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NadN and *e* (P4) Are Essential for Utilization of NAD and Nicotinamide Mononucleotide but Not Nicotinamide Riboside in *Haemophilus influenzae*

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Haemophilus influenzae **has an absolute requirement for NAD (factor V) because it lacks almost all the biosynthetic enzymes necessary for the de novo synthesis of that cofactor. Factor V can be provided as either nicotinamide adenosine dinucleotide (NAD), nicotinamide mononucleotide (NMN), or nicotinamide riboside (NR) in vitro, but little is known about the source or the mechanism of uptake of these substrates in vivo. As shown by us earlier, at least two gene products are involved in the uptake of NAD, the outer membrane lipoprotein *e* (P4), which has phosphatase activity and is encoded by *hel*, and a periplasmic NAD nucleotidase, encoded by *nadN*. It has also been observed that the latter gene product is essential for *H. influenzae* growth on media supplemented with NAD. In this report, we describe the functions and substrates of these two proteins as they act together in an NAD utilization pathway. Data are provided which indicate that NadN harbors not only NAD pyrophosphatase but also NMN 5′-nucleotidase activity. The *e* (P4) protein is also shown to have NMN 5′-nucleotidase activity, recognizing NMN as a substrate and releasing NR as its product. Insertion mutants of *nadN* or deletion and site-directed mutants of *hel* had attenuated growth and a reduced uptake phenotype when NMN served as substrate. A *hel* and *nadN* double mutant was only able to grow in the presence of NR, whereas no uptake of NMN was observed.**

Haemophilus influenzae, a gram-negative facultative anaerobic bacterium, is responsible for significant morbidity and mortality in young children (9, 35). In order to cultivate *H. influenzae*, complex medium is required, and if it is not blood based, it must contain two growth factors: nicotinamide adenine dinucleotide (NAD) and hemin (6). Early biochemical investigations established that nicotinamide mononucleotide (NMN) and nicotinamide riboside (NR) can substitute for NAD, whereas nicotinamide, niacin, or other nicotine-based intermediates of the Preiss-Handler pathway cannot (10, 20, 31). The NAD dependency of *H. influenzae* was confirmed by the absence of the genes encoding the enzymes necessary for the de novo biosynthesis of NAD (8). Accumulation of nicotinamide nucleotides derived from NAD or NR has been demonstrated in *H. influenzae* and *Haemophilus parainfluenzae* (4, 11). For *H. parainfluenzae* the K_m for transport is about 0.55 μM for NAD and 0.14 μM for NR, while the V_{max} for NR is about four times that of NAD (4). This implies that NR is the substrate for an as-yet-unidentified inner membrane transporter, a proposal that is supported by the observation that NAD cannot be taken up into the cytosolic compartment as an intact molecule. Limited NAD salvage capacity resides within the *H. influenzae* cytosol, which can be demonstrated if cell extracts are incubated with NR or NMN, indicating the presence of an NMN adenylyl transferase or an NAD pyrophosphorylase activity (5, 16).

In other bacteria, NAD is degraded into NMN or NR prior to uptake. In *Salmonella enterica* serovar Typhimurium, an inner membrane-associated NAD pyrophosphatase is present with its activity expressed in the periplasm (7). The encoding gene is *pnuE* (22), and PnuE is needed to utilize NAD and to produce NMN. NMN is transported across the cytoplasmic membrane via two independent routes. One system resembles that of an active transport, consisting of two gene products encoded by *pnuC* and *nadR*. PnuC was characterized as an integral membrane protein essential for transport (37), and NadR was characterized in several ways. It is a repressor protein involved in feedback regulation coupled with de novo biosynthesis of NAD (14, 24), and it participates in NAD uptake mechanisms acting on PnuC (37), but it is nonessential (38). Recently, it was shown that in *Escherichia coli* NadR itself has NAD pyrophosphorylase activity (25). In addition, a second NAD transport route exists, in which a membrane-bound NMN glycohydrolase, presumably an inner membrane protein facing the periplasm, effects the release of nicotinamide, which is then assumed to diffuse across the inner membrane (for a review, see reference 23).

Recently, our investigators presented data showing that two gene products appear to be involved in the NAD utilization pathway of *H. influenzae* (26). The genes were identified as the *hel*-encoding outer membrane lipoprotein *e* (P4) and a periplasm-encoded gene product termed NadN. Knockout mutations of both genes resulted in growth-deficient phenotypes

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TABLE 1. Relevant strains and plasmids used in this study

Strain or plasmid	Phenotype or genotype[a]	Reference or source
Strains		
H. influenzae Rd KW20	Genome determined	8
R906	NTHi	3
REI1010	Strain Rd (*nadN*::*cat*)	26
REI1012	Strain Rd (Δ*hel*::*kan*) Kan^r	This study
GK02	Strain Rd (hel_{D86L}), with exchange of D to L on aa position 86; Cm^r	This study
GK03	Strain Rd (hel_{D84A}), with exchange of D to A on aa position 84, Cm^r	This study
GK04	Strain Rd (*nadN*::*cat* Δ*hel*::*kan*) Cm^r Kan^r	This study
Plasmids		
pACYC177	Amp^r Kan^r	New England Biolabs
pACYC184	Cm^r Tet^r	New England Biolabs
phel1	*Eco*RI fragment of ATCC GhiGU90 into pBKS	28
pGK01	pACYC184, Cm^r *hel*$^+$ (*cat* in *Swa*I site)	This study
pSEhel	pACYC184, Cm^r *hel*$^+$	This study
pSEΔhel	pACYC184, Cm^r Δ*hel*	This study
pSEΔhelkan	pACYC184, Kan^r/Cm^r Δ*hel*	This study
pSE2	pACYC177, Kan^r HI0205$^+$ *nadN*::*cat*	26

[a] aa, amino acid.

which were dependent on the NAD concentrations provided in the growth medium. The enzymatic activities of both proteins were partially characterized. It was shown by Reidl, Reilly, and colleagues (26–28) that *e* (P4) is an acid phosphatase, while NadN-enriched protein fractions have NAD pyrophosphatase activity. NadN is thought to be identical to a 64-kDa periplasmic NAD pyrophosphatase described earlier (16). In nontypeable *H. influenzae* (NTHi), *nadN* was also identified but was named *nucA*. The protein product of *nucA* possessed a 5′-nucleotidase activity that acted on 5′-phosphorylated nucleosides, e.g., adenosine monophosphate (AMP) (36). Green and coworkers showed that *e* (P4) and NadN proteins are antigenically highly conserved among both typeable *H. influenzae* and NTHi isolates (12, 13, 36), perhaps indicating the physiological importance of these proteins (36).

In this report, we define the functions of NadN and *e* (P4) in the NAD uptake pathway of *H. influenzae*. We present data demonstrating that NadN is identical to NucA and that this protein has the ability to act as an NAD pyrophosphatase as well as an NMN 5′-nucleotidase. Furthermore, we provide data indicating that *e* (P4) also acts as an NMN 5′-nucleotidase, and deletion or point mutants in *hel* affect the growth of *H. influenzae* and the uptake of NAD and NMN. Finally, a *nadN hel* double mutant was constructed and shown to be unable to utilize NAD or NMN and only able to survive when NR was provided in the growth medium.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. All plasmid constructs were cloned in *E. coli* strains XL-1 and LE392 (New England Biolabs, Frankfurt, Germany). Reference strain *H. influenzae* Rd KW20 was obtained from A. Wright (Tufts University, Boston, Mass.), and strain R906 (3) was from A. Smith (University of Missouri, Columbia). These strains were used for the construction of mutants in *hel* and *nadN* (Table 1). *H. influenzae* was grown at 37°C under aerobic conditions on 3.8% brain heart infusion (BHI) agar (Difco Laboratories, Detroit, Mich.) supplemented with NAD (15 to 30 μM) and hemin-chloride (20 μg/ml) (Sigma, Deisenhofen, Germany). *H. influenzae* mutants REI1010 (*nadN*::*cat*), REI1012 (Δ*hel*::*kan*), GK02 (hel_{D86L}), and GK03 (hel_{D84A}) were grown on BHI agar supplemented with NMN (30 μM). The double mutant GK04 (*nadN*::*cat* Δ*hel*::*kan*) was grown on NR (30 μM). Antibiotics were used for *H. influenzae* and *E. coli* as follows: chloramphenicol (Cm), 2 and 30 μg/ml, respectively; kanamycin (Kan), 10 and 50 μg/ml; ampicillin (Amp), 6 and 100 μg/ml (Sigma). Plasmids used are listed in Table 1. Plasmids were isolated according to the Qiagen kit protocol (Qiagen, Hilden, Germany).

Construction of *H. influenzae* mutant strains REI1012 (Δ*hel*::*kan*), GK02 (hel_{D86L}), GK03 (hel_{D84A}), and GK04 (*nadN*::*cat* Δ*hel*::*kan*). All primers used for cloning and constructions were synthesized by MWG-Biotech, Ebersberg, Germany. For construction of strain REI1012, a chromosomal DNA fragment encoding *hel* (HI0693) along with adjacent gene sequences was generated by PCR from strain Rd chromosomal DNA with primers hel-5′ and helEcoRV-3′ (Table 2). PCR amplification was carried out with the Thermoprime PCR kit (Advanced Biotechnologies, Hamburg, Germany), using the protocol of Mullis and Faloona (21). Primer helEcoRV-3′ contained an *Eco*RV site for further subcloning. After PCR amplification, a 3.1-kbp DNA fragment containing bp 736123 to 739333 of the Rd genome (8) was purified, digested with *Eco*RV and *Bam*HI (a *Bam*HI site is located at bp 736160 on the chromosome, 37 bp in from the 5′ end of the PCR fragment), and ligated into *Bam*HI- and *Eco*RV-digested pACYC184 (30). The resulting construct was named pSEhel (Fig. 1A). The *hel* gene was deleted in plasmid pSEhel by inverse PCR using primers designed to contain flanking *Hpa*I sites, ΔP4HpaI-5′ and ΔP4HpaI-3′ (Fig. 1A; Table 2). The resulting 6.35-kbp amplicon was digested with *Hpa*I and then religated to obtain plasmid pSEΔhel. An aminoglycoside 3′ phosphotransferase gene (*kan*), derived from pACYC177 (29), was isolated as a *Hin*cII and *Stu*I fragment and ligated into the *Hpa*I site of pSEΔhel, resulting in plasmid pSEΔhelkan (Fig. 1A). This plasmid served as the template for the amplification of the Δ*hel*::*kan* region by PCR with primers helScaI-5′ and a pACYC184-specific primer, *Bam*HI-3′ (Table 2). The resulting 3.5-kbp DNA fragment was transformed into *H. influenzae* Rd according to the method of Tomb et al. (33). Kan^r transformants were selected on BHI agar containing hemin (20 μg/ml), NMN (30 μM), and kanamycin (10 μg/ml), and the resultant strain was designated REI1012 (Δ*hel*::*kan*). Strain construction was verified by PCR and Southern (data not shown) and Western blot analyses (Fig. 2B).

A D86L point mutation was constructed in *hel*, resulting in mutant strain GK02. A purified *cat* gene, encoding chloramphenicol acetyltransferase derived from pACYC184 (18, 30), was digested with *Sna*BI and *Fsp*I and ligated into the *Swa*I site 7 bp downstream of the *hel* stop codon in pSEhel. The resulting plasmid, pGK01, was digested with *Eco*RV and *Sna*BI, releasing a 2.5-kbp DNA fragment encoding the *cat* gene and 393 bp of the *hel* gene. This was transformed into *H. influenzae* Rd with Cm^r transformants selected on BHI agar containing hemin (20 μg/ml), NMN (30 μM), and chloramphenicol (2 μg/ml). The resulting mutant strain (GK01) encoded *cat* downstream from the 3′ end of *hel* and in the same orientation (Fig. 1B). Two PCR products were generated from the *hel* gene of strain GK01, each encoding an *Xho*I restriction site at either the 5′ or the 3′ end. Normally, no *Xho*I site can be found within *hel*; however, by changing the sequence motif GATGAA at bp 256 to 261 to an *Xho*I site, CTCGAG, the aspartate at position 86 is replaced with leucine. The *hel* 5′ PCR product (1,550 bp) (Fig. 1B), containing the promoter region and the N terminus of *hel* with a

TABLE 2. Primers used for strain constructions

Primer	Sequence (5′ to 3′)
hel-5′	TTGAGCTCTTGTTCCAGCTTTCACAGGCT
helEcoRV-3′	TCGATATCACAAATGCGCTATTCTGACGGG
ΔP4Hpal-5′	CCGTTAACTTGAGGGGCTAAGCTCAGTT
ΔP4Hpal-3′	GGGTTAACGGGTATAGTAAGTCTTTCTG
BamHI-3′	TAAGGGGATGCGTCCGGCGTA
helBamHI5′	GGATTCATATGCACGCGGT
P4XhoI3′	AGTCTCGAGTAAATCAGCCACAAGCGCTTT
P4XhoI5′	TTACTCGAGACTATGTTAGACAACAGCCCT
P4cat3′	TGGTTTCATCCAACGACGACAACG
D84A	GCGGTTGTGGCTGCTTTAGATGAAACTATGTTAG
HI0206-L′	TAATCCCTTACCAATAGGAG
HI0206-R′	ACGCGCTATTTTTAGCCTAT

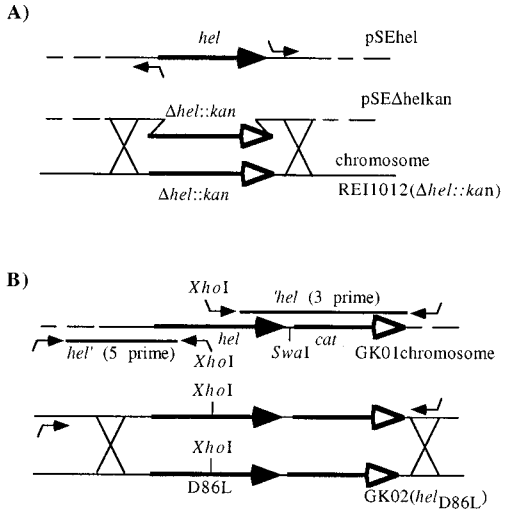

FIG. 1. Construction of *H. influenzae* mutants. (A) REI1012 (Δ*hel*::*kan*); (B) GK02 (hel_{D86L}). Restriction enzymes were *Xho*I, *Swa*I, *Sal*I, and *Kpn*I. DNA crossover constructions are indicated by crossing, encoding regions are indicated as solid arrows, and genes encoding antibiotic resistance are indicated as arrows with open arrowheads; for details see the text.

3′ *Xho*I site, was generated with primers helBamHI5′ and P4XhoI3′ (Table 2). The *hel* 3′ PCR product (1,800 bp; Fig. 1B) was generated with primers P4XhoI5′ and P4cat3′ and started at the 5′ *Xho*I site, contained the C terminus of *hel*, and ended downstream of the *cat* cassette (Table 2). Both *hel*-5′ and *hel*-3′ were digested with *Xho*I and ligated, and the ligation product was PCR amplified with helBamHI5′ and P4cat3′. The amplicon was purified and transformed into REI1012 (Fig. 1B). Cm^r transformants were selected on BHI agar containing hemin (20 μg/ml), NMN (30 μM), and chloramphenicol (2 μg/ml) and replicated to BHI agar containing kanamycin (10 μg/ml). Cm^r and Kan^s colonies were isolated and purified, and the resultant strain, GK02, was verified by PCR and DNA sequence analysis (data not shown). This D86L *hel* point mutation (strain GK02) was characterized by Western blot analysis (Fig. 2B) and phosphatase assays (see Fig. 4).

A D84A point mutation was differently constructed in *hel*, resulting in mutant strain GK03. Wild-type *hel* cloned into the *Eco*RI site of pBluescript and designated phel1 (27) was used as the substrate for PCR mutagenesis. Site-directed mutations were generated using the QuickChange site-directed mutagenesis kit (Stratagene, Amsterdam, The Netherlands) according to the manufacturer's instructions. The primer D84A, containing the degenerate nucleotide (indicated by underlining, Table 2), and the reverse complement primer were used for PCR amplification of plasmid phel1. PCR-mutated phel1 was restricted with *Dpn*I to remove nonmutated phel1, prior to transformation into *E. coli* DH5α. Transformants selected on Luria-Bertani agar containing ampicillin were assayed for phosphomonoesterase activity as described earlier (28). *E. coli* clones lacking phosphomonoesterase activity were isolated and mutated phel1 was recovered (Wizard Plus Miniprep kit; Promega). A 1.3-kb *Hinc*II fragment containing the *cat* gene was cloned into a *Swa*I site 3′ to the *hel* open reading frame in mutant phel1 plasmids. *cat*-containing phel1 plasmid, designated phel1cat, was transformed into DH5α and selected on Luria-Bertani agar containing ampicillin and chloramphenicol. Subsequently, the mutant *hel* gene associated with *cat* was excised from mutant phel1cat with *Eco*RI and transformed into *H. influenzae* strain R906. Transformants were selected on chocolate agar containing chloramphenicol. Selected transformants were analyzed for phosphomonoesterase activity and for the presence of *e* (P4) by Western blot analysis (data not shown). Subsequent transformation of chromosomal DNA from strain R906, containing the *hel* point mutation (D84A) and *cat* gene, into strain REI1012 (Δ*hel*::*kan*) resulted in a Kan^s Cm^r Rd hel_{D84A} mutant, GK03. This strain was also tested for phosphomonoesterase activity (see Fig. 4B) and the presence of the *e* (P4) antigen by Western blot analysis (Fig. 2B).

Plasmid pSE2, containing *nadN*::*cat* (26), was used for the construction of GK04 (*nadN*::*cat* Δ*hel*::*kan*) in strain Rd. A 2.8-kbp *nadN*::*cat* DNA fragment was PCR amplified with primers HI0206-L′ and HI0206-R′ (Table 2) and was transformed into *H. influenzae* strain Rd REI1012. Cm^r colonies of strain GK04 were isolated and purified on BHI agar containing hemin (20 μg/ml), NR (30 μM), and chloramphenicol (2 μg/ml). Isolated clones were verified by PCR and Southern and Western blot analyses (data not shown).

Nicotinamide nucleotide reagents. β-NAD, β-NMN, and AMP were obtained from Sigma. NR was prepared by incubating β-NAD with shrimp alkaline phosphatase in shrimp phosphatase buffer according to the manufacturer's instructions (Amersham Pharmacia Biotech, Freiburg, Germany). Carbonyl-[^{14}C]NAD was obtained from Amersham, and [^{14}C]NMN was prepared from carbonyl-[^{14}C]NAD by treatment with snake venom nucleotide pyrophosphatase (Sigma). [^{14}C]NR was prepared by incubating carbonyl-[^{14}C]NAD, snake venom nucleotide pyrophosphatase, and alkaline phosphatase in alkaline phosphatase buffer for 1 h at 37°C. Enzymes were inactivated by adding 5% trichloroacetic acid, followed by 10-min incubation on ice. Subsequently, the supernatants were recovered after centrifugation (5 min, 16,000 × *g*) and neutralized to pH 7 with 6 N NaOH.

Isolation of OMP and periplasmic protein extracts. Outer membrane proteins (OMPs) were prepared according to a modified (26) protocol of Carlone et al. (2). Samples were kept in HEPES (10 mM) and glycerol (5%) and were stored at −20°C. For the preparation of periplasmic extracts, overnight cultures (5 ml) of strain Rd or REI1010 were harvested by centrifugation and washed once with Tris-HCl (50 mM, pH 8.5) at 4°C. The pellets were resuspended in 200 μl of Tris-HCl (50 mM, pH 8.5) containing polymyxin B (2 mg/ml) and incubated for 10 min on ice. Cells were then centrifuged (20,000 × *g*, 4°C, 10 min) and the supernatant was recovered. These extracts are referred to as periplasmic extracts (15).

Purification of recombinant NadN (NucA) and *e* (P4) protein. Recombinant NadN (NucA), originally derived from nontypeable *H. influenzae* (NTHi) was purified from *E. coli* INVF′(pPX691) as previously described (36). The purified recombinant protein was dialyzed into phosphate-buffered saline (pH 7.2) and

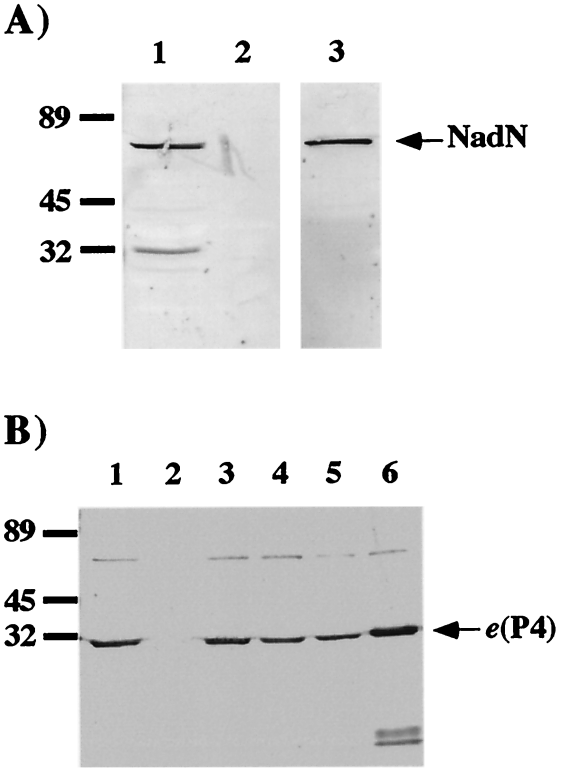

FIG. 2. Western blot analysis of NadN and *e* (P4) of *H. influenzae* Rd. (A) Detection of NadN in periplasmic extracts. Protein sizes (in kilodaltons) are as indicated. Lane 1, Rd extract; lane 2, extract of REI1010 (*nadN*::*cat*); lane 3, purified NadN. (B) Detection of *e* (P4) in OMP extracts. Lane 1, Rd extract; lane 2, extract of REI1012 (Δ*hel*::*kan*); lane 3, extract of REI1012 (Δ*hel*::*kan*) complemented with *e* (P4) on plasmid pJRP4; lane 4, GK02 (hel_{D86L}) extract; lane 5, GK03 (hel_{D84A}) extract; lane 6, purified *e* (P4). Samples were adjusted to a protein concentration of 20 μg/ml and subjected to SDS–12% PAGE.

stored frozen at −20°C. The recombinant *e* (P4) protein used for this study was isolated as described earlier (28).

Protein analysis. Protein concentrations of OMP extracts, periplasmic extracts, and purified recombinant proteins were determined according to the method of Bradford (1), using the Bio-Rad protein assay kit (Bio-Rad, München, Germany). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (19). After SDS-PAGE, proteins were detected by Western blot analysis, according to the methods of Towbin et al. (34), with monoclonal antibodies (MAbs) directed against *e* (P4) and NadN, respectively, as described earlier (12, 36).

Characterization and quantification of NAD pyrophoshatase and 5′-nucleotidase activities. Purified NadN (NucA) (0.12 mg of protein/ml) was incubated with 0.1 μM specific radioactive labeled substrate in shrimp alkaline phosphatase buffer for 1.5 h at 37°C. The enzymatic activity of periplasmic extracts was determined by incubating 1 mg of extract/ml with sample buffer (50 mM $MgCl_2$, pH 8.5) and 0.01 to 0.1 μM concentrations of radioactive ^{14}C-labeled substrate for 1.5 h at 37°C. Reactions were terminated by adding 5% trichloroacetic acid, followed by 10-min incubation on ice. Subsequently, the supernatants were recovered after centrifugation (5 min, 10,000 rpm) and NaOH (6 N) was added to neutralize the samples to pH 7. Aliquots of 2 μl were subsequently analyzed by thin-layer chromatography (TLC). Purified *e* (P4) protein was incubated with [^{14}C]NAD, [^{14}C]NMN, or [^{14}C]NR (a 0.1 μM concentration of each) in HEPES buffer (10 mM, pH 6) for 1.5 h at 37°C. OMP extracts were incubated with sample buffer (50 mM $MgCl_2$, pH 6.0) and [^{14}C]NAD, [^{14}C]NMN, or [^{14}C]NR (each at a 0.1 μM concentration) for 1.5 h at 37°C. Samples were then treated as described for NadN (see above).

Activity of NadN against NMN, NAD, and AMP and of *e* (P4) against NMN was determined by measuring the release of inorganic phosphate from these substrates as previously described (27, 36). The NadN K_{ms} for AMP, NAD, and NMN were determined by using a Lineweaver-Burk double-reciprocal plot based on individual substrate saturation curves. The *e* (P4) K_m for NMN was determined by using the computer-based method of Brooks (1a).

TLC. Radioactively labeled samples were separated by TLC in a solvent system consisting of 1 M ammonium acetate (pH 5) and ethanol (70:30) (17) using Cellulose F plates (Merck, Darmstadt, Germany). After separation, the plates were dried and exposed to radiation-sensitive film (Eastman Kodak Co., Rochester, N.Y.). Spots were identified by comparison with reference samples of [^{14}C]NAD, [^{14}C]NMN, and [^{14}C]NR.

Uptake studies. *H. influenzae* strains from overnight cultures were inoculated in BHI (3.8%) medium supplemented with NR (30 μM) and hemin chloride (20 μg/ml) and grown to an optical density at 490 nm (OD_{490}) of ≈1. After centrifugation (4,000 × *g*, 5 min) the pellets were resuspended in BHI (3.8%) medium without supplements to an OD_{490} of ≈2. Aliquots of 3 ml were incubated on a heating block to 37°C, and radioactively labeled substrates (1 μM concentration) were added; 500-μl samples were removed after 1, 3, 5, 7, and 9 min. The samples were filtered through ME 25 filters (0.45-μm pore size; Schleicher & Schuell, Dassel, Germany) which had been soaked in 0.1 M NaCl. The filters were washed with 5 ml of NaCl (0.1 M) and placed in vials containing 5 ml of scintillation liquid (Emulsifier-Safe; Packard, Dreieich, Germany). Radioactivity was measured with an SL 6000SC scintillation counter (Beckman, München, Germany). Results are expressed as the percent total cellular accumulation of ^{14}C-labeled metabolite, compared to 100% of the ^{14}C-labeled substrate provided in the assay. Results (means) are shown together with the standard deviation from at least three independent experiments.

RESULTS

Characterization of enzymatic activities of NadN. Recently, two new gene loci have been described in typeable *H. influenzae* and NTHi, *nadN* and *nucA*, respectively (26, 36). The *nadN* gene product, NadN, was shown to possess NAD pyrophosphatase activity localized to periplasmic extracts (26). Independently, NucA was purified from an NTHi strain and shown to be a 5′-nucleotidase (36). By comparison of the nucleotide sequences, it was apparent that both proteins were encoded by the same gene.

To demonstrate further that NadN and NucA were the same, periplasmic extracts were isolated from strains Rd and REI1010 and Western blot analysis was performed with a NucA-specific MAb (36). The MAb recognized NadN as a protein of approximately 64 kDa (Fig. 2A). Additional weak bands of about 30 kDa observed only in the Rd extract may have arisen from NadN degradation by copurified proteolytic enzymes in the periplasmic extracts.

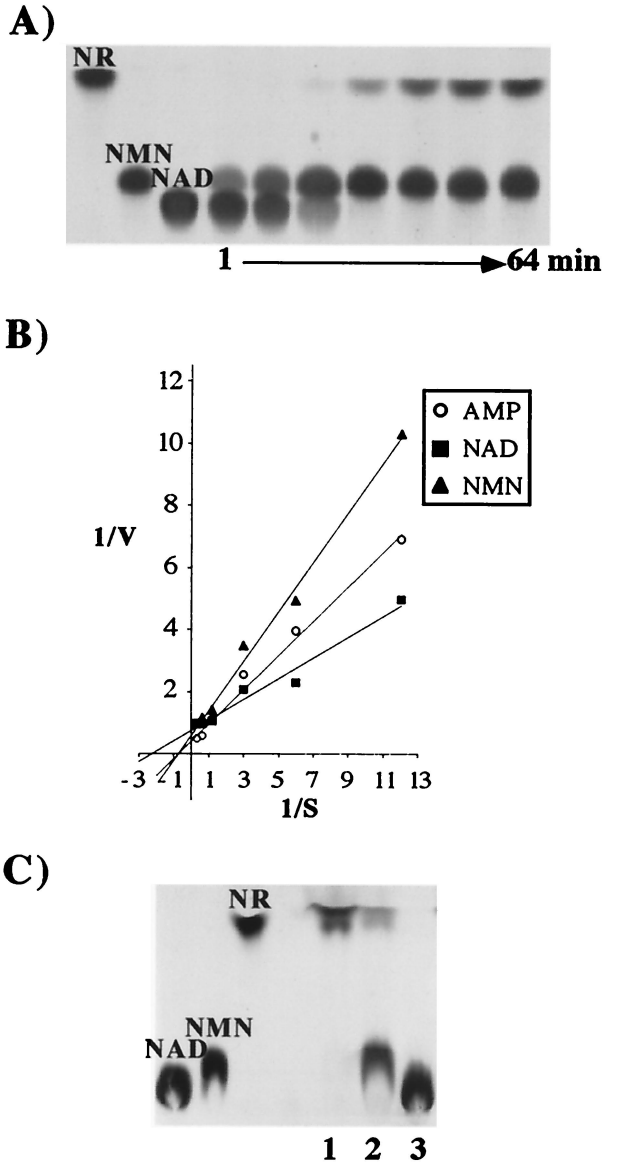

FIG. 3. Enzymatic characterization of NadN. (A) Kinetic analysis of NAD processing by NadN. [^{14}C]NAD (0.1 mM) was incubated with 0.012 mg of NadN/ml. Sampling was done at the indicated time points. Lanes 1 to 7 correspond to reaction times of 1, 2, 4, 8, 16, 32, and 64 min. (B) Determination of K_m values of purified NadN for the substrates NAD, NMN, and AMP; V was determined as change in OD_{660} over 20 min with 0.16 μg of protein. Reaction mixtures were prepared as described earlier (36). (C) TLC of [^{14}C]NAD used as substrate for Rd periplasmic extracts. [^{14}C]NAD (10 nCi; 0.1 mM) was incubated with purified NadN (lane 1), periplasmic extracts of *H. influenzae* Rd (lane 2), and REI1010 (lane 3).

In enzymatic characterization experiments, purified NucA exhibited not just 5′-nucleotidase but also NAD pyrophosphatase activity. Purified NucA sequentially released NMN and then NR from NAD (Fig. 3A). Purified NucA was further assayed to compare the enzyme activity of NAD with alterna-

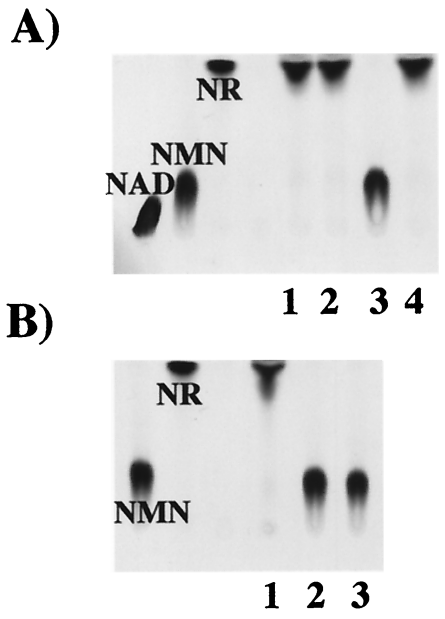

FIG. 4. Enzymatic characterization of *e* (P4). (A) TLC of [^{14}C] NMN (10 nCi; 0.1 μM) incubated with purified *e* (P4) (lane 1) and outer membrane extracts of strains Rd (lane 2), REI1012 (lane 3), and REI1012 (lane 4) complemented with pJRP4. (B) Activity of *e* (P4) phosphatase point mutants, GK02 and GK03. [^{14}C]NMN (10 nCi; 0.1 μM) was incubated with Rd (lane 1), GK02 (lane 2), and GK03 extracts (lane 3).

tive substrates, NMN and AMP. Lineweaver-Burk double-reciprocal plots were used to determine K_m values for each substrate (Fig. 3B). NAD, NMN, and AMP exhibited K_m values of 0.43, 1.26, and 1.09 mM, respectively.

The ability of periplasmic extracts of strains Rd and REI1010 to metabolize [^{14}C]NAD to [^{14}C]NMN and [^{14}C]NR was determined by TLC. The periplasmic extract of Rd hydrolyzed [^{14}C]NAD to [^{14}C]NMN and [^{14}C]NR (Fig. 3C, lane 2), whereas the extract from the *nadN* mutant had no activity against [^{14}C]NAD (Fig. 3C, lane 3).

Characterization of *e* (P4). The OMP *e* (P4) is an acid phosphomonoesterase (26, 27) involved in NAD utilization. To determine a substrate specificity for *e* (P4), purified *e* (P4) and OMP preparations from Rd and REI1012 were incubated with ^{14}C-labeled NAD, NMN, and NR. As shown by TLC (Fig. 4A), NMN was dephosphorylated to NR by purified *e* (P4) and by Rd, but not REI1012, OMP fractions. The absent 5′-nucleotidase activity in the REI1012 OMPs was complemented by *hel* on plasmid pJRP4. Neither the purified *e* (P4) nor Rd OMP fractions possessed the ability to use NAD or NR as substrates (data not shown). Results from kinetic characterization of *e* (P4)-mediated hydrolysis suggest that initial velocity is linearly proportional to enzyme concentration and that the concentration of released inorganic phosphate is directly proportional to the time of incubation for the first 30 min of the reaction at 37°C. The estimated K_m and V_{max} of the *e* (P4)-mediated hydrolysis of NMN was 0.318 mM and 0.033 mmoles of P_i released/min/mg of *e* (P4), respectively. Assays were performed in which substrate concentration [S] were varied from 0.03 to 14.1 times the K_m and V varied from 0.01 to 1.01 times the V_{max} (data not shown).

Growth phenotypes of *H. influenzae* Rd, REI1012 (Δ*hel*::*kan*), and GK04 (*nadN*::*cat* Δ*hel*::*kan*). We previously demonstrated that growth of a *hel* transposon mutant, *hel*::Tn*I0d-bla*, was dependent on the concentration of NAD provided in the growth medium (26). To confirm the role of *e* (P4) in the utilization of NAD, a *hel*-deficient mutant (REI1012) was constructed. The growth of Rd and REI1012 on BHI agar plates supplemented with hemin with various concentrations of NAD, NMN, and NR was tested (Fig. 5).

Compared with Rd, REI1012 had significantly reduced growth with limiting concentrations of NAD (1.5 μM) or NMN (1.5 μM) but had similar growth with high concentrations of NAD (35 μM) and NMN (15 μM) (Fig. 5). However, REI1012 was able to grow as well as strain Rd even with NR, and even when the concentrations were limiting (Fig. 5).

To investigate whether *e* (P4) and NadN are the sole proteins that are able to process NMN to NR, a double mutant, GK04, was constructed. GK04 was unable to grow on BHI agar containing either NAD or NMN at 1.5 or 35 μM (Fig. 5). However, in the presence of NR, GK04 grew as well as strain Rd, even at the low NR concentration (1.5 μM) (Fig. 5).

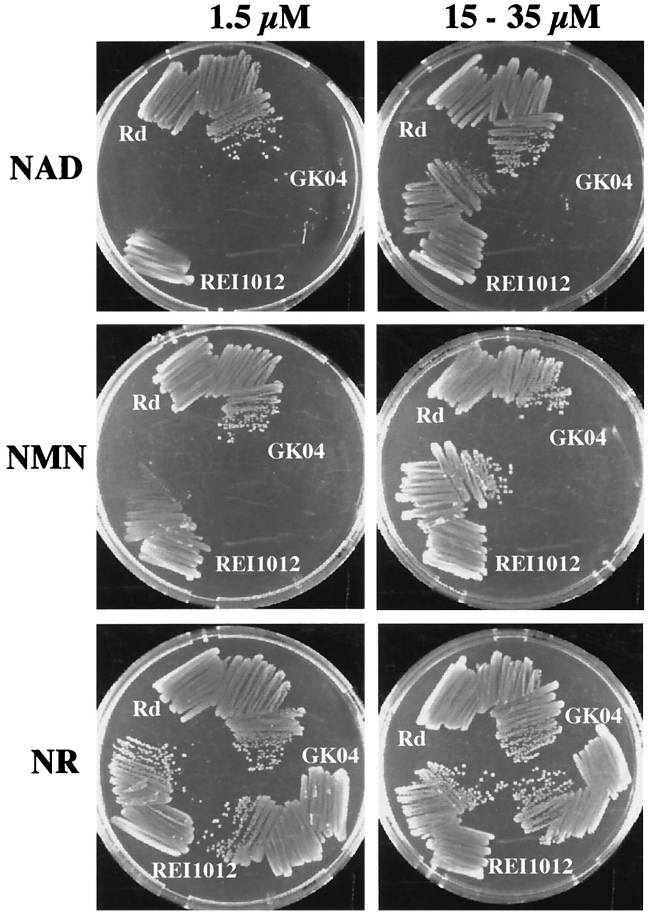

FIG. 5. Growth of mutant *H. influenzae* Rd strains. The strains Rd, REI1012 (Δ*hel*::*kan*), and GK04 (*nadN*::*cat* Δ*hel*::*kan*) were grown on BHI agar plates supplemented with hemin (20 μg/ml) and different nicotinamide nucleotide concentrations and sources. Growth phenotype is shown with 1.5 and 35 μM NAD, with 1.5 and 15 μM NMN, and with 1.5 and 15 μM NR, as indicated.

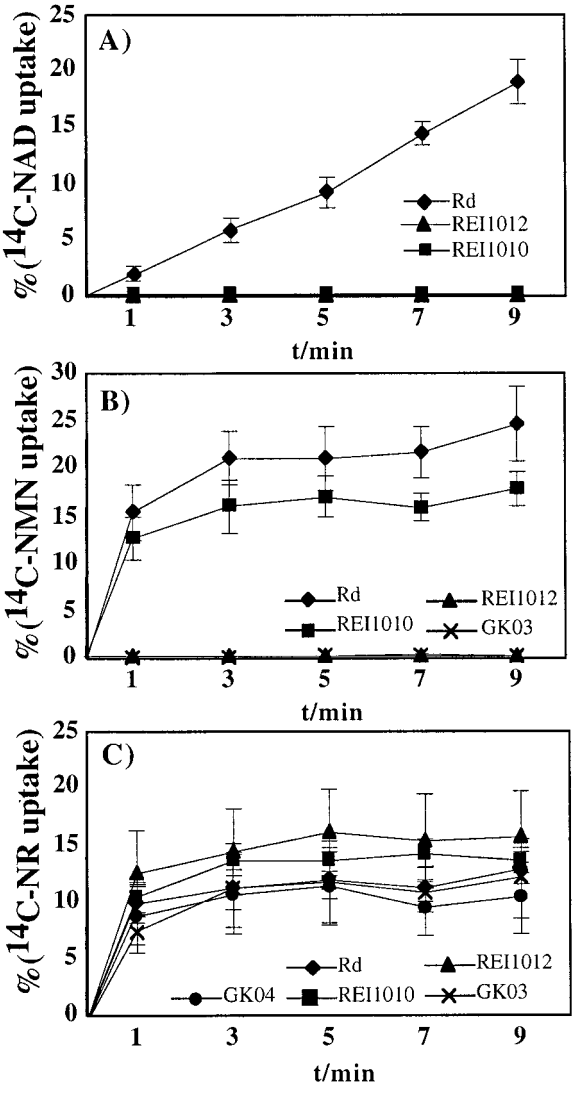

FIG. 6. Uptake of labeled nicotinamide nucleotide substrates by *H. influenzae*. Strains used were *H. influenzae* Rd, REI1010 (*nadN*::*cat*), REI1012 (Δ*hel*::*kan*), GK03 (hel_{D84A}), and GK04 (*nadN*::*cat* Δ*hel*::*kan*). Each point represents the mean value with standard deviation obtained from at least three independent measurements. Uptake is given as the percentage of the initial 1 μM substrate concentration. (A) Uptake of [^{14}C]NAD by Rd, REI1010, and REI1012; (B) uptake of [^{14}C]NMN by Rd, REI1010, REI1012, and GK03; (C) uptake of [^{14}C]NR by Rd, REI1010, REI1012, GK03, and GK04.

Uptake of ^{14}C-labeled nicotinamide nucleotides. The uptakes of ^{14}C-labeled NAD, NMN, and NR by Rd and the various mutants were compared. Rd incorporated about 20% of the available ^{14}C-labeled NAD and NMN within 9 min. REI1010 and REI1012 were unable to take up [^{14}C]NAD (Fig. 6A). Accumulation of [^{14}C]NMN was not observed for REI1012 and decreased uptake was detected for REI1010 (Fig. 6B). Rd, REI1010, REI1012, and GK04 all showed similar accumulations of [^{14}C]NR, at approximately 11 to 15% (Fig. 6C).

Characterization of GK02 (hel_{D86L}) and GK03 (hel_{D84A}). The surface location of *e* (P4), originally described by Green et al. (12), led to the question of whether *e* (P4) acts only as a phosphomonoesterase or possesses other functions necessary for nicotinamide nucleotide utilization, e.g., involvement in substrate binding or transport. To test for other functions, two phosphatase-negative *e* (P4) point mutants, GK02 and GK03, were constructed. These point mutants are predicted to lack acid phosphatase activity, as the mutations modify two of the four conserved aspartates in group C phosphatases (32). The expression of the mutated *e* (P4) was verified by Western blot analysis (Fig. 2B, lanes 4 and 5). The expression of hel_{D86L} was decreased about 30-fold compared to that of hel_{D84A} and wild-type *hel*, perhaps due to instability, decreased expression, or insufficient translocation of the mutated protein. The protein concentrations of these samples were adjusted to yield equivalent concentrations in Western blotting and enzymatic assays.

To verify the loss of the phosphomonoesterase activity of the mutated *e* (P4) proteins, OMP preparations of Rd, GK02, and GK03 were incubated with NMN. As shown by TLC, the membrane fractions of GK02 and GK03 were not able to dephosphorylate NMN (Fig. 4B, lanes 2 and 3). In contrast, the Rd membrane extracts readily hydrolyzed NMN (Fig. 4B, lane 1). OMP preparations of GK02 and GK03 were also unable to hydrolyze pNPP (data not shown). GK02 and GK03 showed attenuated growth on NAD and NMN, comparable with that of REI1012 (Fig. 5), but all strains grew well if NR was the nicotinamide nucleotide source (data not shown).

To confirm the observed phenotypes with the acid phosphatase mutants, we performed uptake studies with [^{14}C]NMN and [^{14}C]NR, comparing Rd with REI1012 and GK03. There was no measurable uptake of ^{14}C-labeled NAD or NMN by GK03, as was seen with REI1012 (Fig. 6A and B). Rd, REI1012, and GK03 all incorporated 12 to 15% of the available [^{14}C]NR (Fig. 6C). We conclude that the phosphatase-negative mutants behave exactly like strain REI1012 in [^{14}C]NAD and [^{14}C]NMN uptake, in their growth, and in their ability to dephosphorylate NMN.

DISCUSSION

Recently, we described two gene products, *e* (P4) and NadN, both involved in the utilization of NAD (26). Individually constructed knockout mutations of both genes gave rise to mutants with growth deficiencies on media containing various concentrations of NAD. We demonstrated that the *nadN* mutant was unable to grow on NAD-supplemented media and had no detectable NAD pyrophosphatase activity (26). Subsequently, a gene in NTHi was characterized and called *nucA*. NucA was purified to homogeneity (36), and its activity was defined as a 5′-nucleotidase acting on phosphorylated nucleosides, especially monophosphate nucleosides (AMP, CMP, GMP, UMP, and TMP) (36). The NucA amino acid sequence matched that of the open reading frame HI0206 (NadN) of strain Rd with nearly complete identity (36); thus, NucA in NTHi and NadN in Rd are encoded by the same gene. Therefore we have renamed *nucA* as *nadN*, for NAD nucleotidase in *H. influenzae*.

In this study we showed that both NAD and NMN are substrates for purified NadN. NadN was shown to exhibit NAD nucleotidase activity with hydrolysis of NAD to NMN and dephosphorylation of NMN to NR. Consequently, loss of NadN function causes a complete inability to utilize NAD.

Furthermore, the *nadN* mutant does not take up as much NR derived from NMN as Rd. However, no difference in accumulation of NR was observed. Earlier, it was postulated that NR might serve as the only nicotinamide nucleotide substrate that is able to cross the cytoplasmic membrane by active transport (4). For *nadN* mutants to be able to accumulate NR derived from NMN, another enzyme must also have NMN 5′-nucleotidase activity. A potential enzyme was the outer membrane lipoprotein *e* (P4), which was recently identified as an acid phosphatase encoded by *hel* (26, 27). We had already demonstrated that dephosphorylation of pNPP by *e* (P4) is strongly inhibited by NMN (26), indicating that NMN might serve as an *e* (P4) substrate. Furthermore, a *hel* knockout mutant had reduced growth if NAD concentrations were low. Therefore, we reasoned that *e* (P4) could be involved in some step in the processing and utilization of NAD.

To test whether NMN, as an intermediate in NAD uptake, is a substrate for *e* (P4), TLC analyses were performed to identify NMN-specific phosphatase activity. The substrates used were ^{14}C-labeled NAD, NMN, and NR. Purified *e* (P4) protein and *e* (P4)-containing OMP fractions were able to catalyze the dephosphorylation of NMN and, subsequently, release of NR was detected. No activity was observed if NAD or NR was added as a substrate (data not shown). A second approach was to quantify the enzyme kinetics, and we determined the Michaelis constant of *e* (P4) for NMN. A third approach addressed the uptake of NAD, NMN, and NR and the ability to use these substrates for growth by comparing the wild type and the *hel* mutant. The uptake analyses indicated that the Δ*hel* strain had lost the ability to utilize NR from NAD and NMN (substrate concentrations used corresponded to limiting concentrations in growth media) but was able to take up NR. Consequently, the Δ*hel* strain could not grow with limiting concentrations of NAD and NMN, but it grew well with NR. The phosphatase activity of *e* (P4) on NMN appears to be important for NMN utilization and hence indirectly also for the utilization of NAD. Therefore, NAD must first be hydrolyzed to NMN, as the only substrate that *e* (P4) can utilize appears to be NMN. To address whether the *hel* mutant phenotype is solely explained by loss of phosphatase activity, site-directed point mutations in *hel* were constructed by replacing amino acid D residues at position 84 or 86 with A and L, respectively. The growth, substrate uptake, and NMN dephosphorylation of these mutants correlated exactly with that of the Δ*hel* mutant. Therefore, we conclude that only the phosphatase activity of *e* (P4) is involved in NMN utilization.

In conclusion, *e* (P4) and NadN are both able to hydrolyze NMN. Mutants of *nadN* or *hel* are able to grow on high concentrations of NMN (26), but under limiting concentrations the uptake of substrate and growth abilities are reduced. A distinct difference between the *nadN* and *hel* mutants can be described as follows: in transport assays a *hel* deletion mutant is unable to accumulate NR derived from NMN; however, in the *nadN* mutant the accumulation is only reduced but not completely abrogated because of the remaining *e* (P4) function. Consistent with that observation, the K_m for NMN of *e* (P4) is lower (0.318 mM) than that of NadN (1.26 mM). Both results may indicate that *e* (P4) rather than NadN is relevant for production of NR from NMN. As *e* (P4) is an outer membrane-located lipoprotein (12), one can speculate that NMN might be a relevant and available nicotinamide nucleotide source in vivo and is mainly recognized by *e* (P4) as a substrate.

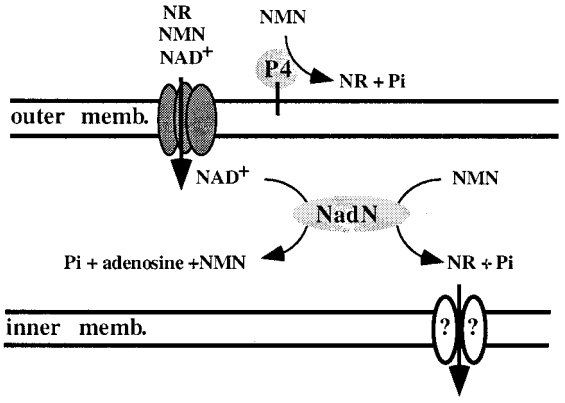

FIG. 7. Model for nicotinamide nucleotide utilization in *H. influenzae*. The outer membrane contains *e* (P4) and a putative diffusion porin, the periplasmic compartment contains NadN, and the inner membrane contains a putative transport complex. The enzymatic activities of the characterized proteins *e* (P4) and NadN, which are illustrated (for details, see text), lead to the uptake of NR.

Since NadN also possesses NMN phosphatase activity, a double mutant of *hel* and *nadN* was characterized to exclude the possibility that any other enzyme of *H. influenzae* participates in the NAD pathway. A double mutant was indeed unable to grow on NMN, even at high concentrations, and was only able to grow with and take up the substrate NR. This result suggests strongly that NR is the final and only substrate which is eventually utilized by *H. influenzae*, as shown in the model (Fig. 7). Based on our deductions from these data, we emphasize that no other enzymatic activity contributes to the release of NR, from either NMN or NAD, and that NR indeed represents the minimal nicotinamide nucleotide requirement and acts as the substrate for an as-yet-unidentified transport system. Both *e* (P4) and NadN are immunodominant, conserved antigens, and both are ubiquitously expressed by typeable and NTHi strains (12, 13, 27, 36). Therefore, it seems predictable that the combined enzyme action of both gene products is needed by the organism to support its parasitic lifestyle and to obtain a broader nicotinamide nucleotide substrate spectrum. We finally conclude that both enzymes, *e* (P4) and NadN, act together in the NAD uptake pathway and are both needed for an efficient processing of NAD and NMN to generate NR.

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