Identification and Functional Analysis of an Immunoreactive DsbA-Like Thio-Disulfide Oxidoreductase of *Ehrlichia* spp.

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Members of the genus *Ehrlichia* exhibit typical gram-negative cell wall structure; however, limited ultrastructural studies suggest that peptidoglycan may be absent. Bacterial outer membranes lacking peptidoglycan may be more dependent on covalent and noncovalent associations of outer membrane proteins. Disulfide bond linkages between cell envelope proteins of organisms in the genus *Ehrlichia* have not been determined, but two ultrastructural forms of *Ehrlichia chaffeensis*, termed reticulate and dense-cored cells, correspond to ultrastructurally similar reticulate and elementary body forms observed in chlamydiae (13), and an increase in disulfide cross-linked proteins has been noted in elementary bodies of *Chlamydia spp.* (3). Intramolecular covalent disulfide bonds between major surface proteins of the related rickettsial pathogen *Anaplasma marginale* have also been described and suggest that disulfide linkages are important in ehrlichial outer membrane structure (16).

Thio-disulfide oxidoreductases have been characterized in the cell envelope of several bacteria (1, 5, 10) and are likely involved in determining the three-dimensional structure of folded outer membrane proteins by catalyzing intra- and intermolecular disulfide bond formation. Disulfide oxidoreductases in *Escherichia coli* include thioredoxin and disulfide bond formation (Dsb) proteins A, B, C, D, and E (9, 14, 15). Some overlap in function occurs among these Dsb proteins, as overexpression of DsbC can alleviate the defects in DsbA mutants (8). We describe in this report functional immunoreactive homologous periplasmic thio-disulfide oxidoreductases, or Dsb proteins, of *E. chaffeensis* and *E. canis*. The role of ehrlichial Dsb proteins in outer membrane supramolecular structure, pathogenesis, and protective immunity remains to be determined.

The *E. canis* dsb gene was identified by immunologic screening of a Lambda Zap II *E. canis* genomic library as described previously (7). Screening of the *E. canis* genomic library identified an immunoreactive 2.4-kb clone with one complete and a second incomplete open reading frame (ORF) 42 bp downstream on the complementary strand. The second ORF was disrupted by a *Hin*P1/*Hpa*II cutting site used to construct the library, but it encoded a protein of at least 309 amino acids (ORF-309). A search of nucleic acid and protein databases did not identify any significant homologous sequences to ORF-309. The *E. chaffeensis* dsb gene was amplified by PCR using forward primer p27nc42 (5’-GAG ATT TCT ACT ATT GAC TTC-3’), targeting the upstream noncoding region, and reverse primer ECa27-700r (5’-CAG CTG CAT GCT GCA CAT-3’), obtained as sequences complementary to the *E. canis* dsb sequence. This primer pair amplified a region beginning upstream of the start codon through nucleotide 700 of the 738-bp ORF. The undetermined carboxy terminus (38 bp) and the primer ECa27-700r annealing region (23 bp) of the *E. chaffeensis* dsb gene were obtained with primer EC27-475 (5’-TTC TAC CAT GCT GCA CTA AAC C-3’), which amplified in the 3’ direction, using a genome walking kit (Clontech, Palo Alto, Calif.) as previously described (17). The *E. chaffeensis* and *E. canis* dsb genes were both 738-bp encoding proteins of 246 amino acids with predicted molecular masses of 27.7 and 27.5 kDa, respectively. The nucleic acid homology between the *Ehrlichia dsb* genes was 84%, but there was no homology with other database sequences.

*Ehrlichia* Dsb (eDsb) amino acid sequences were analyzed by the method of Nielsen et al. (11) for signal sequence recognition by using SignalP (version 1.1) at the Center for Biological Sequence Analysis (http://www.cbs.dtu.dk/services/SignalP/). Homologous domain architecture was determined using the domain architecture retrieval tool (DART) with reverse position-specific BLAST of the conserved domain database at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/Structure/lexington/lexington.cgi?cmd=rps). Nucleotide and deduced amino acid sequences, protein hydrophilicity, and surface probability were determined with LASERGENE software (version 5.0; DNASTAR, Inc., Madison, Wis.), using the Kyte-Doolittle and Emini algorithms. A conserved amino acid domain architecture found in the thioredoxin superfamily was identified in the eDsb proteins, and it was most similar to *E. coli* DsbA according to DART. A conserved cysteine active site was identified in the

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eDsb proteins that was identical to the active site of E. coli DsbC. The eDsb proteins are 87% homologous, contain identical predicted hydrophobic N-terminal signal peptide sequences consisting of 15 amino acids (MLRLFILLSLVILVA), and share some homology with Coxiella burnetii Com1 (31%) (4). The eDsb proteins, C. burnetii Com1, and periplasmic E. coli DsbA exhibited similar hydrophilicity and surface probability. In contrast, cytoplasmic membrane protein DsbB of E. coli had very few hydrophilic regions but had hydrophobic regions indicative of membrane-spanning proteins.

The entire E. chaffeensis dsb ORF was amplified by PCR with primers ECh27f (5'-ATG CTA AGG ATT TTA TTT TTA TTA-3') and ECh27r (5'-TCC TTG CTC ATC TAT TTT ACT TC-3') and cloned directly into the pCR T7/CT TOPO TA expression vector (Invitrogen, Carlsbad, Calif.), which is designed to produce proteins with a native N terminus and a carboxy-terminal polyhistidine region for purification. The resulting construct was designated pECf-dsb. E. chaffeensis and E. canis dsb genes without native N-terminus signal peptide-encoding regions (E. chaffeensis, −75 bp; E. canis, −73 bp) were amplified by PCR using forward primers ECh27-75 (5'-ATG AGC AAA TCT GGT AAA ACT AT-3') and reverse primers ECh27r and ECa27r (5'-TTC TTT CGT CAT ATC TAT TTT AC-3'), cloned into the same expression vector, and designated pECf-Dsb-sp and pECa-Dsb-sp. The recombinant eDsb proteins were purified under denaturing conditions as described previously (7), and the expressed recombinant protein was used for stimulating antibody production and Western blotting experiments. The purified recombinant eDsb proteins’ migration on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) coincided with their predicted molecular masses (Fig. 1A). Both recombinant eDsb proteins reacted with rabbit anti-E. chaffeensis recombinant Dsb, demonstrating the existence of a cross-reactive eDsb epitope (Fig. 1B). Polyclonal antisera produced against the E. chaffeensis recombinant Dsb protein also reacted with native proteins in the whole-cell lysates of E. chaffeensis (26 kDa) and E. canis (25 kDa), but not uninfected DH82 lysate (Fig. 1C). Normal rabbit serum did not react with the ehrlichia or DH82 cell lysate (not shown). Sera from dogs with canine monocytic ehrlichiosis (CME) reacted strongly with the recombinant E. canis Dsb and exhibited weaker reactivity with the recombinant E. chaffeensis Dsb (Fig. 2). Immune sera from human monocytic ehrlichiosis (HME) patients that contained antibodies to E. chaffeensis detected by an immunofluorescent antibody test did not react with the E. chaffeensis recombinant Dsb (Fig. 2).

The eDsb protein function was determined using E. coli strains JCB502 and JCB572 (JCB502 dsbA::kan1), kindly provided by J. Bardwell (University of Michigan), in complementation experiments as reference and mutant strains, respectively (1). E. coli dsbA mutant strain JCM572 carries a kanamycin insertion in the dsbA gene and is immotile due to a defect in flagellar assembly related to a disulfide bond in the flagellar P-ring protein (2). Expression constructs pECf-Dsb and pEFC-Dsb-sp, containing the complete and signal peptide-deficient E. chaffeensis dsb constructs and an expression plasmid control, were electroporated (2.5 kV, 25 μF, 200 Ω) into E. coli strain JCM572 and selected on Luria-Bertani (LB) plates with 100 μg of ampicillin. Mutants were screened for motility on soft agar LB plates (0.22% agar) for 18 h at 37°C. Alkaline phosphatase (AP) activity was determined by culturing cells in minimal medium, and activity was calculated using the formula \[ \text{activity} = \frac{\text{optical density at 420 nm with substrate} - \text{optical density at 420 nm without substrate}}{10^3} \times 10^3 \]. A fluorescent antibody test did not react with the ehrlichia or DH82 cell lysate (not shown). Sera from dogs with canine monocytic ehrlichiosis (CME) reacted strongly with the recombinant E. canis Dsb and exhibited weaker reactivity with the recombinant E. chaffeensis Dsb (Fig. 2). Immune sera from human monocytic ehrlichiosis (HME) patients that contained antibodies to E. chaffeensis detected by an immunofluorescent antibody test did not react with the E. chaffeensis recombinant Dsb (Fig. 2).

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JCB502 and JCB572(pECf-Dsb) exhibited similar AP activities (65 and 63 U), JCB572(pECf-Dsb-sp) had approximately 30% lower AP activity (47 U), and the plasmid control, JCB572(pLacZ) had very low AP activity (5 U).

Localization of eDsb proteins in fixed LR White-embedded ultrathin sections of DH82 cells infected with E. chaffeensis and E. canis was performed as described previously (12). Ultrathin sections were treated with blocking buffer (0.1% bovine serum albumin and 0.01 M glycine in Tris-buffered saline, [TBS]), incubated with rabbit anti-E. chaffeensis recombinant Dsb polyclonal antibody diluted 1:100 in diluting buffer (1% bovine serum albumin in TBS), and washed in blocking buffer, followed by incubation with goat anti-rabbit immunoglobulin G (H+L) labeled with 15-nm colloidal gold particles (AuroProbe EM GAR G15, RPN422; Amersham Life Science, Arlington Heights, Ill.) diluted 1:20 in diluting buffer. Ultrathin sections of E. chaffeensis and E. canis in infected DH82 cells were incubated with rabbit anti-E. chaffeensis recombinant Dsb. eDsb proteins were identified primarily in the cytoplasmic membrane-periplasmic region, with most label appearing to be in the periplasmic space of both E. chaffeensis and E. canis. Cytoplasmic localization was observed, which is consistent with production and transport of eDsb from the cytoplasm to the periplasm, and occasional surface labeling was observed (Fig. 3). No difference was observed in the amount of eDsb in dense-cored and reticulate cells.

Little is known about the mechanism of disulfide bond formation and the role of inter- and intramolecular disulfide bonds in the overall cell envelope structure in Ehrlichia. This is the first report of a thio-disulfide oxidoreductase in Ehrlichia, and it provides evidence that disulfide bond formation may occur, perhaps playing an important role in the ehrlichial life cycle and pathogenesis. Previous studies with the related agent A. marginale demonstrated the importance of intra- and intermolecular disulfide bonds in the supramolecular structure of the cell envelope (16). Disulfide bonds in chlamydiae are involved in the development of ultrastructural forms of the organism (3), which are similar in appearance to the Ehrlichia reticulate and dense-cored forms (12). E. coli Dsb proteins provide some information regarding the possible role of ehrlichial Dsb proteins. It is not clear at this point what role the eDsb proteins have in pathogenesis or immunity, but studies to determine the role of disulfide bonds in cell envelope structure and cell ultrastructure will provide additional insights into the role of eDsb proteins in the ehrlichial life cycle.

Nucleotide sequence accession numbers. The GenBank nucleotide sequence accession numbers for the E. chaffeensis and E. canis dsb genes are AF403710 and AF403711, respectively.

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