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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

Anna M. Raczko,[1] Janusz M. Bujnicki,[2] Marcin Pawłowski,[1,2] Renata Godlewska,[1] Magdalena Lewandowska[1]† and Elżbieta K. Jagusztyn-Krynicka[1]

Correspondence
E. K. Jagusztyn-Krynicka
kjkryn@biol.uw.edu.pl

[1]Department of Bacterial Genetics, Institute of Microbiology, Warsaw University, Miecznikowa 1, 02-096 Warsaw, Poland

[2]Laboratory of Bioinformatics and Protein Engineering, International Institute of Molecular and Cell Biology, Trojdena 4, 02-109 Warsaw, Poland

In Gram-negative bacterial cells, disulfide bond formation occurs in the oxidative environment of the periplasm and is catalysed by Dsb (disulphide bond) proteins found in the periplasm and in the inner membrane. In this report the identification of a new subfamily of disulfide oxidoreductases encoded by a gene denoted *dsbI*, and functional characterization of DsbI proteins from *Campylobacter jejuni* and *Helicobacter pylori*, as well as DsbB from *C. jejuni*, are described. The N-terminal domain of DsbI is related to DsbB proteins and comprises five predicted transmembrane segments, while the C-terminal domain is predicted to locate to the periplasm and to fold into a β-propeller structure. The *dsbI* gene is co-transcribed with a small ORF designated *dba* (*dsbI*-accessory). Based on a series of deletion and complementation experiments it is proposed that DsbB can complement the lack of DsbI but not the converse. In the presence of DsbB, the activity of DsbI was undetectable, hence it probably acts only on a subset of possible substrates of DsbB. To reconstruct the principal events in the evolution of DsbB and DsbI proteins, sequences of all their homologues identifiable in databases were analysed. In the course of this study, previously undetected variations on the common thiol-oxidoreductase theme were identified, such as development of an additional transmembrane helix and loss or migration of the second pair of Cys residues between two distinct periplasmic loops. In conjunction with the experimental characterization of two members of the DsbI lineage, this analysis has resulted in the first comprehensive classification of the DsbB/DsbI family based on structural, functional and evolutionary 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

†Present address: Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Pawińskiego 5a, 02-106 Warsaw, Poland.

Abbreviations: *dba*, *dsbI*-accessory; Dsb, disulphide bond; TM, transmembrane; Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin.

The GenBank/EMBL/DDBJ accession number for the *Campylobacter coli* 72Dz/92 *cjaE* gene sequence reported in this paper is AJ249744.

Data on sensitivity to DTT, electron microscopy of *C. jejuni* mutant strains and 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).

pathogens in humans. Chronic infection of the gastric mucosa by this bacterium results in chronic gastritis, and duodenal and gastric peptic ulcerations. 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 *H. pylori* 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 electroporation 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 *dba* (*dsbI*-accessory) and *dsbI* was amplified from total DNA of *C. jejuni* 81176 with the oligonucleotide pair Cj19LX-2 and Cj16RS. 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 *dba* and *dsbI* was amplified from *H. pylori* J99 chromosomal DNA with the oligonucleotide primers 540RX and 543LS-2. The amplicon was inserted into the plasmid vector pGEM-T Easy. Following digestion of the recombinant plasmid with *Eco*RI, 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 Cj864RX and Cj865RS, followed by insertion into pGEM-T Easy. After digestion of the resulting recombinant plasmid with *Eco*RI, the PCR product was cloned into 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-replicable in *Campylobacter* and *Helicobacter*, to inactivate *dsbI* and *dsbB* genes in *C. jejuni* (*cjdsbI* and *cjdsbD*, respectively) and *dsbI* in *H. pylori* J99 (*hpdsbI*).

To disrupt *cjdsbI*, plasmid pUWM452 (4·8 kb), a derivative of pBluescript II SK carrying *cjdsbI* amplified with Cj16RS/Cj18LM, was used. A 312 bp DNA fragment was removed from pUWM452 by digestion with *Eco*RV. The resulting plasmid DNA was ligated with a *Sma*I-ended Cm^r cassette (0·8 kb *C. jejuni cat* gene from pRY109; Yao *et al.*, 1993), leading to pUWM466 (5·3 kb). *hpdsbI* 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. Godlewska, 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 *Cla*I site. Oligonucleotides 595-1, 595-2, 595-3 and 595-4 were used. Primers contained *Sma*I and *Cla*I restriction site overhangs to facilitate cloning of amplified DNA fragments in the appropriate orientation. A *Cla*I-ended Km^r cassette (1·4 kb *C. jejuni aph* gene from pUOA13; Taylor, 1992) was inserted into the unique *Cla*I site between two fragments of *hp595*.

Table 1. Bacterial strains and plasmids used in this study

Bacterial strains and plasmids	Relevant characteristics	Origin
Strains		
E. coli XL-1 Blue	*recA1 endA1 gyrA96 thi1 hsdR17 supE4 4 relA1lac* [F′ *proAB lacI*^{q}*Z* Δ*M15* Tn*10* (*tet*^{r})]	Stratagene
E. coli DH5α	*recA1 endA1 gyrA96 thi-1 hsdR17* (r_K^- m_K^+) *supE44* Δ*lacU169* F′ (Φ*80dlacZ*M15)	Gibco-BRL
E. coli KM1086	MC4100 *malT*^{c} (λ*malF-lacZ 102*)	Bardwell *et al.* (1991)
E. coli JCB656	KM1086 *dsbB*::*kan5*	Bardwell *et al.* (1993)
C. jejuni 81176	Lior 5; isolated in Canada from a child with bloody diarrhoea	M. J. Blaser, NYU, USA
C. jejuni AR1	*C. jejuni* 81176 *cjdsbI*::Cm	This study
C. jejuni AR2	*C. jejuni* 81176 *cjdsbB*::Km	This study
C. jejuni AR3	*C. jejuni* 81176 *cjdsbI*::Cm; *dsbB*::Km	This study
H. pylori J99	Isolated in the USA (1994) from a patient with a duodenal ulcer; *cagA*$^{+}$ *vacA*$^{+}$	Alm *et al.* (1999)
H. pylori RG1	*H. pylori* J99 *hpdsbI*::Cm	This study
Plasmids		
pBluescript II SK	Ap^{r}, LacZα	Stratagene
pGEM-T Easy	Ap^{r}, LacZα	Promega
pHEL2	Cm^{r}	Heuermann & Haas (1998)
pUWM246	pBluescript II SK/0·8 kb internal fragment of the *C. coli* 72Dz/92 *cjaE* gene	Department of Bacterial Genetics, Warsaw University, Poland
pUWM305	pBluescript II SK/*hp595*::Km	R. Godlewska, unpublished
pUWM333	pBluescript II SK/*hpdba*, *hpdsbI*	This study
pUWM336	pHEL2/*hpdba*, *hpdsbI*	This study
pUWM452	pBluescript II SK/*cjdsbI*	This study
pUWM453	pBluescript II SK/*cjdba*, *cjdsbI*	This study
pUWM466	pBluescript II SK/*cjdsbI*::Cm	This study
pUWM602	pGEM-T Easy/*dsbB C. jejuni*	This study
pUWM607	pGEM-T Easy/*dsbB*::Km *C. jejuni*	This study
pUWM617	pHEL2/*dsbB C. jejuni*	This study

A similar method was used to create a suicide plasmid with the inactivated *cjdsbB* gene. Two fragments of *cjdsbB* 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 *Xba*I, *Bam*HI and *Sal*I restriction site overhangs to facilitate cloning of amplified DNA fragments in the appropriate orientation. A *Bam*HI-ended Km^{r} cassette (1·5 kb *C. jejuni aph* gene from pBF14; University of Utrecht, The Netherlands) was inserted into the unique *Bam*HI 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, 1998). *cjdsbB* was excised from pUWM602 using *Sal*I and inserted into pHEL2 digested with the same enzyme, resulting in pUWM617 (6·3 kb). *H. pylori hpdba* and *hpdsbI* genes were analogously excised from pUWM333 with the same enzyme and ligated with pHEL2. The resulting plasmid was named pUWM336 (7·3 kb).

Transformation of *C. jejuni* and *H. pylori*. *C. jejuni* 81176 electrocompetent cells prepared as described by Wassenaar *et al.* (1993) were mixed with plasmid DNA (1–5 μg) and incubated for 5 min on ice. Electroporation was performed in 1 mm cuvettes (Bio-Rad) by applying 25 μF, 600 Ω and 0·7 kV. Transformants were grown under microaerobic conditions at 37 °C on MH plates for 5 h and then transferred onto blood agar plates (7·5 % horse blood) supplemented with selective antibiotics (Km and/or Cm). The naturally competent *H. pylori* J99 strain was grown under microaerobic conditions at 37 °C on blood agar plates (10 % horse blood) for 24 h. 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 $MgCl_2$ 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 LEO 912 AB electron microscope (Laboratory of Electron Microscopy,

Table 2. Oligonucleotides used in this study

Bold letters indicate *C. jejuni*/*H. pylori* sequences; restriction recognition sequences introduced for cloning purposes are underlined.

Name	Starter sequence (5′–3′)	Orientation	Restriction enzyme
C. jejuni			
Cj19LX-2	AGTTCTAGA**AGTTGGACAGCTTGCTGATA**	Forward	*Xba*I
Cj18Nde	GTACAT**ATGGAGTTTCTTGAACTTTTA**	Forward	*Nde*I
Cj18LM	TATGGATCC**CAGGAGCACTATTAACAATA**	Forward	*Bam*HI
Cj17Nde	GTACAT**ATGAACGAAATCAATAAAAC**	Forward	*Nde*I
Cj17RM	TATGAATTC**AGGAATACCTGTGCTAACAA**	Reverse	*Eco*RI
Cj16RS	GCAGTCGAC**TCAATGAAGGTACGAGTA**	Reverse	*Sal*I
Cj864RX	CGCTCTAGA**AAGCAATGAATGTAAGTAA**	Forward	*Xba*I
Cj865RS	CAGGTCGACC**AATTATTTAAGACATCCTA**	Forward	*Sal*I
Cj865LM	CGTCCCGGGGGATCCTAT**CAAAAGGATTTTCAGAATG**	Reverse	*Sma*I, *Bam*HI
Cj865RM	ATAGGATCCCCCGGGACG**ATTCCTATTTATCCATTTA**	Forward	*Sma*I, *Bam*HI
RT-C.j	GCAGTCGAC**TAGGATCGATAGTAGCTGAA**	Reverse	*Sal*I
H. pylori			
543LS-2	GATGTCGAC**TCTTACCCCACCCTTTCACC**	Reverse	*Sal*I
542Nco	GATCCATGG**ATAAAGAAACCCGATTTTATA**	Forward	*Nco*I
542LM	TATGAATTC**AACTGCGTGTGCAAGCTTAA**	Reverse	*Eco*RI
541Nco	ATAC**CATGGAGTTTTTAGGACTGA**	Forward	*Nco*I
541RM	TGAGGATCC**GTGGTTGGTGGAGCTTATTA**	Forward	*Bam*HI
540RX	AGTTCTAGA**TCTTCCAGACGCGACGCATA**	Forward	*Xba*I
595-1	CCCGGG**ATGCCCAACCTTTC**	Forward	*Sma*I
595-2	ATCGAT**GAAATCCGCCCCCACG**	Reverse	*Cla*I
595-3	ATCGAT**TCGTGGGGGCGGATTTCAA**	Forward	*Sma*I
595-4	**CGTTTCTAAACCCTCCAGCAC**	Reverse	–
RT-H.p	TTTGTCGAC**TTGGGCATTAGGGGC**	Reverse	*Sal*I

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.

Ellman's assay. Ellman's assay is a colorimetric reaction between Ellman's reagent [DTNB, 5,5′-dithiobis(2-nitrobenzoic acid)] and the free thiol groups of proteins (Missiakas *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 Ellman's buffer (1 mM EDTA, 1 mM PMSF in 50 mM phosphate buffer, pH 7·5). The concentration of periplasmic proteins was determined with BCA (bicinchoninic acid). A sample of 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^{-5} 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* DsbI (CjDsbI) and *H. pylori* DsbI (HpDsbI)] 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 HMMTOP (Tusnady & Simon, 2001), TMAP (Persson & Argos, 1997), 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 CjDsbI and HpDsbI, 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 *dsbI* 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 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*/*jhp541*) 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 *dsbI*, respectively. The protein products are referred to as Dba and DsbI. 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 *dsbI* region from *C. jejuni* 81176 was found to be 100 % identical to the corresponding *dsbI* region from *C. jejuni* NCTC 11168. This observation allowed us to design all oligonucleotide primers based on the known sequences of *dsbI* 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 *dsbI* gene generating short intergenic regions of 11 and 9 bp in *C. jejuni* and *H. pylori*, respectively. This suggested that *dba* and *dsbI* 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 *cjdsbI* 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 *dsbI* 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 *cjdsbI*, *cjdsbB* 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 *cjdsbI* 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/*cjdsbI*::Cm and pUWM305/*hpdsbI*::Km) were transformed

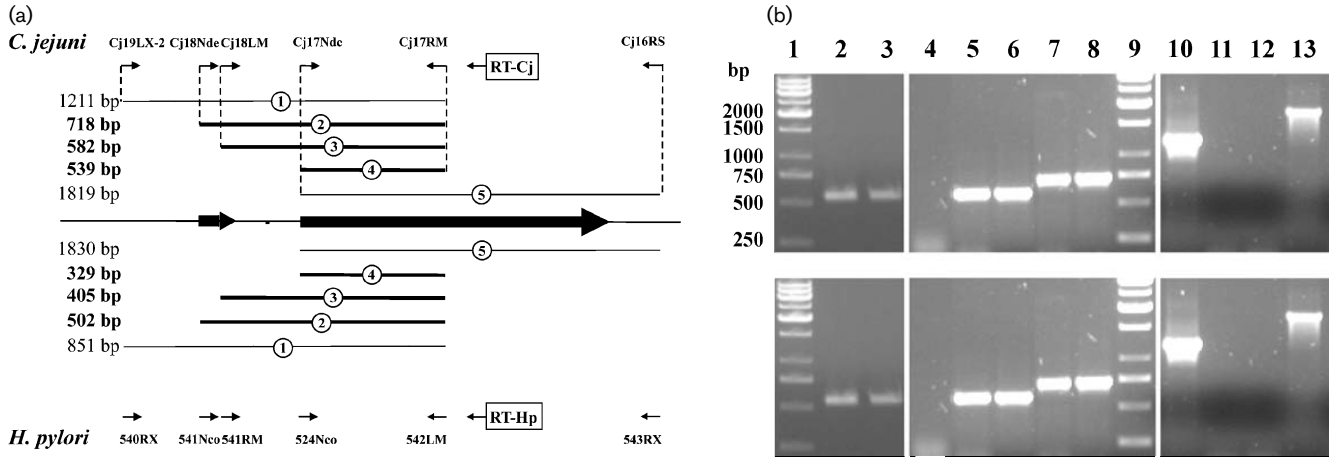

Fig. 1. RT-PCR analysis of transcription of *C. jejuni cjdba/cjdsbI* and their homologues from *H. pylori*. m-RNAs isolated from *C. jejuni* and *H. pylori* cells were reverse-transcribed. The resulting cDNAs were amplified with five pairs of primers. (a) Schematic view of primer location. Reactions 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, 8, 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.

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*$^{-}$ 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.

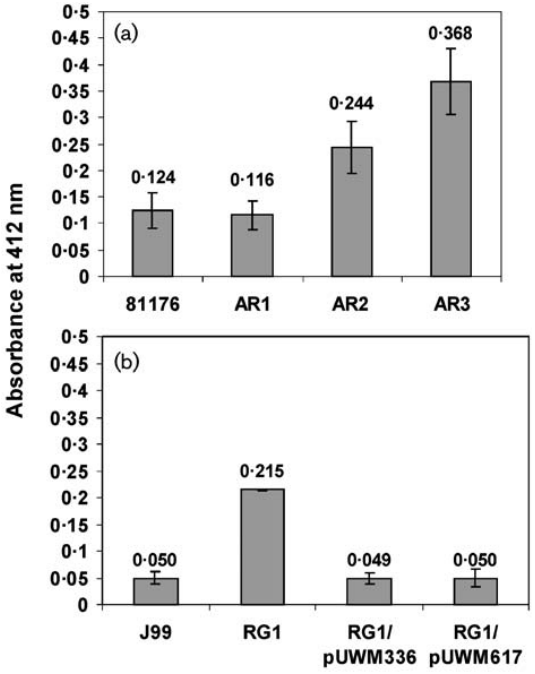

Fig. 2. Accumulation of proteins with reduced cysteines in *C. jejuni* and *H. pylori* mutated strains. Periplasmic proteins isolated from exponential-phase cells were mixed with Ellman's reagent (final concn 0·8 mM). Absorbance was measured at 412 nm. (a) *C. jejuni* (81176, wild-type strain; AR1, *cjdsbI* mutant; AR2, *dsbB* mutant; AR3, *cjdsbI*, *dsbB* double mutant). (b) *H. pylori* (J99, a wild-type strain; RG1, *hpdsbI* mutant; RG1 carrying pUWM336 containing *H. pylori hpdba* and *hpdsbI* genes; RG1 carrying pUWM617 containing *C. jejuni dsbB* gene).

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* DsbI, a complementation test was carried out using *E. coli dsbB*::Km^r (JCB656) as a recipient. In this test we employed pUWM453 and pUWM333 expressing the *dba-dsbI* 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-dsbI* 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 lacZ* 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; MGENTHREADER, 0·44; 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.

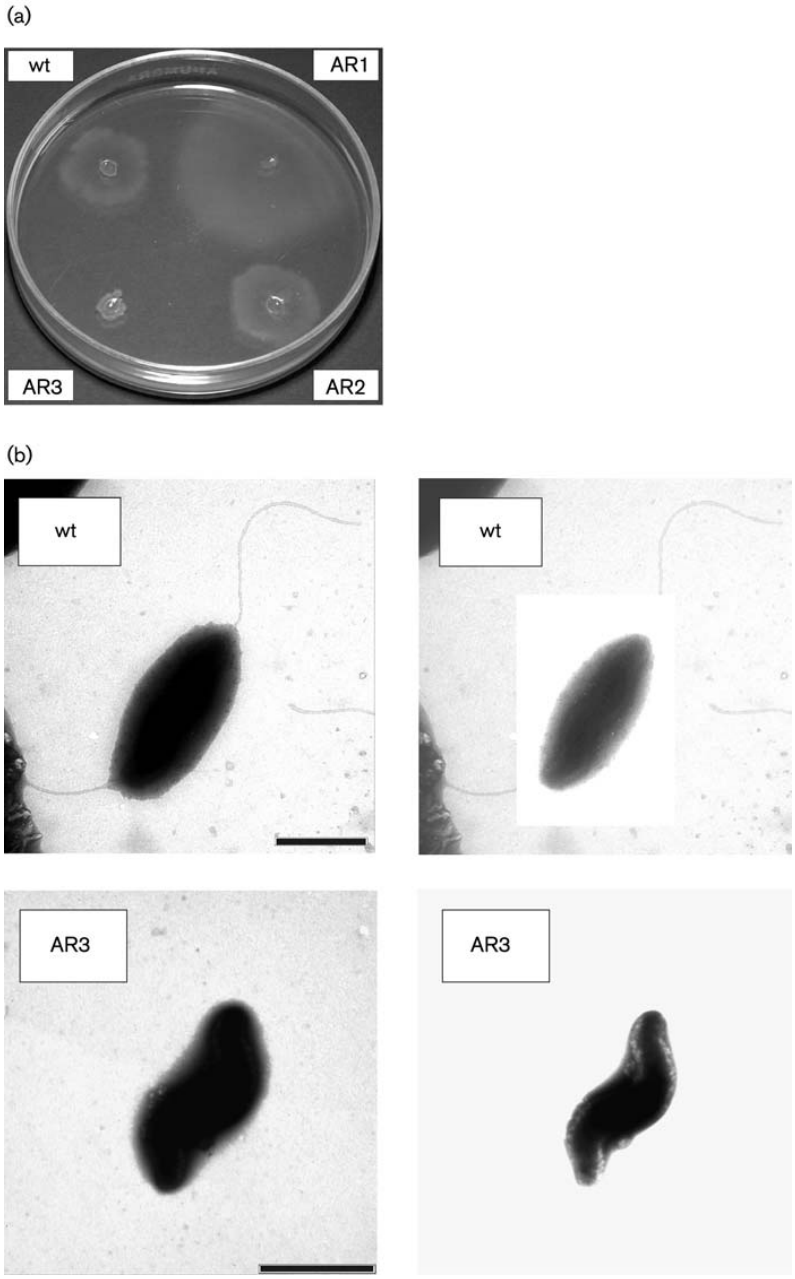

Fig. 3. Motility analysis of *C. jejuni* 81176 and its derivatives lacking *dsbI* and/or *dsbB*. (a) A photograph of MH soft agar after 2 days incubation of the following strains: wt, wild-type; AR1, *cjdsbI* mutant; AR2, *dsbB* mutant; AR3, *cjdsbI-dsbB* double mutant). (b) Electron microscopy of *C. jejuni* 81176 and AR3. Each pair of pictures shows the presence/absence of flagella and cell morphology. The picture in the top right corner was additionally edited to show the spiral shape of the bacterium. Bar 1 μm.

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

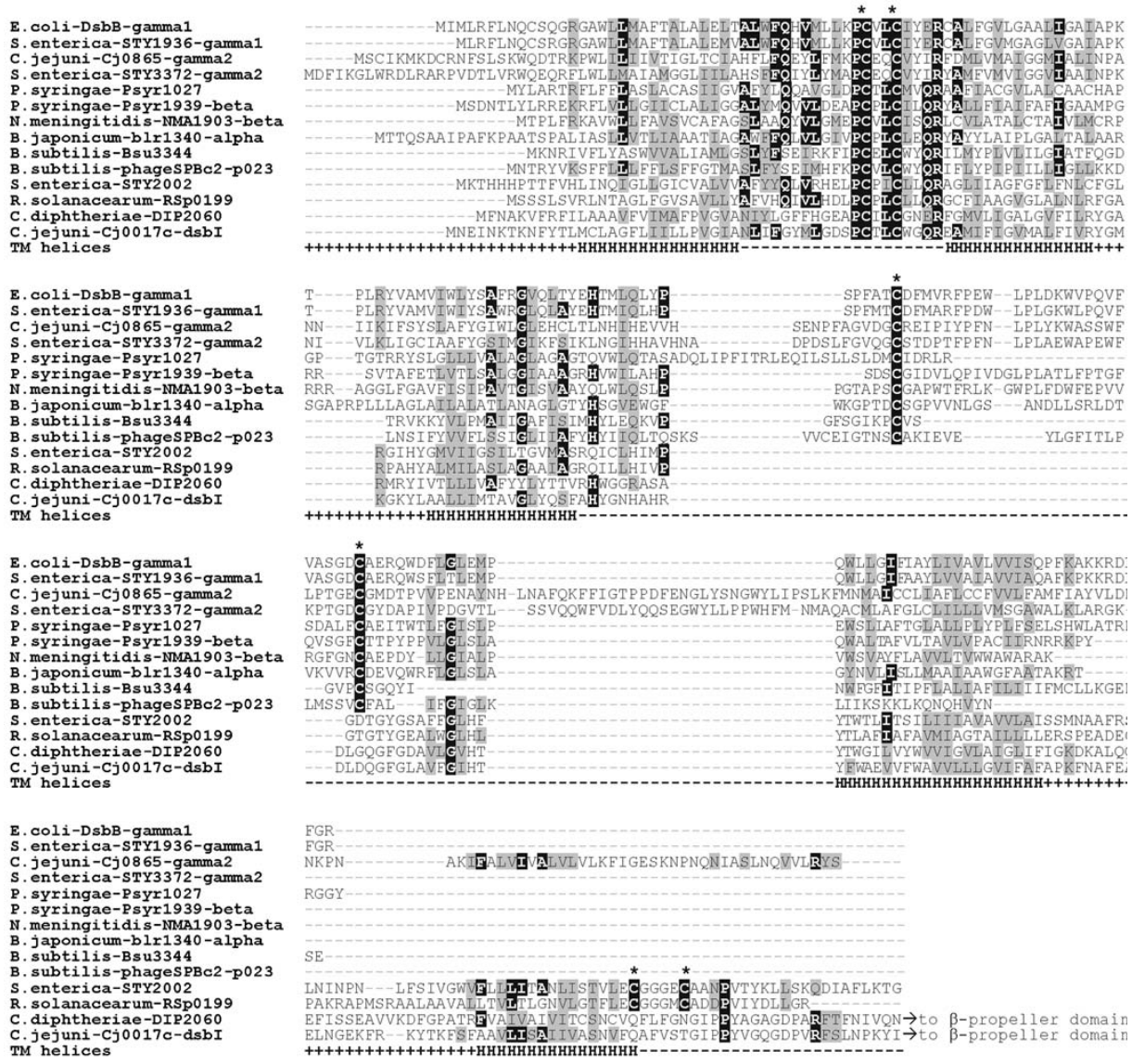

Fig. 4. Sequence alignment of representative members of the DsbB/DsbI family. Only up to two members of each lineage are shown for the clarity of presentation. Grey dashes indicate gaps. Identical residues are shown on a black background. Conserved residues are shown on a grey background. Periplasmic 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 '-'.

loop. 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

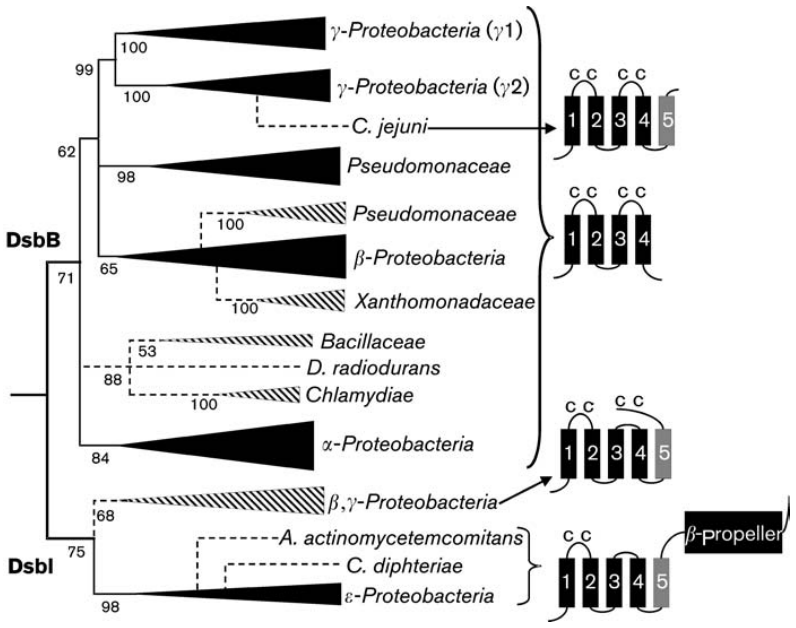

Fig. 5. Phylogenetic tree of the DsbB/DsbI family. Triangles indicate 'compressed' branches, with the names of taxa indicated. Hatched triangles and broken lines indicate subfamilies (or single genes) that have undergone horizontal gene transfer. Numbers at the nodes indicate the bootstrap support in percentile values. Nodes with bootstrap support $<50\%$ are regarded as unresolved and were grouped together with their sister lineages. The right panel illustrates the topology of members of each lineage, including the number of TM segments and the position of periplasmic Cys residues. The periplasmic regions are shown at the top of the helical bundle, the cytoplasmic regions are at the bottom.

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 *Bacillaceae* and *Chlamydiae*, and in *Deinococcus radiodurans* and *Corynebacterium diphtheriae*. It is noteworthy that in *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 γ-proteobacterial lineage of the TM4 subfamily, probably after radiation of the ancestor of *Pseudomonas*. It gave rise to two sister clades (hereafter referred to as $\gamma1$ and $\gamma2$) with paralogous members in many bacteria such as *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 *Pseudomonas* lineages of which one forms an outgroup of the $\gamma1$–$\gamma2$ branch, but the other radiates from within the β-proteobacterial lineage, suggesting the latter was a donor of the horizontally transferred genetic material.

A β-proteobacterium was also a probable donor of a DsbB homologue found in *Xanthomonadaceae* (*γ-Proteobacteria*).

A few β- and γ-proteobacterial members of the TM5 lineage identified in *Ralstonia solanacearum*, *Legionella pneumophila*, *Bordetella pertussis* and in various strains of *Salmonella* were probably obtained by horizontal gene transfer from an ancestor of the ε-proteobacterial lineage. On the other hand, a $\gamma1$ subfamily member from ε-proteobacterium *C. jejuni* (Cj0865) was apparently transferred horizontally from a γ-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 $\gamma2$. 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 $\gamma2$ lineage from *C. jejuni* (Cj0865) can complement a member of the $\gamma1$ lineage from *E. coli* K-12 (the 'orthodox' DsbB), provides strong support for this hypothesis. It will be interesting to study the physiology of those *γ-Proteobacteria* that retained members of both closely related TM4-$\gamma1$ and TM4-$\gamma2$ subfamilies, such as *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. actinomycetemcomitans* 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 *ε-Proteobacteria*. Our analysis indicates significant differences both between the two ε-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 ε-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 DsbIs from β- and γ-*Proteobacteria*, is involved in binding of substrates or

cofactors. One can speculate that the β-propeller domain can act as a platform for recruiting a protein with an active missing second pair of cysteines. The identification of the unusual DsbI 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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REFERENCES

Alm, R. A., Ling, L. S., Moir, D. T. & 20 other authors (1999). Genomic-sequence comparison of two unrelated isolates of the human gastric pathogen *Helicobacter pylori*. *Nature* **397**, 176–180.

Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res* **25**, 3389–3402.

Ames, G. F., Prody, C. & Kutsu, S. (1984). Simple, rapid, and quantitive release of periplasmic proteins by chloroform. *J Bacteriol* **160**, 1181–1183.

Bachoual, R., Ouabdesselam, S., Mory, F., Lascols, C., Soussy, C. J. & Tankovic, J. (2001). Single or double mutational alterations of *gyrA* associated with fluoroquinolone resistance in *Campylobacter jejuni* and *Campylobacter coli*. *Microb Drug Resist* **7**, 257–261.

Bardwell, J. C., McGovern, K. & Beckwith, J. (1991). Identification of a protein required for disulfide bond formation *in vivo*. *Cell* **67**, 581–589.

Bardwell, J. C., Lee, J. O., Jander, G., Martin, N., Belin, D. & Beckwith, J. (1993). A pathway for disulfide bond formation *in vivo*. *Proc Natl Acad Sci U S A* **90**, 1038–1042.

Coker, A. O., Isokpehi, R. D., Thomas, B. N., Amisu, K. O. & Obi, C. L. (2002). Human campylobacteriosis in developing countries. *Emerg Infect Dis* **8**, 237–244.

Collet, J. F. & Bardwell, J. C. (2002). Oxidative protein folding in bacteria. *Mol Microbiol* **44**, 1–8.

Cserzo, M., Wallin, E., Simon, I., von Heijne, G. & Elofsson, A. (1997). Prediction of transmembrane alpha-helices in prokaryotic membrane proteins: the dense alignment surface method. *Protein Eng* **10**, 673–676.

Dunn, B. E., Cohen, H. & Blaser, M. J. (1997). *Helicobacter pylori*. *Clin Microbiol Rev* **10**, 720–741.

Heuermann, D. & Haas, R. (1998). A stable shuttle vector system for efficient genetic complementation of *Helicobacter pylori* strains by transformation and conjugation. *Mol Gen Genet* **257**, 519–528.

Hirokawa, T., Boon-Chieng, S. & Mitaku, S. (1998). SOSUI: classification and secondary structure prediction system for membrane proteins. *Bioinformatics* **14**, 378–379.

Hofmann, K. & Stoffel, W. (1993). TMbase – a database of membrane spanning proteins segments. *Biol Chem* **374**, 166.

Jander, G., Martin, N. L. & Beckwith, J. (1994). Two cysteines in each periplasmic domain of the membrane protein DsbB are required for its function in protein disulfide bond formation. *EMBO J* **13**, 5121–5127.

Jones, D. T. (1999). Protein secondary structure prediction based on position-specific scoring matrices. *J Mol Biol* **292**, 195–202.

Kadokura, H. & Beckwith, J. (2002). Four cysteines of the membrane protein DsbB act in concert to oxidize its substrate DsbA. *EMBO J* **21**, 2354–2363.

Kadokura, H., Bader, M., Tian, H., Bardwell, J. C. & Beckwith, J. (2000). Roles of a conserved arginine residue of DsbB in linking protein disulfide-bond-formation pathway to the respiratory chain of *Escherichia coli*. *Proc Natl Acad Sci U S A* **97**, 10884–10889.

Kadokura, H., Katzen, F. & Beckwith, J. (2003). Protein disulfide bond formation in prokaryotes. *Annu Rev Biochem* **72**, 111–135.

Karplus, K., Karchin, R., Draper, J., Casper, J., Mandel-Gutfreund, Y., Diekhans, M. & Hughey, R. (2003). Combining local-structure, fold-recognition, and new fold methods for protein structure prediction. *Proteins* **53** (suppl. 6), 491–496.

Kimball, R. A., Martin, L. & Saier, M. H., Jr (2003). Reversing transmembrane electron flow: the DsbD and DsbB protein families. *J Mol Microbiol Biotechnol* **5**, 133–149.

Korlath, J. A., Osterholm, M. T., Judy, L. A., Forfang, J. C. & Robinson, R. A. (1985). A point-source outbreak of campylobacteriosis associated with consumption of raw milk. *J Infect Dis* **152**, 592–596.

Kurowski, M. A. & Bujnicki, J. M. (2003). GeneSilico protein structure prediction meta-server. *Nucleic Acids Res* **31**, 3305–3307.

Missiakas, D., Georgopoulos, C. & Raina, S. (1993). Identification and characterization of the *Escherichia coli* gene *dsbB*, whose product is involved in the formation of disulfide bonds *in vivo*. *Proc Natl Acad Sci U S A* **90**, 7084–7088.

Notredame, C., Higgins, D. G. & Heringa, J. (2000). T-Coffee: a novel method for fast and accurate multiple sequence alignment. *J Mol Biol* **302**, 205–217.

Ouali, M. & King, R. D. (2000). Cascaded multiple classifiers for secondary structure prediction. *Protein Sci* **9**, 1162–1176.

Pawelec, D. P., Korsak, D., Wyszynska, A. K., Rozynek, E., Popowski, J. & Jagusztyn-Krynicka, E. K. (2000). Genetic diversity of the *Campylobacter* genes coding immunodominant proteins. *FEMS Microbiol Lett* **185**, 43–49.

Peek, J. A. & Taylor, R. K. (1992). Characterization of a periplasmic thiol : disulfide interchange protein required for the functional maturation of secreted virulence factors of *Vibrio cholerae*. *Proc Natl Acad Sci U S A* **89**, 6210–6214.

Persson, B. & Argos, P. (1997). Prediction of membrane protein topology utilizing multiple sequence alignments. *J Protein Chem* **16**, 453–457.

Raina, S. & Missiakas, D. (1997). Making and breaking disulfide bonds. *Annu Rev Microbiol* **51**, 179–202.

Rietsch, A., Belin, D., Martin, N. & Beckwith, J. (1996). An *in vivo* pathway for disulfide bond isomerization in *Escherichia coli*. *Proc Natl Acad Sci U S A* **93**, 13048–13053.

Saitou, N. & Nei, M. (1987). The neighbour-joining method: a new method for reconstructing phylogenetic trees. *Mol Biol Evol* **4**, 406–425.

Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning. A Laboratory Manual*, 3rd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

Stenson, T. H. & Weiss, A. A. (2002). DsbA and DsbC are required for secretion of pertussis toxin by *Bordetella pertussis*. *Infect Immun* **70**, 2297–2303.

Taylor, D. E. (1992). Genetic analysis of *Campylobacter* spp. In *Campylobacter jejuni. Current Status and Future Trends*, pp. 255–266. Edited by I. Nachamkin, M. J. Blaser & L. S. Tompkins. Washington, DC: American Society for Microbiology.

Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res* **25**, 4876–4882.

Tusnady, G. E. & Simon, I. (2001). The HMMTOP transmembrane topology prediction server. *Bioinformatics* **17**, 849–850.

von Heijne, G. (1992). Membrane protein structure prediction. Hydrophobicity analysis and the positive-inside rule. *J Mol Biol* **225**, 487–494.

Warren, J. R. & Marshall, B. (1983). Unidentified curved bacilli on gastric epithelium in active chronic gastritis. *Lancet* **i**, 1273–1275.

Wassenaar, T. M., Fry, B. N. & van der Zeijst, B. A. (1993). Genetic manipulation of *Campylobacter*: evaluation of natural transformation and electro-transformation. *Gene* **132**, 131–135.

Yao, R., Alm, R. A., Trust, T. J. & Guerry, P. (1993). Construction of new *Campylobacter* cloning vectors and a new mutational *cat* cassette. *Gene* **130**, 127–130.

Yu, J. (1998). Inactivation of DsbA, but not DsbC and DsbD, affects the intracellular survival and virulence of *Shigella flexneri*. *Infect Immun* **66**, 3909–3917.

Yu, J. & Kroll, J. S. (1999). DsbA: a protein-folding catalyst contributing to bacterial virulence. *Microbes Infect* **1**, 1221–1228.