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β-Alanine Auxotrophy Associated with *dfp*, a Locus Affecting DNA Synthesis in *Escherichia coli*

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Strains containing the conditional-lethal *dfp-707* mutation, which have a defect in DNA synthesis at 42°C, were found to require either pantothenate or its precursor, β-alanine, for growth at 30°C. The auxotrophy and conditional lethality were corevertible. Through localized mutagenesis of the *dfp-pyrE* region of *Escherichia coli*, another mutation, *dfp-1*, was obtained. It conferred the auxotrophy but not the conditional lethality of *dfp-707*. Complementation analysis, performed with a set of plasmid-borne deletion and insertion mutations, revealed a correspondence between the complementation of each mutant phenotype and the production of the *dfp* gene product, previously identified as a 45-kilodalton flavoprotein. The *dfp* mutants had a normal level of aspartate-1-decarboxylase, which is the only enzyme known to produce β-alanine in *E. coli* and which is specified by the distant *panD* gene. A prototrophic pseudorevertant of a *dfp-1* strain was found to have retained the *dfp* mutation, to be genetically unstable, and to have an elevated level of aspartate-1-decarboxylase, suggesting that it had acquired a duplication of *panD*. It is not known what steps in pantothenate or DNA metabolism are affected by the mutant *dfp* product or how its flavin moiety may be involved.

The *dfp* gene of *Escherichia coli* is located close to *pyrE* and codes for a 45-kilodalton flavin mononucleotide-containing protein of unknown function (22). The *dfp-707* mutation (originally designated *dna-707*) is conditionally lethal and selectively causes a slow cessation of DNA synthesis at 42°C (21, 22). It could not be determined whether the block was in the initiation or elongation phase of DNA replication. Because the protein contains a flavin cofactor, it may be involved in an oxidative pathway. In this report, we describe an additional unusual property of the gene: it affects pantothenate metabolism.

MATERIALS AND METHODS

Strains and plasmids. The bacterial strains and the parental plasmids used in this study are listed in Table 1. Mutant plasmids are shown in Fig. 1; except for pES40, all have been described previously (22). Plasmid pES40 was produced by digesting pES24 with *Sst*I (an isoschizomer of *Sac*I) and religating it. It contains a deletion extending from the *Sac*I site in *dut* to the *Sac*I site in the proximal end of the inserted Tn*1000*.

Media and chemicals. Tryptone-yeast (TY) medium, VB minimal medium, and nutritional supplements were as described previously (24). VB medium was supplemented with 0.2% Norit-treated (23) or vitamin-free Casamino Acids (Difco Laboratories) when selecting for *pyrE*$^+$ (uracil-independent) strains. Growth and selection of plasmid-bearing strains were performed in TY medium plus ampicillin. Pantoyl lactone, sodium pantothenate, and β-alanine were obtained from Sigma Chemical Co.

Strain and plasmid construction. P1-mediated transduction (24) and transformation with plasmid DNA (7) were as described before. Methods used for restriction enzyme digestion, ligation, and restriction mapping of plasmids were those of Maniatis et al. (16).

Localized mutagenesis of the *pyrE* region. For localized mutagenesis of the *pyrE* region, the method of Hong and Ames (10) was used. Phage P1 *vir* was grown on strain KL16 (*pyrE*$^+$), and the lysate was treated with hydroxylamine to a survival of 10^{-4}. The mutagenized phages and transducing particles were adsorbed to BW322 (*pyrE*) in the presence of Ca^{2+} (24). To permit segregation of recessive markers, the cells were grown overnight at 30°C in TY broth containing 20 mM citrate (to prevent reinfection) before being plated on selective medium (VB plus Norit-treated Casamino Acids) at 30°C. The transductants were replica plated on the same medium at 42°C.

Complementation analysis. The test for complementation of the conditional lethality associated with *dfp-707* was as described previously (22). Briefly, *dfp-707* strains bearing various mutant plasmids were tested for their ability to grow at 30, 37, and 42°C by patch testing diluted cultures on TY plates containing ampicillin. The pantothenate requirement of these strains was tested at 30°C on VB agar containing required amino acids with or without pantothenate (or β-alanine). Plasmid-bearing *dfp-1* strains were similarly tested for growth at 30 and 42°C on VB agar containing Norit-treated vitamin-free Casamino Acids with or without pantothenate (or β-alanine). The cells were also patch tested for growth on TY-ampicillin agar to ensure that lack of complementation was not due to loss of the plasmid.

Analysis of plasmid-specified proteins. Plasmid-specified proteins were radiolabeled via the maxicell technique (20) and visualized after electrophoresis and fluorography (22).

Preparation of extracts and enzyme assays. Strains N100(pKC16) and N100(pWB11), which bear thermoinducible plasmids, were harvested 5 h after heat induction (23). Other bacteria were grown under the stated conditions, and growth was monitored with a Klett colorimeter (23). Cell extracts were prepared as described by Cronan (5), except that a sonicator was used instead of a French press. Protein concentrations were determined by the method of Lowry et

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TABLE 1. *E. coli* strains and plasmids

Designation	Relevant genotype[a]	Source or reference[b]
Bacteria		
AB354	*panD2*	CGSC; 5
AT1371	*panC4*	CGSC; 5
AT2538	*pyrE60*	CGSC
BW230	*zib-205*::Tn*10*	9
BW322	KL16 *pyrE zia-207*::Tn*10*	23
BW412	*pyrE60 zib-205*::Tn*10*	P1(BW230) × AT2538
BW430	BW322 $pyrE^+$ *dfp-1*	This paper
BW447	W3110 *dfp-1*	P1(BW430) × ES31
BW448	AT2538 $pyrE^+$ *dfp-1*	P1(BW430) × BW412
ES31	W3110 *pyrE zia-207*::Tn*10*	22
ES33	W3110 *dfp-707*	P1(SG1019) × ES31
ES37	W3110 *dfp-707 recA56 srlC*::Tn*10*	P1(NK5304) × ES33
ES107	AT2538 $pyrE^+$ *dfp-707*	22
ES108	AT2538 $pyrE^+$	22
KL16	$pyrE^+$	CGSC; 3
N100	*recA*	M. Gottesman
NK5304	*recA56 srlC*::Tn*10*	N. Kleckner
SG1019	*dfp-707*	21
UB1005	pan^+ dfp^+	CGSC; 11
W3110	Prototroph	ATCC; 3
YA139	*panB6*	CGSC; 5
Plasmids		
pKC16	Thermoinducible λ-pKC7[c] chimera; Ap^r *cI857*	18
pWB1	*dfp-dut* region cloned in pKC7; Ap^r	23
pWB11	As pKC16, but containing *dfp-dut* region of pWB1	23

[a] All bacteria are derivatives of *E. coli* K-12. For a more complete description of many of these strains, see reference 22.
[b] CGSC, *E. coli* Genetic Stock Center, Yale University School of Medicine, New Haven, Conn.; ATCC, American Type Culture Collection, Rockville, Md. P1 transductional crosses are described as follows: P1(donor) × recipient.
[c] Plasmid pKC7 (Ap^r) is a derivative of pBR322 (19).

al. (12), with bovine serum albumin as the standard. Aspartate-1-decarboxylase (ADCase) (thin-layer chromatography assay [5, 6]) and pantothenate synthetase (6) were measured as described previously.

RESULTS

Pantothenate requirement of *dfp-707* mutants. The original *dfp* mutant, strain SG1019 (*dfp-707*), was temperature sensitive for growth on rich media due to a defect in DNA synthesis (21, 22). In addition, SG1019 differed from its unmutagenized parent in that it required β-alanine or pantothenate for growth on minimal media at 30°C (B. J. Smith-White, E. Spitzer, and B. Weiss, unpublished data). The conditional lethality of the *dfp-707* mutation did not appear to be a direct result of the auxotrophy; *dfp-707* strains would not grow at 42°C on rich media even when supplemented with 1 mM pantothenate. Preliminary experiments revealed that the auxotrophy could be complemented by plasmid pWB1, which carries the wild-type *dfp* gene. The *dfp-707* allele was transduced into strains with three different genetic backgrounds: W3110, UB1005, and AT2538. In none of these crosses could the β-alanine requirement be separated from the temperature-sensitive growth phenotype.

Isolation of *dfp-1*. In an attempt to isolate new temperature-sensitive *dfp* mutants, we used the localized mutagenesis technique of Hong and Ames (10), taking advantage of the close linkage between *dfp* and *pyrE*. Hydroxylamine-treated P1 phages were used to transduce BW322 to $pyrE^+$ at 30°C. Transductants were then tested for temperature-sensitive growth. Of the 20,000 transductants screened by replica plating, only one was unable to grow on a minimal medium at 42°C. Unlike the *dfp-707* mutants, however, the new mutant was not temperature sensitive for growth on rich (TY) media. The temperature-sensitive auxotrophy could not be satisfied by uracil or thymidine, requirements of mutants for the closely linked *pyrE* and *dut* genes, respectively. The mutation was 94% cotransducible with *pyrE* and was complemented by the dfp^+ plasmid pWB1. Based on

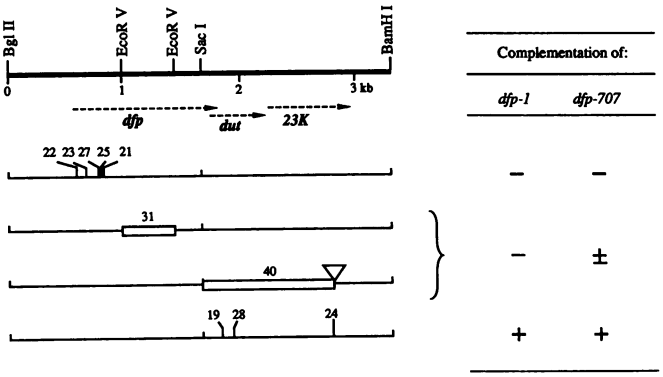

FIG. 1. Complementation of chromosomal *dfp* mutations by mutant plasmids. At the upper left is a restriction map of the chromosomal DNA in plasmid pWB1 (22). The *Bam*HI site is within the *pyrE* gene. The numbers identify the sites of mutation in plasmids identified by the corresponding pES numbers. The numbered vertical lines are Tn*1000* insertions. Rectangles are deletions terminating at restriction sites. The triangle in pES540 represents a remnant of Tn*1000*. + indicates complementation of the auxotrophy of *dfp-1* or of the conditional lethality of *dfp-707* at 42°C. ± (partial complementation) indicates growth at 37°C but not at 42°C. (Data other than that referring to pES40 or to the complementation of *dfp-1* are from reference 22.) kb, Kilobases.

our studies of *dfp-707*, we tested β-alanine and pantothenate and found that both compounds satisfied the temperature-sensitive auxotrophy specified by the new mutation, which we designated *dfp-1*.

Complementation analysis. Nutritional studies and transduction data suggested that the *dfp-1* and *dfp-707* mutations were in the same gene. This was confirmed by complementation analysis with insertion and deletion mutants of pWB1, a recombinant plasmid bearing the chromosomal *dfp* region. The mutant plasmids were tested for complementation of the auxotrophy of *dfp-1* and for complementation of the conditional lethality of *dfp-707* (Fig. 1). All mutations between the insertion site in pES22 and the *Sac*I site affected both *dfp-1* and *dfp-707* complementation, whereas all mutations outside of this region displayed wild-type complementation. Plasmids that failed to complement *dfp-1* were also tested for their ability to complement the auxotrophy of *dfp-707*. Plasmid pES27 failed to complement the auxotrophy at 30°C, whereas pES31, which complements the growth defect of *dfp-707* at 37°C but not at 42°C, complemented the auxotrophy at 30 and 37°C.

Mutations at the 5′ end of the gene led to a complete loss of ability to complement the auxotrophic and growth phenotypes of *dfp-1* and *dfp-707*. Mutations at the 3′ end led to complete loss of *dfp-1* complementation, but only partial loss of the ability to complement the auxotrophy and conditional lethality of *dfp-707*. It is possible that the *dfp* gene product has two functional domains; however, other explanations are possible. There may be partial intra-allelic complementation between the products of *dfp-707* and those of the mutant plasmids, or the auxotrophy might be selectively affected by partial inactivation of the gene.

***dfp* and *dut* overlap.** It was previously shown that mutant plasmids that did not fully complement *dfp-707* also failed to produce a 45-kilodalton protein that was identified as the *dfp* gene product (22). This was also true of pES40, a new plasmid that was similarly studied with maxicell preparations (data not shown). Plasmid pES40, which contains a deletion that extends to the *Sac*I site, failed to complement *dfp-1* and only partially complemented *dfp-707* (Fig. 1). The *Sac*I site is known to be within the *dut* gene promoter (13, 14). Therefore, *dfp* and *dut* probably overlap. This conclusion is consistent with the DNA sequence, which contains a long open reading frame overlapping the first 17 nucleotides of *dut* (14).

Comparison with *pan* mutants. In *E. coli*, the *panB*, *panC*, and *panD* genes are required for pantothenate synthesis (Fig. 2). The *panBDC* cluster is located at 3 min on the genetic map (4), whereas the *dfp* gene is at 82 min (22). The pantothenate auxotrophy of *panB* mutants can be satisfied by pantoic acid and that of *panD* mutants can be satisfied by β-alanine, whereas *panC* mutants grow on neither compound (6). Another locus, *panE*, has been identified in *Salmonella typhimurium*; *panE* mutants can be satisfied by either pantoic acid or pantothenate (17). Both *dfp* and *panD* mutants required a minimum of 0.45 μM pantothenate or 45 μM β-alanine for growth on solid minimal media. D,L-Pantoyl lactone (at 200 μM) permitted the growth of a *panB* mutant but not that of the *dfp* mutants. Therefore, the nutritional requirements of *dfp-707* and *dfp-1* strains correspond to those of *panD* mutants.

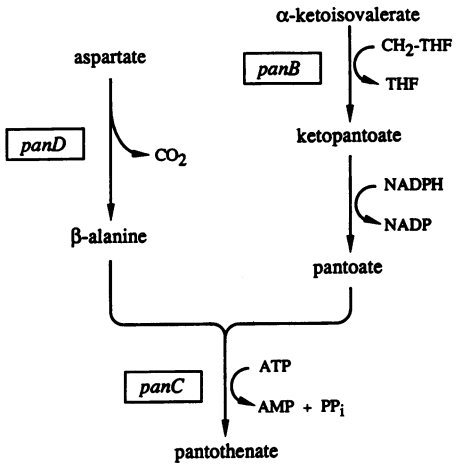

FIG. 2. Pantothenate synthesis in *E. coli*. *panD* codes for ADCase, *panB* codes for ketopantoate hydroxymethyltransferase, and *panC* codes for pantothenate synthetase. Pantoic acid can be formed via ketopantoate reductase or ketopantoyl lactone reductase. (For review, see reference 6.) THF, Tetrahydrofolate.

ADCase activity. Unlike *panD* mutants, which lack ADCase (Fig. 2) (5), *dfp* mutants contained wild-type levels of ADCase, even when grown at 42°C and assayed at 55°C (Table 2). In experiment 3 (Table 2), the cells were collected at a point when they were displaying their phenotypic defect, i.e., cessation of growth due to nutritional limitation. Nevertheless, the *dfp-1* strain had a level of ADCase similar to that of a *panC* (pantothenate synthetase) mutant, the growth of which was similarly limited.

TABLE 2. Levels of ADCase in mutant strains

Expt	Growth conditions	Assay conditions	Strain	Genotype	Sp act (pmol/min per mg of protein)
1	TY medium, 42°C[a]	42°C, 1.5 h	ES108	Wild type	12.2
			BW448	*dfp-1*	16.5
2	TY medium, 30–42°C shift[b]	55°C, 1 h	ES108	Wild type	21.4
			ES107	*dfp-707*	14.0
			AB354	*panD2*	0.2
3	Pantothenate-free medium, 42°C[c]	55°C, 1 h	AT1371	*panC4*	6.4
			AB354	*panD2*	0.4
			BW448	*dfp-1*	5.1

[a] Saturated cultures were diluted into 100 volumes of fresh medium and grown to 150 Klett units (5×10^8 cells per ml).
[b] Cultures were grown at 30°C to 30 Klett units and then at 42°C to 150 Klett units. The growth rates of two strains were similar.
[c] VB minimal medium contained 0.4% glucose, thiamine (1 μg/ml), and 0.2% Norit-treated vitamin-free Casamino Acids. Cultures were grown overnight at room temperature, diluted into fresh medium (50 volumes for BW448, 30 volumes for the others), and then grown to an apparent limit of 80 to 100 Klett units in 4.5 to 5.5 h.

ADCase activity was also measured in a strain that overproduces the *dfp* gene product. Plasmid pWB11 is a derivative of pWB1 that is thermoinducible for replication. Strain N100(pWB11) overproduces dUTPase 300-fold after thermal induction (23) and has been shown to overproduce the 45-kilodalton flavoprotein that was identified as the *dfp* gene product (22). The level of ADCase in heat-induced N100 (pWB11) was 5.6 U/mg, whereas the level in similarly treated N100(pKC16), which lacks the plasmid *dfp* gene, was 6.1 U/mg. Therefore, overproduction of the *dfp* gene product did not result in an increase in ADCase activity.

Prototrophic pseudorevertants. *dfp-1* mutants gave rise to prototrophic revertants at a frequency of 10^{-4} to 10^{-5} when plated at 42°C on minimal media in the absence of β-alanine or pantothenate. The high frequency suggested that the apparent reversion was due to forward mutations at a second site rather than a back mutation at the *dfp* locus. One such revertant of BW447 (*dfp-1*) was further studied and found to still contain the *dfp-1* allele. Thus, when a P1 lysate of the revertant was used to transduce strain ES31 (*pyrE dfp*$^+$) to *pyrE*$^+$, 23 of the 24 transductants tested had become β-alanine auxotrophs at 42°C. Therefore, the *dfp* mutation must have still been present in the revertant (i.e., the donor strain).

Two characteristics of the revertant suggested that it might have arisen via a spontaneous tandem duplication of a chromosomal region (1), thereby producing a compensatory increase in enzyme levels. First, its β-alanine independence was genetically unstable. During growth under nonselective conditions (one passage in TY broth at 30°C), 30 to 85% of the cells reverted to β-alanine auxotrophy. Second, the level of pantothenate synthetase in the revertant was 1.8 times that of its auxotrophic parent BW447 (Table 3). (Its level of ADCase, however, was not significantly elevated.) Unfortunately, the instability of these pseudorevertants precluded further genetic analysis.

Reversion of *dfp-707*. Three independent temperature-resistant revertants of ES107 (*dfp-707*) were obtained after two serial passages of a culture in TY broth at 42°C. This enrichment step was necessary because the leaky Ts phenotype (22) precluded the direct plating of an inoculum large enough to contain the rare revertants. The revertants no longer required β-alanine for growth on minimal medium, suggesting that the temperature sensitivity and auxotrophy were consequences of a single mutation.

The converse experiment, in which selection was for prototrophic revertants, was complicated by the high frequency with which phenotypic revertants arose via presumed gene duplications. Thus, when strain ES33 (*dfp-707*) was grown on minimal agar for 2 days at 30°C, β-alanine-independent colonies arose at a frequency of about 0.5%. After an additional day of incubation, >90% of the remaining cells grew out, although the colonies were smaller than those grown in the presence of pantothenate. If the phenotypic revertants were produced by gene duplications, they should occur much more infrequently in a *recA* strain (2), from which we should then be more likely to isolate intragenic revertants. Accordingly, we found that strain ES37 (*dfp-707 recA*) had an apparent reversion frequency of only 10^{-6}. Twenty-nine prototrophic revertants of ES37 were further analyzed. Two had also regained their ability to grow on rich (TY) media at elevated temperatures. One of them grew on either rich or unsupplemented minimal media at 42°C. The other was a partial revertant for both phenotypes; it grew on TY medium at 37°C but not at 42°C, and it required β-alanine at 37°C but not at 30°C. This partial reversion is reminiscent of the partial complementation obtained with some of the mutant plasmids (Fig. 1). The coreversion observed in these experiments strongly suggests that the conditional lethality and β-alanine auxotrophy of the *dfp-707* mutant resulted from the same mutation.

TABLE 3. Enzymatic characterization of a β-alanine-independent revertant of *dfp-1*

Strain	Relevant genotype	Sp act (U/mg of protein)[a]	
		Pantothenate synthetase	ADCase
W3110	*dfp*$^+$	28	15
BW447	*dfp-1*	30	14
BW447rev	*dfp-1*(Rev)[b]	49	19

[a] Inocula from an overnight culture in TY broth were grown for six to seven generations in a pantothenate-free medium (see Table 2, footnote *c*) at 42°C and harvested at a density of about 5×10^8 cells per ml. One unit of pantothenate synthetase is 1 nmol of product formed per min at 37°C. One unit of the decarboxylase is 1 pmol of product formed per min at 42°C.

[b] β-Alanine-independent phenotypic revertant of BW447 that retained the *dfp-1* allele (see text).

DISCUSSION

Plasmid complementation data strongly suggested that the two *dfp* mutations are allelic. Seven mutants of plasmid pWB1 failed to complement either chromosomal mutation, whereas none of the plasmids displayed wild-type complementation of one mutation and defective complementation of the other. All of the plasmid mutations map in a region that codes for a 45-kilodalton flavoprotein (22). There is insufficient DNA between the deletion endpoint in pES40 and the insertion in pES19 to code for a second gene, and an unidentified gene product was never seen in maxicell preparations (22). Therefore, an inability to complement the auxotrophy cannot be attributed to the polar effects of plasmid insertion mutations on an unknown gene downstream from *dfp*. The coreversion of the auxotrophy and conditional lethality of *dfp-707* provided even stronger evidence that these two mutant phenotypes are specified by the same gene.

In *E. coli*, ADCase is required for the synthesis of β-alanine; *panD* mutants are β-alanine auxotrophs, and prototrophic revertants regain ADCase activity (5, 6, 25). The only known function of β-alanine is to condense with pantoic acid to form pantothenate; when β-[^{3}H]alanine is supplied to a *panD* mutant, all of the label appears to be converted to derivatives of pantothenate (11). It was surprising, therefore, that *dfp* mutants had no apparent deficiency of either ADCase or pantothenate synthetase. It is unlikely that β-alanine is required in a biosynthetic pathway unrelated to pantothenate because the *dfp* mutants can be satisfied by very small amounts of pantothenate alone.

The β-alanine auxotrophy does not necessarily indicate a failure to synthesize the compound in vivo. The block may be in a later step and might be overcome by adding an excess of metabolic precursors. For example, certain temperature-sensitive pantothenate kinase mutants of *S. typhimurium* can be satisfied by a high level of pantothenate (8). Alternatively, the *dfp* mutants may accumulate a toxic metabolite; it is known that inhibitors of ADCase, such as β-hydroxyaspartate and cysteic acid, inhibit the growth of wild-type *E. coli* (15).

Pantothenic acid is a precursor of coenzyme A, a major cofactor in several steps of fatty acid biosynthesis. It is

intriguing to interpret our results as indicating an unsuspected relationship between DNA synthesis and pantothenate metabolism (and hence lipid or membrane biosynthesis); however, this association might be merely incidental. As previously discussed (22), two unrelated biochemical pathways could be inhibited by a toxic product that accumulates in the mutant, different enzymes might require the same gene product for posttranslational activation, or one polypeptide might function in two separate pathways.

In summary, we are left with three puzzling properties of the *dfp* gene. (i) It affects both DNA and pantothenate metabolism, two apparently unrelated pathways. (ii) Mutants behave like ADCase mutants, but they have no measurable defect in the enzyme. (iii) The gene product contains flavin mononucleotide, suggesting involvement in a redox reaction. Future studies with radiolabeled metabolic precursors may provide some insight into the specific function of the *dfp* gene product.

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