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Nicotinamide Ribosyl Uptake Mutants in *Haemophilus influenzae*

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The gene for the nicotinamide riboside (NR) transporter (*pnuC*) was identified in *Haemophilus influenzae*. A *pnuC* mutant had only residual NR uptake and could survive in vitro with high concentrations of NR, but could not survive in vivo. PnuC may represent a target for the development of inhibitors for preventing *H. influenzae* disease.

Haemophilus influenzae does not have the enzymes necessary for the de novo synthesis of NAD^+ (5, 10) and therefore has an absolute requirement for an exogenous source of factor V (6). Most of the factor V uptake pathway in *H. influenzae* has been characterized (11, 14, 16). The organism in vivo can utilize NAD (NAD^+), nicotinamide (NAm) mononucleotide (NMN), and NAm riboside (NR) as factor V sources, but not the precursor of these, NAm (7). The *e*(P4) outer membrane protein and the NadN periplasmic enzyme convert NAD^+ to NMN and NR (11, 14), and only NR is able to cross the inner membrane to the cytoplasm (5, 11), where NadR recycles it back to NAD^+ (13, 17). Through BLAST analysis, we identified the hypothetical gene HI1077.1 as a paralog of the *E. coli pnuC* gene (NT01EC0901), an NMN transporter in *E. coli* but a putative NR transporter in *H. influenzae*. PCR amplification and resequencing of the HI1077.1 gene region (coordinates 1144355 to 1145045) identified two errors with respect to the original genome annotation and restored a complete open reading frame. We reannotated HI1077.1 as *pnuC*, defined by coordinates 1144355 to 1145035, with nucleotides G and A missing at positions 1144511 and 1144972, respectively. The *H. influenzae pnuC* gene encodes a 226-amino-acid protein with 81.1% similarity to the *Pasteurella multocida* putative PnuC protein (PM1838).

H. influenzae pnuC was disrupted. Two DNA fragments flanking the gene were PCR amplified with primers pnuc-F1 (GGTTCTGCAATAAGTGCG), pnuc-R1 (CAAGGATCCATGATTTTGCCGTTATCG), pnuC-F2 (CTTGGATCCTGCTAACCAAGAATCAGG) (underlining indicates restriction enzyme sites used in subsequent cloning of the PCR products), and pnuC-R2 (AGATCCTGAATTGGTGGG); *Bam*HI digested; ligated together with T4 DNA ligase; amplified by PCR for 15 cycles with primers pnuC-F1 and pnuC-R2; and cloned into the pCR4-TOPO cloning vector (Invitrogen). The resulting plasmid was digested with *Bam*HI, dephosphorylated with shrimp alkaline phosphatase, and ligated with the *Bam*HI-cut kanamycin resistance gene of pUC4k (11, 19). The construct was excised from this plasmid with *Eco*RI and transformed (9) into *H. influenzae* strain Rd-b+ (21). A *pnuC* mutant was isolated on brain heart infusion (BHI) agar containing Levinthals medium (90 μM NAD^+).

To complement the *pnuC* mutant, the *H. influenzae pnuC* gene and its promoter region, including a partial Shine-Dalgarno site (genome coordinates 1144090 to1145083), were amplified by PCR with primers pnuC-E and pnuC-KB (sequences AAAGATATCCAATGCGAAAATGGTCACCTC and AAAGGTACCGGATCCCCTTGGTTTGTCGCTTGTCA, respectively). The *pnuC* gene was cloned as an *Eco*RV-*Bam*HI fragment into plasmid pACYC184 (15), and the construct, designated "pSEpnuC," was transformed into the *pnuC* mutant. Growth of the *pnuC* mutant was compared with that of Rd-b+ on BHI medium supplemented with various NR concentrations. In the presence of 0.05 μM NR, the *pnuC* mutant had reduced growth compared with Rd-b+, but with 0.5 μM NR, it had growth similar to that of Rd-b+ (Fig. 1). That a *pnuC* mutant could be created and was viable in vitro indicated the possibility of alternative routes by which NR gains access to the cytoplasm, albeit only in the presence of elevated NR concentrations. The *pnuC* mutant complemented with pSE*pnuC* had growth similar to that of Rd-b+, even in the presence of low NR concentrations.

The uptake of [^{14}C]NAD^+ and [^{14}C]NR was determined in Rd-b+, in the *pnuC* mutant, and in the *pnuC* mutant comple-

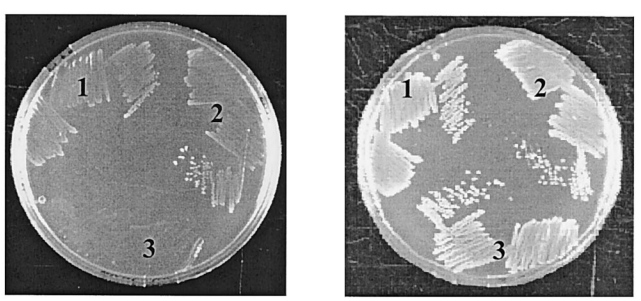

FIG. 1. Growth analysis. Shown is an overnight culture of the following strains on BHI agar plates: 1, Rd-b+; 2, the *pnuC* mutant complemented with pSE*pnuC*; 3, the *pnuC* mutant. (A) BHI agar supplemented with 0.05 μM NR. (B) BHI agar supplemented with 0.5 μM NR.

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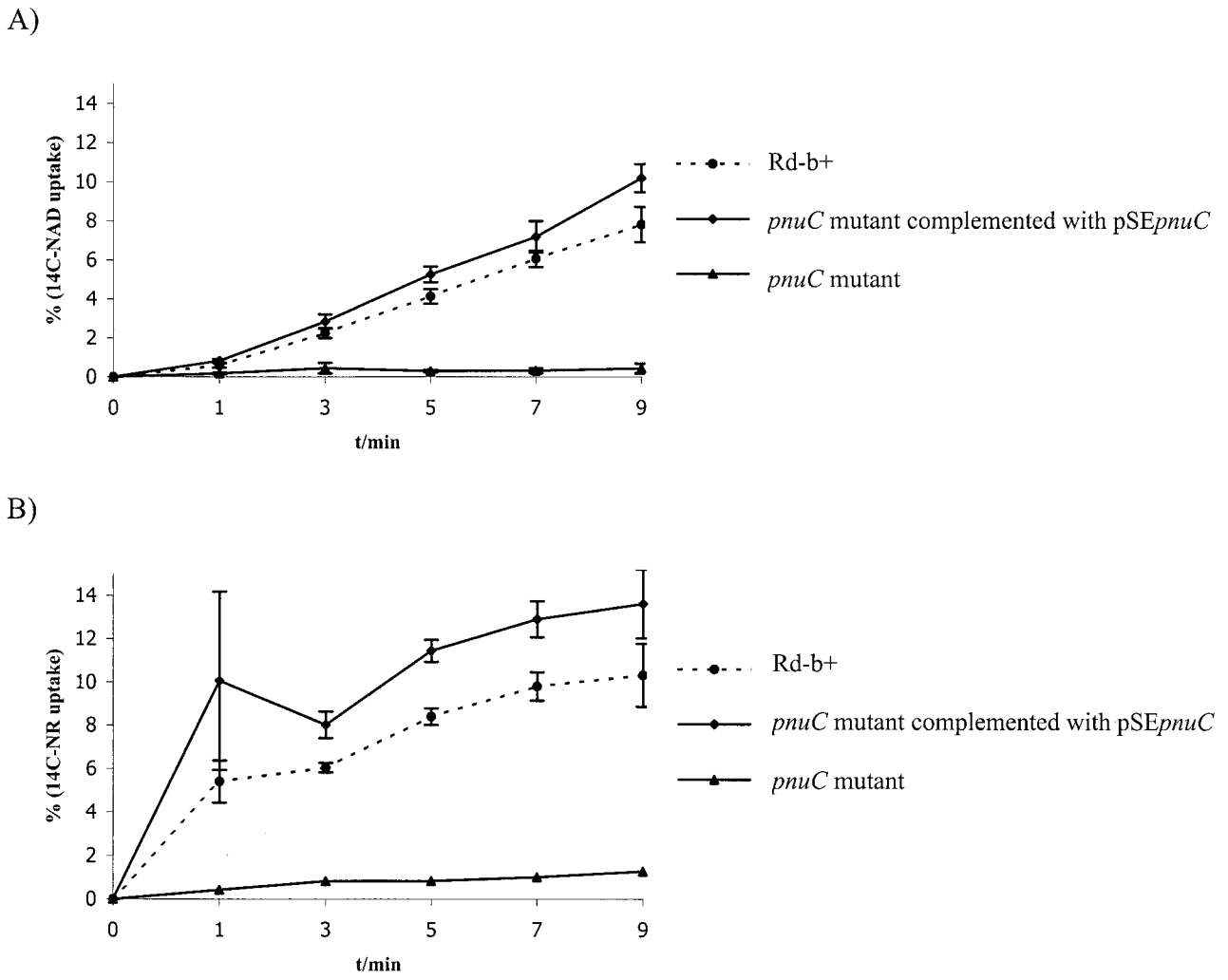

FIG. 2. [^{14}C]NR and [NAD$^+$] uptake by *H. influenzae.* Transport kinetics for 1 μM [^{14}C]NAD$^+$ (A) or [^{14}C]NR (B) in *H. influenzae* strains Rd-b+, the *pnuC* mutant complemented with pSE*pnuC*, and the *pnuC* mutant. All experiments were performed in triplicate. Bars represent the standard deviation.

mented with pSE*pnuC*. The uptake procedure has been reported previously (11). In brief, cells were cultured to an optical density at 490 nm (OD_{490}) of 1, washed, and resuspended in BHI medium to an OD of 2. Samples were incubated with [^{14}C]NAD$^+$ or [^{14}C]NR (1 μM each) (Amersham Pharmacia, Freiburg, Germany), and aliquots were removed at time intervals. Samples were then filtered and washed with an excess volume of NaCl (10 ml [0.1 M]). The ^{14}C uptake was measured in an SL 6000SC scintillation counter (Beckman, Munich, Germany). For both [^{14}C]NAD$^+$ and [^{14}C]NR, the *pnuC* mutant showed a marked decrease in label accumulation compared with that in Rd-b+, an effect that was reversed in the *pnuC* mutant complemented with pSE*pnuC* (Fig. 2). A small increase in label accumulation (up to 1%) could be observed in the *pnuC* mutant over the range of 0 to 9 min, indicating residual uptake ability. Uptake of label derived from [^{14}C]NAD$^+$ was delayed compared with the uptake of [^{14}C]NR, reflecting the dynamics of NAD$^+$ transfer and processing across the outer membrane (1) and degradation to NR (11). Other, possibly low-affinity, NR uptake systems presumably coexist, which may explain the growth of the mutant at high NR or NAD$^+$ concentrations.

To ascertain that the PnuC transporter is required for *H. influenzae* to cause disease in humans, the ability of the mutant to survive in the 5-day old infant rat model was ascertained by competitive index (CI) assay (8). Rats were inoculated with a dual infection of 10^5 CFU of Rd-b+ in combination with either the *pnuC* mutant or the *pnuC* mutant complemented with pSE*pnuC*. Rd-b+ established a bacteremia of $\sim 2 \times 10^6$ CFU/ml in each infant rat ($n = 4$), whereas the *pnuC* mutant did not survive at all (CI < 0.001). The *pnuC* mutant complemented with pSE*pnuC* was partially virulent and produced a bacteremia of $\sim 2 \times 10^5$ CFU/ml (CI = 0.1), indicating that the complemented plasmid-borne *pnuC* partly corrected the deficit produced by disrupting the chromosomal copy of *pnuC* and supporting our contention that the phenotype of the mutant was due to the *pnuC* disruption. Similar results were obtained with the standard infant rat bacteremia model (18; data not

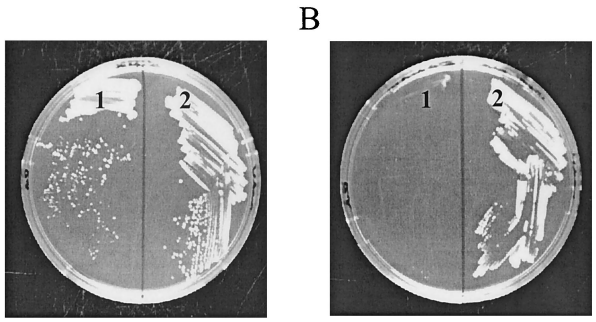

FIG. 3. *nadV*$^+$ growth phenotype of *H. influenzae*. *H. influenzae* mutants were grown for 2 days on a MIc-minimal medium agarose plate supplemented with 0.5 μM NR (A) or 60 μM NAm (B). Section 1, *pnuC* mutant; section 2, *pnuC* mutant complemented with *nadV*$^+$.

shown). The residual uptake ability of the *pnuC* mutant is insufficient to permit survival in vivo, implying that *pnuC* is a potential target for the development of inhibitors that prevent *H. influenzae* disease.

The members of the family *Pasteurellaceae* can be classified into two subgroups (12): the NR-dependent *Pasteurellaceae*, including *H. influenzae*, *Haemophilus parainfluenzae*, *Haemophilus parasuis*, and *Actinobacillus pleuropneumoniae*; and the NR-independent, or NAm-utilizing, *Pasteurellaceae*, including *Pasteurella multocida*, *Mannheimia haemolytica*, *Haemophilus haemoglobulinophilus*, and *Actinobacillus actinomycetemcomitans*. This division depends on whether there is a second mechanism for generating NAD^+, other than NR scavenging. A few NR-independent *Haemophilus* species also exist—for instance, *Haemophilus paragallinarum*, *H. parainfluenzae*, and *Haemophilus ducreyi*—that have been shown to harbor *nadV* (encoding NAm phosphoribosyltransferase) on a plasmid (3, 20). These species can synthesize NMN from NAm and can thus utilize exogenous NAm, which freely diffuses across the membrane. Acquisition of a *nadV*-harboring plasmid could thus transform *H. influenzae* into an NR-independent species. To our knowledge, there are no reports of *H. influenzae* isolates containing *nadV*, but the potential for this phenomenon could undermine a strategy for targeted PnuC inhibition. To examine whether *H. influenzae* could utilize NAm if the uptake of NR were impeded, we complemented the *pnuC* mutant with *nadV* and determined its survival in vivo. A 2-kb *nadV*-containing DNA fragment was PCR amplified from a plasmid preparation derived from *H. ducreyi* strain ATCC 27722 (4, 12) by using primers nadV5EcoRV (TAGATATCAGACTTATG TCTCGGAGTATAACG) and nadV3EcoRV (TTGATATCT CATAGCGTAGTGCGACTAAC). The product was digested with *Eco*RV and ligated into *Eco*RV-cut pACYC184 (15) to yield pSE*nadV*. An *Eco*RV fragment of pSE*nadV* was subcloned into a *Swa*I restriction site of plasmid pSE*hel* (14), to create pSE*helnadV*. The *Swa*I site is located 7 bp downstream of the stop codon of the *hel* gene (HI0693) so that in pSE*helnadV*, *nadV* is subcloned immediately adjacent to *hel* and is flanked by *H. influenzae* DNA preceding HI0694. A *hel-nadV* DNA fragment was amplified from pSE*helnadV* with primers hel5′*Pst*I (AAAACTGCAGCAGAAAGACTTACTATACC CTG) and helEcoRV3′ (TCGATATCACAAATGCGCTATT CTGACGG), and this was transformed into the *pnuC* mutant. Transformants were selected on BHI agar containing only NAm as the factor V source. The *pnuC* mutant complemented with chromosomally integrated *nadV*$^+$ exhibited NR independence when grown on MIc minimal medium (2) (Fig. 3) and was confirmed to have a chromosomal copy of *nadV* (data not shown). We compared the virulence of Rd-b+, the *pnuC* mutant, and the *pnuC* mutant complemented with *nadV* by competitive index (CI) experiments in infant rats. The *pnuC* mutant complemented with *nadV* was as virulent as Rd-b+ (CI = 1), indicating that acquisition of *nadV* permits *H. influenzae* to utilize NAm from host sources during invasive disease.

H. influenzae is defined by its requirement for exogenous NAD^+ or, in absolute terms, its requirement for NR (8, 12, 14). We have demonstrated an NR transport role for PnuC and have supported this by in vitro and in vivo growth analyses and factor V uptake studies. PnuC is therefore a potential target for development of novel methods to prevent disease caused by *H. influenzae*. Acquisition of *nadV* could potentially allow *H. influenzae* to escape the therapeutic effect of a PnuC inhibitor, but, reassuringly, natural *nadV* acquisition has not been described in *H. influenzae*.

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