Characterization of new DsbB-like thiol-oxidoreductases of *Campylobacter jejuni* and *Helicobacter pylori* and classification of the DsbB family based on phylogenomic, structural and functional criteria

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INTRODUCTION

*Campylobacter* and *Helicobacter* spp. are phylogenetically related enteropathogens widely spread among human and animal populations. Members of both genera are Gram-negative, spiral-shaped, microaerophilic and motile bacteria. In spite of many phenotypic similarities, *C. jejuni* and *H. pylori* are causative agents of different disorders of the human intestinal tract. *C. jejuni* is one of the leading causes of bacterial gastroenteritis in humans worldwide (Bachoual et al., 2001; Coker et al., 2002). *H. pylori*, which colonizes human gastric mucosa, is one of the most common bacterial...
pathogens in humans. Chronic infection of the gastric mucosa by this bacterium results in chronic gastritis, and duodenal and gastric peptic ulcers. Furthermore, it may be an initiation factor of gastric adenocarcinoma and B-cell mucosa-associated lymphoid tissue lymphoma (Warren & Marshall, 1983; Dunn et al., 1997).

Many periplasmic proteins of Gram-negative pathogens which contain two or more cysteine residues can fold correctly owing to formation of disulfide bridges, which stabilize the tertiary and quaternary structures of the polypeptide chain. This process in *Escherichia coli* is facilitated by a Dsb (disulphide bond) family of the redox proteins. Up to now, five proteins (DsbA, B, C, D and G) involved in correct introduction of disulfide bonds into proteins in the periplasm have been identified and well characterized through a combination of genetic and biochemical approaches (Raina & Missiakas, 1997; Collet & Bardwell, 2002; Kadokura et al., 2003). A periplasmic enzyme, DsbA, and an integral membrane protein, DsbB, are involved in the oxidation pathway. The isomerization pathway is provided by another periplasmic protein, DsbC, which repairs incorrectly formed disulfide bridges, as well as an inner-membrane protein, DsbD, which transports electrons across the inner membrane from the cytoplasmic thioredoxin. The role of DsbG is not fully understood. Some evidence suggests that this protein is responsible for maintaining the proper redox balance in the periplasm. In recent years, it has been established that Dsb proteins are essential for correct folding or assembly of a number of pathogenic determinants, including toxins, adhesins, components of the TTSS (type III secretion system) and many other proteins. *dsb* gene mutations decrease the rate of disulfide bond formation and very often result in attenuation of pathogens (Peek & Taylor, 1992; Yu, 1998; Yu & Kroll, 1999; Stenson & Weiss, 2002). Thus, there is a great interest in analysing the mechanism of action of the known Dsb proteins as well as in identification of new thiol-oxidoreductases, which could be involved in processes important for bacterial pathogenicity. In this work, we report the identification and characterization of new DsbB-related disulfide oxidoreductases (DsbI) from *C. jejuni* and *E. coli* and a comprehensive classification of the DsbB/DsbI family according to phylogenetic, structural and functional criteria.

**METHODS**

**Bacterial strains, plasmids, media and growth conditions.** Bacterial strains and plasmids used in this study are listed in Table 1. *C. jejuni* strain 81176, kindly provided by M. J. Blaser (Korlath et al., 1985) and *H. pylori* strain J99 (Alm et al., 1999) were grown under microaerobic conditions at 37 °C on Blood Agar Base No. 2 containing 10 and 7.5% horse blood, respectively, or on Mueller–Hinton agar (MH; *C. jejuni*). *C. jejuni* and *H. pylori* mutated strains were grown on media supplemented with antibiotics: kanamycin (Km, 15 μg ml⁻¹) and/or chloramphenicol (Cm, 6 μg ml⁻¹ – *H. pylori*; 15 μg ml⁻¹ – *C. jejuni*). *E. coli* strains were grown at 37 °C in LB broth or on LB agar supplemented with ampicillin (Ap, 50 μg ml⁻¹), Cm (20 μg ml⁻¹) or Km (25 μg ml⁻¹). When needed, DTT of the appropriate concentration was added into the media. A motility test for *C. jejuni* was performed on MH.

**Recombinant DNA techniques.** Procedures for plasmid DNA isolation and DNA analysis (digestion with restriction enzymes, T4 ligation), agarose gel electrophoresis and transformation of *E. coli* competent cells were carried out as described by Sambrook & Russell (2001). Preparation of plasmid DNA for electrophoresis as well as isolation of DNA from agarose gel were performed according to the manufacturer’s instructions (A&A Biotechnology).

DNA sequencing was performed on an ABI Prism 373 automated DNA sequencer (Perkin Elmer) in the DNA Sequencing and Oligonucleotide Synthesis Lab, Institute of Biochemistry and Biophysics, Polish Academy of Sciences. Oligonucleotide primers were synthesized by Sigma-Ark. Sequences of primers are given in Table 2.

**Transcription and translation coupled assay.** The assay was carried out with *E. coli* T7 S30 Extract System for Circular DNA (Promega) following the manufacturer’s instructions.

**Plasmid construction.** The 2449 bp PCR fragment containing the genes *dsb* (*dsbI*-accessory) and *dsbB* was amplified from total DNA of *C. jejuni* 81176 with the oligonucleotide pair Cj19LX-2 and Cj18LM. The PCR product was inserted into the plasmid vector pBluescript II SK in the opposite orientation to the lacZ promoter. The resulting plasmid was named pUWM453 (5·5 kb).

Likewise, the 2277 bp PCR fragment containing the genes *dsb* and *dsbB* was amplified from *H. pylori* J99 chromosomal DNA with the oligonucleotide primers 540RX and 543LR-2. The amplicon was inserted into the plasmid vector pGEM-T Easy. Following digestion of the recombinant plasmid with EcoRI, the product obtained was cloned into pBluescript II SK (orientation opposite to the lacZ gene). The resulting plasmid was named pUWM333 (5·3 kb).

A recombinant plasmid containing the *dsbB* gene from *C. jejuni* 81176 (GenBank accession no. U38280) was constructed by PCR amplification of a 1092 bp fragment from the chromosomal DNA with the oligonucleotide pair Cj16RX-2 and Cj16RS. The PCR product was inserted into the plasmid vector pBluescript II SK in the opposite orientation to the *lacZ* gene. The resulting plasmid was named pUWM602 (4·1 kb).

Three suicide plasmids were designed, based on vectors pBluescript II SK and pGEM-T Easy, which are non-repliable in *Campylobacter* and *Helicobacter*, to inactivate *dsb* and *dsbB* genes in *C. jejuni* (*cjdsbI* and *cjdsbB*, respectively) and *dsbL* in *H. pylori* J99 (*hpdsbl*).

To disrupt *cjdsbL*, plasmid pUWM452 (4·8 kb), a derivative of pBluescript II SK carrying *cjdsbL* amplified with Cj16RS/Cj18LM, was used. A 312 bp DNA fragment was removed from pUWM452 by digestion with EcoRV. The resulting plasmid DNA was ligated with a *SmaI*-ended Cm waits (0·8 kb) in *C. jejuni cat* gene from pRY109; Yao et al., 1993), leading to pUWM466 (5·3 kb). *hpdsbl* disruption in *H. pylori* J99 was achieved with plasmid pUWM305 (5·4 kb) used previously to mutate the orthologous (94% identical at the nucleotide level) hp0595 gene in *H. pylori* 26695 (R. Godlewksa, unpublished). The plasmid was constructed by a two-step PCR. Two internal fragments of hp595 were amplified by PCR and cloned into pBluescript II SK with an inactivated *Clal* site. Oligonucleotides 595-1, 595-2, 595-3 and 595-4 were used. Primers contained *SmaI* and *Clal* restriction site overhangs to facilitate cloning of amplified DNA fragments in the appropriate orientation. A *Clal*–ended Km++ cassette (1·4 kb) in *C. jejuni* aph gene from pUOA13; Taylor, 1992) was inserted into the unique *Clal* site between two fragments of hp595.
Table 1. Bacterial strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Bacterial strains and plasmids</th>
<th>Relevant characteristics</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Strains</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>E. coli XL-1 Blue</td>
<td>recA1 endA1 gyrA96 thi1 hsdR17 supE4 4 relAI lac [F' proAB lacZM AM15 Tn10 (tet')]</td>
<td>Stratagene</td>
</tr>
<tr>
<td>E. coli DH5x</td>
<td>recA1 endA1 gyrA96 thi1 hsdR17 (rK mK) supE44 ΔlacU169 F' (Φ80lacZ2M15)</td>
<td>Gibco-BRL</td>
</tr>
<tr>
<td>E. coli KM1086</td>
<td>MCA100 mAT (small-f-lacZ 102)</td>
<td>Bardwell et al. (1991)</td>
</tr>
<tr>
<td>E. coli JC656</td>
<td>KM1086 dsbB::kan5</td>
<td>Bardwell et al. (1993)</td>
</tr>
<tr>
<td>C. jejuni 81176</td>
<td>Lior 5; isolated in Canada from a child with bloody diarrhoea</td>
<td>M. J. Blaser, NYU, USA</td>
</tr>
<tr>
<td>C. jejuni AR1</td>
<td>C. jejuni 81176 cjdsl::CM</td>
<td>This study</td>
</tr>
<tr>
<td>C. jejuni AR2</td>
<td>C. jejuni 81176 cjdslB::Km</td>
<td>This study</td>
</tr>
<tr>
<td>C. jejuni AR3</td>
<td>C. jejuni 81176 cjdsl::CM; dsbB::Km</td>
<td>This study</td>
</tr>
<tr>
<td>H. pylori J99</td>
<td>Isolated in the USA (1994) from a patient with a duodenal ulcer; cagA+ vacA+</td>
<td>Alm et al. (1999)</td>
</tr>
<tr>
<td>H. pylori RG1</td>
<td>H. pylori J99 hpdsl::CM</td>
<td>This study</td>
</tr>
<tr>
<td><strong>Plasmids</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBluescript II SK</td>
<td>Ap', LacZs</td>
<td>Stratagene</td>
</tr>
<tr>
<td>pGEM-T Easy</td>
<td>Ap', LacZs</td>
<td>Promega</td>
</tr>
<tr>
<td>pHEL2</td>
<td>Cm'</td>
<td>Heuermann &amp; Haas (1998)</td>
</tr>
<tr>
<td>pUWM246</td>
<td>pBluescript II SK/0-8 kb internal fragment of the C. coli 72Dz/92 cjaE gene</td>
<td>Department of Bacterial Genetics, Warsaw University, Poland</td>
</tr>
<tr>
<td>pUWM305</td>
<td>pBluescript II SK/hp595::Km</td>
<td>R. Godlewski, unpublished</td>
</tr>
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<td>pUWM333</td>
<td>pBluescript II SK/hpdba, hpdsbl</td>
<td>This study</td>
</tr>
<tr>
<td>pUWM336</td>
<td>pHEL2/hpdba, hpdsbl</td>
<td>This study</td>
</tr>
<tr>
<td>pUWM452</td>
<td>pBluescript II SK/cjdsl</td>
<td>This study</td>
</tr>
<tr>
<td>pUWM453</td>
<td>pBluescript II SK/cjdsl, cjdsl</td>
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<tr>
<td>pUWM466</td>
<td>pBluescript II SK/cjdsl::CM</td>
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<td>pUWM602</td>
<td>pGEM-T Easy/dsbB C. jejuni</td>
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</tr>
<tr>
<td>pUWM607</td>
<td>pGEM-T Easy/dsbB::Km C. jejuni</td>
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</tr>
<tr>
<td>pUWM617</td>
<td>pHEL2/dsbB C. jejuni</td>
<td>This study</td>
</tr>
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</table>

A similar method was used to create a suicide plasmid with the inactivated cjdslB gene. Two fragments of cjdslB from chromosomal DNA of C. jejuni 81176 were amplified by PCR and cloned into pGEM-T Easy. Oligonucleotides Cj864RX and Cj865LM, Cj865RM and Cj865RS were used. Primers contained XbaI, BamHI and SalI restriction sites to facilitate cloning of amplified DNA fragments in the appropriate orientation. A BamHI-ended Km' cassette (1-5 kb C. jejuni aph gene from pBF14; University of Utrecht, The Netherlands) was inserted into the unique BamHI site. The resulting plasmid was named pUWM607 (5-5 kb).

For the complementation test in H. pylori cells, two plasmids were constructed based on the pHEL2 shuttle vector (Heuermann & Haas, 1991). A similar method was used to create a suicide plasmid with the inactivated cjdslB gene. Two fragments of cjdslB from chromosomal DNA of C. jejuni 81176 were amplified by PCR and cloned into pGEM-T Easy. Oligonucleotides Cj864RX and Cj865LM, Cj865RM and Cj865RS were used. Primers contained XbaI, BamHI and SalI restriction sites to facilitate cloning of amplified DNA fragments in the appropriate orientation. A BamHI-ended Km' cassette (1-5 kb C. jejuni aph gene from pBF14; University of Utrecht, The Netherlands) was inserted into the unique BamHI site. The resulting plasmid was named pUWM607 (5-5 kb).

Thereafter, bacteria were transferred onto a fresh plate for 5 h and incubated with plasmid DNA (1–2 µg) for another 24 h. Afterwards, bacteria were transferred onto a plate with selective antibiotics (Km and/or Cm). C. jejuni as well as H. pylori transformants were grown for 2–5 days. Allelic exchange in mutagenesis was confirmed by PCR.

**RT-PCR.** Total RNAs were extracted from C. jejuni and H. pylori using the standard TRIzol procedure (Invitrogen). After DNase I treatment, RNA was reverse-transcribed using SuperScript II (Gibco-BRL) and primers RT-C.j and RT-H.p. Primers were annealed stepwise (preliminary step at 70°C for 10 min; then 2 min at 70°C; then 1 min each at 65, 60, 55, 50 and 45°C) before adding reverse transcriptase. The reverse transcriptase reaction was performed at 42°C for 50 min followed by five cycles at 50°C for 1 min, 53°C for 1 min and 56°C for 1 min. Reverse transcriptase was inactivated at 70°C for 15 min. A control reaction without reverse transcriptase was used to determine whether RNA was free of contaminating DNA. PCR reactions performed on cDNA were carried out with 2-5 mM MgCl₂ using the following parameters: initial denaturation at 94°C for 5 min; 35 cycles of 30 s denaturation at 94°C, 30 s annealing at 50–60°C, 30–180 s elongation at 72°C and 10 min terminal elongation at 72°C. PCR products were visualized by agarose gel electrophoresis. All other primers used in the RT-PCR experiment to determine the length of C. jejuni and H. pylori transcripts are given below in Results and in Table 2.

**Electron microscopy.** C. jejuni strains were observed using an LEI 912 AB electron microscope (Laboratory of Electron Microscopy, Warsaw University, Poland) at 80 kV.}

http://mic.sgmjournals.org
Table 2. Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Starter sequence (5′–3′)</th>
<th>Orientation</th>
<th>Restriction enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. jejuni</td>
<td><strong>CJ19LX-2</strong> AGTTCTAGAACGTTGACAGCTTGGCTGATA</td>
<td>Forward</td>
<td>XbaI</td>
</tr>
<tr>
<td></td>
<td><strong>CJ18Nde</strong> GTATACATGGAAGTCTTGCAGTACCTTTTA</td>
<td>Forward</td>
<td>NdeI</td>
</tr>
<tr>
<td></td>
<td><strong>CJ18LM</strong> TATTTGGATACGAGACATATTAACAAAT</td>
<td>Forward</td>
<td>BamHI</td>
</tr>
<tr>
<td></td>
<td><strong>CJ17Nde</strong> GATACTAGAGAAGAACTATTAACAAAT</td>
<td>Forward</td>
<td>NdeI</td>
</tr>
<tr>
<td></td>
<td><strong>CJ17RM</strong> TATGAAATTTACATGCTGTAACAA</td>
<td>Reverse</td>
<td>EcoRI</td>
</tr>
<tr>
<td></td>
<td><strong>CJ16RS</strong> GCATCGACTCTAATGGAAGTGACGAGTA</td>
<td>Reverse</td>
<td>SalI</td>
</tr>
<tr>
<td></td>
<td><strong>CJ864RX</strong> CCCGCCTAAAGCAGATGTATGGAAGTGACGAGTA</td>
<td>Forward</td>
<td>XbaI</td>
</tr>
<tr>
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<td><strong>CJ865RS</strong> CGACGTGGCAATTATGAGACATCTTA</td>
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<td>SalI</td>
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<tr>
<td></td>
<td><strong>CJ865LM</strong> CCGCGCCGGGATGCTCTTACAAAAAGGATTTTCAGATG</td>
<td>Reverse</td>
<td>SmaI, BamHI</td>
</tr>
<tr>
<td></td>
<td><strong>CJ865RM</strong> ATTCGGATCAGCGACAGCTGTCGAC</td>
<td>Forward</td>
<td>SmaI, BamHI</td>
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<td></td>
<td><strong>RT-Cj</strong> GCATCGACTAGGATCAGTATGGAAGTGACGAGTA</td>
<td>Reverse</td>
<td>SalI</td>
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<tr>
<td></td>
<td><strong>H. pylori</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td><strong>543LS-2</strong> GATGTCGACTCTTACACCCGCCCTTTACCC</td>
<td>Reverse</td>
<td>SalI</td>
</tr>
<tr>
<td></td>
<td><strong>542Nco</strong> GATCCTAGTGGTAAAGAACGCGATTTTATA</td>
<td>Forward</td>
<td>NolI</td>
</tr>
<tr>
<td></td>
<td><strong>542LM</strong> TATGAAATTTAATCGGATGCAAGCTTAA</td>
<td>Reverse</td>
<td>EcoRI</td>
</tr>
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<td><strong>541Nco</strong> ATACCATGGATTTTATGAGCTGA</td>
<td>Forward</td>
<td>NolI</td>
</tr>
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<td></td>
<td><strong>541LM</strong> TGGAGATCGTGTGTTGGTGACGATTTTA</td>
<td>Forward</td>
<td>BamHI</td>
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<td></td>
<td><strong>540RX</strong> ATGTTCTAGATCTTCAGACGAGCAGGATA</td>
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<td><strong>595-1</strong> CCGGCGAATGCCAACCTTCC</td>
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<td>SmaI</td>
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<td><strong>595-2</strong> ATCGATGAAATCGCCGCCACCG</td>
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<td>Cid</td>
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<td><strong>595-3</strong> ATCGGATGCGCGGCGGAGGCCATTCAA</td>
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<tr>
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<td><strong>595-4</strong> CGGTTCCCAAACCCTCCAGC</td>
<td>Reverse</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td><strong>RT-H.p</strong> TTTCTGACTTGGGCGGATTAGGCC</td>
<td>Reverse</td>
<td>SalI</td>
</tr>
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</table>

Warsaw University, Poland). Bacteria were grown overnight under microaerobic conditions, washed three times with PBS and then suspended in 1:5% glutaraldehyde in 0·1 M phosphate buffer (pH 7·5). After 2 h incubation at room temperature, bacteria were again washed three times and kept in 0·1 M phosphate buffer at 4 °C. Bacteria were visualized by negative staining (0·5% phosphotungstate acid), applying 80 kV.

**Elliott’s assay.** Elliott’s assay is a colorimetric reaction between Elliott’s reagent [DTNB, 5,5′-dithiobis(2-nitrobenzoic acid)] and the free thiol groups of proteins (Misaikas et al., 1993). Periplasmic protein samples from bacteria in the exponential phase of growth were isolated by a chloroform method (Ames et al., 1984) with a small modification. In the final step 10 mM Tris (pH 7·5) was replaced by Elliott’s buffer (1 mM EDTA, 1 mM PMSF in 50 mM phosphate buffer, pH 7·5). The concentration of periplasmic protein (0.5 mg) was taken for a single reaction. The final concentration of DTNB in the reaction mixture was 0.8 mM, providing a necessary excess of the reagent. The absorbance was read at 412 nm after 1 min incubation at room temperature. The assays were carried out in triplicate.

**Bioinformatic analysis.** The amino acid sequence of E. coli DsbB was used to search the non-redundant (nr) database at NCBi (National Center for Biotechnology Information, Bethesda, USA; http://www.ncbi.nlm.nih.gov) with the PSI-BLAST algorithm (Altschul et al., 1997). The search was carried out with an expectation (e) value threshold of 10−3 until no new sequences were reported. Full-length amino acid sequences were retrieved and realigned using CLUSTALX (Thompson et al., 1997). The initial alignment [including the N-terminal domains of C. jejuni DsbB (CjDsbB) and H. pylori DsbB (HpDsbB)] was used to generate a preliminary phylogenetic tree using the neighbour-joining algorithm (Saitou & Nei, 1987) implemented in CLUSTALX. The tree was used to identify several subfamilies of DsbB homologues. Representative members of these subfamilies were used to identify additional members of the DsbB family in incomplete bacterial genomes available from NCBI. The amino acid sequences of DsbB homologues from the nr database and putative products of translation of DsbB-like genes from the ‘unfinished genomes’ were realigned using T_COFFEE (Notredame et al., 2000), followed by calculation of an updated phylogenetic tree and reclassification of subfamilies. We noticed that the automatically generated alignment was still imperfect, probably due to the compositional bias of transmembrane (TM) regions in the DsbB family, enriched in hydrophobic amino acids. To improve the alignment, prediction of TM helices was carried out for each subfamily using HHM_TOM (Tusnady & Simon, 1993, TMpred (Hofmann & Stoffel, 1993), SOSUI (Hirokawa et al., 1998), TOPPRED (von Heijne, 1992) and DAS (Cserzo et al., 1997), available via a single online interface at http://kudlaty.genesilico.pl). The inferred consensus prediction of TM helices was used to improve the alignment and to identify periplasmic and cytoplasmic loops. The final phylogenetic tree was calculated from the unambiguous regions of the refined alignment of the DsbB family; several strongly diverged regions exhibiting insertions or deletions and most likely containing multiple substitutions, such as the N-terminal tail preceding the first TM helix, were omitted from calculations. The stability of the tree was estimated using bootstrap analysis from 1000 pseudo-replications of the sequence alignment. For the C-terminal domains of CjDsbB and HpDsbB, secondary structure prediction was carried out using programs PSI-PRED (Jones, 1999), SAM-T02 (Karplus et al., 2003) and PROFESSOR (Ouali & King, 2000),
Fold-recognition analysis was carried out via the GeneSilico meta-server gateway http://genesilico.pl/meta/ (Kurowski & Bujnicki, 2003).

RESULTS

Identification of the dsbl gene in C. jejuni and H. pylori

The Campylobacter gene originally named cjaE was first identified during an immunological screening of the Campylobacter coli 72Dz/92 genomic library (formerly C. jejuni 72Dz/92). Examination of the nucleotide sequence of the DNA fragment containing the cjaE gene and its surrounding region revealed that it is preceded by a small (168 bp) ORF transcribed in the same orientation. The amino acid sequence of CjaE was 91% identical (96% similar) to the sequence of ORF cj0017c from the genome of C. jejuni NCTC 11168. The presence and the conservation of cjaE among different Campylobacter clinical isolates were studied by the dot-blot method. Strains used in these experiments were described in detail by Pawelec et al. (2000). Under high stringency conditions the probe (pUWM 246) hybridized to the chromosomal DNA of all strains (data not shown).

Database searches (see Methods) revealed the presence of apparent orthologues of both ORFs in two complete genome sequences of H. pylori (ORFs hp0594 and hp0595 in strain 26695, and jhp0541 and jhp0542 in strain J99) as well as in the preliminary genomic data of Corynebacterium diphtheriae and Actinobacillus actinomycetemcomitans (kindly provided by the Sanger Center and Oklahoma University, respectively; see below for sequence analysis). On the one hand, extensive database searches revealed no additional homologues of the small ORFs (cj0018c/hp0594/jhp0541) in genomes other than of the four above-mentioned bacteria. On the other hand, the N-terminal domain of the large ORF (cj0017c/hp0595/jhp0542) was found to belong to the DsbB family (COG1495 in the Clusters of Orthologous Groups Database; http://www.ncbi.nlm.nih.gov/COG/), suggesting that these proteins may exhibit a disulfide oxidoreductase activity. In the initial analyses, the C-terminal domain showed mutual conservation between the large ORFs, but no significant similarity to other proteins in the database.

In this study all experiments were carried out using fully sequenced H. pylori strain J99 and C. jejuni strain 81176. The latter is the best experimentally characterized C. jejuni strain, but its genome is not fully sequenced yet. Genes jhp0541 and jhp0542 from H. pylori J99 and orthologues of cj0018c and cj0017c from C. jejuni 81176 were denoted dba and dsbl, respectively. The product proteins are referred to as Dba and Dsbl. Prefixes Hp or Cj (as in HpDsbI/CjDsbI) are used to denote proteins from H. pylori or C. jejuni, respectively.

The nucleotide sequence of 600 bp of the dsbl region from C. jejuni 81176 was found to be 100% identical to the corresponding dsbl region from C. jejuni NCTC 11168. This observation allowed us to design all oligonucleotide primers based on the known sequences of dsbl and dba from C. jejuni NCTC 11168.

Transcription analysis

Both in C. jejuni NCTC 11168 and in H. pylori J99, the small dba gene precedes the large dsbl gene generating short intergenic regions of 11 and 9 bp in C. jejuni and H. pylori, respectively. This suggested that dba and dsbl might be co-transcribed. To verify this prediction, an RT-PCR experiment was carried out. First, to assess whether the dba gene encodes a protein, an in vitro transcription and translation coupled assay was performed. For this purpose, two recombinant plasmids (pUWM453 for C. jejuni 81176 and pUWM333 for H. pylori J99) were constructed, each with the pair of investigated genes together with their putative promoters. As expected, both recombinant plasmids expressed two proteins of molecular masses 6 and 55 kDa, which could not be observed when the control plasmid pBluescript II SK was analysed (data not shown).

To determine whether investigated genes form a bicistronic operon, total RNAs from C. jejuni and H. pylori cells were reverse-transcribed with appropriate primers (RT-Cj and RT-Hp). Thereafter, the cDNAs obtained were subsequently amplified using five pairs of primers. Fig. 1 illustrates a schematic view and the results of the RT-PCR analysis. The absence of the amplicons in both reactions 1 (Fig. 1a, lane 11) demonstrated that the transcription start site for cjdbsl was located within 493 nt upstream of its start codon and that the transcription site point for hpdsbI was within 348 nt upstream of its start codon. Reactions 2, 3 and 4 on cDNAs derived from C. jejuni and H. pylori mRNAs resulted in amplification of DNA fragments of the expected sizes. A lack of PCR products in both reactions 5 (Fig. 1b, lane 12) as well as the lack of the product in the reaction without reverse transcriptase (Fig. 1b, lane 4) proved that cDNAs were not contaminated with chromosomal DNA. The data presented above document that dba and dsbl genes are co-transcribed. Therefore, the Campylobacter/ Helicobacter DNA fragments containing whole operons were employed in all complementation experiments described below.

Construction and characterization of chromosomal mutants cjdbsl, cjdbsb and hpdsbI

To examine the possible role of CjDsbI and HpDsbI in the formation of the disulfide bond in proteins exported from the cytoplasm, we used homologous recombination to introduce mutations into cjdbsl and hpdsbI loci on the C. jejuni and H. pylori chromosomes. Isogenic mutants were obtained by the allele exchange method, where suicide plasmids carrying interrupted genes (pUWM466/cjdbsl::Cm and pUWM305/hpdsbI::Km) were transformed...
into *C. jejuni* and *H. pylori* cells, respectively. The *C. jejuni* and *H. pylori* dsbI mutant strains were named AR1 and RG1, respectively. To investigate the possible functional cooperation between the CjDsbI and CjDsbB proteins, we also constructed a *C. jejuni* strain (named AR2) in which the wild-type *dsbB* locus was replaced by its disrupted copy (using a suicide plasmid pUWM607/dsbB::Km). In addition to the *dsbI* and *dsbB* mutants, a double *dsbI*-*dsbB* mutant strain (named AR3) was also constructed by introducing the disrupted copy of the *dsbI* locus into a *C. jejuni* *dsbB*2 genome. None of the mutations were lethal and the disruptions of *dsbB* and/or *dsbI* genes did not affect the growth rate of either *C. jejuni* or *H. pylori*.

To determine the effect of disruption of the *dsbI* gene in *C. jejuni* and *H. pylori* on the formation of disulfide bonds in the extracytoplasmic proteins, a quantitative assay of the free thiol groups in the periplasm was performed (Missiakas et al., 1993). The periplasmic protein samples from the wild-type and mutated strains were incubated with an excess of Ellman’s reagent. Results shown in Fig. 2 demonstrate that the products of the *cjdsbI*, *dsbB* and *hpdsbI* genes are indeed involved in the formation of disulfide bonds in extracytoplasmic proteins. The level of the proteins containing reduced cysteine residues in the periplasmic space did not differ between wild-type *C. jejuni* and *C. jejuni* AR1, implying that in the presence of CjDsbB, CjDsbI was dispensable for formation of disulfide bonds. On the contrary, *C. jejuni* AR2 in which the *dsbB* gene was disrupted, accumulated more proteins with free thiols in the periplasm relative to the wild-type cells. Interestingly, the double mutant (*dsbB*-*dsbI*) strain AR3 showed an even higher level of proteins with reduced cysteine residues than the *dsbB* mutant strain AR2, revealing that the DsbI activity can be observed only in the *dsbB*+ background (Fig. 2a). The inactivation of the *dsbI* gene in *H. pylori* (strain RG1), which natively lacks the *dsbB* gene, had also led to the accumulation of proteins with free thiol groups in the periplasmic space (Fig. 2b).

Furthermore, we analysed the complementation of the *H. pylori* *dsbI* mutation by the *C. jejuni* *dsbB* gene and by the *H. pylori* *dba*-*dsbI* operon. In both cases the appropriate DNA fragment containing investigated genes with 5’ regions was amplified from *C. jejuni* or *H. pylori* chromosomal DNAs and inserted into pHel2. The resulting plasmids, designated pUWM617 and pUWM336, respectively, were introduced into *H. pylori* RG1, where both of them substituted for the chromosomal *dsbI* mutation, according to Ellman’s assay (Fig. 2b). This experiment confirmed that the accumulation of proteins with free thiols in the periplasm of *H. pylori* RG1 resulted from the lack of the HpDsbI protein and additionally showed that CjDsbB can complement this deficiency. Despite numerous attempts, we were not able to clone the *C. jejuni* *dba*-*dsbI* operon into pHEL2 and hence it remains to be determined if CjDsbI can also functionally replace HpDsbI. Complementation assays were not carried out in *C. jejuni* 81176 cells due to the fact that all our attempts to introduce recombinant shuttle vectors into this strain were unsuccessful.

As demonstrated by Rietsch *et al.* (1996) and Stenson & Weiss (2002), mutations of *dsb* genes affect the motility of Gram-negative bacteria. Therefore, *C. jejuni* mutants were screened for motility defects on semisolid MH agar plates.

**Fig. 1.** RT-PCR analysis of transcription of *C. jejuni cjdba/cjdsbI* and their homologues from *H. pylori*. m-RNAs isolated from *C. jejuni* and *H. pylori* cells were reverse-transcribed. The resulting cDNAs were amplified with five pairs of primers. (a) Schematic view of primer location. Reactions 2, 3 and 4 (bold) were positive. The length of the intergenic region is not to scale – the little dot between the two genes/gene arrows indicates the real length. (b) *C. jejuni* PCR products resolved on 0.8% agarose. Lanes: 3, 6, 10 and 13, reactions carried out on chromosomal DNA; 2, 5, 7, 11 and 12, reactions carried out on cDNA; 4, reaction carried out without RT; 1 and 9, DNA marker. Lanes: 2, 3, reaction number 4; 4, 5, 6, reaction number 3; 7, 8, reaction number 2; 10, 11, reaction number 1; 12, 13, reaction number 5.
C. jejuni AR1 and AR2 exhibited no defects in motility compared to the wild-type C. jejuni, whereas the AR3 mutant was completely non-motile (Fig. 3a). We also observed the presence of flagella under electron microscopy. C. jejuni AR1 and AR2 strains exhibited full-length polar flagella as does the wild-type, whereas the AR3 mutant was completely devoid of flagella (Fig. 3b; electron micrographs of C. jejuni mutant strains are available as supplementary data with the online version of this paper at http://mic.sgmjournals.org).

**Complementation of the dsbB mutation in E. coli**

To study further the function of C. jejuni H. pylori DsbB, a complementation test was carried out using E. coli dsbB :: Km (JCB656) as a recipient. In this test we employed pUWM453 and pUWM333 expressing the dba-dsbl operons from C. jejuni and H. pylori respectively, as well as pUWM602 expressing the cjdsbB gene. The levels of periplasmic proteins with free thiols in the resulting transformant cells were determined by Ellman’s assay. The dba-dsbl operon from either source did not complement the dsbB mutation in E. coli KM1086. The lack of complementation was not caused by the low affinity of E. coli RNA polymerase to the Campylobacter promoter sequence because replacement of the native promoter by the E. coli lacZ2 promoter did not influence the result of the experiment (data not shown). Only pUWM602, expressing the dsbB gene from C. jejuni, decreased the level of proteins with reduced cysteine residues to that observed in the wild-type E. coli strain (data on the accumulation of proteins with reduced cysteines in E. coli bsbB mutants complemented by C. jejuni are available as supplementary data with the online version of this paper at http://mic.sgmjournals.org). It was shown that mutations in dsb genes of E. coli lead to an increase in sensitivity for reducing agents such as DTT (Missiakas et al., 1993). The result that C. jejuni dsbB is able to complement a defect in disulfide bond formation in E. coli was further supported by the observation that introducing pUWM602 into E. coli JCB656 restored its ability to grow in the presence of 15 mM DTT (DTT sensitivity data are available as supplementary data with the online version of this paper at http://mic.sgmjournals.org).

**Comparative analysis and classification of the DsbB family**

Sequence analysis revealed that CjDsbI and HpDsbI comprise two domains. In PSI-BLAST searches, the N-terminal domain (aa 1–170 of CjDsbI) exhibited significant sequence similarity to proteins from the DsbB family, while the C-terminal domain (aa 171–508; absent from the ‘orthodox’ DsbB proteins) exhibited no sequence similarity to any known protein. Secondary structure prediction suggested that the N-terminal domain forms TM helices, in agreement with its homology to DsbB proteins (Fig. 4). Fold-recognition analysis revealed no close structural similarity of DsbB or the N-terminal domain of DsbI to any known high-resolution structure from the Protein Database.

The C-terminal domain of DsbI was predicted to be localized in the periplasm and, depending on the algorithm used for secondary structure prediction, to comprise 15–25 β-strands. We attempted to predict its three-dimensional structure by scanning the Protein Database for protein structures most compatible with its sequence. The fold-recognition analysis, carried out via the meta-server interface (Kurowski & Bujnicki, 2003), revealed the high propensity of the C-terminal domain to form a β-propeller structure. All programs ranked it as the most probable fold, with medium to significant scores: FFAS, −8.13; FUGUE, 4.16; MGENTHEADER, 0.044; 3D-PSSM, 0.094; SAM-T02, 0.11. The prediction of the β-propeller fold in the C-terminal domain of DsbI is therefore highly confident. However, due to the uncertainty of secondary structure prediction and ambiguities of fold-recognition alignments reported by different methods (matches to β-propellers with different numbers of repeats), the number of ‘blades’ (i.e. β-sheets that form the propeller structure) could not be determined.
Sequence searches of the non-redundant database revealed over 100 members of the DsbB family (representative members shown in Fig. 4; the complete dataset is available from the authors upon request). Notably, we found up to three DsbB homologues in certain bacterial genomes. The alignment of the DsbB family (including the TM domain of DsbI) revealed unexpected patterns of sequence and structural conservation characteristic for the individual subfamilies, but not for the whole family (Fig. 4). As reported previously, the ‘classical’ DsbB homologues comprise only one domain, formed by four TM segments (Jander et al., 1994), here referred to as TM1–TM4. The only exception is the CjDsbB protein, which we found to exhibit a C-terminal extension predicted to form a fifth TM segment (TM5). The two pairs of conserved Cys residues are located in the periplasmic loops connecting segments 1–2 and 3–4. The four cysteines in DsbB were proposed to act in concert to oxidize the substrate DsbA (Kadokura & Beckwith, 2002).

A conserved Arg residue implicated in the process of transfer of electrons from DsbB to membrane-embedded quinones (Kadokura et al., 2000) is also located in the 1–2 loop. We found that the Arg residue and the Cys pair in the 1–2 loop are conserved among all members of the DsbB family (Fig. 4). However, we observed that one subfamily of DsbB homologues, comprising proteins from β- and γ-Proteobacteria, lacks the second Cys pair in the 3–4
Instead, all members of this subfamily possess an additional TM segment in the C terminus (TM5) and exhibit a conserved pair of Cys residues in the apparently periplasmic C terminus. The subfamily comprising CjDsbI and its close homologues also possesses the TM5 segment in the C terminus, followed by the predicted periplasmic β-propeller domain. Strikingly, all four DsbI homologues that exhibit the long C-terminal tail, also completely lack the second pair of Cys residues.

As mentioned above, another feature that distinguishes these proteins from the rest of the DsbB family is the presence of a small protein comprising one potential TM segment, encoded by a small ORF encoded upstream of a large gene. It has been shown in this study that these two ORFs are co-transcribed.

The finding of new members of the DsbB family with unusual structural features prompted us to carry out an evolutionary analysis. Based on the multiple sequence alignment generated for the DsbB family, we inferred the phylogenetic tree. Our results shown in Fig. 5 reveal that the phylogenetic relationships in the DsbB family are rather distinct from the 'traditional' phylogeny of the Thiol-oxidoreductases of C. jejuni and H. pylori.

Fig. 4. Sequence alignment of representative members of the DsbB/DsbI family. Only up to two members of each lineage are shown for the clarity of presentation. Grey dashes indicate gaps. Identical residues are shown on a black background. Periplasmic cysteine residues are indicated by asterisks. The consensus prediction of the protein topology is shown below the alignment. Helical regions are indicated by 'H', cytoplasmic regions are indicated by '+' and periplasmic regions are indicated by '-'.
bacterial branch of the Tree of Life, as calculated from 16S rRNA or protein sequences (http://tolweb.org/tree/phylogeny.html). First, different species have up to three members of the DsbB family located in different branches of the DsbB family tree. Second, proteins from phylogenetically distant species are found clustered together to the exclusion of members from related bacterial taxa. The apparent disagreement between the protein tree and the species tree can be reconciled if horizontal gene transfers and/or duplications and gene losses are identified.

Most members of the DsbB family are found in the Proteobacteria and only a few in bacteria from other taxa, which suggests that the DsbB family evolved in an ancient proteobacterium and that other bacteria acquired a copy of a DsbB-like gene by horizontal gene transfer. Among non-Proteobacteria with completely sequenced genomes, DsbB homologues (one or two copies) were detected in various species and strains of 

\[\text{Bacillaceae and Chlamydiae, and in Deinococcus radiodurans and Corynebacterium diphtheriae.}\]

It is noteworthy that in 

\[\text{Bacillus anthracis and Bacillus subtilis}\]

one of the copies is located on a prophage, which provides strong support for the hypothesis that DsbB homologues were transferred horizontally by association with mobile genetic elements.

The topology of the DsbB family tree suggests an evolutionary scenario with one major duplication and multiple horizontal gene transfers and gene losses. A major duplication has evidently occurred in the \(\gamma\)-proteobacterial lineage of the TM4 subfamily, probably after radiation of the ancestor of \(\text{Pseudomonas}\). It gave rise to two sister clades (hereafter referred to as \(\gamma_1\) and \(\gamma_2\)) with paralogous members in many bacteria such as 

\[\text{E. coli CFT073 (genes c3787 and c1633), Salmonella enterica (genes STY3372 and STY1936) and Shewanella oneidensis MR-1 (genes SO3870, SO2887).}\]

Some bacteria acquired additional copies of DsbB-like proteins via horizontal gene transfer. For instance, there are two \(\text{Pseudomonas}\) lineages of which one forms an outgroup of the \(\gamma_1-\gamma_2\) branch, but the other radiates from within the \(\beta\)-proteobacterial lineage, suggesting the latter was a donor of the horizontally transferred genetic material.

A \(\beta\)-proteobacterium was also a probable donor of a DsbB homologue found in \(\text{Xanthomonadaceae (}\gamma\text{-Proteobacteria)}\).

A few \(\beta\)- and \(\gamma\)-proteobacterial members of the TM5 lineage identified in 

\[\text{Ralstonia solanacearum, Legionella pneumophila, Bordetella pertussis and in various strains of Salmonella}\]

were probably obtained by horizontal gene transfer from an ancestor of the \(\epsilon\)-proteobacterial lineage. On the other hand, a \(\gamma_1\) subfamily member from \(\epsilon\)-proteobacterium 

\[\text{C. jejuni (Cj0865)}\]

was apparently transferred horizontally from a \(\gamma\)-proteobacterium.

The analysis of completely sequenced genomes reveals that most bacteria have lost one or both paralogous gene copies. The gene loss was particularly extensive in subfamily \(\gamma_2\). That only a few bacteria retained more than one copy of a duplicated gene can be explained by the strong purifying selection, which removes functionally redundant additional copies of the DsbB enzyme. Our experimental demonstration that a member of the \(\gamma_2\) lineage from 

\[\text{C. jejuni (Cj0865)}\]

can complement a member of the \(\gamma_1\) lineage from 

\[\text{E. coli K-12}\]

(‘orthodox’ DsbB), provides strong support for this hypothesis. It will be interesting to study the physiology of those \(\gamma\)-Proteobacteria that retained members of both closely related TM4-\(\gamma_1\) and TM4-\(\gamma_2\) subfamilies, such as 

\[\text{E. coli CFT073 genes c3787 and c1633, or even acquired a third copy of a member of the TM5 lineage, such as S. enterica (genes STY2002, STY3372 and STY1936).}\]

We have also analysed completely sequenced bacterial
genomes containing at least one dsbB homologue for the presence of dsbA (which encodes a known substrate of DsbB). We found that the presence or absence of dsbA correlates perfectly with the presence or absence of dsbB, but it has no correlation with the presence of dsbI. No dsbA orthologue could be identified in H. pylori or Corynebacterium diphtheriae, which possess dsbI but lack dsbB; a single dsbA gene was found in C. jejuni, L. pneumophila, R. solanacearum and B. pertussis, which have a single copy of the dsbB gene, while two paralogous dsbA copies were found in S. enterica, which possesses two dsbB paralogues (data not shown). This result suggests that DsbA is probably not a preferred substrate for DsbI.

**DISCUSSION**

Based on phylogenetic and structural considerations, we propose that the DsbB family should be divided into two main classes, TM4 and TM5, which correspond to DsbB homologues with four and five TM segments, respectively (with the notable exception of a TM4-like DsbB protein from C. jejuni, which contains an additional fifth TM segment). Several bacteria possess more than one DsbB homologue of the TM4 class. Our analysis suggests how to classify these multiple copies as orthologues (copies derived by vertical descent), paralogues (copies derived from intragenomic duplications) or xenologues (copies obtained by horizontal gene transfer). In particular, paralogous lineages γ1 and γ2 should be distinguished. Likewise, the TM5 class should be divided into two subclasses, corresponding to the 'long' form with a β-propeller domain and one pair of Cys residues, and the 'short' form with two pairs of Cys residues. It will be interesting to determine to what extent the function of these genes has diverged or if the closely related DsbB homologues remain functionally redundant. Our results suggest that members of lineages γ1 and γ2 can functionally complement each other and that they can complement for the lack of the DsbI protein. However, the DsbI protein acts probably only on a subset of DsbB final substrates, as its activity is undetectable in the presence of DsbB and becomes detectable only in the absence of DsbB.

Currently, a few functionally uncharacterized β- and γ-proteobacterial DsbB orthologues have been annotated in databases as DsbH (PA5256 from *Pseudomonas aeruginosa*, CV3193 from *Chromobacterium violaceum*). In the light of the phylogenetic classification of the DsbB family and without the experimental data to support functional difference from the 'orthodox' enterobacterial DsbB enzyme, this change of nomenclature is unjustified, as they are likely to encode DsbB-like enzymes with very similar, redundant functions. To avoid confusion in the nomenclature, we propose to refer to the 'additional' members of the TM4 family as DsbB2, DsbB3, etc.

While this manuscript was in preparation, another study was published that described a phylogenetic analysis of the DsbB family (Kimball et al., 2003). Our conclusions agree to a large extent with those of these authors concerning the clustering of α-, β- and ϵ-proteobacterial proteins into distinct branches. However, their analysis was carried out on a much smaller set of sequences (28 vs 100 in this work) and has fallen short of identification of the directionality of horizontal gene transfers, thereby leaving the phylogenetic tree of the DsbB family unreconciled. Only the present analysis has collected a sufficient number of representatives of different lineages to perform a meaningful classification. We have reconciled the phylogenetic history of DsbB homologues by identifying a duplication in the γ-proteobacterial lineage and candidates for donors and acceptors of horizontal gene transfers. Moreover, we have identified for the first time a new subclass of DsbB homologues (here renamed as DsbI) with an additional TM5 segment and an unusual lack or different position of the C-terminal pair of Cys residues.

Our analysis revealed that the dsbI genes from *C. jejuni*, *H. pylori*, *Corynebacterium diphtheriae* and *A. actinomycetencomitans* are preceded by small ORFs, denoted as *dba*, potentially encoding 6 kDa proteins comprising a single TM helix. We have verified experimentally that the *dba* and *dsbI* genes from *C. jejuni* and *H. pylori* are co-transcribed. It would be interesting to determine the function of small proteins, whether they act as chaperones for DsbIs or have any contribution to disulfide bond formation.

In this study, we demonstrated that DsbI is a novel component of the Dsb system in *C. jejuni* and *H. pylori*. To date, very little is known about the enzymes catalysing disulfide bond formation in *ω*-Proteobacteria. Our analysis indicates significant differences both between the two ω-proteobacterial pathogens and in their relation to the well-studied γ-proteobacterial DsbB system. Active DsbI is evidently important for disulfide bond formation in periplasmic proteins of *H. pylori*, which lacks the 'classical' DsbB. On the other hand in *C. jejuni*, significant activity of DsbI can be detected only if the DsbB protein is inactivated. We hypothesize that DsbI is more specific than DsbB and probably acts only on a small subset of DsbB substrates. It cannot be excluded that CjDsbI and HpDsbI oxidoreductases co-operate with another ω-proteobacterial periplasmic protein not identified in this work. Such a cofactor could allow them to partially complement the lack of DsbB (as observed in DsbB-less strains of *C. jejuni*), but its absence would render the DsbI protein non-functional (as observed when the complementation of the *E. coli* DsbB mutant by CjDsbI and HpDsbI was attempted). Notably, it was shown that two pairs of Cys residues are essential for the function of DsbB (Kadokura & Beckwith, 2002), while CjDsbI and HpDsbI contain only one such pair. It would be interesting to determine whether the C-terminal domain (β-propeller structure) of CjDsbI and HpDsbI or the second pair of Cys residues present in the C-terminal tail of the shorter TM5 class DsbBs from β- and ω-Proteobacteria, is involved in binding of substrates or...
cofactors. One can speculate that the \( \beta \)-propeller domain can act as a platform for recruiting a protein with an active missing second pair of cysteines. The identification of the unusual DsbB family and characterization of its structural peculiarities and the comparative analysis of DsbB and DsbI proteins presented in this work opens the door to a new line of experiments aimed at detailed understanding of the process of disulfide bond formation in the bacterial periplasm.

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Thiol-oxidoeductases of *C. jejuni* and *H. pylori*


