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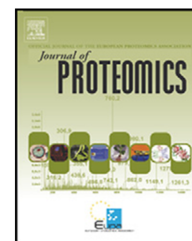

Manganese regulation of virulence factors and oxidative stress resistance in *Neisseria gonorrhoeae*

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ABSTRACT

Neisseria gonorrhoeae has evolved a complex and novel network of oxidative stress responses, including defence mechanisms that are dependent on manganese (Mn). We performed systematic analyses at the transcriptomic and proteomic (1D SDS-PAGE and Isotope-Coded Affinity Tag [ICAT]) levels to investigate the global expression changes that take place in a high Mn environment, which results in a Mn-dependent oxidative stress resistance phenotype. These studies revealed that there were proteins regulated at the post-transcriptional level under conditions of increased Mn concentration, including proteins involved in virulence (e.g., pilin, a key adhesin), oxidative stress defence (e.g., superoxide dismutase), cellular metabolism, protein synthesis, RNA processing and cell division. Mn regulation of inorganic pyrophosphatase (Ppa) indicated the potential involvement of phosphate metabolism in the Mn-dependent oxidative stress defence. A detailed analysis of the role of Ppa and polyphosphate kinase (Ppk) in the gonococcal oxidative stress response revealed that *ppk* and *ppa* mutant strains showed increased resistance to oxidative stress. Investigation of these mutants grown with high Mn suggests that phosphate and pyrophosphate are involved in Mn-dependent oxidative stress resistance.
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Abbreviations: ABC, ATP-binding cassette; BHI, brain heart infusion; CAC, citric acid cycle; ED, Entner–Douderoff; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; IAA, iodoacetamide; Mn, manganese; NCBI, National Center for Biotechnology Information; OM, outer membrane; PEP, phosphoenolpyruvate; PMN, polymorphonuclear leukocyte; PolyP, polyphosphate; Ppa, inorganic pyrophosphatase; RNS, reactive nitrogen species; SOD, superoxide dismutase; TCEP, Tris(carboxyethyl) phosphine; TIGR, The Institute for Genomic Research.

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1. Introduction

Neisseria gonorrhoeae, the causative agent of the sexually transmitted infection gonorrhea, is a facultative aerobe and a host-adapted pathogen that is frequently associated with inflamed urogenital tissues and activated polymorphonuclear leukocytes (PMNs) [1,2]. Hence, *N. gonorrhoeae* is routinely exposed to substantial amounts of superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS), as well as reactive nitrogen species (RNS) [reviewed in 3,4]. Oxidative stress, resulting from the action of ROS and RNS, causes damage to DNA, proteins and lipids [5–7]. The observation that *N. gonorrhoeae* can be isolated from PMN-laden purulent exudates, and can survive in PMNs [8] indicates that this bacterium has highly efficient defence systems to respond to oxidative stress, as previously reviewed [9].

Previous studies have shown that accumulation of manganese (Mn), via the ATP-binding cassette (ABC)-type Mn transporter MntABC, protects *N. gonorrhoeae* from O_2^- and H_2O_2 killing by a mechanism that is independent of superoxide dismutase (SOD) [10] and catalase [11], respectively. The increased resistance seen to oxidative challenge was Mn-specific; no increased resistance was seen when *N. gonorrhoeae* was grown with media supplemented with Co(II), Mg(II) or Zn(II) [10]. MntABC expression in *N. gonorrhoeae* is regulated by PerR, a transcriptional repressor from the Fur family [12]. Both *mntC* and *perR* mutants have reduced intracellular survival in a human cervical epithelial cell model [12]. *Streptococcus pneumoniae* has a similar Mn transport system, PsaBCA, which also plays a role in resistance to O_2^- and H_2O_2, as well as in systemic virulence [13,14].

Mn is now recognised as a key ion in the regulation of metabolism and stress responses and can play a variety of roles in cellular processes in many bacteria. As a consequence, this ion has a major effect on virulence in several bacterial pathogens [reviewed in 15,16]. Mn concentrations vary up to 1000 fold between different sites in the human body [16–18], providing a potential signal for *N. gonorrhoeae* to adapt to microenvironments within the host. Indeed, Mn regulates multiple genes in *S. pneumoniae* via the regulator PsaR, with Mn concentrations signalling expression of virulence factors within different host sites [19,20]. Mn availability also affects the expression of *Streptococcus mutans* virulence genes differentially during planktonic or biofilm culture [21].

To investigate the precise nature of the oxidative stress resistant phenotype observed in *N. gonorrhoeae* grown with a Mn(II) supplement [10], we have used DNA microarray analysis and a shotgun proteomic approach that involved one dimensional sodium dodecyl sulfate-polyacrylamide gel electrophoresis (1D SDS-PAGE) coupled with one dimensional liquid chromatography–tandem mass spectrometry (1D LC–MS/MS) as well as Isotope-Coded Affinity Tag (ICAT) studies coupled with MS/MS. The results of these studies provide new insights into the effect of Mn on the proteome of *N. gonorrhoeae* and the role of this ion in the oxidative stress response.

2. Materials and methods

2.1. Bacterial strains and culture conditions

N. gonorrhoeae strain 1291 was supplied by Dr. Michael Apicella (University of Iowa, USA). Bacteria were grown on brain heart infusion (BHI) agar or broth (Accumedia) supplemented with 10% (v/v) levinthal's base [22] and 1% (v/v) isovitalex (Becton Dickinson) at 37 °C in 5% CO_2. *N. gonorrhoeae* was grown on BHI agar from freezer stocks for about 22 h and approximately ten colonies were passed in supplemented BHI broth. After 18 h, cell density was measured and diluted to optical density at 600 nm (OD_{600}) ~0.5. Then, 500 µl of this culture was inoculated into 5 ml of fresh BHI broth ± 40 µM manganese sulfate ($MnSO_4$) and grown in a flask on a shaking incubator for approximately 5 h to mid-log phase (OD_{600} ~ 0.5).

E. coli DH5α was cultured at 37 °C in a Luria–Bertani (LB) broth or on LB-1.5% bacteriological agar (Difco) plates [23]. Ampicillin (Amp) and kanamycin (Kan) were used at a final concentration of 100 µg/ml and 50 µg/ml, respectively.

2.2. Microarray analysis

Triplicate cultures (biological replicates) of *N. gonorrhoeae* strain 1291 wild type were grown to exponential phase (OD_{600} ~ 0.5) in the presence and absence of 40 µM $MnSO_4$ (as described above). Approximately 100 µg of total RNA was prepared from each sample using the RNeasy Maxi Kit according to the manufacturer's instructions (Qiagen). The triplicate samples were pooled and the integrity and concentration of RNA were determined via micro-fluidic analysis on a bio-analyser (Agilent Technologies).

All microarray analyses were performed on *N. gonorrhoeae/meningitidis* genome arrays (The J. Craig Venter Institute (JCVI), formerly TIGR; http://pfgrc.jcvi.org/index.php/microarray/available_microarrays.html). Each microarray consists of 6389 70mer oligonucleotides representing open reading frames (ORFs) from *N. gonorrhoeae* strains FA1090 and ATCC 700825 (reference strain), and *N. meningitidis* strains Z2491 (serogroup A) and MC58 (serogroup B). Methods and analysis were performed as previously described [24]. 5 µg of each total RNA sample was labelled using random hexamers and direct incorporation of fluorescent Cy3- or Cy5-labelled nucleotides. The hybridisations were performed in triplicate and incorporated a dye-swap to account for dye bias. After 16 h of hybridisation, the arrays were washed and scanned on an Agilent G2565BA microarray scanner at a 5 µm resolution. The resulting images of the hybridisations were analysed using Imagene 5.5 (BioDiscovery Inc.) and the mean foreground, mean background and spot/signal quality were determined.

All primary data were imported into an in-house installation of the comprehensive microarray relational database, BASE (http://kidney.scgap.org/base) (login: Wu 2006, password: Manganese; see experiment Wu 2006, *N. gonorrhoeae* vs manganese). After print-tip intensity independent Lowess normalisation, differential expression was defined using a robust statistical method rather than simple fold change. All genes were ranked using the B statistic method where both fold change and variance of signals in replicates are used to determine the likelihood that genes are truly differentially expressed. A threshold in the *B* statistic of 0.0 was adopted as genes with a *B* score > 0 and a > 50% probability of being truly differentially expressed [25]. The ranked *B*-scores for all genes in each experiment are also maintained in BASE.

2.3. Quantitative real-time PCR

Total RNA was isolated using the RNeasy kit (Qiagen) as described above. The equivalent of 1 μg of the total RNA preparation was treated with RQ1 RNase-free DNase (Promega). RNA was reverse transcribed using random primers and the TaqMan® RT-PCR kit (PE Applied Biosystems) as recommended by the manufacturer. Primers were designed using Primer Express 1.0 software (ABI Prism; PE Biosystems). All quantitative Real-Time PCR (qRT-PCR) reactions were performed in triplicate in a 25 μl mixture containing cDNA (5 μl of 1/5 dilution), 1× SYBR Green buffer (PE Applied Biosystems) and approximately 2 μM of each primer (see Table 1 for primer sequences). 16S rRNA was used as the standard control in each quantitative PCR. Amplification and detection of specific products were performed with the ABI Prism 7700 sequence detection system (PE Applied Biosystems) with the following cycle profile: 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s and 60 °C for 1 min. Data was analysed with ABI Prism 7700 v1.7 analysis software. Relative gene expression between the *N. gonorrhoeae* wild type strain grown in the presence and absence of Mn was determined using the $2^{\Delta\Delta CT}$ relative quantification method. *P* values for statistical significance were determined using Student's *t*-tests.

2.4. Subcellular fractionation

N. gonorrhoeae was grown as described above to mid-log phase. Cultures were harvested by centrifugation at 6000 rpm for 10 min at 4 °C. The cell pellet was washed with phosphate buffered saline (PBS) twice and heat killed at 56 °C for at least 30 min. Cells were sonicated for 30 s at setting 4 in a microson ultrasonic cell disrupter (Misonix Incorporated, New York, USA) followed by cooling on ice for 30 s; this was repeated three times. Cells were centrifuged at 5000 rpm for 10 min to remove debris. The supernatant represented the whole-cell extract. A portion of the whole-cell extract was subjected to further sonication and centrifuged at 100,000×*g* for 60 min; the supernatant contained the cytoplasmic proteins. The pellet was resuspended in 10 mM Tris, pH 8.0, 1% sarcosyl (N-lauroylsarcosine sodium salt) and sonicated as described above. This extract was centrifuged at 100,000×*g* for 60 min; the supernatant contained the cytoplasmic membrane proteins. The pellet containing the outer membrane was resuspended in 100 μl H_2O.

Table 1 – Primers used in this study for PCR and qRT-PCR.

Primer	Sequence (5′–3′)
PilEsignalF	ATGAATACCCTTCAAAAAGGCTTTACCC
PilEsignalR	AGTCTTGGTAGGCGGGAAGGGCGAC
ppa_RTfor	TTACCGAACAACCTCTGGCAAC
ppa_RTrev	GCAGGTACGCAGACGATTTTG
ppsa_RTfor	GGCAAATCGGTAACCAACGTC
ppsa_RTrev	TTTCGATGGTCAGCGCGTA
sodB_RTfor	TCCAAGAAGCGTTCAATGCC
sodB_RTrev	GGCGTTGGAAGTGGAAATCA
Ppk_for	CGTCATGCCGCCTGAAACCGGCGCA
Ppk_rev	GCGGGTCAGCCTCGGAGCAAATC
ppa-KO-F	CCAGCGCGTTTTCGACAAAGG
ppa-KO-R	ATCCTGCTCATCGAACGCAC

2.5. SDS-PAGE

The protein concentration was measured using the Bradford method [26]. The same amount of total cell protein was loaded onto a SDS-PAGE gel with a 5% (v/v) acrylamide stacking gel and a 10, 12.5 or 18% (v/v) resolving gel according to Laemmli [27]. A Low Molecular Weight Protein Standard (Amersham) or Protein Molecular Weight Marker (Fermentas) was used for protein standards. After electrophoresis, the gel was fixed by 10% (v/v) methanol and 7% (v/v) glacial acetic acid for 30 min and then stained by SYPRO® Ruby Protein Gel stain (Sigma) in the dark overnight. The gel was then destained by 10% (v/v) methanol and 7% (v/v) glacial acetic acid for 30 min and washed with distilled water for 10 min.

2.6. In-gel tryptic digestion

The protein bands on 1D gels were manually excised from the gel and cut into pieces. The gel pieces were reduced with 50 mM 1,4-dithioerythreitol (DTE) (Fluka) in 25 mM ammonium bicarbonate, pH 8.5, at 37 °C for 1 h, and subsequently alkylated with 100 mM iodoacetamide (IAA) (Fluka) in 25 mM ammonium bicarbonate, pH 8.5, at room temperature for 1 h. The pieces were then washed twice with 50% acetonitrile (ACN) in 25 mM ammonium bicarbonate, pH 8.5 for 15 min each, dehydrated with 100% ACN for 5 min, dried and then rehydrated with a total of 22.5 ng of sequencing grade modified trypsin (Promega, Madison, WI, USA) in 25 mM ammonium bicarbonate, pH 8.5, at 37 °C for 16 h. Following digestion, tryptic peptides were extracted twice with 50% ACN containing 5% formic acid for 15 min each with moderate sonication. The extracted solutions were pooled and evaporated to dryness under a vacuum, then the samples were analysed by the nanoLC–MS/MS system (Micromass/Waters).

2.7. ICAT

Triplicate cultures (biological replicates) of *N. gonorrhoeae* strain 1291 wild type were grown to exponential phase (OD_{600} ~ 0.5) in the presence and absence of 40 μM $MnSO_4$ (as described above). 500 μg of total protein extracts were quantified by Cleavable ICAT® Reagent Kit for Protein Labelling (Applied Biosystem, Foster City, CA, USA). This was performed according to the manufacturer's instructions. The sample was analysed by the nanoLC–MS/MS system and the data was analysed by the Micromass ProteinLynx™ Global Server (PGS) 2.0 data processing software (Micromass/Waters).

2.8. 1D LC-nanoESI-MS/MS analysis for protein identification

Direct 1D LC-nano electrospray ionization (ESI)-MS/MS analyses were performed on an integrated nanoLC–MS/MS system (Micromass) comprising a 3-pumping Micromass/Waters CapLC™ system with an autosampler, a stream select module configured for precolumn plus analytical capillary column, and a Micromass Q-Tof Ultima™ API mass spectrometer fitted with a nanoLC sprayer, and operated under MassLynx™ 4.0

control. Injected samples were first trapped and desalted isocratically on a C18 precolumn (5 μm, 150 μm I.D.×15 mm; produced in Facilities for Proteomics Research, Academia Sinica, Taiwan) for 2 min with 0.1% formic acid delivered by the auxiliary pump at 15 μl/min after which the peptides were eluted from the precolumn and separated on an analytical C18 capillary column (5 μm, 75 μm I.D.×25 mm) connected inline to the mass spectrometer, at 300 nl/min using a 50 and 240 min gradient of 5% to 80% ACN in 0.1% formic acid for in-gel digestion and ICAT samples, respectively.

The online nanoESI-MS survey scan and data-dependent acquisition of collision-induced dissociation (CID) MS/MS were fully automated and synchronized with the nanoLC runs under the full software control of MassLynx™ 4.0. Prior to online analysis, the nanoLC sprayer and Z-spray source parameters were tuned and optimized with a 50 fmol/μl solution of glufibrinopeptide B in 50% ACN/0.1% formic acid, directly infused at 300 nl/min. Argon maintained at $\sim 4.0\times10^{-5}$ mbar was used as the collision gas for CID MS/MS. Calibration was performed using the product ions generated from the fragmentation of the doubly charged molecular ion of glufibrinopeptide B at *m/z* 785.8. For routine protein identification analysis, the 1 s survey scans were acquired over the mass range *m/z* 400–2000 and a maximum of 3 concurrent MS/MS acquisitions would be triggered for 2+, 3+ and 4+ charged precursors detected at an intensity above the predefined threshold (20 counts/s). MS/MS acquisition is completed and switched back to survey scan when each of the precursor intensity falls below a predefined threshold (3 counts/s) or after a maximum of 6 s acquisition.

After data acquisition, the individual MS/MS spectra acquired for each of the precursors within a single LC run were combined, smoothed, deisotoped (fast option, inclusive of simple transformation of multiply charged peaks) and centroided using the Micromass ProteinLynx™ Global Server (PGS) 2.0 data processing software and output as a single Mascot-searchable peak list (.pkl) file.

2.9. Protein identification and quantification

The peak list files from 1D LC-nanoESI-MS/MS were used to query the National Center for Biotechnology Information (NCBI) database using the Mascot 2.0 program (Matrix Science Ltd) with the following parameters: peptide mass tolerance, 50 ppm; MS/MS ion mass tolerance, 0.25 Da; allow up to one missed cleavage; variable modifications considered were methionine oxidation and cysteine carboxyamidomethylation. Only significant hits as defined by Mascot probability analysis were considered initially. In addition, a minimum total score of 20 comprising at least a peptide match of ion score more than 20 was arbitrarily set as the threshold for acceptance. In the case of data from 1D SDS-PAGE where the same protein may be found in more than one band, the total score for a particular protein hit comprises the best scoring peptide matches from all bands where the same protein hit was found.

2.10. N-terminal protein sequence analysis

For the analysis of N-terminal amino acid sequences, proteins were subjected to SDS-PAGE using the Tris–Tricine system described by Schagger and von Jagow [28]. After electrophoresis, proteins were blotted onto Polyscreen polyvinylidene difluoride (PVDF) Transfer Membrane (NEN Life Science Products, Boston, USA) using CAPS buffer (10 mM 3-[cylcohexylamino]-1-propanesulfonic acid, pH 11) by the method of Towbin et al. [29]. The PVDF membrane was soaked in MilliQ water for 10 min with shaking and stained with 0.1% (w/v) Coomassie Blue R250, 50% (v/v) methanol and 10% (v/v) acetic acid for 5 min. The membrane was destained by 50% (v/v) methanol, and 10% (v/v) acetic acid and rinsed in MilliQ water. The desired protein band was cut out and the N-terminal sequence of the protein was determined using a PE Biosystems 492cLC protein sequencer. "NGOxxxx" Gene ID refers to the annotation of the *N. gonorrhoeae* FA1090 genome strain (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?org=ntng03).

2.11. Immunoblotting

Overnight cultures of *N. gonorrhoeae* were harvested from agar plates into an appropriate volume of PBS and were heat killed at 56 °C for 1 h. Then, cell suspensions were boiled for 5 min and their protein concentrations were measured by absorbance at 280 nm. 500 μg of total cell protein was loaded onto an SDS-PAGE gel with a 5% (v/v) acrylamide stacking gel and a 12% (v/v) resolving gel [27]. The protein standard used was BenchMark™ Prestained Protein Ladder (Invitrogen). Usually, two gels were run simultaneously, one for Coomassie staining to control for equal protein loading, and the other for immunoblotting. Following electrophoresis, polyacrylamide gels were transferred onto nitrocellulose membrane (Bio-Rad) for 1 h at 15 V according to Towbin et al. [29]. The immunological detection was performed by blocking in 5% (w/v) skim milk, followed by incubation with primary antibody (1/100 dilution of pilin specific SM1 [30]) for 1 h, then secondary antibody (1/7500 dilution of anti-mouse alkaline phosphatase conjugate; Sigma) for 1 h and then detection of bound antibody as previously described [31].

2.12. Northern blot

N. gonorrhoeae cells were grown in BHI broth ±100 μM Mn at 37 °C to log phase (about 6 h). RNA was extracted from these cells using 'Rneasy® Midi Kit' (Qiagen) according to the manufacturer's instructions. Northern blot was performed according to standard procedures [23]. Briefly, 1 μg of total RNA was separated with 1% formaldehyde gel, transferred to GeneScreen membrane (NEN Life Science) and hybridised with DIG-labelled probe. Primers, PilEsignalF and PilEsignalR (Table 1), were used in the PCR to amplify the probe, *pilE*, with strain 1291 DNA as the template. The probe was DIG-labelled by using DIG-11-dUTP (Roche). Hybridisation and detection were done using the CDP-Star Kit (Roche) as recommended by the manufacturer. Briefly, filters were prehybridised at 65 °C (high stringency) in a mixture of 5× SSC, 0.02% SDS and 1% blocking reagent (Roche) for 4–6 h. Hybridisations were performed overnight in the same solution and filters were subsequently washed in 1% SDS, and 0.5× SSC for 2×15 min at 65 °C (high stringency) prior to detection.

2.13. Cloning and mutagenesis of the ppk and ppa genes of N. gonorrhoeae

The "NGO" gene designations used below were obtained from the annotated *N. gonorrhoeae* strain FA1090 genome (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?org=ntng03). Knock-out constructs of the *ppk* (NGO0003) and *ppa* (NGO0223) genes were constructed via the insertion of a kanamycin-resistance cassette (pUC4Kan: Amersham Biosciences) into suitable, unique, restriction sites within the coding region of each gene, as described by Tseng et al. [10]. The *ppk* gene was amplified from *N. gonorrhoeae* strain 1291 chromosomal DNA using the primers, Ppk-for and Ppk-rev (see Table 1 for the primer sequences) and cloned into pGEM®-T Easy (Promega). The central 801 bp portion of the *ppk* gene was then deleted by digestion with *Stu*I and *Xcm*I and replaced with a *Hinc*II DNA fragment containing the kanamycin-resistance cassette from plasmid pUC4kan. The *ppa* gene, plus the region 400 bp up- and downstream of this gene, was amplified using the primers, ppa-KO-F and ppa-KO-R, and cloned into pGEM®-T Easy. The kanamycin-resistance cassette was cloned into the *Pfl*MI restriction endonuclease site located within *ppa*. Mutant strains were generated by digesting pGEM*ppk*::*kan* with *Not*I and pGEM*ppa*::*kan* with *Alu*I followed by transforming these linearised fragments into *N. gonorrhoeae* strain 1291. Recombinant strains were selected by growth on BHI agar containing kanamycin (100 μg/ml) as described by Jennings et al. [32]. Previous work has demonstrated that the pUC4kan kanamycin cassette has no promoter nor terminator that is active in *Neisseria* and will neither affect transcription nor have a polar effect on expression of adjacent genes [32,33].

2.14. SOD assay

Cell free extracts of *N. gonorrhoeae* strains were prepared by resuspending an equal amount of cells (as determined by equal optical density of samples at 600 nm) in PBS, followed by three cycles of freezing and thawing. Cell debris was removed by centrifugation at 13,000 rpm for 20 min, and the supernatant was collected. Protein concentration of samples, approximated by absorbance at 280 nm, was used to normalise samples. The SOD assay was performed as described by Crapo et al. [34]. A 500 μl mixture was prepared containing 50 μl of 0.1 mM cytochrome *c* (Sigma), 250 μl of 0.1 mM xanthine and 190 μl of potassium buffer (+/− the sample) and the reaction was initiated by adding 10 μl of 175 mU/ml xanthine oxidase. The rate of increase in absorbance at 550 nm was recorded. A unit is defined as the quantity of SOD required to produce 50% inhibition of the rate of reduction of cytochrome *c* under the specified conditions. *P* values for statistical significance were determined using Student's *t*-tests.

2.15. Pyrophosphatase assay

Samples were prepared as described above for the SOD assay. The pyrophosphatase assay is based on the method described by Heinonen and Lahti [35]. The assay mixture was prepared containing 500 μl of 50 mM Tris–HCl, pH 8 and 1 mM $MgCl_2$, 20 μl 1.7 mM Na-pyrophosphate, and 50 μl of cell free extract. The reaction was incubated at 37 °C for 30 min then stopped with 100 μl of 100 mM citric acid. 120 μl of the assay mix was then mixed with 1 ml of detection solution (1 volume of 10 mM ammonium-molybdate, 1 volume of 5 N sulfuric acid, and 2 volumes of acetone). The amount of phosphomolybdate was determined by measuring the absorbance at 420 nm. *P* values for statistical significance were determined using Student's *t*-tests.

2.16. Oxidative stress killing assays

Paraquat (PQ) [36], xanthine/xanthine oxidase (X/XO) [37] and H_2O_2 [38] killing assays were performed using the established methods described by Tseng et al. [10]. Briefly, cells from agar plates were harvested, resuspended in PBS, and 10^5 to 10^7 cells were added to a solution of BHI broth to a final volume of 100 μl. The killing assay was started by the addition of a final concentration of either 10 mM PQ (Sigma), 4.3 mM xanthine and 300 mU/ml xanthine oxidase (Sigma), or 40 mM H_2O_2 (Riedel-de Haen). Cultures were incubated at 37 °C/5%CO_2. At various time points, samples were taken, serially diluted, plated onto BHI agar and incubated at 37 °C in 5% CO_2 to determine colony forming units (CFU). Experiments were done in triplicate and repeated on at least three occasions, with representative results shown. *P* values were performed using Student's *t*-test. Differences seen between strains and growth conditions were considered significant if the *P* value was $\leq$0.05. Safety considerations: Paraquat is very toxic by inhalation, ingestion and if absorbed through skin.

2.17. Assay for the survival of N. gonorrhoeae in primary human cervical epithelial cells

Primary human cervical epithelial (pex) cells were procured and maintained as described previously [39]. Confluent cell monolayers were challenged with the wild type or mutant gonococci at a multiplicity of infection of 100. Association, invasion, and survival assays were performed as we have described previously using a modified gentamicin-survival assay [24]. Association is defined as the number of extra- and intracellular bacteria associated with pex cells at 90 min post-infection following extensive rinsing of the cell monolayer. Invasion is defined as the number of gonococci that survived gentamicin treatment (100 μg/ml; 30 min) subsequent to a 90 min challenge of pex cells. The ability of gonococci to survive within pex cells was determined by rinsing and then re-incubating (37 °C, 5% CO_2) the infected pex cell monolayers in an antibiotic-free medium for an additional 1 h (*i.e.*, survival 1 h) or 2 h (*i.e.*, survival 2 h) time period following gentamicin treatment. At each time point, viable gonococci were enumerated by counting CFUs obtained from plating serial dilutions of the pex cell lysates. Percent association, invasion, or survival was determined as a function of the original inoculum and the mean number of CFUs calculated for each condition assayed. *P* values were determined using a Kruskal–Wallis non-parametric analysis of variance.

3. Results

3.1. The effect of Mn on the transcriptome and proteome of N. gonorrhoeae

3.1.1. Microarray analysis of Mn-dependent regulation

To examine the effect of Mn on transcription in *N. gonorrhoeae*, gene expression in *N. gonorrhoeae* strain 1291 was investigated

Table 2 – 1D SDS-PAGE analyses comparing the cell fractions of *N. gonorrhoeae* strain 1291 grown in the absence and presence of 40 μM Mn(II).

Gene number [a]	Protein name	Gene name	Class [b]	Theoretical Mr (Da)
Proteins identified in high Mn				
Soluble fractions				
NGO0335	Polyribonucleotide nucleotidyltransferase/ phosphorylase	*pnp*	A	76,448
NGO0199	Transcription termination factor rho	*rho*	A	47,308
NGO1974	Elongation factor EF-Tu (elongation factor TS)	*tsf /EF-Tu*	B	30,345
NGO1843	Translation elongation factor EF-G	*fusA*	B	77,167
NGO1824	30S ribosomal protein S5	*rpsE*	B	18,231
NGO2025	30S ribosomal protein S9	*rpsI*	B	14,363
NGO1838	50S ribosomal protein L3	*rplC*	B	22,663
NGO1837	50S ribosomal protein L4	*rplD*	B	23,288
NGO0584	50S ribosomal protein L9	*rplI*	B	15,687
NGO1853	50S ribosomal protein L10	*rplJ*	B	17,589
NGO1855	50S ribosomal protein L11	*rplK*	B	14,942
NGO0298	50S ribosomal protein L20	*rplT*	B	13,664
NGO0710	A/G-specific adenine glycosylase	*mutY*	E	39,542
NGO0398	Adenylosuccinate synthetise	*purA*	F	45,970
NGO0353	Uracil phosphoribosyltransferase	*upp*	F	22,863
NGO1241	Histidinol-phosphate aminotransferase	*hisC*	G	39,245
NGO1358	Glutamate dehydrogenase	*gdhA*	G	48,462
NGO0040	Glutamate 1-semialdehyde 2,1-aminotransferase	*hemL /gsa*	H	44,999
NGO1931	Glyceraldehyde 3-phosphate dehydrogenase C	*gapC*	I	35,748
NGO1082	Isocitrate dehydrogenase	*idh*	I	79,943
NGO0921	Succinate dehydrogenase flavoprotein subunit	*dhsA /sdhA*	I	64,441
NGO0687	Ferredoxin-NADP reductase	*fenR*	I	29,317
NGO0829	Chaperone protein	*hscA*	J	66,413
NGO0450	Iron-superoxide dismutase [c] [d]	*sodB*	K	17,395
NGO0794	Bacterioferritin A	*bfrA*	L	17,962
NGO0832	Oxidoreductase, short chain reductase family	N/A	M	25,965
NGO1709	Conserved hypothetical protein	N/A	N	21,913
NGO1583	Conserved hypothetical protein	N/A	N	40,892
NGO0361	Conserved hypothetical protein (possible hemY)	N/A	N	45,187
NGO0571	Conserved hypothetical protein (possible MP)	N/A	N	65,933
NGO0156	Conserved hypothetica, possible lipid A synthesis	N/A	N	30,975
NGO1043	Conserved hypothetical protein	N/A	N	11,395
NGO1635	Hypothetical protein	N/A	P	17,060
Membrane fractions				
NGO0096	PilO / pilus assembly protein	*pilO*	O	23,330
NGO1673	Type IV pilus assembly protein (PilF)	*pilF*	O	61,882
NGO1806	UDP-N-acetylglucosamine acyltransferase	*lpxA*	O	28,157
NGO1067 / NGO1972	MafA adhesin–Neisseria-specific	*mafA*	O	34,738
NGO0994	H.8 OMP (azurin-like protein, Laz)	*azu /laz /*H.8	O	18,516
NGO1812	Neisseria-specific major OMP porin P.IB	*pIB*	O	37,174
NGO1780	Probable outer membrane lipoprotein	*omlA*	O	13,904
NG1577	OMP PIII, Omp3 (OMP class 4, RmpM)	*omp3*	O	25,525
NGO2147	ATP synthase delta chain	*atpH*	I	19,482
NGO1363	Multidrug efflux pump channel protein	*mtrE*	Q	50,401
NGO1765	Probable glycosyltransferase	*pglA*	B	41,995
NGO2057	Integral MP	N/A	M	30,694
NGO1800	Conserved hypothetical protein (possible integral MP)	N/A	N	48,088
Proteins identified in low Mn				
Soluble fractions				
NGO0002	DNA polymerase III, beta-subunit	*dnaN*	E	40,857
NGO0259	Ribonuclease III	*rnc*	A	26,934
NGO1858	Translation elongation factor Tu, TufA	*tufA*	B	42,926
NGO1832	30S ribosomal protein S3	*rpsC*	B	25,811
NGO1844	30S ribosomal protein S7	*rpsG*	B	17,629
NGO1826	30S ribosomal protein S8	*rpsH*	B	14,108
NGO1854	50S ribosomal protein L1	*rplA*	B	24,107
NGO1829	50S ribosomal protein L14	*rplN*	B	13,387
NGO1823	50S ribosomal protein L15	*rplO*	B	14,938
NGO18311	50S ribosomal protein L16	*rl16*	B	14,776

Table 2 *(continued)*

Gene number[a]	Protein name	Gene name	Class[b]	Theoretical Mr (Da)
Proteins identified in low Mn				
Soluble fractions				
NGO18241	50S ribosomal protein L18	*rl18*	B	12,781
NGO0171	50S ribosomal protein L19	*rl19*	B	13,751
NGO1676	50S ribosomal protein L21	*rplU*	B	11,431
NGO1828	50S ribosomal protein L24	*rplX*	B	11,591
NGO0442	50S ribosomal protein L25	*rplY*	B	20,939
NGO1454	Lysyl-tRNA synthetase (LysRS)	*lysS*	B	57,370
NGO0799	Inosine-5′-monophosphate dehydrogenase	*imdH*	F	52,429
NGO1667	2,3,4,5-tetrahydropyridine-2-carboxylate N-succinyltransferase, DapD	*dapD*	G	29,291
NGO1238	ATP phosphoribosyltransferase	*hisG*	G	26,232
NGO1134	GTP cyclohydrolase II	*gch2*	H	22,098
NGO1310	Guanylate kinase / GMP kinase	*gmk /kguA*	F	23,412
NGO1668	Glucose-6-phosphate isomerase	*pgi*	I	60,308
NGO0918	Citrate synthase	*cisY /gltA*	I	48,110
NGO0912	Succinyl-CoA synthetase, alpha subunit	*sucD*	I	30,536
NGO1743	NADH dehydrogenase I chain I	*nuoI*	I	18,790
NGO0116	Protein-export protein subunit SecB	*secB*	C	16,317
NGO2141	Chromosome segregation protein SpoOJ (ParB family)	*spoOJ /parB*	D	31,518
NGO1815	Septum site-determining protein	*minD*	D	29,581
NGO1422	Heat shock protein/ HSP-70 cofactor; nucleotide exchange factor	*grpE*	J	21,335
NGO1901	Heat shock protein HSP-40/chaperone DnaJ	*dnaJ*	J	40,590
NGO1378	Transport protein, ExbB	*exbB*	L	25,836
NGO0425	RdgC homolog / phosphatase	*rdgC*	Q	23,134
NGO0561	Conserved hypothetical protein	N/A	N	57,081
NGO0905	Conserved hypothetical protein	N/A	N	25,780
NGO1280	Conserved hypothetical protein	N/A	N	53,783
NGO1709	Conserved hypothetical protein	N/A	N	21,913
NGO1655	Neisseria-specific protein, uncharacterized	N/A	N	28,555
NGO0387	Conserved hypothetical protein	N/A	N	28,733
NGO1656	Possible cell-binding factor (possible protein-export)	N/A	M	31,514
NGO1873	Conserved hypothetical protein	N/A	N	45,436
Membrane fractions				
NGO2061	Pilin (fimbrial protein)	*pilE*	O	18,087
NGO0055	Pilus-associated protein, PilC2	*pilC2*	O	115,036
NGO0094	Pilus secretion protein; OMP-molecular complex, type II secretion pathway D protein	*pilQ*	O	77,943
NGO0070	OMP opacity protein B, opaB	P.II */opaB*	O	29,630
NGO1076	OMP opacity protein (Opa protein)	*opaH /opaK*	O	28,339
NGO0915	Dihydrolipoamide dehydrogenase E3 component	*dldH*	I	50,068
NGO2150	ATP synthase beta chain (ATPase beta-subunit)	*atpD*	I	50,413
NGO1495	Transferrin-binding protein A	*tbp-1 /tbpA*	L	101,940
NGO2059	Peptide methionine sulfoxide reductase, PilB	*msrAB /pilB*	Q	58,047
NGO2118	Conserved hypothetical protein (possible MSD element in ABC transport system)	N/A	N	19,526
NGO0284	Neisseria-specific protein, uncharacterized	N/A	R	15,725

N/A: Not available. OMP: outer membrane protein. MP: membrane protein.

[a] "NGO" gene designations were obtained from the annotated *N. gonorrhoeae* strain FA1090 genome (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?org=ntng03).

[b] Functional classifications as defined by the JCVI website (http://cmr.jcvi.org/tigr-scripts/CMR/shared/RoleList.cgi) are included solely as a reference-point for this large group of proteins. A: Transcription; B: Translation; C: Cellular processes; Protein and peptide secretion; D: Cellular processes; Cell division; E: Replication; DNA replication, restriction, modification, recombination, and repair; F: Purines, pyrimidines, nucleosides, and nucleotides; G: Amino acid biosynthesis; H: Biosynthesis of cofactors, prosthetic groups, and carriers; I: Energy metabolism; J: Cellular processes; Chaperones; K: Cellular processes; Detoxification; L: Transport and binding proteins; M: Unassigned; N: Unknown; O: Cell envelope; P: Hypothetical; Q: Other categories; R: *Neisseria*-specific protein.

[c] Using a biochemical assay for SOD activity it was found that cells grown on Mn had approximately 2.5 fold higher SOD activity than cells grown in the absence of added Mn ($P=0.01$ using a student's t-test).

[d] qRT-PCR was used to confirm microarray results. The ratio of transcription of these genes, wild type:wild type plus Mn, was below 1.5 fold ($P>0.4$).

using *N. gonorrhoeae / N. meningitidis* genome microarrays (JCVI). Total RNA was isolated from wild type strain cultures that had been grown to exponential phase in the presence or absence of added Mn (±40 μM $MnSO_4$). *N. gonorrhoeae* strain 1291 grows at an equal rate in media ± 40 μM $MnSO_4$ (data not shown). Growth in the presence of higher Mn did not result in a significant change in the expression level of any gene within *N. gonorrhoeae*, as determined using a cutoff of 1.5 fold change in expression and a threshold in the *B* statistic of >0 (See Materials and methods). Results from the microarray analysis were confirmed using quantitative real-time (qRT)-PCR on a selection of genes (see footnotes of Tables 2 and 3 for details). This result suggested that the Mn-dependent resistance to oxidative stress that is seen in *N. gonorrhoeae* may be mediated by post-transcriptional mechanisms.

3.1.2. 1D SDS-PAGE analysis of the effect of Mn on protein expression in N. gonorrhoeae

To investigate this possibility of Mn-dependent post-transcriptional regulation, we conducted a detailed analysis of the effect of Mn on the proteome of *N. gonorrhoeae*. A shotgun proteomic approach was used which involved two separate methods to identify Mn-regulated proteins, (1) 1D SDS-PAGE coupled with 1D LC-MS/MS and (2) ICAT studies coupled with MS/MS. This shotgun approach was used rather than 2D-gel based proteome profiling as the development of methods and instrumentation for automated data-dependent ESI MS/MS, in conjunction with nanoLC and database searching, has significantly increased the sensitivity and speed for the identification of gel-separated proteins [40,41]. For 1D SDS-PAGE analysis, cells grown to exponential phase in the presence or absence of added Mn (under the same conditions used for the transcriptomic studies described above) were fractionated into different cell compartments and visualised on 1D gels with varying percentage polyacrylamide and size in order to reduce the complexity of the whole-cell sample. A total of 96 proteins were identified by LC-ESI-MS/MS as having a different level of expression between samples grown in the presence and absence of Mn (Table 2; representative gels are shown in Fig. 1). 1D SDS-PAGE is a semi-quantitative method, therefore Table 2 only includes proteins that have obviously altered expression (i.e., present versus absent) in the presence of low or high Mn and does not include quantitative information, unlike the ICAT results, which quantitatively identify differentially regulated proteins. 46 proteins were present in high Mn that were absent in low Mn. These proteins

Table 3 – Differentially expressed proteins in *N. gonorrhoeae* strain 1291 grown in the absence and presence of 40 μM Mn from ICAT. The proteins listed are either down- or up-regulated by Mn.

Gene number [a]	Protein name	Gene name	Class [b]	Light/high area ratio [c]
Increased expression in the presence of Mn				
Soluble fractions				
NGO0794	Bacterioferritin [d]	*bfrA*	L	0.5
Reduced expression in the presence of Mn				
Soluble fractions				
NGO1845	30S ribosomal protein S12	*rplS*	B	2.1 ± 0.56
NGO1835	50S ribosomal protein L2	*rplB*	B	1.57
NGO1858	Translation elongation factor Tu, TufA [d]	*tufA*	B	2.1 ± 0.44
NGO1227	Cytosol leucyl aminopeptidase, LAP (aminopeptidase A)	*ampA/ pepA /lap*	B	1.9
NGO0564	Dihydrolipoamide S-acetyltransferase complex, E2 component of pyruvate dehydrogenase	*aceF*	I	1.8 ± 0.45
NGO0249	Acetyl-CoA carboxylase, beta-subunit	*accD*	S	2.75
NGO0200	Phosphoenolpyruvate (PEP) synthase [e]	*ppsA*	T	2.2 ± 1.05
NGO0223	Inorganic pyrophosphatase [f] [e]	*ppa*	T	4.7 ± 2.82
NGO1521	Acetate kinase	*ackA*	T	4.1 ± 2.84
NGO0926	Peroxiredoxin 2 family protein/glutaredoxin	*prx*	K	4.0 ± 1.26
NGO2095	Heat shock protein , 60 kD subunit	*groEL*	J	3.6 ± 2.33
NGO1046	Endopeptidase Clp ATP-binding chain B / heat shock protein F84.1	*clpB*	Q	4.2 ± 3.67
NGO0186	Zinc binding alcohol dehydrogenase	*ald*	M	2.9 ± 1.83
Membrane fractions				
NGO0346	Pilus retraction, twitching motility protein	pilT	O	1.91 ± 1.30
NGO0562	Pyruvate E3 component, lipoamide dehydrogenase / glycine cleavage L protein (OMP P64k or PM 6) [d]	*lpdA/ dldH*	T	3.06 ± 2.15
NGO0177	Two-component system transcriptional response regulator OmpR	*ompR /cpxR*	U	3.20

[a] "NGO" gene designations were obtained from the annotated *N. gonorrhoeae* strain FA1090 genome (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?org=ntng03).
[b] See the functional classification in Table 2.
[c] The cutoff of the ICAT experiment is 1.5-fold. The ratio is the mean of three independent ICAT experiments ± standard deviation.
[d] The proteins were also seen to have different expression in response to Mn by 1D SDS-PAGE.
[e] qRT-PCR was used to confirm microarray results. The ratio of transcription of these genes, wild type:wild type plus Mn, was below 1.5 fold ($P > 0.4$).
[f] Using a biochemical assay for Ppa activity it was found that cells grown on Mn had approximately 1.9 fold less activity than cells grown in the absence of added Mn ($P = 0.019$ using a Student's *t*-test).

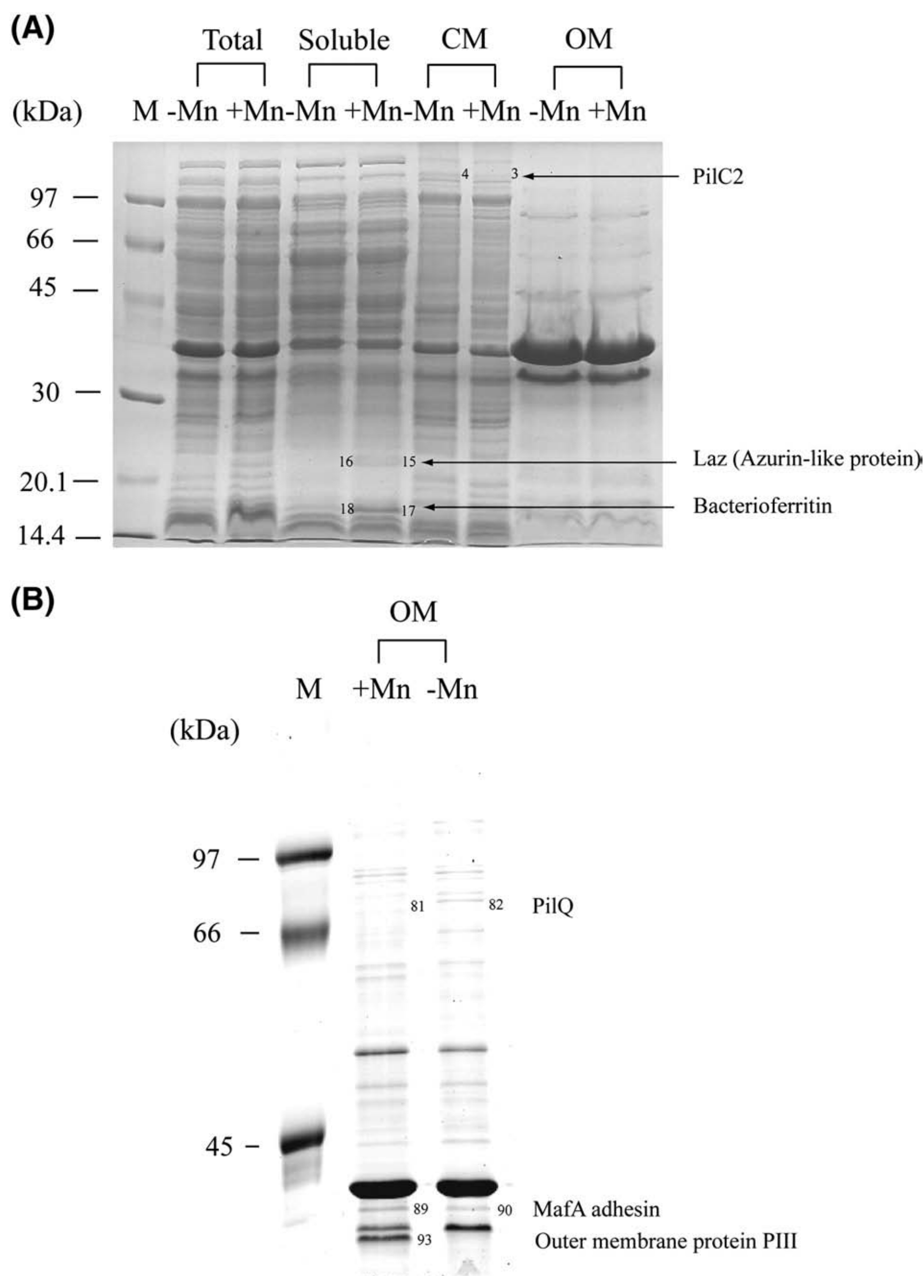


Fig. 1 – Representative 1D SDS-PAGE analyses comparing the soluble fractions of *N. gonorrhoeae* strain 1291 grown in the absence (–) and presence (+) of 40 μM Mn(II) on (A) 12.5% 7 cm or (B) 10% 18 cm gels. Total: total cell lysates; soluble: cytoplasmic and periplasmic proteins. CM: cytoplasmic membrane proteins; OM: outer membrane proteins. The numbers and protein names indicate examples of proteins showing differences in expression level (*i.e.*, present versus absent) between the control and Mn-treated sample.

were found both in the soluble fraction and the membrane fractions and had diverse roles in metabolism, biosynthesis of cellular compounds, protein synthesis, stress defences and virulence (Table 2). Bacterioferritin (Bfr) [42], azurin (Laz) [43] (Figs. 1A and S1) and iron-superoxide dismutase (SodB) [44] (Fig. S1) were present with increased Mn. These proteins are all associated with defence against oxidative stress. However, MsrAB, an outer membrane methionine sulfoxide reductase involved in protection from O_2^- and H_2O_2 [45], were present in low Mn. Several proteins present in high Mn in the membrane fractions were potential virulence factors, including the major gonococcal outer membrane protein (OMP) Porin IB (Fig. S1), and the adhesin MafA (Figs. 1B and S1).

A total of 50 proteins were present in low Mn that were absent in high Mn (Table 2). Again, Mn-regulated proteins in the soluble fraction can be classified into several functional categories including metabolism, biosynthesis, protein synthesis, RNA processing, cell defence, cell division and transport. Several proteins in the membrane fraction that are present in low Mn are involved in pilus biogenesis (Table 2, Figs. 1 and S1), including PilE (structural subunit protein pilin), PilC2 (involved in pilus assembly and biogenesis) and PilQ

(involved in translocation of pili to the cell surface) [46]. However, it is interesting to note that PilO, which functions in competence for transformation [47], was present in high Mn (Table 2). Other OMPs present in low Mn include virulence factors, such as transferrin-binding protein A [48] and Opa [49,50] (Fig. S1). The cytoplasmic stress protein DnaJ [51] (Fig. S1) was also present in low Mn. This stress protein was present in the outer membrane (OM) preparation indicating a contamination of this fraction from the cytoplasmic fraction. Ribosomal proteins present in low Mn were also present in the cytoplasmic membrane fractions instead of the cytoplasmic fraction. Cell fractions were primarily used to enable easier separation and identification of proteins, so cross fraction contamination was not considered to be a significant issue. However, these ribosomal proteins are often highly abundant and may obscure other membrane proteins in SDS-PAGE analysis [52]. These 1D SDS-PAGE results gave us an indication that Mn is an important global regulator in *N. gonorrhoeae* and led to further quantitative investigation.

3.1.3. *ICAT analysis of Mn-dependent regulation in N. gonorrhoeae*

For quantitative proteomics, a combination of ICAT and MS–MS was employed. ICAT is currently one of the most widely adopted isotopic labelling approaches [53,54]. The ICAT proteomic analysis revealed that the level of bacterioferritin was increased by greater than 1.5 fold by Mn. This is consistent with the 1D SDS-PAGE result. On the other hand, 16 proteins were decreased by greater than 1.5 fold in the presence of added Mn, including PilT (involved in pili retraction), peroxiredoxin (Prx; involved in peroxide reduction), OMP P64k (M_r = 64 kDa), OmpR (M_r = 25 kDa) and pyrophosphatase (Ppa) (Table 3). OMP P64k is present in the majority of the meningococcal strains [55], elicits bactericidal antibodies in animal models and has been considered as a potential vaccine candidate [56]. Of particular interest is Ppa, which hydrolyzes pyrophosphate (PPi) to orthophosphate (Pi), since PP_i can stabilize Mn(III) ions [57].

3.2. *Post-transcriptional Mn-dependent regulation of pilin, superoxide dismutase and pyrophosphatase*

The proteomic and transcriptomic analyses described above show that the level of several proteins in *N. gonorrhoeae* is affected by the concentration of Mn present in the growth media, but that transcription of the genes encoding these proteins is unaltered under the same conditions. To further investigate this finding and to confirm that Mn-dependent regulation of protein levels is post-transcriptional, three of the Mn-regulated proteins, pilin, Ppa and SOD, were further examined.

3.2.1. *Pilin expression is down-regulated by Mn*

Our preliminary investigations of the Mn-dependent response of *N. gonorrhoeae* involved an examination of protein expression in wild type strain 1291 cells grown on medium ±40 μM Mn(II) by analysing polypeptide profiles in whole-cell lysates after 1D SDS-PAGE and Coomassie staining. The most striking observation was a decrease in the level of a 17.3 kDa protein in the presence of high Mn(II) (4-fold by densitometry, Fig. 2A). N-terminal amino acid sequencing of this polypeptide was performed and it was determined to be identical to the N-terminal sequence of the pili

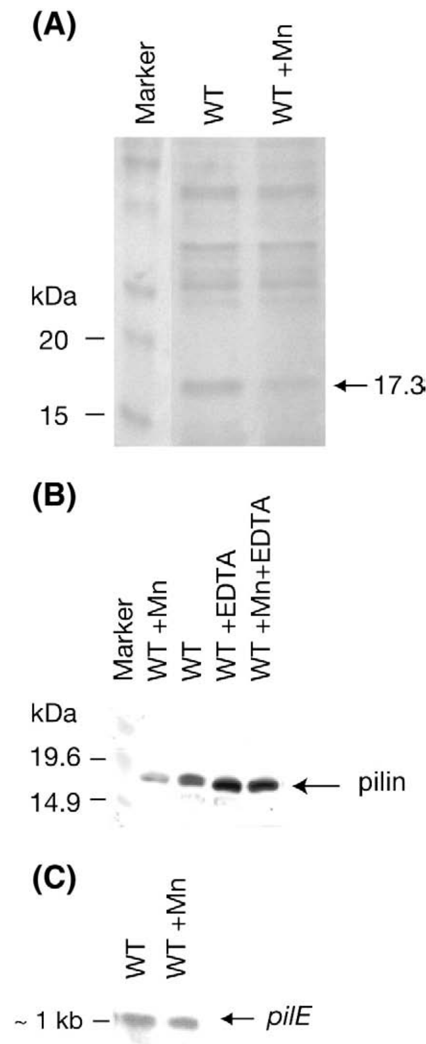


Fig. 2 – (A) Coomassie stained SDS-PAGE, (B) Western blot and (C) Northern blot of Mn(II) regulated proteins in *N. gonorrhoeae*. Panel A, *N. gonorrhoeae* wild type (WT) cells were grown on BHI agar ±40 μM $MnSO_4$. Identical loadings of total cell protein were run on a 12% SDS-PAGE gel and Coomassie stained. A band of 17.3 kDa (calc.) is indicated that displayed a four-fold decrease in the presence of 40 μM Mn (determined by densitometry in several independent experiments). Panel B, Western blot analysis of whole cells of *N. gonorrhoeae* strain 1291. Samples were run on a 12% SDS-PAGE gel, transferred to nitrocellulose and probed with the pilin-specific probe, SM1. Panel C, Northern blot analysis of RNA isolated from *N. gonorrhoeae* strain 1291 WT grown on BHI agar ±40 μM $MnSO_4$, probed with a *pilE* specific probe (amplified by the PilEsignalF and PilEsignalR primers, see Table 1).

subunit protein, pilin, which is encoded by *pilE* (NGO2061). Pili are long polymeric proteins that protrude from the bacterial surface and have a crucial role in both bacterial colonization and adherence to host cells [58,59].

We further investigated pilin expression by Western blot analysis using a pilin-specific monoclonal antibody, SM1, in the wild type strain 1291 grown under different conditions.

The Western blot shows that *N. gonorrhoeae* wild type has decreased pilin production when grown on Mn-supplemented medium (Fig. 2B), which is in agreement with the Coomassie stained SDS-PAGE results described above (Fig. 2A). Addition of EDTA to growth media caused induction of pilin production, presumably by chelating Mn(II) and preventing Mn(II)-dependent suppression of expression (Fig. 2B).

The level of *pilE* transcription in northern blot analysis was similar between *N. gonorrhoeae* 1291 grown with and without Mn(II) supplementation (Fig. 2C), indicating that Mn(II) regulation of pilin expression is post-transcriptional.

3.2.2. *SOD activity is increased in the presence of Mn*

SOD is a major component of the oxidative defence response of the majority of organisms and catalyses the disproportionation of superoxide to hydrogen peroxide and water [60,61]. 1D SDS-PAGE identified SodB when adding Mn to growth media (Table 2 and Fig. S1). Using a biochemical assay for SOD activity it was found that cells grown on Mn had an average of 2.5 fold higher SOD activity than cells grown in the absence of added Mn (P value = 0.01; data not shown). Expression of the *sodB* gene was not significantly altered between cultures grown in the presence versus the absence of Mn (qRT-PCR; 1.21 fold, P value = 0.53), indicating that the difference in activity is a result of Mn-dependent post-transcriptional regulation.

3.2.3. *Pyrophosphatase activity is reduced in the presence of Mn*

Soluble inorganic pyrophosphatases hydrolyse inorganic pyrophosphate (PP_i) into two molecules of orthophosphate (Pi), thus making it possible for many biosynthetic reactions to proceed [62]. ICAT analysis revealed that addition of Mn to growth media resulted in a 4.7 ± 2.82 fold decrease in the level of Ppa (Table 3). Using a biochemical assay for Ppa activity it was found that cells grown on Mn had approximately 2 fold less activity than cells grown in the absence of added Mn ($P = 0.019$; data not shown). In addition to the decreased Ppa protein level seen in the ICAT result, growth in the presence of high Mn levels could result in this decrease in Ppa activity via displacement of Mg by Mn at the active site. The majority of Ppa proteins have higher activity when cofactored with Mg than Mn [63], however some Ppa proteins have a unique requirement for Mn [64]. No significant difference was seen in expression of the *ppa* gene between cultures grown in the presence versus absence of Mn (qRT-PCR: 1.29 fold, P value = 0.47), indicating that the difference in activity is a result of Mn-dependent post-transcriptional regulation.

3.3. *Ppa and Ppk of N. gonorrhoeae*

Interestingly, both ICAT and enzymatic assay demonstrated that in the presence of Mn(II), the level of expression of pyrophosphatase (Ppa) decreased. These results are consistent with previous studies that have outlined the relationships between Mn, PP_i, polyphosphate (polyP) and oxidative stress. In lactic acid bacteria, high concentrations of Mn(II) (about 30 mM) accumulate within the cytoplasm, chelated by polyP, providing a SOD-independent mechanism of resistance to oxidative stress [65,66]. PolyP, a polymer of large numbers (>100) of orthophosphate (P_i), can chelate Mn(II) and is linked to various functions in several pathogens [67]. PolyP is synthesised from P_i by polyphosphate kinase (Ppk), and hydrolysed to P_i by exopolyphosphatase (Ppx). PP_i can also stabilize Mn(III) ions [57], preventing spontaneous dismutation to Mn(II) and Mn(IV). PP_i is hydrolysed to P_i by pyrophosphatase (Ppa). It is established that the pathogenic *Neisseria* can accumulate up to 10% of their phosphate as polyP. In *E. coli*, *ppk* mutants that lack Ppk activity and are unable to synthesize polyP from P_i, are sensitive to oxidative, osmotic and heat stresses and have decreased survival in stationary phase [68]. Some of the properties of a *N. gonorrhoeae* mutant lacking the *ppk* gene have been described [69], although the affect of Ppk on resistance to oxidative stress was not reported.

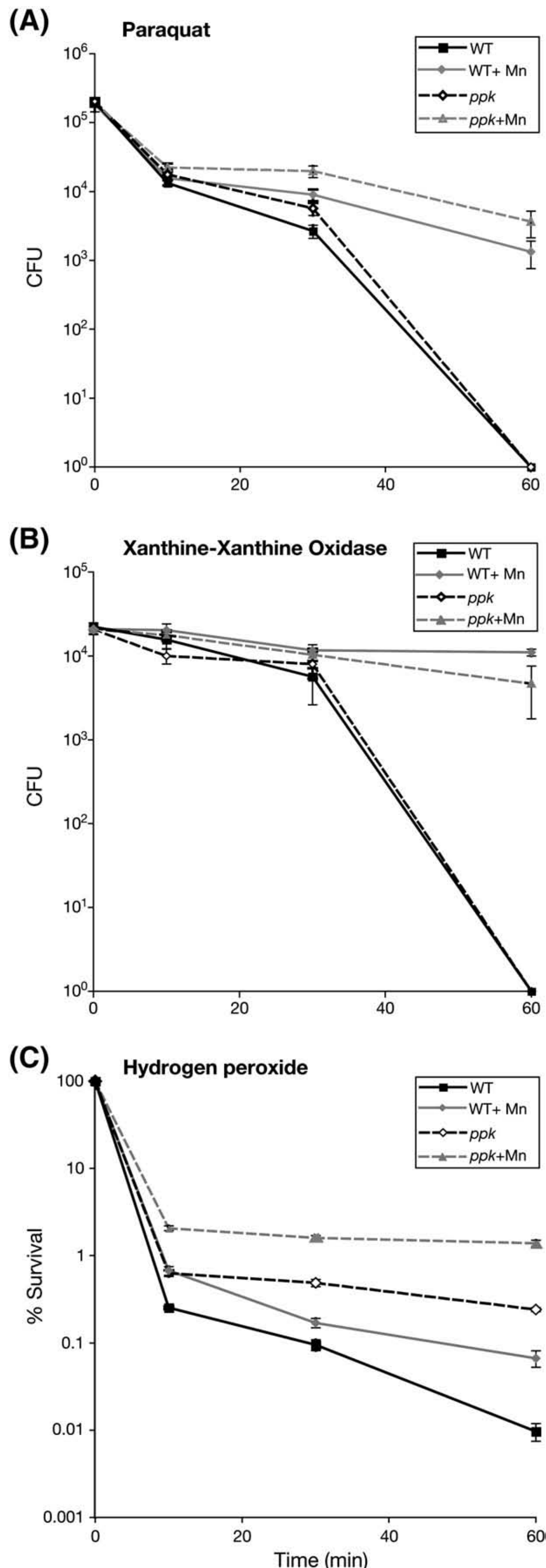
To determine whether PP_i or polyP is involved in the oxidative stress response, in particular the Mn-dependent oxidative stress response of *N. gonorrhoeae*, mutant strains lacking the genes encoding *ppk* or *ppa* were generated. In *E. coli* and *Vibrio cholerae*, the *ppk* and *ppx* genes are adjacent and form an operon [70]. However, in *N. gonorrhoeae ppk* (NGO0003) is flanked by the *dnaN* gene at its 3′ end and by two genes encoding hypothetical proteins at the 5′ end. Thus, it appears that *ppk* is probably monocistronic. The *ppa* gene (NGO0223) is flanked by an uncharacterised gene at its 3′ end and the *ntpA* (nucleoside triphosphate pyrophosphohydrolase) gene at its 5′ end. Kornberg and co-workers have shown that a number of bacteria contain a second *ppk* gene that encodes an enzyme (Ppk2) with distinct biochemical properties, including preference for GTP over ATP as a substrate [71]. In their search of incomplete microbial genomes these researchers established that *N. gonorrhoeae* possessed a single *ppk* gene encoding the enzyme that is the homologue of PPK1. Our more recent search of the annotated *N. gonorrhoeae* genome (http://cmr.jcvi.org/tigr-scripts/CMR/GenomePage.cgi?org=ntng03) confirmed the presence of a single *ppk* gene (NGO0003).

Tinsley and Gotschlich [69] have shown that a *ppk* mutant of *N. gonorrhoeae* was defective in growth on a defined medium. Using the richer growth medium, BHI broth, we observed no significant differences in growth rate and growth yield between wild type and the *ppk* mutant (data not shown). As a consequence, we were able to comparatively analyse differences between these strains with confidence that our data were not the result of differences in growth rate. However, the *ppa* strain did have a slightly increased lag phase with respect to the wild type strain (data not shown).

3.3.1. *Oxidative stress response of N. gonorrhoeae ppk and ppa mutant strains*

To determine whether Ppk and Ppa contribute to the defence of gonococci against oxidative stress, we compared the survival capability of wild type strain 1291 and *ppk* and *ppa* mutant cells after exposure to H_2O_2 as well as to two generators of O_2^-: PQ, which generates O_2^- inside the cell, and X/XO, which generates O_2^- and H_2O_2 external to the cell, as described previously [10].

As mentioned above, we have previously observed that accumulation of Mn(II) by *N. gonorrhoeae* cells correlates with resistance to oxidative killing [10], and Figs. 3 and 4 show that there was a significant increase in survival of wild type cells



grown in the presence of Mn compared to cells grown without Mn supplementation in all assays.

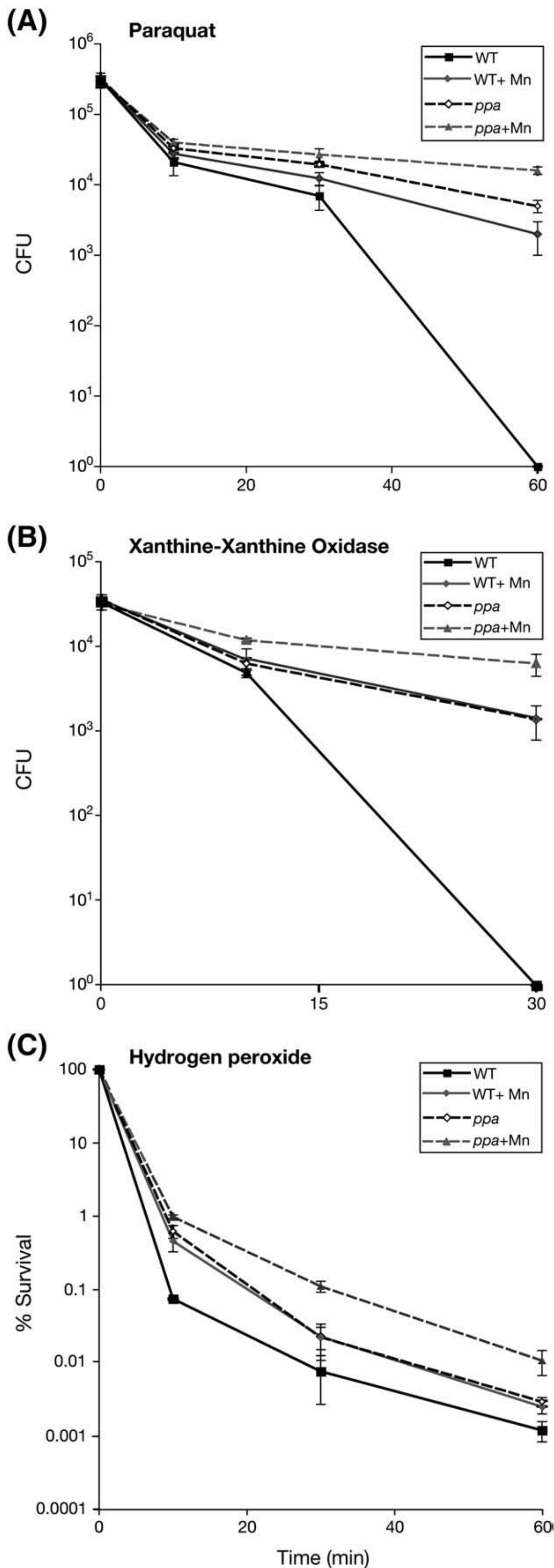
The *ppk* mutant was slightly more resistant than the wild type to PQ (Fig. 3A) with increased survival seen in the absence and the presence of Mn. The *ppk* mutant behaved like the wild type in the X/XO assay (Fig. 3B) but was significantly more resistant to H_2O_2 (Fig. 3C) than was the wild type, with increased survival seen in both the absence and the presence of Mn. The *ppk* mutant grown on Mn also exhibited enhanced survival compared to the *ppk* mutant cells grown on medium without Mn supplementation in all assays (Fig. 3).

The *ppa* mutant was more resistant than was the wild type strain to killing by PQ (Fig. 4A) with an increase in survival seen in both the absence and presence of Mn. Increased resistance was also seen in the *ppa* mutant in the X/XO assay (Fig. 4B) and H_2O_2 assay (Fig. 4C) in both the absence and presence of Mn. However, growth of the *ppa* mutant in the presence of Mn did not provide protection against ROS to the extent typically seen for the wild type in the PQ assay or the X/XO assay and the difference seen in the H_2O_2 assay is not significant as judged by a P value>0.5 (Fig. 4). This decreased Mn-mediated protection against ROS in the *ppa* mutant suggests that the protection of cells against oxidative stress by Mn may involve Mn-PP_i complexes.

3.3.2. *Survival of ppk and ppa mutant gonococci in primary cervical cells*

N. gonorrhoeae adherence to and invasion of epithelial cells is a complex process that is mediated by several factors [72,73]. We evaluated the ability of wild type and *ppk* and *ppa* mutant gonococci to associate with, to invade, and to survive within pex cells, using a modified gentamicin-survival assay as we have described previously [12,24]. Fig. 5 demonstrates that the wild type gonococci and the *ppk* and *ppa* mutants exhibited a similar degree of association with pex cells (P values≥0.67, as determined using a Kruskal–Wallis non-parametric analysis

Fig. 3 – (A) Paraquat, (B) xanthine–xanthine oxidase and (C) hydrogen peroxide oxidative stress killing assays of *N. gonorrhoeae* strain 1291 (wild type) and the *ppk* mutant strains grown on BHI agar or BHI plus 100 μM Mn (Mn). Experiments were performed in triplicate. Error bars indicate ±1 standard deviation of the mean. P values were determined using Student's t-tests. Mn protected the wild type cells in all assays; PQ (on average approximately 2000-fold higher survival at 60 min), X/XO (approximately 6000-fold at 60 min) and H_2O_2 (approximately 5-fold at 60 min; P<0.05 at the final time point for all assays). The *ppk* mutant was slightly more resistant than the wild type to PQ with increased survival seen in the absence of Mn (2.1-fold at 30 min, P=0.016; equal at 60 min, P=0.37) and the presence of Mn (2.2-fold at 30 min, P=0.011; equal at 60 min, P=0.069). The *ppk* mutant was significantly more resistant to H_2O_2 than was the wild type with increased survival seen in both the absence (25-fold at 60 min, P=0.00005) and the presence of Mn (21-fold at 60 min, P=0.0004). Mn protected the *ppk* mutant in all assays; PQ (>3000-fold at 60 min, P=0.014), X/XO (>4000-fold at 60 min, P=0.041) and H_2O_2 (6-fold at 60 min, P=0.001).



of variance). However, both of the mutant gonococcus strains exhibited a distinct phenotype in their ability to invade and to survive within pex cells upon comparison to the wild type bacteria. Consistent with our previous studies, the wild type gonococci were able to invade and survive one-hour post gentamicin treatment of pex. At 2 h following gentamicin treatment the number of viable wild type gonococci had more than doubled, presumably indicating growth within pex cells.

Although the *ppk* mutant gonococci were not significantly impaired in their ability to invade pex cells when compared to the wild type (96% invasion relative to the wild type, $P=0.67$), they had decreased survival within pex cells relative to the wild type at the one-hour post gentamicin treatment time point (75%, P value=0.0001). At the two-hours post gentamicin treatment the number of viable *ppk* gonococci was further decreased both with respect to the previous time point (55%) and the wild type strain (16%, P value=0.0001), suggesting a role for polyP synthesis in the intracellular survival of gonococci.

The *ppa* mutant displayed an increased invasive phenotype relative to the wild type strain (126%, $P=0.0001$) but showed decreased intracellular survival relative to the wild type by one-hour post gentamicin treatment (69%, $P=0.0001$). However, by two-hours post gentamicin treatment the number of viable *ppa* mutant gonococci had increased with respect to the previous time point (141%), but still had decreased survival with respect to the wild type (38%, P value=0.0001).

4. Discussion

Over the last decade Mn has emerged as a trace element of significance in bacterial physiology and virulence, where it can function as a regulator of gene expression and as a modulator of metabolism [15,74]. It has also been reported that Mn levels vary from nM to μM concentrations within the human host [17,18], indicating that Mn could be an important environmental signal for human pathogens. Our previous

Fig. 4 – (A) Paraquat, (B) xanthine–xanthine oxidase and (C) hydrogen peroxide oxidative stress killing assays of *N. gonorrhoeae* strain 1291 (wild type) and the *ppa* mutant strain. Experiments were performed in triplicate. Error bars indicate ±1 standard deviation of the mean. *P* values were determined using Student's *t*-tests. The *ppa* mutant was more resistant than was the wild type strain to killing by PQ in the absence of Mn (>5000-fold at 60 min, $P=0.001$) and in the presence of Mn (8-fold at 60 min, $P=0.004$). Increased resistance was also seen in the *ppa* mutant in the X/XO assay in the absence of Mn (>1300-fold at 60 min, $P=0.016$) and presence of Mn (5-fold increase in survival at 60 min, $P=0.011$), and the H_2O_2 assay in the absence of Mn (2.4-fold at 60 min, $P=0.0043$) and presence of Mn (4.2-fold at 30 min, $P=0.008$; >5000-fold increase at 60 min, $P=0.05$). Growth of the *ppa* mutant in the presence of Mn did not provide the typical level of protection (with respect to the *ppa* mutant on unsupplemented media) in the PQ assay (3-fold at 60 min, $P=0.001$) or the X/XO assay (4.5-fold at 60 min, $P=0.011$) and the difference seen in the H_2O_2 assay is not significant as judged by a P value>0.5 (5-fold at 30 min, $P=0.008$; 4-fold at 60 min, $P=0.055$).

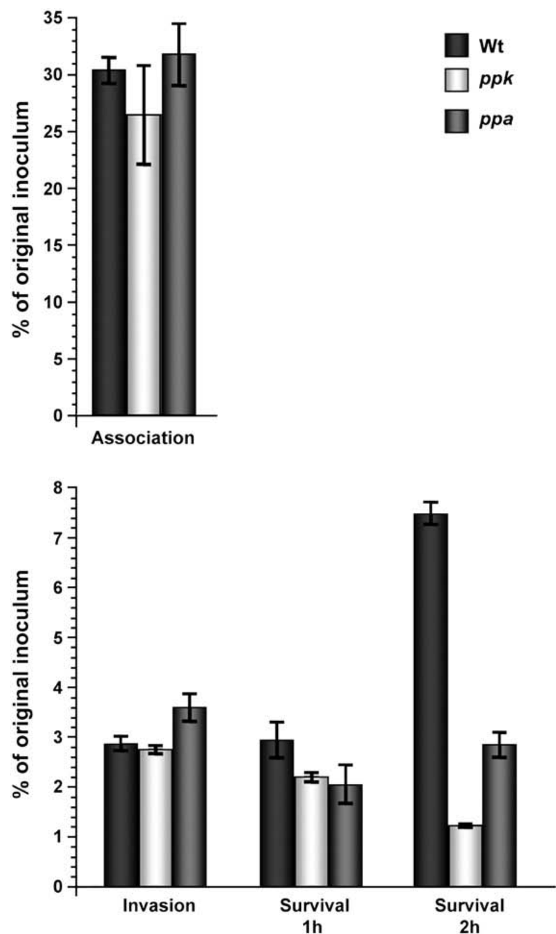


Fig. 5 – Gonococcal association with and intracellular survival within primary human cervical epithelial (pex) cells. The histogram shows the mean percent association, invasion and survival as a function of the original inoculum of the *N. gonorrhoeae* wild type strain (WT), and the *ppk* and *ppa* mutant strains. Data, determined from the number of colony forming units formed upon plating of the cervical cell lysates, were obtained from three experiments performed in triplicate. Y-error bars show +/− 1 variance. *P* values were determined using a Kruskal–Wallis non-parametric analysis of variance.

studies have confirmed a key role of Mn in defence against ROS [10]; however, the mechanism has not been fully resolved. In order to investigate the regulatory and ROS defence roles of Mn in *N. gonorrhoeae*, we used a combined transcriptomic and proteomic approach.

4.1. *Mn-dependent protection against oxidative stress*

Our proteomic study showed that several oxidative stress defence proteins of *N. gonorrhoeae* were increased under high Mn conditions. These proteins included SodB, bacterioferritin and Laz (Tables 2 and 3). SodB is a Fe-dependent enzyme that detoxifies superoxide, while bacterioferritin is an iron storage protein that can also contribute towards protection against oxidative stress [42]. These data indicate that a higher level of Mn may enable higher levels of iron acquisition, which otherwise would be disadvantageous to the cell due to Fenton chemistry [75], and this results in higher levels of these two iron-dependent proteins. Similarly, the Laz protein is a copper-binding protein found in the outer membrane and although its precise function is not known, it is involved in the protection of gonococcus against peroxide killing [43]. Again, the presence of this protein in high Mn may be the result of increased copper acquisition which is facilitated by higher levels of Mn. Conversely, MsrAB, a methionine sulfoxide reductase that is involved in protection from hydrogen peroxide and superoxide [45,76], was present in low Mn (Table 2). This may reflect a decreased requirement for this outer membrane protein under high Mn conditions where other oxidative stress defences become more prominent.

Mn(II) and Mn(III) can scavenge ROS non-enzymatically [77,78] and *N. gonorrhoeae*, like many lactic acid bacteria [65,66,77,79,80], may use accumulated intracellular Mn as a defence against ROS independently of SodB [10] or catalase [11]. Mn complexes, including Mn(II)-PPi and Mn(II)-polyP, have been shown to be potential non-enzymatic antioxidants [81]. Ppa had reduced expression under conditions on high Mn (Table 3), which would result in increased levels of PPi that would be available to form Mn(II)-PPi complexes for antioxidant activity. PPi is generated in cells either as a metabolic byproduct of numerous biochemical and biosynthetic reactions [82] or directly by pyrophosphohydrolysis of the phosphodiesterase I bond in purine and pyrimidine nucleoside triphosphates by members of the phosphodiesterase nucleotide pyrophosphatase (PDNP) family [83]. It is established that the pathogenic *Neisseria* can accumulate up to 10% of their phosphate as polyP. Although most of this polyP is intracellular, there is evidence that polyP is also loosely associated with the extracellular surface where it forms a capsule-like structure around the gonococcus [84]. To investigate the role of PP_i and polyP in oxidative stress, oxidative killing assays were performed using *ppk* and *ppa* mutant strains. The *ppk* and *ppa* mutations cause cells to be more resistant to oxidative killing. The level of Mn-mediated protection against ROS seen in the *ppa* mutant strain was drastically reduced with respect to the wild type strain (Fig. 4). These data suggest that the protection of cells against oxidative stress by Mn may be due to Mn(II)-PP_i complex rather than Mn(II)-polyP complex formation. However, since some Mn(II)-dependent protection was maintained in the *ppa* mutant strain, the Mn(II)-dependent effect is unlikely to completely depend on PP_i. Also, since Mn provides protection in the *ppk* mutant against ROS to a similar extent as that seen in the wild type strain, it can be concluded that Mn is not directly dependent upon polyP for its antioxidant action. Therefore, unlike the situation in *L. plantarum* [66] Mn(II)-polyP complexes do not have a direct role in ROS quenching within *N. gonorrhoeae*.

The role of polyP and PP_i in the invasion of and survival in primary cervical epithelial cells was also investigated. Whereas the *ppk and ppa* mutations did not affect the association of gonococci with pex cells, we did observe increased invasion by the *ppa* mutant. Both mutant strains had decreased survival in the pex cells relative to the wild type. These data show that polyP and PP_i metabolism in *N. gonorrhoeae* have a significant influence on invasion of and survival within cervical epithelial cells. This may be linked to defence against oxidative stress

and varying Mn levels during gonococcal adherence to and invasion of target cells, as outlined in the section above.

4.2. *Mn regulation of pili and other virulence factors*

Mn is involved in virulence in *Salmonella enterica* serovar Typhimurium [15,74] and *S. pneumoniae* [19,85] and regulates the expression of several virulence factors in *N. gonorrhoeae* (Tables 2 and 3). It has also been reported that Mn levels vary from nM to μM concentrations within the human host [17,18]. Thus, differences in Mn concentration between host micro-environments may provide a signal for expression of certain virulence factors at sites of gonococcal infection. Indeed, it has recently been shown that *S. pneumoniae* uses Mn as a signal for the expression of virulence factors within different host sites, and that the disruption of this Mn-dependent regulation reduces virulence in an animal model [19,20]. Pilin (PilE) levels of *N. gonorrhoeae*, as well as several pili associated proteins (PilC2, PilT, PilQ), were reduced in the presence of increased Mn concentrations (Tables 2 and 3). PilC2 is involved in pilus assembly, PilQ is involved in the translocation of pili to the cell surface and PilT is required for pilus retraction [46]. Pili of pathogenic *Neisseria* spp. are typical of a family of adhesins, type IV fimbriae, found in a wide range of Gram-negative pathogens. These long polymeric proteins protrude from the bacterial surface and have a crucial role in both colonization of the host and adhesion to host cells [58,59] and only piliated bacteria are recovered from gonorrhea patients [86]. Piliation increases the ability of gonococci to adhere to numerous cell types including human amniotic cells [87], sperm [88,89], erythrocytes [90,91], buccal epithelial cells, neutrophils [91], vaginal epithelial cells [92] and non-ciliated cells in fallopian-tube organ cultures [93]. Pili are also required for CR3-mediated endocytosis of primary cervical epithelial (pex) cells [72]. Although pili are critical in mediating adhesion to host cells, it is also generally believed that these adhesins are not expressed upon the invasion of a host cell [73,94]. Environmental factors causing pilus release are not known. In light of the role pili plays in gonococcal virulence, Mn-dependent regulation of pilin suggests that Mn may play an important role in pathogenicity of *N. gonorrhoeae* and our data show that this effect is exerted at the post-transcriptional level.

Mn is also linked with regulation of other known and potential virulence factors of *N. gonorrhoeae*, including Porin and the adhesin MafA (increased expression in the presence of Mn) as well as Opa, OMP P64K, OmpR and transferrin-binding protein A (decreased expression in the presence of Mn) (Table 2 and 3). Although development of a vaccine against *N. gonorrhoeae* has been hampered by the variability of antigens between gonococcal strains, research has focused on pilin, porin and transferrin-binding proteins [95,96]. Understanding of Mn-dependent regulation of these proteins may aid in future vaccine development.

4.3. *Mn regulation of carbon metabolism*

Many of the Mn-dependent changes in protein levels observed in this study relate to enzymes involved in intermediary carbon metabolism. Mn has previously been linked to cell metabolism, with higher Mn levels being correlated with slow growing or stationary phase bacterial cells [15]. Kehres and Maguire [15] reviewed several Mn-regulated enzymes that are involved in intermediary carbon metabolism and described a possible network of co-regulated enzymes involved in phosphoenolpyruvate (PEP) and pyruvate metabolism. *N. gonorrhoeae* metabolises glucose via the Entner–Douderoff (ED) pathway and the citric acid cycle (CAC) [97,98], and the ED pathway results in the formation of glyceraldehyde 3-phosphate (GAP) and pyruvate as primary C3 products [97,98].

Changes to the levels of proteins involved in carbon metabolism may be related to the effect of Mn on the redox environment of the cell that is described above, e.g., the sensitivity of GAP dehydrogenase (GAPDH) to inactivation by oxidative stress [99]. Conversely, it would be expected that under conditions of low Mn levels, GAPDH would be lowered and the activity of the enzymes involved in pyruvate and PEP production, via this branch of the ED pathway, would also be lowered. Restriction of production of pyruvate from GAP would mean that the pyruvate produced directly from the ED Pathway would take on greater importance for production of carbon intermediates. PEP production is critical and it is notable that under low Mn conditions there is a higher level of PEP synthase (Table 3). Another consequence of a decreased GAPDH activity, which would be observed under conditions of low Mn, would be an elevation of the level of this intermediate, GAP. C3 sugars (*i.e.,* GAP) cannot cyclise to prevent further oxidation of a carbon atom, and can therefore form dicarbonyls which are highly reactive and toxic species [100,101]. Thus, it is critical to remove accumulating GAP and it is postulated that this function is taken by the zinc-dependent alcohol dehydrogenase which would use NADH to produce glycerol-3-phosphate. Perhaps, this also explains why acetyl-CoA carboxylase is co-ordinately regulated (Table 3) so that production of fatty acyl-CoA intermediates can be used along with glycerol-3-phophate in phospholipid synthesis. It is notable that acetate kinase levels also increase under conditions of low Mn (Table 3) and this provides for increased production of acetyl-P which in turn can lead to formation of acetyl-CoA.

4.4. *Mechanism of Mn-dependent post-transcriptional regulation*

While the mechanism of Mn-dependent post-transcriptional regulation in *N. gonorrhoeae* has not been determined in this study, there are several ways that Mn could regulate the levels of the proteins listed in Tables 2 and 3. Changes in the Mn concentration could result in: (1) modification of activity of the Mn-dependent ppGpp hydrolase (SpoT), which is involved in controlling the level of ppGpp in response to changes in the environment, which in turn globally coordinates mRNA and stable RNA synthesis; (2) binding of Mn instead of Mg to compounds which can alter the catalytic activity of enzymes and tertiary structure of tRNA; (3) binding of Mn to small RNAs involved in translational regulation, leading to RNA structural changes; and (4) decreased protein turnover due to the role of Mn in protection from ROS [reviewed in 15]. There are also the ribosomal proteins present in high or low Mn (Table 2), which might be involved in the change of mRNA synthesis according to the environmental changes indicated by changes in Mn concentration. Or this might be due to the inefficient cell separation, because it has been reported that even after lysis in PBS buffer a

lot of soluble and membrane-associated proteins remain in the membrane fraction (for example, the ribosomal proteins L1/L2/L3/L4/L5 or the elongation factor Tu) [52]. These proteins are often highly abundant and may therefore obscure membrane proteins in the SDS-PAGE. Mn homeostasis and Mn-dependent regulation appear to involve several factors, and more work is necessary to determine the mechanism of Mn regulation in *N. gonorrhoeae* and the role of Mn in pathogenesis in this organism.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jprot.2009.12.001.

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