The moonlighting peroxiredoxin-glutaredoxin in *Neisseria meningitidis* binds plasminogen via a C-terminal lysine residue and contributes to survival in a whole blood model

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Running title: Meningococcal peroxiredoxin (Prx5-Grx)

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Abstract

Neisseria meningitidis is a human-restricted bacterium that can invade the bloodstream and cross the blood-brain barrier resulting in life-threatening sepsis and meningitis. Meningococci express a cytoplasmic peroxiredoxin-glutaredoxin (Prx5-Grx) hybrid protein that has also been identified on the bacterial surface. Here, recombinant Prx5-Grx was confirmed as a plasminogen (Plg)-binding protein, in an interaction which could be inhibited by the lysine analogue ϵ-aminocapronic acid. rPrx5-Grx derivatives bearing a substituted C-terminal lysine residue (rPrx5-Grx^{K244A}), but not the active site cysteine residue (rPrx5-Grx^{C185A}) or the sub-terminal rPrx5-Grx^{K230A} lysine residue, exhibited significantly reduced Plg-binding. The absence of Prx5-Grx did not significantly reduce the ability of whole meningococcal cells to bind Plg, but under hydrogen peroxide-mediated oxidative stress, the *N. meningitidis* Δ*pxn5-grx* mutant survived significantly better than the wild-type or complemented strains. Significantly, using human whole blood as a model of meningococcal bacteremia, it was found that the *N. meningitidis* Δ*pxn5-grx* mutant had a survival defect compared with the parental or complemented strain, confirming an important role for Prx5-Grx in meningococcal pathogenesis.

Keywords: *Neisseria meningitidis*; protein moonlighting; peroxiredoxin; pathogenesis; whole blood model; plasminogen

Highlights:

- Meningococcal Prx5-Grx interacts with plasminogen via a C-terminal lysine residue
- Loss of Prx5-Grx does not reduce the ability of meningococci to bind Plg
- Loss of Prx5-Grx enhances meningococcal survival under oxidative stress
- Loss of Prx5-Grx results in a survival defect in human whole blood

1. Introduction

Neisseria meningitidis is an important cause of sepsis and meningitis in susceptible individuals, with the majority of cases occurring in children and young adults. The organism frequently colonizes the nasopharynx asymptomatically and is transmitted from person-to-person by close contact [1]. Rarely, however, hypervirulent strains may invade the mucosal epithelial barrier to reach subcutaneous tissues and subsequently enter the bloodstream [2]. The ability of the meningococcus to survive in subcutaneous tissues and blood is therefore a prerequisite for invasive meningococcal disease. Various meningococcal components are known to be essential for, or enhance, survival in non-immune blood, including capsular polysaccharide [3, 4], lipooligosaccharide (LOS) [5], the factor H-binding proteins (fHbp and NspA) [5-8] and NalP [8, 9]. Various detoxifying enzymes are also used by *N. meningitidis* to protect against reactive oxygen and nitrogen species produced by polymorphonuclear (PMN) cells and monocytes/macrophages [10, 11]. Up-regulation of catalase (Kat), superoxide dismutase (Sod) and nitrite reductase (AniA) have been demonstrated following incubation in human blood [8]. SodC has previously been shown to protect *N. meningitidis* from phagocytosis [12] and AniA protects *N. gonorrhoeae* in human serum [13], however Kat was shown not to be required for meningococcal survival in human blood [8].

Peroxiredoxins (Prxs) are a large family of cysteine-based peroxidases, present in all kingdoms of life, that catalyse the reaction $ROOH+2e^- \rightarrow ROH+H_2O$ and reduce hydrogen peroxide (H_2O_2), peroxynitrite and a wide range of organic hydroperoxide compounds [14-16]. Prxs are ubiquitous and abundant proteins that are important for antioxidant defence and the regulation of cell signalling pathways [17, 18]. Prxs can be divided into six subfamilies (AhpC-Prx1, BCP-PrxQ, Tpx, Prx5, Prx6 and AhpE) with distinct amino acid sequences, but all contain an absolutely conserved active site cysteine in the motif P-XXX-T(S)-XX-C, which reacts with H_2O_2 to form a cysteine sulfenic acid [19]. Prxs are described as either 1-

Cys Prx or 2-Cys Prx, depending on whether a second cysteine residue participates in the enzymatic reaction [19]. The 2-Cys Prxs are further subdivided into ‘typical’ and ‘atypical’ forms, depending on the location of the second cysteine residue; in typical forms the second cysteine is found in the C-terminus of the partner subunit, whereas in atypical enzymes it is located at other positions [19]. Glutaredoxins (Grxs) are small redox enzymes that use glutathione (GSH) as a cofactor [20]. Grxs possess an active site disulfide bond, which exists in either a reduced or an oxidized form. Grxs act as antioxidants by reducing dehydroascorbate, peroxiredoxins and methionine sulfoxide reductase. Some bacterial species contain tetrameric Prx5-Grx hybrid enzymes in which the C-terminal Grx domain acts as an electron donor for the N-terminal Prx domain [21].

Prx enzymes are increasingly being recognized as multifunctional or moonlighting proteins, which are involved in various aspects of bacterial survival and pathogenesis. In *Helicobacter pylori*, a BCP-PrxQ subfamily Prx was found to contribute to resistance to oxidative stress and host colonization [22] and an AhpC-Prx1 subfamily Prx was shown to oligomerise and acquire protein chaperone activity under oxidative stress conditions [23]. In *Haemophilus influenzae*, a Prx-Grx hybrid enzyme was found to be expressed preferentially during growth in biofilms [24]. A mutant strain was more susceptible to organic hydroperoxides [25] and showed persistence defects in a chinchilla model of otitis media and a murine model of chronic obstructive pulmonary disease [26]. In *Streptococcus agalactiae*, a similar AhpC-Prx1 enzyme was shown to bind haem and participate in its intracellular availability [27]. In *Coxiella burnetii*, a BCP-PrxQ Prx was shown to bind DNA and protect the organism from oxidative stress during the exponential phase of growth [28]. A Prx6 subfamily protein, LsfA, in *Pseudomonas aeruginosa*, was found to reduce the oxidative burst in macrophages and to be important for virulence in a murine model of pneumonia [29]. In *Vibrio vulnificus*, Prx3, a Grx3/GSH-dependent 1-Cys Prx, was shown to be required for

optimal growth in medium containing peroxides and the Prx3 mutant strain was less virulent in mice [30]. A 1-Cys Prx6 enzyme from *Thermococcus kodakarensis*, a thermophilic anaerobic archaeon, was found to act as a molecular chaperone that blocked oxidative stress-mediated aggregation of proteins and DNA [31]. More recently, an AhpC-Prx1 subfamily Prx of *F. tularensis* LVS was shown to confer resistance against a wide range of reactive oxygen and nitrogen species, and to serve as a virulence factor. In the highly virulent strain, *F. tularensis* SchuS4, the enzyme also enhanced intra-macrophage survival [32].

N. meningitidis contains a Prx-Grx hybrid atypical 2-Cys enzyme in the Prx5 subfamily and a BCP-PrxQ subfamily Prx (designated NMB0946 and NMB0750, respectively, in strain MC58) [19, 33]. The Prx5-Grx hybrid enzyme was previously found to be a putative moonlighting protein present on the meningococcal cell surface where it contributes to plasminogen (plg)-binding [34] and in *N. gonorrhoeae*, the Prx5-Grx orthologue was found to play a role in biofilm formation [35]. In this study, we examine the properties and role of Prx5-Grx in meningococcal pathogenesis. We confirm that Prx5-Grx moonlights on the bacterial surface and show that a single lysine residue in the penultimate C-terminal position in Prx5-Grx is required for optimal interactions with human plasminogen. Importantly, we also demonstrate that Prx5-Grx is required for meningococcal survival in human non-immune whole blood.

2. Experimental procedures

2.1. Bacterial strains and culture conditions

Escherichia coli JM109 (Promega) was used as a host strain for the expression of 6 × histidine-tagged rPrx5-Grx and for mutagenic plasmid construction. *E. coli* XL10-Gold ultra-competent cells (Agilent Technologies) were used for site-directed mutagenesis of *rpsL*. NEB 5-alpha competent cells were used for cloning of Gibson assembly reactions. All *E. coli*

strains were grown at 37°C in Lysogeny Broth (LB) or on LB agar supplemented, where appropriate, with ampicillin (100 µg ml^{-1}), kanamycin (30 µg ml^{-1}) or erythromycin (200 µg ml^{-1}). Strains of *Neisseria meningitidis* (Table S1) were grown at 37°C in air plus 5% CO_2 on Columbia agar with chocolated horse blood (Thermo Fisher Scientific), Brain Heart Infusion (BHI) agar or BHI broth supplemented with 1% Vitox (Thermo Fisher Scientific) and kanamycin (50 µg ml^{-1}), streptomycin (100 µg ml^{-1}) or erythromycin (5 µg ml^{-1}), where appropriate.

2.2. Construction of plasmids encoding recombinant Prx5-Grx

The *prx5-grx* gene was amplified from *N. meningitidis* MC58 using oligonucleotides NMB0946F1 and NMB0946R1 (Table S2) using Phusion High-Fidelity DNA polymerase (New England Biolabs). After digestion with BamHI, the PCR product was ligated into BamHI-digested pQE30 to yield pMAJ2 (Table S3). The Prx5-Grx lysine residues (^{230}K, ^{244}K and ^{230}K/^{244}K) and the glutaredoxin active-site cysteine residue (^{185}C) were replaced by alanine residues using the Q5 Site-Directed Mutagenesis Kit (New England BioLabs) following the manufacturer's instructions, yielding pMAJ2-230KA, pMAJ2-244KA, pMAJ2-230244KKAA and pMAJ2-185CA, respectively (Table S3). Oligonucleotides used are given in Table S2.

2.3. Recombinant Prx5-Grx expression and purification

E. coli JM109 strains were grown to log phase, induced with 0.5 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 3 h, and harvested by centrifugation (4,200 × *g* for 10 min). Recombinant 6 × histidine-tagged proteins were then affinity-purified under native conditions. Briefly, *E. coli* cell pellets were resuspended in 30 ml lysis/wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 40 mM imidazole, pH 7.4) followed by sonication using an MSE

SoniPrep 150 sonicator for 8 cycles (30s on, 30s off) on ice. The cell lysate was centrifuged (4,500 × *g* for 15 min) and the cleared lysate was loaded onto a HisTrap FF column (GE Healthcare Lifesciences) pre-packed with Ni Sepharose 6 Fast Flow (GE Healthcare Lifesciences) connected to a ÄKTAprime plus liquid chromatography system (GE Healthcare Lifesciences), equilibrated with 10 column volumes of lysis/wash buffer. Proteins were eluted by step elution using elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 300 mM imidazole, pH 7.4). A HiTrap column pre-packed with five millilitres of Sephadex G-25 Superfine (GE Healthcare Lifesciences) equilibrated with 5 column volumes of phosphate buffered saline (PBS) was used for buffer exchange. Glycerol at a final concentration of 10% was added to inhibit aggregation. A pre-packed Superdex column (GE Healthcare Lifesciences) mounted on an ÄKTAprime plus liquid chromatography system was then used to carry out gel filtration, following the manufacturer's instructions. After equilibration with 150 ml PBS containing 10% glycerol, the IMAC-purified protein sample was injected into the column. Fractions contained the purified protein were collected and concentrated using Vivaspin sample concentrators (Sartorius; 10,000 MWCO).

2.4. Production of a rabbit antiserum against rPrx5-Grx

Rabbit anti-Prx5-Grx antibodies were raised in a New Zealand White female rabbit immunized subcutaneously four times at 2-week intervals with 100 µg of rPrx5-Grx protein emulsified in Freud's complete (first immunization only) or incomplete adjuvant. After three injections, the animal was test bled, boosted once more and sacrificed 7 days later.

2.5. SDS-PAGE and immunoblotting

Proteins were electrophoretically separated using 10% polyacrylamide gels (Mini-Protean III; Bio-Rad) and stained using SimplyBlue™ SafeStain (Thermo Fisher Scientific)

or transferred to nitrocellulose membranes using a Trans-Blot SD semidry transfer cell (Bio-Rad) according to the manufacturer's recommendations. Membranes were probed with mouse anti-pentahistidine antibody (Qiagen) or rabbit anti-Prx5-Grx primary antibody (RαPrx5-Grx) diluted 1:10,000 or 1:1,000, respectively, in blocking buffer (5% [w/v] non-fat dry milk, 0.1% [v/v] Tween 20 in 1 × PBS) and incubated for 2 h. After washing in PBS with 0.1% Tween 20 (PBST), membranes were incubated for 2 h with 1:10,000-diluted goat anti-mouse (or anti-rabbit) IgG-alkaline phosphatase conjugate (Sigma). After washing with PBST, blots were developed using BCIP/NBT-Blue liquid substrate (Sigma).

2.6. ELISA

Microplate wells (Nunc 96-well plates, PolySorp) were coated with 20 pmol glu-plg (human plasma; Calbiochem), laminin (human placenta; Merck Millipore), fibronectin (human plasma; Sigma), collagen I (human placenta; Corning) or 1% BSA in sodium carbonate buffer (142 mM $NaHCO_3$, 8 mM Na_2SO_3, pH 9.0) and incubated overnight at 4°C. After washing with PBS-Tween 20 (0.05%; PBST), wells were blocked with 1% BSA in PBS for 1 h. After removal of the blocking solution, 50 pmol rPrx5-Grx in 1% BSA/PBS was added and incubated for 1 h. Following vigorous washing with PBST, RαPrx5-Grx (diluted 1:2,000 in 1% BSA/PBS) was added for incubation for 1 h. Plates were again vigorously washed, before the addition of goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma; diluted 1:20,000 in 1% BSA/PBS), was added and incubated for 1 h. Plates were again vigorously washed, and the color was developed by adding phosphatase substrate (Sigma), dissolved in buffer containing 0.1 M glycine, 1 mM $ZnCl_2$ and 1 mM $MgCl_2$, pH 10.4, into each well and absorbance at 405nm was measured using a Biotek EL800 spectrophotometer. Alternatively, binding assays were undertaken as above, but using rPrx5-Grx (5 pmol) or 1% BSA as the immobilized ligand to capture glu-plg (5 pmol). Bound glu-plg was detected

using goat anti-human plg (1:10,000; Rockland Immunochemical) and donkey anti-goat IgG-alkaline phosphatase conjugate (1:5,000; Promega). The lysine analogue, ε-aminocaproic acid (EACA; 50 mM) (Sigma) was utilized for inhibition studies.

2.7. Generation of MC58 rpsL$^-$ and MC58 Kan^R rpsL$^+$ rpsL$^-$

A 2.5-kb fragment consisting of *rpsL* and flanking DNA was amplified from *N. meningitidis* MC58 using oligonucleotides rpsL-FlankF and rpsL-FlankR (Table S2). The amplified gene was TA-cloned into pCR4-TOPO to generate plasmid pCF4-TOPOΔrpsL, which was subjected to site-directed mutagenesis, designed to replace ^{43}K with T, using the primers rpsLSD-F and rpsLSD-R (Table S2) and the QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's protocol. Following mutagenesis, the reaction was incubated with DpnI to digest non-mutated parental DNA prior to transformation of *E. coli* XL10-Gold ultra-competent cells. A plasmid extracted from a resulting clone was sequenced and confirmed to contain the desired mutation and designated pCR4-TOPOΔrpsLT and used to transform *N. meningitidis* MC58. Genomic DNA from a resulting clone was sequenced, the desired mutation confirmed, and the resulting mutant designated MC58 *rpsL*$^-$ (Table S1).

An MC58 derivative containing a cassette consisting of a kanamycin resistance gene and a wild-type *rpsL* gene was generated using a protocol based on that described by Gibson [36]. Primer pairs Upstream343_F and Upstream343_R; Downstream343_F and Downstream343_R; rspL_F and rspL_R (Table S2) were designed using the web tool NEBuilder to amplify fragments with appropriate overlaps required for assembly of fragments corresponding to regions upstream and downstream of the gene NMB0343 and the *rpsL* gene from chromosomal DNA of *N. meningitidis* strain MC58. The *aphA-3* gene of plasmid pJMK30 [37] was amplified using the primers Kan cassette_F and Kan cassette_R

and all four fragments, together with pUC19 plasmid DNA linearized with EcoRI and BamHI, was performed using 0.2-0.25 pmol of each DNA fragment, 10 µl of 2 × Gibson assembly master mix (NEB) and dH_2O to 20 µl. After incubation at 50˚C, the assembly reaction was used to transform NEB 5-alpha competent *E. coli* cells (NEB). The resulting plasmid was subsequently used to transform MC58 *rpsL*$^-$ by selection for kanamycin resistance and screening for streptomycin sensitivity. The resulting strain was designated MC58 *Kan*^{R} *rpsL*$^+$ *rpsL*$^-$ (Table S1).

2.8. Construction of a markerless Δprx5-grx mutant

A 2.7-kb fragment consisting of *prx5-grx* and flanking DNA was amplified using oligonucleotides UprxDF1 and UprxDR1 (Table S2) from *N. meningitidis* MC58. The amplified DNA was TA-cloned into pGEM-T Easy to generate pGUD1 (Table S3). This was then subjected to inverse PCR using oligonucleotides DeprxF1 and DeprxR1 (Table S2) resulting in the amplification of a *ca.* 5-kb amplicon in which the *prx5-grx* coding sequence was deleted and a unique BamHI restriction site had been introduced. The BamHI site was used to introduce a DNA fragment containing a kanamycin resistance cassette and *rpsL*$^+$ (a dominant selectable marker) which was generated by PCR from *N. meningitidis* MC58 *Kan*^{R} *rpsL*$^+$ *rpsL*$^-$ (Table S1) using oligonucleotides KnsLF1 and KnsLR1 (Table S2), in place of *prx5-grx*, resulting in plasmid pGUD2. Plasmid pGUD3 was generated by self-ligation of the same inverse PCR product.

pGUD2, containing the resistance cassette in the same orientation as the deleted gene, was subsequently used to mutate meningococcal strain MC58 *rpsL*$^-$ by natural transformation and allelic exchange as previously described [38] generating MC58 *Kan*^{R} *rpsL*$^+$ *rpsL*$^-$ Δ*prx5-grx*. pGUD3 was then used mutate MC58 *Kan*^{R} *rpsL*$^+$ *rpsL* $^-$ Δ*prx5-grx* to generate the markerless mutant MC58 *rpsL*$^-$ Δ*prx5-grx.*

242

243 *2.9. Complementation of prx5-grx*

244 A fragment corresponding to the *prx5-grx* coding sequence and upstream sequence was
245 amplified from strain MC58 using oligonucleotides PerCompF1 and PerCompR1 (Table S2)
246 incorporating BglII sites into the amplified fragment. The BglII-digested fragment was then
247 introduced into a unique BglII-site in pYHS25 (Table S3). This vector contains an
248 erythromycin resistance gene flanked by the MC58 genes NMB0102 and NMB0103. The
249 resulting plasmid, pLD1, was used to transform MC58 *rpsL*$^{-}$ Δ*prx5-grx* by natural
250 transformation, thus introducing a single chromosomal copy of *prx5-grx* and the downstream
251 erythromycin resistance cassette into the intergenic region between NMB0102 and
252 NMB0103, generating MC58 *rpsL*$^{-}$ Δ*prx5-grx prx5-grx*^{ECT}.

253

254 *2.10. Whole cell ELISA*

255 Mid-log phase ($OD_{600} \geq 0.5$) liquid cultures of meningococcal strains were
256 centrifuged at 2087 × *g* at 4°C for 10 min. After discarding the supernatant, the bacterial
257 pellet was washed twice with sterile-filtered PBS and resuspended in 0.5% v/v formaldehyde
258 in PBS. The cells were incubated at 4°C for 30 min with gentle agitation. Cells were then
259 centrifuged at 2087 × *g* for 10 min at 4°C, washed one more time with PBS, resuspended in
260 sodium carbonate buffer and the OD_{600} adjusted to 0.2. 100 µl aliquots of formaldehyde-fixed
261 meningococcal cells in carbonate buffer were then used to coat microplate wells overnight at
262 4˚C with gentle agitation. Plates were then washed three times with PBST and further steps
263 were carried out as described in section 2.6.

264

265 *2.11. Hydrogen peroxide survival assay*

Sensitivity to hydrogen peroxide stress was determined by a method based on that described by Tala *et al* [39]. Briefly, meningococcal strains were grown to mid-log phase ($OD_{600} \geq 0.5$). Suspensions were then adjusted to *ca.* 10^8 CFU ml^{-1} (confirmed retrospectively by plating out serially diluted aliquots). 100 µl aliquots of the meningococcal cell suspension were added to a sterile 96-well polystyrene round bottom microwell plate (Thermo Fisher Scientific) containing 150 µl BHI. Fifty microliters of H_2O_2 was then added to yield a final concentration of 12.5 mM, and the plate was incubated at 37°C in 5% CO_2 atmosphere with gentle shaking for 1 h. At 20 min intervals, 10 µl aliquots were taken, serially diluted, and plated out. After overnight incubation at 37°C in 5% CO_2, colonies were counted and expressed as CFU ml^{-1}.

2.12. Ex vivo human whole blood model of bacteremia

Whole venous blood was collected from healthy individuals (not immunized against *N. meningitidis*) and supplemented with heparin (17 IU ml^{-1}) to inhibit coagulation. Meningococci were grown to mid-log phase and then diluted in BHI broth to approximately 10^8 CFU ml^{-1}. The assay was started by the addition of 190 µl whole human blood to 10 µl of bacterial suspension. Suspensions were incubated at 37°C and 5% CO_2 with gentle agitation for 2 h and at various time points an aliquot of the sample was removed and the number of viable CFU determined by plating out of serial dilutions.

3. Results

3.1. Prx5-Grx is a plasminogen-binding protein

To examine putative host ligand-binding functions of meningococcal Prx5-Grx, and to raise specific antibodies, *prx5-grx* from the group B meningococcal strain MC58 was expressed in *E. coli* and purified under non-denaturing conditions to yield N-terminally 6 ×

291 histidine-tagged recombinant Prx5-Grx (Fig. S1). Since surface-localized bacterial
292 moonlighting proteins are recognized to bind a variety of host proteins [40] we screened the
293 ability of rPrx5-Grx to bind to human plasminogen (glu-plg), fibronectin, laminin and
294 collagen. Of these potential ligands, Prx5-Grx was only able to significantly bind to glu-plg
295 (Fig. 1A). Binding to glu-plg could be inhibited by the lysine analogue, ε-aminocaproic acid
296 (EACA) (Fig. 1B).
297
298 *3.2. Lysine 244 is required for optimal binding of meningococcal Prx5-Grx to plasminogen*
299 Given the likely involvement of lysine residues in the binding of rPrx5-Grx to glu-plg,
300 the two C-terminal lysine residues (^{230}K and ^{244}K) of rPrx5-Grx were individually, or in
301 combination, replaced with alanine using site-directed mutagenesis and the effects on glu-plg
302 binding were examined. rPrx5-Grx^{K244A} exhibited a significantly reduced ability to bind
303 glu-plg, confirming an important role for this lysine residue at the penultimate position in
304 meningococcal Prx5-Grx (Fig. 2). In contrast, mutation of ^{230}K had no significant effect on
305 glu-plg binding, while the double lysine mutant behaved similarly to the ^{244}K mutant (Fig. 2).
306 No statistically significant difference in glu-plg binding was apparent in assays utilizing
307 rPrx5-Grx^{C185A}, in which the second cysteine residue of the Grx active site was substituted,
308 confirming that glutaredoxin activity is not required for the binding of glu-plg (Fig. 2).
309
310 *3.3. Generation of prx5-grx knock out and complemented strains*
311 To examine the roles of Prx5-Grx in the meningococcus, a knockout derivative of *N.*
312 *meningitidis* MC58 was generated using a markerless mutation strategy. Briefly, *prx5-grx*
313 plus flanking DNA was cloned and inverse PCR used to remove the entire ORF. The product
314 was then ligated to a DNA fragment harbouring a kanamycin resistance cassette plus the
315 dominant marker, *rpsL*$^{+}$, (conferring streptomycin sensitivity), and the resulting plasmid used

316 to transform a streptomycin-resistant *N. meningitidis* MC58 derivative (MC58 *rpsL*$^{-}$) yielding
317 MC58 *Kan*^{R} *rpsL*$^{+}$ *rpsL*$^{-}$ Δ*prx5-grx.* The latter strain was then further transformed using the
318 self-ligated inverse PCR product to yield the final streptomycin resistant, but markerless
319 MC58 *rpsL*$^{-}$ Δ*prx5-grx* mutant. Immunoblotting using RαPrx5-Grx showed that a *ca.* 27-kDa
320 protein corresponding to Prx5-Grx could be detected in whole cell lysates of wild-type but
321 not MC58 *rpsL*$^{-}$ Δ*prx5-grx* (Fig. S2), confirming that expression had been abolished in the
322 mutant. To further confirm that the *ca.* 27-kDa immuno-reactive protein was Prx5-Grx, a
323 wild-type copy of *prx5-grx* was introduced *in trans* into MC58 *rpsL*$^{-}$ Δ*prx5-grx* restoring
324 Prx5-Grx expression, albeit at lower expression levels than the wild-type (Fig. S2).
325
326 *3.4. Loss of Prx5-Grx does not significantly reduce the ability of meningococci to bind*
327 *plasminogen*
328 A whole cell ELISA assay suggested localisation of meningococcal Prx5-Grx on the
329 surface of wild-type, MC58 *rpsL*$^{-}$ and the complemented mutant (Fig. 3), with the latter
330 exhibiting reduced levels of anti-Prx5-Grx reactivity compared to the parental strain in line
331 with reduced overall Prx5-Grx expression. Importantly, there was no significant difference in
332 the Plg-binding ability of Δ*prx5-grx* compared to strains expressing surface-exposed Prx5-
333 Grx (Fig. 4) consistent with previous observations that meningococci possess a range of
334 surface-exposed glu-plg ligands which may be functionally redundant [34, 41].
335
336 *3.5. Prx-Grx-deficient meningococci are protected from killing in a hydrogen peroxide assay*
337 Given the likely involvement of Prx5-Grx in oxidative stress responses, hydrogen
338 peroxide (H_2O_2) killing assays were performed. After 20 min of exposure to H_2O_2, survival of
339 MC58 *rpsL*$^{-}$ Δ*prx5-grx* was significantly enhanced compared to the parental or
340 complemented strains (Fig. 5). With the exception of the *prx5-grx* mutant, each strain tested

was undetectable at the 40 and 60 min time points. In contrast, growth curve assays carried out in the absence of H_2O_2 showed no significant differences between strains (Fig. S3). Taken together, the absence of Prx5-Grx expression had no effect on *in vitro* growth under standard conditions, but Prx-Grx-deficient meningococci were protected from killing when exposed to H_2O_2.

3.6. Prx5-Grx is important for survival in an ex vivo human whole blood model of meningococcal septicemia

An ex vivo human whole blood model of meningococcal bacteremia was used to assess the contribution of Prx5-Grx to survival on exposure to cellular and humoral mechanisms of killing. In both donors tested, there was a $\geq$ 2-$\log_{10}$ difference in CFU between MC58 *rpsL*$^-$ Δ*prx5-grx* and the parental strain at 30 and 60 min time points. Survival of the complemented mutant was similar to that of the parental strain, suggesting that even the lower level of expression of Prx5-Grx observed in the complemented strain was sufficient to provide full protection against killing in human blood (Fig. 6).

4. Discussion

Prx5-Grx is a highly conserved protein in *Neisseria* species [42] and a previous study demonstrated that both the Prx and Grx domains are biochemically functional in the meningococcal enzyme [33]. Prx5-Grx is upregulated in response to heat shock in *N. meningitidis* [43] and a deletion mutant in *N. gonorrhoeae* was found to have a defect in biofilm formation [35]. In *N. meningitidis*, Prx5-Grx was also identified as a putative plg-binding protein on the bacterial surface [34]. In this study, we confirm that Prx5-Grx is a multifunctional protein with plg-binding activity, but show that it is functionally redundant on the bacterial cell surface in the presence of alternative plg-binding proteins. Importantly,

366 we demonstrate that Prx5-Grx is also required, however, for survival of *N. meningitidis* in
367 non-immune blood.

368 Having confirmed that meningococcal Prx5-Grx is able to bind human plg, we
369 investigated which amino acid residues in Prx5-Grx were required for the plg-binding. We
370 observed that binding of plg was sensitive to the lysine analogue EACA, suggesting that
371 certain lysine residues in Prx5-Grx were responsible for the interaction. Knaust *et al*. reported
372 that internal lysine residues (rather than the lysine residues in terminal or penultimate
373 positions) might be important for plg-binding by enolase, DnaK and Prx5-Grx [34]. In
374 contrast, we demonstrated that the lysine residue in the penultimate position in Prx5-Grx
375 rather than an internal lysine residue was primarily responsible for plg-binding. The
376 difference in findings may be explained by our use of an EIA-based assay to detect plg-
377 binding to non-denatured rPrx5-Grx rather than a dot blot assay, or possibly the replacement
378 of both the penultimate lysine residue and the asparagine residue in the terminal position of
379 Prx5-Grx in the study by Knaust *et al* [34].

380 *N. meningitidis* normally inhabits the mucosal surface of the human nasopharynx or
381 subcutaneous tissues. In these niches, meningococcal cells are continuously exposed to
382 reactive oxygen species (ROS), produced by internal metabolic functions as a natural by-
383 product of aerobic respiration, by other commensal microorganisms, or by host metabolism
384 and immune responses, and must adapt rapidly to varying levels of oxidative stress [44]. In
385 the upper respiratory tract (and in the bloodstream), macrophages and polymorphonuclear
386 cells, which utilize oxygen-dependent pathways to generate large quantities of ROS, facilitate
387 the clearance of invading organisms [45].

388 The response to oxidative stress in pathogenic *Neisseria* is controlled by the OxyR
389 regulon, which has been studied in *N. gonorrhoeae* [35], *N. meningitidis* [46] and
390 *H. influenzae* [47], and has been shown to operate in a distinctive manner in these organisms

391 compared to the OxyR regulon in *Escherichia coli*. The OxyR regulon controls expression of
392 *kat* in response to H_2O_2 [48, 49], however, it was found that *oxyR* mutant strains of
393 *N. gonorrhoeae* [35, 50], *N. meningitidis* [46] and *H. influenzae* [47] have considerably
394 higher levels of *kat* expression and are significantly more resistant to H_2O_2 killing than their
395 respective wild-type strains. In *N. gonorrhoeae*, the OxyR regulon was suggested to be
396 limited to *kat* and two other genes: *prx* (designated *prx5-grx* in this study) and the
397 downstream gene, *gor* [35]. In *H. influenzae*, the Prx5-Grx homologue was suggested to be
398 involved in scavenging low levels of endogenous H_2O_2, thus limiting the activation of *kat*,
399 and, thereby, enabling the response to oxidative stress to be tightly controlled at a level
400 proportionate to the stress [25, 47]. In this study, we found that the meningococcal *prx5-grx*
401 mutant is also more resistant to exogenously supplied H_2O_2 compared to the wild-type and
402 complemented strains, which is in keeping with the findings described in *N. gonorrhoeae* and
403 *H. influenzae* [25, 35]. Increased catalase expression was determined in both the *N.*
404 *gonorrhoeae* and *H. influenzae* mutants lacking Prx5-Grx expression and suggested as a
405 likely explanation for increased H_2O_2 resistance [25, 35]. Further experimentation will be
406 required to establish if catalase expression is also elevated in the meningococcal *prx5-grx*
407 mutant. Interestingly, in *H. influenzae*, the majority of scavenging of metabolically-generated
408 H_2O_2 was shown to be due to expression of *kat* or the *prx5-grx* homologue, however, deletion
409 of both genes did not impair virulence in the infant rat model of infection [51].
410 Ex-vivo whole blood survival assays have been used widely as models of invasive
411 meningococcal disease [52-55]. They are considered to be useful as they are relatively facile
412 and reproduce many of the important constituents of the host-pathogen interaction using cells
413 from the host relevant to this highly human-adapted pathogen. Killing of *N. meningitidis* in a
414 whole blood survival assay is mediated by both cellular and humoral components of the
415 immune system, although capsulated *N. meningitidis* are protected from complement-

mediated killing in the absence of specific antibody. It has also been shown recently that the presence of heparin, as used in our assay to prevent coagulation, may also reduce complement-mediated killing [56]. The observation that the *prx5-grx* mutant was more sensitive to killing than its wild-type parent is unlikely to be explained by differences in sensitivity to H_2O_2 as this mutant was less sensitive in an H_2O_2 killing assay. It also cannot be explained by changes in levels of plasminogen binding, which might act to inhibit interactions with phagocytes, as the mutant cells bound as much plasminogen as their wild-type parents in ELISA assays, presumably due to the presence of other identified plasminogen-binding proteins on the meningococcal surface. The mechanism by which Prx5-Grx promotes meningococcal survival remains to be determined, but our findings demonstrate that it is likely to play an important role in invasive meningococcal disease.

Funding

This work was financially supported by the Higher Committee for Education Development (HCED), Iraq.

Conflicts of interest

The authors declare that there are no conflicts of interest.

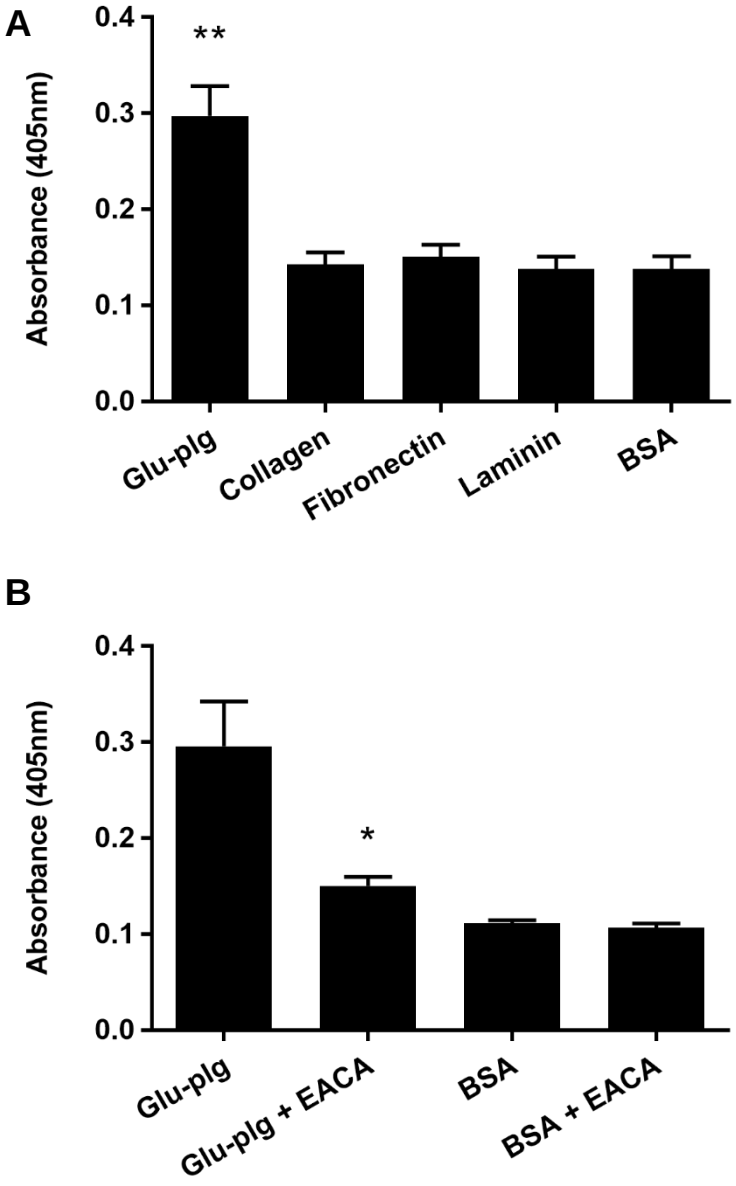
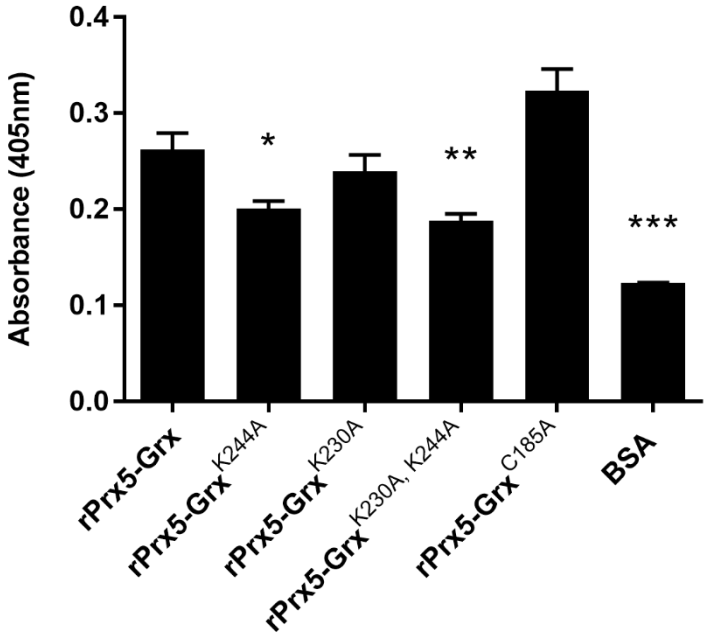


Fig. 1. rPrx5-Grx is a plasminogen-binding protein. (A) Binding of rPrx5-Grx to immobilized human ligands examined by ELISA. rPrx5-Grx bound significantly to glu-plg compared to the negative control immobilized ligand (BSA). ** *P* value < 0.01 (Student's *t*-test). Binding of rPrx5-Grx to laminin, fibronectin or collagen was not significantly different to BSA. (B) Binding of rPrx5-Grx to glu-plg in the presence or absence of 50 mM ϵ-aminocapronic acid (EACA). * *P* value < 0.05 (Student's *t*-test) compared to binding in the absence of EACA. Means are from ≥ 4 independent experiments carried out in triplicate wells. Error bars indicate SE.



444

445 **Fig. 2. Lysine 244 is required for optimal binding of rPrx5-Grx to plasminogen.** No
446 significant differences were detected in the ability of rPrx5-Grx^{K230A} and rPrx5-Grx^{C185A} to
447 bind glu-plg compared to rPrx5-Grx as determined by ELISA. The significant reduction in
448 binding of rPrx5-Grx$^{K230A, K244A}$ and rPrx5-Grx^{K244A} shows that lysine 244 is required for
449 optimal glu-plg binding. BSA was used as a negative control. *, **, *** denote *P* values <
450 0.05, < 0.01 and < 0.001, respectively (Student's *t*-test) compared to rPrx5-Grx. Means are
451 from ≥ 4 independent experiments carried out in triplicate wells. Error bars indicate SE.

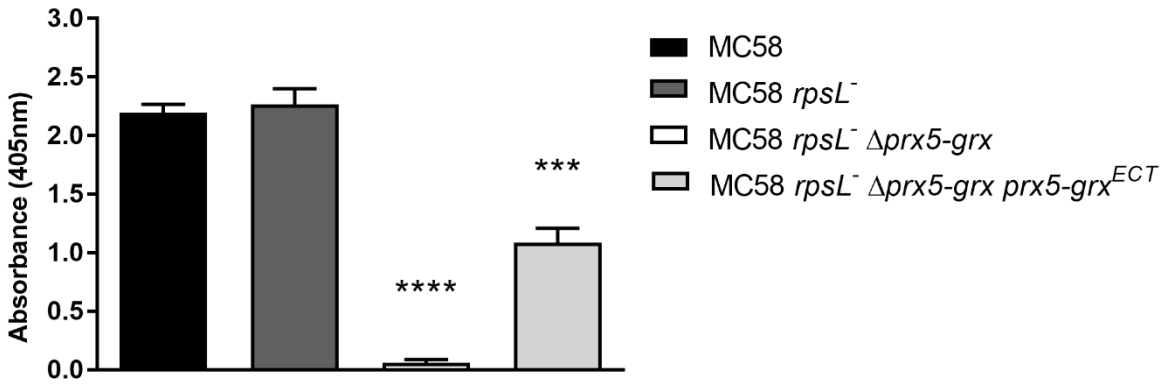


Fig. 3. Detection of surface-exposed meningococcal Prx5-Grx by whole-cell ELISA. Wells were coated with formaldehyde-fixed whole meningococcal cells before being probed with rabbit anti-rPrx5-Grx antibodies. Binding of the antiserum was significantly reduced in strains lacking wild-type levels of rPrx5-Grx expression. Complementation of *prx5-grx* restored expression, albeit not to wild-type levels. Values shown are minus the values obtained from control wells coated with 1% BSA. *** and **** denote *P* values < 0.001 and < 0.0001, respectively (Student's *t*-test) compared to MC58. Means are from ≥ 4 independent experiments carried out in triplicate wells. Error bars indicate SE.

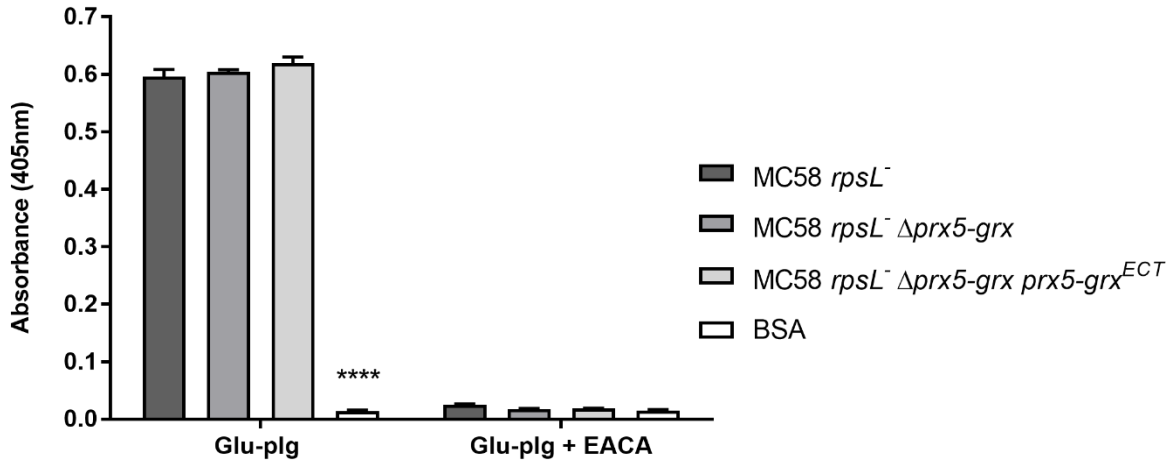


Fig. 4. Loss of surface-localized Prx5-Grx does not significantly reduce the ability of meningococci to bind plasminogen. No significant differences were detected in the ability of the MC58 strains tested to bind glu-plg. In each case, the addition of ϵ-aminocapronic acid (EACA) abolished the interaction confirming that all significant interactions at the meningococcal surface with glu-plg are lysine-mediated. **** denote *P* value < 0.0001 (Student's *t*-test) compared to MC58 *rpsL*$^{-}$. Means are from ≥ 3 independent experiments carried out in triplicate wells. Error bars indicate SE.

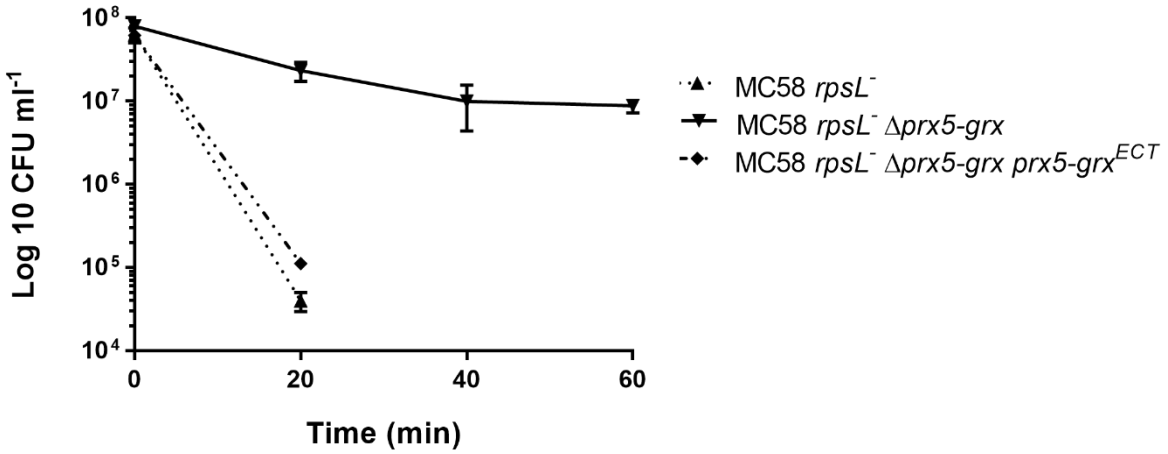


Fig. 5. Prx5-Grx deficient meningococci have increased tolerance to hydrogen peroxide. Meningococcal cells were resuspended in BHI broth and exposed to a final concentration of 12.5 mM H_2O_2. After 20 min of exposure, the survival of MC58 *rpsL*$^-$ Δ*prx5-grx* was significantly higher (Student's *t*-test *p* value < 0.05) than MC58 *rpsL*$^-$. With the exception of MC58 *rpsL*$^-$ Δ*prx5-grx*, all strains were undetectable at 40 and 60 min. Three experiments, each in triplicate, were performed. Error bars indicate SE of the mean.

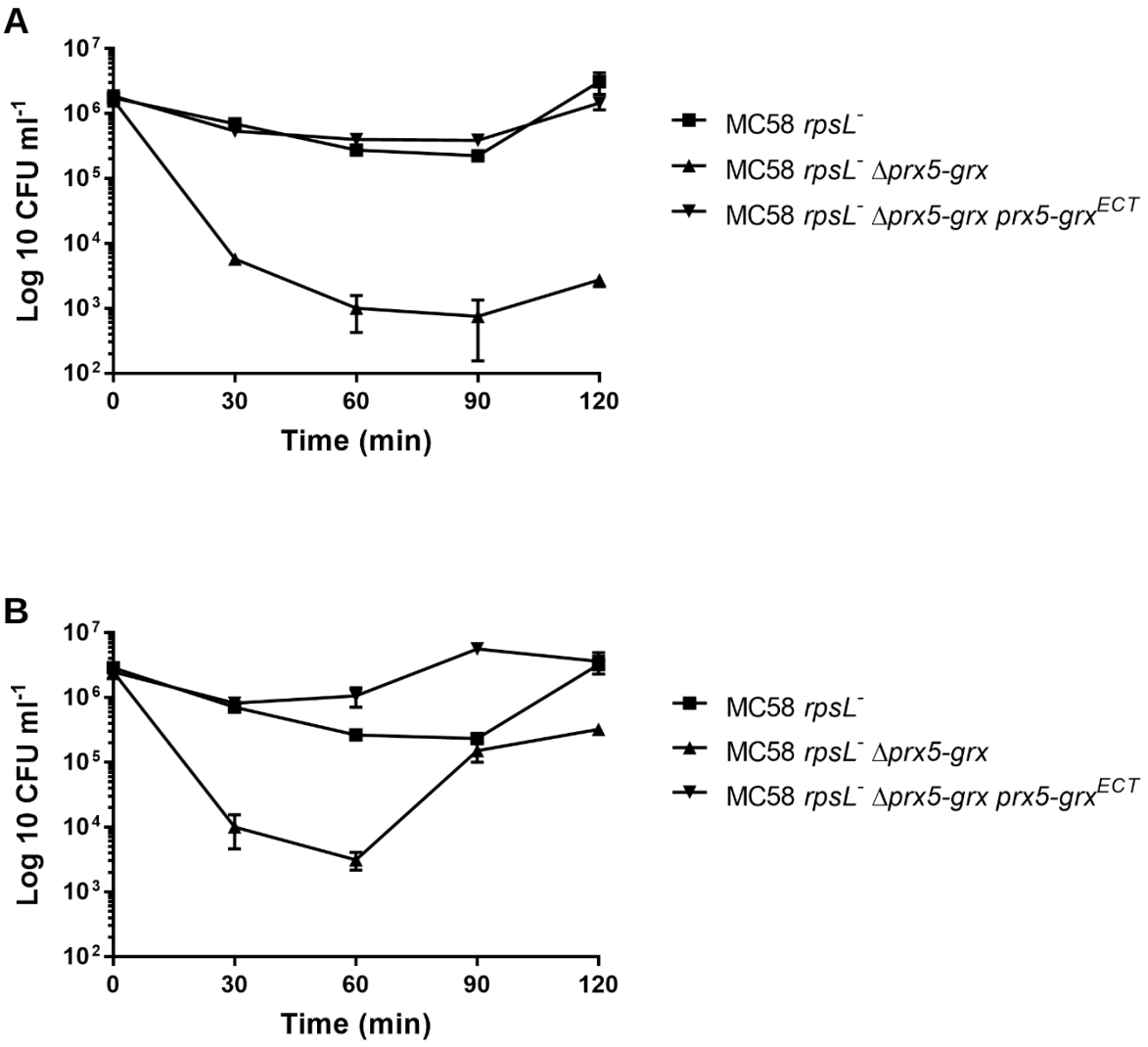


Fig. 6. Prx5-Grx is important for survival in an ex vivo human whole blood model of meningococcal septicemia. Results show the survival of strains in human whole blood from two different donors (A and B) over the course of 2 h. There was a $\geq$ 2-$\log_{10}$ difference in CFU between MC58 *rpsL*$^-$ Δ*prx5-grx* and the parental or complemented strain at the 30 and 60 min time points. Each donor sample was tested twice in duplicate. Error bars indicate SE of the mean.

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