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Isolation of NAD Cycle Mutants Defective in Nicotinamide Mononucleotide Deamidase in *Salmonella typhimurium*

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The NAD or pyridine nucleotide cycle is the sequence of reactions involved in the breakdown of NAD to nicotinamide mononucleotide (NMN) and regeneration of NAD. This cycle is fivefold more active during aerobic growth of *Salmonella typhimurium* and under this condition breaks down half of the NAD pool every 90 min. DNA ligase is known to convert NAD to NMN but is only a minor contributor to the NAD cycle during aerobic growth. The dominant aerobic route of NMN formation is otherwise uncharacterized. Accumulated NMN generated by either of these routes is potentially dangerous in that it can inhibit the essential enzyme DNA ligase. The reactions which recycle NMN to NAD may serve to minimize the inhibition of ligase and other enzymes by accumulated NMN. The predominant recycling reaction in *S. typhimurium* appears to be NMN deamidase, which converts NMN directly to the biosynthetic intermediate nicotinic acid mononucleotide. Mutants defective in this recycling step were isolated and characterized. By starting with a ligase-deficient (*lig* mutant) parent strain that requires deamidase to assimilate exogenous NMN, two classes of mutants that are unable to grow on minimal NMN media were isolated. One class (*pncC*) maps at 83.7 min and shows only 2% of the wild-type levels of NMN deamidase. Under aerobic conditions, a *lig*$^+$ allele allows a *pncC* mutant to grow on NMN and restores some deamidase activity. This growth ability and enzyme activity are not found in *lig*$^+$ strains grown without oxygen. This suggests the existence of a second NMN deamidase (*pncL*) dependent on ligase and stimulated during aerobic growth. The second class of mutants (*pncD*) gains a requirement for isoleucine plus valine with growth in the presence of exogenous NMN. We propose that *pncD* mutations reduce the activity of an *ilv* biosynthetic enzyme that is naturally sensitive to inhibition by NMN.

NAD and NADP are cofactors for numerous anabolic and catabolic reactions. The current understanding of NAD metabolism in *Salmonella typhimurium* is summarized in Fig. 1 and has previously been reviewed (11, 32). In *S. typhimurium*, NAD metabolism involves a de novo synthetic pathway and two recycling pathways. Pools of NAD and NADP have been shown to turn over in all of the organisms tested (9, 17, 21, 22, 28). The pyridine nucleotide cycle in several organisms has been described previously (10, 13, 14, 25, 27). Two pathways for recycling are found in *Escherichia coli* and *S. typhimurium* (13, 19, 23). The predominant path in both bacteria involves nicotinamide mononucleotide (NMN) deamidase (21, 23, 24), which converts NMN to the biosynthetic intermediate nicotinic acid mononucleotide (NaMN). NMN deamidase has been assayed and partially purified in both *E. coli* and *S. typhimurium* (9). The *Salmonella* NMN deamidase has a pH optimum of 8.7 and its activity shows sigmoidal dependence on substrate concentration, while *E. coli* NMN deamidase has a pH optimum of 9.0 and exhibits linear kinetics (9).

While NMN is known to be produced by DNA ligase, this activity is a very minor contributor to the NMN recycled during aerobic growth. About 80% of cycle activity is eliminated in the absence of oxygen (26). It is not clear what reactions contribute to oxygen-stimulated NMN production. We have previously suggested that the oxygen-stimulated cycle involves the formation and breakdown of NADP, as shown in Fig. 1 (6). The reactions which recycle NMN to NAD may serve to minimize the inhibition of ligase and other enzymes by accumulated NMN. We describe here the isolation and characterization of mutants defective in NMN deamidase, the main NMN recycling activity of *S. typhimurium.*

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MATERIALS AND METHODS

Bacterial strains. All of the strains used in this study are derived from *S. typhimurium* LT2 and are listed in Table 1. Bacteriophage Mu*d*A is the conditionally transposition-defective element (Mu*d*1-8) (16) derived from the original operon-fusion-forming phage Mu*d*1(Lac Ap^r) of Casadaban and Cohen (2). The Mu*dJ* element is the transposition-defective mini-Mu phage constructed by Castilho et al. (3); this element lacks transposition functions and encodes kanamycin resistance. The Tn*10 d*(Tc) and Tn*10 d*(Cm) elements are small transposition-defective derivatives of transposon Tn*10* (8, 34).

Chemicals and media. Labeled (carbonyl-^{14}C) NAD was purchased from Amersham. All other chemicals were purchased from Sigma Chemicals. The E medium of Vogel and Bonner (33), supplemented with 0.2% glucose, was used as minimal medium. Difco nutrient broth (NB; 8 g/liter) was used as rich medium with added NaCl (0.5%). Difco agar was added to a final concentration of 1.5% to make solid media. Nutrients were added at the concentrations described by Davis et al. (7); all exceptions are indicated below. Antibiotics were added to media at the following final concentrations (in micrograms per milliliter): ampicillin (sodium salt), 30 (NB) or 15 (E medium); tetracycline hydrochloride, 20 (NB) or 10 (E medium); kanamycin sulfate, 50 (NB) or 125 (E medium).

Transductional methods. The high-frequency, generalized transducing mutant of bacteriophage P22 (HT 105/1 *int-201*) was used for all transductional crosses. This phage was derived by G. Roberts (28a) from the P22 HT 105/1 phage of Schmieger (30). To select for inheritance of the Kn^r marker of Mu*dJ* and the Cm^r marker of Tn*10 d*(Cm), a mixture of cells and phage was spread on NB plates and incubated overnight before replica printing to selective medium. In all other crosses, selective plates were spread directly with 2×10^8 cells and 10^8 to 10^9 phage. Transductants were purified and made phage free by streaking on nonselective green indicator plates (4).

Mutagenesis and initial isolation of *pncC* and *pncD* mutations. Diethyl sulfate mutagenesis of cells was done as described previously (29). Hydroxylamine mutagenesis of P22 transducing phage was performed as described by Davis et al. (7). The general method for localized mutagenesis was that of Hong and Ames (15).

Twenty independent cultures of parent strain TT18391 were mutagenized by incubation for 1 h at 37°C in minimal medium saturated with diethyl sulfate. After exposure to the mutagen, cells were diluted 20-fold in fresh NB and grown at 37°C for 2 h. This culture was diluted 10^{-5}-fold, and a 0.1-ml aliquot was plated on each of 10 plates, yielding 200 to 250 colonies per plate. By replica printing to supplemented minimal medium, clones that could use nicotinic acid (NA) but not NMN as an NAD precusor were identified. No more than one mutant from each of the 20 original cultures was tested further. This hunt yielded

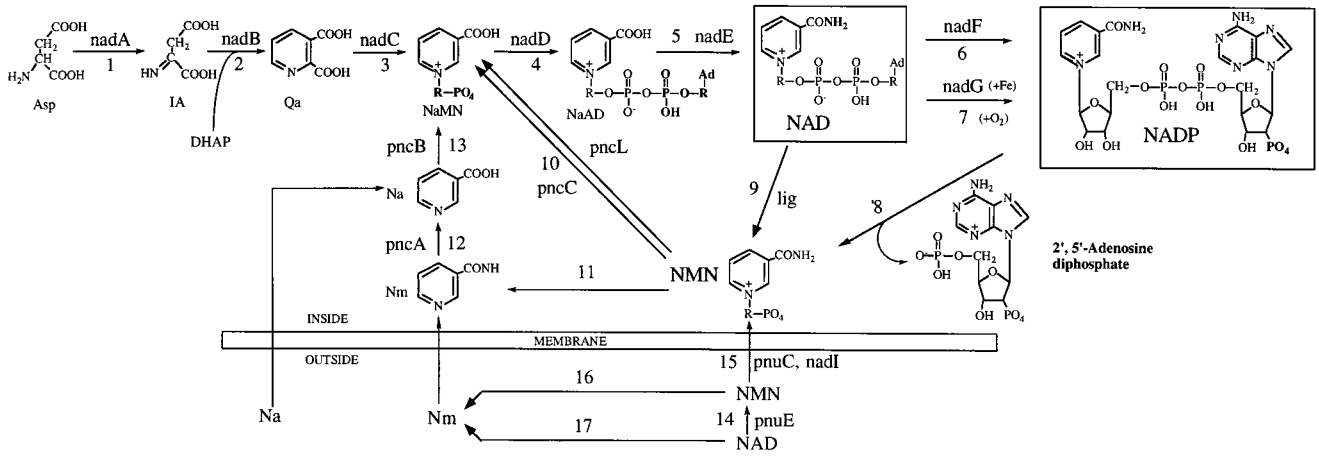

FIG. 1. The NAD metabolic pathways of *S. typhimurium*. The steps and enzymes included are as follows: 1, L-aspartate (Asp) oxidase; 2, quinolinic acid (Qa) synthetase; 3, quinolinic acid phosphoribosyltransferase; 4, NaMN adenylyltransferase; 5, NAD synthetase; 6 and 7, NAD kinase (the NadG enzyme is induced by O_2 and activated by Fe^{2+}); 8, NADP pyrophosphatase; 9, DNA ligase (lig); 10, NMN deamidase; 11 and 16, NMN glycohydrolase; 12, nicotinamide (Nm) deamidase; 13, NA phosphoribosyltransferase; 14, NAD pyrophosphatase; 15, NMN transporter; 17, NAD glycohydrolase. Abbreviations: DHAP, dihydroxyacetone phosphate; IA, iminoaspartate; PRPP, 5-phosphoribosyl-1-PP_i. Genetic loci corresponding to enzymatic steps are indicated above reaction arrows. Mutants for the glyhydrolases (reactions 11, 16, and 17) have not been identified. The NADP glycohydrolase (reaction 8) is inferred but has not been demonstrated.

two *nadI* mutants and four *pnuC* mutants in addition to the two *pncC* mutants and one *pncD* mutant described here.

Isolation and mapping of Tn*10 d*(Tc) insertions linked to the *pncC* or *pncD* gene. A *pncC* mutant and a *pncD* mutant were transduced to tetracycline resistance by using P22 phage grown on a random pool of 10^4 Tn*10 d*(Tc) insertion mutants (7, 8). Transductants (Tc^r) were screened for the loss of recipient NMN auxotrophy, indicative of coinheritance of a $pncC^+$ or $pncD^+$ allele with the selected Tn*10* element. Linked Tn*10* insertions were then transduced back into the original *pnc* mutant to test the linkage of the Tn*10 d*(Tc) element to the *pncC* or *pncD* mutation. The chromosomal locations of the *pncC* and *pncD* genes were determined by mapping the nearby Tn*10* insertions by the method of Benson and Goldman (1). More accurate mapping was done by testing transductional linkage to known markers in the region identified.

Preparation of [carbonyl-^{14}C]NMN from [carbonyl-^{14}C]NAD. Radioactive [carbonyl-^{14}C]NMN was prepared by pyrophosphorolysis of [carbonyl-^{14}C]NAD with the enzyme nucleotide pyrophosphatase (36). The reaction mixture (1 ml) was 47.5 μM NAD–4.75 mM $MgCl_2$–9.5 mM Tris-HCl (pH 7.4) and contained 0.2 U of Crotalus nucleotide pyrophosphatase type II (Sigma) and 9.5 μCi of [carbonyl-^{14}C]NAD. The reaction was carried out at 37°C for 60 min and monitored by spotting 2 μl of the reaction mix onto a thin-layer chromatography (TLC) plate (plastic PEI-Cellulose F; EM Science), with 2 μl of 10 mM NAD–50 mM NMN solution spotted at the same place as markers. The TLC separations were done with 1 M LiCl. After the plate dried, the NAD and NMN control spots were located under UV light and cut out to assay radioactivity. Greater than 98% of the NAD was converted to NMN. The NMN spot was removed from the plate, debris was removed by centrifugation, and the supernatant was used in the assay described below.

Preparation of cell extracts. Cells in a 500-ml, exponentially growing culture were pelleted by centrifugation, washed, and resuspended in 5 ml of 0.02 M Tris buffer (pH 7.5). Cells were lysed with a French press at 20,000 lb/in^2. The crude extract was centrifuged at 17,000 × *g* for 30 min to remove debris. Glycerol was added to each extract to a final concentration of 15%, and the extract was divided into small samples (500 μl), which were quickly frozen in dry ice-ethanol and stored at −70°C. Extracts stored in this way retained full NMN deamidase activity for over 1 month. Protein concentrations were determined by the method of Lowry et al. (20) with bovine serum albumin as the standard.

Assay of NMN deamidase. The standard reaction mixture (100 μl) contained 10 μmol of Tris-HCl (pH 8.0), 50 nmol of NMN, 2.5×10^{-3} μCi of [carbonyl-^{14}C]NMN, 1 μmol of EDTA, 0.2 μmol of dithiothreitol, and 100 μg of crude extract protein. The reaction proceeded at 37°C for 10 min and was stopped by boiling for 30 s. Denatured protein was pelleted in a microcentrifuge, and the supernatant was spotted on a TLC chromatography plate (plastic PEI-Cellulose F; EM Science), with 1 μl of 10 mM NaMN–50 mM NMN solution spotted at the same place as samples. The TLC plates were run with 0.25 M NH_4SO_4. After the plate was dried, the distribution of radioactivity on the plate was determined with a PhosphorImager. The specific NMN deamidase activity was calculated as nanomoles of NaMN formed per minute per milligram of extract protein added. All specific activities reported are based on the amount of produced NaMN that fell within the linear dependence of the assay for time and protein.

RESULTS

Isolation of NMN deamidase mutants. Mutants were isolated in a parent strain with multiple mutations (TT18391) whose genotype is diagrammed in Fig. 2. This strain requires an external precursor for pyridine nucleotide and can use either NA or NMN. Because of the *pncA* mutation, the ability to use NMN depends on the enzyme NMN deamidase (Fig. 2 [diagonal arrow]). The bold arrows in Fig. 2 show the route of NMN utilization in parent strain TT18391. We screened mutagenized cells for mutants that could use NA but not NMN as an NAD precursor. When this strategy had been used previously, all of the recovered mutants were defective in NMN transport (*nadI pnuC*); none lacked NMN deamidase (9, 18, 36). We suspected that the failure to recover NMN deamidase mutants was due to toxic inhibition of ligase by accumulated NMN. (Below we show that the real reason is the presence of two NMN deamidases.) Pursuing our original suspicion, we added to the strain a ligase mutation and a plasmid encoding the ATP-dependent ligase of phage T4. It was thought that the ATP-dependent ligase in this strain should not be subject to the toxic effects of NMN.

The parent strain (TT18391) was mutagenized with diethyl sulfate and plated for single colonies on nutrient agar at 37°C. Mutagenized clones were replica printed to minimal media with 1 μM NA and with 0.1 mM NMN. Plates were screened for clones that grew only on NA. The mutants isolated included *pnuC* and *nadI* mutants, as expected from previous results (18, 31, 35); these previously known classes were identified by genetic mapping and were not studied further. Two new types of mutants were isolated, and their phenotypes are presented in Table 2. Both types grew on NB medium and on minimal medium with 1 μM NA. The first mutant type (*pncC*) could grow on medium with NA even in the presence of NMN and thus was not inhibited by NMN. The second mutant type (*pncD*) failed to grow in the presence of NMN even when NA was also provided. This suggested that the growth of *pncD* mutants is inhibited by NMN. This inhibition was proved to be corrected by adding isoleucine and valine, suggesting that NMN induces an *ilv* auxotrophy in these mutants. On the basis

TABLE 1. List of strains used in this study

Strain	Genotype(s)
TT13259	*lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18417	*ilvC16*
TT10855	*metE205 ara-9 cya-961*::Tn*10 d*(Tc)
TT11558	*metE2362*::Mu*d*J
TT12990	*nadB103 pncA278*::Tn*10 d*(Cm)
TT17453	*metE ara-9 zid-3732*::Tn*10 d*(Cm) (92% linked to *cya*)
TT18391	*nadB103 pncA278*::Tn*10 d*(Cm) *lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18392	*nadB103 pncA278*::Tn*10 d*(Cm) *pncC328 lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18393	*nadB103 pncA278*::Tn*10 d*(Cm) *pncD329 lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18394	*nadB103 pncA278*::Tn*10 d*(Cm) *pncC330 lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18395	*nadB103 pncA278*::Tn*10 d*(Cm) *zic-3767*::Tn*10 d*(Tc) *lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18396	*nadB103 pncA278*::Tn*10 d*(Cm) *pncD329 zic-3767*::Tn*10 d*(Tc) *lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18397	*nadB103 pncA278*::Tn*10 d*(Cm) *zie-3782*::Tn*10 d*(Tc) *lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18398	*nadB103 pncA278*::Tn*10 d*(Cm) *zie-3782*::Tn*10 d*(Tc) *pncC328 lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18400	*nadB103 pncA278*::Tn*10 d*(Cm) *pncC330 zie-3782*::Tn*10 d*(Tc) *lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18401	*nadB103 pncA278*::Tn*10 d*(Cm) *pncC331*(Ts) *zie-3782*::Tn*10 d*(Tc) *lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18402	*nadB103 pncA278*::Tn*10 d*(Cm) *pncC332*(Ts) *zie-3782*::Tn*10 d*(Tc) *lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18403	*nadB103 pncA278*::Tn*10 d*(Cm) *pncC333*(Ts) *zie-3782*::Tn*10 d*(Tc) *lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18404	*nadB103 pncA278*::Tn*10 d*(Cm) *pncC334*(Ts) *zie-3782*::Tn*10 d*(Tc) *lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18405	*nadB103 pncA278*::Tn*10 d*(Cm) *pncC331*(Ts) *zie-3782*::Tn*10 d*(Tc)
TT18406	*nadB103 pncA278*::Tn*10 d*(Cm) *pncC332*(Ts) *zie-3782*::Tn*10 d*(Tc)
TT18407	*nadB103 pncA278*::Tn*10 d*(Cm) *pncC333*(Ts) *zie-3782*::Tn*10 d*(Tc)
TT18408	*nadB103 pncA278*::Tn*10 d*(Cm) *pncC334*(Ts) *zie-3782*::Tn*10 d*(Tc)
TT18409	*nadB103 pncA278*::Tn*10 d*(Cm) *zic-3767*::Tn*10 d*(Tc) *pncD337 lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18410	*nadB103 pncA278*::Tn*10 d*(Cm) *zic-3767*::Tn*10 d*(Tc) *pncD338 lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18411	*nadB103 pncA278*::Tn*10 d*(Cm) *zic-3767*::Tn*10 d*(Tc) *pncD339 lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18412	*nadB103 pncA278*::Tn*10 d*(Cm) *zic-3767*::Tn*10 d*(Tc) *pncD340 lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18413	*nadB103 pncA278*::Tn*10 d*(Cm) *zic-3767*::Tn*10 d*(Tc) *pncD341 lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18414	*nadB103 pncA278*::Tn*10 d*(Cm) *zic-3767*::Tn*10 d*(Tc) *pncD342 lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18418	*ilvD27*
TT18419	*ilvA8*
TT18420	*ilvB7*
TT18443	*nadB103 pncA278*::Tn*10 d*(Cm) *zic-3767*::Tn*10 d*(Tc) *pncD343 lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18444	*nadB103 pncA278*::Tn*10 d*(Cm) *zic-3767*::Tn*10 d*(Tc) *pncD344 lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18445	*nadB103 pncA278*::Tn*10 d*(Cm) *zic-3767*::Tn*10 d*(Tc) *pncD345 lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18446	*nadB103 pncA278*::Tn*10 d*(Cm) *zic-3767*::Tn*10 d*(Tc) *pncD346 lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)
TT18447	*nadB103 pncA278*::Tn*10 d*(Cm) *zic-3767*::Tn*10 d*(Tc) *pncD347 lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r)

of the genetic tests described below, the two mutant types were found to affect different chromosomal loci. Strains with a Tn*10 d*(Tc) insertion near the *pncC* or *pncD* locus were isolated as described in Materials and Methods. By using these nearby insertions as selective markers for local mutagenesis, several additional *pncC* and *pncD* point mutants were isolated. The growth phenotypes of all *pncC* and *pncD* mutants are included in Table 2.

Genetic characterizations of the *pncC* and *pncD* loci. To locate the *pncC* and *pncD* loci on the genetic map, we mapped the linked Tn*10* elements. Insertion *zie-3782*::Tn*10* is 89% cotransducible with a *pncC* mutation, and insertion *zie-3767*::Tn*10* is 86% cotransducible with a *pncD* mutation. These Tn*10* insertions were roughly mapped by the method of Benson and Goldman (1), which involves transducing the Tn*10* (Tc^r) insertion mutant to tetracycline sensitivity by using a series of donor

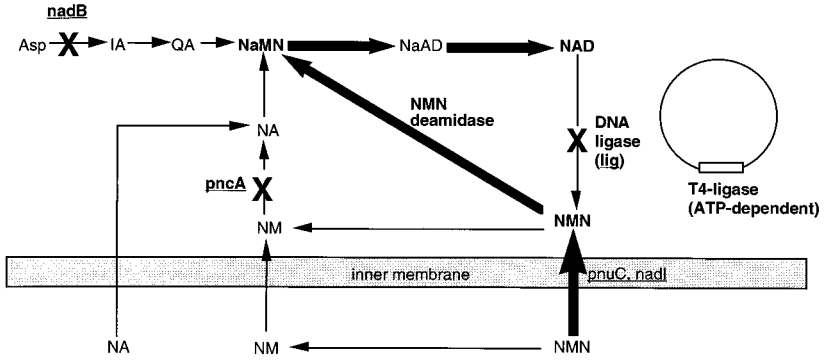

FIG. 2. Parent strain used for the *pncC* mutant hunt. The genotype of this strain (TT18391) is *nadB103 pncA278*::Tn*10 d*(Cm) *lig-2*::Mu*d*J (pBR313/598/8/1b = T4 *lig*$^+$ Amp^r). The growth of this strain on minimal medium with NMN depends on NMN deamidase. Bold arrows indicate the pathway of NMN assimilation. Asp, L-aspartate oxidase; IA, iminoaspartate; QA, quinolinic acid synthetase.

TABLE 2. Growth phenotypes of *pncC* and *pncD* mutants

Strain	Relevant genotype[a]	Growth on minimal medium under indicated conditions[b]											
		NA[c]		NMN[d]		NMN + IV		NA + IV		NA + NMN		NA + NMN + IV	
		30°C	42°C	30°C	42°C	30°C	42°C	30°C	42°C	30°C	42°C	30°C	42°C
TT18391	Wild type	+	+	+	+	+	+	+	+	+	+	+	+
TT18392[e]	*pncC328*	+	+	−	−	−	−	+	+	+	+	+	+
TT18394	*pncC330*	+	+	−	−	−	−	+	+	+	+	+	+
TT18401	*pncC331*(Ts)	+	+	+	−	+	−	+	+	+	+	+	+
TT18402	*pncC332*(Ts)	+	+	+	−	+	−	+	+	+	+	+	+
TT18403	*pncC333*(Ts)	+	+	+	−	+	−	+	+	+	+	+	+
TT18404	*pncC334*(Ts)	+	+	+	−	+	−	+	+	+	+	+	+
TT18393[e]	*pncD329*	+	+	−	−	+	+	+	+	−	−	+	+
TT18410	*pncD338*	+	+	−	−	+	+	+	+	−	−	+	+
TT18409	*pncD337*(Ts)	+	−	−	−	+	+	+	+	−	−	+	+
TT18410	*pncD338*(Ts)	+	−	−	−	+	+	+	+	−	−	+	+
TT18411	*pncD339*(Ts)	+	−	−	−	+	+	+	+	−	−	+	+
TT18413	*pncD341*(Ts)	+	+	+	−	+	+	+	+	+	−	+	+
TT18414	*pncD342*(Ts)	+	+	+	−	+	+	+	+	+	−	+	+

[a] All strains carry the mutations *nadB103 pncA278*::Tn*10 d*(Cm) *lig-2*::Mu*d*J and plasmid pBR313/598/8/1b (= T4 *lig*$^+$ Amp^r).
[b] +, growth; −, no growth. IV, isoleucine and valine (0.3 mM).
[c] *nadB pncA* auxotrophs grow on minimal medium with the addition of NA (10^{-6} M) or NMN as an NAD precursor.
[d] *nadB pncA* auxotrophs grow on minimal medium with the addition of NMN (10^{-4} M).
[e] These are the original mutants; all others were isolated by local mutagenesis of the *pncC* or *pncD* region.

lysates prepared by inducing a well-mapped Mu*d* P22 lysogen. Each such donor lysate transduces a characteristic region of the chromosome with high frequency. The *pncC* locus mapped between 83 and 87 min on the *S. typhimurium* chromosome, and the *pncD* locus mapped between 81 and 83 min.

Transduction crosses were performed to test the linkage of *pncC* and *pncD* mutations to mutations known to lie in these regions of the chromosome. The results, summarized in Fig. 3, place the *pncC* locus between the *cva* and *metE* loci at 83.8 min. The *pncD* locus appears to coincide with the *ilv* operon at 83 min (Fig. 3).

Local mutagenesis of the *pncD* region. Using a Tn*10* insertion near the *pncD* gene as a selective marker, we locally mutagenized the *pncD* locus. Tetracycline-resistant transductant colonies were selected on NB agar plates supplemented with tetracycline by using a hydroxylamine-treated phage lysate; transductant colonies were then replica printed to minimal NA and NMN plates with and without isoleucine and valine; such plates were scored at 30 and 42°C. This revealed mutants that were *ilv* auxotrophs on either NA or NMN medium or on both. By this procedure, we isolated 76 mutants with some sort of *ilv* auxotrophy. Of these, 41 showed NMN-induced auxotrophy at some temperature. The phenotypes of these mutants are summarized in Table 3.

Assay of NMN deamidase in *S. typhimurium*. Cell extracts prepared from the parent strain (TT18391), a *pncA nadB lig*

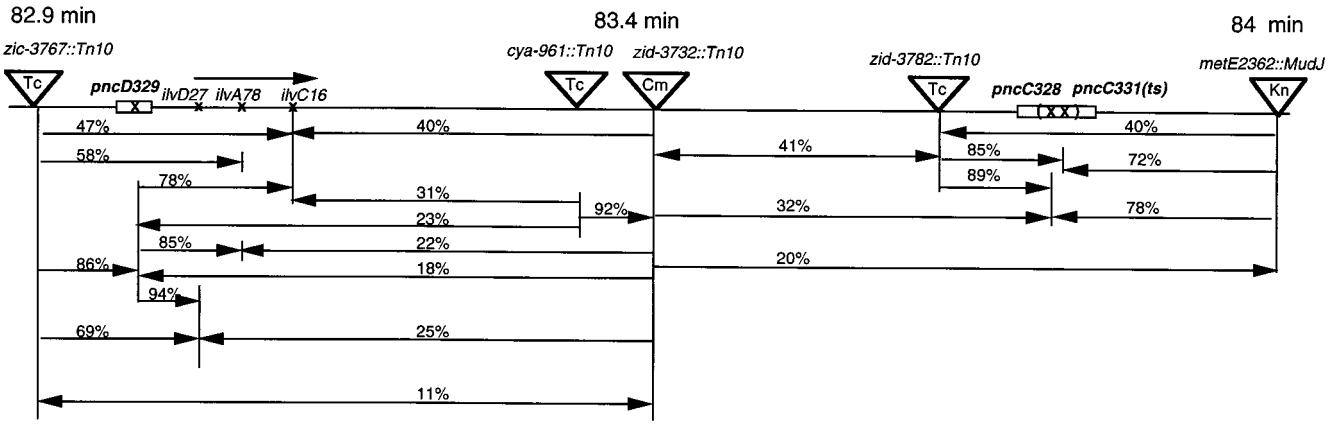

FIG. 3. Map positions of the *pncC* and *pncD* genes on the *S. typhimurium* chromosome. The strains used in crosses were *pncC328* (TT18392), *zie-3782*::Tn*10* (TT18397), *pncC331*(Ts) (TT18401), *metE2362*::Mu*d*J (TT11558), *cya-961*::Tn*10* (TT10855), *zid-3732*::Tn*10* (TT17453), *pncD329* (TT18392), *zic-3767*::Tn*10* (TT18396), *ilvD27* (TT18418), *ilvA8* (TT18419), and *ilvC16* (TT18417). All linkages were determined by P22-mediated transduction crosses. For each cross, the phenotypes of 250 transductants were scored. Arrowheads point to the donor marker selected in each cross.

TABLE 3. Growth phenotypes of *ilv* mutants isolated by local mutagenesis of the *pncD* region[a]

Mutation type	Representative strain	Phenotype[b]	% of mutants tested[c]	Growth on minimal medium under indicated conditions[d]									
				NA[e]		NMN[f]		NMN + IV		NA + IV		NA + NMN	
				30°C	42°C	30°C	42°C	30°C	42°C	30°C	42°C	30°C	42°C
I	TT18443	Ilv^-	38	−	−	−	−	+	+	+	+	−	−
II	TT18444	Ilv^-(Ts)	21	+	−	+	−	+	+	+	+	+	−
III	TT18445	Ilv^{42} NMN^{30}	31	+	−	−	−	+	+	+	+	−	−
IV	TT18446	NMN	5	+	+	−	−	+	+	+	+	−	−
V	TT18447	NMN^{42}	5	+	+	+	−	+	+	+	+	+	−

[a] All strains carry the mutations *nadB103 pncA278*::Tn*10 d*(Cm) *lig-2*::Mu*dJ* and plasmid pBR313/598/8/1b (= T4 lig^+ Amp^r).
[b] Ilv^{42}, isoleucine-plus-valine auxotrophy at 42°C; NMN^{30}, sensitivity to growth inhibition by NMN at 30°C and full auxotrophy at 42°C; NMN, sensitivity to growth inhibition by NMN at both 30 and 42°C; NMN^{42}, sensitivity to growth inhibition by NMN at 42°C and prototrophy at 30°C.
[c] The total number of *ilv* mutants tested was 76.
[d] +, growth; −, no growth. IV, isoleucine and valine (0.3 mM).
[e] The parent strain (*nadB pncA*) grows on minimal medium with the addition of NA (10^{-6} M).
[f] The parent strain (*nadB pncA*) grows on minimal medium with the addition of NMN (10^{-4} M).

mutant, were assayed for NMN deamidase activity as described in Materials and Methods. The assay was linear with added enzyme (up to about 100 μg of added protein) and with time (for 10 min). The enzyme showed a pH optimum of 8.5. This value is similar to the optimum of the *E. coli* enzyme (pH 9) but differs greatly from that (pH 5.6) observed for *Propionibacterium shermanii* (12, 18). Our data for the dependence of the reaction rate on substrate concentration at the optimum pH suggested sigmoidal kinetics. The sigmoidal dependence of activity on substrate concentration for *S. typhimurium* (18) and *E. coli* (5) NMN deamidase makes accurate determinations of K_m and maximum rate of metabolism difficult. The data suggest that the NMN deamidase in *S. typhimurium* is under some form of allosteric control. Crude extracts with strong NMN deamidase activities showed no ability to deamidate NAD.

Assay of NMN deamidase in *pncC* and *pncD* mutants. To test if the growth phenotypes of *pncC* and *pncD* mutants are correlated with a defect in NMN deamidase, this enzyme was assayed in extracts of both mutant types. As seen in Fig. 4, the *pncD* mutant retained 95% of wild-type NMN deamidase activity while *pncC* mutants have only 5% of wild-type activity. The phenotype, genetic map position, and enzyme assays suggest that *pncD* mutations are actually *ilv* mutations which render some isoleucine-valine biosynthetic enzyme sensitive to inhibition by NMN. The *pncC* mutation appears to affect the level of NMN deamidase. As seen in Fig. 4, the NMN deamidase activities of all *pncC*(Ts) mutants were temperature sensitive while the NMN deamidase activities of all *pncD*(Ts) mutants were not strongly affected by growth temperature.

Heat inactivation of PncC enzyme from temperature-sensitive mutants. To determine whether *pncC* mutations affect the structure gene for NMN deamidase, the heat stability of NMN deamidase from *pncC*(Ts) mutants was tested. Extracts were made from cells grown at 30°C in NB. Extracts were incubated at 65°C, and samples were withdrawn periodically and added directly to the reaction mixture to measure the NMN deamidase activity. As seen in Fig. 5, the NMN deamidase activity of a *pncC*(Ts) strain was more rapidly inactivated than that of a $pncC^+$ strain. We conclude that a *pncC*(Ts) mutation changes the quality of NMN deamidase activity and is likely to affect the structural gene for this enzyme.

Phenotypes of *pncC* mutations in a lig^+ background. Two *pncC*(Ts) mutations were moved into a lig^+ strain (TT12990) by P22 transduction, selecting for inheritance of a Tn*10* insertion linked to the *pncC*(Ts) mutation. Under anaerobic conditions, the *pncC* mutation caused a failure to use NMN in both lig^+ and *lig* mutant strains. However, in the presence of oxygen, a lig^+ *pncC* mutant is able to use NMN as an NAD precursor. The results are summarized in Table 4.

NMN deamidase activities in lig^+ *pncC* mutants. We tested directly the possibility that a second NMN deamidase activity was activated or induced by oxygen in a lig^+ *pncC* mutant. This

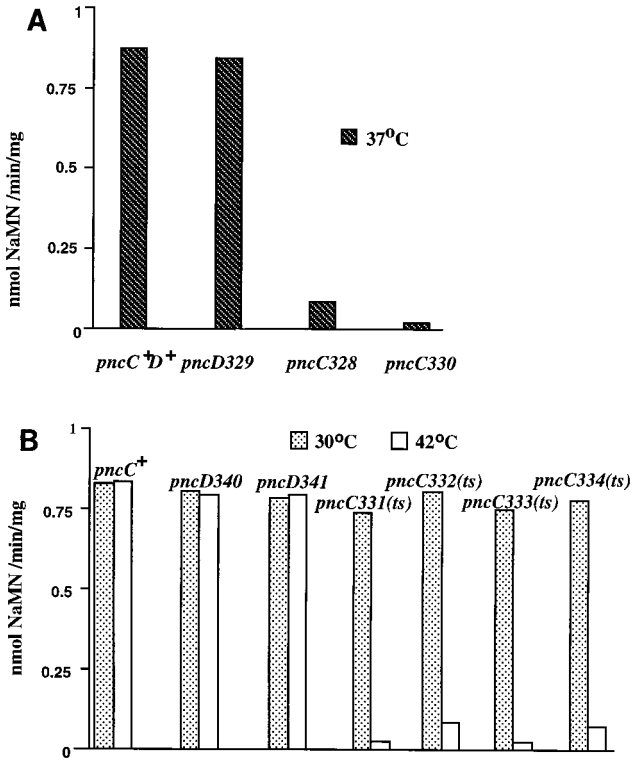

FIG. 4. Assays of NMN deamidase activities of *pncC* and *pncD* mutants. All cells were grown in NB at 37°C and harvested at a density of about 5×10^8. Extract procedures and assay conditions are described in Materials and Methods. (A) All assays were run at 37°C for 10 min. The strains assayed were the $pncC^+D^+$ parent (TT18391), *pncD329* (TT18393), *pncC328* (TT18392), and *pncC330* (TT18394). (B) For assays of temperature-sensitive mutants, all cells were grown in minimal medium with 10^{-6} M NA at 30 or 42°C and harvested at a density of 100 Klett units. Extract preparations and assay conditions are described in Materials and Methods. All assays were run at 37°C for 10 min. The strains assayed were the $pncC^+$ parent (TT18391), *pncD340* (TT18412), *pncD341* (TT18413), *pncC331*(Ts) (TT18401), *pncC332*(Ts) (TT18402), *pncC333*(Ts) (TT18403), and *pncC334*(Ts) (TT18404). Full genotypes are listed in Table 1.

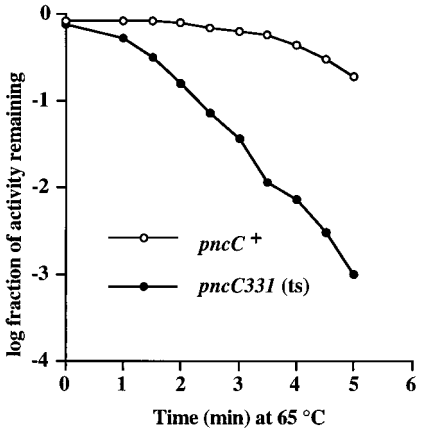

FIG. 5. Heat inactivation of NMN deamidase. Extracts were made from cells grown in minimal medium with 10^{-6} M NA at 30°C. Extracts were incubated at 65°C for the times indicated; samples were withdrawn and added directly to the reaction mixture to measure the NMN deamidase activity at 37°C. The initial wild-type activity is 0.828 U, and the initial mutant activity is 0.738 U. The strains used were the *pncC*$^+$ parent (TT18391) and *pncC331*(Ts) (TT18401). Full genotypes are listed in Table 1.

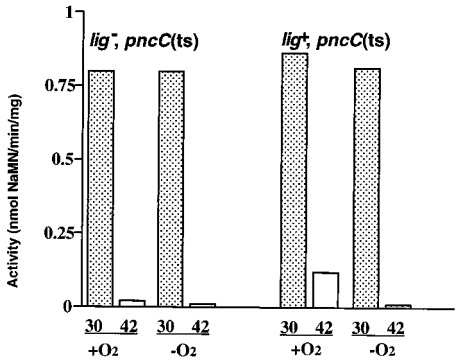

FIG. 6. Effects of ligase deficiency and oxygen on the NMN deamidase activities of *pncC* mutants. Strains were grown aerobically or anaerobically at 30 or 42°C in minimal medium with 10^{-6} M NA. Assays were done under standard conditions (37°C), as described in Materials and Methods. The strains assayed were a *lig pncC*(Ts) mutant (TT18401) and a *lig*$^+$ *pncC*(Ts) mutant (TT18405) (Table 1).

activity in a *lig*$^+$ *pncC*(Ts) mutant strain was assayed under various conditions. The results are summarized in Fig. 6.

In a *pncC*(Ts) *lig* mutant strain, NMN deamidase activity was about 2% of the wild-type level for cells grown at 40°C with or without oxygen. The *lig*$^+$ *pncC*(Ts) mutant strain showed the same temperature-sensitive activity when grown anaerobically. However, when cells were grown aerobically at a high temperature, a *lig*$^+$ *pncC*(Ts) mutant strain showed 10 to 15% of the wild-type activity. This suggested that another NMN deamidase activity might be induced by oxygen in a *lig*$^+$ strain, accounting for the growth phenotype of the *lig*$^+$ *pncC* mutant strain (Table 4).

DISCUSSION

To investigate the NAD cycle in *S. typhimurium*, we isolated mutants defective in NMN deamidase. A parental strain (*nadB pncA*) that required an NAD precursor and could use exogenous NMN as that precursor only if NMN deamidase activity was present was constructed. Two classes of mutants that could not grow on NMN medium were found. One type, *pncC*, is deficient in NMN deamidase. The other type, *pncD*, appears to carry an *ilv* mutation which renders the synthesis of branched-chain amino acids sensitive to inhibition by exogenous NMN.

Previously, this mutant isolation strategy yielded only NMN transport mutants (*nadI pnuC*) (9, 36). In the mutant isolation procedure described here, the parental strain lacked the essential enzyme NAD-dependent DNA ligase and was kept alive by an alternative ligase from T4 that uses ATP instead of NAD as a cofactor. Characterizations of the *pncC* mutations suggest that the success of the hunt depended on the *lig* mutation. Under aerobic conditions in a *lig*$^+$ strain, a second NMN deamidase activity (*pncL*) appears and obscures the NMN utilization defects of *pncC* mutants. We are currently characterizing this oxygen-activated, ligase-dependent deamidase activity.

Salmonella NAD-dependent DNA ligase is an essential enzyme and is sensitive to inhibition by NMN. The sensitivity of ligase to NMN is due to product inhibition of the first ligase-catalyzed reaction (E [ligase] + NAD = E-AMP + NMN), which has a very unfavorable equilibrium. The two NMN deamidases may be important to maintain a small NMN pool size. NMN is generated in minor part by DNA ligase; the major source is an oxygen-stimulated, ligase-independent route which we suspect is pyrophosphorylation of NADP (Fig. 1). When oxygen stimulates the NAD cycle, the ligase-dependent deamidase (*pncL*) may serve to protect DNA ligase from in-

TABLE 4. Effect of a *lig* mutation on the PncC phenotype

Strain	Relevant genotype[a]	Growth on minimal medium under indicated conditions[b]							
		Aerobic				Anerobic			
		NA[c]		NMN[d]		NA[c]		NMN[d]	
		30°C	42°C	30°C	42°C	30°C	42°C	30°C	42°C
TT12990	*pncC*$^+$ *lig*$^+$	+	+	+	+	+	+	+	+
TT18391	*pncC*$^+$ *lig-2*::MudJ	+	+	+	+	+	+	+	+
TT18402	*pncC332*(Ts) *lig-2*::MudJ	+	+	+	−	+	+	+	−
TT18401	*pncC331*(Ts) *lig-2*::MudJ	+	+	+	−	+	+	+	−
TT18405	*pncC331*(Ts) *lig*$^+$	+	+	+	+	+	+	+	−
TT18406	*pncC332*(Ts) *lig*$^+$	+	+	+	+	+	+	+	−

[a] All strains carry the mutations *nadB103 pncA278*::Tn*10 d*(Cm), as diagrammed in Fig. 2. All strains with *lig-2*::Mu*d*J also carry plasmid pBR313/598/8/1b (= T4 *lig*$^+$ Amp^r).

[b] +, growth; −, no growth.

[c] *nadB pncA* auxotrophs grow on minimal medium with the addition of NA (10^{-6} M).

[d] *nadB pncA* auxotrophs grow on minimal medium with the addition of NMN (10^{-6} M).

hibition by accumulated NMN and drives the ligase reaction under these adverse conditions. We suggest that the PncC activity serves mainly for assimilation of exogenous NMN.

The map positions and phenotypes of *pncD* mutations suggested that their NMN-induced auxotrophy was due to the inhibition of an *ilv* biosynthetic enzyme by NMN. While we did not expect this mutant type, its existence (in retrospect) is not surprising. Many enzymes use NAD or NADP as a cofactor and NMN might be expected to compete with these cofactors for binding to enzymes. Mutations with the *pncD* phenotype are not rare. Of 76 *ilv* mutants produced by local mutagenesis of this locus, 41 showed sensitivity to NMN inhibition (Table 3). Particularly revealing are the mutants that are NMN sensitive only at 42°C (class V) and the mutants that show NMN-induced auxotrophy at 30°C and become normal *ilv* auxotrophs at 42°C (class III). Both mutant types suggest that NMN sensitivity is associated with a loss of activity rather than with a specific novel property of a mutant protein; this conclusion is supported by the high frequency of NMN-sensitive *ilv* auxotrophs. We propose that any mutation which reduces the level of the critical enzyme allows the natural NMN sensitivity of the wild-type enzyme to produce a detectable growth phenotype. The first enzyme (IlvC) of the biosynthetic pathway common to isoleucine and valine is a reductase which uses NADPH as a cofactor. Since NMN is an analog of NADPH, we speculate that the IlvC enzyme is a likely target enzyme for this inhibition.

In the original global mutant hunt described here, we isolated three mutants unable to grow on minimal medium with NMN. Two had defects in NMN deamidase (*pncC*), and the other showed an NMN-induced auxotrophy (*ilv*). So few mutants do not allow us to evaluate how common NMN-sensitive mutants are for a variety of other enzymes that use NAD(P) as a cofactor. If many enzymes that use pyridine nucleotides are sensitive to NMN inhibition, such NMN-sensitive mutations may be common. The existence of many NMN-sensitive enzymes would add further importance to the control of internal NMN levels.

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