Published in final edited form as:

Can J Microbiol. 2008 August ; 54(8): 639–646. doi:10.1139/w08-057.

Differences in nitric oxide steady-states between arginine, hypoxanthine, uracil auxotrophs (AHU) and non-AHU strains of *Neisseria gonorrhoeae* during anaerobic respiration in the presence of nitrite

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Abstract

Neisseria gonorrhoeae can grow by anaerobic respiration using nitrite as an alternative electron acceptor. Under these growth conditions, *N. gonorrhoeae* produces and degrades nitric oxide (NO), an important host defense molecule. Laboratory strain F62 has been shown to establish and maintain a NO steady-state level that is a function of the nitrite reductase/nitric oxide reductase ratio and is independent of cell number. The nitrite reductase activities (122–197 nmoles NO₂ reduced/ min-OD₆₀₀) and nitric oxide reductase activities (88–155 nmoles NO reduced/ min-OD₆₀₀) in a variety of gonococcal clinical isolates were similar to the specific activities seen in F62 (241 nmoles NO₂ reduced/ min-OD and 88 nmoles NO reduced/ min-OD, respectively). In 7 gonococcal strains, the NO steady state levels established in the presence of nitrite were similar to that of F62 (801–2121 nM NO), while 6 of the strains, identified as arginine, hypoxanthine, and uracil auxotrophs (AHU), that cause asymptomatic infection in men, had either a 2- to 3-fold (373–579 nM NO) or about 100-fold (13–24 nM NO) lower NO steady state concentrations. All tested strains in the presence of a NO-donor, DETA/NO, quickly lowered and maintained NO levels in the non-inflammatory range of NO (<300 nM). The generation of a NO steady-state concentration was directly affected by alterations in respiratory control in both F62 and an AHU strain, although differences in membrane function are suspected to be responsible for NO steady-state level differences in AHU strains.

Keywords

Neisseria gonorrhoeae; nitric oxide; *norB*; *aniA*; denitrification

Introduction

Neisseria gonorrhoeae is a gram-negative diplococcus that causes the sexually transmitted disease gonorrhea. It is a strictly human pathogen capable of colonizing a variety of mucosal surfaces, and accounts for more than 700,000 new cases of gonorrhea a year in the U.S., as estimated by the Centers for Disease Control and Prevention. The establishment of growth in association with these different epithelial surfaces can lead to a spectrum of clinical manifestations predominately involving the genitourinary tract, along with the conjunctiva, pharynx, and rectal mucosa. If the gonococcus is controlled to a defined environment, urethritis or cervicitis develops, while other serious complications occur in the event of bacterial spread.

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These include disseminated gonococcal infection (DGI), which can lead to septic arthritis (Ghosn and Kibbi 2004; Mayer *et al.* 1977), and pelvic inflammatory disease (PID), of which 1 in 10 of all women suffer (Aral *et al.* 1991). PID is a major cause of infertility and ectopic pregnancies.

Under oxygen limited conditions, gonococcal growth occurs through anaerobic respiration (Knapp and Clark 1984), where nitrite or nitric oxide acts as an alternative electron acceptor. *N. gonorrhoeae* was shown to contain only a partial denitrification system, including AniA, a copper containing nitrite reductase (Nir) (Cardinale Ph.D thesis 1999; Boulanger and Murphy 2002), that reduces nitrite to nitric oxide, and NorB, a nitric oxide reductase (Nor) (Householder *et al.* 2000) that reduces nitric oxide to nitrous oxide (Lissenden *et al.* 2000).

The presence of the denitrification pathway in *N. gonorrhoeae* allows the ability to both produce and degrade NO. NO occupies a central role in mammalian biology, where it has direct functions for cell-to-cell signaling, along with being an integral part of the host response to infection (Nakatsuka *et al.* 2003; Togashi *et al.* 1997; Guzik *et al.* 2003). For an in-depth review of how NO levels correlate with immune system activation state and the potential impact of gonococcal NO metabolism, refer to Cardinale and Clark 2005.

We previously investigated the kinetic parameters for Nir and Nor in *N. gonorrhoeae* laboratory strain F62. This strain produced a steady-state level of NO when using nitrite as a terminal electron acceptor, which was dependent on pH but independent of cell number. The NO steady-state in the presence of saturating levels of nitrite was directly dependent on the ratio of Nir/Nor, while the NO steady-state concentration in the presence of an exogenous NO source, to mimic an *in vivo* environment, was directly dependent on the NO concentration generated by the NO donor (Cardinale and Clark 2005). Furthermore, F62 was able to rapidly modulate NO levels from a pro-inflammatory level (>1 μ M) to a non-inflammatory level (<100 nM) in under 30 minutes, within an *in vitro* reaction vessel. These studies illustrate the ability of *N. gonorrhoeae* to produce and degrade nitric oxide, suggesting a possible role for NO metabolism in the immunosuppressive effects of gonococcal infection.

We explore in this report the ability of other gonococcal strains to produce and degrade nitric oxide, to establish a nitric oxide steady-state concentration, and to investigate if respiratory control (the coupling of ATP phosphorylation with electron flow through the respiratory chain) affects the NO steady-state level.

Materials and Methods

Bacterial strains and growth conditions

The strains used in this study were *N. gonorrhoeae* F62, and a collection of clinical isolates acquired from the Neisseria Reference Laboratory, CDC, Atlanta, GA (Table 1). Strains were grown on Difco GC medium base (Becton, Dickinson and Co., Franklin Lakes, NJ) plates with 1% Kellogg's supplement (Kellogg *et al.* 1963), either aerobically in a 5% CO₂ incubator or anaerobically in a Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) with an atmosphere of 85% N₂, 10% H₂, and 5% CO₂, as previously described (Clark *et al.* 1987). Anaerobic plates were subcultured 3 times to ensure full induction of Nir and Nor. Nitrite was provided for anaerobic growth by placing 40 μ l of a 20% (wt/vol) NaNO₂ solution on a sterile cellulose disk in the center of the plate. All incubations were at 37°C.

Measurement of NO concentrations

An Apollo 4000 free radical analyzer with ISO-NOP probes (World Precision Instruments, Sarasota, FL) allowed for real-time measurement of nitric oxide concentrations, as previously described (Cardinale and Clark 2005). The detection is based on the electrochemical response

produced as the free radical is selectively oxidized at the ISO-NOP working electrode surface, resulting in an electrical current. All of the standard curves and reaction assays were performed in a temperature controlled reaction vessel (World Precision Instruments) at a constant 37 °C.

Nitrite reductase and nitric oxide reductase assays

Nir activity was measured by the reduction in nitrite concentration and Nor activity was measured using the short half-life NO donor, 2-(N,N-diethylamino)-diazene-2-oxide (DEA/NO) (Sigma Chemical Company, St. Louis, MO), as previously described (Cardinale and Clark 2005).

Nitrite reductase at steady-state was measured by determining the decrease in nitrite concentration, as previously described (Cardinale and Clark 2005); in cultures that had achieved a steady-state NO level.

Determination of NO steady-state values

Cells were resuspended in GCK broth at the indicated pH and the absorbance at 600 nm was determined. Inoculum was added to a final OD₆₀₀ of 0.02 and cells were allowed to incubate for 10 minutes, when nitrite was added to a final concentration of 2 mM. The same procedure was used when DETA/NO [2,2'-(hydroxynitrosohydrazono) bis-ethanimine; Sigma Chemical Company, St. Louis, MO] was used as a source of exogenous NO, except it was added at a final concentration of 0.3 mM.

DNA sequencing of *aniA* and *norB*

Genomic DNA was isolated from both F62 and RUN 5635 using the Wizard Plus Miniprep kit (Promega Corp, Madison, WI). Gene specific primers to *aniA* and *norB* were designed and used to amplify both gene products in each of the two strains. DNA fragments were purified with the Qiagen PCR purification kit (Qiagen, Valencia, CA) and sent to ACGT Inc. (Wheeling, IL) for sequencing.

Effects of uncoupling agents

F62 and RUN 5635 were selected to investigate the effects of altering respiratory control on the establishment of NO steady-state concentrations in the presence of 2 mM nitrite. For these experiments, the uncoupling agents valinomycin (Sigma Chemical Company) and 2,4-dinitrophenol (Sigma Chemical Company) were dissolved in ethanol and used at a final concentration of 10 µM. The uncoupling agent was added to GCK medium prior to the addition of bacteria. Gonococcal suspensions were allowed to incubate for 10 minutes as done in other assays. We also determined the Nir and Nor specific activities within our uncoupled system. The reaction vessel contained either ethanol alone or the uncoupling agent.

Results and Discussion

Nitrite reductase and nitric oxide reductase specific activities of *N. gonorrhoeae* clinical isolates

To determine the range of activities of the denitrification pathway enzymes in *N. gonorrhoeae*, we chose a set of gonococcal clinical isolates that varied in isolation site and auxotype (Table 1). These strains were grown anaerobically through three passages to ensure the full induction of Nir and Nor. Each strain was then assayed separately for both Nir and Nor specific activities upon resuspension into GCK medium, with subsequent addition into a reaction vessel containing the mounted ISO-NOP electrode.

Upon comparison to laboratory strain F62, that had a Nir activity of 241 nmoles nitrite reduced per minute per OD₆₀₀, each of the nine tested isolates showed no more than a two-fold difference from F62 in Nir activity (122–197 nmoles nitrite reduced per minute per OD₆₀₀) (Fig. 1A). The F62 reference strain had a Nor specific activity of 88 nmoles NO reduced per minute per OD₆₀₀. Similarly, each of the tested isolates showed no more than a two-fold difference from F62 in nitric oxide reductase activity (88–155 nmoles NO reduced per minute per OD₆₀₀) (Fig. 1B). Thus other gonococcal isolates show similar activities for each of the functional reductases involved in denitrification in comparison to a commonly used laboratory strain, independent of auxotrophic phenotype.

Establishment of NO steady-state in presence of exogenous NO

To investigate the *N. gonorrhoeae* response to a steady production of NO, as would normally occur during an active host response, we used a NO-donor, DETA/NO, that has a half-life of 20 hours. Previous work showed that anaerobically grown F62 at an OD₆₀₀ of 0.025 can metabolize a pro-inflammatory level of NO (>1 μ M) and establish a new NO steady-state concentration that is within the non-inflammatory level of NO (~100 nM) within 30 minutes. We tested clinical isolates by the same procedure, where 0.3 mM DETA/NO was added to the medium and produced a constant supply of NO at a concentration of around 1500 nM NO (data not shown), and allowed a new NO steady-state concentration to be established by bacterial denitrification. Figure 2 shows that all of the clinical isolates were equally efficient at metabolizing chemically generated NO and establishing a new NO steady-state level that reduced input concentrations by 79–97%. Maintained NO levels in the nanomolar range could have effects on the host response, leading to immune suppression instead of immune stimulation since pre-treatment of human cells with NO donors that produce low levels of NO prevents LPS-, IL-1 β -, and TNF- α -inducible cytokine and iNOS synthesis (Peng *et al.* 1995; Stefano *et al.* 2000).

Parameters of NO metabolism in the presence of nitrite

The NO steady-state concentration of each of the isolates were measured in the presence of 2 mM nitrite and compared to strain F62. Previous data showed that F62 generates a steady-state level of around 1200 nM NO. With the addition of nitrite, each of the clinical isolates generated a NO steady-state level that remained consistent for several hours (data not shown). Seven of the gonococcal strains established steady-state levels similar to that of F62 (801–2121 nM NO), being near or within the pro-inflammatory range (Fig. 3). However, 2 of the tested strains, NRL 905 and RUN 5635, showed about 100-fold lower NO steady state levels (13 and 24 nM NO, respectively), concentrations that would represent non-inflammatory conditions (Fig. 3).

Upon further investigation, these two unique strains were identified as AHU auxotrophs, requiring arginine, hypoxanthine, and uracil. We also tested 4 additional AHU strains and found that two distinct classes of AHU strains exist. There are those that set about a 2–3-fold lower NO steady-state level in the presence of nitrite (Fig. 3, strains J,K,L,M), while RUN 5635 and NRL 905 set about a 100-fold lower NO steady-state level compared to F62 (Fig. 3, strains H,I).

Parameters of NO metabolism in AHU strains

Effects of pH, cell concentration, and initial nitrite concentrations—We chose one AHU strain for further study (RUN 5635) for its low NO steady-state level in the presence of nitrite that is nearing the detection limit for NO. We have previously shown the elevation in NO steady-state level with increasing nitrite concentration until a saturating level of 1 mM in strain F62 (Cardinale and Clark 2005). A NO steady-state concentration was measured for AHU strain RUN 5635 under a range from 0.1 mM to 2 mM nitrite. RUN 5635 responded much the same as the strains that set high NO steady-state levels by having a dose response to

nitrite concentrations, where increasing amounts of nitrite generated higher levels of NO at the steady-state, albeit at a 100-fold lower level than observed with F62 (data not shown). The NO steady-state level in RUN 5635 was also found to be independent of cell concentration (data not shown), as was found with strain F62 (Cardinale and Clark 2005).

Previous work has shown that gonococcal denitrification in strain F62 is directly dependent on pH, where Nir and Nor show optima at pH 6.5 and 7.5, respectively. As a result, increasing pH causes a lower Nir/Nor ratio, and consequently a lower NO steady-state level (Cardinale and Clark 2005). RUN 5635 was assayed similarly over a range of pH values for NO steady-state level and Nir and Nor activities. Each of the reductases had pH optima that mimicked F62 (data not shown). A plot of the NO steady-state concentration versus Nir/Nor ratio shows a direct correlation for F62 and RUN 5635 (Fig. 4), however other factors must also affect the NO steady-state level, providing two distinct curves.

Sequencing of *aniA* and *norB*

A possible cause for such a distinct difference in NO steady-state levels between F62 and AHU strains would be changes to amino acid sequence of either reductase that altered the K_m value. Upon sequencing of *aniA* and *norB* from RUN 5635, it was identified that *aniA* had only one alteration, a substitution of an asparagine (RUN 5635) for a serine (F62) at residue 323, which maintains the uncharged polar nature of the residue. In addition, this same substitution occurs within another tested isolate, RUN 5640, which has a NO steady-state level at F62 levels (Fig. 3). Furthermore, the *norB* sequence in RUN 5635 is identical to F62, meaning that these differences in NO steady-state concentrations are not due to changes in sequence of either reductase.

Nir activity during steady-state denitrification

The possibility exists that the AHU and non-AHU NO steady-state differences can be attributed to changes in Nir activity during an established denitrification steady-state, as compared to the initial rates of nitrite reduction measured above (Fig. 1A). The low AHU NO steady-state concentration would then be a result of decreased Nir activity as the NO steady-state is reached, decreasing the Nir/Nor ratio. When Nir activity during steady-state NO production was determined, RUN 5635 had a 33% decrease in activity (191 nmoles NO₂ reduced/min-OD₆₀₀ initial rate versus 129 nmoles NO₂ reduced/min-OD₆₀₀ at NO steady-state) while F62 had an 80% decrease in activity (241 nmoles NO₂ reduced/min-OD₆₀₀ initial rate versus 47 nmoles NO₂ reduced/min-OD₆₀₀ at NO steady-state). Thus RUN 5635 Nir activity was about three times higher than F62 at the NO steady-state period (129 versus 47 nmoles NO₂ reduced/min-OD₆₀₀), which would have been predicted to generate a higher not a lower NO steady-state level. Thus, differences in NO inhibition of Nir cannot explain the differences in NO steady-state levels in these two strains. The greater inhibition of Nir activity in strain F62 is most likely due to the higher NO steady-state level generated by nitrite reduction in this strain as compared to RUN 5635. While inhibition of cytochrome *cd₁*Nir by NO is well documented (Kucera 1992; Zumft 1997; Kunak *et al.* 2004), this is the first report suggesting that a copper containing nitrite reductase (Cu-dNir) is inhibited by NO.

Presumably, the NO level at steady-state generated by gonococcal denitrification is a function of the Nir/Nor ratio; i.e., it is a function of the ability to produce NO (Nir activity) and to reduce NO (Nor activity). If the low NO steady-state level in AHU strains is not due to a lower Nir activity at steady state as compared to strain F62, then it must be due to higher Nor activity. Using NO donors to measure Nor activity at steady-state is difficult, due to the complex nature of NO flux into the pathway and possible effects of NO on Nir and/or Nor activities.

Effects of uncoupling agents on NO steady-state levels

Nor is located in the inner membrane of *N. gonorrhoeae* and thus should be under respiratory control. Respiratory control is the modulation of the activity of electron transport systems by the membrane potential, such that respiration is inhibited when the desired membrane potential is achieved. To determine if respiratory control is involved in the NO steady-state level generation, we determined the effect of chemicals that uncouple electron flow from ATP production on the NO steady state level.

In order to examine the effect of respiratory control on the NO steady-state concentration, we used valinomycin to allow Nir and Nor activity to proceed “unchecked”, at a maximal rate that would normally be under strict control. Valinomycin is an ionophore carrier with high selectivity for K⁺ ions but little affinity for other cations (Lauger 1972), and its mechanism of activity has been elucidated (Harold and Altendorf 1974; Henderson *et al.* 1969; Henderson 1971). It functions to shield the positive charge of the ion, allowing K⁺ to move across the lipid bilayer, which reduces the membrane potential of the cell. With valinomycin treatment, both F62 and RUN 5635 showed significant decreases in NO steady-state levels (Fig. 5A).

Uncoupling caused a dramatic decrease in NO steady-state level in the presence of nitrite in F62 (4510 nM NO compared to 532 nM NO), bringing NO levels from a pro-inflammatory to a non-inflammatory range, while RUN 5635 showed a decrease as well (443 nM NO compared to 143 nM NO) (Fig. 5A). The uncoupling agent solvent, ethanol, caused an increase in the NO steady-state level, and this is why the NO steady-state level for both F62 and RUN 5635 are significantly higher in these experiments than the levels determined in the absence of ethanol (Fig. 3). There was also a significant decrease in NO steady-state levels in both F62 and RUN 5635 with uncoupling treatment with 2,4-dinitrophenol (Fig. 5B). Dinitrophenol is a lipophilic weak acid that functions as an uncoupler by dissipating the proton gradient. These data suggest respiratory control is directly involved in modulating the level of NO while denitrification occurs in both F62 and RUN 5635.

Effects of uncoupling agents on Nir and Nor specific activities

The degree to which respiratory control affects NO metabolism within these strains is further evident when we examine Nir and Nor specific activities themselves, in the presence of uncoupling treatment. Nir activity in F62 was limited by respiratory control to a minor extent, as valinomycin treatment resulted in increased Nir activity from 235 to 283 nmoles NO₂ reduced/min-OD₆₀₀, while RUN 5635 showed no significant change (Fig. 5C). This would be expected, as the presence of Nir in the outer membrane (Clark *et al.* 1987) would make the enzyme minimally sensitive to respiratory control. In contrast, there was a much larger influence on Nor activity; F62 Nor activity increased 88 % in the absence of respiratory control (116 compared to 218 nmoles NO reduced/min-OD₆₀₀), while RUN 5635 Nor activity increased 20 % (186 compared to 223 nmoles NO reduced/min-OD₆₀₀) (Fig. 5D). The response of Nir and Nor activity to uncoupler treatment with 2,4-dinitrophenol mimicked that of valinomycin for strains F62 and RUN 5635 (data not shown). This suggests that the lowered NO steady-state concentrations with uncoupling treatment are a result of enhanced Nor activity, which occurs in both F62 and RUN 5635.

Respiratory control is the process by which the electrochemical proton gradient functions to control the rate of electron transport through the respiratory chain. Since bacterial cell membranes are impermeable to protons, ATP production is coupled to electron transport and thus any biological processes requiring an input of electrons is indirectly regulated by respiratory control. Much of the *Pseudomonas stutzeri* NO reductase was found to be inactive *in vivo* (Zumft *et al.* 1994) suggesting that it might be under respiratory control. In *N. gonorrhoeae* the denitrification pathway is under respiratory control in both F62 and the AHU strain RUN 5635.

Known characteristics of AHU strains

In order to hypothesize why AHU strains show different NO steady-state levels, it is helpful to examine how these auxotrophs are unique among gonococcal isolates. Through multilocus sequence typing, it has been established that AHU isolates are an independent lineage, meaning these clones have the majority of diversification occurring through mutation and not recombination events (Bennett *et al.* 2007), where they differed from the predominant type at a single locus (Gutjarh *et al.* 1997). They are unusually susceptible to antibiotics (Koebl and Catlin 1986), and are resistant to human serum killing (Morello and Bornhoff 1989), both of which are uncommon among gonococci of other auxotypes.

There are several studies that give support for AHU strains having membrane defects. AHU strains were uniformly hypersusceptible to vancomycin (Exner *et al.* 1982; Pace and Catlin 1986), suggesting that a mutation of an envelope locus may be responsible. This could mean a number of things are occurring within these strains. Either a decreased amount of peptidoglycan cross-linking or a lowered amount of a major outer membrane protein (Guymon *et al.* 1978) could account for increased sensitivity to vancomycin, as more of the antibiotic is able to progress through the outer membrane. Any genetic alterations within the cytoplasmic membrane architecture may lead to an increase in membrane permeability. Modifications to the bacterial membrane permeability could alter the membrane potential, such that the gonococcal energy-dependent *mtr* efflux pump (Pan and Spratt 1994; Hagman *et al.* 1995) might function at a sub-optimal rate, resulting in a decreased capacity to export vancomycin at the appropriate rates that would lead to normal sensitivities to the antibiotic.

The AHU strains have also been shown to be hypersensitive to fecal fatty acids, by a mechanism that is independent of the Mtr system (McFarland *et al.* 1983). The inhibition of growth of *E. coli* and *Bacillus subtilis* by fatty acids was demonstrated to be due to the uncoupling of oxidative phosphorylation from the electron transport chain (Freese *et al.* 1973). Thus, if the AHU strains are deficient in membrane function, one would predict that their growth might be more sensitive to uncouplers of oxidative phosphorylation.

It is possible that AHU strains have novel genes that function as NO detoxification systems or that there is some level of deregulation in the genetic components involved in NO metabolism. However, all of the phenotypes that have been described for the AHU strains can be linked to membrane defects, suggesting that alterations in membrane function may be responsible for differences in the NO steady-state levels.

Studies have correlated the AHU strains with asymptomatic infections in men (Crawford *et al.* 1977). The majority of gonococcal isolates tested showed generation of a high NO steady-state level, representing over 1 μ M NO, in the presence of 2 mM nitrite. When these strains cause a urethral infection, they would be exposed to an intermittent supply of nitrite through the flushing of urine. This could lead to μ M levels of NO being produced by the gonococcus rather than the host. RUN 5635 and NRL 905, identified as AHU strains showed a drastic 100-fold decrease in NO steady-state levels while in the presence of high levels of nitrite, suggesting that they would not produce high NO levels from nitrite during infection. Their ability to produce NO steady-state concentrations in the non-inflammatory range may be related to their ability to cause asymptomatic infection in men.

In summary, we have shown that while the gonococcal isolates studied showed nearly equivalent Nir and Nor activities, in the presence of nitrite the AHU auxotrophs established a very low NO steady-state concentration, that differed significantly from non-AHU strains, which set a high NO steady-state level. Both types of strains showed a role of respiratory control and thus membrane function on the establishment of a NO steady-state concentration. In addition, each strain was equally adapted to modulate NO levels efficiently in response to a

NO donor, suggesting that this bacterium utilizes its denitrification system to establish a non-inflammatory environment. Current studies are investigating the role of gonococcal NO steady-state level on the suppression of the immune response in host cells.

Acknowledgements

This study was supported by Public Health Service grant R01 AI 11709 from the National Institutes of Health.

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Fig. 1.

Nitrite reductase and nitric oxide reductase specific activity in *Neisseria gonorrhoeae* clinical isolates and in laboratory strain F62. Strains were grown anaerobically through 3 passages on GCK plates to maximally induce Nir or Nor. Activity was measured using an ISO-NOP electrode by either measuring the reduction of nitrite (A) or nitric oxide (B) from the reaction vessel of 3 independent bacterial suspensions, as described in Experimental procedures. Results are presented as the mean + standard error. (A). Average nitrite reductase activity, (B). average nitric oxide reductase activity. Strains are F62 (Pro); A) RUN 4383 (Proto); B) RUN 5632 (PAU); C) RUN 5633 (PAU); D) RUN 5636 (Pro); E) RUN 5638 (Proto); F) RUN 5640 (Proto); G) CDC 86 (Proto); H) RUN 5635 (PAHU); and I) NRL 905 (AHU).

Fig. 2.

Concentration of NO at steady-state under simulated host generated NO conditions. Strains were grown anaerobically through 3 passages on GCK plates and suspended in GCK medium ($OD_{600}=0.02$) for 10 minutes, at which time 0.3 mM DETA/NO, a long term NO donor was added, generating around 1500 nM NO. NO steady states were allowed to establish while the NO concentrations were monitored using an ISO-NOP electrode. Value indicates the average concentration of NO from 3 independent trials + standard error. Strains are F62 (Pro); A) RUN 4383 (Proto); B) RUN 5632 (PAU); C) RUN 5633 (PAU); D) RUN 5636 (Pro); E) RUN 5638 (Proto); F) RUN 5640 (Proto); G) CDC 86 (Proto); H) RUN 5635 (PAHU); and I) NRL 905 (AHU).

Fig. 3.

Concentration of NO at steady-state in the presence of 2 mM nitrite. Strains were grown anaerobically through 3 passages on GCK plates and suspended in GCK medium ($OD_{600}=0.02$) for 10 minutes, at which time 2 mM nitrite was added. NO steady-states were allowed to establish while the NO concentrations were monitored using an ISO-NOP electrode. Each value is the average concentration of NO from 3 independent trials + standard error. Highly significant differences compared to laboratory strain F62 using a student t-test are indicated as * ($p<0.05$) and ** ($p<0.01$), respectively. Strains are F62 (Pro); A) RUN 4383 (Proto); B) RUN 5632 (PAU); C) RUN 5633 (PAU); D) RUN 5636 (Pro); E) RUN 5638 (Proto); F) RUN 5640 (Proto); G) CDC 86 (Proto); H) RUN 5635 (PAHU); and I) NRL 905 (AHU); J) RUN 4002 (AHU); K) RUN 4198 (AHU); L) RUN 4248 (AHU); and M) RUN 4266 (AHU).

Fig. 4. Relationship between nitrite reductase/nitric oxide reductase (Nir/Nor) and $\mu\text{M NO}$ at steady-state. Open circles represents F62, while closed circles represents RUN 5635. Values were obtained by performing Nir and Nor assays and NO steady-state determinations over a pH range of 6.5–8.0.

Fig. 5.

Effects of uncoupling on the NO steady-state levels, Nir activity and Nor activity in F62 and RUN 5635. (A). NO steady-state levels produced by F62 and RUN 5635 in the presence of an ethanol control or 10 μ M valinomycin. NO steady-states were measured as stated previously. Plots are representative of 3 separate experiments. Highly significant differences compared to ethanol controls are indicated by * ($p < 0.005$) or ** ($p < 0.0005$), respectively. (B). NO steady-state levels produced in the presence of an ethanol control or 10 μ M 2,4-Dinitrophenol. Plots are representative of 3 separate experiments. Highly significant differences compared to ethanol controls are indicated by * ($p < 0.05$) or ** ($p < 0.01$), respectively. (C). Nitrite reductase specific activity and (D) Nitric oxide reductase activity of F62 (white bars) and RUN 5635 (gray bars) while in the presence of an ethanol control or 10 μ M valinomycin. Values represent the average of 3 separate determinations + standard error. Highly significant differences compared to the activity in the presence of no valinomycin are indicated as * ($p < 0.05$) and ** ($p < 0.005$). All statistical analysis was done using a student t-test with a p value of < 0.05 as considered significant.

Table 1*Neisseria gonorrhoeae* strains used in this study.

Strain	Isolation Site	Auxotype [*]
F62	Laboratory strain	Pro
RUN 4002	Male Urethra	AHU
RUN 4198	Male Urethra	AHU
RUN 4248	Male Urethra	AHU
RUN 4266	Male Urethra	AHU
RUN 4383	Cervix	Proto
RUN 5632	Endometrium	PAU
RUN 5633	Endometrium	PAU
RUN 5635	Fallopian Tube	PAHU
RUN 5636	Endometrium	Pro
RUN 5638	Cervix	Proto
RUN 5640	Cervix	Proto
CDC 86	Synovial Fluid	Proto
NRL 905	Male Urethra	AHU

* Pro: requires proline; Proto: Prototrophic; PAU: requires proline, arginine and uracil; PAHU: requires proline, arginine, hypoxanthine and uracil; AHU: requires arginine, hypoxanthine and uracil