

Gonococcal Nitric Oxide Reductase Is Encoded by a Single Gene, *norB*, Which Is Required for Anaerobic Growth and Is Induced by Nitric Oxide

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The gene encoding a nitric oxide reductase has been identified in *Neisseria gonorrhoeae*. The *norB* gene product shares significant identity with the nitric oxide reductases in *Ralstonia eutropha* and *Synechocystis sp.* and, like those organisms, the gonococcus lacks a *norC* homolog. The gonococcal *norB* gene was found to be required for anaerobic growth, but the absence of *norB* did not dramatically decrease anaerobic survival. In a wild-type background, induction of *norB* expression was seen anaerobically in the presence of nitrite but not anaerobically without nitrite or aerobically. *norB* expression is not regulated by FNR or NarP, but a functional *aniA* gene (which encodes an anaerobically induced outer membrane nitrite reductase) is necessary for expression. When *aniA* is constitutively expressed, *norB* expression can be induced both anaerobically and aerobically, but only in the presence of nitrite, suggesting that nitric oxide, which is likely to be produced by AniA as a product of nitrite reduction, is the inducing agent. This was confirmed with the use of the nitric oxide donor, spermine-nitric oxide complex, in an *aniA* null background both anaerobically and aerobically. NorB is important for gonococcal adaptation to an anaerobic environment, a physiologically relevant state during gonococcal infection. The presence of this enzyme, which is induced by nitric oxide, may also have implications in immune evasion and immunomodulation in the human host.

Denitrification is the process whereby microorganisms form dinitrogen (N₂) from nitrate (NO₃⁻) via nitrite (NO₂⁻), nitric oxide (NO), and nitrous oxide (N₂O) intermediates. Denitrifiers possess separate reductases which catalyze each intermediate step. Denitrification is usually seen in facultative anaerobes or microaerophilic organisms, but aerobic denitrification has been documented. The process and regulation of denitrification has been extensively studied in pseudomonads, *Paracoccus* spp., and *Rhodobacter* spp.; however, many other organisms, including fungi, denitrify. (See the recent comprehensive review by Zumft [33] for details of this process and the properties of its enzymes.)

Neisseria gonorrhoeae, the etiologic agent of the sexually transmitted disease gonorrhea, is now known to be a facultative anaerobe (17) which possesses a copper-containing nitrite reductase, AniA, in its outer membrane (5). *aniA* is very tightly regulated by oxygen and is only expressed under anaerobic or microaerobic conditions (13, 15). Antibodies to AniA have been found in sera from patients with gonococcal disease, indicating that AniA is expressed within the host and that anaerobiosis is a physiologically relevant environment in gonococcal infections (7). We were interested in denitrifying enzymes because copper-containing nitrite reductases are found in some of the denitrifying bacteria such as *Pseudomonas aureofaciens* and *Rhodobacter sphaeroides*, and these enzymes reduce NO₂⁻ to NO (33).

NO is known to be toxic to bacterial cells, often through its

reaction with dioxygen, superoxide, or the metal centers in various enzymes. Bacteria which produce NO therefore also possess an NO reductase to eliminate the molecule. This two-subunit enzyme reduces NO to N₂O and is encoded by the *norC* and *norB* genes (33).

Since it is likely that AniA produces NO and since it has been reported that gonococci produce nitrous oxide as a result of nitrite reduction (19), it is logical that the gonococcus would also possess an NO reductase. It is known that murine macrophages produce NO, and there is evidence that this is also true in human macrophages (reviewed in references 11 and 20). Therefore, an NO assault could be part of the innate immune system response to a bacterial invader. It was of interest to us to determine if the gonococcus, a strictly human pathogen, possesses this denitrifying enzyme, which could also have a role in the organism's survival in the face of an immune response. This study describes the identification and the basic regulation of the gonococcal *norB* gene and discusses the implications of the presence of the NorB enzyme in the pathogenesis of the organism.

MATERIALS AND METHODS

Growth of gonococcal strains. All gonococcal strains (Table 1) were derived from strain F62 and were grown on GC medium base (Difco Laboratories, Detroit, Mich.) plates with 1% Kellogg's supplement (GCK) (16). When necessary, chloramphenicol or erythromycin was added at 1 or 2 µg ml⁻¹, respectively. Aerobic plate cultures were grown at 37°C in a 5% CO₂ incubator. Aerobic broth cultures were grown in GCK broth plus 0.042% (wt/vol) NaHCO₃ in baffled flasks which were incubated with shaking at 240 rpm in a Gyrotory water bath shaker (New Brunswick Scientific Co., Edison, N.J.) at 37°C. Anaerobic cultures were incubated in a Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, Mich.) at 37°C for 20 h. Nitrite was provided for anaerobically grown cultures by placing 40 µl of a 20% (wt/vol) NaNO₂ solution on a sterile cellulose disk in the center of the plate. Cultures provided with nitrite grow in a characteristic halo around the nitrite disk, while cultures without nitrite remain viable but do not grow (24).

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TABLE 1. Gonococcal strains used in this study

Strain	Relevant genotype	Source or reference
RUG7001 ^a	Wild type	25
RUG7002	<i>ΔaniA</i>	This study
RUG7021	Promoterless <i>lacZ</i>	25
RUG7058	<i>norB::ermC</i>	This study
RUG7060	<i>norB::ermC ΔaniA</i>	This study
RUG7500	<i>norB'-'lacZ</i>	This study
RUG7501	<i>norB'-'lacZ aniA::ermC</i>	This study
RUG7502	<i>norB'-'lacZ tacP::aniA</i>	This study
RUG7503	<i>norB'-'lacZ narP::ermC</i>	This study

^a RUG7001 is an F62 strain which contains *aniA'-'lacZ*.

Gonococcal transformation. Type 1 gonococcal cells were transformed as previously described (15) and plated on GCK plates containing the appropriate antibiotic.

Construction of *norB'-'lacZ* fusion. The region upstream of the *norB* open reading frame (ORF) was amplified by PCR, using primers which created *Bam*HI sites on both ends. The amplified fragment contained 177 bp upstream of the putative start codon and the first three codons of the ORF. This DNA fragment was digested with *Bam*HI and ligated to pLES94 (25), which had also been digested with *Bam*HI, to create a translational *lacZ* fusion. This ligation mixture was used to transform *Escherichia coli* MC1061. Transformants were selected by plating on Luria-Bertani medium plates containing ampicillin at 100 μg ml⁻¹ and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) at 40 μg ml⁻¹. After overnight incubation at 37°C, blue colonies were screened for plasmids containing the insert in the correct orientation. When an appropriate plasmid was identified (pEF1), it was used to transform gonococcal strain F62. Transformants were plated on GCK plates containing chloramphenicol. Chromosomal DNA was isolated from chloramphenicol-resistant colonies (15), and PCR was used to confirm the presence of the reporter construct. The resulting PCR product was sequenced to confirm the desired nucleotide sequence. The resulting strain was designated RUG7500.

Construction of a *norB* null mutant. *norB* was amplified in two fragments by PCR with primers which created a *Clal* site within the coding region. The two DNA fragments were purified and digested with *Clal*. These digested fragments were ligated to an erythromycin resistance cassette (*ermC*) which had been amplified from pHSS23 (30) and digested with *Hpa*II. This ligation mixture was transformed into *N. gonorrhoeae* RUG7001. This resulted in interruption of the *norB* gene with the erythromycin resistance cassette and the creation of strain RUG7058. The interruption of *norB* was confirmed by PCR.

The *norB* null mutant was also combined with an *aniA* null mutation. The insertionally inactivated *norB* was amplified by PCR from strain RUG7058. The resulting PCR product was used to transform RUG7002 which contains a 493-bp deletion in the *aniA* gene and the *aniA'-'lacZ* reporter construct. This *norB aniA* double mutant was designated RUG7060. Both mutations were confirmed by PCR.

Construction of the *aniA* null and constitutive strains. To combine the *norB'-'lacZ* reporter with an *aniA* null background, the *aniA* gene insertionally inactivated with the *ermC* gene from strain RUG7011 (15) was amplified by PCR. This PCR product was used to transform RUG7500, which created strain RUG 7501. The presence of both the reporter construct and the *ermC* cassette was confirmed by PCR. To create the combination with the *aniA* constitutive mutant, pEF1 (*norB'-'lacZ*) was used to transform the F62 strain containing the previously described constitutive *aniA* (15), creating strain RUG7502. PCR was used to confirm the presence of both the reporter construct and the constitutive construct.

Construction of the *narP* null strain. The *norB'-'lacZ* reporter was combined with a *narP* (encodes the response regulator of a two component system [15, 19]) null background by amplifying the insertionally inactivated *narP* from strain RUG7036 (15) by PCR. This PCR product was used to transform RUG7500, creating RUG7503. The presence of both the reporter construct and the *ermC* gene within *narP* was confirmed by PCR.

Anaerobic survival plate counts. Nonplated colonies of desired strains were suspended in GCK broth at an optical density at 600 nm of approximately 0.1. Suspensions were serially diluted, and the appropriate dilutions were plated onto GCK plates in duplicate. Each strain was plated onto GCK with or without 2 mM NaNO₂ and incubated both aerobically and anaerobically. Aerobic cultures were incubated for 48 h before enumeration. Anaerobic cultures were incubated for 24 h anaerobically and then 48 h aerobically to allow for visible colony formation. The number of CFU from the anaerobic cultures was compared to the CFU counts in the corresponding aerobic culture. The results are reported as the percentage of aerobic CFU and are the means of three independent assays for each strain.

Induction of *norB* by NO. For each plate tested, 10 mg of the NO generator, spermine-nitric oxide complex (SPER-NO; Sigma RBI, St. Louis, Mo.) was

dissolved in 50 μl of 0.1 N NaOH and used to saturate a cellulose disk. This disk was placed in the center of a plate which had been inoculated with a heavy suspension of organism, and the plate was incubated for 20 h either in the CO₂ incubator or the anaerobic chamber. Cells were harvested with a sterile swab from around the disk and tested for β-galactosidase (β-Gal) activity. Disks saturated with 50 μl of 0.1 N NaOH were used for the no SPER-NO controls.

β-Gal assays. Gonococcal cultures were assayed for β-Gal by the method of Miller (22) as previously described (15). Activity is reported in Miller units. The results reported are the averages of at least three independent assays for each strain.

Oligonucleotides and DNA sequencing. All oligonucleotide synthesis and DNA sequencing was performed by the University of Rochester Core Nucleic Acid Laboratory.

Molecular biology techniques. General techniques were performed according to standard protocols (2, 23). Plasmid preparations were obtained with the Wizard Plus Minipreps kit (Promega Corp., Madison, Wis.) or the QIAprep Spin Miniprep kit (Qiagen, Inc., Valencia, Calif.). DNA fragments were purified with Wizard PCR Preps kit (Promega), or with GENECLEAN Spin or MERmaid Spin kits (Bio 101, Vista, Calif.).

RESULTS

Identification of the *norB* gene in *N. gonorrhoeae*. The work in our laboratory has focused on the *aniA* gene and its gene product in *N. gonorrhoeae* F62. Since *AniA* is a copper-containing nitrite reductase (5), it is likely that the product of nitrite reduction is NO. The next enzyme in the denitrifying pathway is the NO reductase (i.e., Nor). The sequence of what appears to be the entire coding region of a *norB* homolog was recently completed in the gonococcal genome (Gonococcal Genome Sequencing Project [http://dna1.chem.ou.edu/gono.html]). Surprisingly, *N. gonorrhoeae* does not encode the expected NorCB NO reductase which can be found in denitrifying organisms such as *Pseudomonas aeruginosa* (1), *Pseudomonas stutzeri* (4, 34), *Paracoccus denitrificans* (9), *R. sphaeroides* (3), and others. Instead, the gonococcus seems to encode a single subunit NO reductase, consisting only of NorB.

The predicted NorB protein has a calculated molecular mass of 84.3 kDa and an amino-terminal extension of approximately 260 to 270 amino acids. A BLAST search revealed significant identity to the *Ralstonia eutropha* (*Alcaligenes eutrophus*) and *Synechocystis* NO reductases along the entire length of the protein (61 and 42%, respectively) (Fig. 1). As in the gonococcus, the *R. eutropha norB* and *norZ* (genes encoding two iso-functional NO reductases) and *Synechocystis norB* predicted gene products have amino-terminal extensions, and the organisms lack *norC* genes (8). Like the *R. eutropha* NorB, the gonococcal NorB contains 14 potential transmembrane domains (data not shown) and all six conserved histidine residues (Fig. 1) and lacks a heme *c* binding motif (C-X-X-C-H).

Upstream of the *norB* ORF is a gonococcal ribosome-binding site, AAAGGA, at position -11 from the putative translational start, and a potential σ⁷⁰ -10 sequence, TACAAT, at position -61 (Fig. 2B). In contrast to other NO reductase genes, we did not find a complete FNR binding site, only a potential half site, TTGAA, at position -96.

***aniA* is located upstream of *norB*.** After locating the *norB* gene in the gonococcal genome (contig 17 on 03/03/00), we investigated the other ORFs surrounding *norB*. Considering our work with *aniA*, it was interesting to find *aniA* positioned about 375 bp upstream of *norB*, transcribed in the opposite direction (Fig. 2). This is similar to other organisms in which the *nor* genes are found in proximity to nitrite reductase (*nir*) genes. The two ORFs immediately downstream of *norB* are not homologous to other *nir* or *nor* genes.

***norB* is required for the anaerobic growth, but not the survival, of *N. gonorrhoeae*.** It has been reported previously that *aniA* is required for anaerobic growth in the gonococcus (15, 21). We were interested in determining if *norB* was also re-

Ngo	-----MGOYKK	LWYLLFAVLAVCFITLIGKMG	SEVYKRPPEYEEQVVSASGK	VLMTKDDLLAGQSAWQSTGG	MEVGSILGHGAYQAPDWTAD	86
ReB	-----MGSYRR	LWFLLI AVLAVTFESL LAYYG	AEVYRQAPPMPAKVVAADGR	TLFTGEDILLDGGTAWQSVGG	MQLGSLWGHGAYQAPDWTAD	86
ReZ	-----MGSYRR	LWFLLI AVLAVTFESL LAYYG	VEVYRQAPPMPAKVVTTEGR	TLFTGEEILDGGTAWQSVGG	MQLGSLWGHGAYQAPDWTAD	86
Ssp	MANSTFDLPIATPPRTKSF	LPAWLVLCVVTFSVLLGAG	AAIYKNAPPIEPTVMVSPQQE	IVMTQAEVQRGGQATYLARGG	QHTGSLVNGHGSYLAPDWTAD	100
Ngo	WLHRELSAWLDELTAQQTY--	-GKKFDEVSPPEEQAVLKTRL	ADEYRNQSRIKEDGSSVVISD	TRVKAIESILPYYHGVIYDD	PKLQTTREHFAMKNNLPSA	183
ReB	WLHRELTAWLDLAAQDAY--	-HKPYDKIDAAAARGALREQL	RAEYRGNAADEPATSVLTVSN	RRAQAMAATAGYYDKLFSDA	PELHTSREHFAMKENTLPSA	183
ReZ	WLHRELSAWLFLAAQDAN--	-GRPYAQLDAPAAALREHV	RTEYRGNATDPATNVLTVSK	RRAQAIADTAAYYDQLFSDA	PALHTTREHFAMKENTLPSA	183
Ssp	VLHRWGLATAGVLYDGDANF	SQTDLEALPPVAQAQLQAEV	KQEFKPNRYDEETGILSLTN	AQTQGLAQVFFDYQALLLNG	SATHS-----IPRDFWFEA	194
Ngo	EAREKLFDFEFWTSNSASTN	RPGEVFTYTNWNPHEPLINN	VPTTENYMSFTSVVLLLMG	IQLLMNGYSFLTKHEEVEVP	S--EDPISKIQLTFSQKALG	281
ReB	ERRQQLTHFFFWTAWAAATE	RPFGHEATYTNWNPHEPLIGN	QPTSENVVWSVISVVVLLAG	VGFLVWAWAFIRGHEALPQ	APARDELLSFALTPSORALG	283
ReZ	ERREQLTHFFFWTAWAASTA	RPFGHEATYTNWNPHEPLIGN	QPTSENVVWSVISVVVLLAG	VGFLVWAWAFIRGKEDAPPQ	APARDLLAVALTTPSORALG	283
Ssp	GDRHDVTAFAFAWTAWTAAN	RNSPLSYTANWPHDDLIGN	QAPGQFIVWSIVSVIIVLIA	IALFLFVYLTQEDAEVEVQAV	A---ERPRLRLATFSQRITT	291
*						
Ngo	KYVFLTVALFVQVLLGGLT	AHYTVEGQGFYGIIDEALGFE	MSDWFPYALTRTHIQSALF	WIATGFLTAGLFLAIVNCGG	KDPKFORAGVNFYIALFIV	381
ReB	KYLFVVALFVFOVFLGGFT	AHYTVEGQKPY-----GID	VSQWFPYSLVRTWHIQSALF	WIATGFLAAGLFLARLINGG	KDPKYORLGVDFIWFALVVV	377
ReZ	KYLFVVALFVFOVFLGGFT	AHYTVEGQKPY-----GID	VSQWFPYALVRTWHIQSALF	WIATGFLAAGLFLARLINGG	KDPKYORLGVDFIWFALVVV	377
Ssp	LFFGVAMALFVQVLLMGVMT	AHYAVEGEGEY-----GIP	LQQLVLYAASRTNHLQLAVE	WIATCWLAAAGLYFAPRFG-K	HEPKQQAIGNGLIALALTLV	384
Ngo	VCGSYAGNFFALTHILPEPF	NFWFHGQGYEYLDLGRFWQL	LLMVGLLWFLMLRCTVSA	FKEK-GVDKNNLAI FVASMV	GVGVYAPGDFYCEKSPIAV	480
ReB	VVGSFTGNLATAQKLPNPL	NFWLGHQGYEYVDLGRLLWQI	GKFAGILITWLVLMRGIAPA	ERARSGGDKNDLALLTSSVV	AIGLIFYGAGIFYGERTSLTV	477
ReZ	VVGSFTGNLATAQKLPNPL	NFWLGHQGYEYVDLGRLLWQI	GKFAGILITWLVLMRGIAPA	ERAR-GTDRNLLALLTSSVV	AIGLIFYGAGIYGERTSLTV	476
Ssp	VVGSLVGAWFGIQGYLGNDO	SFWFHGQGYEYVLDLGRLLWQI	LLIGGMVFWLWLMFRALRPA	ERAE-GNKTGLNHFFLYSAI	TILIFYASGLMVTNHTPISV	483
* *						
Ngo	MEYRWWVVHLWVEGFPEVE	ATAAFAEVFNMGFVRRSTA	TASTLAAAAIFMLGGVFGTL	HHLYESGSTSASMAIGACFS	ALEVVELVLGREAHEHWSY	580
ReB	MEYRWWVVHLWVEGFPEVE	ATLALAFIFSTLGLVSRPMA	TAASLASRSLFMLGGIPGTF	HHLYESGTTTPVMVGAFAFS	ALEVVELVLGHEAWDNWRE	577
ReZ	MEYRWWVVHLWVEGFPEVE	ATLALAFIESTLGLVSRPMA	TAASLASASLEMLGGIPGTF	HHLYFAGTTPVMVGAFAFS	ALEVVELVLGHEAWENWSE	576
Ssp	AEYRWWVVHLWVEGFPEVE	ATVAIAAYLCSEIGFLKRSAA	LRATYLTITLYLGSVIGL	HHLYFAGTFVPIAAMGAVAS	ALEVVELTLIGFEVVK--SE	581
* *						
Ngo	QHLSDWAKRLRWPLMCFVAV	AFWNMIGAGVFGFLINPPIS	LYFYQGLNITSAVHAHAALFG	VYGFALGFLVLLVARYLKN	ARFDDKIMTWGFWLLNCGLV	680
ReB	KDRAPWMADEKRWPLMCFVAV	AFWNMIGAGVFGFMINERIS	LYYIQGONTTFVHAHAALFG	VYGFALGFTLLVRYVREPA	YRLSPGLMKTAFWGLNAGLV	677
ReZ	KRRAPWMADEKRWPLMCFVAV	AFWNMIGAGVFGFMINPPIA	LYYIQGONTTFVHAHAALFG	VYGFALGFTLLVRYVREPA	YRLSPTLMKTAFWGLNAGLV	676
Ssp	KLSQEAQGFYRMPKFKFIAT	CVWNLVGAGVFGFLINPPIV	LYYSQGINTEIHAHSAALFG	VYGSALIALMFAALREITPD	QFANERKFNFAFWWNGGLV	681
Ngo	GMIASLLEPVGIQAYASIT	HGLWYARSEEFQMEILDTL	RWVRTAPBLIEIGGATCVAI	QATKIVFGRDK-----	----- 752	
ReB	LMICTSLLEPVGIQFLASVE	HGTWYARSEEFMQPILOTL	RWVRTFGDVVFIIVGAVSFAL	QVVLGLLGKAPALAAPEGRLL	NAAQR--- 762	
ReZ	LMICTSLLEPVGIQFLASVE	HGTWYARSEAFMQPILOTL	RWVRTFGDVVFIIVGAVSFAW	QVVLGLANRTPLAADAVPAG	MKPAAR--- 762	
Ssp	TMLVTEGLIPNGFYQLMSIN	HGTWYARSAEIVIGSSWMQGT	VWLRIPEGDLLFAVGAIVLLY	GMGQAIWITLFOQPTQPQLD	EVQVETPAT 770	

FIG. 1. Amino acid sequence alignment of NorB. The *N. gonorrhoeae* NorB (Ngo) predicted amino acid sequence is compared to the *R. eutropha* NorB (ReB; nucleotide sequence accession no. AF002217), the *R. eutropha* NorZ (ReZ; nucleotide sequence accession no. AF002661), and the *Synechocystis* sp. strain PCC6803 NorB (Ssp; nucleotide sequence accession no. D90917). Identical residues are shaded in gray, and conserved His residues are indicated by asterisks. Sequence alignment was performed using CLUSTAL W (<http://www2.ebi.ac.uk/clustalw/>).

quired. The *norB* gene was insertionally inactivated with the *ermC* gene. The resulting strain, RUG7058, was not attenuated in aerobic growth either in shaken broth cultures or on plates (data not shown) but, like the *aniA* mutant, was unable to grow anaerobically. However, RUG7058 survived anaerobic incubation and grew into visible colonies when transferred to a CO₂ incubator (data not shown).

To quantify anaerobic survival, strains RUG7001 (wild type) and RUG7058 (*norB*) were plated and counted as described in Materials and Methods. Plate counts from the anaerobic plates were compared to the plate counts of the corresponding aerobic cultures. As seen in Table 2, in the absence of nitrite, only approximately 20% of both RUG7001 and RUG7058 (*norB*) survived anaerobic incubation. In contrast, in the presence of nitrite, virtually 100% of RUG7001 survived, while about 65% of RUG7058 (*norB*) survived. The morphology and size of the colonies of the two anaerobically incubated strains were identical when the cultures were subsequently incubated aerobically. Interestingly, this was not the case with the cultures that were grown aerobically. In the absence of nitrite, both strains grew with normal colony morphology and to normal size. In the presence of nitrite, however, the *norB* mutant exhibited a small colony phenotype (0.5 versus 3 mm) only when grown aerobically, while the colonies of the wild-type strain were normal. RUG7058 (*norB*) plates counts did not differ in the

presence or absence of nitrite (data not shown), but the colony morphology was distinct and consistent.

We have previously observed low levels of *aniA* expression in gonococci grown on a plate overnight in the CO₂ incubator (25). In the absence of NorB, the NO produced by AniA in the presence of nitrite could react with O₂, forming toxic compounds such as N₂O₃, which has been found to cause mutations in bacteria (see references 26 and 32 and references therein). This accumulation of toxic molecules could account for the small-colony phenotype of RUG7058 (*norB*) observed only in the presence of nitrite on aerobically grown plates.

We reasoned that, similar to the results in *P. stutzeri* (4), if we eliminated the source of NO, AniA, this would rescue the small-colony phenotype observed aerobically in the presence of nitrite. RUG7002 (*aniA*) and RUG7060 (*aniA norB*) were plated for colony counts aerobically and anaerobically as described above. As we predicted, the colonies of the *aniA norB* double mutant were of normal size both in the presence and in the absence of nitrite when grown aerobically. This suggests that the production of NO in the absence of NorB was the initial cause for the small-colony phenotype. The anaerobic survival results for strains RUG7002 (*aniA*) and RUG7060 (*norB aniA*) were surprising (Table 2). While their survival in the absence of nitrite was comparable to RUG7001 and RUG7058 (*norB*), the anaerobic survival rate of both of the

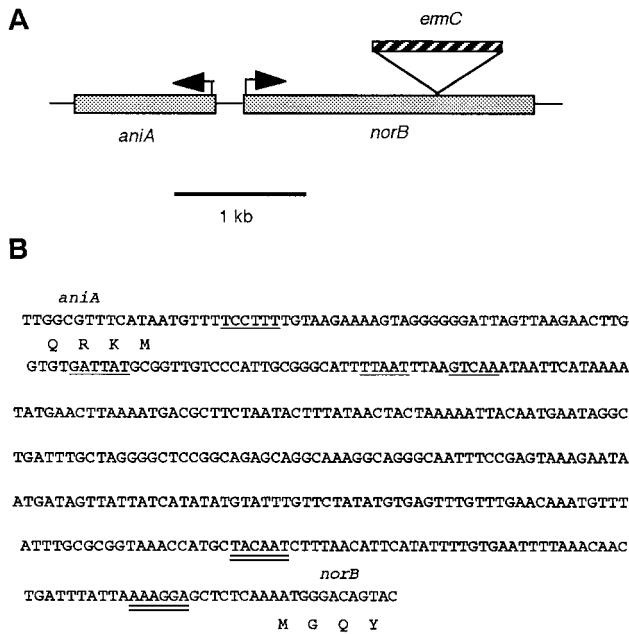


FIG. 2. Orientation of *norB* and *aniA*. (A) Schematic representation of the orientation of the *norB* and *aniA* genes in the gonococcal chromosome. Direction of transcription is indicated by arrows, and the location of the *ermC* insertion in strain RUG7058 is also indicated. (B) Nucleotide sequence of the *aniA-norB* intergenic region from gonococcal strain FA1090 obtained from the Genome Sequencing Project. The previously reported ribosome-binding site, $\sigma^{70} -10$, and FNR binding site for *aniA* (GenBank accession no. AF082184) are marked by a single underline. The putative ribosome-binding site and $\sigma^{70} -10$ for *norB* are indicated by double underlines. The first four amino acids for each gene product are also indicated below the nucleotide sequence.

aniA mutants in the presence of nitrite was only 6%. This seems to indicate that, in anaerobic conditions, 2 mM nitrite is more toxic than NO to the gonococcus.

***norB* is induced anaerobically in the presence of nitrite.** β -Gal assays were performed on RUG7500 (*norB'*-*lacZ*) grown both aerobically and anaerobically measuring *norB* expression (Table 3). In highly aerated broth cultures, the level of *norB* expression was about 20 Miller units both with and without 2 mM NO_2^- . In contrast, when RUG7500 was incubated anaerobically in the presence of nitrite, expression increased to about 1800 Miller units, while levels in cultures without nitrite remained low at 34 Miller units. In all cases the promoterless *lacZ* control (RUG7021) expression was virtually nil.

The levels of *norB* expression exhibited a different pattern than what was observed with *aniA* (15). In the case of *aniA*, we observed significant induction due to anaerobiosis alone, with an additional increase in β -Gal activity when nitrite was added to the cultures, while *norB* remained at a basal level of expression during anaerobiosis with significant induction seen only in the presence of nitrite (Table 3). This suggests a different method of regulation.

***norB* is not regulated by NarP.** Since induction of *norB* was seen in the presence of nitrite, we investigated whether *norB* is activated by NarP. The *narP* gene was insertionally inactivated in the *norB'*-*lacZ* reporter strain. The β -Gal activity obtained from this strain, RUG7503, was not greatly decreased compared to the wild-type background (Table 3), indicating that NarP plays no role in *norB* regulation.

AniA is required for the induction of *norB*. To determine the effects of an *aniA* null mutation on *norB* expression, we created strain RUG7501, as described in Materials and Methods. In-

terestingly, when *aniA* was inactivated, we no longer observed the induction of *norB* in the presence of nitrite. Levels of β -Gal activity were 42 and 29 Miller units with and without NO_2^- , respectively (Table 3). There was no effect aerobically. Since it is likely that AniA produces NO as a product of NO_2^- reduction, these results suggest that NO may be the inducer for *norB*.

Induction of *norB* is seen aerobically when AniA is constitutively expressed. When gonococcal cultures are grown in a highly aerated culture, no induction of the *aniA* gene is detectable due to the requirement for FNR (15). To produce AniA, and thus NO, aerobically and to determine the effects on *norB* expression, we utilized the previously described *aniA* constitutive mutant in which *aniA* is under the control of the *tac* promoter (15). RUG7502 contains the *norB'*-*lacZ* fusion in an *aniA* constitutive background. Anaerobically, RUG7502 produced over 1,000 Miller units of β -Gal activity in the presence of nitrite. In highly aerated cultures, *norB* induction was seen at levels of over 150 Miller units only in the presence of nitrite, while levels in cultures without nitrite remained low. This suggested that AniA was indeed producing NO as a result of nitrite reduction, which in turn induced *norB*.

An NO generator induces *norB* expression in the absence of AniA. To confirm that NO was responsible for *norB* induction, we utilized the NO generator SPER-NO. To eliminate the potential role of AniA in *norB* induction, we tested the NO generator on strain RUG7501, in which *aniA* is insertionally inactivated. For each plate tested, 10 mg of SPER-NO was solubilized in 0.1 N NaOH and was used to saturate a paper disk, a technique similar to the method used for adding nitrite to gonococcal plated cultures. While the actual levels of activity in the anaerobic cultures were somewhat variable, we consistently obtained significant induction of *norB* in the presence of SPER-NO both aerobically and anaerobically. Aerobic β -Gal levels (Miller units \pm the standard error of the mean [SEM]) were 464 ± 91 in the presence of SPER-NO and 24 ± 3.3 in the absence of SPER-NO. Anaerobic levels were 884 ± 199 in the presence of SPER-NO and 28 ± 3.7 in the absence of SPER-NO. This provided convincing data that the gonococcal *norB* gene is induced by NO.

DISCUSSION

As a copper-containing nitrite reductase, it is highly probable that AniA produces NO as a result of its nitrite reduction, and it has been demonstrated that gonococci produce nitrous oxide as the product of nitrite reduction (19). We therefore set out to identify the NO reductase gene and to investigate the role of this enzyme in the anaerobic growth of the organism. It is also intriguing to speculate about the potential role of this enzyme in host immune evasion by the gonococcus.

The gene for the gonococcal NO reductase was identified by

TABLE 2. Anaerobic survival of *N. gonorrhoeae* *norB* and *aniA* mutants

Strain	Relevant genotype	Anaerobic survival ^a (% CFU \pm SEM)	
		+NO ₂	-NO ₂
RUG7001	Wild type	97 \pm 14	16.7 \pm 0.9
RUG7058	<i>norB::ermC</i>	62.7 \pm 8.7	18 \pm 3.6
RUG7002	Δ <i>aniA</i>	6.7 \pm 2.3	26 \pm 9.3
RUG7060	Δ <i>aniA norB::ermC</i>	6.3 \pm 1.5	13.3 \pm 2.4

^a Expressed as a percentage of aerobic CFU ml⁻¹ \pm the SEM; + NO₂, in the presence of 2 mM sodium nitrite; -NO₂, in the absence of sodium nitrite.

TABLE 3. β -Gal activity of the gonococcal *norB'*-*lacZ* fusion

Strain	Relevant genotype	β -Gal activity (Miller units \pm SEM) ^a			
		Aerobic cultures		Anaerobic cultures	
		+NO ₂	-NO ₂	+NO ₂	-NO ₂
RUG7021	Promoterless <i>lacZ</i>	0.1 \pm 0.01	0.1 \pm 0.01	0.4 \pm 0.11	0.1 \pm 0.03
RUG7500	<i>norB'</i> - <i>lacZ</i>	23 \pm 1.5	20 \pm 1.6	1,798 \pm 18.4	34 \pm 1.9
RUG7501	<i>norB'</i> - <i>lacZ aniA::ermC</i>	20 \pm 1.2	21 \pm 0.5	42 \pm 6.4	29 \pm 0.5
RUG7502	<i>norB'</i> - <i>lacZ tacP::aniA</i>	164 \pm 11	19 \pm 0.1	1,094 \pm 33	41 \pm 9.5
RUG7503	<i>norB'</i> - <i>lacZ narP::ermC</i>	21 \pm 0.5	21 \pm 0.4	1,081 \pm 36	22 \pm 2

^a Aerobic cultures were grown in GCK broth with shaking in baffled flasks in the presence (+) or absence (-) of 2 mM sodium nitrite (NO₂). Anaerobic cultures were grown on plates with (+) or without (-) disks saturated with 20% (wt/vol) sodium nitrite (NO₂).

BLAST analysis of the gonococcal genome project. Surprisingly, we found only a *norB* homolog, while most denitrifiers possess a two-subunit enzyme encoded by *norC* and *norB*. The gonococcal NorB has significant identity (61%) to *R. eutropha* NorB and NorZ along the entire length of the predicted protein. In contrast, when gonococcal NorB is compared to the NorB found in *P. denitrificans*, for example, there is only 28% identity, and the gonococcal NorB has an amino-terminal extension of 271 amino acids (data not shown). Cramm et al. speculated that the amino-terminal extension may serve the same role as the NorC subunit and proposed further studies to explore this possibility (8).

When the *norB* gene is inactivated in *N. gonorrhoeae*, the organism can no longer grow anaerobically. However, a *norB* mutant survives anaerobic incubation in the presence of nitrite better than an *aniA* mutant (Table 2). This indicates that NO is not extremely toxic to the gonococcus under anaerobic conditions. In other organisms the inability to grow anaerobically in the absence of an NO reductase was attributed to the toxic effects of NO accumulation (4, 18). This does not seem to be the case in *N. gonorrhoeae* under these culture conditions, as shown by the anaerobic survival data.

Our studies on AniA indicate that it is anchored in the outer membrane of the gonococcus (5, 6, 14). This external location makes it unlikely that AniA is linked to anaerobic respiration and energy production. However, the gonococcal *norB* gene product appears to have multiple transmembrane domains indicative of a protein integral to the cytoplasmic membrane. From this location in the cytoplasmic membrane, it is quite feasible that NorB is involved in anaerobic respiration. Thus, the lack of NorB would result in the inability to respire under anaerobic conditions, preventing the growth of the organism until it is switched to environmental conditions which are permissive for aerobic respiration.

Our initial experiments looking at *norB* regulation revealed significant induction of the gene in the presence of nitrite but none due to anaerobiosis alone (Table 3). This is in contrast to *aniA*, which is induced anaerobically by FNR. Our previous work indicated that *aniA* was the only gene induced by FNR that was required for anaerobic growth (15). It was not surprising, therefore, that we could not locate an FNR binding site upstream of *norB* and that *norB* was not induced due to anaerobiosis alone. The data indicating that *norB* expression can be induced aerobically also rule out involvement of FNR. Since *aniA* requires NarP for the induction due to nitrite (15), we investigated whether or not *norB* also required NarP. Insertionally inactivating *narP* (RUG7503) had only a small effect on *norB* expression (Table 3). This decrease from 1,800 to 1,100 U is most likely due to the decrease in *aniA* expression due to the lack of NarP (15), resulting in diminished NO production and *norB* induction.

The results obtained with the *aniA* null and constitutive strains (Table 3) suggested that *norB* was induced by NO. Confirmation of this was achieved by providing exogenous NO through the use of a NO generator in the absence of AniA. When SPER-NO was added to either aerobically or anaerobically grown plated cultures of RUG7501, significant induction of *norB* was observed (see Results) compared to the low β -Gal activity observed both without an inducer and in the presence of nitrite (Table 3). This observation is not without precedence, since the NO reductases from both *R. sphaeroides* and *P. denitrificans* have been shown to be induced by NO (18, 27, 29).

The question that remains is which activating protein is responding to NO and inducing the gonococcal *norB*? The *R. sphaeroides* and *P. denitrificans* NO reductases are activated by members of the FNR family of proteins (NnrR [27] and NNR [28], respectively). These proteins bind to sequences which are very similar to the FNR consensus in *E. coli* (summarized in reference 33). In *N. gonorrhoeae*, it seems unlikely that a member of the FNR family is involved in *norB* induction since we are unable to locate a full FNR binding site upstream of *norB*, although we cannot rule out a different binding consensus in the gonococcus. We have also searched the gonococcal genome with the protein sequence of several members of the FNR family of proteins and have only found the previously reported FNR (15, 19). Two other well-characterized NO-responsive regulators are SoxR and SoxS in *E. coli* (reviewed in reference 31). Again, BLAST analysis of the gonococcal genome found no homologs to these regulators. Further experimentation will be required, including pinpointing a binding site upstream of *norB*, in order to identify the *norB* activator.

We are intrigued by the possibility that the gonococcal NorB may function in immune evasion. Flavohemoglobin (Hmp), SoxRS, Cu,Zn-superoxide dismutase, and homocysteine have all been found to provide some pathogenic bacteria protection against NO (reviewed in reference 31). Specifically, *E. coli* *soxRS* mutants are more sensitive to macrophage killing, and the *Salmonella enterica* serovar Typhimurium Cu,Zn-superoxide dismutase protects against phagocyte-derived NO. In addition to SoxRS, we have also been unable to locate an Hmp homolog in the gonococcal genome, and although there is a partial coding region in GenBank for a gonococcal *sod* gene (accession no. U11277), the *sod* ORF identified in the genome appears to contain a frameshift, and no one has reported a functional superoxide dismutase in *N. gonorrhoeae*. In the absence of any of these known proteins involved in the defense against NO, it is possible that the gonococcal NorB is the means by which the organism protects itself against NO assault from macrophages.

It is estimated that approximately 50% of all primary gonococcal infections in women are asymptomatic, which leads to

higher rates of complicated gonococcal disease, such as pelvic inflammatory disease and disseminated gonococcal infection (10). The effects of NO on the inflammatory response are dependent on the concentration. Low levels of NO appear to be anti-inflammatory, while higher levels such as those produced by iNOS (NOS-2) in response to bacterial lipopolysaccharide, can be proinflammatory (reviewed in reference 12). Gonococci, with the ability to both produce and reduce NO, may exert a modulatory effect on NO levels and thus on the host inflammatory response. This may result in the development of symptomatic or asymptomatic infections, depending on the relative activities of gonococcal nitrite reductase and NO reductase.

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