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Extracellular Nucleotide Catabolism by the Group B Streptococcus Ectonucleotidase NudP Increases Bacterial Survival in Blood*

Received for publication, December 23, 2013, and in revised form, January 13, 2014. Published, JBC Papers in Press, January 15, 2014, DOI 10.1074/jbc.M113.545632

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Background: Ectonucleotidases regulate extracellular nucleotide concentration.

Results: The NudP ecto-5'-nucleotidase of Streptococcus agalactiae has specific substrate specificities necessary for survival in blood and organ colonization.

Conclusion: Extracellular nucleotide catabolism is involved in the control of Group B streptococcal pathogenesis.

Significance: Bacterial pathogens exploit different enzymatic specificities to subvert extracellular nucleotide signaling.

Streptococcus agalactiae (Group B Streptococcus) is a commensal of the human intestine and vagina of adult women but is the leading cause of invasive infection in neonates. This Gram-positive bacterium displays a set of virulence-associated surface proteins involved in the interaction with the host, such as adhesion to host cells, invasion of tissues, or subversion of the immune system. In this study, we characterized a cell wall-localized protein as an ecto-5'-nucleoside diphosphate phosphohydrolase (NudP) involved in the degradation of extracellular nucleotides which are central mediators of the immune response. Biochemical characterization of recombinant NudP revealed a Mn2+-dependent ecto-5'-nucleotidase activity on ribo- and deoxyribonucleoside 5'-mono- and 5'-diphosphates with a substrate specificity different from that of known orthologous enzymes. Deletion of the gene coding the housekeeping enzyme sortase A led to the release of NudP into the culture supernatant, confirming that this enzyme is anchored to the cell wall by its non-canonical LPXTG motif. The NudP ecto-5'-nucleotidase activity is reminiscent of the reactions performed by the mammalian ectonucleotidases CD39 and CD73 involved in regulating the extracellular level of ATP and adenosine. We further demonstrated that the absence of NudP activity decreases bacterial survival in mouse blood, a process dependent on extracellular adenosine.

In vivo assays in animal models of infection showed that NudP activity is critical for virulence. These results demonstrate that Group B Streptococcus expresses a specific ecto-5'-nucleotidase necessary for its pathogenicity and highlight the diversity of reactions performed by this enzyme family. These results suggest that bacterial pathogens have developed specialized strategies to subvert the mammalian immune response controlled by the extracellular nucleotide signaling pathways.

Pathogenic microorganisms have developed numerous strategies to resist and manipulate the host immune system to avoid recognition and killing. One of them relies on the perturbation of the host purinergic signaling pathway to control the balance between pro- and anti-inflammatory responses (1, 2). This purinergic pathway uses mainly extracellular adenosine triphosphate (eATP) and extracellular adenosine (eAdo) as signaling effectors. In response to infection or cell damage, host cells secrete ATP (3, 4). eATP is a “danger” signal allowing the recruitment of the innate immune system and the autocrine activation of proinflammatory responses (3–8). In contrast, eAdo antagonizes the effect of eATP and is a very potent suppressor of proinflammatory responses (9, 10). eAdo and eATP are recognized by specific cell surface receptors of the P1 and P2 families regulating the balance between anti- and proinflammatory responses as well as numerous cell-cell communication processes and pathological conditions (7, 9–11).

The eATP/eAdo ratio is tightly regulated by ectonucleotidases expressed at the surface of host cells to avoid detrimental overactivation of the proinflammatory response by eATP (12, 13). In mammals, two main ectonucleotidases, CD39 and CD73, allow the sequential degradation of eATP to eAdo (12, 13). The CD39 enzyme is an ectonucleoside triphosphate diphosphohydrolase (ecto-NTPDase) that hydrolyzes the terminal phosphoryl group of nucleoside tri- and diphosphates.

* This work was supported by the Institut Pasteur, the CNRS, the French Government’s Investissement d’Avenir program, Laboratoire d’Excellence “Integrative Biology of Emerging Infectious Diseases” Grant ANR-10-LABX-62-IBIED, and Fondation pour la Recherche Médicale Grant DEQ20130326538.

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2 The abbreviations used are: eATP, extracellular adenosine triphosphate; eAdo, extracellular adenosine; NTPDase, nucleoside triphosphate diphosphohydrolase; GBS, Group B Streptococcus; TH, Todd Hewitt; Bis-Trit, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; SrtA, sortase A; SrtA*, inactive sortase A; NudP, ecto-5'-nucleoside diphosphohydrolase; NudP*, inactive NudP; rNudP, recombinant NudP; eN, ecto-5'-nucleotidase; UshA, UDP-sugar hydrolase.
(NTP and NDP) to nucleoside monophosphates (NMPs), and the unrelated CD73 enzyme is an ecto-5'-nucleotidase that catalyzes the hydrolysis of phosphate esterified at carbon 5' of the ribose and deoxyribose moieties of the NMP molecules to give the corresponding nucleoside (12).

Recently, functional homologues of CD39 have been identified in a number of microbial human pathogens (14), such as Legionella pneumophila (15–17), and proteins belonging to the CD39 family of ecto-5'-nucleotidase have been identified in Staphylococcus aureus, Bacillus anthracis, and Streptococcus sanguinis (18–21). Inactivation of these bacterial ectonucleotidases impairs virulence but not viability, suggesting that their selective inhibition might be a new therapeutic strategy. Of note, each bacterial nucleotidase harbors specific enzymatic activity compared with the related mammalian enzymes. For instance, the S. aureus AdS A hydrolyzes AMP, ADP, and ATP in contrast to the related mammalian CD73 5'-nucleotidase, which hydrolyzes only AMP (12, 19).

In this study, we identified and characterized a putative ecto-nucleotidase of Streptococcus agalactiae, also known as Group B Streptococcus (GBS). GBS is a Gram-positive commensal bacterium of the human intestine and of the vagina of 10–30% of healthy women. However, GBS may turn into a deadly pathogen in neonates and is the leading cause of neonatal pneumonia, for GBS, erythromycin, 10 μg/ml; kanamycin, 1000 μg/ml; ampicillin, 100 μg/ml. GBS was cultured at 37 °C in Todd Hewitt (TH) broth (Difco, BD Biosciences) without agitation and on TH agar or Columbia agar supplemented with 10% horse blood (BioMerieux). Escherichia coli DH5α (Invitrogen), BLR (a recA derivative of BL21), and XL1 Blue (Stratagene) were grown in Luria-Bertani broth (LB) medium. When specified, antibiotics were used at the following concentrations: for E. coli, ampicillin, 100 μg/ml; erythromycin, 150 μg/ml; kanamycin, 25 μg/ml; for GBS, erythromycin, 10 μg/ml; kanamycin, 1000 μg/ml.

**Experimental Procedures**

**Bacterial Strains and Growth Conditions**—GBS strains used in this study are derivatives of NEM316, a fully sequenced ST-23 serotype III clinical isolate (RefSeq accession number NC_004368.1) (32). GBS was cultured at 37 °C in Todd Hewitt (TH) broth (Difco, BD Biosciences) without agitation and on TH agar or Columbia agar supplemented with 10% horse blood (BioMerieux). Escherichia coli DH5α (Invitrogen), BLR (a recA derivative of BL21), and XL1 Blue (Stratagene) were grown in Luria-Bertani broth (LB) medium. When specified, antibiotics were used at the following concentrations: for E. coli, ampicillin, 100 μg/ml; erythromycin, 150 μg/ml; kanamycin, 25 μg/ml; for GBS, erythromycin, 10 μg/ml; kanamycin, 1000 μg/ml.

**Cloning and Purification of Recombinant NudP (rNudP)—**rNudP (residues 28–656) was produced by first cloning a high fidelity PCR product (Phusion DNA polymerase, Thermo Scientific) obtained using GBS NEM316 genomic DNA as template and primers NdP5 and NdP3 (all primers used in this study are listed in Table 1). The resulting BamHI-EcoRI digestion product was cloned into the pMESS plasmid (a gift of J.-M. Betton, Institut Pasteur), a pMalc-p2x (New England Biolabs) expression product was cloned into the pMESS plasmid (a gift of J.-M. Betton, Institut Pasteur), the resulting pMESS_rNudP plasmid was transformed into E. coli BLR cells with ampicillin selection.

Large scale preparations of periplasmic proteins were performed as described (33). Briefly, overnight culture of BLR + pMESS_rNudP was diluted 100 times in 2 liters of LB medium supplemented with ampicillin and incubated at 30 °C. When the cultures reached the exponential phase (\(A_{600} = 0.6\), expres-
sion of rNudP was induced for 3 h by adding 1 mM isopropyl 1-thio-β-D-galactopyranoside. Cells were harvested (5000 rpm, 10 min, 4 °C), resuspended in ice-cold TSE (25 mM Tris-HCl, pH 7, 20% saccharose, 1 mM EDTA), centrifuged (9000 rpm, 10 min, 4 °C), resuspended in ice-cold H2O, and centrifuged (12,000 rpm, 10 min, 4 °C), and the periplasmic proteins were finally precipitated with ammonium sulfate at 4 °C.

For rNudP purification, the precipitated proteins were dia-
lyzed (Spectro/Por membrane cutoff, 6 – 8 kDa; Spectrum Lab-
oratories, Inc.) against 50 mM Bis-Tris, pH 7 at 4 °C. Chromato-
graphic purification was performed with HiTrap QHP columns
using a 0–30% gradient of 1 M NaCl. Fractions containing
rNudP were pooled and concentrated by ammonium sulfate
precipitation. The proteins were resuspended in 50 mM Bis-
Tris, pH 7, NaCl 100 mM and further purified by gel filtration
(HiLoad 16/60 Superdex 200, GE Healthcare) with a flow rate of
1 ml/min. Protein concentrations were determined by UV
absorption at 280 nm.

**Analytical Ultracentrifugation**—Sedimentation velocity ex-
periments were carried out at 20 °C in an XL-I analytical ultra-
centrifuge (Beckman Coulter). Samples were spun using an
An60Ti rotor and 12-mm double sector epoxy centerpieces.
The partial specific volume of NudP (0.738 ml/g) was esti-
mated from their amino acid sequences using the software Sednterp. The same software was used to estimate the buffer viscosity (η = 1.027 centipoises) and density (ρ = 1.004
g/ml), rNudP (400 μl at 5, 9, and 22 μM) was spun at 42,000
rpm, and absorbance profiles were recorded every 5 min. Sedi-
mentation coefficient distributions, c(s), were determined
using the software Sedfit 14.1 (34). Sedimentation coefficients
were extrapolated to zero concentration by linear regression,
and values are presented for standard conditions (in water at
20 °C).

**Enzymatic Activity Assays**—Phosphatase activity was assayed
by measuring the release of inorganic phosphate (P_i) using the
malachite green reagent following the manufacturer’s rec-
ommendations (Biomol Green, Enzo Life Sciences). The reaction
was carried out at 37 °C in 50 mM Bis-Tris adjusted to different
pH values (between 5 and 8.9) containing various concentra-
tions of nucleotides (from 10 to 500 μM), cofactors, and a 1.5 mM
concentration of the rNudP enzyme. After stopping the reac-
tion with 1 ml of Biomol Green reagent, samples were incu-
bated at room temperature for 20–30 min to allow develop-
ment of the green color. P_i concentrations were determined by
spectrophotometric absorbance measurements at 620 nm
against a standard P_i curve.

Substrate degradation and product formation were followed
by rapid resolution high performance liquid chromatography
(HPLC) using a reverse-phase column (Agilent ZORBAX
Eclipse XDB-C18, 2.1 × 50 mm, 1.8 μm). Enzymatic reactions
were performed at 37 °C in 50 mM Bis-Tris, pH 7.5 containing 5
mM MnCl2, 100–200 μM substrates (NTP, NDP, and NMP
from Sigma), and 1.5 mM rNudP or rNudP* or 0.1 μg of cell wall
extracts (see below for NudP* mutagenesis and cell wall prepa-
ration). Products of the reactions were analyzed every 7 min by
rapid resolution HPLC with a flow rate of 0.25 ml/min and a
linear gradient of 1–12% CH3CN (2–13% CH3CN or 1–90% CH3CN) in 20 mM triethylammonium acetate buffer, pH 7.5.

The low resolution mass spectra were obtained by LC/MS (Agil-
ent 1200 series LC with 6120 MS single quadrupole system)
using an atmospheric electrospray ionization system.

**NudP Mutagenesis**—The conserved NudP motif NHE (resi-
dues 126–128) was changed to AGA (alanine-glycine-alanine)
using a splicing by overlap-extension method as described pre-
viously with slight modifications (35, 36). Briefly, two ~280-bp
PCR products flanking the chromosomal NHE region to be
replaced were amplified with oligonucleotides containing the
desired substitution (left product, primers 483 + 337; right
product, primers 338 + 484). The two PCR products were puri-
fied, mixed, denatured, annealed, and then used as template for
a second PCR with the external primers 483 + 484. The resulting
560-bp product was cloned after EcoRI-BamHI digestion
into the thermosensitive shuttle plasmid pG1 to give the
pG+NudP construct propagated into XL1 Blue *E. coli* (Strat-
agene) with kanamycin or erythromycin selection.

After Sanger sequencing (GATC Biotech) of the insert, plas-
mids were introduced in NEM316 by electroproporation. GBS
transformants were selected on erythromycin at 30 °C for
24–48 h to allow episomal replication of the pG+NudP plas-
mid. To select for pG+NudP* chromosomal integration at the
nudP locus, isolated transformants were plated and further iso-
olated on erythromycin at 37 °C for 24–48 h. Isolated colonies
with a stable integration by a single crossover of the pG+NudP* plasmid into the chromosome at the nudP locus, referred to as
integants, were serially replicated (10−4 dilution) two times a
day in TH broth at 30 °C without erythromycin. An aliquot of
each culture was spread on Columbia agar + 10% horse blood
and cultured at 37 °C, and isolated colonies were tested for their
resistance/susceptibility to erythromycin on TH agar at 37 °C
by replica plating in a 96-well format. Erythromycin-sensitive
colonies have lost the plasmid after a second crossover, leaving
the wild-type (WT) nudP sequence or the nudP* mutation.

From the same parental integrant, analytical PCR was car-
ried out to discriminate between WTBk ("WT back sequence") and
nudP* mutants with primers 334 + 341 (= positive PCR prod-
uct for a WT sequence) and primers 334 + 342 (= positive PCR
product for a nudP* sequence). Isogenic WTBk and nudP*
mutants were further confirmed by Sanger sequencing of PCR
products (Phusion) of the nudP locus with primers 334 + 340
designed to anneal outside the genomic region used for the con-
struction of the substitution cassette. Genomic DNA of the
nudP* mutant was further used to clone and express in *E. coli*
the mutated rNudP* protein after amplification with the prim-
ers Nd55 + Nd3 as described above for the WT rNudP allele.

**nudP Chromosomal Deletion**—To confirm the specificity of
antibodies made against NudP, we constructed a ΔnudP deletion
mutant in the NEM316 WT strain. The deletion construct
was designed to delete 2110 bp of chromosomal DNA, starting
from ~125 bp of the nudP start codon and including 1985 bp of
the 2073-bp nudP ORF, using a splicing by overlap-extension
method as described above with primers 184 + 303 and primers
304 + 187. The second PCR product was obtained using the
external primers 184 + 187, digested by KpnI and HindIII, and
cloned into the thermosensitive shuttle plasmid pG1 to give the
pG+ΔnudP construct. After GBS transformation with
pG+ΔnudP (erythromycin, 30 °C) and selection of chromo-
specific Ecto-5'-nucleosidase Activity of S. agalactiae

Somatic integrants (erythromycin, 37 °C), we selected ΔnudP mutant by screening erythromycin-sensitive colonies obtained after five subcultures at 30 °C by PCR with primers 188 + 189. Sanger sequencing was performed to confirm the deletion of the nudP genomic region. To confirm the NudP cell wall anchorage, we used the previously described inactive sortase A (SrtA*) mutant obtained in an NEM316 WT background (37).

NudP Immunodetection—Cell surface and secreted proteins of GBS were prepared as described previously (38) from 50-ml midexponential growth cultures (A_{600} = 0.6) at 37 °C in TH broth buffered with 100 mM Hepes. Cells were centrifuged; washed once in 50 mM Tris-HCl, pH 7.3; resuspended in 1 mL of osmoprotective buffer (50 mM Tris-HCl, pH 7.3, 20% sucrose, Roche Applied Science Complete protease inhibitors) supplemented with 175 units/mL mutanolysin (Sigma–Aldrich); and incubated for 90 min at 37 °C under constant gentle agitation. After centrifugation (13,500 rpm, 15 min, 4 °C), supernatants containing the cell wall proteins were used for the enzyme activity assays or for NudP immunodetection after SDS-PAGE or were kept frozen at −20 °C for further analysis.

For analysis of secreted proteins, supernatants from the same 50-ml cultures were additionally filtered (Millipore filter; pore size, 0.25 μm) to eliminate residual cells. Secreted proteins were precipitated with trichloroacetic acid (TCA; Sigma) overnight at 4 °C, centrifuged (4000 rpm, 30 min, 4 °C), washed with acetone (Sigma), centrifuged (4000 rpm, 30 min, 4 °C), and finally concentrated 100 times in 500 μL of PBS with NaOH (5 mM) to neutralize TCA.

For NudP immunodetection, rabbit-specific polyclonal antibodies directed against rNudP were obtained from Covalab. Immunoglobulins were purified from total serum with protein A (Covalab) follow by an affinity purification step with immobilized rNudP on columns following the manufacturer’s instructions (AminoLink coupling resin and immobilization kit, Thermo Scientific). Total proteins were quantified by the BSA method (Thermo Scientific), and the equivalent of secreted and cell wall proteins from 10^8 bacterial cells was used for NudP immunodetection. SDS-PAGE (Bio-Tris Criterion XT 4–12% gradient gels, Bio-Rad), protein transfer onto nitrocellulose membranes (Hybond-C, Amersham Biosciences), and chemiluminescence detection (Western Pico chemiluminescence, Thermo Scientific) with horseradish peroxidase (HRP)-coupled anti-rabbit secondary antibodies (Zymed Laboratories Inc.) were done following the manufacturers’ instructions.

GBS Survival in Blood—Animal experiments were performed at the Institut Pasteur animal husbandries (Paris, France) in accordance with the policies of the European Union guidelines for the handling of laboratory animals with additional protocols approval by the Institut Pasteur animal care and use committee (Number 04.118). Whole blood was collected by cardiac puncture of 5-week-old female BALB/c mice into tubes containing anticoagulant (Vacuette Premium, Lithium Heparin Ridged, Greiner Bio-One). The blood of 10 mice was pooled and kept for a maximum of 15 min before use.

Overnight cultures of GBS strains were diluted 1:100 into fresh TH broth and grown at 37 °C to midexponential phase (OD = 0.6). Bacterial cells were centrifuged, washed twice, and diluted in PBS to yield 10^8 cfu/mL. A total of 5 × 10^7 bacteria (50 μL) were mixed with mouse blood (250 μL) supplemented with mock (PBS), adenosine, or guanosine (Sigma) at 150 μM final concentration. Bacteria in blood solutions were incubated at 37 °C under constant gentle agitation. Time-dependent bacterial survival was quantified by plating aliquots on TH agar and enumeration of cfu after 24-h incubation. The percentage of GBS survival was calculated as follows: 100 × (cfu after incubation with blood cells/cfu at time 0). Statistical analysis (unpaired t test) from two independent experiments in duplicate was performed with Prism (GraphPad).

In Vivo Virulence Studies—Neonatal Sprague-Dawley rat pups (2 days old; Janvier, France) were randomized in groups of 10. Animals were inoculated intraperitoneally with a solution containing a total of 5 × 10^5 bacteria in 100 μL of PBS prepared from a midexponential phase (OD = 0.6) culture. Mortality curves were determined from two independent experiments by following animal survival over a 5-day period.

Adult animal infections were performed with 5-week-old female BALB/c mice (Charles River). Mice were injected intravenously via the tail vein with 5 × 10^7 bacteria harvested in late exponential phase (OD = 0.6), washed in PBS, and resuspended in 500 μL. At 24 and 48 h after injection, mouse groups (eight by bacterial strains) were sacrificed. Macroscopic observation of the different organs showed no significant difference between the different groups of animals, and bacterial counts in blood and homogenates of liver, spleen, and brain were determined by plating serial dilutions on TH agar plates. A p value less than 0.01 (unpaired t test) was considered statistically significant.

RESULTS

Identification of the NudP 5'-Nucleotidase in GBS—Bioinformatics analysis of the NEM316 WT strain genome reveals an uncharacterized gene (systematic name, gbs1403 or NCBI NP_735840.1) encoding a putative ectonucleotidase. The corresponding protein was renamed hereafter as NudP following its characterization (see below). nudP is a 2073-bp ORF coding for a 690-amino acid polypeptide containing a signal peptide, a putative cell surface localization motif, and the two typical domains of 5'-nucleotidases (Fig. 1A). The amino-terminal region contains the predicted catalytic site within a metallophosphodiesterase motif (metallophosphatase domain (MPP), pfam00149; domain E-value = 1.1e−10) belonging to a large superfamily of distantly related metallophosphatases (12, 39–41). The NudP carboxyl-terminal motif is typical of the substrate-binding domain of 5'-nucleotidases (pfam02872 domain; E-value = 4.6e−34) (40–42).

NudP Is a Mr2+ -dependent NMP/NDP 5'-Phosphodiesterase—NudP is a member of a widespread 5'-nucleotidase family (EC 3.1.3.5) found in prokaryotes and eukaryotes that can hydrolyze a wide range of substrates (12). Among these substrates are the phosphoric ester bonds of 5'-tri-, 5'-di-, and 5'-monophosphate nucleoside, nucleic acids, and phosphoproteins (12). More specifically, NudP is related to the E. coli UDP-sugar hydrolase (UshA; EC 3.6.1.45) periplasmic 5'-nucleotidase (E-value = 1e−21, 26% identities, 43% similarities on 565 residues), which hydrolyzes 5'-phosphonucleotides and UDP-sugar (42), and to the S. aureus AdsA 5'-nucleotidase.
extracellular fluid, and was dependent on Mn\(^2\+)
was found to be at pH 7.5, a pH close to that of the blood and body’s
range of substrates and conditions. The optimal enzymatic activity
activity was low in the presence of Ca\(^2\+)
but not the predicted native peptide
A
Metallophos (32–293)
\(5\textsuperscript{′}-\text{Nucleotid}_{\text{C}}\) (367–531)
NudP (WT) = \(\text{NHE}_{128}\)
NudP\(^\text{cat}\) = \(\text{AGA}_{128}\)
LPKTN (657–661)

![Figure 1. NudP is a monomeric nucleotidase. A, schematic representation of the NudP protein. White boxes highlight the two typical domains of 5\textsuperscript{′}-nucleotidases: the metallophosphatase domain (Metallophos; residues 32–293, pfam00149) and the substrate-binding domain (5\textsuperscript{′}-Nucleotid\(_{\text{C}}\); residues 367–531, pfam02872). Filled black boxes represent the two transmembrane domains (residues 5–27 and 664–683) necessary for secretion (SP, signal peptide; residues 1–28) and LPKTN cell wall anchoring (inverted black triangle; residues 657–661). The position of the conserved NHE motif essential for the stabilization of the transition state of 5\textsuperscript{′}-nucleotidases is highlighted (white triangle; residues 126–128) as well as its corresponding mutation to AGA in the catalytically inactive (cat\(^\text{\textsuperscript{−}}\)) mutant NudP\(^\text{cat}\). B, analytical ultracentrifugation analysis of rNudP. rNudP (residues 28–656) produced and purified from E. coli is a monomer with an elongated shape. Sedimentation coefficients are expressed in Svedberg units where 1 S = 10\(^{\text{−15}}\) S.
](http://www.jbc.org/content/289/9/5483/F1.large.jpg)

(B-value = \(10^{-3}\), 23% identities, 40% similarities on 493 residues), which degrades nucleoside mono-, di-, and triphosphates (19).

To characterize its enzymatic activity, we expressed and purified a NudP-truncated form in E. coli. rNudP (residues 28–656) contains the metallophosphatase and the substrate-binding domains (Fig. 1A) but not the predicted native peptide signal (residues 1–28) or the cell wallanchoring domain (residues 657–690). rNudP was expressed in the E. coli periplasm to avoid interference with intracellular metabolism and further purified by osmotic shock followed by ion exchange and gel filtration. rNudP was produced as a soluble protein that migrates between the 58- and 80-kDa molecular mass markers in agreement with its theoretical 68-kDa mass, and its purity estimated by SDS-PAGE was greater than 95% (data not shown). Analytical gel filtration showed that rNudP elutes at a volume similar to aldolase (~158 kDa), suggesting a dimerization and/or an elongated shape. To further characterize rNudP, sedimentation velocity experiments were performed, revealing an \(s_{20,w}\) of 4.0 S, a frictional ratio of 1.4, and a calculated mass of 66 kDa in agreement with an extended monomeric conformation (Fig. 1B).

The enzymatic activity of the rNudP protein was tested for a range of substrates and conditions. The optimal enzymatic activity was found to be at pH 7.5, a pH close to that of the blood and body’s extracellular fluid, and was dependent on Mn\(^2\+) with an optimum near 5 mM (\(K_m = 2\) mM) (Fig. 2, A and B). Interestingly, the catalytic activity was low in the presence of Ca\(^2\+) (Fig. 2B): a 78% decrease was observed when using 5 mM Ca\(^2\+) instead of 5 mM Mn\(^2\+)\), and it was undetectable (similar to the background level) in the presence of Mg\(^2\+\), Co\(^2\+)\), or Zn\(^2\+)\) (Fig. 2B).

Thus, the kinetic parameters of rNudP were determined in the presence of 5 mM Mn\(^2\+) at pH 7.5 with the different substrates as illustrated in Fig. 2C with adenosine nucleotides. The highest NudP specific activities were obtained with ribonucleoside 5\textsuperscript{′}-mono- and -diphosphates but not with the corresponding triphosphates (Table 2). Michaels constants for AMP, CMP, GMP, and UMP are between 13 and 56 \(\mu\text{M}\), and the \(k_{\text{cat}}/K_m\) values are between 3 \(\times\) 10\(^3\) and 1.8 \(\times\) 10\(^3\) \text{M}^{-1}\text{s}^{-1}\) (Table 3). NudP activity was not dependent on the presence of a 2\textsuperscript{′}-hydroxyl group on the ribose moiety, although NudP activ-
TABLE 2
Substrate specificities of rNudP

<table>
<thead>
<tr>
<th>Ribonucleotides</th>
<th>NTP</th>
<th>&lt;0.1</th>
<th>&lt;0.1</th>
<th>&lt;0.1</th>
<th>&lt;0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>NDP</td>
<td>9.3±0.6</td>
<td>9.6±0.2</td>
<td>9.8±1.4</td>
<td>12.5±0.9</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>NMP</td>
<td>21.5±3.2</td>
<td>8.5±1.1</td>
<td>21.9±3.4</td>
<td>21.1±4.0</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Deoxyribonucleotides</td>
<td>dNTP</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>dNDP</td>
<td>5.3±0.4</td>
<td>2.5±0.7</td>
<td>4.5±0.5</td>
<td>1.0±0.5</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>dNMP</td>
<td>71.8±5.2</td>
<td>64.7±8.0</td>
<td>50.3±4.8</td>
<td>6.9±0.4</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>2’-dN 3’-MP</td>
<td>ND</td>
<td>ND</td>
<td>&lt;1.5</td>
<td>ND</td>
<td>0.6</td>
</tr>
<tr>
<td>Sugar nucleotides</td>
<td>NDP-glucose</td>
<td>ND</td>
<td>ND</td>
<td>&lt;1.5</td>
<td>ND</td>
</tr>
<tr>
<td>NDP-ribose</td>
<td>3.2±0.9</td>
<td>ND</td>
<td>ND</td>
<td>&lt;1.5</td>
<td>ND</td>
</tr>
<tr>
<td>Miscellaneous</td>
<td>A2pA, Ap3A, NAD, pNPP, 5’-PRPP</td>
<td>&lt;0.1</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

TABLE 3
Kinetic parameters of recombinant rNudP on ribonucleoside 5’-monophosphate

<table>
<thead>
<tr>
<th>Kinetic parameters</th>
<th>kcat (s⁻¹)</th>
<th>kcat/Km (s⁻¹mol⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>35±8</td>
<td>6.27±0.3</td>
</tr>
<tr>
<td>CMP</td>
<td>16±7</td>
<td>0.54±0.03</td>
</tr>
<tr>
<td>GMP</td>
<td>56±14</td>
<td>4.36±0.3</td>
</tr>
<tr>
<td>UMP</td>
<td>13±7</td>
<td>2.18±0.2</td>
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</tbody>
</table>

NudP is a Cell Wall-anchored Enzyme Acting on Extracellular Nucleotides—The GBS strain NEM316 encodes 30 putative cell wall-anchored surface proteins that are covalently attached to the peptidoglycan (32, 38). Cell wall-anchored surface proteins contain a characteristic carboxyl-terminal sorting signal composed of the conserved LPXTG motif followed by a hydrophobic domain and a positively charged tail (43–45). Following secretion, the sorting signal is cleaved between the threonyl and glycyl residues of the LPXTG motif, and the threonyl group is covalently attached to the peptidoglycan polymer of the cell wall. The enzyme catalyzing the protease and transpeptidase reactions is a membrane-associated protein called sortase A (SrtA) (38, 43–45).

NudP is one of the two proteins encoded by NEM316 with a degenerated LPXTG motif (Fig. 1A; LPXTN at position 657); the second is the C5a peptidase ScpB (32, 38). To confirm that NudP is anchored to the cell wall, we performed a Western blot analysis of cell wall extracts and concentrated supernatants from NEM316 WT and srtA* mutant strains with polyclonal antibodies made against rNudP. As shown in Fig. 3A, a large amount of NudP was detected in the cell wall extract of the WT strain but not in that of the srtA* mutant. In contrast, we observed that this protein was only present in the culture supernatant of the srtA* mutant and not in that of the WT strain.

To test the consequence of the absence of NudP enzymatic activity, we constructed a GBS mutant expressing an inactive form of NudP. This NudP* mutant has the same mutations (NHE to AGA) as the rNudP* inactive form used above (Figs. 1A and 2C). The NudP* mutant was obtained by mutating the corresponding codons in the chromosome of the WT NEM316 strain in a two-step allelic replacement process. This procedure allowed us to simultaneously select a nudP* mutant and an isogenic strain with a WT nudP sequence, referred to hereafter as the WTbk control strain (see “Experimental Procedures”).

The expression and localization of the NudP* protein were not affected by the mutations introduced in its gene as seen by Western blot analysis (Fig. 3A). Thus, the nudP* mutant expressed an inactive form of NudP at its surface. In addition, cell wall extracts of the WTbk and of the nudP* and srtA* mutants were incubated with AMP, ADP, or ATP in the presence of Mn²⁺. The cell wall extracts of the WTbk, but not of the nudP* and srtA* mutants, hydrolyzed ADP and AMP but not ATP (Fig. 3B and data not shown). Taken together, these results confirm the activity of NudP, its SrtA-dependent cell wall anchoring, and the absence of other cell wall-localized ecto-5’-nucleotidase activity in the tested conditions.

NudP Catalytic Activity Is Necessary to Escape Blood Clearance and to Colonize Internal Organs—In the blood, extracellular nucleotides regulate the balance between the pro- and anti-inflammatory responses (1, 2, 8). Therefore, to test the biological function of NudP, we determined the survival of GBS strains in fresh blood of naïve mice. For the WTbk bacteria, around 40% of GBS cells were killed within 30–60 min in these conditions (Fig. 4A). The absence of NudP activity increased the bacterial killing rate with only 20% of viable nudP* bacteria, compared with 60% of WTbk bacteria, after 90-min incubation in blood (Fig. 4A). When the blood was supplemented with nucleosides (150 μM adenosine or guanosine), a small but reproducible decrease of bacterial survival was observed (Fig. 4A; 40% viable WTbk bacteria after 60–90-min incubation). Importantly, the increased killing of nudP* compared with the WTbk was abrogated when blood was supplemented with adenosine but not with guanosine, linking the observed phenotype to the absence of NudP enzymatic activity (Fig. 4A).
Because the nudP* mutant was killed more efficiently by blood cells in vitro, we tested its in vivo virulence in two models of infection. First, 3-day-old neonate rats were infected with 5 × 10^8 bacteria by intraperitoneal injections. Although all animals died within 3 days upon infection with the WTbk control strain, the absence of NudP activity was associated with a 50% decrease in overall mortality (Fig. 4B). In a second experiment, BALB/c mice were infected intravenously with 5 × 10^7 bacteria, and blood and organ (brain, liver, and spleen) colonization was monitored at 24 and 48 h postinfection. At 24 h, no significant differences were observed between the WTbk and the NudP* mutant (Fig. 4C) even in the blood where an increased killing of the nudP* mutant was observed in vitro (Fig. 4A). However, 48 h postinfection, a higher number (>1 log) of viable bacteria were recovered in all tested organs of mice infected with the WTbk control strain compared with those infected with the nudP* mutant (Fig. 4A). Overall, these in vivo experiments highlight the importance of NudP for bacterial virulence and organ colonization.

DISCUSSION

The main GBS virulence-associated factors identified to date are secreted and surface-exposed molecules (e.g. capsule, hemolysin, lipoproteins, and cell wall-associated proteins) that mediate interactions with host cells (46–50). Among the 30 GBS proteins covalently linked to the cell wall by an LPXTG-type motif (32), several are directly involved in GBS virulence, such as adhesins and immunomodulators (37, 38, 48, 49). In this study, we report the enzymatic activity of a previously uncharacterized cell wall protein of GBS and its function during pathogenesis in animal models of infection. Although NudP has an imperfect LPXTG motif (the terminal glycine is replaced by an asparagine residue), a sequence also found in the Csa peptidase ScpB (32, 38), we observed that NudP is mainly associated to the cell wall by a mechanism dependent on the sortase A enzyme (43–45). This extracellular localization and the biochemical characterization of the recombinant protein demonstrate that NudP belongs to the ecto-5'-nucleotidase (eN) enzyme family (12). Its specificity is unusual for an eN because it hydrolyzes NMP and NDP but not NTPs and specifically requires Mn^{2+} cations for its activity. This is in marked contrast with human ectonucleotidases, which are divided into two major groups: the eN and the NTPDase enzyme families (12). In mammals, these two unrelated enzyme families act sequentially to hydrolyze tri- and diphosphate nucleosides (mostly by the CD39/NTPDase1 enzyme) and monophosphate nucleosides (mostly by the CD73/eN enzyme). Thus, NudP is clearly an eN enzyme characterized by the metallophosphatase and nucleotide-binding domains but shares some substrate specificity with NTPDase (12).

Interestingly, the apyrase conserved regions corresponding to the active domain of NTPDase are almost ubiquitous in eukaryotes and absent in prokaryotes except Legionella pneumophila (15, 16). In contrast, eNs are widespread in bacteria, but only a few of them have been characterized. The homology between bacterial and eukaryotic 5'-nucleotidases is low, but the domain organization and the key residues for catalytic activity are conserved (39). The periplasmic E. coli UshA protein was the first bacterial 5'-nucleotidase characterized. UshA hydrolyzes UDP-glucose and other nucleotide diphosphate sugars to produce sugar 1-phosphate. The main function of UshA was therefore proposed to be as a metabolic enzyme. Thereafter, it was demonstrated that NTP, NDP, NMP, and nucleotide sugars were also UshA substrates, but the biological function of this enzyme remains unclear (39, 42).

More recently, ecto-5'-nucleotidases in bacterial pathogens have been identified, including the S. aureus AdsA enzyme (18–20). AdsA was first described as an adenosine synthase because of its ability to hydrolyze AMP into adenosine (18),

![FIGURE 3. NudP is a cell wall-associated protein acting on extracellular nucleotides. A, immunodetection of NudP by Western analysis of GBS cell wall and secreted proteins. Specific antibodies directed against purified nudP were used to detect NudP in the cell wall (CW) and in the concentrated culture supernatants (SN) of the NEM316 WT strain, the mutant expressing the inactivated AGA substitution form (NudP*), the corresponding isogenic complemented strain (WTbk), the inactive SrtA* mutant, and the nudP deletion mutant (ΔnudP). Similar amounts of total cell wall and secreted proteins, corresponding to the extraction from 10^8 bacterial cells in midexponential phase in TH broth at 37 °C, were loaded. Shown is a representative experiment of at least three independent experiments. Note that NudP is specifically revealed by two bands migrating closely. As both were absent in the ΔnudP extracts, we assume that the smaller band is the result of proteolysis. B, kinetic analysis of the cell wall-associated phosphatase activity in the NudP* mutant, the WTbk complemented strain, and the SrtA* mutant with AMP and ADP as substrates. Experiments were performed at pH 7.5 in the presence of 0.1 μg of cell wall proteins, 5 mM Mn^{2+}, and 200 μM substrates. Substrate degradation and product formation were followed by rapid resolution HPLC (filled triangles, adenosine; empty squares, AMP; empty circles, ADP).](image-url)
whereas further characterization demonstrated that AdsA is an eN enzyme that also hydrolyzes ADP, ATP, GTP, GDP, and GMP as well as 2',3'-deoxyadenosine 3',5'-monophosphate (19, 20).

Homologues of ecto-5'-nucleotidases are present in several Gram-positive pathogens, including *Enterococcus faecalis*, *Bacillus anthracis*, *Listeria monocytogenes*, *Streptococcus pyogenes* (18), and *Streptococcus sanguinis* (21) but absent in the related human pathogen *Streptococcus pneumoniae*.3

A similarity search or pairwise comparison is not effective to predict the biochemical function of bacterial ecto-5'-nucleotidases. Indeed, a BlastP search indicates that NudP is more similar to *E. coli* UshA than to *S. aureus* AdsA protein. However, these homologies are restricted to key amino acid clusters as observed previously by comparing bacterial and eukaryotic enzymes (12, 39). On the other hand, regions and residues critical for substrate specificity and metal coordination are less conserved, suggesting a specific adaptation of each enzyme. For instance, NudP does not hydrolyze triphosphorylated nucleo-

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3 A. Firon and P. Trieu-Cuot, unpublished observation.
ides and specifically requires Mn$^{2+}$. In contrast, AdsA (Mn$^{2+}$ or Mg$^{2+}$) and UshA (Mn$^{2+}$ or Mg$^{2+}$; stimulated by Co$^{2+}$) display different cofactor specificities and are able to hydrolyze these substrates. In addition, NudP has a more restricted pH range of activity (between 6.5 and 7.5) than AdsA (between 4 and 10) (19). Despite these differences, these enzymes share a common catalytic mechanism. Structure-function analysis of the *E. coli* 5′-nucleotidase provided the first insights into substrate binding and catalysis. Among the residues conserved in eukonucleotidases, Asn$^{116}$ and His$^{218}$ were shown to be involved in the stabilization of the transition state, and mutation of His$^{117}$ to Asn diminishes the activity to 0.04% of the wild-type level (12, 40). The absence of catalytic activity of the NudP$^*$ mutant in blood compared with the WT strain was abolished when an exogenous supply of adenosine was provided. However, when tested in vivo in adult animals after intravenous injections, the number of NudP$^*$ mutant cfu in blood was similar to that in the WT strain 24 h after the infection. The NudP$^*$ defects in blood and organs appeared only at the later time point of 48 h, suggesting that NudP is dispensable at early time points of infection. Because NudP acts on extracellular nucleotides, it highlights the role of these extracellular nucleotides in modulating host responses to bacterial infections (2, 8, 14, 18, 51).

The immune cells and signaling pathways affected by extracellular nucleotides during GBS infections remain to be determined. The function and the subversion of the eATP receptors, the P2X family, during infections are mainly documented for intracellular microbial pathogens (14, 51). In contrast, few studies have addressed the function of eATP/eAdo receptors during infections by extracellular pathogens and hence the biological consequences of modulating the eATP/eADP ratio (14, 51). In mammals, the two main eukonucleotidases, CD39 and CD73, expressed at the surface of immune cells control the eATP/eAdo ratio, and their inactivation is detrimental to the clearance of polymicrobial infections (13, 52). This eATP/eAdo ratio serves as a central hub to control the balance between pro- and anti-inflammatory responses. However, P2X receptors and eATP are dispensable in macrophages for caspase-1 activation by *S. pyogenes* (53). As this bacterium encodes a yet-to-be-characterized NudP homologue, it is likely that the involvement of extracellular nucleotides in controlling other signaling pathways is underestimated. In *S. aureus*, it was proposed that the main activity of the AdsA ectonucleotidase is to synthesize adenosine to dampen the proinflammatory response mediated by neutrophils (18) and very recently to inhibit macrophage recruitment and promote immune cell apoptosis following synthesis of deoxyadenosine (20). These multiple tasks of AdsA might be due to the pleiotropic function of extracellular nucleotides in cell signaling (1, 2, 9–11). A key difference between the *S. aureus* AdsA and the *S. agalactiae* NudP, apart from the pH range and the metal requirement, is the inability of NudP to hydrolyze deoxynucleoside 3′-phosphate. Therefore, although the two bacterial species secrete a nuclease involved in neutrophil extracellular trap degradation (20, 54), the deoxyadenosine 3′-phosphate resulting from DNA degradation can be used as a substrate by AdsA but not by NudP.

In conclusion, our study on NudP highlights the diversity of enzymatic reactions performed by a widespread enzyme family and suggests that this diversity might be related to the adaptation of a given organism to specific hosts or environmental niches. Deciphering the precise mechanism(s) and consequence(s) of GBS manipulation of extracellular nucleotides might help to understand and control infections caused by this extracellular pathogen.

Acknowledgments—We thank Stina Linden and Nina Grau for contributions to the work during Master courses. We also thank Shayanoo Dramsi for constructive discussions in the course of this work.

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Specific Ecto-5'-Nucleosidase Activity of S. agalactiae


5488 JOURNAL OF BIOLOGICAL CHEMISTRY VOLUME 289 • NUMBER 9 • FEBRUARY 28, 2014

Extracellular Nucleotide Catabolism by the Group B Streptococcus Ectonucleotidase NudP Increases Bacterial Survival in Blood

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