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The *Neisseria gonorrhoeae* gene *aniA* encodes an inducible nitrite reductase

Abstract The *aniA* gene of *Neisseria gonorrhoeae* encodes an outer membrane lipoprotein which is strongly induced when gonococci are grown anaerobically in vitro in the presence of nitrite. Database searches with the amino acid sequence derived from the *aniA* structural gene revealed significant homologies to copper-containing nitrite reductases from several denitrifying bacteria. We constructed an insertional mutation in the *aniA* locus of strain MS11 by allelic replacement, to determine whether this locus was necessary for growth in oxygen-depleted environments, and to demonstrate that AniA was indeed a nitrite reductase. The mutant was severely impaired in its ability to grow micro-aerophilically in the presence of nitrite, and we observed a loss in viability over several hours of incubation. No measurable nitrite reductase activity was detected in the *aniA* mutant strain, and activity in the strain with a wild-type locus was inducible. Finally, we report investigations to determine whether AniA protein is involved in gonococcal pathogenesis.

Key words Gonococci · *aniA* · Pan1 · Nitrite reductase · Anaerobiosis

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Introduction

The initial event in infection by *Neisseria gonorrhoeae* of its human host is adherence to epithelial cells (Swanson 1973), followed by endocytotic internalisation (McGee et al. 1983; Tijia et al. 1988). Mucosal surfaces of the urogenital tract serve to protect against invading bacteria, providing a reducing barrier which gonococci must penetrate in order to adhere to the underlying epithelial cell layer. Thus, anaerobic, or oxygen-limiting conditions are encountered by *N. gonorrhoeae* in vivo. In support of this conclusion, gonococci are often clinically isolated with facultative and obligate anaerobes (Burnakis and Hildebrandt 1986; Smith 1975).

Infection of mucosal surfaces brings gonococci into a reducing environment, and anaerobiosis alters the expression of at least eight gonococcal outer membrane proteins (Clark et al. 1988). One of these, AniA, is strongly expressed when gonococci are grown anaerobically in vitro (Clark et al. 1987; Hoehn and Clark 1992a) and is tightly regulated by oxygen. AniA is lipid-modified at its N-terminus (Hoehn and Clark 1992b) and reacts strongly with sera isolated from patients suffering from gonococcal infections (Clark et al. 1988), suggesting that AniA is expressed in vivo and supporting further the notion that adaptation to oxygen limitation is an important aspect of *N. gonorrhoeae* virulence. Our laboratory is interested in looking for factors that are induced during infection, and thus might contribute to the pathogenicity of the bacterium. We therefore wished to analyse the physiological role of AniA in more detail as it appears to be immunodominant and the most strongly expressed of the anaerobically induced outer membrane proteins (Clark et al. 1987). To our surprise, the predicted protein sequence of AniA showed significant homologies to copper-containing nitrite reductases from several denitrifying bacteria.

Bacterial denitrification is the sequential reduction of nitrate to nitrite, nitric oxide (NO), nitrous oxide (N_2O), and finally dinitrogen – a process that primarily uses

energy and occurs anoxically. A large body of evidence suggests that nitric oxide is the product of the reduction of nitrite by both heme cytochrome cd_1- and copper-centred nitrite reductases in denitrifying bacteria (Kakutani et al. 1981a, b; Liu et al. 1986; Zumft et al. 1987), though it was only recently concluded that NO is a true chemical intermediate preceding N_2O in this pathway (for review see Zumft 1993). Additional evidence supporting these conclusions is provided by the following observations. Reduction of nitrite to nitric oxide, and nitric oxide to nitrous oxide are independent enzymatic reactions in *Achromobacter cycloclastes* (Shapleigh et al. 1985, 1987). Secondly, the *norC* and *norB* genes encoding the nitric oxide reductase from *Pseudomonas stutzeri* have been isolated and characterised (Braun and Zumft 1992; Zumft et al. 1994). Finally, mutational inactivation of the heme cytochrome cd_1 nitrite reductase in *P. stutzeri* and the copper nitrite reductase in *Pseudomonas* sp. strain G-179 resulted in loss of the ability to reduce nitrite, but nitric oxide still was reduced (Zumft et al. 1988; Ye and Averill 1992).

We report the construction of an insertional mutation in the *aniA* locus of strain MS11, which enabled us to determine whether this locus was necessary for growth in oxygen-depleted environments, and whether AniA was indeed a nitrite reductase. We also demonstrate that AniA-mediated nitrite reductase activity is inducible. Because gonococci encounter anaerobic conditions in vivo we wished to determine the effect of mutation in *aniA* on critical events in gonococcal pathogenesis.

Materials and methods

Bacterial strains

Gonococcal strains used in this study were derived from the *N. gonorrhoeae* strain MS11. N382 (PilE⁻), a non-piliated MS11 derivative deleted for the loci *pilE1* and *pilE2*, which is known to express Opa_{30} (Makino et al. 1991), was used as a positive control in the growth experiments, nitrite reduction, invasion and binding assays. The MS11 variant N318 (P^+ $PilE_{E1}$) was used for the construction of the *aniA* mutant N910. Strain N302 contains an insertional mutation in $opaC_{30}$, eliminating expression of the Opa_{30} protein.

Media and growth conditions

Strains were grown on GC agar plus vitamin supplement (Becton Dickinson) at 37°C and with 5% CO_2, and passaged daily. Anaerobic cultivation was done in anaerobic chambers containing GasPacks (Becton-Dickinson Laboratories) at 37°C, and the reducing environment was monitored using dry anaerobic indicator strips (BBL). For anaerobic growth, gonococci were inoculated onto GC agar, and a sterile filter disk (Schleicher and Schuell) containing 20 μl of a 20% $NaNO_2$ solution was placed in the center of each plate (Knapp and Clark 1984). Liquid cultures were grown in GC medium containing vitamin supplement and 4 ml of $NaHCO_3$ (8.4%) per litre. Bacteria were added to an OD_{550} of 0.1 and incubated at 37°C. Aerobic cultures were grown in liquid medium equivalent to 10% of the volume of the culture flask, and shaken at 120 rpm. To create microaerophilic growth conditions, $NaNO_2$ was supplemented to 2 mM in liquid GC medium, cultures were grown in completely filled flasks to prevent aeration, and incubation proceeded without shaking (Hoehn and Clark 1992b).

Construction of the mutant

The *aniA* gene was cloned via PCR taking advantage of the previously published sequence (Hoehn and Clark 1992a). The 5′ oligo IH4 (GCCTACTCGAGGGATCCAGGCCCGCAATGGGACA-ACCG), with *Xho*I and *Bam*HI sites, and the 3′ oligo IH5 (CCATCAGCTTAGATCTAGGCTGTTGACAAATTCGGC) with *Bgl*II and *Hind*III sites were used to amplify a 1600-bp fragment which was cloned into pBluescript KS II+, creating pIH11. The vector pIH13 was constructed by inserting the *cat* gene containing the *opa* promotor from pDF4 into the *Pst*I and *Sma*I/*Xma*I sites of *aniA*, creating a 1704-bp fragment. All cloning procedures were performed in *E. coli* DH5α. pIH13 then was transformed into the gonococcal strain N318, selecting for antibiotic resistance on GC agar plates containing 10 μg/ml chloramphenicol. The mutation in the chromosomal *aniA* locus was established via homologous recombination, as pIH13 contains no gonococcal origin of replication.

Protein isolation

Gonococci were collected from solid GC medium using a sterile swab and resuspended in 10 mM TRIS-HCl pH 7.6, containing 5 mM EDTA. Gonococci grown in liquid culture were harvested by centrifugation, and then resuspended in the identical solution. Protein quantities were determined by using a Biorad Protein Assay kit, which is based on the method of Bradford (1976). Samples were combined with equal volumes of 2 × sample solution and boiled for 5 min prior to separation on a 10% gel by SDS-PAGE. Protein was detected by silver staining.

Nitrite reductase activity

Nitrite reductase activity was determined as previously described (Abraham et al. 1993). Briefly, bacteria grown on GC agar were harvested and resuspended in phosphate buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ pH 7.0); bacteria grown in liquid culture were pelleted by centrifugation, washed once and resuspended in the identical buffer. $NaNO_2$ was added to bacterial cell suspensions to a final concentration of 0.1 mM and the reduction of nitrite was monitored over time. Methyl viologen was used as a gratuitous electron donor. Nitrite concentrations in solution were determined colorimetrically (Paik 1980) using a standard curve. Protein quantities were measured with the Biorad assay. Care was taken to ensure that activity was measured under conditions where the rate of nitrite utilisation was linear with both time and protein concentration. Specific activity values are reported as μmoles of NO_2^- produced per min/mg protein (U/mg).

Adherence and invasion assays

The gentamicin invasion assay was performed as described previously (Makino et al.1991; Kupsch et al. 1993) with minor modifications. Epithelial cells (Chang conjunctiva, Flow Laboratories, Irvine, Ayrshire, UK) were grown in RPMI 1640 (Biochrom) medium with 5% FCS, 37°C, 5% CO_2 in 24-well culture plates to confluent layers. After washing epithelial cells with RPMI 1640, the monolayers were infected with 5×10^7 gonococci (multiplicity of infection of approximately 100) in the same medium and incubated for the indicated times at 37°C, 5% CO_2. Cell monolayers were washed three times with PBS and extracellular bacteria were killed by incubation with 100 μg/ml gentamicin for 90 min. Epithelial cells were washed three times, then lysed using 1% saponin and bacterial cfu were determined after serial dilution and growth on GC agar.

To assay for adherence of gonococci to Chang epithelial cells, cytochalasin D (Sigma) was added at 2.5 μg/ml to monolayers 5 min prior to infection. This compound inhibits cytoskeletal rearrangement, and thus also inhibits the internalisation of bacteria, serving as a binding control for the gentamicin invasion assay and

allowing one to monitor the adherence characteristics of the *aniA* mutant strain. Elimination of non-adherent bacteria and cfu determinations were performed as described above.

Results

aniA encodes a putative nitrite reductase

AniA of *N. gonorrhoeae* was previously described as an outer membrane lipoprotein (Hoehn and Clark 1992b), though the authors only speculated as to its function. We therefore used a variety of available databases from the Genetics Computer Group (GCG) Software Package (Devereux et al. 1984) to perform homology searches with the amino acid sequence derived from the *aniA* gene.

Significant homologies to several copper-containing nitrite reductases from different bacterial species were detected (Fig. 1). AniA from *N. gonorrhoeae* showed 34% sequence identity to the nitrite reductase of *Pseudomonas aureofaciens* over a stretch of 319 amino acids, to the nitrite reductase of a different Pseudomonas sp. strain G-179 (33%, 370 residues), to the nitrite reductase of *Alcaligenes faecalis* S-6 (34%; 383) and to the nitrite reductase of *Achromobacter cycloclastes* (36%; 318). These enzymes are thought to consist of three identical subunits (Godden et al. 1991) with six copper atoms, and contain type I and type II copper centers. The type II copper sites, which are located at the interfaces between the monomers, are responsible for nitrite binding (Adman et al. 1995), and reduction (Kukimoto et al. 1995). The blue type I copper sites are located intramolecularly, and are involved in interaction with the electron transfer partner (Kashem et al. 1987). The crystal structure of the *A. cycloclastes* NIR was previously determined, and the residues responsible for type I and type II copper binding were identified. All amino acid residues in the *A. cycloclastes* nitrite reductase which were shown to be essential for copper binding, at both the type I and type II sites, were found to be conserved in the AniA sequence of *N. gonorrhoeae* (Fig. 1). For the type I sites, histidines at positions 145 and 185, a methionine at position 189, and a cysteine at position 176 were conserved; for the type II sites, histidines at positions 139, 175 and 349 were conserved. In addition, the aforementioned amino acids which are responsible for copper binding at the type I and type II centres are conserved in all five of the copper nitrite reductase amino acid sequences presented in Fig. 1.

Mutant construction by allelic replacement

Based on these homologies, we hypothesized that *aniA* encoded a gonococcal nitrite reductase. In order to test this hypothesis, a mutation was constructed in *aniA* by allelic replacement. The chloramphenicol acetyltransferase gene, *cat*, was cloned into the *aniA* structural gene, producing plasmid pIH13 as described in Materials
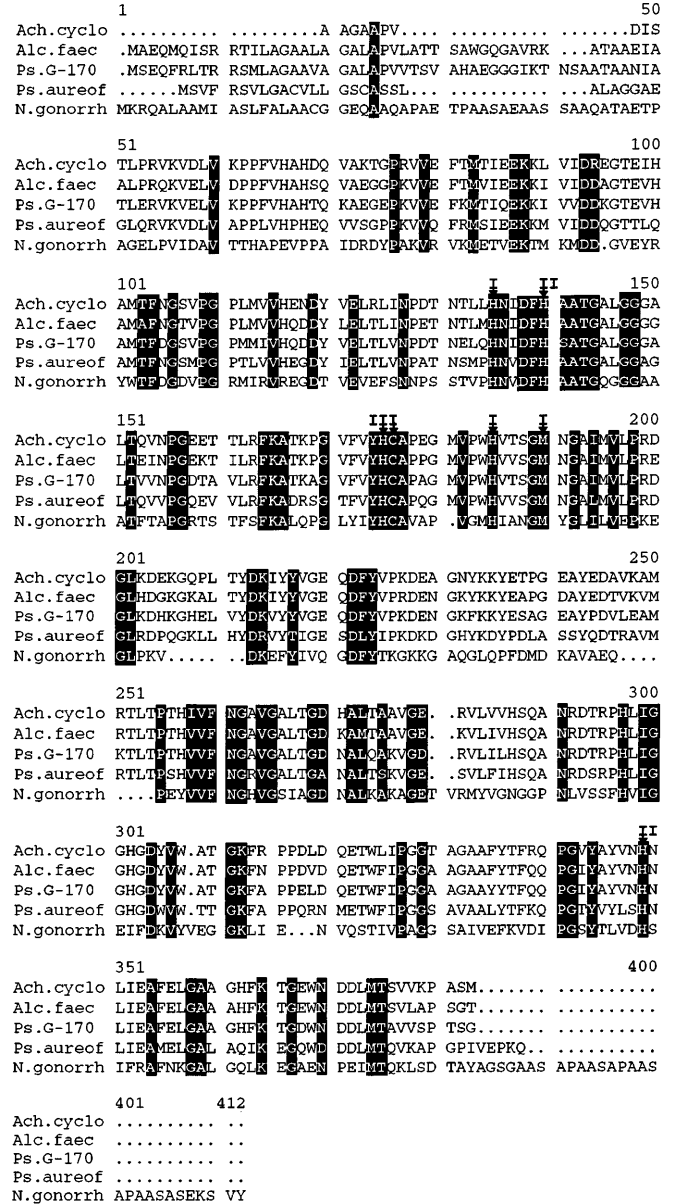
```
            1                                                  50
Ach.cyclo   ........... ..........A AGAAPV.... .......... .......DIS
Alc.faec    .MAEQMQISR RTILAGAALA GALAPVLATT SAWGQGAVRK ...ATAAEIA
Ps.G-170    .MSEQFRLTR RSMLAGAAVA GALAPVVTSV AHAEGGGIKT NSAATAANIA
Ps.aureof   ......MSVF RSVLGACVLL GSCASSL... .......... ...ALAGGAE
N.gonorrh   MKRQALAAMI ASLFALAACG GEQAAQAPAE TPAASAEAAS SAAQATAETP

            51                                                 100
Ach.cyclo   TLPRVKVDLV KPPFVHAHDQ VAKTGPRVVE FTMTIBEKKL VIDREGTEIH
Alc.faec    ALPRQKVELV DPPFVHAHSQ VAEGGPKVVE FTMVIBEKKI VIDDAGTEVH
Ps.G-170    TLERVKVELV KPPFVHAHTQ KAEGEPKVVE FKMTIQEKKI VVDDKGTEVH
Ps.aureof   GLQRVKVDLV APPLVHPHEQ VVSGPPKVVQ FRMSIBEKKM VIDDQGTTLQ
N.gonorrh   AGELPVIDAV TTHAPEVPPA IDRDYPAKVR VKMETVEKIM KMDD.GVEYR

            101                      I     II            150
Ach.cyclo   AMTFNGSVPG PLMVVHENDY VELRLINPDT NTLLHNIDFH AATGALGGCA
Alc.faec    AMAFNGTVPG PLMVVHQDDY LELTLINPET NTLMHNIDFH AATGALGGGG
Ps.G-170    AMTFDGSVPG PMMIVHQDDY VELTLVNPDT NELQHNIDFH SATGALGGGA
Ps.aureof   AMTFNGSMPG PTLVVHEGDY IELTLVNPAT NSMPHNVDFH AATGALGGCA
N.gonorrh   YWTFDGDVPG RMIRVREGDT VEVEFSNNPS STVPHNVDFH AATGQGGGAA

            151            III        I     I           200
Ach.cyclo   LTQVNPGEET TLRFKATKPS VFVYHCAPEG MVPWEVTSGM NCAIMVLERD
Alc.faec    LTEINPGEKT ILRFKATKPG VFVYHCAPPG MVPWEVVSGM NCAIMVLRRE
Ps.G-170    LTVVNPGDTA VLRFKATKAG VFVYHCAPAG MVPWEVTSGM NCAIMVLERD
Ps.aureof   LTQVVPGQEV VLRFKADRSS TFVYHCAPQG MVPWEVVSGM NCAIMVLERD
N.gonorrh   ATFTAPGRTS TFSFKALQPS LYIYHCAVAP .VGMHIANGM YSLILVPEKE

            201                                              250
Ach.cyclo   GLKDEKGQPL TYDKIYYVGE QDFYVPKDEA GNYKKYETPG EAYEDAVKAM
Alc.faec    GLHDGKGKAL TYDKIYYVGE QDFYVPRDEN GKYKKYEAPG DAYEDTVKVM
Ps.G-170    GLKDHKGHEL VYDKVYYVGE QDFYVPKDEN GKFKKYESAG EAYPDVLEAM
Ps.aureof   GLRDPQGKLL HYDRVYTIGE SDLYIPKDKD GHYKDYPDLA SSYQDTRAVM
N.gonorrh   GLPKV..... ..DKEFYIVQ GDFYTKGKKG AQGLQPFDMD KAVAEQ....

            251                                              300
Ach.cyclo   RTLTPTHIVF NGAVGALTGD HALTAAVGE. .RVLVHSQA NRDTRPHLIG
Alc.faec    RTLTPTHVVF NGAVGALTGD KAMTAAVGE. .KVLIVHSQA NRDTRPHLIG
Ps.G-170    KTLTPTHVVF NGAVGALTGD NALQAKVGD. .RVLILHSQA NRDTRPHLIG
Ps.aureof   RTLTPSHVVF NGRVGALTGA NALTSKVGE. .SVLFIHSQA NRDSRPHLIG
N.gonorrh   .....PEYVVF NGHVGSIAGD NALKAKAGET VRMYVGNGGP NLVSSFHVIG

            301                                              II
Ach.cyclo   GHGDYVW.AT GKFR PPDLD QETWLIPGGT AGAAFYTFRQ PGVYAYVNHN
Alc.faec    GHGDYVW.AT GKFN PPDVD QETWFIPGGA AGAAFYTFQQ PGIYAYVNHN
Ps.G-170    GHGDYVW.AT GKFA PPELD QETWFIPGGA AGAAYYTFQQ PGIYAYVNHN
Ps.aureof   GHGDWW.TT GKFA PPQRN METWFIPGGS AVAALYTFKQ PGIYVYLSHN
N.gonorrh   EIFDKMYVEG GKLI E...N VQSTIVPAGG SAIVEFKVDI PGSYTLVDHS

            351                                              400
Ach.cyclo   LIBAFELGAA GHFK TGEWN DDLMTSVVKP ASM....... ..........
Alc.faec    LIBAFELGAA AHFK TGEWN DDLMTSVLAP SGT....... ..........
Ps.G-170    LIBAFELGAA GHFK TGDWN DDLMTAVVSP TSG....... ..........
Ps.aureof   LIBAMELGAL AQIK EGQWD DDLMTQVKAP GPIVEPKQ.. ..........
N.gonorrh   IFRAFNKGAL GQLK EGAEN PEIMTQKLSD TAYAGSGAAS APAASAPAAS

            401         412
Ach.cyclo   .......... ..
Alc.faec    .......... ..
Ps.G-170    .......... ..
Ps.aureof   .......... ..
N.gonorrh   APAASASEKS VY
```

Fig. 1 Comparison of amino acid sequences of nitrite reductases of *A. cycloclastes, A. faecalis, Pseudomonas sp.* (strain G-179), *P. aureofaciens* and AniA of *N. gonorrhoeae* strain MS11. Identical amino acids in all four species are highlighted in *black*, and residues essential for the copper binding are indicated by the *arrows* and labeled I (for the type I copper centre) and II (for the type II copper centre)

and methods (Fig. 2A). Plasmid pIH13 then was transformed into the *N. gonorrhoeae* strain MS11, thus facilitating homologous recombination into the wild-type *aniA* locus and creating strain N910. Recombination of the *cat* gene into the *aniA* locus was confirmed by PCR (data not shown).

AniA is induced when gonococci are cultured anaerobically in vitro, and migrates on SDS gels at a rate equivalent to a molecular weight corresponding to approximately 54 kDa (Clark et al. 1987). Figure 2B demonstrates that AniA was not expressed by the mutant N910 under inducing conditions (lane 3). Induction
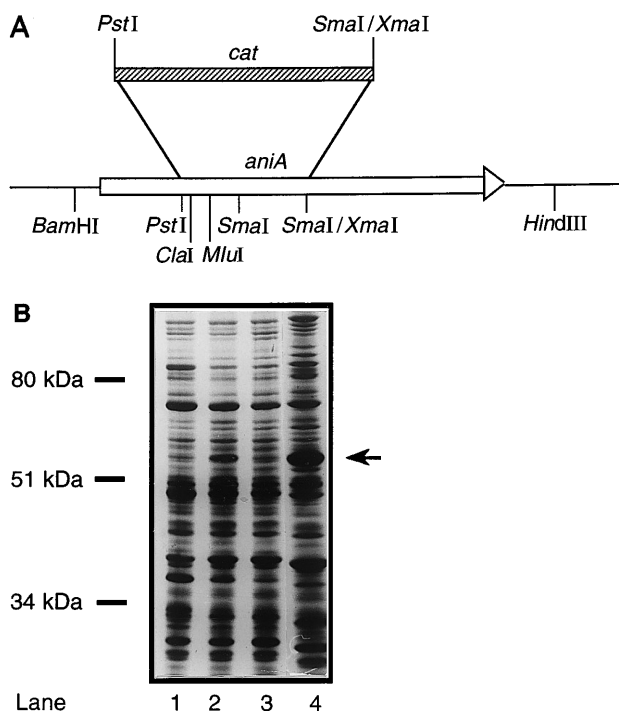
Fig. 2A, B Construction of the *aniA* mutant. **A** Insertion of a *cat* cassette into the *Pst*I and *Sma*I/*Xma*I sites of *aniA*. The *boxed arrow* depicts the *aniA* structural gene. **B** Elimination of AniA expression under inducing conditions in the mutant N910. Lane 3 contains silver-stained whole cell proteins of the mutant N910 isolated after incubation under inducing conditions (microaerophilically in liquid GC medium). Whole cell proteins of gonococcal strain N382 grown aerobically (lane 1) and microaerophilically (lane 2) in liquid GC medium, and anaerobically on GC agar (lane 4) are also presented. The *arrow* points to the AniA protein, with a mobility equivalent to a molecular weight of approximately 54 kDa (Clark et al. 1987)

of AniA in strain N382 under both microaerophilic (lane 2) and anaerobic (lane 4) growth conditions was noted.

The *aniA* mutation affects growth and viability in a microaerophilic environment

It was previously reported that gonococci grow microaerophilically in vitro only in the presence of nitrite (Knapp and Clark 1984). We therefore hypothesised that mutation of *aniA* would eliminate the ability of the gonococcus to grow in this environment. Gonococcal cell growth was monitored by optical density and survival by determining numbers of cfu from subsequent dilutions onto GC agar plates. By both methods, we observed that the strain containing wild-type *aniA* was able to grow in the presence of NO_2^-, whereas the mutant N910 was severely impaired in its ability to grow under the same conditions (Fig. 3A). Figure 3B demonstrates that under microaerophilic conditions, a threefold loss in viability was observed for the mutant strain N910, decreasing from 1.2×10^7 to 3.5×10^6 cfu over 7 h. We observed a similar loss in viability of the
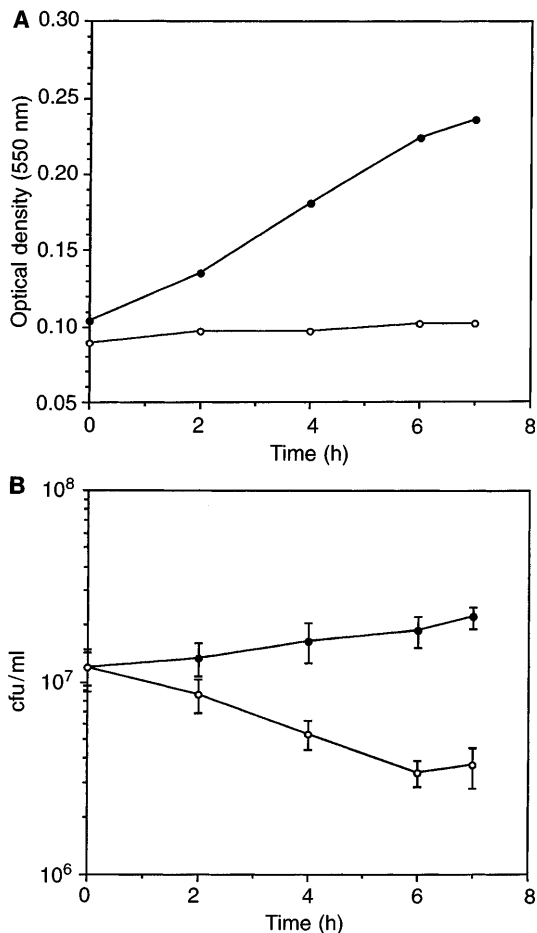
Fig. 3 A mutation in *aniA* affects microaerophilic growth of gonococci. **A** Bacteria were incubated microaerophilically in liquid GC medium and OD_{550} values were monitored at the indicated times. N382 (*filled circles*); N910 (*open circles*). **B** To determine whether the mutant remained viable, aliquots of the cultures in **A** were plated on solid GC medium and incubated for approximately 24 h before the numbers of colony forming units (cfu/ml) were determined. The data are means of four independent experiments and the standard errors are indicated by the *bars*. Strains are indicated as in **A**

mutant in GC liquid medium containing no nitrite (data not shown), indicating that the loss in viability was not simply due to nitrite toxicity. Based on OD_{550} values and cfu determinations the strain N382 undergoes approximately one doubling under these conditions (Fig. 3). No anaerobic growth of strain N910 was observed in the presence of nitrite – after 72 h incubation on GC agar individual colonies were not visible under a dissection microscope (data not shown).

Nitrite reductase activity is inducible

Protein sequence homologies and the inability of strain N910 to grow microaerophilically or anaerobically in the presence of nitrite strongly suggested that the gonococcal *aniA* gene encoded a nitrite reductase. Based on the anaerobic (Clark et al. 1987; Hoehn and Clark

1992a; Fig. 2B) and microaerophilic (Fig. 2B) induction of AniA protein expression, it also seemed likely that this activity was inducible.

To demonstrate whether the gonococcal AniA protein was indeed a nitrite reductase with inducible activity we performed a standard nitrite reduction test (Paik 1980; Abraham et al. 1993). Strains N382 and N910 were grown under microaerophilic conditions in liquid GC medium supplemented with 2 mM $NaNO_2$ as a source of nitrite, and specific activity values were monitored at the indicated times (Fig. 4). In agreement with protein profiles showing induction of AniA when gonococci were grown microaerophilically (Fig. 2B, lane 2), the nitrite reductase activity depicted in Fig. 4 was also inducible. For strain N382, containing the wild-type *aniA* locus, nitrite reductase specific activity increased from approximately 0.15 to 1.5 U/mg, reaching a maximum level of activity after 3 to 4 h in the microaerophilic environment. No nitrite reductase activity was detected for strain N910 under the identical growth conditions.

We grew gonococci anaerobically and aerobically on GC agar to determine the maximal levels of nitrite reductase activity and whether any activity was detectable in aerobically grown strains. Nitrite reductase activity derived from strain N382 was 0.079 and 10.4 U/mg when grown aerobically and anaerobically, respectively (Table 1). The high level of activity detected for strain N382 grown anaerobically was consistent with strong expression of AniA protein under the same growth conditions (Fig. 2B). No detectable nitrite reductase activity was observed when strain N910 was grown aerobically.

Based on protein sequence homologies, the inability of strain N910 to grow anaerobically or microaerophilically (Fig. 3) in the presence of nitrite, the in-
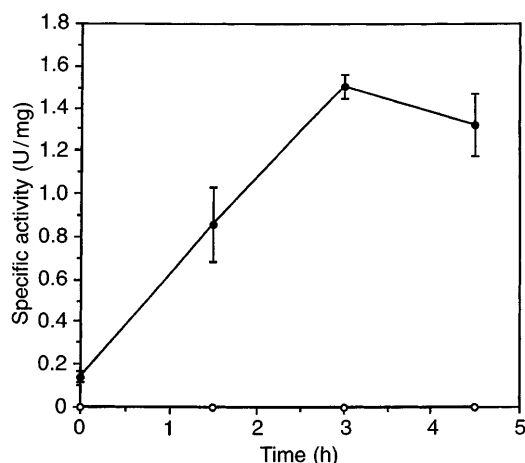
Table 1 Total nitrite reductase activities in resting cells

	Specific activity (U/mg)[a]	
Strain	N382	N910
Growth		
Aerobic	0.079 ± 0.02	–[b]
Anaerobic	10.4 ± 2.4	–[c]

[a] Methyl viologen was used as a gratuitous electron donor. Specific activity values are the average of three independent assays, each performed in duplicate. Standard error values are included
[b] No detectable nitrite reductase activity
[c] Strain N910 does not grow anaerobically on GC agar

ducible total nitrite reductase activities in strain N382 (Fig. 4, Table 1), which were consistent with induction of expression of AniA protein under the same growth conditions (Fig. 2B), and the lack of detectable nitrite reductase activity in strain N910, we concluded that *aniA* encodes an inducible *N. gonorhoeae* nitrite reductase.

Is AniA involved in gonococcal pathogenesis?

Gonococci encounter, and must adapt to, oxygen-limiting conditions in vivo; we therefore wished to determine whether *aniA* was directly involved in the gonococcal infection process. In addition, because AniA is localised in the outer membrane and reacts strongly with sera isolated from patients suffering from gonococcal infections, it was previously proposed that this protein might be a novel adhesin molecule which enables gonococci to adhere to and colonise reduced sites in the urogenital tract (Hoehn and Clark 1992b). We therefore tested whether the mutation in *aniA* affected the ability of gonococci to invade, or adhere to, Chang conjunctival epithelial cells in culture.

In gonococci, the Opa_{30} protein is involved in the intimate attachment to, and internalisation by, epithelial cells in culture (Makino et al. 1991). Western blot analysis showed that strains N382 and N910 produced Opa_{30}, whereas strain N302 did not (data not shown). The mutation in *aniA* did not affect the ability of strain N910 to invade, or survive internalisation by epithelial cells as gentamicin effectively kills extracellular gonococci (Fig. 5A). Figure 5B demonstrates that adherence by the mutant was approximately equal to that of strain N382 after 6 h of infection.

Discussion

Initial studies of AniA of *N. gonorrhoeae* established that this protein bears a lipid modification at the N-terminus, is associated with the outer membrane and its expression is strictly regulated by oxygen (Hoehn and Clark 1992a, b). A physiological function for the *aniA* gene product, however, was not established. The identification of AniA in preparations of gonococcal outer membrane proteins, its strong reaction with sera isolated
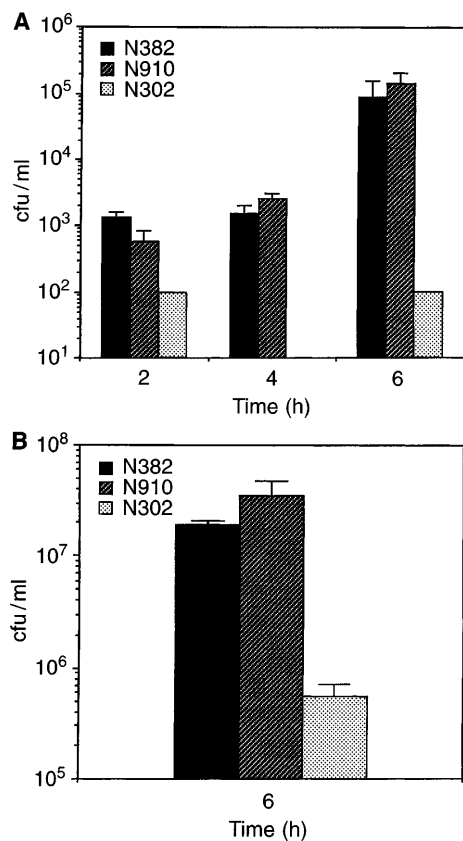

Fig. 4 Nitrite reductase activity is inducible. Strains N382 and N910 were grown microaerophilically, and the specific activity of nitrite reductase was monitored at the indicated times. Data are averages of two independent determinations and the standard error values are presented. Units are defined as μmol NO_2^- per min. *Filled circles* N382; *open circles* N910

Fig. 5A, B Effect of an *aniA* mutation on the gonococcal infection process. **A** Opa$_{30}$-mediated internalisation of gonococci by Chang epithelial cells was monitored by the gentamicin-based invasion assay at 2, 4, and 6 h after initial infection. **B** Opa$_{30}$-mediated adherence after 6 h of infection. Adherence of strains N382 and N910, as assessed by visual inspection under light microscopy (data not shown), was similar at the 2 and 4 h time points. Data are from four independent experiments and standard error values are indicated

from patients suffering from gonococcal infections and the association of clinical isolates with facultative and obligate anaerobes prompted Hoehn and Clark (1992b) to speculate that this protein might be an accessory protein necessary for survival under the anaerobic conditions encountered in vivo, perhaps be involved in establishing infections, or possibly a novel adhesive molecule involved in colonisation (Hoehn and Clark 1992b). AniA sequence homologies with nitrite reductases of denitrifying bacteria, the inability of an *aniA* mutant to grow microaerophilically in the presence of nitrite, the strong induction of AniA protein expression under oxygen limitation, and the results of nitrite reduction assays clearly indicated that the *aniA* gene product is an inducible gonococcal nitrite reductase.

Significant protein sequence homologies to AniA were also found to a lesser degree to the azurin-like protein from *Neisseria* (Gotschlich and Seiff 1987), which shows 24% identity in a stretch of 197 amino acids. In *Alcaligenes faecalis* S-6 (Kukimoto et al. 1995) and *Achromobacter cycloclastes* (Liu et al. 1986) the azurin-like protein is the redox partner of nitrite

reductase, and it was recently reported that the region surrounding the type I copper of pseudoazurin of *A. faecalis* S-6 is responsible for interaction with nitrite reductase (Kukimoto 1995).

AniA is thought to associate with the outer membrane, and both the gonococcal AniA and azurin-like proteins are lipid-modified (Gotschlich and Seiff 1987; Hoehn and Clark 1992b). All copper-containing nitrite reductases whose amino acid sequences appear in Fig. 1 were reported to be soluble periplasmic enzymes. Like their soluble homologues in other bacterial species the, *N. gonorrhoeae* nitrite reductase and azurin-like proteins seem to be extra-cytoplasmic, but the latter are most probably attached to the outer membrane by a lipid anchor. This might explain why *AniA* co-purifies with proteins of the outer membrane, which is a feature unique among known nitrite reductases, and perhaps provides an explanation for why it seems to be an immunodominant protein in gonococcal infections (Clark et al. 1988). Protein sequence homologies and observations on the homologous nitrite reductase systems of denitrifying bacteria indicate that the small blue copper azurin-like protein and the *aniA* gene product in *N. gonorrhoeae* might possess similar electron transfer systems, thus enabling electron transfer between the two copper-containing proteins. Our data clearly support previous observations concerning the inducibility of *aniA* (Hoehn and Clark 1992a), and begin to explain its physiological role during growth in the absence of oxygen.

We have demonstrated that AniA is essential for growth and survival of gonococci in oxygen-depleted environments (Fig. 3). Gonococci cannot grow anaerobically by fermentation, and thus it seems likely that the reduction of nitrite must be essential for maintenance of an oxidation reduction balance in the cell, even if nitrite reduction is not directly associated with ATP synthesis. From gas chromatographic (Zumft et al. 1987) and other analyses of the products of nitrite reduction by related copper-centred enzymes, the product of the reduction of nitrite by AniA is presumed to be nitric oxide. Although this agent is toxic, it is now accepted as a stable intermediate in denitrification. NO is therefore, most probably, rapidly reduced by a separate enzyme in gonococci, possibly by a cytoplasmic-membrane bound protein with a periplasmically oriented active site as proposed in the denitrifier *Pseudomonas stutzeri* (Zumft 1993). That a gonococcal NO reductase may exist is also supported by our finding of homology (35% identity in a stretch of 85 amino acids) between NorB of *P. stutzeri* and gonococcal protein sequences predicted from DNA sequences produced in the course of the *N. gonorrhoeae* Genome Sequencing Project (Gish and States 1993).

Nitrite and NO reduction in *P. stutzeri* are functionally coupled (Zumft 1992; Jüngst and Zumft 1992), and NO reduction in several denitrifying bacterial species is accompanied by proton translocation (Shapleigh and Payne 1985). Perhaps reduction of nitric oxide generates a proton motive force in gonococci, thus supporting anaerobic growth.

Though we observed no activity in the *aniA* strain N910 under any of the conditions tested, we cannot eliminate the possibility that gonococci contain an additional nitrite reductase activity. We did observe measurable nitrite reductase activity in strain N382 grown aerobically. By Western and Northern blot analysis Hoehn and Clark (1992a) did not detect AniA protein or mRNA, respectively, in *N. gonorrhoeae* strain F62 grown aerobically on GC agar. We therefore cannot absolutely conclude that AniA is the only enzyme able to reduce nitrite in gonococci. An alternative explanation for the lack of protein expression but the existence of measurable activity in aerobically grown gonococci is that low-level expression of *aniA* occurs under non-inducing conditions, possibly resulting from subpopulations of bacteria growing under oxygen limitation.

Though the anaerobically inducible nitrite reductase of *N. gonorrhoeae* is probably not directly linked with virulence, as it does not affect the ability of gonococci to bind to, or invade, epithelial cells, adaptation to anaerobic environments is obviously an important aspect of gonococcal pathogenesis where AniA plays an essential role.

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