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The *Salmonella typhimurium nadC* Gene: Sequence Determination by Use of Mud-P22 and Purification of Quinolinate Phosphoribosyltransferase

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The *Salmonella typhimurium nadC* gene and its product, quinolinic acid phosphoribosyltransferase (QAPRTase), were characterized at the molecular and biochemical levels. Fusions of Mud-*lac* elements isolated in the *nadC* gene were converted to Mud-P22 insertions. Starting with six original Mud-*lac* fusions, the entire sequence of the *nadC* gene was readily obtained. The sequence shows a long open reading frame with two potential initiator methionines, one of which is preceded by the Shine-Dalgarno sequence GGAG–7-nucleotide–ATG. The protein predicted from this second open reading frame is 297 residues in length. The *nadC* gene was subcloned into a T7-based expression system, allowing for facile purification of the QAPRTase (EC 2.4.2.19) protein to homogeneity. Upon gel filtration, the protein gave an M_r of 72,000, and sodium dodecyl sulfate-polyacrylamide gel electrophoresis gave a subunit M_r of 35,000. Automated Edman degradation of several tryptic peptides confirmed the amino acid sequence predicted from the DNA sequence. Chromatography of the apparently homogeneous enzyme on reverse-phase high-performance liquid chromatography resolved two protein species. One of these species failed to give an amino-terminal sequence, while the other yielded the amino-terminal sequence predicted by the second open reading frame and lacked the initiator methionine. The mass of the mature protein, predicted from its DNA sequence, was 32,428 Da. Electrospray mass spectrometry gave masses of 32,501 and 32,581 Da for the two peptides. Steady-state kinetics on the purified QAPRTase indicated K_m values of 32 μM for 5-phosphoribosyl-1-pyrophosphate and 20 μM for quinolinate. V_{max} was 0.9 U/mg, similar to values reported for this enzyme by other sources.

The *nadC* gene product, quinolinic acid (QA) phosphoribosyltransferase (PRTase) (EC 2.4.2.19), is required for de novo NAD biosynthesis in both prokaryotes and eukaryotes. The PRTase activity of QAPRTase coupled with decarboxylation of QA results in the formation of nicotinic acid mononucleotide (NAMN), a central intermediate in the de novo and recycling syntheses of NAD (Fig. 1). QAPRTase is a member of the family of PRTases which catalyze the formation of nucleotides from nitrogenous bases and 5-phosphoribosyl-1-pyrophosphate (PRPP) (23). Such enzymes are required for formation of purine and pyrimidine nucleotides, synthesis of histidine and tryptophan, and metabolism of the pyridine nucleotides NAD and NADP. The *nadC* gene has been extensively characterized; a deletion map of *nadC* including 18 deletion intervals has recently been reported (18). The *nadC* gene maps to 3 min on the *Salmonella* chromosome, and together with the unlinked *nadA* and *nadB* genes, it is one of three nonessential genes involved in the de novo biosynthesis of NAD and NADP (28). Both *nadA* and *nadB* are regulated by the *nadI* (or *nadR*) repressor (6, 8, 12, 38), while *nadC* is not known to be under any genetic regulation (11, 31).

The enzymatic reaction catalyzed by QAPRTase is very similar to the salvage reaction catalyzed by the *pncB* gene product, nicotinic acid PRTase (NAPRTase). Like QAPRTase, NAPRTase catalyzes the formation of NAMN. The *pncB* gene of *Salmonella typhimurium* has been previously cloned and sequenced (35). The similarity in the enzymatic reactions that NAPRTase and QAPRTase catalyze would suggest, a priori, similarity in protein sequence and structure. A comparison of the sequence similarities may also provide clues to the evolution of the NAD recycling pathways.

This report describes a method for DNA sequence determination by use of Mud-P22. The method was used in the sequencing of the *nadC* gene of *S. typhimurium*. Once the adjacent DNA was isolated, segments of the *nadC* gene were sequenced with primers which hybridized to the ends of Mu. This technique permits the rapid sequencing of any gene for which Mud-*lac* fusions are available in *Salmonella* spp. and *Escherichia coli*. The *nadC* gene was subcloned into a T7-based vector, and the protein product, QAPRTase, was overexpressed. The enzyme has been purified to homogeneity and physicochemically and kinetically characterized.

MATERIALS AND METHODS

Bacterial strains and plasmids. All strains used in this study are listed in Table 1. All *S. typhimurium* strains were derived from *S. typhimurium* LT2. Several derivatives of the Mu *d*(*lac*) phage described by Casadaban and Cohen (1) were used in this work. Mu *d1-8* refers to a transposition-defective derivative of the original Mu d1(Ap *lac*) phage of Casadaban and Cohen which forms *lac* operon fusions (15). Mu dI1734(Km *lac*) refers to a transposition-defective *lac* operon fusion vector described by Castilho et al. (2). This phage is deleted for transposition functions, and it carries kanamycin resistance. Mu dI1734 insertion mutants are isolated by providing transposition functions in *cis* on a single, P22-transducing fragment (17). MudP and MudQ are

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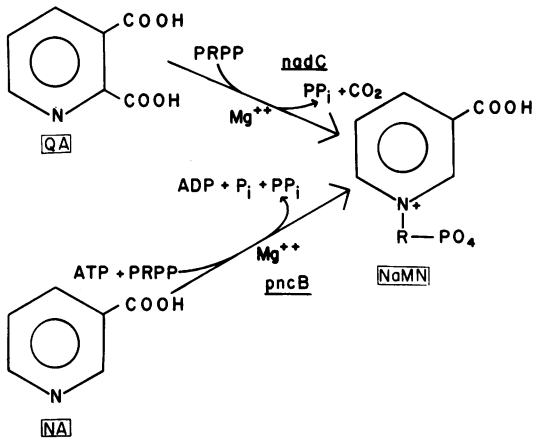

FIG. 1. Comparison of NAPRTase and QAPRTase reactions in synthesis of NAMN in *S. typhimurium*. The similarities between the enzymatic steps for QAPRTase, which is coded for by the *nadC* gene and is required for de novo NAD biosynthesis, and those for NAPRTase, which is coded for by the *pncB* gene and is required for the utilization of exogenously supplied pyridine sources, are depicted. NA, nicotinic acid; R, ribose.

collectively termed Mud-P22 and have been described by Youderian et al. (37). Plasmid derivatives of pBR328 carrying the *nadC* region of the *Salmonella* chromosome were isolated from a library provided by Charles Miller (10). All subcloning of Mud-P22 phage DNA was done into the Bluescript plasmid vector (Stratagene) prior to sequencing and restriction analysis.

Media. The minimal and rich media used in this study and supplements have been described previously (18). Sodium cyanide was purchased from Fisher. Ammonium sulfate was from Research Plus Labs. Other chemicals were from Sigma. The restriction endonucleases *Pst*I and *Sph*I were obtained from U.S. Biochemicals, and *Ava*I came from New England Biolabs. Plasmid DNA (pSP73 and pUC18) was purchased from Promega. [5-^{3}H]QA (11.9 Ci/mmol) from New England Nuclear was a kind gift from B. M. Olivera. Cellulose-precoated chromatography plates were from Brinkman. DEAE-TSK resin was obtained from Supelco, and Liquiscint was from National Diagnostics.

TABLE 1. Bacterial strains and plasmids[a]

Strain or plasmid	Genotype or description	Source
Strains		
E. coli B		
BL21(DE3)	F$^-$ *ompT* r_B^- m_B^-	J. Dunn, Brookhaven
AD100	pAD01/BL21	
S. typhimurium LT2		
MS1868	*leuA414 hsdSB* Fels$^-$	
PY13518	(F'114ts *lac*$^+$ *zzf-20*::Tn*10 tetA*::MudP) *leuA414 hsdSB* Fels$^-$	P. Youderian
PY13518	(F'114ts *lac*$^+$ *zzf-20*::Tn*10 zzf-3553*::MudQ) *leuA414 hsdSB* Fels$^-$	P. Youderian
TT8784	*nadC218*::Mu *d1-8*	
TT8786	*nadC220*::Mu *d1-8*	
TT8787	*nadC221*::Mu *d1-8*	
TT10105	*nadC351*::Mu *d1-8*	
TT10210	*nadC356*::Mu *d1-8*	
TT10493	*nadC368*::Mu dI1734	
Plasmids		
pBS	Bluescript	Stratagene
pKH56	pBR328, *nadC*$^+$	
pAD01	pSP73, *Sph*I-*Pst*I *nadC*$^+$	

[a] Unless indicated otherwise, all strains and plasmids were constructed during the course of this work.

Transductional methods. For all transductional crosses, the high-frequency generalized transducing mutant of bacteriophage P22 (*HT105/1 int-201*) was used (27). Selective plates were spread directly with 2×10^8 cells and 10^8 to 10^9 phage. For transduction of Mu *d1-8*, 10^9 to 10^{10} phage per 2×10^8 cells were used. The Mu *d1-8* prophages are inherited by a two-fragment transductional event and therefore require a higher phage input (14, 16). Transductants were purified, and phage-free clones were isolated by nonselective streaking onto green indicator plates (3). The titers of P22 lysates were determined according to the method of Davis et al. (7).

Construction of expression vectors. Plasmid pKH56, a pBR328-based construct which confers the NadC$^+$ phenotype on TH265, was treated with *Sph*I and *Pst*I; the resulting 2.3-kb fragment was purified in an agarose gel. The fragment was then cloned into a T7 promoter vector, pSP73 (Promega). The resultant construct, pAD01, was used to transform BL21(DE3) (33), and the resulting strain, AD100, was able to overexpress QAPRTase in amounts adequate for purification and kinetic characterization.

Generation of *nadC*::Mud-P22 insertions. Insertions of Mu *d1-8* or Mu dI1734 in *nadC* or any gene are converted by cassette replacement to Mud-P22. First, the Mu *d1-8* or Mu dI1734 insertion mutant is moved into strain MS1868 (this strain lacks the Fels prophage) so that when the Mud-P22 is induced (see below), the resulting lysate is free of Fels phage particles. Phage P22 *int-3* is grown on a *Salmonella* strain harboring either the MudP or MudQ element in the F factor. This lysate is used to transduce a *nadC*::Mud-*lac* recipient to Mud-P22-encoded chloramphenicol resistance. Recombination between the MuL and MuR ends of Mud-P22 and the MuL and MuR ends of Mud-*lac* replaces the internal region of Mud-*lac* with the Mud-P22 sequences (37). Thus, chloramphenicol-resistant transductants that result in the simultaneous loss of either kanamycin resistance in a *nadC*::Mu dI1734 recipient or ampicillin resistance in a *nadC*::Mu *d1*-8 recipient have converted the *nadC*::Mud-*lac* insertion mutant to a *nadC*::Mud-P22 insertion mutant. We have observed that the frequency of conversion varies for different Mud-*lac* recipients. In general, the majority of chloramphenicol-resistant transductants retain the drug resistance of the Mud-*lac* fusion. Previous evidence suggests that these are spontaneous deletions in the donor strains that give rise to mini-F factors that are small enough to be transduced by P22 (37). Although both Mud-P22 elements are lacking the P22 immunity I region and thus are expected to be sensitive to P22 infection, purified MudQ replacement transductants were consistently phage resistant, while the MudP replacement transductants were consistently phage sensitive. A method for isolating P22-sensitive MudQ transductants was found. When the MudQ replacement transductants were streaked for single colonies with a platinum wire on green indicator plates, most of the transductants would have a small region of blue in the streak, indicating phage contamination and cell lysis. The blue area was restreaked on green

plates, and single colonies were retested for sensitivity to P22 infection; phage-sensitive clones were obtained and were shown by further analysis to be MudQ replacement transductants. It is not clear why the MudQ transductants behave in this manner.

Molecular biological techniques. Routine molecular biological techniques were performed essentially as described by Sambrook et al. (26). To prepare miniprep plasmid DNA suitable for reproducible DNA sequence analysis, the following protocol was followed. A 2-ml portion of Luria broth containing 100 μg of ampicillin per ml was inoculated from a single colony of strain JM101 carrying the plasmid to be sequenced with a sterile wooden applicator and left at room temperature overnight. The next day, the tubes were placed at 37°C with shaking for 4 h. A 1.5-ml portion of the culture was centrifuged for 30 s at 12,000 × *g* and resuspended in 300 μl of STET (8% sucrose, 5% Triton X-100, 50 mM EDTA, 50 mM Tris-HCl [pH 8.0]). A 20-μl portion of 10 mg of lysozyme per ml in 50 mM Tris-HCl, pH 8.0, was added, the solution was mixed, and the tubes were incubated at room temperature for 1 to 20 min. The tubes were then placed in a boiling-water bath for 2 min and centrifuged for 5 min at room temperature, and the pellet was removed with a flat toothpick and discarded. A 300-μl portion of 2.5 M ammonium acetate–75% isopropanol was added, and the tubes were inverted 20 times. Afterwards, they were centrifuged for 5 min at room temperature. The pellet was rinsed with 1.5 ml of 70% ethanol–30% TE^{-4} (10 mM Tris-HCl [pH 8.0], 0.1 mM EDTA), vacuum dried, and resuspended in 50 μl of TE^{-4}. A 5-μl portion of 2 N NaOH–2 mM EDTA was added to 50 μl of miniprep plasmid DNA and incubated for 5 min at room temperature. Then, 7.5 μl of 3 M sodium acetate (pH 5) and 17.5 μl of water were added. The solution was mixed, 75 μl of cold 100% ethanol was added, and the solution was incubated at −70°C for 5 to 10 min. The tubes were centrifuged for 15 min at 4°C, and the pellet was rinsed with 1.5 ml of 70% ethanol–30% TE^{-4}, vacuum dried, and resuspended in 25 μl of TE^{-4}. Annealing was done by combining 7 μl of the denatured plasmid DNA with 2 μl of Sequenase buffer (U.S. Biochemicals) and 1 μl of primer (10 ng/ml) and incubating this first at 37°C for 20 min and then at room temperature for 5 to 10 min. DNA sequencing was performed by the method of Sanger et al. (29) with the Sequenase DNA-sequencing kit (U.S. Biochemicals) on miniprep plasmid DNA annealed to primer as described above. To determine the DNA sequence of the *nadC* gene cloned in pKH56, four DNA restriction fragments (*Ssp*I-*Eco*47III, *Eco*47III-*Fsp*I, *Fsp*I-*Sac*II, and *Sac*II-*Xmn*I), which in total cover the entire *nadC* gene, were isolated from pKH56 miniprep DNA on a 1% agarose gel following restriction endonuclease digestion. These fragments were subcloned into the Bluescript vector (Stratagene), and the DNA sequence was determined as described above.

Preparation of *nadC*::Mud-P22 phage DNA. The induction of Mud-P22 lysates and the isolation of DNA were performed as described previously (37).

QAPRTase assay. QAPRTase activity was measured by spectrophotometric quantitation of the cyanide adduct of NAMN (5, 25). The reaction mixture for the assay contained 50 mM phosphate buffer (pH 7.2), 6 mM $MgCl_2$, 0.8 mM QA, and 1 mM PRPP in a final volume of 0.8 ml. Enough enzyme to produce between 0.05 and 0.2 μmol of product was added, and the reaction was stopped after 10 min with the addition of 0.3 ml of 7 M NaCN. The quenched reaction mixture was allowed to stand at 30°C for 10 min; A_{315} was then determined. Blanks included all components except enzyme. One unit of enzyme activity is defined as that amount catalyzing the formation of one μmol of NAMN per min under the specified conditions.

For the kinetic experiments, QAPRTase activity was measured by thin-layer chromatography, in which the conversion of substrate to product was in the range of 3 to 10%. The K_m for PRPP was determined by assays with mixtures which contained 50 mM potassium phosphate (pH 7.2), 6 mM $MgCl_2$, 0.8 mM QA (7,500 cpm/nmol), PRPP concentrations varying between 20 and 150 μM, and 0.19 μg of pure QAPRTase in a final volume of 50 μl at 30°C in a thermostated heat block. The reaction was terminated after 4 min by applying 1 μl of the reaction mixture to a cellulose plate that had been pretreated with a solution of 10% sodium dodecyl sulfate (SDS). An 11-cm-long cellulose thin-layer chromatograph was developed in 1 M ammonium acetate (pH 8.7)–100% ethanol (40:60). Under these conditions, the R_f for QA was 0.37, while that for NAMN was 0.23. Spots were located with a UV lamp set at 265 nm. Plates were then cut into 0.5-cm-long strips, and the coating was scraped into individual plastic scintillation vials. The cellulose scrapings were then washed with 200 μl of water in order to solubilize the compounds, and 3.0 ml of Liquiscint was added to the mixtures. Radioactivity was measured with a Beckman liquid scintillation counter.

The K_m for QA was measured by using the same thin-layer chromatography system and employing 0.13 μg of pure QAPRTase, with QA concentrations ranging from 10 to 25 μM (specific activity, 1.2×10^6 cpm/nmol). The PRPP concentration was constant at 1 mM. The radioactivity was determined as in the PRPP experiments. Kinetic data were analyzed with the use of HYPERO (4).

Purification of QAPRTase. (i) Growth conditions. Strain AD100 was inoculated from a glycerol stock into 200 ml of Luria broth medium containing 100 μg of ampicillin per ml, and the culture was grown overnight at 37°C. After being centrifuged and resuspended in 24 ml of M9 salts (26), each of 12 flasks was inoculated with 2 ml of the cell suspension. Growth occurred for 3.5 h, and then isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM. After an additional 2.5 h of growth, cells were harvested by centrifugation at 10,000 × *g* for 10 min at 4°C. Harvested cells were washed and resuspended in buffer Q (0.1 M Tris brought to pH 8.0 with H_3PO_4–1 mM EDTA–5 mM mercaptoethanol).

Cell lysis was performed with a Sonifier Cell Disrupter (model W185) for 2 min at full power at 4°C. The suspension was allowed to cool on ice for 1 min, and then it was sonicated again for 1 min. Phenylmethylsulfonyl fluoride was added from a stock solution in ethanol immediately prior to sonication, to a final concentration of 1 mM. The soluble fraction was separated from the cell debris by centrifugation at 16,000 × *g* for 20 min at 4°C. To the supernatant, polyethyleneimine (prepared as a 10% [wt/vol] stock and brought to pH 8.0 with HCl) was added to a final concentration of 0.3% (wt/vol). The clear supernatant was recovered after the mixture was centrifuged at 16,000 × *g* for 10 min. Solid ammonium sulfate was then added to the solution to 35% saturation, and after the solution was centrifuged at 16,000 × *g* for 10 min, the pellet was discarded. Additional ammonium sulfate was added to bring the solution to 65% saturation; the precipitate was pelleted by centrifugation at 16,000 × *g* for 20 min and redissolved in buffer Q. It was then desalted by passage through a Sephadex G-50 column (2.5 by 15 cm) which had been preequilibrated with buffer Q.

A DEAE-TSK column (2.5 by 25 cm) was equilibrated

with buffer Q. The enzyme solution from the preceding step was applied to the column, and the resin was washed with 150 ml of buffer Q by use of a Pharmacia FPLC Gradient Programmer at 3.0 ml/min. A 600-ml linear gradient of 0 to 0.5 M Na_2SO_4 in buffer Q was then applied to the column. Fractions (15 ml) were collected, and those showing QAPRTase activity, which eluted at approximately 80% of the gradient, were pooled for further purification. The enzyme was precipitated by the addition of ammonium sulfate to 65% saturation. The same DEAE-TSK column was equilibrated with 0.1 M potassium phosphate–1 mM EDTA–5 mM mercaptoethanol (pH 7.2); other chromatographic steps were performed as described above, except that a linear gradient of 0 to 0.5 M Na_2SO_4 in the phosphate buffer was used to elute the column. Individual fractions containing QAPRTase activity were precipitated with 65% saturated ammonium sulfate and analyzed by SDS-polyacrylamide gel electrophoresis (PAGE).

In order to allow for the separation of minor contaminants from QAPRTase, the ammonium sulfate suspension was centrifuged at 16,000 × *g* for 20 min, and the pellet was redissolved in 0.5 ml of buffer Q. Individual ammonium sulfate crystals were added to the solution with slow stirring at 4°C until slight turbidity was observed. The solution was centrifuged as described above; the pellet was separated from the supernatant, and the process was repeated with the supernatant until the addition of a single crystal caused a large increase in turbidity. This suspension containing pure QAPRTase, as per SDS-PAGE, was used for kinetic and physicochemical characterization. This material appears homogeneous in all kinetic and chromatographic experiments and in SDS gels, and it was used in all kinetic experiments described in Results.

(ii) Molecular sieve chromatography. The apparent M_r of the purified enzyme was determined by using a Sephacryl HR-200 column (3.0 by 65 cm) preequilibrated with 0.1 M potassium phosphate–1 mM EDTA–5 mM mercaptoethanol–100 mM NaCl (pH 7.2). The column was standardized with lactate dehydrogenase, histidinol dehydrogenase, ovalbumin, lactoglobulin, and cytochrome *c*, which have M_rs of 140,000, 92,000, 45,000, 37,000, and 12,400, respectively.

Primary structure of QAPRTase. For amino-terminal sequencing, a portion of the enzyme was chromatographed on a Vydac 214TP54 C4 column. Elution was by a 100-ml linear gradient of 100% A to 100% B, where A was H_2O containing 0.1% trifluoroacetic acid (TFA) and B was acetonitrile containing 0.1% TFA. The effluent was monitored at 214 nm, and chromatographic peaks were collected manually. Two peaks of nearly equal sizes were observed at 68 and 70% B. Amino-terminal protein sequencing was performed by an automated Edman method on a Porton microsequencer with Porton "protein" disks.

For the preparation of tryptic peptides, QAPRTase (3 mg) was dissolved in 0.1 M Tricine–1 mM EDTA (pH 8.0) containing 8 M urea–1 mM dithiothreitol and allowed to incubate for 1 h. Iodoacetic acid was added to a final concentration of 5 mM, and the sample was incubated in the dark for 2 h. The carboxymethylated protein was chromatographed on a column (0.9 by 30 cm) of Sephadex G-50 equilibrated with 50 mM NH_4HCO_3 containing 1 mM $CaCl_2$. Trypsin (tolylsulfonyl phenylalanyl chloromethyl ketone-treated; Worthington) was added at 1:20 (wt/wt), and digestion was allowed to proceed for 17 h at 30°C. The sample was lyophilized and dissolved in 2 ml of H_2O, and a 0.5-ml portion was applied to a Vydac C18 column with elution as described above. Peak fractions were concentrated under vacuum and were subjected to Edman degradation on a "peptide" disk of the Porton microsequencer.

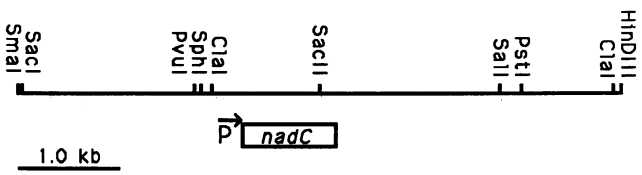

FIG. 2. Restriction map of the *nadC* region of the *S. typhimurium* chromosome. The location of *nadC* was determined by subcloning restriction fragments from plasmid pKH56 and screening for complementation of a chromosomal *nadC* mutant allele. The 2.3-kbp *Sph*I-*Pst*I fragment was cloned into pSP73 (Promega) to produce pAD01.

RESULTS

Isolation of the *nadC* gene of *S. typhimurium*. A plasmid library of *S. typhimurium* DNA cloned in pBR328 (a gift from Charles Miller) transformed into strain TN2540 was obtained (10). P22-transducing phage was grown on the library and was used to transduce a *nadC* mutant to $NadC^+$ and chloramphenicol resistance. Four $nadC^+$ Cam^r transductants were found to contain two different types of plasmid inserts on the basis of restriction analysis. These plasmids were used to transform a *guaC* mutant, and one, pKH56, complemented the *guaC* defect, suggesting that it carried both the *nadC* and *guaC* genes. A restriction endonuclease map of pKH56 is presented in Fig. 2. Through a series of subcloning experiments, it was found that the region of the cloned DNA that complemented *nadC* in TH265 was between the *Sph*I and *Sal*I restriction endonuclease sites. Internal to these sites is a *Sac*II site which, when cut from either end, resulted in the loss of the *nadC*-complementing function, suggesting that it was within *nadC*. Also, we found that the portion of the cloned region to the right of the *Hin*dIII site shown in Fig. 2 complemented a *guaC* mutation (data not shown), suggesting that the *guaC* gene lies in this region. Since transcription of *nadC* has been previously shown to occur in the direction toward *guaC* (18), then the *nadC* promoter region must lie on the opposite side of the *nadC* clone relative to *guaC* near the *Sph*I site.

DNA sequence determination of the *nadC* gene. To sequence the *nadC* gene, we decided to take a novel chromosomal gene-sequencing approach by the use of Mud-P22 elements. P22 *int-3* phage was grown on PY13518 and PY13757, strains with MudP and MudQ elements, respectively, inserted into F-factor DNA, and used to transduce *nadC*::Mud-*lac* insertions to Mud-P22-encoded chloramphenicol resistance (37). Simultaneous transduction to chloramphenicol resistance and loss of the Mud-*lac*-encoded drug resistance (Kan^r for Mu dI1734 and Amp^r for Mu *d1-8*) result from replacement of the Mud-*lac* element with Mud-P22 (37). By this technique, each of the *nadC*::Mud-*lac* insertions was converted to both MudP and MudQ derivatives.

Once *nadC*::Mud-*lac* insertions were converted to Mud-P22 elements, they were induced by mitomycin to obtain phage lysates. The phage genome within the Mud-P22 element cannot excise, and when induced, it produces a replication bubble on the chromosome, amplifying the P22 genome and adjacent chromosomal DNA. The DNA is packaged from a specific site within P22 termed the *pac* site, and on average, three adjacent headfuls of DNA are packaged into phage heads. These three headfuls include about 20

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1     CCACGCACGC TTATCAAAAG GCACATACTG GACGATTTCA CCGTCGCGAC GAATCAGACA ATGGGCCGAA

71    ACACGAAGAT GAGCGATTTC TGCAAAAAAA GGATGGGCGT CTGGATCTAT CGTTCCGGTG AATAATGCAT

141   CGATCCACGG ACCGCCAAAC TCGCCGGGGG GCAGGCTAAT ATTATGCACC ACCAGCAAGG ACGGTTTTTC

211   GTCATCCGGA CGGCAATCAT AATGCGGAGA AGGGACGCGT CGCGCCTCTA CCAACCAACC CTTATCTGGC
                                                  -35                  -10
281   AACATGCGGA GTCTCCTTTT GAGTGGTGCT TATAGCTGCT TCAGAGTAGC ATGTTTCTAC CTTATGATTC
                    S&D
351   GTTAGCAATT TGGAGTTTTA TC

373   ATG CCG CCT CGC CGT TAT AAC CCA GAC GAC CGA CGT GAC GCG CTA CTG GAA CGC ATT AAT
1     Met Pro Pro Arg Arg Tyr Asn Pro Asp Asp Arg Arg Asp Ala Leu Leu Glu Arg Ile Asn

433   CTC GAT ATC CCT GCC GCC GTT GCT CAG GCG CTG CGT GAA GAT TTA GGT GGA GAA GTC GAT
21    Leu Asp Ile Pro Ala Ala Val Ala Gln Ala Leu Arg Glu Asp Leu Gly Gly Glu Val Asp

493   GCT GGC AAC GAT ATC ACC GCG CAA CTT TTG CCG GCA GAT ACG CAA GCC CAT GCC ACG GTG
41    Ala Gly Asn Asp Ile Thr Ala Gln Leu Leu Pro Ala Asp Thr Gln Ala His Ala Thr Val

553   ATC ACT CGT GAA GAC GGC GTT TTC TGC GGC AAG CGC TGG GTT GAA GAG GTC TTC ATC CAA
61    Ile Thr Arg Glu Asp Gly Val Phe Cys Gly Lys Arg Trp Val Glu Glu Val Phe Ile Gln

613   CTG GCG GGC GAT GAT GTG CGC CTC ACC TGG CAT GTC GAT GAC GGC GAC GCC ATT CAC GCC
81    Leu Ala Gly Asp Asp Val Arg Leu Thr Trp His Val Asp Asp Gly Asp Ala Ile His Ala

673   AAC CAA ACA GTG TTT GAA CTG AAC GGC CCG GCT CGC GTA TTG CTG ACC GGC GAG CGC ACG
101   Asn Gln Thr Val Phe Glu Leu Asn Gly Pro Ala Arg Val Leu Leu Thr Gly Glu Arg Thr

733   GCG CTA AAC TTT GTC CAG ACC CTT TCC GGC GTC GCC AGT GAA GTA CGC CGC TAC GTT GGA
121   Ala Val Asn Phe Val Gln Thr Leu Ser Gly Val Ala Ser Glu Val Arg Arg Tyr Val Gly

793   CTG TTG GCT GGC ACC CAA ACC CAG TTG CTC GAC ACG CGT AAA ACG CTG CCG GGT CTG CGC
141   Leu Leu Ala Gly Thr Gln Thr Gln Leu Leu Asp Thr Arg Lys Thr Leu Pro Gly Leu Arg

853   ACC GCG CTC AAA TAT GCG GTT TTA TGC GGC GGC GGC GCC AAT CAT CGT CTG GGC CTC ACT
161   Thr Ala Leu Lys Tyr Ala Val Leu Cys Gly Gly Gly Ala Asn His Arg Leu Gly Leu Thr

913   GAC GCG TTC CTG ATT AAA GAA AAC CAT ATT ATC GCC TCC GGT TCG GTT CGT CAG GCG GTG
181   Asp Ala Phe Leu Ile Lys Glu Asn His Ile Ile Ala Ser Gly Ser Val Arg Gln Ala Val

973   GAA AAA GCG TTC TGG CTA CAT CCG GAC GTG CCG GTA GAA GTC GAG GTC GAA AAT CTG GAT
201   Glu Lys Ala Phe Trp Leu His Pro Asp Val Pro Val Glu Val Glu Val Glu Asn Leu Asp

1033  GAA CTG GAC GAT GCG CTG AAA GCA GGC GCG GAT ATT ATC ATG CTG GAT AAT TTC AAC ACC
221   Glu Leu Asp Asp Ala Leu Lys Ala Gly Ala Asp Ile Ile Met Leu Asp Asn Phe Asn Thr

1093  GAC CAG ATG CGC GAG GCG GTG AAA CGC GTC AAC GGC CAG GCG CGG CTG GAA GTA TCC GGC
241   Asp Gln Met Arg Glu Ala Val Lys Arg Val Asn Gly Gln Ala Arg Leu Glu Val Ser Gly

1153  AAC GTC ACC GCG GAA ACC TTA CGC GAA TTT GCT GAA ACC GGG GTG GAT TTC ATC TCC GTT
261   Asn Val Thr Ala Glu Thr Leu Arg Glu Phe Ala Glu Thr Gly Val Asp Phe Ile Ser Val

1213  GGC GCG CTG ACC AAG CAC GTG CGC GCG CTC GAT CTC TCC ATG CGT TTT TGC TGA
281   Gly Ala Leu Thr Lys His Val Arg Ala Leu Asp Leu Ser Met Arg Phe Cys STOP

1267  TCCTCTCCAC CCGATGGCGC TGCGCCGTCG GGTCTATAAA TCACCTGTCC GTAGATCCGC GTCATTATTC
1337  GGTAAAATAT TCGTAGCGCC TCGCAATAAC GTTGCCGTGT CATGTAACGC AGCGAAAATC TTCAGAA
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FIG. 3. DNA sequence of the *nadC* gene. The predicted amino acid sequence is also depicted. Underlined amino acids are sequenced tryptic fragments from purified QAPRTase.

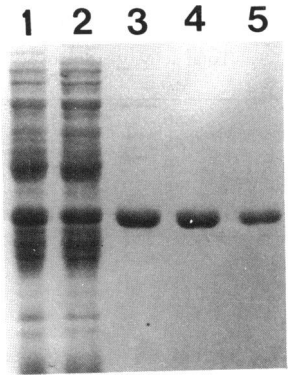

FIG. 4. SDS-PAGE (10% polyacrylamide) of QAPRTase. Lanes: 1, crude cell extract; 2, sample after treatment with polyethyleneimine and $(NH_4)_2SO_4$; 3, QAPRTase fraction after the first ion-exchange chromatographic step; 4, QAPRTase fraction after the second ion-exchange chromatographic step; 5, QAPRTase after selective precipitation with $(NH_4)_2SO_4$.

kb of P22 sequence and 100 kb of adjacent chromosomal DNA. Since MudP and MudQ package in opposite directions, the chromosomal DNA flanking either side of the original Mud-*lac* insert is obtained by using both vectors.

P22 lysates were prepared from each *nadC*::MudP and *nadC*::MudQ insertion. *Xba*I cuts very infrequently in the *S. typhimurium* chromosome, and Mud-P22 introduces new *Xba*I sites (20). Digestion of the DNA with either *Xba*I and *Sac*I or *Xba*I and *Pst*I yielded fragments containing the region including the left or right end of Mu and one end of the *nadC* gene. These fragments were subcloned into the Bluescript plasmid vector and sequenced with either a primer directed to the MuL end, for fragments originating from MudP inserts, or a primer directed to the MuR end, for fragments originating from MudQ inserts. To further verify that the sequence of the *nadC* gene determined with Mud-P22-derived chromosomal DNA was correct, the DNA sequence of the *nadC* gene in pKH56 was also determined (see Materials and Methods). Recently, we have found that the chromosomal DNA isolated from the Mud-P22-induced lysates can be sequenced directly following treatment with T7 gene 6 exonuclease (9a). This eliminates the need for plasmids and subcloning prior to DNA sequence determination.

The results of the DNA sequence analysis are shown in Fig. 3. An open reading frame which could encode either a 311-amino-acid protein beginning at nucleotide 331 or a 297-amino-acid protein beginning at nucleotide 373 because of two possible methionine initiator codons present was observed. An in-frame TGA stop codon occurs at nucleotide 1264. This open reading frame corresponds to the correct transcriptional orientation determined earlier with Mud-*lac* fusions (18). The N-terminal peptide sequence of purified QAPRTase and the molecular weight of purified QAPRTase determined by electrospray mass spectroscopy (described below) show that QAPRTase translation is initiated from the second methionine codon, resulting in a protein 297 amino acids in length. The DNA sequence of *nadC* in pKH56 was found to be identical to that found with Mud-P22-derived chromosomal DNA.

A putative Shine-Dalgarno sequence, GGAG, is found 7 nucleotides 5′ of the second methionine codon. A putative site for RNA transcription can be identified by the sequence (nucleotide [nt] 320) TTCAGAG–16-nt–TATGAT, which provides a good match to the consensus sequence TTGACAT–17-nt–TATAAT (22). No evidence of a *rho*-independent terminator was observed in the sequenced DNA. Previous work has suggested that the sequence element TAAACAA and its inverted repeat, TTGTTTA, may be sites for action of the NadI repressor (35). These sequence elements were not found upstream of the *nadC* gene, in keeping with the previous observation that *nadC* is not under the control of NadI (11).

Overproduction and purification of QAPRTase. Digestion of pKH56 with *Sph*I and *Pst*I followed by ligation of the 2.3-kbp fragment into the T7 promoter vector pSP73 (Promega) previously treated with the same enzymes yielded plasmid pAD01. When harbored in BL21(DE3) (33), pAD01 gave rise to extracts containing QAPRTase activity, which was not detected in extracts of untransformed cells or in transformed $OmpT^+$ cells. From these extracts, QAPRTase was purified to homogeneity by $(NH_4)_2SO_4$ fractionation and ion-exchange chromatography, as described in Materials and Methods (Fig. 4 and Table 2). The method yielded about 40 mg of enzyme.

The purified enzyme migrated on SDS gels with a mobility very similar to that of lactate dehydrogenase from rabbit muscle (M_r, 35,000). On a calibrated column of Sephacryl HR-200, QAPRTase migrated at an apparent M_r of 72,000, which is consistent with a dimeric aggregation state.

Initial velocity kinetics for QAPRTase indicated a V_{max} of 0.9 U/mg and K_ms for QA and PRPP of 19.6 and 32.2 μM,

TABLE 2. Purification of QAPRTase

Step	Total activity (U)	Recovery (%)	Protein (mg)	Sp act (U/mg)
Crude extract	56	100	900	0.06
PEI[a] treatment	45	80	660	0.07
$(NH_4)_2SO_4$ (35 to 65%)	38	68	100	0.37
First ion exchange	25	44	46	0.53
Second ion exchange	35	62	38	0.90

[a] PEI, polyethyleneimine.

respectively (Fig. 5). The purified enzyme did not demonstrate NAPRTase activity (data not shown).

The preparation of tryptic peptides (see Materials and Methods) gave the additional protein sequence data shown in Fig. 3 and helped confirm the DNA sequence.

The purified QAPRTase did not give an amino-terminal sequence when applied directly to the protein disk of the Porton microsequencer, suggesting a blocked amino terminal. However, pretreatment of the protein on a C4 column (see Materials and Methods) resolved two peaks of nearly equal sizes; the first eluting peak gave the amino-terminal sequence H_2N-Pro-Pro-Arg-Arg-Tyr-. The other peak still did not give peptide that could be sequenced. It is possible that the treatment prior to analysis by C4 column chromatography generated the peptide which could be sequenced by releasing the amino-terminal blockage on part of the enzymatic preparation. No enzymatic characterization of these two forms was undertaken.

After resolution of the two QAPRTase forms via C4 chromatography, each QAPRTase peak was analyzed by electrospray mass spectroscopy (32), and the results are shown in Fig. 6. The mass obtained for the first eluting peak corresponded to 32,501 atomic mass units (amu), while that for the second peak was 32,581 amu. This represents a difference of 80 amu between the two forms of the protein. The M_r predicted from the translated DNA sequence which lacked the amino-terminal Met residue is 32,428, 75 amu smaller than the less massive form. It is not clear whether the apparent difference in molecular weight between the two forms has any functional significance.

DISCUSSION

The DNA sequence of the *nadC* gene of *S. typhimurium* was determined with Mud-P22 insertions. Within the DNA sequence of *nadC* lies an open reading frame 297 amino acids in length which corresponds to nucleotides 373 through 1266 in Fig. 3. We believe this to be the correct amino acid sequence for *nadC* by the following criteria. All of the Mud-P22 insertions which result in a NadC$^-$ phenotype lie within this region. Amino acid sequences of tryptic peptides prepared on purified QAPRTase were found in the predicted amino acid sequence. The N-terminal amino acid sequence from purified QAPRTase identified by Edman degradation is identical to the amino terminus predicted from the *nadC* sequence. Also present within the sequence are presumptive promoter and ribosome binding sites. No *rho*-dependent terminator site was located. We also did not detect a NadI repressor binding site. This was not surprising, since the NadI repressor is known to regulate *nadA* and *nadB* gene expression and not *nadC* gene expression (11).

Under nondenaturing conditions, the native enzyme exhibited an M_r of 72,000. Under the denaturing conditions of SDS-PAGE, an M_r of 35,000 was observed. These data were consistent with the enzyme having a dimeric aggregation state which differs from that of hog liver protein (hexameric) (34) and the pseudomonad enzyme (trimeric) (24). The existence of this dimeric state of the *S. typhimurium* QAPRTase enzyme is further supported by the demonstration of intragenic complementation between specific *nadC* alleles (18). Together, these results indicate that the dimeric state is the active form of the enzyme.

The final specific activity of 0.9 U/mg for QAPRTase from *S. typhimurium* is considerably higher than that obtained by Gholson et al. (9) for the mammalian liver enzyme (0.08 U/mg) but similar to the value for castor bean endosperm QAPRTase (0.75 U/mg) (21) and that for the pseudomonad enzyme (0.88 U/mg) (24). The K_m values for both the mammalian liver protein (60 μM for QA and 50 μM for PRPP) (9) and the pseudomonad enzyme (120 μM for QA and 75 μM for PRPP) (25) are somewhat higher than the values obtained for QAPRTase from *S. typhimurium* (20 μM for QA and 32 μM for PRPP). The significance of this difference is not clear.

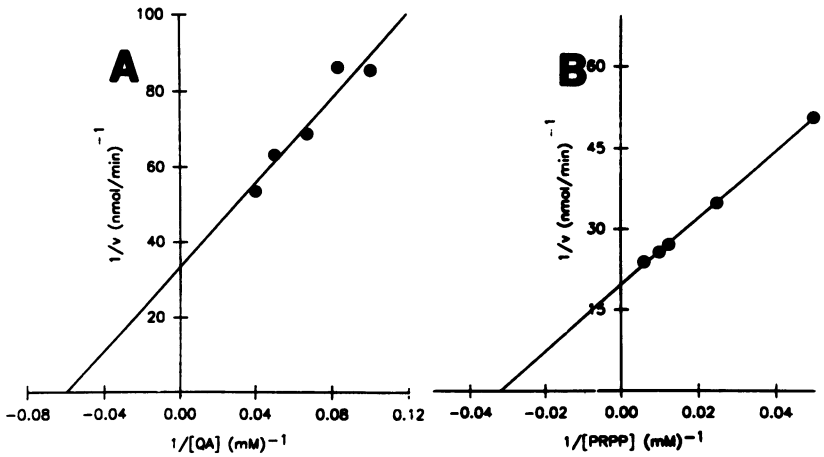

FIG. 5. Double-reciprocal plots of QAPRTase kinetic data. (A) Determination of K_m for PRPP. The PRPP concentrations used were 20, 40, 80, 100, and 150 μM. The QA concentration was maintained at 0.8 mM. The K_m was 32 ± 8 μM. (B) Determination of K_m for QA. The QA concentrations used were 10, 12, 15, 20, and 25 μM. The PRPP concentration was maintained at 1 mM. The K_m was 20 ± 6 μM.

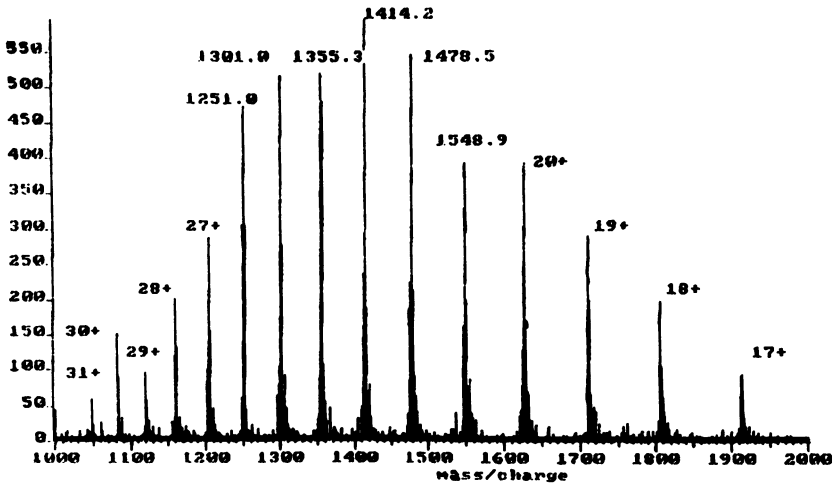

FIG. 6. Electrospray ionization mass spectrometry profile of the first eluting peak from C4 chromatography of QAPRTase. The *y* axis shows intensity in arbitrary units.

The amino acid sequence of QAPRTase does not appear to resemble the NAPRTase amino acid sequences recently reported (35, 36). This was surprising because of the strong similarity between and the identical product of the enzymatic reactions performed by these two enzymes. Only a small stretch of similarity between the two enzymes was found. This includes the region from amino acids 13 through 48 of QAPRTase and the region from amino acids 264 through 298 of NAPRTase (Fig. 7). It is not obvious that this poor similarity represents an evolutionary conservation. The extensive dissimilarity suggests the surprising conclusion that these two enzymes did not evolve from the same precursor. In addition, neither NAPRTase nor QAPRTase shows a consensus PRPP-binding site as identified in PRPP synthetase and several other PRTases (13).

The amino acid sequence of QAPRTase was also compared with those of other proteins by using data bases. The C-terminal 40 amino acids of QAPRTase are highly similar to an open reading frame predicted from the DNA sequence flanking the 3′ end of the *Pseudomonas aeruginosa* pilin gene (Fig. 7) (19, 30). A chromosomal insertion of transposon Tn*5* which maps to the 3′ end flanking the *Pseudomonas* pilin gene was obtained (23a). This insertion mutant was found to be auxotrophic for nicotinic acid and could not use quinolinic acid as an alternative pyridine source (13a). This finding suggests that the *nadC* gene and the pilin-encoding gene are adjacent loci in *P. aeruginosa*.

```
      1         10         20         30         40         50
nadC: MPPRRYNPDDRRDALLERINLDIPAAVAQALREDLGGEVDAGNDITAQLL
                 ** *        *   * ** * *  *  *    *
                 ** *        *   * ** * *  *  *    *
pncB: QLGIALTDCITMDAFLADFGIEF-ASRYQGLRHDSGDPVAWGEKAIAHYE
           260        270        280        290        300

          260        270        280        290
nadC:   VSGNVTAETLREFAETGVDFISVGALTKHVRALDLSMRFC
         **      ***  ****** ** * *** *** *****
         **      ***  ****** ** * *** *** *****
ORF :   ASGGINEGTLRNIAETGVDYISIGTLTKDVRAVDLSMRLT
(P.a.)
```

FIG. 7. Amino acid sequence comparisons. (A) Comparison of protein sequences of *S. typhimurium* QAPRTase (*nadC*) and *S. typhimurium* NAPRTase (*pncB*) (35) reveals a small stretch of similarity of 36 amino acids; 12 between *pncB* and *nadC* are identical (two stars), and another 15 amino acids represent conservative (one star) changes. (B) Comparison of protein sequences of *S. typhimurium* QAPRTase (*nadC*) and an open reading frame (ORF) present at the 3′ end of the *P. aeruginosa* pilin structural gene (30).

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ADDENDUM IN PROOF

An open reading frame at the 5′ end of the *E. coli ampD* gene was reported to possibly be the 5′ end of the *E. coli nadC* gene (S. Lindquist, M. Galleni, F. Lindberg, and S. Normark, Mol. Microbiol. **3**:1091–1102, 1989). Indeed, this open reading frame has an 81% level of identify to the first 115 amino acids of the *S. typhimurium nadC* sequence reported here. Also, an open reading frame divergent to the 5′ end of the *S. typhimurium nadC* gene shares a 93% level of identity with the first 95 amino acids of the *E. coli ampD* gene. These results suggest that the *nadC* and *ampD* genes are adjacent loci in both *E. coli* and *S. typhimurium* and that they are divergently transcribed.

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