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FEMS Microbiology Letters, 363, 2016, fnw181

doi: 10.1093/femsle/fnw181
Advance Access Publication Date: 26 July 2016
Research Letter

RESEARCH LETTER – Physiology & Biochemistry

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

Paula I. Rodas[1,*,†], A. Said Álamos-Musre[1], Francisca P. Álvarez[1], Alejandro Escobar[2], Cecilia V. Tapia[3,4], Eduardo Osorio[5], Carolina Otero[1], Iván L. Calderón[6], Juan A. Fuentes[6], Fernando Gil[6], Daniel Paredes-Sabja[7,8] and Myron Christodoulides[9]

[1]Center for Integrative Medicine and Innovative Sciences, Facultad de Medicina, Universidad Andres Bello, Santiago, Chile, [2]Instituto de Investigación en Ciencias Odontológicas, Facultad de Odontología, Universidad de Chile, Santiago, Chile, [3]Laboratorio Clínica Dávila, Santiago, Chile, [4]Laboratorio de Micología Médica, Programa de Microbiología y Micología, ICBM, Facultad de Medicina, Universidad de Chile, Santiago, Chile, [5]Servicio de Ginecología y Obstetricia, Clínica Dávila, Santiago, Chile, [6]Laboratorio de Genética y Patogénesis Bacteriana, Facultad de Ciencias Biológicas, Universidad Andres Bello, Santiago, Chile, [7]Microbiota-Host Interactions and Clostridia Research Group, Departamento de Ciencias Biológicas, Facultad de Ciencias Biológicas, Universidad Andres Bello, Santiago, Chile, [8]Center for Bioinformatics and Integrative Biology, Facultad de Ciencias Biológicas, Universidad Andres Bello, Santiago, Chile and [9]Neisseria Research Group, Molecular Microbiology, Sir Henry Wellcome Laboratories, Clinical and Experimental Sciences, University of Southampton Faculty of Medicine, Southampton, England

[*]**Corresponding author:** Center for Integrative Medicine and Innovative Sciences, Facultad de Medicina, Universidad Andres Bello, Echaurren 183, Santiago 837 70071, Chile. Tel: +56-22770-3906; Fax: +56-22770-3602; E-mail: paula.rodas@unab.cl
One sentence summary: Characterization of the gonococcal NarE enzyme.
Editor: Dieter Jahn
[†]Paula I. Rodas, http://orcid.org/0000-0002-8071-1229

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 with part of the coding sequence of the 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

Received: 12 February 2016; **Accepted:** 21 July 2016
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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.

Keywords: NarE; *Neisseria gonorrhoeae*; ADP-ribosyltransferase

ABBREVIATIONS

ADPr: ADP-ribosylation
ADPRT: ADP-ribosyltransferase
ART: human ADP-ribosyltransferase
CT: cholera toxin
FTEC: Fallopian tube epithelial cell
gNarE: gonococcal NarE protein
HBD: human antimicrobial peptide β-defensin
HNP-1: human neutrophil peptide-1
LT: heat-labile enterotoxin
mNarE: meningococcal NarE protein
NLRP3: nucleotide-binding domain and leucine-rich-repeat-containing 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, Aktories and Barbieri 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, Shone and Acharya 2006; Lemichez and 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 a 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 Supplementary Table S1. *Neisseria gonorrhoeae* strain P9 was originally isolated in the UK and variants selected and confirmed by colony morphology with stereo-microscopy (Lambden, Robertson and Watt 1980). *Neisseria gonorrhoeae* isolates were cultured from frozen stocks onto GC agar plates supplemented with IsoVitaleX (Becton Dickinson) at 37°C 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}). *Escherichia coli* strains were grown in Luria–Bertani (LB) broth at 37°C 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

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 (Supplementary Table S1 and Fig. 1A). PCR products (~800 bp) were purified (Nucleotide Removal Kit, Qiagen), cloned into pGEM-T easy vector (Promega) (Supplementary 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 were carried out using GeneMarkS (Besemer, Lomsadze and Borodovsky 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 the 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 (3.0×10^9 bacteria mL^{-1}) using Trizol Reagent

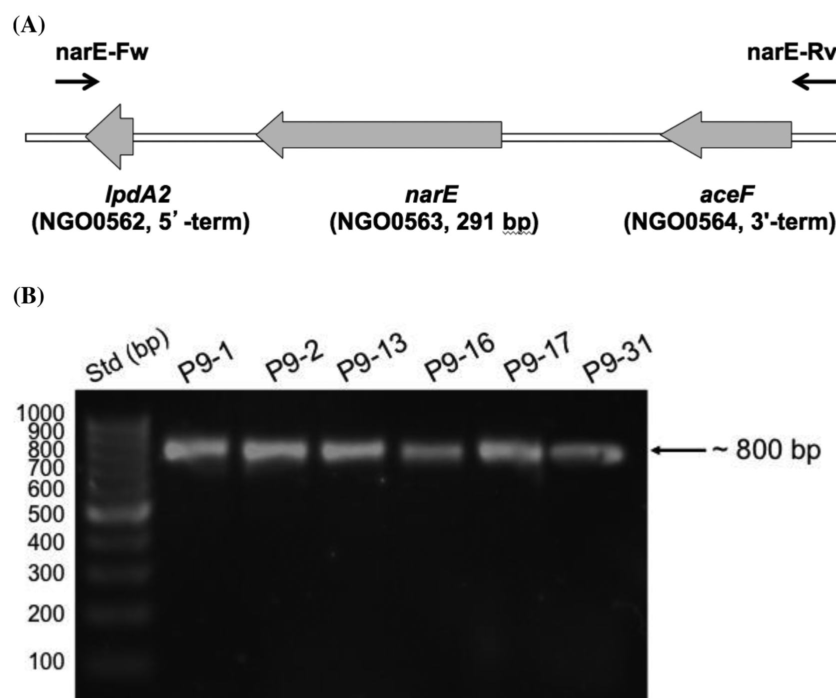

Figure 1. Detection of *narE* locus in *Neisseria gonorrhoeae* isolates. (**A**) Localization of primers used to amplify the gonococcal *narE* locus. (**B**) PCR of gonococcal *narE* locus. PCR products were cloned for subsequent sequencing and bioinformatic analysis.

(Ambion, Life Technologies) (Whitehead *et al.* 2007). Primer sequences are described in Supplementary 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 (94°C for 30 s, 59°C for 45 s and 72°C for 90 s) followed by a 3 min extension at 72°C was carried out. DNAse-treated RNA without reverse transcriptase was used as negative control, while 16S rRNA was included as positive control with 60°C for annealing (Du, Lenz and Arvidson 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 (Supplementary Table S1) yielding plasmid pEC0011, then was purified (Wizard, Promega) and concentrated by phenol–chloroform extraction/ethanol precipitation. *Neisseria gonorrhoeae* P9-17 was transformed with pEC0011 and clones were confirmed by PCR and sequencing.

Western blotting of gNarE–3xFLAG

Neisseria gonorrhoeae P9-17 wild type and *narE*::3xFLAG(Kan^R) strains were grown in 10 mL of GCB and GCB–kanamycin (Kan), respectively, at 1.0 × 10^7 CFU mL^{-1} for 4 h at 37°C and shaking (100 r.p.m.). 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 (Supplementary Table S1). *Escherichia 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 isopropyl β-D-1-thiogalactopyranoside (Invitrogen) at OD_{600} of 0.5–0.6, grown for 4 h at 25°C 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 4°C 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 4°C 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 25°C. Wells were washed with phosphate-buffered saline

(PBS)–0.05% (v/v) Tween 20 (PBST) and were blocked with 5% (w/v) bovine serum albumin (BSA; Millipore) for 2 h; then 100 μL of rabbit anti-agmatine antibody (1/1000) (Millipore) was added to the wells and incubated for 2 h at 25°C. 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-Aldrich) in a final volume of 200 μL. Reactions were incubated for 30 min at 37°C, and then were transferred onto the pre-coated wells and incubated for 2 h at 25°C. The wells were washed and then incubated with 100 μL streptavidin–horseradish peroxidase (HRP) (1/100) (R&D Systems) in 3% (w/v) BSA for 1 h at 25°C. After washing, 100 μL of 3,3′5,5′,-tetramethylbenzidine (Sigma-Aldrich) was added for color development, and the reaction was stopped by adding 50 μL of 1 M 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 95°C) 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 37°C and were stopped by adding NuPAGE LDS sample buffer (4×) (Life Technologies). Immunoblot analysis was performed as described previously (Picchianti *et al.* 2013). Western blotting of gNarE and mNarE was 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 1 h 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

Fallopian tube epithelial cells (FTECs) were washed with PBS and suspended in 500 μL of 20 mM Tris-HCl buffer (pH 7.5) containing 1 mM EDTA, 1 mM DTT, 5 mM $MgCl_2$ and 1× Halt™ Protease Inhibitor Cocktail (EDTA-free). Cells were sonicated using six 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 polyvinylidene difluoride membrane and visualized with peroxidase-coupled streptavidin (R&D Systems) in a chemiluminiscence 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 appropriate 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 performed using pairwise comparisons across groups using *t*-test with pooled variance in agmatine assays and corrected for multiple testing using Bonferroni's correction. A *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, which 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, the *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 (Supplementary 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 the *narE* (locus NEIS2492) amongst the 2263 gonococcal isolates; this 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 (Supplementary Fig. S2).

The previously reported duplication of a tetranucleotide located 12 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 RT-PCR using total RNA of strain P9-17; 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

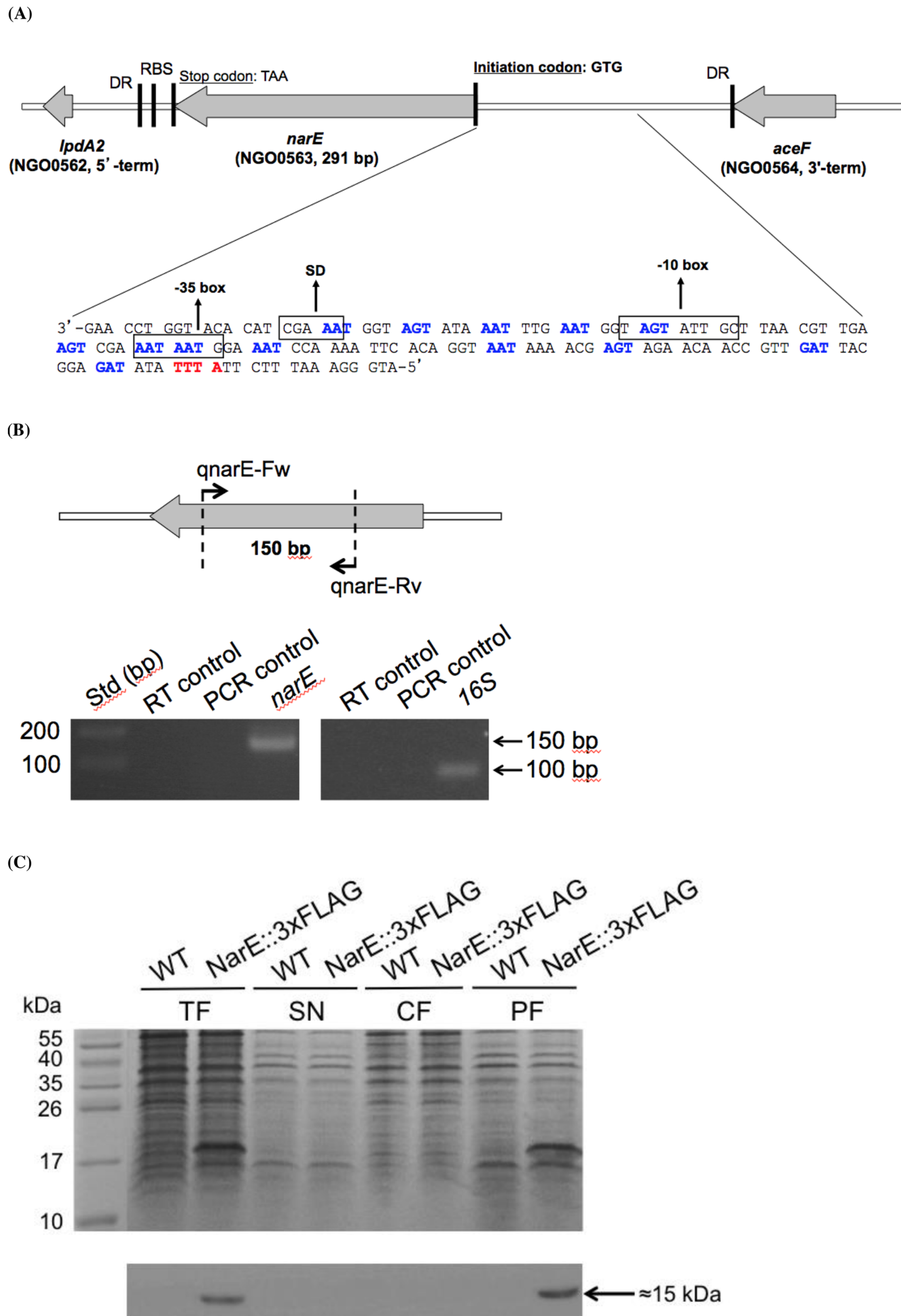

Figure 2. Expression of *Neisseria gonorrhoeae narE* gene. (**A**) Genomic organization of the gonococcal *narE* locus. The diagram was constructed according to the sequences obtained from P9 gonococcal variants. The GTG alternative start codon is shown in bold; red, tetranucleotide sequence; blue, stop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

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, Shone and Acharya 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 an ~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, Collier and Romig 1979; Kannan and 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 and 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, Tudela and Damiani 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 the 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 and Kropinski 2008), and is strongly suggested as a regulatory mechanism of translation (O'Donnell and 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 and Watanabe 2005), the Class 5 outer membrane protein (OMP) *opcA* gene (Zhu, Morelli and Achtman 1999) and the Class I OMP PorA (*porA*) gene (Feavers and 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 an 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 and 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 from 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 the presence of HBDs. Interestingly, HNP-1 can also inhibit ADPRT activity of the mammalian ADPRTs ART-1 and ART-5 (Paone *et al.* 2006) as well as diphtheria toxin 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).

Neisseria gonorrhoeae uses different mechanisms of internalization in epithelial cells depending on the carcinoembryonic antigen-related cellular adhesion molecule (CEACAM) receptor involved (Billker *et al.* 2002). This leads to a redistribution and reorganization of actin cytoskeleton and disruption of cell junction complexes (Wang, Meyer and Rudel 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

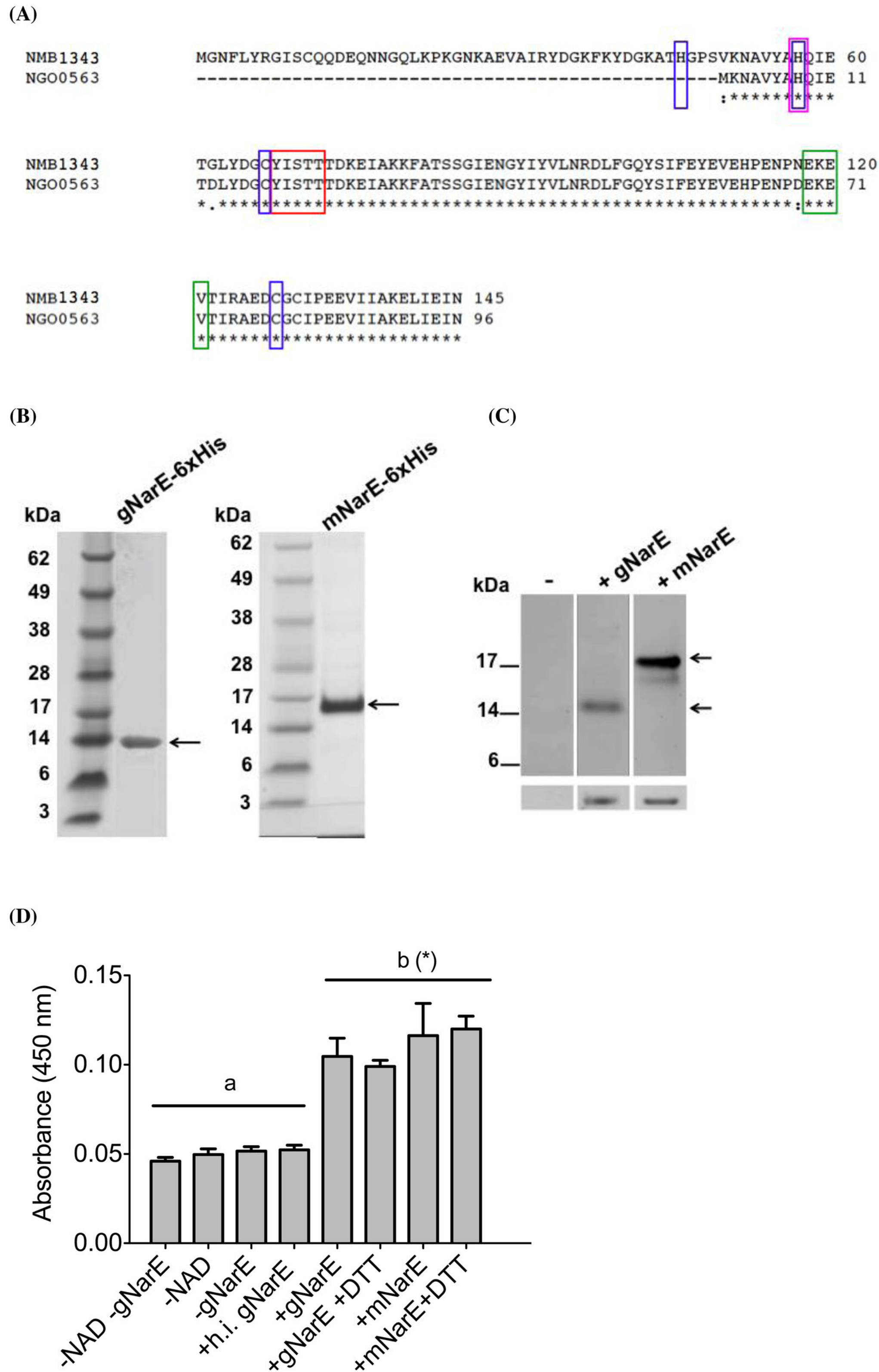

Figure 3. ADP-ribosyltransferase activity of gNarE. (A) Sequence alignment of NarE of *N. gonorrhoeae* and *N. meningitidis*. Blue, Cys_2His_2 residues involved in Fe-S center; fuchsia, pocket for NAD^+; red, NAD^+ binding site; green, catalytic site. (B) SDS-PAGE of purified, recombinant His-tagged NarE proteins. mNarE–6xHis, meningococcal NarE–6xHis fusion protein; gNarE–6xHis, gonococcal NarE fusion protein. (C) Auto-ADP-ribosylation of gNarE. Top, 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, 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 (95°C for 30 min); mNarE, meningococcal NarE–6xHis protein; NAD, 6-biotin-17-NAD. The figure is representative of three experiments carried out in duplicate. *Significant difference between 'a' and 'b'.

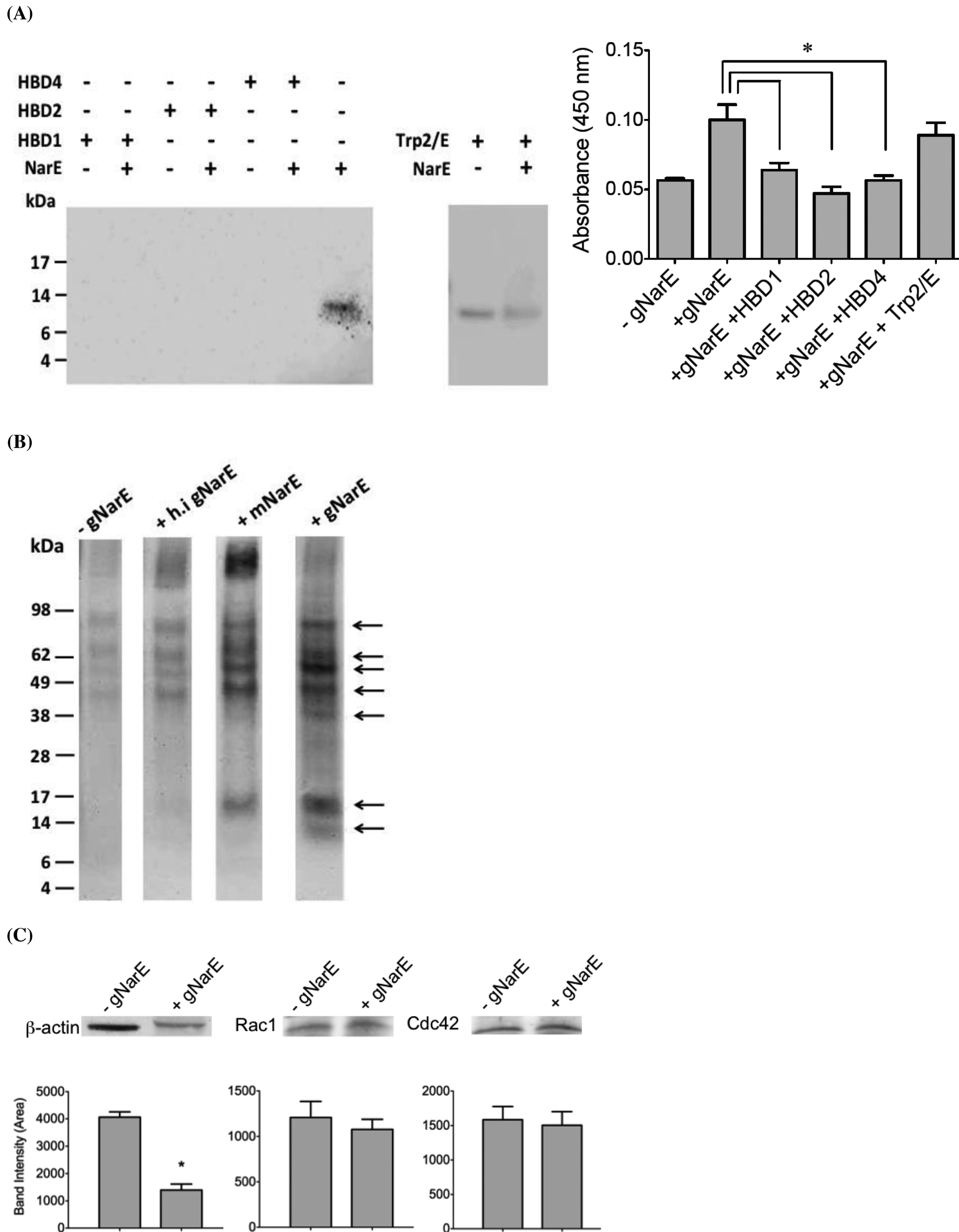

Figure 4. ADP-ribosylation of biological targets by gNarE. (**A**) ADP-ribosylation of human antimicrobial peptides by gNarE. Left, immunoblot of ADPr reactions of human β-defensins HBD1, HBD2, HBD4 and trappin-2/elafin (Trp2/E) in the presence (+) or absence (–) of gNarE or the corresponding antimicrobial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

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 nucleotide-binding domain and leucine-rich-repeat-containing family of pattern-recognition molecules 3 (NLRP3) inflammasome complex, for which ADP-ribosylation leads to subsequent release of interleukin-1β (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 vesicle (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 3 secretion systems and mNarE accumulates in the periplasm, it has been proposed that this enzyme might be delivered to host cells through OMVs and it is possible that *N. gonorrhoeae* use a similar OMV delivery pathway to release gNarE (Masignani *et al.* 2003, 2004; Edwards and 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.

SUPPLEMENTARY DATA

Supplementary data are available at FEMSLE online.

ACKNOWLEDGEMENTS

We thank Marisol Castillo and Ximena Daza (Clínica Dávila) for obtaining human Fallopian tube samples, Yaquelin González (CIMIS, Universidad Andrés Bello) for technical support in processing human Fallopian tube samples and Dr Claudio Pérez-Leighton (CIMIS, Universidad Andrés Bello) for technical support in statistical analysis. This publication made use of the Neisseria Multi Locus Sequence Typing website (http://pubmlst.org/neisseria/) developed by Dr Keith Jolley and sited at the University of Oxford. The development of this site has been funded by the Wellcome Trust and European Union. The authors have no conflicts of interest to declare.

FUNDING

This work was supported by Vicerrectoria de Investigación Universidad Andres Bello (UNAB DI-273-13/R to PIR), Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT 11121262 to PIR, 11110216 to ILC, 11121506 to JAF, 1130074 to FG, 1151025 to DPS) and the Wellcome Trust (WT 090301 to MC).

Conflict of interest. None declared.

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