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Cloning and Genetic Characterization of *Helicobacter pylori* Catalase and Construction of a Catalase-Deficient Mutant Strain

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The N-terminal sequence of a protein, originally described as an adhesin of *Helicobacter pylori*, was used in an oligonucleotide-based screening procedure of an *H. pylori* plasmid library in *Escherichia coli*. Five independent plasmid clones were isolated, all mapping to the same chromosomal region and encoding the *H. pylori* catalase. The gene, designated *katA*, comprises 1,518 nucleotides and encodes a putative protein of 505 amino acids with a predicted M_r of 58,599. A second open reading frame, *orf2*, encoding a putative 32,715-Da protein of unknown function, follows *katA*. The transcriptional start site of *katA* mRNA was determined, but no typical consensus promoter sequence was present. A potential binding site for the Fur protein is located upstream of *katA*. When introduced into the catalase-deficient *E. coli* double-mutant UM255, the cloned gene readily complemented *E. coli* for catalase activity. *H. pylori* KatA is highly homologous to catalases in both prokaryotes and eukaryotes, with the highest homology being shown to *Bordetella pertussis* (64.9%), *Bacteroides fragilis* (59.8%), and *Haemophilus influenzae* (57.9%) catalases. Transposon insertion mutants were generated in three independent *H. pylori* strains by Tn*Max5*-mediated transposon shuttle mutagenesis. In contrast to the wild-type strains, no significant catalase-specific enzymatic activity could be detected in the mutant strains, consistent with the fact that no additional *katA*-homologous gene copies were found in the *H. pylori* chromosome. No significant difference between wild-type and mutant strains for binding to epithelial cells was apparent, suggesting that KatA is not involved in *H. pylori* adhesion. The cloning and genetic characterization of *katA* are essential steps for further investigation of the role of catalase in the defense of *H. pylori* against oxygen-dependent killing mechanisms by polymorphonuclear granulocytes, a process not well understood for this chronically persisting pathogen.

Helicobacter pylori is a slowly growing, spiral-shaped, gram-negative bacterium able to establish chronic infections in the gastric mucosae of humans (for reviews, see references 3, 23, and 45). It causes type B gastritis and contributes to the development of peptic ulcers and probably gastric carcinoma (4, 32) and lymphoma (19, 51). *H. pylori* gastritis usually begins with an acute inflammatory response, predominantly the formation of polymorphonuclear granulocytes (PMNs), which is followed by infiltration with mononuclear cells (44). The bacteria persist in the gastric mucosa despite vigorous specific cellular and humoral responses. This continuous stimulus results in persistent inflammation and chronic gastritis.

Phagocytic cells are one of the first lines of defense in the body against microbial pathogens. Concomitant with phagocytosis, phagocytes produce an oxidative burst, resulting in a number of toxic byproducts. These reactive oxygen intermediates include singlet oxygen ($'O_2$), superoxide anions (O_2^-), hydrogen peroxide (H_2O_2), and the hydroxyl radical ($\cdot OH$), which are part of the bactericidal defense mechanism employed by phagocytic cells. Bacterial enzymes, such as superoxide dismutase and catalase, help bacteria to protect themselves from oxygen toxicity and damage. Superoxide dismutases are metalloenzymes that convert superoxide radicals into H_2O_2 and O_2 (30). The structural gene encoding superoxide dismutase of *H. pylori*, *sod*, has been reported previously (46). The catalase activity is involved in the subsequent step, the detoxification of H_2O_2 by converting it to H_2O and O_2.

Catalase activity has been shown to be important for several bacterial pathogens to survive in PMNs (12, 29, 52). A protein with catalase activity was purified from *H. pylori* and characterized biochemically (17). The enzyme is described as a tetramer of a 50-kDa subunit that is active over a broad pH range and lacks a detectable peroxide activity. We report here the cloning and characterization of the gene encoding catalase (*katA*) and construction of a catalase-deficient mutant, important steps in elucidating a possible protective role of the enzyme in the defense of *H. pylori* against the oxygen-dependent killing mechanism of PMNs of the host.

MATERIALS AND METHODS

Bacterial strains, plasmids, and culture conditions. *Helicobacter* strains were grown on GC agar plates (Difco) supplemented with horse serum (8%), vancomycin (10 mg liter^{-1}), trimethoprim (5 mg liter^{-1}), and nystatin (1 mg liter^{-1}) (serum plates) or in brain heart infusion liquid medium (Difco) supplemented with 10% fetal calf serum or horse serum and incubated for 2 to 3 days in a microaerobic atmosphere (85% N_2, 10% CO_2, 5% O_2) at 37°C. *Escherichia coli* HB101 (6) and DH5α (BRL) were grown on Luria-Bertani agar plates (39) supplemented with ampicillin (100 mg liter^{-1}), chloramphenicol (30 mg liter^{-1}), or tetracycline (15 mg liter^{-1}), as appropriate.

DNA manipulations. Standard cloning and DNA analysis procedures were performed according to the methods of Sambrook et al. (39). Plasmid DNA was purified from *E. coli* by the boiling procedure, and total DNA extractions from *H. pylori* were carried out as described by Leying et al. (24). *E. coli* cells for electroporation were prepared according to the protocol recommended for the Gene Pulser apparatus (Bio-Rad). *H. pylori* P1 was transformed with plasmids according to the procedure described by Haas et al. (16). Southern blotting and hybridizations with DNA fragments as probes were performed by using the

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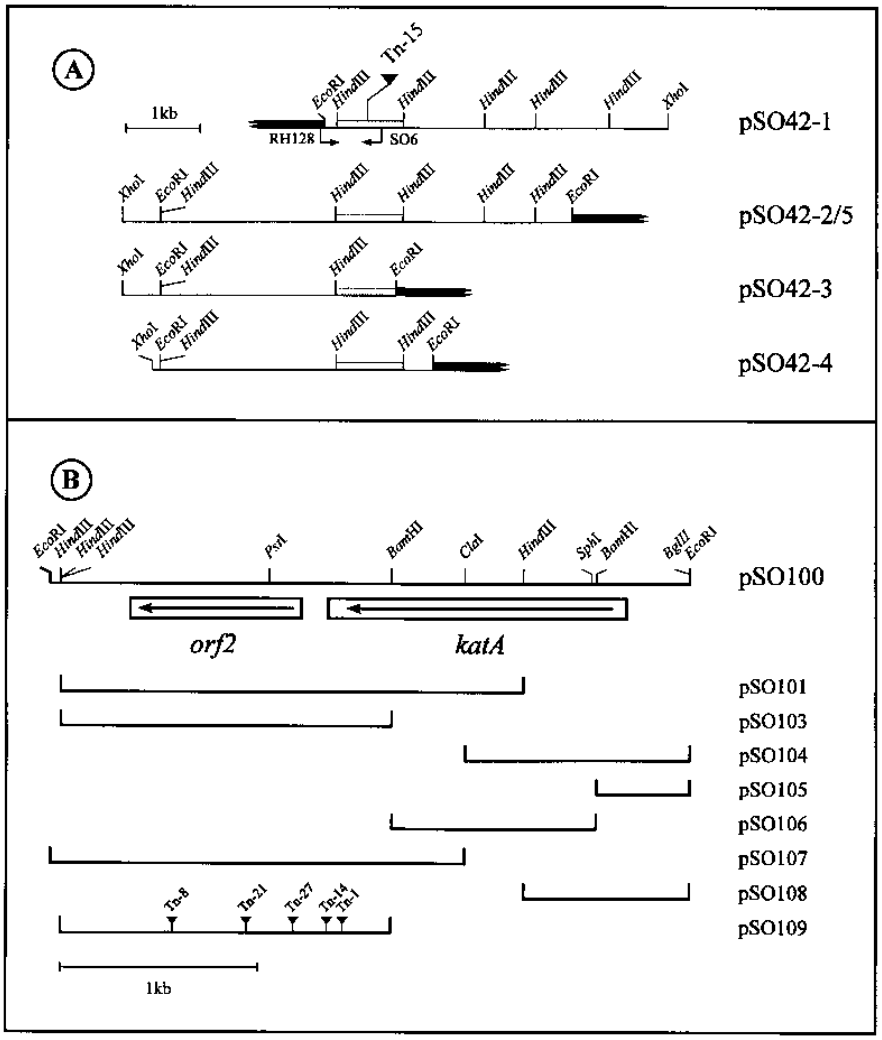

FIG. 1. Cloning and physical map of the *H. pylori katA* gene. (A) Relative alignment of five independent clones carrying *H. pylori katA* gene sequences. The SO6 primer-binding region was localized within the plasmids by PCR with primers RH128 (vector derived) and SO6 (deduced from the N-terminal sequence of KatA) and could be narrowed down to between two *Hin*dIII restriction sites (shaded boxes) common to all five plasmid inserts. The site of the Tn*Max5* insertion into pSO42-1, which leads to pSO42-1::Tn-15, is indicated. Sequences of the cloning vector pMin1 are depicted as filled boxes with a jagged end. *Eco*RI and *Xho*I are restriction enzyme sites derived from the vector and indicate the orientation of the cloned insert. (B) Plasmid pSO100 represents an *Eco*RI subclone of plasmid pSO42-3 (drawn in the same orientation) and was used for construction of subclones pSO101 to pSO109. Plasmid pSO109 served as the target for several Tn*Max5* insertions used to determine the complete sequence of plasmid pSO100 by insertion of mobile sequencing primers (Tn-1, Tn-8, Tn-14, Tn-21, and Tn-27). The locations of *katA* and the putative *orf2* gene are shown as boxes. Arrows within the boxes indicate the orientation of transcription.

enhanced chemiluminescence labeling and detection system according to the manufacturer's protocol (Amersham). For hybridization, 0.5 M NaCl was used; the washing buffer contained 0.5× SSPE (1× SSPE is 180 mM NaCl, 10 mM NaH_2PO_4, 10.5 mM NaOH, and 1 mM EDTA [pH 7.5]), 6 M urea, and 0.4% sodium dodecyl sulfate (SDS) at 42°C. Oligodeoxynucleotides were end labeled with T4 polynucleotide kinase by using [γ-^{32}P]ATP. Southern and colony hybridizations with oligonucleotide SO6 (5′-ATGGTACGTAACTAAGAGACTGTAGCTAAAG-3′; underlined letters indicate wobble sequences at the third positions of codons) were performed under low-stringency conditions, and filters were washed with 2× SSPE–0.05% SDS at 42°C. pBluescript II KS (Stratagene) was used to subclone gene fragments for DNA sequencing and for T7 promoter expression. For construction of plasmids pSO100 and pSO100a, the *Eco*RI fragment of pSO42-3 was ligated into the *Eco*RI site of pBluescript II KS in both orientations. Fragments were separated by preparative agarose gel electrophoresis, and bands were isolated with the GeneClean kit (Bio 101, Inc.). Purified fragments were ligated into the correspondingly prepared vectors. For construction of plasmids, pSO101 to pSO109, corresponding fragments from pSO100 (Fig. 1B) were isolated and ligated into the compatible sites of the pBluescript II KS or pMin1 vectors (20).

DNA sequencing. Sequencing was performed according to the dideoxynucleotide chain termination method (40) with a PRISM ready reaction dye cycle sequencing kit (Applied Biosystems) and fluorescence-labeled M13-FP (5′-TGTAAAACGACGGCCAGT-3′) and M13-RP1 primers (5′-CAGGAAACAGCTATGACC-3′) on an Applied Biosystems model 373A automated DNA sequencer. Linearized, double-stranded plasmids were used as templates. These plasmids carried mobile M13-FP and M13-RP1 primers on the Tn*Max5* minitransposon, which was inserted at different locations in the *katA* gene. On average, 450 nucleotides could be sequenced upstream and downstream from each particular Tn*Max5* insertion, which resulted in an overlapping sequence on both DNA strands. In addition, several restriction fragments covering the *katA* gene were cloned in pBluescript II KS and used for sequencing (Fig. 1B, pSO101 to pSO109). Nucleotide and protein sequence comparisons were performed with the BESTFIT and PILEUP algorithms of the University of Wisconsin's Genetics Computer Group computer program.

Isolation of RNA and primer extension. Total RNA was isolated from *H. pylori* P1 by a modification of a method originally described by Chirgwin et al. (7). Bacteria grown on serum plates for 48 to 72 h were resuspended in 400 μl of ice-cold TES (10 mM Tris-HCl [pH 8], 100 mM EDTA, 100 mM NaCl). After addition of 400 μl of TES containing 1% SDS, a twofold extraction of the suspension with hot phenol (65°C) was performed and the water phase was diluted with 1.2 ml of 4 M guanidinium thiocyanate (0.1 M Tris-HCl [pH 7.5], 2% β-mercaptoethanol, 1% Nonidet P-40) and homogenized by shearing (passaging through a 23G to 25G needle). The homogenate was centrifuged through a 1-ml CsCl cushion (6 M CsCl in 10 mM Tris-HCl [pH 7.5]–1 mM EDTA) at 150,000 ×

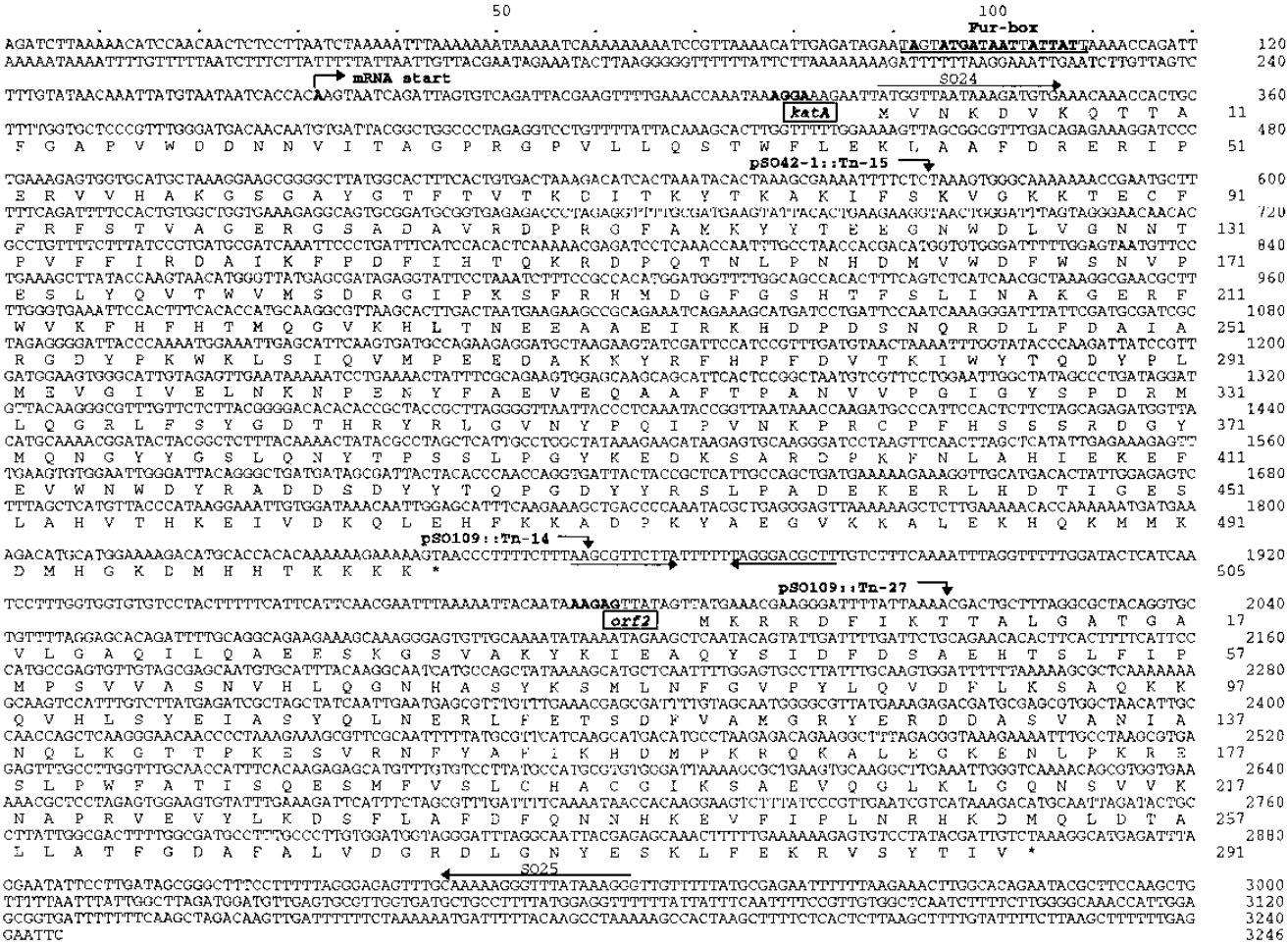

FIG. 2. Nucleotide and deduced amino acid sequences of the *H. pylori* P1 *katA* gene and a putative downstream gene (*orf2*). The transcriptional start site of the *katA* mRNA, as determined by primer extension, is indicated by an arrow. A putative binding site for the Fur protein (Fur-box) is underlined, and nucleotides identical to the perfect consensus sequence are shown in boldface type. Putative ribosome-binding sites for *katA* and *orf2* are printed in boldface type, and a potential transcriptional terminator directly downstream of *katA* is indicated by arrows. The exact positions of Tn*Max5* transposon insertions into the sequence of *katA* or *orf2* and the binding sites of the oligonucleotide primers SO24 and SO25 used for PCR are indicated.

g for 18 to 20 h at 20°C. The RNA pellet was resuspended in water and precipitated in ethanol. Oligonucleotide SO35 (5′-CACATCTTTATTAACCA T-3′), complementary in its sequence to SO24 (Fig. 2), was labeled at its 5′ end with [γ-^{32}P]ATP (1.5 pmol) and hybridized to 20 μg of *H. pylori* RNA in avian myeloblastosis virus buffer (50 mM Tris-HCl [pH 8.3], 40 mM KCl, 5 mM dithiothreitol) by heating at 95°C for 5 min and slow cooling at 42°C for 15 min. The annealing mixture was supplemented with 0.8 mM each the four deoxynucleoside triphosphates (dNTPs). Avian myeloblastosis virus reverse transcriptase (U.S. Biochemicals) was added to extend the primer for 45 min at 42°C. The sequencing reaction was performed with plasmid pSO104 as the template. A Sequenase DNA sequencing kit (version 2.0; U.S. Biochemicals) was used according to the instructions provided by the manufacturer, and the probes were run on a 7% sequencing gel.

Transposon shuttle mutagenesis. Random insertional mutations within cloned *katA* sequences were performed with Tn*Max5* (20). Briefly, plasmids pSO42-1 or pSO109 were transformed into *E. coli* E181 harboring plasmid pTn*Max5*. High-frequency transposition of the minitransposon was induced by growing the bacteria overnight on Luria-Bertani agar containing tetracycline and chloramphenicol to select for maintenance of both plasmids and on 100 μM isopropyl-β-D-thiogalactopyranoside (IPTG). After transposition, total plasmids were transferred into *E. coli* E145 via conjugation. Single transconjugants were analyzed, and the insertion sites of the minitransposons were mapped.

PCR. Amplification of DNA was performed with plasmid or chromosomal DNA templates (1 to 10 ng) in 25 mM TAPS buffer [*N*-Tris-(hydroxymethyl)-methyl-3-amino-propane sulfonic acid] (pH 9.3)–50 mM KCl–2 mM $MgCl_2$–1 mM dithiothreitol–0.25 μM each dNTP–50 pmol of the corresponding oligonucleotide pairs in the presence of 1 U of *Taq* polymerase in a Perkin-Elmer thermal cycler (Cetus model). Cycling was carried out at 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min for 25 cycles.

Mapping of the chromosomal *katA*::Tn*Max* insertion by PCR. The correct insertion of the Tn*Max5*-inactivated *katA* gene into the *H. pylori* chromosome by allelic exchange was verified by PCR. Chromosomal DNAs of strains P1 and P20 were used to amplify the complete *katA* gene with primers SO24 (5′-CGGAAT TCATGGTTAATAAAGATGTG-3′) and SO25 (CCGCTCGAGCCTTTATA AACCCTTTTTG) located upstream and downstream of the *katA* gene, respectively (Fig. 2). The *katA* sequences located upstream or downstream of the Tn*Max5* insertion site were amplified with one of the Tn*Max5*-located M13 primers (M13-FP or M13-RP1) combined with SO24 or SO25.

Determination of catalase activity in bacterial cell extracts. For determining specific catalase activities of *H. pylori* and *E. coli* strains, a slightly modified procedure of the method published by Rupprecht and Schleifer (38) was used. Bacteria grown for 2 days on serum plates (*H. pylori*) or for 1 day on Luria-Bertani agar plates (*E. coli*) were harvested and suspended in 300 μl of phosphate-buffered saline; the solution was sonicated to break the cells, and cell debris was removed by centrifugation (10 min, 13,000 × *g*, 4°C). The supernatant was recovered and kept on ice. To measure the activity of catalase, the conversion of a 10 mM H_2O_2 solution into H_2O and O_2 was determined by decrease of the A_{240}. Measurements were performed under conditions in which the decrease in absorption (0.05 to 0.1 U/min) was linear to the enzyme activity. The volume activity (in units per milliliter) was determined as follows: $\Delta E/\Delta t \times V/v \times \varepsilon^{-1} \times d^{-1}$ (1 U = 1 μmol of H_2O_2 per min) with $\varepsilon(H_2O_2)_{240nm} = 0.0394$ ml μmol^{-1} cm^{-1}, where V is the reaction volume, v is the volume of added cell extract, ΔE is the change in absorption, Δt is the change in the reaction time, ε is the

TABLE 1. Bacterial strains and plasmids used in this study

Strain, plasmid, or bacteriophage	Genotype or characteristic(s)	Reference or source
Strains		
E. coli		
E181	HB101 carrying λCH616 prophage	33
E145	*polA1 su tsx endA1 rnsA100 rpsL* λCH616 *rif*	16
DH5α	F^- ϕ80dlacZΔM15 Δ(*lacZYA-argF*)*U169 endA recA1 hsdR17 deoR thi-1 supE44 gyrA96 relA1*	BRL
HB101	F^- *mcrB mrr hsd-20* (r_B^- m_B^-) *recA13 leuB6 ara-14 proA2 lacY1 galK2 xyl-5 mtl-1 rpsL20 supE44*λ^-	6
UM255	*pro leu rpsL hsdM hsdR endI lacY katG2 katE12*::Tn*10 recA*	31
H. pylori		
NCTC11637	*H. pylori* wild-type strain, type strain	ATCC[a]
P1	*H. pylori* wild-type strain	33
P12	*H. pylori* wild-type strain	33
P20	*H. pylori* P1, *katA* mutant transformed with pSO42-1::Tn-15	This study
P49-1	*H. pylori* wild-type strain; mouse adapted by serial passaging	Unpublished
P70	*H. pylori* P1 transformed with plasmid pSO109::Tn-14	This study
P71	*H. pylori* P1 transformed with plasmid pSO109::Tn-27	This study
P74	*H. pylori* P49-1, *katA* mutant transformed with pSO42-1::Tn-15	This study
P93	NCTC11637, *katA* mutant transformed with pSO42-1::Tn-15	This study
Plasmids		
pTn*Max5*	cat_{GC} *res* ori_{fd} *tnpR tnpA*	20
pMin1	ori_{ColE1} *tet oriT* t_{fd} t_{trpA}	20
pBluescript II KS		Stratagene
pRK2013	ori_{ColE1} *apha-3 oriT tra* genes	9
Bacteriophage		
λCH616	Bacteriophage fd gene 2	13

[a] ATCC, American Type Culture Collection.

absorption coefficient, and *d* is the width of the cuvette. Specific activity (in units per milligram) was calculated as volume activity/protein content.

T7 promoter expression. Plasmids were transformed into *E. coli* K38 (48). An overnight culture of transformants (500 μl), grown at 28°C, was washed three times in M9 minimal medium (39), suspended in 1 ml of M9 minimal medium, and grown aerobically for 45 to 60 min at 30°C. This culture (300 μl) was induced for 15 min at 42°C to allow expression of T7 polymerase, 1.2 μl of rifampin (50 mg/ml) was added to inhibit protein synthesis from bacterial RNA polymerases, and the culture was incubated for 10 min at 42°C and then for 20 min at 30°C. One microliter of [^{35}S]methionine (10 μCi) was added. The cells were incubated for 15 min at 30°C, harvested, and suspended in a 40-μl sample solution (22). Ten microliters of each sample was boiled and subjected to 0.1% SDS–10% polyacrylamide gel electrophoresis; the gel was then dried and autoradiographed.

Nucleotide sequence accession number. The nucleotide sequence data appear in the EMBL, GenBank, and DDBJ nucleotide sequence data libraries under accession number Z70679.

RESULTS

Cloning of an *H. pylori* gene with homologies to prokaryotic and eukaryotic catalases. Interested in the adhesion of *H. pylori* to human gastric cells, we intended to isolate the gene encoding an adhesin specific for phosphatidylethanolamine (PE) on epithelial cells (25). Therefore, the N-terminal protein sequence MVNKDVK, published for a putative 63-kDa PE-specific adhesin of *H. pylori*, was used to synthesize the degenerative oligodeoxynucleotide SO6 (Materials and Methods). Screening of an *H. pylori* P1 gene library established in the minimal vector pMin1 (20) with the ^{32}P-labeled oligonucleotide probe resulted in five independent clones (pSO42-1 to pSO42-5) (Fig. 1A). Mapping of the plasmids by restriction analysis revealed that all five plasmids contained overlapping DNA fragments, with pSO42-2 and pSO42-5 being identical. The binding region of the labeled oligonucleotide was localized on an 850-bp *Hin*dIII fragment (data not shown). The location and orientation of the primer binding site, and thus the orientation of the putative open reading frame (ORF) on plasmid pSO42-1, were determined by PCR with pSO42-1 as the template and oligonucleotides SO6 and RH128 as the primers (Fig. 1A). Partial nucleotide sequences around the primer binding region revealed that the putative translational start codon of an ORF was located 245 nucleotides upstream of the Tn*Max5* insertion Tn-15 (Fig. 1A and 2). The ORF started with exactly the same amino acid sequence as that determined for the putative 63-kDa adhesin, including also the codon positions 8 to 12 of the ORF, which were not covered by the SO6 oligonucleotide sequence.

Surprisingly, a comparison of the first 143 amino acids of the ORF with the GenBank and EMBL databases revealed a strong homology of the ORF with several prokaryotic and eukaryotic catalases. Southern hybridization with chromosomal DNA of *H. pylori* P1 and the cloned gene as the probe clearly demonstrated that only a single gene, corresponding to the published N-terminal protein sequence, was present in the *H. pylori* P1 chromosome (data not shown). Thus, it seemed possible that the protein purified and N-terminally sequenced by Lingwood et al. (27) was actually the *H. pylori* catalase, rather than an adhesin, and that we had cloned the corresponding structural gene.

DNA sequence analysis and genetic characterization of the *H. pylori* catalase gene *katA*. To clarify whether the cloned gene actually encoded a catalase, the complete gene sequence was determined. The insert of plasmid pSO42-3 was further subcloned (Fig. 1B), and random Tn*Max5* insertion mutagenesis was performed to facilitate sequence analysis by the transposon-located sequencing primers (Fig. 1B; Table 1).

The sequence of the complete ORF comprises 1,518 nucleotides, encoding a putative protein with a predicted M_r of 58,600 (Fig. 2). The ORF is preceded by a potential ribosome-

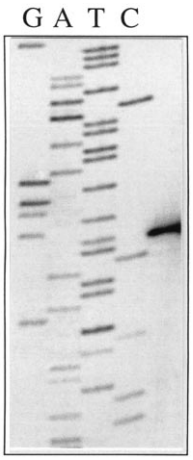

FIG. 3. Mapping of the transcriptional start site of *H. pylori katA* mRNA. The sequencing reaction of the *katA* upstream region (G, A, T, and C) was run together with the primer extension product of the *katA* mRNA on a 7% polyacrylamide–urea sequencing gel.

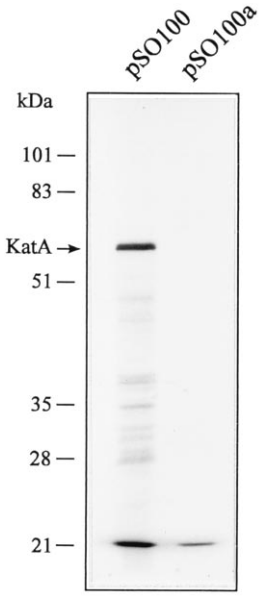

FIG. 4. Expression of the *H. pylori* catalase with the T7 RNA polymerase promoter system. Cell lysates of *E. coli* K-38 (pSO100) (the *katA* gene is behind the T7 promoter) and K38(pSO100a) (the *katA* gene is in the orientation opposite to that of the T7 promoter) were induced for T7 promoter expression and analyzed. The positions of marker proteins and the location of the KatA protein are indicated.

binding site (43), AGGA, starting 11 bp upstream of the putative ATG start codon. A stem-loop structure, which might act as a transcriptional terminator, is located 12 bp downstream of the gene (37). The G+C content of the coding region is 42.3%, which is consistent with the average G+C content for many *H. pylori* genes (21, 24, 42).

To identify the active promoter in *H. pylori*, a primer extension experiment was performed on total mRNA isolated from strain P1. A single primer extension product, represented by a prominent band in the gel, could be identified (Fig. 3), placing the 5′ end of the *katA* mRNA exactly 57 nucleotides upstream of the AUG start codon. Although a potential −10 box was present upstream of the mRNA start site (AATAAT), no corresponding conserved −35 box could be detected in the expected distance to the −10 box. Interestingly, a sequence with strong homology to a Fur box (14 of 19 nucleotides match the perfect consensus sequence) (47) is located upstream of the mRNA start site in a DNA region which might be involved in regulation of *katA* gene expression (Fig. 2). The Fur protein is a transcriptional regulator of iron-regulated genes in many bacteria.

Comparison of the putative catalase sequence with other prokaryotic and eukaryotic catalases revealed that the *H. pylori* protein showed the strongest homology to the *Bordetella pertussis* catalase KatA (64.9% identity) (8), the *Bacteroides fragilis* catalase KatB (59.8% identity) (36), and the *Haemophilus influenzae hktE* gene product (57.9% identity) (2). Further homologous sequences were found in the *Bacillus subtilis* vegetative catalase (57.6% identity) (5), the *Campylobacter jejuni* catalase KatA (54.6% identity) (14) (Table 2), and a range of eukaryotic catalases of yeast, plant, and animal origin. The gene was therefore named *katA*. The pI of the KatA protein, as determined from the amino acid sequence, was 9.2, which is in good agreement with a pI of 9.0 to 9.3, as determined experimentally for the purified 50-kDa *H. pylori* catalase by Hazell and coworkers (17).

TABLE 2. Amino acid sequence comparison of *H. pylori* catalase KatA with other bacterial catalases

Species	Gene product	% Sequence identity[a]	Reference
Bordetella pertussis	KatA	64.9	8
Bacteroides fragilis	KatB	59.8	36
Haemophilus influenzae	KatE	57.9	11
Bacillus subtilis	Kat-19+	57.6	28
Campylobacter jejuni	KatA	54.6	14

[a] The amino acid sequence of *H. pylori* catalase was compared by using the BESTFIT program of the University of Wisconsin's Genetics Computer Group software package.

A second ORF (*orf2*) which starts 145 nucleotides downstream of the *katA* stop codon encodes a putative protein with a predicted M_r of 32,715. The *orf2* is preceded by the putative ribosome-binding site AAGAG. A protein comparison of the putative Orf2 protein did not reveal significant homology to known proteins in the GenBank or EMBL database.

Expression of KatA by the T7 promoter expression system in *E. coli*. The subunit size of the purified catalase of *H. pylori* was 50 kDa, as described by Hazell et al. (17), whereas our cloned gene encoded a catalase with a predicted size of 58.6 kDa. To experimentally determine the size of the protein encoded by the *katA* gene, the T7 promoter expression system (48) was used. The *katA* gene was inserted into the pBluescript II KS vector in both orientations, resulting in plasmids pSO100 and pSO100a. As shown in Fig. 4, a protein of 62 kDa was produced in the clone pSO100, which carries the *katA* gene behind the T7 promoter, but no protein bands were found with plasmid pSO100a, which carries the same insert in the opposite orientation in the expression plasmid. Smaller protein bands in the lane corresponding to pSO100 either represent additional proteins, like the putative 32.7-kDa *orf2* gene product, or stable degradation products of KatA. Thus, we find a protein with a slightly higher molecular mass than that predicted from the gene sequence (62 versus 58.6 kDa) but a considerably higher apparent molecular mass than that of the purified catalase described by Hazell et al. (17) (62 versus 50 kDa). The apparent molecular mass of KatA on the SDS gel is, however, in

agreement with that of the purified protein used for N-terminal sequencing by Lingwood et al. (27) (62 versus 63 kDa).

***H. pylori katA* functionally complements the catalase-deficient *E. coli* UM255 double mutant.** To determine whether the *H. pylori katA* gene encodes a functional catalase, expression of *katA* was tested in the *katE-katG* double-mutant *E. coli* strain UM255 (31), which produces no catalase activity (Table 1). In pSO100a, *katA* was oriented behind the vector-located *lac* promoter; in pSO100, the gene carries its original promoter only. When the plasmids were introduced into *E. coli* UM255, both plasmids complemented *E. coli* UM255 for catalase activity. We observed, however, an approximately 20-fold difference in the specific catalase activity of the recombinant *E. coli* strain carrying pSO100a (17,940 U/mg) versus that of pSO100 (912 U/mg), suggesting that *katA* is weakly expressed, probably from an endogenous promoter. In pSO100a, however, *katA* is under the control of the *lac* promoter. Thus, it was demonstrated that *katA* encodes a catalase which is functional in *E. coli.*

Construction and characterization of catalase-deficient *H. pylori* mutant strains by transposon shuttle mutagenesis. The active catalase purified from fresh *H. pylori* isolates and laboratory-adapted strains by Hazell and coworkers was described as a tetrameric protein of about 50-kDa subunit size (17). Since the product of our cloned catalase gene encoded a protein of considerably larger molecular mass (58.6 kDa as predicted by its gene sequence and 62 kDa as determined by T7 promoter plasmid expression), we wanted to determine whether, in addition to *katA*, a gene encoding the 50-kDa catalase subunit described by Hazell et al. was present in *H. pylori*. Therefore, an isogenic mutant in the *katA* gene was constructed by transposon shuttle mutagenesis with Tn*Max5*. Plasmid pSO42-1::Tn-15, which interrupts the *katA* gene after codon 82, was transformed into *H. pylori* P1. Selection of transformants on chloramphenicol plates allowed the isolation of mutants, which had replaced the wild-type chromosomal *katA* gene with the mutated gene via homologous recombination. One such *H. pylori* mutant, P20, was isolated and characterized further. Insertional inactivation of the chromosomal *katA* gene was verified by Southern hybridization and PCR (Fig. 5). PCR amplification with a transposon-specific and a *katA*-specific primer pair resulted in a 320-bp fragment corresponding to the sequence upstream of the Tn*Max5* insertion site (Fig. 5, lane 4). A fragment of approximately 2.4 kb, corresponding to the Tn*Max5* downstream sequence, was also obtained (SO25–M13-RP1) (Fig. 5, lane 2), thus demonstrating a correct insertional inactivation of the chromosomal *katA* gene. Next, the *katA* mutant P20 was compared with the isogenic wild-type strain P1 for catalase activity. The wild-type strain produced a high level of specific catalase activity (4,422 U/mg), but the *katA* mutant P20 revealed only background activity (5 U/mg). Thus, it was concluded that KatA was the only catalase present in *H. pylori* P1. A chromosomal *katA* mutation was then transferred into two further, independent *H. pylori* strains, NCTC11637 and P49-1, a human isolate and a mouse-adapted *H. pylori* strain, respectively. The resulting mutants also revealed a completely abolished catalase activity, as compared with the activities of the corresponding wild-type strains, indicating that *katA* is expressed in different *H. pylori* isolates and is the only active catalase gene in these *H. pylori* isolates.

Does the downstream *orf2* gene affect catalase activity? The putative *orf2* gene is located closely behind *katA* (145 nucleotides), following a potential transcriptional terminator of the *katA* gene. Sequence comparison of the intergenic region did not reveal a potential promoter sequence for the putative *orf2* gene. Thus, cotranscription of *katA* and the *orf2* gene is possible and the *orf2* gene product might have an influence on the expression or the function of catalase. A regulation of catalase expression on the transcriptional level as an adaptive response to oxidative stress was described for the *E. coli* catalase HPI (41). Therefore, *H. pylori* mutant strains P70 and P71 were generated by transformation of *H. pylori* P1 with plasmids pSO109::Tn-14 and pSO109::Tn-27, respectively (Fig. 1B and 2). They carry a Tn*Max5* transposon either in the intergenic region or at the beginning of the *orf2* gene (Fig. 2). Both mutants revealed full catalase activities, indicating that the *orf2* gene product has no direct measurable influence on catalase activity.

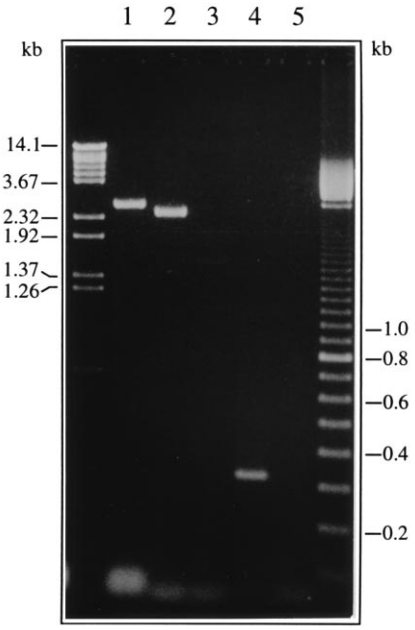

FIG. 5. Genetic characterization of the *H. pylori katA* transposon insertion mutant by PCR. Different regions of the *katA* gene locus were amplified from chromosomal DNA of strains P1 and P20 with various combinations of priming oligonucleotides. Lane 1 shows the *katA-orf2* region amplified with primers SO24 and SO25 from strain P1. PCR fragments from the downstream (lane 2) and upstream (lane 4) sequences of *katA* were obtained in P20 with primer pairs SO25–M13-RP1 and SO24–M13-FP, respectively. No PCR products were obtained with wild-type DNA (P1) as the template when a catalase- or a transposon-specific primer was used for PCR (lane 3, SO25–M13-RP1, and lane 5, SO24–M13-FP). The sizes of the PCR fragments in lanes 2 and 4 correspond well to the position of the Tn*Max5* insertion within the *katA* gene (Fig. 2). Molecular size markers are given in the unmarked lanes.

DISCUSSION

In 1989 Lingwood et al. reported that *H. pylori* is able to bind to a glycerolipid receptor, which was later identified as PE (25, 26). These results were mainly obtained by overlay assays, in which eukaryotic receptor molecules were separated by thin-layer chromatography, and the specific binding of bacteria to defined receptor molecules was determined. In 1993 the same group reported on the receptor affinity purification of a putative 63-kDa lipid-binding adhesin from *H. pylori* using a novel immobilized PE affinity matrix purification procedure (27). They identified a 63-kDa *Pseudomonas aeruginosa* exoenzyme S-like adhesin and determined its N-terminal amino acid sequence (27). We cloned the corresponding gene, *katA.*

There is no doubt that *katA* encodes a catalase of *H. pylori*, a determination based on sequence homology with other prokaryotic and eukaryotic catalases, and the enzymatic activity of

KatA produced in *E. coli* UM255. Furthermore, the enzyme activity was lost in the corresponding isogenic *H. pylori katA* mutants P20, P74, and P93. Although the purified protein was described as an adhesin, we could not find any changes in adhesive properties of the *katA* mutant P20 compared with the isogenic wild-type strain in a thin-layer chromatography overlay assay (27) or in direct binding to epithelial cells. We therefore conclude that the putative PE adhesin purified and published by Lingwood and coworkers represented *H. pylori* catalase, which seems unlinked to *H. pylori* adhesion to PE. One possibility, which could explain the conflicting results, however, would be that the 62-kDa purified protein of Lingwood and coworkers was actually a mixture of catalase and the true PE adhesin, both comigrating in the SDS gel. Assuming that the N terminus of the putative adhesin was blocked, only the sequence of the catalase was determined by N-terminal protein sequencing and believed to be the PE adhesin sequence.

Our DNA hybridization experiments revealed that most probably a single catalase gene is present in the *H. pylori* P1 chromosome. In agreement with that data, defined *katA* mutants generated in three independent *H. pylori* strains did not produce significant catalase activities. In contrast, there are multiple catalases in the gram-positive bacterium *Bacillus subtilis* (28) and in *E. coli* two inducible genes producing catalases (hydroperoxidases), called HPI and HPII, have been described (49, 50). Results from specific mutant strains indicate that in *H. influenzae* (1), *C. jejuni* (14), or *B. pertussis* (8) only a single active catalase gene is present.

The *katA* gene product has a molecular mass of 58.6 kDa, as calculated from its gene sequence. The protein purified from *H. pylori* by Lingwood et al. (27) and the *katA* gene product produced in *E. coli* both run with an apparent molecular mass of 62 or 63 kDa in the SDS gel, which is higher than the calculated molecular mass of the KatA protein described by Hazell and coworkers (17). The discrepancy might be explained by a specific cleavage taking place in *H. pylori* but not in *E. coli*, especially in the T7 promoter expression system, in which there is only a short incubation period after induction. Furthermore, the migration of KatA at an atypical molecular mass in the SDS gel might be a feature of this protein, indicative of a specific intrinsic protein structure. Our genetic data would suggest that the *H. pylori* catalase purified by Hazell and coworkers, which has an apparent molecular mass of 50 kDa, is most likely also the product of the *katA* gene. Whether the size of the protein can vary between *H. pylori* strains to such an extent might be determined when a specific antiserum against this protein is available.

Most bacterial catalases are located in the cytoplasm, where they are involved in protection of DNA from the detrimental effects of H_2O_2. For *E. coli*, however, it has been demonstrated that HPI is present in both the periplasm and the cytoplasm whereas HPII is a cytoplasmic enzyme (18). Our nucleotide sequence data revealed no indication for the presence of a typical export-directing amino acid signal in *H. pylori* KatA. Nevertheless, the protein was isolated from a water extract of *H. pylori*, indicating that it might be surface located. A surface location of catalase in *H. pylori* could, however, assign to the enzyme an important function in the defense against phagocytic attack. A further explanation for this surface location could also be the "altruistic lysis" phenomenon described for *H. pylori*, which is explained by the fact that cytoplasmic proteins are constantly shed into the environment by lysis of bacteria and these proteins apparently bind to the surface of intact *H. pylori*. Such a mechanism has been postulated recently for several *H. pylori* proteins, including urease, a heat shock protein, and catalase (35) as well as an iron-storage protein, the bacterioferritin Pfr (10). A specific transport mechanism of KatA to the *H. pylori* surface, at this stage, however, cannot be excluded.

A DNA sequence with strong homology to the consensus *E. coli* Fur protein-binding site has been identified upstream of the promoter region of *katA*, in a region which could be involved in binding of regulatory proteins. Genes encoding proteins involved in oxidative stress resistance, like catalases or superoxide dismutases, often carry such Fur-binding consensus sequences (14, 15, 34). However, the question of whether a Fur protein homolog is really present in *H. pylori* is still unclear and needs further investigation.

For several pathogenic bacteria, catalase activities are involved in the defense mechanisms against in vivo killing by PMNs. The construction of a catalase-deficient isogenic *H. pylori* mutant is an important first step and a prerequisite for study of the putative role of this enzyme in the defense mechanism of *H. pylori* against PMNs. Future experiments will include infection of wild-type and mutant strains in the *H. pylori* mouse model and/or in vitro phagocytosis experiments using professional phagocytes. Such experiments should help to clarify whether catalase plays a significant role for survival and pathogenesis of these bacteria, which establish chronic infections in their host.

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REFERENCES

1. **Bishai, W. R., N. S. Howard, J. A. Winkelstein, and H. O. Smith.** 1994. Characterization and virulence analysis of catalase mutants of *Haemophilus influenzae*. Infect. Immun. **62:**4855–4860.
2. **Bishai, W. R., H. O. Smith, and G. J. Barcak.** 1994. A peroxide/ascorbate-inducible catalase from *Haemophilus influenzae* is homologous to the *Escherichia coli katE* gene product. J. Bacteriol. **176:**2914–2921.
3. **Blaser, M. J.** 1993. *Helicobacter pylori*: microbiology of a "slow" bacterial infection. Trends Microbiol. **1:**255–260.
4. **Blaser, M. J., G. I. Perez-Perez, H. Kleanthous, T. L. Cover, R. M. Peek, P. H. Chyou, G. N. Stemmermann, and A. Nomura.** 1995. Infection with *Helicobacter pylori* strains possessing *cagA* is associated with an increased risk of developing adenocarcinoma of the stomach. Cancer Res. **55:**2111–2115.
5. **Bol, D. K., and R. E. Yasbin.** 1991. The isolation, cloning and identification of a vegetative catalase gene from *Bacillus subtilis*. Gene **109:**31–37.
6. **Boyer, H. W., and D. Roulland-Dussoix.** 1969. A complementation analysis of the restriction and modification of DNA in *Escherichia coli*. J. Mol. Biol. **41:**459–472.
7. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1979. Isolation of biological active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18:**5294–5299.
8. **DeShazer, D., G. E. Wood, and R. L. Friedman.** 1994. Molecular characterization of catalase from *Bordetella pertussis*: identification of the *katA* promoter in an upstream insertion sequence. Mol. Microbiol. **14:**123–130.
9. **Ditta, M., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Natl. Acad. Sci. USA **77:**7347–7351.
10. **Doig, P., J. W. Austin, and T. J. Trust.** 1993. The *Helicobacter pylori* 19.6-kilodalton protein is an iron-containing protein resembling ferritin. J. Bacteriol. **175:**557–560.
11. **Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, A. R. Kerlavage, C. J. Bult, J. F. Tomb, B. A. Dougherty, J. M. Merrick, et al.** 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. Science **269:**496–512.
12. **Franzon, V. L., J. Arondel, and P. J. Sansonetti.** 1990. Contribution of superoxide dismutase and catalase activities to *Shigella flexneri* pathogenesis. Infect. Immun. **58:**529–535.

13. **Geider, K., C. Hohmeyer, R. Haas, and T. F. Meyer.** 1985. A plasmid cloning system utilizing replication and packaging functions of the filamentous bacteriophage fd. Gene **33:**341–349.
14. **Grant, K. A., and S. F. Park.** 1995. Molecular characterization of *katA* from *Campylobacter jejuni* and generation of a catalase-deficient mutant of *Campylobacter coli* by interspecific allelic exchange. Microbiology **141:**1369–1376.
15. **Haas, A., K. Brehm, J. Kreft, and W. Goebel.** 1991. Cloning, characterization, and expression in *Escherichia coli* of a gene encoding *Listeria seeligeri* catalase, a bacterial enzyme highly homologous to mammalian catalases. J. Bacteriol. **173:**5159–5167.
16. **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.
17. **Hazell, S. L., D. J. Evans, and D. Y. Graham.** 1991. *Helicobacter pylori* catalase. J. Gen. Microbiol. **137:**57–61.
18. **Heimberger, A., and A. Eisenstark.** 1988. Compartmentalization of catalases in *Escherichia coli*. Biochem. Biophys. Res. Commun. **154:**392–397.
19. **Horstmann, M., R. Erttmann, and K. Winkler.** 1994. Relapse of MALT lymphoma associated with *Helicobacter pylori* after antibiotic treatment. Lancet **343:**1098–1099.
20. **Kahrs, A. F., S. Odenbreit, W. Schmitt, D. Heuermann, T. F. Meyer, and R. Haas.** 1995. An improved Tn*Max* mini-transposon system suitable for sequencing, shuttle mutagenesis and gene fusions. Gene **167:**53–57.
21. **Labigne, A., V. Cussac, and P. Courcoux.** 1991. Shuttle cloning of genes responsible for urease activity from *Helicobacter pylori* and nucleotide sequence determination. J. Bacteriol. **173:**1920–1931.
22. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
23. **Lee, A., J. Fox, and S. Hazell.** 1993. Pathogenicity of *Helicobacter pylori*: a perspective. Infect. Immun. **61:**1601–1610.
24. **Leying, H., S. Suerbaum, G. Geis, and R. Haas.** 1992. Characterisation of *flaA*, a *Helicobacter pylori* flagellin gene. Mol. Microbiol. **6:**2863–2874.
25. **Lingwood, C. A., M. Huesca, and A. Kuksis.** 1992. The glycerolipid receptor for *Helicobacter pylori* (and exoenzyme S) is phosphatidylethanolamine. Infect. Immun. **60:**2470–2474.
26. **Lingwood, C. A., A. Pellizzari, H. Law, P. Sherman, and B. Drumm.** 1989. Gastric glycerolipid as a receptor for *Campylobacter pylori*. Lancet **i:**238–241.
27. **Lingwood, C. A., G. Wasfy, H. Han, and M. Huesca.** 1993. Receptor affinity purification of a lipid-binding adhesin from *Helicobacter pylori*. Infect. Immun. **61:**2474–2478.
28. **Loewen, P. C., and J. Switala.** 1987. Multiple catalases in *Bacillus subtilis*. J. Bacteriol. **169:**3601–3607.
29. **Mandell, G. L.** 1975. Catalase, superoxide dismutase, and virulence of *Staphylococcus aureus. In vitro* and *in vivo* studies with emphasis on staphylococcal-leucocyte interaction. J. Clin. Invest. **55:**561–566.
30. **McCord, J. M., and I. Fridovich.** 1969. Superoxide dismutase: an enzymatic function for erythrocuprein. J. Biol. Chem. **244:**6049–6055.
31. **Mulvey, M. R., P. A. Sorby, B. L. Triggs-Raine, and P. C. Loewen.** 1988. Cloning and physical characterization of *katE* and *katF* required for catalase HPI expression in *Escherichia coli*. Gene **73:**337–345.
32. **Nomura, A., G. N. Stemmermann, P. H. Chyou, I. Kato, G. I. Perez-Perez, and M. J. Blaser.** 1991. *Helicobacter pylori* infection and gastric carcinoma among Japanese Americans in Hawaii. N. Engl. J. Med. **325:**1132–1136.
33. **Odenbreit, S., M. Till, and R. Haas.** 1996. Optimized BlaM-transposon shuttle mutagenesis of *Helicobacter pylori* allows the identification of novel genetic loci involved in bacterial virulence. Mol. Microbiol. **20:**361–373.
34. **Pesci, E. C., D. L. Cottle, and C. L. Pickett.** 1994. Genetic, enzymatic, and pathogenic studies of the iron superoxide dismutase of *Campylobacter jejuni*. Infect. Immun. **62:**2687–2694.
35. **Phadnis, S. H., M. H. Parlow, M. Levy, D. Ilver, C. M. Caulkins, J. B. Connors, and B. E. Dunn.** 1996. Surface localization of *Helicobacter pylori* urease and a heat shock protein homolog requires bacterial autolysis. Infect. Immun. **64:**905–912.
36. **Rocha, E. R., and C. J. Smith.** 1995. Biochemical and genetic analyses of a catalase from the anaerobic bacterium *Bacteroides fragilis*. J. Bacteriol. **177:**3111–3119.
37. **Rosenberg, M., and D. Court.** 1979. Regulatory sequences involved in the promotion and termination of RNA transcription. Annu. Rev. Microbiol. **13:**319–353.
38. **Rupprecht, M., and K. H. Schleifer.** 1977. Comparative immunological study of catalases in the genus Micrococcus. Arch. Microbiol. **114:**61–66.
39. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
40. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
41. **Schellhorn, H. E.** 1994. Regulation of hydroperoxidase (catalase) expression in *Escherichia coli*. FEMS Microbiol. Lett. **131:**113–119.
42. **Schmitt, W., and R. Haas.** 1994. Genetic analysis of the *Helicobacter pylori* vacuolating cytotoxin: structural similarities with the IgA protease type of exported protein. Mol. Microbiol. **12:**307–319.
43. **Shine, J., and L. Dalgarno.** 1974. The 3′-terminal sequence of *Escherichia coli* 16S ribosomal RNA: complementarity to nonsense triplets and ribosome binding sites. Proc. Natl. Acad. Sci. USA **71:**1342–1346.
44. **Sobala, G. M., J. E. Crabtree, M. F. Dixon, C. J. Schorah, J. D. Taylor, B. J. Rathbone, R. V. Heatley, and A. T. R. Axon.** 1991. Acute *Helicobacter pylori* infection: clinical features, local and systemic immune response, gastric mucosal histology and gastric juice ascorbic acid concentrations. Gut **32:**1415–1418.
45. **Solnick, J. V., and L. S. Tompkins.** 1993. *Helicobacter pylori* and gastroduodenal disease: pathogenesis and host-parasite interaction. Infect. Agents Dis. **1:**294–309.
46. **Spiegelhalder, C., B. Gerstenecker, A. Kersten, E. Schiltz, and M. Kist.** 1993. Purification of *Helicobacter pylori* superoxide dismutase and cloning and sequencing of the gene. Infect. Immun. **61:**5315–5325.
47. **Stojiljkovic, I., A. J. Bäumler, and K. Hantke.** 1994. Fur regulon in Gram-negative bacteria—identification and characterization of new iron-regulated *Escherichia coli* genes by a Fur titration assay. J. Mol. Biol. **236:**531–545.
48. **Tabor, S., and C. C. Richardson.** 1985. A bacteriophage T7 RNA polymerase/promoter system for controlled exclusive expression of specific genes. Proc. Natl. Acad. Sci. USA **82:**1074–1078.
49. **Triggs-Raine, B. L., B. W. Doble, M. R. Mulvey, P. A. Sorby, and P. C. Loewen.** 1988. Nucleotide sequence of *katG*, encoding catalase HPI of *Escherichia coli*. J. Bacteriol. **170:**4415–4419.
50. **von Ossowski, I., M. R. Mulvey, P. A. Leco, A. Borys, and P. C. Loewen.** 1991. Nucleotide sequence of *Escherichia coli katE*, which encodes catalase HPII. J. Bacteriol. **173:**514–520.
51. **Wotherspoon, A. C., C. Doglioni, T. C. Diss, L. Pan, A. Moschini, M. De-Boni, and P. G. Isaacson.** 1993. Regression of primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of *Helicobacter pylori*. Lancet **342:**575–577.
52. **Zheng, H., D. J. Hasset, K. Bean, and M. S. Cohen.** 1992. Regulation of catalase in *Neisseria gonorrhoeae*. Effects of oxidant stress and exposure to human neutrophils. J. Clin. Invest. **90:**1000–1006.