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The NarE protein of *Neisseria gonorrhoeae* catalyzes ADP-ribosylation of several ADP-ribose acceptors despite an N-terminus deletion

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Abstract

The ADP-ribosylating enzymes are encoded in many pathogenic bacteria in order to affect essential functions of the host. In this study, we show that *Neisseria gonorrhoeae* possess a locus that corresponds to the ADP-ribosyltransferase NarE, a previously characterized enzyme in *N. meningitidis*. The 291 bp coding sequence of gonococcal *narE* shares 100% identity to part of the coding sequence of meningococcal *narE* gene due to a frameshift previously described, thus leading to a 49-amino acid deletion at the N-terminus of gonococcal NarE protein. However, we found a promoter region and a GTG start codon, which allowed expression of the protein as demonstrated by RT-PCR and Western Blot analyses. Using a gonococcal NarE-6xHis fusion protein, we demonstrated that the gonococcal enzyme underwent auto-ADP-ribosylation but to a lower extent than meningococcal NarE. We also observed that gonoccocal NarE exhibited ADP-ribosyltransferase activity using agmatine and cell-free host proteins as ADP-ribose acceptors, but its activity was inhibited by human β-defensins. Taken together, our results showed that NarE of *Neisseria gonorrhoeae* is a functional enzyme that possesses key features of bacterial ADP-ribosylating enzymes.

Abbreviations: ADPRT, ADP-ribosyltransferase; ADPr, ADP-ribosylation; ART-1, human ADP-ribosyltransferase-1; ART-5, human ADP-ribosyltransferase-5; Cam, chloramphenicol; CT, cholera toxin; FTECs, Fallopian tube epithelial cells; gNarE, gonococcal NarE protein; HBD, human antimicrobial peptides β-defensins; HNP-1, human neutrophil peptide-1; Kan, kanamycin; LT, heat-labile enterotoxin; mNarE, meningococcal

NarE protein; NLPR3, nucleotide-binding domain and leucine-rich-repeat-containing (NLR) family of pattern-recognition molecules 3.

Introduction

Pathogenic bacteria possess a subset of cytotoxic products and effectors that target and infect their host cells, including ADP-ribosyltransferases (ADPRTs). These proteins represent a large family of potentially toxic enzymes able to modify or disrupt essential functions of eukaryotic cells (Simon *et al.*, 2014). ADPRTs catalyze the transfer of a single ADP-ribose from β-nicotinamide adenine dinucleotide (NAD^+) onto specific amino acid residues of host cell proteins, releasing nicotinamide (Holbourn *et al.*, 2006; Lemichez & Barbieri, 2013). In *Neisseria meningitidis*, a major causative agent of bacterial meningitis and sepsis in humans, an ADPRT named meningococcal NarE (mNarE) was described based on *in silico* analysis (Masignani *et al.*, 2003, 2004). mNarE (16 kDa) shares structural features with toxins such as cholera toxin (CT) of *Vibrio cholerae* and heat-labile enterotoxin (LT) of enterotoxigenic *Escherichia coli* (ETEC), and hydrolyzes NAD^+ to transfer ADP-ribose to small guanidine compounds like agmatine and arginine analogues (Masignani *et al.*, 2003). In addition, mNarE undergoes auto-ADP ribosylation to regulate enzymatic activity (Picchianti *et al.*, 2013) and binds iron through an iron/zinc-sulfur center (Fe/Zn-S), which is likely involved in regulation of catalytic activity (Del Vecchio *et al.*, 2009; Koehler *et al.*, 2011). The *narE* locus is present only in a subset of hypervirulent lineages of meningococcus (Pizza *et al.*, 2000; Masignani *et al.*, 2003) and the role of the enzyme in pathogenesis is not fully elucidated.

In the case of *Neisseria gonorrhoeae*, the etiological agent of the sexually-transmitted infection gonorrhea, the *narE* locus is also present but the gene has been described as a pseudogene because of duplication of a tetranucleotide (TTAT) occurring 12 bases downstream from the original meningococcal ATG site, which might cause premature interruption of the gene after eight codons (Masignani *et al.*, 2003). In order to analyze the impact of this frameshift on enzymatic activity of NarE, in the current study we characterized the gonococcal *narE* locus and its protein product (gNarE). We show that *narE* of *N. gonorrhoeae* is indeed expressed and the protein conserves the ADPRT activity. However, its auto-ADP-ribosylation is at lower level than the meningococcal protein (mNarE) and the transferase activity is inhibited by human β-defensins. Finally, we demonstrate that gNarE is also able to modify host cell protein targets, where β-actin appears as one of the ADP-ribose acceptors.

Materials and Methods

Bacteria, growth media and culture conditions

The strains and plasmids used are listed in Table S1. *Neisseria gonorrhoeae* strain P9 was originally isolated in the UK and variants selected and confirmed by colony morphology with stereo-microscopy (Lambden *et al.*, 1980). *N. gonorrhoeae* isolates were cultured from frozen stocks onto GC agar plates supplemented with IsoVitaleX (Becton Dickinson) at 37ºC and 5% (v/v) CO_2 for 18-24 h. Liquid cultures of *N. gonorrhoeae* were grown in gonococcal broth (GCB) or GCB supplemented with kanamycin (40 mg l^{-1}). *E. coli* strains were grown in Luria-Bertani (LB) broth at 37ºC with shaking. Solid media were prepared

by addition of 1.5 g (w/v) agar. When required, media were supplemented with chloramphenicol (34 mg l^{-1}) and kanamycin (50 mg l^{-1}).

Culture of primary human Fallopian Tube Epithelial Cells (FTECs)

Human Fallopian tube samples were obtained after informed consent from fertile donors undergoing hysterectomy for reasons unrelated to this study at Servicio de Ginecología y Obstetricia, Clínica Dávila (Santiago, Chile). The Ethics Committee of the Universidad Andres Bello and Clínica Dávila approved all protocols. Procedures for sample processing and cell culture were reported previously (Rodríguez-Tirado *et al.*, 2012).

Cloning and sequencing of *narE* locus

The *narE* locus was amplified by PCR using genomic DNA of *N. gonorrhoeae* P9 variants and *N. meningitidis* MC58 (Table S1 and Figure 1A). PCR products (~ 800 bp) were purified (Nucleotide Removal Kit, Qiagen), cloned into pGEM-T easy vector (Promega) (Tables S1 and S2) and sequenced by Macrogen Corp. (Rockville, MD, USA).

Bioinformatic analysis

Sequences of gonococcal P9 variants were compared with *narE* of *N. gonorrhoeae* strain FA1090 (NGO0563, Genbank ID: 3282906). Detection of the predicted gene and analysis of *narE* locus was carried out using GeneMarkS (Besemer *et al.*, 2001). PSI-BLAST was used to analyze the gNarE predicted amino acid sequence (YP_207708.1). Multiple sequence alignment of the gonococcal strains FA19, FA1090, FA6140, MS11, NCPP11945 and 35/02 were constructed using Clustal Omega Multiple Sequence Alignment. Promoter

analysis was carried out using BPROM program and Vector NT Suite Advance v.10 (Invitrogen).

RT-PCR of gonococcal *narE* gene

Total RNA was extracted from *N. gonorrhoeae* P9-17 (Pil^+, Opa^+) grown in GCB (3,0 x 10^9 bacteria mL^{-1}) using Trizol Reagent (Ambion, Life Technologies) (Whitehead *et al.*, 2007). Primer sequences are described in Table S2. Reverse transcription was performed with 5 μg of DNAse I-treated RNA and Superscript II RT (Invitrogen). A PCR program of 35 cycles (94ºC for 30 s, 59ºC for 45 s, and 72ºC for 90 s) followed by a 3 min extension at 72ºC was carried out. DNAse-treated RNA without reverse transcriptase was used as negative control, while16S rRNA was included as positive control with 60ºC for annealing (Du *et al.*, 2005). Aliquots (15 μL) were resolved on 1.5% (w/v) agarose gels, stained with SYBR Safe DNA Gel Stain (Invitrogen) and visualized with a UV light source.

Generation of chromosomal NarE-3xFLAG fusion in *Neisseria gonorrhoeae*

The *narE*::3xFLAG(kan^R) fusion was generated as described previously (Whitehead *et al.*, 2007). The 3xFLAG fragment was digested with *KpnI* and *XhoI* and ligated into pEC007 (Table S1) yielding plasmid pEC0011, then was purified (Wizard, Promega) and concentrated by phenol-chloroform extraction/ethanol precipitation. *N. gonorrhoeae* P9-17 was transformed with pEC0011 and clones were confirmed by PCR and sequencing.

Western Blotting of gNarE-3xFLAG

N. gonorrhoeae P9-17 wild type and *narE*::3xFLAG (kan^R) strains were grown in 10 mL of GCB and GCB-Kan respectively at 1.0 x 10^7 CFU mL^{-1} for 4 h at 37ºC and shaking (100 rpm). Protein fractionation was done as described previously (Masignani *et al.*, 2003). Proteins were quantified (Pierce® BCA Protein Assay Kit) and 30 µg of proteins were resolved by SDS-PAGE using 16% (w/v) Tris-Tricine gels (Schägger, 2006) and Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific). gNarE-3xFLAG was detected using mouse anti-FLAG Ab M2 (Sigma) and visualized with SuperSignal™ West Pico Chemoluminescent Substrate (Thermo Scientific).

Overexpression and purification of His-tagged NarE proteins of *Neisseriae*

The *narE* gene of *N. gonorrhoeae* P9-17 and *N. meningitidis* MC58 were cloned into pET24b+ plasmid (Novagen) to yield pET plasmids (Table S1). *E. coli* BL21 (DE3) pLysS competent cells (Promega) were transformed with recombinant vectors and selected onto LB plates +kan+cam. Bacterial cultures were induced with 1 mM IPTG (Invitrogen) at OD_{600} of 0.5-0.6, grown for 4h at 25ºC with gentle shaking and harvested by centrifugation. Cells were suspended in 25 mM sodium phosphate buffer (pH 8.0) containing 35 mM NaCl, 10 mM imidazole (GE Healthcare Life Sciences), Halt™ Protease Inhibitor cocktail, EDTA-free (Thermo Scientific), 0.02 mg L^{-1} lysozyme (Thermo Scientific) and lysed at 4ºC by sonication on ice (10 s). Debris and cell membranes were pelleted by centrifugation and supernatants were loaded onto a nickel-chelate affinity column. The column was extensively washed using 25 mM sodium phosphate buffer (pH 8.0) containing 35 mM NaCl and increasing imidazole concentrations (20, 40 and 80 mM) and was eluted with 150 mM imidazole. Protein fractions were analyzed in 4-12% Bis-Tris Novex gels (Invitrogen). Samples were stored at 4ºC until used for enzyme assays (Del Vecchio *et al.*, 2009).

ADP-ribosylation of agmatine by gNarE

A solid-phase assay was carried out with some modifications (Bachran *et al.*, 2007; Picchianti *et al.*, 2013). A U16 Maxisorp Nunc Immuno Module (Thermo Scientific) was coated with goat anti-rabbit IgG H+L (Thermo Scientific) and incubated overnight at 25ºC. Wells were washed with PBS-0.05% (v/v) Tween 20 (PBST) and were blocked with 5% (w/v) bovine serum albumin (BSA, Millipore) for 2h, then 100 µL of rabbit anti-agmatine antibody (1/1,000) (Millipore) were added to the wells and incubated for 2 h at 25ºC. Purified gNarE-6xHis (2.5 µM) was added to 50 mM potassium phosphate buffer (pH 7.4) containing 10 mM 6-biotin-17-NAD (Trevigen) and 75 mM agmatine (Sigma) in a final volume of 200 µL. Reactions were incubated for 30 min at 37ºC, then were transferred onto the pre-coated wells and incubated for 2h at 25ºC. The wells were washed and then incubated with 100 µL streptavidin-HRP (1/100) (R&D Systems) in 3% (w/v) BSA for 1h at 25ºC. After washing, 100 µL of 3,3'5,5,-tetramethylbenzidine (TMB, Sigma) was added for color development, then reaction was stopped by adding 50 µL of 2N H_2SO_4 per well and read at λ_{450} nm. ADPRT activity of gNarE and mNarE were also evaluated in the presence of 20 mM dithiothretol (DTT). Heat-inactivated gNarE (h.i. gNarE, 30 min at 95ºC) was included as a control of enzyme activity (Picchianti *et al.*, 2013).

Auto-ADP-ribosylation of gNarE

Reactions were prepared without agmatine and incubated for 30 min at 37ºC and were stopped by adding NuPAGE LDS sample buffer (4X) (Life Technologies). Immunoblot

analysis was performed as described previously (Picchianti *et al.*, 2013). Western blotting of gNarE and mNarE were carried out using anti-His (C-term) antibody-HRP (1/5000, Invitrogen).

ADP-ribosylation of human antimicrobial peptides by gNarE

Purified gNarE-6xHis (2.5 μM) was mixed with the human antimicrobial peptides β-defensins HBD1 (2 μg, 25.5 μM), HBD2 (2 μg, 23.3 μM), HBD4 (2 μg,17 μM) (US Biological) or trappin-2/elafin (2 μg, 9.1 μM) (R&D Systems) in 50 mM potassium phosphate buffer (pH 7.4) containing 10 mM 6-biotin-17-NAD in a final volume of 20 μL . Reactions were incubated for 1h at 30°C and immunoblot analysis was performed. The experiment was also carried out in the agmatine/ADP-ribosylation assay by adding 2 μg of each peptide.

Cell-free ADP-ribosylation by gNarE

FTECs were washed with PBS and suspended in 500 μL of 20 mM Tris-HCl buffer (pH7.5) containing 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$ and 1X Halt™ Protease Inhibitor cocktail (EDTA-free). Cells were sonicated using 6 pulses of 30 s and were centrifuged (3000 g, 5 min, 4°C). Purified gNarE-6xHis (8.3 μM) was mixed with 25 μg of cell lysate in 50 mM potassium phosphate buffer (pH 7.4) containing 10 μM 6-biotin-17-NAD in a total volume of 50 μL. Reactions were incubated for 30 min at 37°C and then were resolved by SDS-PAGE using 16% tricine gels (Schägger, 2006). Biotin-ADP-ribosylated proteins were transferred to a PDVF membrane and visualized with peroxidase-

coupled streptavidin (R&D Systems) in a chemoluminiscent reaction using Pierce SuperSignal™ West Femto kit (Thermo Scientific). Reactions containing ADP-ribosylated host proteins were also used in Western blots for detection of β-actin and the Rho GTPases Cdc42 and Rac1 using appropiate antibodies (β-actin rabbit polyclonal antibody, abcam8227; Cdc42 rabbit polyclonal antibody, Thermo Scientific PA1-092X; Rac1 rabbit polyclonal antibody, Thermo Scientific PA1-091X). Quantitative densitometric analysis of bands was carried out using ImageJ software (http://imagej.nih.gov/ij/)

Statistics

Statistical analyses (GraphPad Prism v5.0a, GraphPad Software Inc) were done using pairwise comparisons across groups using t-test with pooled variance in agmatine assays and corrected for multiple testing using Bonferroni's correction. P value <0.05 was considered significant.

Results

Analysis of the gonococcal *narE* locus and gene expression

The *narE* gene was previously reported as a pseudogene in *Neisseria gonorrhoeae* (Masignani *et al.*, 2003). In the *N. gonorrhoeae* FA1090 genome, *narE* was located between genes NGO0562 and NGO0564 that encode the putative proteins dihydrolipoamide acetyltransferase and dihydrolipoamide dehydrogenase, respectively (Fig 1A). In *N. meningitidis* MC58 these genes were annotated as *aceF* (NMB1342) and *lpdA2* (NMB1344) respectively, which correspond to the flanking genes of *narE* (NMB1143) (Masignani *et al.*, 2003). In the current study, *narE* locus was identified in a collection of phenotypic variants of gonococcal strain P9 (Fig 1B). Sequencing and multiple sequence

alignment showed a 99% identity between the NGO0563 gene of *N. gonorrhoeae* FA1090 and the *narE* gene in the P9 phenotypic variants, except for P9-16, which contained an A substitution at the 3'-end (Fig S1). However, this substitution did not affect the putative amino acid sequence of gNarE (data not show), suggesting the *narE* gene and the flanked region was conserved. The genome comparator function in the pubmlst.org/*Neisseria* database was used to examine *narE* (locus NEIS2492) amongst the 2263 gonococcal isolates, which showed that no alleles have been assigned to any of these isolates and no amino acid sequences were available to allow further analyses of gNarE conservation (data not shown). However, the full coding sequence of *narE* is 100% conserved among the gonococcal strains available in GeneBank (Fig S2).

The previously reported duplication of a tetranucleotide located12 bases downstream from the meningococcal ATG initiation codon was also found, which generated a series of 13 premature stop codons (Fig. 2A). Nevertheless, -35 and -10 boxes and a Shine-Dalgarno sequence (SD) were found upstream from an alternative start codon (GTG) and a coding sequence of 291 bp, which showed 100% identity with part of the meningococcal coding sequence of *narE* (NMB1343). To assess expression of the gonococcal *narE* gene, we carried out a RT-PCR using total RNA of strain P9-17 where an expected 150 bp fragment was observed, which corresponded to *narE* mRNA (Fig. 2B). In addition, gNarE was detected by Western blot in the periplasmic fraction of *N. gonorrhoeae* (Fig 2C) using a targeted translation fusion of FLAG epitope to the coding sequence of *narE* (~15 kDa).

Enzymatic activity of gNarE

To examine the impact of the frameshift found in the gonococcal *narE* locus, a multiple alignment was done with the amino acid sequences of gNarE and mNarE. gNarE displayed a 49 amino acid deletion at the N-terminus as a consequence of the tetranucleotide duplication in the gonoccocal *narE* locus, but retained the critical domains involved in ADP-ribosylation (ADPr) such as the NAD^+ binding site (His_{57} residue and YISTT domain in mNarE) and a classical catalytic motif previously described in other bacterial ADPRTs (R-Glu-x-Glu) (Holbourn *et al.*, 2006) (Fig. 3A). However, the Cys_2His_2 cluster previously described in the mNarE protein might not be properly assembled because of the absence of His_{46} in gNarE.

To confirm that gNarE is a functional enzyme, the protein was expressed in *E. coli* as a ~ 12 kDa soluble, His-tag fusion protein (gNarE-6xHis) (Fig. 3B). The purified recombinant protein was used to test auto-ADPr and ADPr of agmatine, an arginine peptide and an ADP-ribose acceptor that has been shown previously to be catalytically modified by mNarE (Masignani *et al.*, 2003). A specific band representing biotin-ADP-ribosylated gNarE was observed (Fig. 3C), albeit at a lower band intensity compared with mNarE. Regardless, gNarE transferred ADP-ribose to agmatine in a similar manner as mNarE, since no significant differences in enzyme activity were found (Fig. 3D). In addition, ADPRT activity of gNarE was DTT-independent, suggesting that this enzyme does not require enzymatic activation by reduction of a disulfide bridge as previously reported for other bacterial ADPRTs toxins (Mekalanos *et al.*, 1979; Kannan & Baseman, 2006). Moreover, a subset of human antimicrobial peptides previously reported in the human female genital mucosa (Wira *et al.*, 2011) were not ADP-ribosylated by gNarE, whereas β-defensins

completely inhibited auto-ADPr, suggesting they may act as gNarE inhibitors (Fig. 4A). In contrast, the neutrophil elastase inhibitor trappin-2/elafin did not inhibit auto-ADPr of gNarE (Fig. 4A). These results suggest that gNarE conserves ADPRT activity, which is abolished in the presence of human β-defensins.

In an attempt to detect host cell targets for ADPr by gNarE, we used cell lysates of FTECs as ADP-ribose acceptor. As showed in Fig 4B, six different bands were observed indicating the presence of targets ADP-ribosylated by gNarE. Bacterial ADPRTs such as *Clostridium botulinum* C2 toxin (Aktories *et al.*, 1986) and *Bordetella pertussis* toxin (Xu & Barbieri, 1995) use actin and Rho GTPases as ADP-ribose acceptors to elicit bacterial uptake by cytoskeleton rearrangement. In addition, the small GTPases Cdc42 and Rac1 are involved in internalization of *N. gonorrhoeae* in HeLa cells (Billker *et al.*, 2002; Quintero *et al.*, 2015). Therefore, we performed cell-free ADPr assays and Western Blot to detect any changes in protein levels of β-actin, Rac1 and Cdc42 in FTECs as a consequence of the enzymatic modification. We only observed a significant change in β-actin but not Rac1 and Cdc42 (Fig 4C, lower panel), with a shift that may have been due to the addition of ADP-ribose, which could interfere with antibody recognition.

Discussion

In this report, we characterized the *narE* locus of *Neisseria gonorrhoeae* and the ADP-ribosyltransferase activity of its gene product, gNarE. We found the locus conserved among gonococcal P9 variants at the same region previously reported for *N. meningitidis* strain MC58 (Masignani *et al.*, 2004). Although gonococcal variants used in this study showed

the tetranucleotide duplication that caused a frameshift as previously reported (Masignani *et al.*, 2003), the presence of classical promoter boxes -10 and -35, a Shine-Dalgarno sequence and an alternative initiation codon (GTG) enabled *narE* gene expression. The GTG codon is the most frequent alternative start codon in prokaryotes (Villegas & Kropinski, 2008), and is strongly suggested as a regulatory mechanism of translation (O'Donnell & Janssen, 2001; Kozak, 2005). The latter is an important consideration given that the examination of the pathogenomes of *Neisseria spp* showed that *N. meningitidis* is more closely related to the non-pathogenic members of *Neisseria* than *N. gonorrhoeae* (Putonti *et al.*, 2013). Moreover, pseudogenization of meningococcal genes in the *N. gonorrhoeae* genome have been described previously, such as the *ggt* gonococcal homologue (*ggh*) gene (Takahashi & Watanabe, 2005), the Class 5 outer membrane protein (OMP) *opcA* gene (Zhu *et al.*, 1999) and the Class I OMP PorA (*porA*) gene (Feavers & Maiden, 1998). However, the gonococcal *narE* gene cannot be classified into this group since those genes are either silent or their proteins are not functional, which contrasts with our results.

Arginine auto-ADP-ribosylation has been described as a intramolecular, regulatory mechanism of transferase activity in mNarE of *N. meningitidis*, ExoS of *Pseudomonas aeruginosa*, ChxA of *Vibrio cholera* and eukaryotic mono-ADPRTs (Karlberg *et al.*, 2012; Picchianti *et al.*, 2013; Sung & Tsai, 2014). Since mutagenesis of mNarE at Cys_{67} and Cys_{128} displayed reduced ADP-ribosyltransferase activity (Del Vecchio *et al.*, 2009; Koehler *et al.*, 2011), the lack of Cys_2His_2 assembly in gNarE might explain its reduced auto-modification activity. Moreover, Arg_7 of mNarE has been proposed as the auto-ADP-ribosylation site (Picchianti *et al.*, 2013), but the absence of 49 amino acids at the N-

terminus of gNarE suggests that the gNarE auto-ADPr site is different to that from mNarE. The gNarE protein contains only two arginine residues (Arg_{48} and Arg_{75}) and the ADP-ribosyl-NarE linkage was demonstrated at a single arginine residue in mNarE (Picchianti *et al.*, 2013). Thus, we hypothesize that one of these amino acid residues might be potentially the auto-ADPr site of gNarE, and future studies with site-directed mutation of these residues are required to test this hypothesis.

Human antimicrobial peptides such as α- and β-defensins, which contain several conserved arginine residues, can be ADP-ribosylated by bacterial ADPRTs such as CT and LT toxins (Castagnini *et al.*, 2012). However, mNarE exhibits poor transferase activity using these peptides as acceptors of ADP-ribose, whereas human neutrophil peptide-1 (HNP-1) strongly inhibits mNarE enzymatic activity but enhances auto-ADPr (Castagnini *et al.*, 2012). By contrast, in this work both ADPRT activity and auto-ADPr of gNarE were significantly reduced in presence of HBDs. Interestingly, HNP1 can also inhibit ADPRT activity of the mammalian ADPRTs ART-1 and ART-5 (Paone *et al.*, 2006) as well as diphtheria toxin (DT) and *Pseudomonas* exotoxin A (Kim *et al.*, 2006). Conversely, host cell production of antimicrobial peptides that inhibit gNarE enzyme function might play a defensive role at the epithelium during initial contact with the pathogen. In fact, HNP-1 and HBD2 can inhibit several bacterial toxins through defensin-induced unfolding and subsequent exposure of domains for proteolysis, without causing similar effects on tested mammalian proteins (Kudryashova *et al.*, 2014).

N. gonorrhoeae uses different mechanisms of internalization in epithelial cells depending on the CEACAM receptor (carcinoembryonic antigen-related cellular adhesion molecule) involved (Billker *et al.*, 2002). This leads to a redistribution and reorganization of actin cytoskeleton and disruption of cell junction complexes (Wang *et al.*, 2008; Rodríguez-Tirado *et al.*, 2012). In addition, gonococcal engulfment requires the participation of small GTPases Rac1 and Cdc42 in infected HeLa epithelial cells (Billker *et al.*, 2002). In this work, we also demonstrated that gNarE transferred ADP-ribose moiety to several host cell proteins, with β-actin as one possible acceptor. Valeri *et al*. (2015) reported that purified mNarE triggered loss of epithelial integrity due to ADP-ribosylation of cytoplasmic, cytoskeleton-related proteins in human epithelial cells. It is possible that gonococcal NarE may modify similar host cell targets to gain access to its intracellular niche. Moreover, a novel host cell target of bacterial ADPr has been described for the community-acquired respiratory distress syndrome toxin of *Mycoplasma pneumoniae* and corresponds to the NLRP3 inflammasome complex, for which ADP-ribosylation leads to subsequent release of IL-1β (Bose *et al.*, 2014). Interestingly, *N. gonorrhoeae* can also promote NLRP3 activation and IL-1β secretion in human THP-1 monocytic cells by release of outer membrane vesicles (OMV) cargo molecules such as lipooligosaccharide, although the participation of other gonococcal molecules cannot be excluded (Duncan *et al.*, 2009). Since pathogenic *Neisseria* species lack classical type-three secretion systems and mNarE accumulates in the periplasm, it has been proposed that this enzyme might be delivered to host cells through OMV and it is possible that *N. gonorrhoeae* use a similar OMV delivery pathway to release gNarE (Masignani *et al.*, 2003, 2004; Edwards & Butler, 2011; Valeri *et al.*, 2015).

In summary, our study provides evidence that the *narE* gene of *Neisseria gonorrhoeae* is expressed by the pathogen and the gonococcal enzyme possesses ADP-ribosyltransferase activity. In addition, gNarE can recognize host cell ADP-ribose acceptors *in vitro*. Further studies are required to elucidate the role of *Neisseria* NarE during infection and its delivery into host cells.

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Conflict of interest

None declared

References

Aktories K, Bärmann M, Ohishi I, Tsuyama S, Jakobs KH & Habermann E (1986) Botulinum C2 toxin ADP-ribosylates actin. *Nature* **322**: 390–392.

Bachran C, Sutherland M, Bachran D & Fuchs H (2007) Quantification of diphtheria toxin mediated ADP-ribosylation in a solid-phase assay. *Clin Chem* **53**: 1676–1683.

Besemer J, Lomsadze A & Borodovsky M (2001) GeneMarkS: a self-training method for prediction of gene starts in microbial genomes. Implications for finding sequence motifs in regulatory regions. *Nucleic Acids Res* **29**: 2607–2618.

Billker O, Popp A, Brinkmann V, Wenig G, Schneider J, Caron E & Meyer TF (2002) Distinct mechanisms of internalization of Neisseria gonorrhoeae by members of the CEACAM receptor family involving Rac1- and Cdc42-dependent and -independent pathways. *EMBO J* **21**: 560–571.

Bose S, Segovia JA, Somarajan SR, Chang T-H, Kannan TR & Baseman JB (2014) ADP-ribosylation of NLRP3 by Mycoplasma pneumoniae CARDS toxin regulates inflammasome activity. *MBio* **5**: e02186–14.

Castagnini M, Picchianti M, Talluri E, Biagini M, Del Vecchio M, Di Procolo P, Norais N, Nardi-Dei V & Balducci E (2012) Arginine-specific mono ADP-ribosylation in vitro of antimicrobial peptides by ADP-ribosylating toxins. *PLoS One* **7**: e41417.

Del Vecchio M, Pogni R, Baratto MC, Nobbs A, Rappuoli R, Pizza M & Balducci E (2009)

Identification of an iron-sulfur cluster that modulates the enzymatic activity in NarE, a Neisseria meningitidis ADP-ribosyltransferase. *J Biol Chem* **284**: 33040–33047.

Du Y, Lenz J & Arvidson CG (2005) Global gene expression and the role of sigma factors in Neisseria gonorrhoeae in interactions with epithelial cells. *Infect Immun* **73**: 4834–4845.

Duncan JA, Gao X, Huang MT-H, O'Connor BP, Thomas CE, Willingham SB, Bergstralh DT, Jarvis GA, Sparling PF & Ting JP-Y (2009) Neisseria gonorrhoeae activates the proteinase cathepsin B to mediate the signaling activities of the NLRP3 and ASC-containing inflammasome. *J Immunol* **182**: 6460–6469.

Edwards JL & Butler EK (2011) The Pathobiology of Neisseria gonorrhoeae Lower Female Genital Tract Infection. *Front Microbiol* **2**: 102.

Feavers IM & Maiden MC (1998) A gonococcal porA pseudogene: implications for understanding the evolution and pathogenicity of Neisseria gonorrhoeae. *Mol Microbiol* **30**: 647–656.

Holbourn KP, Shone CC & Acharya KR (2006) A family of killer toxins. Exploring the mechanism of ADP-ribosylating toxins. *FEBS J* **273**: 4579–4593.

Kannan TR & Baseman JB (2006) ADP-ribosylating and vacuolating cytotoxin of Mycoplasma pneumoniae represents unique virulence determinant among bacterial pathogens. *Proc Natl Acad Sci U S A* **103**: 6724–6729.

Karlberg T, Thorsell A-G, Kallas Å & Schüler H (2012) Crystal structure of human ADP-ribose transferase ARTD15/PARP16 reveals a novel putative regulatory domain. *J*

Biol Chem **287**: 24077–24081.

Kim C, Slavinskaya Z, Merrill AR & Kaufmann SHE (2006) Human alpha-defensins neutralize toxins of the mono-ADP-ribosyltransferase family. *Biochem J* **399**: 225–229.

Koehler C, Carlier L, Veggi D, *et al.* (2011) Structural and Biochemical Characterization of NarE, an Iron-containing ADP-ribosyltransferase from Neisseria meningitidis. *J Biol Chem* **286**: 14842–14851.

Kozak M (2005) Regulation of translation via mRNA structure in prokaryotes and eukaryotes. *Gene* **361**: 13–37.

Kudryashova E, Quintyn R, Seveau S, Lu W, Wysocki VH & Kudryashov DS (2014) Human defensins facilitate local unfolding of thermodynamically unstable regions of bacterial protein toxins. *Immunity* **41**: 709–721.

Lambden PR, Robertson JN & Watt PJ (1980) Biological properties of two distinct pilus types produced by isogenic variants of Neisseria gonorrhoeae P9. *J Bacteriol* **141**: 393–396.

Lemichez E & Barbieri JT (2013) General aspects and recent advances on bacterial protein toxins. *Cold Spring Harb Perspect Med* **3**: a013573.

Masignani V, Balducci E, Di Marcello F, *et al.* (2003) NarE: a novel ADP-ribosyltransferase from Neisseria meningitidis. *Mol Microbiol* **50**: 1055–1067.

Masignani V, Balducci E, Serruto D, Veggi D, Aricò B, Comanducci M, Pizza M & Rappuoli R (2004) In silico identification of novel bacterial ADP-ribosyltransferases. *Int J Med Microbiol* **293**: 471–478.

Mekalanos JJ, Collier RJ & Romig WR (1979) Enzymic activity of cholera toxin. I. New method of assay and the mechanism of ADP-ribosyl transfer. *J Biol Chem* **254**: 5849–5854.

O'Donnell SM & Janssen GR (2001) The initiation codon affects ribosome binding and translational efficiency in Escherichia coli of cI mRNA with or without the 5' untranslated leader. *J Bacteriol* **183**: 1277–1283.

Paone G, Stevens LA, Levine RL, Bourgeois C, Steagall WK, Gochuico BR & Moss J (2006) ADP-ribosyltransferase-specific modification of human neutrophil peptide-1. *J Biol Chem* **281**: 17054–17060.

Picchianti M, Del Vecchio M, Di Marcello F, Biagini M, Veggi D, Norais N, Rappuoli R, Pizza M & Balducci E (2013) Auto ADP-ribosylation of NarE, a Neisseria meningitidis ADP-ribosyltransferase, regulates its catalytic activities. *FASEB J* **27**: 4723–4730.

Pizza M, Scarlato V, Masignani V, *et al.* (2000) Identification of vaccine candidates against serogroup B meningococcus by whole-genome sequencing. *Science* **287**: 1816–1820.

Putonti C, Nowicki B, Shaffer M, Fofanov Y & Nowicki S (2013) Where does Neisseria acquire foreign DNA from: an examination of the source of genomic and pathogenic islands and the evolution of the Neisseria genus. *BMC Evol Biol* **13**: 184.

Quintero CA, Tudela JG & Damiani MT (2015) Rho GTPases as pathogen targets: Focus on curable sexually transmitted infections. *Small GTPases* **6**: 108–118. http://www.ncbi.nlm.nih.gov/pubmed/26023809 (Accessed June 16, 2016).

Rodríguez-Tirado C, Maisey K, Rodríguez FE, Reyes-Cerpa S, Reyes-López FE & Imarai

M (2012) Neisseria gonorrhoeae induced disruption of cell junction complexes in epithelial cells of the human genital tract. *Microbes Infect* **14**: 290–300.

Schägger H (2006) Tricine-SDS-PAGE. *Nat Protoc* **1**: 16–22.

Simon NC, Aktories K & Barbieri JT (2014) Novel bacterial ADP-ribosylating toxins: structure and function. *Nat Rev Microbiol* **12**: 599–611.

Sung VM-H & Tsai C-L (2014) ADP-Ribosylargininyl reaction of cholix toxin is mediated through diffusible intermediates. *BMC Biochem* **15**: 26.

Takahashi H & Watanabe H (2005) A gonococcal homologue of meningococcal gamma-glutamyl transpeptidase gene is a new type of bacterial pseudogene that is transcriptionally active but phenotypically silent. *BMC Microbiol* **5**: 56.

Valeri M, Zurli V, Ayala I, Colanzi A, Lapazio L, Corda D, Soriani M, Pizza M & Rossi Paccani S (2015) The Neisseria meningitidis ADP-Ribosyltransferase NarE Enters Human Epithelial Cells and Disrupts Epithelial Monolayer Integrity. *PLoS One* **10**: e0127614.

Villegas A & Kropinski AM (2008) An analysis of initiation codon utilization in the Domain Bacteria - concerns about the quality of bacterial genome annotation. *Microbiology* **154**: 2559–2661.

Wang JA, Meyer TF & Rudel T (2008) Cytoskeleton and motor proteins are required for the transcytosis of Neisseria gonorrhoeae through polarized epithelial cells. *Int J Med Microbiol* **298**: 209–221. http://www.ncbi.nlm.nih.gov/pubmed/17683982 (Accessed June 22, 2016).

Whitehead RN, Overton TW, Snyder LAS, McGowan SJ, Smith H, Cole JA & Saunders

NJ (2007) The small FNR regulon of Neisseria gonorrhoeae: comparison with the larger Escherichia coli FNR regulon and interaction with the NarQ-NarP regulon. *BMC Genomics* **8**: 35.

Wira CR, Patel M V, Ghosh M, Mukura L & Fahey J V (2011) Innate immunity in the human female reproductive tract: endocrine regulation of endogenous antimicrobial protection against HIV and other sexually transmitted infections. *Am J Reprod Immunol* **65**: 196–211.

Xu Y & Barbieri JT (1995) Pertussis toxin-mediated ADP-ribosylation of target proteins in Chinese hamster ovary cells involves a vesicle trafficking mechanism. *Infect Immun* **63**: 825–832.

Zhu P, Morelli G & Achtman M (1999) The opcA and (psi)opcB regions in Neisseria: genes, pseudogenes, deletions, insertion elements and DNA islands. *Mol Microbiol* **33**: 635–650.

Figure 1

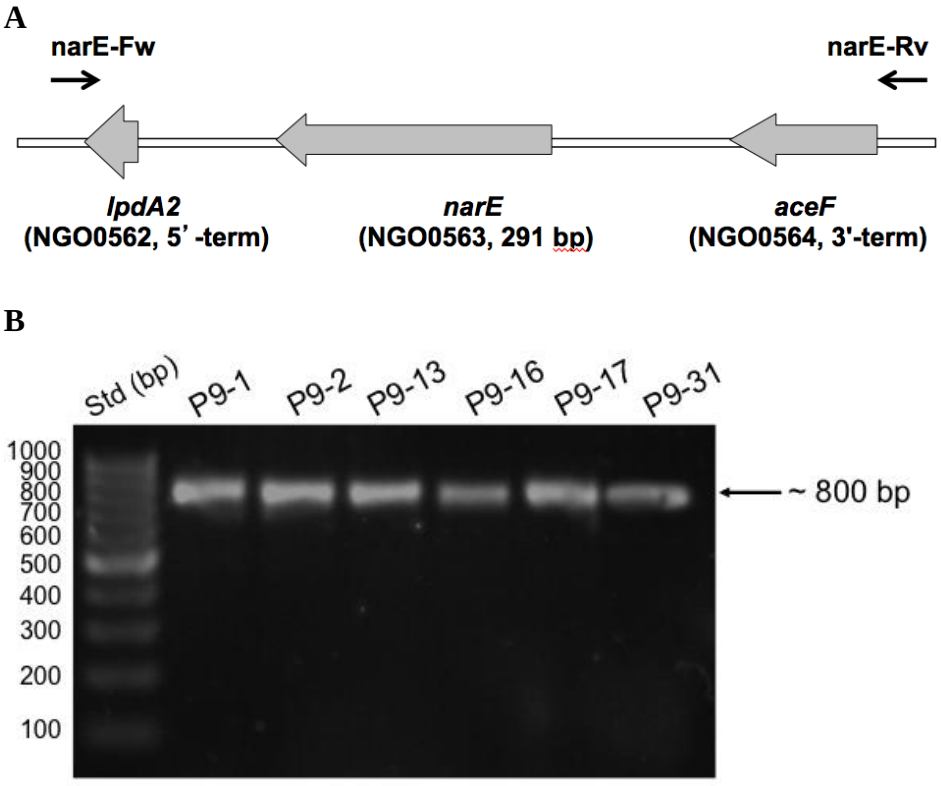
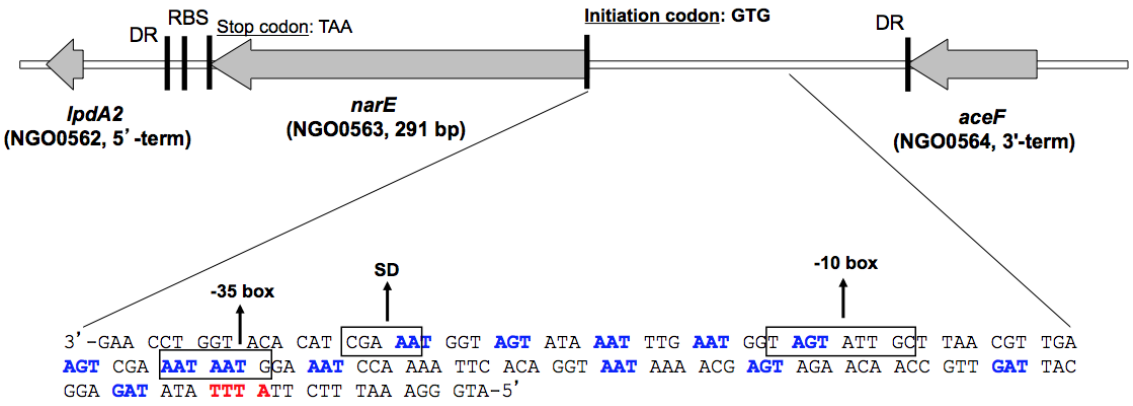


Figure 1. Detection of *narE* locus in *Neisseria gonorrhoeae* isolates. (A) Localization of primers used to amplify the gonococcal *narE* locus. **(B)** PCR of gonococcal *narE* locus. PCR products were cloned for subsequent sequencing and bioinformatic analysis.

Figure 2

A



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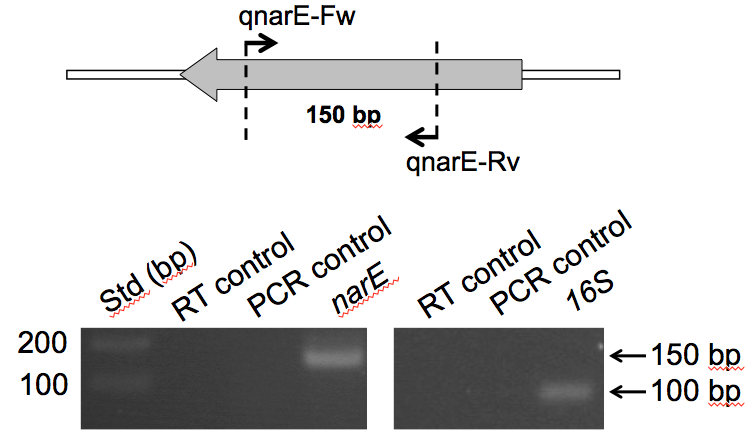

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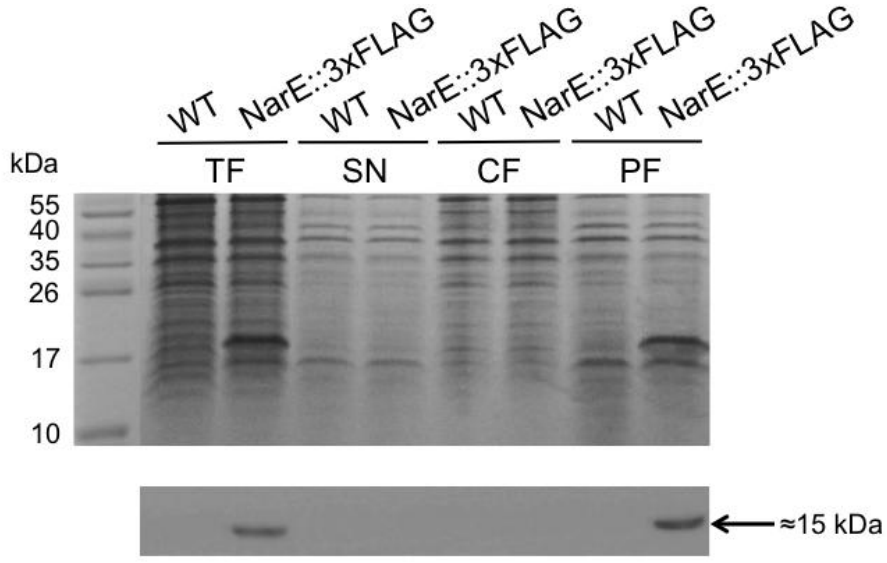


Figure 2. Expression of *Neisseria gonorrhoeae narE* gene. (A) Genomic organization of the gonococcal *narE* locus. The diagram was constructed according to the sequences obtained from P9 gonococcal variants. The GTG alternative start codon is showed in bold; red, tetranucleotide sequence; blue, stop codons; DR, direct repeat; RBS, putative ribosome binding site. **(B)** Reverse transcription (RT) and PCR of gonococcal *narE*. *Top panel*, localization of primers used in reverse transcription of *narE*. *Bottom panel*, RT-PCR of *narE*. RT control, no reverse transcriptase added; PCR control, no DNA polymerase added; 16S, reverse transcription of the 16S rRNA gene. **(C)** Western blot of gonococcal NarE. *N. gonorrhoeae* P9-17 wild type strain (WT, no FLAG-tagged gene) was included as negative control. *Top panel*, SDS-PAGE of gonococcal protein fractions in Tris-tricine gel (16%). Gel was stained with Coomassie blue. *Bottom panel*, Western Blot of gonococcal NarE-3xFLAG fusion protein. TF, total protein fraction; SN, supernatant protein fraction; CF, cytoplasmatic fraction; PF, periplasmic fraction.

Figure 3

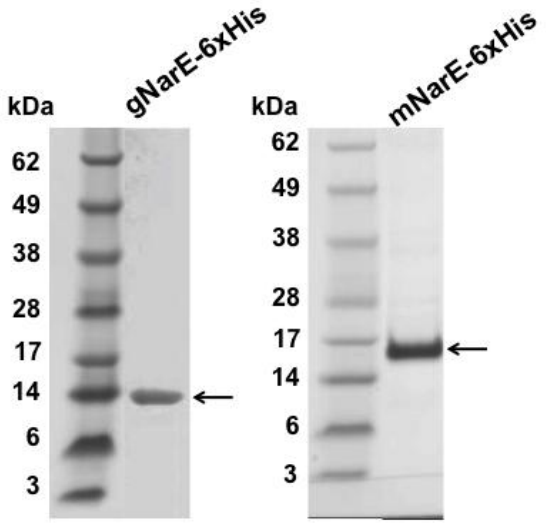
A

```
NMB1343         MGNFLYRGISCQQDEQNNGQLKPKGNKAEVAIRYDGKFKYDGKATHGPSVKNAVYAHQIE 60
NGO0563         ------------------------------------------MKNAVYAHQIE 11
                                                           :**********

NMB1343         TGLYDGCYISTTTDKEIAKKFATSSGIENGYIYVLNRDLFGQYSIFEYEVEHPENPNEKE 120
NGO0563         TDLYDGCYISTTTDKEIAKKFATSSGIENGYIYVLNRDLFGQYSIFEYEVEHPENPDEKE 71
                *.**********************************************:***

NMB1343         VTIRAEDCGCIPEEVIIAKELIEIN 145
NGO0563         VTIRAEDCGCIPEEVIIAKELIEIN 96
                *************************
```

B



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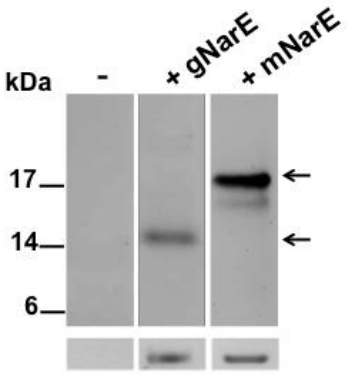

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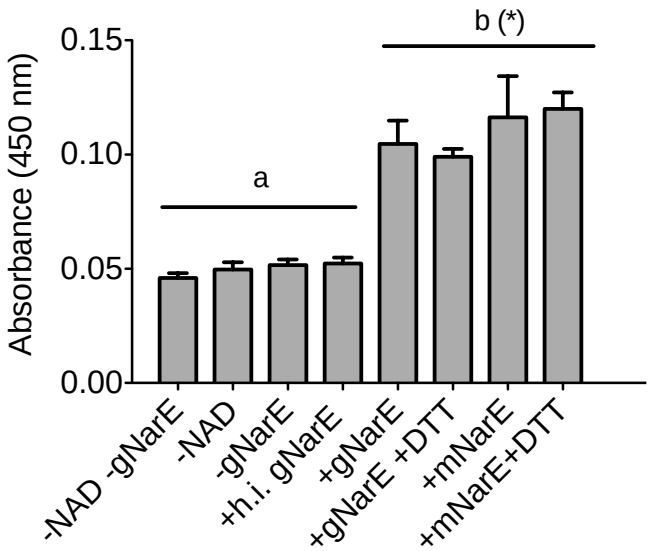
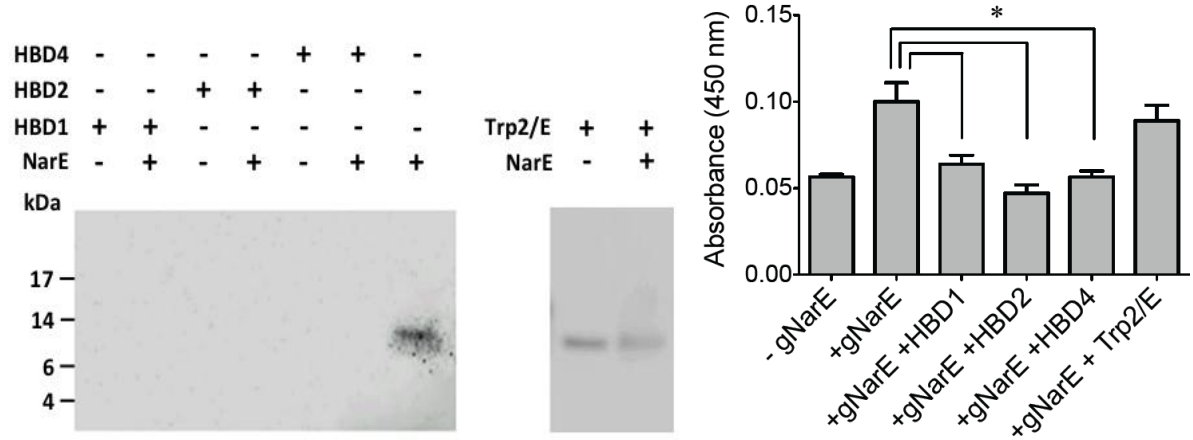


Figure 3. ADP-ribosyltransferase activity of gNarE. (A) Sequence alignment of NarE of *N. gonorrhoeae* and *meningitidis.* Blue, Cys_2His_2 residues involved in Fe-S center; fuchsia, pocket for NAD^+; red, NAD^+ binding site; green, catalytic site. **(B)** SDS-PAGE of purified, recombinant His-tagged NarE proteins. mNarE-6xHis, meningococcal NarE-6xHis fusion protein; gNarE-6xHis, gonococcal NarE fusion protein. **(C)** Auto ADP-ribosylation of gNarE. *Top panel*, Immunoblot of biotin-ADP-ribosylated gNarE. (-), no enzyme added; gNarE, gonococcal NarE-6xHis protein ; mNarE, meningococcal NarE-6xHis protein. Arrows show bands of auto-ADP-ribosylation of both NarE proteins. *Bottom panel*, Western blot of gNarE and mNarE. Anti-His (C-term) antibody was used to detect both NarE proteins. **(D)** ADP-ribosylation of agmatine by gNarE. DTT, dithiothretol; gNarE, gonococcal NarE-6xHis protein; h.i. gNarE, heat inactivated gNarE-6xHis (95ºC for 30 min); mNarE, meningococcal NarE-6xHis protein; NAD, 6-biotin-17-NAD. The graphic is representative of three experiments carried out in duplicated. (*), significant difference between “a” and “b”.

Figure 4

A



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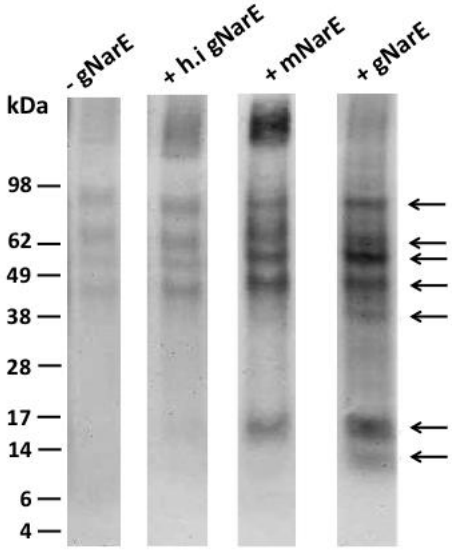

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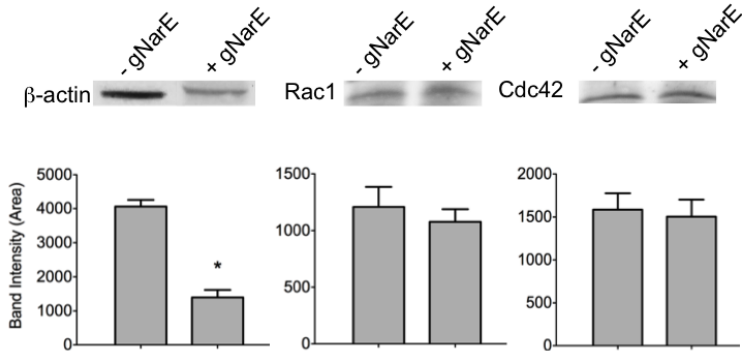

Figure 4. ADP-ribosylation of biological targets by gNarE. (A) ADP-ribosylation of human antimicrobial peptides by gNarE. (*Left*) Immunoblot of ADPr reactions of human β-defensins HBD1, HBD2, HBD4 and trappin-2/elafin (Trp2/E) in presence (+) or absence (-) of gNarE or the corresponding antimicrobial peptide. (*Right*) Inhibition of the ADP-ribosylation of agmatine by gNarE in presence of the human β-defensins HBD1, HBD2, HBD4 and trappin-2/elafin (Trp2/E). **(B)** ADP-ribosylation of host cell proteins present in FTECs lysates by gNarE. -gNarE, no enzyme added, ; h.i. gNarE, heat inactivated gNarE-6xHis (95ºC for 30 min); +gNarE, gonococcal NarE-6xHis protein added to the reaction; mNarE protein, meningococcal NarE-6xHis protein. **(C)** *Top panel*, Western blot of human β-actin, Rac1 and Cdc42 from ADP-ribosylation assays using gNarE. *Bottom panel*, band intensity was analyzed by densitometry using ImageJ software. A representative immunoblot from three independent experiments (n=3) is shown. *$p<0.05$; error bars, SD.