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

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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 LPXTN 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

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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-Tris, 2-[bis(2-hydroxyethyl)aminol]-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.
**Specific Ecto-5′-nucleosidase Activity of S. agalactiae**

TABLE 1

| Name | Sequence (5′ to 3′) | Feature
---|---|---
NdP5 | AAGATCCATGCGACCACTGCAGTATGGCGCTTAAGTGTAGGG | BamHI
NdP3 | AAGATCCATGCGACCACTGCAGTATGGCGCTTAAGTGTAGGG | EcoRI
483 | TCTAGAATTTAATTAAAAGTTTTTTTCAGTATTATGAA | EcoRI
337 | CAAACCTCTCATCAATAATTTGCACTCGTTCAAGTTTAAAGTTTTC | KpnI
338 | AGATATGCACAAATATGCGATCTGGTTGACAGA | AIA
484 | TCTAGAATTTAATTAAAAGTTTTTTTCAGTATTATGAA | BamHI
334 | GTGAGAATTTTTTAAACCAAC | HindIII
341 | AACCTCTCATCAATAATTTGCACTCGTTCAAGTTTAAAGTTTTC | AIA
342 | AACCTCTCATCAATAATTTGCACTCGTTCAAGTTTAAAGTTTTC | AIA
340 | ACTCTTTTGAGCTAAGGGTTT | AIA
184 | AGATATGCACAAATATGCGATCTGGTTGACAGA | AIA
303 | GATAGGGATCCATGGACCAAGTCGGCTCCAAGTTATAGGAA | NdP3
304 | AGATATGCACAAATATGCGATCTGGTTGACAGA | NdP5
187 | TCTAGAATTTAATTAAAAGTTTTTTTCAGTATTATGAA | NdP3
188 | ATCCTAGAATTTAATTAAAAGTTTTTTTCAGTATTATGAA | NdP5
189 | TACCTAGAATTTAATTAAAAGTTTTTTTCAGTATTATGAA | NdP5

*Features include restriction sites (underlined), mutations for AGA substitution (lowercase), and composite primers for chromosomal deletion (underlined lowercase).*

(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 CD3 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* AdsA 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 ectonucleotidase 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, meningitis, and septicaemia in high income countries (22–24). Despite early antimicrobial treatment and improvement in neonatal intensive care, up to 10% of neonatal GBS infections are lethal, and 25–35% of surviving infants with meningitis experience permanent neurological sequelae (25).

Innate immunity against GBS represents the critical first-line barrier of host defenses as newborns have a naive adaptive immune system (26–28). Central to this response are host phagocytic cells, including neutrophils and macrophages, whose activities are dependent on the eATP/eAdo ratio. Moreover, innate immunity of the newborns appears polarized toward an anti-inflammatory response due, at least in part, to an elevated eAdo concentration in the cord and neonate bloods (27–30). Given the putative large effects of the eATP/eAdo ratio on the control of bacterial infections, we studied the GBS ecto-5′-nucleoside diphosphate phosphohydrolase (NudP), which was previously identified as one of the major immunoreactive proteins during bovine mastitis (31). We show that NudP hydrolyzes ribo- and deoxyribonucleoside 5′-di- and -monophosphates but not 5′-triphosphates and is localized at the GBS surface. We demonstrate that inactivation of the NudP enzymatic activity increases the clearance of GBS by blood cells, a process dependent on eAdo. However, the loss of NudP activity impacts bacterial virulence and organ colonization in animal models of infection. We conclude that NudP ectonucleotidase activity is involved in the degradation of extracellular nucleotides and subverts the host immune defenses in favor of bacterial survival.

**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) derivative containing the signal sequence of MalE for targeting the recombinant protein into the periplasm. After Sanger sequencing (GATC Biotech), 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 dialyzed (Spectra/Por membrane cutoff, 6 – 8 kDa; Spectrum Laboratories, Inc.) against 50 mM Bis-Tris, pH 7 at 4 °C. Chromatographic 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 experiments were carried out at 20 °C in an XL-I analytical ultracentrifuge (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 estimated 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-1). rNudP (400 µl at 5, 9, and 22 µm) was spun at 42,000 rpm, and absorbance profiles were recorded every 5 min. Sedimentation coefficient distributions, cs(γ), were determined using the software Sédfit 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) using the malachite green reagent following the manufacturer’s recommendations (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 concentrations of nucleotides (from 10 to 500 mM), cofactors, and a 1.5 nM concentration of the rNudP enzyme. After stopping the reaction with 1 ml of Biomol Green reagent, samples were incubated at room temperature for 20–30 min to allow development of the green color. P concentrations were determined by spectrophotometric absorbance measurements at 620 nm against a standard P 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 nM rNudP or rNudP* or 0.1 µg of cell wall extracts (see below for NudP* mutagenesis and cell wall preparation). 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 (Agilent 1200 series LC with 6120 MS single quadrupole system) using an atmospheric electrospray ionization system.

**NudP Mutagenesis**—The conserved NudP motif NHE (residues 126–128) was changed to AGA (alanine-glycine-alanine) using a splicing by overlap-extension method as described previously 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 purified, 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 (Stratagene) with kanamycin or erythromycin selection.

After Sanger sequencing (GATC Biotech) of the insert, plasmids were introduced in NEM316 by electroporation. GBS transformants were selected on erythromycin at 30 °C for 24 – 48 h to allow episomal replication of the pG+NudP* plasmid. To select for pG+NudP* chromosomal integration at the nudP locus, isolated transformants were plated and further isolated 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 integrants, 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 carried out to discriminate between WTbK (“WT back sequence”) and nudP* mutants with primers 334 + 341 (= positive PCR product 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 construction 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 primers NdP5 + NdP3 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

sominal integrants (erythromycin, 37 °C), we selected ∆nudP 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 (A600 = 0.6) at 37 °C in TH broth buffered with 100 mM Hepes. Cells were centrifuged; osmoprotective buffer (50 mM Tris-HCl, pH 7.3, 20% sucrose, OD fresh TH broth and grown at 37 °C to midexponential phase was pooled and kept for a maximum of 15 min before use.

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 × 10^6 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.

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 (metallothiophosphatase domain (MPP), pfam00149; domain E-value = 1.1e−10) belonging to a large superfamily of distantly related metalloproteases (12, 39–41). The NudP carboxyl-terminal motif is typical of the substrate-binding domain of 5’-nucleotidases (pfam02872 domain; E-value = 4.6e−30) (40–42).

NudP Is a Mr25–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
Specific Ecto-5’-nucleosidase Activity of *S. agalactiae*

![Figure 1](http://www.jbc.org/)

![Figure 2](http://www.jbc.org/)

(E-value = 1e⁻¹³, 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) is a monomeric nucleotidase. A, schematic representation of the NudP protein. White boxes highlight the two typical domains of S'-nucleotidases: the metallophosphatase domain (Metallophos; residues 32–293, pfam00149) and the substrate-binding domain (S'-Nucleotid_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 S'-nucleotidases is highlighted (white triangle; residues 126–128) as well as its corresponding mutation to AGA in the catalytically inactive (cat) mutant NudP*. 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 = 10⁻¹³ S.

Quantification of Pi was performed as in S. agalactiae. A, pH optimum for rNudP phosphatase activity. Release of P_i by the action of the rNudP protein is optimal at pH 7.5. Quantification of P_i was performed with Biolumin Green reagents after incubation of 1.5 nM rNudP with 200 μM AMP in the presence of 5 mM Mn⁴⁺ for 30 min. Mean and S.D. (error bars) are calculated from at least three independent experiments.

Thus, the kinetic parameters of rNudP were determined in the presence of 5 mM Mn⁴⁺ 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'-mono- and -diphosphates but not with the corresponding triphosphates (Table 2). Michaelis constants for AMP, CMP, GMP, and UMP are between 13 and 56 μM, and the $k_{cat}/k_{m}$ values are between $3 \times 10^4$ and $1.8 \times 10^5$ s⁻¹ M⁻¹ (Table 3). NudP activity was not dependent on the presence of a 2'-hydroxyl group on the ribose moiety, although NudP activ-
NudP Is a Cell Wall-anchored Enzyme Acting on Extracellular Nucleotides

The expression and localization of the NudP* protein were reproducible in recombinant NudP* 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).

**TABLE 2**

<table>
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<tr>
<th>Substrate</th>
<th>A</th>
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<td>ND</td>
<td>ND</td>
<td>0.5</td>
<td>1.0</td>
</tr>
</tbody>
</table>

The V_{max} and K_{m} (mean ± S.D.) were obtained from double reciprocal plots of initial velocity measurements with at least five different concentrations of AMP, CMP, GMP, and UMP. The K_{m} (s⁻¹) was calculated assuming a molecular mass of 68 kDa.

NudP is one of the two proteins encoded by NEM316 with a degenerated LPXTG motif (Fig. 1A; LPKTN 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.

We then tested 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.

**TABLE 3**

<table>
<thead>
<tr>
<th>Kinetic parameters of recombinant rNudP on ribonucleoside 5’-monophosphate</th>
<th>K_{m}</th>
<th>K_{cat}</th>
<th>k_{cat}/K_{m}</th>
</tr>
</thead>
<tbody>
<tr>
<td>AMP</td>
<td>35 ± 8</td>
<td>6.27 ± 0.3</td>
<td>1.8 × 10⁶</td>
</tr>
<tr>
<td>CMP</td>
<td>16 ± 7</td>
<td>0.54 ± 0.03</td>
<td>0.3 × 10⁶</td>
</tr>
<tr>
<td>GMP</td>
<td>56 ± 14</td>
<td>4.36 ± 0.3</td>
<td>0.7 × 10⁶</td>
</tr>
<tr>
<td>UMP</td>
<td>13 ± 7</td>
<td>2.18 ± 0.2</td>
<td>1.6 × 10⁵</td>
</tr>
</tbody>
</table>

The K_{m} (μM), K_{cat} (s⁻¹), and K_{cat}/K_{m} (s⁻¹·μM⁻¹) were obtained from double reciprocal plots of initial velocity measurements with at least five different concentrations of AMP, CMP, GMP, and UMP. The K_{m} (s⁻¹) was calculated assuming a molecular mass of 68 kDa.

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**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).
Specific Ecto-5'-nucleosidase Activity of S. agalactiae

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 × 10⁸ 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 × 10⁶ 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 C5a peptidase ScpB (32, 38), we observed that NudP is mainly associated with 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 Mn²⁺ cations for its activity. This is in marked contrast with human euctonucleotidases, 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),
whereas further characterization demonstrated that AdsA is an eN enzyme that also hydrolyzes ADP, ATP, GTP, GDP, and GMP as well as 2'-deoxyadenosine 3'-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'-nucleotidas. 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-

3 A. Firon and P. Trieu-Cuot, unpublished observation.

FIGURE 4. The NudP enzymatic activity contributes to GBS survival in blood and colonization of internal organs. **A**, NudP contributes to GBS survival in blood. 5 × 10^7 bacterial cells of the nudP* mutant (white bars) or the isogenic WTbk complemented strain (black bars) were incubated at 37 °C with fresh blood of BALB/c mice supplemented with 150 μM adenosine (+ Ado) or guanosine (+ Gua). Aliquots were taken at the indicated time points (30 and 60 min), and bacterial survival was calculated after 24-h incubation on an agar plate as the total number of cfu at a given time versus the number of cfu at time 0. Mean and S.D. (error bar) are calculated from two independent experiments in duplicate, and statistical significance is indicated by asterisks (unpaired t test; ***, p < 0.001; **, p < 0.01). **B**, NudP contributes to GBS virulence in neonatal animals. 5 × 10^6 bacterial cells of the nudP* mutant (empty circles) or the isogenic WTbk complemented strain (filled squares) were inoculated intraperitoneally into 2-day-old neonatal Sprague-Dawley rats. Animal survival was followed for 5 days, and the mortality curve is the result of two independent experiments with 2 × 10 animals inoculated with bacterial strains. **C**, NudP contributes to colonization of internal organs. 5 × 10^7 bacterial cells of the nudP* mutant (empty circles) or the isogenic WTbk complemented strain (filled squares) were injected intravenously into 5-week-old BALB/c mice. At 24 and 48 h postinfection, groups of eight mice were sacrificed, and organ colonization was quantified by cfu counting. One representative experiment is shown with statistical significance indicated by asterisks (unpaired t test; ***, p < 0.001; **, p < 0.01).
sides 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 ectonucleotidases, 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* form with an AGA substitution of the NHE motif supports this model.

By analyzing the NudP* inactive mutant, we found that this enzymatic activity is necessary for GBS survival in blood and contributes to organ colonization in animal models of infection. In particular, we observed that the increased killing 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/eADo ratio (14, 51). In mammals, the two main ectonucleotidases, 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 AdS $5'$-nucleotidase 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 AdS might be due to the pleiotropic function of extracellular nucleotides in cell signaling (1, 2, 9 — 11). A key difference between the S. aureus AdS 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 AdS 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.

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45. Tao, A., Disson, O., Bezaire, K.,看上去的文本是中文，这里可能是一个错误。


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Arnaud Firon, Marcia Dinis, Bertrand Raynal, Claire Poyart, Patrick Trieu-Cuot and Pierre Alexandre Kaminski

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