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Purification of *Helicobacter pylori* Superoxide Dismutase and Cloning and Sequencing of the Gene

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The superoxide dismutase (SOD) of *Helicobacter pylori*, a pathogenic bacterium which colonizes the gastric mucosa, evoking a marked inflammatory response, was purified and characterized, and the N-terminal amino acid sequence was determined. The enzyme consists of two identical subunits each with an apparent molecular weight of 24,000. Analysis of the primary structure and inhibition studies revealed that *H. pylori* possesses a typical procaryotic iron-containing enzyme. No other isoenzymes could be detected. Indirect gold immunostaining of *H. pylori* SOD with a polyclonal antibody directed against the iron-containing SOD of *Escherichia coli* showed a surface-associated localization of the enzyme. The *H. pylori* SOD gene was cloned by functional complementation of a SOD-deficient *E. coli* mutant. Sequencing and alignment revealed striking homology to the following facultative intracellular human pathogens: *Listeria ivanovii*, *Listeria monocytogenes*, *Coxiella burnetti*, *Porphyromonas gingivalis*, *Legionella pneumophila*, and *Entamoeba histolytica*. An open reading frame of 642 bp encoding 214 amino acids was determined. There was no leader sequence detectable. Cloning of the *H. pylori* SOD gene is one of the prerequisites to investigation of its pathophysiological role in the defense against antimicrobial mechanisms of polymorphonuclear granulocytes.

The histological features of chronic *Helicobacter pylori* (19, 20) gastritis and ulcer disease are characterized by dense infiltration of the colonized area by polymorphonuclear granulocytes (PMNs), accompanied by accumulation of monocytes and lymphocytes (49). Besides these local effects, *H. pylori* infection induces marked humoral and cellular immune responses. Despite these defense mechanisms, the infection becomes chronic, and the immune system obviously is not able to eliminate the pathogen (28, 29). The potential role of the accumulated phagocytes in the pathogenesis of the infection remains unclear.

Studies concerning the in vitro phagocytosis of *H. pylori* by peripheral neutrophilic granulocytes and monocytes revealed that *H. pylori* was able to survive within the phagocytes, if opsonins are absent. In vitro killing of *H. pylori* by PMNs occurs only when opsonic serum factors are present (2). However, the extent of microbial killing seems to depend on the ratio of *H. pylori* cells to phagocytes. In the presence of immune sera, a 10-fold excess of PMNs is required to achieve complete killing of *H. pylori* (2). Despite this reduced microbicidal effect, phagolysosomal fusion, degranulation, and activation of the oxidative burst seem to take place (31, 42). Therefore, one can assume that *H. pylori* possesses protective defense systems against phagocyte attack.

The oxidative burst is one of the major killing mechanisms of phagocytes. Many pathogens have developed defense mechanisms in response to this oxidative stress, including DNA repair systems, scavenging substrates, and antioxidant enzyme systems (22, 23). One of these antioxidant enzymes is superoxide dismutase (SOD), which catalyzes the dismutation of the primary reaction products of the neutrophilic oxidative burst, the superoxide anions. SODs are not found exclusively in pathogens but are present in nearly all organisms. Three different apoenzymes of SODs exist, all dependent on a metal cofactor. Cu/Zn-containing SODs are the predominant species in eucaryotes, whereas Mn-containing SODs (MnSODs) are present in procaryotes and in mitochondria (3, 18). Iron-utilizing enzymes are present in procaryotes as well as in some eucaryotes. Some procaryotes are able to produce both procaryotic isoenzymes (MnSOD and FeSOD). This is commonly the case for aerobic or facultative anaerobic bacteria; *Escherichia coli*, for example, produces these two isoenzymes under different environmental conditions.

In studies with microbial pathogens, there is some evidence that SODs may be associated with pathogenesis of disease in several cases: *Nocardia asteroides* (4, 5), *Shigella flexneri* (16), *Listeria monocytogenes* (55), *Mycobacterium tuberculosis* (56), and *Porphyromonas gingivalis* (1). In order to establish experimental procedures to elucidate a possible protective role of the SOD from *H. pylori* in the defense against the oxygen-dependent killing mechanism of PMNs, the purified enzyme was characterized and its gene was cloned.

MATERIALS AND METHODS

Bacterial strains and plasmids. Wild-type *H. pylori* 2012, originally isolated from biopsy material of the antral region of the stomach from a symptomatic patient, was stored at −70°C.

All *E. coli* strains used were derivatives of *E. coli* K-12. *E. coli* HB101 was used as the recipient of an *H. pylori* cosmid gene library. *E. coli* TG1 is the parental strain of the SOD-deficient *E. coli* TG1 *sodA sodB*, which was constructed by successive P1 transduction of *sodBΔ2* (8) and *sodA25* (8). The SOD-deficient strain was used in complementation studies, and the parental strain served as a con-

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trol. All strains were kindly provided by D. Touati (Université VII, Institut Jacques Monod, Paris, France).

Reagents, media, and growth conditions. Unless otherwise indicated, all reagents and antibiotics were purchased from Sigma, Deisenhofen, Germany. T4 DNA ligase, restriction endonucleases, and calf intestinal alkaline phosphatase were purchased from Pharmacia LKB, Freiburg, Germany. *H. pylori* 2012 was cultured in brucella broth (Difco Laboratories, Detroit, Mich.) supplemented with 3% fetal calf serum (GIBCO/BRL Life Technologies GMBH, Eggenstein, Germany) in a microaerobic atmosphere of 5% O_2 at 37°C under constant shaking for 48 h (30). *E. coli* strains were grown in Luria-Bertani (LB) broth supplemented with the appropriate antibiotics (ampicillin, 50 μg/ml; kanamycin, 50 μg/ml; or chloramphenicol, 20 μg/ml) at 37°C under rotation. For cultivation on solid media, 1.5% agar (Difco) was added. For the selective growing of recombinant SOD-positive *E. coli* clones, minimal medium agar M63 was prepared by the method of Maniatis et al. (36) and was supplemented with thiamine (1 μg/ml), paraquat (methyl viologen; 10^{-8} M), and the appropriate antibiotics.

Protein purification. *H. pylori* 2012 cells were washed in 50 mM Tris HCl (pH 7.5)–150 mM NaCl–0.1 mM EDTA–100 μM phenylmethylsulfonyl fluoride–1 μM pepstatin A and lysed by sonication for 1 min/ml (Branson Sonifier model 250) at 0 to 4°C. Cell debris was removed by differential centrifugation at 23,500 × *g* and 75,000 × *g* for 1 h at 4°C. The clarified supernatant (soluble fraction) was fractionated further by size exclusion chromatography on a Superose 12 prep-grade HR16/50 column (Pharmacia LKB) with an equilibration buffer of 50 mM Tris HCl (pH 7.5)–150 mM NaCl–0.1 mM EDTA (flow rate, 0.5 ml/min; fraction size, 1.5 ml). Fractions exhibiting SOD activity were pooled and purified further by successive anion-exchange chromatography (AEC) at two different pH levels, using a Mono Q HR5/5 column (Pharmacia LKB) with a flow of 1 ml/min and a fraction size of 1 ml. (i) AEC I at pH 7.5 used starting buffer A (20 mM Tris HCl [pH 7.5], 0.1 mM EDTA) and elution buffer B (buffer A containing 2 M NaCl) and a linear elution gradient to 1 M NaCl (50% buffer B) within 60 min (Fig. 1). (ii) AEC II at pH 9.5 used starting buffer C (20 mM ethanolamine [pH 9.5]) and elution buffer D (buffer C containing 2 M NaCl) and the same linear elution gradient as AEC I. After dialysis against 20 mM Tris HCl (pH 7.5), fractions containing the purified enzyme were stored at 4°C. Protein concentration was determined by the method of Peterson (46). Apparent molecular weight was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (34). Molecular mass standard proteins were purchased from Pharmacia LKB: phosphorylase *b* (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400). Protein was stained with Coomassie brillant blue R-250 (Serva, Heidelberg, Germany).

Native molecular weight was determined by size exclusion chromatography, using a Superose 12 prep-grade HR16/50 column (Pharmacia LKB). The equilibration buffer was 50 mM Tris HCl (pH 7.5)–0.1 mM EDTA–0.3 M NaCl.

Enzyme assays. Soluble SOD activity was determined by the ferricytochrome *c* reduction assay based on that of McCord and Fridovitch (38), which was modified as follows: reaction occurred in a 1-ml sample containing xanthine oxidase (2.5 mU/ml), catalase (100 ng/ml), cytochrome *c* (5 × 10^{-5} M), and hypoxanthine (10^{-4} M) in 10^{-5} M EDTA. Vials were incubated for 30 min at 37°C. Cytochrome *c* reduction was measured at 550 nm. Units were determined by comparison with a control vial containing purchased *E. coli* FeSOD (10 U/ml). The units are not comparable to those determined by McCord and Fridovitch (38).

SOD activity in nondenaturing polyacrylamide gels (12) was visualized by nitroblue tetrazolium negative staining (6). The gels were soaked simultaneously in a solution of 0.2% nitroblue tetrazolium, 0.028 M TEMED (*N,N,N',N'*-tetramethylethylenediamine), and 2.8 × 10^{-5} M riboflavin in 50 mM potassium phosphate buffer (pH 7.8) for 30 min at room temperature. The gels were illuminated until achromatic zones indicating SOD activity were visible in a uniformly blue background. R_f values were determined in 7% polyacrylamide gels.

Determination of the SOD metal cofactor. Metal cofactors were determined by enzyme inhibition studies (52). Soluble cell lysates of *E. coli* and *H. pylori* were separated by nondenaturing PAGE. The gel strips were incubated in 10 mM potassium cyanide (KCN) or 10 mM sodium azide (NaN_3) for 30 min or in 0.5 mM hydrogen peroxide (H_2O_2) for 1 h at room temperature. The control strip was incubated in 50 mM potassium phosphate buffer (pH 7.8)–0.1 mM EDTA. SOD activity was tested by the nitroblue tetrazolium assay (6).

Preparation of *E. coli* cell lysates. Washed *E. coli* cells resuspended in 50 mM potassium phosphate buffer (pH 7.8)–0.1 mM EDTA were lysed by sonication at 0 to 4°C in the presence of 100 μM phenylmethylsulfonyl fluoride. Cell debris was removed by centrifugation at 15,000 × *g* for 10 min at 4°C.

Detection of *H. pylori* SOD by Western immunoblot. After SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes (Millipore, Eschborn, Germany) by the method of Towbin et al. (53). The *H. pylori* SOD was detected by immunological cross-reaction with polyclonal rabbit antibodies directed against the FeSOD from *E. coli*. Polyclonal rabbit antibodies directed against the MnSOD from *E. coli* were used as a control. Both antibodies were kindly provided by D. Touati. The sera were purified by affinity chromatography, using a lysate of *E. coli* TG1 *sodA sodB* coupled to CNBr-activated Sepharose 4B (Pharmacia LKB).

N-terminal amino acid sequencing. A 20-μg amount of the purified enzyme was N-terminally sequenced by Edman degradation, using an automatic sequencer (420A, Applied Biosystems).

Electron microscopy. Immunolocalization studies on the *H. pylori* SOD were performed by using two different cell preparation methods. (i) In the negative staining method of Nermut (40), *H. pylori* cells grown in broth for 24 h were harvested and washed once with phosphate-buffered saline (PBS). Incubation of the bacteria with the polyclonal rabbit antiserum raised against *E. coli* FeSOD diluted 1:10 in PBS buffer–0.1% Triton X-100 was continued for 3 to 4 h at room temperature. After three thorough washing steps with PBS buffer, the bacteria were incubated further with goat anti-rabbit 10-nm gold conjugate (Amersham Buchler, Braunschweig, Germany) diluted 1:10 in PBS buffer–0.1% Triton X-100 for 3 to 4 h at room temperature; this was followed by a final washing procedure (three times) with PBS buffer. The organisms were adsorbed onto Formvar (Merck, Darmstadt, Germany)-coated 100-mesh copper grids (Balzers Union, Brussels, Belgium), air dried, and fixed in 1% glutaraldehyde in 100 mM cacodylate buffer (pH 7.4). The grids were negatively stained with 1% aqueous uranyl acetate.

(ii) In the preembedding immunogold technique of De-

Waele et al. (13), bacteria were prepared as described above. After the final washing step, the bacterial pellet was fixed in glutaraldehyde-osmium tetroxide for 1 h, dehydrated in ethanol, and embedded in LR White acrylic resin (Science Services, Munich, Germany), which was exchanged twice. Polymerization was allowed to occur for 24 h at 60°C, and ultrathin sections were cut with a Reichert-Jung ultramicrotome (Vienna, Austria).

All specimens were examined with a Zeiss EM 9 S 2 transmission electron microscope. To exclude unspecific binding of the gold-labeled goat anti-rabbit immunoglobulin G, the primary antibody was omitted. As a positive control for the different preparation methods, hsp60 surface protein of *H. pylori* was immunogold labeled, using an antiserum raised against the hsp60 protein of *M. tuberculosis* (14) (data not shown).

Isolation of chromosomal *H. pylori* DNA. *H. pylori* 2012 cells were cultured in broth (300 ml; 48 h), harvested, and washed twice in TEN buffer, pH 8.0 (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA, 0.1 M NaCl). The bacteria were resuspended in 10 ml of 10% sucrose solution and lysed by addition of 1 ml of 0.5 M EDTA (pH 8.0), 1 ml of lysozyme (10 mg/ml), and 0.5 ml of RNase A (2 mg/ml). After incubation for 10 min at room temperature, 0.5 ml of 2 M Tris and 1 ml of 10% SDS were added to obtain complete cell lysis. The DNA was collected by centrifugation (12,000 × *g*, 15 min, 4°C) after protein precipitation with 2.5 ml of 3 M KAc/Ac. The supernatant was extracted first with phenol-chloroform and then twice with chloroform-isoamyl alcohol. DNA was precipitated by addition of 0.5 volume of isopropanol (15 min, room temperature) and centrifuged at 12,000 × *g* for 30 min at 4°C. DNA was washed successively with 70 and 100% ethanol and finally redissolved in 200 μl of TE buffer (10 mM Tris HCl [pH 8.0], 0.1 mM EDTA). The chromosomal DNA was stored at 4°C.

Isolation of plasmid DNA. Plasmid DNA was obtained with the Quiagen Plasmid Midi Kit (Diagen GmbH, Quiagen Inc., Hilden, Germany). The isolation was performed in accordance with the manufacturer's instructions.

Cosmid cloning. A cosmid library was prepared by partial digestion of the *H. pylori* DNA with *Sau*3A, resulting in fragments of between 30 and 40 kb. These DNA fragments were ligated into the *Bam*HI-digested and alkaline phosphatase-treated cosmid vector pHC79 (26). Cosmids were packaged into phage lambda particles (Amersham Life Science) and transduced into *E. coli* HB101 (36).

Transformation of the SOD-negative *E. coli* TG1 *sodA sodB*. *E. coli* HB101 clones containing the *H. pylori* genomic fragments were cultured in LB broth supplemented with ampicillin (100 μg/ml) to an optical density at 660 nm of 0.8 to 1.0. For transformation of *E. coli* TG1 *sodA sodB*, a cosmid library was prepared. Competent bacteria were prepared by the $CaCl_2$ method (36).

Screening for *H. pylori* SOD-positive *E. coli* clones by functional complementation. For selection, SOD-negative *E. coli* cells were transformed with the *H. pylori* gene library and then subcultured under aerobic conditions on minimal medium agar supplemented with paraquat and ampicillin. Surviving *E. coli* clones were assessed for the expression of SOD by (i) activity staining in a nondenaturing PAGE and (ii) immune detection with the anti-*E. coli* FeSOD antibody.

Subcloning in pUC18. Cosmid DNA of a recombinant clone containing the *H. pylori* SOD gene inserted in pHC79 was digested by *Hin*dIII. The fragments were ligated into the *Hin*dIII-restricted and alkaline phosphatase-treated vector pUC18 (36). Selection and assessment of clones positive for *H. pylori* SOD were performed as described above.

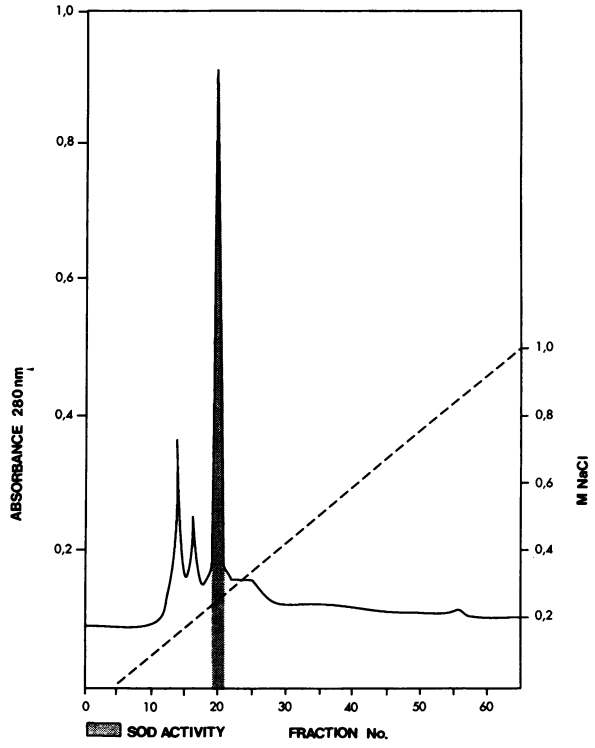


FIG. 1. Elution profile of AEC II at pH 9.5 and corresponding SOD activity. The solid line represents the A_{280} wavelength. The dashed line indicates the increasing NaCl concentration.

Sequencing of the *H. pylori* SOD gene. The nucleotide sequence was determined by the dideoxy chain termination method (47), using an automatic fluorescence sequencer. M13 reverse primer and M13 universal primer were purchased from Pharmacia LKB. Additional oligodeoxyribonucleotide primers in both directions were synthesized on an Applied Biosystems DNA synthesizer, model 394.

Alignment. The nucleotide sequence was aligned to all gene data bases available in the EMBL Nucleotide Sequence Data Library, Heidelberg, Germany, using the FASTA software program (45).

Nucleotide sequence accession number. The nucleotide sequence presented here appears in the EMBL Nucleotide Sequence Data Library under accession number X72618.

RESULTS

Isolation and purification of SOD from *H. pylori*. Cells (*H. pylori* 2012) were lysed by sonication and subjected to differential centrifugation. The resulting supernatant was applied to a gel filtration column. SOD-active fractions were purified further by successive AEC at two different pH levels. The elution profile of the second AEC at pH 9.5 revealed one major protein peak at 240 mM NaCl corresponding to the activity peak of the SOD (Fig. 1). The major SOD-active fraction (fraction 19) showed one single protein band with an apparent molecular weight of 24,000 when analyzed in a reducing (Fig. 2) as well as in a nonreducing (data not shown) SDS-PAGE.

A 624-μg amount of the purified enzyme (0.1% of the initial protein) was recovered from 5.64 g of bacteria (wet

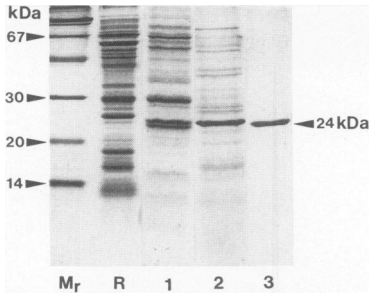


FIG. 2. SDS-PAGE of *H. pylori* SOD at various stages of purification. M_r, apparent molecular mass standard proteins. Lane R, crude cell extract, 15 μg of protein; lane 1, SOD-active fraction after size exclusion chromatography, 15 μg of protein; lane 2, SOD-active fraction after AEC I (pH 7.5), 10 μg of protein; lane 3, SOD-active fraction after AEC II (pH 9.5), 5 μg of protein.

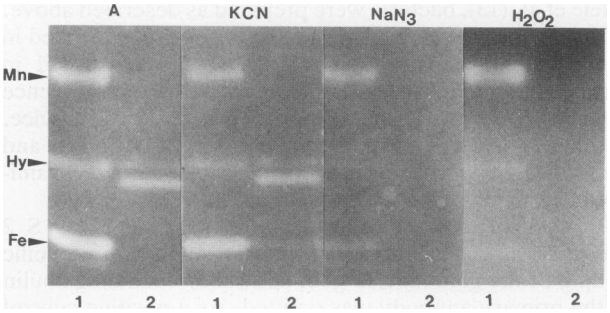


FIG. 3. Determination of the metal cofactor of *H. pylori* SOD by inhibition studies performed in nondenaturing PAGE. SOD activity is visible as achromatic zones on a uniformly dark background. Mn, MnSOD; Hy, hybrid molecule; Fe, FeSOD. Lane 1, *E. coli* TG1 crude cell extract; lane 2, *H. pylori* crude cell extract. There was 50 μg of protein per lane; A, control strip incubated in potassium phosphate buffer without the addition of an inhibitory agent.

weight corresponding to 600 mg of protein). By purification, the specific enzyme activity increased 200-fold from 8.9 to 1,780 U/mg (Table 1).

Characterization of the *H. pylori* SOD. The apparent molecular weight of the purified enzyme was not altered by SDS-PAGE under nonreducing conditions. A nondenaturing PAGE, stained for SOD activity, revealed that, under given growth conditions, *H. pylori* has no isoenzymes. In contrast, *E. coli* expresses a manganese, an iron, and an active hybrid enzyme under aerobic conditions (Fig. 3). To determine the metal cofactor of the *H. pylori* SOD, studies with inhibitors with known effects on SODs containing various cofactors (Cu/Zn, Mn, or Fe) were performed (18). Cyanide, which is known to inhibit Cu/ZnSOD, had no influence on the activity of *H. pylori* SOD, whereas azide and hydrogen peroxide both inhibited SOD activity from *H. pylori* as well as activity of FeSOD from *E. coli*; thus, iron seems to be a metal cofactor of *H. pylori* SOD (Fig. 3).

This observation was supported further by the positive immune reaction with anti-*E. coli* FeSOD antibodies. No reactions were observed with anti-*E. coli* MnSOD antibodies (Fig. 4). The native molecular weight of the enzyme was determined to be 50,000 by size exclusion chromatography, using a Superose 12 column (data not shown), indicating a dimeric structure of the active protein. Since no differences in apparent molecular weight due to a reducing agent were observed, the subunits do not appear to be covalently linked by disulfide bridges, although two cysteine residues are present in the deduced protein sequence. The N-terminal amino acid sequencing of the first 30 amino acids of the SOD from *H. pylori* further confirmed the homogeneity of the protein preparation.

Electron microscopy. *H. pylori* cells were incubated with the polyclonal anti-*E. coli* FeSOD antibody. Bound antibody was detected by an indirect immunogold technique. Neither preparation method allows intracellular detection of the antigen. Gold particles were distributed on the cell envelope of *H. pylori* (Fig. 5b) and on surface-exposed material or cell envelope fragments derived from water-treated organisms (Fig. 5a). Binding of some gold particles was also detectable on the sheath of the flagella (Fig. 5c).

Labeling of the SOD antigen did not occur in every cell of the sample studied. The intracellular localization of the SOD was investigated on ultrathin sections of the bacteria which were subsequently subjected to immunogold labeling. In contrast to the limited number of bacteria that showed extracellular labeling, the enzyme was intracellularly detectable in most of the cells (data not shown).

Cloning the gene. A gene library of *H. pylori* DNA was constructed in the cosmid vector pHC79, packaged into lambda phages, and used for transduction of *E. coli* HB101. The SOD-negative *E. coli* strain was transformed with a cosmid preparation of more than 400 *E. coli* HB101 clones statistically representing the entire *H. pylori* genome. After 48 h of incubation of the transformed cells on minimal medium agar plates supplemented with paraquat and ampicillin, recombinant *E. coli* clones were isolated and subcultured further on paraquat-containing agar plates. Eight recombinant clones were analyzed for expression of the *H.*

TABLE 1. Purification schedule of *H. pylori* SOD

Step	Vol (ml)	Protein (mg)	Yield (%)	Sp act (U/mg)	Purification factor (fold)
Crude extract	20	600.0	100	8.9	1
Soluble extract	16	330.0	55	12.1	1.4
Gel filtration	58	22.6	3.8	158.9	18
AEC I, pH 7.5	12	1.7	0.3	578.5	65
AEC II, pH 9.5	16	0.62	0.1	1,780	200

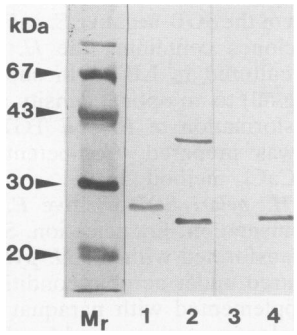


FIG. 4. Immunologic cross-reactivity of *H. pylori* FeSOD with polyclonal rabbit antisera raised against FeSOD from *E. coli* (lane 1), indicating antigenic similarity. No reaction was detectable with the polyclonal antisera raised against the MnSOD from *E. coli* (lane 3). Lanes 1 and 3, *H. pylori* crude cell extract, 10 μg of protein. Lanes 2 and 4, *E. coli* TG1 crude cell extract, 10 μg of protein. M_r, apparent molecular mass standard proteins.

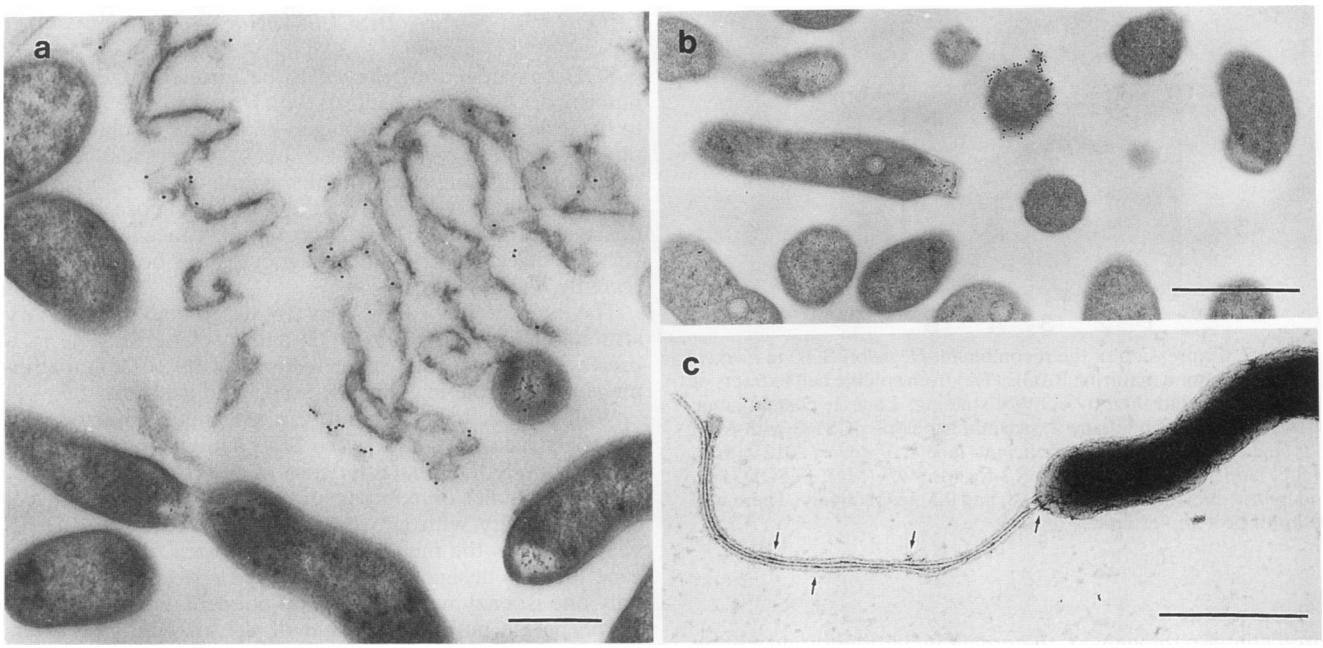


FIG. 5. Indirect immunogold labeling of SOD with anti-*E. coli* FeSOD. (a and b) Ultrathin sections of *H. pylori*: gold particles demonstrating SOD antigen are visible on the surface of the bacteria (b), as well as on surface-exposed material or cell envelope fragments (a) derived from water-treated bacteria. Bars, (a) 0.5 μm; (b) 1 μm. Intracellular SOD antigen is not detected by this preembedding preparation method. (c) Negative staining: labeling of the SOD antigen occurs also at the sheath of the flagella (arrows). Bar, 1 μm.

pylori SOD. By nondenaturing PAGE and activity staining, one single SOD activity with an R_f value of 0.6 could be detected in each of the eight recombinant *E. coli* clones comigrating with the native *H. pylori* SOD. No *E. coli sodA* or *sodB* activity (R_f values, 0.3 and 0.8, respectively) in the recombinant *E. coli* clones was detectable. Immunoblot analysis of the recombinant clones with anti-*E. coli* FeSOD antibodies confirmed the expression of the *H. pylori* SOD in *E. coli* TG1 *sodA sodB*. Cosmid clone pCS335, exhibiting the highest expression of SOD, was chosen for subcloning in pUC18 in order to sequence the gene.

After transformation of *E. coli* TG1 *sodA sodB* and selection on paraquat-containing agar plates, clone pCS188 was isolated and analyzed. The Western immunoblot of this recombinant clone lysate showed immune reactions with anti-*E. coli* FeSOD antibodies with a protein band corresponding to that of the native *H. pylori* SOD (Fig. 6). A nondenaturing PAGE of this recombinant clone revealed a SOD-active protein band which comigrated with neither the *H. pylori* SOD nor the *E. coli* SODs (Fig. 7). The SODs could be easily differentiated from each other by their migration behavior. The R_f values determined in a 7% polyacrylamide gel system were as follows (Fig. 7): 0.3 (MnSOD, *E. coli*), 0.5 (hybrid enzyme, *E. coli*), 0.6 (*H. pylori* SOD), 0.7 (*E. coli* pCS188), and 0.8 (FeSOD, *E. coli*).

Sequencing the gene. A total of 1,587 bases representing the whole insert of pCS188 were sequenced on both strands. An open reading frame of 642 bases coding for a 214-residue protein with a predicted molecular weight of 24,800 was identified. This molecular weight corresponded well to the apparent molecular weight of the native protein (24,000) determined by SDS-PAGE. The calculated pI of the deduced protein sequence was 6.0. The nucleotide sequence, including flanking regions, is shown in Fig. 8. The structural gene has one ATG initiation codon at position 915 and two successive stop codons at position 1557. Upstream of the start codon, a ribosome-binding site with homology to the Shine-Dalgarno consensus sequence of *E. coli* is present. The regions with the closest homology to *E. coli* promoter sequences (σ^{70}) are marked in Fig. 8 (37). The G+C content of the insert is 36.7 mol%, corresponding to the G+C content (36 to 37 mol%) of the entire *H. pylori* genome (7). Analysis of the gene sequence revealed no leader sequence.

Alignments of the *H. pylori* SOD nucleotide sequence with nucleotide sequences of other SODs revealed a high degree of homology with SODs of the following predominantly

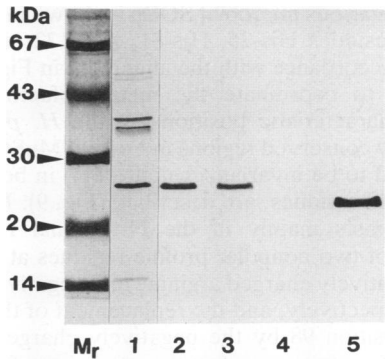


FIG. 6. Immunological cross-reactivity of the recombinant *H. pylori* SOD with polyclonal rabbit FeSOD antibodies: M_r, apparent molecular mass standard proteins. Lane 1, cosmid clone pCS335, 10 μg of protein; lane 2, plasmid subclone pCS188, 8 μg of protein; lane 3, *H. pylori* 2012, 8 μg of protein; lane 4, *E. coli* TG1 SOD deficient, 8 μg of protein; lane 5, *E. coli* TG1, 8 μg of protein.

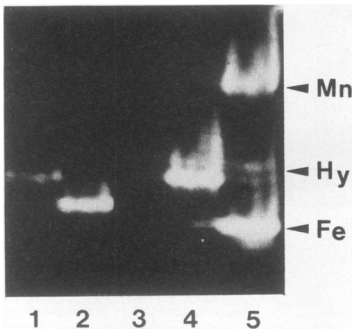


FIG. 7. Expression of the recombinant *H. pylori* SOD in *E. coli*: detection by nondenaturing PAGE (7%) from soluble cell extracts of *E. coli* clones and then by activity staining. Lane 1, cosmid clone pCS335 with R_f of 0.6; lane 2, plasmid subclone pCS188 with R_f of 0.7; lane 3, *E. coli* TG1 SOD deficient; lane 4, *H. pylori* 2012 with R_f of 0.6; lane 5, *E. coli* TG1 with R_fs for MnSOD (Mn), FeSOD (Fe), and hybrid enzyme (Hy) of 0.3, 0.8, and 0.5, respectively. There was 50 μg of protein per lane.

intracellular pathogens: *L. ivanovii* (56.5% identity in 439-bp overlap), *L. monocytogenes* (56.3% identity in 430-bp overlap), *Coxiella burnetti* (58.3% identity in 566-bp overlap), *P. gingivalis* (55.3% identity in 627-bp overlap), *Legionella pneumophila* (58.9% identity in 572-bp overlap), and *Entamoeba histolytica* (54.6% identity in 560-bp overlap).

Alignment of the deduced amino acid sequence. The first deduced 30 amino acids correspond to the 30 amino acid residues determined by N-terminal sequencing of the purified native enzyme. Comparison of the complete predicted amino acid sequence with the Swiss protein sequence data bases (EMBL Sequence Data Library) showed a close homology with *E. coli* FeSOD (53.5%), *C. burnetti* FeSOD (48.9%), *Legionella pneumophila* FeSOD (49.2%), *Pseudomonas ovalis* FeSOD (46.2%), and *P. gingivalis* FeSOD (45.3%). The degree of homology decreases when compared with MnSODs, namely, 31.1% (*E. coli* MnSOD), 31.5% (*L. ivanovii*), and 36.9% (*Bacillus stearothermophilus*). The degree of homology with the FeSOD of *M. tuberculosis* amounts to only 23.2%, but this FeSOD has more resemblance to an MnSOD in structure. The alignment of the deduced amino acid sequence of the *H. pylori* SOD with sequences of various microbial SODs is shown in Fig. 9. The amino acid residues His-26, His-81, Asp-172, and His-176 (numbers in accordance with the alignment in Fig. 9), which are believed to coordinate the metal cofactor (44), are present in characteristic positions in the *H. pylori* SOD. Several highly conserved regions in Fe- and MnSODs, which are postulated to be invariant residues (44) in both position and amino acid residues, are described (Fig. 9). Remarkable divergence exists mainly in the N-terminal region. The replacement of two nonpolar proline residues at positions 5 and 16 by positively charged arginine and negatively charged aspartate, respectively, and the replacement of the nonpolar glycine at position 98 by the negatively charged aspartate could have consequences for the structure, folding, and function of the protein. Four of five amino acid residues (Ala-176, Gln-177, Tyr-184, and Ala-151), which are primary candidates to discriminate between the iron and manganese proteins (44), clearly designate the *H. pylori* SOD as an FeSOD (Fig. 9).

DISCUSSION

SOD enzymes are believed to be virulence factors in a number of pathogenic bacteria which mediate the resistance to bactericidal attacks by oxygen-dependent mechanisms of phagocytes. *H. pylori* is also likely to possess evasion mechanisms against neutrophilic granulocytes, since it is obviously able to persist on the gastric mucosa despite a dense infiltration by PMNs. A putative intracellular in vitro survival of *H. pylori* within phagocytes has been discussed recently (2, 31).

The aim of our study was to determine the primary structure of the *H. pylori* SOD and to clone its gene as a prerequisite for further investigations of its possible pathophysiological role in *H. pylori*-associated gastritis.

With respect to its subunit size, pI, metal cofactor, and dimeric structure, the *H. pylori* SOD appears to be a typical procaryotic SOD. Characteristics of the primary structure, inhibition studies on nondenaturing gels, and immunological cross-reactivity with antisera raised to *E. coli* FeSOD revealed iron as the metal cofactor; therefore, the respective gene locus is designated *sodB*. *H. pylori* seems to produce only one isoenzyme, as shown on nondenaturing gels.

The native molecular weight of the enzyme was determined by size exclusion chromatography to be 50,000, indicating a homodimeric structure with a subunit size of approximately 24,000. This value is in the range of those of other procaryotic SODs (3, 18). Despite the presence of cysteine residues in the protein sequence, subunits are not covalently linked by disulfide bridges, as has been shown also for other microbial SODs (3). Due to a very tight noncovalent subunit binding, native monomers have never been found (3).

The N-terminal amino acid sequence of the purified native protein corresponds exactly to the deduced amino acid sequence, demonstrating the homogeneity of the purified protein and indicating also that no maturation or modification of the N-terminal region is functionally necessary.

To date, the following *H. pylori* genes have been cloned and sequenced: the urease gene cluster (9, 32), a gene encoding a 26,000-Da protein (43), the catalase gene (41), two flagella genes (*flaA* [35] and *flaB* [51]), an adhesin subunit gene (15), the ferritin gene (17), and a cytotoxin-associated protein gene (10, 54).

Functional expression of *H. pylori* genes was reported for the first time by Labigne and coworkers, who were able to clone and express the urease genes in *Campylobacter jejuni* by using a modified shuttle plasmid originally constructed for *C. jejuni* (32, 33). The authors found transcription and translation of the *H. pylori* urease genes in *E. coli* minicells but were unable to detect urease activity. However, Cussac and coworkers recently succeeded in inducing phenotypical expression of the *H. pylori* urease genes in *E. coli* under nitrogen-limiting conditions (11). Expression of the *H. pylori* SOD in a SOD-deficient *E. coli* background resulted in complementation in the presence of paraquat, which induces intracellular oxidative stress conditions. As a consequence, one can conclude that an active *H. pylori* enzyme with sufficient activity for complementation was synthesized in *E. coli*. Clones harboring the SOD gene, which is inserted in pHC79 vectors, expressed a SOD protein which was identical to the native *H. pylori* SOD with respect to relative molecular weight, immune cross-reactivity, and migration behavior in nondenaturing gels. Subcloning of these inserts in pUC18 resulted in an active recombinant protein with unaltered relative molecular weight and antigenic cross-

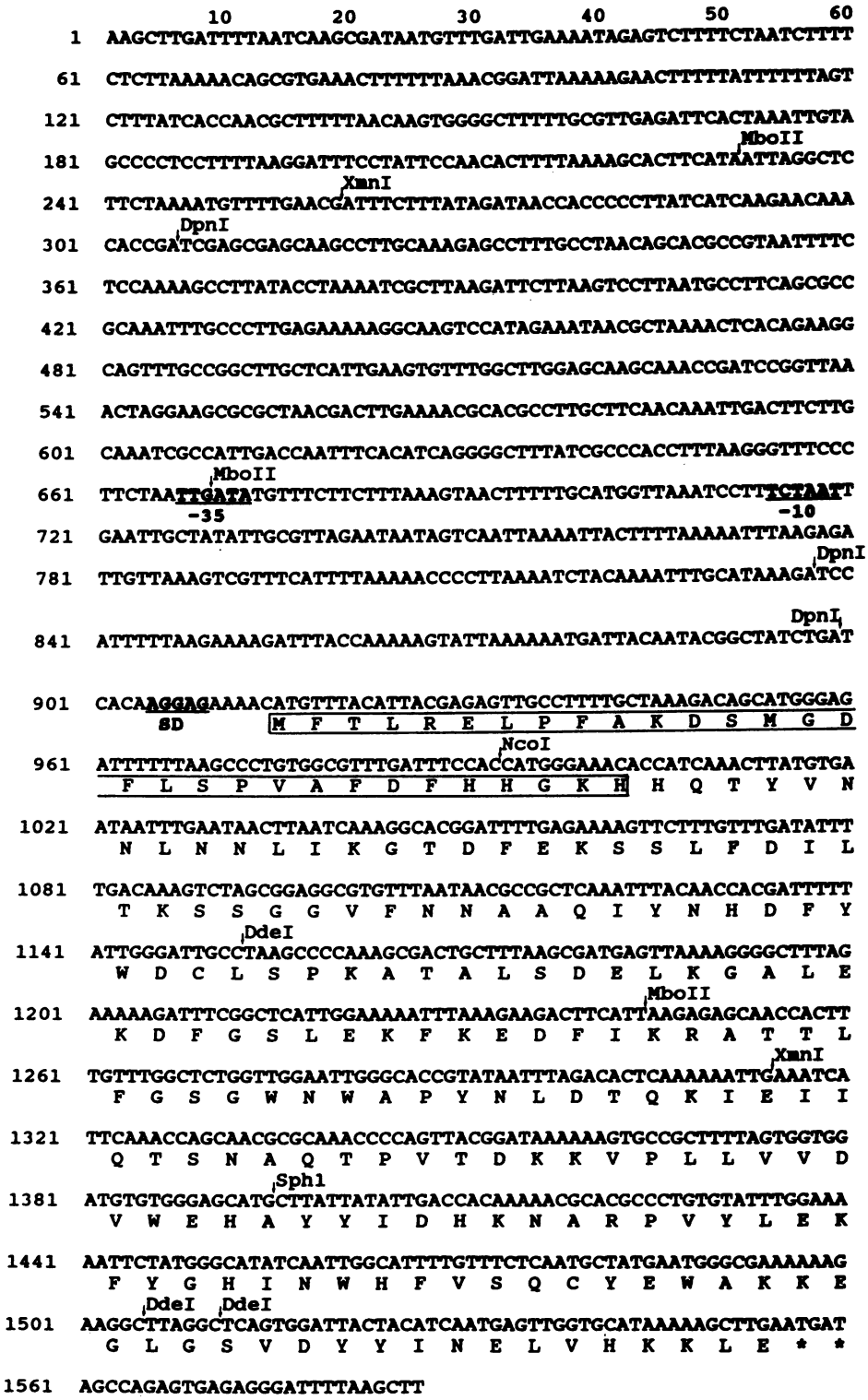
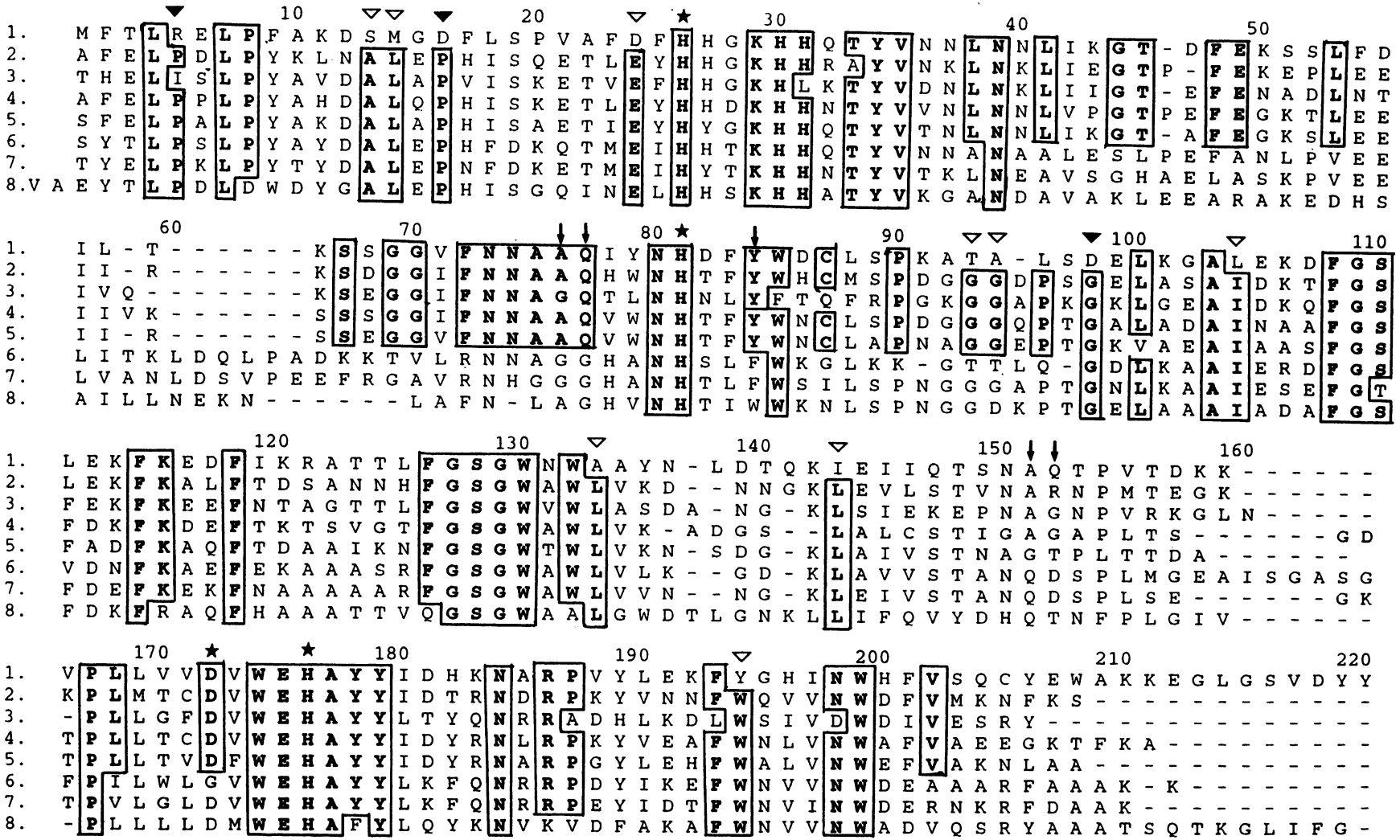


FIG. 8. Nucleotide and deduced amino acid sequences of the *H. pylori* SOD gene, including upstream and downstream flanking regions. The structural gene for SOD (642 bp) begins with an ATG start codon at position 915 and ends with two stop codons at positions 1557 and 1560 (asterisks) encoding a protein of 214 amino acids. The *Hin*dIII site at nucleotide 1548 is believed to be the result of religation of the small fragment (nucleotides 1548 to 1557) to the major part of the gene. Possible −10 and −35 promoter regions and a putative Shine-Dalgarno (SD) sequence are boldfaced and underlined. Boxed amino acid residues indicate the amino-terminal sequence of the purified enzyme determined by protein sequencing. Restriction sites of some endonucleases are indicated (arrows).



1. *Helicobacter pylori* (FeSOD)
2. *Coxiella burnetii* (FeSOD)
3. *Porphyromonas gingivalis* (FeSOD)
4. *Pseudomonas ovalis* (FeSOD)
5. *Escherichia coli* (FeSOD)
6. *Escherichia coli* (MnSOD)
7. *Listeria ivanovii* (MnSOD)
8. *Mycobacterium tuberculosis* (FeSOD)

FIG. 9. Comparison of amino acid sequences of *H. pylori* SOD and the SODs of *C. burnetti* (25), *B. gingivalis* (39), *P. ovalis* (27), *L. ivanovii* (21), and *M. tuberculosis* (56) plus *E. coli* FeSOD (48) and *E. coli* MnSOD (50). Homologous amino acids with FeSOD-specific postulated invariant residues (44) are boldfaced and boxed. Asterisks indicate ligands to the metal cofactor. Primary candidates for discriminating Mn- and FeSODs are identified by closed arrows. Open triangles mark those amino acids of the *H. pylori* SOD which differ from postulated invariant residues (44), and closed triangles point to those differing invariant residues which are exchanged by a distinctly charged amino acid.

reactivity but with a distinct migration behavior in the nondenaturing PAGE when compared with the native protein. The higher R_f value observed might reflect a modification concerning charge and/or folding of the protein, resulting in a more rapid migration in the native gel. A mutation of the *E. coli* SOD genes back to functional proteins can be excluded by exhibition of different R_f values and the presence of the two resistance markers, each of them interrupting one *E. coli* SOD gene (*sodA* or *sodB*). This phenomenon obviously cannot be due to the expression in *E. coli*, because we used the same strain for transformation of pHC79 and pUC18. It is not likely that parts of the gene have been lost, because a start codon with a strong Shine-Dalgarno sequence in the 5′ noncoding region could be identified. In addition, we found two stop codons and good agreement between the calculated molecular weight of the deduced 214 amino acids and the subunit size of the native protein. The reason for the unusual migration behavior mentioned above in the native PAGE remains unclear.

The distance between the putative homologous promoter sequences (−10 and −35 positions) seems to be quite extended (41 bp). For comparison, the distances between the promoterlike structures in other *H. pylori* genes amount to 24 (*ureC*) and 29 (*hpaA*) bp (15, 32), whereas the *E. coli* −10 and −35 promoter sequences usually are separated by 17 bp. Therefore, it is not yet clear whether the presumed structures really possess promoterlike functions. The *Hin*dIII site at position 1,548 of the nucleotide sequence derives presumably from a religation process of a small gene fragment (nucleotides 1,548 to 1,562) to the major part of the gene. Since it has been selected for an active protein, only such clones that harbored the complete gene sequence could be isolated.

Expression of SOD would be more effective as a defense mechanism against phagocyte-derived reactive oxygen species if the SOD was a surface-located or actively secreted enzyme, since superoxide anions are not able to penetrate biological membranes. The immunolocalization studies of the SOD on the ultrastructural level demonstrated that the *H. pylori* SOD is, indeed, located on the surface. It seems even to be attached to the flagellar sheath. However, the expression on the surface appears to be dependent on a certain stage of bacterial growth, since not all of the cells were labeled. This finding is in accordance with the distribution of the surface-exposed hsp60 protein of *H. pylori* (14), used as control, which was also not detectable on every cell.

Interestingly, the *H. pylori* SOD lacks a leader peptide, which is also absent in the secreted urease (24, 32). Therefore, alternative extracellular transport mechanisms must exist. It has been suggested that chaperonins may be responsible for secretion of the urease (14). In this context, the high divergence in the N-terminal region of the *H. pylori* SOD in comparison with other SODs would be of interest: in a comparable analysis of the primary structure of Mn- and FeSODs, Parker and Blake found a high conservation in the amino-terminal region of microbial SODs, presumably from amino acids 1 to 19. This region is involved in the cellular protein transport (44). These workers also found that the positioning of proline and glycine residues is crucial for the development of the tertiary structure of SODs (44). The *H. pylori* SOD primary structure shows three amino acid exchanges with possible biological consequences: two proline and one glycine residue at postulated invariant positions are replaced by charged amino acids. The marked difference between the N-terminal region of the *H. pylori* SOD and that of other microbial SODs could be due to an adaptation to a different protein transport mechanism or to an increasing variability after a decrease in selective pressure. The effect of these important amino acid exchanges on structure, folding, function, and cellular transport of the enzyme remains undetermined.

Computer analysis of the nucleic acid and amino acid sequences of *H. pylori* SOD revealed the greatest homology to facultative intracellular pathogenic microorganisms. This probably indicates a pathogenic role of the *H. pylori* SOD and gives further evidence for its role in resistance to phagocytic attack and killing. Concerning *H. pylori* and defense mechanisms against in vivo killing by PMNs, the pathophysiological role of the SOD must be elucidated in further studies. Therefore, the construction of a SOD-deficient isogenic *H. pylori* mutant and the study of its ability to infect and resist destruction by PMNs will be necessary to answer this question.

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