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Mutations in Genes *cpxA* and *cpxB* of *Escherichia coli* K-12 Cause a Defect in Isoleucine and Valine Syntheses

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Mutations in two chromosomal genes of *Escherichia coli*, *cpxA* and *cpxB*, produced a temperature-sensitive growth defect that was remedied specifically by the addition of isoleucine and valine to the minimal medium. This auxotrophy was manifested only when the medium contained exogenous leucine, suggesting that mutant cells fail to elaborate active acetohydroxy acid synthase, isozyme I. In the presence of leucine, this enzyme was required to catalyze the first reaction common to the biosynthesis of isoleucine and valine. Measurements of enzyme activity in crude extracts showed that mutant cells were seven- to eightfold deficient in active isozyme I when the cells were grown in the presence of leucine. When grown in the absence of leucine, mutant cells contained more acetohydroxy acid synthase activity. We attribute this activity to isozyme III, the product of the *ilvHI* genes, which are derepressed in the absence of exogenous leucine. The *cpxA* and *cpxB* mutations appear to affect the production of active isozyme I, rather than its activity, since (i) neither the *cpxA* nor the *cpxB* gene mapped near the structural gene for isozyme I (*ilvB*), (ii) the growth of mutant cells shifted from the permissive (34°C) to the nonpermissive (41°C) temperature did not immediately cease, but declined gradually over a period corresponding to several normal generation times, and (iii) the enzyme from mutant cells grown at 34°C was as stable at 41°C as the enzyme from *cpx*$^+$ cells.

We recently described two temperature-sensitive, chromosomal mutants of *Escherichia coli* K-12 that are defective in DNA donor and related functions associated with the *tra* genes of the conjugative plasmid F (12). In the preceding communication (13), we showed that full expression of the mutant phenotype requires mutations in two chromosomal genes, *cpxA* and *cpxB*. Furthermore, we showed that the same *cpxA* and *cpxB* mutations that reduce F-related functions prevent the growth of mutant cells in minimal media (13). We show here that this property of mutant cells results from their inability to synthesize two branched-chain amino acids, isoleucine and valine.

In enteric bacteria, such as *E. coli*, four of the five isoleucine and valine synthetic reactions are homologous. Each pair of these homologous reactions, one in the valine biosynthetic pathway and the other in the isoleucine pathway, is catalyzed by the same enzyme. Mutations affecting any one of these enzymes can therefore prevent the synthesis of both amino acids (see 10 and 15 for recent reviews).

The first reaction common to both pathways is the condensation of an active aldehyde moiety derived from pyruvate with either α-ketobutyrate or a second molecule of pyruvate to form homologous acetohydroxy acid precursors of isoleucine and valine, respectively (15). These reactions are catalyzed by acetohydroxy acid synthase (EC 4.1.3.18). *E. coli* K-12 strains carry distinct structural genes for three isozymes of acetohydroxy acid synthase (I, II, and III) (9), though wild-type (*ilvO*$^+$) strains do not elaborate isozyme II (8, 9). In the presence of exogenous leucine, synthesis of isozyme III, the product of the *ilvHI* genes, is repressed (6). Under these conditions, isoleucine and valine syntheses depend entirely on isozyme I, the product of the *ilvB* gene (9). We have exploited these facts to show that the *cpxA* and *cpxB* mutations prevent the elaboration of active acetohydroxy acid synthase isozyme I.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in these studies are described in Table 1.

Media and growth conditions. Cells were grown aerobically in minimal medium E (16) containing 0.2% dextrose and 40 μg each of methionine, arginine, histidine, and thymidine per ml. Where indicated, cultures also contained 40 μg of leucine, isoleucine, or valine per ml. Cell growth was monitored by the optical density (660 nm) of cultures.

Preparation of cell extracts and assay of acetohydroxy acid synthase activity. Cells at an op-

TABLE 1. *Bacterial strains*[a]

Strain	Relevant genotype	Source or comment
AE1031	Hfr *leu-6 cpxA*$^+$ *cpxB*$^+$	Ref. 13
AE1019	Hfr *leu-6 cpxA2 cpxB1*	Ref. 13
AE1061	Hfr *leu-6 cpxA2* *cpxB*$^+$	Ref. 13
AE1010	Hfr *leu-6 cpxA*$^+$ *cpxB1*	Ref. 13
AE1122	Hfr *leu*$^+$ *cpxA*$^+$ *cpxB*$^+$	This study; derived from AE1031 by P1 transduction
AE1124	Hfr *leu*$^+$ *cpxA2* *cpxB1*	This study; derived from AE1019 by P1 transduction

[a] All strains are derivatives of *E. coli* K-12.

tical density of 0.6 to 1 were harvested by centrifugation and frozen at −20°C. Each frozen cell pellet (about 1 mg) was suspended in 2 ml of extraction buffer (0.1 M potassium phosphate, pH 8.1, 10 mM $MgCl_2$, 0.1 mM isoleucine), and the cells were broken by 90 s of intermittent sonic oscillation at 50 W with a Bronson Sonifier microtip. Debris was removed by centrifugation at 24,000 × *g* for 20 min at 4°C. The protein concentration in the supernatant fluids ranged from 4 to 13 mg/ml. Acetohydroxy acid synthase activity was routinely measured by the formation of acetolactate from pyruvate (2). Complete reaction mixtures (0.25 ml) contained 25 μmol of potassium phosphate (pH 8.0), 2.5 μmol of $MgCl_2$, 10 μmol of sodium pyruvate, 20 μg of thiamine pyrophosphate, 0.5 μg of flavin adenine dinucleotide and extract (20 to 35 μg of protein). Incubation was for 10 min at 37°C. The acetolactate formed was converted to acetoin, which was determined colorimetrically as described by Bauerle et al. (2). One unit of enzyme activity is the amount required to catalyze the synthesis of 1 nmol of acetolactate per min.

To compare the in vitro stability of acetohydroxy acid synthases from different cells, we prepared extracts as described above in a buffer containing 10 mM potassium phosphate (pH 7.1), 5 mM $MgCl_2$, 20 μg of flavin adenine dinucleotide, 200 μg of thiamine pyrophosphate per ml and 20% (by volume) glycerol (9). The extracts were then incubated at 41°C. At intervals, samples were removed and assayed for enzyme activity as described above.

Materials. Creatine·H_2O, sodium pyruvate, α-napthol, and thiamine pyrophosphate were obtained from the Sigma Chemical Co.; flavin adenine dinucleotide from Boehringer-Mannheim; and acetoin (3-hydroxy-2-butanone, practical grade), from Eastman Organic Chemicals. The last was washed with ether and air dried before use. Other chemicals and biological media were obtained from standard commercial sources.

RESULTS

Ilv$^-$ phenotype associated with *cpxA* and *cpxB* mutations. The *cpxA* and *cpxB* mutations jointly cause a temperature-sensitive growth defect that is expressed in minimal media but not in nutrient broth (13). To determine whether this property reflects an additional nutritional requirement of mutant cells, we supplemented the minimal medium with combinations of amino acids not required for growth of the parental strain and found that isoleucine and valine were sufficient to restore the growth of *cpxA cpxB* mutant strains at the nonpermissive temperature (41°C).

As shown in Fig. 1, the growth rate of AE1019 (*cpxA2 cpxB1*) after a shift from 34°C to 41°C in the absence of exogenous isoleucine and valine gradually declined over an interval corresponding to several generations of the otherwise isogenic *cpxA*$^+$ *cpxB*$^+$ strain, AE1031. A similar growth defect was observed with the *cpxA1 cpxB1* strain AE1018. In contrast, both AE1031

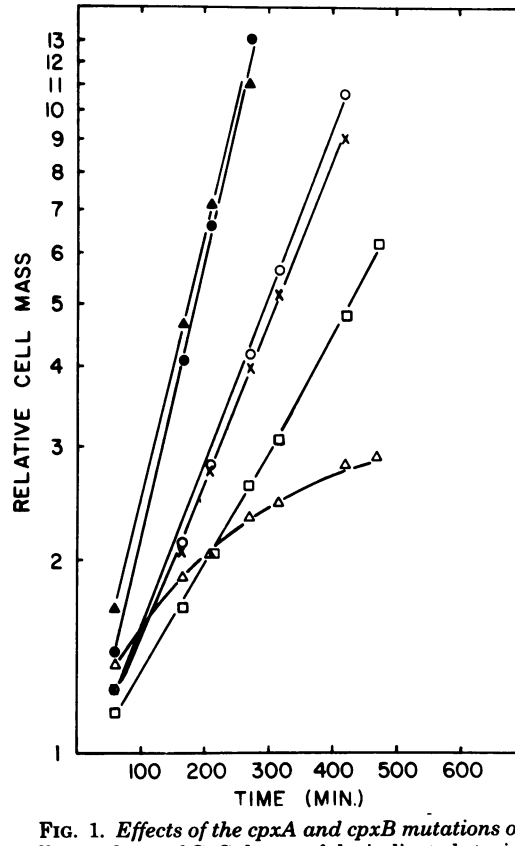

FIG. 1. *Effects of the cpxA and cpxB mutations on cell growth at 41°C. Cultures of the indicated strains grown overnight in minimal medium at 34°C were diluted into minimal medium at 41°C to an optical density of about 0.1. The cultures were incubated with aeration, and at intervals samples were withdrawn for measurement of cell mass as optical density at 660 nm. Symbols: ○, strain AE1031 (cpxA*$^+$ *cpxB*$^+$*); △, strain AE1019 (cpxA2 cpxB1); ×, strain AE1010 (cpxA*$^+$ *cpxB1); □, strain AE1061 (cpxA2 cpxB*$^+$*); ●, strain AE1031 (isoleucine and valine were added to the growth medium); ▲, strain AE1019 (isoleucine and valine were added to the growth medium).*

and AE1019 grew exponentially at 41°C with the same generation time, 70 min, when the medium contained isoleucine and valine (Fig. 1). Isoleucine by itself did not permit the growth of mutant cells (not shown); valine by itself was not tested because our strains, like other K-12 strains of *E. coli*, are valine sensitive (reviewed in 7). The isoleucine and valine auxotrophy of *cpxA cpxB* mutants was temperature sensitive since at 34°C AE1019 grew exponentially and nearly as fast as AE1031, even in the absence of isoleucine and valine (see Table 2).

We also show in Fig. 1 the individual effects of the *cpxA* and *cpxB* mutations on cell growth in the absence of isoleucine and valine. Strain AE1061 (*cpxA2 cpxB*$^+$) grew exponentially at 41°C, but its generation time, 175 min, was longer than that of AE1031, and the final cell density was less. The different growth rates of the two strains were also apparent on solid medium: AE1061 colonies were smaller than AE1031 colonies at 41°C (13). In contrast, the *cpxB1* mutation by itself (strain AE1010) had no detectable effect on growth rate in the absence of isoleucine and valine.

Leucine dependence of the Ilv$^-$ phenotype. The Ilv auxotrophy described above is best explained by an effect of the *cpxA* and *cpxB* mutations on one or more of the reactions that are common to both the isoleucine and valine biosynthetic pathways. In *E. coli* K-12 the first such reaction is catalyzed by any of three acetohydroxy acid synthase isozymes. However, valine-sensitive strains, which include all of the strains analyzed in our studies, do not elaborate isozyme II, the *ilvG* gene product, which is not inhibited by valine (8, 9). Furthermore, the strains examined in Fig. 1 were Leu$^-$, and the medium therefore contained leucine, which represses synthesis of isozyme III (6). Hence, synthesis of isoleucine and valine in these strains depended on isozyme I, whose synthesis is repressed only when both leucine and valine are present (5). A temperature-sensitive defect in isozyme I or in its production could therefore account for the Ilv$^-$ phenotype of *cpxA cpxB* mutants.

As predicted by this hypothesis, the Ilv$^-$ phenotype of *cpxA cpxB* mutants was leucine dependent. The *cpxA*$^+$ *cpxB*$^+$ *leu*$^+$ strain AE1122 grew exponentially at 41°C in the absence of isoleucine and valine, whether or not the medium contained leucine. In contrast, after a shift from 34°C to 41°C in the absence of isoleucine and valine, the *cpxA2 cpxB1 leu*$^+$ strain AE1124 exhibited a gradual decline in growth rate when the medium contained leucine (Fig. 2), as expected from the results shown in Fig. 1, but grew exponentially when the medium lacked leucine.

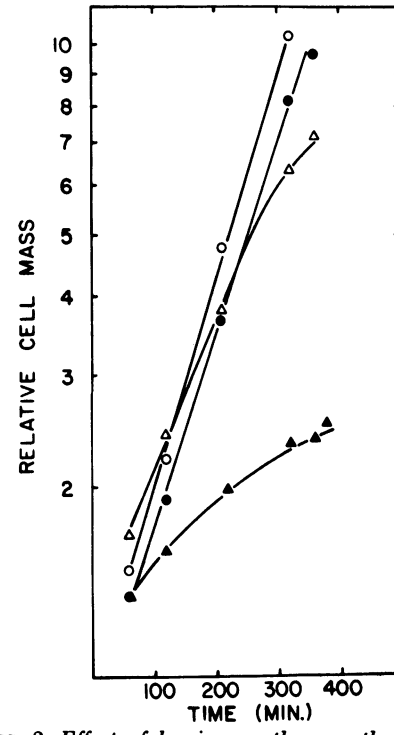

FIG. 2. *Effect of leucine on the growth of cpxA cpxB mutant cells at 41°C. The conditions were as described in the legend to Fig. 1. Symbols:* ○, ●, *strain AE1122 (leu*$^+$ *cpxA*$^+$ *cpxB*$^+$*);* △, ▲, *strain AE1124 (leu*$^+$ *cpxA2 cpxB1);* ●, ▲, *growth in minimal medium containing leucine;* ○, △, *growth in minimal medium lacking leucine.*

Acetohydroxy acid synthase deficiency in *cpxA cpxB* mutants. Measurements of enzyme activity in crude extracts confirmed that the *cpxA* and *cpxB* mutations cause a temperature-sensitive reduction in the steady-state level of active acetohydroxy acid synthase isozyme I. In one set of experiments, Leu$^-$ strains were grown in minimal medium containing leucine (Fig. 1). In these conditions total acetohydroxy acid synthase activity in crude extracts of *ilvO*$^+$ (Val^s) strains should represent the amount of isozyme I in the cells. AE1019, the *cpxA2 cpxB1* mutant grown at 41°C in the absence of isoleucine and valine, was seven- to eightfold deficient in isozyme I as compared with the corresponding culture of the *cpxA*$^+$ *cpxB*$^+$ strain AE1031, whereas both strains maintained comparable levels of isozyme I when they were grown at 34°C (Table 2). A mixture of equal amounts of protein from AE1031 and AE1019 cells grown at 41°C contained as much enzyme activity as the two extracts assayed separately (not shown); hence, AE1019 does not produce an inhibitor of

enzyme activity. Extracts of the *cpxA2 cpxB*$^+$ strain AE1061 grown at 41°C were significantly deficient in isozyme I activity, whereas extracts of AE1010, the *cpxA*$^+$ *cpxB1* strain, contained normal levels of activity (Table 2). These results are consistent with the effects of the individual mutations on cell growth at 41°C in the absence of isoleucine and valine (Fig. 1) and with their effects on the expression of F-plasmid function (13).

AE1019 cells grown in the presence of isoleucine and valine were still deficient in isozyme I activity (Table 2), even though they grew exponentially in these conditions with the same generation time as AE1031 cells (Fig. 1). Hence, the enzyme deficiency of AE1019 is not an indirect result of poor growth in the absence of isoleucine and valine.

Leu$^+$ *cpxA cpxB* mutant cells no longer expressed an Ilv$^-$ phenotype when grown in the absence of leucine (Fig. 2), a result we attribute to elaboration of acetohydroxy acid synthase isozyme III. As expected, extracts of AE1124 (*leu*$^+$ *cpxA2 cpxB1*) contained acetohydroxy acid synthase activity when grown in the absence of leucine, whereas the same cells were deficient when leucine was present in the medium (Table 3). In contrast, AE1122 (*leu*$^+$ *cpxA*$^+$ *cpxB*$^+$) cells contained acetohydroxy acid synthase whether or not they were grown in the presence of leucine. We attribute the higher specific activity of extracts derived from AE1122 grown in the absence of leucine to enhanced expression of both the *ilvB* and *ilvHI* genes. According to published data (9), extracts of cells in the absence of leucine, isoleucine, and valine contained acetohydroxy acid synthase I and III activities in a ratio of about 2.5:1. Using this ratio, we estimate that the isozyme I activity of extracts of strain AE1122 grown in the absence of leucine, isoleucine, and valine is about 40 U/mg, and the isozyme III activity is about 20 U/mg (Table 3). Hence, the total acetohydroxy acid synthase activity in extracts of strain AE1124 grown in the absence of these three amino acids, about 14 U/mg (Table 3), is quantitatively compatible with nearly full expression of the *IlvHI* genes. It therefore appears that the *cpxA* and *cpxB* mutations primarily affect isozyme I of acetohydroxy acid synthase.

Temperature stability of acetohydroxy acid synthase I in vitro. Using conditions employed by Guardiola et al. (9) to demonstrate increased thermolability of acetohydroxy acid

TABLE 3. *Leucine dependence of cpxA cpxB mutant cell growth*

Strain	Genotype	Leucine added (μg/ml)	Growth rate[a] (min)	Enzyme activity[b]
AE1122	*cpxA*$^+$ *cpxB*$^+$	0	95	60.4
		40	95	17.2
AE1124	*cpxA2 cpxB1*	0	130	14.4
		40	NE	<3

[a] See Table 2, footnote *c*, and Fig. 2. Both strains were grown at 41°C in the absence of exogenous isoleucine and valine.

[b] Acetohydroxy acid synthase activity, units per milligram of protein.

TABLE 2. *Acetohydroxy acid synthase isozyme I activity in crude extracts of wild-type and cpx mutant cells*

Strain	Genotype[a]	Growth temp (°C)	Medium[b]		Growth rate[c]	Enzyme activity[d]
			Ile	Val		
AE1031	*cpxA*$^+$ *cpxB*$^+$	41	0	0	115	23.5
		41	40	40	70	27.7
		34	0	0	110	39.5
AE1019	*cpxA2 cpxB1*	41	0	0	NE	3.2
		41	40	40	70	4.4
		34	0	0	140	33.7
AE1061	*cpxA2 cpxB*$^+$	41	0	0	175	15.0
AE1010	*cpxA*$^+$ *cpxB1*	41	0	0	115	24.5

[a] All of the strains carry the *leu-6* mutation and are therefore leucine auxotrophs.

[b] The values indicate the amount of the indicated amino acid (micrograms per milliliter) in the growth medium. Leucine was always present.

[c] Exponential growth rates, in minutes, were derived from the data in Fig. 1 and 2. The symbol "NE" indicates that growth was not exponential.

[d] Average enzyme activity, in units per milligram of protein, in extracts of two clones of each strain grown in the indicated conditions. The cells were harvested after the last measurement of cell mass (see Fig. 1 and 2).

synthase I in extracts of an *ilvB*(Ts) mutant, we tested the stability of this enzyme in an extract of AE1019 cells (*cpxA2 cpxB1*) grown at 34°C in the presence of leucine to repress synthesis of acetohydroxy acid synthase III. After 3 h at 41°C, the extracts retained 67 ± 11% (mean ± standard deviation of the results for three different clones) of their activity, as compared with 79 ± 17% (mean ± standard deviation of the results for four different clones) for extracts of AE1031, a *cpxA*$^+$ *cpxB*$^+$ strain. It therefore seems unlikely that the *cpxA* and *cpxB* mutations affect the thermostability of the enzyme.

DISCUSSION

cpxA cpxB mutants at 41°C are unable to synthesize sufficient isoleucine and valine to sustain growth. The *cpxA* and *cpxB* genes have not previously been identified as *ilv* loci, presumably because both genes must be mutated in order for cells to exhibit an Ilv$^-$ phenotype.

Our data indicate that the mutations prevent the elaboration of active isozyme I of acetohydroxy acid synthase, which catalyzes the first reaction common to the isoleucine and valine synthetic pathways. We believe that the isozyme I deficiency in mutant cells does not reflect a temperature-sensitive defect in the enzyme itself. First, neither the *cpxA* nor the *cpxB* gene maps near the structural gene for isozyme I. This gene, *ilvB*, is the site of mutations leading to a temperature-sensitive isozyme I (9) and maps between *pyrE* and *bgl* (81 to 83 min) on the *E. coli* K-12 linkage map (T. Newman and M. Levinthal, personal communication), whereas the *cpxA* gene is located at 88 min and the *cpxB* gene, at 41 min (13). Second, the growth rate of mutant cells shifted from the permissive to the nonpermissive temperature in the presence of leucine declined gradually over an interval corresponding to several normal generation times. These kinetics are best explained by a defect in the production of active isozyme I at the nonpermissive temperature. In the absence of further production after the shift-up, enzyme in the cell at the time of the shift would continue to function and to support cell growth at a diminishing rate as it became limiting. The same gradual decline in growth rate occurred when leucine was added to an *ilvB* mutant and the amount of isozyme III in the cell became growth limiting (6). These responses differ from the rapid cessation of growth that would be expected for a mutant with a temperature-sensitive enzyme. As expected from this hypothesis, isozyme I in crude extracts of mutant cells grown at 34°C appeared to be as thermostable at 41°C as the enzyme in extracts of *cpx*$^+$ cells.

One explanation of our results is that the *cpxA* and *cpxB* gene products regulate expression of the *ilvB* gene, either independently or as previously unidentified components of the complex *ilv* genetic regulatory system (10, 15). This explanation is not easy to reconcile with the effect of *cpxA* and *cpxB* mutations on the protein composition of the cell envelope (12; J. McEwen and P. Silverman, in preparation). One protein whose content in the outer membrane of mutant cells is greatly reduced, the murein lipoprotein, is nevertheless synthesized in mutant cells at a normal or nearly normal rate (McEwen and Silverman, in preparation). Apparently, the cells fail either to process or to incorporate the lipoprotein properly in the envelope. An analogous effect of these mutations on the F-plasmid *traJ* gene product, another outer membrane protein (1, 11), would explain the failure of mutant cells to express F-plasmid functions (12). The *cpxA* and *cpxB* mutations also lead to a deficiency in several inner membrane proteins (McEwen and Silverman, in preparation), but these have not been identified. These effects of *cpxA* and *cpxB* mutations on the cell envelope suggest an alternative explanation for their effects on isoleucine and valine synthesis, which is that the *ilvB* gene product of *E. coli* must be processed as an envelope protein in order to form enzymatically active acetohydroxy acid synthase and that this processing fails to occur in *cpxA cpxB* mutants. In *Salmonella* spp. (4), *Neurospora* spp. (3), and yeasts (14), biosynthesis of isoleucine and valine may be catalyzed by a multienzyme complex containing the enzymes common to both pathways, including acetohydroxy acid synthase. In *Neurospora* spp. and yeasts, the complex is associated with mitochondria (3, 14), from which it can be released by digitonin (3). In *Salmonella* spp., it appears to be loosely associated with the cell membrane (4). Comparable data for *E. coli* have not been published, but branched-chain amino acid syntheses in *Salmonella* spp. and in *Escherichia* spp. appear to be generally similar. Since the relation between the primary gene product and the active enzyme is not known in either case, the hypothesis that the *cpxA* and *cpxB* mutations prevent the maturation of the *ilvB* gene product to enzymatically active, membrane-associated isozyme I is speculative. Nevertheless, if true, it would explain our result with respect to the effects of the *cpxA* and *cpxB* mutations on isozyme I activity and would also incorporate these totally unexpected effects into the hypothesis that the primary effect of *cpxA* and *cpxB* mutations is on the stable incorporation of a specific set of proteins into the cell envelope.

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