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Copper-Zinc Superoxide Dismutase of *Haemophilus influenzae* and *H. parainfluenzae*

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Copper-zinc superoxide dismutase ([Cu,Zn]-SOD) is widely found in eukaryotes but has only rarely been identified in bacteria. Here we describe *sodC*, encoding [Cu,Zn]-SOD in *Haemophilus influenzae* and *H. parainfluenzae*, frequent colonists and pathogens of the human respiratory tract. In capsulate *H. influenzae*, *sodC* was found in only one division of the bacterial population, and although the protein it encoded was clearly [Cu,Zn]-SOD from its deduced sequence, it lacked enzymatic activity. In *H. parainfluenzae*, in contrast, active enzyme was synthesized which appeared to be secreted beyond the cytoplasm when the gene was expressed in *Escherichia coli* minicells. The origin of gene transcription differed between the *Haemophilus* species, but protein synthesis from cloned genes in vitro was comparable. A C-T transition was found in the *H. influenzae* sequence compared with the *H. parainfluenzae* sequence, leading to a histidine, known to be crucial in eukaryotic [Cu,Zn]-SOD for copper ion coordination and so for enzymatic activity, to be changed to tyrosine. This is speculated to be the cause of inactivity of the *H. influenzae* enzyme. Secreted SODs have only been described in a few bacterial species, and this is the first identification of [Cu,Zn]-SOD in a common human upper respiratory tract colonist. The role of secreted bacterial SODs is unknown, and we speculate that in *Haemophilus* species the enzyme may confer survival advantage by accelerating dismutation of superoxide of environmental origin to hydrogen peroxide, disruptive to the normal mucociliary clearance process in the host.

Superoxide radicals are produced within bacterial cells as oxygen is consumed, and superoxide dismutases (SODs; EC 1.15.1.1), metalloenzymes that play a key role in the enzymatic defense against oxygen toxicity, are correspondingly almost universally distributed (13, 18). Three common forms of the enzyme are known, differing in the metal ion cofactor at the active site. Manganese-containing enzymes ([Mn]-SOD) are found widely in both bacteria and mitochondria (14), while enzymes containing iron ([Fe]-SOD) are found in the cytosols of prokaryotes, in primitive eukaryotes, and in some green plants (52). These SODs are very similar in protein sequence and structure (14, 36), suggesting that they have evolved from a common ancestor. In contrast, enzymes containing copper and zinc ([Cu,Zn]-SODs) show no sequence or structural resemblance to [Mn]-SOD or [Fe]-SOD and appear to have arisen independently. [Cu,Zn]-SODs have been found almost exclusively in the cytosol of eukaryotes, leading to early suggestions that the eukaryotic and prokaryotic evolutionary paths to SOD were entirely distinct and that the first bacterial [Cu,Zn]-SOD described, from the endosymbiotic organism *Photobacterium leiognathi* (37), might have originated by gene transfer from the organism's ponyfish host. This proposition has seemed less likely with the identification of [Cu,Zn]-SODs in a further small number of free-living bacteria: *Caulobacter crescentus* (44), *Paracoccus denitrificans* (53), and *Pseudomonas maltophilia* and *Pseudomonas diminuta* (46). This suggests rather that, in those organisms in which it is found, [Cu,Zn]-SOD plays a role distinct from that of the conventional prokaryotic [Fe]-SOD or [Mn]-SOD that they also contain. Such a role remains to be defined, although evidence in some cases that the enzyme is secreted (47, 48) suggests that it is involved with the dismutation of exogenously rather than endogenously produced superoxide. The recent identification of another secreted bacterial [Cu,Zn]-SOD, in the intracellular pathogen *Brucella abortus* (6), has prompted speculation that here the enzyme might play a part in resistance to bactericidal oxygen radicals generated by the host in the phagolysosome.

While characterizing capsulation (*cap*) genes in *Haemophilus influenzae* type b NCTC8468 (25, 27), we found the 5′ end of an open reading frame adjacent to *cap* which apparently encoded a [Cu,Zn]-SOD. We report here the cloning, characterization, and expression of genes encoding [Cu,Zn]-SOD in *H. influenzae* and *H. parainfluenzae* and speculate on the part that the enzyme may play in the host-parasite relationship between humans and such common commensals of the upper respiratory tract.

MATERIALS AND METHODS

Bacterial strains used: their growth, transformation, and storage. (i) *Haemophilus* strains. *H. influenzae* type b NCTC8468 was obtained from the U.K. National Type Culture Collection. Other *H. influenzae* strains identified in the text, and *H. parainfluenzae* 1391, are clinical isolates from the collections of the Department of Paediatrics and the Public Health Laboratory, John Radcliffe Hospital, Oxford, United Kingdom. Strains were grown in brain heart infusion broth, supplemented with 2 μg of NAD per ml and 10 μg of hemin per ml. Brain heart infusion plates were prepared with 1% agar supplemented with 10% Levinthal base (1). Strains were stored at −80°C in broth after addition of glycerol to 20%.

H. parainfluenzae 1391 was made competent for DNA uptake by growth in supplemented brain heart infusion broth and then by incubation in MIV medium (20) prior to transformation with linearized plasmid encoding a kanamycin resistance gene ligated into *Haemophilus* DNA. Transfor-

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mants were identified by their resistance to kanamycin at a concentration of 10 μg/ml in brain heart infusion plates.

(ii) *Escherichia coli* strains. *E. coli* DH5α (16) was used to propagate constructions in plasmid pUC13. The *sodA sodB* mutant QC779 (34) was kindly provided by Danièle Touati. *E. coli* AA10, a *recA* mutant of the minicell strain P678-54 kindly provided by Staffan Normark, was used for the preparation of minicells. Strains were propagated in Luria broth and stored at −80°C after addition of glycerol to 20%. Standard techniques were used for transformation (30).

Recombinant DNA methods. Total cellular DNA was prepared from 3-ml broth cultures of *Haemophilus* strains as previously described (32). Standard methods were used for restriction digestion, Southern blotting, preparation of plasmid DNA, colony hybridization, and 5′ end labeling of oligonucleotides (30). Southern blots were probed to ~80% stringency in 0.015 M NaCl–0.0015 M sodium citrate–0.1% sodium dodecyl sulfate (SDS) at 45°C for 1 h with three changes of buffer prior to autoradiography done at −70°C. A gene cartridge encoding aminoglycoside phosphotransferase conferring kanamycin resistance, derived from Tn*903* (35) and located on a blunt-ended *Hin*cII DNA fragment, was used for site-directed mutagenesis.

Cloning the *H. parainfluenzae* [Cu,Zn]-SOD gene. Fragments of *Eco*RI-digested chromosomal DNA of strain 1391 between 4 and 7 kb in size were purified from an agarose gel by using Geneclean (Stratech Scientific Ltd.) and ligated to pUC13 linearized with *Eco*RI, and the products of the reaction were used to transform competent *E. coli* DH5α. Recombinants were distinguished by their failure to confer the ability to generate a blue colony in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside and isopropyl-β-D-thiogalactopyranoside. Transformants were picked to an array, and a colony blot was probed with the 360-nucleotide *Hin*dIII-*Nco*I fragment from *sodC* (Fig. 1) to identify clones bearing the *H. parainfluenzae sodC*. pJSK130 and pJSK131, containing the same 5.2-kb *Eco*RI fragment in opposite orientations, were selected for further study.

Nucleotide sequence determination. Nucleotide sequences were determined by the dideoxy chain termination method (41) with denatured plasmid templates (21). [α-^{35}S]dATP was used to label the growing strand. The highly processive modified T7 DNA polymerase Sequenase (U.S. Biochemical Corp.) was used with a sequencing kit according to the manufacturer's instructions. Oligonucleotide primers were the universal forward and reverse sequencing primers (New England Biolabs, CP Laboratories, Bishops Stortford, United Kingdom), and oligonucleotides were prepared with a model 380B DNA synthesizer (Applied Biosystems).

In vitro protein synthesis in *E. coli* minicells. Minicells harboring the appropriate plasmid were isolated essentially by the method of Thompson and Achtman (51) and labeled with [^{35}S]methionine (Amersham). Approximately 300,000 cpm of trichloroacetic acid-acetone-precipitable material was separated electrophoretically in SDS-12.5% polyacrylamide gels. Standard proteins with molecular masses of 14.2 to 66 kDa (Sigma) were run in parallel as markers. Gels were fixed, stained with Coomassie blue to visualize proteins, infiltrated with Amplify (Amersham), dried down on to filter paper, and examined by fluorography.

Periplasmic proteins were extracted from 100 μl of minicell suspension (19) and extracted, and residual proteins were visualized after 12.5% SDS-polyacrylamide gel electrophoresis (PAGE) by silver staining, using a kit purchased from Amersham International plc.

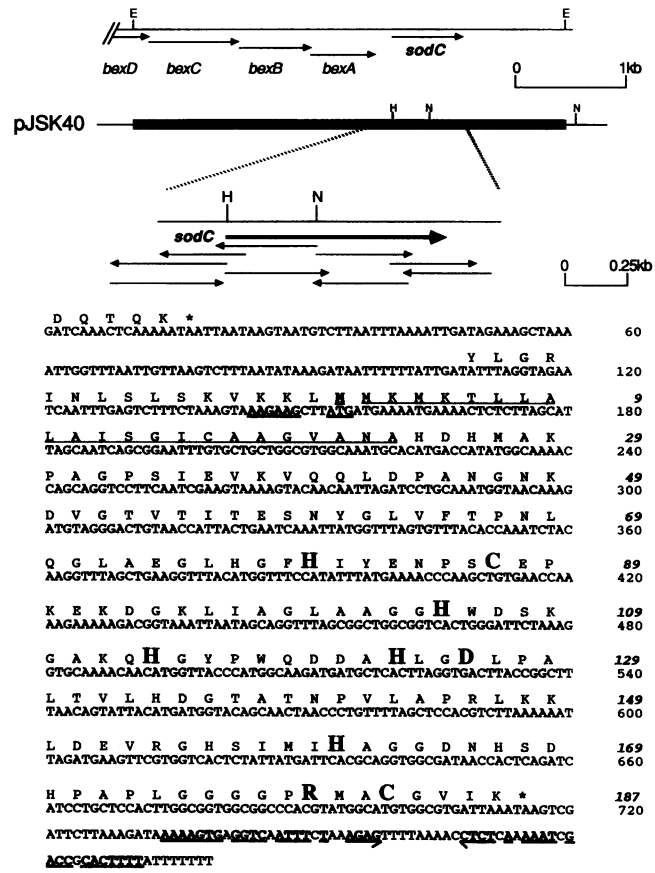


FIG. 1. [Cu,Zn]-SOD gene *sodC* from *H. influenzae* type b NCTC8468. Top: Chromosomal location of *sodC*, downstream of the four *bex* genes of the *cap* locus. The *Eco*RI fragment cloned as pJSK40 is indicated by the bar, with vector DNA as the thin line. Restriction sites are *Eco*RI (E), *Hin*dIII (H), and *Nco*I (N). The sequencing strategy is shown, and thin arrows represent the extent of DNA sequenced in the direction indicated. Bottom: Nucleotide sequence of *sodC* and flanking regions including the 3′ end of *bexA*, and translated protein sequence of the open reading frame (single-letter code). In the DNA sequence, the putative Shine-Dalgarno motif, start codon, and stem-loop rho-independent terminator are underlined. In the protein sequence, the putative N-terminal methionine (M) and leader peptide are underlined. Histidine (H), cysteine (C), aspartate (D), and arginine (R) residues that are highly conserved in [Cu,Zn]-SOD are enlarged. Nucleotide numbering is arbitrary from the beginning of the sequence presented. Amino acid numbering starts with the N-terminal methionine of *sodC*.

Isolation of RNA and primer extension analysis. Total cellular RNA was prepared from 80-ml cultures of exponentially growing cells (31). RNA quality was assessed by electrophoresis in 0.7% agarose gels with and without prior treatment with RNase. RNA was used as a template for the synthesis of cDNA products from 5′-end-labeled synthetic oligonucleotide primers. Primer extension was performed with unlabeled deoxyribonucleotides and avian myeloblastosis virus reverse transcriptase (39). The products of the reaction were visualized by autoradiography after separation in 6% urea-polyacrylamide gels. When unlabeled oligonucleotide primers were used, the products were separated in alkaline 0.7% agarose gels (30) and subjected to the conventional Southern blotting procedure.

Extraction of bacterial cell proteins, gel electrophoresis, and

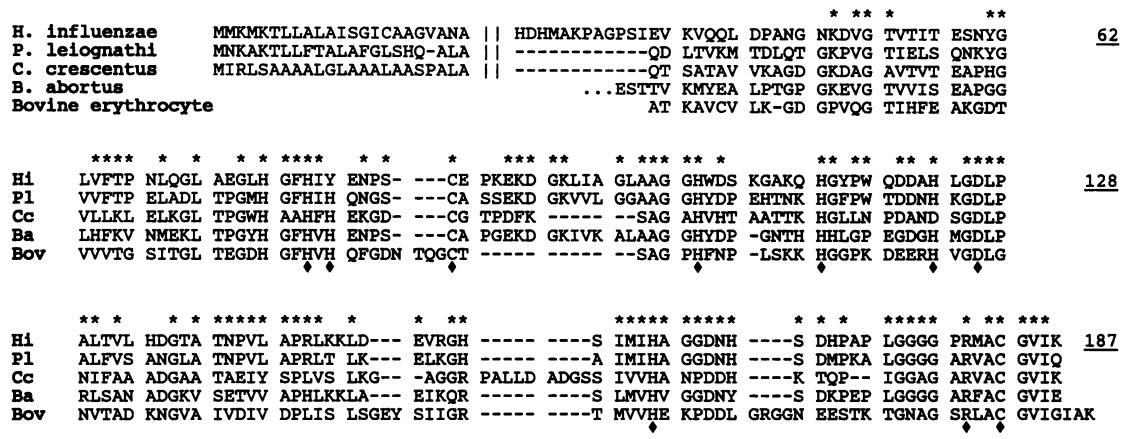


FIG. 2. Amino acid sequence alignment of bacterial and bovine erythrocyte [Cu,Zn]-SODs. Amino acids in the *H. influenzae* sequence are numbered at the end of each line. Leader peptides (where present and of known sequence) are shown: || separates leader peptide from mature [Cu,Zn]-SOD in *Photobacterium leiognathi* and *C. crescentus*, and as proposed in *H. influenzae*. Gaps and insertions are placed to align residues known to be of structural and functional importance in bovine erythrocyte [Cu,Zn]-SOD, indicated by a diamond below the sequence. Residues identical in *H. influenzae* and *Photobacterium leiognathi* are indicated by an asterisk (*). Non-*H. influenzae* sequences are from references 6, 47, and 48.

detection of SOD. The cell pellet from 25-ml exponentially growing aerobic cultures was frozen and thawed and then suspended in 1 ml of 50 mM Tris (pH 7.8)–25 mM benzamidine, sonicated for 1 min on ice, and centrifuged (13,500 × *g* for 10 min), and the supernatant was used directly or stored at −20°C. Protein concentration was measured by using bovine serum albumin as a standard (7, 49).

PAGE conditions were 4.5% stacking gel (pH 8.3), 10% separating gel (pH 8.9), and the buffer system of Davis (11) except that the pH of the upper buffer was raised to 8.9 with 10 M NaOH.

SOD activity in polyacrylamide gels was visualized by the method of Beauchamp and Fridovich (5) as modified by Steinman (46). When used as an inhibitor of SOD activity, hydrogen peroxide or potassium cyanide was added to the riboflavin-TEMED (*N,N,N′,N′*-tetramethylethylenediamine) solution to a final concentration of 5 or 2 mM, respectively. [Cu,Zn]-SODs are usually inactivated by cyanide, and [Fe]-SODs are inactivated by hydrogen peroxide, but [Mn]-SODs are inactivated by neither (10, 12). Bovine erythrocyte [Cu,Zn]-SOD (Sigma) mixed with a sonicate of *E. coli* DH5α containing [Fe]-SOD and [Mn]-SOD was used as a control preparation of all three forms of the enzyme.

RESULTS

Identification of [Cu,Zn]-SOD gene in *H. influenzae* type b NCTC8468. During a study of capsulation genes in *H. influenzae* type b NCTC8468, an open reading frame was identified starting 88 nucleotides (nt) downstream of *bexD-CBA*, the terminal gene cluster of the capsulation locus (23, 25). A potential coding region was defined by a motif resembling the Shine-Dalgarno consensus for ribosome binding 9 nt upstream of an ATG start codon, and a stem-loop structure characteristic of a rho-independent terminator was located 18 nt downstream of the TAA stop codon (Fig. 1). The deduced protein sequence was compared with sequences stored in the PIR data base by using the computer program FASTP on the VAX/VMS operating system. A highly significant match (86 of 187 possible identities with one gap; $Z = 47.3$ [29]) was found to [Cu,Zn]-SOD from *Photobacterium leiognathi* (47). Similar close matches could be shown to the two other sequenced bacterial [Cu,Zn]-SODs, those from *C. crescentus* (48) and *B. abortus* (6). A clear but lesser similarity was found to eukaryotic versions of the enzyme such as bovine [Cu,Zn]-SOD (41 of 187 possible identities) (Fig. 2). There was no significant match to [Fe]-SOD or [Mn]-SOD.

The three-dimensional structure of bovine [Cu,Zn]-SOD is known in detail, and amino acid residues critical for enzymatic activity have been identified (50). The environment of the catalytically important copper ion (Cu^{2+}) and structurally important zinc ion (Zn^{2+}) is defined by six histidines and an aspartate residue that act as ligands to the divalent cations. These have been found to be highly conserved between [Cu,Zn]-SODs, as have an arginine at the entrance to the active site and two cysteines involved in intramolecular bridges (Fig. 2). Five of the six histidines (His-80, His-105, His-114, His-123, His-161) and all four other conserved residues (Asp-126, Arg-180, Cys-87, and Cys-183) are found in the *H. influenzae* sequence. The exception is the Cu^{2+}-coordinating histidine expected at position 82, mutated to tyrosine as the result of a CAT→TAT transition (Fig. 1 and 2). The sequence of the *Haemophilus* protein deduced from the DNA sequence differs from that of the bovine enzyme but resembles the bacterial examples in having an N-terminal 22-amino-acid hydrophobic domain (Fig. 2) characteristic of a signal peptide, suggesting that the enzyme is exported.

Following the naming of genes for prokaryotic [Mn]- and [Fe]-SODs as *sodA* and *sodB*, respectively, in *E. coli*, this *Haemophilus* [Cu,Zn]-SOD gene was named *sodC*.

***sodC* in *H. influenzae* and *H. parainfluenzae*.** The 360-nt *Hin*dIII-*Nco*I DNA fragment containing the 5′ part of *sodC* (nt 147 to 507, Fig. 1) was subcloned as pJSK114 and used as a probe to examine Southern-blotted DNA from other *Haemophilus* strains (Fig. 3). The population of capsulate *Haemophilus* strains has a clonal structure, dividing into two major phylogenetic divisions, I and II (33). DNA from strains belonging to phylogenetic division I (type a, type b, and type c [and type d, not shown]) failed to hybridize to the probe. In contrast, strains of all capsular types segregating to phylogenetic division II (type a, type b, and type f) did

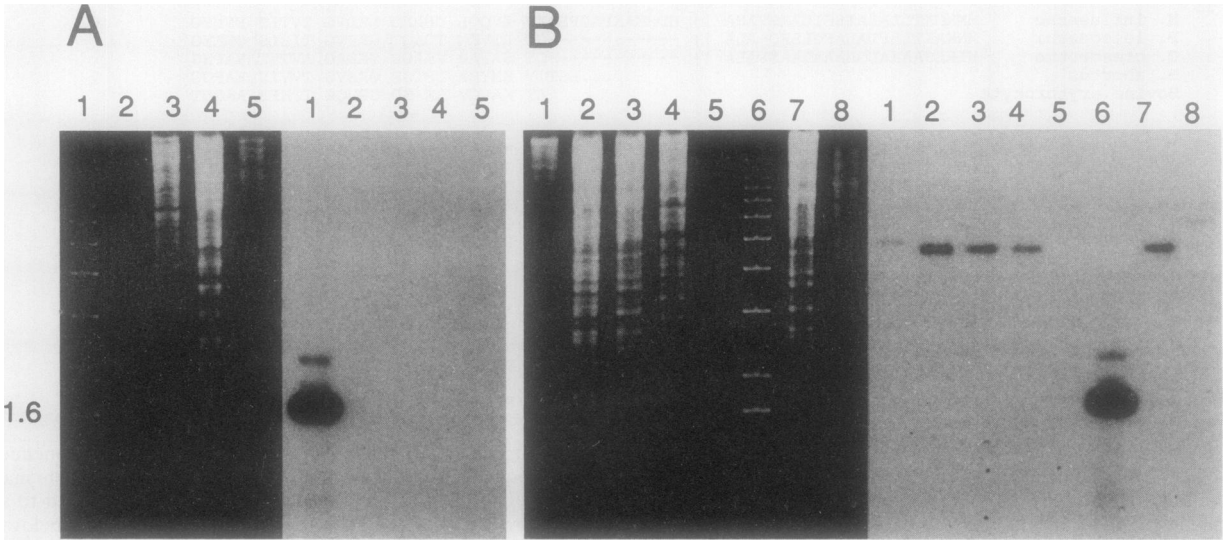


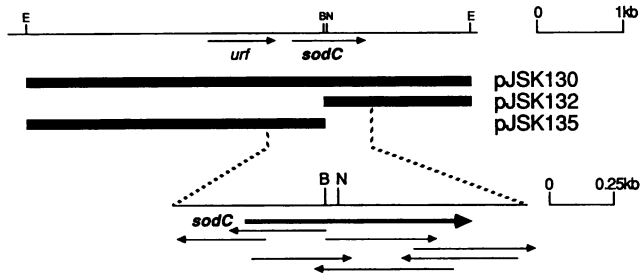
FIG. 3. *sodC* in *H. influenzae* and *H. parainfluenzae*. Ethidium bromide-stained 0.7% agarose gels were aligned with corresponding Southern blots hybridized to the *sodC* probe pJSK114. (A) DNA from *H. influenzae* phylogenetic division I strains. Lanes: 1, 1-kb ladder as marker DNA (1.6-kb fragment, hybridizing to pUC13, is indicated; larger fragments of 2, 3, and 4 kb and more do not hybridize to probe); 2, no DNA; 3, type a strain RM7109; 4, type b strain RM153; 5, type c strain RM8032. (B) DNA from *H. influenzae* type e, from division II strains and from *H. parainfluenzae*. Lanes: 1, type e strain RM6157; 2, type a strain RM107; 3, type b strain NCTC8468; 4, type f strain RM7283; 5, no DNA; 6, 1-kb ladder; 7, type b strain NCTC8468; 8, *H. parainfluenzae* 1391.

hybridize to the probe (NCTC8468 is a division II type b strain). So also did type e strains, only distantly related to strains in either of the main phylogenetic divisions.

DNA downstream of *sodC* in NCTC8468 has previously been shown to be present in strains from both phylogenetic divisions, and the DNA containing the region within which *sodC* would be expected to lie has been cloned from a division I type b strain (26). This cloned DNA was examined by low-stringency hybridization to pJSK114 and by sequencing to rule out the possibility that extensive sequence changes were responsible for the failure of pJSK114 to hybridize to the chromosome of division I strains, but no *sodC* gene was found (data not shown). Thus, the presence or absence of *sodC* DNA broadly correlated with the split of the population of capsulate *Haemophilus* strains into two major phylogenetic divisions.

DNA from 26 nontypeable (noncapsulate) *H. influenzae* clinical isolates was also examined; in 12 cases, weak hybridization to pJSK114 was found (data not shown). The probe also hybridized to DNA from all eight members of a collection of clinical isolates of *H. parainfluenzae*. An example is shown in Fig. 3. A 5.2-kb *Eco*RI fragment from *H. parainfluenzae* 1391 hybridizing to pJSK114 was cloned as pJSK130, and a gene homologous to the *H. influenzae sodC* was identified (Fig. 4). Within the open reading frame, the sequence was 96% identical to the gene from *H. influenzae*. On translation, there were five different amino acids. Glu-88, Ile-97, Ala-98, and Ser-108 of *H. influenzae* were replaced with Asp, Thr, Ser, and Pro, respectively. Most interestingly, amino acid 82 was found to be histidine instead of tyrosine as in *H. influenzae*.

***sodC* expression. (i) SOD activity in *Haemophilus* strains.** Every capsulate *H. influenzae* strain examined from either phylogenetic division showed a single band of SOD activity when electrophoretically separated protein extracts from whole cells were examined in nondenaturing polyacrylamide gels. The activity in samples from representative strains is shown in Fig. 5A. The protein with SOD activity had a mobility similar to that of the [Fe]-SOD of *E. coli*. However, its activity was not inhibited by 5 mM hydrogen peroxide or by 2 mM cyanide, and it was thus more characteristic of [Mn]-SOD, an enzyme which is known to vary in molecular weight and subunit structure between species (45). Catalase staining of duplicate gels (15) confirmed that this lack of inhibition by hydrogen peroxide was not due to the presence of a comigrating catalase (data not shown). Manipulation of the conditions of growth to maximize aerobiosis and incorporation of extra copper into the culture medium (17) failed to elicit any [Cu,Zn]-SOD activity in strains bearing *sodC*. In addition, [Cu,Zn]-SOD activity was not detected in extracts of these strains examined in a solution assay as described elsewhere (28). In contrast, extracts of pJSK114-positive nontypeable *H. influenzae* and *H. parainfluenzae* strains, grown under conditions identical to those for the capsulate *H. influenzae* strains, showed two bands of activity. One diffuse band had the staining characteristics of the putative [Mn]-SOD, but the other was inhibited by 2 mM cyanide and was thus characteristic of [Cu,Zn]-SOD (Fig. 5B). The *H. parainfluenzae* SOD activity with these characteristics was shown unequivocally to be due to the presence of [Cu,Zn]-SOD by site-directed mutagenesis with a gene cartridge encoding aminoglycoside phosphotransferase as used previously (25). The 1.2-kb gene cartridge was ligated into the *Bam*HI site in the middle of *sodC* cloned in pJSK130 to create pJSK137, and the mutation was integrated into the strain 1391 chromosome by transformation and recombination. The kanamycin-resistant transformant was checked by Southern blotting of an *Eco*RI digest, probed with the gene cartridge and with pJSK130 (data not shown). The 5.2-kb *Eco*RI fragment hybridizing to pJSK130 in the wild-type strain was replaced by a single 6.4-kb fragment, indicating recombinant marker exchange. The mutant was assayed as before for SOD activity, and the band identified as [Cu,Zn]-SOD had disappeared (Fig. 5C).

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  D  R  F  D  *                                                    Y  K
GGATAGGTTTGATTGATAGAAAAATCAAATCAATTTAATTGTCAAATATTTAATATAAAC      60

P  L  F  F  Y  W  Y  L  G  R  I  N  L  S  L  S  K  V  K  E
CACTATTTTTTTATTGGTATTTAGGTAGAATTAATTTGAGTCTTTCTAAAGTAAAGGAGC     120

L  M  M  K  M  K  T  L  L  A  L  K  I  S  G  I  C  A  A  G      19
TTATGATGAAAATGAAAACTCTCTTAGCATTAGCAATCAGCGGAATTTGTGCTGCTGGCG     180
                                 ^
V  A  N  A  H  D  H  M  A  K  P  A  G  P  S  I  E  V  K  V      39
TGGCAAATGCACATGACCATATGGCAAAACCAGCAGGTCCTTCAATCGAAGTAAAAGTAC     240

Q  Q  L  D  P  A  N  G  N  K  D  V  G  T  V  T  I  T  E  S      59
AACAATTAGATCCTGCAAATGGTAACAAGGATGTAGGGACTGTAACTATTACTGAATCAA     300

N  Y  G  L  V  F  T  P  N  L  Q  G  L  A  E  G  L  H  G  F      79
ATTATGGTTTAGTATTTACACCAAATCTACAAGGTTTAGCTGAAGGTTTACATGGCTTCC     360

H  I  H  E  N  P  S  C  D  P  K  E  K  D  G  K  L  T  S  G      99
ACATTCATGAAAATCCAAGCTGTGATCCAAAAGAAAAAGACGGTAAATTAACCTCAGGTT     420

L  A  A  G  G  H  W  D  P  K  G  A  K  Q  H  G  Y  P  W  Q     119
TAGCGGCTGGCGGTCACTGGGATCCTAAAGGCGCAAAACAACACGGTTACCCATGGCAAG     480

D  D  A  H  L  G  D  L  P  A  L  T  V  L  H  D  G  T  A  T     139
ATGATGCTCACTTAGGTGACTTACCTGCTTTAACTGTATTACATGATGGTACAGCAACTA     540

N  P  V  L  A  P  R  L  K  K  L  D  E  V  R  G  H  S  I  M     159
ATCCTGTTTTAGCGCCACGTCTTAAAAAATTAGATGAAGTTCGTGGTCATTCTATTATGA     600

I  H  A  G  G  D  N  H  S  D  H  P  A  P  L  G  G  G  G  P     179
TTCACGCTGGTGGTGATAACCACTCAGATCACCCAGCTCCACTTGGCGGTGGCGGCCCAC     660

R  M  A  C  G  V  I  K  *                                       187
GTATGGCATGTGGTGTGATTAAATAAATCTATTTCTAAAGATAAAAAAGTGCGGTCAATT     720
TTGACCGCACTTTGTGTTT....
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FIG. 4. [Cu,Zn]-SOD gene *sodC* from *H. parainfluenzae* 1391. Top: Chromosomal location of *sodC*, showing restriction sites for *Eco*RI (E), *Bam*HI (B), and *Nco*I (N). DNA cloned in pJSK130, pJSK132, and pJSK135 is indicated by the bar. Sequencing strategy is shown as in Fig. 1. Bottom: Nucleotide sequence of *sodC* and flanking regions including the 3′ end of the unknown reading frame (*urf*) upstream, and translated protein sequence of the open reading frame (single-letter code). Sequence motifs and conserved amino acids are indicated as in Fig. 1. As in Fig. 1, nucleotide numbering is arbitrary from the beginning of the sequence presented, and amino acid numbering starts with the N-terminal methionine of *sodC*. Sequence complementary to PARASODPER is underlined.

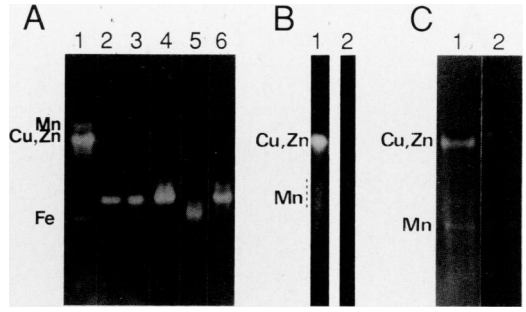

FIG. 5. SODs visualized after electrophoresis in 10% polyacrylamide gels. (A) *H. influenzae* proteins. SOD activity is represented by a decolorized zone against the dark background. Lanes contain 50 μg of protein (except where indicated) from extracts of whole cells from *E. coli* DH5α (20 μg of protein) as the control, with added bovine erythrocyte [Cu,Zn]-SOD (1 McCord-Fridovich unit) (lane 1), type a strain RM7109 (lane 2), type b strain RM153 (lane 3), type c strain RM8032 (lane 4), type b strain NCTC8468 (lane 5), and type f strain RM7283 (lane 6). [Mn]-SOD, [Cu,Zn]-SOD, and [Fe]-SOD activities in lane 1 are indicated. (B) *H. parainfluenzae* 1391 proteins. Samples (50 μg) were electrophoresed and stained for SOD activity immediately (lane 1) or after incubation in 2 mM KCN (lane 2). The [Cu,Zn]-SOD activity is abolished by treatment with KCN. (The putative [Mn]-SOD band was extremely diffuse in this 8% gel, but see panel C.) (C) *H. parainfluenzae* 1391 proteins. Lane 1, 50 μg of extract of wild-type strain; lane 2, 50 μg of extract of strain mutated with pJSK137 to inactivate *sodC*.

(ii) *sodC* mRNA in *Haemophilus* strains. *sodC* mRNA was sought in each species to elucidate this unexpected difference in [Cu,Zn]-SOD activity between *H. parainfluenzae* 1391 and *H. influenzae* NCTC8468. The oligonucleotide primer PARASODPER (Fig. 4), complementary to the sense strand near the 5′ end of *sodC* in both strains, was used in primer extension experiments. With *H. parainfluenzae* RNA, a single product of the reaction could be aligned with sequence derived from denatured pJSK130 DNA by using the same primer (Fig. 6A). This identified the transcriptional start site 25 nt upstream of the putative start codon (Fig. 6C). TAGAAT in the −10 region relative to this start is an excellent match to the *E. coli* promoter consensus for RNA polymerase binding. In the −35 region, however, the sequence ACCACTATT bears no resemblance to the *E. coli* consensus found in constitutive promoters (38). PARASOD PER failed to generate any primer extension reaction product from NCTC8468 RNA comparable to that found with strain 1391 RNA (Fig. 6A). However, high-molecular-weight material was seen at the origin of the 6% urea-acrylamide sequencing gel, so the product of a further primer extension reaction with NCTC8468 RNA and unlabeled PARASOD PER was electrophoresed in a denaturing 0.7% alkaline agarose gel. This showed there to be a cDNA product of approximately 1 kb that hybridized to pJSK40, containing both *sodC* and upstream *bex* genes (Fig. 1), indicating that *sodC* and one or more *bex* genes were cotranscribed on a single mRNA in this strain (Fig. 6B). This was confirmed by control experiments in which no product was detected if PARASODPER was omitted from the primer extension reaction mix, while a band of correspondingly lower molecular weight was detected when the reaction was done with an oligonucleotide primer, BEXAPER, complementary to *bexA* coding sequence 262 nt upstream of PARASODPER (Fig. 6B).

DNA sequence between *bexA* and *sodC* in NCTC8468 was compared with the corresponding region in *H. parainfluenzae* 1391, seeking differences that correlated with the difference in *sodC* transcription. When the sequences were aligned back from the *sodC* start codon, there was 84% identity over the shared span of noncoding DNA (Fig. 6C). The TG dinucleotide corresponding to the transcriptional start in 1391 was present in NCTC8468, as was the −10 consensus, but a cluster of differences was located around the −35 position, with a deletion of 2 nt and alteration of four others within the 9-nt region. With the results of the primer extension experiments, this suggests that in *H. parainfluenzae* the region is involved in the initiation of *sodC* transcription but that in *H. influenzae* transcription initiation at this site is fatally compromised through sequence divergence.

(iii) *sodC* expression in *E. coli*. Despite the lack of [Cu,Zn]-SOD activity in *H. influenzae*, *sodC* has been shown to be transcribed, although differently from its transcription in *H. parainfluenzae*. Among reasons for this failure to make

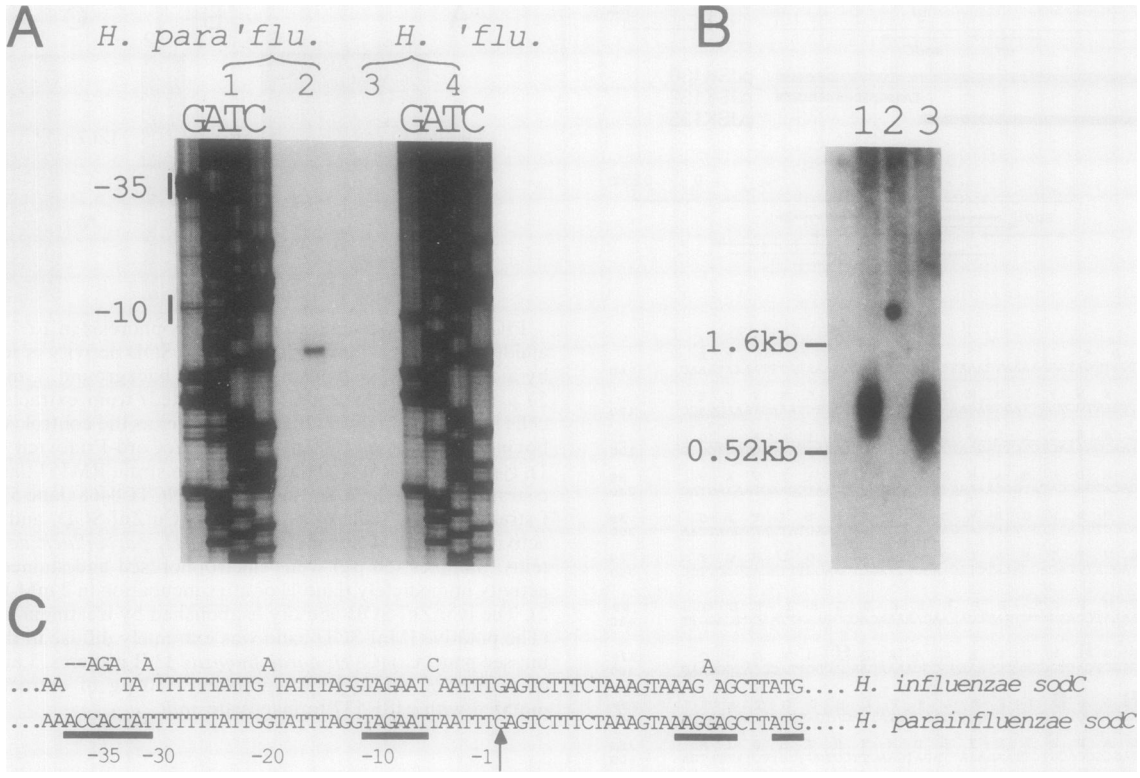

FIG. 6. Primer extension reactions in *H. parainfluenzae* and *H. influenzae*. (A) Autoradiograph of 6% urea-polyacrylamide sequencing gel. Panel 1, *H. parainfluenzae* 1391 DNA cloned in pJSK130, sequenced with PARASODPER primer. Panel 2, cDNA product of primer extension with *H. parainfluenzae* 1391 RNA and labeled PARASODPER as the primer. Panel 3, as in panel 2, but using *H. influenzae* NCTC8468 RNA. Panel 4, as in panel 1, but using *H. influenzae* DNA cloned in pJSK40. Sequences −10 and −35 nt relative to the transcriptional start in *H. parainfluenzae* are shown. (B) Southern blot of 0.7% denaturing agarose gel probed with pJSK40. Lane 1, cDNA product of primer extension with *H. influenzae* NCTC8468 RNA, using PARASODPER as the primer. Lane 2, product of the same reaction with no oligonucleotide primer added. Lane 3, as in lane 1, but using BEXAPER, 262 nt closer to the origin of transcription, as the primer. Relative mobilities of two DNA fragments of known size are shown. (C) Aligned DNA sequence upstream of *sodC* in *H. influenzae* and *H. parainfluenzae*. Differences are indicated by displacement of the *H. influenzae* sequence. The putative start codon and ribosome binding site are underlined, as are the −10 and −35 regions in *H. parainfluenzae*. The transcriptional start site established by primer extension is shown by an arrow.

active enzyme under conditions in which it is made by *H. parainfluenzae* might be differences in mRNA stability, altered translation of the message, or critical differences in the protein sequence. To explore these possibilities, we examined the expression of cloned wild-type and mutated *sodC* genes from both *Haemophilus* species in *E. coli* using various plasmid constructions. Two *Nco*I sites in pJSK40 (Fig. 1), one in the vector downstream of *sodC* and the other in the middle of the gene, were utilized for mutagenesis. A gene cartridge encoding aminoglycoside phosphotransferase was ligated into the vector *Nco*I site to yield pJSK125 and into the insert *Nco*I site to yield pJSK126. Deletion of the DNA between the sites yielded pLNB1. *E. coli* minicells harboring these plasmid constructions were used to synthesize proteins encoded by genes on the sequestered DNA in the presence of [^{35}S]methionine, and labeled proteins were separated electrophoretically in SDS-12.5% polyacrylamide gel (Fig. 7). Labeled proteins synthesized from pBR328 genes are shown in lane 3. An extra ~20-kDa product encoded by the *Haemophilus* DNA in pJSK40 is seen in lane 4, agreeing well with the predicted molecular mass of 19.54 kDa for SodC. This product was also encoded by pJSK125 but not by pJSK126, identifying it unambiguously as SodC. A truncated product was synthesized as expected by pLNB1

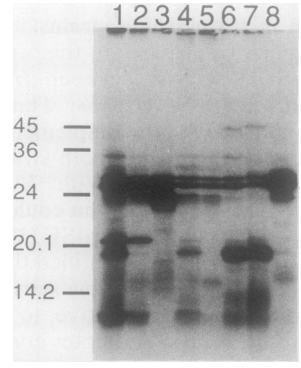

FIG. 7. [^{35}S]methionine-labeled proteins made in *E. coli* minicells. Autoradiograph of an SDS-12.5% polyacrylamide gel carrying labeled proteins encoded by plasmids in lanes as follows: 1, pJSK125; 2, pJSK126; 3, pBR328; 4, pJSK40; 5, pLNB1; 6, pJSK130; 7, pJSK131; 8, pUC13. Relative mobilities of marker proteins (in kilodaltons) are indicated on the left.

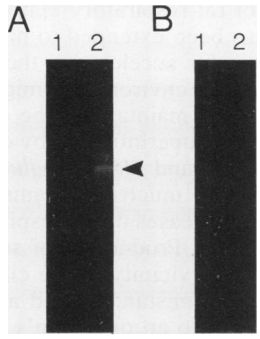

FIG. 8. SOD from *H. influenzae* NCTC8468 overproduced in *E. coli* QC779. Samples were electrophoresed and stained for SOD activity immediately (A) or after incubation in 2 mM KCN (B). Lanes contain 80 μg of protein from extracts of whole cells from QC779 (lanes 1) and QC779 transformed with pJSK40 (lanes 2). The arrowhead marks a band of SOD activity abolished by treatment with KCN. Other faint bands are still just visible after KCN treatment.

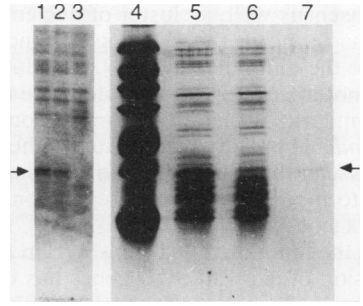

FIG. 9. Cellular localization of *H. parainfluenzae* [Cu,Zn]-SOD in *E. coli* minicells. Lanes 1 to 3, Coomassie-stained SDS-12.5% polyacrylamide gel of separated proteins synthesized by minicells containing plasmids: 1, pJSK130; 2, pJSK131; 3, pUC13. Arrow indicates dominant product, approximately 20-kDa [Cu,Zn]-SOD. Lanes 4 to 7, Silver-stained gel of separated proteins from minicells containing pJSK130 as follows: 4, SDS-7 marker proteins (Sigma); 5, whole minicell proteins; 6, periplasmic protein-depleted minicell proteins; 7, periplasmic proteins. Arrow indicates approximately 20-kDa protein in periplasmic extract correspondingly depleted in lane 6.

in place of intact SodC. The 5.2-kb *Eco*RI fragment bearing *H. parainfluenzae sodC* (Fig. 4) was ligated in each orientation into the polylinker *Eco*RI site of pUC13 (pJSK130, pJSK131). Each construction directed synthesis of the same ~20-kDa protein in minicells (Fig. 7, lanes 6 and 7) in addition to vector products (lane 8).

The possibility that protein synthesis was seen as the result of cloned *sodC* being transcribed differently in *E. coli* from the chromosomal gene in *H. influenzae* was addressed by primer extension analysis with PARASODPER and RNA prepared from *E. coli* DH5α transformed with pJSK40. Again, no low-molecular-weight cDNA product was found (data not presented), indicating that SodC synthesis in such transformants was not occurring as a result of initiation of transcription in the region analogous to the *H. parainfluenzae* start.

The *sodA sodB E. coli* mutant QC779 which makes no SOD was used to seek [Cu,Zn]-SOD enzyme activity derived from cloned *Haemophilus* DNA. The strain was transformed with pJSK40, and a whole-cell extract was examined as before in a nondenaturing polyacrylamide gel (Fig. 8). A very weak band of cyanide-sensitive SOD activity was identified in transformants bearing the multicopy plasmid, not seen in extracts of QC779 alone. Genes cloned upstream of *sodC* in pJSK40 are well-characterized and involved in polysaccharide export rather than oxygen metabolism (25, 27). Thus, the activity detected would appear to arise from *sodC*, suggesting that the failure to detect its product from the single copy of the gene in *H. influenzae* is the consequence of the protein having very low activity.

Cloned *sodC* was manufactured in sufficient quantity by *E. coli* minicells bearing pJSK130 for the protein to be detected by direct staining (Fig. 9). Fractionation of minicells suggested that the protein was localized in the periplasm (Fig. 9), supporting the proposition that the hydrophobic N-terminal end of the deduced protein sequence of *sodC* is a leader peptide and suggesting that the *Haemophilus* [Cu,Zn]-SOD is secreted like other bacterial examples of this enzyme.

DISCUSSION

H. influenzae type b NCTC8468 contains *sodC* with the deduced protein sequence of a [Cu,Zn]-SOD, and Southern blotting data suggest that the same gene is present in other phylogenetic division II capsulate *Haemophilus* strains. A homologous gene was identified by Southern blotting in strains of *H. parainfluenzae* and cloned and sequenced in strain 1391. The corresponding enzyme activity was detected in *H. parainfluenzae* and in some nontypeable strains of *H. influenzae*, but no functional enzyme was found in any capsulate *Haemophilus* strain, and indeed *sodC* has not been found in phylogenetic division I strains of capsulate *H. influenzae*. Despite demonstrable SodC production in *E. coli* minicells harboring the gene cloned from NCTC8468, only a very low level of [Cu,Zn]-SOD activity could be detected in transformed *E. coli*. Studies of gene expression at the mRNA and protein levels, together with comparison of coding and noncoding DNA of these two *sodC* genes, lead us to speculate that the difference in *sodC* phenotype between *H. influenzae* and *H. parainfluenzae* is the consequence of a CAT→TAT transition in the *H. influenzae* gene. This converts to tyrosine a histidine known in bovine [Cu,Zn]-SOD to be critically important for Cu^{2+} coordination and catalytic activity (50), which could affect enzyme stability or activity in various ways. Tyr-82 might itself substitute at low efficiency as a Cu^{2+} ligand. Alternatively, coordination of His-77 to Cu^{2+} might be possible without drastic alteration of protein structure, for the His-80-X-His-82 motif lies at the end of one β strand forming the β-barrel structure of the bovine enzyme subunit (50). The production of SodC from the NCTC8468 gene expressed in *E. coli* minicells argues against the effect of the His→Tyr mutation being to create a highly unstable protein. Whatever the explanation, we infer that the lack of enzymatic activity of the *H. influenzae* protein at normal levels of gene expression reflects an incapacity for normal coordination of Cu^{2+}. It appears that only when the gene is heavily overexpressed, as on a multicopy plasmid, can even weak activity be observed. *sodC* transcription differs in the two *Haemophilus* species, but this does not seem to be responsible for differences in enzyme activity. Cloned *Haemophilus* DNA from either species directs comparable synthesis of SodC in *E. coli* minicells even though the *H. influenzae* version is inactive. While the −10 region relative to the transcriptional start in *H. parainfluenzae* is identical in the two species and matches

the *E. coli* consensus well, a cluster of nucleotide sequence differences was found in the −35 region, and this may be responsible for the difference in mRNA production. If *Haemophilus* promoter structures resemble those of *E. coli*, the complete dissimilarity of this −35 from the consensus would suggest that *sodC* is positively regulated rather than constitutively expressed (38). This finding further suggests a rational approach to investigating *sodC* regulation through site-directed mutagenesis which will prove important to understanding the function of [Cu,Zn]-SOD in *Haemophilus*.

The possession of [Cu,Zn]-SOD genes has been regarded as virtually the exclusive prerogative of eukaryotes, with only five bacterial exceptions known (6, 37, 44, 46, 53). This set can now be expanded considerably, for as well as *H. influenzae* and *H. parainfluenzae* containing *sodC*, a whole series of *Haemophilus* and *Actinobacillus* species, organisms found as commensals and pathogens of the human and animal oropharynx and upper respiratory tract, have been examined and show both *sodC* probe hybridization and enzyme activity (25a, 28a).

In eukaryotic cells, [Cu,Zn]-SOD is a cytosolic enzyme, providing protection against oxygen free radicals produced endogenously during aerobic metabolism. The same is the case for the conventional Mn and Fe enzymes found in bacteria (2). In contrast, [Cu,Zn]-SODs from *Photobacterium*, *Caulobacter*, and *Brucella* species are exported beyond the cytoplasm (6, 47, 48), and preliminary data presented here suggest that the same is true for the *Haemophilus* proteins, at least when expressed in *E. coli* minicells. Preliminary results with SodC–β-lactamase gene fusions (8), which confer ampicillin resistance only if their product is exported beyond the cytoplasm, support this proposition in both *E. coli* and *H. parainfluenzae* (24). Extracellular SODs have been described before in both eukaryotes and prokaryotes (3, 22, 42) and may play a protective role against toxic oxygen species released by macrophages during the respiratory burst. Beaman and Beaman (4) have suggested that *Nocardia asteroides* gains pathogenic potential through a capacity to neutralize superoxide within macrophages by excreting a manganese-containing SOD. In the case of *B. abortus*, however, a very recent study suggests that its capacity to secrete [Cu,Zn]-SOD is not a primary determinant of bacterial virulence (43). Until now, the absence of genetically defined mutants has meant that the case for a translocated SOD, [Cu,Zn] or otherwise, playing a critical role in the pathogenicity of invasive bacteria has remained to be established. Now this is possible for *Haemophilus* species, and on this score, the finding that capsulate *H. influenzae* strains of phylogenetic division I, important causes of invasive infections, do not carry *sodC* at all and that while division II strains do, they do not seem to make active enzyme, argues against the likelihood that [Cu,Zn]-SOD is critical for invasiveness. Examination of the organization of their capsulation loci suggests that division II *H. influenzae* type b strains are phylogenetically older than division I strains (26) and that the gene encoding inactive [Cu,Zn]-SOD is a relic of a noncapsulate ancestral strain in the former, finally eliminated from the chromosome in the latter. The finding on the other hand that *sodC* encodes active enzyme in common oropharyngeal and respiratory commensals suggests a possible function other than facilitating tissue invasion. A translocated SOD could confer a survival advantage on bacteria by catalyzing the production of hydrogen peroxide on the airway mucosal surface. Burman and Martin (9) have demonstrated that low concentrations of hydrogen peroxide lead to a rapid decline in ciliary activity of rat respiratory epithelium in vitro, and this observation has been extended to human tissue (54). A bacterial mechanism for accelerating the production of hydrogen peroxide in this environment might thus be permissive for setting up and maintaining the carrier state in the healthy nasopharynx. Superinfection by organisms like nontypeable *H. influenzae* and *H. parainfluenzae* is a cardinal feature of the chronic mucosal inflammation that is the hallmark of common diseases of the respiratory tract such as chronic bronchitis (40). Production of superoxide from inflammatory cells in the vicinity of the ciliated epithelium is enhanced in these circumstances, and a secreted bacterial SOD might contribute to an organism's capacity to escape host mucociliary clearance mechanisms and establish chronic sinopulmonary infection.

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