Characterization of an ntrX Mutant of Neisseria gonorrhoeae Reveals a Response Regulator That Controls Expression of Respiratory Enzymes in Oxidase-Positive Proteobacteria

Author

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Characterisation of an \( ntrX \) mutant of \textit{Neisseria gonorrhoeae} reveals a response regulator that controls expression of respiratory enzymes in ‘oxidase positive’ \textbf{Proteobacteria}

Running title – \textit{respiratory enzyme regulation in Proteobacteria}

John M. Atack\textsuperscript{1, 2}, Yogitha N. Srikhanta\textsuperscript{1}, Karrera Y. Djoko\textsuperscript{1}, Jessica P. Welch\textsuperscript{1}, Norain H. M. Hasri\textsuperscript{1}, Christopher T. Steichen\textsuperscript{3}, Rachel N. Vanden Hoven\textsuperscript{1}, Sean M. Grimmond\textsuperscript{4}, Dk Seti Maimonah Pg Othman\textsuperscript{1}, Ulrike Kappler\textsuperscript{1}, Michael A. Apicella\textsuperscript{5}, Michael P. Jennings\textsuperscript{1, 2}, Jennifer L. Edwards\textsuperscript{5} and Alastair G. McEwan\textsuperscript{1*}

\textsuperscript{1}School of Chemistry and Molecular Biosciences, The University of Queensland, Brisbane 4072, Australia

\textsuperscript{2}Institute for Glycomics, Griffith University, Gold Coast Campus, QLD 4222, Australia

\textsuperscript{3}Department of Microbiology and Immunology, University of Iowa, Iowa City, Iowa 52242, USA

\textsuperscript{4}Institute for Molecular Bioscience, The University of Queensland, Brisbane 4072, Australia

\textsuperscript{5}Centre for Microbial Pathogenesis, The Research Institute at Nationwide Children’s Hospital, Columbus, Ohio 43205, USA.
*Address for correspondence:

Alastair McEwan
Faculty of Science
The University of Queensland
St Lucia 4072
Australia
Phone: + 61 7 3346 4110
Fax: + 61 7 3365 4620
E-mail address: mcewan@uq.edu.au
Abstract

NtrYX is a sensor-histidine kinase/response regulator two-component system that has had limited characterization in a small number of α-Proteobacteria. Phylogenetic analysis of the response regulator, NtrX, showed that this two-component system is extensively distributed across the bacterial domain and it is present in a variety of β-Proteobacteria, including the human pathogen *Neisseria gonorrhoeae*. Microarray analysis revealed that the expression of several components of the respiratory chain was reduced in a *N. gonorrhoeae* ntrX mutant compared to the isogenic wild-type strain 1291. These included the cytochrome *c* oxidase subunit (ccoP), nitrite reductase (aniA) and nitric oxide reductase (norB). Enzyme activity assays showed decreased cytochrome oxidase and nitrite reductase activities in the ntrX mutant, consistent with microarray data. *N. gonorrhoeae* ntrX mutants had reduced capacity to survive inside primary cervical cells compared to the wild-type, and although they retained the ability to form a biofilm, they exhibited reduced survival within the biofilm compared to wild-type cells, as indicated by live-dead staining. Analyses of an ntrX mutant in a representative α-Proteobacterium, *Rhodobacter capsulatus*, showed that cytochrome oxidase activity was also reduced compared to the wild-type strain, SB1003. Taken together, these data provide evidence that the NtrYX two component-system may be a key regulator in the expression of respiratory enzymes and, in particular, cytochrome *c* oxidase, across a wide range of proteobacteria, including a variety of bacterial pathogens.
Keywords: response regulator, NtrX, cytochrome cbb_3, respiration, ‘oxidase positive bacteria’, Neisseria gonorrhoeae, Rhodobacter capsulatus;

Abbreviations: Cco: cytochrome cbb_3/cytochrome oxidase; TMPD: N, N, N, N-tetra-methyl-p-phenylenediamine; TCS: two component-system; SHK: sensor histidine-kinase; RR: response regulator
Introduction

Respiration is a key process for cellular survival and energy generation. Specific enzymes have evolved to mediate efficient respiration under conditions of varying oxygen availability, and in the absence of this electron acceptor (reviewed in (1)). Recent work has linked cytochrome oxidases to the virulence of several bacteria, including *Neisseria spp.* (2, 3). The cytochrome oxidase mediating these effects is cytochrome *cbb*$_3$ (cytochrome oxidase; Cco), a respiratory oxidase that is present in a wide variety of non-enteric bacteria (4). Cytochrome *cbb*$_3$ accepts electrons from c-type cytochromes and terminates a respiratory chain in which electrons from ubiquinol are transferred to c-type cytochromes via a cytochrome *bc*$_1$ complex. Cytochrome *cbb*$_3$ can use ascorbate/*N, N, N, N*-tetra-methyl-p-phenylenediamine (TMPD) as an artificial electron donor in respiration (5), and its presence results in bacteria staining positive in the classical oxidase test (6, 7). Enteric bacteria that only possess oxidases that directly oxidize ubiquinol, such as *Escherichia coli*, yield a negative oxidase test. An important characteristic of cytochrome *cbb*$_3$ is its remarkable affinity for oxygen, often being in the nM range (5, 8-10), with activity levels being highest under microaerobic or oxygen-limited conditions (8, 11, 12).

Two component-systems (TCSs) composed of a sensor histidine kinase (SHK) and response regulator (RR) have emerged as key signal transducers and global regulators of gene expression in prokaryotes. TCS are known to regulate metabolic and respiratory processes. For example, in enteric bacteria the ArcBA TCS regulates the expression of many metabolic genes (13), including components of the citric acid cycle (e.g., succinate dehydrogenase and malate dehydrogenase (14, 15)) and aerobic quinol oxidase complexes (16, 17). The regulation of cytochrome *cbb*$_3$ expression in proteobacteria has
mostly been studied in *Rhodobacter capsulatus* where the RegBA TCS and FNR are involved (18-20). This suggests that TCS may play a role in modulating cytochrome *cbb*₃ expression in other bacteria. As the RegBA TCS system, like ArcBA, is a master regulator of many cellular functions it is plausible to expect that respiration may be controlled by an alternative TCS(s) in those bacteria possessing a cytochrome *cbb*₃ but lacking RegBA. In contrast to the situation in metabolically versatile bacteria such as *Rhodobacter spp.*, the human-adapted pathogen *Neisseria gonorrhoeae* is known to contain cytochrome *cbb*₃ as its only respiratory oxidase (2). Furthermore, *N. gonorrhoeae* contains only five TCS systems (GenBank AE004969), none of which resemble RegBA. However, one of these five *N. gonorrhoeae* TCSs appears to be an ortholog of the NtrYX system that plays a role in the regulation of nitrogen fixation and metabolism in some bacteria (21, 22). Despite having been implicated in regulating various cellular processes, NtrYX is usually regarded as being associated with the NtrBC system in *α*-Proteobacteria (23) and is typically associated with the regulation of nitrogen metabolism. However, *N. gonorrhoeae* (a *β*-Proteobacterium) does not contain an NtrBC system, raising the question of the functional role of NtrYX in a bacterium that lacks metabolic versatility.

We report herein the role of NtrX in two unrelated ‘oxidase-positive’ bacteria. Characterisation of an *ntrX* mutant in *N. gonorrhoeae* shows that this response regulator has a role in regulating the expression of respiratory enzymes and a key role in the adaptive ability of this bacterium. The effect of an *ntrX* mutation on cytochrome oxidase and respiratory chain activity in the facultative phototroph *R. capsulatus* is also briefly described. These results, together with recent observations on the role of the sensor histidine kinase NtrY in *Brucella abortus* (24), lead us to propose that the NtrYX TCS is
Materials and Methods

Bacterial strains and growth conditions

*N. gonorrhoeae* strains were grown on GC agar supplemented with IsoVitaleX (1% v/v; Becton Dickinson) at 37°C with 5% (v/v) CO₂, or in supplemented BHI or GC (sBHI or sGC respectively) broth supplemented with Levinthal’s base (10% v/v) and IsoVitaleX (1% v/v). *R. capsulatus* SB1003 was routinely grown on either RCV (25) or TYS media (26) at 28°C. Growth media were supplemented with antibiotics as described (27). *E. coli* strains DH5α, JM109 (Promega) and S 17-1 (28) were used to propagate plasmids and were routinely grown at 37°C in Luria-Bertani (LB) medium supplemented with kanamycin (100 μg/ml), or were grown as described previously (27).

Molecular biology

Standard methods, as described by Sambrook *et al.* (1989), were used throughout (29). All enzymes were sourced from New England Biolabs (USA). A *N. gonorrhoeae ntrX::kan* mutant was made by insertional inactivation of the *ntrX* gene in strain 1291 (accession number EEH63133.1) using a promoterless kanamycin-resistance cassette inserted in the same orientation as the *ntrX* transcriptional unit. Briefly, the *ntrX* gene (1.3 kb), including upstream and downstream flanking regions (350 bp each), was amplified by PCR using primers *ntrX*-F (5′-GATACGACCGCCATGCGGCAG-3′) and *ntrX*-R (5′-
CATCCTGAAGCAGCATCAG-3'). The PCR product was A-tailed and sub-cloned into T-tailed pGEM-T Easy vector (Promega) to generate pGEM-T::ntrX. A kanamycin-resistance cassette was obtained from pUC4k (GenBank accession number X06404) by excision with HincII and subsequently inserted into the unique BmgBI restriction site within ntrX to generate pGEM-T::ntrX::kan. The ntrX::kan cassette was finally amplified by PCR using primers DUS-F (5′-TGCCGTCTGAAGACTTCAGACGGCGTAAAACGACGGCAGT-3′) and DUS-R (5′-GGAACAGCTATGACCATG-3′). The resulting PCR product carried the gonococcal DNA uptake sequence GCCGTCTGAA at the 5′ end and was used to transform N. gonorrhoeae strain 1291. Kanamycin-resistant colonies were visible after 1.5 - 2 days of growth on GC agar containing 1% (v/v) IsoVitaleX supplemented with 100 µg/ml kanamycin. Correct insertion into the chromosome was verified by PCR using combinations of cloning primers as well as primers complementary to the kanamycin-resistance cassette: Km-out-F (5′-CATTTGATGCTCGATGAGTTTTCTAA-3′) and Km-out-R (5′-AGACGTTTCCCGTTGAATATGGCTGCAT-3′). This mutant was designated Ng-ntrX.

The Ng-ntrX mutant was complemented in cis by double-crossover of a wild-type copy of the ntrYX locus into the proB locus in the gonococcal chromosome using the complementation construct pCTS32 (30). The entire ntrYX locus, including 300 bp upstream and downstream flanking regions, was amplified from N. gonorrhoeae 1291 wild-type genomic DNA using primers COMP-F (5′-TACAAACTAAGTTTCCATCCG-3′) and COMP-R (5′-AAAGCCGCTTTTCCCGAT-3′). The PCR product was ligated into the SmaI site of pCTS32 to generate pCTS2-ntrYX. The plasmid was linearised with
ScaI and transformed into Ng-ntrX. Positive transformants were selected on GC agar supplemented with both kanamycin (100 µg/mL) and spectinomycin (50 µg/mL). The complemented strain was verified using a combination of PCR and DNA sequencing using primers specific for the ntrYX locus (check-F; 5′-CGGTAGAAACTTATGCGTAG-3′) and the spectinomycin resistance cassette (check-R; 5′-GAATGGTTACAAGAGCTTTA-3′). This strain was designated Ng-ntrX-COMP.

A ntrX mutant in R. capsulatus strain SB1003 was generated by transferring the suicide plasmid pDG9-3II (31) into R. capsulatus SB1003 by conjugation. The identity of the mutant, designated Rc-ntrX, was confirmed by southern blotting using DIG-dUTP (Roche Biochemicals, NSW, Australia) labelled DNA probes as described previously (32). Selection and propagation of Rc-ntrX mutants were carried out aerobically on antibiotic-supplemented TYS medium (26).

### Preparation of cell-free extracts and respiratory enzyme assays

N. gonorrhoeae 1291 wild-type and strain Ng-ntrX were grown aerobically to mid-exponential phase in 100 ml sBHI broth in 250 ml flasks with shaking (37°C, 200 rpm). Cells were harvested by centrifugation (15,000 x g for 20 minutes at 4°C) and resuspended in 50 mM HEPES-Na (pH 7.5). N. gonorrhoeae cytochrome oxidase activity was assayed as a measure of oxygen consumption using an OxyGraph oxygen electrode and the accompanying software (Hansatech), based on the method of Markwell & Lascelles (33). The reaction mixture contained 50 mM HEPES-Na (pH 7.5), 25 µl cell suspension, 50 mM substrate (lactic acid), 10 µM myxothiazol, 10 mM ascorbate and 20 µM TMPD. Reactions were carried out in a total volume of 2 ml at 30°C. Protein
concentrations of cell suspensions were determined using the method of Markwell et al. (34).

R. capsulatus cell free extracts were prepared using late exponential phase cells as described (27). The activity of R. capsulatus cytochrome oxidase was measured using a Rank oxygen electrode, also as described (35). Specific activity of transhydrogenase was measured as described previously (36) with the omission of KCN as experiments were carried out in an anaerobic cabinet (<2 ppm O₂). Specific activity of the NADH:quinone oxidoreductase (also performed in an anaerobic cabinet) was measured by the reduction of decylubiquinone at 340 nm (50 μM decylubiquinone, 100 μM NADH, 2-10 μl chromatophores in 50 mM HEPES-Na pH 7.5 in a total volume of 1 ml).

Nitrite reductase Assays

Methyl viologen-linked nitrite reductase assays were carried out using intact N. gonorrhoeae cells in a Hitachi U3000 spectrophotometer, based on the method of Sellars et al., (37) with slight modifications. N. gonorrhoeae strains were grown aerobically to mid-exponential phase in sBHI. Cells were harvested by centrifugation (15,000 x g for 20 minutes at 4°C) and resuspended in 10 mM Tris-Cl (pH 7.5). The assay was carried out in a total volume of 1 ml and consisted of 10 mM Tris-Cl (pH 7.5) and 100 μM methyl viologen, contained in a screw-topped quartz cuvette (Hellma) fitted with a silicone rubber seal to prevent gas exchange. After the addition of cells and sparging with nitrogen gas for approximately 20 minutes to make the mixture anaerobic, aliquots of freshly prepared sodium dithionite solution were injected into the cuvette until the absorbance at 600 nm was stable at approximately 1.1 absorbance units. The assay was
then started by the addition of sodium nitrite to the cuvette to yield a final concentration of 5 mM. Rates of nitrite reductase activity were calculated using an extinction coefficient for methyl viologen of 8.25 mM$^{-1}$ cm$^{-1}$ at 600 nm. Protein concentrations of cell suspensions were determined using the method of Markwell et al., (34).

Microarray analysis

Triplicate cultures of *N. gonorrhoeae* 1291 wild-type and strain *Ng-ntrX* were grown aerobically to exponential phase (optical density at 600 nm = 0.5 to 0.6) in sGC prior to RNA extraction using the RNeasy Maxi Kit according to manufacturer’s instructions (Qiagen). Growth rates of wild-type and mutant strain pairs used to make RNA for microarray comparison were equivalent, thereby ensuring that RNA isolated for subsequent microarray comparison were obtained from bacteria in the same growth phase. Culture media for RNA isolation was free of antibiotics as the chromosomal ntrX::kan mutation is stable without selection. For each strain, triplicate samples (100 µg RNA each) were isolated, pooled and the integrity and concentration of RNA was determined using a Bio-analyser (Agilent Technologies).

All microarray analysis was performed on *N. gonorrhoeae/meningitidis* genome arrays (TIGR; http://pfgrc.tigr.org/). Each microarray consists of 6,389 70mer oligonucleotides representing open reading frames (ORFs) from *N. gonorrhoeae* strains FA1090 and ATCC 700825 (reference strain), as well as from *N. meningitidis* strains Z2491 (serogroup A) and MC58 (serogroup B). Methods and analysis were as previously described (38). All primary data were imported into an in-house installation of the comprehensive microarray relational database, BASE.
Biofilm formation by *N. gonorrhoeae*

Examination of biofilm formation was carried out via confocal microscopy using a Nikon PCM-2000 confocal microscope scanning system (Nikon, Melville, USA). *N. gonorrhoeae* 1291 wild-type and the isogenic mutant *Ng-ntrX* were transformed with a plasmid, pCmGFP, encoding a green fluorescent protein (39). Formation and analyses of biofilms was as described previously (38, 40), with the exception that cells were grown under the same aerobic conditions as described for the microarray analysis, prior to their inoculation into biofilm growth chambers. Colonies used to inoculate cultures for biofilm assays were assessed for morphology to ensure equivalent level of piliation as described previously (41). Biofilm images are three-dimensional reconstructions of stacked z-series taken at 200X magnification and were rendered by Volocity® (Improvision Inc., Lexington, USA) as described previously (40).

*N. gonorrhoeae* Association, Invasion, Survival Assays.

Primary cervical epithelial (pex) cells were procured from surgical cervical tissue and maintained as described previously (42). Cervical tissue was obtained from pre-menopausal women undergoing hysterectomy at The Ohio State University Medical Center for medically-indicated reasons not related to our study and was provided by the Cooperative Human Tissue Network (The Research Institute at Nationwide Children’s Hospital, Columbus, OH). In accordance with NIH guidelines, these tissues do not constitute human subjects. Quantitative association, invasion, and survival assays were performed as we have described previously (43). Briefly, pex cells were challenged with gonococci at a multiplicity of infection of 100 for 90 min. Gentamicin was then omitted
from (association assays) or added to (invasion and intracellular survival assays) infected pex cell monolayers to kill extracellular cell-associated bacteria. Pex cell monolayers were subsequently lysed or they were subject to a second incubation in antibiotic-free medium before cell lysis (intracellular survival assays). For all assays, serial dilutions of the cell lysates were plated to enumerate viable colony-forming units. The percent association, invasion, or survival was determined as a function of the original inoculum and the number of colonies formed with subsequent plating of the cellular lysate. Each assay was performed in triplicate on at least three separate occasions. A Kruskal-Wallis analysis of variance was used to determine the statistical significance of the calculated percent association, invasion, or survival for each assay. A Student’s t-test was used to determine the significance of the calculated invasion and survival indices.

Phylogenetic analyses

Protein sequences related to the NtrX proteins from N. gonorrhoeae FA1090 (acc. No. YP_208898), R. capsulatus SB1003 (acc. No. YP_0033577952), Pseudovibrio sp. JE062 (ZP_05082799.1), Nitrococcus mobilis Nb-231 (ZP_01126634.1) and Geobacter sulfurreducens PCA (acc. No NP_901868) were identified using the BLAST algorithm (44). All sequences had to have at least 350 amino acids and share the basic domain architecture of the NtrX proteins (N-terminus: Response regulator receiver domain; Middle section: P-Loop ATPase/σ54-interaction domain, C-terminus: DNA binding domain). After the removal of duplicates, sequences were aligned using Mega5.0 (45) followed by construction of phylogenetic trees using the Neighbor Joining, Minimum Evolution, UPGMA and Maximum Likelihood algorithms as embedded in Mega5.0 using
the default settings. Robustness testing was carried out using the bootstrap method and 500 resampling cycles.

Results

Disruption of the N. gonorrhoeae ntrX gene affects expression of genes involved in aerobic and anaerobic respiration

We constructed an ntrX mutant in N. gonorrhoeae strain 1291 by disrupting the ntrX gene (GenBank EEH63133.1; annotated as ntrX in 1291 genome) with a promoterless kanamycin antibiotic-resistance cassette (Fig. 1A) and designated this mutant Ng-ntrX.

To identify NtrX-regulated genes, gene expression in 1291 wild-type and Ng-ntrX were compared by analysis of Neisseria gonorrhoeae/Neisseria meningitidis genome microarrays (TIGR) using total RNA isolated from exponential phase gonococci that had been grown aerobically. Overall, eleven genes were differentially regulated and showed greater than two-fold change in expression (P value < 0.01) between 1291 wild-type and Ng-ntrX (Table 1). Seven genes showed reduced expression in Ng-ntrX compared to the wild-type, with four of these genes (ccoP, aniA, norB, and ccpR) encoding terminal reductases of the Neisseria respiratory chain (Fig. 1B; Table 1). ccoP (NG1371) encodes the tri-haem c-type cytochrome component of the cytochrome cbb₃ oxidase complex (cytochrome oxidase) [GenBank AE004969; (46, 47), and ccpR (NG1767) encodes a cytochrome c peroxidase (a di-haem c-type cytochrome that uses hydrogen peroxide as an electron acceptor (48)); aniA (NG1276) and norB (NG1275) encode a copper-containing nitrite reductase, and a nitric oxide reductase, respectively. These latter two
enzymes make up the partial denitrification pathway present in *N. gonorrhoeae*, which converts nitrite to nitrous oxide with nitric oxide as an intermediate (49). The three remaining genes that also showed reduced expression in the *Ng-ntrX* strain were: 1) NG1024, that shows highest similarity to nitroalkane oxygenases; 2) NG1064, that is related to a carbon starvation protein found in *E. coli*; and 3) NG0718, that is annotated as encoding an *rpiR* family transcription factor, most probably *hexR*, which encodes a repressor of genes involved in glucose catabolism via the Entner-Doudoroff pathway (50, 51), GenBank AE004969]. Of the four genes whose expression was increased in *Ng-ntrX* two (NG0640 & NG0641) encode RmsR and a putative type III restriction/modification methylase (GenBank AE004969), whereas the other two encode hypothetical proteins.

To confirm that a mutation in *ntrX* affects the activity of gonococcal respiratory complexes, the activity of cytochrome oxidase and nitrite reductase in 1291 wild-type and *Ng-ntrX* was determined (Table 1, Fig. S1). Cytochrome oxidase activity and nitrite reductase activity were significantly lower in *Ng-ntrX* when compared to wild-type bacteria, a finding that is consistent with the microarray results. Complementation of the *Ng-ntrX* mutant strain using the entire *nrtYX* operon resulted in a restoration of the wild-type phenotype when using cytochrome oxidase activity as a test for successful mutant complementation (Fig. S1). This confirms that the down-regulation of respiratory chain components in strain *Ng-ntrX* was due to loss of functional NtrX. Taken together these data showed that *N. gonorrhoeae* NtrX has a role in the regulation of terminal reductase expression/activity and, especially, in the regulation of cytochrome *cbb3*. We found no evidence that NtrX affected genes involved in assimilatory nitrogen metabolism in this bacterium.
The gonococcal ntrX mutant is compromised in biofilm formation

The ability to generate energy using respiration or other mechanisms is a key metabolic process, and therefore the effect of the ntrX mutation on growth and survival of Ng-ntrX relative to wild-type was assessed. No difference was observed in the growth of wild-type gonococci or Ng-ntrX during aerobic and microaerobic growth in planktonic culture, and COMSTAT analysis showed no difference in either average thickness or amount of biomass in the biofilms of wild-type vs Ng-ntrX (data not shown). However, when examining gonococcal biofilms, after 48 hours growth on glass, that had been stained using a live/dead stain (Fig. 2), a substantially greater number of dead bacteria were present in the lower strata of the Ng-ntrX biofilm (Fig. 2B) when compared to the biofilm formed by wild-type bacteria (Fig. 2A). It is known that gonococcal growth in a biofilm requires both nitrite reductase and nitric oxide reductase (52, 53), and both of these enzymes, as well as cytochrome cbb3, showed decreased expression in Ng-ntrX. This result suggests that NtrX, and the genes regulated by NtrX, play an important role in gonococcal survival in the oxygen-depleted substrata of gonococcal biofilms.

Gonococcal mutants lacking NtrX show a significant decrease in the ability to invade and survive within primary human cervical epithelial cells

The use of primary human cervical epithelial (pex) cells as a model system of gonococcal cervicitis is well established and has been used to examine various aspects of the cervical-gonococcal interaction (38, 42). To determine the biological significance of the ntrX mutation using this pex cell culture model; we performed quantitative association, invasion, and survival assays using Ng-ntrX and wild-type gonococci (Fig. 3). Ng-ntrX
showed a significant ($P = <0.0001$) decrease in their ability to associate with, invade, and survive within pex cells when compared to wild-type *N. gonorrhoeae* (Fig. 3B). Together with the biofilm data, these results suggest that NtrX plays an important role in the pathobiology of *N. gonorrhoeae* by aiding survival of the bacteria in the context of a cervical cell infection as well as during extracellular biofilm formation.

An *ntrX* mutant of *R. capsulatus* is also affected in cytochrome cbb$_3$ respiratory activity.

Previous studies of NtrYX and its role in the regulation of nitrogen and general metabolism were carried out in $\alpha$-Proteobacteria, including *R. capsulatus* (31). To determine whether NtrYX is also involved in the regulation of specific respiratory processes in *R. capsulatus*, an insertion-deletion mutation in the *ntrX* locus of wild-type *R. capsulatus* SB1003 (designated Rc-*ntrX*) was constructed. To enable comparison of the data derived from the use of Ng-*ntrX*, respiratory activities were measured using an oxygen electrode with chromatophores (inverted membrane vesicles) prepared from *R. capsulatus* grown aerobically with lactate or succinate (carbon source), plus ammonium, (nitrogen source). The activities of the respiratory chain complexes in Rc-*ntrX* were significantly lower in all cases when compared to the wild-type strain (Fig. 4). Respiratory chain activities using NADH, succinate and lactate as electron donors were all reduced by between two- and four-fold. Of particular significance was the low level (reduced by 80%) of respiratory activity observed with the use of ascorbate/TMPD in Rc-*ntrX*, which corresponds to the activity of cytochrome cbb$_3$ and, thus, indicates a
significant decrease in the expression of this terminal oxidase complex in the \textit{ntrX} mutant (Fig 4).

To further confirm the effect of the \textit{ntrX} mutation on the respiratory chain and its associated components the specific activities of two enzymes, NADH:deylubiquinone oxidoreductase (NADH dehydrogenase/complex I) and pyridine nucleotide transhydrogenase (which interconverts NADH and NADPH), were also measured in membranes from wild-type \textit{R. capsulatus} and strain \textit{Rc-ntrX}. The activity of both enzymes was reduced in \textit{Rc-ntrX} when compared to wild-type (NADH dehydrogenase: wild-type: 2.6 ± 0.87; \textit{Rc-ntrX}: 0.80 ± 0.53; Transhydrogenase: wild-type: 0.14 ± 0.021, \textit{Rc-ntrX}: 0.075 ± 0.043; all rates expressed as mU activity/mg protein), indicating a possible role for NtrX in the regulation of these respiratory components as well as cytochrome \textit{cbb}_3 in \textit{R. capsulatus}.

\textit{Comparative analysis of ntrX genes across completed bacterial genomes}

Our data show that \textit{ntrX} mutations in both \textit{N. gonorrhoeae} strain 1291 and \textit{R. capsulatus} SB1003 affected the expression of genes involved in respiration, in spite of the fact that these NtrX proteins originated from two bacteria exhibiting completely different lifestyles and divergent phylogenetic associations. A comparison of the NtrX proteins from \textit{N. gonorrhoeae} and \textit{R. capsulatus} showed that these proteins have an amino acid identity of 30.4\% (similarity: 45.3\%) and share similar domain architectures. In both proteins the N-terminal region (~120 aa) showed strong similarities to a signal receiver domain (Rec domain, cd00156), followed by a P-loop ATPase domain (cl09099;
cd00009)/ $\sigma^{54}$-binding domain (pfam00158), which is less well conserved in the *N. gonorrhoeae* NtrX protein, and a DNA binding domain (Helix-Turn-Helix type) in the C-terminal region. This domain architecture, which closely resembles that of the $\sigma^{54}$ activating NtrC response regulator (54-56) together with the proximity of the *ntrYX* genes to the nitrogen regulatory genes *ntrBC* in many $\alpha$-Proteobacteria (including *R. capsulatus*) led to the original naming of this regulator, as well as to the suggestion that the NtrYX system is involved in nitrogen regulation (22, 31). As the basic function of this regulator appears to be conserved between $\alpha$- and $\beta$-Proteobacteria, we analysed the phylogenetic distribution of *ntrX* genes in sequences available in public databases. A total of 534 NtrX homologues were identified and analyzed. The identified sequences were mostly of proteobacterial origin, with $\alpha$-Proteobacteria species (families Rickettsiales, Rhizobiales and Rhodobacterales) being the most abundant, followed by the Neisseriales ($\beta$-Proteobacteria). Other NtrX related sequences originated from $\gamma$- and $\delta$-Proteobacteria as well as from representatives of the spirochetes and Aquificales (e.g. *Persephonella marina, Sulfurihydrogenibium*). Phylogenetic analyses suggested the presence of four major groups of NtrX sequences (NtrX1-4), with NtrX1, NtrX2 and NtrX4 containing multiple subgroups (Fig. 5). Neighbour Joining and Minimum Evolution analyses returned identical tree topologies using UPGMA analysis to generate a phylogenetic tree, albeit some branch points were changed, leading to an altered placement of some groups. As the outer nodes were less well supported in bootstrap analyses of all trees generated, this is not unusual.

The NtrX sequences of the *N. gonorrhoeae* and *R. capsulatus* are in groups NtrX3 and NtrX2, respectively, whereas NtrX sequences from other bacteria (for which functional
analyses of the NtrYX system have been published) are located in NtrX1 (B. abortus, Azospirillum brasilense, Azospirillum caulinodans) and NtrX4 (Ehrlichia chaffeensis) (21, 24, 57, 58). This indicates that the sequences identified in our database search are true NtrX homologues, although the reasons underlying the sequence divergence are unclear at present and functional analyses and comparisons between representatives of different NtrX groups will be necessary. The NtrX groups did not arise from the differential evolution of taxonomically related species as they all contain representatives of more than one taxonomic unit. For example, NtrX3 is made up of both β-Proteobacterial and γ-Proteobacterial sequences and similar trends are also observed for the other groups.

We also analyzed the correlation of the occurrence of ntrYX genes together with ntrBC genes and found that, with the exception of the Neisseriales and the Rickettsiales, the majority of bacteria that contain ntrYX genes also contain copies of ntrBC genes. Moreover, in Rickettsiales and in Ehrlichia spp., both of which fall into group NtrX4, it appears that the ntrX gene encodes an orphan response regulator, with the NtrY sensor kinase being encoded elsewhere in the genome. It is possible that this is true of additional species within the NtrX4 group, and unlike most other species represented in the phylogenetic tree cco genes appear to be absent from Rickettsiales and Ehrlichia spp. An interesting observation is that whereas most NtrX sequences contain a full length P-loop NTPase domain, in NtrX sequences obtained from Neisseria spp. this domain, which mediates interactions with σ54, appears to be truncated (~ 50 aa instead of ~100 aa). This truncation is likely a specific adaptation that may have occurred in response to the loss of function of the σ54 sigma factor in Neisseria spp. NtrX from N. gonorrhoeae retains the
same functionality as other NtrX proteins as well as the synteny of the \textit{ntrX} and \textit{ntrY} genes, indicating that it is a true NtrX protein.
Discussion

Adaptive responses controlled by a variety of TCS are central to the success of a number of bacterial pathogens in their interactions with the host (reviewed in (59)). One important adaptive response is an adjustment to altered oxygen availability/redox environments, and it is established that the ArcBA TCS is required for the survival of Haemophilus influenzae in a mouse model of bacteremia (60). The ArcBA TCS, however, appears to be restricted to ‘oxidase-negative’ bacteria such as the Enterobacteriaceae and Pasteurellaceae. Our phenotypic characterisation of an ntrX mutant of N. gonorrhoeae suggests that in ‘oxidase-positive’ bacteria the NtrYX TCS may fulfil a similar adaptive role to that of ArcBA in a changing oxygen availability/redox environment. This conclusion is consistent with those of Carrica et al., (2012) (24) who found that an ntrY mutant of B. abortus exhibited reduced expression of those operons encoding enzymes of denitrification (nar, nir, nor, nos). Our results show that in N. gonorrhoeae the expression of the operons encoding the two respiratory complexes of the partial denitrification pathway (aniA and norB) is dependent on NtrX. However, our results also show that in N. gonorrhoeae NtrYX has a broader role in the regulation of respiratory gene expression, as both cytochrome \( cbb_3 \) and cytochrome \( c \) peroxidase were also down-regulated in Ng-ntrX. Although B. abortus does possess a cytochrome \( cbb_3 \) (61) the effect of an ntrY mutation on its expression was not reported by Carrica et al. (2012) (24).

The NtrY sensor kinase (CDD designation COG5000) is predicted to be a membrane-bound protein with cytoplasmic HAMP, PAS and histidine kinase domains. PAS domains are critical sensory components of oxygen/redox sensing systems (62) and Carrica et al.
(2012) have shown that NtrY contains a heme prosthetic group that is involved in sensing redox changes. Our observation that the *Ng-ntrX* mutant biofilm was necrotic beneath the surface layer is consistent with the view that the mutant was unable to adjust to the limitation in oxygen that would occur in the substrata of the biofilm. Together these results suggest that the NtrYX TCS is a system required for adaptation to oxygen limitation. Support for this idea is derived from Carrica *et al.* (2012) (24) who show that NtrY-dependent gene expression increases as cell culture conditions switch from aerobic to anaerobic. The modest difference that we observed in NtrX-dependent gene expression in the wild-type and *Ng-ntrX* gonococci is similar to that observed for *B. abortus* NtrY-dependent gene expression occurring under microaerobic conditions.

It is increasingly clear that cytochrome oxidases with a high affinity for oxygen are required for the intracellular survival of some bacterial pathogens. For example, cytochrome *bd*, is required for the intracellular survival and virulence of the enteric bacterium *Shigella flexneri* (63). In *Brucella suis* cytochrome *cbb*₃ is required for chronic infection of oxygen-limited organs in a murine model (64). Hence, one of the factors that may have contributed to the failure of the gonococcal *ntrX* mutant to survive within pex cells may be a lowered level of cytochrome *cbb*₃ activity. However, the decreased expression of *norB* observed in *Ng-ntrX* also likely played a predominant role in the impaired ability of mutant gonococci to survive within pex cells, as previous data indicate that nitric oxide utilization plays a paramount role in promoting gonococcal survival within pex cells (65, 66). The human and bacterial responses driving the cervical-gonococcal dynamic are complex (66). Therefore, additional, alternative adaptive responses controlled by the NtrYX TCS also may have a contributory role in promoting
the intracellular survival and growth of gonococci in the pex cell model of infection. Carrica et al. (2012) also observed that the ntrY mutant of B. abortus showed lower survival within macrophages, lending further support to the importance of the NtrYX TCS to intracellular survival of bacterial pathogens.

Almost all studies of NtrYX to date have been conducted using bacteria in which the ntrBC genes lie upstream of ntrYX genes. The NtrBC TCS is central to the adaptation of bacteria to changes in the quality of nitrogen source and it is involved in the control of the expression of nif genes in nitrogen fixing bacteria. The NtrYX TCS was first discovered through transposon mutagenesis in Azorhizobium caulinodans where it was described as an NtrBC-related TCS with a role in controlling nitrogen fixation (22). The authors concluded that ntrYX form an operon as ntrY mutations had a polar effect on ntrX. Mutants in both genes were impaired in their ability to use different nitrogen sources and to fix dinitrogen. Further, in nodulation assays, phenotypes similar to fixL and ntrC mutants as well as altered growth characteristics were observed for this A. caulinodans ntrYX double mutant, suggesting that symbiotic growth was affected by mutagenesis (22).

Although NtrYX has been considered to be involved directly in nitrogen metabolism, the phenotype described above for A. caulinodans could have also arisen from a deficiency in the expression of cytochrome cbb3 because this oxidase is essential for symbiotic nitrogen fixation (11). Although we did not investigate Rhizobial species we did observe that in an ntrX mutant of another diazotrophic α-Proteobacterium, R. capsulatus, the activity of cytochrome cbb3 was also reduced. Thus, these data support the idea that control of cco expression is a function shared by the NtrYX TCS from a highly diverse range of bacteria. Several regulators are already known to control Cco expression in R.
capsulatus, including the RegBA TCS and FNR (19). From our data we can now add the
NtrYX TCS to this list, which contributes to the complexity of the regulation of Cco in
this organism. Our observation that NADH dehydrogenase and pyridine nucleotide
transhydrogenase activity were also affected by the mutation in *R. capsulatus ntrX*
suggests that the NtrYX TCS may have a substantive effect on wider bioenergetic
functions in *R. capsulatus*. FNR has been shown to also regulate *cco* and *aniA* expression
in *N. gonorrhoeae* (67). The NarPQ TCS and the repressor NsrR are also involved in the
control of *aniA* expression as part of the partial (AniA-NorB) denitrification pathway in
*N. gonorrhoeae* (68). NorB, AniA and Ccp, all of which are controlled by NtrX, are all
essential for biofilm formation in *N. gonorrhoeae* (53). In the NtrYX-containing
pathogen *Ehrlichia chaffeensis* inhibitors of histidine-kinase activity also reduced the
ability of these bacteria to evade cellular clearance that normally occurs through their
delivery to the lysozomes (58). Clearly, further studies are required to understand the
complex interplay between NtrYX and the other regulators of respiratory gene expression
in *N. gonorrhoeae* and in other bacteria, as well as the impact of an *ntrX* mutation on the
virulence and/or ability of these bacteria to form symbiotic relationships with their
respective hosts.

The activity of the NtrYX system in at least some α-Proteobacteria is also associated with
the alternative sigma factor $\sigma^{54}$ (31, 69). Together these regulators allow a bacterium to
adjust to altered nitrogen status. However, in *N. gonorrhoeae* the locus encoding $\sigma^{54}$ is a
non-functional pseudogene (70). Collectively this is consistent with the view that
adaptations made in response to altered nitrogen availability are not a primary function of
the NtrYX system in this bacterium. Nevertheless, the absence of a functional gene
encoding a $\sigma^{54}$ protein is consistent with the observed truncation of the P-loop NTPase domain of NtrX in Neisseriales in that the P-loop NTPase domain is required for the efficient interaction of NtrC/NtrX type regulators with $\sigma^{54}$ proteins.

Our phylogenetic analysis of NtrX showed that this response regulator is found in four distinct clades, all of which contain studied representatives of the NtrX regulator. NtrYX is present not only in the pathogenic *Neisseria* but also in the intracellular pathogens *Brucella* and *Bartonella* as well as *Ehrlichia* and *Anaplasma*. It is unclear what the exact role of NtrX is in many of these species but the conservation of this regulator in bacterial pathogens that contain very few two component regulatory systems indicates its significance. It is possible that through the control of respiratory gene expression the NtrYX system enables the adaptation and survival of a variety of intracellular ‘oxidase positive’ pathogens in addition to *N. gonorrhoeae*.

In conclusion our results and those of Carrica *et al.*, (2012) (24) indicate that the NtrYX TCS is a novel regulator of respiratory gene expression in a wide range of ‘oxidase-positive’ bacteria and that NtrYX most likely responds to signals arising from oxygen limitation. This TCS may be critical for bacterial survival under conditions where oxygen is limiting, for example within biofilms, intracellular growth within the eukaryotic host ranging from epithelial cells to macrophages and plant root nodules, depending on the host-microbe interaction.

**Acknowledgements**

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References


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A) Construction of an ntrX::kan insertion-deletion mutant in *N. gonorrhoeae* strain 1291, using a non-polar promoterless kanamycin-resistance cassette as detailed in Materials and Methods. The *ntrY* gene is upstream of *ntrX*. No polar effects are expected on the gene downstream of *ntrX*, *dprA*, as there is a large intergenic region of >80 basepairs between these two genes. The *dprA* gene is annotated as DNA processing chain A (NG1865) in the FA1090 genome.

B) Summary of the *N. gonorrhoeae* respiratory chain. A number of different substrates can be used to provide reducing power to the electron transport chain, which transfers electrons from the ubiquinone/ubiquinol pool (UQ pool) to cytochrome *c* (cyt *c*) via the cytochrome *bc*₁ complex. Electrons are then transferred to a number of final electron acceptors – oxygen (cytochrome *cbb₃* oxidase; Cco), hydrogen peroxide (cytochrome *c* peroxidase; CCP), or to nitrite and then nitric oxide (nitrite reductase; AniA, and nitric oxide reductase; NorB, respectively). These latter two enzymes comprise the partial denitrification pathway present in the gonococcus. NorB takes electrons directly from the UQ pool, and is only energy conserving if used in tandem with the Nuo NADH dehydrogenase. All four terminal reductases (Cco, CCP, AniA and NorB) are down-regulated in strain *Ng-ntrX*.

FIG. 2 – Biofilm images of (A) 1291 wild-type and (B) isogenic mutant strain *Ng-ntrX*, using green fluorescent protein to show live/dead cells. Wild-type biofilms contain predominantly live cells, as evidenced by the high amount of GFP produced. *Ng-ntrX* cells by contrast, contain predominantly dead cells, indicating the genes regulated by
NtrX are important in the formation of a live, functional biofilm. Images were taken as 1-D slices at 200x magnification, and stacked to produce a 3-D image using Volocity® software.

**FIG. 3** - A) Pex cell survival assays of 1291 wild-type and Ng-ntrX represented as an index of invasion (invasion/association) and survival (3-hour survival/invasion). $P$-value for invasion index = 0.0016; $P$-value for survival index = 0.0001. Error bars are a measure of variance from the mean; $P$-values for invasion/survival indices were determined using Student’s $t$-test. (B) Data from pex cell association, invasion and survival assays used to generate the data shown in (A), as a percentage of initial inoculum. All data is an average of three separate experiments. $P$-values for association, invasion, and 1-hour and 3-hour survival for 1291 wild-type vs Ng-ntrX are all $\leq 0.0001$. Errors represent variance from the mean; $P$-values were calculated using a Kruskal-Wallis ANOVA. Experiments were performed in triplicate on three separate occasions.

**FIG. 4** – *R. capsulatus* SB1003 wild-type vs Re-ntrX respiratory assays using chromatophores, with NADH, Succinate, Ascorbate/TMPD, and Lactate, provided as substrates. Rates are expressed as a measure of nmol O$_2$ consumed/min/mg protein, and are the result of three separate experiments. Rates were measured using a Rank oxygen electrode as detailed in materials and methods. In all cases there was a statistically significant difference between wild-type and Re-ntrX ($P = <0.0001$), calculated using Student’s $t$-test. Error bars represent one standard error of the mean.
FIG. 5 - Phylogenetic relationships between NtrX sequences. The four NtrX groupings are marked in colour; small arrows indicate NtrX sequences for which experimental data is available, with the NtrX sequences from *Rhodobacter capsulatus* and *Neisseria gonorrhoeae* highlighted with large arrows (Rc NtrX and Ng NtrX respectively).

Sequences were aligned and analyzed in Mega 5.0, the phylogenetic tree shown was constructed using the Neighbor-Joining algorithm and 500 bootstrap resampling cycles. Only bootstrap values above 50% are shown.
Table 1 – Genes that are differentially regulated in Ng-ntrX when compared to 1291 wild-type

<table>
<thead>
<tr>
<th>Gene a</th>
<th>Description</th>
<th>Microarray b</th>
<th>Enzyme activity c</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ntrX:wt</td>
<td>B stat</td>
<td>ntrX:wt</td>
</tr>
<tr>
<td>NGO1276</td>
<td>Nitrite reductase (aniA)</td>
<td>0.33</td>
<td>6.91</td>
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<tr>
<td>NGO1275</td>
<td>Nitric oxide reductase (norB)</td>
<td>0.37</td>
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<tr>
<td>NGO1769</td>
<td>Cytochrome c peroxidase (ccpR)</td>
<td>0.39</td>
<td>4.19</td>
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<tr>
<td>NGO1371</td>
<td>Cytochrome oxidase subunit (ccoP)</td>
<td>0.40</td>
<td>5.23</td>
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<tr>
<td>NGO1024</td>
<td>Hypothetical protein</td>
<td>0.40</td>
<td>5.13</td>
</tr>
<tr>
<td>NGO1064</td>
<td>CstA</td>
<td>0.45</td>
<td>2.18</td>
</tr>
<tr>
<td>NGO0718</td>
<td>Putative RpiR-family transcriptional regulator</td>
<td>0.50</td>
<td>4.77</td>
</tr>
</tbody>
</table>

|        | Putative Type III res/mod methylase      | 2.64         | 1.01              |
| NGO0640| RmsR                                     | 2.62         | 3.70              |
| NGO1712| Hypothetical protein                     | 2.15         | 5.15              |
| NGO1889| Hypothetical protein                     | 2.07         | 3.67              |

a The "NG" gene IDs refers to the annotation of the Neisseria gonorrhoeae strain FA1090 genome in the LosAlamos database (http://www.stdgen.lanl.gov/stdgen/bacteria/ngon/index.html).

b The ratio presented is the mean of mutant:wild-type from six replicate spots on three independent microarrays, incorporating a dye swap. Thus, the expression of each gene was measured six times. Only those genes with an expression value above two-fold (up
regulated), or below 0.5-fold (down regulated), and having a B statistic value above 0.0 were considered significant and included in this study. A threshold in the B statistic of 0.0 was adopted as genes with a B score >0 have a >50% probability of being truly differentially expressed.

Enzyme assays were performed as detailed in materials and methods, and results are detailed in Fig. S1.
**A**

**Invasion Index**

**Survival Index**

<table>
<thead>
<tr>
<th></th>
<th>Wild type 1291</th>
<th>Ngn-trX</th>
</tr>
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<tbody>
<tr>
<td><strong>Association</strong></td>
<td>28.8 ± 0.96</td>
<td>22.8 ± 1.3</td>
</tr>
<tr>
<td><strong>Invasion</strong></td>
<td>2.8 ± 0.19</td>
<td>1.6 ± 0.01</td>
</tr>
<tr>
<td><strong>Survival (1hr)</strong></td>
<td>2.8 ± 0.07</td>
<td>1.4 ± 0.04</td>
</tr>
<tr>
<td><strong>Survival (3hr)</strong></td>
<td>9.0 ± 0.25</td>
<td>0.4 ± 0.06</td>
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