

Prevalence of the *sodC* Gene in Nontypeable *Haemophilus influenzae* and *Haemophilus haemolyticus* by Microarray-Based Hybridization[∇]

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The *sodC* gene has been reported to be a useful marker for differentiating nontypeable (NT) *Haemophilus influenzae* from *Haemophilus haemolyticus* in respiratory-tract samples, but discrepancies exist as to the prevalence of *sodC* in NT *H. influenzae*. Therefore, we used a microarray-based, “library-on-a-slide” method to differentiate the species and found that 21 of 169 (12.4%) NT *H. influenzae* strains and all 110 (100%) *H. haemolyticus* strains possessed the *sodC* gene. Multilocus sequence analysis confirmed that the 21 NT *H. influenzae* strains were *H. influenzae* and not *H. haemolyticus*. An inactive *sodC* gene has been reported in encapsulated *H. influenzae* strains belonging to phylogenetic division II. Capsule-specific Southern hybridization and PCR and a lack of copper/zinc-cofactored superoxide dismutase (CuZnSOD) expression indicated that 6 of the 21 *sodC*-containing NT *H. influenzae* strains in our study were likely capsule-deficient mutants belonging to phylogenetic division II. DNA sequence comparisons of the 21 *H. influenzae sodC* genes with *sodC* from *H. haemolyticus* or encapsulated *H. influenzae* demonstrated that the *sodC* genes of the six *H. influenzae* capsule-deficient mutants were, on average, 99% identical to *sodC* from encapsulated *H. influenzae* but only 85% identical to *sodC* from *H. haemolyticus*. The *sodC* genes from 2/15 NT *H. influenzae* strains were similarly more closely related to *sodC* from encapsulated strains, while *sodC* genes from 13 NT *H. influenzae* strains were almost 95% identical to *sodC* genes from *H. haemolyticus*, suggesting the possibility of interspecies recombination in these strains. In summary, this study demonstrates that *sodC* is not completely absent (9.2%) in true NT *H. influenzae* strains.

Haemophilus influenzae asymptotically colonizes the pharyngeal cavity of humans but may cause either systemic or respiratory-tract infections. Those strains that possess a polysaccharide capsule, serotypes a through f, cause most *H. influenzae* invasive infections (11), whereas nonencapsulated or nonserotypeable (NT) *H. influenzae* strains are associated with localized respiratory-tract diseases, such as otitis media and exacerbation of chronic obstructive pulmonary disease (COPD) (19). In addition, a cryptic genospecies of NT *H. influenzae* is also associated with neonatal invasive infections and adult urogenital infections (36). Phylogenetically, however, the cryptic genospecies is more closely related to *Haemophilus haemolyticus* than to *H. influenzae* of the respiratory tract (26, 27).

H. haemolyticus is a commensal of the pharyngeal cavity, and *H. haemolyticus* and NT *H. influenzae* isolated from the respiratory tract overlap taxonomically and phylogenetically, likely through the exchange of DNA by natural transformation (1, 20, 21, 32). The species have been traditionally differentiated by the ability of *H. haemolyticus* to hemolyze horse blood (11, 25), but recent studies have identified significant numbers of nonhemolytic *H. haemolyticus* in NT *H. influenzae* collections isolated from throat or sputum samples (21, 37). Since sputum samples are used to monitor COPD exacerbation, accurate

differentiation of NT *H. influenzae* from *H. haemolyticus* is clinically important (5, 21).

Fung et al. (9) recently suggested that the presence of the *sodC* gene or activity of its cognate protein, copper/zinc-cofactored superoxide dismutase (CuZnSOD), can differentiate *H. haemolyticus* from NT *H. influenzae*, as *sodC* was present in 20 *H. haemolyticus* isolates and absent in 20 NT *H. influenzae* isolates. In addition, a previous study failed to find the *sodC* gene in 45 NT *H. influenzae* disease isolates (18). Prior to these studies, however, 12 of 26 NT *H. influenzae* strains were found to hybridize weakly with a *sodC* gene probe, and these strains also displayed CuZnSOD activity (13). The discrepancy between these studies has never been explained. Aside from the questionable absence of *sodC* in NT *H. influenzae* of the respiratory tract, the gene and its associated CuZnSOD activity are present in the *H. influenzae* cryptic genospecies associated with neonatal invasive infections and adult urogenital infections (18). In addition, the gene is present among serotype e encapsulated strains and in strains that belong to the capsular phylogenetic division II, a multilocus enzyme electrophoresis (MLEE)-based division that contains some serotype a and b (Hib) strains and all serotype f (Hif) strains (13, 18, 22). When present, the *sodC* gene in encapsulated strains is adjacent to the *bexA* to *-D* genes of the capsule locus. The *sodC* gene of encapsulated *sodC*-containing strains shares significant sequence homology with the gene from *Haemophilus parainfluenzae*, but unlike *H. parainfluenzae*, encapsulated *H. influenzae* lacks CuZnSOD activity, presumably due to a substitution mutation that replaces an active-site histidine with tyrosine (13).

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In the present study, we further evaluated the potential of the *sodC* gene to differentiate a large collection of known NT *H. influenzae* and *H. haemolyticus* respiratory tract isolates using a whole-genome microarray hybridization technology called library on a slide (39).

MATERIALS AND METHODS

Bacterial growth and strains. Bacteria were grown on chocolate agar plates (BD, Franklin Lakes, NJ) at 37°C with 5% CO₂. Bacteria were also grown on Levinthal agar prior to assessment of CuZnSOD activity (34).

Species designation of 169 NT *H. influenzae* and 110 *H. haemolyticus* strains was made previously by phenotypic, genotypic, or phylogenetic assays, and lack of encapsulation of the NT *H. influenzae* strains was determined by serotyping (8, 20, 28, 31). Microarray positive-control *sodC*-containing strains included *H. parainfluenzae* ATCC 33392 and *H. haemolyticus* ATCC 33390, and negative-control *sodC*-lacking strains included genome-sequenced *H. influenzae* strains Rd, 86-028NP, and R2846.

Microarray printing and analysis. Microarray printing of crude genomic DNA and the generation of labeled *sodC* and DNA quantification probes have been described elsewhere (12, 14, 28, 38). The *sodC* gene probe was obtained from *H. haemolyticus* strain 65P28H9 with 5' and 3' UNIVSOD oligonucleotides (14).

A relative measure of probe hybridization to the DNA concentration for each spot was determined as follows. Duplicate slides were hybridized at 65°C in PerfectHyb Plus hybridization buffer (Sigma-Aldrich) with a digoxigenin-labeled control probe, serially washed with low- and high-stringency buffers, and analyzed (12, 28). The slides were then stripped with 4 M NaOH, washed, and rehybridized with a fluorescein-labeled *sodC* probe. As described previously, Spotfinder v.3.1.1 and MIDAS v.2.19 were used for determining probe intensity and spot standardization, respectively, and programs written in the statistical software "R" were used to graph the frequency of log-transformed *sodC* hybridization signal to a concentration-control signal ratio (28).

Southern hybridization. Southern hybridization was performed on the *sodC*-positive strains using *sodC* or *bexA* gene probes. Genomic DNA was digested with EcoRI, electrophoresed on 1% agarose gels, and transferred to nylon membranes (Amersham). Probe labeling and hybridization have been described previously (20).

CuZnSOD activity. CuZnSOD activity was determined by electrophoresis of whole-cell sonicated extracts on isoelectric focusing (IEF) pH 3 to 10 gels (Invitrogen) and visualized using previously described staining protocols (4, 30). CuZnSOD activity was specifically inhibited with 10 mM diethyl dithiocarbamic acid (DEDCA), a copper chelator (17).

DNA isolation, amplification, and sequencing. Isolation of template DNA and PCR amplification have been described previously (20). Oligonucleotides to amplify capsule-specific genes and to create a *bexA* gene probe have been described (7, 35). Intragene oligonucleotides, relative to the 5' and 3' ends of the published *H. influenzae* type b and f and *H. parainfluenzae sodC* gene sequences, were used to amplify partial *sodC* genes from each of the *sodC*-containing *H. influenzae* strains. The primer sequences were F, 5'-ATGATG AAAATGAAACTCTCTAGCATTAGC, and R, 5'-TTATTAATCAAYRC CACATGCCATACG.

DNA sequence analysis and multilocus sequence analysis (MLSA) using partial DNA sequences of the *adhA*, *pgi*, *recA*, *infB*, and 16S rRNA gene have been described previously (20). MLSA was performed on all *sodC*-positive strains (excluding strains Mr27, 32324, and H08-25, which had been done previously [20]). Sequence concatenations were done with Lasergene version 7.0 (DNAStar, Madison, WI), and phylogenetic analysis was done with Mega 3.1 (15). Bootstrap consensus minimum-evolution dendrograms for concatenated or individual sequences were made with 1,000 replicates.

Nucleotide sequence accession numbers. Partial gene sequences for new *H. influenzae* MLSA sequences are available from GenBank under accession numbers GQ358536 to GQ358553 (for *recA*), GQ358554 to GQ358571 (for 16S rRNA genes), GQ358572 to GQ358589 (for *adhA*), GQ358590 to GQ358607 (for *infB*), and GQ358608 to GQ358625 (for *pgi*). Partial *sodC* gene sequences from the *H. haemolyticus* and *H. influenzae* strains described in this study are under accession numbers GU269205 to GU269227.

RESULTS

Microarray hybridization of the *sodC* gene. In whole-genome microarray hybridization, the *H. parainfluenzae* and *H.*

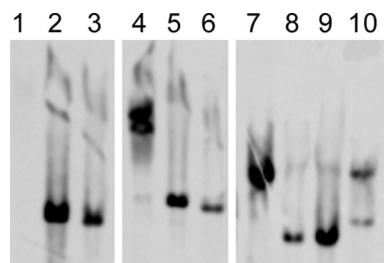
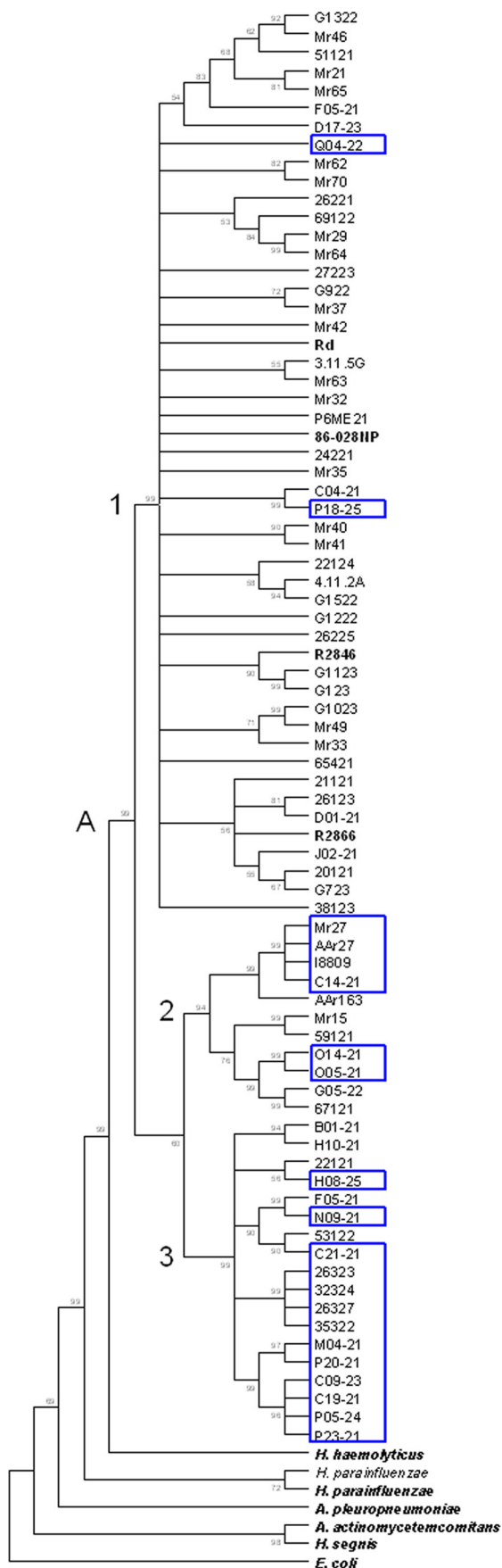


FIG. 1. Southern hybridization of a *sodC* gene probe to microarray-positive NT *H. influenzae* strains. Lane 1, negative-control *H. influenzae* strain Rd; lane 2, positive-control *H. haemolyticus* strain 65P28H9; lanes 3 to 10, eight representative *H. influenzae* strains, P18-25, P05-24, O05-21, I8809, C21-21, O14-21, C14-21, and C09-23, respectively.

haemolyticus positive-control strains hybridized with the *sodC* gene probe while the NT *H. influenzae* negative-control strains did not hybridize with the probe. In addition, 110/110 (100%) *H. haemolyticus* strains and 21/169 (12.4%) NT *H. influenzae* strains hybridized with the *sodC* gene probe. *sodC* hybridization to the 21 NT *H. influenzae* strains was confirmed by Southern blot hybridization. Eight representative NT *H. influenzae* strains, together with an *H. influenzae* negative-control strain, Rd, and an *H. haemolyticus* positive-control strain, 65P28H9, are shown in Fig. 1.

MLSA species confirmation of *sodC*-containing NT *H. influenzae*. Since previous studies have suggested that the *sodC* gene is present in *H. haemolyticus* but not in NT *H. influenzae*, we used MLSA to rule out the possibility that the 21 *sodC*-containing NT *H. influenzae* strains were misidentified in their original species designations (21, 37). DNA sequences (partial gene sequences of *adhA*, *pgi*, *recA*, *infB*, and 16S rRNA genes) obtained from the strains were analyzed according to an MLSA system that was previously developed in our laboratory and designed to differentiate NT *H. influenzae* from hemolytic and nonhemolytic *H. haemolyticus* (20). Generation of a minimum-evolution bootstrap consensus tree using concatenated sequences from the 21 *sodC*-containing NT *H. influenzae* strains, and from the 88 NT *H. influenzae* and 109 *H. haemolyticus* strains used in the original MLSA, demonstrated that all 21 *sodC*-containing strains clustered with known NT *H. influenzae* isolates (a similar tree including all NT *H. influenzae* strains and relevant type strains of other species is shown in Fig. 2). These results confirmed the species identification of the *sodC*-containing NT *H. influenzae* strains.

Presence of capsule DNA among *sodC*-containing NT *H. influenzae* strains. Previous studies suggested that *sodC* is not present in NT *H. influenzae* but is present in encapsulated *H. influenzae* of phylogenetic division II (9, 13, 18). To investigate the possibility that some or all of the 21 *sodC*-containing NT *H. influenzae* strains are capsule-deficient mutants of encapsulated strains, we performed Southern hybridization using a *bexA* gene probe on the strains. The probe hybridized to 6/21 *sodC*-containing NT *H. influenzae* strains (O14-21, O05-21, C14-21, AAR27, I8809, and Mr27). Furthermore, capsule-specific PCR (7) revealed that, of the 6 strains hybridizing to a *bexA* probe, 2 contained type e (O14-21 and O05-21) and 4 contained type f (C14-21, AAR27, I8809, and Mr27) capsular



DNA (Table 1). These results suggest that 6 of the original 21 *sodC*-containing *H. influenzae* strains are capsule-deficient mutants of encapsulated strains and that the remaining 15 strains are true NT *H. influenzae* strains. The results also allow the population prevalence of true *sodC*-containing NT *H. influenzae* strains to be readjusted from 21/169 (12.4%) to 15/163 (9.2%).

CuZnSOD activity among the *sodC*-containing *H. influenzae* strains. The CuZnSOD enzyme has been previously shown to be active in *H. haemolyticus* and *H. parainfluenzae* but inactive in encapsulated *H. influenzae* of phylogenetic division II (9, 18). Therefore, we determined the CuZnSOD activities of the 21 *sodC*-containing *H. influenzae* strains in this study to assess their relationship with the previously described haemophili. Native protein electrophoresis using whole-cell lysates of bacteria run on 3 to 10 IEF gels with subsequent staining for CuZnSOD activity (17) demonstrated that the positive-control *H. haemolyticus* strain, ATCC 33390, and all 15 true NT *H. influenzae* strains containing a *sodC* gene displayed consistent CuZnSOD activity, as evidenced by two achromatic bands, a feature previously observed for *H. haemolyticus* (Fig. 3, left, and Table 1) (9). CuZnSOD activity in the NT *H. influenzae* strains could be specifically inhibited by 10 mM DEDC, a copper-chelating agent (Fig. 3, right). One of the capsule-deficient strains (O14-21) displayed CuZnSOD activity in only one of several experiments. Inhibition with DEDC could not be done with this strain. None of the five remaining capsule-deficient strains had CuZnSOD activity, suggesting that their *sodC* genes are related to the *sodC* gene from encapsulated *H. influenzae* of phylogenetic division II. In addition, the results suggest that *sodC* of NT *H. influenzae* strains differs from the *sodC* of encapsulated strains.

Relationships of the *H. influenzae* and *H. haemolyticus* *sodC* genes. The relationships between the *sodC* gene from NT *H. influenzae* and the *sodC* gene from encapsulated *H. influenzae* or from other *Haemophilus* species are not known. Phylogenetic relationships between the strains and species in the MLSA described above, however, may provide an indication. In our previously published MLSA (20), all 88 NT *H. influenzae* strains were present in a single clade (A) that contained three subclades: A1, A2, and A3. Although 72/88 NT *H. influenzae* strains were found in subclade A1, the remaining 16 strains were divided between subclades A2 and A3. In addition, strains in subclades A2 and A3 were found to possess higher proportions of *H. haemolyticus*-like phenotypic and genotypic traits than NT *H. influenzae* strains present in subclade A1, suggesting the possibility of interspecies recombination for

FIG. 2. Minimum-evolution dendrogram of the *Haemophilus sensu stricto* cluster containing *sodC*-positive *H. influenzae* strains. The tree is rooted by *E. coli* (strain K-12) and is based on concatenated *adk*, *pgi*, *recA*, *infB*, and 16S rRNA gene sequences, with $\geq 50\%$ of 1,000 bootstraps indicated. Node A contains all 88 NT *H. influenzae* strains used in a previous MLSA (15), together with the 21 *H. influenzae* strains that contain a *sodC* gene (blue boxes). The remaining type strains of species bordering *H. influenzae* are shown in boldface and were used in the original dendrogram (15). Clusters 1, 2, and 3 of node A have been previously shown to vary in their distributions of *H. haemolyticus*-like traits (15).

TABLE 1. Characterization of the 21 *sodC*-hybridizing, nonencapsulated *H. influenzae* strains

Strain	MLSA cluster ^a	<i>bexA</i> hybridization ^b	Cap genotype ^b	CuZn-SOD activity ^b	% Identity to Hh <i>sodC</i> ^c	% Identity to Hib-like <i>sodC</i> ^c	<i>sodC</i> gene truncation	Isolation source ^d
P18-25	A1	–	–	+	96.7	84.1	No	Throat
Q04-22	A1	–	–	+	95.5	84.5	No	Throat
C19-21	A3	–	–	+	94.6	83.9	No	Throat
C21-21	A3	–	–	+	94.6	83.9	No	Throat
H08-25	A3	–	–	+	93.8	84.9	No	Throat
M04-21	A3	–	–	+	94.4	83.7	No	Throat
N09-21	A3	–	–	+	93.8	84.9	No	Throat
P05-24	A3	–	–	+	94.6	83.9	No	Throat
P20-21	A3	–	–	+	94.6	83.9	No	Throat
26323	A3	–	–	+	94.6	83.9	No	Throat
26327	A3	–	–	+	94.6	83.9	No	Throat
32324	A3	–	–	+	94.6	83.9	No	Throat
35322	A3	–	–	+	94.6	83.9	No	Throat
C09-23	A3	–	–	+	86.1	95.6	No	Throat
P23-21	A3	–	–	+	84.9	99.6	Yes	Throat
O14-21	A2	+	e	±	84.9	99.6	Yes	Throat
O05-21	A2	+	e	–	84.9	99.6	Yes	Throat
C14-21	A2	+	f	–	84.1	98.8	Yes	Throat
AAr27	A2	+	f	–	84.1	98.8	Yes	ME
I8809	A2	+	f	–	84.1	98.8	Yes	ME
Mr27	A2	+	f	–	84.1	98.8	Yes	ME

^a Subclade from dendrogram (Fig. 2) containing the strain.

^b –, negative; +, positive; ±, positive or negative.

^c Comparison is to the Hh (*H. haemolyticus*) or Hib-like (Hib, Hif, and *H. parainfluenzae*) consensus sequence listed.

^d Throat, isolate obtained from the throat of a healthy child attending day care; ME, middle-ear isolate of a child with otitis media.

strains found in clades A2 and A3 (20). Interestingly, 19 of the 21 *sodC*-containing *H. influenzae* strains in our current study clustered with the NT *H. influenzae* strains originally present in subclades A2 and A3 (Fig. 2 and Table 1). These data suggest the possibility that some *H. influenzae* strains possess a *sodC* gene through interspecies recombination with *H. haemolyticus*.

The *sodC* genes from 6 of the 19 *sodC*-containing strains in subclades A2 and A3, however, would be predicted to be related to encapsulated *H. influenzae* of phylogenetic division II, rather than to the *sodC* gene from *H. haemolyticus*. To investigate these potential differences, we compared the *sodC* gene sequences of our 21 *H. influenzae* strains with *sodC* from *H. haemolyticus* and encapsulated *H. influenzae*. *sodC* genes were PCR amplified from two *H. haemolyticus* strains (ATCC 33390 and 65P28H9) and all 21 *sodC*-containing *H. influenzae* strains using oligonucleotides representing the 5' and 3' ends of *sodC* nucleic acid sequences previously published from encapsulated

Hib and Hif and *H. parainfluenzae* (13, 29). DNA sequences obtained from the 21 *sodC* PCR products of the *H. influenzae* strains in this study revealed an internal 536- to 548-bp fragment, depending on the strain. Sequence comparisons revealed that the two *H. haemolyticus sodC* genes were 95.2% identical to each other, while the *sodC* genes from Hib, Hif, and *H. parainfluenzae* have been previously shown to be 96 to 98.4% identical to each other (13, 29). The *H. haemolyticus sodC* genes were 84.1 to 87.3% identical to a Hib, Hif, and *H. parainfluenzae sodC* consensus sequence (representing an internal 536-bp fragment) (data not shown). Further DNA sequence comparisons revealed that the *sodC* genes from 13 of the 21 *H. influenzae* strains were, on average, 94.7% identical to a consensus sequence generated from the two *H. haemolyticus sodC* gene sequences and 84.1% identical to the Hib, Hif, and *H. parainfluenzae sodC* consensus sequence ($P < 0.05$) (Table 1). All 13 strains were *bexA*-negative true NT *H. influenzae* strains. In contrast, the *sodC* sequences from the remaining 8 strains were on average 84.7% identical to the *H. haemolyticus* consensus sequence but 98.7% identical to the Hib, Hif, and *H. parainfluenzae* consensus sequence (henceforth referred to as Hib-like *sodC*) ($P < 0.05$) (Table 1). Of the eight strains with Hib-like *sodC* genes, six were capsule-deficient *H. influenzae* strains and two (C09-23 and P23-21) were *bexA*-negative NT *H. influenzae* strains. Therefore, *sodC* sequence analysis substantiates the existence of capsule mutant strains in this collection and the possibility that some NT *H. influenzae* strains may have acquired *sodC* through recombination with *H. haemolyticus*.

Interestingly, translation of the partial *sodC* gene sequences from all 21 strains revealed that the *sodC* genes of 7/8 strains with Hib-like *sodC* genes would be inactivated due to various frameshift insertions. This differs from the previously de-

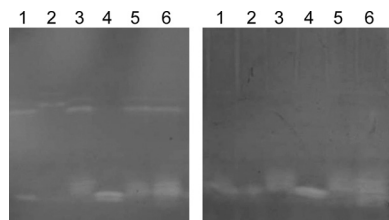


FIG. 3. SOD activities of representative nonencapsulated *H. influenzae* strains. Achromatic zones of SOD activity are shown in the left panel as bands in the upper and lower regions of the gel. Only the two upper bands are absent in the right panel, where the copper chelator DEDC was used to inhibit CuZnSOD activity. The lanes containing sonicated cellular extracts are as follows: lane 1, *H. influenzae* N09-21; lane 2, *H. haemolyticus* ATCC 33390; and lanes 3 to 6, *H. influenzae* strains 35322, Mr27, 26323, and C19-21, respectively.

scribed inactive *sodC* genes of encapsulated strains, where substitution mutations were thought to affect active-site residues (13). Four of the seven strains (AAr27, C14-21, I8809, and Mr27) had a 7-bp tandem duplication at position 169, and three strains (P23-21, O14-21, and O05-21) had a 1-bp insertion at position 302 (both positions are based on the full-length *sodC* gene from Hib, Hif, and *H. parainfluenzae*) (13). In contrast, all 13 strains with *H. haemolyticus*-like *sodC* genes and one Hib-like *sodC* gene possessed open reading frames.

One NT *H. influenzae* strain, C09-23, was atypical in that it possessed a Hib-like *sodC* gene with CuZnSOD activity (data not shown). The *sodC* gene of strain C09-23 differed from other Hib-like *sodC* genes in that it did not contain a truncated reading frame. One explanation is that the *sodC* gene of this strain may have originated from *H. parainfluenzae*. *sodC* in *H. parainfluenzae* is very similar to *sodC* of encapsulated *H. influenzae* and displays CuZnSOD activity (13). Alternatively, NT *H. influenzae* strain C09-23 was also found to possess two EcoRI fragments that hybridized with a *sodC* gene probe (Fig. 1, lane 10), and it is possible that CuZnSOD activity may have come from a second, non-PCR-amplified *sodC* gene. One other strain (P05-24) in this study also contained doublet bands hybridizing to a *sodC* gene probe (Fig. 1, lane 4), suggesting that multiple copies of *sodC* are possible in some *H. influenzae* strains.

In addition to C09-23, two other *sodC*-containing strains were atypical. One NT *H. influenzae* strain, P23-21, and one unencapsulated type e *H. influenzae* strain, O14-21, both displayed CuZnSOD activity despite having Hib-like *sodC* genes that contained frameshift mutations. In repeated experiments, CuZnSOD activity was always present in strain P23-21 but was observed only one time in strain O14-21. A second, unidentified copy of *sodC* would provide an explanation for the discrepancy between CuZnSOD activity and truncated *sodC* genes in these strains. Further studies, however, to confirm and characterize *sodC* copy numbers and expression would be outside the scope of our original objective, which was to identify *sodC*-containing NT *H. influenzae* strains and to estimate the population prevalence of the gene.

DISCUSSION

In this study, microarray hybridization analysis of a large population of respiratory-tract NT *H. influenzae* strains identified two groups of *sodC*-containing, unencapsulated *H. influenzae* strains (Table 1): (i) true NT strains (nonserotypeable strains lacking any capsule-associated genes) that possessed CuZnSOD activity and (ii) capsule-deficient mutants of encapsulated strains (containing *bexA* and capsule-specific DNA) that lacked CuZnSOD activity (with the exception of one type e strain, O14-21, which had inconsistent activity). Preliminary work in our laboratory indicates that the six *bexA*-positive strains represent all of the capsule-deficient mutants among the 169 nonserotypeable *H. influenzae* strains used in this study (unpublished results). Therefore, *sodC* was present in 15/163 (9.2%) true NT *H. influenzae* strains. *sodC* in capsule-deficient type e and f mutant strains are reflective of earlier studies that identified the *sodC* gene with no CuZnSOD activity in phylogenetic division II encapsulated strains (13, 18). The lack of CuZnSOD activity in *sodC*-containing encapsulated strains

was proposed to occur due to a substitution mutation that changed an active-site histidine to a tyrosine. Although all of the *sodC* genes from the capsule derivatives in the current study possessed a tyrosine at the same position (unpublished results), the genes also contained various frameshift insertions that resulted in truncated reading frames.

As mentioned above, previous studies have reported discrepancies in the prevalence of *sodC* among NT *H. influenzae* strains where *sodC* and CuZnSOD activity were found in nearly half of the NT *H. influenzae* strains of one study (13) but were not found among NT *H. influenzae* strains in two later studies (9, 18). Based on our current results, NT *H. influenzae* possessing *sodC* and CuZnSOD activity in the initial report may have been correct. Alternatively, the *sodC*-containing strains may have been nonhemolytic *H. haemolyticus* or capsule-deficient mutants of encapsulated *H. influenzae* that were misclassified as NT *H. influenzae* (21, 40).

Another possible explanation for the discrepancy in the literature, however, may be found in the selection of NT *H. influenzae* strains examined. In our study, 3/56 (5.4%) *H. influenzae* isolates (Mr27, AAr27, and I8809) that contained a *sodC* gene were obtained from the middle ears of children with otitis media, while 18/113 (15.9%) *H. influenzae* isolates that contained a *sodC* gene were obtained from the throats of healthy children or adults (unpublished results) (Table 1). Similarly, Norkov-Lauritsen (23) recently found that only 6/480 (1.25%) clinical *H. influenzae* isolates possessed a *sodC* gene and that all six strains were related to encapsulated phylogenetic division II *H. influenzae* strains. Commensal isolates were not examined in that study. Therefore, the presence or absence of *sodC* and CuZnSOD activity among NT *H. influenzae* strains used in earlier studies may have been due to the virulence potential of the isolates examined (18). These observations also highlight recent studies that document a number of genetic differences between disease and commensal NT *H. influenzae* strains (10, 24, 37).

The role of *sodC* and related CuZnSOD activity in the virulence of Gram-positive and Gram-negative pathogenic bacteria has been the subject of considerable study (2, 6). In pathogenic bacteria that have a functional *sodC* gene, the translated protein is a periplasmic or membrane-bound enzyme thought to inactivate superoxide released during the respiratory burst of phagocytes, thereby contributing to the virulence of the pathogen (2). The role of *sodC* and corresponding CuZnSOD activity in the pathogenesis of NT *H. influenzae* would be expected to be negligible, since only a small proportion of strains carry the gene, and those strains are predominantly commensal isolates. The role of *sodC* in the host interactions of commensal NT *H. influenzae* or in commensal *H. haemolyticus* and *H. parainfluenzae* is not known (14, 16). Aside from superoxide dismutase activity, some bacterial *sodC* genes are known to encode structural variants that contain species-specific insertions, deletions, and substitutions that provide non-cofactor-related, metal-binding properties via their N termini or subunit-subunit interactions (3, 33). Such diverse functional properties of CuZnSOD in *H. haemolyticus*, *H. parainfluenzae*, or *H. influenzae*, however, remain to be determined. The presence of *sodC* in commensal species, such as *H. haemolyticus* and *H. parainfluenzae*, suggests an important role for the gene in the commensal microbiology of

humans. Indeed, a better understanding of commensal microbiology is necessary to differentiate “normal” from disease-related growth of opportunistic pathogens, such as NT *H. influenzae*, a species that resides in humans predominantly as a commensal bacterium but infrequently as a pathogen.

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