatory factors that facilitate the pathogen’s adaptation to varying living conditions. We show that the *dsb* genes are arranged in three operons in the genome, and that expression of those operons responds to an environmental stimulus - iron availability. Although transcription of *dsbB* and *dsbI* are both altered by iron concentration with Fur protein engagement, they are regulated differently. Thus, by changing Dsb protein abundance, the pathogen can regulate the amounts of many extracytoplasmic virulence factors that are substrates of the Dsb system, depending on the environmental conditions. Additionally, results show that synthesis of DsbI oxidoreductase is strongly controlled by the mechanism of translational coupling.

**Methods**

**Bacterial strains, plasmids, media and growth conditions**

Bacterial strains and plasmids used in this study are listed in Table 1. *C. jejuni* strain 81-176 [23], and 480 [24] were grown under microaerobic conditions at 37°C in Mueller Hinton (MH) broth, on MH agar or Blood Agar Base No. 2 (BA) containing 5% horse blood. *E. coli* strains were grown at 37°C in Luria Bertani (LB) broth or on LB agar. When appropriate, the media were supplemented with antibiotics (*Campylobacter* Selective Supplement (Oxoid), ampicillin (100 μg/ml), chloramphenicol (15 μg/ml), kanamycin (30 μg/ml) or tetracycline (10 μg/ml)), iron sulfate Fe₂(SO₄)₃ (40 μM - final concentration in all experiments) or the iron chelator deferoxamine mesylate (20 μM - final concentration in all experiments), X-Gal (13 mg/ml) and/or IPTG (3 mg/ml) in DMF (dimethyl-formamide).

As previously reported [6], growth of the *C. jejuni* NCTC 11168 was slower in the presence of deferoxamine mesylate (iron-restricted conditions) than in the presence of iron sulfate (iron-rich conditions). This was also observed for *C. jejuni* 81-176, so in iron-restricted conditions the strain was cultivated 5–7 hours longer than in iron-sufficient or iron-rich conditions, till the culture reached OD₆₀₀ of about 0.4–0.6. We also noted that growth of the fur::cat mutated *C. jejuni* strains was
Table 1 Bacterial strains and plasmids used in this study

| Strain/plasmid | Genotype or relevant characteristics | Origin |
|----------------|--------------------------------------|--------|
| **C. jejuni strains** | | |
| 81-176 | parental strain; pVir, pTet (Tet^R) | G. Perez - Perez * |
| AG1 | 81-176 dba:aphA-3 | This study |
| AL1 | 81-176 dsbI:cat | This study |
| AG6 | 81-176 Δdba-dsbI:cat | This study |
| AG11 | 81-176 fur:cat | This study |
| 480 | parental strain | J. van Putten ** |
| AL4 | 480 dsbI:cat | This study |
| AG15 | 480 fur:cat | This study |
| **E. coli strains** | | |
| DH5α | F^− lacZ ΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1-1 hsdR17 (rK mK^+ ) phoA supE44 thi-1 gyrA96 relA1 | Gibco BRL |
| TG1 | supE44 hsdS Δ thi (lac- proAB) F^− [traD36 proAB^+ ] lacYI ΔlacZΔM15] | [26] |
| S17-1 | recA pro hsdR RP4-2-Tc::Mu-Km::Tn7 Tmp^R, Spc^R, Str^R | [56] |
| **General cloning/Plasmid vectors** | | |
| pGEM-T Easy | Ap^R; LacZα | Promega |
| pRY107 | Km^R, E. coli/C. jejuni shuttle vector | [27] |
| pRY109 | Cm^R, E. coli/C. jejuni shuttle vector | [27] |
| pRK2013 | Km^R, helper vector for E. coli/C. jejuni conjugation | [28] |
| **Plasmids for gene expression study** | | |
| Cj stands for PCR-amplified C. jejuni 81-176 DNA fragment (PCR primers are given in brackets) | |
| Cc stands for PCR-amplified C. coli 72Dz/92 DNA fragment (PCR primers are given in brackets) | |
| cj stands for C. jejuni 81-176 gene | |
| pUWM471 | pMW10/1300 bp Cc (H0B - H4X) | [39] |
| pUWM803 | pMW10/440 bp Cc (Cj879B - Cj880X) | This study |
| pUWM792 | pMW10/1170 bp Cc (Cj879B - Cj881X) | This study |
| pUWM795 | pMW10/1980 bp Cc (Cj879B - Cj882X) | This study |
| pUWM832 | pMW10/690 bp Cc (Cj880B - Cj880X) | This study |
| pUWM833 | pMW10/750 bp Cc (Cj880B2 - Cj881X) | This study |
| pUWM834 | pMW10/900 bp Cc (Cj881B - Cj882X) | This study |
| pUWM836 | pMW10/660 bp Cc (Cj882B3 - Cj883X2) | This study |
| pUWM827 | pMW10/540 bp Cc (Cj19LX-2 - Cj18Bgl) | This study |
| pUWM828 | pMW10/720 bp Cc (Cj19LX-2 - Cj17Bgl) | This study |
| pUWM858 | pMW10/240 bp Cc (Cj45B - Cj44X) | This study |
| **Plasmids for mutagenesis** | | |
| pAV80 | pBluescript II SK/cjfur:cat | [25] |
| pUWM622 | pBluescript II KS/cjdba:aphA-3 | This study |
| pUWM713 | pGEM-T Easy/cjdsbI:cat | This study |
| pUWM867 | pGEM-T Easy/ΔcjdsbI-cjdsbI:cat | This study |
| **Plasmids for translational coupling study** | | |
| pUWM769 | pRY107/cjdsbI operon | This study |
| pUWM811 | pRY107/cjdba (M1R)-cjdsbI operon | This study |
| pUWM812 | pRY107/cjdba (L29stop)-cjdsbI operon | This study |
| pUWM1072 | pBluescript II SK/promoter of cjdba-cjdsbI operon | This study |
| pUWM1100 | pBluescript II SK/cjdsbI with its own promoter | This study |
| pUWM1103 | pRY107/cjdsbI with its own promoter | This study |
| **Plasmid for recombinant protein synthesis and purification** | | |
| pUWM657 | pET28a/cjdsbI (1100 bp 5′-terminal fragment) | This study |
| pUWM1098 | pET24d/cjfur (fur coding region) | This study |

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markedly slower in iron-rich conditions than that of the wild type strain, but it was not slower in iron-restricted conditions. A similar inhibitory effect of iron chelation on the growth of C. jejuni 11168 was previously reported by van Vliet [6,25].

General DNA procedures
Standard procedures for plasmid DNA isolation and DNA analysis were carried out as described by Sam-brook and Russel [26] or were performed according to the manufacturer’s instructions (A&KA Biotechnology). Synthetic primers synthesis (sequences given in Table 2) and DNA sequencing were performed in the DNA Sequencing and Oligonucleotide Synthesis Laboratory at the Institute of Biochemistry and Biophysics, Polish Academy of Sciences.

Table 2 Oligonucleotides used in the present study

| Name       | Sequence                      | Orientation/restriction site |
|------------|-------------------------------|-----------------------------|
| H0B        | GTCTAGATGATCGTATCCTGATTACT    | Fwd/BamHI                   |
| H4X        | ATCTGCTAGAGCAGACAGGAGCAATTACATCT | Rev/Xbal                   |
| Cj16RS     | GCAGTCGACCTAATGAAGTAAAGTAAG   | Rev/Sall                    |
| Cj17bgl    | CCTAGATCTAGCTGCTCAAACACATTAGT | Rev/BglII                   |
| Cj17Nde    | GTACATAGACGAAAACATAAAAAC      | Fwd/Ndel                    |
| Cj17RbgI   | TCAGAATCTAATGTTTAGGACCAGG    | Fwd/BglII                   |
| Cj17RM     | TGTGAATATGGAGATCTGGAACAAAAC   | Rev/EcoRI                   |
| Cj17LSal   | GTGTGAGATGATGAAAGAAATATTG    | Rev/Sall                    |
| Cj17WDBam-low | GGATCCATGGGGGATGCGATAG     | Rev/BamHI                   |
| Cj17WDBam-up | GGATCCACAGTTACCTCTCTATATAG  | Fwd/BamHI                   |
| Cj18bgI    | CTAGATCTAGATCAGTATCAGGCGA    | Rev/BglII                   |
| Cj18LM29   | CCAAGCTACATTACCCAAACAAAGCAAAT | Rev/Ø                        |
| Cj18LM29.C | ATTTGGCTTCTGTTTGGTTGTAATGCTGTTTGG | Fwd/Ø                        |
| Cj18LM     | TATGGATCAGAGGACTATAAAACAATA  | Fwd/Ø                       |
| Cj18M1R    | AAAGTGAAGAAGAAGCTGAGCTCAAAAAC | Rev/Ø                        |
| Cj18M1R.c  | CAAGAGGAGATACATGGTCTGGAACCTT | Fwd/Ø                       |
| Cj18Nde-Rev | ATACGTAACAGAAGAAACTCATACATCCTC | Rev/PstI, Ndel               |
| Cj18RM     | TACGAGCTACAGCACAAGCTACATACACC | Rev/BamHI                   |
| Cj19LMX-2  | AGTTCTAGAGTTGACAGCTTCTGATA   | Fwd/Xbal                    |
| Cj19cECa   | GCAGAATTCCAGATAGCAGGATTTTGG  | Rev/EcoRI                   |
| Cj19mWL    | CGTGATCCCATGGTGAATCTTATATAATAC | Fwd/Smal, BamHI            |
| Cj19X      | AGTTCTAGAGTTGACAGCTTCTGATA   | Rev/Xbal                    |
| Cj19Sb     | ATCGGATCCGATACATGGGATCTTCTGGA | Fwd/BamHI                   |
| Cj45Dig    | TCAAGAAACTCCATCATGATC        | Rev/Ø                       |
| Cj46       | CATGTTAACTCAAATATATAC        | Fwd/Ø                       |
| Cj46mwr    | ATACCCGGATATCCAAATAGTACCTGAGCAGCTAC | Rev/Smal, BamHI            |
| Cj-RT      | GCAAGTGATTGAGGTCAATGAGCTGAA  | Rev/Sall                    |
| Cj879B     | ATCGGATCCACAAATCTAAAGGCTTTC  | Fwd/BamHI                   |
| Cj880      | CTATCCTAGAAAAATAATATAG       | Fwd/Ø                       |
| Cj880B     | ATCGGATCCATACATGTTTCTTCTTTC  | Fwd/BamHI                   |
| Cj880B2    | ATCGGATCCCTTCCAGAAAAATTAGCAAG | Fwd/BamHI                   |
| Cj880X     | AGTTCTAGACATACCAAATAGATCTTT | Rev/Xbal                    |
| Cj881B     | ATCGGATCCAAATGAAAAAGATATGCGA | Fwd/BamHI                   |
| Cj881X     | AGTTCTAGAAATGTGCTATAACAGTAAG | Rev/Xbal                    |

All vectors containing transcriptional fusions of putative dsb gene promoter regions with a promoterless lacZ gene were constructed using the pMW10 E. coli/C. jejuni shuttle vector. DNA fragments were amplified from C. jejuni 81-176 chromosomal DNA with appropriate pairs of primers (listed in Table 2). Next, PCR products were cloned in the pGEM-T Easy vector (Promega), excised by restriction enzymes and subsequently cloned into pMW10, forming transcriptional fusions with the downstream promoterless lacZ reporter gene. Correct construction of the resulting shuttle plasmids was confirmed by restriction analysis and sequencing. All recombinant plasmids, as well as the empty pMW10, were introduced into C. jejuni 480 cells by electroporation.

Construction of a pUWM1072 plasmid containing dsbl without dba under its native promoter was achieved by
Table 2 Oligonucleotides used in the present study (Continued)

| Oligonucleotide | Sequence | Orientation |
|----------------|----------|-------------|
| Cj882          | AGGTGTAAGTCTTGAGAGGC | Fwd/Ø       |
| Cj882B         | ATGGATACGACCTGCGTAATTCGG | Fwd//BamHI |
| Cj882X         | AGGTCTAGAGATTTTCGTAATGCTCAT | Rev//XbaI |
| Cj882B3        | ATCGAGATCTATGATTATAGCCAAATGG | Fwd//BamHI |
| Cj883X2        | AGGTCTAGAGCTATGGCAAATCTGAATA | Rev//XbaI |
| CM-L           | ATATCGGGATATCCAGGTAACCTTTG | Rev//Ø      |
| CM-R           | GATGAATTACGACCTGGGTTTGC | Fwd//Ø      |
| DLL_dsdB1      | GCTAATGCAAAACTTGAATA | Rev//Ø      |
| DLL_dsdB2X     | CATACTACAAACTGACTCTTG | Rev//Ø      |
| DLL_chuF       | CATATGAGAAATAATGGTATGG | Fwd//Ø      |
| EMSAchuR       | TTTGGTGCAAATTTTACCTC | Rev//Ø      |
| Fur-L          | GTAATTTTATATGTTGATGC | Fwd//Ø      |
| Fur-R          | TCTCTACTCTCTCAATGTTGTC | Rev//Ø      |
| KAN-L          | TATACCTCATTAGTGGTCGTTGG | Rev//Ø      |
| KAN-R          | GGGGATCAAGCTGATGAGGAGA | Fwd//Ø      |
| KM-L1          | GAAATATACCCGGGAATGTA | Fwd//Ø      |
| KM-R1          | CTTCTACACTTCTCGCGACAA | Rev//Ø      |
| lacZ           | AGGTATTGTGGTGTAGATTG | Rev//Ø      |
| lacZ1          | GGAATTCATCGCCGCGTGTGT | Fwd//Ø      |

**Bold letters indicate C. jejuni 81-176 sequences; restriction recognition sites introduced for cloning purposes are underlined, complementary fragments of primers Cj86mWW and Cj43mW are marked with italics.** Point mutated nucleotides in primers are marked with small letters. Orientation of the primers (Fwd states for forward/Rev - for reverse) refers to the orientation of particular C. jejuni gene studied. RT-Cj primer was designed on the basis of C. coli 7202e dsbI nucleotide sequence (there are 2 nucleotide changes compared to the nucleotide sequence of its orthologue from C. jejuni 81-179).

PCR-amplification of the 520 bp chromosomal DNA fragments containing the *dba-dsbI* promoter sequences (primer pair Cj19LX-2 - Cj18Nde-Rev) and cloning it into pBluescript II SK (Stratagene), using XbaI/PstI restriction enzymes. Subsequently the *dsbI* coding sequence (1792 bp) was PCR-amplified using the Cj17Nde - Cj16RS primer pair, cloned into pGEM-T Easy (Promega) and finally, using NdeI/Sall restriction enzymes, transferred into pUWM1072 in the native orientation, generating the plasmid pUWM1100. The whole insert (2316 bp) was then cloned into a shuttle *E. coli/C. jejuni* vector pRY107 [27] using SalI/XbaI restriction enzymes. The resulting, plasmid pUWM1103, whose correct construction was verified by sequencing, was used for complementation assays in *C. jejuni* mutant cells.

Point mutations were generated using a Quick-Change site-directed mutagenesis kit, following the supplier’s recommendations (Stratagene). To construct a *dba* gene with point mutations, the pUWM456 plasmid, containing the *C. jejuni* *dba-dsbI* genes, was used as a template for PCR-mediated mutagenesis. Point mutations M1R and L29stop (replacing a Leu codon with amber stop codon) were introduced using the respective pairs of primers: Cj18M1R - Cj18M1Rc and Cj18L29 - Cj18L29c. The resulting plasmids were introduced into *E. coli* cells by transformation and presence of desired mutations was verified by DNA sequencing. DNA fragments containing the *C. jejuni* *dba-dsbI* operon (with or without a point mutation) were then digested and inserted into the pRY107 shuttle vector. The resulting plasmids were named pUWM769 (containing wt *dba-dsbI*), pUWM811 (*dba*: M1R, wt *dsbI* and pUWM812 (*dba*: L29stop, wt *dsbI*). These plasmids were subsequently introduced into *C. jejuni* 81-176 AL1 (*dsbI::cat*) and *C. jejuni* 81-176 AG6 (*Δ*db*adsbI::cat*) knock-out cells by conjugation [28].

**Construction of bacterial mutant strains**

To inactivate *dba* and *dsbI* genes, three recombinant plasmids were constructed, based on pBluescript II KS (Stratagene) and pGEM-T Easy (Promega) vectors, which are suicide plasmids in *C. jejuni* cells. A. van Vliet kindly furnished the fourth suicide plasmid, pAV80, which was previously used for *C. jejuni* NCTC11168 fur inactivation [25]. Correct construction of all the plasmids was confirmed by restriction analysis and sequencing. 

The plasmid for *C. jejuni* *dba* mutagenesis was generated by PCR-amplification of two *C. jejuni* 81-176 DNA fragments (600 bp and 580 bp long) that contained *dba* gene fragments with their adjacent regions with primer pairs: Cj19LX-2 - Cj18RM and Cj18LM - Cj17RM. Next they were cloned in native orientation in pBluescript II KS (Stratagene). Using BamHI restrictase, the kanamycin resistance cassette (the 1.4 kb *aphA*-3 gene excised from pBF14) was inserted between the cloned *dba* arms in the same transcriptional orientation, generating the suicide plasmid pUWM622.
To obtain the construct for *C. jejuni* dsbI mutagenesis the 1.5 kb DNA fragment containing the *dsbI* gene was PCR-amplified from the *C. jejuni* 81-176 chromosome using primer pair: Cj17LSal - Cj17RBgl and was cloned into pGEM-T Easy (Promega). Subsequently, the internal 300 bp EcoRV-EcoRV region of *dsbI* was replaced by a SmaI-digested chloramphenicol resistance cassette (the 0.8 kb *cat* gene excised from pRY109) [27] inserted in the same transcriptional orientation as the *dsbI* gene, generating the suicide plasmid pUWM713.

To obtain the construct for *C. jejuni* dba-dsbI mutagenesis, the 410 bp and 380 bp DNA fragments, containing dba upstream and dsbI downstream regions were PCR-amplified from the *C. jejuni* 81-176 chromosome using primer pairs: Cj19LSal - Cj46mwR and Cj43mwL. These fragments were directly digested with BamHI restriction enzyme, ligated in a native orientation and used as a template for a subsequent PCR reaction with the external primer pair: Cj19LX-2 - Cj43Eco. This PCR product was cloned into pGEM-T Easy (Promega) and the chloramphenicol resistance cassette (the 0.8 kb *cat* gene excised from pRY109) was inserted in the same transcriptional orientation as *dbadsbI* operon at the BamHI site between the *C. jejuni* DNA fragments, generating suicide plasmid pUWM866.

Gene versions inactivated by insertion of a resistance cassette were introduced into the *C. jejuni* 81-176-480 chromosome by the allele exchange method as described by Wassenaar *et al.* [24]. Construction of the *C. jejuni* 480 fur:*cat* mutant was achieved by natural transformation using *C. jejuni* 81-176 fur:*cat* chromosomal DNA. It should be pointed out that *C. jejuni* 480 was previously described as incapable of accepting chromosomal DNA by natural transformation [24]. Such inconsistency of experimental data might be due to different chromosomal DNA used for natural transformation (*C. jejuni* 81116 vs *C. jejuni* 81-176). The mutant strains were obtained by two- or tri-parental mating experiments performed as described by Labigne-Roussel *et al.* [29] and Davis *et al.* [30]. The constructed mutants were named AG1 (*C. jejuni* 81-176 dba::aphA-3), AL1 (*C. jejuni* 81-176 dsbI::cat), AL4 (*C. jejuni* 480 dsbI::cat), AG6 (*C. jejuni* 81-176 dba-dsbI::cat), AG11 (*C. jejuni* 81-176 fur::cat), and AG15 (*C. jejuni* 480 fur::cat). They demonstrated normal colony morphology and all but two had normal growth rates when cultured on BA plates. Only the *C. jejuni* 81-176 fur::cat and *C. jejuni* 480 fur::cat exhibited slower growth, an observation consistent with other studies on *fur* mutants [25]. Disruption of each gene as a result of double cross-over recombination was verified by PCR with appropriate pairs of primers flanking the insertion site (Table 2). The loss of DsbI synthesis in the constructed mutants was verified by Western blotting of whole-cell protein extracts against specific rabbit polyclonal anti-rDsbI antibodies.

**Protein manipulation, and β-galactosidase and arylsulfate sulfotransferase (AstA) assays**

Preparation of *C. jejuni* protein extracts, SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) and blotting procedures were performed by standard techniques [26].

To obtain recombinant His<sub>6</sub>-DsbI protein, the 1100 bp DNA fragment containing the coding sequence for the predicted periplasmic DsbI C-region was PCR-amplified from the *C. jejuni* 81-176 chromosome using a primer pair: Cj17WDBam-up - Cj17WDBam-low. This fragment was cloned into the pGEM-T Easy vector and then, using BamHI restriction enzyme, into expression vector pET28a (Novagen) to generate plasmid pUWM657, whose correct construction was verified by restriction analysis and sequencing. Cytoplasm-located soluble fusion protein His<sub>6</sub>-DsbI purified from the *E. coli* Rosetta (DE3) Lacl<sup>δ</sup> strain by affinity chromatography was used for rabbit immunization (Institute of Experimental and Clinical Medicine, Polish Academy of Science, Warsaw, Poland). The anti-His<sub>6</sub>-DsbI (anti-rDsbI) serum obtained was highly specific and recognized native DsbI, as verified by Western blot experiments carried out with protein extracts from *C. jejuni* wild type and a *dsbI* mutant strain (data not shown).

To obtain recombinant Fur-His<sub>6</sub> protein, the DNA fragment containing the entire *fur* coding region was PCR-amplified from the *C. jejuni* 81-176 chromosome with primer pair CjFurNcl - CjFurXhol, and then cloned, using Ncol/Xhol restriction enzymes, into pET24d (Novagen). This generated pUWM1098, carrying a fur-his<sub>6</sub> translational fusion. This plasmid was then transformed into *E. coli* BL21 (DE3) cells. Recombinant Fur-His<sub>6</sub> protein was overproduced by addition of 1mM IPTG to the bacterial culture at exponential growth phase and purified under native conditions by affinity chromatography. β-galactosidase activity assays in *C. jejuni* cell extracts were performed three times (each time three independent samples were taken for each strain), as described by Miller [31].

*C. jejuni* transformants grown overnight on BA medium were harvested and resuspended in LB medium to achieve comparable cell densities (OD<sub>600</sub> approx. 0.6). Fresh MH liquid medium (MH supplemented with iron sulfate - iron-rich conditions, MH itself - iron-sufficient and MH with iron chelated by addition of deferoxamine mesylate - iron-restricted conditions) was inoculated with *C. jejuni* (1:10) and incubated overnight (15-22 h depending on the medium) till the culture reached OD<sub>600</sub> of about 0.4-0.6. Since Wright *et al.* documented that *C. jejuni* exhibits a dynamic stationary phase, characterized by switches in
motility, substrate utilization and metabolite production accompanied by concurrent changes in gene expression, exponential phase cultures were used in this experiment to eliminate any stationary phase-dependent physiological switching of gene expression levels [32].

Quantitative assays for AstA arylsulfatase activity were performed three times (each time three independent samples were taken for each strain), using the method described by Hendrixson et al. with one difference: the C. jejuni 81-176 strain was cultivated on MH liquid medium under high- or low-iron conditions [33] (approx. 17 h on MH medium under high iron condition and approx. 22 h on MH medium under low-iron condition). For each experiment, bacterial cultures of the same OD (OD<sub>600</sub> ~ 0.6-0.7) were used.

**RNA analysis**

Total RNAs were extracted from C. jejuni overnight BA culture using the standard TRizol reagent according to the manufacturer’s protocol (Invitrogen). RNA samples were treated with DNaseI to eliminate contaminating DNA and quantified by measurements of OD<sub>260</sub>, RNA were reverse transcribed using Superscript II enzyme (Invitrogen) and RT-primer (Table 2): Cj-RT complementary to the transcribed using Superscript II enzyme (Invitrogen) and RT-primer (Table 2); Cj-RT complementary to the kanamycin-resistance cassette. The RT primer was annealed stepwise before adding the reverse transcriptase. The enzyme was finally inactivated by incubation at 70°C for 15 min. A control reaction without reverse transcriptase enzyme was finally inactivated by incubation at 70°C for 15 min. A control reaction without reverse transcriptase enzyme was finally inactivated by incubation at 70°C for 5 min. at room temperature and subsequently for 5 min. at 37°C in a 20 μl volume of binding buffer routinely used for the Fur-binding assay (10 mM Tris-HCl [pH 7.5], 1 mM MgCl<sub>2</sub>, .05 mM dithiothreitol, 50 mM KCl, 100 μM MnCl<sub>2</sub>, 1 μg poly (dl-dc), 50 μg bovine serum albumin and 5% glycerol). In addition, dsbA2 and dsbA1 promoter regions were incubated with Fur-His protein in binding buffer without Mn<sup>2+</sup>. As negative controls each Dig-labelled DNA fragment was incubated with an unrelated protein (purified H. pylori HP0377- His<sub>6</sub>). Control reactions were performed using competitor DNA - unlabeled promoter DNA region. Samples were run on a 5% non-denaturing Tris-glycine polyacrylamide gel at 4°C. Then DNA was transferred to nylon membranes (Roche) and UV cross-linked. Labelled DNA was detected with anti-DIG antibody using a standard DIG detection protocol (Roche).

**Results**

### In silico analysis of C. jejuni 81-176 dsb gene clusters

C. jejuni 81-176 dsbA2-dsbB-astA-dsbA1 genes (cjj81176_0880-0883) have the same orientation in the chromosome (Figure 1A) and are separated by short intergenic regions - 11 bp, 87 bp, and 85 bp, respectively. Thus, they potentially might be co-transcribed. In silico analysis of the C. jejuni dsbA2-dsbB-astA-dsbA1 cluster revealed the presence of a potential RBS as well as a complete promoter nucleotide sequence upstream of dsbA2, located within the 627 bp intergenic xerD-dsbA2 region [34]. As this DNA fragment consists of -35, -16 and -10 regions (characteristic for the σ<sup>70</sup> binding sequence), it can be recognized by Campylobacter RNAP containing the main sigma factor. Directly upstream of dsbB there is a potential additional RBS sequence but none of the promoter regions were found, suggesting dsbA2-dsbB co-transcription. Upstream of astA and dsbA1 there are putative RBS sequences and incomplete promoter nucleotide sequences, suggesting that astA and dsbA1 might be transcribed separately from dsbA2 and dsbB.

C. jejuni 81-176 dba (cjj81176_0045c) and dsb1 (cjj81176_0044c) have the same orientation in the chromosome (Figure 1B) and their coding sequences are separated by a short intergenic region of 11 bp. An initial RT-PCR experiment carried out on the total C. jejuni RNA documented dba-dsb1 co-transcription in vitro and localization of their promoter within 493 bp DNA upstream of the dba translation start codon [18].

### Transcriptional analysis of two dsb gene clusters

The lacZ reporter gene system was used to determine the dsb gene expression and regulation. Two sets of dsb-lacZ

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**Figure 1**

A: Schematic representation of the dsbA2-dsbB-astA-dsbA1 gene cluster. B: Schematic representation of the dba-dsb1 gene cluster. Both clusters are located in the chromosome of C. jejuni 81-176. The arrows indicate the direction of transcription. **Figure 2**

A: Schematic representation of the dsbA2-dsbB-astA-dsbA1 gene cluster. B: Schematic representation of the dba-dsb1 gene cluster. Both clusters are located in the chromosome of C. jejuni 81-176. The arrows indicate the direction of transcription.
transcriptional fusions were designed based on a promoterless lacZ gene in the shuttle vector pMW10 [34].

The first one comprised of seven plasmids (pUWM792, pUWM803, pUWM795, pUWM832, pUWM833, pUWM834 and pUWM864) employed to study dsbA2/dsbB/astA/dsbA1 expression. The other consisted of three plasmids (pUMM827, pUWM828 and pUWM858) generated to analyze dba/dsbI expression. Details of the recombinant plasmid structures are shown in Figure 1. We successfully prepared all but one of the planned transcriptional fusions - we failed at constructing the longest fusion presented in Figure 1.

β-galactosidase assays indicated that the fusions present in pUWM833, pUWM834 and pUWM858 were not expressed in C. jejuni cells. This documented that the analyzed genes form two polycistronic operons (dsbA2-dsbB-astA and dba-dsbI) and only dsbA1 is independently transcribed. The level of β-galactosidase provided by the dsbA1 promoter was approximately ten times higher than that conferred by the two other promoters that were analyzed (contained in pUWM803 and pUWM827). Thus, three promoters of various strengths and responsible for C. jejuni dsb gene expression were identified: P_{dsbA2dsbB/astA}, P_{dsbA1} and P_{dsbA1}.

**Influence of environmental stimuli on dsb gene expression**

We subsequently tested whether gene expression driven by P_{dsbA2dsbB/astA}, P_{dsbA1} and P_{dsbA1} (C. jejuni 480 strains harbouring pUWM803, pUWM864 or pUWM827) responds to environmental stimuli. While there were no significant differences in β-galactosidase activity between
cells grown at various temperatures (37°C and 42°C) (Figure 2A) or between cells grown in solid and liquid medium (MH broth and MH solidified by agar addition) (data not shown), transcription from each of the analyzed promoters was iron-regulated (Figure 2B). For cells grown in iron-restricted conditions, $P_{dsbA2dsbBastA}$ activity was 10 times lower, $P_{dsbA1}$ activity was about 30% lower, and $P_{dsbA1dsbB}$ activity was four times higher, compared to cells grown under iron-sufficient/iron-rich conditions.

Iron-regulated expression of many Gram-negative bacterial genes is mediated by the ferric uptake regulator (Fur) [35,36]. Classically, the Fur protein first binds to its co-repressor Fe$^{2+}$, and then binds to the conserved DNA sequence (Fur-box) of the regulated promoter, repressing its transcription. However, transcriptomic analyses documented that apo-Fur (without complexed co-repressor) can also influence gene transcription in response to iron concentration [6,36-38].

We therefore decided to evaluate the regulatory function of the Fur protein on $dsb$ gene expression. For this purpose a C. jejuni 480 fur isogenic mutant was constructed. Then, recombinant plasmids containing $dsb$ promoter- lacZ fusions ($p$UWM803, $p$UWM864 and $p$UWM827) were introduced into the C. jejuni 480 fur:cat mutant by electroporation. The results of β-galactosidase assays performed on the constructed strains proved Fur involvement in iron-dependent regulation of the three analyzed $dsb$ gene promoters (Figure 2C). β-galactosidase activity conferred by the $p$UWM827 fusion increased under iron-sufficient/rich conditions in the fur mutant as compared to the wild-type strain, suggesting that inactivation of fur results in derepression of $P_{dsbA1dsbB}$. In contrast, β-galactosidase activities of the $p$UWM803 and $p$UWM864 fusions increased under iron starvation in the fur mutant compared to the wild-type strain. This indicates that low level of iron leads to Fur-mediated repression of the $P_{dsbA2dsbBastA}$ and $P_{dsbA1}$ promoters, since repression was abolished in the fur mutated strain. C. jejuni 480 strain containing $p$UWM471, which harbors cjAA gene promoter fused to a promoterless lacZ gene, was employed as a control in all experiments analyzing the influence of Fur and iron on $dsb$ gene expression. There were no significant differences in β-galactosidase activity between wild type cells harbouring $p$UWM471 grown at various iron concentrations as well as between wt and fur mutated cells containing $p$UWM471. In every case high β-galactosidase levels (about 2000 Miller units) were observed, which is consistent with previously published data that ranked the cjAA promoter as one of the the strongest Campylobacter spp. promoters so far described [39].
Inspection of the nucleotide sequences located upstream of the \textit{dba} translation initiation codon did not reveal the presence of an exact \textit{C. jejuni} Fur-binding site sequence motif [40]. So far, a potential Fur binding site for promoters positively regulated by iron concentration in a Fur-dependent manner has not been determined. Therefore, we used EMSA to gain insight into the mechanism by which \textit{P}_{\text{dba-dsbI}} \textit{P}_{\text{dsbA2-dsbB-astaA}} and \textit{P}_{\text{dsbA1}} are regulated by Fur. To achieve this goal, various primers were designed to amplify a 174-299 bp DNA fragment upstream from the translational start site of each tested operon. The promoter region of the \textit{chuA} gene, which contains the Fur-binding motif and is strongly repressed by iron-complexed Fur, was used as a control [6,40]. Mn$^{2+}$ ions were used in the EMSA in place of Fe$^{2+}$ due to their greater redox stability. It was demonstrated that the Fur-His$_{6}$ was able to bind \textit{in vitro} to the DNA region upstream of the \textit{dba-dsbI} operon only when the regulatory protein was complexed with Mn$^{2+}$, which indicated, in accordance with previously presented data, that this operon is repressed by the iron-complexed form of Fur (Figure 3E). This promoter region interacts with Fur complexed with Mn$^{2+}$ as much as the \textit{chuA} promoter (Figure 3G). In contrast, the upstream DNA region of the \textit{dsbA1} gene did not bind Fur, regardless of the presence of Mn$^{2+}$ in the reaction buffer. This suggested an indirect method of regulation (Figure 3, panel C and D). In the case of the \textit{dsbA2-dsbB-astaA} promoter region, Fur protein bound DNA in the absence of Mn$^{2+}$ as a repressor (Figure 3B), supporting the results obtained in the \textit{P}_{\text{chuA}} promoter (Figure 3G). The formation of DNA/Fur complexes specific for the \textit{dsbA2-dsbB-astaA} promoter region was efficiently inhibited by adding unlabelled DNA containing the same DNA fragment.

To check whether the abundance/activity of Dsb-dependent proteins is conditioned by iron concentration, we compared the arylsulfate sulfotransferase (AstA) activity in \textit{C. jejuni} \textit{Δdba-dsbI} \textit{Dsbl} positive and negative strains grown under iron-restricted conditions as compared to the wild type strain. AstA activity in \textit{C. jejuni} \textit{Δdba-dsbI} cells reached 75-80% of activity observed for the same strain grown under iron-rich conditions. Previously performed \textit{in vitro} transcription/translation coupled assays suggested that \textit{C. jejuni} Dba may influence Dsbl synthesis and/or stability [18]. To reveal details of \textit{dba-dsbI} operon expression we examined whether \textit{dba}/Dba was required for \textit{in vivo} synthesis of Dsbl in \textit{E. coli} cells. It was demonstrated that in \textit{E. coli}, Dsbl underwent partial degradation (for details see Additional file 2 and 3). This result was in agreement with those derived from previous \textit{in vitro} experiments. It is noteworthy that in \textit{C. jejuni} cells, Dsbl is produced in two forms as a result of posttranslational modification by glycan binding (for details see Additional file 2 and 4).

Additionally a \textit{C. jejuni} \textit{Δ81-176} isogenic \textit{dba} mutant was constructed by inserting the kanamycin resistance cassette in the same orientation as \textit{dba} coding sequence. This insertion should not alter the downstream \textit{dsbl} transcription. Nevertheless, inactivation of \textit{C. jejuni} \textit{dba} resulted in the absence of Dsbl, and subsequent RT-PCR experiments, conducted for four independently isolated transformants, also documented the absence of \textit{dsbl} transcript in \textit{dba} mutated cells (data not shown). To further examine the role of \textit{dba} expression in Dsbl synthesis, a double mutant strain - \textit{C. jejuni} \textit{Δdba-dsbI}:\textit{cat} (AG6) - was constructed. The first recombinant shuttle plasmids, pUWM769 (containing the wild type \textit{C. jejuni} \textit{dba-dsbI} operon), pUWM811 and pUWM812 (containing point mutated \textit{dba} - M1R or \textit{dba}: L29stop, respectively, and wild type \textit{dsbl}) were introduced into mutant cells. Transformant cells were screened for Dsbl synthesis by Western blot analysis with specific rabbit anti-Dsbl serum and additionally by RT-PCR for the presence of \textit{dsbl} transcript. Introduction of pUWM769 into \textit{C. jejuni} \textit{AG6} (\textit{Δdsba-dsbl}:\textit{cat}) cells resulted in restoration of Dsbl production in a higher amount compared to the wild type strain (Figure 4, lane 6), due to plasmid-encoded \textit{dba-dsbI} gene expression. When \textit{dba} translation was completely aborted (\textit{C. jejuni} \textit{AG6} carrying pUWM811) and when the truncated 28 aa Dba was produced (\textit{C. jejuni} \textit{AG6}/pUWM812), Dsbl was not synthesized at all (Figure 4, lane 4 and 5, respectively). RT-PCR experiments proved that point mutations in \textit{dba} did not influence \textit{dsbl} transcription, as comparable amounts of \textit{dsbl} mRNA were detected in all but one (AG6) of the strains (Figure 5, lanes: 9, 11-13). Comparable results were obtained for series of \textit{C. jejuni} \textit{dsbl}:\textit{cat} strains carrying pUWM769, pUWM811 and pUWM812 plasmids (data not shown), suggesting that intact, chromosomally-encoded Dba cannot act \textit{in-trans} to ensure \textit{dsbl} mRNA translation.

To further address the role of Dba in \textit{dsbl} expression the recombinant plasmid lacking the \textit{dba} gene but containing the \textit{dsbl} gene transcribed from own promoter was constructed and introduced into the \textit{C. jejuni} \textit{Δdba-dsbI}:\textit{cat} mutant. The \textit{C. jejuni} \textit{Δ81-176 Δdba-dsbI}:\textit{cat}, harbouring pUWM769 was employed as a control. Western experiments showed that an individual expression of the \textit{dsbl} gene from own promoter results in Dsbl
Figure 3 Electrophoretic mobility shift assays of chuA, dba-dsbI, dsbA2 and dsbA1 promoter regions bound by CjFur-His6. 28 fmol of Dig-labelled PCR amplified DNA fragments: dsbA2 (333 bp - panel A and B), dsbA1 (299 bp - panel C and D), dba-dsbI (174 bp - panel E and F) and chuA (216 bp - panel G and H) were incubated with 0, 333, 1000 or 3333 nM of purified Fur protein. The concentration of CjFur-His6 used in the reactions is indicated above the lanes. Binding buffer used in four EMSA studies (panels B, D, F, H) does not contain Mn^{2+}. Panel I presents competition gel mobility shift assay which was performed by incubation of 3333 nmol Fur-His protein with 28 fmol of the labelled promoter region upstream of dsbA2-dsbB-astA operon (dsbA2) and various concentrations of the unlabelled promoter region upstream of dsbA2-dsbB-astA operon (dsbA2*)
The best characterized Dsb oxidative system, that of *E. coli*, consists of two oxidoreductases, periplasmic DsbA and inner membrane DsbB, that are involved in disulfide bond formation *de novo* in the bacterial periplasm. Genes encoding these proteins are located in different chromosomal sites and are transcribed as monocistronic units. Production (Figure 6, lane 2), underlining once more the importance of mRNA secondary structure for the dsbI mRNA translation.

**Discussion**

The best characterized Dsb oxidative system, that of *E. coli* K-12, consists of two oxidoreductases, periplasmic DsbA and inner membrane DsbB, that are involved in disulfide bond formation *de novo* in the bacterial periplasm. Genes encoding these proteins are located in different chromosomal sites and are transcribed as monocistronic units.

The *Campylobacter jejuni* Dsb oxidative pathway is more complex. In the present study we initiated analysis of *C. jejuni* dsb gene organization and regulation. Our results document organization of these genes in two operons, one comprised of *dsbA* and *dsbI*, and another of *dbaA2*, *dsbB* and *astA*. The *dsbA1* gene constitutes a separate monocistronic transcriptional unit. Predictions based on *in silico* analysis by Petersen et al. [44] of the *C. jejuni* NCTC 11168 genome nucleotide sequence stated that the *dba* and *dsbI* genes are cotranscribed. They also indicated that cj0864 (a truncated version of *dsbA2*) and cj0865 (*dsbB*) potentially form an operon. The first T base of the TATA box was predicted to be located 199 bp upstream from the ATG start codon for the *dba-dsbI* operon and 66 bp from the ATG start codon for the *dsbA2-dsbB-astA* operon [44].

Global comparative *C. jejuni* transcriptome or proteome analysis revealed that transcription levels of *dsbA2*, *dsbB* and *astA* increase in strains isolated from a chicken cecum compared with strains grown *in vitro* [5] and they are down-regulated under iron-restricted conditions *in vitro* [6]. Stinzi et al. found that *dsb* gene transcription was not dependent on the temperature of *in vitro* growth (37 vs 42°C) [45]. So far only one transcriptomic study has documented that *dba* and *dsbI* transcript abundance is iron-dependent. Interestingly, the authors stated that the transcription of *dba* and *dsbI* was antagonistically regulated by iron accessibility, depending on the experimental conditions, i.e. iron-activated shortly after iron addition into the medium and iron-repressed in the mid-log phase of growth [40]. All cited transcriptomic experiments were conducted on mRNA derived from *C. jejuni* NCTC 11168, a strain which has the shorter, non-functional *dsbA2* version.

Our experiments, conducted on *C. jejuni* 480 wild type expressing β-galactosidase from different *dsb* gene...
promoters of C. jejuni 81-176, demonstrated that they are all regulated in response to iron availability. Our data are generally consistent with those derived from transcriptomic analysis. The strongest of the analyzed promoters, \( P_{dsbA} \), which was down-regulated in iron starvation conditions, was not identified in comparative transcriptomic experiments conducted by Holmes et al., although that work revealed \( P_{dsbA2dsbBastA} \) iron dependence [6]. Such inconsistency of experimental data might be due to limited sensitivity of the transcriptomic strategy previously used. The transcription level of \( dsbA1 \) is only slightly affected by iron concentration, whereas the transcription level from \( P_{dsbA2dsbBastA} \) decreases about 10-fold in response to iron deficiency. The \( dsb \) gene promoters are antagonistically regulated by iron availability, at least under conditions used in this study. Thus, abundance of both periplasmic oxidoreductases, DsbA1 and DsbA2, decreases when iron becomes restricted, while DsbB and DsbI membrane oxidoreductases are synthesized constitutively, in different extracellular iron concentrations. This might suggest that iron-storage proteins or non-essential iron-using proteins might be direct or indirect targets of the Dsb oxidative pathway involving activity of DsbA1/DsbB or DsbA2/DsbB redox pairs.

In some microorganisms, positive regulation by Fur and iron is provided by action of sRNAs which are themselves regulated by iron-complexed Fur - these sRNAs pair with their target mRNAs and promote their degradation (reviewed in [46]). However, \( P_{dsbA2dsbBastA} \) and \( P_{dsbA1} \) promoters are not regulated that way, since the level of \( \beta \)-galactosidase in iron-sufficient medium is comparable in wild-type and fur mutated cells. This observation proved that these promoters are not induced by iron-bound Fur, as the level of \( \beta \)-galactosidase expressed from these two fusions is higher in response to iron limitation in the fur mutant than in the wild type cells. The most probable explanation of these results is that iron-free Fur is capable of repressing their transcription. Palyada et al. [40] performed in silico analysis aimed at Campylobacter Fur box identification. They inspected 16 DNA fragments located upstream of iron and Fur repressed genes, which allowed them to establish the potential Fur box sequence motif. However, only eleven of the analyzed promoters included this element [40]. So far C. jejuni’s potential Fur box for apo-Fur repressed genes remains undetermined.

In the present study the EMSA assays confirmed that although all the analyzed promoters were members of the Fur regulon, each of them was regulated by a different mechanism. We showed that both iron-free and iron-complexed Fur can act as a repressor. The observed potential dual regulation of the \( P_{dsbA2dsbBastA} \) promoter, dependent on Fur concentration, still remains unclear. An explanation for this phenomenon requires deeper understanding of the C. jejuni fur gene expression. In contrast to E. coli, the C. jejuni fur gene expression is not autoregulated, and additionally, the iron-responsive Fur regulator of C. jejuni is expressed from two separate promoters [47]. Our findings further indicate that transcription under iron-starvation can be controlled by Fur indirectly, as was observed for the \( dsbA1 \) gene. The sophisticated mechanism regulating \( dsb \) gene transcription in response to iron availability may be responsible for subtle changes in the abundance and/or activity of various substrates in the Dsb system. We demonstrated that activity of C. jejuni 81-176 AstA, which is a direct target of Dsb system, is dependent on iron level in the medium. However, as AstA level is dependent on the activities of both DsbA1 and DsbA2 (unpublished results), details of the process remain unclear.

Recently performed comparative Helicobacter pylori and Neisseria gonorrhoeae transcriptomic analysis also indicated that genes included in the Fur regulon can be positively or negatively regulated in response to iron availability [38,48]. Like C. jejuni Fur, H. pylori Fur also binds to some promoters in its iron-free form to repress their expression [38,49-51]. C. jejuni Fur reveals a relatively high degree of amino acid identity with H. pylori Fur. Nonetheless it is not able to complement apo-Fur regulation in an H. pylori fur mutant when delivered in trans [52]. Such unexpected results might be due to subtle differences in conformation of both proteins.

Additional experiments, such as solving the three dimensional structure of C. jejuni Fur, are required to clarify the functional differences between Fur proteins of these closely related species. Although both species have AT-rich genomes and some of their promoters have similar structure, it can not be excluded that the C. jejuni apo-Fur binding nucleotide sequences are not identical as those determined for H. pylori apo-Fur. Also two H. pylori promoters, the pfr and sod gene promoters that are repressed by apo-Fur, exhibited low sequence similarity and revealed different affinities for apo-Fur [38,50].

The second part of our research was aimed at understanding the relationship between \( dba \) and \( dsbI \) expression. Experiments employing point mutated \( dba \) provided evidence for strong translational coupling of the \( dba \) and \( dsbI \) genes. Inhibition or premature termination of \( dba \) mRNA translation resulted in the lack of DsbI. This defect was not complemented by the intact chromosomal \( dba \) gene in C. jejuni 81-176 \( dsbI::cat \). Translational coupling has already been described and is common among functionally related bacterial genes. It was documented that in many cases it involves operons containing overlapping genes as well as genes constituting an operon and divided by short intergenic region
form an active protein complex. It might be a prerequisite for recruiting other proteins to a periplasmic or transmembrane chaperone, providing information about the Dba activity of the Dsb system [6]. Taken together, these results support the notion that iron concentration-through the influence on dsb gene expression - might control abundance of the extracytoplasmic proteins during different stages of infection. Our work further shows that the synthesis of the Dsb membrane oxidoreductase is controlled by a translational coupling mechanism. Among bacterial genomes sequenced so far, those of C. jejuni strains are extremely compact. About 95% of their content is occupied by protein-coding regions and more than 25% of all genes overlap. Presumably, translational coupling occurs during expression of many other C. jejuni operons containing tail-to-head oriented genes with short or no intergenic regions.

Conclusions

The present work documents that iron concentration is a significant factor influencing dsb gene transcription. Preliminary results of proteomic experiments aimed at identification of Campylobacter Dsb system targets suggest that mutations in dsb genes influence the level of a dozen extracytoplasmic proteins (manuscript in preparation). One of them is the periplasmic LivJ protein, which contains four cysteine residues and is involved in the colonization process as shown by Hendrixon and DiRita [55]. Moreover proteomic analysis of iron-regulated C. jejuni protein expression done by Holmes et al. showed that LivJ abundance is iron-dependent. Because LivJ gene transcription is not iron nor Fur dependent, most likely the changes in the abundance of this protein are influenced by activity of the Dsb system [6].

Additional material

Additional file 1: Arylsulfatase (AstA) assay in C. jejuni 81-176 cells
Arylsulfatase (AstA) activity of C. jejuni 81-176 cultivated on MH liquid medium under high- and low-iron conditions (chelator) till the culture reached OD600 ~0.6-0.7. Results are from four assays with each sample performed in triplicate. Values are reported as arylsulfatase units. One unit equals the amount of arylsulfatase required to generate 1 nmol of nitrophenol h$^{-1}$ per OD600 of 1.

Additional file 2: Experiment details concerning DsbI stability and glycosylation
Western blot (anti-rDsbI) analysis of C. jejuni E. coli protein extracts separated by 12% SDS-PAGE. Relative positions of molecular weight markers (lane 1) are listed on the left (in kilodaltons). Lanes 2-7 contain 20 μg of total proteins from: C. jejuni 81-176 wt (2), E. coli/pPLuescript II KS (3), E. coli/plUWM453 (dsb-dsb) (4), E. coli/plUWM454 (dsb) (5), E. coli/plUWM455 (dsb) (6) and E. coli/plUWM456 (dsb-dsb) (7).

Additional file 3: Influence of the dba/DsbA on Dsb stability in E. coli cells
Western blot (anti-rDsbI) analysis of C. jejuni E. coli protein extracts separated by 12% SDS-PAGE. A - proteins isolated from C. jejuni 81-176 wt and pgI8 biogenic mutant. Relative positions of molecular weight markers (lane 1) are listed on the left (in kilodaltons). Lanes 2 and 3 contain 20 μg of total proteins from: C. jejuni 81-176 wt (2) and C. jejuni 81-176 pgI8:cat (3). B - proteins isolated from C. jejuni 480 AL4 (dsb:cat) overexpressing DsbI or the mutated version of the protein DsbI. Relative positions of molecular weight markers (lane 1) are listed on the left (in kilodaltons). Lanes 2-4 contain 20 μg of total proteins from: C. jejuni 480 AL4/pUWM762 (DsbI N292A) (2), AL4/pUWM765 (DsbI N340A) (3) and AL4/pUWM769 (the shuttle plasmid containing a wild type copy of the C. jejuni dsbI gene) (4).

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Authors’ contributions
ADG conducted out most of the laboratory work. MW and MN, working under supervision of EKJK and ADG, contributed to construction of some transcriptional fusion, mutated C. jejuni strains and translational coupling experiments. AM, did RT-PCR experiments for the dsb-dsbI operon as well as expression of dsbI from its own promoter, and was involved in drafting the manuscript. RG performed experiments concerning influence of iron concentration on cpxA gene expression and AsA activity level. PR performed EMSA assays. AW performed experiments concerning DsbI glycosylation. EKJK conceived the study. EKJK and ADG designed the experiments, and were engaged in data interpretation and drafting the manuscript. All authors read and accepted the final version of the manuscript.

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