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6-Aminonicotinamide-Resistant Mutants of *Salmonella typhimurium*

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Resistance to the nicotinamide analog 6-aminonicotinamide has been used to identify the following three new classes of mutants in pyridine nucleotide metabolism. (i) *pncX* mutants have Tn*10* insertion mutations near the *pncA* locus which reduce but do not eliminate the *pncA* product, nicotinamide deamidase. (ii) *nadB* (6-aminonicotinamide-resistant) mutants have dominant alleles of the *nadB* gene, which we propose are altered in feedback inhibition of the *nadB* enzyme, L-aspartate oxidase. Many of these mutants also exhibit a temperature-sensitive nicotinamide requirement phenotype. (iii) *nadD* mutants have mutations that affect a new gene involved in pyridine nucleotide metabolism. Since a high proportion of *nadD* mutations are temperature-sensitive lethal mutations, this appears to be an essential gene for NAD and NADP biosynthesis. In vivo labeling experiments indicate that in all the above cases, resistance is gained by increasing the ratio of NAD to 6-aminonicotinamide adenine dinucleotide. 6-Aminonicotinamide adenine dinucleotide turns over significantly more slowly in vivo than does normal NAD.

Two widely used analogs of nicotinamide and nicotinic acid are 6- aminonicotinamide and 6-aminonicotinic acid (Fig. 1). In coliform bacteria, these analogs have been useful in selecting for mutants in pyridine nucleotide metabolism (5, 8, 10). The analogs inhibit growth of wild-type bacteria; this inhibition is corrected by the addition of either nicotinic acid or nicotinamide to the medium, suggesting that the analogs mimic these normal pyridine metabolites and thereby interfere with metabolism. The generally accepted pathway of pyridine nucleotide synthesis, utilization, and recycling is presented in Fig. 2.

Nicotinamide (or its analog 6-aminonicotinamide) must be deamidated to nicotinic acid (or 6-aminonicotinic acid) before it can be converted to NAD and NADP (or 6-aminonicotinamide adenine dinucleotide [6-aminoNAD] and 6-aminoNADP). It is thought that the toxic effects of the analogs are due to abnormal behavior of these final cofactors. Thus, *pncA* mutants, defective in deamidation of nicotinamide or its analog, are resistant only to 6-aminonicotinamide; *pncB* mutants are resistant to both 6-aminonicotinic acid and 6-aminonicotinamide since this step is required to metabolize both analogs. Thus, by the addition of the appropriate analogs to selection media, it has been possible to select and identify *pncA* or *pncB* mutants (8).

In this study, we have isolated a variety of new mutant types that are 6-aminonicotinamide resistant, but that do not have standard mutations at either the *pncA* or the *pncB* locus. We have also more directly tested the hypothesis (5) that these analogs exert their bacteriostatic effects by being metabolized by the enzymes of the Preiss-Handler pathway (16) to 6-aminoNAD. We demonstrate that 6-aminonicotinamide-resistant mutants make a lower proportion of 6-aminoNAD to normal NAD than do sensitive strains.

MATERIALS AND METHODS

Bacterial strains. All strains used in this study and their sources are listed in Table 1. All strains are derived from *Salmonella typhimurium* LT2.

Media. The E medium of Vogel and Bonner (20), supplemented with 0.2% dextrose, was used as minimal medium. Alternative carbon sources were supplemented to 0.2% in E medium lacking citrate (17). Difco nutrient broth (8 g/liter), with NaCl added (5 g/liter), was used as rich medium. To purify transductants, single colonies were isolated on the green indicator agar of Chan et al. (2). The following additives were included in media as needed (final concentrations given): amino acids (approximately 0.3 mM), lipoic acid (5 ng/ml), tetracycline (25 μg/ml in rich medium or 10 μg/ml in minimal medium), nicotinic acid and nicotinamide (2 μg/ml), 6-aminonicotinic acid and 6-aminonicotinamide (50 μg/ml), and quinolinic acid (5 mM, recrystallized in cold 40% acetic acid before use).

Transductional methods. The high-frequency general transducing mutant bacteriophage P22 (HT 105/1) *int201* (1) was used for all transductional crosses.

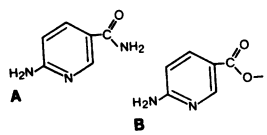

FIG. 1. Structures of (A) 6-aminonicotinamide and (B) 6-aminonicotinic acid.

Selective plates were spread with 10^8 to 10^9 cells and 10^8 to 10^9 phage. Transductants were purified and made phage free by streaking nonselectively onto green agar. Putative phage-free clones were then checked for phage sensitivity by cross-streaking with P22 H5 (clear plaque mutant) phage.

Tn*10* mutagenesis. A culture of wild-type *S. typhimurium* strain LT2 was transduced to tetracycline resistance (Tet^r) on rich medium by using donor phage grown on a pool of over 10,000 randomly isolated Tn*10* insertion mutants (6, 13). These Tet^r transductants were replica printed to minimal agar containing 50 μg of 6-aminonicotinamide per ml and 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*-tetraacetic acid, and also to nutrient broth-tetracycline agar. Transductant colonies resistant to 6-aminonicotinamide were purified on green agar, and P22 phage-sensitive colonies were saved. P22 generalized transducing phage was grown on all of the 6-aminonicotinamide-resistant Tet^r isolates. LT2 was then transduced to Tet^r, and 6-aminonicotinamide resistance was scored. All donors for which 100% of the Tet^r transductants became 6-aminonicotinamide resistant were assumed to have an insertion of Tn*10* in a gene critical to some aspect of pyridine nucleotide metabolism. These Tn*10* insertions were further characterized by transducing Tet^r into Tr5985 (*nadB*) and then determining the ability of the double mutant to grow on various pyridine ring sources. Of the 35 6-aminonicotinamide resistance Tn*10* insertion mutations, 11 rendered the *nadB*$^-$ strain unable to utilize nicotinamide or both nicotinic acid and nicotinamide. These mutants carried insertions of Tn*10* in the *pncA* or *pncB* genes and were not analyzed further. The remaining 24 insertion mutations left the *nadB* strain still able to utilize nicotinamide, nicotinic acid, quinolinate, or nicotinamide mononucleotide as a pyridine source. Thus, they retained functional *pncA* and *pncB* genes and must owe their analog resistance to other mutations affecting pyridine nucleotide metabolism.

Localized mutagenesis. Localized mutagenesis was carried out by the procedure of Hong and Ames (12) by utilizing the presence of Tn*10* near *nadB* as the selectable marker (18).

Labeling of NAD and 6-aminoNAD with [^{14}C]adenine. Overnight cultures were diluted twofold and grown in E medium supplemented with 0.2% glucose containing [^{3}H]nicotinic acid (~ 10 μCi/ml, 0.8 Ci/mol) until the optical density was 0.5. The culture was centrifuged at 7,500 rpm for 10 min in an RC2B Sorvall centrifuge with an SS34 rotor. Cells were washed once with E medium containing unlabeled 6-aminonicotinamide (100 μg/ml) and were suspended in 10 ml of E medium containing 0.2% glucose and 100 μg of 6-aminonicotinamide per ml. After 10 min of

TABLE 1. List of strains

Strain	Genotype	Source
LT2		
TR2577	*aroA124 hisD8457*	Laboratory collection
TR3682	*Δhis-3050 nag-1*/F′ *his-2385*	Laboratory collection
TR3933	*lip-2 hisD8531*	Laboratory collection
TR5129	*his-63 pi-401*	Anderson and Roth (1)
TR5284	*pyrD121 put-521*	Ratzkin and Roth (17)
TR5654	*thrA9 strA1*	Davis et al. (6)
TR5655	*leu485 strA1*	Davis et al. (6)
TR5656	*proA36 strA1*	Davis et al. (6)
TR5657	*purE8 strA1*	Davis et al. (6)
TR5658	*pyrC7 strA1*	Davis et al. (6)
TR5660	*pyrF146 strA1*	Davis et al. (6)
TR5661	*aroD5 strA1*	Davis et al. (6)
TR5662	*hisO1242 Δhis-2236 strA1*	Davis et al. (6)
TR5663	*ΔpurF145 strA1*	Davis et al. (6)
TR5664	*cysA533 strA1*	Davis et al. (6)
TR5665	*cysC519 strA1*	Davis et al. (6)
TR5666	*serA13 strA1*	Davis et al. (6)
TR5667	*cysG439 strA1*	Davis et al. (6)
TR5668	*cysE396 strA1*	Davis et al. (6)
TR5669	*ilv-508 strA1*	Davis et al. (6)
TR5670	*metA53 strA1*	Davis et al. (6)
TR5671	*pyrB64 strA1*	Davis et al. (6)
TR5922	*nadD191* ($6AN^r$, $6ANa^r$)[a]	This work
TR5929	*nadD192* ($6AN^r$, $6ANa^r$)[a]	This work
TR5934	*nadD190* ($6AN^r$, $6ANa^r$)[a]	This work
TR5936	*nadD193* ($6AN^r$, $6ANa^r$)[a]	This work
TR5937	*nadD194* ($6AN^r$, $6ANa^r$)[a]	This work
TR5985	*nadB32 purC7 proA46 ilv-405 rha-461 strR* M10 Fla-56 *frm*	J. Foster

TABLE 1—*Continued*

Strain	Genotype	Source
TR5987	*nadB31 pncA15 trpA49*	J. Foster
TR6082	*nadA56 trpA49 pnuA11*	This work
TR6416	*nadD157* (6AN^r, 6ANa^r, Ts)[a]	This work
TR6417	*nadD158* (6AN^r, 6ANa^r, Ts)[a]	This work
TR6418	*nadD159* (6AN^r, 6ANa^r, Ts)[a]	This work
TR6465	*nadD187* (6AN^r, 6ANa^r, Ts)[a]	This work
TR6466	*nadD188* (6AN^r, 6ANa^r, Ts)[a]	This work
TR6467	*nadD189* (6AN^r, 6ANa^r, Ts)[a]	This work
TR6480	*nadB200* (6AN^r, 6ANa^r, Ts)[a]	This work
TR6481	*nadB201* (6AN^r, 6ANa^r, Ts)[a]	This work
TR6482	*nadB202* (6AN^r, 6ANa^r, Ts)[a]	This work
TR6483	*nadB203* (6AN^r, 6ANa^r, Ts)[a]	This work
TR6484	*nadB204* (6AN^r, 6ANa^r, Ts)[a]	This work
TR6485	*nadB205* (6AN^r, 6ANa^r, Ts)[a]	This work
TR6486	*nadB206* (6AN^r, 6ANa^r, Ts)[a]	This work
TR6487	*nadB207* (6AN^r, 6ANa^r, Ts)[a]	This work
TR6488	*nadB208* (6AN^r, 6ANa^r, Ts)[a]	This work
TT627	*strA1 pyrC7*/F′ 114-Ts *lac*$^+$ *zzf-20*::Tn*10*(A)	Chumley et al. (3)
TT628	*strA1 pyrC7*/F′ 114-Ts *lac*$^+$ *zzf-21*::Tn*10*(B)	Chumley et al. (3)
TT629	*strA1 pyrC7*/F′ 114-Ts *lac*$^+$ *zzf-22*::Tn*10*(A)	Chumley et al. (3)
TT6195	*pncA148*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6196	*pncX149*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6200	*pncX153*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6203	*pncX156*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6204	*pncX157*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6205	*pncX158*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6206	*pncX159*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6207	*pncX160*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6208	*pncA161*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6209	*pncX162*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6210	*pncX163*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6211	*pncX164*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6213	*pncX166*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6214	*pncX167*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6215	*pncX168*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6216	*pncX169*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6218	*pncX171*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6221	*pncX174*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6222	*pncX175*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6223	*pncX176*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6224	*pncX177*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6226	*pncX179*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6230	*pncX183*::Tn*10* (6AN^r, 6ANa^s)	This work
TT6581	*zfi-789*::Tn*10* (Tn*10* 88% linked to *nadB*$^+$)	This work
TT6974	*zfi-789*::Tn*10 nadB131* (6AN^r, 6ANa^r)	This work
TT6975	*zfi-789*::Tn*10 nadB132* (6AN^r, 6ANa^r)	This work
TT6976	*zfi-789*::Tn*10 nadB133* (6AN^r, 6ANa^r)	This work
TT6977	*zfi-789*::Tn*10 nadB134* (6AN^r, 6ANa^r)	This work
TT6978	*zfi-789*::Tn*10 nadB135* (6AN^r, 6ANa^r)	This work
TT6979	*zfi-789*::Tn*10 nadB136* (6AN^r, 6ANa^r)	This work
TT6980	*zfi-789*::Tn*10 nadB137* (6AN^r, 6ANa^r)	This work
TT6981	*zfi-789*::Tn*10 nadB138* (6AN^r, 6ANa^r)	This work
TT6982	*zfi-789*::Tn*10 nadB139* (6AN^r, 6ANa^r)	This work
TT6983	*zfi-789*::Tn*10 nadB140* (6AN^r, 6ANa^r)	This work
TT6984	*zfi-789*::Tn*10 nadB141* (6AN^r, 6ANa^r)	This work
TT6985	*zfi-789*::Tn*10 nadB142* (6AN^r, 6ANa^r)	This work
TT6986	*zfi-789*::Tn*10 nadB143* (6AN^r, 6ANa^r)	This work
TT7247	*zbe-1023*::Tn*10* (Tn*10* 55% linked to *nadD*)	
TT8052	*zbe-1023*::Tn*10*/F′114-Ts *lac*$^+$ *zzf-20*::Tn*10*(A)	This work
TT8053	*zbe-1023*::Tn*10*/F′114-Ts *lac*$^+$ *zzf-21*::Tn*10*(B)	This work
TT8054	*zbe-1023*::Tn*10*/F′114-Ts *lac*$^+$ *zzf-22*::Tn*10*(A)	This work

[a] 6AN^r, 6-aminonicotinamide resistant; 6ANa, 6-aminonicotinic acid resistant; Ts, temperature-sensitive allele.

growth, [^{14}C]adenine (specific activity, ca. 30 to 70 Ci/mmol) was added. The final concentration was 7.5 μCi/ml for 90-min pulses and 15 μCi/ml for 15-min pulses. After labeling with [^{14}C]adenine, cells were centrifuged and washed with E medium, and the pellet was suspended in 0.4 ml of 0.3 M HCl and left overnight at 0°C. The acid extract was centrifuged in an Eppendorf centrifuge 5412 to remove cell debris. The supernatant was neutralized with 1 M Tris (pH 8) and diluted to 1 ml with 0.01 M Tris (pH 8). A commercial preparation of bacterial alkaline phosphatase (Worthington Diagnostics; BAP-C, 10 μl) was added, and the mixture was incubated for 15 min at 37°C. Then, a second sample of BAP-C was added, and the mixture was incubated for an additional 15 min at 37°C. The phosphatase-treated extract was purified by chromatography on a diethylaminoethyl Sephadex column (2.5 by 25 cm). The column was washed with 5 mM ammonium acetate before the cell extract was applied. After application, elution was carried out with 30 ml each of 0.005, 0.01, 0.02, 0.07, 0.1, and 0.2 M ammonium acetate. Samples (0.2 ml) from each fraction (~5 ml, total) were counted in Aquasol to locate radioactivity. The labeled NAD and 6-aminoNAD were eluted with 0.07 M ammonium acetate.

Samples (0.06 ml) were taken from the fractions with maximum radioactivity and pooled. The pool was applied to DE81 paper including AMP, NMN, NAD, 6-aminonicotinamide, and nicotinamide as markers, and chromatography was carried out by using 0.25 M ammonium bicarbonate to develop the chromatogram. Although NAD and 6-aminoNAD essentially overlap on the diethylaminoethyl Sephadex column, they are well resolved on diethylaminoethyl paper under these conditions.

Preparation of cell extracts. A culture of either *S. typhimurium* LT2, the *pncA* strain TT6195, or the *pncX* strain TT6196 was grown in E medium plus glucose to an absorbance of 0.8 at 650 nm. The cells were harvested by centrifugation and washed twice with E medium, and the cell pellet was suspended in 2 ml of 0.05 M phosphate buffer (pH 8). The cells were broken by the use of a French press. For assays of enzymatic activity, a 0.1-ml sample of each cell extract obtained was used.

Assay for conversion of nicotinamide to nicotinic acid. To assay for conversion of radioactive nicotinamide to nicotinic acid, an acid extract or culture supernatant was spotted on DE81 paper. The chromatogram was developed with 0.25 M ammonium bicarbonate for 4 to 5 h. Nicotinamide and nicotinic acid markers (10 mg/ml) were spotted, the chromatogram was cut into 1-cm strips, and the strips were counted on an L200 Beckman scintillation counter with a toluene-based scintillation fluid.

RESULTS

Screening for 6-aminonicotinamide-resistant mutants. The use of pyridine nucleotide analogs 6-aminonicotinamide and 6-aminonicotinic acid has permitted isolation of *pncA* and *pncB* mutants. These mutants are resistant to inhibition by the analog because they are unable to convert exogenous nicotinamide to NAD. To obtain 6-aminonicotinamide resistance mutations that are distinct from classes previously characterized, analog-resistant mutants were selected which retain the ability to use exogenous nicotinamide as a precursor for NAD.

In the first screen, 6-aminonicotinamide-resistant mutants were isolated by using transposon Tn*10* insertion mutagenesis (see above). Since analog resistance is due to Tn*10* insertion into a gene, the resistance marker could be transduced into other strains by selecting for inheritance of the Tn*10*-encoded tetracycline resistance. When 6-aminonicotinamide resistance Tn*10* insertions were introduced into a *nadB* strain, the expected *pncA*::Tn*10* and *pncB*::Tn*10* insertion mutants were observed (by their failure to utilize nicotinamide as a pyridine source). In addition, a new class of mutants was seen. This class (class A) is resistant to 6-aminonicotinamide and sensitive to 6-aminonicotinic acid; in this respect it is similar to *pncA* mutants. However, when a class A mutation is introduced into a *nadB* strain the resulting transductants retain the ability to use nicotinamide as an exogenous pyridine source. Thus class A mutations have not caused complete loss of *pncA* function.

In a second experiment, spontaneous 6-aminonicotinamide-resistant mutants (not Tn*10* generated) were selected. In this screen no strains with class a resistance mutations were isolated; however, along with the expected *pncA* and *pncB* mutants, an additional class was isolated. These new mutants are resistant to both 6-aminonicotinamide and 6-aminonicotinic acid (as are *pncB* mutants), but when an *nadA*::Tn*10* mutation is introduced into these strains, they retain the ability to utilize nicotinamide and nicotinic acid as exogenous pyridine sources.

Finally, a third screening procedure was carried out, looking for mutants with temperature-sensitive lethal mutations and which are resistant to 6-aminonicotinamide on minimal medium at the permissive temperature. Mutants resistant to 6-aminonicotinamide were selected at 30°C and were then screened for their ability to grow at the high temperature (42°C). Some of the resulting temperature-sensitive mutants grew at the high temperature in the presence of nicotinamide (class B), whereas the rest did not. Mutants in which temperature sensitivity is not corrected by nicotinamide proved to have a mutation in the same gene as several of the spontaneous mutations described above. This type of mutation was placed in class C.

In summary, class A mutant were all 6-aminonicotinamide resistant, but sensitive to 6-aminonicotinic acid. Class B and class C mutants were resistant to both 6-aminonicotinamide and 6-aminonicotinic acid. Only Tn*10* insertion mutations fell in the class A group. The temperature-sensitive mutants were found for both the class

B and class C mutants. However, all class B mutants that were temperature sensitive could be rescued by the addition of nicotinamide or nicotinic acid at the nonpermissive temperature. Temperature-sensitive class C mutants fail to grow at 42°C regardless of the medium. A list of strains belonging to each class is given in Table 1.

Characterization of class A mutants. The class A mutants, all of which have Tn*10* insertions, were mapped by Tn*10*-mediated Hfr formation to a region between min 22 and 33 of the *S. typhimurium* chromosome map. Since the *pncA* locus maps in the same region, and because of the sensitivity of these mutants to 6-aminonicotinic acid, we suspected that they might affect expression of the *pncA* gene. A transduction analysis demonstrated that 22 of 23 class A mutants were linked by transduction to *pncA15*; linkage varied between 26 and 92% depending on the class A allele used.

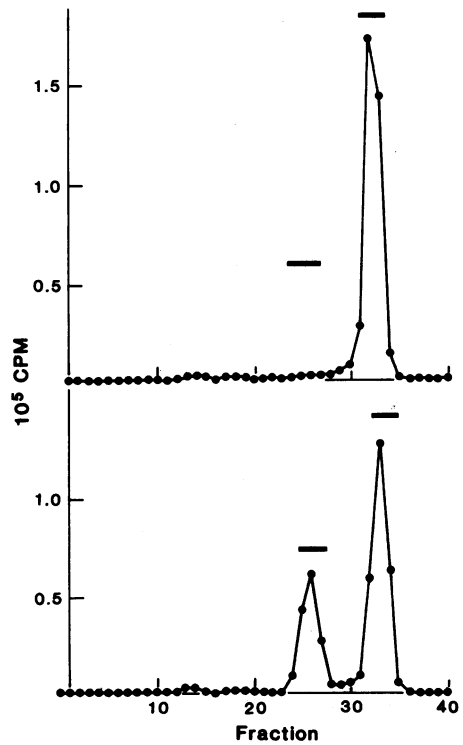

FIG. 2. Excretion of nicotinic acid in vivo. Two cultures (6 ml) of *S. typhimurium* LT2 (top) and TT6196 (a class A mutant—see text) (bottom) were grown in E medium plus 0.2% glucose to an absorbance of 0.5. [^{14}C]nicotinamide was added (17.5 μCi, 0.3 μmol), and the culture was incubated for 2 h at 37°C. The cells were centrifuged, and the culture supernatant (0.2 ml) was spotted on diethylaminoethyl paper and analyzed as described in the text. The nicotinamide (fractions 31 through 34) and nicotinic acid (fractions 25 through 27) markers are indicated by the horizontal bars.

TABLE 2. Deamidase activity[a]

Strain	Incubation time (min)	% Conversion to nicotinic acid	Enzyme activity (nmol/min per mg of protein)
LT2	10	12.0	5.17
	60	83.3	5.98
TT6195	10	<0.5	
(*pncA*::Tn*10*)	60	<0.5	
TT6196	10	1.24	0.446
(*pncX*::Tn*10*)	60	6.88	0.415

[a] Extracts of strains LT2 (*pncA*$^+$ *pncX*$^+$), TT6195 (*pncA*), and the class A mutant TT6196 (*pncX*) were prepared as described in the text. To 0.1 ml of the crude extract was added to 0.3 ml of a [^{14}C]nicotinamide solution containing 130 nmol of nicotinamide and 7.5 μCi of ^{14}C. The extracts were incubated for either 10 min or 1 h; after incubation, the percent radioactivity converted to nicotinic acid was analyzed as described in the text. Two separate experiments are shown.

The ability of these strains to convert nicotinamide to nicotinic acid was tested in vivo. The experiment shown in Fig. 2 demonstrates that class A mutants did not convert exogenous nicotinamide to nicotinic acid (in the medium) at a detectable level; a corresponding experiment with a wild-type strain shows rapid excretion of nicotinic acid produced from exogenous nicotinamide. However, some conversion of nicotinamide to nicotinic acid must occur in these cells, since strains harboring a class A resistance mutation and an *nadA* mutation can utilize nicotinamide as a source of the pyridine ring. Enzymatic assays for nicotinamide deamidase were carried out on cell extracts to determine how much enzyme was actually present in these mutants. The deamidase activity in a class A mutant was compared with that in isogenic *pncA* and wild-type strains. The class A mutant had detectable activity, but that activity was about 10-fold lower than the activity of nicotinamide deamidase detected in wild-type cells. However, the deamidase levels are at least 10-fold higher than in a *pncA* strain; the latter had no detectable activity (Table 2). These results suggest that the class A Tn*10* insertions reduce, but do not eliminate, expression of the nearby *pncA* gene. This could be due to substitution of the Tn*10* outward promoter (4) for the normal *pncA* promoter. Alternatively, the insertions may inactivate a positive regulatory gene for *pncA*. These possibilities are currently being tested.

We have tentatively designated all class A mutants *pncX*; whether these are all mutated in the same gene is not clear. Similar mutants have

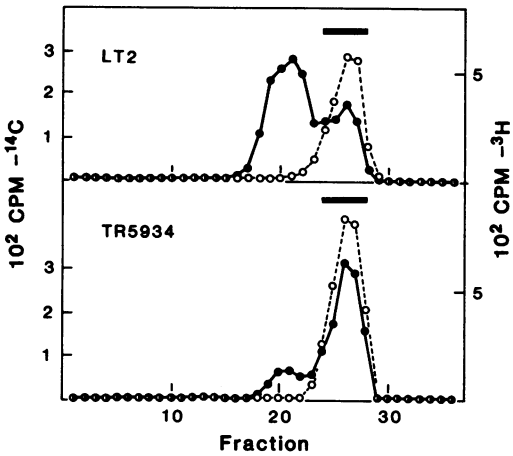

FIG. 3. Cultures of *S. typhimurium* LT2 and TR5934, a class C (*nadD*) mutant, were grown in the presence of [^{3}H]nicotinic acid, treated with 6-aminonicotinamide (100 μg/ml), and then pulsed with [^{14}C]adenine to label both the NAD and 6-aminoNAD pools as described in the text. The labeling time in [^{14}C]adenine was 90 min at 37°C in this experiment. The NAD and 6-aminoNAD were then purified (see text). The chromatography of NAD and 6-aminoNAD from extracts of the two strains is shown. An authentic NAD marker traveled in fractions 24 through 28 (horizontal bar). Identical samples that had been pretreated with venom phosphodiesterase were also analyzed; under these conditions, all the ^{14}C radioactivity migrated with authentic AMP.

been isolated in J. Foster's laboratory (personal communication).

Basis for the 6-aminonicotinamide resistance of the class B and class C mutants. The resistance of class A mutants to 6-aminonicotinamide can readily be explained by poor conversion of 6-aminonicotinamide to 6-aminonicotinic acid, consistent with the sensitivity of such mutants to 6-aminonicotinic acid. Similar tests for class B and class C mutants showed that in these strains, conversion of nicotinamide to nicotinic acid was normal. (This metabolic step is catalyzed by nicotinamide deamidase, coded for by the *pncA* gene.) When grown in a medium containing nicotinamide, mutants in these classes excreted nicotinic acid to the same degree as the wild-type LT2 strains. This result and the fact that these mutants are resistant to 6-aminonicotinic acid as well as 6-aminonicotinamide also suggest that class B and C mutants must be affected at a later point in analog metabolism.

The basis for the 6-aminonicotinamide resistance was investigated. Since 6-aminonicotinamide and 6-aminonicotinic acid may be bacteriostatic because of their conversion to 6-aminoNAD, we developed an assay to determine the relative levels of NAD and 6-aminoNAD. If cells are grown in the presence of 6-aminonicotinamide and pulse labeled with [^{14}C]adenine, a labeled compound appears that copurifies with NAD upon diethylaminoethyl Sephadex chromatography, but has a slightly different R_f on paper chromatography. This material is 6-aminoNAD by several criteria: it is insensitive to phosphatase, yields [^{14}C]AMP upon venom phosphodiesterase treatment, and is totally absent if 6-aminonicotinamide is withdrawn from the medium. Furthermore, it inhibits and is not reduced by yeast alcohol dehydrogenase and has spectral properties consistent with 6-aminoNAD. If cells are grown in 6-aminonicotinamide and pulsed with [^{14}C]adenine, and 6-aminoNAD and NAD are purified from all other adenine-containing compounds, the ratio between [^{14}C]adenine label in NAD and label in 6-aminoNAD can be determined. Experiments of this type are shown in Fig. 3 and 4.

As shown in Fig. 3, a class C mutant has a much lower ratio of 6-aminoNAD to NAD than does the wild type. Similar results are found for the class B mutants. However, all of these measurements are complicated by the fact that different ratios of 6-aminoNAD to NAD are obtained depending on the precise labeling protocol used. It appears that the ratios change as a function of time of labeling because 6-aminoNAD turns over much less rapidly than NAD. Thus, the specific activity of 6-aminoNAD increases with respect to NAD the longer the labeling period. This effect is shown in Fig. 4. As shown in Table 3, however, at all times the 6-aminonicotinamide-resistant mutant shows a

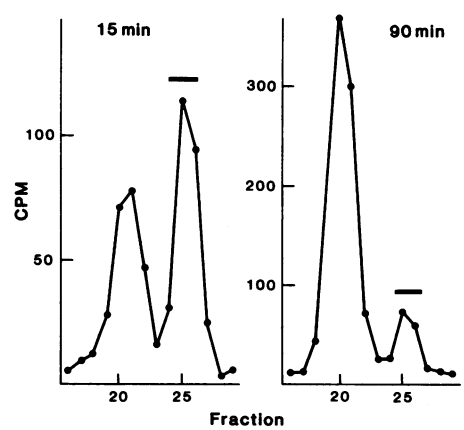

FIG. 4. A culture of *S. typhimurium* TT6975 (*nadB*, 6-aminonicotinamide resistant) was grown in the presence of 6-aminonicotinamide and pulse-labeled with [^{14}C]adenine for 15 and 90 min, and the labeled 6-aminoNAD and NAD were purified as described in the text and analyzed as described in the legend to Fig. 3. The horizontal bar (fractions 24 through 26) represents the position of an authentic NAD marker.

TABLE 3. NAD/6-aminoNAD ratios[a]

Expt	[^{14}C]NAD/[^{14}C]6-aminoNAD	
	15 min	90 min
I TT6581 (*nadB*$^+$)	1.12	0.18
TT6975 (*nadB* 6AN^r)	1.86	0.57
II TR5634 (*nadD*)		3.68
LT2 (wild type)		0.39

[a] The ratio of [^{14}C]adenine radioactivity in NAD and 6-aminoNAD was determined as described in the text. The data are calculated from experiments similar to those shown in Fig. 3 and 4. 6AN^r, 6-aminonicotinamide resistant.

higher ratio of NAD to 6-aminoNAD than does the parental 6-aminonicotinamide-sensitive strain.

We conclude on the basis of these results that the 6-aminonicotinamide resistance of class B and class C mutants is consistent with a higher ratio of normal NAD to 6-aminoNAD in the presence of the precursor 6-aminonicotinamide. Therefore the mutations must in some way bias synthesis of NAD against the analog.

Characterization of class B mutants. The class B mutants have been further characterized. Class B mutants, which exhibit a temperature-sensitive phenotype in minimal medium, grow at 42°C if nicotinamide is added as a supplement. This result suggests that these mutants produced an abnormally low level of NAD through the endogenous pathway, i.e., had mutations in for the *nadA*, *nadB*, or *nadC* genes.

Transduction experiments show that the class B mutations all map at the *nadB* locus. None mapped near the *nadA* or *nadC* locus (9, 10). A large number of class B mutants were also generated by localized mutagenesis of the *nadB* region (see above). A fine structure mapping of these mutations has shown that all class B mutations examined map within the *nadB* gene (B. Cookson and J. Roth, unpublished data).

The dominance of the *nadB* (6-aminonicotinamide resistance) mutations to a wild-type allele was shown in chromosomal duplications of the *nadB* region. Strain TR5129 carries a tandem chromosomal duplication of the *nadB* region, which can be selectively maintained by growth on histidinol. Selection and manipulation of these duplications is described elsewhere (1). This strain was transduced to Tet^r with phage grown on an *nadB* (6-aminonicotinamide resistant) host harboring a Tn*10* insertion close to the *nadB* gene. Approximately 65% of all Tet^r transductants were made resistant to 6-aminonicotinic acid. The high frequency of 6-aminonicotinamide-resistant colonies indicates that the *nadB* (6-aminonicotinamide resistance) allele is dominant over the *nadB*$^+$ phenotype. To insure that the strains actually carried duplications and were heterozygous, containing both the *nadB* (6-

TABLE 4. Hfr mapping of *zbe-1023*::Tn*10*[a]

Recipient genotype	No. of prototrophic recombinant colonies[b]			
	Donor TT8052	Donor TT8053	Donor TT8054	Control (no donor)
TR5654 (*thrA strA*)	6	32	5	2
TR5655 (*leu strA*)	0	43	1	0
TR5656 (*proA strA*)	4	33	0	0
TR5657 (*purE strA*)	3	59	0	0
TR5658 (*pyrC strA*)	631	5	56	12
TR5660 (*pyrF strA*)	172	1	16	0
TR5661 (*aroD strA*)	28	4	6	0
TR5662 (*his strA*)	10	12	1	0
TR5663 (*purF strA*)	21	0	1	1
TR5664 (*cysA strA*)	0	0	0	0
TR5665 (*cysC strA*)	2	0	0	0
TR5666 (*serA strA*)	5	1	3	0
TR5667 (*cysG strA*)	0	0	0	0
TR5668 (*cysE strA*)	0	1	0	0
TR5669 (*ilv strA*)	0	1	1	5
TR5670 (*metA strA*)	2	23	0	0
TR5671 (*pyrB strA*)	1	25	0	0
Control (no recipient)	0	0	0	

[a] Hfr mapping shows that Tn*10* lies between min 12 (*purE*) and min 22 (*pyrC*). Hfr strains were prepared from donor strains as described by Chumley et al. (3). Hfr mapping of the *zbe-1023*::Tn*10* insertion, present in all donors, that is linked by a 55% P22 cotransduction frequency to *nadD157*. Homologous recombination between F'114-ts containing *zzf-20*::Tn*10* (TT8052) or *zzf-22*::Tn*10* (TT8054); both of which are in the A orientation, result in Hfrs with direction of transfer opposite that of F'114-ts containing *zzf-21*::Tn*10* (TT8053), which is in the B orientation (Chumley et al. [3]). The Hfr strains were mated with auxotrophic strains defective in genes throughout the chromosome. The results show that *zbe-1023*::Tn*10* lies between *purE* and *pyrC* on the chromosome.

[b] 10^8 donor and recipient cells were used for each mating.

TABLE 5. Linkage of *zbe-1023*::Tn*10* to markers between and including *purE* and *pyrC*[a]

Donor (TT7247)	Recipient	Map position (min) of auxotrophic marker	Selected marker (no. scored)	No. with unselected marker
zbe-1023	TR5658	22.3	Tc^r (1,000)	0 *pyrC*$^+$
			pyrC$^+$ (1,000)	0 Tc^r
zbe-1023	TR5284	21.5	Tc^r (1,000)	0 *put*$^+$
		20.2		0 *pyrD*$^+$
			pyrD$^+$ (1,000)	0 Tc^r
zbe-1023	TR2577	19.3	Tc^r (300)	0 *aro*$^+$
			aro$^+$ (500)	0 Tc^r
zbe-1023	TR6082	17.4	Tc^r (1,000)	0 *nadA*$^+$
			nadA$^+$ (1,000)	0 Tc^r
zbe-1023	TR3682	15.0	Tc^r (500)	0 *nag*$^+$
			nag$^+$ (1,000)	0 Tc^r
zbe-1023	TR3933	14.0	Tc^r (104)	103 *lip*$^+$
			lip$^+$ (30)	29 Tc^r
zbe-1023	TR5657	12.5	Tc^r (1,00)	0 *purE*$^+$
			purE$^+$ (500)	0 Tc^r

[a] P22 transduction shows linkage of Tn*10* to *lip* and not *nadA*. We tested P22 cotransduction frequencies between *zbe-1023*::Tn*10* and various genes that lie between *purE* and *pyrC* on the chromosome. The results show that the Tn*10* insertion is linked to lip at min 14 and not to *nadA* at min 17, indicating that *nadB* is unlinked to any known *nad* genes and constitutes a new locus on the *S. typhimurium* genetic map.

aminonicotinamide resistant) and the wild-type allele, the phenotype of spontaneous haploid segregants was determined. The duplications tested were grown overnight on nutrient broth and then streaked on minimal medium containing histidinol and low histidine. Parent duplication types form large colonies on this medium, and haploid segregants form small colonies. Twenty-five colonies were picked, patched, and printed to minimal medium, minimal medium plus histidinol, and minimal medium plus histidinol and 6-aminonicotinic acid. Both 6-aminonicotinamide-sensitive and 6-aminonicotinamide-resistant strains were found among the haploid segregants, insuring that the strains actually harbored both *nadB*$^+$ and *nadB* (6-aminonicotinamide resistance) alleles of the locus.

Characterization of class C mutants. All mutants with lethal temperature-sensitive and 6-aminonicotinamide resistance mutations and which were not rescued by the addition of exogenous nicotinamide or nicotinic acid were class C mutants. Hfr mapping showed that these mutations mapped between min 12 and 22 on the *S. typhimurium* chromosome (between *purE* and *pyrC*) (Table 4) (19). Transduction experiments show that all class C mutations which have been tested map at a new position, closely linked to the *lip* locus at min 14 (Table 5). Since all 6-aminonicotinamide-resistant mutants isolated that have temperature-sensitive lethal mutations are mutated in this gene, this locus may be an essential gene of NAD metabolism. Mutants in this class are currently being characterized. Preliminary results indicate that this new locus, here designated *nadD*, may be the structural gene for the enzyme nicotinic acid adenine dinucleotide (NaAD) pyrophosphorylase (nicotinic acid mononucleotide [NaMN] adenyltransferase) (Fig. 5).

DISCUSSION

The nicotinamide and nicotinic acid analogs 6-aminonicotinamide and 6-aminonicotinic acid are believed to cause bacteriostasis because the 6-aminoNAD formed from these analogs interferes with many NAD-utilizing reactions in general metabolism. Previous studies indicate that 6-aminoNAD cannot act as an electron acceptor for several dehydrogenases (7). Thus, Cobb and co-workers suggested that for 6-aminonicotinamide to cause growth inhibition, it must first be converted to 6-aminoNAD (5). A mutation that knocks out either the nicotinamidase or nicotinic acid phosphoribosyl transferase activities (which catalyze the first two steps in the Preiss-Handler pathway and are coded for by the *pncA* and *pncB* genes, respectively) gives complete resistance to this analog. These are the mutants that have been previously isolated as 6-aminonicotinamide resistant.

In this report, we have screened for novel types of 6-aminonicotinamide- resistant mutants of *S. typhimurium* which are not the standard *pncA* and *pncB* strains. Three new classes of 6-aminonicotinamide-resistant mutants were found. Of these, only one is related to the metabolic steps above. We have designated this locus *pncX*. Mutations of the *pncX* type cause a 10-fold lowering of the levels of nicotinamidase. However, there is enough enzyme present so

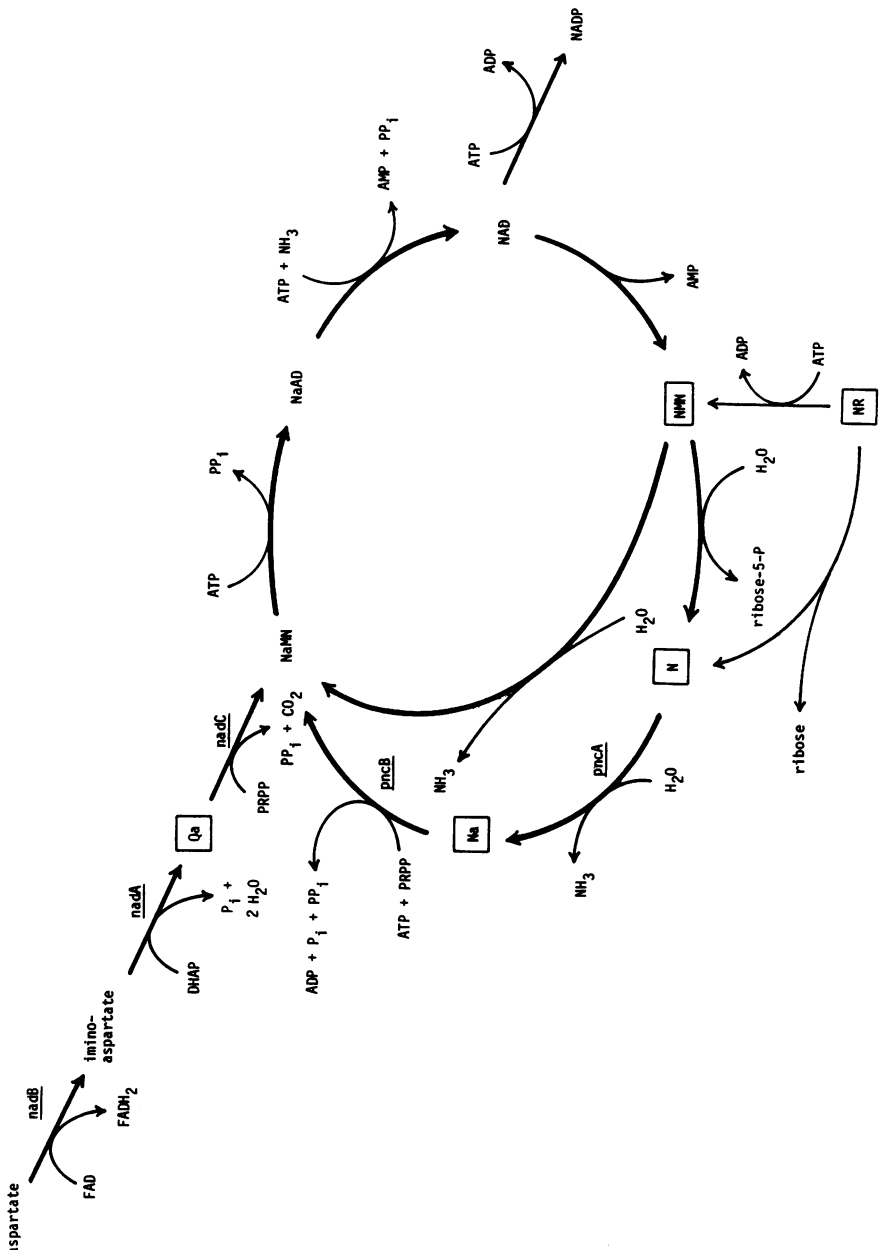

FIG. 5. Biosynthesis and turnover pathway of pyridine nucleotides. Abbreviations: Qa, quinolinic acid; DHAP, dihydroxyacetone phosphate; NaMN, nicotinic acid mononucleotide; NaAD, nicotinic acid adenine dinucleotide; NMN, nicotinamide mononucleotide; NR, nicotinamide ribonucleaside; PRPP, phosphoribosylpyrophosphate.

that the cells can grow on nicotinamide as the only source of the pyridine ring. Nevertheless, because of the drastic decrease in enzymatic levels, these strains do not rapidly convert nicotinamide to nicotinic acid in the medium as is observed for normal wild-type *S. typhimurium*. The precise defect in *pncX* mutants is not understood at this time. It is noteworthy that *pncX* mutants are obtained by using Tn*10* insertions and have not been found among spontaneous resistant mutants.

The second class of mutants found, with mutations mapping in the *nadB* gene, are of considerable interest. The *nadB* product is involved in the endogenous biosynthetic pathway and would not be directly involved in the conversion of 6-aminonicotinamide to 6-aminoNAD. The simplest explanation for the dominance of the 6-aminonicotinamide-resistance mutations in the *nadB* gene is that the *nadB* enzyme, L-aspartate oxidase, is regulated by feed back inhibition, and that the 6-aminonicotinamide-resistant mutants are defective in such feed back regulation (14). We suggest that 6-aminonicotinamide is not only a precursor for the bacteriostatic metabolite 6-aminoNAD, but may, in addition, depress synthesis of normal NAD by direct or indirect feedback on endogenous synthesis. It is known that *Escherichia coli*, given a choice between an exogenous precursor and endogenous synthesis, will preferentially use the exogenous source of the pyridine ring. How this regulation is achieved is not known in detail. The results with *S. typhimurium* suggest, however, that the *nadB* gene is an important component of the regulation. If 6-aminonicotinamide normally caused inhibition of *nadB* function, then endogenous synthesis would be depressed and the levels of 6-aminoNAD would be very high compared with normal NAD. However, in the 6-aminonicotinamide-resistant *nadB* mutants, the presence of 6-aminonicotinamide apparently does not cause as great a depression of endogenous synthesis. As a consequence, high levels of endogenous NAD synthesis continue even in the presence of the analog accounting for the relative resistance of such strains. The isolation of these mutants in the *nadB* locus, but not in the *nadA* or *nadC* locus, indicates that *nadB* is the primary site of regulation of endogenous synthesis. Reports in the literature indicate that NAD inhibits *nadB* function (11, 14). However, a detailed comparison of wild-type and mutant *nadB* gene products will be necessary to definitively identify which metabolite is relevant in this regulation.

The third class of 6-aminonicotinamide resistance mutations are in a previously undefined genetic locus. We have presented evidence that these do not fall in any of the known loci for *nad* or *pnc* genes. The 6-aminonicotinamide-resistant mutations that are also temperature-sensitive lethal mutations map at this locus. The precise map location of these mutations and the precise biochemical function affected are currently being determined. Our preliminary results strongly suggest that mutants with mutations at this locus (which we have designated *nadD*) are defective in the enzyme NaAD pyrophosphorylase, which converts NaMN to NaAD (Fig. 5). A comprehensive study of these *nadD* mutants is currently under way.

The in vivo determination of 6-aminoNAD demonstrates that resistant strains have a lower 6-aminoNAD/NAD ratio than sensitive strains. These results support the suggestion of Cobb and co-workers (5) that it is the 6-aminoNAD and 6-aminoNADP analogs that account for bacteriostatic effects in vivo. In this study, we also obtained evidence that 6-aminoNAD turns over much more slowly than does the normal dinucleotide. Thus, it appears that in addition to its well known inability to act as an electron acceptor in reactions catalyzed by a number of dehydrogenases, 6-aminoNAD is also a poor substrate for enzymes that catalyze the degradation of NAD in vivo. This raises the possibility that the limiting reaction for bacterial growth may not be a dehydrogenase, but rather one in which NAD is a substrate such as DNA ligase (15).

The results of this study suggest that any mutation that leads to a lower 6-aminoNAD/NAD ratio may confer some degree of resistance to the analog. The most easily isolated mutants have mutations at the *pncA* and *pncB* loci that completely block formation of 6-aminoNAD from 6-aminonicotinamide. In this work, the mutants we have isolated have mutations that do not completely block 6-aminoNAD formation, but in different ways, cause a relative decrease in the level of the analog compared to the normal dinucleotide in vivo. These mutants, as well as perhaps even more subtle 6-aminonicotinamide-resistant phenotypes, should not only be useful in identifying new genetic functions affecting pyridine nucleotide metabolism, but also in unscrambling the regulatory features of the complex pathways of the biosynthesis, and the breakdown and turnover of NAD and NADP.

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