Characterization of the *Streptococcus pneumoniae* NADH oxidase that is required for infection

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*Streptococcus pneumoniae* is an important human pathogen capable of causing serious infections. NADH oxidase, a factor necessary for infection, was previously identified as part of a signature-tagged mutagenesis screen of a *S. pneumoniae* clinical isolate, 0100993. The mutant, with a plasmid insertion disrupting the nox gene, was attenuated for virulence in a murine respiratory tract infection model. A complete refined nox deletion mutant was generated by allelic-replacement mutagenesis and found to be attenuated for virulence 105-fold in the murine respiratory tract infection model and at least 104-fold in a Mongolian gerbil otitis media infection model, confirming the importance of the NADH oxidase for both types of *S. pneumoniae* infection. NADH oxidase converts O2 to H2O. If O2 is not fully reduced, it can form superoxide anion (O2−) and hydrogen peroxide (H2O2), both of which can be toxic to cells. Bacterial cell extracts from the allelic-replacement mutant were found to lack NADH oxidase activity and the mutant was unable to grow exponentially under conditions of vigorous aeration. In contrast, the mutant displayed normal growth characteristics under conditions of limited aeration. The *S. pneumoniae* nox gene was cloned and expressed in *E. coli*. The purified His-tagged NADH oxidase was shown to oxidize NADH with a *Km* of 32 µM, but was unable to oxidize NADPH. Oxidation of NADH was independent of exogenous FAD or FMN.

**Keywords:** nox gene, reactive oxygen species, virulence

**INTRODUCTION**

*Streptococcus pneumoniae* is an important cause of pneumonia, meningitis and bacteraemia, as well as of otitis media in young children. The pathogenic mechanisms have been extensively investigated (AlonsoDeVelasco et al., 1995; Bruyn et al., 1992; Paton et al., 1993). The polysaccharide capsule has been shown to be an important virulence factor for infection (Kelly et al., 1994; Watson & Musher, 1990). In addition, several pneumococcal proteins, such as pneumolysin, autolysin and surface protein A, as well as some pneumococcal enzymes, such as neuraminidase, hyaluronidase, IgA1 protease and pyruvate oxidase, are also considered to play important roles in *S. pneumoniae* pathogenesis (Briles et al., 1998; Paton et al., 1993; Spellerberg et al., 1996).

*S. pneumoniae* is classified as a facultative anaerobe. Although it can grow in the presence or absence of oxygen, its energy metabolism is of an anaerobic type regardless of growth conditions (Konings & Otto, 1983; Poolman, 1993). However, if molecular oxygen (O2) is not fully reduced (four-electron reduction) to H2O, it can undergo one- or two-electron reductions to form reactive superoxide anion (O2−) and hydrogen peroxide (H2O2), both of which are toxic to cells. The enzyme that converts O2 to H2O is an NADH oxidase called Nox. It has been identified and characterized from...
Streptococcus faecalis (Badway & Karnovsky, 1980), Mycoplasma pneumoniae (Himmelfreich et al., 1996), Brachyspira hyodysenteriae (Stanton et al., 1999), Streptococcus pyogenes (Gibson et al., 2000) and Streptococcus mutans (Higuchi et al., 1993) and in S. pneumoniae it has been shown to be important for virulence in a murine model using an intraperitoneal challenge (Auzat et al., 1999). The enzyme was proposed to have a protective role in defense against O₂ toxicity (Higuchi, 1992), or to function as an oxygen sensor (Auzat et al., 1999). In S. mutans, a minor H₂O₂-forming NADH oxidase called Nox1 has also been described (Higuchi et al., 1993).

In this study, we confirm the importance of NADH oxidase for S. pneumoniae virulence by showing significant attenuation for a nox allelic-replacement mutant in two additional S. pneumoniae models of infection. In addition, we have cloned, expressed and purified this important factor for virulence, confirmed its NADH oxidase activity and performed preliminary biochemical characterization of the enzyme.

METHODS

Bacterial strains and growth conditions. S. pneumoniae human clinical isolate 0100993 (serotype 3) was used as the parent strain for the construction of a nox allelic-replacement mutant. S. pneumoniae strain R6 is a single-colony isolate of strain R36A obtained from Avery’s strain D39 (Avery et al., 1944; Ottolenghi & Hotchkiss, 1962). S. pneumoniae strains were grown at 37 °C with 5% CO₂ in Todd–Hewitt broth with 0.5% yeast extract or on tryptic soy agar plates containing 5% sheep blood, supplemented with 1 µg erythromycin ml⁻¹ when appropriate. To grow S. pneumoniae under different aeration conditions, 20 ml culture in a 250 ml flask was shaken at 200 r.p.m. (vigorous aeration) or kept static (limited aeration). The anaerobic environment (H₂ gas) was shaken at 200 r.p.m. (vigorous aeration) or kept static (limited aeration). The anaerobic environment (H₂ gas) was used as the host for plasmid construction and strain BL21 (DE3) was used for expression of the recombinant protein.

Phylogenetic analysis. Sequences were aligned with CLUSTALX (Thompson et al., 1994) followed by minimal manual editing. All gapped segments were removed from the analysis, leaving 389 positions. The phylogenetic tree was calculated by PUZZLE 4.02 (Strimmer & von Haeseler, 1996). Ten thousand quartet puzzling steps were performed with the BLOSSUM 62 model and eight gamma-distributed substitution rate categories.

Construction of a nox allelic-replacement mutant. An allelic-replacement cassette comprising a 511 bp fragment of upstream nox sequence, a 1234 bp fragment containing an ermA gene encoding resistance to erythromycin and a 465 bp fragment of directly downstream nox sequence was generated by PCR. This 2.2 kb cassette was purified and 500 ng was used to transform competent S. pneumoniae strain R6. Cells (10 × 10⁶) were incubated at 30 °C for 30 min followed by a 37 °C incubation for 90 min to allow expression of the erythromycin-resistance gene. Bacteria were plated in agar containing 1 µg erythromycin ml⁻¹. Following incubation at 37 °C for 48 h, any visible colonies were picked and grown overnight in Todd–Hewitt broth supplemented with 0.5% yeast extract. Chromosomal DNA from the allelic-replacement S. pneumoniae strain R6 mutant was isolated and used to transform S. pneumoniae strain 0100993. The transformation procedure was identical to that for S. pneumoniae strain R6 except that a competence-stimulating heptadecapeptide (Havarstein et al., 1995) was added at a concentration of 1 µg ml⁻¹ in the initial transformation mix. Mutants were selected by their abilities to grow in agar containing 1 µg erythromycin ml⁻¹.

NADH oxidase assay. NADH oxidase activities of the crude S. pneumoniae extracts or purified His-tagged Nox protein were measured spectrophotometrically at 30 °C using a Spectra-MaxPlus (Molecular Devices). The reactions were performed in the wells of a 96-well microtitre plate. Each well was preloaded with 180 µl 0.17 mM NADH in 50 mM potassium phosphate buffer pH 7.4. The reaction was initiated by adding 20 µl crude extracts or purified enzyme to a final volume of 200 µl. One unit of NADH oxidase activity was defined as the amount of enzyme (mg protein) that catalysed the oxidation of 1 µmol NADH to NAD⁺ per min at 30 °C.

H₂O₂ production. The H₂O₂ production assay was based on the method of Pick & Keisari (1980). The S. pneumoniae pellet was resuspended in 1 ml freshly made H₂O₂ working solution (5 mM KH₂PO₄, 1 mM KHP₀₄, 140 mM NaCl and 0.5 mM glucose, pH 7.4). Prior to the assay, phenol red and horseradish peroxidase were added to a final concentration of 0.46 mM and 0.046 U ml⁻¹ respectively. After incubation at 37 °C for 30 min with shaking, 10 µl 10 M NaOH was added to stop the reaction. The reaction mix was centrifuged and 200 µl supernatant was transferred to the wells of a 96-well plate. Absorbance was measured at 610 nm using a Spectra-MaxPlus. H₂O₂ production was described as nmol H₂O₂ produced per mg bacterial protein per 30 min.

Murine respiratory tract infection model. Bacteria for infection were prepared by inoculation of tryptic soy agar plates containing 5% sheep blood from frozen stocks followed by overnight growth at 37 °C in 5% CO₂. Bacteria were recovered from plates, resuspended in PBS and adjusted to OD₅₆₀ 0.95. Animals (male CBA/J mice, 14–16 g) were anaesthetized with isoflurane (3%) and 50 µl bacterial inoculum (7.0 × 10⁶ c.f.u.) was administered by intranasal instillation using a Pipetman (Rainin Instruction Co.). The mice were allowed to recover and given food and water ad libitum. Animals were observed three times daily and those unlikely to survive the challenge (i.e. exhibiting cyanosis, hypothermia, staring coat, or being moribund) were killed by CO₂ overdose. Surviving animals were killed at 6–48 h post-infection by CO₂ overdose and their lungs were aseptically removed and then homogenized in 1 ml of PBS. The viable bacteria were serially diluted in PBS, spread on tryptic soy agar plates containing 5% sheep blood and enumerated after incubation at 37 °C supplemented with 5% CO₂ overnight.

Mongolian gerbil otitis media infection model. Male Mongolian gerbils (40–60 g) were anaesthetized with isoflurane (3%) and the area around the left ear bulla was prepared by swabbing with ethanol. Forty microlitres (1.0 × 10⁵ c.f.u.) of S. pneumoniae (isolate 0100993 or the isogenic nox mutant) was prepared as described previously and injected through the bone of the left bulla, and the animals were allowed to recover under observation. Food and water were provided ad libitum and the gerbils were killed 96 h post-inoculation by CO₂ overdose. The tympanic membrane was then examined and middle-ear aspirates were obtained by injecting 250 µl PBS into the middle ear cavity and withdrawing the fluid contained
NADH oxidase of S. pneumoniae

RESULTS

Identification of the nox gene

A S. pneumoniae strain 0100993 plasmid-insertion mutant with a disrupted nox gene was previously identified as attenuated for virulence in a murine respiratory tract infection model when tested in a pool of 96 uniquely tagged mutants in a STM study (G. Lau and others, unpublished data). When tested individually in this model, the plasmid-insertion mutant was found to be attenuated 10^5-fold compared with the parental strain 0100993. DNA sequencing of the excised plasmid from the chromosone of the mutant revealed that the plasmid had inserted between codon 255/366 of an ORF that had 95.5% identity to the Nox NADH oxidase of 459 amino acids. The full-length nox gene from strain 0100993 was subsequently cloned and sequenced. The Nox protein sequence was 98.7% identical to that of Nox NADH oxidase from S. pneumoniae strain 1015 (GenBank entry AAC26485), with six amino acid differences at D265G, G288D, I320N, N340I, Y345N and K459N.

Phylogenetic analysis of Nox protein sequences (Fig. 1) revealed that the NADH oxidase of S. pneumoniae 0100993 was closely related to the NADH oxidases from S. mutans, spirochaetes, mycoplasmas, Enterococcus faecalis (both an NADH oxidase and an NADH peroxidase) and archaea. The Treponema pallidum and streptococcal sequences are more closely related to each other than to other members of their respective families, indicating possible convergence or lateral gene transfer. The redox-active cysteine of the Ent. faecalis NADH peroxidase (Ahmed & Claiborne, 1989), which corresponds to Cys44 in S. pneumoniae 0100993 NADH oxidase, is also conserved in all the NADH oxidase sequences analysed.

Isolation of a S. pneumoniae nox allelic-replacement mutant

The nox plasmid-insertion mutant identified from the STM study was not ideal for virulence studies, because of the possibility of residual activity being expressed by the relatively large truncated nox gene product (amino acid residues 1–366 intact). Moreover, insertion-inactivation mutants often generate polarity effects on neighbouring genes. Hence, an allelic-replacement mutant was generated in strain 0100993 where the complete nox gene was deleted and replaced by ermAM, a selectable erythromycin-resistance determinant. This resistance gene lacked a downstream transcription terminator and was inserted so that transcription from its promoter was in the same direction as the nox gene in an attempt to minimize polar effects. There is a strong ρ-independent terminator (AG = −21.2 kcal mol⁻¹, −88.7 kJ mol⁻¹) (Tinoco et al., 1973) immediately
strain R6 had significant NADH oxidase activities of aerobic and anaerobic strains regardless of the growth conditions (Fig. 2), compared to the activity in cell extracts of its parent (data not shown).

To examine if the nox allelic-replacement mutant lacked NADH oxidase activity, crude cell extracts were prepared from aerobically or anaerobically grown cells of the allelic-replacement mutant, its pathogenic wild-type parent strain 0100993 and a non-pathogenic wild-type strain R6. As expected, the NADH oxidase activity was almost undetectable in cell extracts of the nox mutant compared to the activity in cell extracts of its parent strain regardless of the growth conditions (Fig. 2), demonstrating the effectiveness of the deletion replacement construct. The parental strain 0100993 and the strain R6 had significant NADH oxidase activities of 0.28 ± 0.06 and 0.31 ± 0.05 U mg⁻¹ respectively when grown in aerobic conditions (means ± SD, n = 3). Interestingly, they had similar levels when grown anaerobically, suggesting that NADH oxidase activity is not inducible by O₂ in S. pneumoniae. The residual activity suggests that a truncated protein was produced by the plasmid-insertion mutant.

In vitro growth characteristics of the nox allelic-replacement mutant

As NADH oxidase is involved in the reduction of potentially toxic O₂ to H₂O₂, we tested whether the nox allelic-replacement mutant was affected in its growth under different aeration conditions. In liquid culture under conditions of vigorous aeration (200 r.p.m.), growth of the allelic-replacement mutant was dramatically inhibited (Fig. 3) compared with that of the parent strain, suggesting that Nox NADH oxidase was required for growth of S. pneumoniae under these conditions. Under conditions of limited aeration, growth of the parent strain was much improved compared with growth under vigorous aeration. There was no significant difference in growth of the allelic-replacement

NADH oxidase activity and H₂O₂ production by the nox allelic-replacement mutant

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Under aerobic conditions, O₂ can be reduced to H₂O by the Nox NADH oxidase, or it can be partially reduced to O₂⁻ or H₂O₂. The possibility that the lack of NADH oxidase in the nox allelic-replacement mutant caused a change of H₂O₂ production was examined. H₂O₂ was produced at the rate of 22.2 ± 6.7 and 24.1 ± 5.8 nmol per 30 min per mg by the nox allelic-replacement mutant and the parent strain, respectively, indicating that there was no significant difference in H₂O₂ production by the mutant compared with the parent strain.
mutant compared with that of the parent under limited aeration conditions.

Virulence testing of the nox allelic-replacement mutant in animal infection models

The requirement of the Nox NADH oxidase for S. pneumoniae infection was tested by comparing the virulence of the allelic-replacement nox mutant and its parent strain 0100993 in two different animal infection models. In the murine respiratory tract infection model, 7-0 × 10^6 c.f.u. parent or mutant bacteria that had been grown in vitro under aerobic conditions, were administered intranasally into CBA/J mice. After 48 h, a mean of 7-0 × 10^6 c.f.u. per mouse was recovered from lungs infected with the parental wild-type strain (Fig. 4a). In contrast, only 80 c.f.u. per mouse was isolated from lungs infected with the nox mutant, representing a 10^8-fold attenuation level. A time-course analysis revealed that the nox mutant was 10^2- to 10^3-fold attenuated for virulence compared with the parent, even during the first 24 h of infection (Fig. 4a).

In a Mongolian gerbil otitis media infection model, two groups of ten animals were infected with 1-0 × 10^6 c.f.u. of S. pneumoniae 0100993 or the nox allelic-replacement mutant. A mean of 7-0 × 10^6 c.f.u. bacteria was recovered from middle ear aspirates of gerbils infected with the parent strain after 96 h of infection. In contrast, no bacteria were recovered (limit of detection) from the middle ear aspirates of any gerbils infected with the nox mutant (Fig. 4b). This suggested that the mutant was at least 10^4-fold attenuated in this model of infection. This was the maximum level of virulence attenuation achievable with this model. Thus, the Nox NADH oxidase was required for S. pneumoniae virulence in two different models of infection.

Isolation and characterization of the NADH oxidase

The nox gene from S. pneumoniae 0100993 was cloned and expressed in E. coli as an N-terminally His-tagged fusion protein. The predicted molecular mass was 52.355 Da and a strongly induced protein of approximately 53 kDa was observed on an SDS-polyacrylamide gel (data not shown). Single passage of an induced bacterial lysate through a Ni²⁺ column yielded a 53 kDa protein of greater than 95% purity as determined by SDS-PAGE.

The purified His-tagged protein exhibited a specific NADH oxidase activity of 241.3 ± 2.0 U mg⁻¹ (mean ± SD, n = 4), suggesting that this protein was indeed the Nox NADH oxidase. The oxidation of NADH was independent of exogenous FMN (220.2 ± 27.0 U mg⁻¹) or FAD (239.2 ± 13.1 U mg⁻¹). The K_m of the protein for NADH was 32 μM. As expected, NADPH was not oxidized by the NADH oxidase. The optimum pH for NADH oxidase activity was at 7.0–7.5 in 50 mM potassium phosphate buffer.

DISCUSSION

The Nox NADH oxidase may function as a housekeeping enzyme to reduce molecular oxygen to water, thereby avoiding the production of partially reduced reactive oxygen radicals, such as superoxide (O²⁻) and hydrogen peroxide (H₂O₂) both of which are toxic to cells. In the presence of metals such as iron or copper, O²⁻ and H₂O₂ can react with each other to form other active oxygen species, such as hydroxyl radicals (OH⁻), which are highly reactive with DNA, membrane lipids and other essential cell components (Fridovich, 1978; Thomas & Pera, 1983). Several reports have demonstrated that in the presence of copper or iron, the combination of O²⁻, H₂O₂ and other oxygen radical species caused the inactivation of various cellular enzymes, including fructosyltransferase (Abbe et al., 1986), penicillinase (Samuni et al., 1981), acetylcholine esterase (Shinar et al., 1983), superoxide dismutase (Hodgson & Fridovich, 1975) and glutamine synthetase
(Oliver et al., 1984). Thus, a deletion of Nox NADH oxidase activity would be expected to impair the ability of bacteria to grow under oxygen rich conditions both in vitro and in vivo.

The requirement of the Nox NADH oxidase for S. pneumoniae infection was indicated by the isolation of a nox plasmid-insertion mutant in a STM virulence attenuation screen (G. Lau and others, unpublished data). When compared with its parent strain 0100993, the S. pneumoniae mutant was 10³-fold attenuated for virulence in a murine respiratory tract infection model. However, this plasmid-insertion mutant was capable of expressing a truncated protein of 366 amino acid residues (~ 80% of the full-length protein of 459 amino acid residues) that contained the conserved cysteine at residue 44 that has been implicated in enzymic activity. The 11% residual NADH oxidase activity found in extracts of this mutant is likely to be due to activity from the truncated Nox produced by this mutant. Thus, a refined mutant was generated by allelic-replacement mutagenesis where the entire nox gene was deleted. This nox deletion mutant lacked any significant NADH oxidase activity. When tested in the same respiratory tract infection model, this allelic-replacement mutant was 10⁴-fold attenuated for virulence. The increase in the attenuation level between this mutant and the original plasmid-insertion mutant correlated with the elimination of residual NADH oxidase activity. The nox mutant was also at least 10⁴-fold attenuated for virulence in the Mongolian gerbil otitis media model, which is the maximum level of virulence attenuation achievable with this model. Thus, Nox is required for S. pneumoniae infection in both models tested, each of which deals with environments that are O₂ rich (lungs and middle ear). Although we do not consider NADH oxidase as a classical virulence factor like an adhesin or a toxin, it is nevertheless required for infection in the models tested. These results are in agreement with those of Auzat et al. (1999), where NADH oxidase was also shown to be an oxygen sensor for S. pneumoniae infection. A murine intra-peritoneal challenge was used by Auzat et al. (1999), while our challenge was intranasal. It is unlikely that virulence attenuation is due to a polar effect of the allelic-replacement mutation on transcription of a downstream gene, as there appears to be a strong transcription terminator directly downstream of nox. We attempted to clone the S. pneumoniae nox locus in E. coli, for subsequent transfer to S. pneumoniae for complementation studies, but were repeatedly unsuccessful. Another group has also reported difficulty in cloning the S. pneumoniae nox locus in E. coli and suggested that upstream and/or downstream sequences might be toxic or unstable (Auzat et al., 1998).

Lack of NADH oxidase activity has been associated with oxygen-related in vitro growth inhibition in some S. mutans strains. When growing cells were shifted from anaerobic to vigorous aerobic growth conditions, the growth of oxygen-tolerant S. mutans strains was unaffected, but the growth of oxygen-sensitive strains was severely inhibited (Higuchi, 1984). Expression of the

Nox NADH oxidase was found to be induced by oxygen in oxygen-tolerant strains (i.e. from 0.052 to 0.985 U mg⁻¹) but was not induced in oxygen-sensitive strains (i.e. from 0.050 to 0.059 U mg⁻¹) (Higuchi, 1984) and was thus postulated to be important in protecting bacteria against oxygen-related toxicity (Higuchi, 1992). The growth characteristics of the S. pneumoniae strains used in this study (0100993 and R6) were similar to those of the oxygen-sensitive S. mutans strains described previously. Growth was significantly inhibited under conditions of vigorous aeration, probably because the low-level, non-inducible NADH oxidase (0.26 U mg⁻¹ under anaerobic conditions and 0.28 U mg⁻¹ under aerobic conditions) was not sufficient fully to protect bacteria against oxygen toxicity. The results from another laboratory also support our findings that NADH oxidase is required for S. pyogenes growth in oxygen-rich conditions (Gibson et al., 2000). The in vitro growth inhibition was almost complete when the gene for NADH oxidase was deleted in the nox allelic-replacement mutant. This nox mutant did grow normally in conditions of limited aeration, suggesting that other enzymes (e.g. pyruvate oxidase), were available to reduce oxygen during static growth (limited aeration) or that lower levels of O₂ were not toxic. Apparently, the residual oxygen tolerance conferred by non-NADH oxidase enzymes is not sufficient to support growth of the NADH oxidase-deficient mutant either in vitro under vigorous aeration conditions or during infection.

Examination of public sequence databases revealed that Nox is present in S. pneumoniae, S. mutans, Ent. faecalis, mycoplasmas and spirochaetes and is broadly distributed in archaea. Interestingly, the Nox NADH oxidases appear to be related at the protein sequence level to the NADH peroxidase from Ent. faecalis strain 10C1, the X-ray crystal structure of which has been solved (Stehle et al., 1991). The proposed active redox centre and the putative FAD- and NADH-binding motifs of the Ent. faecalis NADH peroxidase (Ross & Claiborne, 1992) are conserved in the various NADH oxidases, including S. pneumoniae Nox NADH oxidase, suggesting that these NADH oxidases might be functionally related. Auzat et al. (1999) mutated the catalytic Cys44 residue and the mutated protein, which was detected by Western blot from crude extracts, did not have measurable NADH oxidase activity.

The nox gene from S. pneumoniae 0100993 was cloned, expressed and purified as an N-terminal His-tagged fusion protein. The enzymes from S. pneumoniae 0100993 (this study) and S. mutans (Higuchi et al., 1993) oxidized NADH, but not NADPH, at similar Kₘ values (32 and 25 μM respectively). Their activities were independent of exogenously added FAD or FMN. In contrast, exogenous FAD was required for maximum activities of NADH oxidases of Ent. faecalis (Schmidt et al., 1986), Leuconostoc mesenteroides (Koike et al., 1985) and Mycoplasma capricolum (Klomkes et al., 1985). Thus S. pneumoniae Nox NADH oxidase has biochemical characteristics similar to but distinct from other NADH oxidases.
In summary, we have demonstrated that the S. pneumoniae Nox NADH oxidase is required for in vitro growth in oxygen-rich conditions and, importantly, for virulence in two distinct model systems of infection. For the allelic-replacement nox mutant, it is likely that loss of a protective function from NADH oxidase resulted in in vitro cell growth impairment and in vivo infection attenuation. The precise mechanism by which the Nox NADH oxidase is involved in the pathogenesis of S. pneumoniae remains to be explored.

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