

Action of the Thiamine Antagonist Bacimethrin on Thiamine Biosynthesis

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Received 17 April 2000/Accepted 17 July 2000

Bacimethrin is an analog of the 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) moiety of thiamine and inhibits the growth of *Salmonella enterica* serovar Typhimurium on a defined medium. Two classes of mutants that had increased bacimethrin resistance were isolated and characterized. Results showed that overexpression of the *thi* operon or specific lesions in *thiD* resulted in a bacimethrin-resistant phenotype. Phenotypic analyses of the *thiD* mutants suggested that they had a specific defect in one of the two kinase activities associated with this gene product and, further, that ThiD and not PdxK was primarily responsible for salvage of HMP from the medium.

Thiamine is synthesized *de novo* by many bacteria. Although the general outline of the thiamine biosynthetic pathway in enteric microorganisms is known, the individual reactions in this pathway are not yet fully understood. Thiamine pyrophosphate (TPP), the biologically active cofactor, is generated by the condensation of two independently synthesized intermediates, 4-methyl-5-(β -hydroxyethyl)thiazole (THZ) phosphate and 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) pyrophosphate. *In vivo* labeling studies demonstrated that the carbon atoms in HMP were derived from the purine intermediate aminoimidazole ribonucleotide (AIR), although the biochemical details of this conversion have not been elucidated (2). Mutations causing an unconditional requirement for exogenous HMP or thiamine map to a single gene, *thiC*. *In vivo* labeling also determined the metabolic precursors to the THZ moiety, and several genes have been implicated in this synthesis (2, 18). Our current understanding of the synthesis and salvage of TPP is presented in Fig. 1.

While the low cellular requirement for thiamine has complicated the detailed analysis of its biosynthetic pathway, this characteristic has made thiamine synthesis an attractive model system for dissecting the integration of metabolic pathways (25). Characterization of mutations that perturbed, but did not eliminate, thiamine synthesis has resulted in the identification of a number of loci required for optimal synthesis of this vitamin and has increased our understanding of the integration of thiamine biosynthesis with central cellular processes (1, 5, 6, 8, 10–12, 24b, 25, 27).

Use of metabolic inhibitors, or analogs, has provided insight in the studies of a number of metabolic pathways, including histidine (17), folate (3), and branched-chain amino acids (19–21). Bacimethrin is a thiamine antagonist produced by gram-positive bacteria, including *Streptomyces albus* (7) and *Bacillus megaterium* (29) (Fig. 1). Previous reports showed that the inhibitory effect of bacimethrin on defined medium could be

overcome by exogenous addition of thiamine or the HMP moiety (7).

Wild-type *Salmonella enterica* serovar Typhimurium strain LT2 was unable to grow on solid minimal medium in the presence of 130 nM bacimethrin, and resistant mutants arose spontaneously at a frequency of $\sim 10^{-7}$ (data not shown). Growth of the wild-type strain was restored by the addition of either HMP or thiamine, indicating a specific antagonism for the pyrimidine moiety of thiamine (7). Mutations allowing the growth of LT2 in the presence of 130 nM bacimethrin were isolated, either spontaneously or following localized chemical mutagenesis (15), in the *thi* loci at 90 min (*thiCEFSGH*) and 46 min (*thiMD*).

Increased transcription of the *thiCEFSGH* operon results in bacimethrin resistance. Two bacimethrin-resistant mutants with causative lesions at 90 min on the chromosome were characterized to determine if expression of the *thiCEFSGH* operon was altered. A DNA fragment containing the 400 bp upstream of the *thiC* initiating codon was PCR amplified from wild-type strain LT2 and bacimethrin-resistant strains DM4078 (*thi-1132*) and DM4081 (*thi-1133*). The resulting fragments were ligated in front of the promoterless *lacZ* gene in pKC1 (4), generating plasmids pThi, pThi4, and pThi8, respectively. Strains containing these plasmids, grown on medium in the presence or absence of thiamine, were assayed for β -galactosidase activity and the results are shown in Table 1. Two points could be made from these data: (i) transcription from the mutant constructs was three- to fourfold increased compared to the wild-type construct, and (ii) in both the wild-type and mutant constructs, exogenous thiamine repressed transcription. These results suggested that the causative mutations affected primarily promoter strength rather than regulation of the operon.

To confirm that increased expression of the *thiCEFSGH* operon was responsible for the bacimethrin-resistant phenotype of these strains, a multicopy plasmid carrying the entire *thiCEFSGH* operon from *Escherichia coli* was electroporated into strain LT2. This plasmid, pVJS717 (30), and not the parent plasmid (pGEM) allowed growth in the presence of 130 nM bacimethrin. Since bacimethrin is structurally similar to HMP, ThiC and ThiE were potential targets for inhibition. To address whether overexpression of *thiC* or *thiE* alone was sufficient to cause bacimethrin resistance, multicopy plasmids containing either *thiC* (pThiC) or *thiE* (pThiE) were generated, with expression of the *thi* gene from the *lacZ* promoter of

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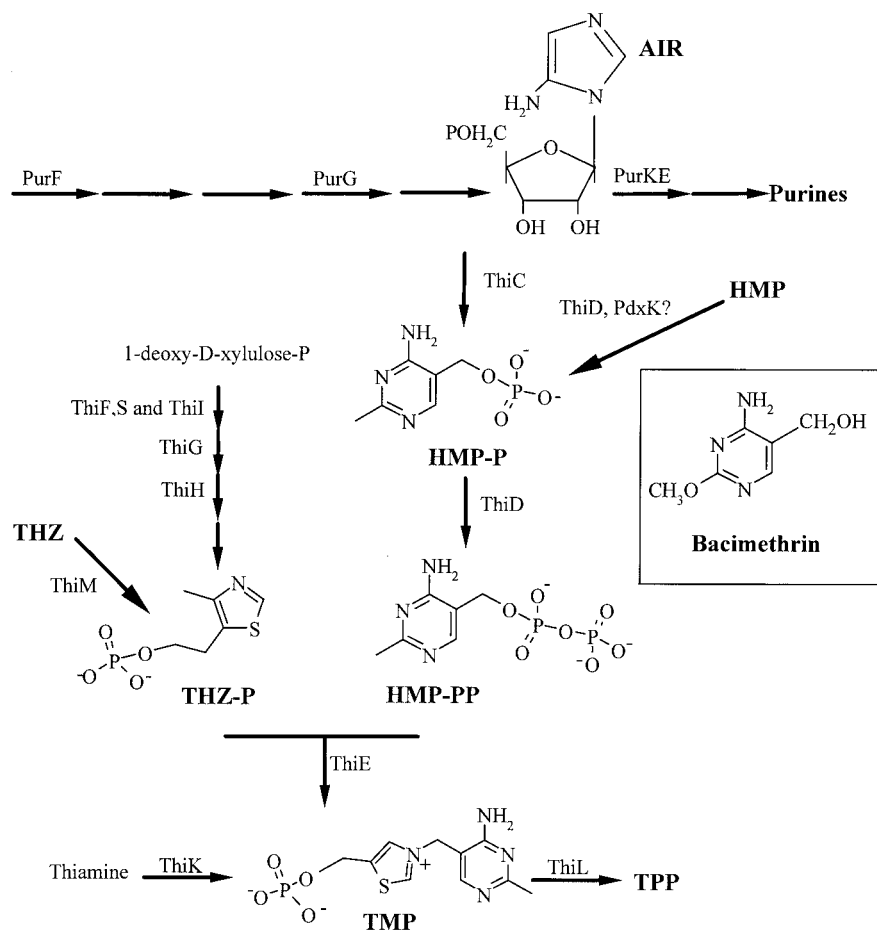


FIG. 1. Biosynthetic and salvage pathways for thiamine. The biosynthetic and salvage pathways for thiamine in *S. enterica*, including gene products where known, is represented. The structure of bacimethrin is shown in the inset. TMP, thiamine monophosphate. Other terms are as defined in the text.

pSU19 (22). Neither strain DM5717 (LT2/pThiE) nor strain DM5654 (LT2/pThiC) was able to grow on solid medium in the presence of 130 nM bacimethrin. These results suggested either that expression of the respective genes from the *lacZ* promoter in these constructs was not high enough or that overexpression of more than one gene was required to generate the bacimethrin-resistant phenotype.

Mutations in *thiD* can result in a bacimethrin-resistant phenotype. Two bacimethrin-resistant strains whose lesions mapped to 46 min on the *S. enterica* chromosome were examined in detail (Table 2). Single gene clones of the mutant alleles were generated by PCR amplifying *thiD* from the chromosome of DM2983 (*thiD1126*) and DM4147 (*thiD1125*) and ligating it into pSU19, resulting in plasmids pThiD1126 and pThiD1125, respectively. Each plasmid was electroporated into a strain carrying a null mutation in *thiD* (DM456). Both of the resulting strains (DM5556 and DM5557), but not a strain with a plasmid carrying the wild-type *thiD* gene, were able to grow on solid minimal medium in the presence of 130 nM bacimethrin. These results demonstrated that the bacimethrin-resistant mutations in strains DM2983 and DM4147 were alleles of *thiD*. Sequence analyses of plasmids pThiD1126 and pThiD1125 identified a single base change in each *ThiD* coding sequence that would result in the amino acid changes P186Q and N15S, respectively.

The *thiD* gene product has been shown to catalyze two

kinase reactions relevant to thiamine biosynthesis (2, 24a). Since both the original bacimethrin-resistant *thiD* mutants and the plasmid-containing strains were prototrophic, we concluded that the HMP-P kinase activity of *ThiD* required for de novo thiamine synthesis was present.

Increased bacimethrin resistance could theoretically result from mutant forms of *ThiD* with increased catalytic activity or

TABLE 1. *thi* promoter region from bacimethrin-resistant mutants directs increased expression^a

Plasmid	β-Galactosidase activity ^b ± SD		Fold repression
	Without thiamine	With thiamine	
pThi	2,402 ± 219	756 ± 31	3.2
pThi4	8,947 ± 1,303	1,685 ± 353	5.3
pThi8	8,933 ± 1,825	2,036 ± 515	4.4

^a Derivatives of LT2 containing the indicated plasmid were grown overnight in minimal medium containing chloramphenicol and subcultured into minimal medium with or without thiamine. Subcultures were grown to 0.3 to 0.4 A_{650} before being assayed. β-Galactosidase activity was assayed as previously described (9, 24), except that volumes were reduced 10-fold, and absorbance was read in a microtiter plate by a Spectramax Plus (Molecular Devices).

^b Activities are reported as nanomoles of *o*-nitrophenyl-β-D-galactopyranoside hydrolyzed/minute/ A_{650} ± 1 standard deviation of the mean.

TABLE 2. Phenotype of bacimethrin-resistant mutants

Strain	Genotype	Zone of inhibition (cm) with bacimethrin ^a :	
		0.13	1.3
LT2	Wild type	2.4	3.4
DM2983	<i>thiD1126</i>	0	0.2
DM4147	<i>thiD1125</i>	0	0.5

^a The diameter of the zone of inhibition was measured when 1 or 10 μ l, containing 0.13 or 1.3 pmol of bacimethrin, respectively, was spotted onto a soft agar overlay of cells on minimal medium.

increased substrate specificity. In both of these scenarios, the mutant *thiD* would be dominant to wild type. However, when plasmids pThiD1126 and pThiD1125 were electroporated into LT2, the resulting strains remained sensitive to bacimethrin, indicating that the mutant alleles of *thiD* were recessive. We reasoned that loss of the HMP kinase, but not the HMP-P kinase activity of ThiD, would eliminate the initial phosphorylation of bacimethrin, preventing it from interfering with thiamine synthesis (T. Begley, personal communication). This scenario predicted that the bacimethrin-resistant *thiD* mutants would be defective in the incorporation of exogenous HMP to thiamine. To address this, de novo HMP synthesis was blocked in strains containing *thiD1125*, *thiD1126*, and the wild-type strain LT2 by introducing a *purG* insertion mutation (Fig. 1). The minimal concentration of HMP required for growth of the resulting strains was determined. As shown by the data in Table 3, each of the strains containing a bacimethrin-resistant allele of *thiD* required an increased amount of HMP (compared to the *purG* mutant) for growth. Specifically, the *thiD1125*-containing strain required 100-fold more HMP than the *purG* single mutant. It was noted that the mutant most resistant to bacimethrin (DM2983, containing *thiD1126*) did not contain the allele that caused the most dramatic increase in HMP requirement (*thiD1125* in DM5570). There are a number of formal possibilities to explain this apparent inconsistency, including different affinities for HMP compared to bacimethrin.

These results strongly suggested that *thiD* alleles resulting in bacimethrin resistance were specifically defective in the HMP kinase activity. If confirmed by in vitro kinetic analyses of the mutant proteins, this model provides a positive selection for *thiD* mutants defective in one of the two kinase activities and supports the prediction that the active sites for the two phosphorylation reactions would be quite different (26).

A role for PdxK in HMP salvage? The prediction that the phenotypes shown in Tables 2 and 3 were caused by lack of HMP kinase activity caused us to consider the role of PdxK in HMP salvage. The *pdxK* gene was identified by the role of its gene product in the salvage of pyridoxine in the synthesis of pyridoxal 5'-phosphate synthesis (30). The in vitro analysis of PdxK demonstrated that it phosphorylated HMP with the same general kinetic parameters as it had for pyridoxine (26). Further, the K_m for HMP of PdxK was \sim 5-fold lower, and the k_{cat}/K_m ratio was 10-fold higher than the same parameters of ThiD, suggesting that in vivo PdxK would play a major role in HMP salvage (26, 30). The identification of mutations in *thiD* that resulted in a significantly increased requirement for HMP in strains carrying a wild-type *pdxK* locus suggested that if PdxK plays a role in HMP salvage, it is a minor one.

Sensitivity to bacimethrin reflects flux through the HMP branch of the thiamine biosynthetic pathway. From the above results it was formally possible that bacimethrin uptake was

inhibited by HMP and that its target was at a different metabolic step. A correlation of endogenous HMP synthesis and bacimethrin resistance would eliminate this possibility. Strains not auxotrophic for thiamine but likely to have increased or decreased HMP synthesis were assessed for bacimethrin resistance. The growth data from some of these analyses are presented in Fig. 2 and indicated that the sensitivity of a strain to bacimethrin correlated with the anticipated flux through the HMP biosynthetic pathway. The growth of wild-type strain LT2 in liquid minimal medium was not significantly affected by 516 nM bacimethrin (Fig. 2A). The difference in effective concentrations on a solid medium versus a liquid medium was noted but was not pursued since the strain differences were qualitatively the same in the two media.

Exogenous addition of purines to the growth medium has been shown to reduce flux through the biosynthetic pathway \sim 90%, due to the combined transcriptional and allosteric regulatory effects of purines on the pathway (14, 16, 23, 31). This flux reduction does not generate a requirement for thiamine in a wild-type strain (J. Zilles and D. M. Downs, submitted for publication). When purines (e.g., adenine) were included in the growth medium, wild-type LT2 was more sensitive to bacimethrin (Fig. 2B). A *purE* mutant, blocked immediately after the purine-thiamine branch point, was not sensitive to bacimethrin under the same conditions (Fig. 2C) (25).

This analysis was extended to mutants with a less-characterized effect on HMP synthesis. Mutations in *panE* reduce internal coenzyme A pools 10-fold, which results in a thiamine requirement only if flux through the purine pathway is also reduced (11). When *panE* mutants are grown on minimal medium, no growth defect is measurable, and internal metabolite pools of TPP are wild type (11). However, as shown in Fig. 2D, the *panE* mutant was sensitive to bacimethrin. A similar response was found with an *apbC* mutant that is thought to be defective in HMP synthesis (Fig. 2E) (13, 24b). Taken together, these results suggest that bacimethrin sensitivity can provide a qualitative measure of flux through the HMP biosynthetic pathway.

Conclusions. Results from this study have increased our understanding of the antagonistic effects of bacimethrin in *S. enterica* by identifying two distinct lesions resulting in resistance to this analog. We showed that increased expression of the *thi* operon or specific lesions in *thiD* allow cells to grow in the presence of increased bacimethrin. Phenotypic analysis of the *thiD* mutants suggested that the mutant proteins were defective in one of the two kinase activities associated with this protein. Analyses of these mutants suggested that the HMP-kinase activity of PdxK was the primary route for HMP salvage (26).

We thank T. Begley for providing us with bacimethrin and for

TABLE 3. Bacimethrin-resistant mutants have increased requirement for exogenous HMP

Strain	Genotype	Diam of growth (cm) after addition of HMP ^a :					
		100	50	10	5	1	0.5
DM40	<i>purG</i>	3.2	3.0	2.3	2.0	0.8	0.7 ^b
DM5571	<i>purG thiD1126</i>	2.7	2.1	1.2	1	NG	NG
DM5570	<i>purG thiD1125</i>	1.6	0.9	NG	NG	NG	NG

^a Diameter of growth zone resulting from the addition of the indicated amount of HMP (in picomoles) to a soft agar overlay of cells on minimal medium containing 0.4 mM adenine. NG, no growth.

^b Strain DM40 (*purG*) failed to grow with 0.1 pmol of HMP.

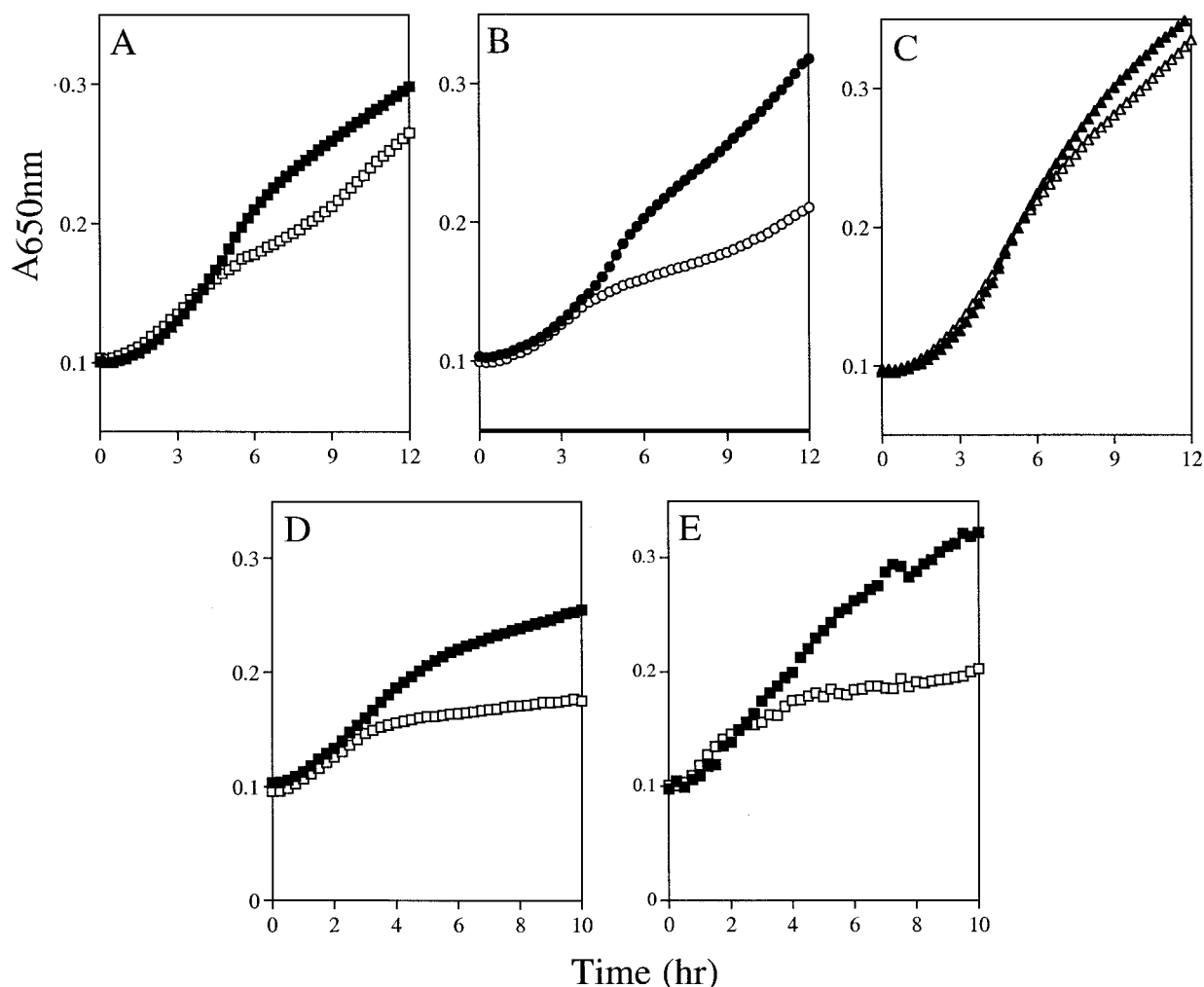


FIG. 2. Phenotypes of representative strains. Strains were grown aerobically in a microtiter plate on glucose minimal medium, and the absorbance was monitored at 650 nm on a Spectramax plate reader (Molecular Devices, Sunnyvale, Calif.). In panels B and C adenine was included at 0.4 mM. All panels compare growth on minimal medium (closed symbols) with growth in the presence of 516 nM bacimethrin (open symbols). The strains include LT2 (A and B), *purE* (C), *panE* (D), and *apbC* (E).

helpful discussion and S. Weber for technical assistance. Plasmid pVJS717 was obtained from V. Stewart.

This work was supported by competitive grant MCB9723830 from the National Science Foundation and a Shaw Scientists Award from the Milwaukee Foundation. J.L.Z. was supported by a National Science Foundation Graduate Fellowship and a Wisconsin Alumni Research Foundation Annual Fellowship.

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