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Role of Manganese-Containing Superoxide Dismutase in Oxidative Stress and Virulence of *Streptococcus pneumoniae*

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***Streptococcus pneumoniae* was shown to contain two types of superoxide dismutase, MnSOD and FeSOD. Levels of MnSOD increased during growth in an aerobic environment. The *sodA* gene, encoding MnSOD, of virulent *S. pneumoniae* type 2 strain D39 was inactivated to give mutant D39HY1. Aerobically, D39HY1 had a lower growth rate than the wild type and exhibited susceptibility to the redox-active compound paraquat, but anaerobic growth of D39HY1 was identical to that of the wild type. Virulence studies showed that the median survival time of mice infected intranasally with D39HY1 was significantly longer than that of mice infected with the wild-type pneumococcus. In contrast to the wild type, D39HY1 did not multiply in lungs during the first 24 h but thereafter grew at the same rate as the wild type. Appearance in the bloodstream was also delayed, but growth in the blood was unimpaired by the *sodA* mutation. The pattern of inflammation in lungs infected with D39HY1 differed from that in wild-type-infected mice. After infection with D39HY1, neutrophils were densely packed around bronchioles, in contrast to the wild-type infection, where neutrophils were more diffusely localized.**

Streptococcus pneumoniae is the etiological agent of pneumonia, otitis media, and meningitis in humans (1, 36). Increasing antibiotic resistance (26, 39) and drawbacks of the current vaccine (2) have increased the need for a better understanding of the pathogenesis of pneumococcal diseases. Several bacterial components, including the capsule, the cell wall, and some pneumococcal protein products, such as pneumococcal surface protein A, pneumolysin, and neuraminidase, are thought to play a role in the virulence of pneumococci (36). There are, however, many questions about pathogenic mechanisms and the basic metabolism of the pneumococcus that remain to be answered. One of them is how the pneumococcus copes with oxygen and its reactive derivatives.

Reactive oxygen intermediates, including superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH^-$), have many deleterious effects on living organisms, ranging from DNA strand damage to peroxidation of membrane lipids (15, 25, 47). Sources of reactive oxygen intermediates are abundant and include incomplete reduction of oxygen during respiration, exposure to radiation or to redox-active compounds, and the respiratory burst of phagocytes (18). Aerobic organisms, however, have developed several enzymatic and nonenzymatic mechanisms to detoxify these very active compounds. Enzymatically, oxygen radicals are removed mainly by the action of four enzymes: superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase (7).

Superoxide dismutases (SODs) are metalloenzymes that catalyze the conversion of superoxide molecules to hydrogen peroxide and molecular oxygen and therefore form one of the cell's major defense mechanisms against oxidative stress (33). In prokaryotes, depending on the metal cofactor, three types of SODs have been described: Cu-Zn- (SodC), Fe- (SodB), or Mn-type (SodA) SODs. FeSOD and MnSOD are characteristically prokaryote enzymes, but MnSOD is also present in mitochondria of eukaryotes. Cu-ZnSOD, on the other hand, is primarily found in the cytosol of many eukaryotic organisms (19). However, several prokaryotes containing Cu-ZnSOD have recently been reported (29, 45). As well as being important in the detoxification of superoxide radical during aerobic metabolism, SOD has also been shown to be a virulence factor for several pathogenic bacteria. SOD in *Nocardia asteroides* was found to protect the bacterium from the oxidative killing mechanisms of neutrophils during infection (3). In another study done with a *Shigella flexneri* Δ*sodB* mutant, the mutant strain was less virulent than wild-type bacteria in the rabbit ligated ileal loop model (16). Pesci and coworkers (37) showed that a *sodB* insertion mutation in *Campylobacter jejuni* caused a significant reduction in the ability of the bacterium to invade an embryonic intestinal cell line. Inactivation of *sodA* in *Yersinia enterocolitica* resulted in a marked reduction in virulence of the organism in a mouse infection model after intravenous infection when compared to its parental strain (40). More recently, it was reported that a Cu-ZnSOD mutant strain of *Neisseria meningitidis* was less virulent in a mouse intraperitoneal infection model than the wild-type strain (46). On the other hand, a *sodA* deletion mutation of *Bordetella bronchiseptica* and *Bordetella pertussis* did not affect the virulence of these species in mice following intranasal infection (17).

S. pneumoniae is a facultative anaerobe that lacks catalase (18a). Thus, the absence of this enzyme suggests that SOD may play a critical role against oxidative stress, affecting both the survival and, consequently, the virulence of the organism. In this paper, we report some characterization of a pneumococcal SOD. An isogenic *sodA* insertion mutant strain of *S. pneumoniae* was constructed in order to study the role of the en-

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zyme during in vitro growth, and the mutant also was tested in a mouse model of bacteremia and pneumonia.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. Virulent, capsular type 2 *S. pneumoniae* strain D39 was used to construct the isogenic *sodA*-deficient mutant. The parent strain and its *sodA*-deficient derivative were routinely grown in brain heart infusion (BHI) broth or on blood agar base containing 7% (vol/vol) horse blood. Where appropriate, a defined medium containing 0.4% (wt/vol) sucrose, originally described by Lacks (27), was used. Overnight incubation of the pneumococcal cultures was done at 37°C in an anaerobic jar (Gas Pak; Becton Dickinson) or on a shaker (aerobic growth). *Escherichia coli* strains DH5α and XL1 Blue were grown in Luria-Bertani broth or on Luria-Bertani agar plates. Where appropriate, spectinomycin and ampicillin were added to growth media at the following concentrations: ampicillin at 100 μg/ml and spectinomycin at 200 μg/ml for *E. coli* or at 1 mg/ml for *S. pneumoniae*.

Recombinant DNA techniques. Plasmid DNA preparation from *E. coli* was done using ion exchange columns from QIAGEN (Hilden, Germany). Pneumococcal chromosomal DNA was isolated as described previously (41). Standard methods were followed for restriction digestion, ligation, gel electrophoresis of DNA, and Southern blotting (42). DNA was introduced in *E. coli* by electrotransformation (48). Competence-stimulating peptide was used to transform encapsulated *S. pneumoniae* (23). PCR amplifications were carried out in a thermal cycler (Omnigene HBTR3CM; Hybaid Ltd., Teddington, United Kingdom) with 50 ng of template DNA primers at a final concentration of 0.1 μM, each deoxynucleotide at 200 μM, 1× reaction buffer (20 mM Tris-HCl, pH 8.4, and 50 mM KCl), 2 mM $MgCl_2$, and 2 U of *Taq* DNA polymerase. The PCR mixture was subjected to a denaturation step (3 min at 95°C), followed by 35 cycles of amplification (30 s of denaturation at 95°C, 2 min of annealing at 50°C, 90 s of elongation at 72°C) and elongation (12 min at 72°C) steps. Sequence analyses were done manually by using a Sequenase 2.0 sequencing kit (U. S. Biochemicals).

Primers. A 480-bp internal region of the *sodA* gene (sod_{int}) was amplified by PCR from *S. pneumoniae* by using the degenerate primers HYK1 (5′-CCGTA [CT]GCGTA[CT]GA[CT]GCG[CT]TGGA[AG]CC-3′) and HYK2 (5′-A[AG] [AG]TA[AG]TAGGC[AG]TG[CT]TCCCAGAC[AG]TC-3′), whose sequences were based on conserved amino acids from microbial SODs PY(PAT)YDALEP and DVWEHAYYL, respectively (38). (Nucleotides in brackets were used as alternatives to the one left of the bracket; for example, C and T could be used instead of A.) The primer SOD4 (5′-GGCGCGCCCTCCTTCAGCAGAACTG GCA-3′) anneals to nucleotides 257 to 276 within sod_{int} and generated an *Asc*I site. Primer SOD5 (5′-GGCGCGCCCAATTCCCAGAAAAGAGCGT-3′) binds to nucleotides 244 to 263 of the complementary strand of sod_{int} and created an *Asc*I site.

The oligonucleotide primers spec1 (5′-CAGGCGCGCCATCGATTTTCGTT CGTGAAT-3′) and spec2 (5′-AAGGCGCGCCCATATGCAAGGGTTTATT GT-3′), which add *Asc*I sites to 5′ and 3′ ends, respectively, were used to amplify a spectinomycin resistance gene from pDL278 (30). The SOD6 (5′-TAGCAGC TGCGTACAATTCAT-3′) primer based on the 3′ end of the entire *sod* gene, which was discovered during the course of this work (data not shown), was used to detect insertion of the spectinomycin resistance cassette into the pneumococcal chromosome. When this primer was used in combination with primer HYK1, a 577-bp *sod* gene fragment was amplified.

Construction of *S. pneumoniae* Δ*sodA* mutant. A 480-bp internal fragment of the *sodA* gene (sod_{int}), representing approximately 85% of the gene, was obtained by PCR by using the primers HYK1 and HYK2. This fragment was cloned into pPCR-Script Amp SK(+) to produce pSOD1. The primers SOD4 and SOD5 were used to introduce a unique *Asc*I site into the sod_{int} fragment in pSOD1 by reverse PCR. This site was needed to ligate a spectinomycin resistance gene cassette that was used as a selective marker for transformants. The resulting plasmid was designated pSOD2. A spectinomycin resistance gene carrying 5′ and 3′ *Asc*I sites was amplified from pDL278 (14) with the spec1 and spec2 primers. Following digestion with *Asc*I, pSOD2 and the spectinomycin gene were ligated, and the resulting construct was designated pHY2. Pneumococci were transformed with pHY2, and spectinomycin-resistant colonies were selected. Because pHY2 is unable to replicate in gram-positive bacteria, recombinants were anticipated to be spectinomycin resistant by virtue of the cassette entering the chromosome by homologous recombination via *sod* sequences.

Sequencing strategy. The sequence of *sodA* in D39 that corresponded to that reported by Poyart et al. (38) was determined by sequencing PCR fragments generated with primers HYK1 and HYK2. To obtain the 5′ end of the gene, we took advantage of the presence of a spectinomycin resistance gene cassette within *sodA* of D39HY1. *Pst*I-digested DNA from D39HY1 was inserted into pBluescript SK(+), selecting for spectinomycin-resistant recombinants. The 5′ sequence of *sodA* was obtained from analysis of the plasmid (pPSTSOD) from one of these recombinants. The sequence of the 3′ end of the gene was obtained by first screening an available lambda ZAP expression library of a type 1 pneumococcus by using radiolabelled sod_{int} fragment as the probe. As a result, a strongly hybridizing clone, designated pHYSOD1, was isolated. Sequence analysis of pHYSOD1 provided the 3′ end, and downstream sequences, of *sodA* in the type 1 strain. From this information, a primer downstream of *sodA* was used with a primer from within the gene to amplify by PCR the 3′ end of *sodA* from D39. These PCR fragments were then sequenced.

Preparation of bacterial cell protein. All manipulations were carried out at 4°C. Cell pellets of late-log-phase cultures were collected by centrifugation at 2,000 × *g* for 15 min and were resuspended in 50 mM phosphate buffer, pH 7. This cell suspension was sonicated by using a Sanyo Soniprep model 150 sonicator, and cell debris was removed by centrifugation at 22,000 × *g* for 30 min. Total protein concentration of the lysate was determined by the Bradford assay; bovine serum albumin was used as a standard (5).

SOD activity assays. Extracts of *S. pneumoniae*, prepared as described above, were separated by polyacrylamide gel electrophoresis (PAGE) under nondenaturing conditions. Electrophoresis was carried out by a modification of the Davis (12) procedure as described by Langford et al. (28). The gels were then stained for SOD activity by nitroblue tetrazolium, as described previously (4). To assess the type of SOD, 5 mM KCN or 5 mM H_2O_2 was added to the staining solution (11). Where appropriate, a cell extract of *E. coli* QC771, as a source of MnSOD and FeSOD (prepared as described above), or bovine erythrocyte Cu-ZnSOD (Sigma) was used as a control. SOD was assayed quantitatively by the xanthine oxidase-cytochrome *c* method (33).

Hydrogen peroxide assay. Bacteria were grown until mid-log phase, were harvested by centrifugation, and were resuspended in an equal volume of a solution containing 50 mM Tris-HCl, pH 7.5, and 10 mM glucose. The bacterial culture was incubated at 37°C for 30 min, and cells were removed by centrifugation, as described by Spellerberg et al. (44). Bacterial cells (4×10^7) were used to measure hydrogen peroxide production fluorimetrically by the method of Jackett et al. (24), in which the level of H_2O_2 was assayed by following the oxidation of *p*-hydroxyphenyl acetic acid by H_2O_2 catalyzed by horseradish peroxidase.

In vivo virulence test. Female MFI outbred mice, weighing 30 to 35 g, were obtained from Harlan Olac (Bicester, United Kingdom). A standardized inoculum was prepared as follows: 200 μl of an overnight culture of pneumococcal strains was injected into the peritoneal cavity of mice and was recovered 24 h later from cardiac blood. This was done to standardize the virulence of inocula. Passaged bacteria were grown overnight at 37°C in BHI broth, were harvested by centrifugation, were resuspended in 1 ml of BHI containing 20% (vol/vol) fetal calf serum, and then were diluted with fresh BHI-fetal calf serum to give an A_{600} of 0.7. The culture was incubated at 37°C for 4 to 5 h. The viability of the suspension was determined by serial dilution in sterile nanopure water and plating on blood agar plates in duplicate. Aliquots of the suspension were stored at −70°C until required. Under these conditions, the viability of strains was unaffected for at least 1 month. When needed, an aliquot was thawed slowly at room temperature, and bacteria were harvested by centrifugation before resuspension in sterile phosphate-buffered saline (PBS).

To determine the virulence of pneumococcal strains, mice were lightly anesthetized with 2.5% (vol/vol) fluothane (Zeneca, Macclesfield, United Kingdom) over oxygen (1.5 to 2 liters/min), administered with a calibrated vaporizer. A 50-μl sample of PBS containing 10^6 CFU of *S. pneumoniae* was given into the nostrils. Mice were monitored for clinical symptoms (progressively starry coat, hunched, lethargic, and then moribund) for 14 days, and those that became moribund were accepted to have reached the end point of the assay and were killed humanely.

To measure in vivo growth of bacteria, preselected groups of mice were deeply anesthetized with 5% (vol/vol) fluothane at prechosen intervals following infection and blood was collected by cardiac puncture. Following this, mice were killed by cervical dislocation. The lungs were removed into 10 ml of sterile distilled water, were weighed, and then were homogenized in a stomacher laboratory blender (Seward Medical, London, United Kingdom). Viable counts in homogenates and in blood were determined as above on selective and nonselective media. In the case of intravenous infection, the desired number of bacteria was given in 100 μl of sterile PBS through a tail vein.

Histology. Lung samples were embedded in Tissue Tek OCT (Miles) tissue embedding compound, were snap-frozen in liquid nitrogen, and were stored at −70°C until required. Before sectioning, samples were kept at −20°C overnight. Sections (20 μm) were cut with a cryostat and were subsequently stained with hematoxylin and eosin. One observer did a histological analysis of sections, blinded to the identity of samples, by using bright field microscopy.

Statistical analysis. Data were analyzed by analysis of variance followed by Tukey-Kramer multiple comparisons test or by Mann-Whitney U test, as appropriate. Growth rates were analyzed by comparison of regression slopes.

Nucleotide sequence accession number. The sequence of pneumococcal *sod* has been deposited in GenBank under accession no. AF162664.

RESULTS

Construction of *S. pneumoniae* Δ*sodA* mutant. A Δ*sodA* mutant was made in the virulent type 2 strain D39, as described in Materials and Methods. PCR and Southern blot analysis (data not shown) confirmed disruption of the *sod* gene in the chromosome of transformants. Furthermore, none of the transformants hybridized with the pPCR-Script Amp SK(+) probe,

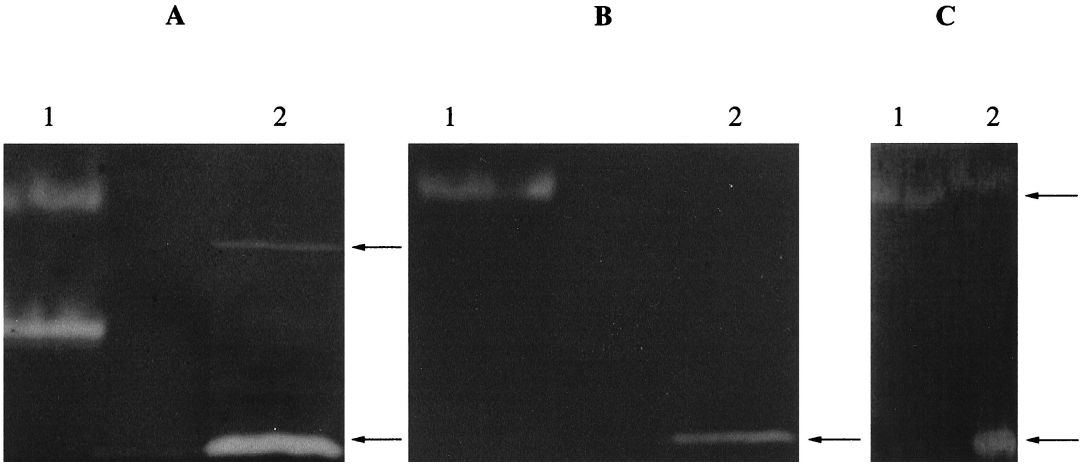

FIG. 1. Type inhibition assay and insertional inactivation of SOD activity. Nondenaturing PAGE gels for determination of SOD activity were treated (B) or not treated (A) with 5 mM H_2O_2. (A and B) Lanes 1 contain *E. coli* extract and lanes 2 contain *S. pneumoniae* extract. (C) Extract of the wild type (lane 2) or mutant (D39HY1) (lane 1) was separated on nondenaturing PAGE gels. The lane containing the wild type had two bands of activity, a minor and a major band, whereas D39HY1 had only the minor activity band. Arrows indicate the minor and major SODs.

indicating that a double-crossover event had occurred (data not shown). One transformant, designated D39HY1, was chosen for further study.

Sequence of the *sodA* gene and site of insertion in D39HY1. A partial sequence of pneumococcal *sod* had been published (38). We determined the sequence of the remaining parts of the gene and deposited the information in GenBank (accession no. AF162664). The internal sequence of *sodA* agreed exactly with the sod_{int} sequence of Poyart et al. (38). By comparison, the pneumococcal SOD most resembled SodA of known bacterial species (data not shown). Furthermore, analysis of the amino acid sequence of the pneumococcal SOD showed that it contained residues that are unique to MnSODs (35), such as glycine at positions 76 and 77 and phenylalanine, glutamine, and aspartic acid at positions 85, 151, and 152, respectively (data not shown). A putative rho-independent transcription terminator (GGAGGGAAGAATTGTTCTTCTCTTT; $\Delta G = -12.04$ kcal/mol) occurs downstream of *sodA*. The sequencing also showed that the site of insertion of the spectinomycin resistance gene was between nucleotides 279 and 280.

SOD activity detection in D39HY1 and in the wild-type strain. Separation of protein extracts from the whole cells under nondenaturing polyacrylamide gel conditions showed two electrophoretically distinct activity bands for the wild type (Fig. 1A, lane 2). The intensities of these bands were considerably different, as were their mobilities. The activity of these bands was not affected by treatment with 5 mM KCN, indicating the lack of a Cu-ZnSOD in the pneumococcus (data not shown). However, H_2O_2 inhibited the activity of the minor band in the wild type (Fig. 1B, lane 2) and in D39HY1 (data not shown). This suggests that the minor band was an iron-cofactored SOD. The major band was partially inhibited by H_2O_2. By exclusion, the major SOD in the pneumococcus appears to be MnSOD; MnSOD is not affected by KCN and is either fully or partially resistant to H_2O_2 (7, 11, 32). In the mutant strain, however, only the minor activity band was seen (Fig. 1C, compare lanes 1 and 2), confirming that D39HY1 is a *sodA* mutant.

Aeration increases the expression of SOD. The SOD activity of wild-type D39 grown anaerobically was 2.4 U of protein per mg (± 0.1; $n = 3$), whereas aerated cultures had 4.1 U of protein per mg (± 0.49; $n = 3$) ($P < 0.05$). In contrast, the SOD activity of D39HY1 was not statistically different ($P > 0.05$) when grown anaerobically or aerobically: 0.5 U/mg (± 0.04; $n = 3$) anaerobically and 1.0 U/mg (± 0.06; $n = 3$) aerobically. This activity in D39HY1 represents the activity of the FeSOD.

Hydrogen peroxide production in D39HY1. Since the catalytic activity of superoxide dismutase generates H_2O_2, we measured the ability of D39HY1 to generate this molecule. H_2O_2 production was quantified in the mid-log-phase cultures by a fluorimetric assay. There was no statistical difference ($P > 0.05$) between the mutant and the wild type in H_2O_2 production, the mean of the four experiments being 51 nmol/10^7 bacteria (± 1.72) for D39HY1 and 50 nmol/10^7 bacteria (± 1.35) for the wild type.

In vitro growth characteristics of the D39HY1. The colony size of the mutant strain was comparable to that of the wild type on blood agar plates kept under anaerobic conditions. Aerobically, however, D39HY1 grew slower than wild type. D39HY1 formed alpha-hemolytic colonies under both conditions on blood agar. To examine possible effects of the *sodA* mutation, the SOD-deficient strain was tested under aerobic and anaerobic growth conditions in broth culture. In addition, the redox-active compound paraquat was added to the culture medium as a superoxide generator to assess any effect of excess superoxide on the mutant strain.

The rate of increase in the optical density (OD) of the wild-type strain did not change under aerobic or anaerobic conditions ($P > 0.05$) (Fig. 2A and B). However, D39HY1 had a lower rate of aerobic increase than anaerobic increase ($P < 0.001$) (Fig. 2A and B). D39HY1 had a rate statistically similar to the wild type under anaerobic conditions ($P > 0.05$), but aerobically, it had a significantly lower rate of increase in OD than the wild type ($P < 0.001$). The rate of increase of the wild-type strain was not significantly affected by any concentration of paraquat used, under either aerobic or anaerobic conditions ($P > 0.05$) (Fig. 2A and B), but addition of 0.001 M paraquat affected the rate of increase of D39HY1 in both conditions ($P < 0.001$).

In addition to growth characteristics in complex medium, we investigated growth of the mutant strain in defined medium (Fig. 2C). Anaerobically, the wild type and D39HY1 showed statistically similar rates of increase of OD ($P > 0.05$), but

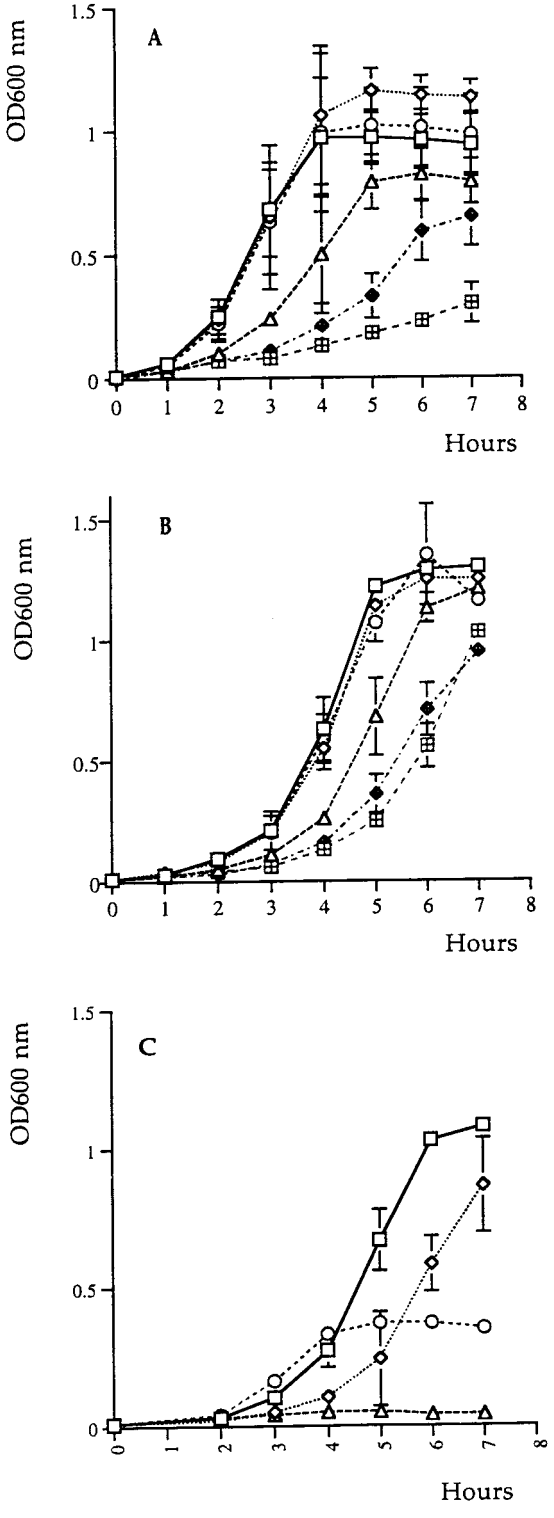

FIG. 2. In vitro growth of wild type and *sod* mutant strain (D39HY1). Aerobic growth (A) and anaerobic growth (B) in BHI broth with and without paraquat and growth in defined medium (C) are shown. (A and B) Strains and concentrations of paraquat are represented with symbols as follows: wild type with no paraquat (-□-), wild type with 0.001 M paraquat (-◇-), wild type with 0.0001 M paraquat (···○···), D39HY1 with no paraquat (-△-), D39HY1 with 0.001 M paraquat (-□··), and D39HY1 with 0.0001 M paraquat (···◆···). (C) Strains and growth conditions are represented with symbols as follows: wild type, anaerobic conditions (-□-); wild type, aerobic conditions (···○···); D39HY1, anaerobic conditions (-◇-); and D39HY1, aerobic conditions (-△-). Each point is the mean of three experiments.

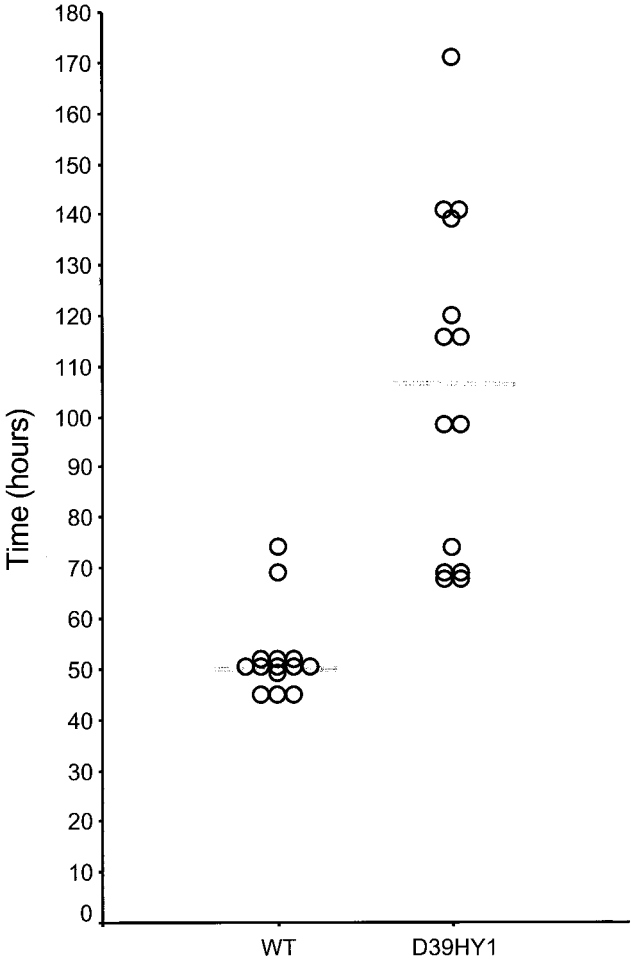

FIG. 3. Survival of mice infected intranasally with D39HY1 or the wild-type (WT) pneumococcus. Symbols show the time that individual mice became moribund. The horizontal bars mark the median time to the moribund state.

D39HY1 was unable to grow under aerobic conditions in defined medium.

In vivo studies. The median survival time of mice was determined after intranasal infection with D39HY1 or the wild type to investigate if the *sodA* mutation had any effect on the ability of *S. pneumoniae* to cause disease. Infected mice were observed for 7 days, and the development of clinical symptoms was recorded. Around 19 h postinfection, individual mice within the wild-type-infected group began to show symptoms (starry coat), and all mice in this group showed symptoms by 45 h. However, none of the D39HY1-infected mice showed any sign of illness by 45 h postinfection, at which point three mice in the wild-type-infected group were moribund. No animal in the D39HY1-infected group showed the initial symptoms until 47 h postinfection, and the first moribund case in this group was not recorded until 69 h after infection. Within 7 days, all 14 mice in each group became moribund (the end point of the assay). Statistical analysis showed that the D39HY1-infected group survived significantly longer (107 h) than the wild-type-infected mice (50 h) ($P < 0.001$) (Fig. 3).

To further investigate the nature of impaired virulence, we went on to investigate the growth of bacteria in blood and lungs of mice. Significantly fewer bacteria were recovered in the lungs of mice infected with D39HY1 than in the wild-type-

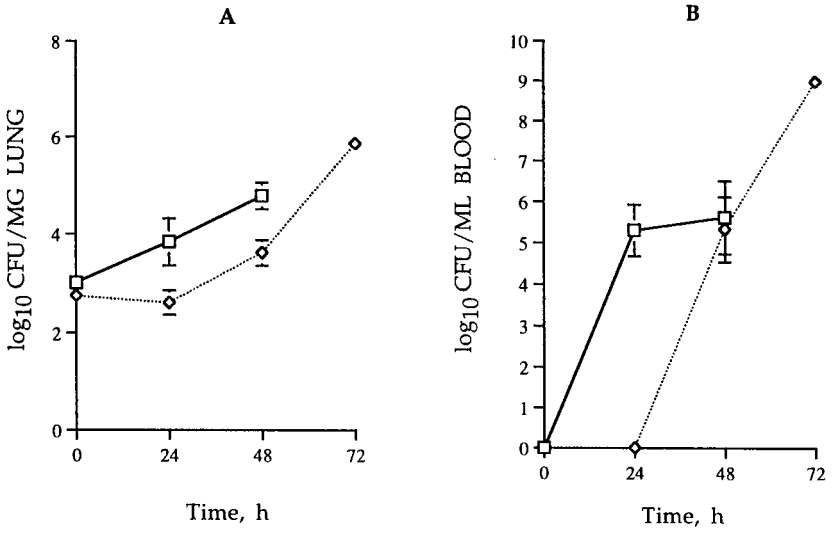

FIG. 4. Time course of bacterial growth in the lungs (A) and blood (B) of mice infected intranasally with D39HY1 or the wild-type pneumococcus. Symbols indicate mice infected with the wild type (-□-) or D39HY1 (-◇-). Each point is the mean log data from five MF1 female mice except 72 h, which is from three mice. Error bars show the standard errors of the means.

infected mice 24 and 48 h after infection ($P < 0.05$) (Fig. 4A). The explanation for this appears to be a lag in the first 24 h with D39HY1, which did not occur with the wild type. After 24 h, the rates of growth in the lungs of D39HY1 and the wild type were not significantly different (0.15 h^{-1} and 0.09 h^{-1}, respectively; $P > 0.05$). D39HY1 is apparently less invasive than the wild type, since, in contrast to the wild type, the mutant was not recovered from blood at 24 h (Fig. 4B). However, by 48 h, the level of D39HY1 in the blood was similar to that of the wild type, and the concentration of D39HY1 in blood had reached approximately 10^9 bacteria/ml by 72 h. The maximum growth rates for D39HY1 and the wild type were the same (0.51 h^{-1}).

Changes in growth rate due to emergence of revertants were discounted because at each time point the number of colonies was the same when determined on selective or nonselective medium (data not shown). Furthermore, Southern blotting of mutants recovered from lungs, using the spectinomycin resistance cassette as the probe, confirmed the presence of the antibiotic resistance gene (data not shown).

In addition to intranasal challenge, the virulence of the mutant strain was tested by observing survival for 7 days after intravenous infection. All mice survived infection with 50 CFU, but both of the two mice in each group infected with 5×10^2 and 1×10^3 CFU became moribund. Following intravenous infection with 10^4 CFU, the rates of growth of the wild type and D39HY1 were found to be similar: 0.17 and 0.22 h^{-1}, respectively ($P > 0.05$). There also was no difference seen in the median survival times of the mice infected intravenously with D39HY1 (46 h) and those infected with the wild type (45 h) ($P > 0.05$).

Histological examination of lung tissue. Histological analysis of lung tissue sections from mice infected with wild-type pneumococci showed inflammation and cellular infiltration centered around bronchioles and perivascular areas (Fig. 5A). The foci of inflammation were restricted to certain bronchioles and perivascular areas close to these bronchioles at 24 h postinfection. Inflammation presented itself as bronchiole wall thickening and heavy cellular infiltration around such bronchioles.

By 48 h postinfection, bronchiole wall thickening had increased, along with the emergence of fibrous tissue (Fig. 5B). Additionally, cellular infiltration had increased with the extension of inflammatory cells from bronchioles and perivascular areas into the surrounding lung parenchyma, with focal areas of consolidation becoming larger and more diffuse. The presence of alveoli in lung sections at this time point was hardly distinguishable due to intensive tissue edema.

Mice infected with D39HY1 showed less tissue inflammation and cellular infiltration into perivascular areas between infected bronchioles than wild-type-infected mice at both time points (Fig. 5C and D). However, despite this and lower levels of pathologic tissue damage, some bronchioles exhibited levels of inflammation comparable to wild-type-infected mice at 24 h. Bronchioles also exhibited substantial levels of cellular infiltration 48 h postinfection. Compared to wild-type-infected mice, however, the cellular infiltration around such bronchioles appeared to be more intense and localized and did not extend into the perivascular areas.

DISCUSSION

Our data revealed that the pneumococcus has two types of SOD, an MnSOD as the major fraction and an FeSOD as a minor fraction. Identification of the minor band as FeSOD was on the basis of its sensitivity to inhibition by H_2O_2. The occurrence of this band from D39HY1 confirmed that it was a product of a separate gene and not due to Fe cofactoring the *sodA* product. The major band was identified as an MnSOD because of the extent of its resistance to H_2O_2 and on the basis of the gene sequence. MnSODs are classically described as being completely resistant to H_2O_2, but the pneumococcal SOD appeared to be slightly sensitive to H_2O_2. This might be because a proportion of the SOD in the major band is in the form of a hybrid SOD, cofactored in the main by Mn but with some Fe also. Such a hybrid would have limited sensitivity to H_2O_2 (10). However, the expectation that a hybrid would have a pI different from those of the other two SODs and appear as a third band (5) contradicts this idea.

Lack of Cu-ZnSOD in the pneumococcal whole-cell extract

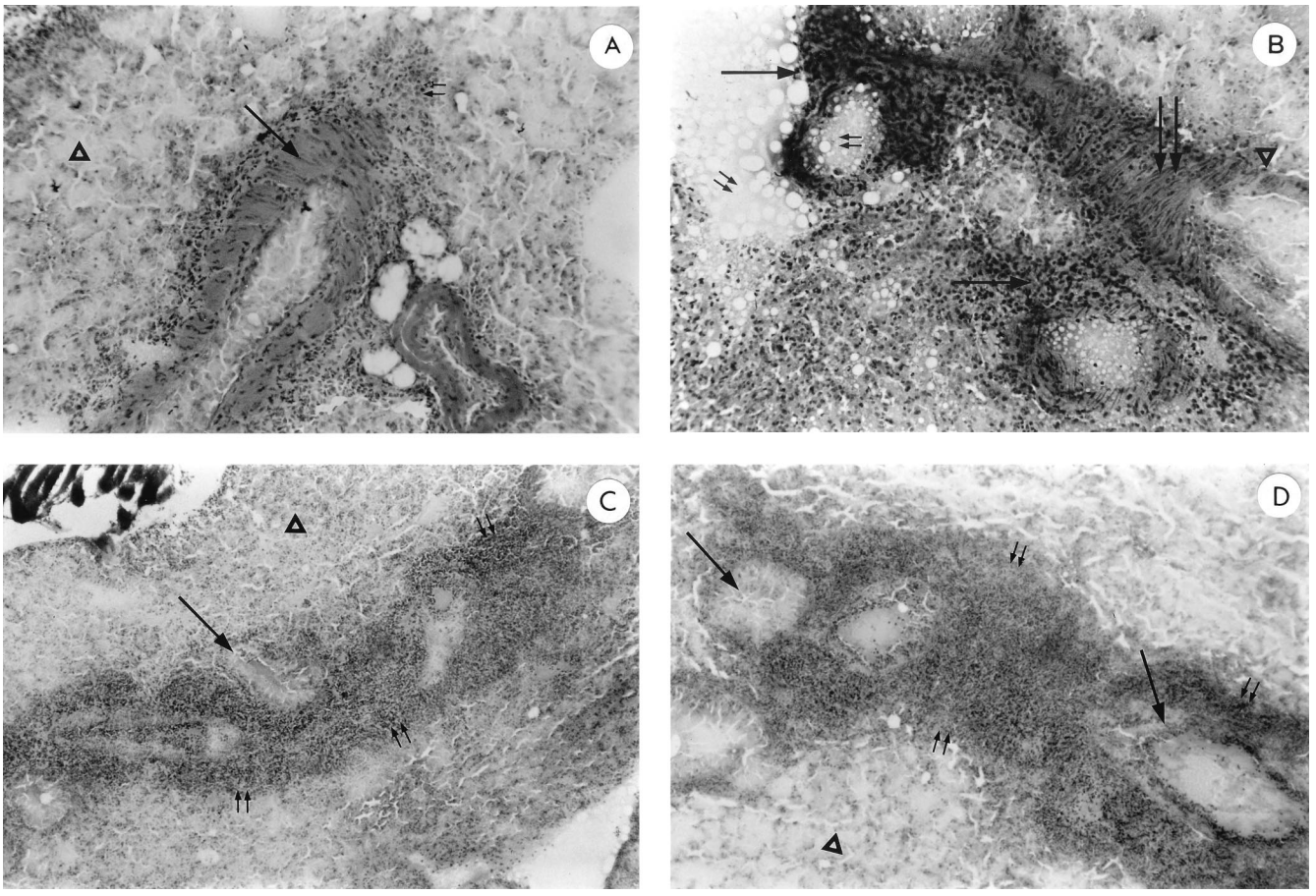

FIG. 5. Lung histology. Light microscopy of cryostat cut frozen sections (20 μm) of lung tissue from mice infected with 10^6 wild-type bacteria and sacrificed at 24 h (panel A; magnification, ×400; large arrow, bronchiole wall; pair of small arrows, cellular infiltration; open arrowhead, perivascular space) and 48 h (panel B; magnification, ×400; large arrow, heavy cellular infiltration; pair of large arrows, fibrosis; pair of small arrows, edema; open arrowhead, bronchiole wall) and from mice infected with 10^6 D39HY1 bacteria and sacrificed at 24 h (panel C; magnification, ×300; large arrow, bronchiole wall; pair of small arrows, heavy cellular infiltration; open arrowhead, perivascular space) and 48 h (panel D; magnification, ×400; large arrow, bronchiole wall; pair of small arrows, heavy cellular infiltration; open arrowhead, perivascular space). Sections were stained with hematoxylin and eosin.

was suggested by the type inhibition assays. This conclusion was supported by an analysis of the pneumococcal genome, available from The Institute for Genomic Research through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov), by using two Cu-ZnSOD consensus sequences: GFHIHENGSCG and GGGGARIACGVI. No significant similarities were found.

The amount of SOD in pneumococci varied depending on aeration. Although no significant change in the amount of FeSOD occurred, more MnSOD activity could be detected under aerobic conditions than under anaerobic conditions. These findings are consistent with observations with other bacteria (13, 20, 21). We took this observation as suggesting that the ability to adapt to oxidative stress was part of the virulence of this respiratory pathogen. Thus, to examine the role of the MnSOD for pneumococci, we mutated the *sodA* gene of *S. pneumoniae*. Our supposition was supported by the observation that the mutant, D39HY1, had an impaired ability to survive oxidative stress either from an oxygenated environment or due to a flux of superoxide from paraquat. In a defined medium, which presumably lacks the superoxide scavengers present in complex media, inactivation of *sodA* was lethal for pneumococci in oxygen. Impaired growth appears to be a common characteristic of bacteria deficient in cytosolic SOD, being also observed in *sodA* mutants of *Streptococcus mutans* (34), *Haemophilus influenzae* (13), *Pseudomonas aeruginosa* (22), and *Lactobacillus lactis* (43) and in *E. coli sodA sodB* mutants (9).

The susceptibility of D39HY1 to paraquat under anaerobic conditions was unexpected, because in the absence of oxygen, paraquat does not give rise to superoxide (31). An explanation is that strict anaerobic conditions were not achieved in this study. If this is so, then we are probably underestimating the increase in SodA when pneumococci move from anaerobic to aerobic conditions.

In vivo analysis of D39HY1 in a mouse model of pneumonia and bacteremia showed that *sod* mutation reduced the virulence of *S. pneumoniae* after intranasal infection. Reduction in virulence was dependent, however, on the route of infection, as no difference was seen between the wild type and D39HY1 when infection was intravenous. These observations presumably reflect differences in oxidative stress for pneumococci in the lungs and the blood. When infection is by the intranasal route, the crucial oxidative stress is found within the lungs, since once the bacteria invaded the bloodstream, no effect of impairment of growth in oxygen was seen. The conclusion that SodA protects against externally derived oxidative stress rather than against pneumococcus-derived superoxide is supported by

the identity in peroxide production by D39HY1 and the wild type. This observation was consistent with the report (44) that 99% of the peroxide produced by the pneumococci is derived from pyruvate oxidase.

It was during the early stages after intranasal infection of the lungs that the effect of impairment of ability to grow in an aerobic environment was seen. Only after the first 24 h in the lungs did D39HY1 begin to grow in the lungs, in contrast to the wild type. This could reflect the time taken for the bacteria to move from oxygen-rich environments in the alveoli and bronchioles into more microaerophilic areas. The ability to survive in oxygen-rich areas appears to be part of the repertoire for invasion. Thus, wild-type pneumococci, with oxygen resistance mechanisms intact, rapidly move from the bronchioles and invade the bloodstream, whereas pneumococci without SodA remain for a time within the lungs. Nevertheless, these pneumococci do eventually become invasive, although not as a result of the loss of the mutagenizing antibiotic resistance gene. Why D39HY1 should eventually begin to grow within the lungs and also become invasive is not clear. The microenvironment of the pneumococci may become anoxygenic due to the pathological processes and enable invasive mechanisms to operate. Alternatively, the pneumococci become adapted to the in vivo oxygen environment. The nature of any adaptive mechanisms occurring during this period and the effect of oxygen and oxygen radicals on pneumococcal virulence mechanisms will be of interest.

The different patterns of cellular infiltration into the lungs may be explained by the different invasive properties of the pneumococci. D39HY1 remains in the bronchioles longer than the wild type, and inflammatory cells congregate around these areas of the lung, whereas wild-type pneumococci spread throughout the lung tissue and, therefore, the distribution of neutrophils is more diffuse. Experiments to determine the precise localization of pneumococci in the lungs have been started.

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