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cis- and *trans*-Acting Elements Involved in Regulation of *aniA*, the Gene Encoding the Major Anaerobically Induced Outer Membrane Protein in *Neisseria gonorrhoeae*

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AniA (formerly Pan1) is the major anaerobically induced outer membrane protein in *Neisseria gonorrhoeae*. AniA has been shown to be a major antigen in patients with gonococcal disease, and we have been studying its regulation in order to understand the gonococcal response to anaerobiosis and its potential role in virulence. This study presents a genetic analysis of *aniA* regulation. Through deletion analysis of the upstream region, we have determined the minimal promoter region necessary for *aniA* expression. This 130-bp region contains a sigma 70-type promoter and an FNR (fumarate and nitrate reductase regulator protein) binding site, both of which are absolutely required for anaerobic expression. Also located in the minimal promoter region are three T-rich direct repeats and several potential NarP binding sites. This 80-bp region is required for induction by nitrite. By site-directed mutagenesis of promoter sequences, we have determined that the transcription of *aniA* is initiated only from the sigma 70-type promoter. The gearbox promoter, previously believed to be the major promoter, does not appear to be active during anaerobiosis. The gonococcal FNR and NarP homologs are involved in the regulation of *aniA*, and we demonstrate that placing *aniA* under the control of the *tac* promoter compensates for the inability of a gonococcal *fnr* mutant to grow anaerobically.

Neisseria gonorrhoeae, like other pathogenic bacteria, regulates the expression of proteins in response to environmental stimuli. While previously considered to be an obligate aerobe (39), *N. gonorrhoeae* has been shown to grow anaerobically in the laboratory when provided with nitrite as a terminal electron acceptor for anaerobic respiration (24). We are particularly interested in how the gonococcus alters protein expression in response to anaerobiosis. It has been found that at least three gonococcal outer membrane proteins (OMPs) are induced and that at least five OMPs are repressed by anaerobic growth in gonococcal strain F62. AniA (formerly Pan1), the major anaerobically induced OMP, is tightly regulated, and its expression is restricted to anaerobically grown cells (7).

Western blot analyses with sera from patients with gonococcal disease indicated that AniA was a major antigen in patients with both complicated and uncomplicated diseases (8). These results suggested that AniA is expressed in the host and that the gonococcus encounters an anaerobic environment during infection. An antigenically related anaerobically induced OMP was detected in all strains of gonococci tested and in a number of commensal *Neisseria* strains but was poorly expressed in *N. meningitidis* strains (19).

The initial cloning and characterization of the *aniA* gene have been reported (20). In that study, Northern analysis demonstrated the lack of an *aniA* message in aerobically grown cells. The primer extension data from anaerobically grown cells suggested the presence of two RNA transcripts differing in length by only 9 bp. Consistent with this finding, two overlapping corresponding promoter sequences were proposed. The −10 sequence of the promoter for the longer, less abundant message was homologous to the sequence of *Escherichia coli* σ^{70} promoters, while the sequence of the promoter for the shorter, more abundant message shared 11 of 14 bases with the *E. coli* gearbox promoter consensus sequence (20). Gearbox promoters were named for their characteristic of producing a gene product at a rate inversely proportional to the growth rate of the cell. These promoters are induced during the stationary phase in *E. coli*, and some are dependent on σ^s, a stationary-phase sigma factor (1, 2, 25, 40). When *aniA* was initially sequenced, there were no homologous proteins for AniA in the databases; it has since been reported that AniA shares significant identity with copper-containing nitrite reductases (6, 29).

In this paper, we present the nucleotide sequence of the region upstream of the *aniA* gene and an initial characterization of the elements involved in the regulation of *aniA* in strain F62.

(This work was presented in part at the 97th General Meeting of the American Society for Microbiology, Miami Beach, Fla., 4 to 8 May 1997 [33a].)

MATERIALS AND METHODS

Growth of gonococcal strains. All gonococcal strains were derived from strain F62 and were grown on plates containing GC medium base (Difco Laboratories, Detroit, Mich.) with 1% Kellogg's supplement (GCK) (23). When necessary, chloramphenicol was added at 1 μg ml^{-1}, erythromycin was added at 2 μg ml^{-1}, or kanamycin was added at 40 μg ml^{-1}. Aerobic cultures were grown at 37°C in a 5% CO_2 incubator. Anaerobic cultures were incubated in a Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, Mich.) at 37°C for 20 h in an atmosphere of 85% N_2–10% H_2–5% CO_2. Nitrite was provided for anaerobically grown cultures by placing 40 μl of a 20% (wt/vol) $NaNO_2$ solution on a sterile cellulose disk in the center of a plate. Cultures with nitrite grow in a characteristic halo around the nitrite disk, while cultures without nitrite remain viable but do not grow (35).

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TABLE 1. Primer sequences used in the construction of deletions and mutations

Primer	Sequence (5′ to 3′)[a]	Location[b]
P1	CCTTTTAA**GGATCCGCCGTCTG**AAAATTCACAAAATATGAATGTTA	−364
P2	GGCTAAT**GGATCC**CGTTTCATAATGTTTTCCTTTTG	+31
P3	GATGAAACGCCGAGTTAACGCC	+470[c]
P4	ACTTAA**GGATCC**ATGCCCGCAATGGGACAACC	−15
P5	ATGAAT**GGATCC**GATTGTAGCATGGTTTACCG	−250
P6	ACCGCA**ATTA**CACACCAAGTTCTTAACTAATC	−7
P7	TGTG**TAAT**TGCGGTTGTCCCATTGCGGGC	+15
P8	TCACAC**GTTCA**TCTTAACTAATCC	+15
P9	AAGA**TGAAC**GTGTGATTATGCGGTTG	−19
P10	**CAGG**TTATTTGAC**G**TAAATTAAAATGC	−31
P11	**C**GTCAAATAA**CCTG**TAAAATATGAAC	−69
P12	AATC**TCTAG**ACACCAAGTTCTTAACTAATC	+14
P13	TGGTGT**CTAGA**GATTATGCGGTTGTCCCATTG	−27
P14	AAAGGA**TCAAATA**ATGAAACGGGATCCCGTCG	+65[C]
P15	**GA**TCCTTT**GTATA**GAAAAGTAGGGGGGATTAG	+9
P16	CTTAAAA**TTTT**ATGCCCGCAATGGGAC	−19
P17	GGGCATA**AAAAT**TTTAAGTCAAATAATTC	−46
P18	CCGGAG**GGATCC**GCAAATCAGCCTATTCATTG	−111
P19	ATGAATTATC**TA**ACTTAAATTAAAATGC	−31
P20	TAAGT**TAG**ATAATTCATAAAATATGAAC	−69
P21	TTTTAT**GGATCC**TTTGACTTAAATTAAAATGC	−31
P22	GTCATTTT**GGATCC**ATATTTTATGAATTATTTGAC	−45
P23	ATCGTA**CTCGAG**CCAGTAGTTCGGGCGGCCTTT	+2650
P24	TAATGT**CTCGAG**TTTGTAAGAAAAGTAGGGGGGAT	+12
P25	ACAAAA**GGATCC**CATTATGAAACGCCAAGCAT	+62
P26	TTATAA**GGATCC**AGAAGCGTCATTTTAAGTTC	−65

[a] Changes in the sequence from the wild-type sequence are indicated by bold type.
[b] Location of the 3′ end of the primer relative to the sigma 70-type promoter transcription start site.
[c] Relative to the sigma 70-type promoter transcription start site in the *lacZ* fusion.

Gonococcal transformation. A light suspension of type 1 cells (23) was made with 2 ml of GCK broth containing 0.042% $NaHCO_3$ and 10 mM $MgCl_2$. Purified DNA or a ligation mixture was added, and cultures were grown for 5 to 6 h with shaking at 37°C. Cultures were then plated on GCK plates containing the appropriate antibiotic.

Extraction of gonococcal chromosomal DNA. Gonococci were harvested from plates, suspended in 0.5 ml of 50 mM Tris-HCl (pH 8.5)–50 mM EDTA–15% sucrose–1 mg of lysozyme ml^{-1}, and incubated at room temperature for 10 min. Sodium dodecyl sulfate was added to 0.4% to lyse the cells, and the solution was mixed by inversion of the tube. After incubation for 5 min at 70°C, 100 μl of 5 M potassium acetate was added, and the mixture was chilled on ice for 30 min. The precipitated proteins were pelleted by centrifugation at 12,000 × *g* for 15 min. The supernatant containing the DNA was removed to a clean tube, to which 2 volumes of cold 95% ethanol was added. This mixture was centrifuged for 5 min at maximum speed in an SS-34 rotor of an RC-5B centrifuge (Sorvall, Newtown, Conn.). The supernatant was decanted, and the pellet was allowed to dry. The pellet was then resuspended in 50 μl of TE buffer (10 mM Tris-Cl [pH 8.0], 1 mM EDTA [pH 8.0]) containing 10 μg of RNase A ml^{-1}.

PCR. The primers used for the creation of reporter constructs by PCR are listed in Table 1.

Nuclease protection assay. A PCR product amplified from pLES940 (36) and containing approximately 350 bp of the *aniA* upstream region, the junction, and about 20 bp of *lacZ* was cloned into the Bluescript II SK(+) phagemid (Stratagene, LaJolla, Calif.), which had been digested with *Xba*I and *Eco*RI. The phagemid containing the insert was linearized by restriction digestion with *Eco*RI. An [α-^{32}P]UTP-labeled antisense RNA probe was generated by in vitro transcription with T3 RNA polymerase and a MAXIscript kit (Ambion Inc., Austin, Tex.) in accordance with the manufacturer's instructions. An RNeasy total RNA kit (Qiagen, Chatsworth, Calif.) was used to prepare total RNA from RUG7001 (wild-type *aniA′-′lacZ* fusion in a wild-type background; see below) harvested from anaerobic plates with nitrite disks.

Nuclease protection was performed with an RPA II kit (Ambion), substituting a 1:50 dilution of S1 nuclease for the RNase and 10× S1 nuclease buffer (300 mM Na acetate [pH 4.6], 10 mM Zn acetate, 500 mM NaCl, 50% glycerol) for buffer Bx. Hybridization was performed overnight at 42°C, and the nuclease reaction was carried out at 37°C for 45 min. The reaction products were electrophoresed along with a sequence of the probe made with a T3 primer and the dsDNA cycle sequencing system (Life Technologies Inc., Gaithersburg, Md.).

Construction of *lacZ* fusions. Deletions and mutations of the *aniA* upstream region were created by PCR with the primer pairs listed in Table 2. Translational *lacZ* fusions were made with pLES94 (36). Gonococcal strain F62 chromosomal DNA was used as the template for PCR. PCR products and pLES94 were cut with *Bam*HI. Digested insert and plasmid were ligated and transformed into *E. coli* MC1061. Transformants were selected on Luria-Bertani medium plates containing ampicillin at 100 μg ml^{-1} and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) at 40 μg ml^{-1}. After overnight incubation at 37°C, blue colonies were identified, and their plasmids were extracted. Plasmids were checked for the presence and orientation of the insert by PCR. Plasmids containing an insert in the correct orientation were used to transform gonococcal strain F62. Transformants were plated on GCK plates containing chloramphenicol. Chromosomal DNA was prepared from chloramphenicol-resistant colonies, and PCR was used to confirm the presence of the reporter construct. The PCR product was sequenced to ensure that the desired alteration had been made.

Construction of deletions. Deletions were created by PCR with primers containing *Bam*HI sites at the desired deletion sites in the *aniA* upstream region. The inserts were amplified with these upstream primers and primer P2 (Table 2).

TABLE 2. Primer pairs and plasmids used in the construction of reporter strains

Strain	Plasmid	Primer pair(s)
RUG7001	pLES940	P1, P2
RUG7004	pTCH1	P4, P2
RUG7005	pTCH2	P5, P2
RUG7006	pTCH3	P1, P7; P6, P2
RUG7007	pTCH4	P1, P9; P8, P2
RUG7008	pTCH5	P1, P11; P10, P2
RUG7009	pTCH6	P1, P13; P12, P2
RUG7012	pLES943	P22, P2
RUG7014	pLES945	P1, P2
RUG7015	pLES946	P1, P2
RUG7020	pTCH8	P1, P17; P16, P2
RUG7023	pTCH9	P1, P15; P14, P3
RUG7024	pTCH10	P18, P2
RUG7040	pTCH11	P1, P20; P19, P2
RUG7041	pTCH12	P21, P2
RUG7045	pTCH13	P26, P2

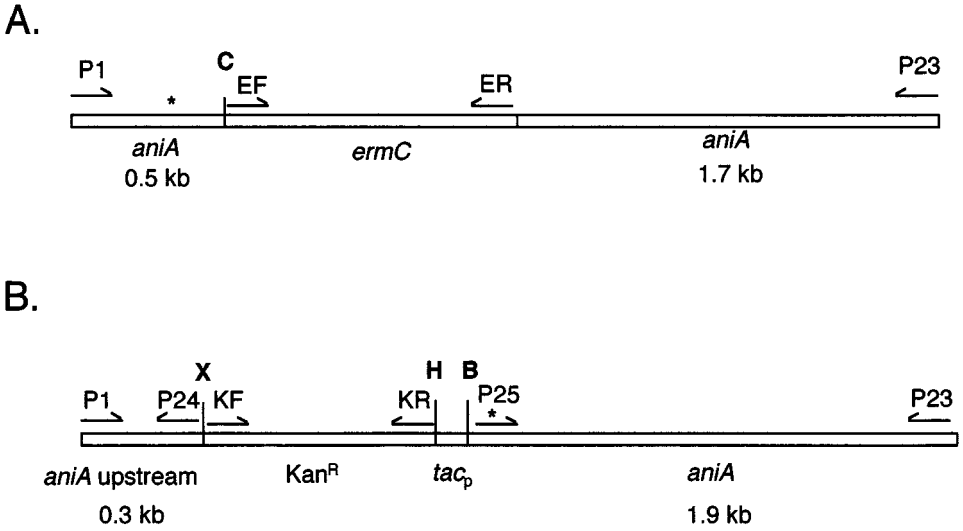

FIG. 1. Maps of the *aniA* null (A) and constitutive (B) mutants. Primers used to amplify the fragments are indicated above the genes. An asterisk denotes the location of the ATG start codon in each construct. Maps are not drawn to scale. C, *Cla*I; X, *Xho*I; H, *Hin*dIII; B, *Bam*HI.

Site-directed mutagenesis. All mutations in the *aniA* upstream region were created by PCR overlap extension (18, 32). Briefly, two PCRs were used to create 5′ and 3′ fragments whose sequences overlapped by several base pairs at the mutation. Both fragments were used as the template for a third PCR. The plus strand from one fragment and the minus strand from the other fragment annealed, acting as both a primer and a template. When the product was extended, it created the full-length insert; this insert was then amplified with primers P1 and P2, which were included in the PCR. Due to the proximity of the symmetric repeat to the P2 primer site, the pTCH9 insert, which contains a mutation of the symmetric repeat, was created with a plasmid pLES940 preparation as a template and the P3 primer instead of P2.

The reporter constructs in strains RUG7014 and RUG7015 contained the *aniA* upstream regions amplified from *N. meningitidis* RUN5645 and RUN5646, respectively (19).

Construction of an *aniA* null mutant. With F62 chromosomal DNA as a template, *aniA* was amplified by PCR with primers P1 and P23. The PCR product was digested with *Cla*I, creating two fragments 0.5 and 1.7 kb long. The ends of the 1.7-kb fragment were made flush with the Klenow fragment of *E. coli* DNA polymerase I. The *ermC* gene, which confers erythromycin resistance, was amplified from pHSS23 (41) (gift from Joe Dillard) with primers EF (5′-ATGTT TGCGCCGGTATCATCGATAAGCTTTGGC-3′) and ER (5′-CTGGATGAT CCTCCAGCGCG-5′) and digested with *Cla*I and *Sma*I. The *ermC* fragment was ligated to the two *aniA* fragments, a step which resulted in *ermC* interrupting *aniA*. The ligation mixture was transformed into strain RUG7001. Transformants were plated on GCK plates containing erythromycin, and the insertion was confirmed by PCR. This process created strain RUG7011 (Fig. 1A).

Construction of a constitutive *aniA* mutant. With F62 chromosomal DNA as a template, the upstream region and the coding region of *aniA* were amplified by PCR into two separate fragments. The upstream region (335 bp) was amplified with primers P1 and P24 and then digested with *Xho*I. The coding region (1,894 bp), including the ATG start codon, was amplified with primers P25 and P23 and then digested with *Bam*HI. The kanamycin resistance gene was amplified from pHSS23 (41) with primers KF (5′-TGAGCGAAGCTTCGGAAGAGCGCCTG ATGCGG-3′) and KR (5′-AGAACTCTCGAGTGAGATCCCCGCGCTGGA GG-3′) and digested with *Xho*I and *Hin*dIII. The *tac* promoter (Pharmacia Biotech Inc., Piscataway, N.J.) was cloned into the *Hin*dIII and *Bam*HI sites of pBluescript II SK(+) (Stratagene). The kanamycin resistance gene was then cloned into the *Xho*I and *Hin*dIII sites, placing it upstream of the *tac* promoter. The kanamycin resistance gene-*tac* promoter fragment was amplified by PCR from this construct and digested with *Xho*I and *Bam*HI. The fragment was ligated to the two *aniA* fragments, and the ligation mixture was transformed into F62. This step inserted the kanamycin resistance gene-*tac* promoter fragment between the upstream region and the coding region of *aniA* and placed the *tac* promoter and the ribosome binding site in the proper orientation relative to the ATG start codon of the *aniA* coding region (Fig. 1B).

To transfer constitutive *aniA* to strain RUG7001, the entire construct was amplified with primers P1 and P23. The PCR product was used to transform RUG7001, creating RUG7035. This PCR product was also transformed into *fnr* and *narP* mutants (27) containing the *aniA′-′lacZ* fusion (creating strains RUG7025 and RUG7039, respectively). For all strains containing the constitutive *aniA* mutation, transformants were selected on GCK plates containing kanamycin, and the presence of the kanamycin resistance gene and the *tac* promoter was confirmed by PCR.

β-gal assays. Anaerobically grown and anaerobically incubated cultures were assayed for β-galactosidase (β-gal) activity by the method of Miller (30). For β-gal assays with plate cultures, the plates were inoculated with 100 μl of a cell suspension with an A_{600} of approximately 10, and the incubation time was standardized to 20 h. These cultures were harvested with sterile swabs and suspended in Z buffer (30). When nitrite disks were used, only the halo of growth around the nitrite disks was harvested; the full plate was harvested for cultures incubated in the absence of nitrite. Samples containing broth were centrifuged to remove the medium, and the cell pellets were resuspended in Z buffer. Cells were lysed with toluene–0.1% sodium dodecyl sulfate and assayed as described (30). Activity is reported in Miller units. Results reported are the averages of at least three independent assays performed in triplicate on each day that the cultures were grown for each strain.

Time course for *aniA* induction. Gonococcal cultures were grown in GCK broth containing 0.042% $NaHCO_3$ in a Gyrotory water bath shaker (New Brunswick Scientific Co., Edison, N.J.) at 37°C to an A_{600} of approximately 1.0. A sample of each culture was taken for initial measurements of β-gal activity. The remaining culture was transferred into the anaerobic chamber, where 3.3 ml was added to 6.6 ml of prereduced supplemented GCK broth in 12-ml serum vials containing stir bars. The cultures were allowed to equilibrate for 15 min on a stir plate at 37°C. $NaNO_2$ (5 mM, final concentration) was added to appropriate vials. All vials were stirred for 5 min, removed from the anaerobic chamber, and placed on a stir plate in a 37°C room. Vial caps were loosened to create an environment with reduced oxygen tension. Samples were removed each hour for 4 h with a needle and syringe through the septa in the caps and assayed for β-gal activity. RUG7001 served as the wild-type control.

Oligonucleotides and DNA sequencing. All oligonucleotide syntheses and confirmatory DNA sequencing were performed at the University of Rochester Core Nucleic Acid Laboratory.

Molecular biology techniques. General techniques were performed in accordance with standard protocols (3, 4, 33). Plasmid preparations were obtained with Wizard Plus Minipreps or Wizard Plus SV Minipreps kits (Promega Corp., Madison, Wis.). DNA fragments were purified with a Wizard PCR Preps kit (Promega), by the freeze-squeeze technique, or with a MERmaid kit (Bio 101, Inc., Vista, Calif.).

Nucleotide sequence accession number. The sequence upstream of *aniA* has been deposited in GenBank under accession no. AF082184.

RESULTS

Anaerobic growth of *N. gonorrhoeae*. Unlike organisms such as *E. coli*, most strains of gonococci, including F62, do not grow anaerobically in broth cultures. It is possible to grow F62 in broth under oxygen-limiting conditions and to obtain the induction of *aniA* (21), but in order to study the regulation of *aniA* under completely anaerobic conditions, it was necessary to utilize plate cultures. It has been reported that gonococci

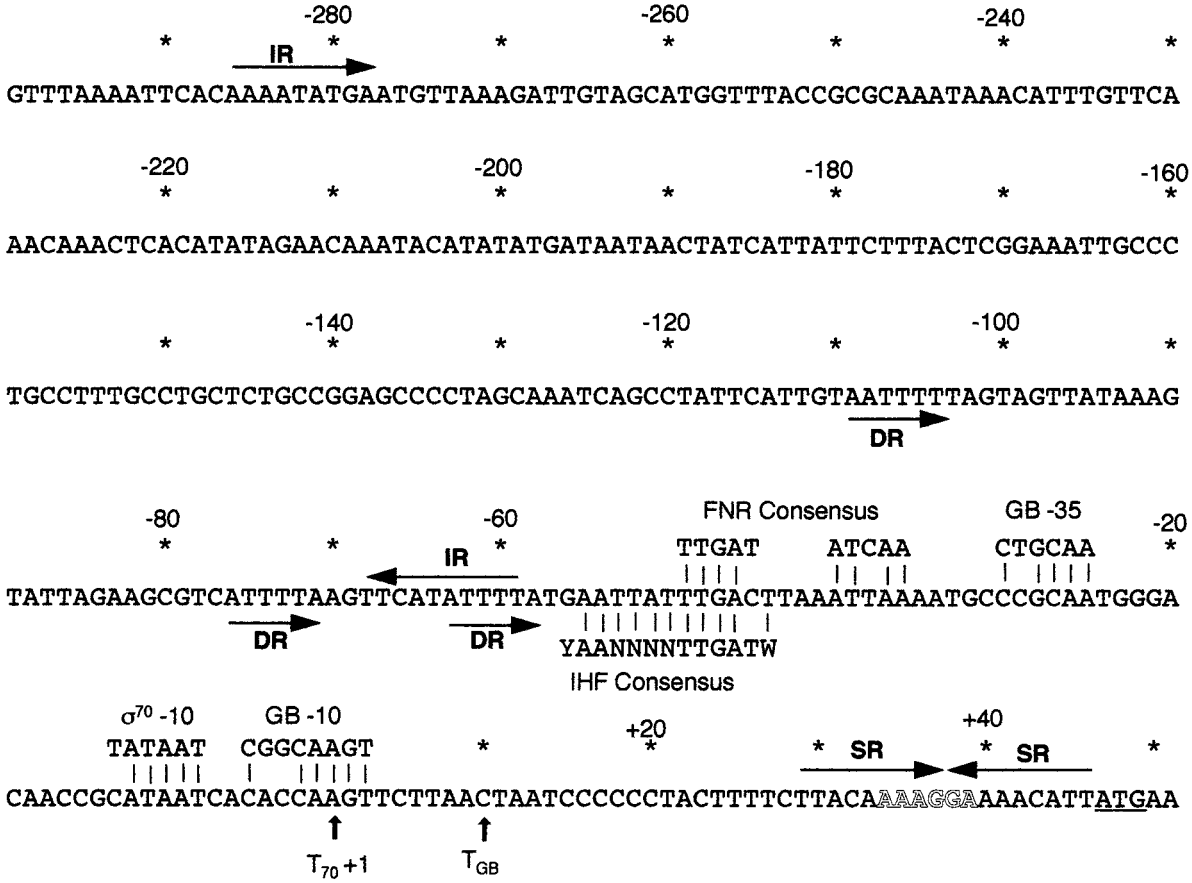

FIG. 2. Nucleotide sequence of the *aniA* upstream region. The sequence is numbered in relation to the sigma 70-type promoter transcription start site. The ATG start codon is indicated by underlining, and the ribosome binding site is indicated by outlined letters. The previously reported transcription start sites are indicated by vertical arrows beneath the sequence: T_{70}, start of transcription from the putative σ^{70} promoter; T_{GB}, start of transcription from the putative gearbox (GB) promoter. The inverted and symmetric repeats (IR and SR, respectively) are labeled with arrows above the sequence, and the direct repeats (DR) are indicated by arrows beneath the sequence. Sequences homologous to known promoters and consensus binding sites are indicated; matching bases within these sequences are indicated with vertical lines.

require a continuous supply of low levels of nitrite to support anaerobic growth, and this was obtained by placing a nitrite disk on the plate (24). While not completely ideal conditions, this method produced visible growth, and β-gal assays performed on these cultures were reproducible between assays and laboratory personnel. This method was used in this study as well as those previously reported from our laboratory (7, 8, 19–21, 36).

Sequence upstream of *aniA*. An approximately 350-bp sequence upstream of the *aniA* start codon was cloned, and analysis of the sequence (Fig. 2) revealed the presence of several interesting motifs. One base upstream of the ATG is a 9-bp symmetric repeat which contains the Shine-Dalgarno sequence (centered at +37.5 from the σ^{70} transcription start site). There are two overlapping putative promoters; the sequence homologous to *E. coli* gearbox promoters contains both −10 and −35 consensus sequences, while the sigma 70-type promoter has a potential FNR (fumarate and nitrate reductase regulator protein) consensus binding site (14, 37) in lieu of a −35 sequence (centered at −42.5). An IHF (integration host factor) consensus binding site (10) overlaps the FNR consensus. A T-rich region is located just upstream of the IHF consensus and contains three direct repeats (at −105, −73.5, and −60.5). Finally, there is a 10-bp inverted repeat with approximately 200 bp separating the two halves (at −280.5 and −63.5).

Nuclease protection assay of *aniA* transcripts. Previous primer extension data suggested the presence of two *aniA* transcripts (20). To confirm these results, nuclease protection was performed on strain RUG7001, which contains both the parental *aniA* and the *aniA'-'lacZ* fusion. The RNA probe was designed to contain the *aniA* upstream region fused to a portion of *lacZ* to enable us to detect transcripts from both the parental and the fusion promoters (expected sizes of 46/55 bp and 68/77 bp [gearbox promoter/σ^{70} promoter], respectively). The results from this assay indicated the presence of two RNA transcripts, one corresponding to each of the putative promoters, and that the shorter fragment accounted for the majority of the *aniA* message (Fig. 3). The lengths of the protected fragments (46/54 bp and 69/80 bp) correlated well with the primer extension data. In addition, it was evident from these data that the levels of message from the fusion promoter were similar to those from the parental promoter, thus validating our use of the *lacZ* fusion to measure the induction of *aniA*. The minor bands present in Fig. 3 can be attributed to the difficulty in obtaining clean results when one is working with very short protected fragments in a nuclease protection assay.

Deletion analysis of the *aniA* upstream region. To determine which *cis* elements are important in the regulation of *aniA*, we began with a deletion analysis of the upstream region. We had previously cloned 360 bp of the *aniA* upstream region, including the ATG start codon and the codons for the first 3 amino

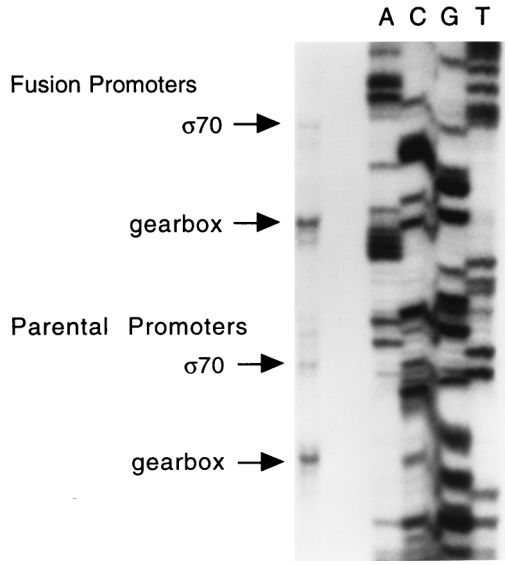

FIG. 3. Nuclease protection. Autoradiogram of S1 nuclease protection products from both fusion and parental promoters. The larger products of each set correspond to the sigma 70-type promoter, while the smaller products correspond to the gearbox promoter. A sequence generated by the same primer as that used to produce the probe is used as a size marker (lanes A, C, G, and T).

acids, into vector pLES94, forming a translational *lacZ* fusion (36). The resulting plasmid, pLES940, was transformed into gonococcal strain F62. The *lacZ* fusion was integrated into the chromosome by homologous recombination into the *proAB* genes, creating a single-copy reporter system for the expression of *aniA*. The resulting strain, RUG7001, contains what is designated the "wild-type" *lacZ* fusion in a wild-type background. While β-gal activity was negligible in aerobic cultures (<10 Miller units for all strains reported in this study), when cultures of RUG7001 were incubated anaerobically, β-gal activity increased to approximately 900 Miller units. When nitrite was provided to the cultures to allow anaerobic growth, β-gal activity increased about threefold to yield 2,500 Miller units (Fig. 4).

Six *lacZ* fusions containing *aniA* deletion mutations were constructed and placed into a wild-type F62 background. These are depicted schematically in Fig. 4A; the β-gal assay results are shown in Fig. 4B. The fusion in RUG7005 contains 334 bp and eliminates the 5′ half of the inverted repeat; β-gal activity was not changed from that of the wild type. The fusion in RUG7024 contains 195 bp of the *aniA* upstream region and eliminates everything upstream of the T-rich region. As shown in Fig. 4B, β-gal activity was the same as that in the wild type. The fusion in RUG7045 contains 150 bp and deletes the 5′ half of the inverted repeat and the T-rich region located at −102 to −108, while the fusion in RUG7012 contains 131 bp and deletes the 5′ half of the inverted repeat, most of the T-rich region, and a portion of the 3′ half of the inverted repeat; β-gal activity in these two strains grown in the presence of nitrite decreased to approximately the levels seen in the absence of nitrite. This result suggests that an element present in the RUG7024 fusion but not in the RUG7045 fusion is involved in responding to a second induction signal. This signal is most likely nitrite. The RUG7041 fusion contains 115 bp of the *aniA* upstream region, and the deletion was made just upstream of the FNR consensus binding site, eliminating the inverted repeat and the T-rich region; β-gal activity in this strain was essentially the same in the presence or absence of nitrite but was lower than has been seen previously (300 Miller units). The RUG7004 fusion contains only 99 bp of the *aniA* upstream region, and the deletion eliminated a required element, as evidenced by the absence of β-gal activity in cultures grown either with or without nitrite.

Mutation of the *aniA* promoter. Data from a previous primer extension experiment (20) and the nuclease protection assay (Fig. 3) suggested the presence of two transcripts corresponding to two putative promoters. The shorter, more abundant message corresponded to sequences homologous to *E. coli* gearbox promoters, and the longer, less abundant message corresponded to a sigma 70-type promoter. We were particularly interested in confirming the presence of two active promoters. To evaluate the roles of the two putative promoters, mutations were made in each promoter separately and were cloned into the *lacZ* reporter system. To determine the role of the sigma 70-type promoter, the −10 sequence was changed from CATAAT to CAATTA (RUG7006). This change eliminated virtually all activity from the *lacZ* fusion, indicating an absolute requirement for this promoter (Fig. 5B).

The mutation analysis of the sigma 70-type promoter suggested that there was a single active promoter, while the primer extension analysis and nuclease protection assay suggested that the gearbox was the major promoter. Although it was impossible to evaluate the gearbox promoter without an active sigma 70-type promoter, we performed mutation analysis on the gearbox sequences to resolve the conflict of data that we had obtained. We initially used two *aniA* promoters from strains of *N. meningitidis*. The fusions in RUG7015 and RUG7014 contain sequences amplified from meningococcal strains RUN5646 and RUN5645, respectively. These are *N. meningitidis* strains which possess the *aniA* gene (6a) but in which the AniA protein is undetectable by Western blotting (19). When compared to that of F62, the *aniA* upstream region of these meningococcal strains contained nucleotide differences in the gearbox promoter sequences (Fig. 5A). We hypothesized that these differences may be responsible for the lack of AniA in these strains.

The single-base-pair change in the gearbox −35 sequence in RUG7015 resulted in no decrease in β-gal activity when cultures were grown in the presence of nitrite (Fig. 5B). Cultures incubated anaerobically without nitrite produced about one-third the β-gal activity of the wild type. Similarly, the 3-bp change in the −35 sequence and the single-base-pair deletion in the −10 sequence in RUG7014 did not produce a dramatic decrease in β-gal activity. Site-directed mutagenesis was used to change the gearbox −10 sequence from CACCAAGT to CACGTTCA (RUG7007). This mutation decreased β-gal activity by only one-half. Altering the number of bases between the −10 and −35 sequences is known to significantly affect expression from *E. coli* promoters (31 and references therein). We therefore introduced an additional 5 bp between the gearbox −10 and −35 sequences without changing the sigma 70-type promoter spacing (RUG7009). This mutation decreased β-gal activity in anaerobically grown cultures by only one-third. All of these results indicate that the gearbox promoter is not an active promoter during anaerobic growth.

Mutation of the IHF and FNR consensus binding sites. As the putative IHF and FNR binding sites overlap, it was impossible to completely mutate one site without potentially affecting the other. Bases which matched one consensus and were not a part of the other consensus were mutated as shown in Fig. 6. Mutation of the IHF consensus only slightly affected β-gal activity (RUG7008). In contrast, mutation of the 3′ half of the FNR consensus decreased β-gal activity to almost zero (RUG7020). To further investigate the putative FNR binding site, the first and third bases of the consensus, which are known

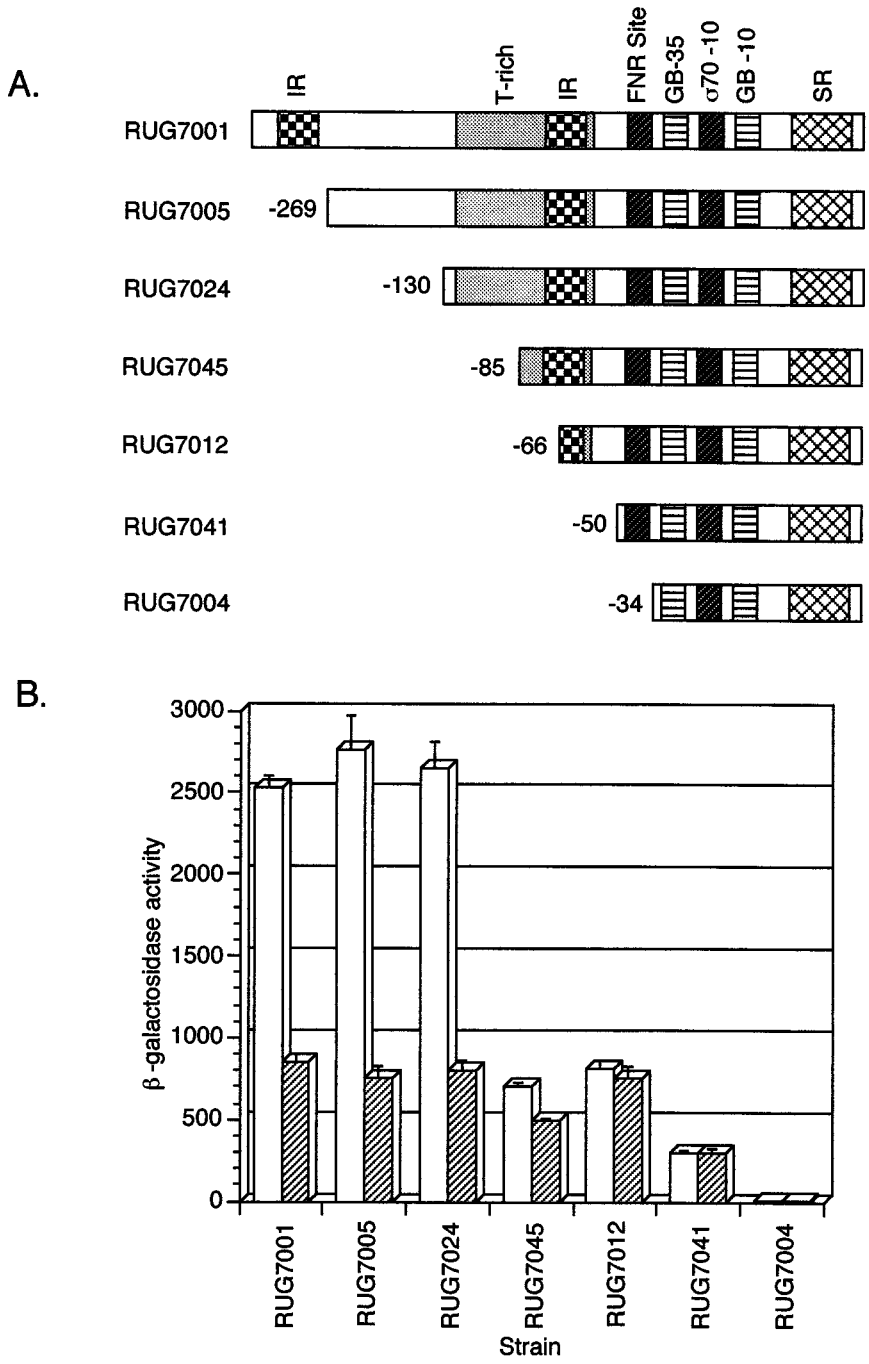

FIG. 4. Deletion results. (A) Schematic of the deletions constructed in the *lacZ* reporter fusions (not drawn to scale). The 5′ ends of the deletions are numbered in relation to the sigma 70-type promoter transcription start site. IR, inverted repeats; GB, gearbox promoter sequences; SR, symmetric repeat. (B) Anaerobic β-gal assay results (mean ± standard error of the mean Miller units). Cultures grown with nitrite are indicated by open bars; cultures incubated without nitrite are indicated by hatched bars.

to be required for the *E. coli nirB* promoter (5), were mutated. This mutation (RUG7040) also dramatically decreased β-gal activity.

Evaluating the role of the symmetric repeat. Positioned around the ribosome binding site, the symmetric repeat was a possible repressor binding site (reviewed in references 9 and 16). When bound to the DNA, a protein at that position could prevent procession of the RNA polymerase or could prevent the binding of the ribosome by binding to the message. To investigate this possibility, the sequence of the repeat was scrambled, except for the 6-bp Shine-Dalgarno sequence, changing wild-type TTACAAAAGGAAAACATT to TATAC AAAGGATCAAATA (RUG7023). This mutation had little effect on β-gal activity. The β-gal activity of cultures grown in the presence of nitrite was 1,930 ± 65 Miller units, while the β-gal activity of cultures incubated without nitrite was 511 ± 19 Miller units.

Requirement for an FNR homolog. Mutation analysis of the *aniA* promoter indicated that there is an absolute requirement for the FNR binding site (Fig. 6). The gonococcal FNR ho-

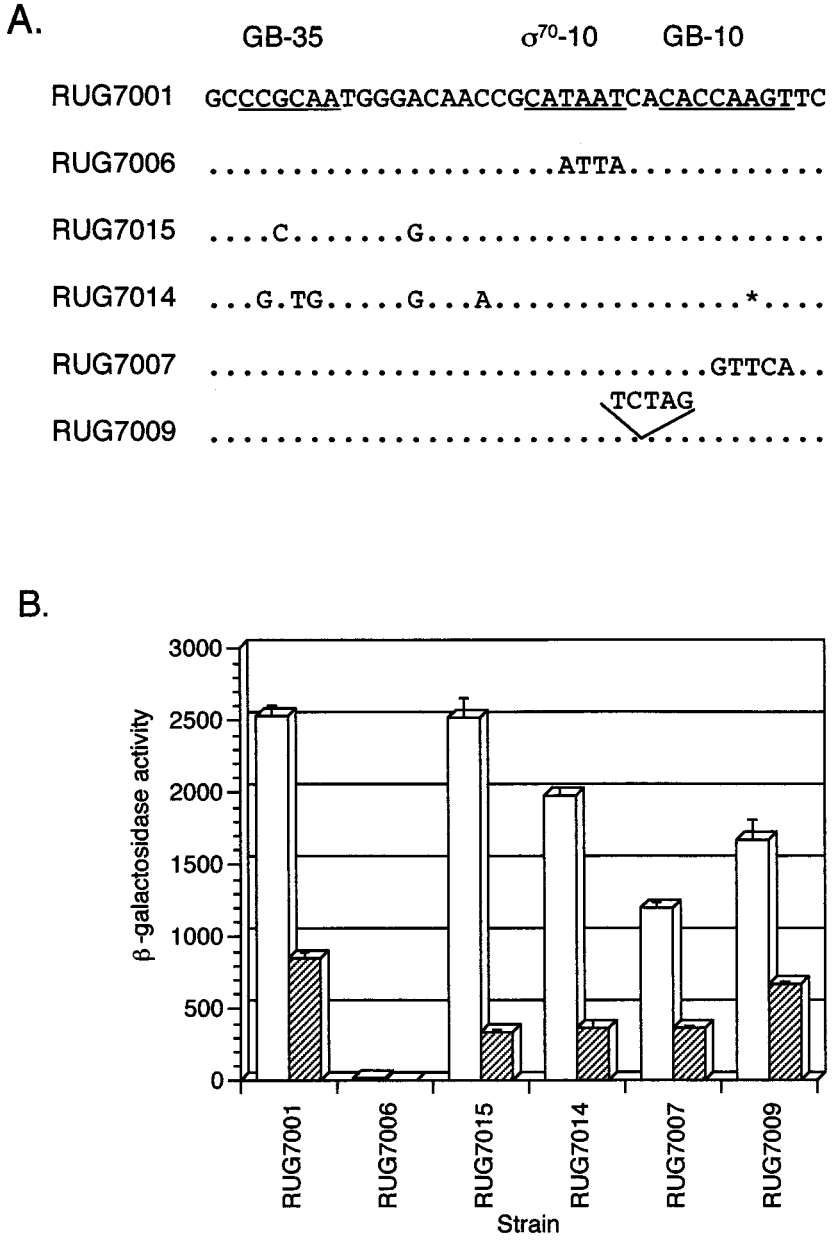

FIG. 5. Mutation analysis of promoter sequences. (A) Putative −10 and −35 promoter sequences are underlined. Base changes in each strain are indicated. An asterisk represents a base deletion. GB, gearbox. (B) Anaerobic β-gal assay results (mean ± standard error of the mean Miller units). Cultures grown with nitrite are indicated by open bars; cultures incubated without nitrite are indicated by hatched bars.

molog was recently identified and will be reported elsewhere (27). When the gonococcal *fnr* gene was insertionally inactivated, strain F62 could no longer grow anaerobically. A wild-type promoter fusion in this strain (RUG7022) resulted in negligible β-gal activity (Table 3). Placing the *aniA* gene under the control of the *tac* promoter in this background (RUG7025) restored the ability to grow anaerobically but had no effect on β-gal activity.

Full induction requires a NarP homolog. Nitrate and nitrite regulation of genes in *E. coli* is mediated by dual two-component systems. The NarX-NarL and NarQ-NarP systems respond differentially to nitrate and nitrite (reviewed in reference 11). Since *aniA* seems to respond to a nitrite signal, we were interested in these regulatory systems. The gonococcal NarP homolog has been identified (27). Insertionally inactivating the *narP* gene resulted in a strain (RUG7036) that would grow anaerobically but that produced decreased β-gal activity from the *aniA′-′lacZ* fusion. As shown in Table 3, β-gal activity was diminished in cultures both with and without nitrite. Placing *aniA* under the control of the *tac* promoter in this strain (RUG7039) had no significant effect on β-gal activity.

Autoregulation. As reported for gonococcal strain MS11 (29), an *aniA* null mutant was unable to grow anaerobically. This F62 derivative (RUG7011) had one-fifth the wild-type β-gal activity in the presence of nitrite (Table 3). A strain with *aniA* constitutively expressed from the *tac* promoter was also created (RUG7035). This strain grew very well anaerobically; β-gal activity in this strain was increased over that in the wild-type strain when cultures were grown in the presence of nitrite but were unchanged when cultures were incubated without

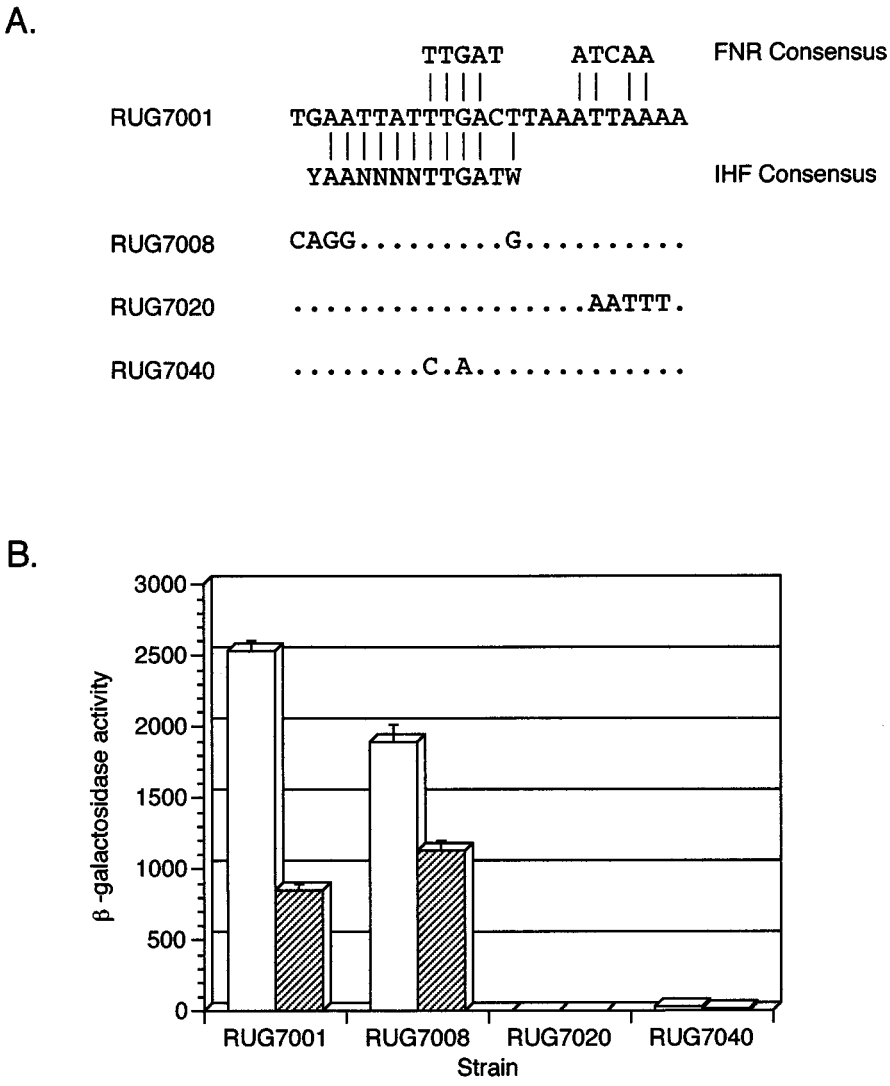

FIG. 6. Mutation of FNR and IHF binding sites. (A) Base changes in each strain are indicated. (B) Anaerobic β-gal assay results (mean ± standard error of the mean Miller units). Cultures grown with nitrite are indicated by open bars; cultures incubated without nitrite are indicated by hatched bars.

nitrite (Table 3). These results can be attributed either to autogenous regulation or to the growth characteristics of these strains.

While we have reported β-gal activity after 20 h of incubation, it was desirable to look at the initial induction of the *aniA* promoter under conditions in which the cultures could grow. A 4-h induction assay was performed on the *aniA* null mutant (RUG7011), and the results were compared to those for wild-type strain RUG7001 in the same assay. The A_{600} of cultures of RUG7001 without nitrite doubled on average during this time period, while the A_{600} of cultures of RUG7001 with nitrite increased by 2.7-fold. The increases in the A_{600} for strain RUG7011 without and with nitrite were 1.6- and 1.4-fold, respectively. The β-gal data (Fig. 7) indicated that the *aniA* null mutant responded to the presence of nitrite in the same manner as the wild-type strain and that its decreased 20-h β-gal levels were due to its inability to grow anaerobically.

TABLE 3. Anaerobic β-gal activity and growth characteristics of *aniA*, *fnr*, and *narP* mutants

Strain	Relevant genotype	β-gal activity in the presence of[a]:		Anaerobic growth[b]
		$-O_2$ $+NO_2$	$-O_2$ $-NO_2$	
RUG7001	Wild type	2,530 ± 70	853 ± 46	+
RUG7011	*aniA*::*ermC*	493 ± 30	1,003 ± 102	−
RUG7035	*tacp*::*aniA*	3,182 ± 109	740 ± 64	+
RUG7022	*fnr*::*ermC*	6 ± 0.6	5 ± 0.2	−
RUG7025	*fnr*::*ermC tacp*::*aniA*	12 ± 0.3	7 ± 0.5	+
RUG7036	*narP*::*ermC*	669 ± 96	101 ± 8	+
RUG7039	*narP*::*ermC tacp*::*aniA*	472 ± 19	72 ± 7	+

[a] Expressed in Miller units as mean ± standard error of the mean. $-O_2$, no oxygen; $+NO_2$, nitrite; $-NO_2$, no nitrite.

[b] Presence (+) or absence (−) of growth around the nitrite disk on GCK plates.

DISCUSSION

Pathogenic bacteria often coordinately regulate virulence genes in response to environmental stimuli (recently reviewed in reference 28). Previous studies on *N. gonorrhoeae* indicated that anaerobiosis is likely a physiologically relevant state in human infection (8) and that the organism alters outer membrane protein expression in response to oxygen levels (7). We

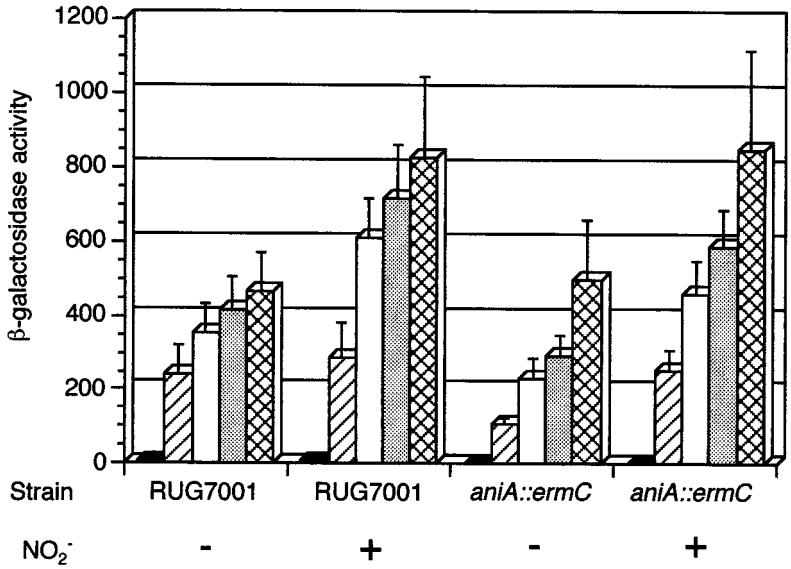

FIG. 7. Time course for *aniA* induction. Aerobic cultures of RUG7001 and RUG7011 were shifted to oxygen-limited conditions in the absence (−) or presence (+) of nitrite as indicated. Samples were removed before the shift (solid bar), at 1 h (hatched bar), at 2 h (open bar), at 3 h (stippled bar), and at 4 h (cross-hatched bar) and assayed for β-gal activity (expressed as mean ± standard error of the mean Miller units).

have undertaken the study of how *aniA* is regulated to better understand the gonococcal response to anaerobiosis and to evaluate its potential role in virulence.

An examination of the sequence upstream of *aniA* revealed several interesting motifs which have been the main focus of this report. We were most interested in the status of the two putative promoters. The mutations that we constructed indicated that the sigma 70-type promoter is the only active promoter during anaerobiosis. Mutating the sigma 70-type promoter −10 sequence eliminated expression, while mutations in the gearbox sequences decreased expression relatively slightly. These results were surprising considering the primer extension data (20) and nuclease protection assay data (Fig. 3), which indicated that the gearbox was the major promoter. This conflict in the data can be resolved when one takes into account that both primer extension and nuclease protection detect not only initiated transcripts but also degraded and processed RNA species. Given the distinct products seen with both of these biochemical techniques, it is possible that specific cleavage of 9 bp from the 5′ end of the message occurs, although the purpose of such processing is unknown at this time. The fact that changing the spacing in the gearbox promoter (RUG7009) does not cause a significant decrease in β-gal activity makes it clear that the gearbox sequences do not act as a promoter.

In conjunction with the requirement for the sigma 70-type promoter is the requirement for the FNR binding site. It is common for anaerobically induced genes to possess an FNR binding site in place of the traditional −35 sequence, so it is not at all surprising that this motif is present in *aniA*. The FNR binding site is centered at −42.5 and is therefore in position to act as a conventional class II transcription activator (22). It is still unclear if there is a role for the IHF binding site which overlaps the FNR binding site. IHF is known to coordinate with FNR and NarL in the regulation of the nitrate reductase operon in *E. coli* (34). Gonococcal IHF has been shown to bind to the *pilE* promoter in gel mobility shift assays (17), and mutation of the IHF binding site results in a large decrease in expression from the $pilEp_1$ promoter (15). However, mutation of the consensus sequence in *aniA* resulted in little change in β-gal activity (Fig. 6). The decrease seen may have been due to an alteration of the context of the FNR binding site. It is therefore unlikely that IHF functions in the regulation of *aniA*, but it remains to be determined if IHF actually binds to the *aniA* promoter.

The deletion studies reported here indicated that the length of the upstream region required for full induction is approximately 170 bp. Although repeated sequences within promoter regions have often proved to be binding sites for either activators or repressors, our deletion and mutation analyses of the inverted and symmetric repeats in the *aniA* promoter region failed to reveal any function for these two motifs. The direct repeats, however, are part of the region which seems to respond to a second induction signal and is required for full expression during anaerobic growth. As with other nitrite reductases, it is likely that this second signal is nitrite.

The requirement for the gonococcal homologs of FNR and NarP was not unexpected. We had an indication of the necessity for FNR through the mutation studies of the putative FNR binding site, and NarP was a likely candidate for an activator, considering its role in the regulation of other genes induced by nitrite (recently reviewed in reference 11). By placing *aniA* under the control of the *tac* promoter, we restored the ability of the *fnr* mutant to grow anaerobically. This result indicates that *aniA* is the only gene regulated by FNR that is essential for anaerobic growth. The fact that the *narP* mutant had decreased β-gal activity due to anaerobiosis alone suggests that NarP and FNR may act in synergy to regulate *aniA*, as has been noted for *E. coli nirB* (38).

The data that we obtained from the deletion analysis and the *narP* mutant analysis suggest that NarP interacts directly with the *aniA* promoter at the T-rich region. We closely scrutinized this region for the presence of potential NarP binding sites. In *E. coli*, NarL and NarP bind to a consensus sequence, TACY NMT (Y, C or T; M, A or C), which has been called the NarL heptamer. Such heptamers can be found as direct repeats, inverted repeats of the form 7-2-7 (heptamers separated by 2 bp), or individually as half sites (11, 13, 26, 38). It was recently reported that NarP binds only to the 7-2-7 motif, while NarL

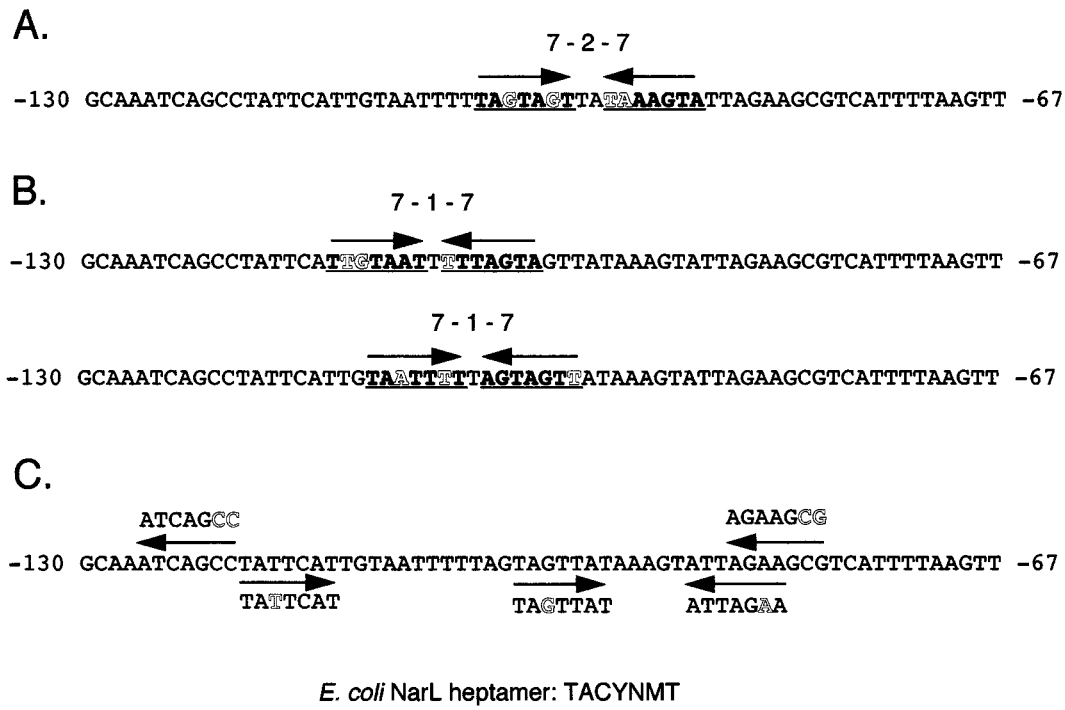

FIG. 8. Potential NarP binding sites in the *aniA* upstream region. The plus-strand sequence of the region from −130 to −67 from the sigma 70-type promoter transcription start site is presented. NarL heptamers and their direction are indicated by arrows. Nucleotides which do not match the *E. coli* consensus are indicated by outlined letters. The *E. coli* consensus is indicated at the bottom (Y, C or T; M, C or A). As the binding site sequence for gonococcal NarP is unknown, any of these sites is a candidate. (A) A single 7-2-7 motif consisting of an inverted repeat of the heptamer separated by 2 bp was found. Heptamers are also indicated by bold type and underlining. (B) Two similar heptamer inverted repeats in a 7-1-7 arrangement (heptamers separated by 1 bp). Heptamers are also indicated by bold type and underlining. (C) Remaining unpaired half sites.

can bind to any heptamer arrangement (12). We found no less than 11 heptamers in the region from −67 to −130. As shown in Fig. 8, these heptamers deviated from the *E. coli* consensus by only 1 or 2 bases each and were found as a single 7-2-7 motif, as two 7-1-7 motifs (heptamers separated by 1 bp), and as several half sites. *aniA* is the first gonococcal gene found to be regulated by NarP; therefore, the binding site sequence is unknown. However, the deletion in RUG7045 pinpoints the region responsible for nitrite induction to between −85 and −130, a region which contains the majority of the NarL heptamers. This finding suggests that gonococcal NarP may have a binding site similar to that of NarL or NarP in *E. coli*. It will be necessary to perform further mutagenesis studies as well as gel mobility shift assays to determine to which, if any, of these sites NarP can bind. These studies, as well as those necessary to confirm that FNR interacts directly with the FNR consensus binding site in the *aniA* promoter, are in progress.

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