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 10^5-fold in the murine respiratory tract infection model and at least 10^4-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 O_2 to H_2O. If O_2 is not fully reduced, it can form superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), 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 K_m 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 (O_2) is not fully reduced (four-electron reduction) to H_2O, it can undergo one- or two-electron reductions to form reactive superoxide anion (O_2^-) and hydrogen peroxide (H_2O_2), both of which are toxic to cells. The enzyme that converts O_2 to H_2O is an NADH oxidase called Nox. It has been identified and characterized from

Streptococcus faecalis (Badway & Karnovsky, 1980), *Mycoplasma pneumoniae* (Himmerlreich *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 defence against O_2 toxicity (Higuchi, 1992), or to function as an oxygen sensor (Auzat *et al.*, 1999). In *S. mutans*, a minor H_2O_2-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 % CO_2 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 (H_2 + 5 % CO_2) was generated and maintained by the GasPak Plus anaerobic system (Becton Dickinson). *Escherichia coli* strain DH10B 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 *ermAM* 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 ($1·0 \times 10^6$) 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 36 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.

H_2O_2 production. The H_2O_2 production assay was based on the method of Pick & Keisari (1980). The *S. pneumoniae* pellet was resuspended in 1 ml freshly made H_2O_2 working solution (5 mM K_2HPO_4, 1 mM KH_2PO_4, 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. H_2O_2 production was described as nmol H_2O_2 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 % CO_2. Bacteria were recovered from plates, resuspended in PBS and adjusted to OD_{600} 0·95. Animals (male CBA/J mice, 14–16 g) were anaesthetized with isoflurane (3 %) and 50 μl bacterial inoculum ($7·0 \times 10^7$ 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 CO_2 overdose. Surviving animals were killed at 6–48 h post-infection by CO_2 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 % CO_2 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 ($1·0 \times 10^5$ 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 CO_2 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

therein. Aspirates were then serially diluted and evaluated for viable bacteria.

Cloning, expression and purification of His-tagged NADH oxidase. The sequence of the *nox* gene and its flanking region was obtained from the SmithKline Beecham database and from GenBank (accession number AAC26485). The full-length *nox* coding region was amplified by *Pfu* DNA polymerase (Stratagene) from *S. pneumoniae* strain 0100993 chromosomal DNA with primers noxup (5′-AGG AAA TTC ATA TGA GTA AAA TCG TTG TA-3′) and noxdown (5′-AGT CAT TTG TTG GAT CCT CAT CA-3′). The purified *nox* fragment was digested with *Nde*I and *Bam*HI (these sites were included in the primers noxup and noxdown respectively). A 1547 bp *Nde*I/*Bam*HI-digested *nox* fragment was ligated with the expression vector pET28a (Novagen) that was also digested with *Nde*I and *Bam*HI. The ligated plasmid was electroporated into competent cells of *E. coli* ElectroMax DH10B. After the *nox* sequence was confirmed using appropriate primers, the resulting plasmid pET28nox was introduced into *E. coli* strain BL21 (DE3) for the expression of His-tagged Nox.

The pET28nox-bearing BL21 (DE3) was grown to OD_{600} 0·4 in Luria broth at 37 °C. Expression of His-tagged Nox was induced by addition of 1 mM IPTG, and after 3 h, the bacterial cells were collected by centrifugation. The pellet was resuspended in 10 ml 1 × binding buffer from the His-tagged protein purification kit (Novagen). The bacteria were disrupted on ice by sonication for 30 s at amplitude 10 with 30 s intervals for a total of 6 min using a Soniprep150 (Sanyo). The His-tagged Nox protein in the supernatant was purified using a Ni^{2+} column according to the manufacturer's instructions. The concentration of purified protein was determined with a BCA kit (Pierce), with bovine serum albumin as standard. The purity of protein was examined with 10 % NuPAGE gel from Novex.

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 $10^{3·5}$-fold compared with the parental strain 0100993. DNA sequencing of the excised plasmid from the chromosome 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*
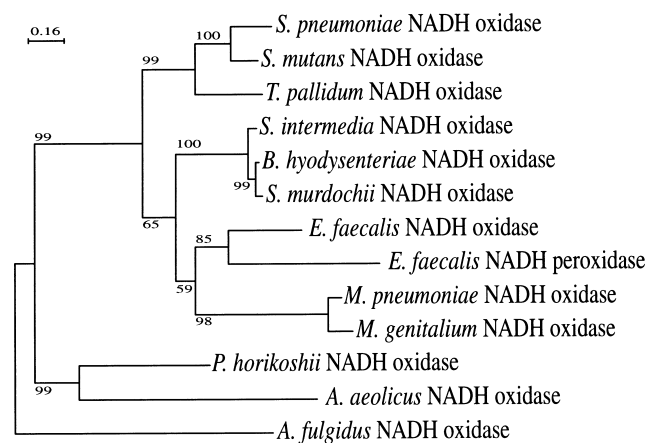

Fig. 1. Phylogenetic tree comparing the relativeness of Nox NADH oxidase protein sequences. Branch lengths are proportional to the amount of sequence divergence. Support values for internal branches are shown as percentages. The following sequences were included in the phylogenetic analysis, with GenBank (GB) or SWISS-PROT (SP) accessions: *Streptococcus pneumoniae* (this study), *Streptococcus mutans* (GB: D49951), *Treponema pallidum* (GB: AE001260), *Serpulina intermedia* (GB: AF060812), *Brachyspira hyodysenteriae* (GB: AF060802), *Serpulina murdochii* (GB: AF060813), *Enterococcus faecalis* (SP: P37061), *Enterococcus faecalis* NADH peroxidase (SP: P37062), *Mycoplasma pneumoniae* (SP: P75389), *Mycoplasma genitalium* (SP: Q49408), *Pyrococcus horikoshii* (GB: AP000006), *Aquifex aeolicus* (GB: AE000709), *Archaeoglobus fulgidus* NADH oxidase *noxA-1* (GB: AE001088).

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, insertional 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 ($\Delta G = -21\cdot2$ kcal mol^{-1}, $-88\cdot7$ kJ mol^{-1}) (Tinoco *et al.*, 1973) immediately
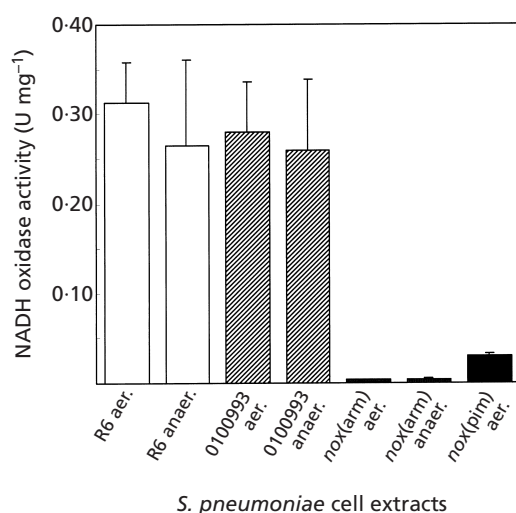
downstream of the *nox* gene, suggesting that *nox* is not co-transcribed with its nearest downstream ORF (152 bp downstream), which encodes a hypothetical protein. There is a 324 bp gap between *nox* and its nearest upstream gene that is transcribed in the opposite direction to the *nox* gene. The chromosomal disruption of *nox* in the allelic-replacement mutant was confirmed by diagnostic PCR and Southern hybridization analysis (data not shown).

NADH oxidase activity and H_2O_2 production by the *nox* allelic-replacement mutant

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 $\pm$ SD, $n = 3$). Interestingly, they had similar levels when grown anaerobically, suggesting that NADH oxidase activity is not inducible by O_2 in *S. pneumoniae*. The NADH oxidase activity of the STM-derived *nox* plasmid-insertion mutant was also determined (Fig. 2) to be $0.030 \pm$
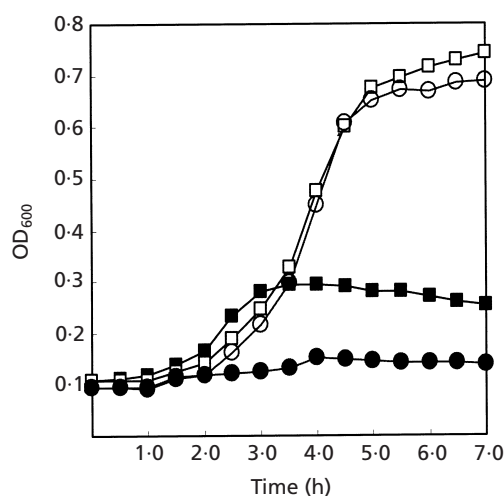
0.003 U mg^{-1}, which represents 10 % of the activity in the wild-type parent and significantly more activity than was found in the allelic-replacement mutant. This residual activity suggests that a truncated protein was being produced by the plasmid-insertion mutant.

Under aerobic conditions, O_2 can be reduced to H_2O by the Nox NADH oxidase, or it can be partially reduced to O_2^- or H_2O_2. The possibility that the lack of NADH oxidase in the *nox* allelic-replacement mutant caused a change of H_2O_2 production was examined. H_2O_2 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 H_2O_2 production by the mutant compared with the parent strain.

In vitro growth characteristics of the *nox* allelic-replacement mutant

As NADH oxidase is involved in the reduction of potentially toxic O_2 to H_2O, 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
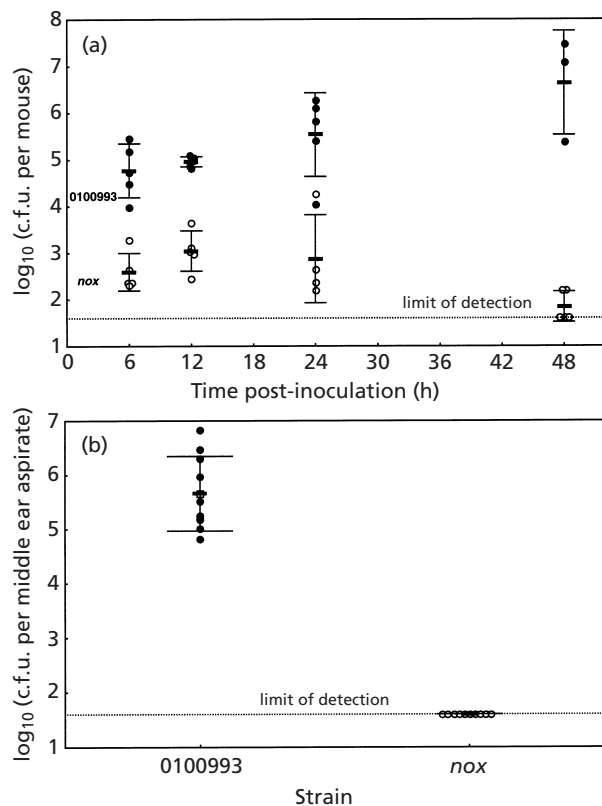
Fig. 4. Virulence testing of the *nox* allelic-replacement in animal models. (a) Murine respiratory tract infection model. For each time point (6, 12, 24 and 48 hours post-inoculation), five animals were infected intranasally with $7·0 \times 10^7$ c.f.u. per mouse of either the *nox* mutant (○) or its parent strain 0100993 (●). (b) Mongolian gerbil otitis media infection model. For each group, ten animals were infected with $1·0 \times 10^5$ c.f.u. per gerbil of either the *nox* mutant (○) or its parent strain 0100993 (●). For details of the animal infection models see text.

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, $7·0 \times 10^7$ 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 $7·0 \times 10^6$ 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 10^5-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 $1·0 \times 10^5$ c.f.u. of *S. pneumoniae* 0100993 or the *nox* allelic-replacement mutant. A mean of $7·0 \times 10^5$ 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 Ni^{2+} 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 $241·3 \pm 2·0$ U mg^{-1} (mean $\pm$ SD, $n = 4$), suggesting that this protein was indeed the Nox NADH oxidase. The oxidation of NADH was independent of exogenous FMN ($220·2 \pm 27·0$ U mg^{-1}) or FAD ($239·2 \pm 13·1$ 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 (O_2^-) and hydrogen peroxide (H_2O_2) both of which are toxic to cells. In the presence of metals such as iron or copper, O_2^- and H_2O_2 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 O_2^-, H_2O_2 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 depletion 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 $10^{3 \cdot 5}$-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 ($\sim 80\%$ 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 10^5-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 10^4-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 O_2 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 O_2 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 K_m 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 caprocolum* (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 models 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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