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Genetic Analysis of Diaminopimelic Acid- and Lysine-Requiring Mutants of *Escherichia coli*[1]

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Several diaminopimelic acid (DAP)- and lysine-requiring mutants of *Escherichia coli* were isolated and studied by genetic, physiological, and biochemical means. The genes concerned with DAP-lysine synthesis map at several different sites on the *E. coli* chromosome and, therefore, do not constitute a single operon. Three separate loci affecting DAP synthesis are located in the 0 to 2.5 min region of the genetic map. The order of the loci in this region is *thr-dapB-pyrA-ara-leu-pan-dapC-tonA-dapD*. Two additional DAP genes map in the region between min 47 and 48, with the gene order being *gua-dapA-dapE-ctr*. The *lys* locus at min 55 determines the synthesis of the enzyme DAP decarboxylase, which catalyzes the conversion of DAP into lysine. The order of the genes in this region is *serA-lysA-thyA*.

It is now well established that the biosynthesis of lysine occurs by two entirely different routes. In yeasts and fungi, L-aminoadipic acid serves as a key intermediate, whereas bacteria and algae form lysine by the decarboxylation of the *meso* isomer of α-ϵ-diaminopimelic acid (DAP), which, in turn is derived from L-aspartate (33, 38). Since aspartate contributes to the carbon skeleton of DAP, lysine, methionine, threonine, and isoleucine, these amino acids are collectively termed the aspartate family of amino acids in bacteria (4, 26, 31). The mechanism of lysine biosynthesis in bacteria generated interest not only because of its relevance to studies on the regulation of branched biosynthetic pathways but also because of the association of DAP with uniquely bacterial cell structures. Since its discovery by Work (37), DAP has been found to occur in the cell wall and spore mucopeptides of various bacterial species (16, 24). Dihydrodipicolinic acid (DHDP), an intermediate of the DAP-lysine pathway, also acts as the precursor of dipicolinic acid which is a substantial constituent of bacterial spores (7).

Although the biochemistry of the DAP-lysine pathway has been worked out in detail (2, 6, 10, 11, 13, 22, 32, 41), very little is known about the distribution and function of the gene loci involved in DAP-lysine synthesis on the linkage group of *Escherichia coli*. In the only genetic study involving DAP mutants of *E. coli* reported so far, Cohen and co-workers (4) presented preliminary evidence that three separate mutations leading to deficiencies in DHDP synthetase, succinyl DAP deacylase, and DAP decarboxylase map in the region between *his* at min 39 and *strA* at min 64 on the linkage map of *E. coli* (29).

To map and to analyze genetically the loci for DAP-lysine synthesis, we isolated several DAP- and lysine-requiring mutants of *E. coli* K-12. This paper describes the genetic mapping of six distinct genes which affect the synthesis of DAP and lysine.

MATERIALS AND METHODS

Bacterial and bacteriophage strains. The bacterial strains used in this work are derivatives of *E. coli* K-12, except the mutant M-203 (6). Table 1 lists the key genetic stocks employed in this study. To avoid unnecessary repetitions, the relevant genotypes of several additional strains are supplied in Tables 2 and 3. The points of origin of chromosome transfer for the Hfr strains used were described elsewhere (29). Two strains of phage P1, P1*kc* and P1*vir* (obtained from A. J. Clark), were used in transduction experiments. Temperate phage Mu-1 was described by Taylor (27).

Genetic nomenclature. Genetic nomenclature used in this paper conforms to the rules proposed by Demerec et al. (5), and a detailed description of the genotypic and phenotypic symbols appears elsewhere (28). Capital letters denoting distinct *dap* loci were assigned in order of the enzymatic steps involved wherever the information was available. Thus, *dapA* designates the locus for DHDP synthetase, the enzyme catalyzing the first step in DAP synthesis (Fig. 1), and *dapB* codes for DHDP reductase, the second enzyme of the pathway. Phage Mu-1 induced mutations are designated by the sign *Mu*$^+$ after the genotypic symbol.

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TABLE 1. *Bacterial strains used*

Strain	Sex	Genotype[a]
AB257	HfrC	*metB2*
AB712	F⁻	*thr-4 leu-8 proA2 ara-14 thi-1*
KL16	Hfr	Prototroph (obtained from B. Low)
KL98	Hfr	Prototroph (obtained from B. Low)
Pa3306C	F⁻	*nadB2 argH1 purI66 ctr-2* (obtained from M. L. Morse)
AT793	HfrH	*ara-16*
AT941	HfrC	*tonA3 thyA12 metB2*
AT955	F⁻	*dapD12Mu⁺ purF1 proA2 strA20*
AT957	F⁻	*dapD12Mu⁺ thr-4 strA20*
AT978	HfrKL16	*dapE9*
AT984	F⁺	*dapA6*
AT998	HfrKL16	*dapA16*
AT999	HfrKL16	*dapB17Mu⁺*
AT1002	HfrC	*dapD12Mu⁺ tonA4 thyA12*
AT1009	F⁻	*tonA7 his-4 pan-4 argE3*
AT1015	F⁻	*dapA16 his-21 ilv-18 strA10*
AT1018	F⁻	*dapE9 his-21 proC22 strA10*
AT1055	F⁻	*dapA16 nadB2 argH1*
AT1056	HfrH	*dapE9*
AT1057	HfrH	*dapA16*
AT1062	F⁻	*dapE9 purF1 guaA5*
AT1063	F⁻	*dapA16 purF1 guaA5*
AT1371	F⁻	*his-4 pan-4 argE3*
AT2457	HfrH	*glyA6*
AT2465	HfrH	*guaA5*
AT2363	F⁻	*thr-4 leu-8 proA2 ara-16 thi-1 pyrA49Mu⁺*
AT3006	F⁻	*nadB2 argH1 purI66* (Pa3306 obtained from R. Lavallé)
AT3055	F⁻	*thr-14 proC22 pyrC73 pyrA76 his-21 purE73 ilv-18 pdxC2 met-75 strA10* (strain X961 obtained from R. Curtiss)
AT3154		*dapB19* (*E. coli* W mutant M-203 obtained from C. Gilvarg)

[a] All gene symbols are defined in reference 28.

μg/ml, with vitamins at 2 μg/ml, and with 2% agar in solid media. Glucose at a final concentration of 0.5% was used for growing bacteria in liquid culture. A tryptone-yeast extract medium (L broth) was used for routine cultivation of bacteria as well as the diluent for phages (15). Agar (1%) was added to L broth for agar plates. For cultivation of DAP-requiring auxotrophs, L broth was supplemented with DAP at a concentration of 50 μg/ml. Soft agar for top layers contained L broth with either 0.5% agar (for phage lysates) or 0.7% agar (for scoring lysogeny by replica plating). All incubations were carried out at 37 C.

Mutagenesis. Bacteria were mutagenized with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) under conditions described by Adelberg et al. (1). Treated cells were washed and transferred to MM supplemented with DAP and lysine to permit outgrowth of DAP- or lysine-requiring mutants. Mutants were selected by two cycles of ampicillin treatment as described by Molholt (18). To prevent possible lysis of DAP auxotrophs in the presence of lysine only, the bacteria were always starved in unsupplemented MM before treatment with ampicillin (Ayerst Laboratories, New York). To detect DAP-requiring (Dap⁻) mutants, the treated cells were plated on L agar + DAP; after overnight incubation, the colonies were replica plated onto L agar without DAP. Colonies that grew poorly or not at all on the latter medium were selected for further study. In a few cases, mutations were induced with phage Mu-1 as by the method of Taylor (27).

Nutritional tests. Mutant strains were routinely examined for requirements by pouring 0.1 ml of washed cell suspension in a soft agar layer (0.7% agar in water) onto MM plates. A few crystals of the nutrient to be tested were placed on the surface of the plate, or 0.1 ml of 1 to 10% solution of the nutrient was added to a filter paper disc on the agar surface. The growth response around the crystals or disc was checked after incubation for 12 to 36 hr.

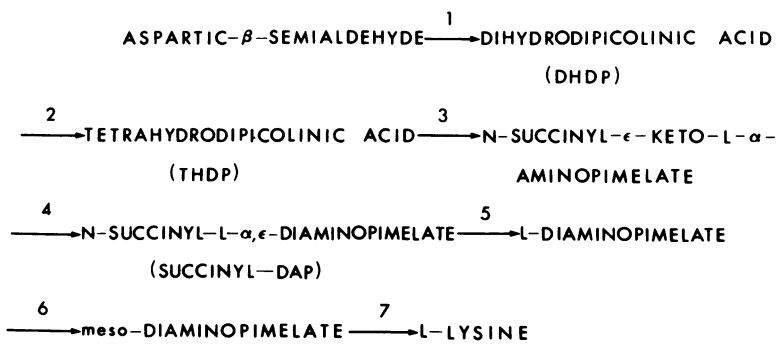

FIG. 1. *Pathway of lysine biosynthesis in E. coli. Numbers refer to the following enzymes: (1) DHDP synthetase, (2) DHDP reductase, (3) THDP acylase, (4) succinyl DAP-glutamic acid transaminase, (5) succinyl DAP deacylase, (6) DAP epimerase, (7) DAP decarboxylase.*

Media and growth conditions. Minimal medium (MM) was a half-strength preparation of medium 56 described by Monod et al. (19) with glucose (or other carbon sources) at a final concentration of 0.2%. It was supplemented with DAP at 75 μg/ml, with other required amino acids, purines, and pyrimidines at predetermined optimal concentrations varying from 20 to 140

Genetic techniques and procedures. Conjugation and transduction procedures, mating conditions, and the method for interruption of conjugation were described elsewhere (29).

Biochemical procedures: preparation of cell-free ex-

tracts. A small volume (5 ml) of overnight broth culture was centrifuged and suspended in 1 ml of sterile saline. This cell suspension (0.5 ml) was used to inoculate 250 ml of MM supplemented with appropriate nutrients in 1-liter flasks and incubated with shaking. Cells were harvested at the end of exponential growth phase, washed twice with 0.02 M phosphate buffer (*p*H 6.8), and suspended in 3 to 5 ml of 0.1 M phosphate buffer (*p*H 6.8). Cell-free extracts were obtained by disrupting cells with a Branson sonic oscillator at maximum power output for 3 min at −10 C, followed by centrifugation at 6,000 rev/min for 20 min to remove cell debris. The extracts were immediately assayed for enzyme activities.

Estimation of DAP and protein. DAP was measured in all experiments by using a specific colorimetric assay in which lysine does not interfere (40). Absorbancy was measured at 440 nm in a Zeiss PMQ II spectrophotometer. Protein was estimated by the method of Lowry et al. (14), by using bovine serum albumin (Sigma Chemical Co.) as the standard.

Enzyme assays. DAP decarboxylase activity was determined by measuring the rate of disappearance of *meso*-DAP with the specific colorimetric assay for DAP (35). The presence of DAP epimerase was detected by coupling the reaction with DAP decarboxylase in 0.1 M phosphate buffer (*p*H 7.2). A mixture of *meso* and L-DAP is comletely decarboxylated under the conditions of DAP decarboxylase assay. Absence of DAP epimerase leaves L-DAP intact since it is not attacked by DAP decarboxylase (39). L-DAP was determined by paper chromatography after 1 and 12 hr of incubation of the assay mixture. A methanol-water-10 N HCl-pyridine (80:17.5:2.5:10) solvent system was used to resolve *meso* and L isomers of DAP (25).

DAP accumulation studies: in vitro and in vivo synthesis of DAP. The levels of DAP in cell-free extracts were routinely measured by the method of Work (40). A more elaborate procedure to detect the synthesis and accumulation of DAP by various mutants was devised by exploiting the resting cell system for DAP synthesis described by Gilvarg (8). Cells were grown in MM, 30 μg of lysine/ml, and 10 μg of DAP/ml; harvested; washed, and suspended as described under preparation of cell-free extracts. To 2 ml of cell suspension (2×10^{11} cells/ml) was added 0.1 ml of 20% glucose, 0.1 ml of Mg aspartate (0.2 M), 0.1 ml of sodium pyruvate (0.4 M), and 0.1 ml of potassium glutamate (0.04 M). The mixture was incubated at 37 C for 48 hr. Streptomycin (40 μg/ml) was added to prevent contamination. DAP was estimated in 0.5-ml samples either after centrifugation (DAP excretion) or after boiling the cell suspension for 30 min (total DAP). In vitro synthesis of DAP (DAP synthesis from asparate by cell-free extracts) was studied by the method of Gilvarg (8).

Chemicals. Most of the chemicals used were of reagent grade available commercially. DAP was obtained from Sigma Chemical Co. as a synthetic mixture of the D, L, and *meso* isomers. *Meso*-DAP was separated from this product by fractional crystallization (36).

RESULTS

Isolation and preliminary characterization of mutants. A number of independent DAP- or lysine-requiring auxotrophs of *E. coli* K-12 were isolated from cultures subjected to NTG or temperate phage Mu-1 mutagenesis. Nineteen of these mutants were chosen for further study on the basis of their relative nonleakiness and low rate of backmutation to prototrophy. This collection included 12 nonsibling mutants of Hfr strain KL16 that showed an absolute requirement for DAP, and 7 lysine specific auxotrophs (mutants which grow only on lysine and not on DAP). Two of the Dap$^-$ mutants and five of the Lys$^-$ mutants were recovered from cultures which were infected with phage Mu-1. Linkage of prophage Mu-1 to the mutation site in all presumptive phage-induced mutants was verified by transduction crosses as follows. Mutant strains were infected with phage P1 grown on wild-type cells. The transductants, selected for the wild-type marker, were examined for Mu-1 lysogeny by replica plating onto a lawn of cells sensitive to Mu-1. Inheritance of the wild-type allele with the concomitant removal of prophage was taken to mean that Mu-1 was linked to the mutation site. Alternatively, the mutant strains were used as P1 donors, and transductants were selected for suitable markers near the mutation in question. Linkage of Mu-1 to the mutation site is indicated by 100% coinheritance of prophage and the mutant phenotype as unselected markers.

The Dap$^-$ strain AT915 (*dapD12Mu*$^+$) differs from all other Dap$^-$ mutants in that it produces normal looking colonies on L agar containing no DAP, indicating that it can fulfill the requirement for DAP when grown on complex media. When colonies of the other Dap$^-$ mutants are replica plated onto L agar without DAP, some strains produce highly mucoid colonies after overnight incubation, some produce a slight mucoid growth, and some produce no evidence of residual growth. The variable appearance of these mutants on L agar probably reflects different degrees of impairment of DAP synthesis in the individual mutants. However, all of the mutants, including AT915, specifically require DAP for growth in MM, although optimal growth is obtained in MM plus DAP plus lysine. In MM plus lysine only, lysis of the bacteria is observed in most cases.

To study further the capacity of strain AT915 for residual DAP synthesis, we constructed a double mutant of the genotype *dapD12Mu*$^+$ *lysA8Mu*$^+$. This strain produces no active DAP decarboxylase and no longer requires DAP if it is provided with lysine in the growth medium. Thus, it seems that the *dapD12Mu*$^+$ mutant can synthesize a sufficient amount of DAP to satisfy its requirement when none of the DAP is utilized for decarboxylation to lysine. In the in vitro system

for DAP synthesis, the cell-free extract from the double mutant was found to promote the formation of a surprisingly large amount of DAP, equivalent to about 50% of the amount formed by a Dap$^+$ *lysA8Mu*$^+$ strain. The *lysA8Mu*$^+$ derivatives of other Dap$^-$ mutants were also examined for DAP synthesis by measuring accumulation of DAP in the resting cell system previously described. All were found to accumulate DAP in amounts ranging from 20 to 40% of the amount formed by a Dap$^+$ *lysA8Mu*$^+$ strain. In contrast to the *dapD12Mu*$^+$ *lysA8Mu*$^+$ strain, these double mutants required DAP in addition to lysine to achieve a significant rate of growth in MM. We conclude that none of the Dap$^-$ mutants isolated has a complete block in DAP synthesis and that mutants which retain the ability to make substantial amounts of DAP nonetheless behave as strict DAP auxotrophs in MM.

Screening for DAP epimerase mutants. Lysine-requiring mutants defective in DAP decarboxylase accumulate both L and *meso* isomers of DAP (12, 40). A block at the DAP epimerase step (Fig. 1) would be expected to result in the accumulation of L-DAP only. A mutant of this kind has been reported in *Staphylococcus aureus* (3). To detect DAP epimerase mutants, samples of the resting cell suspensions of Dap$^-$ mutants from the accumulation studies described above were examined by paper chromatography for the presence of L and *meso* isomers. As expected, the Lys$^-$ Dap$^+$ mutants formed large amounts of both L and *meso*-DAP, but none of the Lys$^+$ Dap$^-$ mutants accumulated detectable amounts of L-DAP. Furthermore, cell-free extracts from all of these mutants were able to convert L-DAP into lysine in the qualitative assay for DAP epimerase previously described. We conclude, therefore, that none of the Dap$^-$ mutants isolated in this study is blocked at the DAP epimerase step.

Mapping of the gene coding for DAP decarboxylase. The position of the *lysA* locus in *E. coli* is precisely known. A mutation, *lys-10*, maps between *serA* and *thyA* and has been shown to cotransduce with *thyA* at a frequency of about 80% (29). It was assumed that strains with mutations at this locus have defective DAP decarboxylase, the enzyme which is responsible for the conversion of DAP into lysine. To verify this assumption, we determined the DAP decarboxylase activities of seven independent Lys$^-$ mutants, the *lys*$^-$ alleles of which are known to be cotransduced with *thyA*. The data presented in Table 2 show that mutations which result in the elimination of DAP decarboxylase activity do indeed map at this locus. However, we found that another distinct phenotype can be generated by mutations at this locus. The lysine-requiring auxotrophs, AT1050 and AT2453, show no reduction in DAP decarboxylase activity as judged by in vitro assay (Table 2). These mutants can grow on either lysine or pyridoxine (vitamin B6) and are thus different from other mutants which show no response to pyridoxine. Additional genetic and biochemical studies on the *lysA* locus and on the enzyme DAP decarboxylase are presented in an accompanying report (3*a*).

TABLE 2. *Diaminopimelic acid (DAP) decarboxylase activity in Lys$^-$ mutants*

Strain	DAP decarboxylase activity[a]	Growth response to
AB257 (wild type)	1,760	
AT2373 (*lysA8Mu*$^+$)	0	Lysine only
AT875 (*lysA13Mu*$^+$)	0	Lysine only
AT992 (*lysA16Mu*$^+$)	0	Lysine only
AT974 (*lysA14*)	60	Lysine only
AT988 (*lysA17Mu*$^+$)	154	Lysine only
AT1050 (*lys-9*)	2,040	Lysine or pyridoxine
AT2453 (*lys-10*)	1,453	Lysine or pyridoxine

[a] Nanomoles of DAP converted per hour per milligram of protein. Cells were grown in minimal medium plus lysine (30 μg/ml), except for the wild type which was grown without lysine.

Approximate mapping of the dap loci. Cohen et al. (4) raised the question whether all loci concerned with lysine synthesis constitute an operon. To test this possibility all *dap* markers of the DAP auxotrophs were screened for cotransduction with *thyA*, the marker nearest to *lysA*. None was found to contransduce with *thyA*. Interrupted conjugation experiments, in which AT793 (HfrH) and AB257 (HfrC) were mated with F$^-$ *dapD12Mu*$^+$ recipient strains, showed that this *dap* marker is located near min 3 on the linkage map, between the *thr* and *proA* genes (Fig. 2). The *tonA* (T1 resistance) locus, the nearest available marker in this region, was found to cotransduce with the wild-type allele of *dapD12Mu*$^+$ at a frequency of 80%. The remaining Dap$^-$ mutants were therefore examined for cotransduction with *tonA*. Out of 11 tested, 7 *dap*$^-$ markers were cotransduced with *tonA*, at frequencies ranging from 42% to 91% (Table 3).

The remaining four mutants, AT978, AT984, AT998, and AT999, were mated with the F$^-$ recipient strain AT3055. The inheritance of *dap*$^-$ with *his*, *proC*, *pyrA* and *thr* markers was determined in 75-min uninterrupted conjugation crosses. The cross with AT984 failed, apparently owing to loss of the Hfr mating type in this strain. The results with strain AT999 demonstrated a close linkage between its *dap*$^-$ mutation and the *pyrA* and *thr* markers near min 1 on the

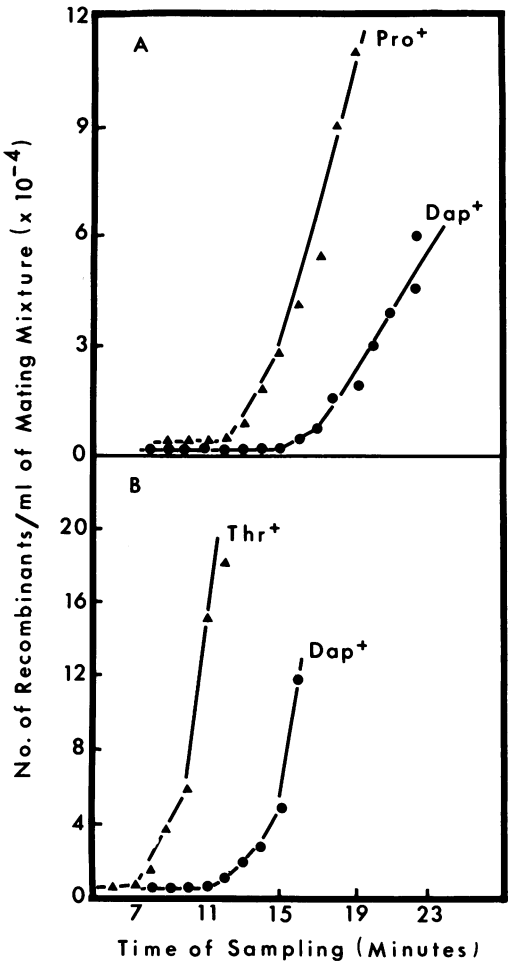

FIG. 2. *Kinetics of recombinant formation in interrupted conjugation crosses. (A) AB257 (HfrC) × AT955, selection for Pro+ Str^R and DAP+ Str^R recombinants. (B) AT793 (HfrH) × AT957, selection for Thr+ Str^R and Dap+ Str^R recombinants.*

linkage map. The *dap*⁻ mutations of AT978 and AT998 were found to be located at a different position, between *his* and the point of origin of chromosomal transfer by Hfr KL16 (i.e., between min 39 and 56).

Identification and precise mapping of dapA and dapE. The location of the *dap*⁻ mutations in AT978 and AT998 at min 39 to 56 roughly corresponds to the preliminary mapping results of Cohen et al. (4) for genes controlling succinyl-DAP deacylase (*dapE*) and DHDP synthetase (*dapA*). The *dap*⁻ mutation of the infertile strain AT984 was also shown to map in this region by transduction experiments described below. Gilvarg (9, 10) has shown that a mutant deficient in succinyl-DAP deacylase excretes *N*-succinyl-L-DAP, a substance which is chromogenic in the DAP colorimetric assay and which can be slowly converted to L-DAP in the acidified medium. It was thought possible to exploit Gilvarg's finding to ascertain whether AT978, AT984, and AT998 lacked one or both of the enzymes mentioned above since a mutation in the *dapE* locus would result in accumulation of *N*-succinyl-DAP, whereas mutants with *dapA* mutations would not show this accumulation. Strain AT978 (and all other strains carrying its *dap*⁻ marker) was found to accumulate material chromogenic in the specific DAP assay. Furthermore, L-DAP could be detected in the acidified medium by paper chromatography. There was also a residual amount of *meso*-DAP, as found in other Dap⁻ mutants. On the other hand, AT984 and AT998 (and all other strains carrying their specific *dap*⁻ markers) did not accumulate significant amounts of chromogenic material under these conditions. Therefore, it appears that AT978 is a mutant at the *dapE* locus whereas AT984 and AT998 probably represent blocks at *dapA*, the gene for the first step of DAP synthesis from ASA.

TABLE 3. *Cotransduction of dap alleles with tonA*
P1 donor: AT941 *thyA12 tonA3*

Recipient	No. of Dap+ transductants examined	Per cent of transductants which score as T1 resistant
AT980 (*dapD2*)	90	65
AT982 (*dapD4*)	78	91
AT983 (*dapD5*)	150	84
AT985 (*dapD7*)	100	85
AT986 (*dapD8*)	125	80
AT915 (*dapD12Mu+*)	96	84
AT997 (*dapC15*)	196	42
AT1001 (*dapD18*)	80	85

To locate these genes more precisely on the linkage map, interrupted conjugation experiments were done with Hfr KL98 and Hfr KL16 by using F⁻ recipients with *dapA16* and *dapE9* mutations. Hfr KL98 transferred neither of these markers early. Hfr KL16 did transfer both markers early, and the results shown in Fig. 3 placed both at min 46–48, i.e., about 7 to 9 min clockwise from the *his* locus at min 39.

An attempt was made to cotransduce these markers with known nearby gene loci. The standard linkage map of *E. coli* places *glyA*, *gua*, and *purC*, *ctr* and *nadB* (formerly *nicB*) in this region (29). Results of the transduction analyses and linkage relationships of various markers in this chromosomal segment are summarized in Table 4. Experiments with *dapA16* and *dapE9* strains as P1 donors and AT2465 (*guaA5*) as recipient showed that 18 to 20% of Gua+ transduc-

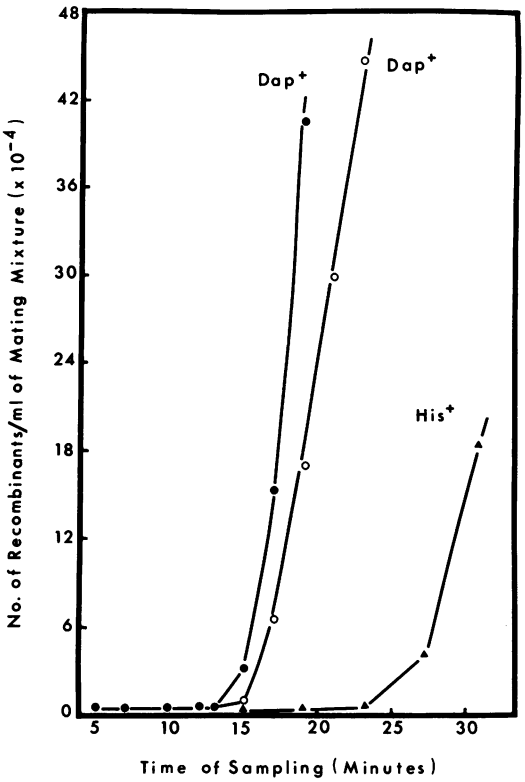

FIG. 3. *Kinetics of recombinant formation in interrupted conjugation crosses of Hfr KL16 × AT1018 with selection for Dap⁺ Str^R recombinants* (○) *and Hfr KL16 × AT1015 with selection for Dap⁺ Str^R and His⁺ Str^R recombinants.*

TABLE 4. *Linkage between various loci in the 46 to 50 min region*

PI donor	Recipient	Selected marker	Unselected marker	Per cent of cotransduction	
AT998	AT2465	Gua⁺	*dapA16*	20	(40/200)
AT978	AT2465	Gua⁺	*dapE9*	18	(36/200)
AT984	AT2465	Gua⁺	*dapA6*	18	(19/104)
AT978	AT2457	Gly⁺	*dapE9*	0	(0/200)
AT998	AT2457	Gly⁺	*dapA16*	0.3	(1/300)
AT1055	AT2457	Gly⁺	*nadB2*	13	(35/264)
AT1055	AT2465	Gua⁺	*nadB2*	0.5	(1/204)
Pa3306C	AT1056	Dap⁺	*ctr-2*	11	(24/214)
Pa3306C	AT1057	Dap⁺	*ctr-2*	8.5	(20/230)
AT998	AT3006	Pur⁺	*dapA16*	0.3	(1/300)
AT1018	AT3006	Pur⁺	*dapE9*	0	(0/300)

tants inherited the *dapA16* and *dapE9* markers. Similar experiments with AT2457 (*glyA6*) as P1 recipient revealed no cotransduction of *dapE9* with *glyA6* and 0.3% cotransduction of *dapA16* with *glyA*. Since there is 14% cotransduction between *glyA* and *guaA* (29), it follows that *dapA16* and *dapE9* must be on the opposite side of *guaA* with respect to *glyA*. AT984, which could not be used in conjugation experiments, was also found to contain a mutation (*dapA6*) which cotransduced with *guaA5* at a frequency of 18%. Other crosses listed in Table 4 showed that the locus of *ctr* mutations described by Wang, Morse, and Morse (34) cotransduces 11 and 8.5% with *dapE* and *dapA*, respectively. Since *ctr* is not cotransducible with *guaA* (34), it follows that the two *dap* genes must lie between *guaA* and *ctr*. The remaining crosses in Table 4 confirm the recent findings of Tritz et al. (30), who demonstrated the gene order in this region to be *pheA-nadB-pur-glyA-guaA*.

Orientation of dapA and dapE. Reciprocal three-factor transduction crosses were set up to determine the orientation of *dapA16* and *dapE9* with respect to *guaA5*. Crosses and the results obtained are shown in Table 5. The ratios of Gua⁺ Dap⁺/Gua⁺ transductants in the control crosses A and B are 0.33 and 0.43, respectively, as compared to 0.026 in cross C and 0.058 in cross D. Comparable results were obtained when the Dap⁺ trait was scored as an unselected marker (Table 5, column 7). Thus, Gua⁺ Dap⁺ transductants are more frequent in cross D (*dapE9* × *guaA5 dapA16*) than in cross C (*dapA16* × *guaA5 dapE9*). Assuming that recombination which demands quadruple crossovers would generate the least number of Gua⁺ Dap⁺ transductants, the order of these gene loci must be *guaA-dapA-dapE*. The gene order and map distances in the 46 to 50 min segment of the linkage map, as inferred from the above results, are depicted in Fig. 4A.

Identification and mapping of the dapB locus. Farkas and Gilvarg (6) described an auxotroph of *E. coli* W, strain M-203, which is defective in DHDP reductase, the enzyme catalyzing the second reaction in the conversion of aspartic-β-aminaldehyde (ASA) to DAP. The evidence presented below will show that mutations in M-203 and AT999 map at the same locus. AT999 is therefore also likely to have a lesion in DHDP reductase.

Interrupted conjugation experiments with HfrH and an F⁻ strain which carried the *dap*⁻ mutation from AT999 showed that Dap⁺ recombinants first appear about 7 to 8 min after the onset of mating. Since the *thr* marker of Hfr H is also known to enter at 8 min, the *dapB* locus is evidently close to *thr*. Transduction crosses were performed to determine the exact position of *dapB* in the *thr-pyrA-ara-leu* region. The results are given in Table 6. In cross A, the distribution of unselected donor markers among Dap⁺ transductants suggests that *dapB17Mu*⁺ is nearer to *thr* than to *ara* or *leu*. The fact that 18 out of 19 Leu⁻ transductants are also Ara⁻ indicates that

TABLE 5. *Order of the dapA, dapE, and guaA genes*[a]

Genotype of strains:			
KL16	+	+	+
AT1062	*guaA5*	+	*dapE9*
AT1063	*guaA5*	*dapA16*	+
AT998	+	*dapA16*	+
AT978	+	+	*dapE9*

Cross	P1 donor	Recipient	Selection	No. of selected transductants obtained/ml	Gua⁺ Dap⁺/Gua⁺	Per cent of Gua⁺ transductants which score as Dap⁺
A	KL16	AT1062	Gua⁺	4.4×10^3	0.33	30
			Gua⁺ Dap⁺	1.7×10^3		
B	KL16	AT1063	Gua⁺	5.8×10^3	0.43	40
			Gua⁺ Dap⁺	2.4×10^3		
C	AT998	AT1062	Gua⁺	1.9×10^3	0.026	3
			Gua⁺ Dap⁺	5.0×10^1		
D	AT978	AT1063	Gua⁺	2.6×10^3	0.058	9.5
			Gua⁺ Dap⁺	1.6×10^2		

[a] Gua⁺ Dap⁺ recombinants were either selected directly (column 5) or Gua⁺ transductants were selected and tested for Dap⁺ trait as an unselected marker by replica plating. In each case, 200 Gua⁺ transductants were examined for the inheritance of Dap⁺ (column 7).

TABLE 6. *Mapping of the dapB locus*

P1 donors: AB712 *thr-4 ara-14 leu-8*
AT2363 *thr-4 ara-14 leu-8 pyrA49Mu⁺*
Recipients: AT999 *dapB17Mu⁺*
AT3154 *dapB* (*E. coli* W strain M-203)

Cross	P1 donor	Recipient	No. of Dap⁺ transductants scored	No. of Dap⁺ transductants that score as								
				Thr⁻	Pyr⁻	Leu⁻	Ara⁻	Leu⁻ Ara⁻	Leu⁻ Thr⁻	Thr⁻ Ara	Thr⁻ Pyr	Ara Pyr
A	AB712	AT999	196	79		19	33	18	2	4		
B	AT2363	AT999	218	73	73	1	10	1	0	0	18	9
C	AB712	AT3154	50	11		2						
D	AT2363	AT3154	160	29	5	1						

ara lies between *dapB* and *leu*. Furthermore, *dapB* apparently lies between *thr* and *ara* because the 17% (33/196) cotransduction of *ara* with *dapB* is significantly higher than the known 3 to 7% cotransduction frequency of *ara* with *thr* (29). In cross B with AT2363 (*thr-4 pyrA49Mu⁺ ara-14 leu-8*) as the P1 donor, coinheritance of both Thr⁻ and Pyr⁻ characters with Dap⁺ is 33.5% (73/218), whereas Ara⁻ and Leu⁻ are cotransduced at frequencies of about 5% (10/218) and 0.5% (1/218), respectively. There is some reduction in cotransduction frequencies due to the presence of prophage Mu-1 at the *pyrA49* site; *pyrA* is therefore closer to *dapB* than the 33.5% figure indicates. Since 9 out of 10 Dap⁺ Ara⁻ clones are also Pyr⁻, we conclude that *pyrA* is situated between *ara* and *dapB*. The overall gene order is therefore *thr-dapB-pyrA-ara-leu*.

Similar experiments were done with the mutant M-203 (Table 6, crosses C and D). Although there is restriction of P1 phage in this *E. coli* W strain which precludes its use as a donor, it can be used successfully as a recipient in transduction crosses. The reduction in cotransduction frequencies recorded in Table 6 might be the result of this restriction. However, the results clearly indicate that the mutation in M-203 maps at the same place as *dapB17Mu⁺*. Figure 4B depicts the linkage relationship of *dapB* with other markers of this region.

Mapping of dap genes in the tonA region. The results presented in Table 3 demonstrated that 8 out of 12 independent *dap⁻* mutations are clustered near the *tonA* locus, their cotransduction frequencies with *tonA* varying over a wide range. This raised the possibility that the chromosomal segment near *tonA* might contain more than one gene involved in DAP synthesis. Three-factor transduction crosses were set up to determine the orientation of the *dap⁻* mutations with respect to *tonA* and a second nearby marker, *pan* (29). Table 7 gives the results of such crosses.

Part I lists the crosses in which AT1009 (*pan-4, tonA7*) was the P1 donor. With AT980 (*dapD2*) and AT982 (*dapD4*) as the recipients, inheritance of T1 resistance among Dap⁺ transductants was

TABLE 7. *Order of dapD2, dapD4, dapD12Mu⁺, and dapC15 with respect to tonA and pan*

Part	P1 donor	Recipient	Selected marker	No. scored	Per cent of transductants that score as				Per cent of Pan⁻ transductants that are	
					T1ᴿ		Pan⁻		T1ᴿ	T1ˢ
I	AT1009 (*pan-4 tonA7*)	AT980 (*dapD2*)	Dap⁺	75	63		29		86	
		AT982 (*dapD4*)	Dap⁺	150	90		33		98	
		AT997 (*dapC15*)	Dap⁺	150	55		60		35	
					T1ˢ	Pan⁻	T1ᴿ	Dap⁻	T1ᴿ	T1ˢ
II	AT1371 (*pan-4*)	AT1002 (*dapD12Mu⁺ tonA4*)	Dap⁺	208	52	20				84
	AT1002 (*dapD12Mu⁺ tonA4*)	AT1371 (*pan-4*)	Pan⁺	362			63	0.3		
					Per cent of transductants that inherit					
					tonA4				*dapD12Mu⁺*	
III	AT1002	AT997	Dap⁺	125	50				<1	

much greater than inheritance of the Pan⁻ trait, showing that *tonA* is nearer to the *dap* mutations than *pan*. In addition most of the Pan⁻ transductants (86 and 98%) were also T1 resistant; thus, the transducing fragments which cover the *dap* and *pan* markers also frequently include the *tonA* locus. The inferred gene order is therefore *pan-tonA-(dapD2, dapD4)*. Similar results, not shown in Table 7, were obtained for the *dapD5, D7, D8,* and *D18* mutations. On the other hand, when AT997 (*dapC15*) was used as the recipient, only 35% of the Pan⁻ transductants were also T1 resistant. Moreover, the *pan-4* and *tonA7* markers were cotransduced with Dap⁺ at roughly equal frequencies of 55 and 60%, respectively. These results suggest that *dapC15* may be located between *pan* and *tonA*.

Part II of Table 7 describes reciprocal crosses between AT1371 (*pan-4*) and AT1002 (*dapD12Mu⁺ tonA4*). With AT1371 as the donor, 52% of Dap⁺ transductants are T1 sensitive as compared to 20% which are Pan⁻. A large proportion of the transductants (84%) which inherit the *pan-4* marker of the donor also acquire T1 sensitivity. This distribution would be expected if *tonA4* were located between the *pan* and *dapD12Mu⁺* mutations. This conclusion is supported by the reciprocal cross in which AT1002 is the donor. Among Pan⁺ transductants, 63% are T1 resistant and only 0.3% are Dap⁻. (Extremely low cotransduction of *dapD12Mu⁺* with *pan⁺* is due to the presence of prophage Mu-1 at the mutation site.) The gene order determined from these crosses is *pan-tonA4-dapD12Mu⁺*.

The cross outlined in part III of Table 7 gives further evidence that *dapD12Mu⁺* and *dapC15* lie on opposite sides of *tonA*. L agar was used instead of MM for selecting Dap⁺ transductants in this cross. As noted earlier, *dapD12Mu⁺* strains grow satisfactorily on L agar and one can therefore score *dapD12Mu⁺* as an unselected marker among Dap⁺ clones selected on L-agar plates. Of 126 Dap⁺ transductants examined, none inherited the *dapD12Mu⁺* marker, whereas cotransduction of *tonA* and *dapC* (50%) was unaffected. This excludes the possibility that *dapD12Mu⁺* lies between *tonA* and *dapC*, since in that case the cotransduction of *tonA* and *dapC* would have been severely reduced. Taking into account the previous crosses which showed the gene order to be *pan-dapC-tonA* and *pan-tonA-dapD12Mu⁺*, it must be concluded that there are two distinct regions, one on each side of *tonA*, which affect the synthesis of DAP. The overall sequence which emerges from the above results is *pan-dapC15-tonA dapD(12Mu⁺, 2, 4)*.

The enzymatic blocks resulting from the mutations in the *tonA-dap* region are not known, and the assignment of gene symbols *dapC* and *dapD* to the loci on the opposite sides of *tonA* is therefore arbitrary. Linkage relationships of markers in the *pan-tonA* region are summarized in Fig. 4C.

Genetic analysis of Dap⁺ revertants of AT915 (dapD12Mu⁺). All Dap⁻ mutants examined so far, including the two Mu-1 induced mutants, generate prototrophic revertants. Apparent reversions of phage-induced mutations are of special interest because these mutations are usually found to be very stable (32). For this reason, some of the revertants of *dapD12Mu⁺* strains were characterized genetically.

Revertants of *dapD12Mu⁺* strains which no longer require DAP occur at a frequency of 1 to 10 per 10^8 cells plated and can be isolated on MM plates after 2 to 3 days of incubation. These revertants grow more slowly than their wild-type ancestors in MM, suggesting that normal physio-

logical functions are only partially restored in these mutants. To map the reversion sites, 11 independent Dap$^+$ revertants of AT915 were employed as P1 donors in backcrosses to recipient strain AT1002 (*dapD12Mu*$^+$, *tonA4*). In each cross, Dap$^+$ transductants were selected and scored for coinheritance of the donor T1 sensitive trait. All of the reversion mutations (designated here as *rev-1* through *rev-11*) mapped in the *tonA* region because each donor showed measurable cotransduction of T1 sensitivity with the selected Dap$^+$ marker. Cotransduction frequencies of the 11 *rev* mutations with *tonA* ranged from 29 to 53%. These values are lower than the usual 80–85% cotransduction obtained with wild-type Dap$^+$ donors (Table 3), which may mean that the *rev* mutations are produced at secondary sites.

Proof that reversions of the *dapD12Mu*$^+$ mutant involve mutations at secondary sites was obtained by recovering the *dapD12Mu*$^+$ marker from revertants in the following manner. Phage P1 grown on the revertant strains (*dapD12Mu*$^+$, *rev-1* to *rev-11*) was used to infect recipient strain AT896 (*dapD8*). Taking advantage of the phenotypic difference between the donor and recipient Dap$^-$ traits, we selected for Dap$^+$ transductants on L agar with the expectation that *dapD12Mu*$^+$ would be recovered as an unselected marker on this medium. When transductants were scored for DAP requirement on MM, clones with the *dapD12Mu*$^+$ phenotype were detected in every case. These results indicate that the original mutation is still present in the revertants and that all reversions examined so far involve suppressor mutations at sites which are close to, but distinct from, the original *dapD12Mu*$^+$ site.

DISCUSSION

The results demonstrate that most of the genes concerned with DAP-lysine synthesis are scattered on *E. coli* chromosome and, therefore, do not constitute an operon. There are four general chromosomal regions, widely separated from each other, which contain the genes for DAP-lysine formation beginning from aspartate.

The genes for lysine-sensitive aspartokinase (*apk*) and aspartic semialdehyde dehydrogenase (*asd*) are located in the 66 to 67 min region near the *mal* locus (21, 25). These two enzymes catalyze reactions before the specific branch leading from aspartic semialdehyde to lysine, and their reaction products contribute to the pool of intermediates common for lysine, threonine, and methionine biosynthesis.

The second region of DAP-lysine synthesis consists of the *lysA* locus at min 55 which determines the synthesis of DAP decarboxylase, the enzyme for the terminal step of lysine synthesis. Mutations in the *lysA* region give rise to two distinct phenotypes. One phenotypic class is devoid of DAP decarboxylase activity, whereas the second class produces an enzyme protein which is inactive only in the absence of pyridoxal 5′-phosphate (3*a*).

The third region consists of genes coding for DHDP synthetase (*dapA*) and succinyl-DAP deacylase (*dapE*) which were identified on the basis of earlier observations by Cohen et al. (4). These markers are located at approximately 47.5 min on the genetic map. They map closely together between the *gua* and *ctr* loci and may possibly represent an operon; it has not been proven, however, that *dapA* and *dapE* are contiguous genes.

The fourth and most complex region lies in the 0 to 2.5 min segment of the genetic map (Fig. 4).

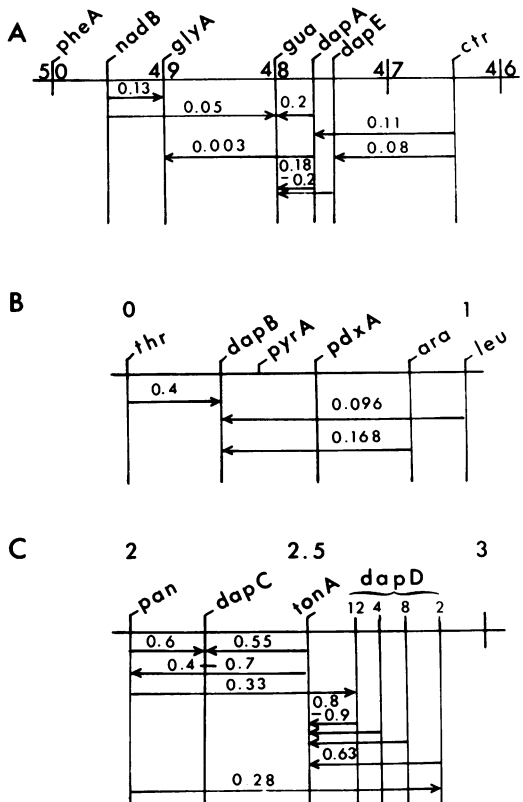

FIG. 4. *Linkage of dap genes to various reference loci in (A) the 46 to 50 min segment of the standard linkage map, (B) the 0 to 1 min segment, and (C) the 2 to 3 min segment. Cotransduction frequencies are given as the ratio: number of recombinants inheriting the unselected marker per number of selected transductants examined. For each cross, the selected marker is at the head of the arrow and the unselected marker is at the tail.*

The *dapB* gene, which codes for DHDP reductase in *E. coli* W (6), is located between *thr* and *pyrA* near min 0.5. The *dapC* gene maps near min 2, between *pan* and *tonA*. A third region, designated *dapD* is also located close to *tonA* but on the side away from *pan*. The functional roles of the *dapC* and *dapD* loci have not yet been identified because of the unavailability of substrates for the middle enzymes of the DAP pathway. The assignment of letters *C* and *D* to these loci is therefore arbitrary and does not necessarily reflect the order of the enzymatic steps involved. The *dapD* locus seems to be a complicated genetic region, possibly consisting of more than one gene. Although no complementation tests have been done to investigate this point directly, there is some indirect evidence for multiple loci. First, a disproportionately large share of mutations resulting in DAP auxotrophy (about 60% of all mutants studied) map in the *dapD* region. Second, the *dapD* mutants show a broad range of cotransduction frequencies with *tonA* (65 to 91% in Table 3), a result which suggests that the mutations cover a large genetic region. Third, mutations in this region can lead to distinctly different phenotypes, as exemplified by the different growth responses of *dapD12Mu*$^+$ and *dapD8* strains on L agar. Fourth, partial restoration of DAP synthesis in a *dapD* mutant can occur by mutations at secondary sites in this region.

Resolution of the *dapD* region must await further enzymological and genetic characterization of the mutants involved. For the present, the six gene loci described in this study are sufficient to account for all of the known steps involved in the conversion of ASA to lysine (Fig. 1). No mutant blocked at the DAP epimerase step has been isolated and, therefore, the location of the DAP epimerase gene remains unknown.

It should be noted that the chromosomal region near *tonA*, where most of the DAP mutations were mapped, apparently plays an important role in the overall construction of the cell wall of *E. coli*. Normark et al. (20) described a locus, *envA*, at 2 to 4 min, in which mutations lead to incomplete septum formation and abnormal cell fission. Matsuzawa et al. (17) recently provided direct evidence that two genes affecting the synthesis of cell wall peptidoglycan are located between *ara* and *lac*. Their data, indicating that the distance between these loci and *ara* is approximately 0.21 of the distance between *ara* and *lac*, places these loci in the *tonA-dap* region. Indeed, the *tonA* locus itself is concerned in some way with the outer surface of the cell as it determines the receptor sites for phages T1 and T5. Many of the known genes in the 2 to 5 min segment of the *E. coli* chromosome thus appear to be specifically involved in cell envelope formation.

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