Metal Binding Specificity of the MntABC Permease of *Neisseria gonorrhoeae* and Its Influence on Bacterial Growth and Interaction with Cervical Epithelial Cells[∇][†]

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mntABC from *Neisseria gonorrhoeae* encodes an ABC permease which includes a periplasmic divalent cation binding receptor protein of the cluster IX family, encoded by *mntC*. Analysis of an *mntC* mutant showed that growth of *N. gonorrhoeae* could be stimulated by addition of either manganese(II) or zinc(II) ions, suggesting that the MntABC system could transport both ions. In contrast, growth of the *mntAB* mutant in liquid culture was possible only when the medium was supplemented with an antioxidant such as mannitol, consistent with the view that ion transport via MntABC is essential for protection of *N. gonorrhoeae* against oxidative stress. Using recombinant MntC, we determined that MntC binds Zn^{2+} and Mn^{2+} with almost equal affinity (dissociation constant of ~0.1 µM). Competition assays with the metallochromic zinc indicator 4-(2-pyridylazo)resorcinol showed that MntC binds Mn^{2+} and Zn^{2+} at the same binding site. Analysis of the *N. gonorrhoeae* genome showed that MntC is the only Mn/Zn metal binding receptor protein cluster IX in this bacterium, in contrast to the situation in many other bacteria which have systems with dedicated Mn and Zn binding proteins as part of distinctive ABC cassette permeases. Both the *mntC* and *mntAB* mutants had reduced intracellular survival in a human cervical epithelial cell model and showed reduced ability to form a biofilm. These data suggest that the MntABC transporter is of importance for survival of *Neisseria gonorrhoeae* in the human host.

Neisseria gonorrhoeae is a mucosal pathogen often associated with the genitourinary tract, and it is the etiological agent of the sexually transmitted disease gonorrhea (31). Gonorrhea is often characterized by a localized inflammatory response involving inflamed urogenital tissues and activated polymorphonuclear leukocytes (PMNs) (26). This innate immune response includes production of superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), and reactive nitrogen species. The accumulation of reactive oxygen species around the site of gonococcal infection can exert a cytotoxic effect, which results from oxidant interaction with proteins, lipids, and nucleic acids (18, 32). Despite the constant environmental stress elicited by $O_2^{\cdot-}$ and H_2O_2, gonococci are routinely isolated from PMN-laden purulent exudates. Furthermore, studies suggest that gonococci not only survive within PMNs but may also replicate within these cells (27, 29, 36). The ability of gonococci to survive in environments high in reactive oxygen species suggests that this bacterium has an efficient antioxidant defense system.

Previous studies show that the accumulation of manganese (Mn), via the Mn transporter, MntABC, is important for protection of *N. gonorrhoeae* from $O_2^{\cdot-}$ and H_2O_2 killing via a mechanism that is independent of superoxide dismutase (34) and catalase (30). MntABC is an *A*TP *b*inding *c*assette (ABC) permease, and it contains a periplasmic metal binding receptor protein (MBR), MntC (34). Protein phylogenetic analysis indicates that MntC is a member of the cluster IX family of solute binding proteins that interact with metal ions (9). Generally, cluster IX MBRs demonstrate specificity for Zn^{2+}, Mn^{2+}, and (occasionally) Fe^{2+} (7). The high-resolution crystal structures of several cluster IX MBRs have been determined. These include ZnuA, from *Synechocystis* sp. strain 6803 (3); PsaA, from *Streptococcus pneumoniae* (20) and TroA, from *Treponema pallidum* (21). PsaA consists of two $(\beta/\alpha)_4$ domains linked by a single α-helical "backbone" (20). A single Zn^{2+} ion was found to be coordinated between the two domains. The presence of a single linking helix was a significant departure from previously characterized solute binding proteins associated with ABC permeases. Solute binding proteins involved in binding large substrates such as SO_4^{2-} invariably have a flexible hinge region linking the two domains which allows binding and release of the substrate and may signal transport. A single helical linker in the MBRs limits the ability of the domains to move, which may stabilize the protein and allow metal binding. Since the structure of PsaA was solved, others, including *T. pallidum* TroA (21), *Synechocystis* ZnuA (3) and MntC (28), and *Escherichia coli* ZnuA (22), have all been found to have two domains linked by a long helix. In all structures the metal

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ion (Zn^{2+} or Mn^{2+}) is bound at the interface between the domains, and it is not clear how the structure influences metal specificity. An interesting feature of some of these proteins is the presence of an unstructured, flexible histidine-rich sequence that is located near the metal binding site. Studies with ZnuA (*Synechocystis*) have shown that this loop has a very weak affinity for Zn^{2+} but does not affect the main high-affinity site (37). It has been suggested that this loop may be involved in the sensing of zinc concentrations rather than directing Zn^{2+} to the main site. The histidine-rich region is absent in zinc transporters such as TroA and varies in length. Thus, its exact role remains unclear.

Although purified PsaA contains Zn, all of the available evidence indicates that in *S. pneumoniae* the PsaABC permease transports Mn^{2+} into the cell and is required for protection against oxidative stress (23). In *S. pneumoniae* a distinct ABC transporter, AdcBCA, is required for Zn^{2+} transport as well as optimal growth of *S. pneumoniae* (10). In *T. pallidum*, the TroABCD permease was reported to transport both Zn^{2+} and Mn^{2+} with similar affinities, suggesting multiple metal specificities of TroA.

Our in vitro experiments have suggested that MntABC is important in defense against oxidative stress in the gonococcus (34), and this defense was dependent upon the presence of Mn^{2+} ions. To provide a better understanding of the influence of metal ions on oxidative stress defense mechanisms in the gonococcus, we have purified recombinant MntC (rMntC), investigated the metal binding properties of this protein, and characterized further the phenotypic properties of *mntC* and *mntAB* mutants in relation to the ability of gonococci to invade human cervical epithelial cells and form a biofilm.

MATERIALS AND METHODS

Bacterial media and growth. *N. gonorrhoeae* 1291 strains were cultured on solid brain heart infusion (BHI) agar (Becton Dickinson) with IsoVitaleX (Becton Dickinson) and levithal (1) and incubated at 37°C in a 5% elevated carbon dioxide chamber overnight. Fresh colonies were subsequently transferred to BHI broth (Oxoid) with shaking at 180 rpm at 37°C overnight. For growth studies, the gonococcus colonies were selected from agar, grown in BHI broth until mid-log phase, and then pelleted and resuspended in same medium to a desired starting optical density at 600 nm (OD_{600}) of 0.001. Various concentrations of Zn(II) and Mn(II) sulfate and mannitol were added for gonococcus phenotype rescue experiments. The construction of *mntC* and *mntAB* mutants has been described previously (34, 38). Both mutations were constructed by insertion of a kanamycin resistance cassette inserted in an orientation to allow transcriptional readthrough into the distal genes. In this way polar effects of the insertional mutagenesis are avoided. The cell growth was monitored at regular intervals by measuring the OD_{600}. *E. coli* cells were grown aerobically in Luria-Bertani (LB) medium with appropriate antibiotic and shaking (180 rpm) at 37°C.

Chemicals. All buffers were prepared using MilliQ deionized water. MES (morpholineethanesulfonic acid), Tris and HEPES buffer salts, and 4-(2-pyridylazo)resorcinol (PAR) were obtained from Sigma. All chromatography materials were obtained from GE Healthcare.

Cloning of the *mntC* gene. The *mntC* coding sequence was PCR amplified from *N. gonorrhoeae* 1291 using forward primer 5′-AAA GAG CTC GCA CCC CTT CCG GT-3′ and reverse primer 5′-ACC CTG CAG TTA TTG CTT CAT CGC-3′, creating the SacI and PstI restriction sites. The purified PCR product was double digested with SacI and PstI and subsequently cloned into the SacI and PstI sites of expression vector pBad/His (Invitrogen). The plasmid was transformed into competent *E. coli* Top10 cells. Transformants were selected on LB agar containing 100 μg/ml ampicillin.

Expression and purification of rMntC in *E. coli*. *E. coli* Top10 cells carrying the recombinant pBad-*mntC* plasmid were grown overnight in LB and ampillicin at 37°C. The overnight culture was diluted 1:100 into fresh LB medium and incubated at 37°C with shaking at 180 rpm until an OD_{600} of 0.4 was obtained.

His-tagged MntC expression was induced by adding 0.2% (wt/vol) L-arabinose, and the cells were allowed to grow overnight. Cells were harvested by centrifugation, and the cell pellets were resuspended in 20 ml lysis buffer (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl, and 10 mM imidazole). The cells were lysed by passage through a French press (1.4×10^8 lb/in²) and centrifuged twice to remove any unlysed cells and cell debris. The resultant supernatant was loaded into a preequilibrated nickel-chelated Sepharose fast-flow gel column (GE Healthcare). Purification of His-tagged MntC was conducted using methods described in the ProBond purification kit (Invitrogen). The purified proteins were analyzed by polyacrylamide gel electrophoresis. Electrophoretic analysis of protein samples was conducted using either 10% (wt/vol) or 12% (wt/vol) sodium dodecyl sulfate-polyacrylamide gels. Purified MntC was then dialyzed against storage buffer (50 mM MES, pH 6.0). The protein concentration was determined using the calculated molar extinction coefficient at 280 nm of 23,380 M^{-1} cm^{-1} (12). Protein samples were concentrated using Centricon Ultra 15 centrifugal filters with YM-10 MW membranes (10,000-molecular-weight cutoff) (Millipore) in accordance with manufacturer's instructions.

Cleavage and purification of MntC. The hexahistidine tag from His-MntC was cleaved using an enterokinase, EKMAX (Invitrogen). Cleaved MntC was purified using a PD-10 column (GE Healthcare). The eluate was collected in 0.2-ml fractions and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis with Coomassie blue staining. Fractions containing protein were pooled and stored at −80°C. Western blot analysis was used to determine the presence or absence of the hexahistidine tags in the expressed recombinant protein. Proteins were transferred to a nitrocellulose membrane (Protran; Schleicher & Schuell) after polyacrylamide gel electrophoresis, using a Hoeffer minigel system. The membrane was then treated according to the manufacturer's instructions for monoclonal antipolyhistidine clone His-1 (Sigma, St. Louis, MO).

Production of apoprotein. Metal-free protein was formed by denaturation and refolding the native MntC protein. The native protein was denatured by dialysis using a Slide-A-Lyzer (Pierce) with denaturing buffer [6 M guanidine-HCl, 2× $N,N,N′,N′$-tetrakis-(2-pyridylmethyl)ethylenediamine, 2 mM Na_2EDTA, dithiothreitol [DTT]) overnight at 25°C. The denatured protein was then refolded through a series of dialyses performed at 4°C with pH 7.5 solutions: (i) buffer A (6 M urea, 2 mM Na_2EDTA, and 1 mM DTT) for 4 hours, (ii) buffer B (2 M urea, 2 mM Na_2EDTA, and 1 mM DTT) for 4 hours, and (iii) buffer C (2 mM Na_2EDTA and 0.75 ml of 1 M DTT solution) overnight.

Determination of metal content using ICP-MS. To determine the metal content of *N. gonorrhoeae*, an acid digestion was performed prior to inductively coupled plasma-mass spectrometry (ICP-MS) sample analysis. The culture was grown overnight in BHI broth, pelleted, and resuspended in 1 ml BHI broth, and then 0.5 ml of the culture was transferred to aluminum foil and left to dry overnight in 68°C oven. The dry weight of the culture was noted, and 1 ml of concentrated nitric acid was added to 0.5 ml of the remaining liquid culture. The sample was then allowed to stand at room temperature for 20 min or until the effervescence ceased. Subsequently, the sample was placed in a boiling water bath for 9 h. The sample was then diluted to a final solution of 2% nitric acid, and samples were analyzed by EnTox (The University of Queensland, Brisbane, Australia). The results were corrected using appropriate buffers as references.

Determination of relative metal affinity of MntC by monitoring of fluorescence quenching. Fluorescence studies were carried out using a Perkin-Elmer model LS55 spectrofluorimeter. Apo-MntC was prepared as described above. Titration with Mn(II) and Zn(II) sulfate salts was carried out at room temperature in 50 mM MES, pH 6.0. Aliquots of metal salts solutions were added from a gas-tight Hamilton syringe to protein solutions. Following each addition, the sample solution was mixed well, and the fluorescence emission spectrum was collected. The sample was excited at 280 nm, and the emission was scanned between 320 nm and 400 nm. The dilution effect after each addition of metal salt solution was taken into account. Spectra were fitted using Graphpad Prism software (33) using different binding models.

PAR competition assays. PAR was employed in a competition assay with MntC. PAR (200 μM) was mixed with $ZnSO_4$ (50 μM) in 50 mM HEPES (pH 7.4), and the optical spectrum was recorded. Small aliquots of MntC were added, and the spectrum was recorded after each addition. Spectra were corrected for dilution effects and by subtraction of the "apo-spectrum." For the Mn^{2+} competition assay, MntC (50 μM) was loaded with one equivalent of Zn^{2+} (50 μM) in the presence of PAR (200 μM) in 50 mM HEPES, pH 7.4. Mn sulfate salt was subsequently titrated into the solution. Following each titration of Mn^{2+}, the spectrum was recorded and corrected as described above.

EPR spectroscopy. Electron paramagnetic resonance (EPR) spectra were acquired on a Bruker Elexsys E580 instrument operating in continuous-wave mode at X-band frequency (~9.4 GHz). Calibration of the magnetic field was achieved with an ER036TM teslameter. Room temperature spectra were collected using

a Super High-Q cavity incorporating a Bruker AquaX flow cell. The flow cell was connected in-line to a peristaltic pump. To monitor Mn(II) binding, apo-MntC was titrated with Mn(II) sulfate salts (12.5 μM MntC and 2.5 to 50 μM Mn^{2+}), and the solution mixed by pumping through the flow cell for 5 min. The pump was stopped prior to acquiring EPR data. All spectra were zeroed and baseline corrected prior to analysis.

Assay for survival of *N. gonorrhoeae* in primary human cervical epithelial cells. Primary human cervical epithelial (pex) cells were procured and maintained as described previously (11), and cell monolayers were grown to confluence in 35-mm tissue culture dishes (Falcon). Wild-type and mutant *N. gonorrhoeae* cells that were allowed to grow overnight (37°C, 5% CO$_2$) on GC-IsoVitaleX agar plates were harvested using a sterile swab and resuspended in sterile saline. Culture density was determined spectrophotometrically, where an OD$_{600}$ of 1 was equivalent to 10^9 bacteria ml^{-1} of cell culture. Bacterial cells were then used to challenge 10^5 pex cells at a multiplicity of infection of 100 (i.e., 10^7 bacteria). To confirm the actual number of bacteria used to challenge pex cells, the inoculum used for each *N. gonorrhoeae* strain (1291, 1291 *mntAB*, or 1291 *mntC*) was quantitated by plating the bacterial suspension and enumerating CFU. To determine the ability of *N. gonorrhoeae* wild-type and mutant strains to associate with, invade, and survive within pex cells, the infection was then allowed to progress at 37°C in a 5% CO$_2$ atmosphere. For association assays, the infection medium was removed at 90 min postinfection and the cells rinsed with phosphate-buffered saline (PBS). For invasion assays, pex cells were incubated for a further 30 min with medium containing 100 μg of gentamicin (Gibco) per ml to kill extracellular bacteria. Survival assays were performed in a similar manner with the exception that following gentamicin treatment, the infected cell monolayers were again rinsed with PBS. Fresh antibiotic-free medium was then added to each infected cell monolayer before 1 h or 3 h of incubation. Following each assay, pex cells were lysed with 0.5% saponin to release invasive bacteria, and serial dilutions were plated to determine CFU. Association, invasion, and survival assays in which the same wild-type or mutant bacterial solution was used to infect pex cell monolayers were performed in parallel on three separate occasions. The percent association, invasion, or survival was determined as a function of the original inoculum and the number of colonies formed with subsequent plating of the cellular lysate. *P* values were determined using a Kruskal-Wallis nonparametric analysis of variance.

Assay for biofilm formation. For examination of biofilm formation via confocal microscopy, the *N. gonorrhoeae* 1291 strain and the *mntAB* and *mntC* mutants were transformed with a plasmid encoding green fluorescent protein (GFP) (pLES98 containing GFP was a gift from Virginia Clark, University of Rochester, NY). Strains were propagated from frozen stock cultures on GC agar with IsoVitaleX (Becton Dickinson) and incubated at 37°C with 5% CO$_2$. Overnight plate cultures were used to create cell suspensions for inoculation of biofilm flow chambers. *N. gonorrhoeae* was grown in continuous-flow chambers in 1:10 GC broth (19) diluted in PBS with 1% IsoVitaleX, 100 mM sodium nitrite, 5 mM mannitol, and 5 mg/ml chloramphenicol to maintain pGFP. Approximately 2 × 10^8 CFU/ml of each strain was suspended in biofilm medium, and 1 ml of each suspension was used to inoculate two flow cell chamber wells (37 by 5 by 5 mm). Flow chambers were incubated under static conditions at 37°C for 1 h postinoculation to facilitate bacterial attachment before flow was initiated at 180 ml/min. After 48 h, the biofilm effluent was cultured to confirm purity, and biofilm formation was assessed via confocal microscopy.

Z-series photomicrographs of flow chamber biofilms were taken with the Nikon PCM-2000 confocal microscope scanning system (Nikon, Melville, NY) using a modified stage for flow cell microscopy. GFP was excited at 450 to 490 nm for biofilm imaging. Three-dimensional images of the biofilms were created from each z series using Volocity High Performance 3D Imaging software (Improvision Inc., Lexington, MA). The images were adjusted to incorporate the pixel sizes for the *x*, *y*, and *z* axes of each image stack. Quantitative analysis of each z series was performed using COMSTAT (16) (http://www.cbm.biocentrum.dtu.dk /English/Services/Resources/COMSTAT.aspx). An information file was created for each z series to adjust for the pixel sizes of the x, y, and z axes and the number of images in each z series. COMSTAT was then used to threshold the images to reduce background. The biomass and average and maximum thicknesses in each z series were calculated by COMSTAT from the threshold images.

RESULTS

Comparison of gonococcal MntC with known Zn^{2+} and Mn^{2+} binding proteins of the cluster IX family. Initially, we searched the *N. gonorrhoeae* FA1090 genome for open reading frames that encoded an MBR of the cluster IX family (Gono-

coccal Genome Sequencing Project, University of Oklahoma GenBank accession number AE004969). In contrast to the situation in many other bacterial pathogens, *mntC* (NGO0168) was the only gene encoding a cluster IX MBR that could be identified in the gonococcal genome. Gonococcal MntC exhibited 23% sequence identity to MntC from *Synechocystis* sp. strain PC6803 and PsaA from *Streptococcus pneumoniae*, which are MBR proteins of known Mn permease systems, and it showed 22% and 24% sequence identity, respectively, to *S. pneumoniae* AdaC and *Synechocystis* sp. strain PC6803 ZnuA, which are MBRs from Zn permease systems. However, MntC showed the highest sequence identity (27%) to *T. pallidum* TroA, an MBR that is known to bind Zn or Mn with similar affinity (14). TroA coordinates a single Zn(II) ion in a pentavalent complex using three histidine ligands and a bidentate aspartate side chain, while ZnuA binds Zn(II) in a distorted tetrahedral coordinated geometry with a water molecule providing the fourth coordinating ligand (15). Alignment of the primary structures of gonococcal MntC with those of TroA and ZnuA (see Fig. S1 in the supplemental material) showed that these histidine residues are present in MntC. The conserved aspartate is also present in gonococcal MntC. In ZnuA this aspartate residue points away from the metal binding site, but it is a fourth ligand in TroA and Mn binding MBRs. It has also been suggested that a key difference between TroA and ZnuA may lie in the amino acid sequence between the conserved aspartate ligand and the conserved glutamate (E290 in ZnuA) that is part of the secondary shell of interacting residues. Alignment of sequences indicated that gonococcal MntC had highest sequence similarity in this region to Mn binding MBRs such as *Synechocystis* MntC. Based on the above analysis, these data suggested that the gonococcal MntC should bind Mn^{2+} since it exhibits homology to Mn^{2+} binding MBRs. However, a feature of some zinc binding MBRs such as ZnuA is the presence of a histidine-rich loop that is located near the metal ion binding cleft. This histidine-rich loop is present in gonococcal MntC, suggesting that gonococcal MntC might also bind Zn^{2+}.

Zn^{2+} or Mn^{2+} can restore optimal growth to an *mntC* mutant, but growth of an *mntAB* mutant requires the presence of an antioxidant. The presence of only one MBR of the cluster IX family in *N. gonorrhoeae* raised the question of whether the MntABC permease could transport both Zn and Mn. In an earlier publication we observed that Mn ions could protect *N. gonorrhoeae* against killing via oxidative stress and that this protection was dependent upon MntC (34). During measurements of growth of gonococci in liquid culture, we observed that the *mntC* mutant was unable to grow in BHI medium from a small inoculum unless it was supplemented with either Zn^{2+} (Fig. 1A) or Mn^{2+} (Fig. 1B). Optimal growth was achieved with a minimum of 3 μM Zn^{2+} and 5 μM Mn^{2+}. It was previously observed that the *mntAB* mutant exhibited a more severe growth defect than the *mntC* mutant; the *mntAB* mutant will form only small colonies on solid medium and will not grow in BHI liquid medium (34). We now extend this work by showing that the *mntAB* mutant, in liquid medium, cannot be rescued by addition of Zn^{2+} or Mn^{2+} (Fig. 1A and B). However, we observed that addition of the antioxidant mannitol could restore growth of this mutant (Fig. 1C). This observation supports our previous conclusion that mutation of the *mntABC* system leads to oxidative stress in *N. gonorrhoeae*.
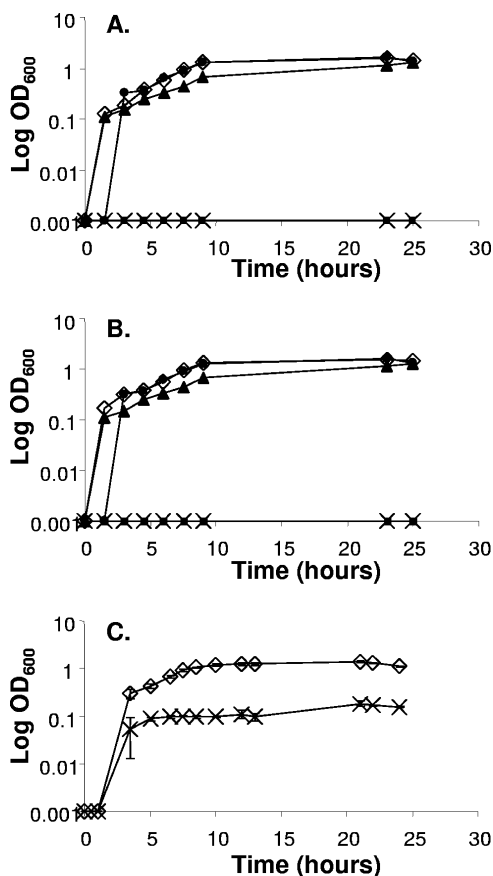
FIG. 1. Growth of wild-type *N. gonorrhoeae* 1291 and the *mntAB* and *mntC* mutants in liquid culture on BHI medium supplemented with zinc sulfate (A), manganese sulfate (B), or mannitol (C). (A) $\diamond$, *N. gonorrhoeae* wild-type 1291; $\times$, *mntAB* mutant with 100 μM Zn^{2+}; $\blacksquare$, *mntC* mutant; $\blacktriangle$, *mntC* mutant with 3 μM Zn^{2+}; $\bullet$, *mntC* mutant with 100 μM Zn^{2+}. (B) $\diamond$, wild-type *N. gonorrhoeae* 1291; $\times$, *mntAB* mutant with 100 μM Mn^{2+}; $\blacksquare$, *mntC* mutant; $\blacktriangle$, *mntC* mutant with 5 μM Mn^{2+}; $\bullet$, *mntC* mutant with 100 μM Mn^{2+}. (C) $\diamond$, *N. gonorrhoeae* 1291 with 5 mM mannitol; $\times$, *mntAB* mutant with 5 mM mannitol. The data are the means ± standard errors from three independent experiments.

FIG. 2. MntC titration with Zn^{2+} and Mn^{2+}. (A) Relative change in fluorescence emission at 340 nm during Zn^{2+} titration. Inset, decrease in fluorescence emission spectrum as increasing concentrations of Zn^{2+} were added to 25 μM MntC. (B) Relative change in fluorescence emission at 340 nm during Mn^{2+} titration. Inset, decrease in fluorescence emission spectrum as increasing concentrations of Mn^{2+} were added to 25 μM MntC. The arrow denotes the decrease in the relative fluorescence emission at 340 nm upon Zn^{2+} (A) or Mn^{2+} (B) titration into MntC.

MntC binds Zn^{2+} and Mn^{2+} with similar affinities. The growth assays indicated that gonococcal MntABC permease is able to transport both Zn and Mn ions, suggesting that MntC should be able to bind both of these ions. This is observed for TroA, which has very similar affinities for Zn^{2+} and Mn^{2+} (15). To investigate metal binding to MntC, we overexpressed and purified the protein from *E. coli*. In order to ensure that any bound transition metal ions were removed from rMntC, the protein was denatured, dialyzed against metal-free buffers, and then refolded. The secondary structure of the refolded rMntC was similar to that of native rMntC, as indicated by circular dichroism spectroscopy (data not shown). Metal analysis using ICP-MS revealed that refolded rMntC did not contain any transition metal ions; this allowed us to investigate metal binding to rMntC.

MntC contains a single tryptophan residue (Trp 138) located near the putative metal binding site between the $(\beta/\alpha)_4$ domains. This allowed us to use changes in tryptophan fluores-
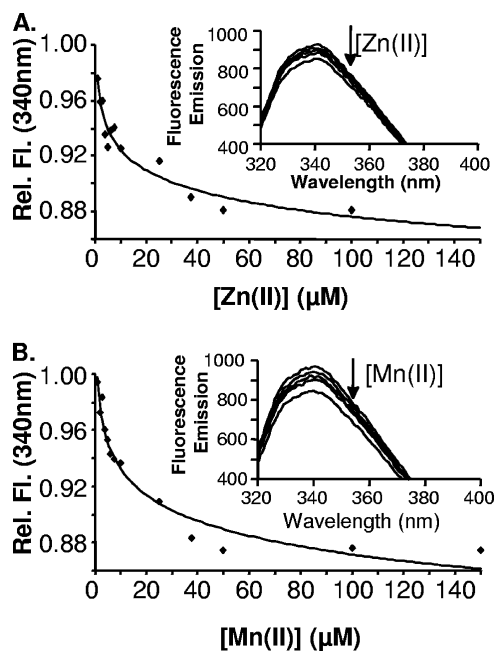
cence emission at 340 nm to monitor metal ion binding to rMntC. Addition of Zn^{2+} or Mn^{2+} to rMntC caused a decrease in fluorescence at 340 nm (Fig. 2A and B), which suggests a metal ion-induced alteration to the tryptophan environment within the protein. The change in fluorescence quenching was measured as a function of metal ion concentration, and this yielded curves that fitted best to a 1:1 binding model for the binding of Zn^{2+} or Mn^{2+} to rMntC (Fig. 2A and B, insets). The dissociation constants for the interaction of Zn^{2+} and Mn^{2+} with rMntC were calculated to be 104 ± 5 nM and 100 ± 8 nM, respectively. These values are of a similar magnitude to those described for binding of Zn^{2+} and Mn^{2+} to TroA (8). Circular dichroism spectra of native rMntC and metal-saturated rMntC from fluorescence quenching were identical and show that metal binding does not induce changes to major secondary structural elements (data not shown). This is supported by the fluorescence data that show no change to the maximum tryptophan wavelength as Zn^{2+} or Mn^{2+} is titrated in. Indeed, the 340-nm maximum absorption indicates that the tryptophan is initially in a solvent-shielded environment and remains so after metal addition. To determine whether the observed tryptophan quenching was due to a specific interaction between Zn^{2+} and Mn^{2+} with MntC, we titrated Fe(II). Titration of MntC with Fe^{2+} showed no quenching of tryptophan fluorescence, suggesting a lack of binding (data not shown). This result suggests that rMntC is specific for Zn^{2+} and Mn^{2+}, similar to previous results for the homologue TroA (15).
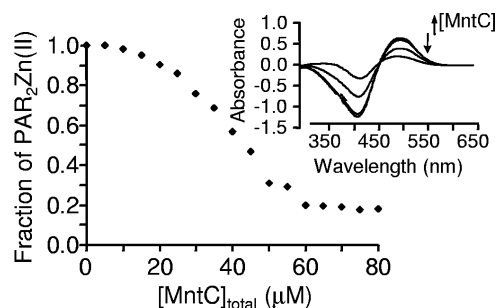
FIG. 3. Measurement of Zn^{2+} binding to MntC via a PAR competition assay. A binding isotherm was generated from 200 μM PAR and 50 μM Zn^{2+} titration with increasing concentration of MntC (0 to 80 μM). Inset, representative spectra obtained during MntC titration. The downward arrow denotes the decrease in absorbance at 500 nm, indicating the removal of Zn^{2+} from the PAR_2Zn complex by adding (upward arrow) various concentration of MntC to form MntC-Zn^{2+}.

FIG. 4. Displacement of Zn^{2+} from MntC by Mn^{2+}, showing spectra of Mn^{2+} (0 to 100 μM) titrated into MntC-Zn^{2+} (50 μM) and excess PAR (200 μM). The arrow indicates the increase in absorbance at 500 nm, indicating the generation of a PAR_2Zn complex.

The binding of Mn(II) to MntC was confirmed using EPR spectroscopy. Mn(II) is a paramagnetic metal ion for which $S = 5/2$ and $I = 5/2$. Hexaquo-Mn(II) has a distinctive six-line EPR signal at room temperature. When Mn(II) binds to a protein, solvent ligands are displaced and the room temperature X-band signal disappears. The loss of signal is due to broadening via the zero field splitting arising from disturbances to the ligand field of the bound metal. Thus, Mn(II) resonances observed in room temperature solution EPR spectra in the presence of MntC arise only from unbound Mn(II) ions free in solution. Upon addition of MntC to the Mn(II) solution, there was a decrease in the amplitude of the EPR signal, consistent with the binding of Mn(II) to MntC (see Fig. S2 in the supplemental material).

In order to qualitatively verify that Zn^{2+} was bound by MntC, a competition assay with the metallochromic Zn^{2+} indicator PAR was performed. Under solution conditions (pH 7.4), PAR is calculated to form 1:1 and 2:1 complexes with Zn^{2+} with stepwise affinity constants of 9.7×10^6 and 6.1×10^5 M^{-1}, respectively (17). Thus, an excess of PAR was used in the experiments described here to ensure that $[Zn(PAR)_2]$ was the only Zn(II) complex in solution prior the addition of protein. The addition of Zn^{2+} to PAR results in an intense absorption band at 500 nm. Upon the addition of MntC, a decrease in the absorption band at 500 nm was observed (Fig. 3). This indicates that Zn^{2+} was displaced from $[Zn(PAR)_2]$ to presumably form an MntC-Zn complex and is consistent with our fluorescence measurements (Fig. 2A) that indicated that MntC has a higher affinity for Zn^{2+} than PAR. This observation is consistent with the Zn dissociation constant value for MntC obtained from the fluorescence spectra data (nanomolar to micromolar).

Zn and Mn ions compete for the same site in MntC. The observation that MntC would displace Zn(II) from [Zn-(PAR)$_2$] allowed us to use a PAR-based competitive assay to determine whether MntC has a single binding site for both Mn^{2+} and Zn^{2+}. The displacement assay was performed using a 1:1 MntC-Zn complex in the presence of excess PAR. Increasing concentrations of Mn^{2+} were titrated into the MntC-Zn and PAR reaction mixture. The titration of Mn(II) resulted in an absorbance increase at 500 nm, indicative of
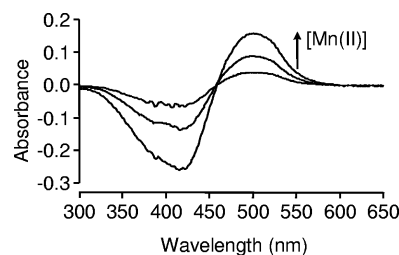
$[Zn(PAR)_2]$ formation (Fig. 4). These data show that Mn(II) can displace Zn(II) from the MntC-Zn complex to form an MntC-Mn complex. This result suggests that Zn^{2+} and Mn^{2+} utilize the same ligands (binding site) to coordinate MntC. Furthermore, these data indicate that Mn^{2+} has a higher affinity for the site than does Zn^{2+}.

***mntC* and *mntAB* mutants have a reduced ability to invade primary ectocervical cells.** *N. gonorrhoeae* adherence to and invasion of cervical epithelial cells is a complex process that is mediated by several factors (11, 24). The ability of wild-type and mutant gonococci to associate with, invade, and survive within primary cervical (pex) cells was determined as described previously (38). Figure 5 shows that both the *mntAB* and *mntC* mutants showed a similar level of association to pex cells as did wild-type cells; however, there was a significant reduction in the level of invasion, and this was particularly noticeable for the *mntC* mutant. Extending the study of the invasion produced some interesting results. After 1 h there was no difference in CFU for wild-type cells, while CFU for the *mntAB* mutant were reduced by 50%. The CFU count of the *mntC* mutant was the lowest of all three strains tested after 1 h of invasion, and this value essentially did not change after 3 h of invasion. Similarly, the *mntAB* mutant CFU count did not increase after 3 h, but, in contrast, the wild-type cells had shown clear evidence of growth, as indicated by recovery of CFU. Taken together, these data show that the MntABC permease is critically important for intracellular survival of *N. gonorrhoeae* in cervical epithelial cells.

***mntC* and *mntAB* mutants are deficient in forming biofilms.** Studies performed in continuous-flow chambers have recently shown that *N. gonorrhoeae* strain 1291 can form a biofilm on glass coverslips as well as on primary cervical cells without loss of viability of the epithelial cells (13). The abilities of the *N. gonorrhoeae mntAB* and *mntC* mutant strains to form biofilm compared to that of the wild-type strain were evaluated after 2 days of growth under continuous-flow conditions (Fig. 6). COMSTAT (16) was used to quantitatively assess the biomass and the average and maximum thicknesses of confocal z-series photomicrographs taken for each flow chamber. COMSTAT analysis showed that both the *mntAB* and *mntC* mutants are deficient in biofilm formation compared to the wild type (Fig. 6B). Specifically, the *mntAB* mutant had 19.8% of the biomass of the wild type, while the *mntC* mutant had 7.0% of the biomass of the wild type. Both mutants also form biofilms with lower average thicknesses than the wild type. The *mntAB* and
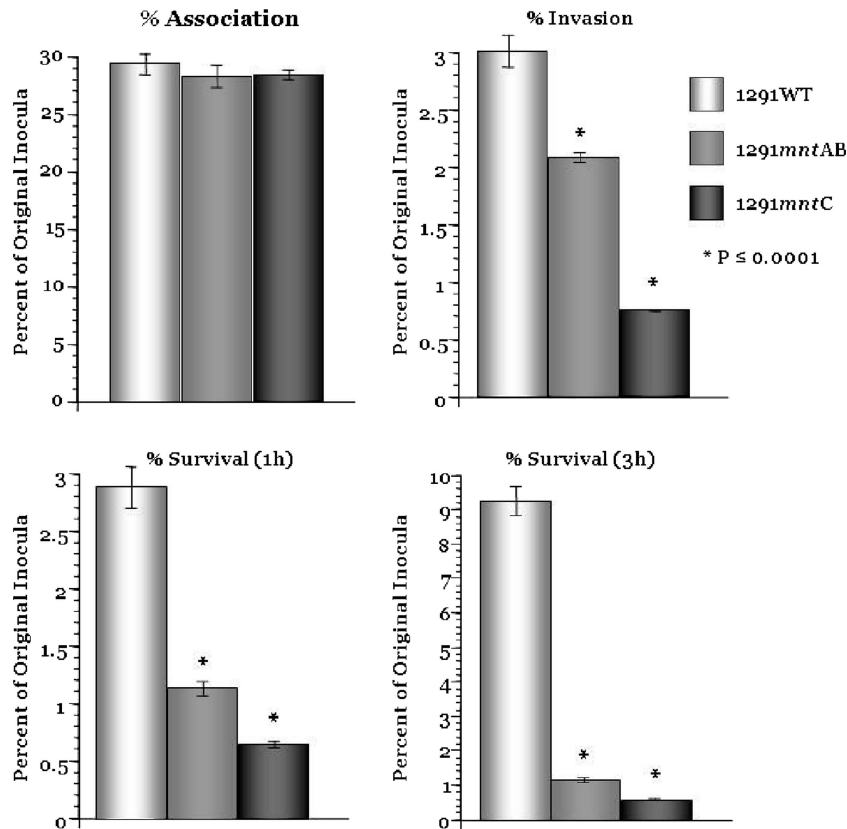
FIG. 5. Association/invasion/survival assays performed using wild-type (WT) *N. gonorrhoeae* 1291, 1291 *mntAB*, and 1291 *mntC* with 90 min of infection without (association) or with (invasion) 30 min of gentamicin treatment (100 μg/ml) and then a 1-h or 3-h second incubation (survival) in antibiotic-free medium. Gonococcal association, invasion, and survival within pex cells was determined in parallel in a single assay; however, the data obtained have been plotted as individual panels. Data shown are the means (variances) obtained from three separate assays performed in triplicate.

mntC mutants possessed 10.0% and 11.7% the average thickness of the wild type, respectively. Differences in biomass and average thickness were determined to be statistically significant using a Student *t* test ($P < 0.000001$). Three-dimensional images of these biofilms were created in Volocity. These images show that overall both mutants form thinner and more diffuse biofilms with large gaps between biofilm clusters compared to wild type.

DISCUSSION

ABC permeases which contain an MBR of the cluster IX family have emerged as key transporters that are required for virulence in several bacterial pathogens. In a number of bacteria, for example, *S. pneumoniae*, there are two distinct permeases which appear to have biologically distinct roles as transporters of Zn or Mn ions (9). The presence of a single MBR of the cluster IX family in *N. gonorrhoeae* raised the question of its metal binding specificity in relation to its role in growth and survival of this bacterium. We have previously shown that MntC is required for accumulation of Mn^{2+} in gonococci to levels that are sufficient to confer protection against killing via oxidative stress (34). The present data showed that in the absence of MntC, gonococci required addition of either Zn^{2+} or Mn^{2+} to liquid medium in order to facilitate growth. In an earlier report, Chen and Morse (5) identified an ABC permease in *N. gonorrhoeae* strain F62 which was essential for uptake of zinc. This permease, described as ZnuABC, is homologous to the MntABC that we have described in the present study. We have made a similar observation in the case of a *psaA* mutant of *S. pneumoniae*, where we observed that Mn^{2+} supplementation of liquid media was required to promote growth (23). These results are consistent with the expected biological role of MBRs, which is to confer a high affinity on the permease system. In the absence of the MBR, the permease can still function, but it will have a lower affinity for metal. The loss of *mntAB* produced a more severe phenotype with respect to growth of gonococci on plates and in liquid culture, and it was not possible to facilitate growth of the *mntAB* mutant with Zn or Mn supplementation. These data suggest that the MntABC system is the dominant Mn and Zn transporter in gonococci. The *mntAB* mutant could be rescued by addition of the antioxidant mannitol or tiron, suggesting that the effect of Mn^{2+} and Zn^{2+} transport by the MntABC system is to protect *N. gonorrhoeae* against oxidative stress. We have previously focused on Mn-dependent protection of *N. gonorrhoeae* against oxidative stress and have shown that MntC is required for this phenomenon. Mn and Zn are both known to possess antioxidant properties, but their modes of action are quite different. Mn is able to cycle between the
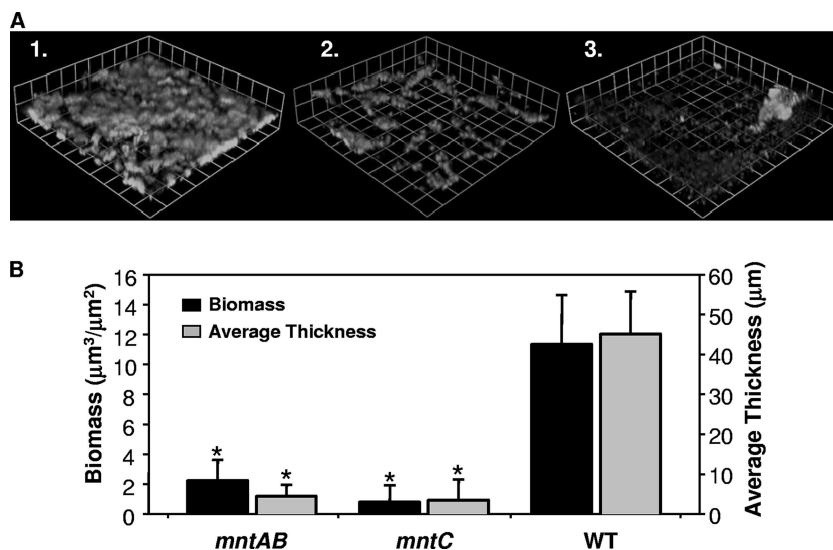
FIG. 6. Biofilm formation by wild-type *N. gonorrhoeae* 1291 and the *mntAB* and *mntC* mutants. (A) Biofilm mass over 2 days of growth for (1) wild-type *N. gonorrhoeae* 1291, (2) the *mntAB* mutant, and (3) the *mntC* mutant. These images are stacked z series taken at a magnification of ×200. (B) COMSTAT analysis of the biomass and average thickness of biofilm stacks. The error bars represent ±1 standard deviation of the mean. Asterisks denote statistically significant differences in biomass and average thickness compared to the wild type. These experiments were repeated on three different occasions, and a representative result is shown. There is a statistically significant difference in the mean biomasses of the *mntAB* and *mntC* mutants relative to the wild-type (P values of 3.1×10^{-7} and 7.1×10^{-8}, respectively, as determined using Student's t test). There is also a statistically significant difference in the average thicknesses of the biofilm of the *mntAB* and *mntC* mutants relative to the wild type (P values of 1.3×10^{-8} and 1.5×10^{-9}, respectively).

Mn^{2+} and Mn^{3+} states and can quench both superoxide ions and hydrogen peroxide. In many bacteria Mn is a component of superoxide dismutase (SodA), although in *N. gonorrhoeae* the sole type of superoxide dismutase appears to be the Fe-Sod (SodB). However, Mn can provide protection against oxidative stress to a *sodB* mutant of *N. gonorrhoeae*, consistent with the view that chemical quenching of superoxide by Mn has a potentially important role as an antioxidant in this bacterium.

Zn lacks redox activity, and it has ligand binding properties different from those of Mn. Zn^{2+} is known to bind cysteine thiolates as well as N ligands (imidazole). As such, it is able to stabilize dithiols in a reduced state and prevent disulfide bridge formation (4). It is established that release of Zn^{2+} from a ribosomal pool is an important part of the oxidative stress response in *Bacillus subtilis* (25). Zn^{2+} is bound via at least one ribosomal protein, L31, but during oxidative stress expression of an L31 protein paralogue which lacks the cysteine residues capable of binding Zn leads to displacement of the Zn-L31 from the ribosome and an increase in cytoplasmic Zn. *N. gonorrhoeae* possesses both forms of L31, and expression of the Zn-free L31 has been shown to be increased in a PerR mutant of this bacterium (38). Although we have named this Fur paralogue in *N. gonorrhoeae* PerR, it seems more likely that this transcriptional repressor is responding to Zn^{2+}. This transcription factor also represses *mntC* expression, suggesting that it is Zn that controls expression of MntABC. A similar situation has been observed in *T. pallidum*, where the repressor of *troABCD* expression responds to Zn^{2+} levels but not to Mn^{2+} within a physiological range (8).

In *S. pneumoniae* it is established that PsaA is a virulence factor that is required for disease (9). The importance of MntC in the pathogenesis of the gonococcus, in vivo, is more difficult to assess. Nevertheless, our observation that MntC is required for invasion and survival within primary cervical epithelial cells as well as biofilm formation is strong evidence that Mn and/or Zn transport via the MntABC permease is important in gonococcal pathogenesis. Our results show that MntC from *N. gonorrhoeae* is able to bind Mn^{2+} or Zn^{2+} with almost equal affinity. This situation has also been reported for TroA from *T. pallidum*, where a single ABC cassette system containing a cluster IX MBR was able to transport both Mn^{2+} and Zn^{2+} (8). Unlike *T. pallidum*, gonococci do not possess a dedicated Zn(II) transporter. It is possible that transport of both Zn^{2+} and Mn^{2+} via the MntABC transporter is relevant to growth and survival of gonococci. However, the bioavailability of manganese and zinc in different settings is hard to assess. Lactic acid bacteria are present as commensals in the female genitourinary tract, and they may act to concentrate Mn^{2+} (2). It is possible that this may lead to increased bioavailability of Mn^{2+} in that environment. Although zinc is more abundant, in human serum the total Zn concentration is approximately 20 μM and the concentration of Mn is near 100 nM (6, 35); it is also the case that Zn^{2+} is usually bound tightly to proteins, while Mn^{2+} exchanges ligands rapidly. The presence of the histidine-rich loop in gonococcal MntC may be an additional feature that allows Zn^{2+} to be acquired.

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