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An Indispensable Gene for NAD Biosynthesis in *Salmonella typhimurium*

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We have located the *nadD* locus between *lip* and *leuS* at 14 min on the *Salmonella typhimurium* chromosome, and we have shown it to be the structural gene for nicotinic acid mononucleotide adenylyltransferase. This is the first indispensable gene of pyridine nucleotide metabolism that has been identified. Mutants altered at this locus, isolated by their 6-aminonicotinamide resistance phenotype, accumulate abnormally large pools of nicotinic acid mononucleotide in vivo; many exhibit a temperature-sensitive lethal phenotype. Enzyme assays reveal markedly lower transferase activity in mutant extracts than in $nadD^+$ extracts. The partial dominance of *nadD* mutants when placed in a $nadD^+/nadD$ diploid suggests that nicotinic acid mononucleotide adenylyltransferase is a multimeric enzyme.

NAD and NADP and their reduced forms, NADH and NADPH, are the major electron acceptors and donors in cellular metabolism. The pathways for the biosynthesis of these key cofactors are complex. In bacteria such as *Salmonella typhimurium* and *Escherichia coli*, there is both a de novo pathway, starting with aspartate and dihydroxyacetone phosphate, and a complex series of salvage pathways. Thus, *S. typhimurium* can use nicotinamide, nicotinic acid, nicotinamide ribonucleoside, and even nicotinamide mononucleotide as exogenous precursors for the synthesis of NAD. The presently defined pathways for NAD biosynthesis in *S. typhimurium* are shown in Fig. 1.

There is a preference hierarchy for the different pathways of NAD biosynthesis in bacteria; when an exogenous source of the pyridine ring is available, endogenous synthesis is suppressed (6). However, little is known about the regulatory interactions between these pathways. All of the known genes of pyridine nucleotide metabolism map at widely separated locations in both the *S. typhimurium* and *E. coli* chromosomes (2, 14).

Inspection of the biosynthetic pathways diagrammed in Fig. 1 shows that all of the presently identified mutations are in genes in the branches of the pathway which can be bypassed (for a review, see reference 10). Thus, a lesion in the *nadA*, *nadB*, or *nadC* gene causes auxotrophy, but NAD and NADP can still be synthesized from exogenous precursors. Mutations in the *pncA* and *pncB* genes are viable since de novo synthesis, as well as nucleoside salvage, can still occur. Even double mutants in the *nad* and *pnc* genes survive if given nicotinamide ribonucleoside or nicotinamide mononucleotide as a pyridine nucleotide source.

These branches of the pathway which can be bypassed converge to the key metabolite, nicotinic acid mononucleotide (NaMN). NaMN can be formed by three different enzymatic reactions: in the de novo pathway from quinolinate, in the Preiss-Handler salvage pathway from nicotinic acid, and in the nucleoside salvage pathway by deamidation of nicotinamide mononucleotide (10). In bacteria, there are no known alternatives for the metabolic steps between NaMN and NADP. Mutants blocked in these steps cannot be recovered as auxotrophs since the required metabolites are not taken up by cells. One of the initial aims of our study of pyridine nucleotide metabolism is to obtain mutants in this essential branch of the biosynthetic pathway for NAD and NADP. In this report, we present evidence that the recently described *nadD* locus in *S. typhimurium* (11) is the structural gene for the first essential enzyme of pyridine nucleotide biosynthesis, NaMN adenylyltransferase.

MATERIALS AND METHODS

Bacterial strains. All strains used in this study are listed with their sources in Table 1. All strains were derived from *S. typhimurium* LT2.

Media. The E medium of Vogel and Bonner (18), supplemented with 0.2% glucose, was used as minimal medium. Difco nutrient broth (NB; 8 g/liter), with NaCl (5 g/liter) added, was used as rich medium. Difco agar was added to a final concentration of 1.5% for solid medium. The following additives were included in media as needed (final concentrations): amino acids

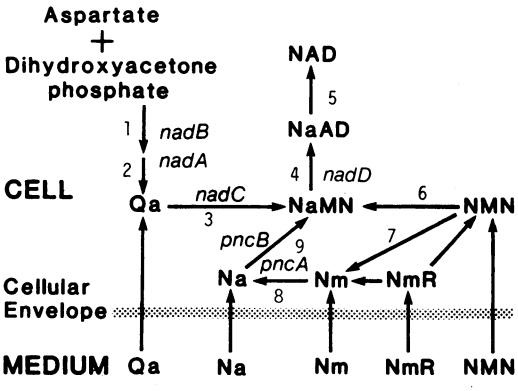

FIG. 1. NAD metabolism in *S.* and *E. coli*. The enzyme designations are: 1, L-aspartate oxidase; 2, quinolinic acid synthetase; 3, quinolinic acid phosphoribosyltransferase, 4, NaMN adenylyltransferase; 5, NAD synthetase; 6, nicotinamide mononucleotide deamidase; 7, nicotinamide mononucleotide glycohydrolase; 8, nicotinamide deamidase; 9, nicotinic acid phosphoribosyltransferase. The relationship of known genetic markers is given. Abbreviations: Na, nicotinic acid; Nm, nicotinamide; NMN, nicotinamide mononucleotide; NmR, nicotinamide ribonucleoside; Qa, qumolinic acid.

(approximately 0.3 mM), adenine and uracil (0.4 mM), lipoic acid (5 ng/ml), tetracycline hydrochloride (25 μg/ml in rich medium or 10 μg/ml in minimal medium), kanamycin sulfate (50 μg/ml in rich medium or 125 μg/ml in minimal medium), 5',5',5'-trifluoro-DL-leucine (250 μg/ml), nicotinic acid and nicotinamide (2 μg/ml), 6-aminonicotinamide (50 μg/ml), 6-aminonicotinic acid (50 μg/ml), and quinolinic acid (5 mM), which was recrystallized in cold 40% acetic acid before use. Plates for the assay of leucine excretion were prepared as suggested by J. Calvo (personal communication). A 10-ml portion of E-glucose agar was poured into petri dishes and allowed to solidify. Another 10-ml portion of the same agar was cooled to 48°C, and the *leu-447* mutant was added to a concentration of 10^8 cells per ml, mixed, poured on top, and allowed to solidify. A third 10-ml layer of E-glucose agar cooled to 48°C was then poured on top.

Transductional methods. The high-frequency generalized transducing bacteriophage P22 mutant (HT105/1) (15) *int-201* (1) was used for all transductional crosses. Selective plates were spread with 10^8 cells and 10^8 to 10^9 phage. Transductants were purified and made phage free by streaking nonselectively on green indicator plates (5). Phage-free clones were then checked for phage sensitivity by cross-streaking with P22 H5 (clear-plaque mutant) phage.

Conjugational methods. F^- recipients were grown overnight in nutrient broth before mating. F' donors were grown overnight in selective minimal media. Plate matings were performed as described by Miller (12). Transconjugants were purified by two successive single-colony isolations on selective plates. F' merodiploids were cured of their episome with acridine orange by the method of Miller (12).

Construction and characterization of tandem *nadD* merodiploids. Merodiploids for the *nadD* region were constructed by using Tn*10* insertions as regions for homologous recombination (8). Tn*10* insertions that mapped near the *nadD* gene were isolated, and proximity was estimated by cotransductional frequency of linkage to the *lip* gene. Two Tn*10* insertions (*zbe-1023* and *zbe-1028*) were isolated and were found to be on opposite sides of *nadD* by three-factor crosses (data not shown). Hfr strains were constructed with these Tn*10* insertions as sites for integration via homologous recombination with Tn*10* on several F plasmids (7). Hfr matings determined the Tn*10* insertions to be in the A orientation (7) on the chromosome (data not shown). These Tn*10* insertions near *nadD* were used to generate tandem duplication merodiploids ($nadD^{+/-}$/$nadD^{+/-}$) as diagrammed in Fig. 4. The *nadD*/*nadD* merodiploids for complementation tests were also constructed as diagrammed in Fig. 4, except that the *nadD189*(Ts) allele was used as donor.

Putative merodiploid strains were grown overnight in NB liquid medium to full density (2×10^9 cells per ml). These strains were then diluted 10^6-fold and were plated for single colonies on NB plates. After overnight incubation, single colonies were replica plated to NB and NB + tetracycline plates. The accumulation of tetracycline-sensitive clones and the presence of either $nadD^+$ or *nadD* mutants (thermosensitive lethal, 6-aminonicotinamide resistant) were used as indications of merodiploidy.

In the cases in which segregrants of *nadD*/*nadD* merodiploids had identical phenotypes, specific alleles were determined by recombinational tests. For example, each segregant from a *nadD189*/*nadD157* diploid would harbor only one of the two alleles. The ability of phage grown on a segregant to recombine with a strain carrying the *nadD157* allele and not with a strain carrying the *nadD189* allele indicated that the segregant harbored only the *nadD189* allele. The ability of *nadD* mutant diploids to segregate two different alleles was used to demonstrate that heterozygosity of the duplicated region had been achieved.

Isolation of frameshift mutations in the *E. coli nadD* gene present on episome F'254. Strain TT7513 [*nadD*(Ts)/(F'$nadD^+$)] was grown overnight in selective minimal medium and plated onto selective minimal agar containing 6-aminonicotinic acid. A drop of ICR-191 (1 mg/ml) was added to the center of each plate. Two classes of 6-aminonicotinic acid-resistant mutants were expected. First, those lacking the *pncB* gene product would be resistant because conversion of 6-aminonicotinic acid to 6-amino NAD would be blocked; this class would be analog resistant and would grow at 42°C. The second mutant class would include those lacking the F'254 *E. coli* $nadD^+$ gene product. These would be temperature sensitive and analog resistant owing to the 6-aminonicotinic acid resistance phenotype characteristic of the chromosomal *nadD*(Ts) haploid, which is recessive to the F'254 $nadD^+$ allele (see below). The 6-aminonicotinic acid-resistant colonies that arose around the ICR-191 spot were picked and screened for temperature sensitivity at 42°C. They were then screened for ICR-191 revertibility to growth at 42°C with concomitant recovery of analog sensitivity. An identifying characteristic of frameshift mutations is their revertibility by the frameshift mutagen ICR-191 (13). The *nadD* frameshift mutants isolated were then screened for the inability to

TABLE 1. List of strains

Strain	Genotype	Source
TR3682	*his-3050 nag-1*(F'*his-2385*)	Lab collection
TR3933	*lip-2 hisD8531*	Lab collection
TR5349	*trpA8 purE801*(F'254)	Lab collection
TR5657	*purE8 strA1*	Lab collection
TR5934	*nadD190*	This work
TR6416	*nadD157*	This work
TR6417	*nadD158*	This work
TR6418	*nadD159*	This work
TR6419	*ara-9 gal-205 leuS2*	J. Calvo (4)
TR6465	*nadD187*	This work
TR6466	*nadD188*	This work
TR6467	*nadD189*	This work
TT627	*strA1 pyrC7*(F'114-Ts *lac*$^+$ *zzf-20*::Tn*10*(A))	Chumley and Roth (8)
TT628	*strA1 pyrC7*(F'114-Ts *lac*$^+$ *zzf*-21::Tn*10*(B))	Chumley and Roth (8)
TT629	*strA1 pyrC7*(F'114-Ts *lac*$^+$ *zzf*-22::Tn*10*(A))	Chumley and Roth (8)
TT7247	*zbe-1023*::Tn*10*	This work
TT7252	*zbe-1028*::Tn*10*	This work
TT7260	*lip-2 zbe-1023*::Tn*10 hisD8531*	This work
TT7261	*nadD157 lip-2 zbe-1023*::Tn*10*	This work
TT7453	*nadD158 lip-2 zbe-1023*::Tn*10*	This work
TT7454	*nadD159 lip-2 zbe-1023*::Tn*10*	This work
TT7455	*nadD187 lip-2 zbe-1023*::Tn*10*	This work
TT7456	*nadD188 lip-2 zbe-1023*::Tn*10*	This work
TT7457	*nadD189 lip-2 zbe-1023*::Tn*10*	This work
TT7459	*lip-2 zbe-1023*::Tn*10*	This work
TT7466	*nadD157 zbe-1028*::Tn*10*	This work
TT7467	*nadD158 zbe-1028*::Tn*10*	This work
TT7468	*nadD159 zbe-1028*::Tn*10*	This work
TT7469	*nadD187 zbe-1028*::Tn*10*	This work
TT7470	*nadD188 zbe-1028*::Tn*10*	This work
TT7471	*nadD189 zbe-1028*::Tn*10*	This work
TT7473	(*nadD*$^+$)Tn*10*(*nadD*$^+$)	This work
TT7477	(*nadD*$^+$)Tn*10*(*nadD157*)	This work
TT7481	(*nadD*$^+$)Tn*10*(*nadD158*)	This work
TT7485	(*nadD*$^+$)Tn*10*(*nadD159*)	This work
TT7513	*nadD157 lip-2 zbe-1023*::Tn*10*(F'254 *lac*$^+$ *nadD*$^+$)	This work
TT7514	*nadD157 lip-2 zbe-1023*::Tn*10*(F'254 *lac*$^+$ *nadD200*)	This work
TT7587	(*nadD*$^+$)Tn*10*(*nadD187*)	This work
TT7591	(*nadD*$^+$)Tn*10*(*nadD188*)	This work
TT7595	(*nadD*$^+$)Tn*10*(*nadD189*)	This work
TT7599	(*nadD*$^+$)Tn*10*(*nadD190*)	This work
TT7643	(*nadD189*)Tn*10*(*nadD157*)	This work
TT7644	(*nadD189*)Tn*10*(*nadD158*)	This work
TT7645	(*nadD189*)Tn*10*(*nadD159*)	This work
TT7646	(*nadD189*)Tn*10*(*nadD187*)	This work
TT7647	(*nadD189*)Tn*10*(*nadD188*)	This work
TT7648	(*nadD189*)Tn*10*(*nadD189*)	This work
TT7649	(*nadD189*)Tn*10*(*nadD190*)	This work
TT7658	*lip-2 zbe-1023*::Tn*10*(F'254 *lac*$^+$ *nadD200*)	This work
TT7659	*nadD157 lip-2 zbe-1023*::Tn*10*(F'254 *lac*$^+$ *nadD200*)	This work
TT7660	*nadD158 lip-2 zbe-1023*::Tn*10*(F'254 *lac*$^+$ *nadD200*)	This work
TT7661	*nadD159 lip-2 zbe-1023*::Tn*10*(F'254 *lac*$^+$ *nadD200*)	This work
TT7662	*nadD187 lip-2 zbe-1023*::Tn*10*(F'254 *lac*$^+$ *nadD200*)	This work
TT7663	*nadD188 lip-2 zbe-1023*::Tn*10*(F'254 *lac*$^+$ *nadD200*)	This work
TT7664	*nadD189 lip-2 zbe-1023*::Tn*10*(F'254 *lac*$^+$ *nadD200*)	This work
TT7665	*nadD190 lip-2 zbe-1023*::Tn*10*(F'254 *lac*$^+$ *nadD200*)	This work
TT7666	*lip-2 zbe-1023*::Tn*10*(F'254 *lac*$^+$ *nadD*$^+$)	This work
TT7667	*nadD157 lip-2 zbe-1023*::Tn*10*(F'254 *lac*$^+$ *nadD*$^+$)	This work
TT7668	*nadD158 lip-2 zbe-1023*::Tn*10*(F'254 *lac*$^+$ *nadD*$^+$)	This work
TT7669	*nadD159 lip-2 zbe-1023*::Tn*10*(F'254 *lac*$^+$ *nadD*$^+$)	This work
TT7670	*nadD187 lip-2 zbe-1023*::Tn*10*(F'254 *lac*$^+$ *nadD*$^+$)	This work
TT7671	*nadD188 lip-2 zbe-1023*::Tn*10*(F'254 *lac*$^+$ *nadD*$^+$)	This work
TT7672	*nadD189 lip-2 zbe-1023*::Tn*10*(F'254 *lac*$^+$ *nadD*$^+$)	This work
TT7673	*nadD190 lip-2 zbe-1023*::Tn*10*(F'254 *lac*$^+$ *nadD*$^+$)	This work

complement the *nadD157* allele when the episome carrying the mutation was introduced into a strain carrying this allele.

Preparation of [^{14}C]NaAD. Radiolabeled [carboxyl-^{14}C]nicotinic acid adenine dinucleotide ([carboxyl-^{14}C]NaAD) was prepared by the nicotinamide adenine dinucleotidase-catalyzed exchange reaction between [carboxyl-^{14}C]nicotinic acid and unlabeled NAD. The reaction mixture contained 74 mg of NAD, 0.3 μmol of [carboxyl-^{14}C]nicotinic acid (New England Nuclear Corp.; specific activity, 50.4 mCi/mmol), 0.1 mmols of Tris-hydrocholoride (pH 8.0), and 23 mg of nicotinamide adenine dinucleotidase (Sigma Chemical Co.) in a reaction volume of 1 ml. The reaction was incubated at 37°C with shaking and was monitored by spotting 1 μl onto GFC-filter disks and then washing in 7:3 (vol/vol) acetone-ethanol (this washes off nicotinic acid while retaining NaAD). The reaction was terminated when the production of [^{14}C]NaAD leveled off (2 to 3 h). The entire reaction mixture was spread on Whatman 3MM filter paper and was chromatographed with citrate-ethanol used as the developing solvent (19). The chromatogram was dried, and the NaAD band was cut into 1.5-cm strips. These were desalted by suspension in methanol for 15 min and then dried. The [^{14}C]NaAD was concentrated to one end of the strip by ascending chromatography with distilled water as the solvent. When the solvent front reached the top, the strips were centrifuged at 5,000 rpm for 20 min, and the eluate was collected.

Preparation of [^{14}C]NaMN. Radiolabeled [carboxyl-^{14}C]NaMN was prepared from [^{14}C]NaAD (above) by the action of snake venom phosphodiesterase (Sigma). Before using a commercial preparation of the phosphodiesterase, contaminating 5′-nucleotidase was inactivated (17). The reaction mixture for the production of [^{14}C]NaMN was 10 μmol of Tris-hydrochloride (pH 8.6), 0.1 μmol of $MgSO_4$, 1 μg of snake venom phosphodiesterase, and 30 pmol of [^{14}C]NaAD in a total volume of 1 ml. The reaction was monitored on polyethyleneimine-impregnated cellulose by thin-layer chromatography (solvent: 100 ml of sodium phosphate buffer, pH 6.8, 60 g of NH_4SO_4, and 2 ml of *n*-propanol). When thin-layer chromatography indicated that the reaction was complete ([^{14}C]NaMN production leveled off), the reaction mixture was spread on 3MM chromatography paper and chromatographed with a citrate-ethanol buffer system. The chromatogram was dried, and the NaMN band was cut out and desalted by suspension in methanol for 15 min. It was then cut into 1.5-cm strips, and the [^{14}C]NaMN was isolated as described for [^{14}C]NaAD above.

Preparation of crude extracts. Overnight cultures of strains to be assayed were grown in NB or (for merodiploids) NB + 15 μg of tetracycline per ml. These cultures were used to inoculate 100 ml of NB or NB + tetracycline to a cell density of approximately 10 Klett units. The cultures were grown to 100 Klett units (ca. 6×10^8 cells per ml), harvested by centrifugation, washed once with E medium and a second time in 0.2 M Tris-hydrochloride buffer (pH 7.5), and suspended in 2.5 ml of 0.2 M Tris (pH 7.5). The cells were lysed by use of a French press. The extract was centrifuged at 15,000 rpm for 25 to 30 min to remove debris. Samples of extract (200 μl) were quick-frozen in dry ice-methanol and were kept at −70°C until needed for NAD synthetase assays. All NaMN adenylyltransferase assays were done with freshly prepared extracts. All NAD synthetase assays reported were done with extracts frozen at −70°C for 1 week. No significant NAD synthetase or NaMN adenylyltransferase activity was lost after the extracts had been frozen for 2 weeks at −70°C. Protein concentrations were determined by a Coomassie dye-binding assay (3) with commercial reagents (Bio-Rad Laboratories). Bovine serum albumin (Sigma) was used as a standard.

Assay for NaMN adenylyltransferase. NaMN adenylyltransferase was assayed essentially as described by Dahmen et al. (9), except that the NaMN concentration used was 80 μM. After termination, the reaction mixture was spread onto 3MM chromatography paper and was chromatographed with the citrate-ethanol buffer system. The chromatogram was cut into 1.5-cm strips, which were counted with a Beckman LS200 liquid scintillation counter.

Assay for NAD synthetase. NAD synthetase was assayed as described by Spencer and Priess (16). However, unlabeled NaAD was added to a final concentration of 10^{-5} M. Upon completion, the reaction mixture was spread onto DEAE chromatography paper and was chromatographed with 0.25 M ammonium bicarbonate. The chromatogram was cut into 1.5-cm strips and counted as described above.

Analysis of pyridine nucleotide pools in vivo. Cultures of wild-type *S. typhimurium* (Nad^+) or *nadD* mutants were grown in E medium containing 0.2% glucose to an absorbance at 650 nm of 0.4. Cultures were then labeled with [^{3}H]nicotinic acid (to a final concentration of 0.05 mCi/ml, 0.8 Ci/mmol), and the cells were harvested after 15 min at 37°C or, in the case of the temperature-sensitive mutants, 1 h at 42°C. The cultures were harvested by centrifugation in an RC2B Sorvall centrifuge, and the cell pellet was washed twice with E medium containing 0.2% glucose, 10 μg of nicotinic acid per ml, and 10 μg of nicotinamide per ml. The cell pellet was suspended in 0.4 ml of 0.3 M HCl and kept overnight at 0°C.

Chromatography was performed by using either DEAE-paper (Whatman DE81) with 0.25 M NH_4HCO_3 as the developing system, or the citrate ethanol system of Witholt (19). Before chromatography on DEAE-paper, the cell extract was neutralized with 1 M Tris-hydrochloride (pH 8). A commercial preparation of bacterial alkaline phosphatase (Worthington Diagnostics; BAF-F; 3 μl) was added, and the mixture was incubated for 15 min at 37°C. A second 3-μl sample of alkaline phosphatase was then added, and a second incubation for 15 min at 37°C was carried out. The phosphatase-treated extract (in which NADP is converted to NAD and NaMN is converted to nicotinic acid riboside) was then chromatographed on DEAE-paper, and the radioactivity was analyzed as previously described. This is a rapid method for assessing the fraction of NaMN present.

Assay for leucine excretion. Excretion of leucine was determined qualitatively. Colonies were transferred with sterile toothpicks to the surface of the leucine excretion assay plates described above. After incubation for 36 to 48 h, leucine excretion was detected as growth of the *leu-447* mutant forming a halo of growth beneath the transferred colonies. Both leucine excretion and resistance to trifluoroleucine were used to screen for the presence of the *leuS* marker (4).

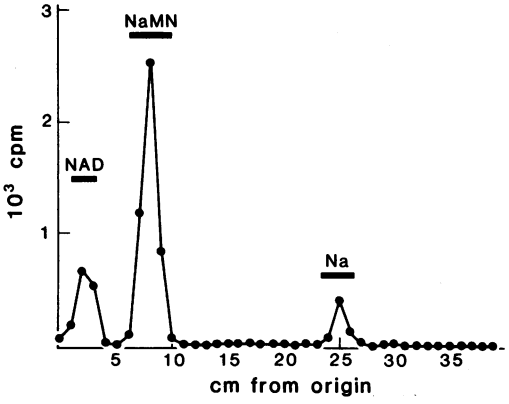

FIG. 2. Analysis of nicotinic acid metabolites in an *S. typhimurium nadD* strain shifted to the nonpermissive temperature. *S. typhimurium* TR6418, which carries the *nadD159*(Ts) allele, was grown at 30°C to an absorbance at 650 nm of 0.4. The culture was then shifted to 42°C and incubated for 1 h at 42°C in the presence of [^{3}H]nicotinic acid. The cells were then harvested, and the internal pools of pyridine nucleotides were analyzed as described in the text. The chromatography system used is the citrate ethanol system described by Witholt (19). Marker positions are indicated by the bars. NADP migrates near the origin, overlapping the NAD peak in this chromatographic system.

RESULTS

Isolation and characterization of mutants defective in NaMN adenylyltransferase activity. The isolation of 6-aminonicotinamide-resistant mutants of *S. typhimurium*, including mutants that were also thermosensitive lethals, has been described (10). All thermosensitive lethal mutants and some which are simply 6-aminonicotinamide resistant were found to be closely linked to the *lip* locus of *S. typhimurium*. This previously unmapped locus involved in pyridine nucleotide metabolism was designated the *nadD* locus. We have further characterized *nadD* strains by labeling pyridine nucleotide metabolites in vivo.

To identify the metabolic defect in these *nadD* strains, the mutant cells were grown in medium containing [^{3}H]nicotinic acid, and the intracellular pyridine pools were analyzed (Fig. 2). In a wild-type strain, all intracellular radioactivity is found in fractions near the origin in the chromatographic system used, consistent with the NAD and NADP pools being the only significantly labeled metabolites. However, in the *nadD* mutant, a major fraction of the intracellular radioactivity was present as NaMN (Fig. 2). A variety of biochemical criteria established that the material accumulating was NaMN. The radioactive material comigrated with the authentic nucleotide (NaMN) in two separate chromatography systems. After treatment with alkaline phosphatase, the radioactivity comigrated with authentic nicotinic acid ribonucleoside. In the particular *nadD* mutant shown in Fig. 2, there was more NaMN than NAD; in *nadD*$^+$ strains, the NaMN pool was below detectable limits. In addition to a large pool of NaMN, this *nadD*(Ts) mutant also accumulates a substantial nicotinic acid pool under these conditions.

Although the largest pools of NaMN are found in the thermosensitive-lethal *nadD* mutants, a significant pool of NaMN accumulates even in the non-temperature-sensitive *nadD190* mutant, which has a pool of NaMN which is 20% of the total pyridine nucleotide pool. No NaMN is detectable in the wild-type strain (results not shown). These results are consistent with a defect in NaMN adenylyltransferase (also called NaAD pyrophosphorylase). The fact that many *nadD* mutants are temperature-sensitive lethals is also consistent with this assignment in that the enzyme catalyzing the conversion of NaMN to NaAD would be an indispensable enzyme for NAD biosynthesis, since none of the subsequent intermediates in the pathway is taken up and used as a nutritional supplement.

Enzyme assays. The accumulation of NaMN suggests that a defect in NaMN adenylyltransferase in these mutants causes the analog resistance and temperature sensitivity. Crude extracts of all *nadD* mutants were assayed for NaMN adenylyltransferase activities (Table 2). All *nadD* temperature-sensitive strains have less than 2% of the NaMN adenylyltransferase activity of the wild-type strain, even when grown and assayed at the permissive temperature. The *nadD190* mutant, which does not show a temperature-sensitive phenotype, had less than 25% of wild-type activity. As a control, NAD synthetase activity was also assayed. There was no significant difference in the NAD synthetase activities between the mutants and the wild type. These assays are strong evidence that the *nadD* gene codes for NaMN adenylyltransferase.

Genetic mapping of the *nadD* gene. Our previous studies have shown that the *nadD* locus is tightly linked to the *lip* gene (located at 14 min) by P22-mediated transductional crosses (11). Three-factor crosses were used to locate the precise position of the *nadD* gene. The *nadD* gene is located between *lip* and the *leuS* locus (Table 3). A detailed map of the region is shown in Fig. 3. This map includes P22 cotransduction frequencies between genetic markers in the *nadD* region. The locations of the two Tn*10* insertions used to generate *nadD* tandem diploid strains (discussed below) are also included.

Complementation and recombination studies. Tandem duplications of the *nadD* region were

TABLE 2. Enzyme assays

Strain[a]	NaMN adenyl transferase ([14C]NaMN → [14C]NaAD)		NAD synthetase ([14C]NaAD → [14C]NAD)		Transferase/ synthetase ratio
	% Conversion	Relative enzyme activity[b]	% Conversion	Relative enzyme activity[b]	
LT2	31.8	1	26	1	1.29
TR5934	6.35	0.24	21	0.93	0.32
TR6416	<0.5	<0.02	16	0.77	<0.02
TR6417	<0.5	<0.02	20	1	<0.02
TR6418	<0.5	<0.02	16	0.89	<0.02
TR6465	<0.5	<0.02	17	0.78	<0.02
TR6466	<0.5	<0.02	22	1.21	<0.02
TR6467	<0.5	<0.02	19	0.88	<0.02

[a] All strains were grown in NB medium at 30°C before assay.
[b] The value of 1 represents a specific activity of 868 and 672 pmol/min per mg of protein, respectively, for the transferase and synthetase.

constructed by homologous recombination of Tn*10* insertions as described by Chumley and Roth (8) (Fig. 4). Homologous recombination generated a small duplication of the *nadD* region with a Tn*10* insertion betwen the two copies. The duplication is maintained by selection of tetracycline resistance; in the absence of tetracycline, tetracycline-sensitive clones arise via homologous recombination, causing deletion of the Tn*10* and retention of only one of the two *nadD* alleles. In the first set of complementation experiments, one *nadD*(Ts) allele (*nadD189*) was used in the donor strain, and diploids of this allele and all other *nadD* mutant alleles were constructed to test for complementation.

All of the tandem duplication complementation diploids listed in Table 4 are able to grow and show analog resistance at 30°C, as do the parental haploid strains. At the nonpermissive temperature (42°C); which is lethal for all temperature-sensitive haploids, the diploids are unable to grow, except for the diploid which includes the *nadD190* (temperature resistant) allelle (Table 4). This demonstrates that all *nadD*(Ts) alleles tested belong to a single complementation group.

All of the *nadD*(Ts)/*nadD*(Ts) merodiploids in Table 4A (lines b through g) recombine to give occasional haploid *nadD*$^+$ (tetracycline-sensitive) segregants, except for *nadD189*/*nadD158* and *nadD189*/*nadD189*. All *nadD*(Ts) alleles were tested in standard transductional tests for the ability to recombine with each other to yield *nadD*$^+$ recombinants (data not shown). The *nadD*(Ts) alleles were found to belong to three recombinational groups: (i) *nadD157*, *nadD187*, and *nadD188*; (ii) *nadD158* and *nadD189*; and (iii) *nadD159*. Since all these belong to a single complementation group, these results suggest that the *nadD*(Ts), 6-aminonicotinamide-resistant mutants affect three local regions within a single complementation group.

TABLE 3. Three-factor crosses: position of *nadD* with respect to *lip* and *leuS*[a]

Cross	Selected marker (no. scored)	Resulting characters			No. with indicated genotype	Relative frequency (% of total)
		lip	*nadD*	*leuS*		
1	*lip*$^+$ (298)	+	+	−	94	31
		+	+	+	32	11
		+	−	−	2	1
		+	−	+	170	57
2	*nadD*$^+$ (208)	+	+	−	90	43
		+	+	+	15	7
		−	+	−	78	38
		−	+	+	25	12

[a] Donor was strain TR6419 (*lip-2*$^+$ *nadD157*$^+$ *leuS2*); recipient was strain TT7261 (*lip-2 nadD157 leuS2*$^+$).

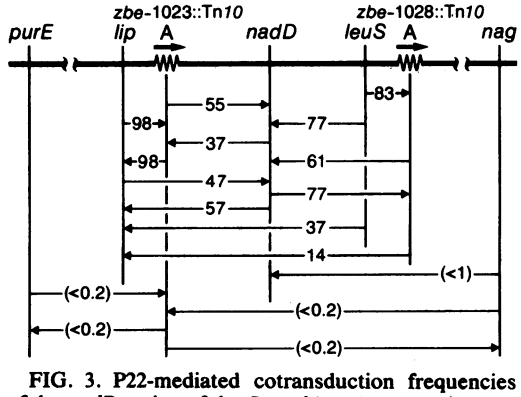

FIG. 3. P22-mediated cotransduction frequencies of the *nadD* region of the *S. typhimurium* genetic map. A scaled portion of the map from 12 to 15 min is illustrated here. Included in the map are the locations of the two Tn*10* insertions used to generate tandem duplications of the *nadD* region (see the text). The *nadD157* allele was used in all mapping transductions. The orientations of the Tn*10* insertions were determined as described by Chumley et al. (7).

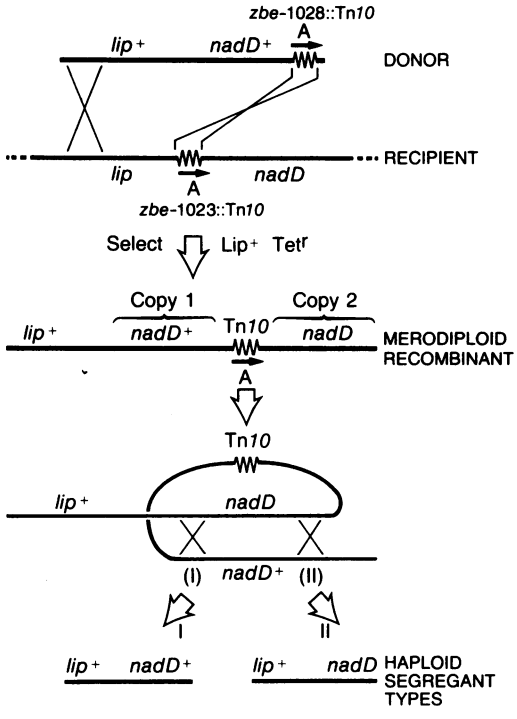

FIG. 4. Generation of tandem duplications of the *nadD* region. Unequal recombination between Tn*10* elements yields *lip*+ *nadD*+ recombinants containing two copies of the *nadD* region. The Tn*10* insertions in the donor and recipient strains have the same orientation as determined by Tn*10*-directed Hfr formation. When selection for tetracycline resistance is removed, tetracycline-sensitive clones arise in which homologous recombination between the duplicated region has occurred. This results in the loss of Tn*10* and one of the two *nadD* alleles. Recombination event I results in a tetracycline-sensitive *nadD*+ haploid segregant; recombination event II results in a tetracycline-sensitive *nadD* haploid segregant.

A second set of complementation experiments was carried out with an *E. coli* episome (F'254) which is known to carry the homologous region of the *E. coli* chromosome (from 7 to 14 min) (see Fig. 5) (2). This *E. coli* episome corrects the temperature sensitivity phenotype when introduced into a *nadD*(Ts) mutant and restores sensitivity to 6-aminonicotinamide. This episome therefore presumably carries an *E. coli* *nadD*+ gene (Table 4B, lines b through g). To test for complementation, a frameshift mutation was introduced into the *E. coli nadD* allele by using ICR-191 mutagenesis of a merodiploid strain carrying *nadD157* in the chromosome and the *E. coli* F'254 *nadD*+ episome (13). Such a mutant (*nadD*) episome no longer complemented the *nadD157* allele of *S. typhimurium* (Table 4B, line j), resulting in 6-aminonicotinamide resistance (the episomal *nadD* mutation is induced to revert by ICR-191, so it is not a large deletion mutation). The mutant episome was tested for its ability to complement all other mutant *Salmonella nadD* alleles. As shown in Table 4B, lines j through p, the mutant *E. coli nadD* frameshift mutant allele failed to complement any of the *Salmonella nadD* mutant alleles, consistent with all mutations belonging to a single complementation group.

Dominance studies. Merodiploids containing tandem duplications with both the *nadD*+ and *nadD* mutant alleles were constructed as shown in Fig. 4. The *nadD*+/*nadD* merodiploids were characterized, and as expected, the *nadD*+ allele was found to be dominant to the temperature-sensitive mutant alleles at 42°C (i.e., the merodiploids were viable at 42°C). However, the second phenotype, 6-aminonicotinamide resistance, was partially dominant at the permissive temperature for all *nadD*(Ts) and nontemperature-sensitive alleles tested. Wild-type *nadD*+ strains do not grow on 6-aminonicotinamide-containing plates. The *nadD* mutants form colonies that are visible after approximately 12 h at

TABLE 4. Complementation studies

Complementation	Growth on: Glucose (30°C)	Glucose (42°C)	Glucose + 6ANm[a] (30°C)
A. Tandem duplication			
a. *nadD+/nadD189*	+	+	+
b. *nadD189/nadD157*	+	−	+
c. *nadD189/nadD158*	+	−	+
d. *nadD189/nadD159*	+	−	+
e. *nadD189/nadD187*	+	−	+
f. *nadD189/nadD188*	+	−	+
g. *nadD189/nadD189*	+	−	+
h. *nadD189/nadD190*[b]	+	+	+
B. *E. coli* F'			
a. F'254 *nadD+/nadD+*	+	+	−
b. F'254 *nadD+/nadD157*	+	+	−
c. F'254 *nadD+/nadD158*	+	+	−
d. F'254 *nadD+/nadD159*	+	+	−
e. F'254 *nadD+/nadD187*	+	+	−
f. F'254 *nadD+/nadD188*	+	+	−
g. F'254 *nadD+/nadD189*	+	+	−
h. F'254 *nadD+/nadD190*	+	+	−
i. F'254 *nadD200/nadD+*	+	+	−
j. F'254 *nadD200/nadD157*	+	−	+
k. F'254 *nadD200/nadD158*	+	−	+
l. F'254 *nadD200/nadD159*	+	−	+
m. F'254 *nadD200/nadD187*	+	−	+
n. F'254 *nadD200/nadD188*	+	−	+
o. F'254 *nadD200/nadD189*	+	−	+
p. F'254 *nadD200/nadD190*	+	+	+

[a] 6ANm, 6-Aminonicotinamide.
[b] All *nadD* mutant alleles used are temperature sensitive except for *nadD190*.

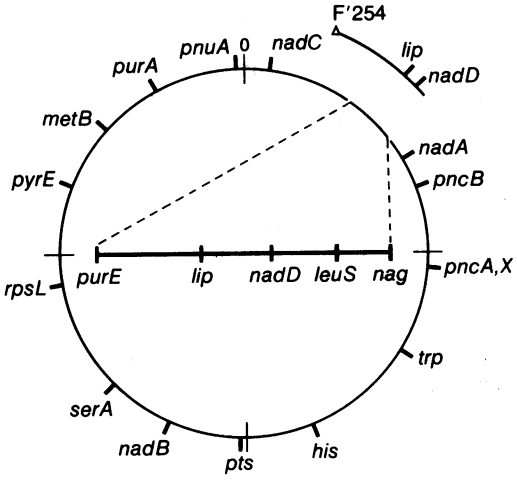

FIG. 5. Chromosome of *S. typhimurium* with relevant markers. Included in the figure is the *E. coli* episome F'254, which harbors the *nadD* region on the *E. coli* chromosome (2).

30°C; the *nadD*$^+$/*nadD*(Ts) merodiploid forms colonies that are detectable at 24 h, and the *nadD*$^+$/*nadD190* (temperature resistant) merodiploid forms colonies that appear at 36 h at the same temperature. At 42°C, no *nadD*$^+$/*nadD*(Ts) merodiploid grew on 6-aminonicotinamide-containing plates. Thus, *nadD*$^+$ is dominant over *nadD*(Ts) at 42°C but is codominant with respect to 6-aminonicotinamide resistance at permissive temperatures. In contrast, the *E. coli* episome F'254, which complemented all mutant *nadD* alleles, was dominant to *Salmonella nadD* alleles for both temperature sensitivity and analog resistance. These results suggest that the *Salmonella nadD* product is a multimeric enzyme and that the *nadD*(Ts) mutants produce no functional product at 42°C. At 30°C, the mutant protein may be able to form active but abnormal multimers with the wild-type *Salmonella* protein and allow 6-aminonicotinamide resistance. The *E. coli* product may be unable to form mixed multimers with the *Salmonella nadD* product. This leads to dominance of the *E. coli nadD*$^+$ allele for both the temperature sensitivity and 6-aminonicotinamide resistance phenotypes.

Enzyme assays were also performed on a merodiploid containing the *nadD*$^+$/*nadD*(Ts) alleles grown at various temperatures. If the *nadD*$^+$/*nadD*(Ts) merodiploid was grown and assayed at 30°C, enzyme activity was significantly lower than with the wild type. However, if the diploid was pregrown at 42°C, the enzymatic activity detected was essentially wild type (Table 5). Under our assay conditions, all merodiploids showed significant enzyme activity, but the specific activity was decreased in *nadD*$^+$/*nadD*(Ts) cells grown at 30°C as compared to the same strains grown at 42°C, consistent with the codominant phenotype.

DISCUSSION

In a previous report, we described three new classes of mutants that are resistant to 6-aminonicotinamide (11). One of these classes, which we designated *nadD*, mapped at a previously undefined locus of pyridine nucleotide metabolism and yielded a high proportion of mutants which were both temperature-sensitive lethal and 6-aminonicotinamide resistant; in fact, all temperature-sensitive lethals obtained by this selection map at the *nadD* locus. In the present study, the *nadD* gene and the encoded product have been more precisely defined, both genetically and biochemically. Additional mapping data demonstrate that *nadD* maps between the *lip* and *leuS* loci at 14 min on the *Salmonella* chromosome.

We have also presented several lines of evidence in this report that the *nadD* gene codes for an enzyme in the essential branch of the NAD biosynthetic pathway, NaMN adenylyltransferase (EC 2.7.7.18), an enzyme catalyzing the metabolic reaction: NaMN + ATP → NaAD + PP_i.

First, in vivo studies show that mutants in the *nadD* locus accumulate NaMN, which would be expected if the transferase is the defective en-

TABLE 5. NaMN adenylyltransferase assays of *nadD* diploids

Strain[a]	Genotype	Activity at:				42°C cells/ 30°C cells
		30°C		42°C		
		Specific[b]	Relative	Specific[b]	Relative	
LT2	*nadD*$^+$	868	0.83			
TT7473	*nadD*$^+$/*nadD*$^+$	1,040	1	1,330	1	1.28
TT7477	*nadD*$^+$/*nadD157*	487	0.47	1,130	0.85	2.32

[a] Strain LT2 was grown in NB medium before assay. The *nadD* diploid strains were grown in NB plus 15 μg of tetracycline per ml to maintain the duplication. All assays were performed at 30°C.
[b] Specific activity expressed in picomoles per minute per milligram of protein.

zyme. Second, enzymatic assays of mutant extracts show reduced NaMN adenylyltransferase activity. Indeed, all mutants which were both 6-aminonicotinamide resistant and temperature-sensitive lethal had no detectable activity for this enzyme either at the permissive or nonpermissive temperature. The one mutant studied that was 6-aminonicotinamide resistant but not temperature-sensitive lethal had 25% of the activity of the wild-type strain. Finally, merodiploids with both the *nadD*$^+$ and *nadD*(Ts) alleles are viable at 42°C (*nadD*$^+$ is dominant) and possess enzymatic activity.

The *nadD* locus comprises a single complementation group. As expected, *nadD*$^+$ is dominant over *nadD*(Ts) with respect to viability at 42°C. An unexpected observation is that *nadD*$^+$/nadD(Ts) merodiploids were codominant with respect to 6-aminonicotinamide resistance at the permissive temperature. In contrast, the merodiploids at the nonpermissive temperature were 6-aminonicotinamide sensitive. The most straightforward explanation for this result is that NaMN adenylyltransferase is a multimeric enzyme. At the nonpermissive temperature, protein coded for by the temperature-sensitive allele of the *nadD* gene cannot be assembled. Thus, all transferase activity found is wild type, leaving the cell temperature resistant and 6-aminonicotinamide sensitive. At the permissive temperature, mixed-subunit enzyme is made, and the mixed-multimer protein causes the intermediate phenotype observed. The results with *nadD*$^+$/nadD(Ts) diploids constructed by introducing the *E. coli* F'254 episome are consistent with the above hypothesis if it is assumed that mutant *Salmonella* subunits do not assemble into multimers with heterologous, wild-type *E. coli* subunits so that wild-type *E. coli* multimers confer a 6-aminonicotinamide sensitivity phenotype on the interspecies merodiploid at all temperatures.

The identification of the *nadD* locus as a structural gene for NaMN adenylyltransferase should permit a detailed examination of how expression of the essential branch of the pyridine nucleotide biosynthetic pathway is regulated.

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