JOURNAL OF BACTERIOLOGY, May 1988, p. 2113–2120
0021-9193/88/052113-08$02.00/0

Vol. 170, No. 5

Structural Gene for NAD Synthetase in *Salmonella typhimurium*

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Received 8 October 1987/Accepted 11 January 1988

We have identified the structural gene for NAD synthetase, which catalyzes the final metabolic step in NAD biosynthesis. This gene, designated *nadE*, is located between *gdh* and *nit* at 27 min on the *Salmonella typhimurium* chromosome. Mutants of *nadE* include those with a temperature-sensitive lethal phenotype; these strains accumulate large internal pools of nicotinic acid adenine dinucleotide, the substrate for NAD synthetase. Native gel electrophoresis experiments suggest that NAD synthetase is a multimeric enzyme of at least two subunits and that subunits from *Escherichia coli* and *S. typhimurium* interact to form an active heteromultimer.

NAD is a central compound in metabolism; in addition to its well-known role as an oxidation-reduction cofactor, NAD has a variety of other metabolic roles in bacterial cells. It can serve as the energy source for DNA ligation and as a substrate for protein modification (i.e., ADP ribosylation). In addition, an intermediate in NAD biosynthesis, nicotinic acid mononucleotide (NaMN), provides the biosynthetic source of ribose for the synthesis of the vitamin B_{12} coenzyme (for a recent review, see reference 11).

In our laboratories we have carried out a genetic and biochemical characterization of NAD metabolism in the bacterium *Salmonella typhimurium*. A number of genes controlling metabolic steps in the biosynthesis of NAD had already been characterized. NAD can be synthesized either de novo or from pyridines acquired through a salvage pathway. These two pathways converge at the key metabolic intermediate NaMN (Fig. 1). Three metabolic steps have been defined for the de novo biosynthesis of NaMN in the enteric bacteria *Salmonella typhimurium* and *Escherichia coli*; these are the *nadB*, *nadA*, and *nadC* genes, which are the structural genes for L-aspartate oxidase, quinolinate synthetase, and quinolinic acid phosphoribosyl transferase, respectively (6, 25). A defect in any one of these genetic loci leads to a nutritional requirement for an exogenous pyridine ring source; either nicotinamide, nicotinic acid or NMN can serve as a source of the pyridine ring. Two genes control the salvage pathway for nicotinamide and nicotinic acid: *pncA* and *pncB* loci, which code for nicotinamide deamidase and nicotinic acid phosphoribosyl transferase, respectively (1, 10, 19). NMN can also be converted directly to NaMN by the enzyme NMN deamidase, for which a gene has not yet been identified (12).

Although there are three alternative pathways for the biosynthesis of NaMN, the metabolic conversion of NaMN to NAD can only proceed through the "essential branch" of the NAD biosynthetic pathway (22), comprising two metabolic steps. First, NaMN is adenylylated, to yield nicotinic acid adenine dinucleotide (NaAD); the enzyme catalyzing this reaction is NaAD pyrophosphorylase. We previously defined the structural gene for this enzyme, the *nadD* locus (15). It was shown that this gene is essential, in that strains with temperature-sensitive lethal mutations in this gene were recovered.

The final metabolic step in the pathway is conversion of NaAD to NAD, a step catalyzed by the enzyme NAD synthetase. This is the only synthetic step in the pathway to which a genetic locus has not been assigned. In this report, we describe the isolation of mutants which are defective in NAD synthetase. We designate the gene encoding this enzyme *nadE*; like the *nadD* gene, *nadE* appears to be essential for viability in *S. typhimurium*.

MATERIALS AND METHODS

Bacterial strains. All strains used in this study are listed in Table 1. All *S. typhimurium* strains were derived from *S. typhimurium* LT2. Plasmids with the transposase gene of Tn*10* were gifts from Nancy Kleckner.

Media. The E medium of Vogel and Bonner (26), supplemented with 0.2% glucose, was used as the minimal medium. For testing other carbon sources, NCE medium (9) was used. Difco nutrient broth (NB; 8 g/liter), with NaCl (5 g/liter) added, was used as the rich medium for growing cells. Luria-Bertani medium (LB) (9), made with E medium in place of water (0.2% glucose), was used for growing P22 phage lysates. Difco agar was added to a final concentration of 1.5% for solid medium. Auxotrophic supplements were included in media at the final concentrations suggested by Davis et al. (9). The following additives were included in media as needed (final concentrations given): 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), ampicillin (30 μg/ml in rich medium or 15 μg/ml in minimal medium), and chloramphenicol (25 μg/ml in rich medium or 5 μg/ml in minimal medium).

Transductional methods. For all transductional crosses, the high-frequency generalized transducing mutant of bacteriophage P22(*HT105/1 int-201*) was used (18). Selective plates were spread directly with 2×10^8 cells and 10^8 to 10^9 phage. For transduction of Mu dA, 10^9 to 10^{10} phage were used per 2×10^8 cells. The Mu dA prophages are inherited by a two-fragment transductional event and therefore require a higher phage input (17). Transductants were purified, and phage-free clones were isolated by streaking nonselectively onto green indicator plates (5). P22 lysates were titered by the method of Davis et al. (9).

Conjugational methods. F^- recipients were grown overnight in nutrient broth before mating. F′ donors were grown overnight in selective minimal medium. Plate matings were performed as described by Miller (20). A drop of donor culture was added to a drop of recipient culture directly on selective plates, allowed to dry, and then streaked for single

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TABLE 1. List of strains

Strain	Genotype	Source[a]
CV4017	*leu-4017* [Δ(*leuA-ppsB*)] *ara-9 gal-205*	J. Calvo via SGSC[b]
JB1994	*dhuA1 hisJ5601 gal-2394* Δ(*gltB832-cod*) *gdhA71*	J. Brenchley
SB3073	F'142/*guaA1 his-3217*	SGSC
SB3184	F'14/*metB36 purG302*	SGSC
SB3185	F'143/*aroCS cysC1111*	SGSC
SB3188	F'140/*proAB47 argD459*	SGSC
TH993	*zch-3518*::Tn*10 nadE381 nadB499*::Mu dJ *leu-4017 ara-9 gal-205*	
TH994	*zch-3518*::Tn*10 nadB499*::Mu dJ *leu-4017 ara-9 gal-205*	
TR1604	F'118/*tyr-294 thr-115 pyrB92*	Strain collection
TR1741	F'110/*his01242 hisF3704 thi-502 ilv-567*	Strain colleciton
TR2977	F'123/*proC90 trp-1001* r^- m^-	Strain collection
TR5345	F'116/*serA790 lys-554 his-644*	Strain collection
TR5346	F'117/*pyrB64 mel hisD6414*	Strain collection
TR5347	F'133/*hisD2421 metE338 ilvC401 ara-9*	Strain collection
TR5348	F'129/*his01242 hisC3737 aroC5 purF145 glpT*	Strain collection
TR5353	F'112/*pyrB642 argI537 his-6421*	Strain collection
TR6534	F'254/LT2	
TR6653	F'104/*pyrB655 proAB47*	
TR6659	*dhuA1 hisJ5601* Δ(*gltB832-cod*) *gdhA71*	
TT6159	F'128/*hsi072*	Strain collection
TT10199	F'152-2/*nadA56*	
TT10389	F'506/*aroD553*::Tn*10*	
TT10396	F'500/*nadA56 pncA15 trpA49 hisD9953*::Mu dJ	
TT10397	F'150/*nadA56 pncA15 trpA49 hisD9953*::Mu dJ	
TT10414	F'126/*nadA56 pncA15 trpA49 purB1744*::Tn*10*	
TT10423	*proAB47*/F'128 *zzf-1831*::Tn*10*dTet	T. Elliot
TT10427	pNK972/LT2	T. Elliot
TT10490	F'148/*pncA15 trpA49 hisD9953*::Mu dJ	
TT10604	*proAB47*/F'128 *zzf-1836*::Tn*10*dCam	T. Elliot
TT10739	*nadB499*::Mu dJ *leu-4017* [Δ(*leuA-ppsB*)] *ara-9 gal-205*	
TT10740	*nadE381 nadB499*::Mu dJ *leu-4017* [Δ(*leuA-ppsB*)] *ara-9 gal-205*	
TT10741	*zch-1838*::Tn*10 nadB499*::Mu dJ *leu-4017* [Δ(*leuA-ppsB*)] *ara-9 gal-205*	
TT10742	*zch-1839*::Tn*10 nadB499*::Mu dJ *leu-4017* [Δ(*leuA-ppsB*)] *ara-9 gal-205*	
TT10743	*zch-1838*::Tn*10 nadE381 nadB499*::Mu dJ *leu-4017 ara-9 gal-205*	
TT10744	*zch-1839*::Tn*10 nadE381 nadB499*::Mu dJ *leu-4017 ara-9 gal-205*	
TT10745	*zch-1839*::Tn*10*	
TT10746	*zch-1839*::Tn*10 nadE381*	
TT10757	*pncA*::Tn*10*dTet	
TT10758	*pncX*::Tn*10*dTet	
TT10759	*pncA*::Tn*10*dCam	
TT10760	*pncX*::Tn*10*dCam	
TT11113	*nadB499*::Mu dJ *dhuA1 hisJ5601* Δ(*gltB832-cod*)	
TT11114	*nadE500 nadB499*::Mu dJ *dhuA1 hisJ5601* Δ(*gltB832-cod*)	
TT11115	*nadE501 nadB499*::Mu dJ *dhuA1 hisJ5601* Δ(*gltB832-cod*)	
TT11116	*nadE502 nadB499*::Mu dJ *dhuA1 hisJ5601* Δ(*gltB832-cod*)	
TT11117	*nadE503 nadB499*::Mu dJ *dhuA1 hisJ5601* Δ(*gltB832-cod*)	
TT11118	*nadE504 nadB499*::Mu dJ *dhuA1 hisJ5601* Δ(*gltB832-cod*)	
TT11119	*nadE505 nadB499*::Mu dJ *dhuA1 hisJ5601* Δ(*gltB832-cod*)	
TT11138	*nadE381 nadB499*::Mu dJ *pncA180*::Tn*10 leu-4017 ara-9 gal-205*	
TT13492	*nadE500 nadB499*::Mu dJ *pncA180*::Tn*10*	
TT13493	*nadE501 nadB499*::Mu dJ *pncA180*::Tn*10*	
TT13494	*nadE502 nadB499*::Mu dJ *pncA180*::Tn*10*	
TT13495	*nadE503 nadB499*::Mu dJ *pncA180*::Tn*10*	
TT13496	*nadE504 nadB499*::Mu dJ *pncA180*::Tn*10*	
TT13497	*nadE505 nadB499*::Mu dJ *pncA180*::Tn*10*	
TT13498	*nadB499*::Mu dJ *pncA180*::Tn*10*	
TT13499	*nadE381 nadB499*::Mu dJ *pncA180*::Tn*10*	

[a] Unless indicated otherwise all strains were constructed during the course of this work.
[b] SGSC, *Salmonella* Genetic Stock Center.

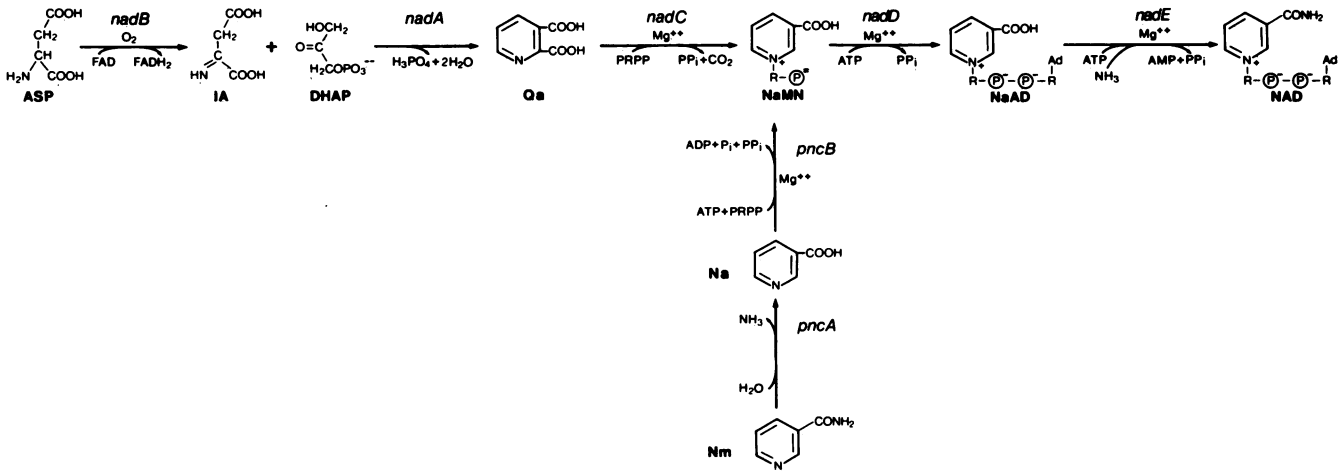

FIG. 1. NAD metabolism in *S. typhimurium* and *E. coli*. The de novo pathway for the biosynthesis of NAD and the Preiss-Handler pathway for the salvage of the pyridine ring are shown. Abbreviations: Na, nicotinic acid; Nm, nicotinamide; ASP, aspartate; IA, iminoaspartate; DHAP, dihydroxyacetone phosphate; Qa, quinolate; Ad, adenine; R, ribose.

transconjugants. Transconjugants were purified by two successive single-colony isolations on selective plates. F′ merodiploids were cured of their episomes by growing under nonselective growth conditions or, when necessary, with acridine orange (15) followed by screening for loss of F′ markers.

Localized mutagenesis. The localized mutagenesis procedure used was that of Hong and Ames (14). Mutants in *nadE* were isolated by using hydroxylamine-mutagenized donor phage grown on a wild-type donor. The *gdh*$^+$ locus was used as a nearby selectable marker (23).

Transposon mutagenesis. The isolation of Tn*10* insertions near *nadE* was done with donor phage grown on pools of random Tn*10* insertions in the *Salmonella* chromosome (9, 24), selecting for inheritance of Tn*10*-encoded tetracycline resistance by a *nadE*(Ts) recipient. Those Tet^r transductants that also become *nadE*$^+$ were kept as putative *nadE*-linked insertion mutants.

For isolating insertions of Tn*10*dTet (27) and Tn*10*dCam (T. Elliot and J. Roth, unpublished procedure) in *pncA* and *pncX*, a culture of wild-type *S. typhimurium* LT2 carrying Tn*10* transposase-producing plasmid pNK972 (21) was transduced to mini-Tn*10*-encoded drug resistance (Tet^r or Cam^r. The mini-Tn*10* derivatives used, Tn*10*dTet and Tn*10*dCam, are derivatives of Tn*10* that have deletions of the entire transposase gene but retain the ends of Tn*10*, which can be acted on by Tn*10* transposase provided in *trans* from plasmid pNK972 (21). The Tn*10*dTet and Tn*10*dCam elements were introduced into LT2 carrying pNK972 by P22 transduction with strains TT10423 and TT10604 as donors. These donors harbor the insertion elements on *E. coli* F′128. The elements are in the F DNA and cannot be inherited by transduction into LT2 strains which do not carry an F′ plasmid. This is because there is no homology between the recipient chromosome and the sequences flanking the insertion elements in the F′ plasmid. When the elements are introduced into LT2 (pNK972), Tn*10* transposase acts on the elements causing their transposition into the chromosome. Since there is no DNA homology between the DNA flanking the insertion elements and the recipient chromosome, all of the drug-resistant transductants result from mini-Tn*10* transposition events. To screen for insertions into the *pnc* genes, antibiotic-resistant transductants were replica printed to minimal medium containing 6-aminonicotinamide, an analog of nicotinamide. Only insertions which have transposed into the *pncA*, *pncB*, or *pncX* gene confer 6-aminonicotinamide resistance (15). Analog-resistant transductants were purified and transduced into LT2, selecting for antibiotic resistance and screening 6-aminonicotinamide resistance. Mutations in donors for which 100% of the Tet^r or Cam^r transductants also became resistant to 6-aminonicotinamide were assigned to the *pncA*, *pncB*, or *pncX* locus as described previously (15).

Analysis of pyridine pools in vivo. Cultures of *Salmonella* cells were grown in E medium containing 0.2% glucose to 100 Klett units (4×10^8 cells per ml) at 30°C. The cultures were then labeled with [^{14}C]nicotinamide (final concentration, 0.05 mCi/ml [0.8 Ci/mmol]) and shifted to 42°C. After 1 h at 42°C the cells were pelleted by centrifugation and washed twice in ice-cold E medium. The cell pellet was suspended in 0.4 ml of 0.3 M HCl and kept overnight at 0°C.

The cell extracts were chromatographed on either DEAE paper (Whatman DE81) with 0.25 M NH_4HCO_3 as the developing solvent or on cellulose (Whatman 3MM) with the citrate-ethanol developing system devised by Witholt (28). Before chromatography, unlabeled markers of nicotinamide, nicotinic acid, NMN, NaMN, NAD, NaAD, and NADP were spotted and run with the extract to serve as standards for identification of the pyridines present in the extract. After chromatography the chromatograms were cut into 1-cm (for DEAE) or 1.5-cm (for 3MM) strips and counted on an L200 Beckman scintillation counter with a toluene-based scintillation fluid. The position of standard pyridines was determined by UV absorption.

Preparation of crude extracts. Overnight cultures of strains to be assayed were used to inoculate 100 ml of fresh medium. The cultures were grown to a density of 100 to 120 Klett units (ca. 6×10^8 cells per ml), harvested by centrifugation, washed once with 0.85% NaCl, washed a second time with 10 mM potassium phosphate (pH 7.5), and suspended in 2.5 ml of the same phosphate buffer solution. The cells were lysed with a French press. The extract was centifuged at 15,000 rpm in a Sorvall GSA rotor for 25 to 30 min to remove debris. Samples of extract (200 μl) were quick-frozen in dry ice-ethanol and kept at −70°C until needed. Protein concentrations were determined by a Coomassie dye-binding assay (4) with commercial reagents (Bio-Rad Laboratories). Bo-

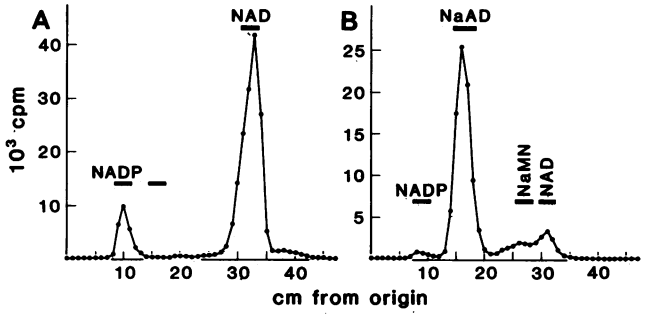

FIG. 2. Effect of a *nadE* gene defect on nicotinic acid-derived metabolites. Wild-type cells (A) and cells of the isogenic mutant *nadE381* (TT11138) (B) were labeled with [^{14}C]nicotinic acid, and their radioactive metabolite pools were compared as previously described (15). Fresh cultures were diluted 20-fold and grown at 30°C to a density of 20 Klett units, [^{14}C]nicotinic acid was added, and growth was continued at 30°C until the culture reached 65 Klett units. The culture was then shifted to 42°C and grown for 1.5 h before harvesting. Chromatography of the cell extract was done by using the system of Witholt (28). The positions of marker compounds are indicated by the horizontal bars. The accumulation of NaAD by the *nadE* mutant is the noteworthy feature of the results.

vine serum albumin (Sigma Chemical Co.) was used as a standard.

NAD synthetase assays. NAD synthetase was assayed essentially as described by Spencer and Priess by following the conversion of [^{14}C]NaAD to [^{14}C]NAD. The reaction mixture for the assay of NAD synthetase contained the following: $MgCl_2$, 0.5 μmol; ATP, 0.6 μmol; Tris hydrochloride (pH 7.5), 10 μmol; [^{14}C]NaAD, 100 nmol (17); and 20 μl of extract in a final volume of 100 μl. Reactions were done for 30 min at 37°C and terminated by immersion into a boiling water bath for 45 s. After termination of the reaction, the mixture was spread onto DEAE-paper (Whatman DE81) and chromatographed with 0.25 M NH_4CO_3 as the developing system. The chromatogram was cut into 1-cm strips and counted with a Beckman LS7500 liquid scintillation counter.

RESULTS

Selection method for *nadE* mutants. In an earlier study, we reported the isolation of temperature-sensitive lethal *nadD* mutants defective in NaMN adenylyl transferase activity. NaMN adenylyl transferase is the first essential step of the NAD metabolic pathway; the product of this step, NaAD, cannot be taken up by the cell. Mutations in *nadD* appear to limit NAD synthesis and result in derepressed transcription from the *nadB* promoter (8, 13). The *nadB* gene encodes the first enzymatic step in de novo NAD biosynthesis and is known to be transcriptionally regulated in response to pyridine availability. For cells growing on excess exogenous pyridine the presence of a *nadD* mutation causes a 20-fold derepression in β-galactosidase level (8). If, as we infer, limitation of NAD synthesis causes this derepression, then partial defects in the *nadE* gene should also cause derepression of the *nadB* gene.

The rationale used for isolating NAD synthetase (*nadE*) mutants was to select for Lac$^+$ derivatives of a *nadB*::Mu dA insertion mutant. The parental insertion mutant is Lac$^-$ when grown on nicotinamide or nicotinic acid. These Lac$^+$ revertants were selected at 30°C and screened for those which are lethal at 42°C in hopes of isolating mutants that are partially blocked at 30°C but completely defective at 42°C. This class would be expected to include *nadD* mutants and mutants defective in NAD synthetase.

When selecting for increased *lac* expression, the usual screen involves selection for growth on lactose as the sole carbon source. Since many mutants constitutive for *nadB* expression do not produce enough β-galactosidase in a *nadB*::Mu dA fusion background to permit utilization of lactose as a sole carbon source, we developed selection conditions that are satisfied by lower β-galactosidase levels. We selected for the ability to utilize lactose as a source of hexoses normally supplied by gluconeogenesis. A *ppsB* mutant, which is defective in phosphoenolpyruvate synthase, cannot convert pyruvate to phosphoenolpyruvate, a required step in bacterial gluconeogenesis. Consequently, these mutants cannot grow on many short-chain metabolites such as acetate and tricarboxylic acid cycle intermediates because they cannot convert them to glucose and other larger carbohydrate intermediates necessary for growth. A *ppsB* mutant can grow on acetate if a sugar such as glucose is provided even in small amounts. By selecting for growth on lactose plus acetate as the source for carbon and energy in a *ppsB* mutant background, one demands only enough β-galactosidase activity to supply hexoses normally provided by gluconeogenesis. The result of this selection with a *ppsB nadB*::Mu dA double mutant strain was that five times as many revertants appeared on plates containing acetate plus lactose than on lactose alone. In addition, a *nadD nadB*::Mu dA double mutant which could not grow on lactose as a sole carbon source could grow on acetate plus lactose in a *ppsB* mutant background. Thus, the selection permits isolation of constitutive mutants with low-level expression of genes whose promoters are fused to the *lac* operon in addition to mutants that express β-galactosidase at high levels.

Isolation and characterization of an isolate with a *nadE* temperature-sensitive lethal mutation. The selection described above was tested to determine whether we could isolate, in a *nadB*::Mu dA parent strain, mutants able to grow on lactose as a hexose source but not as a sole carbon source. A 0.1-ml sample from each of 10 independent overnight cultures of TT10739 (*ppsB nadB*::Mu dA) were plated onto NCE plates with acetate and lactose as carbon sources; nicotinic acid (10^{-4} M) and leucine were present to supply the growth requirements of the strain. This high concentration of nicotinic acid results in repression of transcription of the *nadB* genes rendering the parent strain phenotypically Lac$^-$ (8, 13). After 3 days of incubation at 30°C, about 300 colonies per plate appeared on acetate plus lactose as the carbon source, whereas only about 50 colonies per plate appeared when lactose was the sole carbon source. Most of the colonies which grew on lactose plus acetate grew poorly on lactose alone (data not shown). The lactose-acetate plates were replica printed selectively at 30 and 42°C. Six temperature-sensitive mutants were isolated which were also temperature sensitive when grown on NB plates. Of these six, five mutants were temperature sensitive only on nicotinic acid as a pyridine source but not when quinolinic acid was used; these proved to have temperature-sensitive defects in the *pncB* step of the pathway. Only one strain was temperature sensitive on both nicotinic acid and quinolinic acid and thus a candidate for a mutant of the *nadD* or NAD synthetase. The new mutant, which is temperature sensitive under all growth conditions, was found to be unlinked to *zbe-1023*::Tn*10*, an insertion 50% linked to *nadD* by P22 transduction (16). Thus the mutation represents a new gene which has been designated *nadE* and is shown below to affect NAD synthetase activity.

Analysis of pyridine nucleotide pools in vivo. To character-

ize the defect in the *nadE* mutant strain, cells were grown in minimal medium containing [^{14}C]nicotinic acid, and the intracellular pyridine pools were analyzed (Fig. 2). In a *nadE*$^+$ strain, all of the intracellular label comigrated with either NAD or NADP. However, in the *nadE* mutant, a major fraction of the intracellular label was present in NaAD in addition to NAD and NADP. The *nadE*$^+$ strain showed no detectable intracellular accumulation of NaAD. Since NaAD is the substrate for NAD synthetase, these results are consistent with the *nadE* mutant being defective in NAD synthetase.

Chromosomal location of the NAD synthetase structural gene. We found that NAD synthetases from *E. coli* and *S. typhimurium* have different electrophoretic mobilities in agarose. We used an activity stain to locate the NAD synthetase band on a native agarose gel of crude extracts. The substrate NaAD is converted by NAD synthetase to NAD, which, in the presence of ethanol and alcohol dehydrogenase, can be reduced to NADH. The NADH formed reduces nitroblue tetrazolium to a purple precipitate, thus producing a purple band at the position on the gel to which the NAD synthetase enzyme has migrated. The *E. coli* enzyme is more negatively charged, thus migrating faster toward the anode (Fig. 3, lanes A and B). Thus, we have found a difference between the *E. coli* and *S. typhimurium* forms of NAD synthetase which can be used in the F′ plasmid mapping of the *nadE* structural gene.

A set of *S. typhimurium* strains carrying different *E. coli* F′ plasmids which cover the entire *E. coli* chromosome were assayed by agarose gel electrophoresis followed by an activity staining procedure specific for NAD synthetase (see Materials and Methods). We presumed that any strain carrying both copies of the NAD synthetase gene from *E. coli* and *S. typhimurium* would express both enzymes and produce two NAD synthetase bands corresponding to the different bacterial species. Of the 20 partially diploid strains assayed, 2 expressed both NAD synthetase activites. In addition, a third band which migrated between the other two was present in both cases (Fig. 3). This is consistent with the NAD synthetase enzyme comprising at least two subunits; the *E. coli* and *S. typhimurium* subunits can presumably interact to form an active hybrid enzyme.

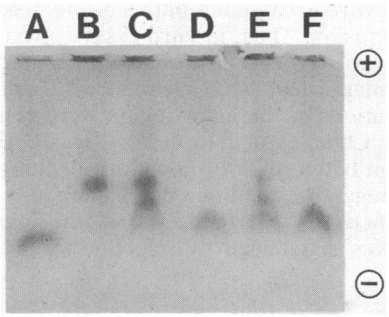

FIG. 3. Electrophoretic identification of NAD synthetase activity. Crude extracts prepared with a French press (~4 mg of protein per ml) were analyzed by agarose gel electrophoresis (1% agarose) in a Tris-borate buffer (54 mM Tris base mixed with 82.5 mM boric acid). Electrophoresis was carried out on 5 μl of each extract for 30 min at 200 V. After electrophoresis, the gel was soaked in a solution of 20 mM NH_4Cl, 100 mM Tris (pH 9.0), 5 mM $MgCl_2$, 2 mM ATP, 0.5 mM NaAD, and 140 mM KCl. The NAD synthetase present in the gel converts the NaAD to NAD, which is assayed by including in the reaction mixture 100 μg of yeast alcohol dehydrogenase per ml, 200 mM ethanol, 100 μg of phenazine methosulfate per ml, and 100 μg of nitroblue tetrazolium per ml. Any NAD produced by NAD synthetase is reduced by the coupled alcohol dehydrogenase reaction to NADH. NADH is oxidized by pherazine methosulfate; the reduced pherazine methosulfate is oxidized by nitroblue tetrazolium, which, when reduced, forms a blue precipitate. Lanes: A, *S. typhimurium* LT2; B, *E. coli* K12; C through F, *S. typhimurium* strains carrying *E. coli* F′148 (C), F′150 (D), F′500 (E), or F′506 (F).

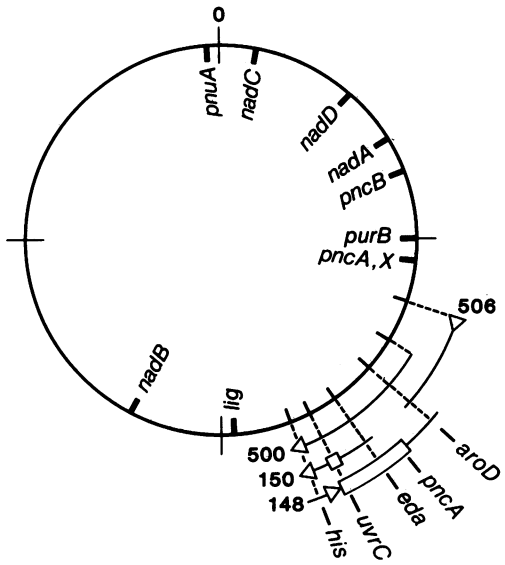

FIG. 4. Chromosome of *S. typhimurium* showing marker genes and the relevant *E. coli* F′ plasmids described in the text.

The two F′ plasmids which carry the *E. coli* NAD synthetase gene are F′148 and F′500 (Fig. 4). Plasmid F′506, which overlaps F′500 counterclockwise of the *aroD* gene, and plasmid F′150, which overlaps F′148 clockwise of the *eda* gene, do not carry the NAD synthetase gene (F′148 carries a deletion of the material from *pncA* through *cheC* [Fig. 4]) (3). Since the overlapping regions from F′150 and F′506 do not include NAD synthetase, and F′148 carries a deletion from *pncA* through *cheC*, the region of the chromosome inferred to include the NAD synthetase structural gene lies between *aroD* and *pncA* in *E. coli*. The genetic maps of *S. typhimurium* and *E. coli* are nearly identical (2, 24) except for a region near the terminus of replication, where one or more inversions of the chromosome has presumably occurred during the evolution of *E. coli* and *S. typhimurium* from a common ancestor. Unfortunately, one of these inversion breakpoints occurs between *pncA* and *aroD*. Thus, whereas *pncA* and *aroD* are less than 2 map units (min) apart on the *E. coli* chromosome (1 min corresponds to 1% of the entire chromosome), in *S. typhimurium* they are separated by 9 min. Thus, the NAD synthetase gene of *S. typhimurium* should be closely linked to either *aroD* or *pncA*, depending on where the inversion breakpoint occurred.

Mapping of *nadE*. To facilitate mapping of the *nadE* mutation, Tn*10* insertion mutants linked to the *nadE* temperature-sensitive mutation were isolated (see Materials and Methods). Two of these insertions were checked for linkage to the *aroD* and *pncA* genes in *Salmonella*; both were found to be linked to the *pncA* region of the chromosome. Multiple three-factor transduction crosses mediated by P22 were performed to determine the gene order; *pncA*, *pnxX*, *gdh*, *zch-1839*::Tn*10*, *nadE*, *zch-3518* (data not shown). Figure 5 presents this order with cotransduction frequencies seen for mutations in these genes. The *nadE* gene mapped between

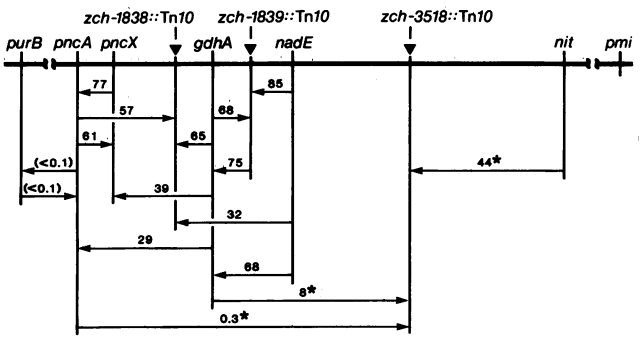

FIG. 5. P22-mediated cotransduction frequencies of the *nadE* region of the *S. typhimurium* genetic map. A portion of the map from *purB* to *pmi* (25 to 27 min) is illustrated here. Included in the map are the locations of the Tn*10* insertions used to facilitate mapping and in the generation of tandem duplications of the *nadE* region (*zch-3518*::Tn*10*). The orientations of the Tn*10* insertions were determined as described by Chumley and Roth (7). Linkages marked with an asterisk are taken from Rosenfeld et al. (23).

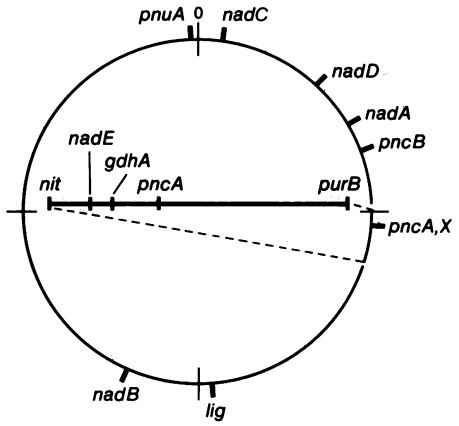

FIG. 6. Map of *S. typhimurium* chromosome showing the *pncA* region expanded and the location of the *nadE* gene.

gdh and *nit* at 27 min on the *Salmonella* chromosome (Fig. 6).

Isolation of *nadE* mutants by localized mutagenesis. The determination of the *nadE* gene position on the chromosome facilitated the rapid isolation of more *nadE* mutants by localized mutagenesis. P22 grown on wild-type LT2 was mutagenized with hydroxylamine and used to transduce a *nadB*::Mu dA *gdh* recipient to *gdh*$^+$ on minimal medium containing X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) and a high concentration of nicotinic acid (10^{-4} M). This high level of exogenous nicotinic acid results in repression of *nadB* transcription, and the colonies are white (Lac$^-$) in the presence of the X-gal indicator (8, 13). When these cells are transduced to *gdh*$^+$, 70% of the *gdh*$^+$ transductants coinherit the *nadE* gene from the donor transduced fragment. If the coinherited *nadE* gene were partially defective as a result of mutagenesis, NAD levels would drop, causing derepression of the *nadB-lac* fusion, and give rise to blue (Lac$^+$) colonies in the presence of X-gal and high nicotinic acid levels.

For the isolation of *nadE* mutants by localized mutagenesis, a culture of TT11112 (*nadB-lac gdh*) was transduced to *gdh*$^+$ on minimal medium with high nicotinic acid plus X-gal at 30°C with hydroxylamine-mutagenized donor phage grown on a wild-type donor. Of approximately 3,000 transductants screened, 13 colonies arose which were dark blue on X-gal. Six of these colonies were also red on MacConkey-lactose indicator plates at 37°C and were temperature sensitive for growth at 42°C. Colonies which are red on MacConkey-lactose indicator plates express the *lac* operon at high levels and indicate a high level of derepression of the *nadB-lac* operon fusion due to the introduced *gdh*-linked mutation. This is consistent with a defect in NAD synthetase which cannot produce wild-type levels of NAD even in the presence of high amounts of exogenous nicotinic acid. Conditional lethality is also consistent with a defect in NAD synthetase. NAD synthetase is an essential enzyme in *S. typhimurium* because the metabolic product of this step, NAD, cannot be taken up by the cell. The conditional-lethal mutations linked to *gdh* which affect transcription of a *nadB-lac* fusion were characterized further as putative NAD synthetase mutants.

Enzyme assays. The accumulation of NaAD by the *nadE381* mutant and the mapping of *nadE* to a locus distinct from all previously known *nad* loci suggested that *nadE* is the structural gene for NAD synthetase. Crude extracts of the conditionally defective *nadE* mutants were assayed for NAD synthetase activity. All of the *nadE* alleles tested were defective in their in vitro NAD synthetase activities; the activities of crude extracts varied from less than 0.1% to 25% of the wild-type level (Table 2). Thus, the *nadE* mutations which cause derepression of de novo NAD biosynthesis cause a deficiency in NAD synthetase activity in crude extracts.

In addition to crude extract assays in vitro, levels of NAD synthetase in vivo were estimated by assaying levels of derepression of the *nadB-lac* fusions resulting from the *nadE* defect. The levels of derepression did not always correspond with the in vitro assays (Table 2). For example, the *nadE502* mutant showed 8% of wild-type NAD synthetase activity in crude extracts with a 25-fold derepression of the *nadB-lac* fusion in vivo whereas the *nadE504* mutant showed no detectable in vitro activity but only a 3-fold derepression of the *nadB-lac* fusion. Thus, in vitro assays of crude extracts do not necessarily reflect the extent of the defect in vivo.

Complementation and dominance studies. Tandem chromosomal duplications of the *nadE* region were constructed as described by Chumley and Roth (7) (Fig. 7). Homologous recombination between Tn*10* insertions flanking both sides of the *nadE* region resulted in a duplication with two copies of the *nadE* locus and a Tn*10* insertion at the joint point. The duplication was maintained by selection for Tn*10*-encoded

TABLE 2. Effects of *nadE* mutations on in vitro NAD synthetase activity and on regulation of de novo NAD biosynthesis

Strain	Relevant genotype	Relative NAD synthetase activity in vitro[a] (%)	β-Galactosidase activity[b]
TT11113	*nadE*$^+$ *nadB-lac*	100	19
TT11114	*nadE500 nadB-lac*	<0.1	630
TT11115	*nadE501 nadB-lac*	<0.1	170
TT11116	*nadE502 nadB-lac*	6.3	480
TT11117	*nadE503 nadB-lac*	20	43
TT11118	*nadE504 nadB-lac*	<0.1	61
TT11119	*nadE505 nadB-lac*	<0.1	200
TT11138	*nadE381 nadB-lac*	<0.1	440

[a] 100% = 4.3 nmol/min per mg of protein.

[b] β-Galactosidase activity is expressed as nanomoles per minute per optical density unit (650 nm). Cells were grown in the presence of high levels (10^{-4} M) of nicotinic acid before the assay.

tetracycline resistance. When drug selection was removed, homologous recombination occurred between the duplicated segments, resulting in the loss of the Tn*10* at the joint point and retention of one of the two *nadE* loci. A set of duplications was constructed in which each carried a *nadE*$^+$ allele and one of the *nadE*(Ts) alleles in a *nadB-lac* fusion background. *nadE*$^+$ was dominant for growth at 42°C (Table 3). At 30°C dominance was tested by screening for β-galactosidase from the *nadB-lac* fusion. If the *nadE* mutations showed any codominant effect on *nadE*$^+$, the merodiploids might be expected to show a derepression of the *lac* (Lac$^+$) genes compared with *nadE*$^+$ alone (Lac$^-$). All diploids showed *lac* expression similar to that of the *nadE*$^+$ haploid strain (Table 3). Thus, *nadE*$^+$ was completely dominant to the *nadE* mutant alleles. A set of diploids was also constructed which carried the *nadE381*(Ts) allele and each of the other *nadE*(Ts) alleles in a *nadB-lac* fusion background. None of the *nadE*(Ts) alleles was able to complement the *nadE381* allele for either repression of a *nadB-lac* fusion at 30°C or growth at 42°C (Table 3). Thus, all *nadE*(Ts) alleles tested belong to a single complementation group.

Analog sensitivity of *nadE* mutants. In a previous report, we described mutations which affect *nadD*, the structural gene for NaMN adenylytransferase (Fig. 1). The *nadD* mutants were found by screening resistance to the analogs 6-aminonicotinamide and 6-aminonicotinic acid (16). The analog-resistant *nadD* mutants were derepressed for de novo NAD biosynthesis and accumulate large internal pools of NaMN and smaller internal pools of nicotinic acid. The *nadE* mutants reported here are also derepressed for de novo NAD biosynthesis but, unlike the *nadD* mutants, remain susceptible to the analogs. This suggests that the accumulation of either NaMN or nicotinic acid in the *nadD* mutants is responsible for the analog resistance phenotype. The affinity of either the *pncB* enzyme or the *nadD* enzyme for its normal substrate must be much greater than its affinity for the 6-amino derivative. The NAD synthetase, on the other hand, probably utilizes either NaAD or 6-amino NaAD with comparable efficiency. This would account for the sensitivity of the *nadE* mutants to the analogs even when transcription of the de novo pathway is derepressed.

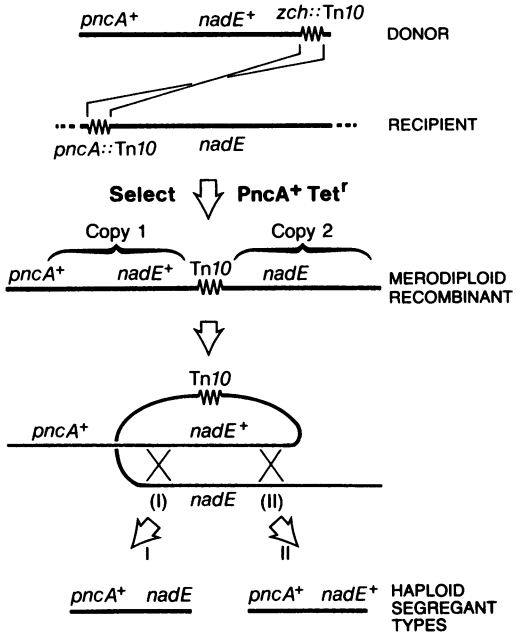

FIG. 7. Generation of tandem duplications of the *nadE* region. Unequal recombination between Tn*10* elements yields *pncA*$^+$ *nadE*$^+$ recombinants containing two copies of the *nadE* region. The Tn*10* insertions and the donor recipient strains have the same orientation as determined by Tn*10*-directed Hfr formation. Once 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 *nadE* alleles. Recombination event I results in a Tet^s *nadE* haploid segregant; recombination event II results in a Tet^s *nadE*$^+$ haploid segregant.

TABLE 3. *nadE* complementation and dominance studies

Relevant diploid genotype[a]	Lactose utilization phenotype[b]	Growth[c]	
		30°C	42°C
nadE$^+$/*nadE*$^+$	−	+	+
nadE$^+$/*nadE381*	−	+	+
nadE$^+$/*nadE500*	−	+	+
nadE$^+$/*nadE501*	−	+	+
nadE$^+$/*nadE502*	−	+	+
nadE$^+$/*nadE503*	−	+	+
nadE$^+$/*nadE504*	−	+	+
nadE$^+$/*nadE505*	−	+	+
nadE381/*nadE381*	+	+	−
nadE381/*nadE500*	+	+	−
nadE381/*nadE501*	+	+	−
nadE381/*nadE502*	+	+	−
nadE381/*nadE503*	+	+	−
nadE381/*nadE504*	+	+	−
nadE381/*nadE505*	+	+	−

[a] All strains were in a *nadB-lac* fusion background.
[b] +, Blue on X-gal indicator plates at 30°C; −, white on X-gal indicator plates at 30°C. Plates contained minimal E medium with 10^{-4} M nicotinic acid.
[c] Growth was tested on minimal E medium with nicotinic acid.

DISCUSSION

In this work, we identified and mapped the gene for the last step in the metabolic pathway for the biosynthesis of NAD, the structural gene for NAD synthetase. To identify the *nadE* locus, two technical developments were essential. One was the ability to identify mutants which are inappropriate derepressed for de novo NAD biosynthesis. By using a *lac* operon fusion to the *nadB* gene which codes for the first enzyme in the biosynthetic pathway, transcription of the presumptive pacemaker enzyme in the do novo pathway could be monitored. In the presence of an exogenous pyridine source such as nicotinic acid, the de novo pathway is normally shut off. A mutation at the *nadE* locus is one of a class of mutations which continue to express the *nadB* gene even in the presence of exogenous pyridine nucleotides. Other mutants in this class carry *nadD* mutations as well as true regulatory mutations such as that at the structural gene for the repressor of NAD biosynthetic pathway, *nadI*. Thus, NAD biosynthesis is derepressed when the end product (NAD or some derivative) is in short supply, or if the *nadI* repressor is nonfunctional.

The second technical feature that made it possible to identify the *nadE* locus was the ability to select for mutants expressing β-galactosidase at a modest level; technically, this was accomplished by providing lactose only as a hexose source but not as a source of all carbon and energy. In a strain carrying a *ppsB* mutation, a much lower level expression of the *nadB*::*lac* fusion could be selected. This approach was essential to the mutant isolation described above and

should be useful in screening for mutations which affect the transcription of genes with relatively weak promoters.

The *nadD* and *nadE* genes encode the enzymes which constitute the essential branch of the NAD biosynthetic pathway, i.e., the metabolic steps between NaMN and NAD. Like the *nadD* gene, the *nadE* locus is indispensable, and a high proportion of the mutants have temperature-sensitive lethal mutations; presumably a null mutation would not be viable. With the identification of the *nadE* locus, all metabolic steps in both the de novo biosynthetic pathway for NAD and the nicotinamide salvage pathway have an associated genetic locus.

The *nadE* locus has been located between *gdh* and *nit* at 27 min on the *S. typhimurium* chromosome. It is noteworthy that none of the structural genes for enzymes involved in NAD biosynthesis is contiguous (Fig. 6); each gene is independently transcribed. The regulation of transcription of these genes needs to be understood; it is already clear that the *nadI* repressor regulates the transcription of both *nadA* and *nadB*, although the operator regions are not identical (29).

The question of why certain biosynthetic pathways are organized into operons or clusters of genes (such as *his* or *trp* or the vitamin B_{12} operons), whereas the NAD biosynthetic pathway and the purine and pyrimidine genes are scattered all over the chromosome, remains an intriguing and unanswered question. It is noteworthy that the purine, pyrimidine, and NAD biosynthetic genes have been largely conserved with respect to their location in the *S. typhimurium* and *E. coli* chromosomes; it is believed that these organisms diverged 120 million years ago. The advantages of multigene operons seem straightforward: coordinate regulation and metabolic economy of transcription and translation. It is not obvious why the NAD and purine genes remain scattered throughout the chromosome or why this is the strategy used in most eucaryotic metabolic pathways as well.

ACKNOWLEDGMENTS

We thank Jean Brenchley and Ken Sanderson for their generous gift of strains.

This work was supported by Public Health Service grants GM25654 (to B.M.O.) and GM23408 (to J.R.R.) from the National Institutes of Health. K.T.H. was supported by Public Health Service predoctoral training grant T32-GM07537 from the National Institutes of Health.

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