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Construction and Characterization of a *Helicobacter pylori clpB* Mutant and Role of the Gene in the Stress Response

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Antiserum raised against whole *Helicobacter pylori* cells identified a novel 94-kDa antigen. The nucleotide sequence of the gene encoding the 94-kDa antigen was determined, and analysis of the deduced amino acid sequence revealed structural features typical of the ClpB ATPase family of stress response proteins. An isogenic *H. pylori clpB* mutant showed increased sensitivity to high-temperature stress, indicating that the *clpB* gene product functions as a stress response protein in *H. pylori*.

Helicobacter pylori is the causative agent of chronic active gastritis and is associated with the development of peptic ulcer disease and gastric cancer (1). *H. pylori* is unique among bacterial pathogens in that it can tolerate exposure to a range of physiological and biological stresses encountered in the human stomach. In response to stress conditions, bacteria transiently increase the synthesis of stress response proteins which are thought to protect the cell from stress-induced damage by preventing denaturation of cellular proteins, reactivating once-inactivated proteins, and regulating the degradation of irreversibly denatured proteins (20). Studies of the immune response to stress proteins have demonstrated that they are major antigens of many bacterial pathogens (2, 11, 25), suggesting that they are abundant in the bacterial cell during infection. The HtrA (high-temperature-requirement) stress proteins are essential for the virulence of intracellular bacterial pathogens, presumably against the toxic effects of oxidative killing within host phagocytes (3, 10, 15). The Clp ATPase protein family, which comprises ClpA-, ClpB-, ClpC-, and ClpX-like proteins, includes several stress response proteins (22). Conditions which induce expression of the Clp stress proteins include high temperature, high salt or ethanol concentration, oxygen limitation, and iron limitation. Bacterial *clp* mutants show increased sensitivity to a range of stress conditions in vitro (13, 21–23); e.g., *Listeria monocytogenes clpC* mutants are sensitive to high temperature, high osmolarity, iron limitation, and oxidative stress and are attenuated in mice (21).

Despite the ability of *H. pylori* to survive in a stressful environment, only the urease-associated HspA and HspB and the HtrA stress proteins of *H. pylori* have been characterized to date (2, 4, 12, 25). This report describes the cloning and nucleotide sequence analysis of an *H. pylori* gene encoding a homolog of the ClpB stress response proteins, identified by screening a λ ZAP genomic library of *H. pylori* NCTC 11637 for clones reactive with an antiserum raised against whole *H. pylori* cells. To determine the role of the *clpB* gene product in *H. pylori*, an isogenic *clpB* mutant was constructed and compared with the parental strain for survival at high temperature.

Bacterial strains and growth conditions. *H. pylori* was grown on *Helicobacter*-selective agar, consisting of blood agar base No. 2 (Oxoid) supplemented with 7% lysed horse blood and Dent's selective supplement (10 μg of vancomycin/ml, 5 μg of trimethoprim/ml, 5 μg of cefsulodin/ml, and 5 μg of amphotericin/ml; Oxoid), in a microaerobic atmosphere for 48 h at 37°C. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth or on LB agar. The antibiotics used for selection of clones were ampicillin (100 μg/ml) and kanamycin (50 μg/ml).

Screening of genomic library and recovery of pBK-CMV plasmids. An antiserum raised against whole cells of *H. pylori* Roberts (16) and a λ ZAP II Express genomic library of *H. pylori* NCTC 11637 were kindly provided by C. W. Penn, University of Birmingham, Birmingham, United Kingdom. Anti-whole-cell antiserum was raised in two adult New Zealand White rabbits each injected intravenously twice weekly for 3 weeks with a heat-killed suspension of *H. pylori* containing between 5×10^6 and 1.6×10^8 bacteria. The animals were bled 2 weeks after the final dose, and the sera were pooled. The genomic library was constructed by ligation of *Sau*3AI partial digest fragments of 2 to 10 kb with *Bam*HI arms of the λ ZAP II Express (Stratagene, Cambridge, United Kingdom). To screen for clones reactive with the anti-whole-cell antiserum, the library was plated on *E. coli* XL1-Blue MRF′ and incubated at 42°C for 3.5 h to allow plaque formation and then induced with IPTG (isopropyl-β-D-thiogalactopyranoside) at 37°C for gene expression. The plaques were transferred to nitrocellulose filters and reacted with a 1:200 dilution of anti-whole-cell antiserum as previously described (27). Of 4×10^3 plaques screened, 54 clones expressing *H. pylori* antigens were identified. To recover the recombinant pBK-CMV plasmids from these clones, *E. coli* XL1-Blue MRF′ was infected simultaneously with an f1 helper phage (Exassist; Stratagene) and the recombinant λ ZAP phage (according to the instructions provided with Exassist). Recombinant pBK-CMV plasmids were transformed into *E. coli* XLOLR and plated on LB-kanamycin agar. Lysates of the *E. coli* clones were analyzed by immunoblotting, with *E. coli* XLOLR containing pBK-CMV alone (CP1) used as a negative control. Of 27 *E. coli* XLOLR clones examined by immunoblotting, 10 clones which reacted strongly with the antiserum were characterized. Three clones encoded the structural subunits of urease (UreA and UreB) (14), two encoded UreA alone, and one each encoded the flagellar hook protein (19), catalase (18), HspB (25), and a

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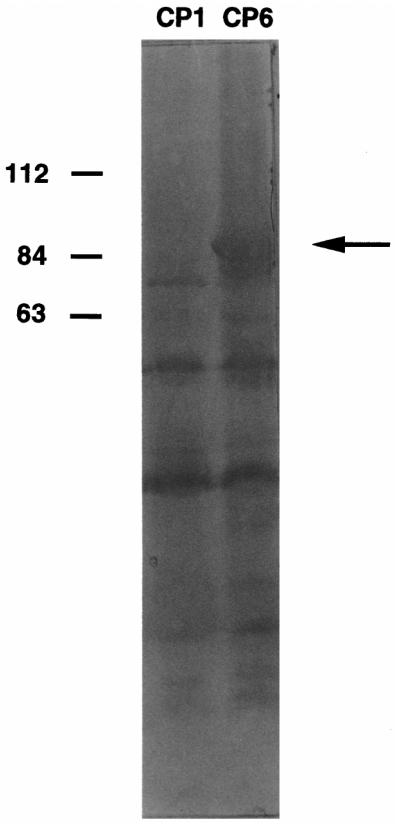

FIG. 1. Immunoblot analysis of *E. coli* XLOLR containing either pBK-CMV with a 3.2-kb insert of *H. pylori* genomic DNA (CP6) or pBK-CMV alone (CP1). Blots were probed with rabbit anti-*H. pylori* whole-cell antiserum and detected with alkaline phosphatase-conjugated anti-rabbit antibody. The numbers to the left indicate the sizes of the protein standards in kilodaltons. The arrow to the right indicates the 94-kDa antigen expressed by *E. coli* CP6.

homolog of bacterial methyl-accepting chemotaxis proteins (9). The remaining clone, *E. coli* CP6, produced a strongly immunoreactive reactive polypeptide of 94 kDa (Fig. 1) and was characterized further.

Nucleotide sequence and conservation of the gene encoding the 94-kDa antigen. The region encoding the 94-kDa antigen was localized to a 3-kb fragment of the insert in pCP6 by immunoblot analysis of a set of nested deletion subclones, constructed by using a Promega (Southampton, United Kingdom) Erase-A-Base kit. Analysis of the nucleotide sequence of the 3-kb insert revealed an open reading frame of 2,571 nucleotides with the potential to code for 856 amino acids. The calculated molecular mass of this protein is 94 kDa, which agrees with the mass of the antigen expressed in *E. coli* CP6 and determined by immunoblotting. The sequence TTGAGA-15-TATTTT, which resembles an *E. coli* σ^{70} promoter (8), is located 94 bp upstream of the putative ATG start codon. Southern blot and PCR analyses of 15 *H. pylori* strains isolated at St. Bartholomew's Hospital and 8 isolated at St. James's Hospital, Dublin, Ireland, showed that the *clpB* gene was present in all strains examined (results not shown).

Analysis of the predicted amino acid sequence. Comparison with protein sequences in the NBRF (National BioMedical Research Foundation) database demonstrated that the deduced amino acid sequence shows structural features typical of the Clp ATPase family, with two nucleotide-binding regions (N1 and N2) containing segment A and B nucleotide-binding motifs separated by a spacer region and enclosed between a leader sequence and a trailer sequence. The length of the spacer region of the *H. pylori* Clp protein is 126 amino acids, indicating that it belongs to the ClpB subfamily (22). *H. pylori* ClpB showed extensive amino acid identity with the ClpB proteins of *Dichelobacter* (*Bacteroides*) *nodosus* (17) (41% identity in an 856-amino-acid overlap), *Haemophilus influenzae* (6) (39.2%), and *E. coli* (22) (38.6%).

Construction of *H. pylori clpB* mutant. The insert from pCP6 was subcloned into pUC18, generating pIP6. A 935-bp deletion and a unique *Bgl*II site were introduced into the *clpB* gene in pIP6 by the inverse-PCR mutagenesis (IPCRM) procedure (28) with the oligonucleotides 5′-AAAAAGAGTGGTGGG GCAAGA-3′ and 5′-CTCTTCAAACTCGCCTCTGTA-3′, each of which included a 5′-terminal *Bgl*II restriction site. For IPCRM, 0.25 to 25 ng of pIP6 was added to a PCR mixture which was subjected to 40 cycles of 1 min of denaturation at 94°C, 30 s of annealing at 55°C, and 5 min of extension at 72°C in an Omnigene thermal cycler. PCR products were digested with *Bgl*II, self-ligated (to form pIP7), and transformed into *E. coli* XL2-Blue (Stratagene). A 1.4-kb *Bam*HI restriction fragment of plasmid pJMK30 containing a gene encoding resistance to kanamycin (*aph*3′-III [5]) was ligated in each orientation into the unique *Bgl*II site of pIP7, generating pIP18 and pIP19. These plasmids were introduced into *H. pylori* N6 (5) by natural transformation as previously described (7). No difference in transformation frequency was observed for the two plasmids, each giving rise to 2×10^4 kanamycin-resistant (Km^r) transformants per μg of DNA. PCR, with *clpB*-specific primers (5′-TTAAAAATTCCGGCTTGAAG-3′ and 5′-GTT GATAATGAATTTATTTGA-3′), on genomic DNA isolated from the parental strain and from two Km^r transformants, one obtained by using pIP18 (N6.1) and one by using pIP19 (N15.8), amplified a 2,262-bp product from N6 and a 2,827-bp product from N6.1 and N15.8, consistent with deletion of 935 bp and insertion of the 1.4-kb kanamycin cassette in strains N6.1 and N15.8. Southern blot analysis of N6.1 and N15.8 using a 1.4-kb *H. pylori clpB* probe provided further confirmation of allelic replacement (data not shown). The failure to isolate mutants in genes encoding stress response proteins, including *hspA* and *hspB* of *H. pylori*, indicates a vital role for these proteins in normal cell growth (24). Our ability to construct a *clpB* mutant indicates a nonessential function for this gene in *H. pylori*.

Characterization of *H. pylori clpB* mutant. The *H. pylori clpB* mutant N6.1 was microaerophilic and oxidase, catalase, and urease positive. No difference in the rate of growth was apparent between mutant and wild-type bacteria on *Helicobacter*-selective agar at 30, 37, or 40°C. For temperature stress experiments, wild-type and mutant bacteria, grown on *Helicobacter*-selective agar at 30°C for 48 h, were harvested, washed, and resuspended in phosphate-buffered saline (pH 7.24). Approximately 10^8 bacteria (in 50 μl) were transferred to 1-ml volumes of phosphate-buffered saline, (pH 7.24) prewarmed to 50°C, and were incubated for 15 min. Samples (20 μl) were removed at 6, 8, 10, and 15 min, diluted 10-fold serially, and plated on *Helicobacter*-selective agar to determine the number of CFU at increasing time intervals. Statistical significance was determined by using the Wilcoxon rank sum test (STATA statistical package; Stata Corporation, College Station, Tex.). In five independent experiments the sizes of the initial inocula of mutant and wild-type bacteria did not differ significantly; the median values (ranges) were 1.7×10^8 CFU/ml (1.0×10^8 to 2.4×10^8) and 1.8×10^8 CFU/ml (9.5×10^7 to 4.2×10^8) for the wild type and mutant, respectively ($P = 0.754$) (Fig. 2). In each of the five experiments, the viable count for the *clpB*

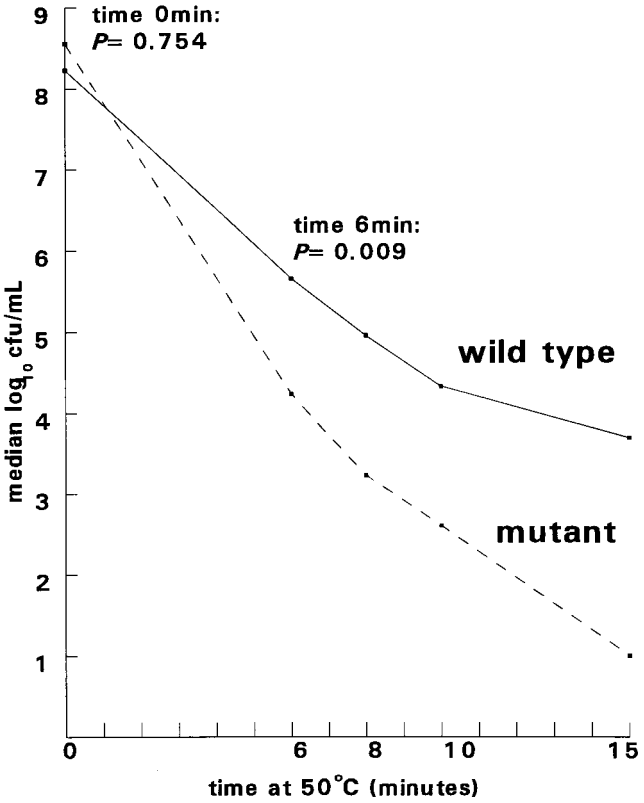

FIG. 2. Survival at 50°C of wild-type *H. pylori* (N6) and the *H. pylori clpB* mutant (N6.1). Median values for five experiments are shown.

mutant was lower than that of the wild-type strain at each subsequent sampling time (Fig. 2). The difference in viability was most apparent in the first 6 min: the median viable counts (ranges) for the wild type and mutant were 4.5×10^5 CFU/ml (8.2×10^4 to 1.2×10^8) and 1.7×10^4 CFU/ml (1.1×10^4 to 2.2×10^4), respectively ($P = 0.009$). Thereafter the difference in viability was less marked, although the counts for the mutant remained significantly lower than those for the wild type at 8, 10, and 15 min.

Summary. This work has identified a conserved *H. pylori* gene encoding a novel immunodominant antigen. The predicted amino acid sequence of this antigen has structural features typical of the ClpB stress response proteins. The clone encoding the *H. pylori* ClpB protein was identified in a λ ZAP genomic library by virtue of its reactivity with antiserum raised against *H. pylori* whole cells. Our ability to construct a *clpB* mutant indicates that the *clpB* gene is not essential for growth of *H. pylori*. The *clpB* mutant shows increased sensitivity to high-temperature stress, indicating that the *clpB* gene product is a stress response protein which may be important for survival of *H. pylori* in the hostile environment of the human stomach. As *H. pylori* lacks a heat shock sigma 32 (26), transcriptional control of this gene is likely to be different from that in *E. coli*. Further experiments are required to define the promoter for this gene.

Nucleotide sequence accession number. The nucleotide sequence of the *H. pylori clpB* gene has been submitted to the EMBL database under accession no. YO8238.

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