

# Lesions in the *nuo* Operon, Encoding NADH Dehydrogenase Complex I, Prevent PurF-Independent Thiamine Synthesis and Reduce Flux through the Oxidative Pentose Phosphate Pathway in *Salmonella enterica* Serovar Typhimurium

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**In *Salmonella enterica* serovar Typhimurium, PurF-independent thiamine synthesis (or alternative pyrimidine biosynthesis) allows strains, under some growth conditions, to synthesize thiamine in the absence of the first step in the purine biosynthetic pathway. Mutations have been isolated in a number of loci that prevent this synthesis and thus result in an  $\text{Apb}^-$  phenotype. Here we identify a new class of mutations that prevent PurF-independent thiamine synthesis and show that they are defective in the *nuo* genes, which encode the major, energy-generating NADH dehydrogenase of the cell. Data presented here indicated that a *nuo* mutant has reduced flux through the oxidative pentose phosphate pathway that may contribute to, but is not sufficient to cause, the observed thiamine requirement. We suggest that reduction of the oxidative pentose phosphate pathway capacity in a *nuo* mutant is an attempt to restore the ratio between reduced and oxidized pyridine nucleotide pools.**

As our knowledge of discrete biochemical pathways increases, it has become important to understand how these pathways are integrated to result in an effective physiology. We have examined thiamine synthesis in *Salmonella enterica* serovar Typhimurium as a model system for detecting metabolic pathway integration (8, 13, 15, 23). Thiamine monophosphate is synthesized by the condensation of 4-amino-5-hydroxymethyl-2-methyl pyrimidine (HMP) pyrophosphate and 4-methyl-5-( $\beta$ -hydroxyethyl)-thiazole phosphate. Thiamine monophosphate phosphorylation produces thiamine pyrophosphate, the coenzymic form of the vitamin (5, 6). As depicted in Fig. 1A, HMP pyrophosphate and 4-methyl-5-( $\beta$ -hydroxyethyl)-thiazole phosphate are synthesized by independent pathways. Despite rigorous *in vivo* labeling experiments, the biochemical reactions that result in the synthesis of these moieties are not defined.

The HMP moiety of thiamine is synthesized from the *de novo* purine pathway intermediate aminoimidazole ribotide, the last intermediate common to the two pathways (16, 20, 21). Work in our laboratory demonstrated that thiamine synthesis could occur independently of the *purF* gene product, and this synthesis was attributed to the alternative pyrimidine biosynthetic (APB) pathway (10, 12). Recent work has demonstrated that the four purine biosynthetic reactions after that catalyzed by PurF are needed for HMP synthesis via the APB pathway, making it synonymous with PurF-independent thiamine synthesis (13). However, the proposed step(s) forming phosphoribosylamine (PRA) in the absence of PurF has not been defined genetically or biochemically. To date, mutations preventing PurF-independent thiamine synthesis ( $\text{Apb}^-$ ) have been involved in the pantothenate biosynthetic pathway (e.g., *panE*) (11, 17), in loci thought to affect the conversion of aminoimidazole ribotide to HMP (i.e., *apbC* and *apbE*) (4, 22),

or in genes encoding enzymes of the oxidative pentose phosphate (OPP) pathway (e.g., *gnd* and *zwf*) (15, 23). Based on analyses of the last class of mutants, it was proposed that the OPP pathway contributed ribose-5-phosphate for the formation of PRA via the APB pathway (14, 24). Work presented here was initiated to identify genes encoding functions needed to synthesize PRA in the absence of PurF.

Herein we describe a new class of mutations that block PurF-independent thiamine synthesis. These lesions were located in the *nuo* operon, which encodes NADH dehydrogenase I (NADH dHI). NADH dHI transfers electrons to ubiquinone in the electron transport chain and, unlike NADH dHII (encoded by *ndh*), produces a proton motive force (18). Two general classes of metabolic phenotypes have been reported for *nuo* mutants: those due to lack of energy generation (2, 27) and those due to the resulting imbalance in the ratio of reduced to oxidized pyridine nucleotide pools (25). We report here that, in addition to having a defect in thiamine synthesis, *nuo* mutants have a reduced capacity for flux through the OPP pathway and suggest that this is a consequence of the imbalance in pyridine nucleotide pools in these mutants.

**Rationale for mutant isolation.** In the majority of mutants isolated for their defect in PurF-independent thiamine synthesis, a *purE* block was able to restore thiamine-independent growth to the levels of wild-type strains (4, 22, 24). To eliminate this predominant class of mutants, we screened Mud-J(Km) insertion mutations for those that prevented strain DM2353 (*purF purE*) (Table 1) from growing on gluconate-adenine medium lacking thiamine. Of the approximately 35,000 Km<sup>r</sup> transductants screened, 11 mutants carried insertions that blocked thiamine synthesis in a *purF purE* mutant but that had no effect on the ability of a wild-type strain to grow on minimal medium. Three of these mutants had lesions in *gnd* (encoding gluconate-6-phosphate dehydrogenase), and one had a lesion in *panE* (which encodes ketopantoate reductase), both of which were expected based on previously described phenotypes (11, 15, 17). The remaining seven mutants had insertions that were unlinked to loci previously shown to pre-

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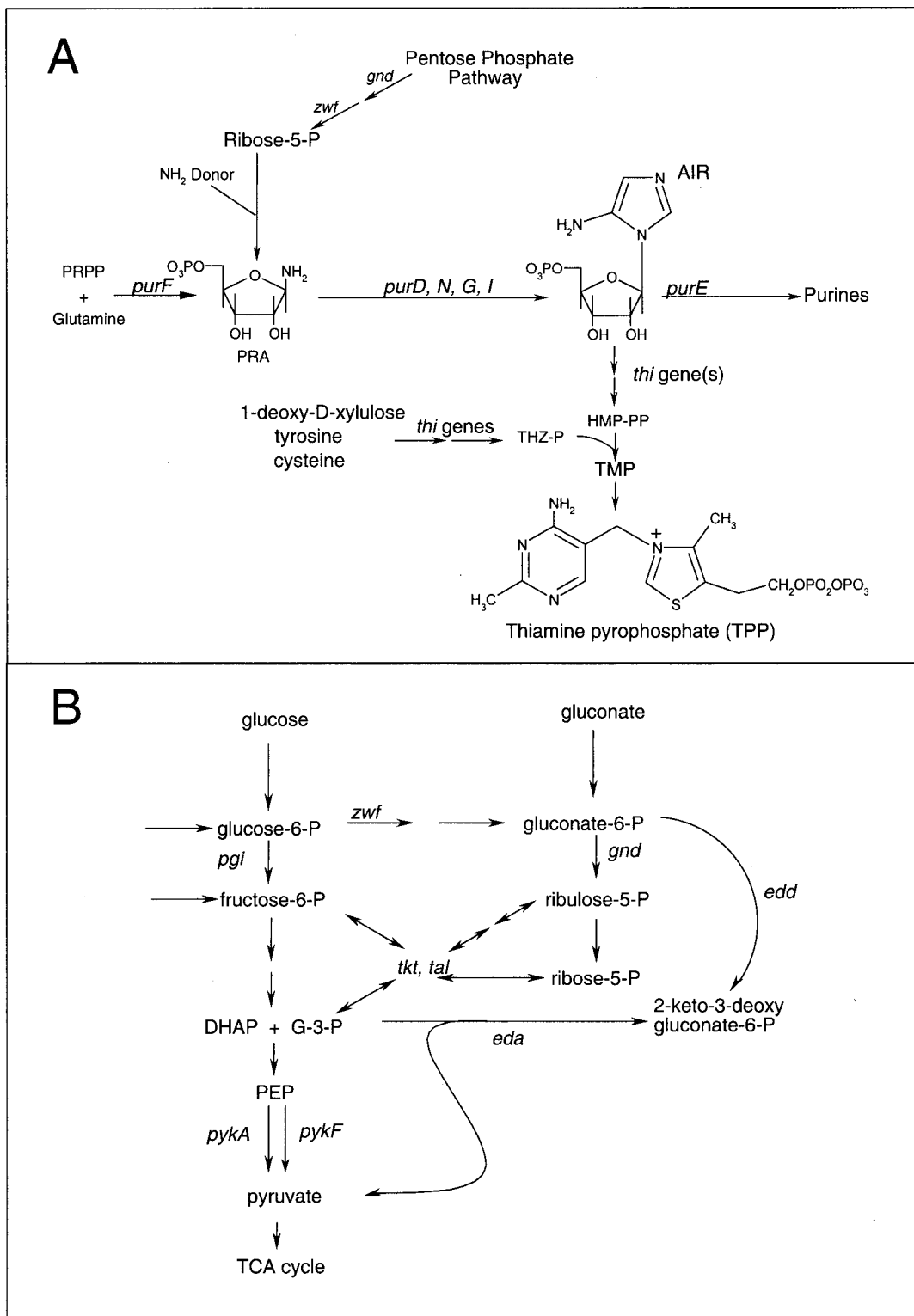


FIG. 1. Relevant metabolic pathways. (A) Illustrated are the biosynthetic pathways involved in purine and thiamine synthesis. Names of genes whose products are known are indicated. The reaction(s) involved in the formation of PRA independent of PurF has not been defined biochemically or genetically. (B) Glycolysis, the pentose phosphate pathway, and the Entner-Doudoroff pathway are schematically represented. Names of genes whose products are required are indicated. Abbreviations: P, phosphate; PRPP, 5-phosphoribosyl-1-pyrophosphate; AIR, aminoimidazole ribotide; PP, pyrophosphate; THZ-P, 4-methyl-5-(β-hydroxyethyl)-thiazole phosphate; TMP, thiamine monophosphate; G-3-P, glyceraldehyde-3-phosphate; DHAP, dihydroxyacetone phosphate; PEP, phosphoenolpyruvate; TCA, tricarboxylic acid.

TABLE 1. Strain list

Strain	Genotype
LT2	Wild type
DM574	<i>purF2085 gnd175::Tn10d(Tc)</i>
DM837	<i>nuo-351::Tn10d(Tc)</i>
DM1936	<i>purF2085</i>
DM2353	<i>purF2085 purE3041</i>
DM3702	<i>zec-8611::MudJ edd</i>
DM4134	<i>purF2085 purE3041 nuo-353::MudJ</i>
DM4484	<i>purF2085 purE3041 nuoN355::MudJ</i>
DM4485	<i>nuoN355::MudJ</i>
DM4489	<i>nuoA357::MudJ</i>
DM4615	<i>purF2085 nuoN355::MudJ</i>
DM5247	<i>nuo-351::Tn10d(Tc) zec-8611::MudJ edd</i>
DM5248	<i>purF2085 ndh::Km<sup>r</sup></i>
DM5399	<i>edd zec-8611::MudJ nuo-351::Tn10d(Tc)</i>
DM5400	<i>zec-8611::MudJ nuo-351::Tn10d(Tc)</i>
DM5411 <sup>a</sup>	<i>edd zec-8611::MudJ nuo-351::Tn10d(Tc)/pMN6 (Gnd)</i>
DM5479	<i>purF2085 nuoN355::MudJ/pMN6 (Gnd)</i>
MWC233 <sup>b</sup>	<i>cyd ndh::Km<sup>r</sup> (E. coli)</i>

<sup>a</sup> pMN6 was obtained from R. E. Wolf, Jr. (19).

<sup>b</sup> *E. coli* MWC233 was obtained from Robert Gennis.

vent PurF-independent thiamine synthesis and thus represented a new class of mutants.

**Mutations in the *nuo* operon prevent PurF-independent thiamine synthesis.** A *Tn10d(Tc)* insertion [*zeg-8661::Tn10d(Tc)*] was isolated 50% linked to MudJ(Km) in strain DM4134 (*purF2085 purE3041 nuo-354::MudJ*), and found to be linked to the remaining six MudJ insertion mutations, suggesting that the seven mutants were defective in the same locus.

To identify the physical locations of the insertions, sequence adjacent to two representative insertions was determined by sequencing a PCR product produced with degenerate primers and primers derived from the insertion sequence (7). Based on homology alignment with BLASTX (1), the MudJ(Km) insertions in DM4489 and DM4485 were positioned in *nuoA* and *nuoN*, respectively. These genes are the first and last, respectively, in an operon (*nuoA-nuoN*) located in the minute 51 region of the *Salmonella* serovar Typhimurium chromosome. The 14-gene *nuo* operon encodes the subunits of the major energy-generating NADH dehydrogenase complex (NADH dHI) (3). Since the insertions chosen for sequencing disrupted a gene at the beginning and end of the *nuo* operon and resulted in the same phenotype, it was unlikely that polarity effects were responsible for the observed phenotypes.

The mutations described here were isolated for their ability to prevent growth of a *purF purE* double mutant on solid medium containing adenine and lacking thiamine with gluconate as the sole carbon and energy source. The insertions were transduced into additional genetic backgrounds, and the resulting phenotypes are illustrated by the growth rates ( $\mu$ ) shown in Table 2. These data reflect two significant points: (i) that mutations in *nuo* eliminated thiamine synthesis in a *purF* mutant when gluconate (Table 2) or other sugars such as fructose, glycerol, and mannitol were used as sole carbon sources (data not shown) and (ii) that although growth of a *nuo purF purE* triple mutant was scored as negative on solid adenine gluconate medium, thiamine-independent growth was detectable in liquid ( $\mu = 0.15$ ), which reflects the sensitivity for quantification afforded by liquid medium, rather than a medium-specific growth.

Thiamine-independent growth in previously described mutant classes was either completely restored (i.e., *apbE* and *apbC*) or unaffected (i.e., *gnd* and *zwf*) by mutations in *purE*

TABLE 2. A *nuo* mutation prevents PurF-independent thiamine synthesis

Strain	Relevant genotype	Specific $\mu$ with <sup>a</sup> :	
		Adenine	Adenine + thiamine
DM1936	<i>purF</i>	0.20	0.34
DM6415	<i>purF nuo</i>	0.03	0.26
DM2353	<i>purF purE</i>	0.28	0.27
DM4484	<i>purF purE nuo</i>	0.15	0.27

<sup>a</sup> Gluconate was used as the sole carbon source, and growth curves were determined as described previously (8). Data shown here are from a representative experiment.

when mutants were tested under the above-described conditions. From these results we considered that mutants defective in *nuo* represented a new, phenotypically distinct class of mutants defective in PurF-independent thiamine synthesis. Alternatively, *nuo* mutants may have a unique phenotype because thiamine synthesis was being affected in more than one way by a lack of the major NADH dehydrogenase.

**Mutations in *nuo* reduce flux through the OPP pathway in vivo.** Previously we showed that flux through the OPP pathway was required for PurF-independent thiamine synthesis (14). Therefore, we tested whether *nuo* mutations resulted in reduced flux through the OPP pathway and, if so, whether the reduction was sufficient to cause the observed thiamine requirement. We reasoned that eliminating the major NADH dehydrogenase activity would result in a higher cellular NADH/NAD pool ratio, an idea previously proposed by others (25). If this assumption was correct, the cell might compensate by decreasing NADPH pools in an attempt to restore internal redox balance. Since the OPP pathway provides a source of NADPH, this pathway may be a site for such regulation. Results from the following experiments indicated that the flux capacity of the OPP pathway was reduced in a *nuo* mutant but that this reduction was not solely responsible for the resulting thiamine requirement.

Gluconate is a non-PTS hexose that is taken up by the cell through a specific gluconate transporter and phosphorylated by an ATP-dependent kinase (26). Gluconate-6-phosphate can then enter central metabolism by one of two routes: (i) it can enter the OPP pathway, where gluconate-6-phosphate dehydrogenase (encoded by *gnd*) converts it to ribulose-5-phosphate, after which it can enter central metabolism by the actions of transketolase and transaldolase, or (ii) it can be utilized via the gluconate-inducible Entner-Doudoroff pathway, producing pyruvate and glyceraldehyde-3-phosphate (Fig. 1B) (9).

In the presence of an *edd* block, gluconate will be catabolized exclusively via the OPP pathway and require Gnd (gluconate-6-phosphate dehydrogenase) activity. Thus, a *gnd edd* double mutant is unable to utilize gluconate as a sole carbon source ( $\mu < 0.03$  [data not shown]) (9). To test whether *nuo* mutants had reduced the flux of the OPP pathway in vivo, we assessed the ability of a *nuo edd* strain to utilize gluconate as a sole carbon source. The data in Table 3 clearly show that the *nuo edd* double mutant had a >2-fold reduction in growth rate compared to that of either single parental mutant when gluconate was used as the sole carbon source. Importantly, the growth rate of the double mutant was indistinguishable from that of either of the single mutants when glucose was provided as the sole carbon source (Table 3). The latter result demonstrated that *nuo* mutations were not simply causing an overall decrease in growth rate independently of the carbon source.

TABLE 3. A *nuo edd* double mutant is unable to efficiently catabolize gluconate

Strain	Relevant genotype	Specific $\mu$ on <sup>a</sup> :	
		Gluconate	Glucose
DM5400	<i>nuo</i>	0.23	0.18
DM3702	<i>edd</i>	0.16	0.18
DM5399	<i>edd nuo</i>	0.07	0.17
DM5411	<i>edd nuo/pGnd</i> <sup>+</sup>	0.16	0.17

<sup>a</sup> Growth curves were determined as described previously (8), and data are from a representative experiment.

Since the structural genes for enzymes of the OPP were intact in the *edd nuo* double mutant, these results suggested that a gene(s) and/or enzyme(s) of the OPP pathway was down regulated in the *nuo* mutant.

#### Gnd is the limiting step of the OPP pathway in *nuo* mutants.

Based on the described routes for gluconate catabolism, the above results indicated that a *nuo* mutation resulted in reduced flux through the OPP pathway. The rationale for the model presented above suggested that Gnd, the enzyme directly responsible for production of NADPH, may be the limiting step in gluconate utilization in the *nuo edd* mutant. To address this possibility, pMN6 (Gnd<sup>+</sup>) (19) was introduced into DM5399 (*nuo edd*) and growth of the resulting strain (DM5400) was assessed with gluconate as the sole carbon source. Representative data from these experiments, shown in Table 3, demonstrated that pMN6 restored a wild-type growth rate to the double mutant. Assays in cell-free extracts determined that strains carrying the pMN6 plasmid had ~10-fold more gluconate-6-phosphate dehydrogenase activity than those with only chromosomal *gnd* (data not shown). These results were consistent with a reduction of Gnd activity preventing a *nuo edd* double mutant from utilizing gluconate.

**Reduced flux through the OPP pathway does not cause the Thi<sup>-</sup> phenotype of *nuo* mutants.** Having determined that *nuo* mutants had reduced flux through the OPP pathway, we asked whether this reduction was responsible for the thiamine requirement observed in a *purF nuo* mutant. Two results indicated that the defect in the OPP pathway was not sufficient to generate the thiamine requirement of a *purF nuo* mutant. First, the presence of pMN6(Gnd<sup>+</sup>) in a *purF nuo* double mutant failed to restore thiamine-independent growth. The specific growth rates of the strain with and without this plasmid were not significantly different in medium with gluconate as the sole carbon and energy source and containing adenine ( $\mu = 0.134$  and 0.198, respectively). That the pMN6 plasmid restored flux through the OPP pathway suggested that an additional effect of the *nuo* mutation was involved in causing the thiamine requirement. Second, when exogenous ribose is spotted on a top agar lawn of *purF gnd* cells on gluconate adenine medium, thiamine-independent growth is restored (14). Exogenous ribose was unable to restore thiamine synthesis in either DM4615 (*purF nuo*) or DM4484 (*purF nuo purE*) when the strains were tested in the same way (data not shown). This result also supported the conclusion that a *nuo* mutation did not cause a thiamine requirement solely by reducing flux through the OPP pathway.

**Summary.** The studies described here made two contributions to our understanding of metabolism in *Salmonella* serovar Typhimurium. First, we have shown that mutations in the *nuo* operon are unable to perform PurF-independent thiamine synthesis. Second, we showed that strains defective in the major energy-generating NADH dehydrogenase complex are im-

paired in the OPP pathway. Our data are consistent with the model that lesions in this complex result in an increased NADH/NAD ratio that inhibits the activity of Gnd. Such an inhibition may be designed to reduce the NADPH/NADP ratio and thus restore the balance of reduced to oxidized pyridine nucleotide pools in the cell.

We further showed that the reduced flux through the OPP pathway caused by a *nuo* mutation was not sufficient to cause the observed thiamine requirement in this strain. This finding predicts that an additional metabolic perturbation(s) caused by a *nuo* mutation inhibits PurF-independent thiamine synthesis. It is tempting to speculate that the role of the *nuo* locus in energy generation is involved in causing this phenotype. For instance, in a *purF* mutant, thiamine synthesis may have an energy requirement that cannot be met in the absence of a functional NADH DHI. Since *nuo* mutants are proficient at thiamine synthesis in a PurF<sup>+</sup> background, such an energy requirement would be specific for conditions of low flux through the purine (and thus the HMP) pathway.

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