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Isolation and Characterization of *Bacillus subtilis* Mutants Blocked in the Synthesis of Pantothenic Acid

MARIO BAIGORI, ROBERTO GRAU, HECTOR R. MORBIDONI, AND DIEGO DE MENDOZA*

Departamento de Microbiologia, Facultad de Ciencias Bioquimicas y Farmaceuticas, Universidad Nacional de Rosario, Suipacha 531, 2000 Rosario, Republica Argentina

Received 15 March 1991/Accepted 24 April 1991

We have produced and characterized by physiological and enzymatic analyses pantothenate (*pan*) auxotrophs of *Bacillus subtilis*. *panB* auxotrophs are deficient in ketopantoate hydroxymethyltransferase, whereas *panE* mutants lack ketopantoic acid reductase. The *pan* mutations were mapped by phage PBS1-mediated two-factor crosses and found to be located in the interval *purE-tre* of the genetic map of *B. subtilis*.

D-Pantothenate, a vitamin of the B group, is formed by condensation of β-alanine and D-pantoic acid (1) and utilized primarily for the biosynthesis of coenzyme A and acyl carrier protein. These coenzymes function in the metabolism of acyl moieties which form thioesters with the sulfhydryl group of the 4′-phosphopantetheine portion of these molecules (1).

The structural genes of all the enzymes involved in the biosynthesis of pantothenic acid in *Salmonella typhimurium* and *Escherichia coli* (1) have been identified. This is not the case for *Bacillus subtilis*, a gram-positive bacterium widely used in genetic analysis (8). Because of our interest in the biosynthesis of fatty acids in this organism and taking into account the fact that auxotrophs in the pantothenate pathway of *E. coli* greatly facilitated both the study of regulation of biosynthesis of coenzyme A (4) and the biochemical characterization of acyl carrier protein (10, 11), we attempted the isolation of pantothenate auxotrophs of *B. subtilis*. In this article, we report the isolation and characterization of two *B. subtilis* mutants blocked in the biosynthesis of pantoic acid.

The strains of *B. subtilis* used in this study are listed in Table 1. The strains were grown in LB media (7) or the mineral salts medium of Spizizen (12) supplemented with either 0.5% glucose or 0.1% trehalose. Supplements required for auxotrophs were added at 50 μg/ml for amino acids, 100 μg/ml for adenine, and 1 μg/ml for thiamine. Pantothenate and its precursors were used at 0.02%. For enzymatic analysis, cell extracts of the different *B. subtilis* strains were prepared as described previously (2). Ketopantoate hydroxymethyltransferase was assayed by the method of Teller et al. (13) as modified by Cronan et al. (2), and ketopantoate reductase was assayed by the method of King and Wilken (5). Excreted and intracellular pantothenates were purified and quantified as described by Jackowski and Rock (4).

B. subtilis 168 and BD170 were mutagenized with *N*-methyl-*N*′-nitrosoguanidine (7) and enriched for pantothenate auxotrophs with ampicillin and streptozotocin by using procedures described by Miller (7) and Lengeler (6), respectively. The isolated auxotrophs were of two distinct classes and were designated *panB* and *panE*, respectively. *panB* mutants can grow on pantothenic acid, pantoic acid, and ketopantoic acid but do not respond to β-alanine or α-ketoisovaleric acid. *panE* mutants responded only to pantoate or pantothenate. Accordingly, the two types of mutants we isolated appear to be blocked in the pathway of pantoic acid synthesis. This metabolite is formed in *E. coli* and *S. typhimurium* from α-ketoisovaleric acid by a hydroxymethyltransferase and an NADPH-dependent reductase (Fig. 1).

The results of enzymatic analysis confirm those expected from the nutritional data (Table 2). *panB* mutants were severely deficient in ketopantoate hydroxymethyltransferase, and *panE* mutants possessed about 20% of ketopantoic acid reductase activity of *panE*$^+$ strains (Table 2). Moreover, as reported in the table, no pleiotropic effects were found, each class of auxotrophs being deficient in only one enzyme. Mixtures of extracts from the mutant with active extracts gave additional activities, indicating that the effects observed could not be ascribed to an inhibitor present in *panE* or *panB* extracts (data not shown). *pan*$^+$ revertants of *panB* and *panE* mutants regained normal transferase and reductase activity, respectively (Table 2). Moreover, *pan*$^+$ transformants of strains UR1 and UR2 (the DNA was obtained from strain BD170) also showed normal levels of transferase and reductase activity, respectively (data not shown). These results indicate that a single mutation is responsible for the lack of transferase and reductase activities in *panB* and *panE* mutants, respectively.

Figure 2 shows the growth response of the *panB* strain UR2 to pantothenate. Maximum growth was achieved at 800 μM pantothenate, and similar results were obtained with a *panE* strain (data not shown). These results indicate that pantothenate requirements of *B. subtilis pan* auxotrophs are about 1,000-fold higher than those observed for *E. coli* or *S. typhimurium pan* mutants (2).

The generalized transducing phage PBS1, grown either in the *panB* or *panE* mutant, was used to transduce all the markers present in *B. subtilis* strains constructed for mapping by Dedonder et al. (3). Both the *panB* and the *panE* mutations were located in the interval *purE-tre* (Fig. 3). The linkage between *panE* and *purE* (29%) was closer than that between *panE* and *tre* (5%) (Fig. 3). *panB* was closer to *panE* (28%) than to *purE* (11%) or *tre* (12%) (Fig. 3), suggesting

* Corresponding author.

TABLE 1. Bacterial strains

Strain	Relevant genotype	Source[a] or reference
168	*trpC2*	BGSC (1A1)
BD170	*trpC2 thr-5*	BGSC (1A42)
CU4139	(*spβc*$_2$) *liv-1-82*::Tn*917 trpC2*	BGSC (1A619)
QB861	*glpK21 purE1 sacA231*	BGSC (1A156)
QB870	*glpK21 glyB133 thiA78 tre12*	BGSC (1A122)
UR1	*trpC2 thr-5 panE*	NTG mutagenesis of BD170
UR2	*trpC2 panB*	NTG mutagenesis of 168
UR3	*pan*$^+$ revertant of UR1	This study[b]
UR4	*pan*$^+$ revertant of UR2	This study
UR5	*trpC2 thr-5 panE liv-1-82*::Tn*917*	UR1 td CU4139

[a] Abbreviations: BGSC, *Bacillus* Genetic Stock Center, Ohio State University, Columbus, Ohio; td, first strain was transduced by phage PBS1 grown in the second strain; NTG, *N*-methyl-*N'*-nitrosoguanidine.

[b] *pan*$^+$ revertants of *panE* or *panB* mutants were obtained by diluting overnight cultures into saline and plating 10^8 cells onto minimal agar medium not supplemented with pantothenate.

TABLE 2. Enzymatic characterization of pantothenate auxotrophs[a]

Strain	Characteristic(s)	Sp act (U/mg of protein)	
		Transferase	Reductase
168	*pan*$^+$	2.62	42.35
BD170	*pan*$^+$	2.50	31.08
UR1	*panE*	2.54	6.43
UR2	*panB*	0.02	32.00
UR3	*pan*$^+$ revertant of UR1	ND[b]	25.37
UR4	*pan*$^+$ revertant of UR2	2.58	ND
UR5	*panE liv-1*	ND	0.00

[a] The enzymes assayed were ketopantoate hydroxymethytransferase and ketopantoic acid reductase. A unit of the first enzyme is 1 nmol of $H^{14}CHO$ incorporated per min, whereas a unit of the reductase is 1 nmol of $NADP^+$ formed per min.

[b] ND, not determined.

that the order is *purE-panE-panB-tre*. We performed only two-factor transduction crosses since no markers with phenotypes selectable in transduction have been reported in the region between 65° and 75° of the genetic linkage map of *B. subtilis* (8).

The results presented in this article define two new genetic loci encoding enzymes involved in the biosynthesis of pantothenic acid in *B. subtilis*. Physiological and enzymatic criteria indicate that *panB* mutants are deficient in ketopantoate hydroxymethyltransferase and that *panE* mutants lack ketopantoic acid reductase. In *S. typhimurium*, the reduction of ketopantoate to pantoate can be catalyzed by both ketopantoate reductase and acetohydroxy acid isomeroreductase (the product of the *ilvC* gene which catalyzes the second common step in isoleucine and valine biosynthesis) (9). Accordingly, for the isolation of *S. typhimurium panE* auxotrophs, it was necessary to first introduce a mutation in the *ilvC* gene (9). As shown in this study, the expression of the *panE* phenotype in *B. subtilis* does not require the use of an *ilvC* mutation (Table 1). The low levels of ketopantoate reduction in UR1 extracts (Table 2) are probably due to isomeroreductase activity, since extracts of strain UR5, which is derived from UR1 and bears an *ilvBC* deletion, completely lacked ketopantoate reductase activity (Table 2). However, *panE* mutants of *B. subtilis* are unable to grow in the absence of pantothenate even with 2 mM α-ketopantoate in the culture media (data not shown). These results indicate that unlike *S. typhimurium* (9), *B. subtilis* does not use the

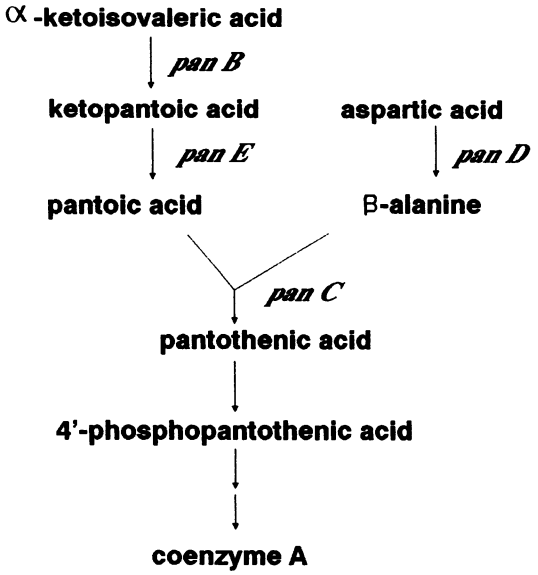

FIG. 1. Pantothenate synthesis in *E. coli* and *S. typhimurium*. The loci believed to code for the enzymes catalyzing the steps are in italics. The *panB* step is catalyzed by ketopantoate hydroxymethyltransferase; the *panE* step is catalyzed by ketopantoate reductase; the *panC* step is catalyzed by pantothenate synthetase; and the *panD* step is catalyzed by aspartate 1-decarboxylase.

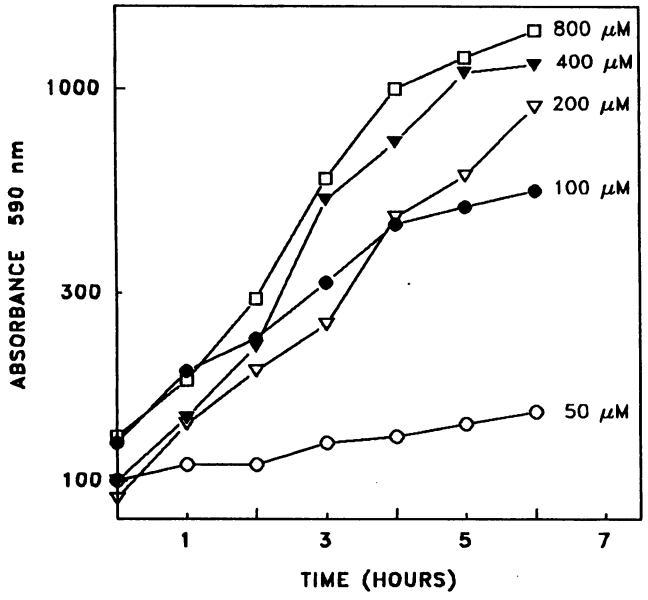

FIG. 2. Growth response of UR2 to pantothenate. Strain UR2 was grown overnight at 37°C in minimal medium containing 800 μM pantothenate. Cells were washed, suspended in the same medium without pantothenate, and incubated for 6 h until growth had ceased. Approximately 10^6 cells were inoculated into 10 ml of minimal medium containing the indicated amount of filter-sterilized pantothenate. Cultures were incubated at 37°C, and bacterial growth was monitored spectrophotometrically at 590 nm.

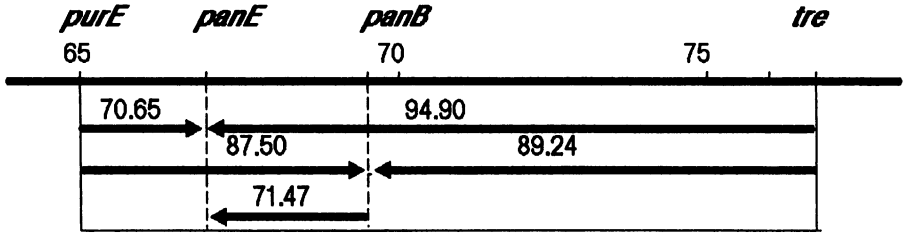

FIG. 3. Portion of the genetic map of *B. subtilis* containing the *pan* mutations. The numbers represent the percentages of recombination, which are obtained with the equation 1−(cotransduction frequency) × 100. Arrows are drawn from selected to unselected markers.

isomeroreductase as an alternative enzyme to catalyze the conversion of ketopantoate to pantoate even in the presence of abundant quantities of α-ketopantoate in the culture media.

Maximum growth of *B. subtilis pan* mutants was achieved at 800 μM pantothenate (Fig. 2). This and the fact that we could not detect appreciable transport of D-[1-^{14}C]pantothenate using a variety of conditions and strains (unpublished observations) suggest the absence of an efficient transport system for this metabolite in *B. subtilis*. We have also determined that the medium from stationary-phase cultures of strain 168 contains about 1,000 pmol of pantothenate per 10^8 cells, while the intracellular pool of pantothenate is about 5 pmol/10^8 cells (data not shown). These data indicate that pantothenate is efficiently effluxed from *B. subtilis*, a situation similar to that found with *E. coli* (4). It is also worth mentioning that *panF* mutants of *E. coli* are defective in pantothenate uptake but conserve a mechanism for efficient pantothenate efflux (14). These results suggest that a similar mechanism operates in *B. subtilis*, in which a separate carrier may catalyze efflux. It is necessary to note that the *pan* mutants we have isolated will not be suitable for specific labeling of intracellular pools of coenzyme A and acyl carrier protein because of the high concentrations of pantothenate required to supplement the cells (Fig. 2). For this purpose, it would be necessary to isolate β-alanine auxotrophs.

Genetic and enzymological experiments indicate that a single mutation is responsible for the lack of transferase and reductase in *panB* and *panE* mutants, respectively, and that both genes are located in the interval *purE-tre* of the *B. subtilis* chromosome (Fig. 3). An analysis of DNA fragments adjacent to these *pan* genes will help to clarify whether other *pan* loci are clustered in this region.

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