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Isolation of Temperature-Sensitive Pantothenate Kinase Mutants of *Salmonella typhimurium* and Mapping of the *coaA* Gene

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Temperature-sensitive pantothenate kinase mutants of *Salmonella typhimurium* LT2 were selected by using the excretion of pantothenate at the nonpermissive temperature as a screening method. Thermolability of the pantothenate kinase activity in extracts of the mutants was demonstrated. The mutations were mapped at min 89 of the *Salmonella* chromosome, near *rpoB*, by transduction. As pantothenate kinase catalyzes the first step in the biosynthesis of coenzyme A from pantothenate, the new genetic locus has been designated *coaA*.

Coenzyme A is biosynthesized from pantothenic acid via a five-step pathway (14). The first step of the pathway is the phosphorylation of pantothenate to produce 4'-phosphopantothenate, a reaction catalyzed by pantothenate kinase (EC 2.7.1.33) (6). Pantothenate kinase activity has been studied in extracts prepared from bacteria, including *Escherichia coli*, *Proteus morganii*, and *Lactobacillus arabinosus* (6, 13, 20), and from mammalian sources, such as rat liver (1, 3). Abiko and co-workers have extensively studied the other enzymes involved in the synthesis of coenzyme A in rat liver (1, 2, 4, 18).

Mutants of both *E. coli* and *Salmonella typhimurium* unable to synthesize pantothenate have been isolated, and the genetic loci have been mapped (7, 8, 12). However, there have been no previous reports of mutants of these organisms which lack the ability to synthesize coenzyme A from pantothenate. It is possible that these bacteria are unable to transport coenzyme A or any of the biosynthetic intermediates, which would make such mutations lethal unless they were of a conditional nature.

In the course of a study of the pantothenate transport system of *S. typhimurium* LT2, we observed rapid metabolism of the accumulated pantothenate. To avoid this complication, we isolated temperature-sensitive pantothenate kinase mutants for use in the uptake studies. As these mutations affect the first step in the biosynthesis of coenzyme A, the genetic locus has been designated *coaA*. The isolation and characterization of these mutants and the genetic mapping of *coaA* are described in this paper.

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MATERIALS AND METHODS

Bacterial strains and growth medium. The *argE94*, *panC3*, *rpoB43*, and *thi-502* derivatives of *S. typhimurium* LT2 were kindly provided by G. F.-L. Ames.

The minimal medium (VBCG) used throughout this study was the citrate medium E of Vogel and Bonner (19) supplemented with 0.4% glucose and the trace element mixture described by Ames (5).

Selection of mutants. Wild-type *S. typhimurium* LT2 was mutagenized to a 30% survival rate with 1-methyl-3-nitro-1-nitrosoguanidine by the method described by Roth (16). The cells were grown at 27°C on medium VBCG overnight, diluted to a density of 6×10^6 cells per ml in fresh medium at 40°C, and grown for 4 h at that temperature. The culture was then enriched for temperature-sensitive mutants by growth in the presence of penicillin (20,000 U/ml) for 2 h at 40°C. Survivors were screened for pantothenate excretion by the following method: approximately 500 of the survivors were mixed with 5×10^8 cells of the *panC3* strain in molten top agar and poured onto VBCG plates. The plates were incubated at 30°C for 36 h to allow temperature-sensitive colonies to develop; then they were shifted to 40°C for further incubation. Periodically over the next 2 days the plates were inspected with a dissecting microscope for the presence of colonies which had stimulated growth of the lawn of the *panC3* strain around them. Apparent pantothenate excretors were purified by streaking on nutrient agar plates and tested for temperature sensitivity and pantothenate excretion.

Assay of pantothenate kinase activity. For the preparation of cell-free extracts for pantothenate kinase studies, 1-liter cultures were grown with vigorous shaking at 30°C to late log phase. The cells were harvested, washed, suspended in 2 volumes of 250 mM Tris-hydrochloride, pH 7.5, and broken by sonication for 2 min. After centrifugation for 40 min at 36,000 × *g*, the supernatant solutions were dialyzed for 8 h against 50 volumes of 25 mM Tris-hydrochloride, pH 7.5, at 4°C.

Pantothenate kinase activity was assayed by following the conversion of [1-^{14}C]pantothenate to 4'-phospho[1-^{14}C]pantothenate. Crude extract (0.2 to 0.6 mg of protein) was added to 0.2 ml of 25 mM Tris-hydrochloride, pH 7.5, containing 2 mM $MgCl_2$. The reaction was started by adding ATP and [1-^{14}C]pantothenate (10 mCi/mmol) to final concentrations of 2 mM and 10 μM, respectively. The final volume was 0.25 ml. After 5 and 10 min, 0.1-ml samples were mixed with 0.5 ml of 50% ethanol–50% 0.1 M acetic acid to quench the reaction. Each sample was analyzed on a column (0.5 by 2 cm) of Bio-Rad AG1-X2 (Cl^- form). The loaded columns were washed with 10 ml of 0.1 M acetic acid to elute the labeled pantothenate. The labeled 4'-phosphopantothenate was eluted with 0.1 N HCl and counted with 10 ml of toluene–Triton X-100 scintillation fluid (9). The assay was reasonably linear up to protein concentrations of 4 mg/ml and for times up to 30 min.

Other methods. Temperature sensitivity was tested by applying cells to medium VBCG plates and scoring for growth after incubation at 30 or 40°C for 24 h. Rifampin resistance was tested by streaking cells away from a filter disk containing 40 μg of rifampin on a medium VBCG plate.

Complementation of mutations by episomes was tested by mixing drops of the episome donors and the temperature-sensitive strains on a plate which was incubated at 40°C. Complementation was indicated by the growth of discrete colonies where the strains were mixed.

Phage P22-mediated transductional crosses were done by spreading 0.1 ml of a freshly grown culture of the recipient strain and 10^9 phages grown on the donor strain on a medium VBCG plate. Strain DD12 was obtained by mixing the *argE94* strain with phage grown on strain DD2 [*coaA2*(Ts)] and screening for temperature-sensitive transductants.

Protein was determined by the method of Lowry et al. (11).

RESULTS

Mutant selection and characterization. Of 6,000 colonies screened, 11 which stimulated haloes of growth of the *panC3* lawn were observed. Due to the small size of the colonies and haloes, a dissecting microscope was helpful. Seven of the mutants, DD1, DD2, DD6, and DD8 through DD11, were temperature sensitive, growing well at 30°C, but not at 40°C. The temperature sensitivity and pantothenate excretion of these strains were retained through repeated purification. The other mutants grew very slowly at 30°C and were not studied further.

As many enzymes are protected from thermal denaturation by the presence of their substrates, the effect of a high level of pantothenate (1 mM) on the temperature sensitivity of the strains was tested in liquid culture. Strains DD6, DD9, and DD10 grew at 40°C in medium VBCG containing the vitamin, but did not grow in unsupplemented medium at this temperature. The temperature sensitivity of the other strains was unaffected by the pantothenate. None of the strains was able to grow at 40°C in medium containing coenzyme A at concentrations of up to 100 μM. In contrast, pantothenate-requiring strains, such as *panC3*, grow normally in the presence of 1 μM pantothenate.

Thermolability of mutant pantothenate kinase in vitro. The pantothenate kinase activity in crude extracts of the mutants and the parental strain LT2 was assayed at 30 and 40°C (Table 1). As the thermolability of the enzymes might not be expressed immediately, the diluted extracts were incubated at the assay temperature for 10 min before addition of the substrates. The results demonstrate a pronounced thermolability of the mutant enzymes (strain DD9 excepted) at the nonpermissive temperature, whereas the wild-type enzyme showed a temperature coefficient of the expected magnitude for a stable enzyme. Even at 30°C, the permissive temperature, the kinase activity of the mutants was much lower than that of the wild type. This may simply reflect an instability of the mutant enzymes, as they were sensitive to extended dialysis or freezing, whereas the parental activity was relatively unaffected by these treatments.

Genetic mapping. Initial mapping of *coaA2* in strain DD2 was done with a set of episome donors carrying sections of the chromosome on their F' factors. The *coaA2* mutation was complemented by F' 111 (carried by *E. coli* CGSC4258), which covers min 80 to 91 of the *Salmonella* map.

In transduction crosses mediated by phage P22, *coaA2* was cotransducible with *argE94*, *thi-502*, and *rpoB43* at min 89 (Table 2). This section of the chromosome has been studied by Hong and Ames (10), who found that *rpoB* lies between *argE* and *thi*. The cotransduction fre-

TABLE 1. *Thermolability of the pantothenate kinase activity of the temperature-sensitive mutants*

Strain	Pantothenate kinase activity[a] at:		40:30[b]
	30°C	40°C	
LT2	123	203	1.7
DD1	25.4	4.8	0.18
DD2	26.4	4.1	0.15
DD6	34.0	4.3	0.12
DD8	52.4	7.5	0.14
DD9	88.0	142	1.6
DD10	23.2	12.0	0.51
DD11	23.3	12.8	0.54

[a] 4'-P-pantothenate formed (picomoles per minute per milligram of protein).

[b] Ratio of activities at the two temperatures.

TABLE 2. *Mapping of mutations by transduction*[a]

Donor strain	Recipient strain	Recombinants (donor phenotype/total)	Cotransduction (%)
DD1 [*coaA1*(Ts)]	*argE94*	4/31	13
DD2 [*coaA2*(Ts)]	*argE94*	15/82	18
DD2 [*coaA2*(Ts)]	*thi-502*	6/73	8
DD6 [*coaA6*(Ts)]	*argE94*	3/24	13
DD8 [*coaA8*(Ts)]	*argE94*	2/26	8
DD9 (?)	*argE94*	34/150	23
DD10 [*coaA10*(Ts)]	*argE94*	3/25	12
DD11 [*coaA11*(Ts)]	*argE94*	3/25	12
rpoB43	DD12 [*coaA2* (Ts)]	49/73	67

[a] All crosses were incubated at 30°C, except *rpoB43* × DD12, which was incubated at 40°C.

quencies shown in Table 2 are consistent with their map and indicate that *coaA* is located very near *rpoB*. All of the other *coaA* mutations, and also the mutation in strain DD9, were cotransducible with *argE94*.

DISCUSSION

The screening method devised for the isolation of pantothenate kinase mutants was based on the assumption that in the absence of this activity the internal level of pantothenate would rise due to continued biosynthesis and that some of the vitamin would leak from the cells. The validity of this assumption is supported by the observations made by Powers and Snell (15) in a study of ketopantoate hydroxymethyltransferase, the first enzyme unique to pantothenate biosynthesis. They found that relatively high levels of pantothenate were required for effective feedback inhibition of this enzyme. In addition, the enzyme was not subject to repression or induction. These results suggested that a substantial increase in intracellular pantothenate levels would not prevent further synthesis of the vitamin. The lawn of the *panC3* strain provided a sensitive indicator for identifying colonies which excrete pantothenate. This strain is well suited for this application, since it neither forms haloes around wild-type colonies, as *panB* mutants do (S. D. Dunn and E. E. Snell, unpublished observations), nor reverts.

As shown in Table 1, the pantothenate kinase activity in crude extracts of six of the seven mutants was thermolabile, indicating that those mutations are in the structural gene for pantothenate kinase. Three of the genes coding for pantothenate biosynthesis map in a cluster, *panABC*, at min 5 of the *Salmonella* chromosome (7, 17). A fourth gene, *panD*, which is required for the biosynthesis of the β-alanine moiety of pantothenate, maps at min 89 (12), as does *coaA*. It remains to be determined whether other genes involved in coenzyme A biosynthesis are clustered at this position. The isolation of mutants which are blocked in these other steps may not be straightforward, however, as the inability of coenzyme A to support the growth of the pantothenate kinase mutants indicates that this cofactor cannot be accumulated from the medium by *S. typhimurium*.

Apparently, the mutation of strain DD9 does not affect the *coaA* gene, as the pantothenate kinase activity of this mutant was not thermolabile in vitro. However, three lines of evidence suggest that the defect of this strain is related to pantothenate and coenzyme A: (i) pantothenate is excreted at the nonpermissive temperature; (ii) the temperature sensitivity of the strain is suppressed in medium containing 1 mM pantothenate; (iii) the mutation maps near *coaA*. As many sets of related genes map in clusters, it is possible that strain DD9 is blocked at the nonpermissive temperature either in the synthesis of pantothenate kinase or in some subsequent step of coenzyme A biosynthesis. In the latter case, feedback inhibition of pantothenate kinase by the accumulated intermediate might account for the excretion of pantothenate at the nonpermissive temperature. In this regard, the pantothenate kinase of rat liver is strongly inhibited by both pantothenoylcysteine 4′-phosphate and pantetheine 4′-phosphate (3).

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