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Isolation and Characterization of Mutants of *Escherichia coli* Defective in Pyridine Nucleotide Cycle Enzymes

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Abstract. Thirty-eight analogs of nicotinic acid and nicotinamide were tested for their ability to inhibit growth of wild-type *Escherichia coli* K-12. Two of the compounds tested, 6-aminonicotinic acid and 6-aminonicotinamide were strongly inhibitory to growth of the organism. Mutants resistant to these compounds were isolated and characterized by cross-feeding experiments. All of the mutants isolated by their resistance to these analogs were found to excrete a metabolite which supported growth of *nadA*, *nadB*, or *nadC* strains of *E. coli* on a minimal medium. Wild-type strains failed to exhibit this cross-feeding ability. The *pncB*$^+$ locus codes for nicotinic acid phosphoribosyl transferase and maps near minute 23 on the chromosome.

It is well established that nicotinamide adenine dinucleotide (NAD/$NADH_2$) and nicotinamide adenine dinucleotide phosphate (NADP/$NADPH_2$) play fundamental roles in metabolism as oxidation-reduction couples.

In addition to this role, some nonoxidative functions have been recently established [1,2,3,11,12,13]. Although biologists have been aware of these compounds and their physiological role for many years, the metabolism of NAD or NADP has not been clearly elucidated in bacterial systems. The proposed pathway for the biosynthesis of NAD or NADP is shown in Fig. 1. The cell can make these universal proton-electron donors/acceptors de novo from the condensation of aspartate and dihydroxyacetone phosphate. However, when nicotinic acid or nicotinamide is available the de novo pathway is repressed and these compounds are metabolized via the pyridine cycle (Fig. 1).

One approach which can be taken to investigate the metabolism and regulation of the metabolism of NAD and NADP is through the isolation of mutants blocked in the pyridine nucleotide cycle. We describe here a simple procedure for obtaining mutants unable to utilize exogenously supplied nicotinic acid or nicotinamide. The procedure is based on the sensitivity of *Escherichia coli* K-12 to analogs of nicotinic acid or nicotinamide.

Materials and Methods

All bacterial strains used in this study were derivatives of *Escherichia coli* K-12: UTH 7094 (F^-) represents the wild-type organism, UTH 4460 (F^-, *nadA*, *arg*, *xyl*, *mtl*, *lac*, *str*^{r}), UTH 4471 (F^+, *nadB*), UTH 4673 (F^+, *nadC*), UTH 7073 (*HfrH*, *str*^{s}), and UTH 123 (F^-, *proC*$^-$, *thr*, *leu*).

Chemicals. Nicotinic acid, nicotinamide, quinolinic acid and 6-aminonicotinamide were purchased from the Sigma Chemical Co., St. Louis, Missouri. Nicotinic acid -7^{14}-C was purchased from the New England Nuclear Corp., Boston, Massachusetts. 6-Aminonicotinic acid was synthesized following the procedure outlined below.

1. 1 g of 6-aminonicotinamide was refluxed in 10 ml of 6 N HCl for 10 h.
2. The solution was lyophilized and suspended in 25 ml of deionized water. The pH was adjusted to 7.5–8.0 and the solution was diluted to a concentration of approximately 3×10^{-3} M.
3. The material was applied to a Dowex-1 (Sigma) formate (8% cross-linked 100–200 dry mesh) column and washed with 500 ml of deionized water.
4. The 6-aminonicotinic acid was eluted from the column with 1,200 ml of 0.1 N formic acid, evaporated to a smaller volume, and lyophilized.
5. A small portion of lyophilized powder was dissolved in deionized water to a concentration of approximately 10^{-4} M (pH 7.5–8.0). This material was then checked for purity by one-dimensional descending partition chromatography employing Whatman no. 1 chromatography paper and a solvent system consisting of 95% ethanol and concentrated ammonium hydroxide mixed 95:5. Under these chromatographic conditions, 6-aminonicotinic acid and 6-aminonicotinamide have R_f values of 0.12 and 0.40, respectively.

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Media. The minimal medium of Davis and Mingioli [4] was supplemented with 10 mg of thiamine per liter and appropriate amino acid(s) at 50 μg per ml and/or vitamin(s) at 10 μg per ml. Streptomycin-sulfate was added to minimal medium, when required, to a concentration of 100 μg per ml. The vitamin analogs, 6-aminonicotinic acid and 6-aminonicotinamide, were added to the medium to concentrations of 1×10^{-4}M (13.8 μg/ml for 6-aminonicotinic acid and 13.7 μg/ml for 6-aminonicotinamide) unless otherwise noted. Penassay medium consisted of 17.5 g of antibiotic assay medium no. 3 (Difco Laboratories, Detroit, Michigan) per liter of deionized water. L-medium was prepared as described by Lennox [8]. Hard agar plates of the above media contained 15 g agar per liter. Soft agar contained 6 g agar per liter.

The minimal medium without glucose, thiamine, or any other organic additions was utilized as a basal salts solution. Unless stated otherwise, this solution was used for washing the suspension of cells. Eluting fluid, used for harvesting P_1 phage, contained 8 g of sodium chloride and 3 g of peptone (Difco) per liter of water.

Minimal inhibitory concentration (MIC). The MIC for 6-aminonicotinic acid and 6-aminonicotinamide was determined by the agar plate dilution method [8]. The MIC was defined as the lowest concentration of analog which inhibited the development of visible growth on a plate which had been inoculated with a 10^{-2} dilution of an overnight culture grown in minimal medium.

Isolation of mutants. All 6-aminonicotinic acid-resistant (*pncB*) and 6-aminonicotinamide-resistant (*pncA*) strains were spontaneous mutants selected by plating a 10^{-2} dilution of an 18-h culture of the parent strain on minimal medium containing 1×10^{-4} M of the respective analog. Resistant colonies began to appear after 48 h. Individual colonies were picked and purified by restreaking for isolation on identical selection media.

Cross-feeding. Cross-feeding experiments were performed on solid medium. The medium was designed to allow growth of the organism tested but lacked a constituent or included an inhibitor which prevented growth of the indicator organism. The test and indicator organisms were streaked in opposing semicircular patterns as shown in Fig. 2. The plates were incubated at 37°C for at least 48 h.

Preparation of cell-free extracts. Cultures were grown overnight in minimal medium at 37°C. The cells were harvested by centrifugation and washed twice. The drained pellets were disrupted by alumina grinding with a mortar and pestle (2.5 g of levigated alumina per 1 g wet weight of cells). During the grinding procedure, 100 mM Tris-phosphate (pH 8.0) was slowly added to a final volume of 8 ml per 1 g wet weight of cells. The mixture was centrifuged for 30 min at 20,000 × *g*, and the slightly turbid supernatant was used immediately in assays for nicotinic acid phosphoribosyltransferase activity. The temperature was maintained at 0–4°C during each step in the preparation of the extracts.

Nicotinic acid phosphoribosyltransferase assay. Nicotinic acid phosphoribosyltransferase was assayed using a modification of the procedure of Imsande [6]. The complete reaction mixture was contained in a total volume of 1.0 ml; 20 μmol $MgSO_4$, 3 μmol of ATP, 1 μmol of PRPP, 0.1 μmol of nicotinic acid-7-C^{14} (4.55 mCi/mmol), 1 μmol of reduced glutathione, 100 μmol of Tris-phosphate buffer, pH 8.5, and 0.25 ml of enzyme preparation. Protein concentration of the enzyme preparation was measured according to the method of Layne [7].

The reaction mixture was incubated at 37°C without agitation. At various times, 0.01-ml samples were withdrawn and applied to Whatman no.1 chromatography paper. The samples were air-dried and separated by ascending partition chromatography in a solvent system which was the upper phase of 250 ml of *n*-butanol, 60 ml of glacial acetic acid, and 25 ml of deionized water. Under these chromatographic conditions, nicotinic acid migrated away from the point of sample application and was separated from the reaction products which remained at the origin. The origin was cut from the paper and was transferred to a scintillation vial containing 10 ml scintillation cocktail, and the radioactivity was determined in a liquid scintillation spectrophotometer.

Genetic studies. Donor P_1 phage stocks for transduction were propagated by seeding 3 ml of molten (45°C) soft L-agar with the donor bacterium and inoculating this dilute suspension with approximately 10^5 phage particles. This mixture was overlayed on L-agar plates and incubated at 37°C for 6 h. The soft agar layer was transferred to 12 ml centrifuge tubes. Three milliliters of eluting fluid were added and the contents were mixed vigorously for 1 min. Debris was removed by centrifugation and the supernatant was stored over chloroform at 4°C.

Transductions were performed according to the procedure of Miller [10].

Conjugation experiments were performed according to the membrane filter technique of Matney and Achenback [9], except that the membranes which supported the mating cells were incubated on soft Penassay agar plates instead of soft minimal medium plates.

Results

Isolation and initial characterization by cross-feeding of mutants. The approach chosen for the isolation procedure involved the use of analogs of nicotinic acid and nicotinamide. Thirty-eight potential analogs of nicotinic acid and nicotinamide were screened for their ability to satisfy two basic qualitative criteria: (i) significant inhibition of wild-type strains of *Escherichia coli*; and (ii) easy and rapid isolation of mutants resistant to the analog. Two of the compounds tested, 6-aminonicotinic acid and 6-aminonicotinamide exhibited relatively low MICs as shown in Table 1. The strain with a functional pyridine nucleotide cycle, UTH 7094 (*pnc*$^+$), was representative of several *E. coli* K-12 strains tested with respect to MIC.

Two classes of mutants were obtained corresponding to resistance patterns toward the two different analogs. All mutants were characterized by MICs

Table 1. Minimal inhibitory concentration (MIC) of 6-aminonicotinic acid and 6-aminonicotinamide for *Escherichia coli*.

		MIC[a]	
Strain	Genotype	6-amino-nicotinic acid	6-amino-nicotinamide
UTH 7094	*pnc*$^+$	25 M	25 μM
UTH 7095	*pncB*$^-$	>5 mM	>5 mM
UTH 7109	*pncA*$^-$	25 μM	>5 mM

[a] Plate sensitivity test.

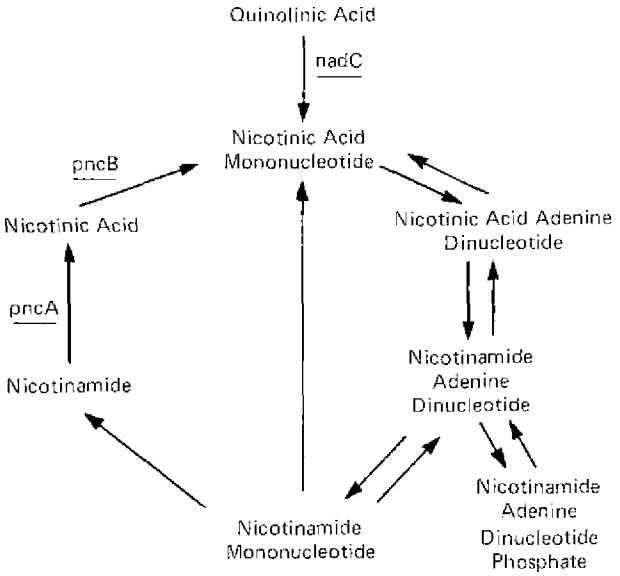

Fig. 1. Proposed routes of biosynthesis of NAD or NADP in *Escherichia coli*. The upper portion leading to NMN represents the de novo pathway while the lower cycle represents the pyridine nucleotide cycle.

Table 2. Reversal of the inhibitory effects of 50 μM 6-aminonicotinic acid on the growth of wild-type *Escherichia coli* by nicotinic acid.

	Molar concn of nicotinic acid				
	0	10^{-6}	10^{-5}	10^{-4}	10^{-3}
CFU[a]	47.6	43.4	+[b]	+	+

[a] CFU, colony-forming units of a 10^{-2} dilution of culture.
[b] +, Too numerous to count.

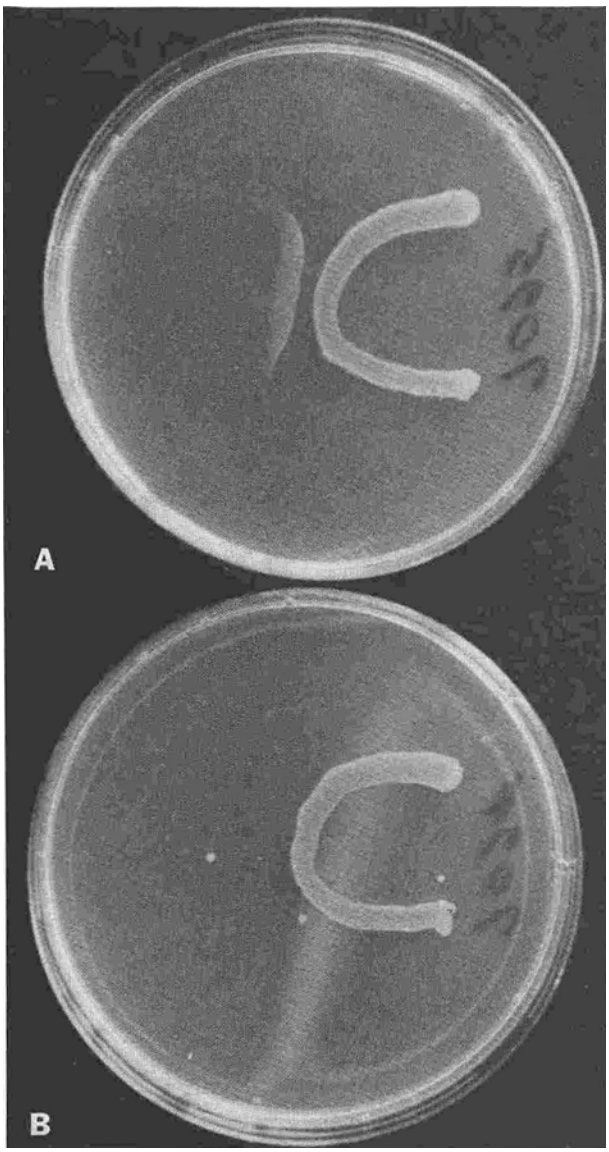

Fig. 2. Demonstration of a metabolite(s) excreted by a *pncB* strain of *Escherichia coli* which supports the growth of mutants defective in the de novo NAD pathway. (A) UTH 7095 (*pncB*$^-$) cross-feeding; (B) UTH 7094 (*pnc*$^+$) no cross-feeding.

200-fold or greater than that found for wild-type strains. Strains designated *pncA* (by virtue of a mutation near minute 39 which results in a defect in the synthesis of nicotinamide deaminase; see [1]) were found to be sensitive to 6-aminonicotinic acid and resistant to 6-aminonicotinamide; those strains designated *pncB* were resistant to both of these analogs (Table 1).

In order to obtain evidence to support the premise that 6-aminonicotinic acid was inhibitory to the pyridine nucleotide cycle, an experiment was designed to test whether nicotinic acid would effect a reversal of the inhibitory action of the compound. Nicotinic acid, incorporated in the medium at a concentration of 1×10^{-5} M, was found to reverse the effects of 5×10^{-5} M 6-aminonicotinic acid.

This reversal by nicotinic acid was further defined by the results of the tube dilution experiment outlined in Table 2. Employing this technique, the MIC was approximately 1×10^{-5} M 6-aminonicotinic acid for the *pnc*$^+$ strain, and greater than 2.5×10^{-3} M for one of our *pncB*$^-$ mutants. The addition of 1×10^{-5} M nicotinic acid resulted in complete phenotypic resistance of the *pnc*$^+$ strain, while 1×10^{-6} M nicotinic acid resulted in a 10-fold increase in the MIC of the analog.

The plates shown in Fig. 2 demonstrate the presence of a diffusible metabolite excreted from both classes of *pnc*$^-$ strains. The excretion product(s) was found to cross-feed mutants defective in the de novo

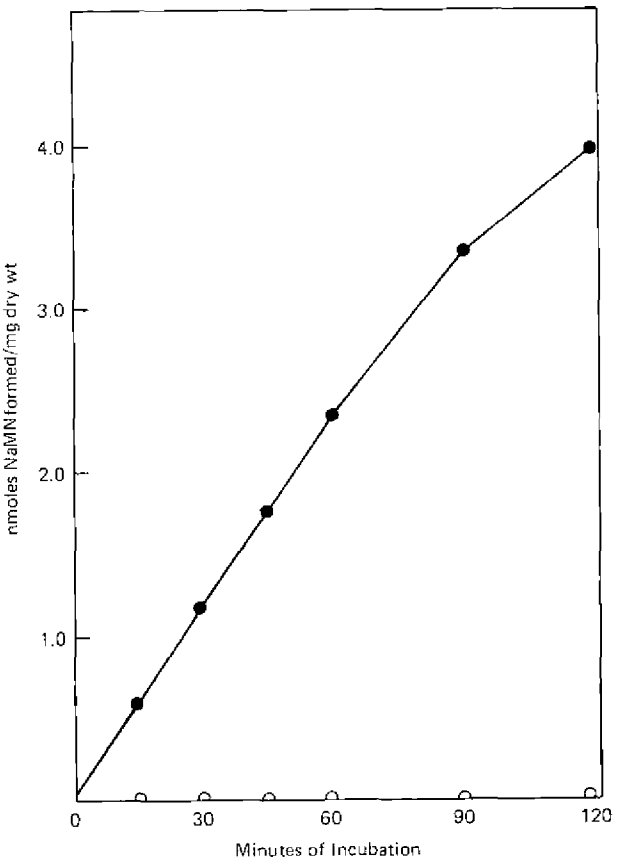

Fig. 3. Time course of nicotinic acid phosphoribosyltransferase activity in extracts of wild-type and *pncB* mutants of *Escherichia coli*. ●, UTH 7094 (wild type); ○, UTH 7095 (*pncB*).

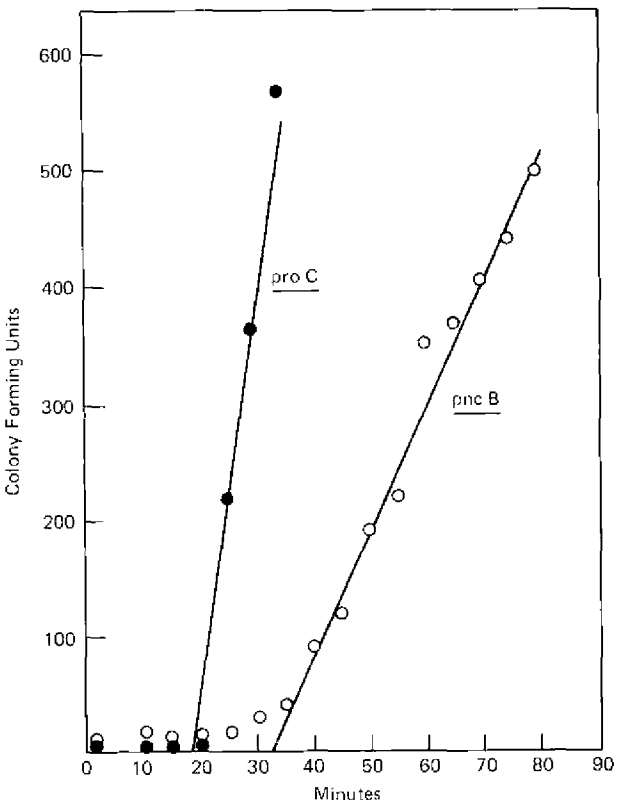

Fig. 4. Time entry of *pncB* marker as determined by conjugation in *Escherichia coli*. Donor UTH 7110 (Hfr *pncB*); recipient UTH 123 (F^- *proC thr leu*).

NAD pathway, and also to reverse the effect of the analog on the wild-type strain.

This qualitative cross-feeding procedure was utilized to screen 20 independent mutant isolates (10 resistant to 6-aminonicotinic acid and 10 resistant to 6-aminonicotinamide). All 20 mutant strains were found to excrete a metabolite which supported the growth of *nadA*$^-$, *nadB*$^-$, and *nadC*$^-$ strains in nicotinic acid–free medium. Wild-type strains failed to exhibit this cross-feeding behavior.

Nicotinic acid phosphoribosyltransferase activity in the wild-type and *pncB* mutants. The relationship between mutations designated *pncB* and nicotinic acid phosphoribosyltransferase activity was examined by enzyme analysis of cell-free extracts from the wild-type and mutant organisms. Fig. 3 illustrates a time course of nicotinic acid phosphoribosyl-transferase activity in a *pnc*$^+$ strain. The enzyme reaction rate observed for this strain was approximately 2.5 nmol of nicotinic acid mononucleotide per h per mg dry weight of cell-free preparation. Also presented are the results obtained with a *pncB*$^-$ mutant. Ten other independently isolated *pncB* mutants were screened, all of which failed to show detectable nicotinic acid phosphoribosyltransferase activity.

Genetic studies. Genetic experiments were performed in an attempt to further characterize the enzymatic lesion resulting in 6-aminonicotinic acid resistance (*pncB*). A transduction between a *purI*$^+$ *nadB pncB*$^+$ donor and a *purI nadB*$^+$ *pncB* recipient was carried out whereby *purI*$^+$ transductants were selected in a minimal medium containing nicotinic acid. Control experiments indicated that one could expect at least 10% of these *purI*$^+$ transductants to incorporate the adjacent *nadB*$^-$ allele; published figures [13] indicate a 56% cotransduction frequency. However, an analysis of over 2,000 *purI*$^+$ transductants, from 13 separate donor strains of the indicated genotype, indicated that not a single *purI*$^+$ transductant recovered contained the *nadB* allele. In other words, the *nadB* and *pncB* alleles never occurred within the same cell. This indicates that the incorporation of the *nadB* and *pncB* alleles into the same cell is a lethal event. This event would only be lethal if both the de novo NAD biosynthetic pathway and the pyridine nucleotide cycle were blocked. Since it is known that *nadB*$^+$ codes for an enzyme in-

volved in quinolinic acid synthesis, the *pncB*$^+$ gene must code for a pyridine nucleotide cycle enzyme which converts nicotinic acid into NAD, i.e., nicotinic acid phosphoribosyl transferase.

A high spontaneous mutation frequency to 6-aminonicotinic acid resistance of approximately 10^{-5} precluded the accurate mapping of these markers by transduction methods. However, conjugation experiments employing the membrane filter method were used to approximate the position on the chromosome of *E. coli*. Fig. 4 shows the kinetics of recombinant formation for 6-aminonicotinic acid resistance and the *proC* marker. The donor utilized was an Hfr Hayes *pncB* derivative. The time of entry difference between the *proC* marker at 9 min on the chromosome and the *pncB*$^-$ marker was approximately 14 min which places the *pncB*$^+$ locus at approximately 23 min on the chromosome of *E. coli*.

Discussion

Mutants of *Escherichia coli* that are defective at the *pncA* or *pncB* locus exhibit the dual phenotype of pyridine analog resistance and excretion of a metabolite(s) that cross-feeds *nad* mutants. It is quite easy to select for, and isolate, these types of mutants. It should be noted that a *pncA* or a *pncB* mutation in a cell that is wild type for NAD biosynthesis will not result in a phenotypic nutritional requirement for that strain. However, a double mutant such as *nad pncA* or *nad pncB* exhibits a nutritional requirement for NAD or one of its more immediate precursors. NAD is not permeable to the cell [5] and the nucleotide precursors of NAD are probably not permeable or have very limited permeability due to the highly polar phosphate groups. Thus, while it is possible to isolate a *nad pncA* double mutant by satisfying the resulting nutritional requirement with nicotinic acid, it has not been possible to isolate a *nad pncB* double mutant because of the inability of the pyridine nucleotides to penetrate the cell. Quinolinic acid, at physiological concentrations and higher (up to at least 10^{-3} M) does not enter the cell in sufficient quantity to support the growth of a *nad pncB* mutant. Therefore a *nad pncB* double mutation is a lethal event.

Since the *pncB* mutation does not confer a nutritional requirement upon the cell, the finding that the wild-type organism is sensitive to the nicotinic acid analog, 6-aminonicotinic acid, indicates the possibility that the inhibition of cell proliferation is due to the conversion of 6-aminonicotinic acid to 6-aminonicotinamide adenine dinucleotide and the subsequent effect of the dinucleotide analog on NAD-requiring metabolic reactions.

Our data indicate that the *pncB* locus maps at minute 23 on the chromosome; *pncA* has been mapped at minute 39 [1]. Therefore, the *pnc* loci do not fall within an operon and any control mechanism operational on the system must affect each locus independently.

The availability of *pnc* mutants singly and in combination with *nad* mutants presents a means of manipulating the pyridine nucleotide cycle in order to study the genetics and metabolism of the pathway.

ACKNOWLEDGMENTS

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