**INTRODUCTION**

*Neisseria gonorrhoeae*, a Gram-negative diplococcus, is the causative agent of the sexually transmitted disease gonorrhea, one of the most prevalent diseases in the world. In the USA alone over 330 000 new cases of gonorrhoea were reported in 2004, and antimicrobial resistance remains an important consideration in treatment (http://www.cdc.gov). Infection may cause several complications, including pelvic inflammatory disease (PID), a leading cause of female sterility, and disseminated gonococcal infection (DGI), which can lead to dermatitis and migratory polyarthritis. Prompt identification and treatment are essential to prevent permanent host damage; however, the initial mucosal infection in gonococcal disease is often asymptomatic, hampering effective control (Garnett et al., 1999).

*N. gonorrhoeae* is a facultative anaerobe (Knapp & Clark, 1984) that possesses a copper-containing nitrate reductase, AniA, in its outer membrane that is only expressed under anaerobic conditions (Householder et al., 2000). Anaerobically grown gonococci both induce and repress the expression of several outer-membrane proteins, and antibody to AniA is found in sera from women with gonococcal infections, demonstrating that this protein is expressed *in vivo* and that anaerobic growth is a physiologically significant state for this organism (Clark et al., 1987, 1988). We have previously reported that *aniA* is regulated by a gonococcal homologue of fumarate nitrate reductase regulator (FNR) in response to anaerobiosis and by NarP in response to nitrite (Householder et al., 1999; Lissenden et al., 2000). However, new research suggests...
that NarP does not directly sense nitrite; rather its activation function is blocked by the presence of NsrR, a repressor that binds downstream of NarP and is induced by nitric oxide (NO) (Overton et al., 2006; Rock et al., 2007). We have also identified another gene, norB (norZ), which encodes a single-component nitrite reductase that is induced by NO (Householder et al., 2000). NsrR has also recently been shown to play a role in regulation of norB (Overton et al., 2006; Rock et al., 2007). Both AniA and NorB are essential for anaerobic growth (Householder et al., 1999, 2000). The gonococcus is thus capable of anaerobic respiration using nitrite (NO$_2^-$) or NO as a terminal electron acceptor.

NO is toxic to bacterial cells through its reaction with molecular oxygen, superoxide radicals, or the metal centres in various enzymes (Davis et al., 2001). In mice and humans, NO has also been shown to be an important modulator of the cellular events that form the immune response. Low basal levels of NO are anti-inflammatory, while high levels of NO, generated by inducible nitric oxide synthase (iNOS), are pro-inflammatory (Stefano et al., 2007; Tunbridge et al., 2007). The role of NO metabolism during gonococcal infection is largely unknown, but the reduction of host-produced NO to anti-inflammatory levels could contribute to asymptomatic infections by this organism in women (Cardinale & Clark, 2005).

A comprehensive understanding of NO metabolism in N. gonorrhoeae requires that the complete mechanism of norB regulation be elucidated. We have previously demonstrated that the gonococcal norB gene is not regulated by FNR or NarP (Householder et al., 2000), as is in other denitrifiers (Rinaldo et al., 2006), and that it is induced by NO. Aside from NsrR regulation of norB (Overton et al., 2006; Rock et al., 2007), it has also been suggested that the ferric uptake regulator (Fur) protein is responsible for direct transcriptional activation of norB in N. meningitidis, despite the fact that Fur is generally thought to act as a repressor (Delany et al., 2004).

We present here an analysis of the nucleotide sequence of the region upstream of norB to characterize elements involved in transcription and regulation. We confirm and further develop recent work showing that nsoR, a gene encoding an Rrf2-type transcriptional repressor in the winged-helix superfamily, controls norB regulation in N. gonorrhoeae (Overton et al., 2006). We also show that NsR senses NO directly. Furthermore, we show that Fur is not involved in the direct regulation of norB, but rather its role in controlling norB expression is through a novel mechanism of indirect activation. Fur competes for binding to its operator with a gonococcal ArsR homologue, a repressor protein with an overlapping binding site that inhibits norB transcription in the absence of bound Fur.

**METHODS**

**Growth of gonococcal strains.** All gonococcal strains were derived from strain F62 (Table 1) and were grown on Difco GC medium base (Becton Dickinson) plates with 1% Kellogg’s supplement (GCK) (Kellogg et al., 1963). When necessary, chloramphenicol, kanamycin or erythromycin was added at 1, 50 or 2 μg ml$^{-1}$, respectively. Aerobic plate cultures were grown in a 37 °C incubator supplying 5% CO$_2$. Anaerobic cultures were incubated in a Coy anaerobic chamber (Coy Laboratory Products) at 37 °C for 20 h in an atmosphere of 85% N$_2$, 10% H$_2$ and 5% CO$_2$. Nitrite was provided for anaerobic cultures by placing 40 μl of 20% (w/v) NaNO$_2$ solution on a sterile cellulose disk in the centre of a plate inoculated for confluent growth (Knapp & Clark, 1984). Cultures capable of responding to nitrite grow in a characteristic halo around the nitrite disk.

**PCR.** Genomic DNA from gonococcal strain F62 was isolated for use as a PCR template. Promoter sequences for lacZ fusions and genes amplified for insertion inactivation or complementation were amplified with either iProof high-fidelity polymerase (Bio-Rad) or the GC-rich PCR system (Roche). Clones were screened by PCR for the presence and orientation of the insert using AmpliTaq (Applied Biosystems). Primer sequences used for all constructs are available from the authors upon request.

**Construction of lacZ fusions.** Deletions and mutations of the norB upstream region were created by PCR. Translational lacZ fusions were constructed with pLES94 (Silver & Clark, 1995). Genomic DNA from F62 was used as the template. PCR fragments were cut with BamHI. Digested insert and plasmid were ligated and cloned into Escherichia coli MC1061. Transformants were selected on LB medium plates containing chloramphenicol (25 μg ml$^{-1}$) and X-Gal (40 μg ml$^{-1}$; Invitrogen). Plasmids were checked for the presence and orientation of the insert by PCR, and those plasmids that contained an insert in the correct orientation were used to transform F62. Colony PCR was performed on chloramphenicol-resistant colonies to confirm the presence of the reporter construct. The PCR product was also sequenced to ensure that the appropriate fusion had been made.

**Construction of mutants.** RUG7600 was constructed by deleting 226 bp of nsoR and inserting an erm resistance cassette within the gene. Two fragments were amplified using GC-Rich Taq (Roche). The 5’ fragment began 378 bp upstream from the nsoR start site and included 102 bp of the coding region, with a PstI restriction site on the 3’ end. The second fragment had an XhoI restriction site on the 5’ end, and began 328 bp into the gene and ended 495 bp downstream of the TAA stop codon. The two fragments, 490 bp (PstI-cut) and 616 bp (XhoI-cut), were ligated with a complementarily digested erm resistance cassette and transformed directly into RUG7500. Clones were verified by colony PCR. The arsR mutant was constructed in a similar fashion, by deleting 162 bp of the gene and inserting an aph (kanamycin) resistance cassette. The 5’ fragment began 704 bp upstream of the arsR start site and included 97 bp of the coding region, with a HindIII restriction site on the 3’ end. The second fragment had an XhoI site on the 5’ end, and began 251 bp into the gene and ended 924 bp downstream of the TAG stop codon. The fragments were cut with HindIII or XhoI, and ligated with a complementarily digested aph resistance cassette and transformed into RUG7526. The gonococcal fur mutant was constructed as previously described for the generation of the meningococcal fur mutant (Delany et al., 2004).
### Table 1. Plasmids and bacterial strains used in this study

<table>
<thead>
<tr>
<th>Plasmid or strain</th>
<th>Relevant genotype or properties</th>
<th>Source or reference</th>
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<tr>
<td><strong>Plasmids</strong></td>
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<tr>
<td>pEXT22</td>
<td>Low-copy-number expression vector (1.5 per cell); Km'</td>
<td>Dykxhoorn et al. (1996)</td>
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<td>Promoterless lacZ vector; Ap', Cm'</td>
<td>Silver &amp; Clark (1995)</td>
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<td>pLES94/full norB upstream from −150 to +9</td>
<td>Householder et al. (1999)</td>
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<td>pKB1</td>
<td>pEF1 extended −10 changed from TG to GT</td>
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<td>pLES94/aniA upstream, deleted narP binding site</td>
<td>Householder et al. (1999)</td>
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<td>pVI1</td>
<td>pEXT22/norB upstream fused to lacZ</td>
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<td>pVI8</td>
<td>pLES94/norB::aniA::lacZ hybrid promoter</td>
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<td>pVI19</td>
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<td>pLES94/norB upstream from −131 to +9</td>
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<td>pWL48</td>
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<tr>
<td>RUG7726</td>
<td>F62 arsR::kan transformed with pVI26</td>
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**Complementation of the ΔnsrR mutant.** To complement the ΔnsrR mutation, RUG7605 was created by inserting a copy of nsrR into the nosX pseudogene. The nsrR gene was amplified from 415 bp upstream of the ATG start site to 174 bp downstream of the TAA stop codon (to preserve the gonococcal uptake signal sequence, 5'-GCCGCTCTGAA-3'). The fragment contained BamHI and EcoRI sites on the 5' and 3' ends, respectively. The fragment was digested and a ligation was performed with this fragment, a 550 bp amplified fragment containing the upstream sequence and 5' end of the coding sequence of the nosX gene digested with BamHI, and a 2.2 kb fragment containing a kanamycin resistance cassette along with the 3' coding region and downstream sequence of nosX digested with EcoRI. Gonococcal strain F62 was transformed with this ligation mix, and construction of the complemented mutant was confirmed by PCR.

**Gonococcal transformation.** A light suspension of type I cells (Kellogg et al., 1963) was prepared in 1 ml GCK broth containing 0.042 % NaHCO₃ and 10 mM MgCl₂. Purified plasmid DNA or ligation mixture was added, and 100 μl of the suspension was plated onto two GCK plates that were incubated right side up for between 6 and 9 h at 37 °C. Cells were then harvested from the plates and streaked on GCK plates containing the appropriate antibiotic for selection of clones.

**β-Galactosidase assays.** Gene reporter activity was determined by β-galactosidase assays from cultures grown under various conditions (Miller, 1972). For gonococcal cultures, sterile swabs were used to harvest cells from overnight plate cultures, and cells were resuspended in Z buffer (Miller, 1972). When cells were grown with nitrite disks, only the halo of gonococcal growth around the nitrite disk was used (Householder et al., 1999). Cells were lysed with chloroform and 0.1 % SDS and assayed as described elsewhere (Miller, 1972). Activity was reported in Miller units and the results were reported as the average of at least three assays performed in duplicate from each day the cultures were grown. For E. coli, overnight broth cultures were resuspended to OD₆₀₀ ~0.05 in LB containing kanamycin (50 μg ml⁻¹) to which 15 mM diethylenetriamine/nitric oxide adduct (DETA/NO; Sigma) was added as indicated, and cultures were grown to OD₆₀₀ ~0.5. Cells were spun down and resuspended in Z buffer, and β-galactosidase activity was measured as described above.

**Nitric oxide reductase (Nor) assays.** Aerobic and anaerobic Nor activity of F62, the ΔnsrR knockout and the nsrR-complemented mutant were measured as previously described (Cardinale & Clark, 2005).

**Repression by gonococcal NsrR in E. coli.** Plasmid pEXT22 (Dykshoorn et al., 1996) and the norB::lacZ fusion fragment from RUG7500 (Fig. 1) were digested with XbaI and HindIII and ligated. The construct (pVI1) was transformed into E. coli strain MC1061 and selected on LB plates containing kanamycin (50 μg ml⁻¹) and X-Gal (40 μg ml⁻¹). Following overnight incubation at 37 °C, plasmids were extracted from blue colonies and screened by PCR for the presence of the insert. Plasmid pEXT22 was also digested with BamHI and SstI, and the nsrR insert (obtained by PCR from genomic F62 DNA) was digested with BglII and SstI, ligated, and transformed into E. coli MC1061 and selected on LB plates containing kanamycin (50 μg ml⁻¹). The norB::lacZ fusion was then cloned into the XbaI and HindIII sites of this construct as described above, making pV118. Chemically competent MC1061 was transformed with either pVI1 or pV118 and selected on LB plates containing kanamycin (50 μg ml⁻¹). Both norB::lacZ and nsrR were under the control of their own promoters.

**Isolation of DNA-binding protein by affinity chromatography.** The protein that bound to the conserved motif upstream of the −35 sequence (Fig. 1a) was isolated by a method based on that described for IscR, with several modifications (Yeo et al., 2006). The putative repressor binding site was amplified by PCR from RUG7526 (Fig. 1a) using an upstream primer located in the proline region and a downstream primer ending at the −35 sequence, making a 100 bp fragment. This fragment was cut with BamHI and cloned into pLE594. A clone containing a plasmid with two repeats of the BamHI fragment was selected, and outside primers were used to generate a 320 bp affinity probe by PCR with the downstream primer, complementary to lacZ in the pLE594 backbone, end-labelled with biotin. This 320 bp fragment was purified and incubated with 25 μl streptavidin-coated agarose beads (Pierce) in Tris-buffered saline (TBS; 50 mM Tris, 150 mM NaCl, pH 7.4) with rotation for 1 h at room temperature. The beads were then washed with TBS to remove unbound DNA, according to the manufacturer’s instructions.

Gonococcal cultures (100 ml) of strain F62 were grown aerobically in a shaking incubator at 37 °C in GCK broth to OD₆₀₀ 0.5. Cells were spun down, resuspended in 5 ml TBS, and lysed by sonication (Branson 150D) to shear the genomic DNA into approximately 0.5 kb pieces. Lysates were incubated with the DNA-coated streptavidin beads for 1.5 h with rotation at 4 °C. The beads were washed four times with 5 ml TBS to remove non-specific proteins, resuspended in 25 μl SDS–PAGE running buffer (Bio-Rad), and incubated in boiling water for 10 min. The eluates were recovered and analysed on SDS–PAGE gels.

**Site-specific mutagenesis of NsrR binding sites.** Splice overlap extension (SOE) PCR (Ho et al., 1989) was used to change the 29 bp inverted repeat sequence of the putative norB NsrR binding site in strain F62 to match the consensus NsrR binding sites from other gonococcal genes. The primers used to generate the norB::lacZ fusion in strain RUG7500 were used as outside primers in the PCR reaction. Fragments were cut with BamHI, and cloned as described above for the construction of lacZ fusions.

**Oligonucleotide and DNA sequencing.** All synthesized oligonucleotides were obtained from Invitrogen, and confirmatory DNA sequencing was performed at ACGT (Wheeling, IL).

**Molecular biology techniques.** Cloning and PCR techniques were performed in accordance with standard protocols (Ausubel et al., 1987, 1992; Sambrook et al., 1989). Plasmid preparations were obtained with Wizard Plus SV Miniprep kits (Promega). DNA fragments were purified with QIAquick PCR purification or QIAquick gel extraction kits (Qiagen).

**RESULTS**

**Sequence upstream of norB**

A 367 bp intergenic sequence separates the divergently transcribed denitrification genes norB and aniA (Householder et al., 2000). The sequence 150 bp upstream of the norB ATG start codon contains several interesting motifs and was selected for further characterization (Fig. 1a). The existence of a Fur binding motif between positions −75 and −95 relative to the −10 promoter element (positions −130 and −150 relative to the start codon; Fig. 1a) suggests a possible role for Fur in transcriptional activation of gonococcal norB, as has been proposed in N. meningitidis (Delany et al., 2004). The −35 region contains a poor match to the E. coli −35 consensus, while there is a TG dinucleotide 3 bp upstream of the −10
sequence that is typical of "extended −10" promoters that do not require a strong −35 sequence for efficient transcription (Keilty & Rosenberg, 1987). The extra TG dinucleotide stabilizes open complex formation by providing critical contacts with region 3.0 of RNA polymerase (Gruber & Gross, 2003; Murakami & Darst, 2003).

Mutational analysis of the norB promoter

We performed a functional analysis of the norB promoter in order to understand its mode of regulation and to define the transcriptional elements. Translational lacZ fusions were constructed by cloning different segments of the norB

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**Fig. 1.** Sequence of the norB promoter/operator region and β-galactosidase activities of norB::lacZ fusion strains in the wild-type and ΔnsrR mutant in strain F62. (a) Numbers below the sequence refer to nucleotides from the ATG translation start site. The C at position −53 is the norB transcription start site identified in N. meningitidis (Delany et al., 2004), indicated by an asterisk above the sequence, and the putative −10 and −35 sequences are based on this site. The inverted repeat described in the text is underlined, while the putative NsrR binding site predicted by Rodionov et al. (2005) within this repeat is highlighted in light grey. A 6 bp conserved motif present as one copy in the forward and two copies in the inverted orientation is highlighted in dark grey, and this sequence is predicted to bind a gonococcal ArsR homologue. The RBS is indicated by double underlining. The deletions and the site-specific mutations in the norB::lacZ fusions described in the text are indicated under the sequence.

(b) A schematic representation of the upstream region of norB::lacZ constructs described in (a) is shown to the left of the β-galactosidase activities of those strains, in Miller units, determined in cells grown aerobically (+O₂) and cells grown anaerobically in the presence of nitrite (−O₂/−NO₂), as described in Methods. Results are presented as the mean ± SD of six determinations, and the significance of the data is discussed in the text. Strains in the RUG7500 series have a wild-type background and those in the RUG7600 series contain the ΔnsrR insertion/deletion mutation.
upstream sequence into vector pLES94 followed by chromosomal integration into the proAB pseudogenes by homologous recombination, creating a single-copy reporter system (Silver & Clark, 1995). These fusions contained the RBS and first three codons of norB.

First, site-specific mutagenesis was performed on the TG dinucleotide in the extended −10 promoter, changing it from 5′-TGTCTACAAT-3′ to 5′-GTGTCTACAAT-3′ (RUG7513 in Fig. 1). This alteration caused a decrease in promoter activity of approximately 20-fold under aerobic conditions and 80-fold under anaerobic conditions (P<0.005; Fig. 1b), but the promoter was still induced under conditions of NO generation (cells grown anaerobically with nitrite). These results demonstrate the importance of the extended −10 TG dinucleotide in norB expression.

A series of deletions from the 5′ end of the norB promoter were constructed in order to locate sequences involved in regulation (Fig. 1). A deletion eliminating the Fur binding site (RUG7512) had no effect on norB::lacZ activity or NO induction (Fig. 1b). This suggested that the Fur protein is not involved in direct activation of norB. Further deletion from the 5′ end of the upstream region up to the −35 sequence (RUG7531) also had no effect on norB regulation. A deletion that eliminated the −35 element, leaving only the extended −10 sequence (RUG7523), caused an approximately 10-fold reduction in promoter activity (P<0.005), showing that the −35 element is important for expression even with its low level of similarity to the consensus sequence, but it had no effect on induction by NO. These results suggest that norB is not regulated by an activator that binds upstream of the extended −10 element.

To determine whether the region downstream of the extended −10 element was involved in norB regulation, a lacZ fusion was constructed that replaced norB sequence downstream of the −10 promoter element with aniA promoter sequence (RUG7508 in Fig. 1). We have previously demonstrated that the sequence used for this replacement contains no regulatory elements (Householder et al., 1999); rather it merely acts to maintain spacing and provide an RBS. The mean level of aerobic norB::lacZ activity from strain RUG7508 was higher than that observed with RUG7500 grown anaerobically (Fig. 1b), confirming that the norB sequence required for NO induction is found downstream of the extended −10 sequence, that the transcriptional regulator is indeed a repressor, and that norB::lacZ is not fully derepressed during anaerobic growth.

Confirmation of NsrR as a transcriptional repressor

Using Regulatory Sequence Analysis Tools (RSAT) (http://rsat.ulb.ac.be/rsat/), examination of the norB sequence downstream of the extended −10 element revealed a region of dyad symmetry with an imperfect inverted repeat sequence of TTTAAACATTTCTTTTGGTTAATTTTAA (Fig. 1a). This inverted repeat sequence also overlapped with the 19 bp NsrR binding consensus sequence predicted by Rodionov et al. (2005). The lacZ reporter fusion in RUG7508 had the effect of removing the putative NsrR binding site. To determine whether this inverted repeat sequence, including the binding site predicted by Rodionov et al. (2005), was responsible for norB regulation, a lacZ fusion was constructed that replaced the norB sequence with that of aniA downstream of this inverted repeat (RUG7520 in Fig. 1). The reporter fusion in strain RUG7520 restored repression of norB expression and displayed levels of norB::lacZ activity comparable to those of RUG7500 (Fig. 1b). These data show that the DNA sequence involved in norB regulation is, or is contained within, this inverted repeat.

We insertionally inactivated the nsrR gene in N. gonorrhoeae to demonstrate its role in norB regulation (Fig. 2a). The aerobic expression of norB in the ΔnsrR mutant (1542 ± 89 Miller units) was higher than the level expressed under NO-generating conditions in the wild-type strain (1317 ± 56 Miller units; P<0.005). While insertional inactivation of nsrR suggests that it is the norB repressor, the gonococcal genome annotation (http://stdgen.northwestern.edu/) reveals that nsrR is completely contained within another putative ORF, NGO1518, which is transcribed in the opposite direction, and that this ORF would also be inactivated in a ΔnsrR knockout. To complement the ΔnsrR mutation in single-copy in N. gonorrhoeae, nsrR, but not the 5′ and 3′ ends of NGO1518, was used to transform RUG7600. The nsrR gene was inserted into the non-functional nosX gene through homologous recombination (RUG7605, see Methods) and assayed for β-galactosidase activity from the norB::lacZ fusion (Fig. 2a). This complementation restored the wild-type phenotype and repressed the aerobic expression of norB, proving that nsrR and not NGO1518 is responsible for regulating norB expression.

Nitric oxide reductase activity was assayed directly in strain F62 and the ΔnsrR mutant. Activity was determined by addition of the NO donor DEA/NO to gonococcal cultures followed by measuring the rate of NO disappearance. The aerobic nitric oxide reductase activity of the ΔnsrR mutant was found to be 16-fold higher than the corresponding levels in the wild-type strain (Fig. 2b). In agreement with results of the norB::lacZ fusions, these data further confirm the role of NsrR in norB regulation.

NsrR regulation of norB in E. coli

To confirm that NsrR is involved in direct regulation of norB, we constructed a system that recapitulated its repression of norB in E. coli. The norB::lacZ fusion in RUG7500 was amplified and cloned into the low-copy-number plasmid pEXT22 (Dykxhoorn et al., 1996), to make pVII. The entire nsrR gene, including 415 bp upstream of the ATG start codon, was cloned upstream of norB in pVII to make pVII18. β-Galactosidase activity was determined aerobically in strains carrying pVII1 and

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The presence of nsrR in the strain carrying pVI18 significantly repressed norB expression (1805 ± 267 Miller units in the strain carrying pVI1 vs 174 ± 13 Miller units in the strain carrying pVI18, P < 0.005). Thus NsrR directly regulates norB and does not act through other gonococcal regulatory proteins.

We next confirmed that NO is the signalling molecule responsible for directly inactivating NsrR (Overton et al., 2006; Rock et al., 2007). Expression of norB::lacZ in the strain carrying pVI18 (nsrR) was induced to levels comparable to those of a strain carrying pVI1 by the addition of 15 mM DETA/NO, a long-half-life NO donor (1738 ± 184 Miller units in the strain carrying pVI1 vs 1933 ± 589 Miller units in the strain carrying pVI18). This shows that NsrR is directly responsive to nitric oxide and that norB becomes derepressed in its presence. Derepression of the norB::lacZ fusion could not be achieved by addition of nitrite (data not shown). The addition of NO to the strain containing pVI1 did not increase norB expression levels, indicating that E. coli NsrR did not repress gonococcal norB, confirming the prediction of Rodionov et al. (2005) that the Neisserial NsrR binding site was different from that found in other bacteria. We also supported this by demonstrating that mutation of the E. coli nsrR gene did not affect expression of gonococcal norB in the strain carrying pVI1 (data not shown).

**norB expression in a gonococcal ΔnsrR mutant**

We have shown that NsrR represses norB in *N. gonorrhoeae* when the entire 150 bp upstream of the ATG start site is present. To determine whether there were any other *trans*-acting factors regulating norB that respond to NO or anaerobiosis, the norB::lacZ constructs analysed in wild-type F62 were transformed into a ΔnsrR mutant (all 7600-series constructs in Fig. 1). The mean levels of aerobic β-galactosidase activity from these constructs were high and most exceeded the anaerobic expression level observed in a wild-type background (Fig. 1b). The lower norB expression when the −35 consensus or the extended −10 dinucleotide was deleted or mutated in the ΔnsrR mutant (RUG7623 and RUG7613, respectively) can be attributed to a decrease in promoter strength rather than to lack of regulation. A comparison of the results from RUG7508 and RUG7600 showed nearly identical levels of both aerobic and anaerobic expression in the two strains, demonstrating that the elimination of the repressor binding site (RUG7508) has the same effect on norB expression as the inactivation of the repressor (RUG7600).

**Indirect regulation of norB by Fur**

Elimination of the Fur binding site in RUG7512 suggested that Fur had no role in the regulation of norB, despite several reports to the contrary in *N. gonorrhoeae* (Sebastian et al., 2002) and *N. meningitidis* (Grifantini et al., 2003, 2004; Delany et al., 2004). We therefore investigated the expression of norB::lacZ fusions in a Δfur mutant (Fig. 3). While deletion of the Fur Box had no apparent effect on norB expression, a Δfur mutant in reporter strain RUG7500 (RUG7550) resulted in a 60% reduction in anaerobic expression (P < 0.005). This effect was independent of nsrR, as expression in a norB::lacZ fusion strain in a ΔnsrR Δfur double mutant (RUG7650) and in the norB::lacZ fusion
strain missing the NsrR binding site in a Δfur mutant (RUG7558) was decreased by 65 and 72%, respectively (P<0.005). In reporter strains with deletions of the upstream region of the norB promoter that eliminated the Fur box (RUG7512 and RUG7531), the effect of the fur mutation (RUG7562 and RUG7581) was to increase norB expression by 41 and 36%, respectively, suggesting that the fur mutation had indirect effects. The loss of apparent activation by Fur suggested that there was a repressor that binds in the upstream region of the norB promoter whose effect was decreased by Fur binding. There are three copies of a 6 bp conserved motif in the norB upstream region that partially overlaps the Fur box (Fig. 1a). Since the fusion in RUG7512 lacks part of this repeat, we constructed a norB::lacZ fusion that deleted all but 2 bp of the Fur box and retained this inverted repeat (RUG7526 in Fig. 1a). The anaerobic expression of this fusion was only 18% of that observed with RUG7500 (P<0.005), and the Δfur mutation had no effect. These results suggest that norB is regulated by a repressor that binds upstream of the promoter and presumably tethers the RNA polymerase. The binding site for this repressor partially overlaps the Fur binding site, and the apparent activation of norB expression by Fur may be due to its inhibition of binding of this repressor. This would be a novel mechanism for indirect activation by Fur, by which Fur competes with a second repressor for binding to the norB upstream region.

Identification of an ArsR-like repressor of norB

In an attempt to isolate the putative repressor of norB that binds between the −35 promoter and the Fur binding site, a fragment containing the three inverted repeat sequences located in that region (Fig. 1a) was amplified with a biotin end-labelled primer and used to coat streptavidin beads (see Methods). After incubation in a gonococcal lysate, the eluate recovered from DNA-coated beads was analysed by SDS-PAGE. A negative control was run in parallel, which consisted of streptavidin beads coated with biotin-labelled non-specific DNA. Only one enriched protein was found in the eluate from beads containing the inverted repeats compared to the negative control (gel not shown). The enriched band was approximately 10.5 kDa, and comparison to a list of annotated gonococcal proteins with helix-turn-helix motifs revealed only one protein of that size. NGO1562 encodes an arsR family transcriptional regulator with a predicted molecular mass of 10.6 kDa. Genome-wide RSAT analysis (http://rsat.ulb.ac.be/rsat/) of the gonococcal intergenic regions was performed with query CATATAnnTATTG, the first two inverted repeats in Fig. 1(a). This analysis revealed one other hit, upstream of NGO1411, which encodes a conserved protein homologous to an ArsB efflux pump. These two facts, along with the high degree of similarity between this putative binding site and the E. coli chromosomal ArsR binding site (Xu et al., 1996), suggested that this ArsR-like protein may bind to the norB upstream region. The 10.6 kDa gonococcal arsR homologue was insertionally inactivated in strain RUG7526, generating RUG7726 (Fig. 1a). This mutation alleviated the observed repression of norB::lacZ in RUG7526, and restored high-level expression of norB::lacZ under anaerobic conditions in the presence of nitrite (Fig. 4), confirming that this ArsR-like protein represses norB in the absence of bound Fur. A second arsR family transcriptional regulator exists in the gonococcal genome (NGO1185), but insertional inactivation of this
gene in strain RUG7526 had no effect on norB:: lacZ activity (data not shown).

NsrR regulation of aniA and dnrN

Overton et al. (2006) demonstrated regulation of aniA and dnrN under microaerobic conditions by using an aniA:: lacZ fusion and quantitative real-time PCR, respectively. We confirmed the regulation of these genes by NsrR in anaerobically grown cells using promoter:: lacZ fusions (Fig. 5). The effect of the ΔnsrR mutation in the aniA:: lacZ reporter strain RUG7001 (RUG7601) was to increase aniA expression in both the absence and presence of nitrite (P < 0.005). This effect was also seen in the ΔnsrR mutant of reporter strain RUG7045 (RUG7643) which lacked the NarP binding site. Thus, we confirm the findings of Overton et al. (2006) that the nitrite enhancement of aniA expression is due to derepression of NsrR rather than nitrite stimulation of the NarQP two-component system. There was a slightly higher mean level of expression of aniA:: lacZ in the ΔnsrR mutant in the absence of nitrite versus its presence, which is probably due to partial inhibition of FNR by NO (Cruz-Ramos et al., 2002). The nearly absent level of aerobic aniA:: lacZ expression in the ΔnsrR mutant also confirms the requirement for FNR for aniA transcription (Householder et al., 1999).

The gonococcal dnrN gene is homologous to the ytfE gene of E. coli and the norA gene of Ralstonia eutropha. The mechanism of action of the ytfE gene product is unknown; however, it has been shown to be regulated by the E. coli NsrR homologue (Bodenmiller & Spiro, 2006; Overton et al., 2006). E. coli strains with a mutation in the ytfE gene demonstrate increased sensitivity to NO (Justino et al., 2005; Bodenmiller & Spiro, 2006). It has also been suggested that YtfE may play an important role in the biogenesis and repair of stress-damaged Fe–S clusters (Justino et al., 2006, 2007). In R. eutropha, norA is believed to be a cytoplasmic NO-binding protein that regulates gene transcription by lowering the free cytoplasmic concentration of NO (Strube et al., 2007). We confirmed that dnrN is regulated by NsrR, as its aerobic expression increased 11-fold in the ΔnsrR mutant (Fig. 5b). However, the almost fourfold higher expression of the dnrN:: lacZ fusion in the ΔnsrR mutant when grown anaerobically rather than aerobically suggests that other transcription factors may also control this gene.

We constructed an nsrR:: lacZ promoter fusion and transformed it into the wild-type and ΔnsrR mutant to determine whether nsrR is autoregulated. Aerobic β-galactosidase activities of the fusion in the ΔnsrR mutant were 95-fold higher than those observed in the wild-type (Fig. 5c), indicating that the nsrR gene is indeed regulated by NsrR.

Analysis of the NsrR binding site

Rodionov et al. (2005) predicted a 19 bp consensus sequence to be involved in NsrR binding, though the deletion analysis we used to examine the F62 norB upstream region identified a larger, imperfect inverted repeat sequence spanning 29 bp and completely overlapping the site predicted by Rodionov et al. (2005), extending it by 5 bp on each side. In order to further characterize the NsrR binding site and thus determine the bases likely to be important in protein–DNA interaction, we analysed the 29 bp putative NsrR binding sites from genes known to be regulated by NsrR (dnrN, aniA and norR), as well as narQP, which has been shown to have a site containing sequence homology to that of the other NsrR binding sites, but not to be regulated by NsrR (Overton et al., 2006). Site-directed mutagenesis was used to clone these putative NsrR binding motifs into the norB:: lacZ fusion, replacing the wild-type NsrR binding sequence and allowing for determination of the strength of the different binding sites by measuring β-galactosidase levels in the context of the norB promoter (Fig. 6). The putative NsrR binding site from the norB upstream region in strain FA1090 had the best match to an inverted repeat sequence and also displayed the lowest anaerobic norB:: lacZ activity, presumably because the presence of the near perfect inverted repeat was ideal for high-strength interaction between NsrR and the DNA. The NsrR binding site from F62 norB contains a single base pair change from that of FA1090 at the second base (T→A), and this single change, making the inverted repeat less perfect, was enough to allow a 28% higher level of anaerobic expression. This suggested that bases outside of the 19 bp consensus predicted by

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**Fig. 4.** β-Galactosidase activities of norB:: lacZ fusion strains in the wild-type and ΔarsR homologue mutant. RUG7500, RUG7526 and RUG7726 (RUG7526; ΔarsR homologue) were grown aerobically (black bars) and anaerobically with nitrite (light-grey bars) as described in Methods, and β-galactosidase was measured. The mutation in the arsR homologue alleviates the observed repression in RUG7726. Results are presented as the mean + SD of six determinations. **Highly significant difference (P < 0.005) from the anaerobically grown parental strain F62.**
Rodionov et al. (2005) were important for binding. The *dnrN* NsrR binding site particularly lacked similarity to the *norB* FA1090 inverted repeat sequence at the upstream end, with a few other altered bases in the central region and the downstream end. The *aniA* NsrR binding site displayed less similarity to the *norB* FA1090 inverted repeat sequence at the downstream end, with a few altered bases towards the upstream end. These NsrR binding sites were not as capable as the FA1090 *norB* or F62 *norB* NsrR binding sites of repressing *norB* expression aerobically and also displayed higher levels of anaerobic expression, probably due to a lower strength of binding by NsrR. Interestingly, the *norB*:::lacZ fusion carrying the *narQP* NsrR binding site was observed to be repressed aerobically and induced 10-fold anaerobically, even though *narQP* does not appear to be regulated by NsrR. The NsrR site from the *nsrR* gene showed the lowest similarity to that of the *norB* FA1090 inverted repeat sequence, but still retained some ability to repress *norB* expression aerobically and to become induced anaerobically, though only by approximately fourfold.

**DISCUSSION**

In this paper we have confirmed the results of Overton et al. (2006) and Rock et al. (2007) and the prediction of Rodionov et al. (2005) that the gonococcal nitric oxide reductase gene *norB* is repressed by NsrR and that this repression is lifted in the presence of NO. We have extended the results of these investigators by demonstrating that the putative NsrR binding site is contained within the 29 bp inverted repeat found directly downstream of an extended −10 promoter. Complementation of the gonococcal *nsrR* in single copy in *trans* restores anaerobic expression of the *norB* gene.
Fig. 6. Alignment of the putative NsrR binding sites of NsrR-regulated genes. The 29 bp inverted repeat was aligned with the upstream regions from aniA, dnrN, nsrR and narQP using Multalin (http://bioinfo.genopole-toulouse.prd.fr/multalin/). Bases conserved in all putative NsrR binding sites are highlighted in dark grey and bases conserved with respect to the FA1090 norB NsrR binding site are highlighted in light grey. Asterisks above the sequence represent bases making up an inverted repeat in the FA1090 NsrR binding site. In strain F62, the 29 bp wild-type NsrR binding site in a norB::lacZ fusion was replaced with the norBFA1090, aniA, dnrN, nsrR or narQP NsrR binding site using site-directed mutagenesis, and β-galactosidase activity was measured. The fold induction presented is the ratio of β-galactosidase activity (−O2/NO2): +O2 within each strain. Results are presented as the mean ± SD of four determinations.

regulation of both norB transcription and nitric oxide reductase activity. Gonococcal NsrR regulation of norB by NO can be reconstituted in E. coli. We have also determined that nsrR is autoregulated. Finally, we have identified a novel mechanism for indirect regulation of norB by Fur. Fur indirectly activates norB by preventing the binding of another repressor, an ArsR homologue, whose putative binding site partially overlaps the Fur binding site.

We present a model for anaerobic regulation of aniA and norB (Fig. 7). When gonococci are aerobically grown, there are only basal levels of expression of aniA and norB due to the inactivity of FNR in the presence of oxygen and the activity of NsrR in the absence of NO respectively. When cells are shifted to an anaerobic environment in the absence of nitrite, FNR becomes active and aniA is partially induced while norB is still repressed due to the lack of NO. When nitrite is added to the anaerobic cells, nitrite reductase (AniA) reduces nitrite to NO, which in turn inactivates NsrR. This results in high levels of expression of norB and an increase in expression of aniA as NsrR-mediated repression is relieved. The presence of NO also derepresses nsrR, resulting in an increase in NsrR. This explains the lower level of anaerobic norB expression in the wild-type as compared to the nsrR mutant, and raises the possibility that NsrR may act as a detoxifying NO sink. We also propose that while aniA cannot be expressed aerobically due to the absolute requirement for FNR (Householder et al., 1999), norB can be expressed aerobically in the presence of NO, as might be encountered in vivo during infection. The norB gene is subject to a second level of regulation by Fur and a gonococcal ArsR homologue. In the presence of iron, Fur binds to its operator and inhibits the binding of the ArsR-like protein. However, when iron is limiting, Fur is not bound to its operator allowing the ArsR-like protein to bind and repress norB expression.

An alignment of the putative NsrR binding sites from the NsrR-regulated genes, norB, aniA, dnrN and nsrR and from narQP, which has been shown not to be regulated by NsrR (Overton et al., 2006), shows a high degree of similarity. As shown in Fig. 6 we observed that differences in the sequence of NsrR binding sites have a large effect on the ability of NsrR to regulate transcription aerobically or in the presence of NO. Regulation by NsrR allows a dynamic range of expression; the more perfect the repeat, the more tightly NsrR binds. Rodionov et al. (2005) predicted a 19 bp NsrR binding site that would be centred within the 29 bp sequence. This predicted NsrR binding site is not a functional regulatory site as this sequence bound NsrR weakly. Nevertheless, this suggests that NsrR can regulate transcription in the absence of NO. Overton et al. (2006) determined that although narQP contained a putative NsrR binding site, the genes were not regulated by NsrR. We have demonstrated that the putative binding site found in narQP is recognized by NsrR. Presumably it is not functional in regulating the narQP genes because it is positioned such that it does not inhibit either RNA polymerase binding or promoter clearance. We found that nsrR was autoregulated, with a 50-fold increase in expression in an nsrR mutant. However, we found that the sequence of the nsrR upstream region that is similar to that of the norB NsrR binding site is most likely not the functional regulatory site as this sequence bound NsrR weakly. IscR was also found to be autoregulated, and the
IscR binding site in the *iscR* gene had little similarity to the IscR binding sequence found in other genes (Giel *et al.*, 2006).

Fur has been extensively studied as an iron-responsive repressor of gene transcription in both Gram-positive and Gram-negative bacteria. Fur regulates the transcription of genes whose proteins take up and metabolize iron to maintain homeostasis within the cell. Beyond this function, for which its role in regulation was initially defined, Fur has been implicated in the regulation of genes important in oxidative stress and acid resistance, as well as in virulence (Hantke, 2001). Fur acts to repress transcription by binding to a 19 bp consensus sequence (the Fur Box) and blocking RNA polymerase entrance to the promoter or promoter clearance (Ochsner & Vasil, 1996; Escolar *et al.*, 1999; Zheleznova *et al.*, 2000). Though generally thought of as a repressor, there are a few examples showing that Fur acts as an activator of transcription (Escolar *et al.*, 1999; Hantke, 2001). However, this activation function in *E. coli* was later shown to be an indirect result of the repression of an antisense regulatory RNA (Masse & Gottesman, 2002). There have been several reports that *norB* is activated by Fur aerobically (Sebastian *et al.*, 2002; Grifantini *et al.*, 2003, 2004); however, most of the data that support a role for Fur in activation of *norB* are based on demonstrating that Fur can bind to the site. In an *in vitro* transcription assay, Delany *et al.* (2004) provide evidence that Fur may directly activate the meningococcal *norB* promoter, although our *in vivo* translational fusion analysis of the gonococcal *norB* upstream region shows no direct Fur activation (Fig. 1). We present a novel mechanism for indirect activation of *norB* by Fur, by inhibiting the binding of a gonococcal ArsR homologue due to competition for overlapping binding sites.

The ArsR family of transcriptional regulators generally controls the expression of genes required for metal-ion detoxification and efflux (Busenlehner *et al.*, 2003; Pennella & Giedroc, 2005). Unlike Fur, ArsR family proteins bind to their operator regions in the absence of their inducing metal ligands (Busenlehner *et al.*, 2003). The metal ligand presumably coordinated by this gonococcal ArsR homologue is unknown, and the amino acid sequence of the protein shows little similarity to any proposed metal-binding sites in functionally characterized ArsR proteins from other organisms (Busenlehner *et al.*, 2003). The reason *norB* would be subject to an additional level of regulation by metal availability is not understood, but identification of the actual ligand recognized by this regulator is required before hypotheses can be formed. However, it would be expected that this ArsR-like protein plays an important role in regulating *norB* during infection, when iron is limiting and Fur would not always be bound to its operator.

Fig. 7. Schematic representation of *aniA* and *norB* regulation. In this model of regulation of *aniA* and *norB*, ‘+’ indicates activation while ‘−’ indicates repression. The hatched squares represent the promoters of *aniA* and *norB*. See Discussion for details.
It has recently been shown that NsrR controls the expression of NO detoxification systems in several pathogenic and environmental organisms, and that genes in the NsrR regulon contribute to resistance to nitrosative stress (Rodionov et al., 2005; Overton et al., 2006; Filenko et al., 2007; Gilberthorpe et al., 2007; Rock et al., 2007; Rogstam et al., 2007). NsrR also regulates NO metabolism in *N. meningitidis*, and meningococcal reduction of NO produced by activated macrophages has been shown to greatly modulate the resulting cytokine and chemokine response, enhance intracellular survival and inhibit macrophage apoptosis (Stevanin et al., 2005, 2007; Tunbridge et al., 2006). We wish to determine the effect of gonococcal NO metabolism during infection. To do this we will attempt to define more completely the NsrR binding site in the *norB* and *nsrR* promoters to help identify the full gonococcal NsrR regulon. Identification of other genes in addition to *norB*, *ania*, *dnrN* and *nsrR* that are regulated by NsrR in response to NO may not only increase our knowledge of anaerobic respiration in *N. gonorrhoeae* but also enhance our understanding of the response of this organism to nitrosative stress. We are also working to characterize the ArsR-like *norB* regulator by defining its complete binding site and determining its cognate ligand.

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