A Mutant Allele of *rpoD* Results in Increased Conversion of Aminoimidazole Ribotide to Hydroxymethyl Pyrimidine in *Salmonella enterica*

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An allele of *rpoD* (*rpoD1181*) that results in increased synthesis of the pyrimidine moiety of thiamine in *Salmonella enterica* was identified. The S508Y substitution caused by *rpoD1181* is analogous to the S506F derivative of the *Escherichia coli* protein. The properties of this *E. coli* mutant protein have been well characterized in vitro. Identification of a metabolic phenotype caused by the *rpoD1181* allele of *S. enterica* allows past in vitro results to be incorporated in continuing efforts to understand cellular processes that are integrated with the thiamine biosynthetic pathway.

The essential cofactor thiamine pyrophosphate is generated by the condensation of two independently synthesized molecules, 4-methyl-5-(β-hydroxyethyl) thiazole phosphate and 4-amino-5-hydroxymethyl-2-methylpyrimidine pyrophosphate (HMP-PP) (Fig. 1) (3). The precursor of the pyrimidine moiety is 5-aminoimidazole ribotide (AIR), an intermediate in the purine biosynthetic pathway. HMP-P synthesis involves a complex intramolecular rearrangement and requires at least the product of the *thiC* gene (24, 26). Reconstitution of HMP-P synthesis in vitro has not yet been reported.

Past work has identified loci other than *thi* biosynthetic enzymes that are required for efficient HMP-P synthesis. Characterization of these loci has implicated the oxidative pentose phosphate pathway, pantothenate (and/or coenzyme A [CoA]), and iron-sulfur cluster metabolism in the synthesis of HMP (10, 13, 14, 21, 23).

Work described here was initiated to probe the integration of distinct metabolic processes with thiamine synthesis. Genetic analysis identified an allele of *rpoD* (*rpoD1181*) that allowed several HMP-requiring mutant strains to grow in the absence of thiamine. Our working model suggests that the *rpoD1181* (S508Y) allele restores thiamine-independent growth by altering expression of an unidentified gene(s) whose product is required for efficient HMP-P synthesis.

Thiamine synthesis in a *purF iscA* strain is restored by an *rpoD* allele. *Salmonella enterica* strain DM6176 (*purF iscA*) is unable to grow in the absence of thiamine (23). The *iscA* allele used is an insertion that is polar on *hscAB* (23). Mutations that suppressed the thiamine synthetic defect caused by the *iscA* lesion were identified. A 0.1-ml aliquot of an overnight culture of DM6176 was spread on a plate containing minimal medium plus gluconate and adenine. After overnight incubation at 37°C, more than 300 colonies that no longer required thiamine for growth were found. Ten random colonies were chosen for further analysis. In nine of the mutants, an insertion near the

gltA locus, *zbg-6391*::Tn*10d*(Tc), was genetically linked to the causative lesion. On the basis of the proximity of *gltA* to the *sdh* genes and the knowledge that null mutations in *sdh* spared cellular thiamine pools (11), succinate dehydrogenase activity was measured in the nine mutants. Each of the nine mutants lacked detectable succinate dehydrogenase activity (data not shown), and they were not analyzed further.

Multiple transposon insertions (MudJ, Tn*10d*) linked to the suppressor mutation in the remaining mutant (*S. enterica* DM6229) were identified. The chromosomal location of each transposon insertion was determined by a PCR-based protocol using degenerate primers (5, 25). Linkage analyses and three-factor cross data focused attention on a small region of the chromosome (~10 kb) at approximately 70 min that contained five open reading frames. Each of the open reading frames was amplified from wild-type (DM6176) and mutant (DM6229) strains of *S. enterica* and sequenced. The causative lesion was found to be a CG-to-AT transversion in *rpoD*, resulting in the substitution of a tyrosine for serine at residue 508. Subsequently, *rpoD* was amplified and the PCR product was sequenced from the chromosome of DM6229 eight independent times to confirm the causative lesion.

Residue 508 is in conserved region 3.1 of RpoD and is analogous to S506 in the *Escherichia coli* protein. In *E. coli*, the similar *rpoD* (S506F) allele was isolated as a suppressor of the multiple amino acid auxotrophy of a *relA spoT* double mutant (15) and subsequently shown to reduce abortive transcription at several promoters in vitro (6, 16, 22). This substitution belonged to a class of mutants that responded as if ppGpp were always present. Most characterized members of this mutant class contained lesions in *rpoBC*. An *rpoB* (T563P) allele that belongs to this class (16) was isolated as a suppressor of the nutritional requirements of an *E. coli dksA* mutant (4). One interpretation of these results was that reduction in abortive transcription at a global set of promoters suppressed the nutritional requirements of the *dksA* mutant. To complete the correlation between this defined class of polymerase mutants and the *Salmonella rpoD1181* allele, an isogenic pair of strains DM7088 (*dksA3*::MudJ) and DM7087 (*dksA3*::MudJ *rpoD1181*) was constructed. Doubling times on minimal medium contain-

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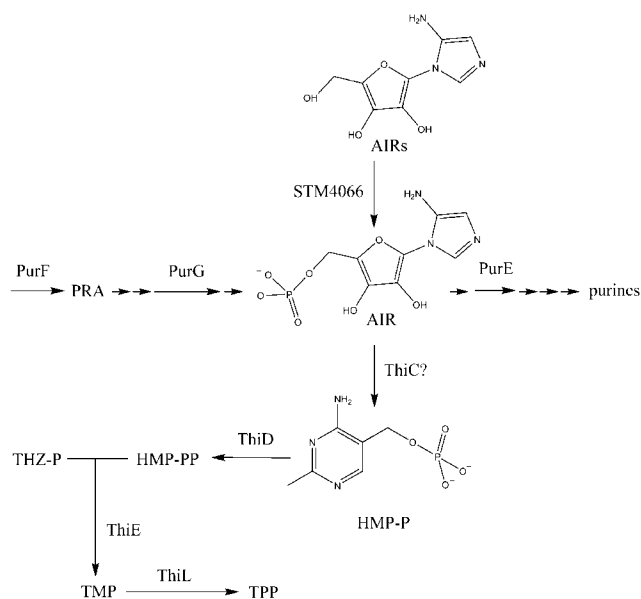
FIG. 1. Purine mononucleotide and thiamine pyrophosphate biosynthetic pathways in *S. enterica*. Relevant intermediates are indicated. The documented phosphorylation of AIRs to AIR by STM4066 (8) is represented. Abbreviations: THZ-P, 4-methyl-5(β-hydroxyethyl) thiazole phosphate; TMP, thiamine phosphate; TPP, thiamine pyrophosphate.

ing glucose (240 and 100 min, respectively) determined that the *rpoD1181* allele substantially relieved the nutritional requirements of the *dksA* mutant.

The *rpoD* (S508Y) allele alters transcription on a global level. β-Galactosidase activity was measured from several MudJ transcriptional fusions in the presence or absence of the *rpoD1181* allele (Table 1). Fusions to several loci involved in thiamine biosynthesis and CoA biosynthesis were used, as well as fusions to loci not expected to affect these pathways directly. The data show that expression from several promoters was slightly, but significantly, increased by the *rpoD1181* allele. In particular, it was noted that expression from the *panBCD* fusion (1.6-fold), *ilvC* fusion (1.5-fold), and *thiC* fusion (1.6-fold) was increased (Table 1).

The *rpoD1181* allele suppresses several conditional thiamine auxotrophs. Multiple loci that prevent PurF-independent thiamine synthesis have been identified (9, 10, 18). In general, mutations in these loci are thought to reduce either (i) PurF-independent formation of phosphoribosylamine (PRA) or (ii) efficiency of conversion of AIR to HMP (M. J. Dougherty and D. M. Downs, unpublished data). The *rpoD1181* allele was transduced into mutants representing both classes, and thiamine-independent growth was monitored. Results from these experiments are shown in Table 2. With the exception of the *purF gnd* mutant, each of the strains that contained the *rpoD1181* allele regained thiamine-independent growth. Significantly, the *purF gnd* mutant has a defect in PRA formation (10), while the other mutants have been shown genetically to have defective conversion of AIR to HMP (Dougherty and Downs, unpublished).

The *rpoD1181* allele specifically affects the conversion of AIR to HMP-P. The above results suggested a model in which altered gene expression caused by the *rpoD1181* allele resulted

in increased conversion of AIR to HMP, thus suppressing strains that are compromised in that step. The effect of the *rpoD1181* allele on the efficiency of AIR to HMP conversion was monitored in vivo. Isogenic strains, DM7256 (*purG purE*

TABLE 1. Expression from transcriptional fusions is affected by *rpoD1181*

Strain	*rpoD* allele	Locus[a]	β-Galactosidase activity (Miller units)[b]	Relative activity[c]
DM7358	*rpoD1181*	*ilvC*[d]	352 ± 5	1.5
DM7359	wt[e]	*ilvC*[d]	235 ± 6	
DM7081	*rpoD1181*	*panC*[d]	38.9 ± 1.3	1.6
DM7082	wt	*panC*[d]	23.6 ± 0.4	
DM7114	*rpoD1181*	*thiC*	104 ± 4	1.6
DM7115	wt	*thiC*	63.0 ± 1.4	
DM7106	*rpoD1181*	*dksA*	144 ± 2	1.3
DM7107	wt	*dksA*	107 ± 4	
DM7108	*rpoD1181*	*stm4065*	3.7 ± 0.0	0.8
DM7109	wt	*stm4065*	4.5 ± 0.1	
DM7201	*rpoD1181*	*gnd*	27.3 ± 0.9	1.0
DM7202	wt	*gnd*	26.1 ± 1.9	
DM7203	*rpoD1181*	*yigF*	48.4 ± 0.2	1.0
DM7204	wt	*yjgF*	48.9 ± 1.9	
DM7205	*rpoD1181*	*hisD*	22.6 ± 3.0	1.5
DM7206	wt	*hisD*	15.3 ± 1.0	
DM7207	*rpoD1181*	*ilvD*	26.3 ± 0.3	1.1
DM7208	wt	*ilvD*	24.5 ± 1.5	

[a] Strains carried a MudJ fusion (7) inserted in the indicated locus.
[b] Cells from a full-density nutrient broth (NB) culture were inoculated into NB and grown to an optical density at 650 nm of ≈0.3. β-Galactosidase activity was determined as previously described (12, 17). A Miller unit is defined as 1 nmol of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed per min.
[c] β-Galactosidase activity in the *rpoD1181* strain divided by activity of the wild-type strain.
[d] Gene products from the *panBCD* operon are required for synthesis of pantothenate. IlvC function is required for pantothenate synthesis when *panE* is absent (20).
[e] wt, wild type.

TABLE 2. The *rpoD1181* allele alters thiamine-independent growth and CoA levels

Strain	Relevant genotype	Doubling time (min)[a]	Total CoA thioester[b]
DM6293	*purF iscA*[c] rpoD1181	97 ± 6	0.115 ± 0.035
DM6294	*purF iscA*	NG[d]	0.082 ± 0.004
DM6324	*purF rpoD1181*	80 ± 1	0.072 ± 0.002
DM6325	*purF*	NG	0.155 ± 0.007
DM6326	*purF panE rpoD1181*	82 ± 5	0.024 ± 0.002
DM6327	*purF panE*	NG	0.003 ± 0.005
DM6328	*panE rpoD1181*	95 ± 2	ND[e]
DM6329	*panE*	NG	ND
DM7291	*purF apbC rpoD1181*	68 ± 4	0.058 ± 0.000
DM7292	*purF apbC*	NG	0.090 ± 0.010
DM7360	*purF apbE rpoD1181*	74 ± 1	0.038 ± 0.004
DM7361	*purF apbE*	NG	0.079 ± 0.001
DM7362	*purF gshA rpoD1181*	103 ± 2	0.033 ± 0.004
DM7363	*purF gshA*	NG	0.044 ± 0.003
DM7289	*purF gnd rpoD1181*	NG	0.079 ± 0.003
DM7290	*purF gnd*	NG	0.136 ± 0.006

[a] Growth in liquid medium was assessed as described previously (18) using NCE (no carbon E medium) with 11 mM glucose or gluconate as the carbon source and 0.4 mM adenine.
[b] Total amount of CoA thioester is given in nanomoles of CoA per milligram (dry weight). CoA levels were determined by the method of Allred and Guy (2).
[c] *iscA* mutation is a polar insertion affecting *iscA* and *hscAB* (23).
[d] NG, no growth. The absorbance at 650 nm did not increase during the course of the experiment.
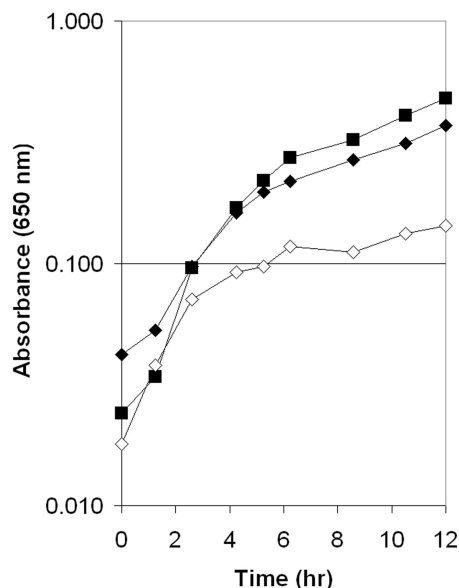[e] ND, not done.

FIG. 2. The *rpoD1181* allele increases the efficiency of the conversion of AIR to HMP-P. Growth in liquid medium was assessed as described previously (19) using NCE (no carbon E medium) with 11 mM glucose and 0.4 mM adenine; 10 nM AIRs and 100 µM pantothenate were added as indicated. The *stm4068-6*::Tn*10d*(Tc) insertion results in an ~100-fold increase in expression of *stm4066* (8). Representative growth for strain DM7256 [*purG3111 purE3043 stm4068-6*::Tn*10d*(Tc) *zxx-9149*::MudJ *rpoD1181*] grown with 10 nM AIRs (filled diamonds) or with 10 nM AIRs plus 100 µM pantothenate (filled squares) and strain DM7257 [*purG3111 purE3043 stm4068-6*::Tn*10d*(Tc) *zxx-9149*::MudJ] grown with 10 nM AIRs (open diamonds) is depicted.

stm4068 rpoD1181) and DM7257 (*purG purE stm4068*) were constructed. These strains are blocked in the de novo purine biosynthetic pathway before and after the thiamine precursor AIR (Fig. 1). An insertion in *stm4068* allows efficient utilization of aminoimidazole riboside (AIRs) as a source of thiamine by increasing the expression of a kinase that converts AIRs to AIR (8).

The two relevant strains were tested for growth on minimal medium containing glucose and adenine supplemented with variable amounts of AIRs. A 10 nM concentration of AIRs was determined empirically to be limiting for thiamine synthesis in strain DM7257, and this concentration was used in growth analyses. Results of the growth analyses (Fig. 2) showed that when 10 nM AIRs was provided as the source of HMP, the *rpoD1181* allele slightly increased the growth rate and significantly increased the final yield of the strain. This result was interpreted to reflect increased conversion efficiency of AIR to HMP. In a similar assay, pantothenate was shown to stimulate the conversion of AIR to HMP (1). As shown in Fig. 2, addition of pantothenate to the *rpoD* mutant strain failed to further stimulate growth, suggesting that the same cellular process was being targeted by each. Although the *rpoD1181* allele increased the transcription of the *thi* operon (Table 1), increasing the level of ThiC fails to result in thiamine-independent growth in the mutant backgrounds suppressed by the *rpoD1181* allele (Table 2) (S. Allen and D. M. Downs, unpublished data).

The *rpoD* allele alters the level of total CoA thioesters. The thiamine requirement of each strain suppressed by the *rpoD1181* allele could be satisfied by pantothenate (D. M. Downs, un-

published data). Past work showed that in some cases, the effect of pantothenate on thiamine synthesis was via an increase in CoA levels (21). Taken together, these results suggested that the *rpoD* mutation could be altering endogenous CoA levels to allow growth, possibly by increasing expression of the *panBCD* operon (Table 1). Total CoA levels were determined in multiple isogenic strains differing in the allele of *rpoD* present (Table 2). In the majority of strain backgrounds, the mutant *rpoD* allele decreased CoA levels approximately twofold. This result was not consistent with increased CoA levels being responsible for restoring thiamine-independent growth.

In contrast to other strains, the *rpoD1181* allele in the *panE* mutants increased total CoA levels approximately eightfold. However, even with this increase, the total CoA level in the *panE* strains was well below that found in the strains unable to grow independent of thiamine (Table 2). Strains lacking *panE* are proficient in pantothenate synthesis due to the ability of the IlvC enzyme to catalyze the conversion of α-ketopantoate to pantoate at a low efficiency (20). Thus, increased expression of the *panBCD* operon and the *ilvC* gene by the *rpoD1181* allele (Table 1) could be responsible for increased pantothenate (and thus CoA) levels in this strain.

The working model implicates an additional factor(s) in the conversion of AIR to HMP. Taken together, the above data do not support a model in which the *rpoD1181* allele restores thiamine synthesis simply by elevating the cellular level of CoA thioesters. In considering data presented here and elsewhere, a working model was developed. This model suggests that the expression of a gene required for optimal conversion of AIR to HMP is increased by the *rpoD1181* allele. In this scenario, the resulting gene product is involved (directly or indirectly) in the conversion of AIR to HMP and its function requires a Fe-S cluster and involves a CoA thioester.

Conclusions. This work has identified a metabolic phenotype caused by the *rpoD1181* allele. Results presented herein, in addition to previous work with the analogous *E. coli* allele, emphasize that small changes in global gene expression can result in metabolic flux changes significant enough to generate clear growth phenotypes. The suppressor allele described herein (*rpoD1181*) mediates its effect by stimulating the conversion of AIR to HMP by an as yet undefined mechanism. On the basis of the small magnitude of the transcriptional effect, the growth phenotype defined here provides the best means to identify cellular factors involved in HMP-P synthesis in this genetic background that may have eluded more standard genetic approaches.

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