Coordinated Regulation of the Neisseria gonorrhoeae-truncated Denitrification Pathway by the Nitric Oxide-sensitive Repressor, NsrR, and Nitrite-insensitive NarQ-NarP*§

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Neisseria gonorrhoeae survives anaerobically by reducing nitrite to nitrous oxide catalyzed by the nitrite and nitric oxide reductases, AniA and NorB. PaniA is activated by FNR (regulator of fumarate and nitrate reduction), the two-component regulatory system NarQ–NarP, and induced by nitrite; PnorB is induced by NO independently of FNR by an uncharacterized mechanism. We report the results of microarray analysis, bioinformatic analysis, and chromatin immunoprecipitation, which revealed that only five genes with readily identified NarP-binding sites are differentially expressed in narP* and narP strains. These include three genes implicated in the truncated gonococcal denitrification pathway: aniA, norB, and narQ. We also report that (i) nitrite induces aniA transcription in a narP mutant; (ii) nitrite induction involves indirect inactivation by nitrite of a gonococcal repressor, NsrR, identified from a multigenome bioinformatic study; (iii) in an nfrR mutant, aniA, norB, and dnrN (encoding a putative reactive nitrogen species response protein) were expressed constitutively in the absence of nitrite, suggesting that NsrR is the only NO-sensing transcription factor in N. gonorrhoeae; and (iv) NO rather than nitrite is the ligand to which NsrR responds. When expressed in Escherichia coli, gonococcal NarQ and chimaeras of E. coli and gonococcal NarQ are ligand-insensitive and constitutively active: a “locked-on” phenotype. We conclude that genes involved in the truncated denitrification pathway of N. gonorrhoeae are key components of the small NarQP regulon, that NarP indirectly regulates $P_{norB}$ by stimulating NO production by AniA, and that NsrR plays a critical role in enabling gonococci to evade NO generated as a host defense mechanism.

In contrast to Escherichia coli that can inhabit a variety of environments and utilize numerous carbon sources and electron acceptors, some niche dwellers such as the obligate human pathogen Neisseria gonorrhoeae are far less versatile. The gonococcus can grow aerobically using glucose, lactate, or pyruvate as carbon sources and electron donors, and for many years it was thought to be an obligate aerobe. However, following the isolation of gonococci from patients alongside obligate anaerobes, it became clear that they could survive in the absence of oxygen in vivo using nitrite as an alternative electron acceptor (1, 2). Although gonococci express both a copper-containing nitrite reductase, AniA (NGO1276), and a single subunit nitric oxide reductase, NorB (NGO1275), which reduce nitrite via nitric oxide to nitrous oxide (2–5), denitrification is incomplete, because they lack genes for nitrate reduction, and there is a premature stop codon in the nitrous oxide reductase gene (nosZ, XNG1300), and the putative regulator of the nitrous oxide reduction genes, nosR (XNG1301), is also degenerate (see Fig. 1A). During oxygen-limited or anaerobic growth, AniA is the major anaerobically induced outer membrane protein (6). It is expressed by bacteria infecting patients, confirming that oxygen-limited conditions are encountered during pathogenesis (7, 8). Because the gonococcus is capable of surviving oxygen limitation during colonization of the host, study of the gonococcal response to oxygen limitation is physiologically relevant to the pathogenesis of gonorrhea. Nitrite reduction via nitric oxide to nitrous oxide is clearly the critical biochemical pathway for this mode of survival.

We previously reported that expression of aniA is activated by the oxygen-sensitive transcription regulator FNR2 (NGO1579) and by a two-component regulatory system that we designated NarQ–NarP (5, 9). Although aniA transcription is further activated during growth in the presence of nitrite, but not nitrate (5), it is unknown whether nitrite is sensed directly by NarQ or indirectly by another transcription factor. In contrast to aniA, expression of norB encoding the nitric oxide reductase is independent of FNR but is induced by nitric oxide by an unknown mechanism (4). Recent studies have identified members of the Rrf2 family of transcription factors that, in

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† The on-line version of this article (available at http://www.jbc.org) contains supplemental Tables S1 and S2.

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§ The abbreviations used are: FNR, regulator of fumarate and nitrate reduction; CHIP, chromatin immunoprecipitation; NsrR, nitrosative stress response regulator; HAMP, histidine kinase, adenylate cyclase, methyl-accepting protein, and phosphotransferase domain; TMII, second transmembrane region; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; pON, probability that the probe-signal in that channel is genuine.
Regulation of Denitrification in N. gonorrhoeae

**TABLE 1**

| Strain | Description | Source |
|--------|-------------|--------|
| N. gonorrhoeae strains | | |
| F62 | Parental strain | Laboratory stocks |
| RUG7001 | F62 proAB panA: lacZ | 38 |
| RUG7022 | F62 proAB panA: lacZ, fer | 9 |
| RUG7036 | F62 proAB panA: lacZ narP | 9 |
| JCCG501 | F62 narP: 3xFLAG Kan<sup>+</sup> | This work |
| JCCG701 | F62 fer | This work |
| JCCG702 | F62 proAB panA: lacZ narR narP | This work |
| JCCG703 | F62 aniA | This work |
| JCCG751 | F62 nrS | This work |
| E. coli strains | | |
| RV | Parental strain | Laboratory stocks |
| JCB12 | RV narX narQ frdA: lacZ | This work |

Plasmids

| Plasmid | Description | Source |
|---------|-------------|--------|
| pGEM T-Easy | Cloning vector | Promega |
| pGEM T- Easy containing the sequence upstream of aniA | This work |
| pGEM T- Easy containing the sequence downstream of aniA | This work |
| pGEM T- Easy containing the sequences upstream and downstream of aniA joined by a BamHI restriction site | This work |
| pGEM T- Easy containing sequences upstream and downstream of aniA interrupted by a kanamycin resistance cassette | This work |
| pGEM NarR | pGEM T- Easy containing the sequences upstream and downstream of narR joined by an AgeI restriction site | This work |
| pGEMNarR-KO | This work |
| pSUB11 | Epitope tagging plasmid carrying 3xFLAG tag and kanamycin resistance cassette | The work |
| pBAD Myc-His A | Cloning vector containing arabinose-inducible araBAD promoter | Invitrogen |
| pBADdetQ | Gonococcal narQ gene under the control of the araBAD promoter cloned into pBAD myc-His A | This work |
| pBADgQ | E. coli narQ gene under the control of the araBAD promoter cloned into pBAD myc-His A | This work |
| pRNW200 | E. coli narQ N terminus fused to gonococcal narQ C terminus in the P<sup>+</sup> box, cloned into pBAD/myc-hisA | This work |
| pRNW201 | E. coli narQ N terminus fused to gonococcal narQ C terminus in the P<sup>+</sup> box, cloned into pBAD/myc-hisA | This work |
| pRNW202 | E. coli narQ with a BamHI restriction site in the P<sup>+</sup> box, resulting in substitutions A143G and E144S, cloned into pBAD/myc-hisA | This work |
| pRNW203 | Gonococcal narQ with a BamHI restriction site in the P<sup>+</sup> box, resulting in substitution E156S, cloned into pBAD/myc-hisA | This work |
| pRNW300 | E. coli narQ N terminus fused to gonococcal narQ C terminus in TMII, cloned into pBAD/mhc-hisA | This work |
| pRNW301 | Gonococcal narQ N terminus fused to E. coli narQ C terminus in TMII, cloned into pBAD/mhc-hisA | This work |
| pRNW302 | E. coli narQ with a BamHI restriction site in TMII, resulting in substitutions F167W and T168I, cloned into pBAD/mhc-hisA | This work |
| pRNW303 | Gonococcal narQ with a BamHI restriction site in TMII, resulting in substitutions L179R and M180I, cloned into pBAD/mhc-hisA | This work |
| pRNW400 | E. coli narQ N terminus fused to gonococcal narQ C terminus in the HAMP linker, cloned into pBAD/myc-hisA | This work |
| pRNW401 | Gonococcal narQ N terminus fused to E. coli narQ C terminus in the HAMP linker, cloned into pBAD/myc-hisA | This work |
| pRNW402 | E. coli narQ with an NdeI restriction site in the HAMP linker, resulting in substitution E217H, cloned into pBAD/myc-hisA | This work |
| pRNW403 | Gonococcal narQ with an NdeI restriction site in the HAMP linker, resulting in substitution E228H, cloned into pBAD/myc-hisA | This work |
| pRNW500 | E. coli narQ N terminus fused to gonococcal narQ C terminus in the Y box, cloned into pBAD/mhc-hisA | This work |
| pRNW501 | Gonococcal narQ N terminus fused to E. coli narQ C terminus in the Y box, cloned into pBAD/mhc-hisA | This work |
| pRNW502 | E. coli narQ with a BstBI restriction site in the Y box, resulting in substitution L229F, cloned into pBAD/mhc-hisA | This work |
| pRNW503 | Gonococcal narQ with a BstBI restriction site in the Y box, resulting in substitution L240F, cloned into pBAD/mhc-hisA | This work |

**EXPERIMENTAL PROCEDURES**

Strains, Plasmids, Oligonucleotide Primers, and Gene Identification Numbers Used in This Study—Strains and plasmids used in this study are listed in Table 1. Sequences of oligonucleotide primers are available on-line in supplemental Table S1. All newly constructed plasmids were checked by sequencing to ensure that unanticipated changes had not been introduced during their construction. The XNG gene identification numbers used for some genes in this study (not annotated in the GenBank<sup>™</sup> data base) refer to the genome sequence annotation of N. gonorrhoeae strain FA1090 that was used in the design of the pan-Neisseria microarray (13) and pan-Neisseria microarray version 2 (14). A GBrowse data base containing this annotation comparatively presented against other neisserial genome annotations can be found at http://www.compbio.ox.ac.uk/data. Clusters of orthologous genes were identified using the NCBI Conserved Domain Search tool (15).

*Generation of nsrR, fnr, and aniA Mutants of N. gonorrhoeae—* Crossover PCR was used to generate an nsrR knock-out mutant of N. gonorrhoeae (16). Primers NsrRA plus NsrRB and NsrRC plus NsrRD were used to generate DNA fragments upstream and downstream of the nsrR gene (NGO1519). The flanking fragments were cleaned and combined in a crossover PCR reaction with primers NsrRA and NsrRD, yielding a single fragment with an AgeI restriction site between the upstream and down-
stream sequences. The crossover PCR product was cloned into pGEM T-Easy, yielding pGEMNsrR. A kanamycin resistance cassette was amplified from pSUB11 by PCR using primers KanAgelFwd and KanAgelRev, which introduced Agel sites at each end of the resultant fragment and was ligated into Agel-digested pGEMNsrR, yielding pGEMNsrR-KO. The nskr::kanR fragment was generated by digestion of pGEMNsrR-KO with EcoRI and was transformed, as previously described (5), into piliated N. gonorrhoeae strain F62, yielding strain JGC701 and into strain RUG7036 (paniA::lacZ narP), yielding strain JGC702. An ania mutant was also generated. Primers AniAA plus AniAB and AniAC plus AniAD were used to amplify PCR products upstream and downstream of the ania gene, which were cloned into pGEM T-Easy yielding pGEMAniAUP and pGEMAniADN. Candidate plasmids were screened for inserts in the correct orientation by double digestion with BamHI, engineered into the PCR products by primers AniAB and AniAC, and PstI, carried on pGEM T-Easy, such that pGEMA-
niAUP contained BamHI and PstI sites at the 3’-end of the ania upstream fragment, and pGEMAniADN contained a BamHI site at the 5’-end and a PstI site at the 3’-end of the ania downstream fragment. The downstream fragment was ligated into BamHI- and PstI-digested pGEMAniAUP yielding pGEMAniA, containing the sequences upstream and downstream if the ania gene separated by a BamHI site. A kanamycin resistance cassette was amplified from pSUB11 by PCR using primers KanBamHI and KanBamHIRev, which introduced BamHI restriction sites into pGEMAniA, forming pGEMAniA-KO. The resultant ania::kanR construct was generated by digesting pGEMAniA-KO with EcoRI, which was transformed into piliated N. gonorrhoeae strain F62, yielding strain JGC703. Strain JGC701 was generated by transforming strain F62 with an fnr::eryR cassette amplified from N. gonorrhoeae strain RUG7022 using primers FnrA and FnrB.

Construction of a frdA::lacZ Reporter Strain—To construct the NarL-repressed frdA::lacZ reporter strain, the Lac− E. coli strain RV was first transduced with bacteriophage P1 that had been propagated on a strain carrying an frdA::lacZ fusion. Because strain RV cannot metabolize lactose, only Lac− transductants were able to express β-galactosidase activity. Transductants were enriched during overnight growth in LB supplemented with lactose, streaked onto MacConkey-lactose agar and incubated overnight at 37 °C. Areas of growth showing a red, lactose-positive phenotype were streaked for purification by single colony isolation. Lac− transductants were checked for repression of transcription from the frdA promoter during anaerobic growth in the presence, but not in the absence, of nitrate (17). The narX and narQ mutations were then introduced by successive rounds of transduction with bacteriophage P1 that had been propagated on insertion-deletion mutants that had been generated by the method of Datsenko and Wanner (18), followed by curing of the antibiotic resistance determinant to leave unmarked deletions in narQ and narX as described previously (18).

Construction of Plasmids Expressing Gonococcal and E. coli narQP—To construct plasmid pBADgcQ, the gonococcal narQ gene was cloned into the arabinose-inducible pBAD myc-hisA overexpression vector using primers NgNarQNcoI and NgNar-QHindIII to generate an Ncol-HindIII narQ fragment, which was ligated into Ncol/HindIII-digested pBAD myc-hisA (Invitrogen). Similarly, pBADecQ contained the E. coli narQ gene cloned into pBAD myc-hisA. Primers EcNarQHindIII and EcNarQ HindIII were used to generate an Ncol-BamHI E. coli narQ fragment, which was cloned into pBAD myc-hisA. The QuiChange site-directed mutagenesis system (Stratagene) was used to generate specific mutations in narQ genes using primers listed in supplemental Table S1.

Chimeric NarQ proteins were generated by introducing restriction sites into pBADgcQ and pBADecQ, restriction digestion, and ligation of resultant fragments. For P’-box hybrids, BamHI sites were introduced at codons 143–144 of E. coli narQ (introducing substitutions A143G and E144S in plasmid pRNW202) and the corresponding codons 155 and 156 of gonococcal narQ (introducing substitution E156S in pRNW203). The resultant plasmids were digested with BamHI: the gonococcal narQ gene (from the P’-box to the stop codon) was ligated into the vector fragment containing the E. coli narQ N-terminal fragment, yielding the E. coli-gonococcal P’-box fusion encoded by pRNW200. The E. coli narQ gene fragment was likewise ligated into the vector fragment containing the gonococcal narQ N-terminal, yielding the gonococcal-E. coli P’-box fusion (pRNW201). Similarly, the TMII chimaeras were created by introducing BamHI restriction sites into E. coli narQ at codons 167 and 168 (resulting in substitutions F167W and T168L: pRNW302) and gonococcal narQ codons 179–180 (substitutions L179R M180I: pRNW303). The HAMP linker chimaeras were created by introducing Ndel sites into E. coli narQ at codon 217 (resulting in substitution E217H: pRNW402) and into gonococcal narQ at codon 229 (generating substitution E229H: pRNW403). The Y-box chimaeras were created by introducing BstBI sites into E. coli narQ at codon 229 (resulting in substitution L229F: pRNW502) and into gonococcal narQ at codon 240 (generating substitution L240F: pRNW503).

Growth of N. gonorrhoeae—N. gonorrhoeae was grown on gonococcal agar plates and in gonococcal broth (Beckton Dickinson UK Ltd.). Solid and liquid media were supplemented with 1% (v/v) Kellogg’s Supplement (19). For liquid cultures, 2 µl of a stock of N. gonorrhoeae was plated onto a gonococcal agar plate and incubated in a candle jar at 37 °C for 24 h. Bacteria from this plate were swabbed onto a second plate and incubated in the same way for a further 16 h. The entire bacterial growth from this second plate was swabbed into 10 ml of gonococcal broth and incubated at 37 °C in an orbital shaker at 100 rpm for 1 h. This 10-ml pre-culture was then tipped into 50 ml of gonococcal broth in a 100-ml conical flask and incubated in the same way for 1 h. The entire bacterial growth from this second plate was swabbed into 10 ml of gonococcal broth and incubated at 37 °C in an orbital shaker at 100 rpm for 1 h.

Preparation of RNA for Microarray Experiments—RNA was extracted from five independent cultures of each strain during exponential growth. Samples (10 ml) of bacterial culture were mixed with an equal volume of RNAlater (Ambion), and the bacteria were pelleted by centrifugation, resuspended in 0.5 ml of RNAlater, and stored at 4 °C overnight. Bacteria were collected by centrifugation and resuspended in TRIzol (Invitrogen) by vortexing for 10 min. Chloroform
was added, the phases were separated, and the aqueous phase was transferred to a clean tube. Crude RNA in the aqueous phase was precipitated with isopropanol and cleaned using an RNeasy kit (Qiagen). Purified RNA was eluted in RNase-free water with 2% (v/v) SuperaseIN RNase inhibitor (Ambion). RNA was quantified using a Nanodrop ND-1000 spectrophotometer (Labtech), and integrity was checked using an Agilent 2100 Bioanalyzer and RNA Nano Chips, according to manufacturer’s instructions.

**cDNA Generation, Labeling, and Microarray Hybridization**—Reagents and enzymes for the preparation of materials for microarray hybridizations were sourced from the 3DNA Array 900-MPX kit (Genisphere, Hatfield, PA) unless otherwise stated. One microgram of RNA was reverse-transcribed into unlabeled cDNA using SuperScript III reverse transcriptase (Invitrogen) at 42 °C for 2 h. The cDNA was cleaned using a Clean & Concentrate-5 column (Zymo Research) and poly-T tailed with terminal deoxynucleotidyl transferase. Dye-specific capture sequences were ligated to the poly-T tails, and the tagged cDNAs were cleaned using a Clean & Concentrate-5 column. The pan-*Neisseria* microarray version 2 (14), containing probes to *N. gonorrhoeae* and *Neisseria meningitidis* genes, was used for these experiments. Microarray slides were prehybridized in 3.5 × SSC, 0.1% SDS, and 10 mg ml⁻¹ bovine serum albumin for 65 °C for 20 min, washed with water and isopropanol, dried with an airbrush, and pre-scanned to check for array defects. The capture sequence-tagged cDNAs were hybridized onto the microarray slide for 16 h at 60 °C in a SlideBooster (Adalvityx) with the power setting at 25 and a pulse/pause ratio of 3:7. Following the first hybridization, the slides were washed in 2× SSC, 0.2% SDS for 10 min at 60 °C, followed by washes in 2× SSC and 0.2× SSC for 10 min, each at room temperature. The slides were dried with an airbrush and hybridized with the Cy3 and Cy5 capture reagents at 55 °C for 4 h in a SlideBooster. The slides were again washed in 2× SSC, 0.2% SDS (10 min at 60 °C) followed by 10-min room temperature washes in 2× SSC and 0.2× SSC (10 min at room temperature) and dried with an airbrush. Dried slides were scanned using a ScanArray ExpressHT (PerkinElmer Life Sciences) using autocalibration to obtain optimized non-saturating images for each fluorophore.

**Microarray Data Analysis**—Scanned microarray images were straightened, if necessary, with ImageViewer (Blue-Gnome) and analyzed using BlueFuse for Microarrays (Blue-Gnome). Spot data were extracted from images and manually flagged to remove artifacts before fusion. Fused data were filtered according to the pON value (20). Spots with pON values <0.5 in both channels were excluded to eliminate the bias generated by the inclusion of unhybridized spots in the statistical interpretation of the data, and the data were globally adjusted such that the mean rRNA ratio was 1.0. The data were then analyzed using BASE. For each pairwise comparison, gene expression median -fold changes were calculated from the biological replicates using the MGH fold-change algorithm, and the Student’s t test was used to assess statistical significance. For the NarP microarray experiment, a cutoff p value of 0.05 was used. Genes whose transcript levels did not change consistently (i.e. with an expression ratio greater than or less than one in all five replicate experiments) in all the biological replicates for each experiment were discarded. Data were also analyzed using a locally prepared implementation of the Cyber-T algorithm within BASE; the results from this analysis are available online (gbrowse.molbiol.ox.ac.uk/cgi-bin/gbrowse/NarPQ/; username: NarPQ; password: reviewOnly). Total microarray data have been deposited in the ArrayExpress data base (www.ebi.ac.uk/arrayexpress) with the accession number “E-MEXP-726” (hybridizations were PNA8_36 to PNA8_41).

**Generation of a Chromosomal 3×FLAG Fusion in N. gonorrhoeae**—Codons for a 3×FLAG tag, (DYKDDDDK)₃, were linked in-frame to the 3′-end of the narP gene on the chromosome of *N. gonorrhoeae* strain F62 using crossover PCR (16). Three fragments corresponding to the 3′-end of the gonococcal narP gene, the region immediately downstream of the narP gene, and the FLAG tag and kanamycin resistance cassette carried on pSUB11 were generated by PCR using primer pairs NarPXO1 plus NarPXO2, NarPXO3 plus NarPXO4, and pSUBXO1 plus pSUBXO2, respectively. The three PCR products were cleaned and combined in a second PCR using primers NarPXO1 and NarPXO4. This reaction generated a 3-kb product containing the narP gene fused to a 3×FLAG tag, a kanamycin resistance cassette, and the region downstream of the narP gene. This fragment was transformed into piliated *N. gonorrhoeae* strain F62 generating strain JCGC501, carrying a chromosomal narP:FLAG fusion.

**Western Blotting**—Gonococcal proteins separated by Tris/Tricine SDS-PAGE using a 15% polyacrylamide gel were blotted onto a polyvinylidene difluoride membrane, and FLAG-tagged NarP protein was detected using anti-FLAG monoclonal antibodies (Sigma) and the ECL-Plus chemiluminescence detection system (GE Healthcare Life Sciences).

**Chromatin Immunoprecipitation**—Interactions between NarP and promoter DNA were studied in vivo by chromatin immunoprecipitation (ChIP) as described in ref. 21. Oxygen-limited cultures of *N. gonorrhoeae* strain JCGC501 were grown in the presence of 5 mM NaNO₂ to late exponential phase. Protein-DNA cross-linking, DNA-protein complex preparations, and immunoprecipitations were as described previously except that the tagged protein was immunoprecipitated with anti-FLAG monoclonal antibodies (Sigma) for 16 h at 4 °C. The concentration of immunoprecipitated promoter fragments was measured using quantitative real-time PCR (22). Primers for each promoter were designed using PrimerExpress (Applied Biosystems) and are listed in supplemental Table S1. The promoter of the *hpt* (NG2035) gene, which is not regulated by NarP and is not preceded by a NarP binding site, was a negative control used to normalize the data. Promoter fragments enriched by 50% or more in at least two independent ChIP experiments, relative to the hpt promoter fragment, were scored positive.

**Quantitative Real-time PCR Analysis of Gene Expression**—RNA was stabilized by mixing 500 μl of bacterial culture with 900 μl of RNAlater (Ambion). After 5-min incubation at room temperature, the bacteria were harvested by centrifugation at 3000 × g for 10 min. RNA was isolated from the pellet using an RNeasy mini kit (Qiagen) using the manufacturer’s protocol. Genomic DNA was removed from the purified RNA using TURBO DNase (Ambion). The RNA was reverse-transcribed...
TABLE 2

Genes differentially expressed in \textit{narP} and \textit{narP} strains for which there is direct evidence for NarP binding

The ratio of transcript intensity, \textit{narP}/\textit{narP}, and Student’s \textit{t}-test \textit{p} value are shown for each gene. NarP binding sites with at most two mismatches to the consensus \textit{E. coli} NarP binding site, TACYNMNNAKNGTA, are displayed with their location with respect to the translation start of each gene. ChIP enrichments are expressed as a percentage of enrichment of the promoter fragment in the ChIP sample containing anti-FLAG antibodies compared to the no antibody control. All ChIP data are normalized to the hpt promoter fragment, which contains no NarP binding site and whose expression is not regulated by NarP. Values stated are the mean \pm S.D. of two to four independent experiments. The \textit{norP} promoter, which contains no putative NarP binding site, was enriched <1% in all experiments.

| Expression ratio | \textit{narP}/\textit{narP} | \textit{p} value | Gene | Function | NarP binding site | ChIP enrichment |
|------------------|-----------------|----------------|------|----------|------------------|---------------|
| Number | Name | | | | | |
| | | | | | | |
| 3.98 | 0.022 | NGO1275 | \textit{norB} | Nitric oxide reductase | TAAACTCATAAACTA | 63 \pm 17* |
| 3.22 | 0.001 | NGO1276 | \textit{aniA} | Nitrite reductase | TAACTTACATAAATTA | 63 \pm 17* |
| 0.25 | 1.05 \times 10^{-4} | NGO1215 | Conserved hypothetical protein (COG2847) | TAAACCTCATAAGATTA | 82 \pm 25 |
| 0.24 | 0.001 | NGO0753 | \textit{narQ} | Sensor kinase | TACCGAGAGCGGTC | 69 \pm 5 |
| 0.23 | 0.001 | NGO1370 | Conserved membrane protein (COG3182) | TAGTCATGAGAATTA | 70 \pm 14 |

* The \textit{norB} and \textit{aniA} promoters are divergent and contain a single NarP binding site.

\* With respect to mapped transcription start site; all other positions are relative to the translation start.

to cDNA using a Superscript first-strand synthesis kit (Invitrogen). For each sample, a control to check for DNA contamination in the RNA preparation was included from which reverse transcriptase was omitted. Transcript levels were measured by quantitative real-time PCR using SensiMix with SYBR green detection (Quantace) and an ABI 7000 sequence analyzer (Applied Biosystems). Primers, designed using PrimerExpress (Applied Biosystems), are described in supplemental Table S1 (available online). Transcript levels were quantified using the \Delta\Delta\textit{Ct} method (23) relative to expression of the \textit{polA} gene. For each experiment, quantitative real-time PCR was used to determine transcript levels in triplicate on three independent cDNA samples derived from three independent cultures. Error bars show standard deviations. Expression levels were normalized to the parental strain in the absence of nitrite or nitric oxide.

\beta-Galactosidase Assay—\textit{E. coli} was grown at 37 °C in 10 ml of minimal medium (24) supplemented with 40 mM sodium fumarate, 10% LB, and 0.4% glycerol. Where stated, cultures were supplemented with 20 mM NaNO\textsubscript{3} or 2.5 mM NaNO\textsubscript{2}. 2-ml aliquots of bacterial cultures were lysed by the addition of 30 \mu l of each toluene and 2% (w/v) sodium deoxycholate and aerated at 30 °C for 20 min. Lysates were assayed for \beta-galactosidase activity as previously described (25).

Sequence Pattern Searching—Potential NarP binding sites were located in promoter regions using Findpatterns in the GCG suite (Accelrys, Cambridge, UK). The consensus \textit{E. coli} binding site for NarP, TACYNMNNAKNRGTAGA, was used to search the gonococcal DNA sequences.

RESULTS

Microarray and ChIP Analysis of the NarP Regulon—From previous gene-specific studies, the \textit{N. gonorrhoeae} NarP has been suggested to regulate only two promoters, activating transcription of \textit{P\_aniA} and repressing transcription at the cytochrome \textit{C} peroxidase promoter, \textit{P\_ccp}. Microarray analysis was used to determine whether NarP regulates major aspects of gonococcal metabolism other than nitrite reduction. However, because it has not proved possible to grow gonococci anaerobically in liquid medium, there is currently no known method for growing a \textit{narP} parental strain and a \textit{narP} mutant anaerobically at the same rate under conditions in which NarP-activated genes would be optimally expressed. Consequently, many small differences in transcript levels due to growth rate effects between the two strains were anticipated. During oxygen-limited growth of \textit{N. gonorrhoeae} strains RUG7001 (\textit{narP}) and RUG7036 (\textit{narP}) in the presence of nitrite, the \textit{narP} mutant grew more slowly than the wild-type strain, presumably due to its diminished ability to express \textit{aniA}, and thus reduce nitrite. RNA was extracted from five independent cultures of each strain during exponential growth, reverse-transcribed to cDNA, and hybridized to the pan-\textit{Neisseria} microarray version 2 (14). Data were analyzed according to -fold change (>2-fold difference between the two strains) and Student’s \textit{t} test (\textit{p} < 0.05): 40 transcripts were consistently found to be more abundant in the \textit{narP} strain compared with the \textit{narP} mutant, whereas 8 transcripts were less abundant (available online in supplemental Table S2). As expected, given the growth rate differences between these strains, many of the genes found to be more highly expressed in the \textit{narP} strain encoded ribosomal and other growth rate-dependent proteins.

Potential NarP-binding sites (at least a 12/14 match to the \textit{E. coli} consensus NarP binding site, TACYNMNNAKNRGTAGA) were identified within 400 bp upstream of only five genes differentially expressed in the \textit{narP} and \textit{narP} strains (Table 2): nitric oxide reductase \textit{norB}, nitrite reductase \textit{aniA}, putative sensor kinase \textit{narQ}, and two hypothetical genes, NGO1215 and NGO1370. Because there is a common divergent regulatory region between two of these genes, \textit{aniA} and \textit{norB}, expression of these genes is regulated from four promoter regions. A strain containing a chromosomal \textit{narP}:3×\textit{FLAG} fusion was constructed (\textit{N. gonorrhoeae} strain JCCG501) and anti-\textit{FLAG} antibodies were used to immunoprecipitate NarP-DNA complexes from formaldehyde-cross-linked chromatin. All four promoter regions with potential NarP sites were enriched in ChIP experiments, confirming that NarP binds to the target promoter fragments. The \textit{aniA-norB} promoter region contains one potential NarP binding site, centered between bases 95 and 96 upstream of the mapped \textit{aniA} transcription start (Fig. 1B). Thus, NarP is likely to act at this site, activating \textit{aniA} expression. Because \textit{norB} expression is induced by NO, the product of nitrite reduction by \textit{aniA}, the differential expression of \textit{norB} observed in the microarray is likely to be an indirect effect, caused by the synthesis of more NO in the \textit{narP} strain than in the \textit{narP} mutant. This is also the likely explanation for the induction of \textit{nosZ} transcription, which appears to
**Regulation of Denitrification in N. gonorrhoeae**

A. 

\[
\text{NO}_2^- \rightarrow \text{NO} \rightarrow \text{N}_2 \]

B. 

| Gene | NarP | NsrR |
|------|------|------|
| AniA | +    | -    |
| NorB | +    | +    |
| NosZ | -    | -    |

C. 

**TABLE 3**

| Strain   | Genotype | \( \beta \)-Galactosidase activity (OD420 min⁻¹ mg⁻¹ protein) | Nitrile induction ratio |
|----------|----------|---------------------------------------------------------------|------------------------|
| RUG7001  | wt       | 1600 ± 90/358 ± 55/41.7 ± 3 | 4.7/7.7 |
| RUG7036  | narP     | 323 ± 37/41.7 ± 3 | 7.7 |

**FIGURE 1.** The *aniA* promoter is regulated by NsrR in response to nitrate. A, the truncated denitrification pathway of *N. gonorrhoeae*. Nitrite reductase AniA reduces nitrite to nitric oxide, which is reduced to dinitrogen by NarB. Nitrous oxide is not reduced to dinitrogen, because the nitrous oxide reductase structural gene, *nsrR*, is interrupted by a frameshift mutation. B, the *aniA* promoter sequence showing the mapped transcription start site and proposed NarP, NsrR, and FNR binding sites. C, oxygen-limited cultures of *N. gonorrhoeae* strains F62 (wt) and JCGC751 (*nsrR*) were grown in the presence or absence of 5 mM nitrite. RNA was isolated at an *A_{550}* of 0.5, the expression of *aniA* was determined using quantitative real-time PCR, and normalized to the level of expression in the parental strain in the absence of nitrite. D, *aniA* expression was not induced by nitrite and was comparable to expression in the presence of nitrite in the absence of nitrite. Error bars show the standard deviation of at least three independent cultures.

**Gonococcal NsrR, Not NarP, Regulates the *aniA* Promoter in Response to Nitrite**—Previous studies had suggested that, even in the absence of NarP, the *aniA* promoter might still respond to nitrite, so it was not established whether nitrite directly activates the *aniA* expression by another mechanism. Because the previous study relied on *β*-galactosidase assays using a heterogeneous population of plategrown bacteria, the assays were repeated using homogeneous liquid cultures.

Oxygen-limited cultures of *N. gonorrhoeae* strains RUG7001 and RUG7036 (*narP*), both carrying chromosomal *aniA::lacZ* fusions, were grown in the presence and absence of 5 mM sodium nitrite. In the presence of nitrite, the *β*-galactosidase activity of the *narP* strain, RUG7001, was far higher than in the absence of nitrite (Table 3). When grown in the presence of nitrite, the *β*-galactosidase activity of the *narP* strain RUG7036 was comparable to that of the *narP* strain in the absence of nitrite, but was even lower during growth in the absence of nitrite. These results established that, although the *aniA* promoter is activated by NarP and by nitrite, NarP is not essential for nitrite activation.

The open reading frame encoding the gonococcal *nsrR* homologue (NGO1519) was interrupted with a kanamycin resistance cassette in strain F62, yielding the *nsrR* mutant, strain JCGC751. Oxygen-limited cultures of strains F62 and JCGC751 were grown in the presence and absence of 5 mM sodium nitrite, RNA was extracted and reverse-transcribed to cDNA, and the quantity of *aniA* transcript was measured using quantitative real-time PCR (Fig. 1C). Expression of *aniA* was induced ~2-fold by nitrite in the *nsrR* strain, whereas in the *nsrR* strain, *aniA* expression was not induced by nitrite and was comparable to expression in the presence of nitrite in the *nsrR* strain.

The *nsrR* gene was also deleted from *N. gonorrhoeae* strain RUG7036 (*aniA::lacZ narP*) yielding strain JCGC702. Both strains were grown in the presence and absence of 5 mM nitrite, and the *β*-galactosidase activity was assayed when each culture had become oxygen-limited (Fig. 1D). The *β*-galactosidase activity of strain JCGC702 (*narP nsrR*) was essentially identical in the presence and absence of nitrite and similar to strain

was reported to be induced by iron in a previous microarray study (26).

be NarP-dependent in the microarray data, but has no NarP binding site (and so is not listed in Table 2) and was not enriched in ChIP experiments (the enrichment ratio was <1% in all experiments). Because NarP was not detected to bind to the *nosZ* promoter, it acts as a negative control for the ChIP experiment.

Of the four ChIP-confirmed NarP-regulated genes, only *aniA* had previously been reported to be NarP activated. The observation that NarP represses transcription of the *narQP* operon is unsurprising, because many transcription regulators are autoregulated. NGO1370 encodes a conserved hypothetical protein predicted to be an integral membrane protein. Homologs of NG1370 in other bacteria have been identified as iron-inducible (COG3182, PiuB conserved domain), but neither NGO1370 nor its homologue in *N. meningitidis* (NMB1721)
Regulation of Denitrification in N. gonorrhoeae

NarP Synthesis Is Not Regulated by the Presence of Nitrite—An alternative or additional possibility was that NsrR regulates expression of narQ-narP, and hence controls aniA expression indirectly by regulating the quantity of NarP. To investigate this possibility, the effect of an nsrR mutation on narQ transcription was first assayed by quantitative real-time PCR. No differences in narQ transcript abundance were detected between the mutant and the parental strain (Fig. 2A). To confirm the above conclusion, a strain containing a chromosomal narP::3XFLAG fusion was constructed (N. gonorrhoeae strain JCGC501), and the quantity of NarP accumulated during growth in the presence or absence of nitrite was assayed by Western analysis using commercial anti-FLAG antibodies. The concentration of NarP was essentially constant throughout growth, and similar in cultures grown in the presence or absence of nitrite (Fig. 2B). Thus two independent approaches confirmed that NsrR regulates aniA transcription directly in response to the availability of nitrite, rather than indirectly by modulating the cellular level of the transcription activator, NarP.

Hybrid Gonococcal E. coli NarQ Proteins Exhibit Unusual Sensing and Kinase Characteristics—Demonstration that the mechanism of nitrite induction at PaniA depends on relief of repression by NsrR in response to availability of nitrite indicated marked differences between the E. coli and gonococcal environmental sensors that have been designated NarQ. Only a very limited range of genetic techniques are available to investigate sensor kinases by site-directed mutagenesis and gene deletions in the gonococcus. To investigate the ligand sensing and signal transduction characteristics of the gonococcal NarQ protein, the gonococcal narQ gene was expressed in E. coli from plasmid pBAD-myc His, and the ability of the gonococcal NarQ to phosphorylate E. coli NarL was investigated using the NarL-repressed frdA promoter as a reporter of protein binding to DNA.

Hybrid sensor kinases have been constructed previously by fusing the sensing and kinase domains of two sensor kinases, resulting in chimaeras with the ligand-sensing and kinase specificities of the two fused proteins (27, 28). In this study, the N. gonorrhoeae and E. coli NarQ proteins were fused at four points: the end of the P’-box; at the C-terminal end of the second transmembrane domain; in amphipathic sequence 2 in the HAMP linker; and in the Y-box. For each fusion, two hybrids were created; one with E. coli NarQ N-terminal and N. gonorrhoeae C-terminal regions, the other with N. gonorrhoeae N-terminal and E. coli C-terminal regions (Table 4). Because the construction of each fusion required the introduction of a restriction site that resulted in amino acid substitutions, the function of the E. coli and N. gonorrhoeae NarQ proteins substituted at the site of the restriction site were also assessed. E. coli strain JCB12 (narX narQ frdA::lacZ) was transformed with plasmids expressing chimeric or restriction site-mutated NarQ proteins. Transformants were grown anaerobically in the presence or absence of nitrate or nitrite, and β-galactosidase activities were measured to determine the ability of the mutated NarQ proteins to phosphorylate NarL and repress frdA expression (Table 4).

The E. coli NarQ proteins substituted with the four restriction sites used in this experiment were capable of sensing nitrate and nitrite and phosphorylating NarL, thus repressing frdA. In contrast, the N. gonorrhoeae NarQ proteins were all “locked-on,” phosphorylating NarL even in the absence of ligand. This provided the first direct evidence that gonococcal NarQ might be a constitutively active ligand-insensitive kinase rather than an environmental sensor protein.

The P’-box and TMII fusions were “locked-off” in gonococcal N-terminal and E. coli C-terminal chimaeras, and locked-on in E. coli N-terminal and N. gonorrhoeae C-terminal hybrids. The N terminus of N. gonorrhoeae NarQ appears to be either ligand-insensitive, or incapable of transducing a “no ligand” signal to the C terminus of E. coli NarQ, whereas the C terminus of N. gonorrhoeae NarQ appears to be constitutively active. All of the HAMP linker and Y-box chimaeras were locked-on, possi-
Regulation of Denitrification in N. gonorrhoeae

Ability of chimeric NarQ proteins to activate NarL-dependent repression of the E. coli fumurate reductase promoter revealed using a chromosomal frdA::lacZ fusion

E. coli strain JC12 (narQ::frdA::lacZ) transformed with plasmids expressing chimeric and restriction site-modified NarQ proteins, as listed in Table I, was grown anaerobically in minimal medium supplemented with 40 mM fumarate, 10% LB, 0.4% glycerol, and either 20 mM NaNO₃ or 2.5 mM NaN₃, and β-galactosidase activities were measured. Units of β-galactosidase activity are nmol ONPG hydrolyzed/min/mg of dry mass.

Table 4

| Site of fusion | β-Galactosidase activity of cultures expressing NarQ proteins |
|---------------|-------------------------------------------------------------|
|               | E. coli | Ec-Gc chimaera | Ge-Ec chimaera | N. gonorrhoeae |
| P'-box        |         |               |               |               |
| Plasmid       | pRNW202 | pRNW200       | pRNW201       | pRNW203       |
| O₁          | 3400 ± 29 | 1800 ± 2     | 5590 ± 24     | 760 ± 8       |
| O₁ + NO₂      | 2640 ± 37 | 1430 ± 2      | 4760 ± 27     | 660 ± 1       |
| Phenotype     | NO₂/NO₃-sensitive | Locked on/slight NO₂/NO₃ response | Locked off/slight NO₂/NO₃ response | Locked on |
| TMII          |         |               |               |               |
| Plasmid       | pRNW302 | pRNW300       | pRNW301       | pRNW303       |
| O₁          | 4390 ± 19 | 1290 ± 7      | 4920 ± 35     | 1420 ± 4      |
| O₁ + NO₂      | 2040 ± 12 | 1330 ± 4      | 4710 ± 53     | 1490 ± 32     |
| Phenotype     | NO₂/NO₃-sensitive | Locked on/slight NO₂/NO₃ response | Locked off/slight NO₂/NO₃ response | Locked on |
| HAMP linker   |         |               |               |               |
| Plasmid       | pRNW402 | pRNW400       | pRNW401       | pRNW403       |
| O₁          | 2660 ± 4  | 600 ± 2       | 450 ± 1       | 630 ± 17      |
| O₁ + NO₂      | 1120 ± 6  | 670 ± 15      | 630 ± 6       | 590 ± 3       |
| Phenotype     | NO₂/NO₃-sensitive | Locked on | Locked on | Locked on |
| Y-box         |         |               |               |               |
| Plasmid       | pRNW502 | pRNW500       | pRNW501       | pRNW503       |
| O₁          | 2860 ± 32 | 600 ± 6       | 500 ± 4       | 760 ± 2       |
| O₁ + NO₂      | 1870 ± 42 | 530 ± 6       | 570 ± 1       | 610 ± 19      |
| Phenotype     | NO₂/NO₃-sensitive | Locked on | Locked on | Locked on |

NsrR Regulates Expression of the Nitric Oxide Reductase—Expression of the N. gonorrhoeae norB gene encoding the nitric oxide reductase is independent of FNR, but is induced by nitric oxide via an unknown mechanism (4). In addition to the NsrR binding site located at the aniA promoter, sites were also identified at the norB promoter, and the dnrN promoter, which regulates expression of a putative reactive nitrogen species-responsive gene homologous to the E. coli ytfE gene (Fig. 3A) (12, 29). Real-time quantitative PCR was used to determine the relative amounts of the norB and dnrN transcripts in N. gonorrhoeae strains F62 (narR⁺) and JCGC751 (nsrR) grown in the presence and absence of nitrite (Fig. 3, B and C). In the norR⁺ strain, expression of both genes was highly induced during growth in the presence of nitrite under conditions in which nitric oxide is generated from nitrite by AniA. In the nsrR strain, both genes were expressed constitutively at high level even in the absence of nitrite, suggesting that NsrR represses both norB and dnrN, the repression being lifted upon exposure to reactive nitrogen species.

Growth Response of the NsrR Mutant to Sudden Exposure to Toxic Concentrations of Nitrite—The growth phenotype of the nsrR strain JCGC751 provided preliminary evidence that NsrR plays a critical role in NO homeostasis. Although there was no difference in growth phenotype between the mutant and the parental strain during oxygen-limited growth in the presence or absence of nitrite, opposite responses were seen when uninduced cultures were suddenly exposed to a high concentration bly reflecting the importance of interactions between the two helices comprising the HAMP linker and the Y-box. From these experiments in E. coli, it was concluded that the N. gonorrhoeae NarQ protein is ligand-insensitive and constitutively active in a locked-on phenotype.

NsrR Regulates Expression of the Nitric Oxide Reductase—Expression of the N. gonorrhoeae norB gene encoding the nitric oxide reductase is independent of FNR, but is induced by nitric oxide via an unknown mechanism (4). In addition to the NsrR binding site located at the aniA promoter, sites were also identified at the norB promoter, and the dnrN promoter, which regulates expression of a putative reactive nitrogen species-responsive gene homologous to the E. coli ytfE gene (Fig. 3A) (12, 29). Real-time quantitative PCR was used to determine the relative amounts of the norB and dnrN transcripts in N. gonorrhoeae strains F62 (narR⁺) and JCGC751 (nsrR) grown in the presence and absence of nitrite (Fig. 3, B and C). In the norR⁺ strain, expression of both genes was highly induced during growth in the presence of nitrite under conditions in which nitric oxide is generated from nitrite by AniA. In the nsrR strain, both genes were expressed constitutively at high level even in the absence of nitrite, suggesting that NsrR represses both norB and dnrN, the repression being lifted upon exposure to reactive nitrogen species.

Growth Response of the NsrR Mutant to Sudden Exposure to Toxic Concentrations of Nitrite—The growth phenotype of the nsrR strain JCGC751 provided preliminary evidence that NsrR plays a critical role in NO homeostasis. Although there was no difference in growth phenotype between the mutant and the parental strain during oxygen-limited growth in the presence or absence of nitrite, opposite responses were seen when uninduced cultures were suddenly exposed to a high concentration
Regulation of Denitrification in N. gonorrhoeae

NsrR Responds to Nitric Oxide Rather than Nitrite—NsrR proteins in other bacteria have been shown to regulate gene expression in response to reactive nitrogen species. Although both nitrite and nitric oxide have been suggested to act as the ligand to NsrR, the exact signal to which NsrR responds is unknown (10–12). To determine whether N. gonorrhoeae NsrR responds to nitric oxide, oxygen-limited cultures of strains F62, JCGC701 (fnr) and JCGC751 (nsrR), were grown without nitrite to an 

\[ A_{650} \] of between 0.3 and 0.4, and the shaker speed was decreased to 50 rpm to decrease the oxygen concentration in the cultures. After 20 min, nitric oxide-saturated water was added to half of the cultures to a final NO concentration of 10 \( \mu M \). An equal volume of sterile water was added to the control cultures. After 20 min, RNA was isolated from the cultures and reverse-transcribed to cDNA, and the quantities of ania, norB, and dnrN transcripts were determined by quantitative real-time PCR (Fig. 5, A–C). Because nitric oxide can react with oxygen to produce nitrite, a qualitative nitrite assay was used to show that no nitrite was present in NO-shocked cultures of the parental strain and the nsrR mutant. A trace of nitrite was detected in cultures of the fnr mutant. This indicates that, although some nitrite is likely to be generated during treatment with NO, it is immediately reduced to NO by AniA in the parental and nsrR strains. Because the fnr strain is unable to synthesize AniA, any nitrite generated chemically would accumulate in the growth medium. The norB and dnrN promoters were induced by NO in an NsrR-dependent manner. The ania promoter was NsrR-repressed but not NO-induced, possibly because FNR was inactivated by NO. However, as in cultures grown in the presence and absence of nitrite, ania, norB, and dnrN were expressed constitutively in the nsrR strain at a high level even in the absence of NO. Whereas ania expression was FNR-dependent, neither norB nor dnrN expression in the presence of NO was affected by an fnr mutation, confirming that NsrR is the primary regulator of the norB and dnrN promoters.

Furthermore, this confirms that differences in transcript levels were not simply due to differences in growth rate.

To confirm that NsrR responds to nitric oxide rather than nitrite generated chemically during treatment of cultures with NO, the response of norB expression to nitrite and nitric oxide in a parental strain, F62, and its ania mutant, JCGC703, were compared. Both strains were grown in the absence of nitrite to an 

\[ A_{650} \] of 0.4 and pulsed with either NO-saturated water, or sterile water. RNA was isolated, and quantitative real-time PCR was used to assay norB expression (Fig. 5D). In both the ania- and ania strains, NO induced expression of norB. Strains F62

FIGURE 4. The nsrR mutant is more resistant to toxic concentrations of nitrite than the parental strain. The optical density at 650 nm was measured at hourly intervals of oxygen-limited cultures of N. gonorrhoeae strains F62 (A), JCGC751 (nsrR, B) and JCGC701 (fnr, C) grown in liquid medium without nitrite (gray diamonds, dashed line), with nitrite (black diamonds, solid line, 1 mM NaNO2 added after 1 h of growth and 4 mM NaNO2, after 2 h) or without nitrite followed by a nitrite shock (white diamonds, dotted line; 5 mM of NaNO2 added at the indicated point). Error bars show standard deviation of duplicate cultures.

of nitrite (Fig. 4, A and B). When oxygen-limited cultures were first grown in the absence of nitrite to an 

\[ A_{650} \] of 0.4, the addition of 5 mM nitrite completely inhibited growth of the wild-type strain but enhanced the growth rate of the nsrR mutant. This difference was most probably due to the sudden production of nitric oxide. In the absence of nitrite, the parental strain would synthesize nitrite reductase AniA but not NO reductase NorB, so upon addition of nitrite, nitric oxide would accumulate and inhibit growth. In contrast, constitutive synthesis of NorB by the nsrR strain would prevent the accumulation of nitric oxide, and hence its growth would not be inhibited.

Expression of ania is totally dependent upon FNR (5): an fnr mutant is therefore unable to generate NO on addition of nitrite and provides a method to determine whether growth inhibition of the parental strain is due to NO rather than nitrite. To confirm that growth inhibition was due to NO rather than nitrite, oxygen-limited cultures of an fnr mutant were also grown in the presence and absence of nitrite, and uninduced cultures were pulsed with 5 mM nitrite when oxygen became growth-limiting. Growth of the fnr mutant was neither enhanced nor inhibited by nitrite irrespective of the stage of growth at which it was added to the culture (Fig. 4C). This confirmed that NO generated by the activity of AniA is responsible for the growth inhibition when nitrite is suddenly added to cultures of N. gonorrhoeae.

Addition of 5 mM nitrite completely inhibited growth of the nitrite than the parental strain.
Regulation of Denitrification in N. gonorrhoeae

FIGURE 5. Regulation of NsrR-repressed promoters in the presence of nitric oxide. A–C, oxygen-limited cultures of strains F62, JCGC701 (fnr) and JCGC751 (nsrR) were grown in the absence of nitrite to an A650 of between 0.3 and 0.4. The shaker speed was decreased to 50 rpm to decrease the oxygen supply. After 20 min, cultures were treated with either NO-saturated water resulting in an NO concentration of 10 μM (NO-treated, gray bars) or an equal volume of sterile water (untreated, white bars). After 10 min, samples were taken, RNA was extracted and quantitative real-time PCR was used to measure the level of aniA (A), norB (B), and dnrN (C) expression. Note that the y-axes of each graph are different and that expression of norB is induced by exposure to NO to a greater extent than dnrN and aniA. D and E, NsrR responds to NO rather than nitrite. Oxygen-limited cultures of strains F62 (aniA−) and JCGC703 (aniA+) were grown in the absence of nitrite (D), then treated with either NO-saturated water or sterile water, and RNA was extracted after 10 min as described above. Strains F62 and JCGC703 were also grown in the presence and absence of 5 mM nitrite, and RNA was extracted during exponential growth (E). Expression of norB was measured using quantitative real-time PCR.

and JCGC703 were also grown in the presence and absence of nitrite, RNA was isolated during exponential growth, and quantitative real-time PCR again used to measure the amount of norB transcript (Fig. 5E). In the aniA− strain, as observed previously, nitrite induced expression of norB. However, in the aniA mutant that does not express nitrite reductase and so cannot reduce nitrite to nitric oxide, nitrite did not induce norB expression. It is clear from these data that nitric oxide, not nitrite, is the reactive nitrogen species sensed by NsrR in the gonococcus.

DISCUSSION

Contrasts between the N. gonorrhoeae and E. coli NarP Regulons—Once growth rate-dependent transcripts for genes such as those for ribosomal proteins had been deleted from the list of transcripts differentially expressed in the narP mutant and its parent, it became apparent that the gonococcal NarP regulon is extremely small compared with the NarP regulon of E. coli (30). Inverted repeat sequences similar to the binding site for E. coli NarP were readily identified upstream of five genes that fulfilled the statistical tests used to be considered to be NarP-regulated, and binding of FLAG-tagged NarP upstream of these genes was confirmed by ChIP. Although there is only one credible NarP-binding site in the intergenic region between the divergently transcribed aniA and norB genes, expression of both of these genes was stimulated by NarP. It is therefore likely that increased expression of norB by NarP is an indirect effect of the increased expression of AniA, which would result in increased production of NO, the signal to which norB transcription responds (Fig. 5) (5). Two of the three transcripts that were repressed by NarP encode conserved hypothetical proteins of unknown function. NGO1215 encodes a putative periplasmic protein that is present in many other bacteria, suggesting that this family of proteins might be important during survival in microaerobic environments. The third transcript repressed by NarP was narQ, so expression of gonococcal narQ-narP is autoregulated by feedback repression, as has been found for many other transcription factors.

A Key Role for NsrR in Protection of Gonococci from Nitric Oxide—The original identification of NarQP as the possible nitrite sensor in the gonococcus was based upon the observation that deletion of the region of the aniA promoter containing the NarP binding site resulted in loss of nitrite induction (5). However, data in Fig. 1 confirm preliminary results from a previous experiment, which indicated that the gonococcal NarQ-NarP two-component system is not essential for activation of aniA transcription by nitrite (see Table 3 of Ref. 9). This raises two questions. First, what is the alternative mechanism by which gonococci respond to nitrite? Second, to what signal, if any, does the gonococcal NarQ respond?

The first question was definitively answered by showing that NsrR is both essential and sufficient for transcription activation by nitrite at the aniA promoter. The explanation for the earlier discrepancy is that the NarP-binding site deletion also removed the NsrR binding site. The signal to which NsrR responds is the product of nitrite reduction, nitric oxide, rather than nitrite itself, as shown in Fig. 6. Two other promoters, norB and dnrN, were also repressed by NsrR but were derepressed in the presence of nitric oxide. As in other bacteria, these results identify gonococcal NsrR as the central regulator of the reactive nitrogen species response (Fig. 6). NsrR proteins are also related to the IscR family of transcription factors, which regulate the expression of genes involved in iron-sulfur center metabolism (31). IscR contains a [2Fe-2S] center coordinated by cysteine...
Regulation of Denitrification in N. gonorrhoeae

residues thought to sense the Fe-S center assembly status of the bacteria. NsrR also contains conserved cysteine residues and is proposed to contain an iron-sulfur center that senses reactive nitrogen species (11). NsrR homologues in other bacteria have also been shown to respond to other stimuli, such as iron limitation (11). This “cross-sensing” of stimuli might reflect the need for bacterial transcription factors to sense not one but a variety of environmental signals, integrate these “inputs” and regulate gene expression accordingly. Another example of this is FNR, primarily an oxygen sensor but also capable of sensing reactive nitrogen species (and possibly reactive oxygen species) via damage to its iron-sulfur center (32, 33).

NarQP proteins are restricted to members of the β- and γ-proteobacteria, and only three other NarQ family proteins have been characterized: NarQ and NarX in E. coli; and NarX in Pseudomonas stutzeri (34). The two E. coli proteins sense nitrate and nitrite and regulate expression of a large number of genes; the Pseudomonas protein is thought to sense nitrate and regulate expression of a nitrate reductase. It is entirely possible that gonococcal NarQ responds to a ligand that was present during all of the assays in the current study. If so, the growth requirements of gonococci and their sensitivity to stress imposed by changes in growth environment might make identification of such a ligand difficult. We believe an alternative explanation to be more likely, namely that the gonococcal NarQ is a ligand-insensitive kinase locked in the phosphorylation mode, as indicated by the domain swap and functional studies using the E. coli frrd promoter as a model system (Table 4).

Implications for Pathogenesis—Previous studies have identified possible links between anaerobiosis and gonococcal pathogenesis. Anaerobically grown gonococci are more resistant to anaerobiosis and can exploit host defense mechanisms for their survival, for example, the ability to sialylate their lipid-oligosaccharide via host-derived CMP-N-acetylneuraminic acid (36, 37). Induction of norB by NO generated by the host would provide a potential source of energy during oxygen-limited growth, enhancing gonococcal survival and growth even during oxygen starvation.

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FIGURE 6. The truncated denitrification pathway of N. gonorrhoeae and its regulation by FNR, NarQ-NarP, and NsrR. Plus and minus signs indicate transcription activation or repression by the transcription factor shown. NosZ and nosZ indicate that there is a frameshift mutation in the nosZ gene, so nitrous oxide is the end product of gonococcal nitrite reduction.
Regulation of Denitrification in *N. gonorrhoeae*

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