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Evidence for Distinctive Mechanisms of *S*-Nitrosoglutathione Metabolism by AdhC in Two Closely Related Species, *Neisseria gonorrhoeae* and *Neisseria meningitidis*[▽]

Adam J. Potter, Stephen P. Kidd, Michael P. Jennings, and Alastair G. McEwan*

Australian Bacterial Pathogenesis Program, Centre for Metals in Biology, School of Molecular and Microbial Sciences, University of Queensland, Brisbane, QLD 4072, Australia

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The *adhC* gene from 11 strains of *Neisseria gonorrhoeae* was distinguished from its homologue in *Neisseria meningitidis* by the presence of a premature stop codon caused by a single base insertion. Mutational analysis showed that NADH *S*-nitrosoglutathione oxidoreductase activity was associated with *adhC* in *Neisseria meningitidis* but not in *Neisseria gonorrhoeae*.

Neisseria gonorrhoeae and *Neisseria meningitidis* are closely related obligate human pathogens (3) with conservation of the majority of genes (1, 7). While both organisms are associated with mucosal tissues, meningococcus is associated primarily with the nasopharynx, where it is carried asymptomatically. In rare cases, *N. meningitidis* causes invasive disease resulting in meningitis and septicemia. Gonococcus usually inhabits the urogenital tract, but it can also infect the throat or rectum. Gonococcus is typically linked to inflammation and purulent discharge but may be carried asymptomatically. Unlike *N. meningitidis*, gonococcus is not usually associated with septicemia. The differences in the interaction with the human host that the two bacteria exhibit in both colonization and disease states lead to the expectation that the bacteria should also exhibit genetic and biochemical differences. An example of differences between the two bacterial species is in their defenses against oxidative stress (11).

Recently, we described a regulon in *N. gonorrhoeae* which is controlled by NmlR, a transcription factor of the MerR family (6). NmlR controls the expression of *adhC*, which encodes a class III alcohol dehydrogenase, an enzyme which is conserved from bacteria to mammals and is known to protect cells against nitrosative stress by catalyzing the NADH-dependent reduction of *S*-nitrosoglutathione (GSNO) (2, 5, 9). It was postulated the AdhC might be part of a defense system that protected *N. gonorrhoeae* from killing by nitric oxide (6). AdhC in bacteria is usually encoded by a single gene. However, in the *N. gonorrhoeae* genome (strain FA1090), this gene is annotated as two open reading frames, *adhC1* and *adhC2*. In view of the presence of phenotypically silent genes (pseudogenes) in bacteria that arise from mutational events, we investigated the gonococcal *adhC* locus in more detail and compared it to the *adhC* gene in *N. meningitidis*. The results suggest that there are critical differences between meningococcus and gonococcus in the way that they metabolize glutathione and *S*-nitrosoglutathione.

The *adhC* gene from all gonococcal strains contains a premature stop codon. The genetic organization of the *nmlR*-*adhC* loci in *N. gonorrhoeae* FA1090 and *N. meningitidis* MC58 is shown in Fig. 1. The meningococcal *adhC* gene is a single locus of 1,137 nucleotides. However, the gonococcal *adhC* gene is interrupted by a stop codon arising from a single base pair insertion at nucleotide 764 (Fig. 1). The single base insertion causes a frameshift mutation, with the complete *adhC* coding sequence continuing in an alternate reading frame terminating at the same position as the meningococcal *adhC* coding sequence (Fig. 1). This nucleotide sequence is unlikely to be expressed or to encode a functional polypeptide. Thus, it appears that the *adhC* locus in gonococcus is likely to produce a single truncated gene product. To determine whether this altered sequence is conserved across different *N. gonorrhoeae* strains, the *adhC* gene was sequenced in wild-type strain 1291 as well as 10 clinical isolates representing a broad spectrum of infection sites, geographical locations, and isolation dates (P. M. Power et al., submitted for publication). Sequence data identified the presence of the single base pair insertion in each strain, consistent with *N. gonorrhoeae* strain FA1090.

Mutation of the *adhC* gene in *N. gonorrhoeae* is not correlated with the loss of NADH GSNO oxidoreductase activity. To determine whether the *N. gonorrhoeae adhC* gene encoded an active enzyme, we mutated the gene in *N. gonorrhoeae* 1291 and *N. meningitidis* MC58 ¢3 (an acapsulate version of MC58) by marker exchange mutagenesis. The *N. gonorrhoeae* 1291 *adhC* gene was amplified with primers adhC-KO-F1 (5′-CAAGGAAAGGCGTTCTTCAATGGT-3′) and adhC-KO-R1 (5′-TTTGCCGTTGGTAGGAAAATGCTC-3′), cloned into pGEM-T Easy (Promega), and interrupted by the insertion of the kanamycin resistance cassette from pUC4kan into the unique AvaI site (Fig. 1). The resulting plasmid, pGEM-T:*adhC*:kan, was linearized with XmnI and used to transform *N. gonorrhoeae* 1291 and *N. meningitidis* MC58¢3. Correct insertion into the chromosome was verified by PCR using primers external to the construct used for mutagenesis: adhC-F-check (5′-AGCAAGCAACGGATTAGAGC-3′) and adhC-R-check (5′-GAGGCTTGGCGATAAAATAGG-3′). GSNO reduc-

* Corresponding author. Mailing address: Australian Bacterial Pathogenesis Program, Centre for Metals in Biology, School of Molecular and Microbial Sciences, University of Queensland, Brisbane, QLD 4072, Australia. Phone: 61 7 3365 4878. Fax: 61 7 3365 4520. E-mail: mcewan@uq.edu.au.

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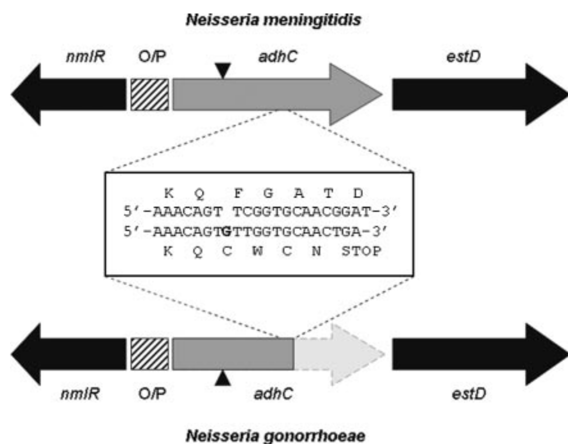

FIG. 1. Comparison of the *adhC* genes of *Neisseria meningitidis* and *Neisseria gonorrhoeae*. The inset shows a nucleotide and amino acid sequence alignment which contains the frameshift mutation (G insertion) in *N. gonorrhoeae* that leads to a premature stop codon. The light-gray region after the frameshift indicates the remainder of the full-length *adhC* open reading frame continuing in an alternate reading frame. ▼ and ▲ indicate the insertion of the kanamycin resistance cassette into the unique AvaI restriction site for *adhC* mutant strains. O/P, operator/promoter.

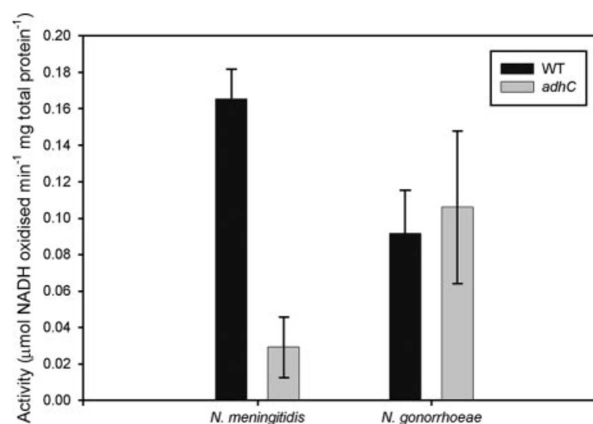

FIG. 2. GSNO reductase activity of *N. meningitidis* and *N. gonorrhoeae* wild-type and *adhC* strains. Error bars indicate ±1 standard deviation from the mean. Experiments were conducted using triplicate cultures and repeated at least three times. The data shown are representative results. There is a statistically significant difference in activity between *N. meningitidis* wild-type (WT) and *adhC* mutant strains ($P = 0.001$); there is no statistically significant difference between *N. gonorrhoeae* wild-type and *adhC* mutant strains ($P = 0.633$), as determined using Student's *t* test.

tase assays were then performed on wild-type and *adhC* mutant cell lysates. Strains were grown overnight on brain heart infusion agar (Acumedia) with 10% Levinthal's base at 37°C in 5% CO_2. Medium for *N. gonorrhoeae* was also supplemented with IsoVitaleX (Becton Dickinson). Cells were resuspended in 1 ml phosphate-buffered saline and lysed by subjecting the suspension to five freeze-thaw cycles. Cell debris was removed by centrifugation for 15 min at 18,000 × *g* and the solutions sterilized by passing them through a 0.22-μm filter (Millipore). The total protein concentration of the supernatant was determined spectrophotometrically using the following equation: protein (mg/ml) = $1.55 \times A_{280} - 0.76 \times A_{260}$ (8). The GSNO reductase activity of cell lysates was then determined using a method similar to that described by Liu et al. (9). NADH (0.2 mM; Roche), 1 mM GSNO (prepared according to the method described by Sahoo et al. [10]), and 200 μg total protein were combined in a 1-ml reaction mixture, and the decrease in absorbance at 340 nm was measured. GSNO reductase activity was expressed as μmol NADH oxidized per minute per mg total protein. The *N. meningitidis adhC* mutant was found to exhibit much lower GSNO reductase activity than the wild type (Fig. 2). There was a background of NADH GSNO oxidoreductase activity in *N. gonorrhoeae*, but the *adhC* mutant exhibited activity similar to that of its wild-type counterpart (Fig. 2). This indicates that the GSNO reductase activity in *N. gonorrhoeae* is not associated with the *adhC* gene and must arise from a distinct and thus-far-unidentified enzyme(s).

Expression of the neisserial *adhC* gene in *Escherichia coli*. To further investigate the properties of *N. gonorrhoeae* and *N. meningitidis* AdhC, the *adhC* gene of each was cloned into the expression vector pPROEX (Life Technologies) under the control of an IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter. *adhC* was PCR amplified using primers adhC-F1 (5′-AACCATGGAAATGAAACAAACCG-3′) and adhC-R1 (5′-GGGTCGACCTTAGTAATGAATAA-3′) and cloned into pPROEX by using NcoI and SalI. The resulting plasmids, pPROEX:Ng-*adhC* and pPROEX:Nm-*adhC*, were transformed into competent *E. coli* BL21 (DE3) cells. *E. coli* was grown at 37°C in LB containing 100 μg ml^{-1} ampicillin. Gene expression was induced by adding 0.5 mM IPTG to 50 ml of exponentially growing cells and shaking (180 rpm) at 22°C for 5 h. Noninduced samples were treated as described above but without IPTG addition. Cells were harvested by centrifugation at 4,470 × *g* (Universal 16R) for 10 min and resuspended in 10 ml phosphate-buffered saline before being broken by sonication. Clarified lysates were prepared, and GSNO reductase activity in *E. coli* was determined as previously described. Induction of *N. meningitidis adhC* expression was

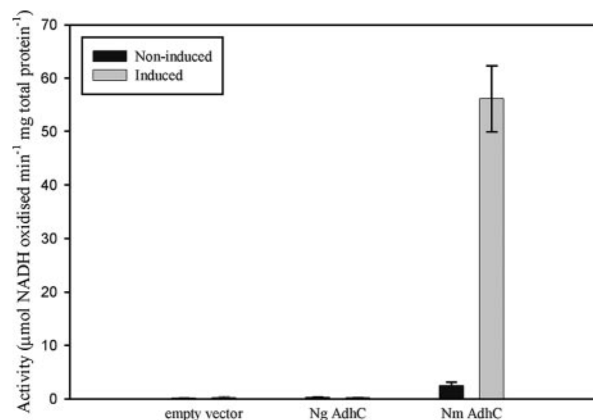

FIG. 3. GSNO reductase activity of *E. coli* BL21 (DE3) cells carrying the pPROEX overexpression vector with either *N. meningitidis* (Nm) or *N. gonorrhoeae* (Ng) *adhC* cloned downstream of its IPTG-inducible promoter. Cells carrying empty vector were used as a negative control. Error bars indicate ±1 standard deviation from the mean. Experiments were conducted using triplicate cultures and repeated at least three times. The data shown are representative results.

found to result in >20-fold increased GSNO reductase activity compared with the noninduced sample (Fig. 3). In contrast, induction of *N. gonorrhoeae adhC* had no effect on activity (Fig. 3) and further analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blotting revealed that most of the *N. gonorrhoeae* AdhC protein expressed in *E. coli* formed insoluble inclusion bodies (data not shown).

The results presented herein indicate that *N. gonorrhoeae adhC* may be a pseudogene. It is already established that in *N. gonorrhoeae*, the *ggt* gene, encoding a γ-glutamyl transpeptidase, is also a pseudogene, although the meningococcal homologue encodes an active enzyme (12). Like the gonococcal *ggt* gene, the *adhC* gene is transcriptionally active (6) but phenotypically silent. The present results may relate to differences in the way that the two species handle nitric oxide. We note that γ-glutamyl transpeptidase is able to accelerate the decomposition of GSNO by hydrolyzing the γ-glutamyl moiety (4). Similarly, an active AdhC protein in this bacterium would accelerate the removal of GSNO. We speculate that selective pressure for the loss of a functional *ggt* and *adhC* in *N. gonorrhoeae* is associated with a distinctive mechanism of handling nitric oxide that is suited to an interaction with the human host which allows the bacterium to respond to the acute inflammatory response. The setting in which AdhC has an important role for survival of *N. meningitidis* has not yet been identified.

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