A genetic screen reveals a periplasmic copper chaperone required for nitrite reductase activity in pathogenic *Neisseria*

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ABSTRACT Under conditions of low oxygen availability, *Neisseria meningitidis* and *Neisseria gonorrhoeae* are able to respire via a partial denitrification pathway in which nitrite is converted to nitrous oxide. In this process, nitrite reductase (AniA), a copper (Cu)-containing protein converts nitrite to NO, and this product is converted to nitrous oxide by nitric oxide reductase (NorB). NorB also confers protection against toxic NO, and so we devised a conditional lethal screen, using a *norB* mutant, to identify mutants that were resistant to nitrite-dependent killing. After random-deletion mutagenesis of *N. meningitidis*, this genetic screen identified a gene encoding a Cu chaperone that is essential for AniA function, AccA. Purified AccA binds one Cu (I) ion and also possesses a second binding site for Cu (II). This novel periplasmic Cu chaperone (AccA) appears to be essential for provision of Cu ions to AniA of pathogenic *Neisseria* to generate an active nitrite reductase. Apart from the *Neisseria* genus, AccA is distributed across a wide range of environmental Proteobacteria species.—Jen, F. E.-C., Djoko, K. Y., Bent, S. J., Day, C. J., McEwan, A. G., Jennings, M. P. A genetic screen reveals a periplasmic copper chaperone required for nitrite reductase activity in pathogenic *Neisseria*. *FASEB J.* 29, 3828–3838 (2015). www.fasebj.org

Key Words: meningitis • gonorrhea • denitrification

NEISSERIA GONORRHOAE AND *NEISSERIA MENINGITIDIS* are closely related pathogenic species that colonize only humans. *N. meningitidis* (meningococcus) can be carried asymptotically, but can also cause rapidly progressing, life-threatening meningitis and sepsis when it crosses the epithelium and enters the blood. Vaccines are available to prevent invasive meningococcal disease. *N. gonorrhoeae* primarily colonizes the human

genitourinary tract, causing the sexually transmitted disease, gonorrhea, a major public health concern. The emergence of gonococcal resistance toward antibiotics has recently become a significant public health problem (1). Development of a vaccine to prevent gonorrhea is the ideal solution to this emerging public health threat.

Anaerobically induced protein A [AniA (2); previously known as Pan1 (3)], is a nitrite reductase (NirK) that plays a key role in the biology of *N. meningitidis* and *N. gonorrhoeae*. These organisms cannot use nitrate, but can use nitrite as a terminal electron acceptor to grow anaerobically (4). AniA converts nitrite (NO₂[−]) to nitric oxide (NO), which is then reduced to nitrous oxide (N₂O) by nitric oxide reductase (NorB) (Fig. 1A). The gene encoding nitrous oxide reductase, *nosZ*, is a pseudogene in pathogenic *Neisseria*, so the denitrification pathway terminates at N₂O, rather than at the dinitrogen gas N₂ (5). Both AniA and NorB are essential for *N. gonorrhoeae* biofilm formation on human cervical cells (6). The expression of AniA in *N. meningitidis* is up-regulated during the host infection in the bloodstream (7).

AniA is a copper (Cu)-containing NirK that has both type I and II Cu centers (8). The type II center catalyses nitrite reduction, whereas the plastocyanin-like type I center is involved in mediating electron transfer from an electron donor, which is usually a c-type cytochrome or a cupredoxin such as azurin (9). Most Gram-negative bacteria express NirK (usually called NirK) in the periplasm (10, 11). In contrast, AniA of pathogenic *Neisseria* has a long N-terminal sequence that directs its export to the outer surface of the outer membrane, where it is anchored as a lipoprotein (12). AniA of the pathogenic *Neisseria* also has a long, C-terminal extension that is glycosylated (13). The localization of AniA has led to investigations of this outer membrane glycoprotein as a vaccine candidate to prevent gonococcal infections (12). This unique outer membrane localization raises questions in relation to AniA folding, export, and Cu loading and about

Abbreviations: AccA, AniA Cu chaperone; AniA, nitrite reductase in *Neisseria*; BCS, bathocuproine disulfonate; COG, class of orthologous group; *kan*, kanamycin; KLH, keyhole limpet hemocyanin; NirK, nitrite reductase; NorB, nitric oxide reductase; orf, open reading frame; PAR, 4-(2-pyridylazo)resorcinol; PCu_AC, periplasmic Cu_A chaperone; SOD, superoxide dismutase; SPR, surface plasmon resonance

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Figure 1. A) Bacterial denitrification pathway. B) Serial dilutions of bacterial growth on the media, with or without nitrite (2 mM NaNO₂). C) $\phi 3$ *norB::tet* genomic DNA and *kan* cassette were digested by the same set of restriction enzymes and ligated to have a mixture of random insertions of the *kan* cassette in NorB genomic DNA (mutagenesis library). Homologous recombination transformation of the mutagenesis library allowed the *kan* cassette to be randomly inserted into the NorB mutant. Over 150,000 kanamycin-resistant colonies were screened. Colonies grew on medium containing 2 mM NaNO₂ indicating loss of AniA function. D*i*) All the kanamycin- and nitrite-resistant colonies were picked and patched on new kanamycin (100 μ g/ml)/nitrite (2 mM NaNO₂) plates to confirm the phenotype. D*ii*) To eliminate the colonies that had these phenotypes due to the loss of AniA, PCR of the *aniA* gene and Western blot probing with anti-AniA were performed. D*iii*) NirK activity assays of the screened mutant confirmed the loss of AniA activity, even when AniA protein was present. ***P* = 0.001.

the identity of its cognate electron donor on the outside of the outer membrane. The accessory proteins needed for these processes have not been defined. In this study, we used random-deletion mutagenesis and a novel screen for loss of NirK activity to identify the genes necessary for AniA function. This screen revealed a Cu chaperone that is essential for AniA activity named AniA Cu chaperone A (*AccA*).

MATERIALS AND METHODS

Bacterial strains and growth conditions

N. meningitidis strains $\phi 3$ (14) and *N. gonorrhoeae* 1291 (15) were used in this study. The *N. meningitidis* strains were grown on brain-heart infusion (BHI; Oxoid, Heidelberg West, VIC, Australia) 1% agar and 10% Levinthal's Medium Base at 37°C with 5% CO₂,

with either kanamycin (100 µg/ml) or tetracycline (5 µg/ml), as required. *N. gonorrhoeae* strains were grown on GC agar (Oxoid) with 1% IsoVitalEx (BD Biosciences, North Ryde, NSW, Australia).

Generation of mutant strains

N. gonorrhoeae strain 1291 *norB::tet* and *N. meningitidis* strain *ϵ3norB::tet* mutants were generated by a homologous recombination resulting in allelic replacement of the active gene with the inactive allele in the chromosome of these strains. The *norB* coding sequence (with 186 bp upstream and 494 bp downstream) was amplified from strain 1291 using primers NorBFor and NorBRev, and the resulting PCR product was cloned into pGEM-T Easy (Promega, Alexandria, NSW, Australia), to generate pGemT*norB*. The pGemT*norB* plasmid was digested with *Bsa*BI to remove most of the *norB* coding sequence (1323 bp) and was replaced by a *Hinc*II-digested *tetM* cassette from pGem*tetM* (16), to generate pGemT*norB::tet*. The construct pGemT*norB::tet* was linearized with *Not*I and transformed into the 1291 and *ϵ3* strains to generate the 1291 *norB::tet* and *ϵ3norB::tet* mutant strains. *N. gonorrhoeae* strain 1291 *accA::kanamycin (kan)* and *N. meningitidis* strain *ϵ3accA::kan* mutants were generated by a similar process. The *nmb1557* coding sequence was cloned into pGEMT-T Easy to generate pGemT*accA*. A Mutation Generation System (MGS) kit (Thermo Fisher Scientific, Scoresby, VIC, Australia) was used according to the manufacturer's instructions, to insert a transposon containing a *kanR3* gene into the pGemT*accA* to generate pGemT*accA::kan*. The location and orientation of the *kanR3* insertion in *accA* was determined by sequencing. The pGemT*accA::kan* construct was linearized by *Xmn*I and transformed into strains 1291 and *ϵ3* by homologous recombination to generate 1291 *accA::kan* and *ϵ3accA::kan* mutant strains. To produce the AccA complemented version of these mutant strains, we first made 2 complementation plasmid constructs. In the first of these constructs, the *N. meningitidis accA* gene (*nmb1557*) was amplified and cloned between *nmb1428* and *nmb1429* in plasmid pComPind (17). In the second construct, the *N. gonorrhoeae accA* gene (*ngo1215*) was amplified and cloned into plasmid pCTS32 (18). Plasmid pComPind*nmb1557* and pCTS32*ngo1215* were linearized and transformed into *ϵ3accA::kan* and 1291 *accA::kan* mutants, respectively. The primers used in the processes described above are listed in Table 1.

Random mutagenesis

The random mutagenesis method used in this study was originally described by van der Ley *et al.* (19). Chromosomal DNA of strain *ϵ3norB::tet* was isolated and digested with 1 of

the 8 restriction enzymes: *Pst*II, *Eco*RI, *Bsp*HI, *Pci*I, *Sph*I, *Nsp*I, *Spe*I, or *Xba*I. A set (pairs selected from the 8 restriction enzymes) of digested chromosomal DNA samples was subsequently mixed and ligated to the 1.2 kb kanamycin-resistance marker (*kan* cassette) digested with the same pair of restriction enzymes (see Fig. 1B). Strain *ϵ3norB::tet* was then transformed with the ligation mix. Kanamycin resistance was used to select for random insertion of the *kan* cassette into the chromosome.

AccA expression and purification

The AccA protein (amino acid residues 20–157) was amplified from *N. meningitidis* strain *ϵ3* using primers 1557NSFor_*Nco*I and 1557HisRev_*Nde*I and cloned into pET15b (Novagen-EMD Biosciences, Madison, WI, USA) between the *Nco*I and *Nde*I sites, to create plasmid pET15bAccAHis. This plasmid was transformed into *Escherichia coli* BL21 (DE3) (NEB) for overexpression. AccA was overexpressed in Luria-Bertani (LB) broth at 25°C for 24 h after addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Bacteria were lysed in buffer A (50 mM Tris-HCl, 0.1 mg/ml DNaseI, and 1 mM MgCl₂) *via* 3 passages through a French pressure cell (15,000 kPa). AccA was purified by using TALON Metal Affinity Resin (BD Biosciences). After the sample passed through the affinity resin column, the column was washed with 10 column volumes of buffer A and 2 column volumes of buffer A with 10 mM imidazole. The protein was eluted by collecting 1 ml fractions over 10 column volumes of buffer A with 150 mM imidazole. The His-tag was removed by cleavage with AcTEV Protease (Thermo Fisher Scientific, Waltham, MA, USA) prior to surface plasmon resonance (SPR) studies. The protease was removed after digestion using His-tag affinity chromatography. Removal of the His-tag from AccA was confirmed by the molecular weight shift in SDS-PAGE. After this process, the yield of AccA was sufficient for SPR analysis, but not for later Cu-binding studies.

To generate sufficient AccA protein for Cu-binding studies, we took a second approach to purifying AccA, by using its histidine-rich C terminus as a natural affinity tag. The *accA* gene was amplified from *N. meningitidis* strain *ϵ3* using primers *accA-Nde*I-F/*accA-Bam*HI-R, cloned into vector pET-11b and then transformed into *E. coli* BL21 Star (DE3)pLysS (both from Novagen) for overexpression. AccA was expressed as described above and purified from crude lysates on an Ni(II)-NTA His-Trap affinity column (GE Healthcare, Brisbane, QLD, Australia). AccA (without the synthetic His-tag) bound efficiently to the resin and was eluted with a gradient of 0–250 mM imidazole in buffer B [50 mM Tris, 150 mM NaCl, and 5%

TABLE 1. Primers

Primer	Sequence	Construct
NorBFor	CAGAGCAGGCAAAGGCAG	pGemT <i>norB</i>
NorBRev	GAACAGCCCTACCGCATC	
1557ComNdeI_F	GCAGACATATGAAAAAATTATTGGCGGC	pComPind <i>accA</i>
1557ComNsiI_R	ATTTTCATGCATTTAGTGCTGATGCGCTTC	
1215Cmp_A/III	CAGCACCTTAAGAGAATCGTTCTCTTTG	pCST32 <i>accA</i>
1215Cmp_SmaIR	TTTAAACCCGGGCATAAAAATGCCGTC	
1557KOFor	GCCGTCTGAAGGCAACGCTGTACTGGTTTTTG	pGemT <i>accA</i>
1557KORev	TTCAGACGGCCAGATTAGTGCTGATGCGCTTC	
1557NSFor_ <i>Nco</i> I	CGGTTTCCATGGATGAAAGCCGGAGTCCACGTT GAGGACGG	pET15bAccAHis
1557HisRev_ <i>Nde</i> I	TTTTACATATGTTAGTGATGATGATGATGGTG CTGATGCGCTTCGCCGTGGTGATGACC	
<i>accA-Nde</i> I-F	CTGGAACATATGAAAAAATTATTGGCGG	pET11bAccA
<i>accA-Bam</i> HI-R	CAGGTAGGATCCTTAGTGCTGATGCGCTTC	

glycerol v/v, pH 8.2]. Fractions containing AccA were combined and treated with 5 mM EDTA. Subsequent elution on a Superdex75 size-exclusion column (GE Healthcare) in buffer C [50 mM HEPES and 150 mM NaCl, pH 7.6] yielded pure AccA protein in a monomeric and unmetallated *apo* form. The purity of AccA preparations was >99% as determined by SDS-PAGE. Final protein yields were ~200 mg/L culture. Electrospray ionization/mass spectrometry confirmed a final protein mass of 15,161 Da, indicating that the signal peptide was cleaved correctly between Ala¹⁹ and Ala²⁰ by the *E. coli* host strain. This purified AccA protein was used in Cu-binding studies. AniA protein was expressed and purified as described in Shewell *et al.* (12). Protein concentrations were estimated using the solution absorbance at 280 nm ($\epsilon_{280-6300} \text{ M}^{-1} \cdot \text{cm}^{-1}$).

Antibody production

Polyclonal antisera, α -AccA, were produced by inoculating mice with the peptide-conjugated keyhole limpet hemocyanin (KLH). Peptide ³²KQDFLLGGSPVADRVEVHT⁵¹ of AccA was synthesized and conjugated to KLH by Mimotopes (Clayton, VIC, Australia).

SPR

SPR experiments were performed on a Biacore T100 system (GE Healthcare) at 25°C. Recombinant AniA protein was immobilized onto a CM5 series S sensor chip with an *N*-hydroxysuccinimide capture kit (GE Healthcare). This process covalently linked the AniA protein to the surface of the chip *via* free amine groups. The AccA protein, with and without Cu, were run over the chip at concentrations ranging from 6.3 to 100 $\mu\text{g/ml}$ in PBS, and the data were analyzed by using single-cycle kinetics as described by DuMont *et al.* (20). At least 3 experiments were performed for each interaction.

Superoxide dismutase activity stain

The superoxide dismutase (SOD) assay used in this study was originally described by Beauchamp and Fridovich (21). Cell-free extracts were prepared by resuspending cultures of *N. meningitidis* strain $\epsilon 3$, strain $\epsilon 3$ *sodC::kan*, and strain $\epsilon 3$ *accA::kan* in PBS overnight and then lysing the cells by TissueLyserLT (Qiagen, Chadstone Centre, VIC, Australia). Unbroken cells and cell debris were removed by centrifugation, and the supernatant was passed through a 0.45 μm -pore membrane filter (Millipore, North Ryde, NSW, Australia) and collected. Protein concentration in cell extracts was determined by absorbance at 280 nm. Total proteins (~100 μg) from each sample was run on a Novex pH3–10 IEF protein gel (Life Technologies). After electrophoresis, the gel was soaked in solution A [0.025% (w/v) nitroblue tetrazolium (NBT; Sigma-Aldrich, Castle Hill, NSW, Australia) and 0.01% (w/v) riboflavin (Sigma-Aldrich) in MilliQ water (Millipore)] for 20 min and then in solution B [1% (w/v) tetramethylethylenediamine (Sigma-Aldrich)] for 20 min. After the gel was exposed to a light source, the SOD activity appeared as a colorless band against a purple background.

Cytochrome *cbb*₃ oxidase activity assay

N. gonorrhoeae strain 1291 and derivatives were grown in aerated BHI cultures (50 ml) for 5 h. The bacteria were collected by centrifugation (4000 g, 5 min) and washed twice with ice-cold PBS

containing magnesium chloride (10 mM). The final bacterial pellets were resuspended in the same buffer and kept on ice. Consumption of molecular oxygen was measured at 35°C in PBS (2 ml) in the presence of sodium ascorbate (1 mM) and *N,N,N',N'*-tetramethylphenylenediamine (0.2 mM) as a gratuitous electron donor to cytochrome *cbb*₃ oxidase. Reactions were monitored with an S1/Mini Clark-type oxygen electrode (Hansatech Instruments, King's Lynn, United Kingdom) in conjunction with an Oxytherm control unit. The rates of oxygen reduction (1 U = 1 nmol O₂/min) were plotted by using the Oxygraph Plus program (both from Hansatech), and the results were normalized to total protein content (in milligrams).

Phylogenetic distribution of AccA across the bacterial domains

The protein sequence of the *accA* gene from *N. meningitidis* strain $\epsilon 3$ was used as a tblastn query against the Representative Genomes nucleotide database from the National Center for Biotechnology (NCBI; National Institutes of Health, Bethesda, MD, USA), by using the BLAST+ command-line executable suite (22) and the BLOSUM45 matrix, with a custom output format of type 6 and the maximum target sequences set to 5000. The genomes from this database with AccA tblastn *E* values less than 0.01 were termed positive, and the representation of positive taxa across the phylogenetic tree was used to identify groups containing positive taxa at the level of family, order, class, or phylum. To minimize any potential artifacts due to the arbitrary 0.01 *E* value cutoff, we also calculated the geometric mean of the positive *E* value scores within each group.

To provide a backbone for representation of the distribution of *accA* gene homologs in bacteria, a 16S rRNA phylogenetic tree was generated. One representative from each of 23 bacterial phyla was taken for which whole genome sequences are available, plus an additional 4 representatives from the Alpha-, Beta-, Delta-, and Gammaproteobacteria classes, to represent the groups containing clear homologs of *accA*. We generated a maximum-likelihood tree using the GTR+I+G substitution model in Mega 6.06 (23) and indicated on the leaf nodes the proportion of *accA*-positive taxa with sequenced genomes and geometric mean *E* value from the AccA BLAST search.

RESULTS

Use of a novel genetic screen to identify an essential gene for NirK activity

It has been established that a *norB* mutant of *Neisseria* does not tolerate the presence of nitrite on the growth medium, presumably because the accumulation of NO leads to cell death (24). This lethal phenotype of the *norB* mutant can be suppressed by loss of AniA function (24) as shown in Fig. 1C. This observation allowed the development of a genetic screen in which *norB* mutant cells could survive only if they lost AniA activity. Construction of a kanamycin-resistant random deletion mutagenesis library in *N. meningitidis* strain $\epsilon 3$ *norB::tet*, followed by selection for growth in the presence of 2 mM nitrite, resulted in selection of kanamycin-resistant, deletion mutants with an AniA-negative phenotype (Fig. 1C).

Over 150,000 kanamycin-resistant colonies were screened on the selective media, resulting in 197 colonies that showed growth on 2 mM nitrite. These mutants could be categorized into 2 distinct types (Fig. 1DI, II): the first

type ($n=184$) had a deletion in the *aniA* gene, confirmed by loss of AniA expression in Western blot analysis (Fig. 1DII). In the second type ($n=13$), the AniA protein was expressed at wild-type levels, but this class of mutant lacked NirK activity (Fig. 1DIII). The chromosomal DNA from each of the second class of mutants (AniA expression+NirK activity) was isolated, digested with *Hae*II, and self-ligated. Inverse-PCR of these self-ligated DNA fragments using outward-facing primers, anchored in the kanamycin-resistance gene, all generated the same ~ 2 kbp PCR product. Sequence analysis of these PCR products revealed that the kanamycin-resistance cassette ligation event had deleted 2 genes: *nmb1557* and *nmb1558*. According to the Kyoto Encyclopedia of Genes and Genomes (KEGG; <http://www.genome.jp/kegg/>), NMB1558 encodes a diacylglycerol kinase (EC: 2.7.1.107). An NCBI blast search of the NMB1557 (*N. meningitidis*) [or analogous *N. gonorrhoeae* open reading frame (orf); NGO1215] showed homology with a hypothetical protein found in many species that contains a class of orthologous groups (COG) domain, COG2847, which is found in Cu(I)-binding proteins. Signal P (<http://www.cbs.dtu.dk/services/SignalP/>, Center for Biological Sequencing Analysis, Technical University of Denmark, Lyngby, Denmark) analysis of the NMB1557 orf predicts that NMB1557 is a periplasmic protein with a N-terminal 19-amino-acid signal peptide.

Cu suppresses the loss of AniA activity in mutants lacking NMB1557 and NGO1215

Because AniA contains Cu centers types I and II (8), we hypothesized that NMB1557 (and NGO1215) is involved in the transfer of Cu to AniA. To investigate the role of NMB1557 (and NGO1215) in AniA function, knockout strains and the complemented versions of the knockout strains were constructed in $\phi 3$ ($\phi 3accA::kan$; $\phi 3accA::kanComP$) and 1291 ($1291accA::kan$; $1291accA::kanComP$). The phenotype of these mutants was examined by growing them in selective medium and by conducting NirK assays. In nitrite-containing growth medium (Fig. 2A), nitrite had no effect on the growth of $\phi 3accA::kan$ mutant, and the lethal phenotype of the $\phi 3norB::tet$ mutant was rescued by loss of NMB1557 function ($\phi 3accA::kan/norB::tet$). The NirK activity assay in Fig. 2B showed that the $\phi 3accA::kan$ mutant had no NirK activity, but activity could then be restored by supplementing growth medium with excess Cu ($5 \mu\text{M}$ CuSO_4), suggesting that AniA is an *apo*-enzyme in the $\phi 3accA::kan$ mutant strain. Some NirK activity was restored when the $\phi 3accA::kan$ strain was grown in growth medium containing 2 mM NaNO_2 and $5 \mu\text{M}$ CuSO_4 , resulting in a lethal phenotype (Fig. 2A). The double-knockout mutant strain, $\phi 3accA::kan/norB::tet$ showed the same phenotype as the $\phi 3norB::tet$ strain in this growth

Figure 2. A) Ten-fold serial dilutions of $\phi 3$ and 1291 WT and mutant strains growing on the media with or without nitrite (2 mM NaNO_2) and with or without Cu ($5 \mu\text{M}$ CuSO_4). B) NirK activity assays of $\phi 3$ and 1291 WT and knockout mutants (AniA and AccA) strains. AccA mutant strain growing on Cu-supplemented media had a restored AniA activity. $**P = 0.0013$; $***P < 0.0001$. The expressions of AniA and AccA were confirmed by Western blot analysis.

condition. Parallel studies in *N. gonorrhoeae* 1291 revealed that the same NMB1557 (called NGO1215 in strain 1291; 98% identity) chaperone is also necessary for AniA function in *N. gonorrhoeae*. In view of these results NMB1557 and NGO1215 were named AniA Cu chaperone A (AccA).

AccA has a direct interaction with AniA

It is clear that AniA requires AccA for NirK activity and that exogenous Cu can complement the absence of AccA in AniA function. To determine whether AccA can deliver Cu ions through a direct interaction with AniA, SPR was used. Recombinant AccA and AniA were expressed and purified. AniA protein was immobilized onto a series S sensor chip CM5. The binding affinity (K_d) of AccA (without Cu) to AniA from triplicate experiments was $3.00 \pm 0.61 \mu\text{M}$ (Table 2). The sensogram in Fig. 3 shows that AccA without Cu (as shown in the blue curve) associated and dissociated slowly from AniA. The off rate of this interaction (the dissociation rate in inverse time, k_{off}) was $9.49 \times 10^{-10} \pm 4.1 \times 10^{-10}$ (1/s). The proposed function of AccA is to deliver Cu to AniA. The red curve in the sensogram in Fig. 3 shows that in the presence of Cu, the response unit dropped significantly after AccA and AniA association. With Cu, the K_d of AccA to AniA was $2.13 \pm 1.2 \mu\text{M}$, but it had a much faster on and off rate [$k_{\text{on}} = 0.0005$ (1/ms) and $k_{\text{off}} = 0.004$ (1/s); Table 2]. This result suggests that after AccA loading Cu to AniA, it rapidly dissociates from AniA. Other metals, Zn, Co, Fe, and Mn, were also tested, and none of them had effects similar to Cu in binding AccA to AniA (Table 2).

The role of AccA in SodC and cytochrome *cbb*₃ oxidase function

Apart from AniA, both pathogenic *Neisseria* species possess a cytochrome *cbb*₃ oxidase that contains a Cu_B center, but not a Cu_A center (25), whereas *N. meningitidis* possesses SodC, a periplasmic SOD protein that contains a type II Cu center (26). To examine whether AccA is also associated with the protein function of SodC, an SOD assay was performed (27). Strains *N. meningitidis* $\phi 3$, $\phi 3\text{accA}::kan$, and $\phi 3\text{SodC}::kan$ were examined, and the results showed that SodC activity remained in $\phi 3\text{accA}::kan$ (Fig. 4A). On the other hand, compared to the wild-type strain, the 1291 *accA::kan* mutant displayed a small and reproducible decrease in the activity of cytochrome *cbb*₃ oxidase (Fig. 4B). The activity of this oxidase in the complemented

strain returned to wild-type levels. This result indicates that AccA is not essential, but it does have an effect on the assembly of the Cu_B center in this oxidase.

Homology modeling predicts that AccA contains a Cu(I) center

The amino acid sequence of AccA was used as a template for homology modeling with Swiss-Model (<http://swissmodel.expasy.org/>; Swiss Institute of Bioinformatics, Basel, Switzerland) (28) to conduct a library search of experimental protein structures to identify suitable templates. Two structurally homologous proteins of AccA with known structures were found: TTHA1943 and DR1885. TTHA1943 from *Thermus thermophilus*, donates Cu(I) ions to subunit II of the cytochrome *ba*₃ oxidase (Cox2) to generate a functional Cu_A center (29). DR1885 from *Deinococcus radiodurans*, is predicted to have a role in delivering Cu (I) to the Cu_A center of cytochrome *c* oxidase (30). The modeled structure, as shown in Fig. 5, revealed that AccA should contain a Cu(I) binding site through the conserved methionine and histidine motif HX¹⁰MX²²HXM.

AccA is a Cu(I)- and Cu(II)-binding protein

The AccA protein was purified in its *apo*-form (see Materials and Methods), as determined by metal analyses using excess 4-(2-pyridylazo)resorcinol (PAR) as a colorimetric ligand for Cu(II), Co(II), Mn(II), Ni(II), and Zn(II) (λ_{max} , 450 – 550 nm), in denaturing conditions (8 M urea). Incubation of *apo*-AccA with 4 M equivalents of CuCl₂ in the presence of 100 equivalents of sodium ascorbate, followed by elution on a PD-10 desalting column, led to the isolation of a metallated form. Cu analyses using excess bathocuproine disulfonate (BCS) as a colorimetric ligand for Cu(I) (λ_{max} , 483 nm; ϵ_{483} , 13,000 M⁻¹ · cm⁻¹) (31) in denaturing conditions detected 1.00 ± 0.06 equivalent of bound Cu(I) (Table 3). Addition of ascorbate to reduce any bound Cu(II) led to an increase in the Cu stoichiometry [Cu(I) + Cu(II)] to 2.0 ± 0.1 . These results indicate that AccA possesses 2 binding sites, 1 for Cu(I) and 1 for Cu(II). *apo*-AccA was also metallated by Zn(II) (~1 equivalent), Ni(II) (~1 equivalent), Co(II) (~0.5 equivalent), or Mn(II) (~0.5 equivalent), as determined by metal analyses with PAR, against concentration standards (Table 4). However, these metal ions were unable to compete with

TABLE 2. Kinetics and affinity analysis of AccA for binding to AniA

Binding to AniA ± metal	K_d (M) ^a	k_{off} (1/s) ^b
AccA	$3.00 \times 10^{-06} \pm 6.1 \times 10^{-07}$	$9.49 \times 10^{-06} \pm 4.1 \times 10^{-10}$
AccA + Cu	$2.13 \times 10^{-06} \pm 1.2 \times 10^{-06}$	$4.01 \times 10^{-03} \pm 1.4 \times 10^{-05}$
AccA + Co	$9.87 \times 10^{-06} \pm 1.3 \times 10^{-06}$	$1.21 \times 10^{-06} \pm 6.2 \times 10^{-07}$
AccA + Fe	$6.27 \times 10^{-05} \pm 2.2 \times 10^{-05}$	ND
AccA + Zn	$1.86 \times 10^{-04} \pm 2.4 \times 10^{-05}$	ND
AccA + Mn	No binding	—

^aAffinity of the interaction. ^bDisassociation rate in inverse time. ND, not determined [Biacore software (GE Healthcare) could not uniquely determine the off rate, because it was outside the range of detection (less than 1×10^{-10})].

Figure 3. The binding affinity of AccA to AniA protein was measured by SPR. Blue curve: binding of apo-AccA to apo-AniA in PBS. Without Cu, AccA bound to AniA and did not dissociate after binding. Red curve: binding of apo-AccA to apo-AniA in PBS with 25 mM CuSO₄. In the presence of Cu, AccA bound to AniA and dissociated after binding.

and replace bound Cu(I) or Cu(II) in AccA, suggesting that AccA is a Cu-specific binding protein.

Phylogenetic distribution of AccA across the bacterial domain

At the phylum level, homologs for AccA were found across the Proteobacteria, and in Actinobacteria, Aquificae, Chlorobi, Chloroflexi, Deinococcus-Thermus, and Gemmatimonadetes. There were also patterns of representation at the family, class, and order levels within the Aquificae, Epsilonproteobacteria, Chloroflexi, and Gammaproteobacteria (**Fig. 6A**; **Table 5**), with Aquificaceae, Campylobacteraceae, non-Dehalococcoides (in Chloroflexi), and non-Enterobacteriaceae (in Gammaproteobacteria) containing all of the positive species in those phyla. In summary, some pathogenic bacteria such as *Pseudomonas aeruginosa*, *Burkholderia pseudomallei*, *Bordetella parapertussis*, and *Bordetella pertussis* contain AccA homologs; however, most strains containing AccA homologs are

environmental bacteria, especially the diazotrophic bacteria, such as *Azospirillum lipoferum*, *Rhizobium*, and *Azotobacter vinelandii*.

AccA has a structural homology with TTHA1943 in *T. thermophilus*; however, it differs from this cytochrome *c* oxidase Cu chaperone, in that TTHA1943 does not have a C-terminal histidine rich Cu(II) binding site. A multiple alignment of AccA homologs is shown in Fig. 6B. All these homologs from different bacteria contain an N-terminal signal peptide (sequence underlined, predicted using Signal P), a conserved Cu(I)-binding site (red box) and a C-terminal Cu(II)-binding site (blue box). This alignment suggests that, apart from *Neisseria*, AccA is a common Cu chaperone found in a diverse range of bacteria. Furthermore, these predicted Cu chaperone homologs may have the same role as AccA.

DISCUSSION

Cu is a key redox center in enzymes involved in aerobic respiration, as well as a variety of reactions involving molecular oxygen (32). However, it is also a highly toxic metal ion that can exert its toxicity, either *via* promotion of oxidative stress or insertion into sites that normally would be occupied by other transition metal ions (33, 34). These biochemical properties have driven the evolution of complex pathways of Cu handling in both eukaryotic and prokaryotic cells, to avoid the toxic effects of this metal ion (35). These functions include the regulation of Cu movement across membranes *via* P-type ATPases and the control of Cu transfer between proteins *via* the use of Cu-binding proteins (chaperones) (35, 36). In prokaryotes, the focus of research has been the biogenesis of the Cu centers associated with respiratory enzymes of the heme-Cu oxidase superfamily (37) or on the Cu tolerance system (Cue) that protects the cell against excess Cu (38). In addition to its ability to catalyze reactions involving molecular oxygen, Cu is involved in reactions with nitrogen oxides, and 2 of the enzymes involved in denitrification are Cu enzymes: nitrous oxide reductase and NirK, the subject of the current study.

In this work, we identified a gene encoding a Cu chaperone, AccA, that is necessary for the biogenesis of functional NirK. From homology modeling, it appears that AccA has a Cu(I) center that is very similar to that described

Figure 4. A) Detection of Cu/Zn Sod activity showed that SodC activity was not affected by the loss of AccA. B) A cytochrome *cbb*₃ oxidase activity assay showed that AccA was not essential, but that it had an effect on the assembly of the Cu_B center in this oxidase.

Figure 5. The structure of AccA, modeled by Swiss-Model. A) Secondary structure alignment with a known Cu(I) chaperone, TTHA1943, from *T. thermophilus*. Purple boxes: binding motif of Cu(I). B) Modeled AccA 3D structure (gray) aligns to the TTHA1943 Cu(I) chaperone. Yellow: Cu(I); purple: binding motif.

in TTHA1943 from *T. thermophilus* (30) and DR1885 from *D. radiodurans* (30), in that the Cu(I)-coordinating amino acids found in DR1885 (2 Met, 2 His) are conserved in AccA. Banci *et al.* (30) have demonstrated biochemically that DR1885 from *D. radiodurans* donates Cu(I) to a water-soluble apo-Cu_A fragment of the CoxII protein of the cytochrome *ba*₃ oxidase from the same bacterium leading to the assembly of a Cu_A center. For this reason, it was named the periplasmic Cu_A chaperone (PCu_AC). However, our data show for the first time that a structural analog protein of PCu_AC, AccA, has an essential role in the assembly of an active nitrite reductase in pathogenic *Neisseria*, suggesting that there may be more diverse functions for this family of Cu chaperones in the periplasm of Gram-negative bacteria. In the case of *N. gonorrhoeae*, we observed that mutation of *accA* led to a small but significant effect on cytochrome oxidase activity. Because *N. gonorrhoeae* contains only cytochrome *cbb*₃, it can be concluded that AccA may also enhance the efficiency of the biogenesis of the heme-Cu center, but it is not essential. Pathogenic *Neisseria*

lack cytochrome *aa*₃ and do not express a functional nitrous oxide, and so it was not possible to determine whether AccA is involved in the assembly of the Cu_A center in this bacterium. Given the established role of PCu_AC in Cu_A assembly in cytochrome oxidases that possess this redox center, it would be interesting to test its role in commensal *Neisseria* species that possess a functional nitrous oxide reductase.

AccA did not appear to be essential for assembly of SodC in *N. meningitidis*, although our assay would not allow us to determine whether there was a small effect on activity. The *aniA*[−] phenotype could be suppressed by addition of excess Cu, and similar observations have been made with regard to the biochemical suppression of mutations that affect the transfer of Cu to cytochrome oxidase in yeast (39). This finding suggests that a Cu chaperone is not obligatory for the biogenesis of Cu centers, but it is necessary in some cases at physiologically relevant Cu concentrations. The observation that mutation of PCu_AC in *Bradyrhizobium* led to restricted

TABLE 3. Cu analyses of AccA

No.	Abs ₂₈₀	AccA (μM)	Abs ₄₈₃		Cu ^I (μM)	Cu ^I +Cu ^{II} (μM)	Cu ^I /AccA ^a	(Cu ^I +Cu ^{II})/(AccA) ^b
			−asc	+asc				
1	0.147	17.1	0.222	0.451	17.1	34.7	1.0	2.0
2	0.203	23.6	0.271	0.603	20.8	46.4	0.9	2.0
3	0.153	17.8	0.235	0.472	18.1	36.3	1.0	2.0
4	0.109	15.6	0.194	0.416	14.9	32.0	1.0	2.0
5	0.135	19.3	0.241	0.529	18.5	40.7	1.0	2.1
6	0.095	13.6	0.178	0.394	13.7	30.3	1.0	2.2
7	0.116	13.4	0.181	0.321	13.9	24.7	1.0	1.8
8	0.131	15.2	0.203	0.397	15.6	30.5	1.0	2.0
9	0.110	12.7	0.177	0.334	13.6	25.7	1.1	2.0

Apo-AccA was incubated with CuCl₂ (4 M equivalent) and sodium ascorbate (100 M equivalent) and desalted. Metal analyses were performed with BCS. asc, sodium ascorbate. ^aAverage Cu^I/AccA = 1.00 ± 0.06.

^bAverage Cu^I + Cu^{II}/AccA = 2.0 ± 0.1.

M ^{IIa}	No.	Abs ₂₈₀ ^b	AccA (μM ^c)	Abs ₅₀₀	M ^{II} (μM ^d)	M ^{II} /AccA	
						Each	Average
Zn	1	0.423	6.1	0.589	5.3	0.88	0.87 ± 0.04
	2	0.529	7.6	0.699	6.3	0.83	
	3	0.406	5.8	0.586	5.3	0.91	
Co	1	0.425	6.1	0.181	2.2	0.36	0.39 ± 0.03
	2	0.530	7.6	0.257	3.1	0.41	
	3	0.400	5.7	0.189	2.3	0.40	
Ni	1	0.415	5.9	0.578	5.9	0.99	0.97 ± 0.02
	2	0.525	7.5	0.702	7.2	0.95	
	3	0.409	5.9	0.551	5.6	0.96	
Mn	1	0.416	6.0	0.067	3.3	0.55	0.49 ± 0.08
	2	0.531	7.6	0.062	3.1	0.40	
	3	0.411	5.9	0.063	3.1	0.53	

Production of reactive nitrogen species is one of the host defenses against pathogens. In *N. gonorrhoeae*, anaerobic metabolism of nitrite and NO is particularly important in colonization of the mucosal surface and may have led to the location of AniA at the bacterial surface. The unique location of AniA provides a challenge for the efficient distribution of Cu to this enzyme, and for this reason, it appears that there is a high dependence on AccA for AniA biogenesis. SPR revealed a direct and high-affinity protein-protein interaction between AccA and AniA *in*

TABLE 5. Patterns of *AccA* homolog present in phyla containing at least 1 positive sequenced species

Phylum	Frequency of <i>accA</i>	Geometric mean		Positive taxa	Positive taxa <i>accA</i> frequency
			<i>E</i> value		
Actinobacteria	41/115		6E-8	Various	
Alphaproteobacteria	94/109		1E-12	Various	
Aquificae	3/6		3E-12	Aquificaceae	3/3
Betaproteobacteria	50/65		2E-20	Various	
Chlorobi	1/11 ^a		2E-10	<i>Ignavibacterium album</i>	
Chloroflexi	9/11		5E-12	Non-Dehalococcoides	9/9
Deinococcus-Thermus	7/13		2E-7	Various	
Deltaproteobacteria	1/36 ^a		3E3 ^a	<i>Stigmatella aurantiaca</i>	
Epsilonproteobacteria	5/21		2E-11	Campylobacteraceae	5/6
Gammaproteobacteria	68/153		3E-11	Non-Enterobacteriaceae	68/108
Gemmatimonadetes	1/1 ^a		1E-6	<i>Gemmatimonas aurantiaca</i>	

Patterns at the family, class, and order level were noted in Aquificae, Chloroflexi, Epsilonproteobacteria, and Gammaproteobacteria. ^aWeak support for presence, such as when a single positive representative was found (Chlorobi, Deltaproteobacteria, and Gemmatimonadetes) and when the tblastn *E* value was weak (Deltaproteobacteria).

vitro that is consistent with the view that, in pathogenesis, *Neisseria* has evolved as a dedicated biogenetic pathway for NirK. Apart from *Neisseria*, the *AccA* homolog was observed in some pathogenic bacteria but mainly in environmental bacteria that catalyze denitrification. [F]

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