Salmonella typhimurium Mutants Lacking NAD Pyrophosphatase

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NAD can serve as both a purine and a pyridine source for *Salmonella typhimurium*. Exogenous NAD is rapidly broken down into nicotinamide mononucleotide and AMP by an NAD pyrophosphatase, the first step in the pathway for the assimilation of exogenous NAD. We isolated and characterized mutants of *S. typhimurium* lacking NAD pyrophosphatase activity; such mutants were identified by their failure to use exogenous NAD as a purine source. These mutants carry mutations that map at a new locus, designated *pnuE*, between 86 and 87 min on the *Salmonella* chromosome.

NAD has a central metabolic role as a cofactor in oxidation-reduction reactions. In bacterial cells, NAD is synthesized de novo or from exogenous pyridines through two salvage pathways (12, 16). These salvage pathways also serve to regenerate NAD from nicotinamide mononucleotide (NMN) formed by DNA ligase and other reactions that break down NAD (13; for reviews, see references 7, 8, and 15). In *Salmonella typhimurium*, NAD pyrophosphatase is apparently the first enzyme in assimilation of exogenous NAD, catalyzing the cleavage of NAD to NMN and AMP. NAD cannot pass the inner membrane; however, NMN is taken up by *S. typhimurium* intact (12). Alternatively, NMN can be broken down before uptake, generating nicotinamide (1), which can traverse the cell membrane. Intracellular NMN or nicotinamide can then be used for the synthesis of NAD by pathways shown in Fig. 1. The AMP moiety of NAD is also assimilated and can serve as a source of purine rings.

NAD pyrophosphatase activity can be demonstrated with intact cells; the enzyme is apparently bound to the cytoplasmic membrane, facing outward (6). This enzyme is the major contributor to the breakdown of NAD in crude extracts of *S. typhimurium*. In this report, we describe the isolation of mutants deficient in NAD pyrophosphatase and the mapping of the NAD pyrophosphatase (*pnuE*) gene.

MATERIALS AND METHODS

Materials. [^{14}C]NAD, [^3H]ATP, and [^{14}C]nicotinamide were purchased from Amersham Corp. Snake venom phosphodiesterase was the product of Sigma Chemical Co.

Bacterial strains. All strains used in this study (Table 1) were derived from *S. typhimurium* LT2.

Media. The low-phosphate morpholinepropanesulfonic acid medium of Neidhardt et al. (14), with added NAD or AMP, was used for the NAD pyrophosphatase mutant isolation. In all other cases, the E medium of Vogel and Bonner (21) was used as minimal medium supplemented with 0.2% glucose as the carbon source. E medium lacking citrate was used when cells were grown on carbon sources other than glucose. Nutrient broth (8 g/liter) with NaCl (5 g/liter) was used as the rich medium. Difco agar (1.5%) was added to make solid media. The following additives were included in media as needed (final concentrations): amino acids (0.3 mM), adenine (0.4 mM), AMP (0.5 mM), NAD (0.5 mM), kanamycin sulfate (25 $\mu\text{g}/\text{ml}$ in rich medium, 125 $\mu\text{g}/\text{ml}$ in minimal medium), tetracycline sulfate (20 $\mu\text{g}/\text{ml}$ in rich

medium, 10 $\mu\text{g}/\text{ml}$ in minimal medium), nicotinic acid (2 $\mu\text{g}/\text{ml}$), and streptomycin sulfate (200 $\mu\text{g}/\text{ml}$ in rich medium, 1 mg/ml in minimal medium).

Transductions. P22 bacteriophage and *S. typhimurium* cells were spread directly on selection plates: 10^8 to 10^9 cells were plated with an equal number of phage particles. For the transduction of kanamycin resistance, phage and cell mixtures were preincubated for 30 min at 37°C before being spread on plates. The high-frequency, general transducing mutant of bacteriophage P22 (HT105/1) *int201* (17) was used for all transductional crosses. Transductants were isolated as single colonies on green indicator agar (3) to identify phage-free clones; these clones were checked for phage sensitivity by cross-streaking with clear plaque P22 mutant H5.

***mudJ* mutagenesis.** Overnight cultures of strain TT13263 (*purE nadB*) were transduced to kanamycin resistance on rich plates by using donor phage grown on a pool of over 10,000 random *mudJ* insertion mutants (8a). The transductant colonies were replica printed to NAD-minimal and AMP-minimal plates. Mutants able to use AMP but not NAD as a purine source were purified and checked for phage sensitivity.

Isolation of Tn10d Tet insertion linked to *pnuE*. A culture of a *pnuE::mudJ* insertion mutant was transduced to tetracycline resistance by using P22 phage grown on a random pool of 10^4 Tn10d Tet insertion mutants (22). Transductants were replica printed to plates containing both tetracycline and kanamycin. Kanamycin-susceptible colonies, which had lost *pnuE::mudJ*, were identified.

Several putative clones with Tn10d linked to *pnuE* were purified on green plates and checked for phage sensitivity. Linkage of this Tn10d Tet insertion to *pnuE::mudJ* was measured by P22 transduction. One such clone, which showed 96% linkage, has been designated *zgi-3651::Tn10d Tet*.

Isolation of *pnuE* deletion mutants. A double mutant having both *zgi-3651::Tn10d* and *pnuE::mudJ* was constructed by P22 transduction. Cells (10^8 to 10^9) of the double mutant were spread on Bochner plates (2), which allow growth only of tetracycline-susceptible colonies. Survivors selected on Bochner plates were patched to Bochner plates and replica printed to kanamycin plates and to tetracycline plates. Several mutants which had lost both kanamycin and tetracycline resistance were isolated on Bochner plates and characterized.

Preparation of cell extracts. Exponentially growing cultures (100 ml) of strains to be analyzed were centrifuged to

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FIG. 1. Assimilation of exogenous NAD by *S. typhimurium*, showing the role of NAD pyrophosphatase. The major pathways for the assimilation of exogenous NAD, showing resynthesis into endogenous NAD and nucleic acids, are present. The breakdown of NAD to AMP and NMN and eventually to adenosine and nicotinamide takes place in the periplasmic space. The pyridine and purine moieties can return to resynthesize NAD by the routes shown; the pyridine moiety almost exclusively goes toward synthesis of NAD. However, only a small proportion (ca. 1%) of purine biosynthesis goes into the NAD pool; a vast majority either stays as ATP or is used for nucleic acid biosynthesis. Abbreviations: Ado, adenosine; Nm, nicotinamide; Na, nicotinic acid; NaMN, nicotinic acid mononucleotide; NaAD, nicotinic acid adenine dinucleotide.

pellet cells, washed, and suspended in 3 ml of 0.02 M Tris buffer (pH 7.5). The cells were lysed with a French press at 20,000 lb/in². Crude extracts were divided into small samples (200 µl), quickly frozen in dry ice-methanol, and stored at -70°C.

Enzyme assays. NAD pyrophosphatase in crude extracts was assayed in a reaction mixture (0.15 ml) containing 0.02 M Tris buffer (pH 7.5), 3 mM [¹⁴C]NAD (total radioactivity per assay, 75 nCi), and 70 to 100 µg of crude extract protein; the reaction proceeded for 30 min at 37°C. The NAD pyrophosphatase activity of whole cells was measured by using log-phase cells grown to an absorbance of 0.5. Samples of washed cells were incubated in the reaction mixture at 30°C for 20 to 50 min. The reaction mixture (0.12 ml) contained [¹⁴C]NAD (30 nmol; 0.2 µCi) and, in some experiments, [³H]ATP (30 nmol; 4 µCi), 0.05 M Tris buffer (pH 8.5), and 30 µl of the whole-cell preparation (around 150 µg of protein). Reactions were stopped by cooling the reaction mixture to 0°C and adding HCl to a final concentration of 0.1 M.

Samples of the pyrophosphatase reaction mixtures were spotted on 3MM chromatography paper and developed with the citrate-ethanol system of Witholt (23) for 14 to 17 h. Fully developed chromatograms were cut into 1.0- or 1.5-cm strips and counted for both ³H and ¹⁴C to determine the rate at which each substrate is hydrolyzed.

Nicotinamide deamidase was assayed at 37°C for 30 min in a reaction mixture (0.1 ml) containing 1 mM [¹⁴C]nicotinamide (250 nCi per assay)-0.02 M Tris buffer (pH 7.5). The reaction was stopped by adding HCl. [¹⁴C]nicotinic acid

TABLE 1. List of *S. typhimurium* strains

Strain	Genotype	Source
TR139	<i>metE338 ilvC441</i>	Laboratory collection
TR305	<i>metE2 cys-7</i>	Laboratory collection
TR310	<i>metB160 ser-793</i>	Laboratory collection
TR2344	<i>hisC483 ilvC401 metE338 rha-12</i>	Laboratory collection
TR5654	<i>thrA9 strA1</i>	Davis et al. (5)
TR5655	<i>leu485 strA1</i>	Davis et al. (5)
TR5656	<i>proA36 strA1</i>	Davis et al. (5)
TR5658	<i>pyrC7 strA1</i>	Davis et al. (5)
TR5660	<i>pyrF146 strA1</i>	Davis et al. (5)
TR5662	<i>hisO1242 Δhis-2236 strA1</i>	Davis et al. (5)
TR5663	<i>ΔpurF145 strA1</i>	Davis et al. (5)
TR5664	<i>cysA533 strA1</i>	Davis et al. (5)
TR5665	<i>cysC519 strA1</i>	Davis et al. (5)
TR5666	<i>serA13 strA1</i>	Davis et al. (5)
TR5667	<i>cysG439 strA1</i>	Davis et al. (5)
TR5670	<i>metA53 strA1</i>	Davis et al. (5)
TR5671	<i>pyrB64 strA1</i>	Davis et al. (5)
TT218	<i>met-862::tet-218 (MetE⁻)</i>	Laboratory collection
TT628	<i>strA1 pyrC7/F'114ts lac⁺ zzf-21::Tn10(B)</i>	Chumley et al. (4)
TT629	<i>strA1 pyrC7/F'114ts lac⁺ zzf-22::Tn10(A)</i>	Chumley et al. (4)
TT13263	<i>purE884::Tn10 nadB51</i>	This work
TT13264	<i>purE884::Tn10 nadB51 z?:mudJ</i> (possible porin mutant)	This work
TT13265	<i>pnuE84::mudJ purE884::Tn10 nadB51</i>	This work
TT13266	<i>pnuE41::mudJ purE884::Tn10 nadB51</i>	This work
TT13267	<i>zgi-3642::Tn10 (Tn10 96% linked to pnuE)</i>	This work
TT13268	<i>zgi-3642::Tn10/F'114ts lac⁺ zzf-21::Tn10(B)</i>	This work
TT13269	<i>zgi-3642::Tn10/F'114ts lac⁺ zzf-22::Tn10(A)</i>	This work
TT13270	<i>pnuE84::mudJ</i>	This work
TT13272	<i>purE8 ΔpnuE1</i>	This work
TT13273	<i>purE8 ΔpnuE2</i>	This work
TT13488	<i>purE8 zgi-3650::Tn10d (Tn10d 32% linked to glpK)</i>	This work
TT13489	<i>hisC483 ilvC401 metE338 rha-12 pnuE84::mudJ</i>	This work
TT13490	<i>glpK7 serA13 pnuE84::mudJ</i>	This work
TT13491	<i>glpK7 hisC483 ilvC401 metE338 rha-12 pnuE84::mudJ</i>	This work
TT13500	<i>purE8 zgi-3651::Tn10d (Tn10d 96% linked to pnuE)</i>	This work

and nicotinamide were separated by DEAE paper chromatography developed with 0.25 M ammonium bicarbonate solution for 6 to 8 h. The nicotinamide and nicotinic acid spots were cut from the DEAE paper and counted; unlabeled samples of both pyridine bases (10 mg/ml) were included in all chromatography lanes.

Protein radiolabeled with [³²P]NAD. Crude extract (30 µl, 25 to ~60 µg) was added to a reaction mixture (0.1 ml) which contained 1 µM [³²P]NAD (1.2 µCi), 4 mM MgCl₂, and 40 mM Tris buffer (pH 8.0). Reaction mixtures were incubated at 37°C for 12 min and stopped by the addition of an equal volume of sodium dodecyl sulfate sample buffer (11) followed by boiling for 2 min.

Electrophoresis of a portion of the above samples was performed by the protocol of Laemmli with a 3% stacking gel (1.0 by 12 by 0.1 cm) and 14% separating gel (9.5 by 12 by 0.1 cm) for 2 1/2 h at 35 mA. The gel was then fixed overnight in

TABLE 2. Enzyme assays

Strain	Sp act (nmol/min per mg)	
	NAD pyrophosphatase ^a	Nicotinamide deamidase ^b
TT13263 (<i>purE::Tn10</i>)	11.4	1.44
TT13265 (<i>purE::Tn10</i> <i>pnuE::mudJ</i>)	<0.2	1.66
TT13266 (<i>purE::Tn10</i> <i>pnuE::mudJ</i>)	<0.01	2.00
TT13264	14.1	1.73
TT13272 (<i>pnuE</i> deletion)	<0.01	Not determined

^a Extracts of strains were prepared with a French press as described in Materials and Methods. NAD pyrophosphatase activity was assayed at 37°C for 30 min in a reaction mixture (0.15 ml) containing 0.02 M Tris buffer (pH 7.5) and 3 mM [¹⁴C]NAD (total radioactivity per assay, 75 nCi).

^b Nicotinamide deamidase was assayed with the same crude extract in a reaction (0.1 ml) with 1 mM [¹⁴C]nicotinamide (250 nCi per assay)–0.02 M Tris buffer (pH 7.5).

45% isopropanol and 9.1% glacial acetic acid and stained for 2 h in a 0.25% Coomassie blue–45% isopropanol–9.1% acetic acid. Destaining was done in 10% isopropanol–acetic acid for 2 to 4 h. The vacuum-dried gel was exposed to Kodak X-Omat AR film at –70°C.

RESULTS

Isolation of mutants deficient in pyrophosphatase. The identification of mutants deficient in NAD pyrophosphatase was based on the fact that this enzyme cleaves NAD to NMN plus AMP in the periplasmic space. NMN traverses the inner membrane intact or is degraded to nicotinamide; both are precursors for NAD biosynthesis. AMP is hydrolyzed to adenosine by phosphatases; the adenosine can be utilized as a purine source. Thus, NAD can be used as a source of both purine and pyridine rings in the presence of NAD pyrophosphatase.

We identified three insertion mutants which did not use exogenous NAD as an adenine source, but could use AMP. Phage P22 grown on a pool of *mudJ* random insertion mutants (Hughes and Roth, unpublished observations) was used to transduce an adenine-requiring strain (*PurE*[–]) to kanamycin resistance. From 40,000 selected Kan^r colonies, three were found which did not grow on minimal NAD medium but grew normally on medium containing AMP. An enzyme assay was carried out on crude extracts of these mutants to determine whether they were, in fact, deficient in NAD pyrophosphatase. Two strains (TT13265 and TT13266) showed no pyrophosphatase activity (<1% of the level seen in the parent strain). The third mutant (TT13264) showed the same activity as the parental strain; we have not identified the defect preventing this strain from utilizing NAD. As a control, nicotinamide deamidase activity was also monitored; the mutants and wild type had comparable deamidase activity (Table 2). The mutations causing a loss of NAD pyrophosphatase activity have been designated *pnuE* (pyridine nucleotide utilization).

A deletion of the *pnuE* gene was isolated as described in Materials and Methods. As expected, the deletion strain (TT13272) also possessed no detectable NAD pyrophosphatase activity (<1% of the level found in the parental strain). A comparison of the rate of NMN accumulation by whole cells for a wild-type strain and a *pnuE* mutant is shown in Fig. 2.

FIG. 2. Effect of a *pnuE* mutation on NMN accumulation from NAD in the medium. Both *PnuE*⁺ and *PnuE*[–] cells were grown to an *A*₅₉₅ of 0.5 and incubated with [¹⁴C]NAD (each assay containing 0.2 μCi and 30 nmol) for 120 min as described in Materials and Methods. Cultures were analyzed by paper chromatography as described in Materials and Methods. The dotted lines indicate the radioactivity at 0 min with no incubation.

Mapping. We constructed strains with *Tn10* insertions linked to the *pnuE* locus as described in Materials and Methods. Both of the independently isolated *pnuE* mutations are closely linked to the same *Tn10* insertions. We mapped one of these *Tn10* insertions (*zgi-3642*) by the method of Chumley et al. (4), which involves formation of an Hfr by *Flac* integration into the *Tn10* element to be mapped. Initial Hfr mapping crosses showed that insertion *zgi-3642::Tn10* (96% linked to the *pnuE* gene by P22 transduction) maps between 73 and 90 min on the *S. typhimurium* chromosome (Table 3). Subsequent Hfr mapping with recipient mutations in this region narrowed the position of the *Tn10* to the region between unit 86 (*rha*) and unit 87 (*glpK*). We then tested the possibility of transductional linkage between insertions *pnuE::mudJ* and *glpK*, *rha*, and other markers in this region (Table 4). Figure 3 shows a detailed map of the region including the cotransduction frequencies obtained. The *pnuE* gene is not linked to any known genes involved in pyridine nucleotide metabolism.

A *pnuE* deletion constructed as described in Materials and Methods was also cotransducible with *glp* and *rha* mutations. The deletion mutant (TT13272) is defective in the utilization of mannitol and glucose. This defect was 100% cotransducible with the *PnuE*[–] phenotype. In *Escherichia coli*, the *pfkA* locus (the phosphofructokinase structural gene) is located between *rha* and *glpK* (20); the *E. coli* *pfkA* mutants have the same sugar utilization as our deletion mutant, TT13272 (i.e., a deficiency in mannitol and glucose utilization). Thus, the deletion in TT13272 seems to remove both the *pfkA* and *pnuE* genes.

TABLE 3. Hfr crosses^a

Expt	Recipient strain (genotype)	Map position of recipient marker (min)	No. of recombinants from cross with indicated donor		
			TT13268 (B orientation)	TT13269 (A orientation)	Control (no donor)
1	st Hfr mapping				
	TR5654 (<i>thrA strA</i>)	0	25	11	0
	TR5655 (<i>leu strA</i>)	3	35	50	0
	TR5656 (<i>proA strA</i>)	7	8	2	0
	TR5658 (<i>pyrC strA</i>)	22	0	0	0
	TR5660 (<i>purF strA</i>)	34	0	11	0
	TR5662 (<i>his strA</i>)	44	0	5	0
	TR5663 (<i>purF strA</i>)	49	0	9	0
	TR5664 (<i>cysA strA</i>)	50	0	3	0
	TR5665 (<i>cysC strA</i>)	60	0	124	0
	TR5666 (<i>serA strA</i>)	63	0	170	2
	TR5667 (<i>cysG strA</i>)	73	0	500	1
	TR5670 (<i>metA strA</i>)	90	184	30	0
TR5671 (<i>pyrB strA</i>)	98	81	30	2	
	No recipient		0	0	
2	TR5667 (<i>cysG</i>)	73	8	522	0
	TR139 (<i>ilvB</i>)	81	30	760	0
	TR305 (<i>metE</i>)	84	20	800	0
	TR2344 (<i>rha-12</i>)	86	4	700	0
	TR6595 (<i>glpK</i>)	87	800	2	0
	TR310 (<i>metB</i>)	87.5	400	5	0
	TR5670 (<i>metA</i>)	89	484	0	0

^a Hfr donor TT13268 contains *zgi-3642::Tn10*, which is 96% linked to *pnuE* and *zzf-21::Tn10* on F'114ts as described by Chumley et al. (4). Another donor, TT13269, has *zzf-22::Tn10* on F'114ts and *zgi-3642::Tn10*. Homologous recombination between *zzf-21::Tn10* of F'114ts and *zgi-3642::Tn10* results in Hfrs with directions of transfer opposite to that generated between *zzf-22::Tn10* and *zgi::Tn10*. In the first mapping experiment, recipients were auxotrophic strains defective in genes throughout the chromosome; in the second experiment, recipients were strains with markers between *cysG* and *metA*.

NAD pyrophosphatase is not a general phosphodiesterase. We have determined whether the NAD pyrophosphatase studied here might have a more general phosphodiesterase activity like snake venom phosphodiesterase. The same concentration of [³H]ATP and [¹⁴C]NAD was incubated with whole cells of *PnuE*⁺ and *PnuE*⁻ strains. Very little phosphodiesterase activity against ATP was found. Whole cells were harvested when the *A*₅₉₅ was 0.5 and suspended in 0.05 M Tris buffer (pH 8.5) after being washed. Activity was measured after 20 to 50 min of incubation at 30°C. Reaction mixtures (0.12 ml) contained [¹⁴C]NAD (30 nmol, 0.2 μCi), [³H]ATP (30 nmol, 4 μCi), and 0.05 M Tris buffer (pH 8.5). The protein concentration was between 3.5 and 5.0 mg/ml. The rates of hydrolysis of NAD pyrophosphatase from the *PnuE*⁺ strain were 0.68 nmol/min per mg for NAD and <0.03 nmol/min per mg for ATP. The respective rates of hydrolysis for snake venom commercial phosphodiesterase (0.6 μg) added instead of whole cells in the same reaction mixture was 1,200 and 1,900 nmol/min per mg. NAD was broken down to NMN and AMP at a rate 20-fold greater than that at which ATP was converted to AMP by wild-type cells. After prolonged incubation, [³H]AMP was detectable; how-

ever, under these conditions, almost all of the [³H]ATP was converted to [³H]ADP. Mutant cells lacking NAD pyrophosphatase broke down the same amount of ATP to [³H]ADP and [³H]AMP as did the wild-type cells. In contrast, the breakdown of [¹⁴C]NAD in mutant strains was completely abolished. The conversion of ATP to ADP and AMP may be due to nonspecific acid phosphatase (9) located in the periplasmic space of *S. typhimurium*. Since most nonspecific phosphodiesterases (see above) are more active against ATP than NAD, these results suggest that the NAD pyrophosphate activity of *S. typhimurium* is not a general phosphodiesterase. We have deferred more detailed characterization of the specificity of this enzyme until it has been purified.

DISCUSSION

The isolation of mutants defective in NAD pyrophosphatase has demonstrated that the *pnuE* locus is the probable structural gene for this enzyme. As is characteristic of genetic loci involved in NAD metabolism, this gene is not linked to any other known locus involved in pyridine nucleotide metabolism. Genes of NAD metabolism have been

TABLE 4. Linkage by P22 transduction

Donor	Recipient	Map position of marker (min)	Selected marker (no. selected)	No. with unselected marker
TT218 (<i>MetE::Tn10</i>)	TT13270 (<i>pnuE::mudJ</i>)	84	Tc ^r (352)	0 Kan ^s
TT13270 (<i>pnuE::mudJ</i>)	TR2344 (<i>rha</i>)	86	Kan ^r (274)	8 Rha ⁺
LT2	TT13489 (<i>rha pnuE::mudJ</i>)	86	Rha ⁺ (101)	10 Kan ^s
TT13488 (<i>Tn10d</i> 32% linked to <i>glpK</i>)	TT13270 (<i>pnuE::mudJ</i>)	86	Tc ^r (344)	187 Kan ^s
TT13488 (<i>Tn10d</i> 32% linked to <i>glpK</i>)	TT13273 (<i>pnuE</i> deletion, <i>purE</i>)	87	Tc ^r (297)	137 <i>pnuE</i> (<i>pfKA</i>)
TT13270 (<i>pnuE::mudJ</i>)	TT6595 (<i>glpK</i>)	87	Kan ^r (880)	62 GlpK ⁺
LT2	TT13490 (<i>glpK pnuE::mudJ</i>)	87	GlpK ⁺ (213)	11 Kan ^s
TT13273 (<i>pnuE</i> deletion)	TR13490 (<i>glpK pnuE::mudJ</i>)	87	GlpK ⁺ (299)	108 Kan ^s (<i>pfKA</i>)
TT233 (<i>metF::Tn10</i>)	TT13270	87	Tc ^r (465)	0 Kan ^s

FIG. 3. P22-mediated cotransduction frequencies of relevant markers in the *pnuE* region. The region from min 86 (*rha*) to min 87 (*glpK*) is illustrated.

assigned to three general groups: the *nad* genes are directly involved in de novo biosynthesis of NAD (*nadA*, *B*, *C*, *D*, and *E*), the *pnc* genes are involved in pyridine nucleotide cycles, and the *pnu* genes are involved in utilization of exogenous pyridine nucleotides. This nomenclature is somewhat confusing, since many of the genes that were originally postulated to be involved in the pyridine nucleotide cycle are also involved in pyridine nucleotide utilization. In their original description of *pnuA*, Foster and co-workers used the *pnu* designation to describe pyridine nucleotide uptake (10); as a broader classification of such genes, we redesignate *pnu* to stand for pyridine nucleotide utilization. The present locus is involved in utilization of exogenous pyridine nucleotides, a prerequisite to uptake, but is not directly involved in uptake per se. Since this enzyme is extracellular, it should not be involved in intracellular pyridine nucleotide cycles; the rate of intracellular NAD turnover is the same in *pnuE*⁺ and *pnuE* strains (Uhnme Park, unpublished results).

It appears that the two best characterized enteric bacteria have different pathways for utilizing exogenous NAD as a source of the pyridine ring; both *E. coli* and *S. typhimurium* can use NAD as an exogenous pyridine source, but the major pathway for utilization of NAD appears to differ significantly. In *E. coli*, NMN is never observed to accumulate, and the major pathway appears to be a glycohydrolase mechanism in which NAD is directly cleaved to nicotinamide and the amide is subsequently hydrolyzed to nicotinic acid (7, 8). In *S. typhimurium*, NAD pyrophosphatase is the dominant enzyme with exogenous NAD as a substrate; significant amounts of NMN accumulate in culture media when exogenous NAD is provided (Fig. 2). NMN can then be directly taken up by the cells, and at least two genetic loci (*pnuA* and *pnuC*) (19) appear to be involved in NMN uptake. Alternatively, NMN can be hydrolyzed by NMN glycohydrolase to yield nicotinamide (1). It is unclear at the present time under what conditions or in what proportion these two alternative pathways for the utilization of NMN as a pyridine source are used. In this work, we deleted the NAD pyrophosphatase locus, and we detected no remaining NAD pyrophosphatase activity. Under these conditions, it is clear that there is an NAD glycohydrolase activity in *S. typhimurium*, although it is quantitatively minor compared with the pyrophosphatase activity (Fig. 2). In addition, strains lacking pyrophosphatase can be grown on NAD as a pyridine source if they have an intact salvage pathway for nicotinamide (*PncA*⁺ *PncB*⁺) (8). Thus, there appear to be alternative pathways for the utilization of NAD. It is clear, however, that NAD pyrophosphatase must be the major pathway for salvage of the purine ring of NAD, since mutants lacking this enzyme are unable to grow on NAD as a purine source.

Recent data from other enteric bacteria (e.g., *Shigella*

flexneri) suggest that a mechanism important in pathogenesis is depletion of the NAD pool of phagocytes by the bacteria (18; P. J. Sansonetti, personal communication). The availability of NAD pyrophosphatase mutants and the mapping of the *pnuE* gene should provide an opportunity to evaluate the role of NAD pyrophosphatase in pathogenesis. In addition, *Salmonella* strains lacking NAD pyrophosphatase make it possible to assay other pathways involved in both endogenous and exogenous NAD breakdown with far more sensitivity. The pyrophosphatase encoded by *pnuE* is the dominant NAD breakdown activity seen in crude extracts. Because of this, minor activities resulting in NAD breakdown are masked in *pnuE*⁺ strains. The NAD glycohydrolase activity, which cannot be clearly detected in the presence of NAD pyrophosphatase, becomes an easily detectable enzymatic activity in these mutants. We are presently examining mutant extracts to define other NAD breakdown activities, as part of our long-term goal to define the metabolic processes that play a role in intracellular pyridine nucleotide turnover.

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