***Neisseria gonorrhoeae* bacterioferritin: structural heterogeneity, involvement in iron storage and protection against oxidative stress**

Cheng-Yen Chen and Stephen A. Morse

Author for correspondence: Cheng-Yen Chen. Tel: +1 404 639 3154. Fax: +1 404 639 3976.
e-mail: cyc1@cdc.gov

Division of AIDS, Sexually Transmitted Diseases and Tuberculosis Laboratory Research, National Centers for Infectious Disease, Centers for Disease Control and Prevention, 1600 Clifton Rd, Atlanta, GA 30333, USA

The iron-storage protein bacterioferritin (Bfr) from *Neisseria gonorrhoeae* strain F62 was identified in cell-free extracts and subsequently purified by column chromatography. Gonococcal Bfr had an estimated molecular mass of 400 kDa by gel filtration; however, analysis by SDS-PAGE revealed that it was composed of 18 kDa (BfrA) and 22 kDa (BfrB) subunits. DNA encoding BfrB was amplified by PCR using degenerate primers derived from the N-terminal amino acid sequence of BfrB and from a C-terminal amino acid sequence of *Escherichia coli* Bfr. The DNA sequence of *bfrA* was subsequently obtained by genome walking using single-specific-primer PCR. The two Bfr genes were located in tandem with an intervening gap of 27 bp. A potential Fur-binding sequence (12 of 19 bp identical to the consensus neisserial *fur* sequence) was located within the 5' flanking region of *bfrA* in front of a putative –35 hexamer. The homology between the DNA sequences of *bfrA* and *bfrB* was 55.7%; the deduced amino acid sequences of BfrA (154 residues) and BfrB (157 residues) showed 39.7% identity, and showed 41.3% and 56.1% identity, respectively, to *E. coli* Bfr. Expression of recombinant BfrA and BfrB in *E. coli* strain DH5 α was detected on Western blots probed with polyclonal anti-*E. coli* Bfr antiserum. Most Bfrs are homopolymers with identical subunits; however, the evidence presented here suggests that gonococcal Bfr was composed of two similar but not identical subunits, both of which appear to be required for the formation of a functional Bfr. A Bfr-deficient mutant was constructed by inserting the Ω fragment into the BfrB gene. The growth of the BfrB-deficient mutant in complex medium was reduced under iron-limited conditions. The BfrB-deficient mutant was also more sensitive to killing by H₂O₂ and paraquat than the isogenic parent strain. These results demonstrate that gonococcal Bfr plays an important role in iron storage and protection from iron-mediated oxidative stress.

Keywords: *Neisseria gonorrhoeae*, iron-storage protein, bacterioferritin

INTRODUCTION

Iron is important for the growth of bacteria, as evidenced by the wide variety of molecules and mechanisms that have evolved to capture and retain this essential element. However, in the presence of O₂, iron is potentially toxic because of its catalytic generation of cell-damaging free

radicals (Flitter *et al.*, 1983; Halliwell & Gutteridge, 1984; Dunford, 1987). To sequester iron for essential requirements and to avoid toxicity from iron overload, aerobic organisms have evolved well-coordinated systems controlling the acquisition, transportation and storage of this element (Briat, 1992).

Two types of iron-storage proteins have been identified in bacteria (Harrison & Arosio, 1996): bacterioferritin (Bfr), which contains non-covalently bound haem groups in addition to a non-haem iron core (Stiefel & Watt, 1979); and ferritin, which does not possess intrinsic haem groups and resembles the iron-containing

Abbreviations: Bfr, bacterioferritin; DF, Desferal; SSP-PCR, single-specific-primer PCR.

The GenBank accession numbers for the sequences reported in this paper are U76633 and U76634.

protein (H-type subunit) of mammalian cells (Ford *et al.*, 1984). Bfrs are characteristically composed of 24 identical 18–22 kDa subunits, which are assembled into a spherical protein shell containing 0.2–20% (approx. 600–2400 iron atoms per molecule) by weight of non-haem iron and 3–12 non-covalently bound protohaem IX groups (Yariv *et al.*, 1981; Ford *et al.*, 1984; Frolow *et al.*, 1994). Subunits are tightly assembled to form a molecule with fourfold, threefold and twofold symmetry axes (432 symmetry) (Ford *et al.*, 1984). Results of electron-microscopic studies have indicated that the iron core can be either crystalline (ferrihydrate) or amorphous. Although the mechanism of iron storage *in vivo* is uncertain, *in vitro*, iron-core formation involves the oxidation of Fe(II) and hydrolytic polymerization of Fe(III) (Andrews *et al.*, 1993; Hudson *et al.*, 1993).

Studies on *Neisseria gonorrhoeae* have focused primarily on the receptor-mediated mechanisms that this organism uses to obtain iron from host sources; very little is known about the fate of intracellular iron. In this study, we report the identification and purification of a Bfr from *N. gonorrhoeae* composed of two non-identical subunits; the genes encoding these subunits have been cloned and sequenced. A BfrB-deficient mutant was constructed to examine the biological role of gonococcal Bfr.

METHODS

Organisms and growth conditions. *N. gonorrhoeae* strain F62 was used for purification of Bfr. This strain was maintained on GC agar (Difco Laboratories) supplemented with 1% (v/v) IsoVitalEx (Baltimore Biological Laboratories). To determine the effect of iron limitation on the growth of a gonococcal BfrB-deficient mutant, cells were grown in GC broth (Mietzner *et al.*, 1984) supplemented with 1% IsoVitalEx, 0.04% (w/v) NaHCO₃, and with or without 30 µM Desferal (DF) (Ciba-Geigy). Broth cultures were incubated at 37 °C in a gyratory shaker and growth was followed for 8 h by monitoring the optical density of the culture at 600 nm in a model DU-70 spectrophotometer (Beckman Instruments). *Escherichia coli* strain DH5α was used as a host for recombinant plasmids and was grown on Luria broth (LB) agar. The Ω fragment was purified from *E. coli* strain HB101 containing pHP45Ω. This was accomplished by purifying pHP45Ω with a plasmid midi-kit (Qiagen), digesting the plasmid with *Hind*III and then by gel purifying the 2 kb DNA band containing the Ω fragment. All cultures were grown at 37 °C in a humidified atmosphere containing 5% CO₂. pUC18 was purchased from Bethesda Research Laboratories.

Purification of gonococcal Bfr. Overnight growth of *N. gonorrhoeae* strain F62 was harvested from GC agar plates and resuspended in 20 mM Tris/HCl buffer (pH 7.2). The cell suspension was sonicated (30 s, 6 times) and then centrifuged at 10000 g for 20 min. The supernatant was decanted, heated to 65–70 °C for 15 min and centrifuged at 10000 g for 20 min. The heat-treated supernatant was then fractionated by precipitation with (NH₄)₂SO₄. The fraction that precipitated between 30 and 60% saturation was collected by centrifugation (10000 g, 15 min), dissolved in 20 mM Tris/HCl buffer (pH 7.2) and desalted by dialysis against the same buffer overnight at 4 °C. The dialysed material was applied to a Sephacryl S-200-HR (Sigma) gel-filtration column (0.9 × 60 cm) that had been equilibrated with 20 mM Tris/

HCl buffer (pH 7.4) containing 0.15 M NaCl. The molecular mass standards (Bio-Rad) used were thyroglobulin (670 kDa), bovine gamma globulin (158 kDa), chicken ovalbumin (44 kDa), equine myoglobin (17 kDa) and vitamin B₁₂ (1.35 kDa). The iron-stain-positive fractions, corresponding to 300–500 kDa molecular mass, were pooled and concentrated by centrifugation (Centriprep-30; Amicon). The retentate was dialysed overnight against 20 mM Tris/HCl buffer (pH 7.2) and then fractionated by anion-exchange chromatography using a DEAE Sepharose CL-6B (Sigma) column (2.8 × 11 cm). Proteins were eluted with a linear gradient of 0.1–0.6 M NaCl; Bfr eluted at approximately 0.3 M NaCl. The purity of Bfr was assessed by SDS-PAGE and by iron-specific staining of non-denaturing polyacrylamide gels.

Iron-specific stain. After electrophoresis of cell-free extracts in non-denaturing polyacrylamide gels, iron-containing proteins were visualized by staining with a solution of 0.75 mM 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine, sodium salt (Ferrozine; Sigma) and 15 mM thioglycolic acid in 2% (v/v) acetic acid (Chung, 1985). To locate column fractions containing iron, an aliquot of each column fraction (200 µl) was added to the Ferrozine reagent (5 µl) and the absorbance at 562 nm was measured after 15 min. Iron-containing protein bands or column fractions exhibited a visible red colour within 20 min of staining. The iron-stained gel was subsequently stained with Coomassie blue and destained with 10% (v/v) acetic acid in order to visualize the presence of any other cellular proteins.

N-terminal sequence analysis. Purified Bfr was subjected to SDS-PAGE and electro-transferred onto a PVDF membrane (Schleicher & Schuell). Protein bands were excised from the transblot and sequenced by standard Edman chemistry on a Beckman PI2090 Integrated Micro-sequencing System.

PCR and DNA sequence analysis. *bfrB* was PCR-amplified using a forward degenerate primer (964FP, Table 1), deduced from the N-terminal amino acid sequence of gonococcal BfrB, and a reverse degenerate primer (966RP, Table 1), based on a conserved region in the C-terminal of the *E. coli* Bfr (amino acids 128–134) (Andrews *et al.*, 1989; Denoel *et al.*, 1995). PCR conditions consisted of 30 cycles of denaturation at 94 °C for 1.5 min, annealing at 62 °C for 2 min and extension at 72 °C for 2 min. The flanking regions and the remaining sequences of gonococcal *bfrA* and *bfrB* were completed by using single-specific-primer PCR (SSP-PCR) (Shyamala & Ames, 1989). The Applied Biosystems model 373 DNA sequencing system (Perkin-Elmer) was used for sequencing the PCR products according to the manufacturer's cycle sequencing protocol by using dye-terminator chemistry (Sanger *et al.*, 1977).

Cloning of Bfr genes. The entire *bfrA* and *bfrB* coding sequences were amplified by PCR using synthetic primers designed to introduce an *Eco*RI restriction site at the start codon (FPEco1, Table 1) and a *Bam*HI site just downstream of the stop codon (RPBam1, Table 1). The PCR product was double-digested with *Bam*HI and *Eco*RI and ligated into pUC18. Competent *E. coli* DH5α cells were transformed and ampicillin-resistant transformants selected on LB medium containing 50 µg ampicillin ml⁻¹. Plasmid minipreps from several transformants were prepared and sequenced to confirm that the *bfr* sequence was correct and in-frame with *lacZ*. The recombinant plasmid in one of the transformants was selected and designated pCYC-1.

Expression of gonococcal Bfr. Whole-cell lysates prepared from *E. coli* DH5α, DH5α with pUC18, or DH5α with pCYC-1 were fractionated by SDS-PAGE and electro-transferred

Table 1. Primer sequences of oligonucleotides used in this study

Primer designation	Sequence*	Use
964FP	GTIATTCGIGAAGCTIAACAAAAA	Amplification of <i>bfrB</i>
966RP	CCAITCIATITGICCTCITC	Same as above
FPEco1	CCCGAATTCATGCAAGGCAATCAAGCTGTT	Amplification of <i>bfrA</i> and <i>bfrB</i>
RPBam1	CCCGGATCCGTCCTCTTGCGCCGCTGTTTG	Same as above
FPEco2	CCCAATTCACCGTTTGCGAGGAAGCCTC	Amplification of <i>bfrA</i> and <i>bfrB</i> and flanking regions
RPBam2	CCCGGATCCCATATCGGTATTATCAAACAA	Same as above

* I represents inosine.

onto a nitrocellulose membrane (Schleicher & Schuell). The blot was probed with anti-*E. coli* Bfr polyclonal antiserum (1:1000) and bound antibody detected with horseradish-peroxidase-labelled goat anti-rabbit IgG conjugate (Bio-Rad).

Construction of a BfrB-deficient mutant of *N. gonorrhoeae*.

The sequence encompassing *bfrA* and *bfrB* and their corresponding flanking regions were amplified by PCR using synthetic oligonucleotide primers (FPEco2 and RPBam2, Table 1) designed to introduce an *EcoRI* restriction site 1.1 kb upstream of the start codon of *bfrA* and a *BamHI* site 163 bp downstream of the stop codon of *bfrB*. The resulting 2.4 kb PCR fragment was double-digested with *BamHI* and *EcoRI* and ligated into pUC18. The single *HindIII* restriction site on pUC18 had been previously eliminated by digesting with the enzyme, end-filled using the Klenow fragment and religated. Competent *E. coli* DH5 α cells were subsequently used for transformation. Ampicillin-resistant transformants were obtained on LB medium containing 50 μ g ampicillin ml⁻¹. Plasmid minipreps were prepared to confirm the presence of the 2.4 kb DNA insert. The recombinant plasmid, designated pCYC-2, was digested with *HindIII*, which cuts only once in the coding region of *bfrB*, and ligated with the purified Ω fragment prepared with *HindIII* ends. Competent *E. coli* DH5 α cells were transformed as previously described and transformants selected on LB medium containing 30 μ g ampicillin ml⁻¹ and 30 μ g spectinomycin ml⁻¹. The pCYC-2 containing the insertionally inactivated *bfrB*, designated pCYC-3, was purified from several transformants to confirm the insertion of the Ω fragment and then linearized with *EcoRI* prior to transforming pilated cells of *N. gonorrhoeae* strain F62. Gonococcal transformants were selected on GC agar plates containing 30 μ g streptomycin ml⁻¹ and 30 μ g spectinomycin ml⁻¹. The presence of the Ω fragment in *bfrB* was confirmed by PCR amplification of the BfrB structural gene. One of the transformants, designated F62BfrB Ω , was selected for further studies.

Sensitivity to H₂O₂ and paraquat. The sensitivity of *N. gonorrhoeae* strain F62 and its isogenic BfrB-deficient mutant to H₂O₂ and paraquat was determined by a disc-diffusion assay. Gonococci were grown overnight on GC agar plates and resuspended in GC broth to a concentration of 10⁸ c.f.u ml⁻¹ and 100 μ l of the cell suspension was spread onto GC agar plates. Filter paper discs (1/4 in. in diameter, Schleicher & Schuell), containing various amounts of H₂O₂ (0–5 μ mol) or paraquat (0–3 μ mol), were placed on the surface of the plate. After 24 h incubation at 35 °C with 5% CO₂, plates were examined for zones of growth inhibition. The zone of growth

inhibition was determined by measuring the diameter (cm) of the clear zone surrounding the disc minus the diameter of the disc. The mean zone of inhibition $\pm$ standard deviation was calculated from the results of three separate experiments.

RESULTS

Purification of gonococcal Bfr

Bfr was initially purified by size-exclusion chromatography and its location identified by the addition of Ferrozine to each column fraction and monitoring of OD at 562 nm. Bfr-containing fractions exhibited the highest OD₅₆₂ and were subjected to non-denaturing PAGE followed by the iron-specific stain. Subsequent staining of the same gel with Coomassie blue revealed the presence of several protein-containing bands; however, Bfr was the only protein that could be visualized by the iron-specific stain. The molecular mass of Bfr, as estimated by gel filtration, was between 300 and 500 kDa. DEAE-Sepharose column chromatography was subsequently used for further purification of Bfr. Fractions eluted by a NaCl gradient, which exhibited the highest OD at 562 nm in the presence of Ferrozine, were assessed by SDS-PAGE (12% acrylamide) and Coomassie blue staining. The data in Fig. 1 show that, without heating, purified Bfr migrated as a single protein-containing band at a slower rate than the 97 kDa molecular mass standard (lane 1). However, after heating at 100 °C for 10 min in sample loading buffer, Bfr migrated as two smaller bands with estimated molecular masses of 18 and 22 kDa, respectively (lane 2, shown with arrows). It was noted that after heating and electrophoresis, Bfr could not be visualized by the iron-specific stain (data not shown).

N-terminal sequence analysis

The bands containing the 18 and 22 kDa proteins were eluted from the gels and the sequence of the first 10 (MQGNQAVVDY) N-terminal residues of the 18 kDa protein and the first 15 (MKGDRLVIRELNKNL) N-terminal residues of the 22 kDa protein were obtained by automated Edman degradation. These N-terminal sequences showed similarities (30–66%) to the N-

Fig. 1. Coomassie blue-stained SDS-PAGE (12 % acrylamide) of the multimeric (lane 1) and monomeric (lane 2) forms of *N. gonorrhoeae* Bfr. Lanes: 1, purified Bfr without heating at 100 °C; 2, purified Bfr heated at 100 °C for 10 min in sample loading buffer. Arrows indicate band sizes mentioned in the text.

terminal sequences of several bacterial Bfrs, for example *E. coli*, *Nitrobacter winogradskyi*, *Azotobacter vinelandii*, *Brucella melitensis*, *Synechocystis* spp., *Mycobacterium leprae*, *Mycobacterium paratuberculosis* and *Mycobacterium avium*, and were designated as BfrA (18 kDa) and BfrB (22 kDa).

PCR and DNA sequence analysis

Degenerate primers deduced from the N-terminal amino acid sequence of the gonococcal BfrB and from a region of *E. coli* Bfr (amino acids 128–134) were used to PCR-amplify the majority of the BfrB gene. The flanking regions and the remaining sequence of the BfrB gene were obtained by SSP-PCR. Results of this additional sequencing indicated that the DNA sequence encoding *bfrA* was upstream of *bfrB*. The complete nucleotide and deduced amino acid sequences of the gonococcal Bfrs indicated two ORFs of 462 bp and 471 bp, corresponding to positions 148–609 and 640–1110, respectively. The two Bfr genes were located in tandem with an intervening gap of 27 bases. Potential Shine–Dalgarno ribosome-binding sites (GAGAG for *bfrA* and AGGAG for *bfrB*) were located 6–10 bp upstream from the ATG start codon of *bfrA* and *bfrB*, respectively. Putative –10 and –35 promoter regions were also identified and a GC-rich region of dyad symmetry located 43 bp downstream from the stop codon in *bfrB* could function as a rho-independent transcription terminator. There was a potential Fur-binding sequence (12 of 19 bases were identical to the consensus neisserial

Fig. 2. Expression of gonococcal Bfr in *E. coli* DH5 α . Whole-cell lysates from DH5 α with or without gonococcal Bfr were separated by SDS-PAGE (a), electro-transferred and probed with anti-*E. coli* Bfr polyclonal antiserum (b). Lanes: 1, DH5 α ; 2, DH5 α with pUC18; 3, DH5 α alone with pCYC-1.

fur sequences; Genco & Desai, 1996) in front of the –35 hexamer. There was 55.7 % homology between the DNA sequences of *bfrA* and *bfrB*; and 39.7 % identity for the amino acid sequences of BfrA and BfrB. The deduced amino acid sequence of BfrA consisted of 154 amino acids with a predicted molecular mass of 17961 Da and an isoelectric point (pI) of 4.57; BfrB consisted of 157 amino acids, and had a molecular mass of 18014 Da and a pI of 4.58. The deduced amino acid sequences of BfrA and BfrB showed 41.3 % and 56.1 % identity to *E. coli* Bfr, respectively.

Cloning and expression of Bfr

The entire coding sequence encompassing *bfrA* and *bfrB* was amplified by PCR and ligated into pUC18 and in-frame with *lacZ*. Competent *E. coli* DH5 α cells were

Fig. 3. Effect of iron limitation on the growth of *N. gonorrhoeae* strain F62 and Bfr-deficient strain F62BfrB Ω . Cells were grown in GC broth supplemented with IsoVitalX and with or without 30 μ M DF. Broth cultures were incubated at 37 °C in a gyratory shaker and the growth was monitored by measuring OD₆₀₀ over a period of 8 h. ○, F62 + Fe; △, F62BfrB Ω + Fe; □, F62 + DF; ▽, F62BfrB Ω + DF.

transformed, and transformants containing pCYC-1 were analysed for expression of gonococcal Bfr on Western blots probed with anti-*E. coli* Bfr polyclonal antiserum. The results in Fig. 2 show that *E. coli* DH5 α or *E. coli* DH5 α containing pUC18 exhibited a single Bfr subunit of 20 kDa. The transformants expressed two additional proteins corresponding in size to the gonococcal BfrA and B (Fig. 2a, lane 3), and both proteins reacted with the polyclonal antiserum to *E. coli* Bfr (Fig. 2b, lane 3). These additional proteins were not observed in *E. coli* DH5 α or *E. coli* DH5 α containing pUC18.

Construction of a BfrB-deficient mutant of *N. gonorrhoeae*

A BfrB-deficient mutant was constructed to determine the biological function of gonococcal Bfr. Attempts to construct a gonococcal BfrA-deficient mutant, or a double knock out by either insertion or deletion inactivation were not successful because of the lack of restriction sites in *bfrA*. Therefore, pCYC-2 was disrupted by the insertion of the Ω fragment from pHP45 Ω into the single *Hind*III site present in *bfrB*. The resulting plasmid, pCYC-3, which contains the insertionally inactivated *bfrB*, was linearized with *Eco*RI prior to transforming pilated *N. gonorrhoeae* strain F62. One of the several spectinomycin- and streptomycin-resistant transformants, F62BfrB Ω , was selected for further studies. The presence of the Ω fragment in the BfrB gene of F62BfrB Ω was confirmed by PCR amplification of *bfrA* and *bfrB* individually. The *bfrA* amplicon from F62BfrB Ω was the same size as that from strain F62; however, the *bfrB* amplicon from F62BfrB Ω was 2 kb larger than that of strain F62, indicating that the Ω

Fig. 4. Sensitivity of *N. gonorrhoeae* BfrB-deficient mutant to H₂O₂ and paraquat. The disc-diffusion assays were conducted on GC agar plates as described in Methods. Various amounts of H₂O₂ or paraquat were placed on the filter-paper discs. The zone of growth inhibition was determined by measuring the diameter of the clear zone surrounding the disc minus the diameter of the disc. Data shown are the mean and standard deviation from three separate experiments. ●, Strain F62; ○, Bfr-deficient strain F62BfrB Ω .

fragment was inserted into *bfrB* (data not shown). The absence of BfrB subunits in the mutant was also confirmed by immunoblot analysis with anti-*E. coli* Bfr antiserum (data not shown).

Effect of iron on the growth of BfrB-deficient mutant

N. gonorrhoeae strains F62 and F62BfrB Ω were grown in liquid GC medium with or without 30 μ M DF. Growth of both strains was markedly reduced under iron-limited conditions (Fig. 3); however, growth of

strain F62BfrB Ω was inhibited to a greater degree than was the growth of strain F62 under identical growth conditions.

Sensitivity of BfrB-deficient mutant to H₂O₂ and paraquat

To assess the contribution of BfrB to the oxidative stress resistance of *N. gonorrhoeae*, the sensitivities of strains F62 and F62BfrB Ω to H₂O₂ and paraquat were compared in a disc-diffusion assay. The results (Fig. 4a) show that the growth of F62BfrB Ω was inhibited by H₂O₂ in a dose-dependent fashion, whereas the growth of wild-type parent strain was inhibited to a lesser degree by amounts of H₂O₂ up to 2.5 μ mol; both F62 and F62BfrB Ω exhibited a similar growth inhibition at 5 μ mol H₂O₂. Paraquat is a redox-cycling agent that readily diffuses across the cell envelopes and generates superoxide within the cytosol. Both strains F62 and F62BfrB Ω appeared to be more sensitive to inhibition by paraquat than H₂O₂ and the growth of F62BfrB Ω was inhibited to a greater extent than strain F62 even at the lowest concentration of paraquat tested (Fig. 4b).

DISCUSSION

Iron is an essential element for the growth of almost all bacteria. Many of the molecular details of iron assimilation have been explored (Briat, 1992; Wooldridge & Williams, 1993); however, most of these studies have focused on the variety of strategies that bacterial pathogens employ to obtain iron from the host. Relatively little attention has been given to the intracellular sequestration, mobilization and storage of iron. Under aerobic conditions, excess iron may contribute to the generation of compounds by stimulating the production of highly reactive oxygen species by the iron-catalysed Haber–Weiss reaction (Haber & Weiss, 1934). The resulting hydroxyl radicals can cause lipid peroxidation, DNA damage and degradation of other biomolecules (Halliwell & Gutteridge, 1984; Touati *et al.*, 1995). Thus, strict regulation of assimilation and storage of iron in both eukaryotic and prokaryotic cells is crucial to prevent the accumulation of free intracellular iron and to protect cells from iron toxicity (Klausner *et al.*, 1993). Protection from iron toxicity may be mediated, in part, by ferritins and Bfr, which are capable of sequestering a few thousand iron atoms in their central cavity in a soluble, non-toxic, bioavailable form (Grossman *et al.*, 1992).

In this study, we have identified and purified a Bfr from *N. gonorrhoeae*, and subsequently cloned and sequenced the corresponding genes. The identification of gonococcal Bfr was facilitated by an iron-specific stain, which is based on the interaction between the iron atoms stored in the Bfr and a sensitive chromogenic ligand, Ferrozine, which results in the formation of a reddish-coloured complex. Bfr was the only protein in non-denaturing PAGE of supernatants of sonicated cell suspensions that could be readily stained with Ferrozine,

indicating the high content of iron molecules within this protein. Additional data indicating that this protein is Bfr include heat stability (65–70 °C for 15 min) (Smith *et al.*, 1988; Andrews *et al.*, 1993), molecular mass between 300 and 500 kDa for the holoprotein and between 18 and 22 kDa for its subunits, N-terminal sequence homology to Bfr subunits from other bacteria, and immunological cross-reactivity with anti-*E. coli* Bfr antiserum.

Unlike most Bfrs, which are homopolymers composed of identical subunits, gonococcal Bfr comprises two similar, but not identical, subunits. Harker & Wullstein (1985) reported that the Bfr from *A. vinelandii* comprised two non-identical subunits; however, Grossman *et al.* (1992) were able to clone only one gene for this protein. More convincing data for structural heterogeneity of Bfr were obtained from *Pseudomonas aeruginosa*, in which the first 69 and 55 residues of the N-terminal amino acid sequence of the α and β subunits differed considerably (Moore *et al.*, 1994). The corresponding genes for the α and β subunits of *P. aeruginosa* Bfr have not been cloned to further substantiate these findings. The results of our study are the first to locate genes encoding different Bfr subunits and verify the existence of structural heterogeneity in this iron-storage protein. Structural heterogeneity of Bfr may be less complicated than in mammalian ferritins, where the structural complexity is due to combinations of various ratios of two subunits, heavy (H) and light (L), which differ in size, amino acid composition, surface charge and immunoreactivity (Theil, 1987; Harrison & Arosio, 1996).

How the biosynthesis of Bfr or prokaryotic ferritin is regulated by iron awaits elucidation. In *E. coli*, the induction of *bfr* expression by iron was found to be dependent on the ferric uptake regulator protein (Fur), although not to the direct interaction between Fur and the *bfr* gene (Harrison & Arosio, 1996). It is not clear how Fur acts as an intermediate and what other cellular components are involved in this regulation. The genes encoding the two gonococcal Bfr subunits were located in tandem with an intervening gap of only 27 bp, suggesting that they might be co-transcribed and co-regulated. The presence of a potential iron box within the 5' region of *bfrA* in front of a putative –35 hexamer suggests that gonococcal Bfr might be negatively regulated by iron. However, this contradicts results obtained with other micro-organisms, suggesting that in spite of the presence of potential iron boxes the production of Bfr was either up-regulated by, or indifferent to, the iron concentration present in the growth medium (Grossman *et al.*, 1992; Cristina *et al.*, 1994; Evans *et al.*, 1995). The genetic regulation of gonococcal Bfr is currently under investigation.

Despite the structural similarity between ferritins and Bfrs, they share little homology in their amino acid sequences and exhibit no immunological cross-reactivity (Wai *et al.*, 1995), suggesting that they have different origins. Most studies suggest micro-organisms possess

	1				50
NgBfrA	MKGKPAVVDY	MNELLSGELA	ARDQYFIHSR	LYSEWGYTKL	FERLNHEMEE
SyBfr	MKGKPAVLAQ	LHKLLRGELA	ARDQYFIHSR	MYQDWGLEKL	YSRIDHEMQD
AvBfr	MKGDKVIOH	LNKLLGNELI	AINQYFLHAR	MYEDWGLEKL	GKHEYHESID
EcBfr	MKGDTKVINY	LNKLLGNELV	AINQYFLHAR	MFKNWGLKRL	NDVEYHESID
NgBfrB	MKGDRLVIRE	LKNLGLLLV	TINQYFLHAR	ILKNWGFTEL	GEHFFKQSIY
MIBfr	MQGDPEVLRL	LNEQLTSELT	AINQYFLHSK	MQDNWGFTEL	AERTRVESFD
MpBfr	MQGDPEVLRL	LNEQLTSELT	AINQYFLHSK	MQDNWGFTEL	AE.....
MaBfr	MQGDPEVLRL	LNEQLTSELT	AINQYFLHSK	MQDNWGFTEL	AEHTRAESFD
NwBfr	MKGDPKVIDY	LNKALRHETL	AINQYWLHYR	LLDNWGIKDL	AKKWAESIE
BmBfr	MKGEPKVIER	LNDALFLELG	AVNQYWLHYR	LLDNWGYTRL	AKKERESIE
	51				100
NgBfrA	ETTHAEDFIR	RILMLGGTPK	MAR.AELNIG	TDVVSCLKAD	LQTEYEVDRDA
SyBfr	E.THA.....				
AvBfr	EMKHADKLIK	RILFLEGLPN	LQELGKLLIG	EHTKEMLECD	LKLEQAGLPD
EcBfr	EMKHADRYIE	RILFLEGLPN	LQDLGKLLIG	EDVEMLRSD	LALELDGAKN
NgBfrB	EMKAADDLIE	RILFLEGLPN	LQELGKLLIG	ESTEEIIACD	LTKEQEKHEA
MIBfr	EMRHAEAITD	RILLLDGLPN	YQRIGSLRVG	QTLREQFEAD	LAIEYEVMSR
MpBfr					
MaBfr	EMRHAEAITD	RILLLDGLPN	YQRLFSLRIG	QTLREQFEAD	LAIEYEVMDR
NwBfr					
BmBfr	EMHHADKLIN	RIIFFEFGFPN	LQTVSPLRIG	QNVKEVLEAD	LKGEYDARAS
	101				150
NgBfrA	LKKGIKLCEE	AQDYVTRDLM	VAQLKDTEED	HAHWLEQQLR	LIELIGEGNY
SyBfr					
AvBfr	LKAAIAYCES	VGDYASRELL	EDILESEED	HIDWLETQLD	LIDKIGLENY
EcBfr	LREAIGYADS	VHDYVSRDMM	IEILRDEEG	HIDWLETQLD	LIQKMGLONY
NgBfrB	LLAAIATAEA	QDDYVSRDLM	EKQKDTNEK	HIDWLETQOE	LIGKIGLPNY
MIBfr	LKPGIIMCRE	KQDSTSAVLL	EKIVADEEE	HIDWLETQEA	LMGQLGEELY
MpBfr					
MaBfr	LKPAIILCRE	KQDSTATATF	EKIVADEEK	HIDWLETQLE	LMKDLGEVLY
NwBfr					
BmBfr	YKESREICDK	LGDYVSKQLF	DELLADEEG	HIDFLETQLD	LLAKIGEERY
	151				163
NgBfrA	YQSGL.....				
SyBfr					
AvBfr	LQSQMDE.....				
EcBfr	LQAQIREEG.....				
NgBfrB	LQTAQAQED.....				
MIBfr	SAQCVSRPPS.....				
MpBfr					
MaBfr	SAQCVSRPPS.....				
NwBfr					
BmBfr	GQLNAAPADE AE*				

Fig. 5. Comparison of the deduced amino acid sequence of *N. gonorrhoeae* Bfr to other bacterial Bfrs. From top to bottom, the sequences presented are Bfrs from: *N. gonorrhoeae* (NgBfrA and NgBfrB), *Synechocystis* PCC 6308 (SyBfr), *A. vinelandii* (AvBfr), *E. coli* (EcBfr), *M. leprae* (MIBfr), *M. paratuberculosis* (MpBfr), *M. avium* (MaBfr), *Nitrobacter winogradskyi* (NwBfr) and *B. melitensis* (BmBfr). ●, Putative ferroxidase-centre residues; ▼, methionine, haem-iron axial ligand.

either a ferritin or a Bfr homologue (Striefel & Watt, 1979; Moore *et al.*, 1986; Kurokwa *et al.*, 1989; Brooks *et al.*, 1991; Rocha *et al.*, 1992; Laulhère *et al.*, 1993; Cristina *et al.*, 1994; Inglis *et al.*, 1994; Denoel *et al.*, 1995; Evans *et al.*, 1995; Penfold *et al.*, 1996). However, the presence of both ferritin and Bfr has been reported in *E. coli* (Yariv *et al.*, 1981; Izuhara *et al.*, 1991) and *Helicobacter pylori* (Doig *et al.*, 1993; Evans *et al.*, 1995). It is not clear why relatively few bacteria possess both types of iron-storage proteins and what advantage this apparent redundancy has. *In vitro* data indicate that the rate at which iron is incorporated into apoBfr is approximately fivefold faster than into apoBfr, suggesting that ferritin might compete for iron *in vivo* more efficiently than Bfr (Andrews *et al.*, 1993). We cannot rule out the possibility that the two biochemically and genetically different iron-storage proteins might have other specific or essential functions. For example, *E. coli* Bfr is also known as cytochrome *b*₁ and may additionally function as an electron-storage

molecule (Smith *et al.*, 1988). We have been unable to identify a potential ferritin homologue in *N. gonorrhoeae* by homology searches comparing the sequences of the *E. coli* and *Haemophilus influenzae* ferritin against the gonococcal (University of Oklahoma, *N. gonorrhoeae* strain FA1090) and meningococcal (The Sanger Center, *Neisseria meningitidis* serogroup A strain Z2491) genome databases. Alternatively, the gonococcus may have a ferritin gene that has little homology to those of *E. coli* or *H. influenzae*.

The homology of the amino acid sequences of gonococcal BfrA and BfrB to those of other Bfrs (Andrews *et al.*, 1989; Kurokwa *et al.*, 1989; Brooks *et al.*, 1991; Grossman *et al.*, 1992; Laulhère *et al.*, 1993; Inglis *et al.*, 1994; Denoel *et al.*, 1995) for which full-length or partial sequences are available is shown in Fig. 5. Gonococcal BfrA and BfrB shared 39.7% identity. The percentage identity compared to other bacterial Bfrs ranged from 39.5% to 59.6% for BfrA, and from 41.6% to 62.2% for BfrB. A seven amino acid motif consisting of Glu-18, -51, -94, -127, His-54, -130 and Tyr-25, which constitutes the ferroxidase centre (Andrews *et al.*, 1991; Brun *et al.*, 1995) and is associated with the binuclear metal-binding site, was fully conserved in BfrA (Fig. 5); however, only four of these seven residues were conserved in BfrB. Conversely, the methionine residue (Met-52), which provides the axial ligand for haem binding (Grossman *et al.*, 1992; Cheesman *et al.*, 1993), was conserved only in BfrB. These data demonstrate that the structure and function of Bfr are conserved among prokaryotes and suggest that both subunits are required to form a functional Bfr, with BfrA providing the ferroxidase centre and BfrB the haem-binding ligand. Interestingly, the seven amino acids which constitute the ferroxidase centre were conserved in *E. coli* ferritin but only five were retained in *E. coli* Bfr (Andrews *et al.*, 1991, 1992). It has been suggested that the ferritin and Bfr of *E. coli* are the bacterial counterparts of the mammalian H-rich and L-rich ferritins with respective roles in short-term iron flux and long-term iron storage (Andrews *et al.*, 1993). It is not clear whether gonococcal Bfr comprises equal molar ratios of the A and B subunits, or whether different Bfr subunits associate to form a Bfr that is either BfrA-rich or BfrB-rich. However, the absence of iron-specific staining of Bfr by Ferrozine in the F62BfrB Ω mutant (data not shown) suggests that BfrA subunit alone can not form a functional iron-storage protein.

Based on X-ray crystallographic data, the structure of *E. coli* Bfr was shown to consist of 24 identical subunits that are assembled into a compact spherical protein shell containing approximately 12 haem groups per 24 subunits, with each haem bound in a pocket formed by the interface between a pair of symmetry-related subunits (Frolow *et al.*, 1994). A stoichiometry of one haem per four subunits has been found in the Bfr from the cyanobacterium *Synechocystis* PCC6803 (Laulhère *et al.*, 1993) and three to nine haem groups per 24 subunits in the Bfr from *P. aeruginosa* (Kadir & Moore, 1990). We have yet to determine the number of haem groups

per subunit in the gonococcal Bfr. However, it is likely that this value is less than 12 since only BfrB contains the essential methionine residue for haem binding.

Eukaryotic ferritins function by buffering the free-iron concentration in the cells, thus protecting cells from harmful iron-catalysed oxidative damage (Harrison & Arosio, 1996). Similarly, a ferritin homologue from *Campylobacter jejuni* was recently reported to contribute to protection against oxidative stress (Wai *et al.*, 1996). We investigated the role of the gonococcal Bfr in protecting against oxidative stress by insertionally inactivating BfrB and examining the sensitivity of a BfrB-deficient mutant to hydrogen peroxide and paraquat. The growth of the BfrB-deficient mutant of *N. gonorrhoeae* was less than that of the parent strain under both iron-sufficient and iron-limiting conditions, suggesting that Bfr plays a role in iron storage. In addition, the BfrB-deficient mutant was more sensitive to H₂O₂ and paraquat than the parent strain, suggesting that the accumulation of intracellular free iron caused by the absence of BfrB may lead to hypersensitivity to H₂O₂. Furthermore, BfrA alone was not capable of protecting the cell from oxidative injury.

A ferritin- and Bfr-deficient double mutant of *E. coli* grew poorly under iron-limited conditions (Simon Andrews, personal communication), suggesting the possibility of another internal iron pool. In our study, insertional inactivation of BfrB was not lethal to the growth of the gonococcus and provides further support for an internal iron pool. However, questions remain as to how this iron pool interacts with iron-storage or iron-requiring proteins. Although ferritins and Bfrs are involved in iron storage and in protection against oxidative stress, the exact role of ferritin and Bfr in iron metabolism has yet to be elucidated since the intracellular levels of these proteins present are considered too low to serve as major iron-storage compounds (Braun, 1997).

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