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Escherichia coli phnN, Encoding Ribose 1,5-Bisphosphokinase Activity (Phosphoribosyl Diphosphate Forming): Dual Role in Phosphonate Degradation and NAD Biosynthesis Pathways

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An enzymatic pathway for synthesis of 5-phospho-D-ribosyl α-1-diphosphate (PRPP) without the participation of PRPP synthase was analyzed in *Escherichia coli*. This pathway was revealed by selection for suppression of the NAD requirement of strains with a deletion of the *prs* gene, the gene encoding PRPP synthase (B. Hove-Jensen, J. Bacteriol. 178:714-722, 1996). The new pathway requires three enzymes: phosphopentomutase, ribose 1-phosphokinase, and ribose 1,5-bisphosphokinase. The latter activity is encoded by *phnN*; the product of this gene is required for phosphonate degradation, but its enzymatic activity has not been determined previously. The reaction sequence is ribose 5-phosphate → ribose 1-phosphate → ribose 1,5-bisphosphate → PRPP. Alternatively, the synthesis of ribose 1-phosphate in the first step, catalyzed by phosphopentomutase, can proceed via phosphorolysis of a nucleoside, as follows: guanosine + P_i → guanine + ribose 1-phosphate. The ribose 1,5-bisphosphokinase-catalyzed phosphorylation of ribose 1,5-bisphosphate is a novel reaction and represents the first assignment of a specific chemical reaction to a polypeptide required for cleavage of a carbon-phosphorus (C—P) bond by a C-P lyase. The *phnN* gene was manipulated in vitro to encode a variant of ribose 1,5-bisphosphokinase with a tail consisting of six histidine residues at the carboxy-terminal end. PhnN was purified almost to homogeneity and characterized. The enzyme accepted ATP but not GTP as a phosphoryl donor, and it used ribose 1,5-bisphosphate but not ribose, ribose 1-phosphate, or ribose 5-phosphate as a phosphoryl acceptor. The identity of the reaction product as PRPP was confirmed by coupling the ribose 1,5-bisphosphokinase activity to the activity of xanthine phosphoribosyltransferase in the presence of xanthine, which resulted in the formation of 5′-XMP, and by cochromatography of the reaction product with authentic PRPP.

NAD biosynthesis in *Escherichia coli* usually proceeds by consumption of 5-phospho-D-ribosyl α-1-diphosphate (PRPP). NAD is synthesized from aspartate and dihydroxyacetone phosphate. A de novo pathway and a number of salvage pathways for the reutilization of nicotinamide mononucleotide and nicotinamide exist, as shown in Fig. 1 (32). Two of the enzymatic reactions, the reactions catalyzed by quinolinate and nicotinate phosphoribosyltransferases, require PRPP. PRPP-less mutants with a deletion of the *prs* gene, encoding PRPP synthase, consequently require NAD or nicotinamide mononucleotide. Δ*prs* strains also require guanosine, uridine, histidine, and tryptophan, which are likewise synthesized with PRPP as an intermediate (14, 15). Nevertheless, mutants that suppress the NAD requirement are easily obtained by selecting for growth of Δ*prs* cells on medium lacking NAD. These mutants still require guanosine, uridine, histidine, and tryptophan. All such NAD-suppressed mutants were previously shown to have lesions in the *pst-phoU* operon (17), which leads to high-level constitutive expression of genes belonging to the phosphate (Pho) regulon (39, 40). Furthermore, *pst*, *phoU*, or *phoR* also suppressed the NAD requirement of Δ*prs* strains. Suppression by *pst*, *phoU*, or *phoR* mutations was eliminated by a *phoB* mutation, while suppression by a *phoR* mutation was also eliminated by a *creC* mutation. These data show that synthesis of a Pho regulon gene product is apparently responsible for suppression of the NAD requirement (17).

We show here that suppression of the NAD requirement requires at least three polypeptides, one of which is specified by the *phnN* gene. The *phnN* gene lies within a 14-cistron operon, *phnCDEFGHIJKLMNOP*, which encodes proteins for uptake, breakdown, and regulation of phosphonate degradation by carbon-phosphorus (C-P) lyase (7, 29, 39). In this paper we describe the first detection of an in vitro activity associated with a component of a C-P lyase.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The *E. coli* K-12 strains used are listed in Table 1. Cells were grown in AB minimal medium (8) or in NZY medium (containing [per liter] 10 g of NZ-amin [Struers, Copenhagen, Denmark], 5 g of yeast extract [Oxoid], and 5 g of NaCl; pH adjusted to 7.5) (18). Carbon sources were used at a concentration of 0.2% (glucose, galactose, melibiose) or 0.1% (thymidine). Thiamine (1 mg/liter) was routinely added to minimal media. The following other compounds were added when necessary: guanosine (30 mg/liter), uridine (20 mg/liter), and amino acids and δ-aminolevulinate (40 mg/liter each). NAD was added to both NZY and AB media at a concentration of 25 mg/liter. Ampicillin was used at a concentration of 25 or 100 mg/liter, whereas kanamycin and tetracycline were used at concentrations of 30 and 10

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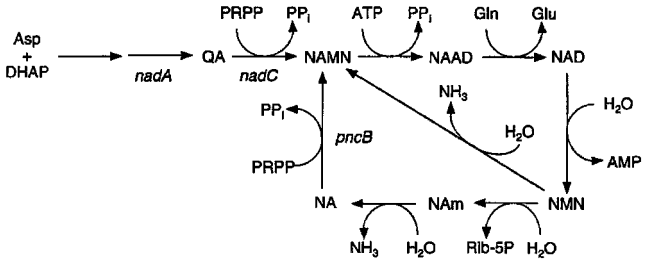

FIG. 1. NAD metabolism of *E. coli*. The de novo and salvage pathways are shown. Abbreviations: Asp, aspartate; DHAP, dihydroxyacetone phosphate; Gln, glutamine; Glu, glutamate; NA, nicotinate; NAm, nicotinamide; NAMN, nicotinate mononucleotide; NAAD; nicotinate adenine dinucleotide; NMN, nicotinamide mononucleotide; QA, quinolinate; Rib-5P, ribose 5-phosphate. Relevant enzyme-catalyzed reactions are indicated by gene designations (*nadA*, quinolinate synthase; *nadC*, quinolinate phosphoribosyltransferase; *pncB*, nicotinate phosphoribosyltransferase).

mg/liter, respectively. Cell growth was monitored by determining the optical density at 436 nm (OD_{436}) with an Eppendorf PCP6121 spectrophotometer. An OD_{436} of 1 (path length, 1 cm) corresponds to approximately 3×10^{11} cells per liter.

Genetic techniques. Previously described procedures were used for transduction (30) and transformation (26). Gene conversion by homologous recombination was performed by using a *recD* strain (35). Integration of pAH126 at *att*λ was performed as described elsewhere (10).

DNA technology and plasmids. Plasmid DNA was isolated by the procedure of Birnboim and Doly (4) or by commercial procedures (Qiagen). Restriction and ligation of DNA were performed as described by the vendors of restriction endonucleases (Amersham, Promega, and Roche) or T4 DNA ligase (Amersham). The plasmids containing *deo* cistrons were constructed as follows. Plasmid pBR*deo*, provided by J. Neuhard (University of Copenhagen), consists of the entire *deo* operon (*deoC*$^+$ *deoA*$^+$ *deoB*$^+$ *deoD*$^+$) as a 5,660-bp *Eco*RI-*Pvu*II-digested DNA fragment ligated to similarly digested DNA of pBR322 (5). To delete *deoD*, DNA of pBR*deo* was digested by restriction endonucleases *Pvu*II and *Hpa*I, and this was followed by ligation, resulting in pHO365 (*deoC*$^+$ *deoA*$^+$ *deoB*$^+$). To delete *deoB* and *deoD*, DNA of pBR*deo* was digested by restriction endonucleases *Pvu*II and *Hin*dIII, and this was followed by S1 nuclease treatment and ligation, resulting in pHO366 (*deoC*$^+$ *deoA*$^+$). To delete *deoA*, *deoB*, and *deoD*, DNA of pBR*deo* was digested by restriction endonucleases *Pvu*II and *Nco*I, and this was followed by S1 nuclease treatment and ligation, resulting in pHO367 (*deoC*$^+$). The *deo* operon is expressed from the *deoP2* promoter in these plasmids (37). To construct a plasmid containing only *deoB*, DNA of pBR*deo* was digested by restriction endonuclease *Bcl*I. This treatment liberated a 1,551-bp DNA fragment containing *deoB*, as well as four other DNA fragments. This DNA was ligated to *Bam*HI-digested DNA of pBR322. Recombinant plasmids containing *deoB*$^+$ were obtained by transformation of the ligated DNA to strain HO1077 (*deoB*) and selection for *deoB*$^+$ on medium with thymidine as the carbon source. The resulting plasmid, which presumably expressed *deoB* from the *tet* promoter, was pHO368 (*deoB*$^+$). To replace *deoB* and *deoD* with a kanamycin resistance-encoding DNA fragment, DNA of pBR*deo* was digested by restriction endonuclease *Hin*dIII, and this was followed by S1 nuclease treatment and digestion with *Hpa*I. This DNA was ligated to DNA of pUC4-K (44) previously digested with *Hin*dII, which liberated the kanamycin resistance-encoding DNA fragment, as well as two other DNA fragments. The desired plasmid was obtained by transformation of the ligated DNA to strain HO1183 (*deoA*) and selection for *deoA*$^+$ on medium containing kanamycin and thymidine as the carbon source. This procedure resulted in pHO370 (*deoC*$^+$ *deoA*$^+$ Δ*deoBD*::Kan^r). This plasmid contained approximately 1,000 bp downstream of the *Hpa*I site to allow for homologous recombination.

Plasmids containing *phnN* were constructed as follows. Wild-type *phnN* was PCR amplified by using the oligodeoxyribonucleotides 5′-GAAGATCTCAT-ATGATGGGAAAAACTGATTTGG and 5′-GCGAGCTCT-ACAAGCAGGCATGGTGTTTC as the primers, DNA of pBW120 (41) as the template, the four deoxyribonucleotides, and Vent DNA polymerase (New England Biolabs) (the nucleotides preceding hyphens indicate noncomplementary extensions). The PCR product was cloned into pSK50-Δ*uidA2* (9) by using *Nde*I and *Sac*I, resulting in pCP-*phnN*. The insert was verified by automated DNA sequencing of both strands. The *phnN* gene was then subcloned into the conditional-replication and modular plasmid pCAH56 (10) by using *Nde*I and *Nhe*I, resulting in pAH126. This plasmid contained *phnN* under control of P_{tac} and could be readily integrated into the chromosome. In addition, *phnN* was amplified with the oligodeoxyribonucleotides 5′-GAGAATTCATTAAAGAGGAGAAATTAACT-ATGATGGGAAAACTGATTTGGTTAATGG (phnNupUHE) and 5′-TGGTTGGGATCCCGAGCCATGGTTATTACAAG-CAGGCATGGTGTTTCTCC as the primers, DNA of pAH126 as the template, the four deoxyribonucleotides, and DNA polymerase of *Pyrococcus furiosum* (Gibco-BRL). The resulting DNA was digested by restriction endonucleases *Eco*RI and *Nco*I, ligated to similarly digested DNA of pUHE23-2 (H. Bujard, personal communication), and transformed to strain MC1061, resulting in pHO500. A variant of *phnN*, specifying a polypeptide with a six-histidine tail at the carboxy-terminal end, was constructed by PCR with the oligodeoxyribonucleotides phnNupUHE and 5′-TGGTTGGGATCCCGAGCCATGGTTATTAATGGTGATGGTGA TGGTGCAAG-CAGGCATGGTGTTTCTCC as the primers and DNA of pAH126 as the template, and this was followed by *Eco*RI and *Nco*I digestion, ligation, and transformation as described above for pHO500. The resulting plasmid was designated pTR553. The correct inserts of the plasmids were confirmed by automated nucleotide sequencing. Plasmid pGD248, a derivative of pSU18 (3), harbors a *lac* promoter-expressed *xapAB* operon, which encodes xanthosine phosphorylase and xanthosine permease (G. Dandanell, personal communication).

Expression of recombinant *phnN* and purification of histidine-tailed ribose 1,5-bisphosphokinase. Strain HO1088 (Δ*prs*)/pTR553 (*phnN*) was grown in NZY broth supplemented with ampicillin and tetracycline. At an OD_{436} of approximately 1, isopropyl-1-thio-β-D-galactopyranoside (IPTG) was added at a concentration of 0.5 mM to induce expression of the *phnN* gene. After 2 h of incubation cells were harvested by centrifugation, washed with unsupplemented AB minimal medium, and broken by ultrasonic treatment. Debris was removed by centrifugation, and the supernatant fluid was applied to a Co^{2+}-containing Talon metal affinity resin. After the column was washed, ribose 1,5-bisphosphokinase was eluted with imidazole as described by the resin supplier (Clontech).

Enzyme assays. The activity of ribose 1,5-bisphosphokinase was assayed as follows. A 100-μl reaction mixture contained 50 mM Tris-HCl (pH 7.5), 0.6 mM ribose 1,5-bisphosphate, 1 mM (120-GBq/mol) [γ-^{32}P]ATP, and 2 mM magnesium chloride. The reaction was initiated by the addition of ribose 1,5-bisphosphokinase (0.8 nmol/min). Samples (10 μl) were removed after 1, 3, and 10 min of incubation at 30°C and mixed with 5 μl of 0.33 M formic acid. This 15 μl was applied to polyethyleneimine-coated cellulose thin-layer chromatographic plates (Baker-flex; J. T. Baker). After drying, the chromatogram was developed in 0.85 M KH_2PO_4 which had been previously adjusted to pH 3.4 with 0.85 M H_3PO_4 (20). Radioactivity was quantitated with an Instant Imager (Packard). Occasionally, 1 mM (120-GBq/mol) [α-^{32}P]ATP or 1 mM (120-GBq/mol) [α-^{32}P]GTP was used instead of [γ-^{32}P]ATP. Production of PRPP with purified *E. coli* PRPP synthase was performed as previously described (42). When unlabeled ribose 1,5-bisphosphokinase reaction product or PRPP synthase reaction product was synthesized, the same procedures were employed, except that the labeled nucleotide was omitted, and heating at 90°C for 1 min terminated the reaction. Consumption of labeled ribose 1,5-bisphosphokinase reaction product or PRPP synthase reaction product by xanthine phosphoribosyltransferase (XPRTase) was performed as follows. A 50-μl reaction mixture contained 40 mM Tris-HCl (pH 7.5), 0.1 mM xanthine, ^{32}P-labeled ribose 1,5-bisphosphokinase reaction product or ^{32}P-labeled PRPP (approximately 0.1 kBq), 10 mM magnesium chloride, and XPRTase (30 nmol/min). A 10-μl sample was removed after 10 min of incubation at 37°C, mixed with formic acid, and examined by thin-layer chromatography as described above. The formation of 5′-XMP with unlabeled ribose 1,5-bisphosphokinase reaction product or PRPP synthase reaction product was assayed by a similar procedure. A 50-μl reaction mixture contained 40 mM Tris-HCl (pH 7.5), 0.1 mM (185-GBq/mol) xanthine, unlabeled ribose 1,5-bisphosphokinase reaction product or PRPP synthase reaction product (the same volume as described above), 10 mM magnesium chloride, and XPRTase (30 nmol/min). A 10-μl sample was removed after 10 min of incubation at 37°C and applied to a polyethyleneimine thin-layer plate. After drying, the chromatogram was developed in methanol (2 cm), followed by 1 M acetic acid (2 cm) and 0.9 M acetic acid–0.3 M lithium chloride (16 cm) (16, 34). Protein content was determined as previously described (23).

Materials. [α-^{32}P]ATP (15 TBq/mmol) and [α-^{32}P]GTP (110 TBq/mmol) were obtained from Amersham-Pharmacia Biotech. [γ-^{32}P]ATP was prepared as previously described (20). [8-^{14}C]xanthine was obtained from New England Nuclear. Unlabeled nucleotides were obtained from Roche. Xanthine, adenine, ribose, ribose 1-phosphate, ribose 5-phosphate, and PRPP were obtained from Sigma. Ribose 1,5-bisphosphate was supplied by H. Klenow (University of Copenhagen)

TABLE 1. Bacterial strains

Strain	Genotype	Source, reference, or construction
BW12057	*araD* Δ(*lac*)*U169 rpsL aroB crp creC thi psiD31*::*lacZ* (Mu*d*I-1734)	Laboratory strain
BW14001	Δ(*lac*)χ*74* Δ*phoA532* Δ(*mel-proP-phnCDEFGHIJKLMNOP*)*2*::Tn*5*seq1/*132*(*tet*)	28
BW14894	Δ(*lac*)χ*74* Δ(*phnC?DEFGHIJKLMNOP*)*33-30*	43
BW15089	Δ(*lac*)χ*74 robA creC* Δ(*gal-att*λ*-bio*)*76 zbh-283*::Tn*10*	Laboratory strain
BW17471	Δ(*lac*)χ*74* Δ*phoA532 phnM28*::Tn*phoA′-1 phn-10*::*uidA2-aadA*	Laboratory strain
BW17562	Δ(*lac*)χ*74* Δ*phoA532 phnF23*::Tn*phoA′-9 phn-10*::*uidA2-aadA*	Laboratory strain
BW18524	Δ(*lac*)χ*74* Δ*phoA532 phnCp*Δ(*phnCDE*)*59*	Laboratory strain
BW20274	Δ(*lac*)χ*74* Δ*phoA532 phnN45*::Tn*phoA′-3 phn-10*::*uidA2-aadA*	Laboratory strain
BW24388	Δ(*lac*)χ*74* Δ(*phnC?DEFGHIJKLMNOP*)*33-30 att*λ::pCAH56	Laboratory strain
BW24389	Δ(*lac*)χ*74* Δ(*phnC?DEFGHIJKLMNOP*)*33-30 att*λ::pAH126 (P_{tac}-*phnN*)	Laboratory strain
CAG5051	*thi relA spoT supQ nadA*::Tn*10*	C. Gross[b]
CAG18430	*zjj-202*::Tn*10*	C. Gross[b]
HO340	—[a]	31
HO402	*metB zcg-2402*::Tn*10*	13
HO650	*supF relA spoT rpsL lamB metB deoD udp gsk-3 pncA hemA*	15
HO677	*zcg*::Tn*10 supF relA spoT rpsL lamB metB deoD udp gsk-3 pncA hemA*	P1(HO402) × HO650, Tet^r
HO770	*deoD gsk-3 udp*[a]	33
HO773	*deoD gsk-3 udp* Δ(*prs-4*)::Kan^r[a]	33
HO861	*deoD gsk-3 udp* Δ(*prs-4*)::Kan^r (*pst-phoU*)*1001*[a]	17
HO878	*deoD gsk-3 udp* Δ(*prs-4*)::Kan^r (*pst-phoU*)*1001 nadA*::Tn*10*[a]	17
HO959	*zjj-202*::Tn*10* Δ(*deoCABD*) Δ*lac thi upp udp ton*	P1(CAG18430) × SØ928, Tet^r
HO965	*zjj-202*::Tn*10* Δ(*deoCABD*) *gsk-3 udp* Δ(*prs-4*)::Kan^r[a]	P1(HO959) × HO773, Tet^r
HO967	*zjj-202*::Tn*10* Δ(*deoCABD*) *gsk-3 udp* Δ(*prs-4*)::Kan^r (*pst-phoU*)*1001*[a]	P1(HO959) × HO861, Tet^r
HO1077	*supF relA spoT rpsL lamB metB guaA deoD deoB thyA zjj-202::*Tn*10*	P1(CAG18430) × SØ489, Tet^r
HO1088	*deoD gsk-3 udp* Δ(*prs-4*)::Kan^r/F *zzf*::Tn*10 lacI*^{q}	23
HO1123	*zjj-202*::Tn*10 thi galE* Δ(*att*λ*-bio*) *deoC56 deoB*::λc*I857*	P1(CAG18430) × SØ1055, Tet^r
HO1150	*zjj-202*::Tn*10* Δ(*gal-deoR*) *thi bio upp deoC*$^+$*A*$^+$*B*$^+$ Δ(*deoD-thr*)	P1(CAG18430) × SØ1068, Tet^r
HO1156	*zjj-202*::Tn*10* Δ(*deoD-thr*) *gsk-3 udp* Δ(*prs-4*)::Kan^r[a]	P1(HO1150) × HO861, Tet^r
HO1171	*zjj-202*::Tn*10 thi galE* Δ(*att*λ*-bio*) *deoA103 deoC lysA argA cytR*	P1(CAG18430) × SØ1108, Tet^r
HO1183	*zjj-202*::Tn*10 deoA103 deoD gsk-3 udp*[a]	P1(HO1171) × HO770, Tet^r
HO1187	*zjj-202*::Tn*10 deoA103 deoD gsk-3 udp* Δ(*prs-4*)::Kan^r[a]	P1(HO1183) × HO861, Tet^r
HO1357	*zjj-202*::Tn*10 deoC*[a]	P1(HO1123) × HO340, Tet^r
HO1358	*zjj-202*::Tn*10 deoC deoD gsk-3 udp*[a]	P1(HO1357) × HO770, Tet^r
HO1359	*zjj-202*::Tn*10 deoC deoD gsk-3 udp* Δ(*prs-4*)::Kan^r (*pst-phoU*)*1001*[a]	P1(HO1358) × HO861, Tet^r
HO1403	*hemA zcg*::Tn*10 deoD gsk-3 udp* (*pst-phoU*)*1001*[a]	P1(HO677) × HO861, Tet^r
HO1404	*psiD31*::*lacZ* (Mu*d*I-1734) *hemA zcg*::Tn*10 deoD gsk-3 udp* (*pst-phoU*)*1001*[a]	P1(BW12057) × HO1403, Kan^r
HO1408	*psiD31*::*lacZ* (Mu*d*I-1734) *deoD gsk-3 udp* Δ(*prs-4*)::Kan^r (*pst-phoU*)*1001*[a]	P1(HO773) × HO1404, Hem$^+$
HO1494	Δ(*mel-proP-phnCDEFGHIJKLMNOP*)*2*::Tn*5*seq1/*132*(*tet*) *deoD gsk-3 udp* Δ(*prs-4*)::Kan^r (*pst-phoU*)*1001*[a]	P1(BW14001) × HO861, Tet^r
HO1496	*phnCp*Δ(*phnCDE*)*59 deoD gsk-3 udp* Δ(*prs-4*)::Kan^r (*pst-phoU*)*1001*[a]	P1(BW18524) × HO1494, Mel$^+$
HO1559	*phnM28*::Tn*phoA′-1 phn-10*::*uidA2-aadA deoD gsk-3 udp* Δ(*prs-4*)::Kan^r (*pst-phoU*)*1001*[a]	P1(BW17471) × HO1494, Mel$^+$
HO1565	*phnF23*::Tn*phoA′-9 phn-10*::*uidA2-aadA deoD gsk-3 udp* Δ(*prs-4*)::Kan^r (*pst-phoU*)*1001*[a]	P1(BW17562) × HO1494, Mel$^+$
HO1569	*phnN45*::Tn*phoA′-3 phn-10*::*uidA2-aadA deoD gsk-3 udp* Δ(*prs-4*)::Kan^r (*pst-phoU*)*1001*[a]	P1(BW20274) × HO1494, Mel$^+$
HO1652	Δ(*gal-att*λ*-bio*)*76 zbh-283*::Tn*10 deoD gsk-3 udp* Δ(*prs-4*)::Kan^r[a]	P1(BW15089) × HO773, Tet^r
HO1654	Δ(*phnC?DEFGHIJKLMNOP*)*33-30 deoD gsk-3 udp* Δ(*prs-4*)::Kan^r (*pst-phoU*)*1001*[a]	P1(BW14894) × HO1494, Mel$^+$
HO1655	Δ(*gal-att*λ*-bio*)*76 zbh-283*::Tn*10* Δ(*phnC?DEFGHIJKLMNOP*)*33-30 deoD gsk-3 udp* Δ(*prs-4*)::Kan^r (*pst-phoU*)*1001*[a]	P1(BW15089) × HO1654, Tet^r
HO1877	*att*λ::pCAH56 Δ(*phnC?DEFGHIJKLMNOP*)*33-30 deoD gsk-3 udp* Δ(*prs-4*)::Kan^r (*pst-phoU*)*1001*[a]	P1(BW24388) × HO1655, Gal$^+$
HO1878	*att*λ::pAH126 (P_{tac}-*phnN*) Δ(*phnC?DEFGHIJKLMNOP*)*33-30 deoD gsk-3 udp* Δ(*prs-4*)::Kan^r (*pst-phoU*)*1001*[a]	P1(BW24389) × HO1655, Gal$^+$
HO1879	*att*λ::pCAH56 *deoD gsk-3 udp* Δ(*prs-4*)::Kan^r[a]	P1(BW24388) × HO1652, Gal$^+$
HO1902	*att*λ::pAH126 (P_{tac}-*phnN*) *deoD gsk-3 udp* Δ(*prs-4*)::Kan^r	P1(BW24389) × HO1652, Gal$^+$
HO1908	*nadA*::Tn*10 att*λ::pAH126 (P_{tac}-*phnN*) Δ(*phnC?DEFGHIJKLMNOP*)*33-30 deoD gsk-3 udp* Δ(*prs-4*)::Kan^r (*pst-phoU*)*1001*[a]	P1(CAG5051) × HO1878, Tet^r
HO1909	*nadA*::Tn*10 att*λ::pAH126 (P_{tac}-*phnN*) *deoD gsk-3 udp* Δ(*prs-4*)::Kan^r[a]	P1(CAG5051) × HO1902, Tet^r
HO1910	*zjj-202*::Tn*10* Δ(*deoCABD*) *att*λ::pAH126 (P_{tac}-*phnN*) Δ(*phnC?DEFGHIJKLMNOP*)*3-30 gsk-3 udp* Δ(*prs-4*)::Kan^r (*pst-phoU*)*1001*[a]	P1(HO959) × HO1878, Tet^r
HO1911	*zjj-202*::Tn*10* Δ(*deoCABD*) *att*λ::pAH126 (P_{tac}-*phnN*) *gsk-3 udp* Δ(*prs-4*)::Kan^r[a]	P1(HO959) × HO1902, Tet^r
HO1935	Δ(*deoBD*)::Kan^r *recD1903*::mini-Tet Δ(*lac*)χ*74* Δ*phoA phnCp*(*phnCDE*)*59*	Homologous recombination with linearized DNA of pHO370, Kan^r, via TP1904
HO1938	Δ(*deoBD*)::Kan^r *hemA zcg*::Tn*10 gsk-3 udp* (*pst-phoU*)*1001*[a]	P1(HO1935) × HO1403, Kan^r
HO1949	Δ(*prs-4*)::Kan^r Δ(*deoBD*)::Kan^r *gsk-3 udp* (*pst-phoU*)*1001*[a]	P1 (HO773) × HO1938, Hem$^+$
MC1061	*araD139* Δ(*ara-leu*) Δ(*lac*)χ*74 galE15 galK16 rpsL*	6
SØ489	*supF relA spoT rpsL lamB metB guaA deoD deoB thyA*	P. Nygaard, University of Copenhagen
SØ928	Δ(*deoCABD*) Δ*lac thi upp udp ton*	K. Hammer[c]
SØ1055	*thi galE* Δ(*att*λ*-bio*) *deoC56 deoB*::λc*I857*	K. Hammer, Danish Technical University
SØ1068	Δ(*gal-deoR*) *thi bio upp deoC*$^+$*A*$^+$*B*$^+$ Δ(*deoD-thr*)	K. Hammer, Danish Technical University
SØ1108	*thi galE* Δ(*att*λ*-bio*) *deoA103 deoC lysA argA cytR*	K. Hammer, Danish Technical University
TP1904	*recD1903*::mini-Tet Δ(*lac*)χ*74* Δ*phoA phnCp*(*phnCDE*)*59*	T. Poulsen, University of Copenhagen

[a] The strain also contains *araC*(Am) *araD* Δ(*lac*)*U169 trp*(Am) *mal*(Am) *rpsL relA thi supF*.
[b] See reference 36.
[c] See reference 38.

TABLE 2. Effect of *deo* alleles on NAD suppression phenotype[a]

Strain	Genotype and/or plasmid	Pyridine source	
		None	NAD
HO773	Δ*prs deoD*	−	+
HO861	Δ*prs pst-phoU deoD*	+	+
HO967	Δ*prs pst-phoU* Δ(*deoCABD*)	−	+
HO1156	Δ*prs pst-phoU* Δ(*deoD-thr*)	+	+
HO1359	Δ*prs pst-phoU deoD deoC56*	+	+
HO1187	Δ*prs pst-phoU deoD deoA103*	+	+
HO1949	Δ*prs pst-phoU* Δ(*deoBD*)	−	+
HO967	pHO365 (*deoC*$^+$ *A*$^+$ *B*$^+$ *bla*$^+$)	+	+
HO967	pHO366 (*deoC*$^+$ *A*$^+$ *bla*$^+$)	+	+
HO967	pHO367 (*deoC*$^+$ *bla*$^+$)	−	+
HO967	pHO368 (*deoB*$^+$ *bla*$^+$)	+	+
HO967	pBR322 (*bla*$^+$)	−	+
HO967	pGD248 (*xapA*$^+$*B*$^+$ *cat*$^+$)	+	+
HO967	pSU18 (*cat*$^+$)	−	+
HO773	pHO368 (*deoB*$^+$ *bla*$^+$)	−	+
HO773	pGD248 (*xapA*$^+$*B*$^+$ *cat*$^+$)	−	+

[a] Cells were grown at 32°C in glucose minimal medium supplemented with guanosine, uridine, histidine, tryptophan, and NAD as indicated. Plasmid-containing strains were grown with ampicillin or chloramphenicol present, +, growth; −, no growth.

(22). Purified recombinant *Bacillus subtilis* XPRTase was supplied by K. F. Jensen (University of Copenhagen).

RESULTS

Genes necessary for the NAD suppression phenotype. It has been shown previously that the NAD suppression phenotype requires a wild-type allele of the *nadC* gene, which encodes quinolinate phosphoribosyltransferase. Similarly, utilization of nicotinate by cells with the NAD suppression phenotype requires a wild-type allele of the *pncB* gene, which encodes nicotinate phosphoribosyltransferase (17).

Involvement of *deoB*. In general, conversion of purine or pyrimidine bases to the corresponding ribonucleoside 5′-monophosphates may occur by either of two pathways: (i) by phosphoribosylation or (ii) by ribosylation followed by phosphorylation. The former reaction, catalyzed by base-specific phosphoribosyltransferases, requires PRPP. The latter reaction occurs, with guanine as an example, as follows: guanine + ribose 1-phosphate → guanosine + P_i. The latter reaction is catalyzed by purine nucleoside phosphorylase. In a second reaction, guanosine is phosphorylated to GMP by guanosine kinase. Ribose 1-phosphate may be generated from ribose 5-phosphate in an isomerization reaction catalyzed by phosphoribomutase, which is encoded by the *deoB* gene. To test if the NAD suppression phenotype requires phosphoribomutase, several mutant strains and plasmids were constructed (Table 2). First, removal of the entire *deo* operon resulted in generation of an NAD requirement (compare strains HO861 and HO967). Although the extent of the *deo* deletion is unknown, introduction of a Δ(*deoD-thr*) allele (strain HO1156), a *deoC* allele (HO1359), or a *deoA* allele (HO1187) had no effect on the NAD phenotype, as these strains, like HO861, were NAD prototrophs. In contrast, the *deoB* strain HO1949 was an NAD auxotroph, indicating that *deoB* is required for the NAD suppression phenotype. Second, plasmids bearing various *deo* cistrons were introduced into strain HO967 [Δ*deo*(*CABD*)]. These results showed that either *deoB* or *deoA* restored the NAD suppression phenotype to strain HO967 (compare strains HO967, HO967/pHO365, HO967/pHO366, and HO967/pHO368). The phosphoribomutase (*deoB*) may provide ribose 1-phosphate by isomerization of ribose 5-phosphate. Thymidine phosphorylase (*deoA*) may also provide ribose 1-phosphate, although by a different mechanism, namely, by phosphorolysis of uridine: uridine + P_i → uracil + ribose 1-phosphate. It has been shown that thymidine phosphorylase has specificity for uridine (11). Finally, we showed that xanthosine phosphorylase (*xapA*) was able to restore the NAD suppression phenotype (compare strains HO967/pSU18 and HO967/pGD248). Xanthosine phosphorylase has specificity for guanosine, as well as for xanthosine, and it catalyzes the following reaction: guanosine + P_i → guanine + ribose 1-phosphate. Both uridine and guanosine were present in the growth medium, and the strains are wild type for *deoA* and *xapA* in the chromosome. However, apparently due to the low affinity of thymidine phosphorylase towards uridine, the *deoA* gene must be present in a high-copy-number plasmid to have an effect on the NAD suppression phenotype. While xanthosine phosphorylase is normally synthesized only in the presence of xanthosine (12), *xapA* gene expression is independent of xanthosine in pGD248, in which it is behind a *lac* promoter.

Involvement of *phnN*. The NAD suppression phenotype results from Pho regulon-controlling mutations (17). We therefore sought to identify which component of the Pho regulon is responsible for the suppression phenotype. First, several mutant phosphate starvation-inducible (*psi*) alleles were introduced into strain HO861, including *psiC*, *psiD*, *psiE*, and *psiF*

TABLE 3. Effect of *phn* mutations on NAD suppression phenotype[a]

Strain	Genotype	Pyridine source		
		None	Quinolinate or nicotinate	NAD
HO861	Δ*prs pst-phoU*	+	+	+
HO1408	Δ*prs pst-phoU psiD31*	−	NT[b]	+
HO1655	Δ*prs pst-phoU* Δ*phn*	−	−	+
HO1559	Δ*prs pst-phoU phnM28*	−	NT	+
HO1569	Δ*prs pst-phoU phnN45*[c]	−	NT	+
HO1496	Δ*prs pst-phoU* Δ(*phnC-E*)	+	NT	+
HO1565	Δ*prs pst-phoU phnF23*[c]	+	NT	+
HO1877	Δ*prs pst-phoU* Δ*phn att*λ::pCAH56	−	−	+
HO1878	Δ*prs pst-phoU* Δ*phn att*λ::pAH126 (*P*$_{tac}$-*phnN*)	+	+	+
HO1908	Δ*prs pst-phoU* Δ*phn att*λ::pAH126 (*P*$_{tac}$-*phnN*) *nadA*	−	+	+
HO878	Δ*prs pst-phoU nadA*	−	+	+
HO1910	Δ*prs pst-phoU* Δ*phn att*λ::pAH126 (*P*$_{tac}$-*phnN*) Δ*deo*	−	−	+
HO967	Δ*prs pst-phoU* Δ*deo*	−	−	+
HO773	Δ*prs*	−	−	+
HO1879	Δ*prs att*λ::pCAH56	−	−	+
HO1902	Δ*prs att*λ::pAH126 (*P*$_{tac}$-*phnN*)	+	+	+
HO1909	Δ*prs att*λ::pAH126 (*P*$_{tac}$-*phnN*) *nadA*	−	+	+
HO1911	Δ*prs att*λ::pAH126 (*P*$_{tac}$-*phnN*) Δ*deo*	−	−	+
HO965	Δ*prs* Δ*deo*	−	−	+

[a] Cells were grown at 32°C in glucose minimal medium supplemented with guanosine, uridine, histidine, tryptophan, and the pyridine source indicated. Growth was read after 48 h of incubation; +, growth; −, no growth.

[b] NT, not tested.

[c] The *phnF* and *phnN* alleles are nonpolar.

alleles. One of these, *psiD31*, restored the NAD requirement (Table 3). The others had no effect. The *psiD31* allele (also called *phnD31*) is a polar insertion at the *phn* locus (27), encoding enzymes involved in degradation of phosphonates (7, 41). As expected, deletion of the *phn* operon also restored the NAD requirement (strain HO1655). We therefore tested null alleles of every *phn* cistron, most of which are nonpolar (28), in the NAD suppression strain. Introduction of *phnM28* or *phnN45* eliminated suppression; i.e., both the Δ*prs pst-phoU phnM* and Δ*prs pst-phoU phnN* strains were NAD auxotrophs (Table 3). Among the entire collection of *phn* mutations analyzed, the *phnM* mutation was the only polar mutation. We therefore inferred that the effect of *phnM* was due to polarity on *phnN*. Furthermore, a mutation eliminating the phosphonate transport system (Δ*phnCDE*) had no effect on the NAD suppression phenotype (strain HO1496). This was also the case for a mutation in *phnF* (HO1565) (Table 3), mutations in *phnGHIJKL*, and mutations in *phnO* and *phnP* (data not shown).

To show that *phnN* was sufficient for establishing the NAD suppression phenotype, *phnN* was cloned into a plasmid (pAH126), and a single copy of this plasmid was subsequently integrated into the chromosome of the NAD auxotrophic strain HO1655 (Δ*prs pst-phoU* Δ*phn*). Introduction of *phnN* alone (strain HO1878) resulted in NAD prototrophy, whereas the vector had no effect (HO1877). Similarly, insertion of *phnN* into the NAD auxotrophic strain HO773 (Δ*prs* [*pst-phoU*$^+$]) resulted in NAD prototrophy. As expected, introduction of a mutant *nad* allele resulted in a pyridine requirement that could be satisfied by quinolinate or nicotinate in addition to NAD, whereas introduction of a Δ*deo*(*CABD*) allele resulted in a pyridine requirement that could be satisfied only by NAD (Table 3). Finally, a plasmid-harbored *phnN* allele, either a wild-type allele (pHO500) or a mutant allele specifying a histidine-tailed version of PhnN (pTR553), also suppressed the NAD requirement.

We conclude that the NAD suppression phenotype is caused by derepression of expression of the *phn* operon, which results in synthesis of the *phnN* gene product. This gene product, together with quinolinate phosphoribosyltransferase and phosphoribomutase, is necessary for the NAD suppression phenotype. The latter two enzymes were also present in the unsuppressed parental strain.

Reaction catalyzed by the *phnN* gene product. The ribose moiety of ribose 1-phosphate formed by phosphoribomutase eventually ends up as the ribose moiety of nicotinate mononucleotide (NAMN) in reactions which are catalyzed by the *phnN* gene product and by quinolinate phosphoribosyltransferase. One or more additional enzymes might be involved as well. We therefore considered the possibility that *phnN* encodes an enzyme with ribose 1-phosphokinase activity (ribose 1-phosphate + ATP → ribose 1,5-bisphosphate + ADP) or with ribose 1,5-bisphosphokinase activity (ribose 1,5-bisphosphate + ATP → PRPP + ADP). To do this, the *phnN* gene was altered to specify a version with a histidine tail, and this variant protein was purified as described in Materials and Methods. The activity of the enzyme was assayed by incubation with the ribose compounds of interest in the presence of [γ-^{32}P]ATP, followed by separation of the reaction mixture by thin-layer chromatography and finally by identification of novel radioactive compounds. Indeed, incubation of PhnN with [γ-^{32}P]ATP and ribose 1,5-bisphosphate resulted in the formation of a radioactive compound, which comigrated with PRPP in one-dimensional thin-layer chromatography (Fig. 2A). This compound also comigrated with PRPP in two-dimensional thin-layer chromatography (data not shown). In contrast, no novel radioactive compound could be detected by incubation of PhnN with [γ-^{32}P]ATP and ribose, ribose 1-phosphate, or ribose 5-phosphate (Fig. 2A).

To confirm that the compound formed was PRPP, we used a purified preparation of *B. subtilis* XPRTase, which catalyzes the reaction xanthine + PRPP → 5′-XMP + PP_i. Thus, if the compound synthesized by PhnN is PRPP, it should be consumed by XPRTase in the presence of xanthine. This is exactly what was observed (Fig. 2B, lanes 1 to 4). Furthermore, addition of unlabeled PRPP prevented consumption of the radiolabeled PhnN-synthesized compound by XPRTase (Fig. 2B, lane 5). Similar results were obtained by incubation of authentic PRPP synthesized by using highly purified *E. coli* PRPP synthase (Fig. 2B, lanes 5 to 10). In these experiments PRPP was presumably labeled in the β position of the diphosphoryl group. Upon reaction with XPRTase, this diphosphoryl group was released as PP_i. Eventually, the formation of small amounts of PP_i could be seen (Fig. 2B), although most of the PP_i was presumably converted to P_i by a pyrophosphatase present in the XPRTase preparation.

To reinforce the finding that the compound synthesized by PhnN was a substrate for XPRTase, the compound was synthesized in unlabeled form and again used as a substrate, together with [8-^{14}C]xanthine and XPRTase; in this way 5′-[^{14}C]XMP was generated (Fig. 2C, lane 5). Similarly, 5′-[^{14}C]XMP was generated by incubation of [8-^{14}C]xanthine and XPRTase with PRPP synthesized by *E. coli* PRPP synthase or with commercial PRPP (Fig. 2C, lanes 6 and 7). The results of some additional control reactions are shown in Fig. 2C. Formation of 5′-XMP required PRPP and XPRTase (Fig. 2C, lanes 1 to 4). Including either ribose 1,5-bisphosphate and ATP or ribose 5-phosphate and ATP (i.e., the substrates of ribose 1,5-bisphosphokinase and PRPP synthase, respectively) in the XPRTase assay mixture resulted in abolishing 5′-XMP production at levels above background levels (Fig. 2C, compare lanes 8 and 9 to lane 1). Thus, the XPRTase preparation contained no PRPP-forming contaminant. In a similar way we showed that the compound synthesized by PhnN could be converted to [^{14}C]UMP in the presence of highly purified uracil phosphoribosyltransferase and [^{14}C]uracil (data not shown). When ribose 1,5-bisphosphate and PhnN were incubated with [α-^{32}P]ATP, the labeled nucleotide product was ADP rather than AMP. This showed that the enzyme transferred a phosphoryl group rather than a diphosphoryl group. The PhnN protein was unable to use GTP as a phosphoryl donor, as measured with [α-^{32}P]GTP, in which case no labeled compound besides GTP appeared on the thin-layer chromatogram (data not shown). We conclude from these experiments that the product of the PhnN-catalyzed reaction is PRPP and consequently, that PhnN is an enzyme with ribose 1,5-bisphosphokinase activity.

To further show that phosphorylation of ribose 1,5-bisphosphate was catalyzed by the *phnN* gene product, we compared the band intensity of the PhnN polypeptide during purification

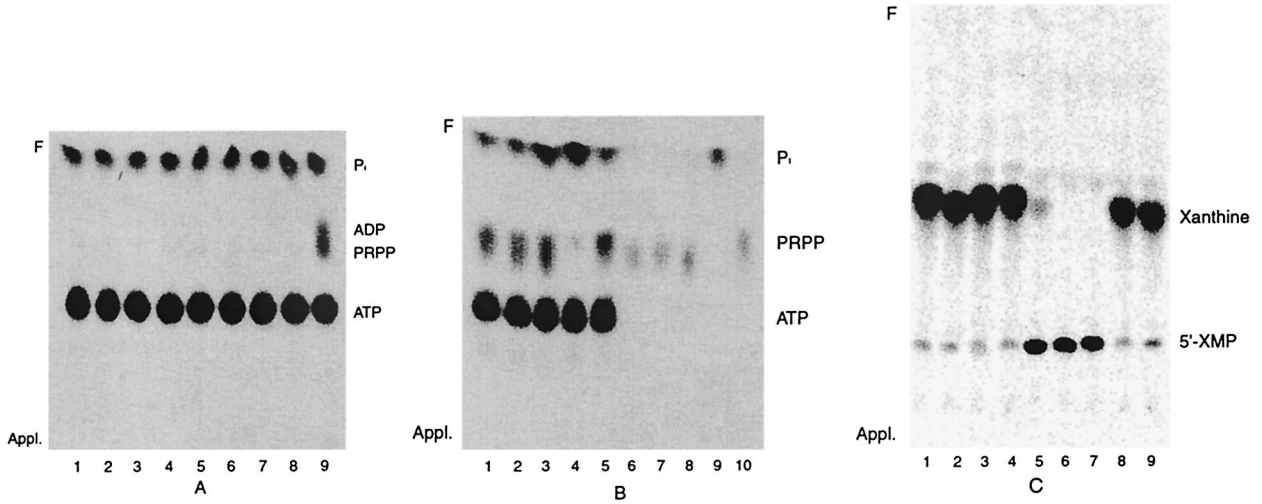

FIG. 2. Identification of PRPP as the product of the PhnN-catalyzed reaction. Incubation and one-dimensional chromatography were performed as described in Materials and Methods. Abbreviations: Appl., application line; F, solvent front. (A) Incubation of PhnN with various ribose compounds and [γ-^{32}P]ATP. All of the incubation mixtures contained [γ-^{32}P]ATP. Lanes 1 and 6 to 9 contained PhnN, whereas PhnN was omitted from lanes 2 to 5. Other additions were as follows: lane 1, no ribose compound; lanes 2 and 6, ribose; lanes 3 and 7, ribose 1-phosphate; lanes 4 and 8, ribose 5-phosphate; and lanes 5 and 9, ribose 1,5-bisphosphate. The positions of ATP, PRPP, ADP, and P_i are indicated on the right. In the reaction mixture shown in lane 9 a small amount of ADP was formed in addition to PRPP, presumably due to the presence of traces of [β-^{32}P]ATP in the [γ-^{32}P]ATP preparation. (B) Consumption of ^{32}P-labeled PhnN reaction product (lanes 1 to 5) or PRPP synthase-generated [^{32}P]PRPP (lanes 6 to 10) by XPRTase. Production of labeled compounds and incubation with XPRTase were performed as described in Materials and Methods. The reaction mixture analyzed in each lane contained reaction cocktail with the following supplement(s): lanes 1 and 6, no supplement; lanes 2 and 7, xanthine; lanes 3 and 8, XPRTase; lanes 4 and 9, xanthine and XPRTase; lanes 5 and 10, xanthine, XPRTase, and unlabeled PRPP (5 mM). The positions of ATP, PRPP, and P_i are indicated on the right. Lanes 1 to 5 contained appreciable amounts of ATP, whereas no ATP was present in lanes 6 to 10. We were unable to identify conditions that allowed complete consumption of ATP by PhnN, whereas ATP was readily consumed by PRPP synthase. (C) Conversion of unlabeled PhnN reaction product or PRPP to 5′-[^{14}C]XMP in the presence of XPRTase and [^{14}C]xanthine. The assay conditions are described in Materials and Methods. The reaction mixture analyzed in each lane contained reaction cocktail consisting of [^{14}C]xanthine with the following addition(s): lane 1, no addition; lane 2, XPRTase; lane 3, PhnN reaction product; lane 4, PRPP (synthesized by PRPP synthase); lane 5, XPRTase and PhnN reaction product; lane 6, XPRTase and PRPP (synthesized by PRPP synthase); lane 7, XPRTase and commercial PRPP (5 mM); lane 8, XPRTase and a sample of PhnN assay mixture to which no PhnN enzyme was added (the mixture contained ribose 1,5-bisphosphate and ATP); and lane 9, XPRTase and a sample of PRPP synthase assay to which no PRPP synthase was added (the mixture contained ribose 5-phosphate and ATP). The positions of xanthine and 5′-XMP are indicated on the right. Spots were visualized under a UV mineral lamp at 254 nm.

with the ribose 1,5-bisphosphokinase activity of the same fractions (Fig. 3). We were unable to determine the ribose 1,5-bisphosphokinase activity in a crude extract of strain HO1088/pTR553 grown in the absence of IPTG (i.e., the activity was less than 0.01 μmol/min/mg of protein), and similarly, no PhnN band was visible in the gel. In contrast, appreciable specific activity was observed in a crude extract of cells grown in the presence of IPTG, and there was a further increase in the specific activity of the purified enzyme. The relative intensity in the gel of the PhnN band increased from the crude extract to the purified enzyme. Thus, there was clear agreement between enzyme activity and band intensity of the PhnN polypeptide following gel electrophoresis in dodecyl-containing polyacrylamide. We therefore concluded that the band identified in Fig. 3 represents PhnN and that this polypeptide is responsible for ribose 1,5-bisphosphate phosphorylation. The relative molecular weight of this polypeptide was 23,500, which is in agreement with the value (21,408) calculated from the deduced nucleotide sequence of the allele specifying the histidine-tailed polypeptide. Lane 5 in Fig. 3 contained extract of a strain expressing wild-type *phnN*. As expected, the PhnN polypeptide was slightly smaller than the histidine-tailed polypeptide. The relative intensity of the PhnN band was somewhat less than that of the histidine-tailed PhnN (Fig. 3, lane 2). However, the ribose 1,5-bisphosphokinase activity of the former was significantly lower than that of the latter. We therefore concluded that the wild-type PhnN and the histidine-tailed PhnN have similar specific activities.

Formation in vivo of PRPP in NAD suppression mutants. It was shown previously that the PRPP content of an *E. coli* Δ*prs pst-phoU* strain was less than 5% of that of a wild-type strain (i.e., PRPP was undetectable) (17). Strain HO1088 (Δ*prs*)/pTR553 (*phnN*) is NAD prototrophic, presumably due to increased expression of *phnN*. However, in the presence of adenine (100 mg/liter) this strain became NAD auxotrophic. Adenine is a potent scavenger of PRPP in cells that contain a wild-type allele of the *apt* gene, which encodes adenine phosphoribosyltransferase (2). In addition, the strain formed colonies in the absence of tryptophan, although at a much lower rate than it formed colonies in the absence of NAD. These data suggest that PRPP indeed is synthesized in strain HO1088/pTR553. We were unable to cultivate strain HO1088/pTR553 in a low-phosphate medium, and thus, we were unable to determine the sizes of the intracellular pools of PRPP or nucleotides.

DISCUSSION

Pathway for NAMN synthesis in NAD suppression mutants. Our results show that PRPP can be synthesized from ribose 5-phosphate by the following pathway: ribose 5-phosphate →

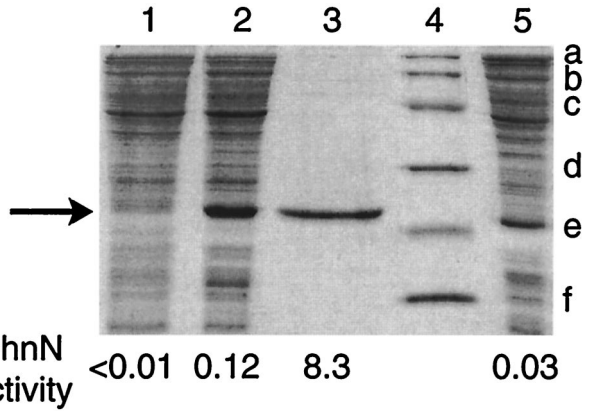

FIG. 3. Purification and activity of histidine-tailed ribose 1,5-bisphosphokinase. Cells of strain HO1088 (Δ*prs*)/pTR553 (specifying histidine-tailed PhnN) were grown in NZY medium, *phnN* gene expression was induced by IPTG, and ribose 1,5-bisphosphokinase was purified and assayed as described in Materials and Methods. In addition, a culture sample was removed before addition of IPTG. Furthermore, strain HO1088/pHO500 (specifying wild-type PhnN) was grown in parallel with strain HO1088/pTR553. Extracts and purified enzyme were subjected to gel electrophoresis in 17.5% polyacrylamide containing sodium dodecyl sulfate. Lane 1, crude extract (10 μg of protein) of HO1088/pTR553 cells before IPTG addition; lane 2, crude extract (10 μg) of HO1088/pTR553 cells after IPTG addition; lane 3, purified PhnN protein (1.3 μg); lane 4, molecular weight markers (positions indicated on the right, as follows: a, phosphorylase *b* [molecular weight, 97,400]; b, serum albumin [66,200]; c, ovalbumin [45,000]; d, carbonic anhydrase [31,000]; e, trypsin inhibitor [21,500]; f, lysozyme [14,400]); lane 5, crude extract (10 μg) of HO1088/pHO500 cells after IPTG addition. The arrow on the left indicates the position of the PhnN band. The ribose 1,5-bisphosphokinase activity of each fraction (in micromoles per minute per milligram of protein) is indicated at the bottom.

ribose 1-phosphate → ribose 1,5-bisphosphate → PRPP in reactions catalyzed by the sequential action of phosphoribomutase (*deoB*), ribose 1-phosphokinase, and ribose 1,5-bisphosphokinase (*phnN*) (Fig. 4). The activity of the enzyme catalyzing the second step, ribose 1-phosphokinase, remains to be identified, along with the corresponding gene. However, our data leave no doubt that ribose 1-phosphate is indeed involved in the pathway. NAD suppression strictly requires the production of ribose 1-phosphate, an intermediate of cellular metabolism only under certain conditions, as follows: either (i) when ribose 5-phosphate and phosphoribomutase are present (ribose

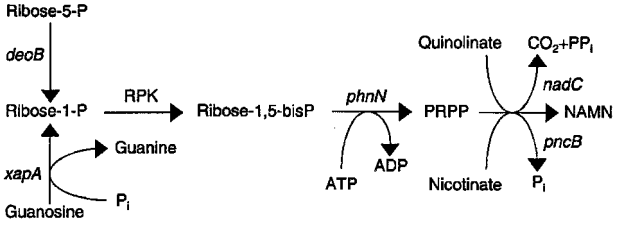

FIG. 4. Proposed pathway for conversion of ribose 5-phosphate to the 5′-phosphoribosyl moiety of NAMN. Abbreviations: P, phosphate; RPK, ribose 1-phosphokinase. Other enzyme-catalyzed reactions are indicated by gene designations (*deoB*, phosphoribomutase; *xapA*, xanthosine phosphorylase; *phnN*, ribose 1,5-bisphosphokinase activity; *nadC*, quinolinate phosphoribosyltransferase; *pncB*, nicotinate phosphoribosyltransferase.)

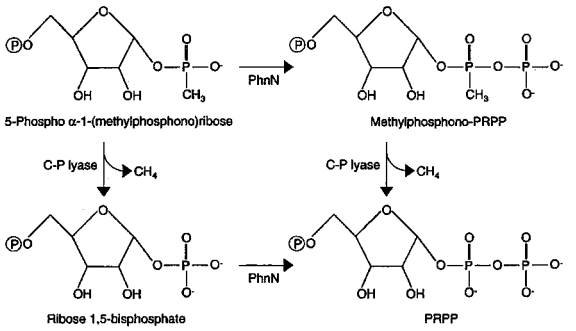

FIG. 5. Hypothetical reactions involved in the degradation of methylphosphonate by the C-P lyase pathway. An intermediate in the pathway is 5-phospho-α-1-(methylphosphono)ribose. This compound may be either demethylated to ribose 1,5-bisphosphate or phosphorylated to methylphosphono-PRPP. Ribose 1,5-bisphosphate or methylphosphono-PRPP may be phosphorylated or demethylated, which results in the formation of PRPP.

5-phosphate → ribose 1-phosphate) or (ii) when a ribonucleoside can be phosphorolyzed (e.g., guanosine in the presence of xanthosine phosphorylase [guanosine + P_i → guanine + ribose 1-phosphate]). Both of these reactions are included in Fig. 4.

The physiological role of ribose 1,5-bisphosphate is poorly understood. Its only known function is as a coenzyme for phosphopentomutase of *E. coli* and other organisms (22, 24). The pathway for ribose 1,5-bisphosphate synthesis has not been described previously, except that macrophages synthesize the compound by dephosphorylation of PRPP (21).

Role of the *phnN* gene product in phosphonate degradation by the C-P lyase pathway. We have shown that the PhnN protein is capable of catalyzing the phosphorylation of ribose 1,5-bisphosphate to PRPP; i.e., it is a ribose 1,5-bisphosphokinase. This is not only the first assignment of a chemical reaction to a specific polypeptide specified by the *phn* operon, but it is the first report of characterization of an enzyme with this activity. The specific activity of the enzyme with ribose 1,5-bisphosphate and ATP as substrates was rather low (i.e., undetectable) in an extract of uninduced cells. This low activity presumably provides an explanation for the retention in the NAD suppression mutants of requirements for purine and pyrimidine compounds. In wild-type *E. coli* cells approximately 80% of the PRPP formed is spent on purine and pyrimidine nucleotide synthesis, approximately 10% is spent on synthesis of the amino acid histidine, approximately 10% is spent on synthesis of the amino acid tryptophan, and approximately 1% is spent on NAD synthesis (19). Thus, only a small amount of PRPP may be required to satisfy the need for NAD and tryptophan biosynthesis. In addition, the elevated ribose 1,5-bisphosphokinase activity may not increase the PRPP pool to an extent that satisfies the amount required for nucleotide and histidine synthesis, as the level of the enzyme's substrate, ribose 1,5-bisphosphate, may not be elevated.

The biochemistry of phosphonate degradation by the *E. coli* C-P lyase pathway is poorly understood. Methyl phosphonate is converted to methane and some other unknown phosphorus-containing compound(s). Furthermore, methane formation appears to occur at the outside of the cell as the reaction may occur in cells which are deficient in the phosphonate ABC transporter specified by *phnCDE* (43; W. W. Metcalf and B. L.

Wanner, unpublished data). It has also been shown that wild-type *E. coli* K-12 cells, which are unable to degrade phosphonates due to a transport defect (25), accumulate [^{32}P]α-1-(ethylphosphono)ribose, when they are supplied with [^{32}P] ethyl phosphonate; i.e., the ethyl phosphonate is attached to ribose at the C-1 hydroxyl as a phosphate ester. Most of this ribose derivative is found in the culture fluid. Phosphonate degradation-proficient cells fail to accumulate the compound, presumably because the compound is further degraded (1). A relationship between this phosphono-ribose compound and the activity of the *phnN* gene product, if any, remains to be established. However, it is possible that the α-1-(ethylphosphono)ribose is a dephosphorylation product of 5-phospho-α-1-(ethylphosphono)ribose. As noted above, α-1-(ethylphosphono)ribose was detected primarily in the culture fluid, possibly resulting from dephosphosphorylation of 5-phospho-α-1-(ethylphosphono)ribose by a nonspecific periplasmic phosphatase. Hypothetical reactions involving ribose 1,5-bisphosphokinase are shown in Fig. 5. We suggest that 5-phospho-α-1-(methylphosphono)ribose is an intermediate in methylphosphonate utilization. 5-Phospho-α-1-(methylphosphono)ribose may be demethylated by the C-P lyase, yielding methane and ribose 1,5-bisphosphate. The latter compound then may be phosphorylated to PRPP by PhnN, as was shown to occur in vitro in the present work. Alternatively, PhnN may phosphorylate 5-phospho-α-1-(methylphosphono)ribose to methylphosphono-PRPP, which in turn may be demethylated by the C-P lyase, yielding methane and PRPP.

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REFERENCES

1. **Avilla, L. Z., K. M. Draths, and J. W. Frost.** 1991. Metabolites associated with organophosphonate C-P bond cleavage: chemical synthesis and microbial degradation of [^{32}P]-ethylphosphonic acid. Bioorg. Med. Chem. Lett. **1:**51–54.
2. **Bagnara, A. S., and L. R. Finch.** 1974. The effects of bases and nucleosides on the intracellular contents of nucleotides and 5-phosphoribosyl 1-pyrophosphate in *Escherichia coli*. Eur. J. Biochem. **41:**421–430.
3. **Bartolomé, B., Y. Jubete, E. Martinez, and F. de la Cruz.** 1991. Construction and properties of a family of pACYC184-derived cloning vectors compatible with pBR322 and its derivatives. Gene **102:**75–78.
4. **Birnboim, H. C., and J. Doly.** 1979. A rapid alkaline extraction procedure for screening recombinant plasmid DNA. Nucleic Acids Res. **7:**1513–1523.
5. **Bolivar, F., R. L. Rodriguez, P. J. Greene, M. C. Betlach, H. L. Heynecker, H. W. Boyer, J. H. Crosa, and S. Falkow.** 1977. Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. Gene **2:**95–113.
6. **Casadaban, M. J., A. Martinez-Arias, S. K. Shapiro, and J. Chou.** 1983. β-Galactosidase gene fusion for analyzing gene expression in *Escherichia coli* and yeast. Methods Enzymol. **100:**293–308.
7. **Chen, C.-M., Q.-Z. Ye, Z. Zhu, B. L. Wanner, and C. T. Walsh.** 1990. Molecular biology of carbon-phosphorus bond cleavage. Cloning and sequencing of the *phn* (*psiD*) genes involved in alkylphosphonate uptake and C-P lyase activity in *Escherichia coli* B. J. Biol. Chem. **265:**4461–4471.
8. **Clark, D. J., and O. Maaløe.** 1967. DNA replication and the division cycle in *Escherichia coli*. J. Mol. Biol. **23:**99–112.
9. **Haldimann, A., M. K. Prahalad, S. L. Fisher, S.-K. Kim, C. T. Walsh, and B. L. Wanner.** 1996. Altered recognition mutants of the response regulator PhoB: a new genetic strategy for studying protein-protein interactions. Proc. Natl. Acad. Sci. USA **93:**14361–14366.
10. **Haldimann, A., and B. L. Wanner.** 2001. Conditional-replication, integration, excision, and retrieval plasmid-host systems for gene structure-function studies in bacteria. J. Bacteriol. **183:**6384–6393.
11. **Hammer-Jespersen, K.** 1983. Nucleoside catabolism, p. 203–258. *In* A. Munch-Petersen (ed.), Metabolism of nucleotides, nucleosides and nucleobases in microorganisms. Academic Press, Inc., London, United Kingdom.
12. **Hammer-Jespersen, K., R. S. Buxton, and T. D. Hansen.** 1980. A second purine nucleoside phosphorylase in *Escherichia coli* K-12. II. Properties of xanthosine phosphorylase and its induction by xanthosine. Mol. Gen. Genet. **179:**341–348.
13. **Hove-Jensen, B.** 1983. Chromosomal location of the gene encoding phosphoribosylpyrophosphate synthetase in *Escherichia coli*. J. Bacteriol. **154:** 177–184.
14. **Hove-Jensen, B.** 1988. Mutation in the phosphoribosylpyrophosphate synthetase gene (*prs*) that results in simultaneous requirements for purine and pyrimidine nucleosides, nicotinamide nucleotide, histidine and tryptophan in *Escherichia coli.* J. Bacteriol. **170:**1148–1152.
15. **Hove-Jensen, B.** 1989. Phosphoribosylpyrophosphate (PRPP)-less mutants of *Escherichia coli*. Mol. Microbiol. **3:**1487–1492.
16. **Hove-Jensen, B.** 1992. Identification of *tms-26* as an allele of the *gcaD* gene, which encodes *N*-acetylglucosamine 1-phosphate uridyltransferase in *Bacillus subtilis*. J. Bacteriol. **174:**6852–6856.
17. **Hove-Jensen, B.** 1996. Phosphoribosyl diphosphate synthetase-independent NAD de novo synthesis in *Escherichia coli*: a new phenotype of phosphate regulon mutants. J. Bacteriol. **178:**714–722.
18. **Hove-Jensen, B., and M. Maigaard.** 1993. *Escherichia coli rpiA* gene encoding ribose phosphate isomerase A. J. Bacteriol. **175:**5628–5635.
19. **Jensen, K. F.** 1983. Metabolism of 5-phosphoribosyl 1-pyrophosphate (PRPP) in *Escherichia coli* and *Salmonella typhimurium*, p. 1–25. *In* A. Munch-Petersen (ed.), Metabolism of nucleotides, nucleosides and nucleobases in microorganisms. Academic Press, Inc., London, United Kingdom.
20. **Jensen, K. F., U. Houlberg, and P. Nygaard.** 1979. Thin-layer chromatographic methods to isolate ^{32}P-labelled 5-phosphoribosyl-α-1-pyrophosphate (PRPP): determination of cellular PRPP pools and assay of PRPP synthetase activity. Anal. Biochem. **98:**254–263.
21. **Kawaguchi, T., T. L. Veech, and K. Uyeda.** 2001. Regulation of energy metabolism in macrophages during hypoxia. Roles of fructose 2,6-bisphoshate and ribose 1,5-bisphosphate. J. Biol. Chem. **276:**28554–28561.
22. **Klenow, H.** 1953. Some properties of the phosphoribomutase reaction. Arch. Biochem. Biophys. **46:**186–200.
23. **Krath, B. N., and B. Hove-Jensen.** 2001. Class II recombinant phosphoribosyl diphosphate synthase from spinach. Phosphate independence and diphosphoryl donor specificity. J. Biol. Chem. **276:**17851–17856.
24. **Leer, J. C., and K. Hammer-Jespersen.** 1975. Multiple forms of phosphodeoxyribomutase from *Escherichia coli*. Physical and chemical characterization. Biochemistry **14:**599–607.
25. **Makino, K., S.-K. Kim, H. Shinagawa, M. Amemura, and A. Nakata.** 1991. Molecular analysis of the cryptic and functional *phn* operons for phosphonate use in *Escherichia coli* K-12. J. Bacteriol. **173:**2665–2672.
26. **Mandel, M., and A. Higa.** 1970. Calcium-dependent bacteriophage DNA infection. J. Mol. Biol. **53:**159–162.
27. **Metcalf, W. W., P. M. Steed, and B. L. Wanner.** 1990. Identification of phosphate-starvation-inducible genes in *Escherichia coli* K-12 by DNA sequence analysis of *psi*::*lacZ*(Mud1) transcriptional fusions. J. Bacteriol. **172:** 3191–3200.
28. **Metcalf, W. W., and B. L. Wanner.** 1991. Involvement of the *Escherichia coli phn* (*psiD*) gene cluster in assimilation of phosphorus in the form of phosphonates, phosphite, P_i esters, and P_i. J. Bacteriol. **173:**587–600.
29. **Metcalf, W. W., and B. L. Wanner.** 1993. Evidence for a fourteen-gene, *phnC* to *phnP* locus for phosphonate metabolism in *Escherichia coli*. Gene **129:** 27–32.
30. **Miller, J. H.** 1992. A short course in bacterial genetics. Cold Harbor Laboratory Press, Cold Spring Harbor, N.Y.
31. **Nilsson, D., and B. Hove-Jensen.** 1987. Phosphoribosylpyrophosphate synthetase of *Bacillus subtilis*. Cloning, characterization and chromosomal mapping of the *prs* gene. Gene **53:**247–255.
32. **Penfound, T., and J. W. Foster.** 1996. Biosynthesis and recycling of NAD, p. 721–730. *In* F. C. Neidhart, R. Curtis III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. American Society for Microbiology, Washington, D.C.
33. **Post, D. A., R. L. Switzer, and B. Hove-Jensen.** 1996. The defective phosphoribosyl diphosphate synthase in a temperature-sensitive *prs-2* mutant of *Escherichia coli* is compensated by increased enzyme synthesis. Microbiology **142:**359–365.
34. **Randerath, K., and E. Randerath.** 1965. Ion-exchange thin-layer chromatography. XIV. Separation of nucleotide sugars and nucleoside monophosphates on PEI-cellulose. Anal. Biochem. **13:**575–579.
35. **Shevell, D. E., A. M. Abou-Zamzam, B. Demple, and G. C. Walker.** 1988. Construction of an *Escherichia coli* K-12 *ada* deletion by gene replacement in

a *recD* strain reveals a second methyltransferase that repairs alkylated DNA. J. Bacteriol. **170:**3294–3296.

36. **Singer, M., T. A. Baker, G. Schnitzler, S. M. Deischel, M. Goel, W. Dove, K. J. Jaacks, A. D. Grossman, J. W. Erickson, and C. A. Gross.** 1989. A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping in *Escherichia coli*. Microbiol. Rev. **53:**1–24.
37. **Valentin-Hansen, P., H. Aiba, and D. Schümperli.** 1982. The structure of tandem regulatory regions in the *deo* operon of *Escherichia coli* K12. EMBO J. **1:**317–322.
38. **Valentin-Hansen, P., B. A. Svenningsen, A. Munch-Petersen, and K. Hammer-Jespersen.** 1978. Regulation of the *deo* operon in *Escherichia coli*. The double negative control of the *deo* operon by the *cytR* and *deoR* repressors in a DNA directed in vitro system. Mol. Gen. Genet. **159:**191–202.
39. **Wanner, B. L.** 1996. Phosphorus assimilation and control of the phosphate regulon, p. 1357–1381. *In* F. C. Neidhart, R. Curtis III, J. L. Ingraham, E. C. C. Lin, K. B. Low, B. Magasanik, W. S. Reznikoff, M. Riley, M. Schaechter, and H. E. Umbarger (ed.), *Escherichia coli* and *Salmonella*: cellular and molecular biology, 2nd ed. American Society for Microbiology, Washington, D.C.
40. **Wanner, B. L.** 1997. Phosphate signaling and the control of gene expression in *Escherichia coli*, p. 104–128. *In* S. Silver and W. Walden (ed.), Metal ions in gene regulation. Chapman and Hall, New York, N.Y.
41. **Wanner, B. L., and J. A. Boline.** 1990. Mapping and molecular cloning of the *phn* (*psiD*) locus for phosphonate utilization in *Escherichia coli*. J. Bacteriol. **172:**1186–1196.
42. **Willemoës, M., B. Hove-Jensen, and S. Larsen.** 2000. Steady state kinetic model for the binding of substrates and allosteric effectors to *Escherichia coli* phosphoribosyl-diphosphate synthase. J. Biol. Chem. **275:**35408–35412.
43. **Yakovleva, G. M., S.-K. Kim, and B. L. Wanner.** 1998. Phosphate-independent expression of the carbon-phosphorus activity of *Escherichia coli*. Appl. Microbiol. Biotechnol. **49:**573–578.
44. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.