

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

ELAINE ALLAN,* PETER MULLANY,† AND SOAD TABAQCHALI

Department of Medical Microbiology, St. Bartholomew's and the Royal London School of Medicine and Dentistry, West Smithfield, London, EC1A 7BE, United Kingdom

Received 11 July 1997/Accepted 12 November 1997

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 μ g of vancomycin/ml, 5 μ g of trimethoprim/ml, 5 μ g of cefsulodin/ml, and 5 μ g 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 μ g/ml) and kanamycin (50 μ g/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* XL0LR and plated on LB-kanamycin agar. Lysates of the *E. coli* clones were analyzed by immunoblotting, with *E. coli* XL0LR containing pBK-CMV alone (CP1) used as a negative control. Of 27 *E. coli* XL0LR 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

* Corresponding author. Mailing address: Department of Medical Microbiology, St. Bartholomew's Hospital, West Smithfield, London EC1A 7BE, United Kingdom. Phone: (44) 1716018410. Fax: (44) 1716018409. E-mail: e.allan@mds.qmw.ac.uk.

† Present address: Department of Molecular and Cell Biology, Institute of Medical Sciences, University of Aberdeen, Foresterhill, Aberdeen AB25 2ZB, Scotland.

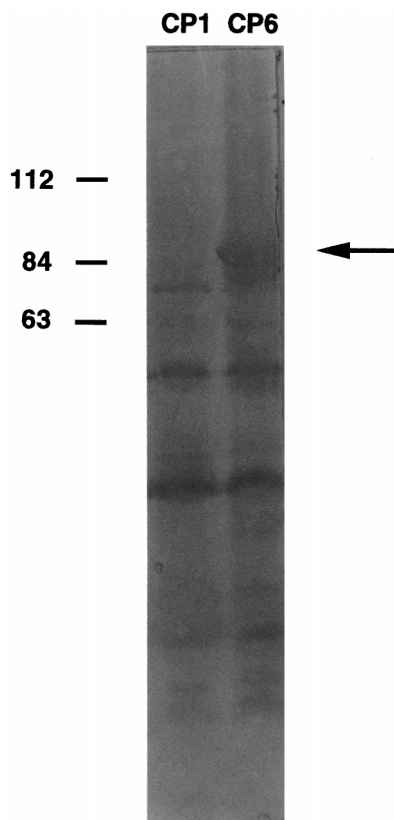


FIG. 1. Immunoblot analysis of *E. coli* XL0LR containing either pBK-CMV with a 3.2-kb insert of *H. pylori* genomic DNA (CP6) or pBK-CMV alone (CP1). Blots were probed with rabbit anti-*H. pylori* whole-cell antiserum and detected with alkaline phosphatase-conjugated anti-rabbit antibody. The numbers 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'-CTCTTCAAACCTCGCTCTGTA-3', each of which included a 5'-terminal *Bgl*III 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 μ g of DNA. PCR, with *clpB*-specific primers (5'-TTAAAATTCCGGCTTGAAG-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 μ l) 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 μ l) 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*

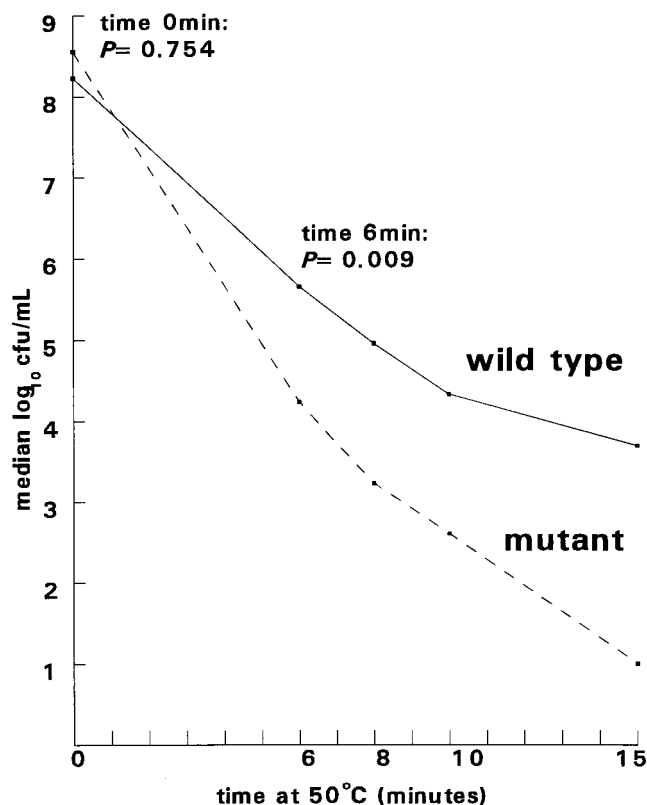


FIG. 2. Survival at 50°C of wild-type *H. pylori* (N6) and the *H. pylori clpB* mutant (N6.1). Median values for five experiments are shown.

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 1.2×10^6) 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.

We gratefully acknowledge Charles Penn for his generous gift of the *H. pylori* λ ZAP II Express library and rabbit anti-whole cell antiserum. We are also grateful to C. A. O'Morain for donating clinical isolates

and to Richard Ferrero for *H. pylori* N6. We thank Nick Dorrell for help with IPCRM and Brendan Wren for critical review of the manuscript.

This work was supported in part by Oravax Inc., Cambridge, Mass.

REFERENCES

- Blaser, M. J. 1992. *Helicobacter pylori*: its role in disease. *Clin. Infect. Dis.* **15**:386-393.
- Dunn, B. E., R. M. Roop II, C. C. Sung, S. A. Sharma, G. I. Perez-Perez, and M. J. Blaser. 1992. Identification and purification of a cpn60 heat shock protein homolog from *Helicobacter pylori*. *Infect. Immun.* **60**:1946-1951.
- Elzer, P. H., R. W. Phillips, M. E. Kovach, K. M. Peterson, and R. M. Roop II. 1994. Characterization and genetic complementation of a *Brucella abortus* high-temperature-requirement A (*htrA*) deletion mutant. *Infect. Immun.* **62**:4135-4139.
- Evans, D. J., Jr., D. G. Evans, L. Engstrand, and D. Y. Graham. 1992. Urease-associated heat shock protein of *Helicobacter pylori*. *Infect. Immun.* **60**:2125-2127.
- Ferrero, R. L., V. Cussac, P. Courcoux, and A. Labigne. 1992. Construction of isogenic urease-negative mutants of *Helicobacter pylori* by allelic exchange. *J. Bacteriol.* **174**:4212-4217.
- Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, et al. 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269**:496-512.
- Haas, R., T. F. Meyer, and J. P. M. van Putten. 1993. Aflagellated mutants of *Helicobacter pylori* generated by genetic transformation of naturally competent strains using transposon shuttle mutagenesis. *Mol. Microbiol.* **8**:753-760.
- Hawley, D. K., and W. R. McClure. 1983. Compilation and analysis of *Escherichia coli* promoter DNA sequences. *Nucleic Acids Res.* **11**:2237-2255.
- Hazelbauer, G. L. 1992. Bacterial chemoreceptors. *Curr. Opin. Struct. Biol.* **2**:505-510.
- Johnson, K., I. Charles, G. Dougan, D. Pickard, P. O'Gaora, G. Costa, T. A. I. Miller, and C. E. Hormaeche. 1991. The role of a stress-response protein in *Salmonella typhimurium* virulence. *Mol. Microbiol.* **5**:401-407.
- Kaufmann, S. H. E., and B. Schoel. 1994. Heat shock proteins as antigens in immunity against infection and self, p. 495-531. In R. I. Morimoto, A. Tisieres, and C. Georgopoulos (ed.), *The biology of heat shock proteins and molecular chaperones*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Kleanthous, H., C. L. Clayton, D. D. Morgan, M. J. Pallen, and S. Tabaqchali. 1993. Molecular cloning and nucleotide sequence determination of *htrA*, a gene encoding a 48-kDa stress protein of *Helicobacter pylori*, p. 195-202. In G. Gasbarrini and S. Pretolani (ed.), *Basic and clinical aspects of H. pylori* infection. Springer-Verlag, Berlin, Germany.
- Kruger, E., U. Volker, and M. Hecker. 1994. Stress induction of *clpC* in *Bacillus subtilis* and its involvement in stress tolerance. *J. Bacteriol.* **176**:3360-3367.
- Labigne, A. F., V. Cussac, and P. Courcoux. 1991. Shuttle cloning and nucleotide sequences of *Helicobacter pylori* genes responsible for urease activity. *Infect. Immun.* **173**:1920-1931.
- Li, S.-R., N. Dorrell, P. H. Everest, G. Dougan, and B. W. Wren. 1996. Construction and characterization of a *Yersinia enterocolitica* O:8 high-temperature requirement (*htrA*) isogenic mutant. *Infect. Immun.* **64**:2088-2094.
- Luke, C. J., E. Kubiak, A. Cockayne, T. S. J. Elliott, and C. W. Penn. 1990. Identification of flagellar and associated polypeptides of *Helicobacter* (formerly *Campylobacter*) *pylori*. *FEMS Microbiol. Lett.* **71**:225-230.
- Mattick, J. S., B. J. Anderson, P. T. Cox, B. P. Dalrymple, M. M. Bills, M. Hobbs, and J. R. Egerton. 1991. Gene sequences and comparison of the fibrial subunits representative of *Bacteroides nodosus* serogroups A to I: class I and class II strains. *Mol. Microbiol.* **5**:561-573.
- Odenbreit, S., B. Wieland, and R. Haas. 1996. Cloning and genetic characterization of *Helicobacter pylori* catalase and construction of a catalase-deficient mutant strain. *J. Bacteriol.* **178**:6960-6967.
- O'Toole, P. W., M. Kostrzynska, and T. J. Trust. 1994. Non-motile mutants of *Helicobacter pylori* defective in flagellar hook production. *Mol. Microbiol.* **14**:691-703.
- Parsell, D. A., and S. Lindquist. 1993. The function of heat-shock proteins in stress tolerance: degradation and reactivation of damaged proteins. *Annu. Rev. Genet.* **27**:437-496.
- Rouquette, C., M. T. Ripio, E. Pellegrini, J. M. Bolla, R. I. Tascon, J. A. Vazquez-Boland, and P. Berche. 1996. Identification of a ClpC ATPase required for stress tolerance and *in vivo* survival of *Listeria monocytogenes*. *Mol. Microbiol.* **21**:977-987.
- Squires, C., and C. L. Squires. 1992. The Clp proteins: proteolysis regulators or molecular chaperones? *J. Bacteriol.* **174**:1081-1085.
- Squires, C. L., S. Pedersen, B. M. Ross, and C. Squires. 1991. ClpB is the *Escherichia coli* heat shock protein F84.1. *J. Bacteriol.* **173**:4254-4262.
- Suerbaum, S., and A. Labigne. 1993. *Helicobacter pylori hspA-B* heat shock operon: nucleotide sequence, expression, and putative role. *Acta Gastroenterol. Belg.* **56**(Suppl.):51.
- Suerbaum, S., J. M. Thiberge, I. Kansau, R. L. Ferrero, and A. Labigne.

1994. *Helicobacter pylori hspA-hspB* heat-shock gene cluster: nucleotide sequence, expression, putative function and immunogenicity. *Mol. Microbiol.* **14**:959-974.
26. Tomb, J. F., O. White, A. R. Kerlavage, R. A. Clayton, G. G. Sutton, R. D. Fleischmann, K. A. Ketchum, H. P. Klenk, S. Gill, B. A. Dougherty, K. Nelson, J. Quackenbush, L. Zhou, E. F. Kirkness, S. Peterson, B. Loftus, D. Richardson, R. Dodson, H. G. Khalak, A. Glodek, K. McKenney, L. M. Fitzgerald, N. Lee, M. D. Adams, E. K. Hickey, D. E. Berg, J. D. Gocayne, T. R. Utterback, J. D. Peterson, J. M. Kelley, M. D. Cotton, J. M. Weidman, C. Fujii, C. Bowman, L. Watthey, E. Wallin, W. S. Hayes, M. Borodovsky, P. D. Karp, H. O. Smith, C. M. Fraser, and J. C. Venter. 1997. The complete genome sequence of the gastric pathogen *Helicobacter pylori*. *Nature* **388**:539-547.
27. Wren, B. W., C. L. Clayton, P. Mullany, and S. Tabaqchali. 1987. Molecular cloning and expression of *Clostridium difficile* toxin A in *Escherichia coli* K12. *FEBS Lett.* **225**:82-86.
28. Wren, B. W., J. Henderson, and J. M. Ketley. 1994. A PCR-based strategy for the rapid construction of defined bacterial deletion mutants. *BioTechniques* **16**:994-996.