# Microaerobic denitrification in *Neisseria meningitidis*

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## Abstract

**The major aetiological agent of human bacterial meningitis is *Neisseria meningitidis*. During the course of disease and host colonization, the bacterium has to withstand limited oxygen availability. Nitrogen oxide and nitrogen oxyanions are thought to be present, which may constitute an alternative sink for electrons from the *N. meningitidis* respiratory chain. A partial denitrification pathway is encoded by the *aniA* nitrite reductase gene and the *norB* nitric oxide reductase gene. Analysis of the completed genome sequences of two *N. meningitidis* strains is used to generate a model for the membrane-associated respiratory chain of this organism. Analysis of *aniA* expression indicates it to be controlled primarily by oxygen and secondarily by nitrite. The ability of *N. meningitidis* to denitrify relies on microaerobic growth conditions. Here we show that under microaerobic conditions nitrite supplements oxygen as an alternative respiratory substrate.**

## Introduction

*Neisseria meningitidis* is a major cause of mortality and morbidity to infants and adolescents. This organism causes two diseases, meningitis and septicaemia, both characterized by a rapid onset and high death rates. The natural habitat of *N. meningitidis* is the human nasopharynx, where it normally resides asymptomatically, and it has no other known natural reservoir. The nasopharynx is rich in microflora, and strict aerobes and strict anaerobic bacteria have been isolated from this environment [1]. *N. meningitidis* is traditionally treated as a strict aerobe, and is hence cultured under fully aerobic conditions. We have previously shown, however, that the organism is capable of growing in the absence of extensive aeration, and that under these conditions nitrite can be removed from cultures through a denitrification pathway [2]. It was not clear from our earlier work the extent to which denitrification was able to supplement growth under oxygen-limiting conditions, or whether the denitrification pathway was operating mainly as a detoxification pathway. Nitrite may be an important source of respiratory substrate for *N. meningitidis* living in an environment where oxygen availability is limited and variable.

## Genomic analysis

Complete genome sequences are available for *N. meningitidis* MC58 (a serogroup B strain) and Z2491 (a serogroup A strain). The sequences were analysed to model the respiratory chain of *N. meningitidis*. The meningococcus possesses homologues of respiratory complexes I, II and III, suggesting that electrons enter the respiratory chain through a proton-translocating NADH dehydrogenase or a succinate dehydrogenase, and transfer those electrons to the cytochrome $bc_1$ complex through ubiquinone (the meningococcus can synthesize ubiquinone but not menaquinone).

*N. meningitidis* appears to be capable of synthesizing only a single oxygen-utilizing terminal reductase: a cytochrome $cbb_3$ oxidase. Cytochrome $cbb_3$ in other organisms typically has a high oxygen affinity [3], and is associated with growth under oxygen-limiting conditions. This implies that *N. meningitidis* is adapted for microaerobic growth. There are two alternative terminal electron acceptor reductases, a nitrite reductase and a nitric oxide reductase. The nitrite reductase, AniA, was originally identified as a major anaerobically inducible outer membrane protein. The protein is predicted to encode a Cu-type nitrite reductase and is somewhat unusual in being predicted to associate covalently with the outer membrane. The N-terminal section of the translation product of *aniA* is predicted to be cleaved by a signal peptidase 2 and the resultant N-terminal cysteine bonds covalently to an outer membrane lipid. This attachment site is followed by a highly repetitive region, which may form a linker that allows the globular copper nitrite reductase enzyme to have mobility within the periplasm. The NO reductase is most closely related to the NO reductase enzyme from *Ralstonia eutropha* that has been shown to be a quinol-oxidizing enzyme [4]. It is predicted that meningococcus has a branched respiratory chain with nitrite- and oxygen-reducing enzymes receiving electrons downstream from the $bc_1$ complex, and the NO reductase receiving electrons directly from the quinol pool (Figure 1).

Meningococcus contains a gene for a lipid-modified azurin (Laz), which like AniA, is predicted to be attached to the outer membrane through a linker domain. The copper centre of this cupredoxin may be involved in electron transfer from the $bc_1$ complex to AniA and/or $cbb_3$ oxidase. Alternatively, there are a number of *c*-type cytochromes that may fulfil this role. A putative periplasmic cytochrome ('$c_{552}$') encoded by the gene NMB0717

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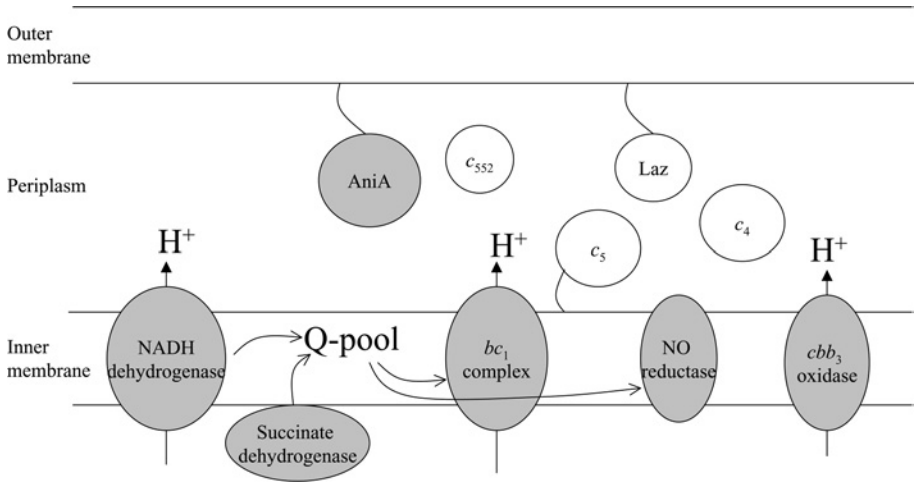
**Figure 1 | Predicted organization of the meningococcal respiratory chain**

Components of the respiratory chain are shown and electron transfer pathways are represented as curved arrows.



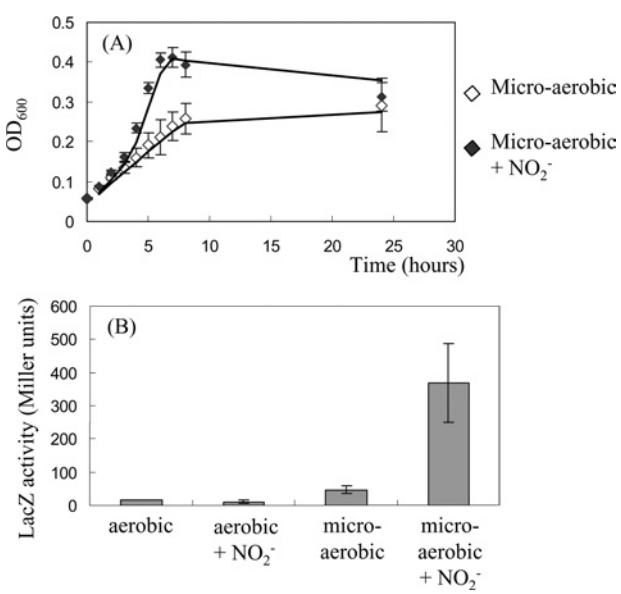

(*N. meningitidis* MC58 notation) is most similar to a domain from *Bdellovibrio bacteriovorans* Bd2608. The latter gene is annotated as a major anaerobically induced transmembrane protein/putative multicopper oxidase, but LipoP [5] predicts the protein to be periplasmic, and BLAST homology searching indicates that the protein consists of two domains: an N-terminal Cu-type nitrite reductase and a C-terminal cytochrome domain. NMB0717 may be a specific partner of AniA, involved in the transfer of electrons to this enzyme. Two predicted dihaem *c*-type cytochromes, periplasmic cytochrome $c_4$ encoded by NMB1805 and membrane-associated cytochrome $c_5$ encoded by NMB1677, may be involved in electron transfer in the respiratory chain of meningococcus, but this obviously requires some experimental tests.

## Role of AniA and nitrite in the growth of meningococcus

We have demonstrated previously [2] that inclusion of nitrite in liquid media enhances the growth of *N. meningitidis* in stationary (not agitated) cultures. However, the observed growth enhancement was small and variable. Here we show that incubating cultures with continuous slow agitation led to the establishment of culture conditions that allowed visualization of a consistent nitrite-dependent *N. meningitidis* growth stimulation under oxygen-limiting conditions. *N. meningitidis* MC58 was inoculated to a $D_{600}$ of 0.05 into 20 ml of Mueller–Hinton broth (containing 10 mM $NaHCO_3$) in 25 ml McCartney flasks ±5 mM $NaNO_2$. Cultures were incubated at 37°C with shaking at 100 rev./min and the $D_{600}$ monitored over a 24 h period. Figure 2(A) shows a clear increase in growth rate of nitrite-supplemented cultures compared with unsupplemented cultures. This growth stimulation is coincident with depletion of nitrite from the growth medium and the duration of growth stimulation depends on the concentration of nitrite initially present (results not shown). In the absence of nitrite, growth is linear, indicative of oxygen limitation, whereas growth is exponential in the presence of nitrite.

**Figure 2 | The response of *N. meningitidis* to the presence of nitrite during oxygen limitation**

(**A**) Shows the growth of *N. meningitidis* MC58 in oxygen-limited conditions ±5 mM nitrite. Error bars on both curves represent the standard error of each data point. (**B**) Shows differences in *aniA* expression of an *N. meningitidis aniA* promoter/*lacZ* fusion ($p_{aniA}$) grown for 6 h under aerobic or microaerobic conditions in the presence and absence of 5 mM nitrite.




The *aniA* gene of *N. meningitidis* is similar to *aniA* from *Neisseria gonorrhoeae*, where it has been shown to

be involved in nitrite reduction [6]. Owing to the possible involvement of the *N. meningitidis aniA* gene in the nitrite-dependent growth stimulation during microaerobic conditions, a strain containing an *aniA* promoter *lacZ* fusion ($p_{aniA}$) was cultured at 37°C in both aerobic (5 ml culture, shaking at 180 rev./min) and microaerobic (20 ml culture, stationary) conditions in the presence and absence of 5 mM nitrite. The LacZ activities of 1 ml culture samples were measured at 1 h intervals for the 24 h duration of this experiment. The LacZ activities at 6 h intervals are shown in Figure 2(B). Oxygen limitation stimulated *aniA* expression and this expression was increased further by the presence of nitrite. Under aerobic conditions, nitrite has no effect and *aniA* expression remains very low, indicating that oxygen availability must become limited before nitrite can exert a regulatory effect on $p_{aniA}$. The presence of a functional FNR (oxygen sensor-regulator) and NarQP (nitrate/nitrite two-component sensor regulator) in *N. meningitidis* leads us to believe that these proteins may be involved in regulating the expression of *aniA* in response to the redox state and substrate presence, as has been previously shown for *N. gonorrhoeae* [7]. Although NarQP is involved in nitrate-dependent regulation in *Escherichia coli* [8], *N. meningitidis* does not possess a known nitrate reductase and the presence of 40 mM nitrate does not stimulate growth or *aniA* expression (results not shown).

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