JOURNAL OF BACTERIOLOGY, Feb. 1991, p. 1311–1320
0021-9193/91/031311-10$02.00/0

Vol. 173, No. 3

Activity of the Nicotinamide Mononucleotide Transport System Is Regulated in *Salmonella typhimurium*

NING ZHU, BALDOMERO M. OLIVERA, AND JOHN R. ROTH*
Department of Biology, University of Utah, Salt Lake City, Utah 84112

Received 21 August 1990/Accepted 29 November 1990

Transport of nicotinamide mononucleotide (NMN) requires two functions, NadI(T) and PnuC. The PnuC protein is membrane associated, as judged by isolation of active Tn*phoA* gene fusions and demonstration that the fusion protein is membrane associated. The PnuC function appears to be the major component of the transport system, since mutant alleles of the *pnuC* gene permit NMN transport in the absence of NadI(T) function. We present evidence that the activity of the NMN transport system varies in response to internal pyridine levels (presumably NAD). This control mechanism requires NadI(T) function, which is provided by a bifunctional protein encoded by the *nadI* gene (called *nadR* by Foster and co-workers [J. W. Foster, Y. K. Park, T. Fenger, and M. P. Spector, J. Bacteriol. 172:4187–4196]). The *nadI* protein regulates transcription of the *nadA* and *nadB* biosynthetic genes and modulates activity of the NMN permease; both regulatory activities respond to the internal pyridine nucleotide level.

Salmonella typhimurium can derive pyridines from exogenous nicotinamide mononucleotide (NMN) by several routes. The first route, which does not require NMN transport, involves cleavage of NMN by a periplasmic glycohydrolase to yield nicotinamide (NM), which can be taken up and converted to NAD by the sequential action of the *pncA*, *pncB*, *nadD*, and *nadE* gene products (2, 15, 17, 18, 22, 23) (Fig. 1). The second route requires transport of intact NMN (10, 17, 30, 31, 46, 51). Internal NMN can be deaminated to yield nicotinic acid mononucleotide (NAMN), which is converted to NAD by the sequential action of the *nadD* and *nadE* gene products (18, 22, 23). Some internal NMN may also be cleaved by an internal glycohydrolase to yield NM (Fig. 1).

Two classes of mutations, *pnuA* and *pnuC*, have been described which fail to use exogenous NMN as a pyridine source. It has been inferred that these mutants fail to transport NMN (17, 30, 46, 51). The *pnuC* gene maps at min 17 of the *Salmonella* chromosome (46, 51) and is located promoter distal to the *nadA* gene in a regulated operon (51). The *pnuC* gene is expressed by both the regulated main promoter of this operon and a weaker constitutive promoter located within the operon (51). The *pnuA* mutations map at min 99, immediately adjacent to *nadI* mutations which eliminate a repressor; this repressor mediates transcriptional control of the *nadB* gene and the *nadA-pnuC* operon (10, 16, 20, 50). Genetic studies and DNA sequencing of the *nadI-pnuA* region indicate that both types of mutations affect a single gene that encodes a bifunctional protein, acting both as a repressor (NadI function) and as a component of the NMN transport system (PnuA function) (17a, 52). We will designate this gene *nadI* and refer to the R (repression) and T (transport) functions. Mutations causing only a defect in transport will be designated *nadI*(R^+ T^-); mutations eliminating both transport and repression will be designated *nadI*(R^- T^-). Mutants defective only in repression will be designated *nadI*(R^- T^+). (Foster and co-workers [17a] refer to this gene as *nadR*.)

In this report, we present evidence that NMN transport is provided by the *pnuC* gene product and that this transport activity is regulated by the *nadI*(T^+) function, probably in response to internal NAD levels. We propose that the bifunctional NadI protein senses internal NAD and controls both transcription of synthetic genes and the activity of the NMN transport system.

MATERIALS AND METHODS

Bacterial strains. All strains used in this study are derived from *S. typhimurium* LT2 and are listed in Table 1. Mu dA refers to a conditionally transposition-defective derivative (24) of the original Mu d1(Lac Ap^r) phage of Casadaban and Cohen which forms operon fusions (5). Mu dJ refers to a transposition-defective mini-Mu phage, Mu d1-1734(Lac Km^r), constructed by Castilho et al. (6). This phage lacks transposition functions and carries kanamycin resistance. Mu dP refers to a transposable P22 prophage flanked by ends of the Mu prophage (49). Tn*10d*(Tc) refers to a small transposition-defective derivative of Tn*10* (Tn*10* Del16 Del17 Tet^r) constructed by Way et al. (48). Tn*10d*(Cm) refers to a transposition-defective derivative of transposon Tn*10* constructed by Elliott and Roth (14).

Media. The E medium of Vogel and Bonner (47), supplemented with 0.2% glucose, was used as the minimal medium. Difco nutrient broth (NB; 8 g/liter with 5 g of NaCl per liter) was used as the rich medium. Difco agar was added at a final concentration of 1.5% for solid medium. Nutrients to feed auxotrophs were included in minimal media at final concentrations described by Davis et al. (12); exceptions are indicated in the text. Antibiotics were added to media at the following final concentrations: ampicillin (sodium salt), 30 μg/ml in NB and 15 μg/ml in E medium; tetracycline hydrochloride and chloramphenicol, 20 μg/ml in NB and 10 μg/ml in E medium; and kanamycin sulfate, 50 μg/ml in NB and 125 μg/ml in E medium. All antibiotics were obtained from Sigma Chemical Co. Media containing ampicillin were always prepared fresh before use. 5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) dissolved in *N,N*-dimethyl formamide (20 mg/ml) was added to media at a final concentration of 25 μg/ml. 5-Bromo-4-chloro-3-indolylphosphate, toluidine salt (X-P), also dissolved in *N,N*-dimethyl formam-

* Corresponding author.

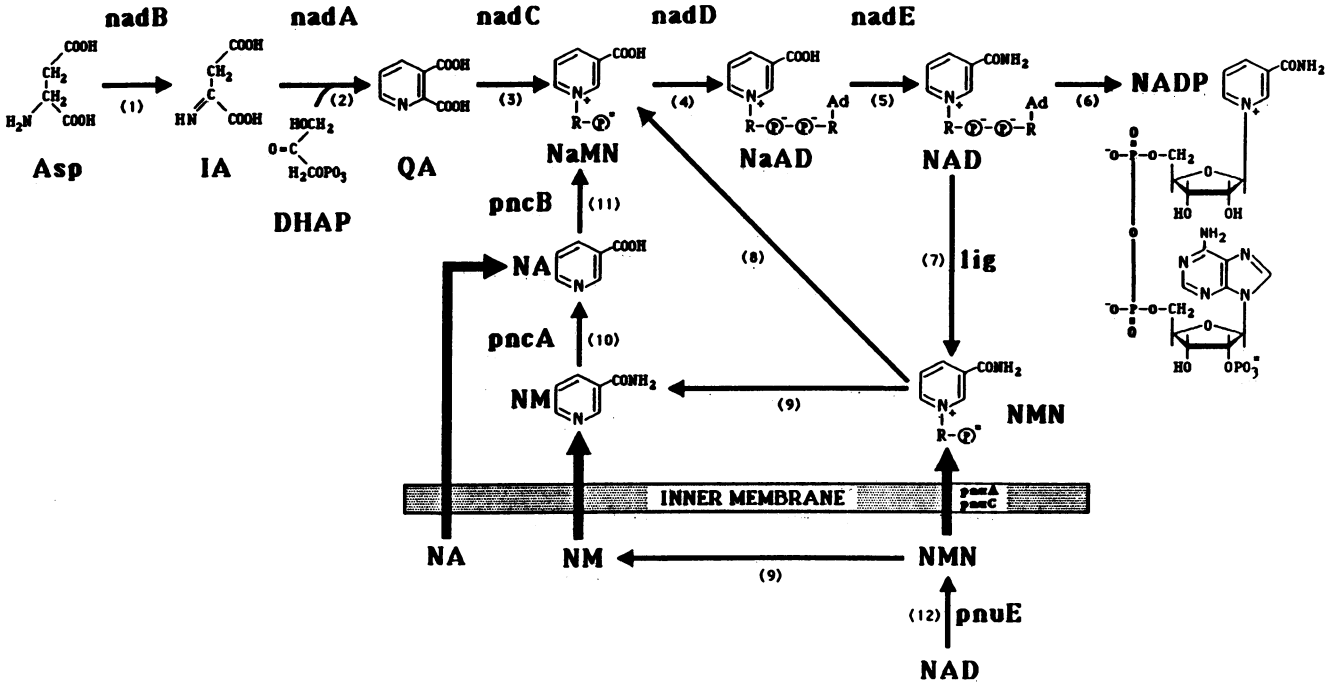

FIG. 1. The NAD metabolic pathway of *S. typhimurium*. The enzymes included are (1) L-aspartate (Asp) oxidase, (2) quinolinic acid (QA) synthetase, (3) quinolinic acid phosphoribosyltransferase, (4) nicotinic acid mononucleotide (NaMN) adenyltransferase, (5) NAD synthetase, (6) NAD kinase, (7) DNA ligase (lig), (8) NMN deamidase, (9) NMN glycohydrolase, (10) NM deamidase, (11) NA phosphoribosyltransferase, and (12) NAD pyrophosphatase. DHAP, Dihydroxyacetone phosphate; IA, iminoaspartate; PRPP, 5-phosphoribosyl-1-pyrophosphate. Genetic loci corresponding to enzymatic steps are indicated above the reaction arrows.

ide (20 mg/ml) was added to media at a final concentration of 50 μg/ml.

Transductional methods. All transductional crosses were mediated by a mutant of phage P22 (HT105/1 *int-201*) which performs generalized transduction with high frequency. The *int* mutation of this phage was added by Roberts (39a) to the P22 HT105/1 phage of Schmieger (45). To select for the inheritance of the Km^r marker of Mu dJ and the Cm^r marker of Tn*10d*(Cm), the mixture of recipient cells and donor phages was spread on NB plates and incubated overnight before replica printing to selective plates. In all other crosses, selective plates were spread directly with 2×10^8 cells and 10^8 to 10^9 phages. Transductants were purified, and phage-free clones were isolated by being streaked nonselectively onto green indicator plates (7). Phage-free clones were identified on green indicator plates and then tested for phage sensitivity by cross-streaking with P22 H5 phage, a clear-plaque mutant of P22.

Mutagenesis. Hydroxylamine mutagenesis of localized regions of the *Salmonella* chromosome was done by treating P22 transducing phage as described by Davis et al. (12). By selecting for inheritance of fragments carrying a particular region, one can obtain strains mutagenized for a single small region of the chromosome. The general method is that of Hong and Ames (21).

Diethyl sulfate mutagenesis of cells was done as described by Roth (41) with some modifications. Minimal medium (5 ml) with no added carbon source was equilibrated with 0.2 ml of diethyl sulfate at 37°C for 1 h before addition of 0.1 ml of an overnight culture. After mutagenesis for 30 min at 37°C, an aliquot was removed, diluted 1:50 into fresh medium without mutagen, and grown to saturation. Mutants can be isolated either by direct selection or by screening among single colonies derived from the culture.

Insertion mutagenesis with the Mu dJ and Mu dK transposons was done by the *cis* complementation method described previously (25).

Tn*phoA* transposition mutagenesis. The nonspecific acid phosphatase of *S. typhimurium* (29) is sufficiently active to make all strains score positive for the chromogenic phosphatase substrate, X-P. Therefore, all Tn*phoA* insertion mutagenesis (32) is done in strains carrying a *phoN* or a *phoP* mutation that eliminates this activity (53). To introduce the Tn*phoA* element for transposition mutagenesis, a donor strain (TT15088) was constructed which carries the Tn*phoA* element near a properly oriented locked-in P22 prophage (Mu d-P22) (49); this strain also carries a plasmid which produces P22 tail protein. When the P22 prophage is induced, the resulting lysate has a very high frequency of transducing particles that include Tn*phoA*. This lysate is a suitable donor of Tn*phoA* for mutant isolation; the lysate is used to transduce a recipient strain that carries a *phoN* or *phoP* mutation. Selection is made for Km^r, and transductant colonies are screened for interesting insertion mutations (2a). $PhoA^+$ insertion mutants were initially identified as blue colonies on media containing X-P; they were then assayed for alkaline phosphatase activity.

Selection for revertants of NMN transport mutants. A *pnuA* or *pnuC* mutant strain was grown overnight in NB, and cells were washed once with E medium lacking glucose. A 0.1-ml aliquot of washed cell suspension was plated on medium containing 10^{-4} M NMN. Revertants able to transport NMN grow up as colonies after incubation at 37°C for 2 days.

Enzyme assay. All enzymatic activity assays were de-

TABLE 1. Strains

Strain[a]	Genotype[b]
TT315	*purG1739*::Tn*10*
TT13007	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm)
TT13121	*nadB215*::Tn*10* *pncA278*::Tn*10d*(Cm) *pnuA101*::Mu dJ
TT13123	*nadB215*::Tn*10* *pncA278*::Tn*10d*(Cm) *pnuC103*::Mu dJ
TT13124	*nadB215*::Tn*10* *pncA278*::Tn*10d*(Cm) *pnuC104*::Mu dJ
TT13125	*nadB215*::Tn*10* *pncA278*::Tn*10d*(Cm) *pnuC105*::Mu dJ
TT13493	*nadB499*::Mu dJ *pncA180*::Tn*10* *nadE501*
TT13521	*pncA278*::Tn*10d*(Cm) *nadI116*::Mu dK
TT13562	*nadA542* *pncA278*::Tn*10d*(Cm) *phoN61*
TT14946	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *nadI312*(T^- R^+)
TT15088	*hisD10088*::Tn*phoA* *his(HA)9556*::Mu dP/pPB13
TT15335	*nadB499*::Mu dJ *pncA286*::Tn*10d*(Tc)
TT15336	*nadA219*::Mu dJ *pncA286*::Tn*10d*(Tc)
TT15478	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *DEL1085*(*serB-nadI*)
TT15480	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *DEL1085*(*serB-nadI*) *pnuC*127*
TT15486	*nadB215*::Tn*10* *pncA278*::Tn*10d*(Cm) *pnuA101*::Mu dJ *pnuC*127*
TT15500	*DEL1081*(*nadA–zbh-3652*) *pncA278*::Tn*10d*(Cm) *nadI563*::Mu dJ (T^- R^-)
TT15507	*nadB215*::Tn*10* *pncA278*::Tn*10d*(Cm) *pnuC*127*
TT15508	*nadB215*::Tn*10* *pncA278*::Tn*10d*(Cm) *pnuC*128*
TT15521	*nadB103* *pncA278*::Tn*10d*(Cm) *DEL794*(*serB-nadI*) *DUP1034*[(*pnuC*$^+$ *aroG*$^+$)*Tn*10**(*pnuC*$^+$ *aroG*$^+$)]
TT15522	*nadB103* *pncA278*::Tn*10d*(Cm) *DEL794*(*serB-nadI*) *DUP1034*[(*pnuC*127* *aroG583*::Mu dJ)*Tn*10**(*pnuC*$^+$ *aroG*$^+$)]
TT15527	*nadB215*::Tn*10* *pncA278*::Tn*10d*(Cm) *pnuC131*::Mu dK *serB9*
TT15539	*nadB215*::Tn*10* *pncA278*::Tn*10d*(Cm) *DEL1085*(*serB-nadI*) *nadA*^{c}*532*
TT15556	*nadA219*::Mu dJ *pncA278*::Tn*10d*(Cm) *pnuC*127*
TT15558	*nadA379*::Tn*10d*(Tc) *pncA278*::Tn*10d*(Cm) *pnuC*127*
TT15593	*nadA542* *pncA278*::Tn*10d*(Cm) *phoN61* *pnuC149*::Tn*phoA*
TT15598	*nadA379*::Tn*10d*(Tc) *pncA278*::Tn*10d*(Cm) *phoN61* *pnuC149*::Tn*phoA*
TT15599	*nadA542* *pncA278*::Tn*10d*(Cm) *phoN61* *pnuC149*::Tn*phoA* *nadI242*::Tn*10* (T^- R^-)
TT15604	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *nadA*^{c}*532*
TT15611	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *nadI*^{s}*511* *nadA*^{c}*532* *pnuC*152*
TT15643	*nadB214*::Tn*10* *pncA278*::Tn*10d*(Cm) *pnuC104*::Mu dJ
TT15644	*nadB214*::Tn*10* *pncA278*::Tn*10d*(Cm) *pnuC104*::Mu dJ *nadA*^{c}*532*
TT15869	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm)
TT15870	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *nadI260* (R^- T^+)
TT15871	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *nadI275* (R^- T^-)
TT15872	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *nadI312* (R^+ T^-)
TT15873	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *nadI*^{s}*511* (R^s T^-)
TT15874	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *pnuC*127*
TT15875	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *pnuC*127* *nadI260* (R^- T^+)
TT15876	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *pnuC*127* *nadI275* (R^- T^-)
TT15877	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *pnuC*127* *nadI312* (R^+ T^-)
TT15878	*nadB499*::Mu dJ *pncA278*::Tn*10d*(Cm) *pnuC*127* *nadI*^{s}*511* (R^s T^-)
TT15602	*nadB227*::Mu dA *pncA278*::Tn*10d*(Cm) *phoN61* *pnuC149*::Tn*phoA*

[a] All strains are derivatives of *S. typhimurium* LT2 and were either constructed in the course of this work or obtained from the *Salmonella* strain collection, University of Utah.

[b] Nomenclature is as described by Demerec et al. (13), Chumley et al. (8), and Schmid and Roth (44).

scribed previously: the NADH-ferricyanide oxidoreductase assay is that of Jaworowski et al. (28); the glutamate dehydrogenase assay is that of Coulton and Kapoor (11); β-lactamase activity was assayed as described by Sargent (42) and Sawai et al. (43). The units of β-lactamase activity are reported as nanomoles of substrate hydrolyzed per minute by enzymes from 1 ml of original cell culture, based on the calculation described by Sawai et al. (43). The β-galactosidase activity assay is that of Miller (35), and the β-galactosidase activity reported is nanomoles per minute per optical density unit (at 650 nm) of cells. The alkaline phosphatase assay is that of Brickman and Beckwith (4) and Manoil (31b), and the units of phosphatase were calculated as described by Brickman and Beckwith (4).

Cell fractionation. Cell fractionation was done by the method of Manoil and Beckwith (33). The modifications of the method are that cells were resuspended in 1/6 volume of cold spheroplast buffer containing 40% sucrose before osmotic shock, and lysed spheroplasts were centrifuged for 60 min at 40,000 rpm in a 45 Ti rotor to separate the membrane and cytoplasmic fractions. Each fraction was assayed for glutamate dehydrogenase, NADH-ferricyanide oxidoreductase, and β-lactamase. These enzymes are known to be associated with cytoplasmic, membrane, and periplasmic fractions, respectively (11, 28, 42, 43).

Preparation of carbonyl-^{14}C-NMN. Carbonyl-^{14}C-NMN was made from carbonyl-^{14}C-NAD by a nucleotide pyrophosphatase reaction (31a). The reaction mixture contains 47.5 μM NAD, 9.5 μCi of carbonyl-^{14}C-NAD per ml (1 μCi/20 μl), 4.75 mM $MgCl_2$, 9.5 mM Tris hydrochloride (pH 7.4), and 0.2 U of nucleotide pyrophosphatase (type II [from *Crotalus* sp.]; Sigma). The reactions were carried out at 37°C for 60 min and monitored by spotting 2 μl of reaction mix onto a thin-layer chromatograph plate (plastic polyethyleneimine-cellulose F; EM Science) with 2 μl of 10 mM NAD–50 mM NMN solution spotted at the same place as markers.

The thin-layer chromatography plates were developed by 1 M LiCl. After drying, the NAD and NMN spots were located under UV light and cut out to count radioactivity. Greater than 96% of the NAD was converted to NMN.

NMN uptake assay. Strains were grown overnight in E medium containing 10^{-6} M nicotinic acid (NA), diluted 10-fold in the same medium, and grown to mid-log phase (140 Klett units). Cells were harvested by centrifugation and washed twice in E salt solution before being resuspended into a half volume of E medium containing glucose (0.2%). Cells were preincubated by shaking for 20 min at 37°C in a water bath. The assay was started by addition of ^{14}C-NMN to a final concentration of 10 μM (0.57 μCi/nmol). One milliliter of sample was removed at each time point, and cells were harvested by filtration through Millipore filters (0.45-μm pore size). Filters were washed three times with 1.5 ml of E salt solution and then dried before determination of radioactivity. The effect of NA on NMN uptake was tested by adding NA to a final concentration of 2×10^{-4} M; NA was added 20 min after addition of ^{14}C-NMN. The effect of chloramphenicol was tested by adding the antibiotic (20 mg/ml in 95% ethanol) to a final concentration of 20 μg/ml. Chloramphenicol was added with ^{14}C-NMN when the assay was started.

Assay of protein synthesis. The method was modified from that of Imamoto (27). Cells to be assayed were prepared as for NMN uptake assays (see above). The assay was started by adding ^{14}C-leucine (251 mCi/mmol) and nonradioactive NMN to cell suspensions to final concentrations of 4 nCi/ml and 10 μM, respectively. A 0.5-ml sample was removed at each time point and mixed with 0.5 ml of 10% trichloroacetic acid (200 μg of leucine per ml). Protein precipitates were recovered by filtration on Millipore filters (0.45-μm pore size) and washed three times with 1.5 ml of deionized H_2O. Filter disks were dried before radioactivity was measured. The effect of chloramphenicol on protein synthesis was tested by adding the antibiotic (20 mg/ml in 95% ethanol) to a final concentration of 20 μg/ml; chloramphenicol was added at the initiation of the reaction. An equal volume of 95% ethanol was added to controls.

RESULTS

Both *nadI*(R$^+$ T$^-$) and *pnuC* mutants are deficient in NMN transport. Mutants defective in NMN transport were originally isolated in parent strains that required exogenous pyridine (due to a *nadB* mutation) and could not utilize free NM (due to a *pncA* mutation) (17, 30, 46, 51). These parental strains can use NMN as a pyridine source and apparently transport it intact before converting it to NAMN and ultimately to NAD (Fig. 1). Mutants defective in NMN utilization fell into two classes, *nadI*(T$^-$) (originally called *pnuA*) and *pnuC* mutants. Both mutant types possessed NMN deamidase activity and were inferred to be defective in transport of NMN (30, 46).

To clarify the roles played by the *nadI* and *pnuC* gene products, we measured the NMN uptake of *nadI*(R$^+$ T$^-$) and *pnuC* mutants (TT13124 and TT14946) and a wild-type strain (TT15335) (Fig. 2). In agreement with previous conclusions (30, 46), no NMN uptake could be detected in either the *nadI*(R$^+$ T$^-$) mutant or the *pnuC* mutant. The NMN uptake by a *nadA*::Mu dJ insertion mutant (TT15336) was normal and could not be distinguished from that of a *nadB*::Mu dJ insertion strain, TT15335 (data not shown). Thus, the polar effect of the *nadA* insertion on the *pnuC* gene (in the same operon) does not limit NMN transport; apparently, the *pnuC* gene expression provided by the internal promoter, downstream of the Mu dJ insertion, is sufficient to provide transport activity (51). The *pnuC** mutant in Fig. 2 will be described later.

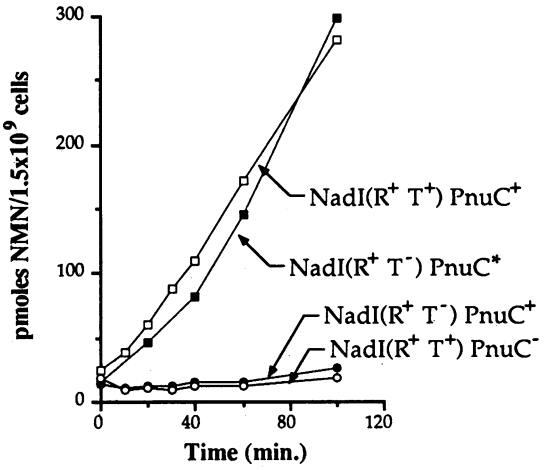

FIG. 2. NMN uptake in a *pnuA*$^+$ *pnuC*$^+$ strain (TT15335) (□), a *nadI*(R$^+$ T$^-$) *pnuC** strain (TT15877) (■).

Neither NadI(T$^+$) nor PnuC function is essential for expression of the other. Since two distinct mutations eliminate NMN transport, we tested the possibility that the NMN transport deficiency of one mutant type could be due to a regulatory effect on expression of a single transport function encoded by the other. Operon and gene fusions to the *nadI* and *pnuC* genes were isolated by insertion of Mu dJ and Mu dK elements into a *nadB pncA* mutant strain. All mutants unable to use NMN map at either the *nadI* or the *pnuC* locus; no other class of mutants was found. Since Mu dJ and Mu dK insertions generate *lac* operon fusions and protein fusions, respectively, the transcription and translation of the *nadI* and *pnuC* genes can be monitored by assaying the levels of β-galactosidase from these strains [e.g., TT13121 for *nadI*::Mu dJ(R$^-$ T$^-$), TT13124 for *pnuC*::Mu dJ, TT13521 for *nadI*::Mu dK(R$^-$ T$^-$), and TT15527 for *pnuC*::Mu dK]. These NMN$^-$ mutants were scored for their Lac phenotype, and the effect of various mutations in the other gene was scored.

The expression of *nadI*::Mu dJ and *nadI*::Mu dK operon and protein fusions is constitutive and is not affected either by the concentration of exogenous NA or NMN or by the presence of *pnuC* mutations, including the *pnuC** mutation (data not shown). [The *pnuC** mutation allows NMN transport without a *nadI*(T$^+$) function and will be described below.]

Because the *nadA-pnuC* operon is regulated by the *nadI* repressor, assaying the influence of *nadI* mutations on *pnuC* gene expression is complicated. We have tested all three phenotypic classes of *nadI* mutations: R$^+$ T$^-$, R$^-$ T$^+$, and R$^-$ T$^-$. Both R$^-$ T$^+$ and R$^-$ T$^-$ mutations cause derepression of the *pnuC* gene; this finding confirms the previous conclusion that the *pnuC* gene is expressed by transcription from the *nadA* promoter and is therefore regulated by the NadI repressor (46, 51). However, *nadI*(R$^+$ T$^-$) mutations, which eliminate NMN transport, have no effect on the expression of the *pnuC* gene at a transcriptional or translational level from either the *nadA* promoter or the *pnuC* promoter (51; other data not shown). Spector et al. had previously shown that *nadI*(T$^-$) mutations have no effect on transcription of the *pnuC* gene (46). On the basis of all of

these results, we conclude that the NMN transport defect of *nadI* and *pnuC* mutants is not due to a failure to express the other gene.

An alteration of PnuC function permits NMN transport without NadI(T^+) function. To investigate the relationship between the NadI(T^+) and PnuC functions, revertants of a *nadI*(R^- T^-) deletion mutant (TT15478) were selected; these revertants can use 10^{-4} M NMN as a pyridine source despite the lack of the NadI protein. The revertants were selected in a strain that carries both a *nadB* and a *pncA* insertion mutation; both parental mutations are retained by the revertants. The revertant frequency was about 10^{-8}. All 16 revertants tested map at the *pnuC* locus and are phenotypically $NadA^+$. The revertants were designated *pnuC** (e.g., TT15480). The *pnuC** mutants can be distinguished from *pnuB* mutants reported previously (31) by map position and by the fact that the *pnuC** mutants are able to grow on 10^{-4} M but not 10^{-5} M NMN as a pyridine source, while *pnuB* mutants are selected for their ability to use the lower concentration of NMN.

Suppression of the *nadI* defect by *pnuC** is not due to increased expression of the *pnuC* gene. We conclude this because known constitutive mutations for the *nadA-pnuC* operon (*nadA*^{c}) do not suppress the transport defect of *nadI*(T^-) mutants. That is, a strain (TT15539) of genotype *nadA*^{c} *nadB pncA nadI*(R^- T^-) is unable to grow on 10^{-4} M NMN.

The ability of *pnuC** mutations to suppress the transport defect of *nadI*(T^-) mutations is not allele specific. Even a deletion mutant lacking the entire *nadI* locus is able to transport NMN following introduction of a *pnuC** mutation (Table 2, lines 1 and 2; Fig. 2). Similarly, *nadI* point mutants with either a simple transport defect (R^+ T^-) or lacking both transport and repressor functions (R^- T^-) are corrected for transport by all *pnuC** mutations (Table 2, lines 3 to 6).

A *pnuC** mutation alone causes no transport defect. This was tested in strains with a normal *nadI*(R^+ T^+) region (Table 2, lines 7 and 8) and in strains with a *nadI* regulatory mutation (R^- T^+) causing constitutive expression of *nadA-pnuC* and *nadB* (Table 2, lines 9 and 10).

Superrepressing *nadI*(R^s) mutations all cause an NMN transport defect that is not corrected by *pnuC**. This is apparently due to reduced expression of the *pnuC** mutant gene, caused by the superrepressing *nadI*(R^s) allele. The repression effect of *nadI*(R^s) can be circumvented by a mutation causing constitutive expression of the *nadA-pnuC* operon (*nadA*^{c}); under these conditions, the *pnuC** mutation can provide NMN transport (Table 2, lines 11 to 13). This situation is expected since *nadI*(R^s) mutations (superrepressor) cause a failure to express the *nadA-pnuC* operon from its main promoter. When the *pnuC** mutant gene is not expressed at a high level, there is apparently not enough *pnuC** gene product for NMN transport.

The need for high levels of the *pnuC** product is confirmed by the effect of a *nadA219*::Mu dJ insertion mutation.

TABLE 2. Phenotypes of mutants on 10^{-4} M NMN medium

Line	Strain	Relevant genotype	Growth on 10^{-4} M NMN
1	TT15478	*DEL1085(serB-nadI)*	−
2	TT15480	*DEL1085(serB-nadI) pnuC*127*	+
3	TT15872	*nadI312*(T^- R^+)	−
4	TT15877	*nadI312*(T^- R^+) *pnuC*127*	+
5	TT15871	*nadI275*(T^- R^-)	−
6	TT15618	*nadI275*(T^- R^-) *pnuC*127*	+
7	TT15869	*nadI*$^+$(T^+ R^+)	+
8	TT15874	*nadI*$^+$(T^+ R^+) *pnuC*127*	+
9	TT15870	*nadI260*(T^+ R^-)	+
10	TT15875	*nadI260*(T^+ R^-) *pnuC*127*	+
11	TT15873	*nadI*^{s}*511*(T^-R^s)	−
12	TT15878	*nadI*^{s}*511*(T^- R^s) *pnuC*127*	−
13	TT15611	*nadI*^{s}*511*(T^- R^s) *nadA*^{c}*532 pnuC*127*	+
14	TT13008	*nadA219*::Mu dJ	+
15	TT15556	*nadA219*::Mu dJ *pnuC*127*	−
16	TT16346	*nadA219*::Mu dJ *pnuC*127 DEL794(nadI)*	−

Although this insertion is located upstream of the *pnuC* gene within the same operon, it does not reduce *pnuC* gene expression sufficiently to prevent NMN transport in an otherwise normal strain (*nadI*$^+$ *pnuC*$^+$). This is due to the constitutive internal promoter within the *nadA-pnuC* operon downstream of the *nadA219* insertion; this promoter can provide sufficient *pnuC*$^+$ function to allow *nadI*(T^+)-dependent transport. However, in a *pnuC** strain, a *nadA* insertion prevents NMN transport with or without *nadI*(T^+) function (Table 2, lines 14 to 16). The internal promoter is apparently not sufficient to permit a *pnuC** allele to provide transport. Presumably, NMN transport by the PnuC* protein with or without nadI(T^+) is less efficient than transport by the wild-type protein aided by the *nadI*(T) function.

The *pnuC mutations are dominant.** Since the *pnuC* gene must be expressed at a high level to permit *pnuC** to correct a *nadI*(T^-) defect, one would predict that the *pnuC** mutation might be dominant. A dominance test was done in a strain carrying a tandem duplication of the chromosome segment from *pyrC* (11 min) to *purE* (23 min) and a Tn*10* element at the duplication join point (TT15521) (51). This strain carries a *nadI*(R^- T^-) deletion (outside the duplicated region) and is diploid for the *pnuC*$^+$ allele. A *pnuC** mutation was introduced into one copy of the duplication by using the linked marker, *aroG*::Mu dJ. The resulting *pnuC*$^+$/*pnuC** heterozygote (TT15522) was able to grow on 10^{-4} M NMN. The genotype of this heterozygous strain (*pnuC*$^+$/*pnuC**) was verified by scoring the phenotypes of haploid segregants.

Evidence that the *pnuC* gene product is membrane associated. We have isolated a Tn*phoA* insertion mutant of the

TABLE 3. Effects of *nadA* insertion and *nadI* mutation on expression of the *pnuC149*::Tn*phoA* fusion

Line	Strain	Relevant genotype	Alkaline phosphatase activity (U) in medium with indicated NA concn	
			10^{-6} M	2×10^{-4} M
1	TT15593	*pnuC149*::Tn*phoA*	13	1
2	TT15599	*pnuC149*::Tn*phoA nadI242*::Tn*10*	13	13
3	TT15598	*nadA379*::Tn*10d*(Tc) *pnuC149*::Tn*phoA*	1	1

TABLE 4. Cellular location of PnuC::PhoA fusion protein

Enzyme assayed	Enzyme activity[a]				
	In whole-cell extract[b]	Recovery after fractionation	Distribution of recovered activity in indicated fraction		
			Periplasm	Membrane	Cytoplasm
β-Lactamase	18	16 (89)	15 (94)	1 (6)	0 (0)
NADH-ferricyanide oxidoreductase	43	37 (86)	2 (5.5)	33 (89)	2 (5.5)
Glutamate dehydrogenase	9.8	9.3 (95)	0.5 (5)	0.1 (1)	8.7 (94)
PnuC::PhoA alkaline phosphatase	4.3	3.6 (84)	0.8 (22)	2.5 (70)	0.3 (8)

[a] Normalized as activity units in the amount of extract or fraction prepared from 1 ml of starting cell culture. The optical density at 650 nm of the starting cell culture in this experiment was 0.553. Numbers in parentheses are percentages. Data are from one fractionation experiment in which all activities were assayed.

pnuC gene; this mutant expresses alkaline phosphatase activity. The Tn*phoA* element, constructed by Manoil and Beckwith (32), has the gene for alkaline phosphatase (*phoA*) near one end of the transposable sequence; this *phoA* gene lacks a translation initiation site and the signal sequence required for its secretion to the periplasm (32, 34). Alkaline phosphatase is active only if the protein can be transported outside of the cytoplasmic membrane. To produce active phosphatase, the Tn*phoA* element must insert in the proper reading frame into a structure gene that can provide both a translational initiation site and a functional signal sequence. This *phoA* fusion technique has been used extensively to study the topology of membrane proteins and the process of protein transport (1, 3, 9, 19, 33). An insertion mutation which expresses alkaline phosphatase indicates that the target gene has a signal sequence and that its product is probably located in either the membrane or the periplasm.

The *pnuC149*::Tn*phoA* mutation appears to be a simple insertion within the *pnuC* gene; it does not affect the promoter-proximal *nadA* gene. Furthermore, the expressed alkaline phosphatase is regulated in response to pyridine levels as are *lac* operon fusions to the *pnuC* gene. Data for regulation of *phoA* are in Table 3; all of the strains described carry a *phoN* mutation which eliminates the *Salmonella* nonspecific acid phosphatase. The first two strains in Table 3 carry the nonpolar *nadA542* mutation to block the synthetic pathway. Alkaline phosphatase is repressed in response to an increased level of exogenous nicotinic acid (Table 3, line 1). Alkaline phosphatase is constitutively expressed when a *nadI*(R^- T^-) mutation is introduced (line 2). A Tn*10d*(Tc) insertion in *nadA* exerts a polar effect, reducing expression of the *pnuC*::Tn*phoA* fusion (line 3).

The cellular location of the hybrid PnuC-PhoA protein produced by the *pnuC149*::Tn*phoA* fusion gene was determined by cell fractionation as described in Materials and Methods. Each fraction was assayed for alkaline phosphatase and for the marker proteins β-lactamase (periplasmic), NADH-ferricyanide oxidoreductase (membrane associated), and glutamate dehydrogenase (cytoplasmic). Most alkaline phosphatase is associated with membranes, suggesting that the *phoA* sequences have been fused to a protein that is anchored in the membrane (Table 4). This finding suggests that the *pnuC* gene encodes a membrane-associated protein.

A model for the role of the NadI protein in regulation of transcription of de novo pathway and NMN transport. To explain the effects of *nadI* mutations on both transport and transcription, we propose that the NadI protein is allosteric, alternating between two distinct regulatory states (Fig. 3). When NAD levels are low, the protein assumes a conformation that stimulates transport of NMN and allows increased

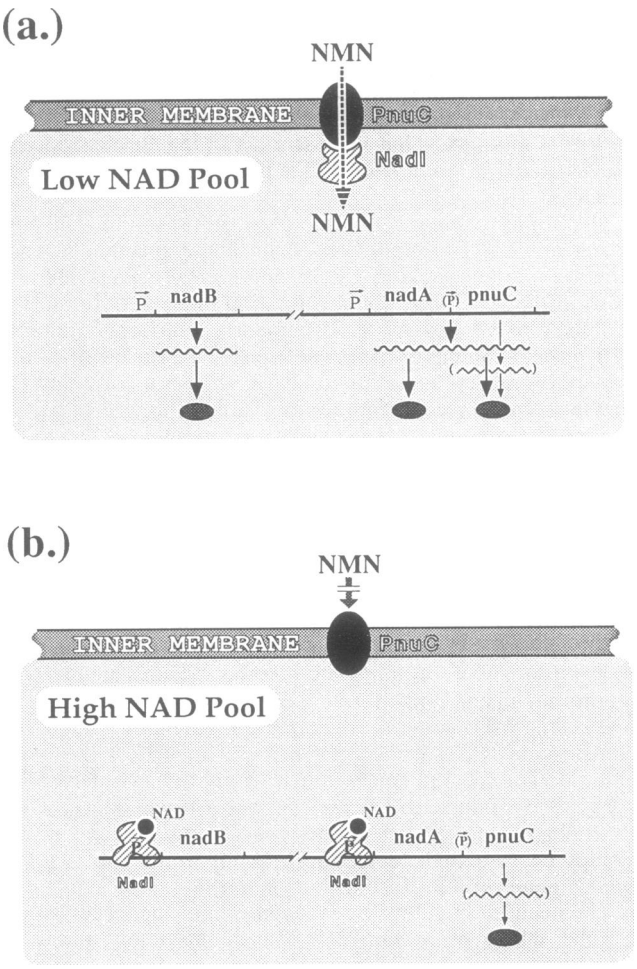

FIG. 3. Model for coordinate control of NMN uptake and transcription of the NAD de novo biosynthetic genes. (a) When the internal NAD level is low, NadI bifunctional protein is in a conformation that is able to bind with the membrane protein, PnuC, to form functional NMN transport systems but is not able to bind with the *nadA* and *nadB* promoters. The *nadA* and *nadB* genes are expressed. (b) When the internal NAD level is high, NadI protein binds NAD, stabilizing a conformation of the protein that is able to bind to operator sites in DNA but unable to activate transport. As a result, both NMN uptake and the expression of biosynthetic genes *nadA* and *nadB* stop.

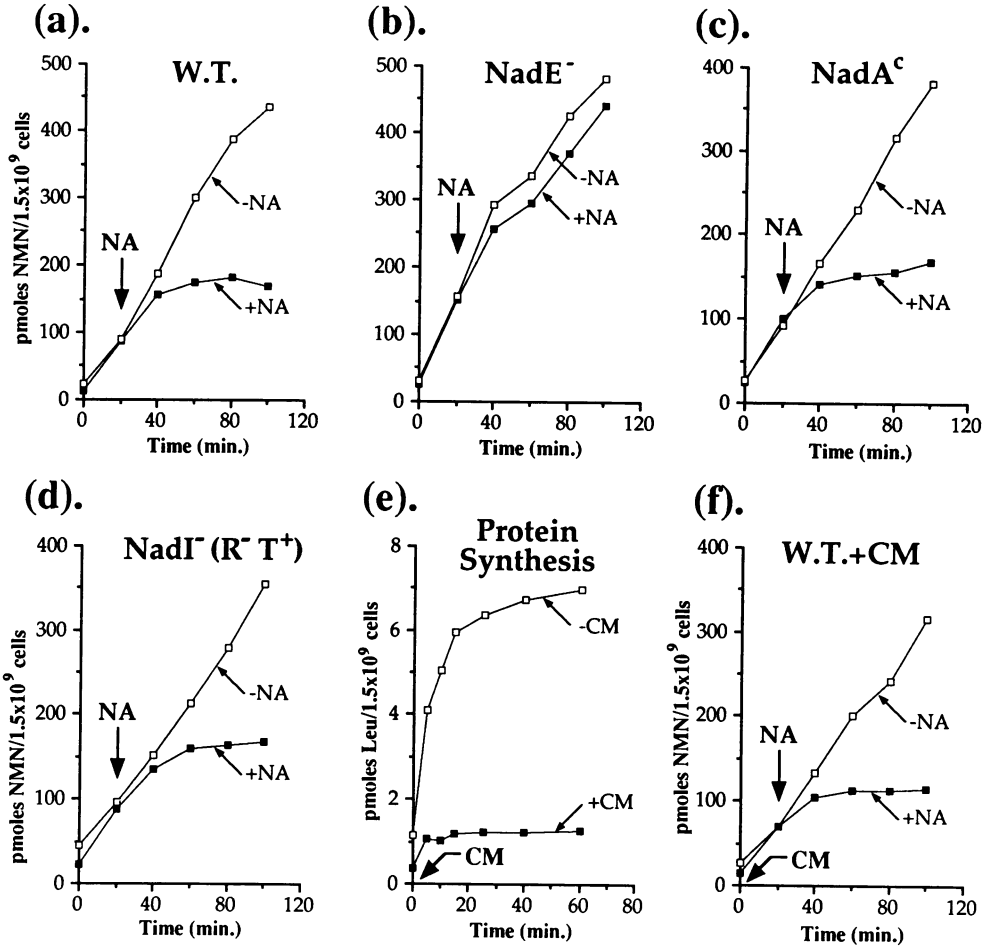

FIG. 4. Effects of NA and chloramphenicol on NMN uptake. (a) A wild-type (W.T.) strain (TT15335) with (■) and without (□) addition of 2×10^{-4} M NA at 20 min, assayed at 43°C; (b) a *nadE* strain (TT13493) with (■) and without (□) addition of 2×10^{-4} M NA at 20 min, assayed at 43°C; (c) a *nadA* promoter constitutive mutant strain (TT15604) with (■) and without (□) addition of 2×10^{-4} M NA at 20 min; (d), a *nadI*($R^- T^+$) *pnuC*$^+$ (TT15870) with (■) and without (□) addition of 2×10^{-4} M NA at 20 min; (e) protein synthesis by a wild-type strain (TT15335) with (■) and without (□) addition of chloramphenicol (CM); (f) NMN uptake of a wild-type strain (TT15335) in the presence of chloramphenicol with (■) and without (□) addition of 2×10^{-4} M NA at 20 min.

expression of biosynthetic genes (Fig. 3a). When NAD levels are high, the protein represses transcription of the *nadB* and *nadA* genes and ceases to stimulate NMN transport (Fig. 3b). The role of NadI(T^+) in modulating activity of the transort system is a functional parallel with its role as a transcriptional repressor (52). Depending on the supply of NAD, two routes of pyridine acquisition (synthesis and transport) are either restricted or increased. The model makes several testable predictions. Most important of these is that the activity of the transport system should vary in response to internal pyridine levels. We have tested this prediction.

Regulation of NMN transport function. Transport of NMN was assayed for a strain (TT15335) in which the pyridine moiety of NMN is assimilated only by the NadI(T^+)-PnuC route (any NM generated by a periplasmic glycohydrolase cannot be assimilated). Following addition of a high concentration of NA, transport of NMN stops (Fig. 4a). This cessation of NMN transport is a consequence of internal NAD accumulation (not competition between NMN and NA for a common transport system), since NA has no regulatory effect in a *nadE* (NAD synthetase) mutant which is blocked in the last step of NAD synthesis (Fig. 4b). The *nadE* mutation used is a temperature-sensitive mutation known to stop NAD synthesis and accumulate nicotinic acid adenine dinucleotide (NaAD) at high temperatures (23); the assay presented was performed at 42°C. The cessation of NMN transport following NA addition is not due to repression of the *nadA-pnuC* operon by the NadI function, since the NA effect still occurs in strains which carry a *nadA*^{c} or *nadI*($R^- T^+$) mutation, both of which cause constitutively high expression of the *nadA-pnuC* operon (Fig. 4c and d). The regulatory effect of NA on NMN transport does not require protein synthesis. When protein synthesis is blocked by chloramphenicol, NA still shows its negative effect on NMN transport (Fig. 4e and f).

The variation in transport activity seems to depend on the NadI(T^+) function. Transport is eliminated by *nadI*($R^+ T^-$) mutations (Fig. 2). Furthermore, regulation of transport is lost in *pnuC** mutants which are insensitive to NadI(T^+). NMN transport in the *pnuC** *nadI*($R^- T^-$) and *pnuC** *nadI*($R^+ T^-$) mutants (Fig. 5b and c) is comparable to that of

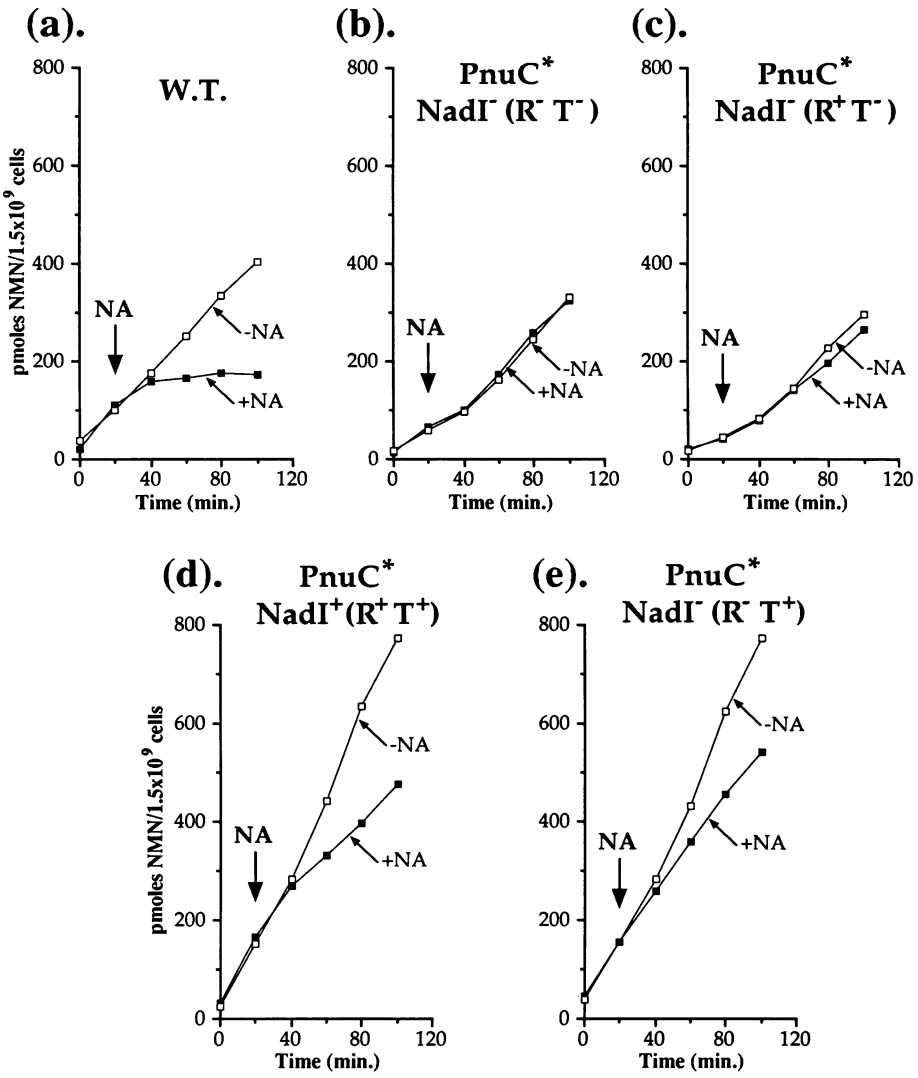

FIG. 5. Effects of *nadI* mutations on NMN uptake by *pnuC** mutant strains. (a) A wild-type (W.T.) strain (*pnuC*$^+$ *nadI*$^+$) (TT15869) with (■) and without (□) addition of 2×10^{-4} M NA at 20 min; (b) a *pnuC** *nadI*(R$^-$ T$^-$) strain (TT15876) with (■) and without (□) addition of 2×10^{-4} M NA at 20 min; (c) a *pnuC** *nadI*(R$^+$ T$^-$) strain (TT15877) with (■) and without (□) addition of 2×10^{-4} M NA at 20 min; (d) a *pnuC** *nadI*$^+$ strain (TT15874) with (■) and without (□) addition of 2×10^{-4} M NA at 20 min; (e) a *pnuC** *nadI*(R$^-$ T$^+$) strain (TT15875) with (■) and without (□) addition of 2×10^{-4} M NA at 20 min.

a wild-type strain under starvation conditions (Fig. 5a) but has become insensitive to NA addition. Since *pnuC** mutants have simultaneously become independent of *nadI*(T$^+$) and have lost regulation of NMN transport by NA, we conclude that the regulation of transport is via the NadI(T$^+$) function. Strains with both a *pnuC** mutation and a *nadI*(T$^+$) allele have a hyperactive NMN transport system. This activity is reduced to the slower PnuC* rate when NA is added (Fig. 5d and e).

We conclude that the regulatory effect of NA on NMN transport is due to internal accumulation of NAD which causes the NadI(T$^+$) function to stop activating the PnuC transport system. The transport system is fully shut off within about 20 min of NA addition. According to the model (Fig. 3), this time is required for NA to be converted to NAD, for the increased NAD to be sensed by the NadI protein, and for the *pnuC* transport system to return to its inactive state.

DISCUSSION

We have presented a model for regulation of pyridine levels (Fig. 3) that includes a single bifunctional protein which acts to vary transcription of biosynthetic genes and to modulate the rate of transport of exogenous pyridines. The most novel aspect of this model is the idea of varying the activity of an uptake system. Below we discuss the history of this model, the pieces of evidence that support it, and reasons why this elaborate mechanism might be particularly important to the pyridine pathway.

Previous work has demonstrated that mutants defective in both repression and NMN transport map at a single locus (*nadI*) on the *Salmonella* chromosome (10, 17, 20, 30, 50). Foster and co-workers found that the DNA sequence of this locus includes a single open reading frame (16, 17a). On the basis of these sequence data and the phenotypes of mutants, a model was presented in which a single protein acts as a

repressor of biosynthetic genes and a component of the NMN transport system (17a). The accompanying report (52) provides in vivo data that the transport and repression functions are provided by a single bifunctional protein. We present here an independently developed model (Fig. 3) that is similar to that of Foster et al. but includes several specific new features for which we can provide evidence. The new features and the evidence for each are listed below.

(i) The *nadI*(T) function is not an essential component of the NMN transport system but rather a regulatory modulator of the activity of the *pnuC* transport system. We conclude this because *pnuC** mutants transport NMN even if the *nadI* gene is completely deleted. Thus, the PnuC protein with only minor modification can independently serve to transport NMN.

(ii) We provide evidence that the activity of this transport system does in fact vary in response to cell physiology. The regulatory signal is likely to be NAD or NADP, since *nadE* mutants (blocked in NAD synthetase) are unable to vary NMN transport activity in response to exogenous NA. Mutations that limit *nadE* function also cause increased transcription of biosynthetic genes, suggesting that NAD (or NADP) is also the effector for the repressor activity of the NadI protein (23).

(iii) The role of the NadI protein in varying NMN transport activity is supported by the fact that *pnuC** mutants show unregulated high transport activity and have lost the need for *nadI*(T) function to stimulate activity. Conversely, *nadI*(T^-) mutants show unregulated low transport activity.

(iv) The NadI protein is probably an allosteric protein. One form is able to repress biosynthetic genes but not activate transport; the other can not repress transcription but is able to activate transport. These two states correspond to the *nadI* mutant phenotypes: R^+ T^- and R^- T^+. Two lines of evidence support this conclusion. The main support is the fact that all *nadI*(R^s) (superrepressor) mutants have simultaneously lost transport activity. This is expected of an allosteric protein which is locked in the repressing conformation; it represses under all conditions and fails, under all conditions, to stimulate transport. An alternative possibility, that repression is lifted when NadI protein binds and is sequestered by transport sites, is rendered unlikely because deletion mutants of *pnuC* which lack the transport protein show normal transcriptional regulation.

The model for *nadI* functions is presented in simplest form in Fig. 3. In this diagram, the NadI protein (without NAD) binds directly to PnuC to activate transport. It may be more likely that the regulatory protein will prove to be a kinase or ADP ribosyltransferase which activates PnuC by covalent modification. Covalent protein modification (phosphorylation-dephosphorylation) has been shown to be a regulatory mechanism in many bacterial systems such as chemotaxis, control of outer membrane (Omp) proteins, the phosphate regulon, and nitrogen assimilation functions of *Escherichia coli* (26, 36, 39, 40). Regulation of NMN transport would be achieved if the NadI protein varied between a repressor and a modifier conformation. The PnuC protein might then be active or inactive, depending on its state of chemical modification. Following NA addition, there is a lag of about 20 min before NMN transport ceases; this lag may include time for removal or spontaneous breakdown of a (*nadI*-catalyzed) modification that activates the PnuC protein.

We suspect that control of pyridine transport is important to *Salmonella* spp. because it permits cells to assimilate NMN and yet avoid raising internal pools of NMN to excessive levels. The essential enzyme DNA ligase is known to be inhibited by NMN (37, 38); some dehydrogenases may also be sensitive to inhibition. The mechanism described here permits NMN transport only under conditions of NAD limitation. This would prevent accumulation of a large, potentially inhibitory internal NMN pool following exposure to exogenous NMN. Regulated transport systems of this type might be expected for any valuable nutrient that is toxic at high concentrations.

REFERENCES

1. **Akiyama, Y., and K. Ito.** 1987. Topology analysis of the SecY protein, an integral membrane protein involved in protein export in *Escherichia coli*. EMBO J. **6**:3465–3470.
2. **Andreoli, A. J., T. W. Okita, R. Bloom, and T. A. Grover.** 1972. The pyridine nucleotide cycle: presence of a nicotinamide mononucleotide-specific glycohydrolase in *Escherichia coli*. Biochem. Biophys. Res. Commun. **49**:264–269.

2a. **Ardehali, H., N. Zhu, D. Roof, and J. R. Roth.** Unpublished data.

3. **Boyd, D., C. Manoil, and J. Beckwith.** 1987. Determinants of membrane protein topology. Proc. Natl. Acad. Sci. USA **84**: 8525–8529.
4. **Brickman, E., and J. Beckwith.** 1975. Analysis of the regulation of *Escherichia coli* alkaline phosphatase synthesis using deletions and ϕ80 transducing phages. J. Mol. Biol. **96**:307–316.
5. **Casadaban, M. J., and S. N. Cohen.** 1979. Lactose genes fused to exogenous promoters in one step using a Mu-*lac* bacteriophage: *in vivo* probe for transcriptional control sequences. Proc. Natl. Acad. Sci. USA **76**:4530–4533.
6. **Castilho, B. A., P. Olfson, and M. J. Casadaban.** 1984. Plasmid insertion mutagenesis and *lac* gene fusion with mini-Mu bacteriophage transposons. J. Bacteriol. **158**:488–495.
7. **Chan, R. K., D. Botstein, T. Watanabe, and Y. Ogata.** 1972. Specialized transduction of tetracycline resistance by phage P22 in *Salmonella typhimurium*. II. Properties of a high frequency transducing lysate. Virology **50**:883–898.
8. **Chumley, F., R. Menzel, and J. R. Roth.** 1979. Hfr formation directed by Tn*10*. Genetics **91**:639–655.
9. **Chun, S. Y., and J. S. Parkinson.** 1988. Bacterial motility: membrane topology of the *Escherichia coli* MotB protein. Science **239**:276–278.
10. **Cookson, B. T., B. M. Olivera, and J. R. Roth.** 1987. Genetic characterization and regulation of the *nadB* locus of *Salmonella typhimurium*. J. Bacteriol. **169**:4285–4293.
11. **Coulton, J. W., and M. Kapoor.** 1973. Purification and some properties of the glutamate dehydrogenase of *Salmonella typhimurium*. Can. J. Microbiol. **19**:427–438.
12. **Davis, R. W., D. Botstein, and J. R. Roth.** 1980. Advanced bacterial genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
13. **Demerec, M., E. Adelberg, A. J. Clark, and P. E. Hartman.** 1966. A proposal for a uniform nomenclature in bacterial genetics. Genetics **54**:61–76.
14. **Elliott, T., and J. R. Roth.** 1988. Characterization of Tn*10d*-Cam: a transposition-defective Tn*10* specifying chloramphenicol resistance. Mol. Gen. Genet. **213**:332–338.
15. **Foster, J. W.** 1981. Pyridine nucleotide cycle of *Salmonella typhimurium*: in vitro demonstration of nicotinamide adenine dinucleotide glycohydrolase, nicotinamide mononucleotide glycohydrolase, and nicotinamide adenine dinucleotide pyrophosphatase activities. J. Bacteriol. **145**:1002–1009.
16. **Foster, J. W., E. A. Holley-Guthrie, and F. Warren.** 1987. Regulation of NAD metabolism in *Salmonella typhimurium* genetic analysis and cloning of the *nadR* repressor locus. Mol. Gen. Genet. **208**:279–287.
17. **Foster, J. W., D. M. Kinney, and A. G. Moat.** 1979. Pyridine nucleotide cycle of *Salmonella typhimurium*: isolation and characterization of *pncA*, *pncB*, and *pncC* mutations and utilization of exogenous nicotinamide adenine dinucleotide. J. Bacteriol. **137**:1165–1175.

17a. **Foster, J. W., Y. K. Park, T. Fenger, and M. P. Spector.** 1990. Regulation of NAD metabolism in *Salmonella typhimurium*:

molecular sequence analysis of the bifunctional *nadR* regulator and the *nadA-pnuC* operon. J. Bacteriol. **172:**4187–4196.
18. **Hillyard, D., M. Rechsteiner, P. Manlapaz-Ramos, J. S. Imperial, L. J. Cruz, and B. M. Olivera.** 1981. The pyridine nucleotide cycle. Studies in *Escherichia coli* and the human cell line D98/AH2. J. Biol. Chem. **256:**8491–8497.
19. **Hoffman, C. S., and A. Wright.** 1985. Fusions of secreted proteins to alkaline phosphatase: an approach for studying protein secretion. Proc. Natl. Acad. Sci. USA **82:**5107–5111.
20. **Holley, E. A., M. P. Spector, and J. W. Foster.** 1985. Regulation of NAD biosynthesis in *Salmonella typhimurium*: expression of *nad-lac* gene fusions and identification of a *nad* regulatory locus. J. Gen. Microbiol. **131:**2759–2770.
21. **Hong, J.-S., and B. N. Ames.** 1971. Localized mutagenesis of any specific small region of the bacterial chromosomes. Proc. Natl. Acad. Sci. USA **68:**3158–3162.
22. **Hughes, K. T., D. Ladika, J. R. Roth, and B. M. Olivera.** 1983. An indispensable gene for NAD biosynthesis in *Salmonella typhimurium*. J. Bacteriol. **155:**213–221.
23. **Hughes, K. T., B. M. Olivera, and J. R. Roth.** 1988. Structural gene for NAD synthetase in *Salmonella typhimurium*. J. Bacteriol. **170:**2113–2120.
24. **Hughes, K. T., and J. R. Roth.** 1984. Conditional transposition-defective derivative of Mu dI(Amp Lac). J. Bacteriol. **159:**130–137.
25. **Hughes, K. T., and J. R. Roth.** 1988. Transitory *cis* complementation: a method for providing transposition function to defective transposons. Genetics **119:**9–12.
26. **Igo, M. M., A. J. Ninfa, J. B. Stock, and T. J. Silhavy.** 1989. Phosphorylation and dephosphorylation of a bacterial transcriptional activator by a transmembrane receptor. Genes Dev. **3:**1725–1734.
27. **Imamoto, F.** 1973. Diversity of regulation of genetic transcription. I. Effect of antibiotics which inhibit the process of translation on RNA metabolism in *Escherichia coli*. J. Mol. Biol. **74:**113–136.
28. **Jaworowski, A., H. D. Campbell, M. I. Poulis, and I. G. Young.** 1981. Genetic identification and purification of the respiratory NADH dehydrogenase of *Escherichia coli*. Biochemistry **20:**2041–2047.
29. **Kier, L. D., R. Weppelman, and B. N. Ames.** 1979. Regulation of nonspecific acid phosphatase in *Salmonella*: *phoN* and *phoP* genes. J. Bacteriol. **138:**155–161.
30. **Kinney, D. M., J. W. Foster, and A. G. Moat.** 1979. Pyridine nucleotide cycle of *Salmonella typhimurium*: in vitro demonstration of nicotinamide mononucleotide deamidase and characterization of *pnuA* mutants defective in nicotinamide mononucleotide transport. J. Bacteriol. **140:**607–611.
31. **Liu, G.-P., J. Foster, P. Manlapaz-Ramos, and B. M. Olivera.** 1982. Nucleoside salvage pathway for NAD biosynthesis in *Salmonella typhimurium*. J. Bacteriol. **152:**1111–1116.
31a. **Lowery, R.** Personal communication.
31b. **Manoil, C.** Personal communication.
32. **Manoil, C., and J. Beckwith.** 1985. Tn*phoA*: a transposon probe for protein export signals. Proc. Natl. Acad. Sci. USA **82:**8129–8133.
33. **Manoil, C., and J. Beckwith.** 1986. A genetic approach to analyzing membrane protein topology. Science **233:**1403–1408.
34. **Michaelis, S., J. F. Hunt, and J. Beckwith.** 1986. Effects of signal sequence mutations on the kinetics of alkaline phosphatase export to the periplasm in *Escherichia coli*. J. Bacteriol. **167:**160–167.
35. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
36. **Ninfa, A. J., and B. Magasanik.** 1986. Covalent modification of the *glnG* product, NR_I, by the *glnL* product, NR_{II}, regulates the transcription of the *glnALG* operon in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **83:**5909–5913.
37. **Olivera, B. M., and F. Bonhoeffer.** 1972. Discontinuous DNA replication *in vitro*. I. Two distinct size classes of intermediates. Nature (London) New Biol. **240:**233–235.
38. **Olivera, B. M., and F. Bonhoeffer.** 1972. Replication of ϕX174 DNA by *Escherichia coli polA in vitro*. Proc. Natl. Acad. Sci. USA **69:**25–29.
39. **Parkinson, J. S.** 1988. Protein phosphorylation in bacterial chemotaxis. Cell **53:**1–2.
39a. **Roberts, G.** Unpublished data.
40. **Ronson, C. W., B. T. Nixon, and F. M. Ausubel.** 1987. Conserved domains in bacterial regulatory proteins that respond to environmental stimuli. Cell **49:**579–581.
41. **Roth, J. R.** 1970. Genetic techniques in studies of bacterial metabolism. Methods Enzymol. **17A:**3–35.
42. **Sargent, M. G.** 1968. Rapid fixed-time assay for penicillinase. J. Bacteriol. **95:**1493–1494.
43. **Sawai, T., I. Takahashi, and S. Yamagishi.** 1978. Iodometric assay method for beta-lactamase with various beta-lactam antibiotics as substrates. Antimicrob. Agents Chemother. **13:**910–913.
44. **Schmid, M. B., and J. R. Roth.** 1983. Genetic methods for analysis and manipulation of inversion mutations in bacteria. Genetics **105:**517–537.
45. **Schmieger, H.** 1971. A method for detection of phage mutants with altered transducing ability. Mol. Gen. Genet. **100:**378–381.
46. **Spector, M. P., J. M. Hill, E. A. Holley, and J. W. Foster.** 1985. Genetic characterization of pyridine nucleotide uptake mutants of *Salmonella typhimurium*. J. Gen. Microbiol. **131:**1313–1322.
47. **Vogel, H. J., and D. M. Bonner.** 1956. Acetylornithinase of *Escherichia coli*: partial purification and some properties. J. Biol. Chem. **218:**97–106.
48. **Way, J. C., M. A. Davis, D. Morisato, D. E. Roberts, and N. Kleckner.** 1984. New Tn*10* derivatives for transposon mutagenesis and for construction of *lacZ* operon fusions by transposition. Gene **32:**369–379.
49. **Youderian, P., P. Sugiono, K. L. Brewer, N. P. Higgins, and T. Elliott.** 1988. Packaging specific segments of the *Salmonella* chromosome with locked-in Mu*d*-P22 prophages. Genetics **118:**581–592.
50. **Zhu, N., B. M. Olivera, and J. R. Roth.** 1988. Identification of a repressor gene involved in the regulation of NAD de novo biosynthesis in *Salmonella typhimurium*. J. Bacteriol. **170:**117–125.
51. **Zhu, N., B. M. Olivera, and J. R. Roth.** 1989. Genetic characterization of the *pnuC* gene, which encodes a component of the nicotinamide mononucleotide transport system in *Salmonella typhimurium*. J. Bacteriol. **171:**4402–4409.
52. **Zhu, N., and J. R. Roth.** 1991. The *nadI* region of *Salmonella typhimurium* encodes a bifunctional regulatory protein. J. Bacteriol. **173:**1302–1310.
53. **Zhu, N., and J. R. Roth.** Unpublished data.