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Mutant Strains of *Escherichia coli* K12 That Use D-Amino Acids

(histidine/phenylalanine/tryptophan/keto acid/deamination/membrane transport)

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ABSTRACT **A series of mutations has been isolated that confer upon amino-acid auxotrophs of *Escherichia coli* K-12 the ability to grow when fed various D-amino acids. Several distinct systems, mediating cellular use of the D-isomers of leucine, histidine, phenylalanine, tyrosine, tryptophan, isoleucine, and valine, can be mutationally activated. Mutations leading to D-tryptophan use (*dadR*) all map near *purB*. They result in high activities of an enzyme that deaminates D-amino acids. Neither the enzymes of the tryptophan biosynthetic pathway nor tryptophanase (EC 4.2.1.e) are involved in D-tryptophan utilization.**

D-amino acids occur infrequently in nature, usually as constituents of bacterial cell walls (1) or in polypeptide antibiotics (2). *Escherichia coli* strains can metabolize several D-amino acids, including D-alanine and D-glutamic acid, which are integral components of the cell wall (3). D-alanine is formed from L-alanine by alanine racemase (EC 5.1.1.1) (4). In *E. coli*, *cys* or *met* mutants are capable of using either isomer of cysteine or methionine for growth. All other amino-acid mutants normally require the appropriate L form. Use of D-methionine by methionine auxotrophs was studied by Cooper (5), who showed that the use of the D form was oxygen dependent; he isolated mutants unable to use D-methionine. The enzymatic basis of this conversion remains unknown, but it seems reasonable to assume that an oxidative deamination is one of the steps.

MATERIALS AND METHODS

Isolation of Mutants Able to Use A Particular D-Amino Acid. The parental strains that gave the mutants described in this paper were all ultraviolet-induced auxotrophs isolated by penicillin selection from various laboratory strains of *E. coli* K12 (W1485 or Y_{mel}). All of them mutated to give small numbers of prototrophic revertants, in addition to derivatives capable of using D-amino acids to fulfill their nutritional requirements. The appropriate L-amino-acid auxotrophs were grown overnight in 10 ml of L broth (6), harvested, washed with saline (0.85%), and resuspended in 0.2 ml of saline. A portion (0.1 ml from each tube) was plated on a minimal agar (7) plate containing 20 μg/ml of the corresponding D-amino acid. A total of 1–2 × 10^{11} cells were plated for each amino-acid requirement tested. After incubation at 37°C for 3–10 days, all colonies were picked and tested with and without the D-amino-acid supplement. Auxotrophic strains capable of using either isomer were purified by successive single-colony isolations. To ensure that each mutant arose independently, 20 separate cultures were grown for each mutant preparation, and all cultures were started from freshly isolated single colonies. Only one mutant per tube was kept.

Genetic Mapping by Transduction with Bacteriophage P1kc. Conditions for the growth of bacterial recipients, preparations of donor P1 phage, and performance and scoring of the crosses were those of Yanofsky and Lennox (8). Minimal media plus indole (10 μg/ml) were used to select for transductants in the crosses with a *trp* deletion as a recipient. To score for segregation of *dadR*, the transductants were replica-plated to media containing D-tryptophan. When a *trpE trpA purB* strain was the recipient, *purB*$^+$ transductants, selected on minimal media plus L-tryptophan (20 μg/ml), were replica-plated to medium containing D-tryptophan. In crosses involving selection for *cysB* and *pyrF*, both donor and recipient carried the same *trpE* allele, and *dadR* was the unselected marker. To determine a precise cotransduction frequency between *dadR* and the *trp* operon, the recipient was *trpE* T3 *trpA* and the donor was *trpE* T3 *dadR*. Selection was for *trpA*$^+$, and the segregation of the unselected *dadR* mutation was determined by replication. Controls for reversion or *trp*$^+$-recombinant formation were negative in each experiment.

Growth of Strains for Enzyme Determinations, Preparation of Extracts, and Assay of D-Amino-Acid Deaminase. All strains were grown in minimal medium containing either glucose or glycerol (0.2%) as the carbon source. When added, D- or L-tryptophan was present at 20 μg/ml. After the cells were harvested by centrifugation, they were disrupted in a Branson W-185 C sonifier, then centrifuged for 15 min at 20,000 rpm to remove all cell debris. To measure D-amino acid oxidase activity the following assay was performed (T. Klopotowski, personal communication): 20 μmol of substrate, 500 μmol of sodium phosphate (pH 8.0), and 0.2 ml of crude extract were mixed in a total volume of 5.0 ml. Samples (2.0 ml) were removed at 30 sec and at an appropriate termination time (1.5–30 min) and pipetted into 2.0 ml of 1 N NaOH. When D-histidine was the substrate, incubation was continued for an additional 30 min after the addition of NaOH to allow chromogen formation. Ice-cold 1 N NaOH was used to terminate reactions with D-phenylalanine as substrate. Solutions were kept cold until absorbance determinations to prevent chromogen breakdown. A relative activity of 1.0 equals an absorbancy change of 0.054 per mg of protein in 30 min at 310 nm with D-histidine as the substrate, and 0.61 per mg of protein in 30 min at 320 nm with D-phenylalanine as the substrate (see below for a description of the chromogen in this assay).

RESULTS

We have found that many amino-acid auxotrophs of *E. coli* K12 can undergo mutations that allow them to use D-amino

acids for growth as efficiently as they use the corresponding L isomers. In all likelihood, the D-amino acid is converted to the L-isomer, rather than directly incorporated into protein, since it is a generally accepted tenet of biology that all optically active amino acids found in proteins are of the L-configuration (9).

As shown in Table 1, we have obtained mutants able to use the D- and L-isomers of histidine, phenylalanine, tryptophan, tyrosine, leucine, isoleucine, and valine. Two classes of D-histidine-utilizing histidine-requiring strains were obtained. The first had a growth rate on agar plates similar to the wild type, while the other grew at about one-third of this rate. A similar result has been found for D-histidine-utilizing (*dhu*) mutants of *Salmonella typhimurium* (10). Mutations that permit D-histidine utilization are encountered relatively frequently; every tube gave mutants of both kinds. Mutants able to use D-phenylalanine (*dfu*)*, D-tryptophan (*dadR*), D-tyrosine (*dyu*), and D-leucine (*dlu*) arise less frequently. Mutants that can utilize both D-valine (*dvu*) and D-isoleucine were rare.

Since the genetics, regulation, and enzymology of the tryptophan pathway in *E. coli* have been extensively characterized (11), the D-tryptophan-utilizing mutants were further analyzed. These *dadR* mutations were selected from parental strains with lesions in various genes of the tryptophan operon. That any *trp* gene is itself required for conversion of D-tryptophan was ruled out by performing a transduction cross using a *cysB trpE dadR* strain as a recipient, and phage P1kc grown on a T1-resistant trp^{A-E} deletion (8) as donor. $CysB^+$ recombinants that had incorporated the trp^{A-E} deletion from the donor strain were selected. All grew on D-tryptophan. In separate experiments, spontaneous mutations to *dadR* were selected in an *E. coli* strain that had lost by means of a deletion mutation, all the genes in the *trpE–tonB* region, which includes the entire *trp* operon. Although they were rare, several were obtained.

In *E. coli*, tryptophan can be degraded by tryptophanase to indole, pyruvic acid, and ammonia. Although crystalline tryptophanase has no activity on the D-isomer (12), it could not be ruled out *a priori* that tryptophanase was involved *in vivo;* this enzyme catalyzes several minor reactions involving tryptophan, indole, and some of their derivatives. Triple mutants that were tryptophanase-less and tryptophan-requiring, yet were capable of using D- or L-tryptophan were synthesized. We verified that the multiple-mutant strains we constructed lacked tryptophanase *in vivo* by showing that they excreted no indole upon growth in tryptone broth. When tryptophanase-positive cultures are tested with acidic *p*-dimethylaminobenzaldehyde after growth in broth, an immediate cherry-red color, diagnostic for indole, appears. It therefore seems unlikely that tryptophanase is involved in the utilization of D-tryptophan.

* The basis for our notation, which may undergo change as the biochemical basis for the observed phenotypes becomes better understood, is as follows: e.g., for leucine, dlu: d (for D-amino acid), l (for leucine), u (for utilization. The single-letter designations for each amino acid are those suggested by the IUPAC-IUB Commission on Biochemical Nomenclature, *J. Biol. Chem.*, **243**, 3557 (1968).

† Rahmanian, M., and D. L. Oxender, *Fed. Proc.*, **30**, 1061 (1971).

‡ Thorne, G. M., and L. M. Corwin, *Bacteriol. Proc.*, 21 (1970); Thorne, G. M., and L. M. Corwin, *Fed. Proc.*, **30**, 1115 (1971).

TABLE 1. *Mutations that allow auxotrophs to use the pertinent* D *isomer*

Obtained	Not obtained
Histidine	Arginine
Isoleucine	Lysine
Leucine	Proline
Phenylalanine	Serine
Tryptophan	Threonine
Tyrosine	
Valine	

The gene or genes responsible for allowing *trp* mutants to use the D-isomer is closely linked to the *trp* operon and to *purB*. A summary of some representative data for nine mutants is given in Table 2. A map of the *pyrF* to *purB* region of the *E. coli* genetic map, and the inkage relationship of certain markers to *dadR* is shown in Fig. 1. Since a transducing fragment has a maximum length of about 2 min (16) in terms of conjugational time-of-entry differences, the position of *dadR* on the conjugational map should be at about 23.3 min, because the *pyrF* to *dadR* distance is about equal to the maximum length of a transducing fragment. No gene involved in tryptophan metabolism or regulation is known to reside in the *purB* region.

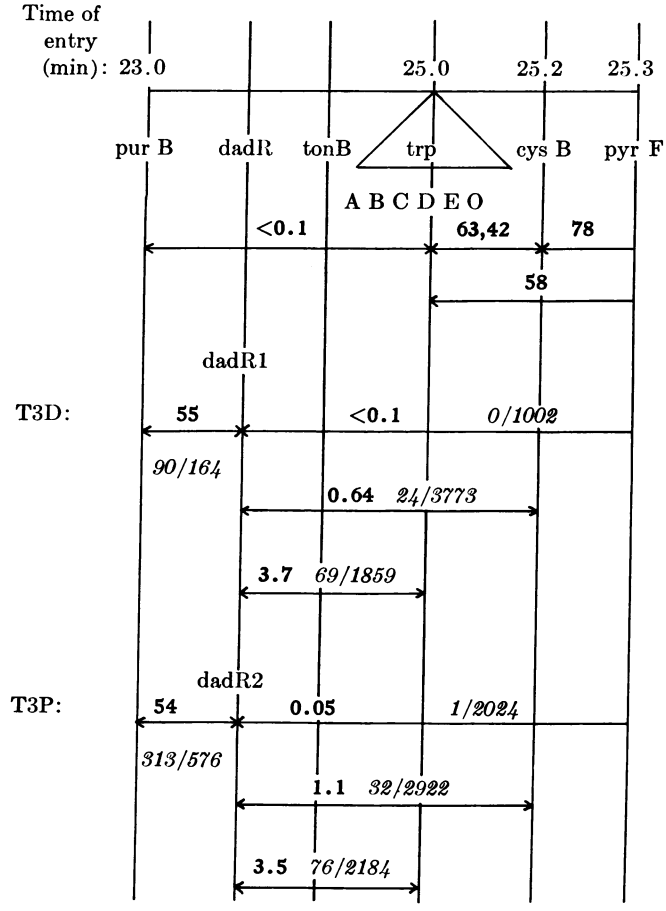

FIG. 1. Transductional map of the *purB-pyrF* region in *E. coli*. The data for the top half of the figure is from refs. 13–15. Numbers in bold-face type represent per cent cotransduction. The actual data is given as fractions (italicized). Explanation of abbreviations and description of these loci are given in ref. 16.

TABLE 2. *Genetic linkage between dadR and the trp operon, and between dadR and purB*

Donor strain	*dadR* mutation	*trp* mutation	*trp* deletion recipient		*pur B* recipient	
			Transductant colonies	Cotransduction (%)	Transductant colonies	Cotransduction (%)
T3D	*dadR1*	*E*	126	2.4	164	54.9
T3P	*dadR2*	*E*	70	1.4	576	54.3
DT80A	*dadR3*	*D*	543	2.6	345	47.5
DT80B	*dadR4*	*D*	100	1.0	301	48.8
DT80C	*dadR5*	*D*	286	1.7	282	56.7
CT20E	*dadR6*	*C*	1105	4.0	303	51.5
CT24D	*dadR7*	*C*	1817	5.4	301	48.5
CT24E	*dadR8*	*C*	2174	4.5	309	51.5
CT24F	*dadR9*	*C*	406	4.7	222	50.5
Average	—	—	—	4.3	—	51.6

Enzymes such as D-amino-acid oxidase (17) catalyze the oxidative deamination of a number of D-amino acids in higher organisms. The possibility therefore existed that mutational changes in a common system resulted in the aquisition of the ability to use the D-isomers of histidine, tryptophan, leucine, isoleucine, valine, phenylalanine, and tyrosine. To examine this question, we constructed a number of double auxotrophs that carried, in addition, a single mutation conferring the capability to use D-tryptophan. These strains were then checked to see whether the other requirement could be satisfied by its D-isomer, e.g., *trp his dadR* was tested for growth on minimal medium containing L-tryptophan and D-histidine. The presence of *dadR* in a strain does not permit auxotrophs requiring L-lysine, L-arginine, L-threonine, L-isoleucine, or L-tyrosine to use the pertinent D-isomer. However, D-phenylalanine, D-leucine, and D-histidine are used by several *dadR* strains. The mutations we have so far selected that confer *dlu*, *dhu*, *dvu*, and *dfu* phenotypes are unable to grow on D-tryptophan. It seems certain, therefore, that a number of different systems are involved in D-amino-acid utilization.

Mutant strains that carry *dadR* have elevated activities of an enzyme system capable of deaminating D-histidine and D-phenylalanine. D-histidine and D-phenylalanine are convenient substrates, because their deamination products, imidazole pyruvic acid and phenylpyruvic acid, yield relatively stable enol tautomers in alkali. These enol tautomers, because of conjugation with the aromatic ring, show strong absorption bands at about 310 nm. The keto acid corresponding to tryptophan, indole pyruvic acid, is very unstable and cannot be accurately quantitated spectrophotometrically.

Table 3 shows the D-amino-acid oxidase activities observed in crude extracts of several different strains grown under various growth conditions. The assays of enzymatic activity were always performed under conditions where the extent of the reaction was linear with time and with enzyme concentration. The protein concentrations of the cell-free extracts were all about equal, so that the results are unlikely to be a consequence of the disruption of the cells themselves. It is clear from these data that the presence of the *dadR* (D-amino-acid deaminase; regulatory) mutation leads to a 16- to 95-fold increase in the enzyme activities found in crude extracts. Growth with glycerol as the carbon source (*lines 4 to 6*) rather than glucose (*lines 1 to 3*) has little or no effect on enzyme activity. In the lower half of the table (*lines 7 to 12*) are shown data for a number of *trp*$^+$ derivatives with a complete, wild-type, tryptophan operon carried on an episome. This method of eliminating tryptophan auxotropy was chosen over transduction with phage P1kc to avoid obtaining *dadR*$^+$ recombinants (see Fig. 1 and Table 2). These *trp*$^+$ strains were used to see whether the presence of D-tryptophan affected the formation of D-amino-acid oxidase. It is clear from the data (*lines 7–12*) that D-tryptophan does not induce, and exogenous L-tryptophan does not repress, the enzyme under these conditions. The activity of enzyme in strains that carry *dadR1* and *dadR2* remained relatively constant, so the various growth conditions examined seem to be unimportant in determining enzyme activity when a *dad R* mutation is present. Finally, the con-

TABLE 3. *The effect of genotype and growth conditions on* D-*amino-acid deaminase*

Genotype	Carbon source	Additions	Relative D-His activity	Relative D-Phe activity	D-Phe activity / D-His activity
trp E	Glucose	L-Trp	1.0	1.0	11.3
trp E dadR1	Glucose	L-Trp	28.8	26.1	10.2
trp E dadR2	Glucose	L-Trp	26.7	15.9	6.7
trp E	Glycerol	L-Trp	2.3	2.0	9.9
trp E dadR1	Glycerol	L-Trp	78.0	95.8	13.8
trp E dadR1	Glycerol	L-Trp	30.7	30.2	11.1
trp E/F'trp	Glucose	D-Trp	3.6	3.0	9.5
trp E dadR1/F'trp	Glucose	D-Trp	28.4	33.7	13.4
trp E dadR2/F'trp	Glucose	D-Trp	21.5	30.2	15.9
trp E/F'trp	Glucose	None	0.9	1.0	12.8
trp E dadR1/F'trp	Glucose	None	23.0	28.9	14.2
trp E dadR2/F'trp	Glucose	None	19.1	19.7	11.7

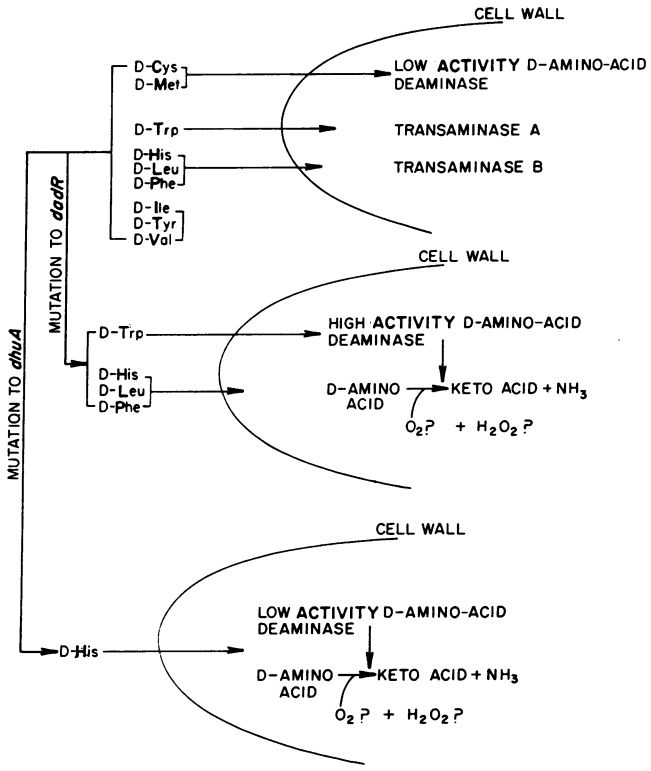

FIG. 2. Model for Utilization of D-amino acids by Mutant and Wild-Type *E. coli* K12. *Top:* The situation thought to exist in wild-type *E. coli* cells. *Arrows* represent permeation of certain amino acids; the length of that portion of the arrow inside the cell represents the relative extent of such permeation. *Middle:* the effect of mutation to *dadR* on the cell. Whereas in the top, only D-Met and D-Cys could be used (because the combination of poor transport and low activity of D-amino-acid deaminase prevented D-Trp, D-His, D-Phe, and D-Leu utilization), in the *dad R* cell the high enzyme activity is enough to efficiently convert to keto acids the small amount of D-Trp, D-His, D-Leu, or D-Phe that permeates the cell. *Bottom:* the effect of mutation to *dhuA* on the cell. The mutation leads to an enormous increase in the transport capability of the cell for D-histidine. Mutations leading to *dyu*, *dfu*, *dlu*, *dvu*, and *dhuB* genotypes may result in similar effects on transport of particular D-amino acids.

stancy in the ratio of activities with D-phenylalanine versus D-histidine as the substrate with a given extract suggests either that a single enzyme with broad substrate specificity is involved, or that two separate enzymes are coordinately synthesized in *dad R*$^+$ and *dad R* strains.

DISCUSSION

Recently, it has been shown (18) in *Salmonella* that mutations allowing D-histidine utilization, *dhuA*, lead to a five-fold increase in the production of a histidine-binding protein (*his J*-gene product) thought to be a component of the membrane transport system for L-histidine. Other workers (10) have found that the presence of a *dhuA* mutation confers the ability to transport D-histidine, which is very poorly transported, if at all, in *dhuA*$^+$ strains. The *dhuA* locus was found to map near *hisP*. We predict that our fast-growing *dhu* mutants will resemble these *dhuA* mutants.

Examination of mutants that utilize D-leucine, isolated by our methods, has demonstrated that some *dlu* mutations affect the ability of *E. coli* K12 to transport D-leucine†. Thorne and Corwin‡ have suggested that a tryptophan-permease gene (*trpP*) is located at 25 min of the *E. coli* map, between *tonB* and *trpA*. The locus we have studied, which produced the *dadR* phenotype, is clearly distinct from *trpP*, since the two loci are separated by almost 2 min on the map.

Fig. 2 shows our model for the way in which *E. coli* uses (or does not use) D-amino acids to meet the requirements of the relevant auxotroph in otherwise wild-type strains. The process must consist of at least three steps: transport of a D-amino acid to the cellular interior; deamination to the corresponding keto acid, and transmination of the keto acid to the corresponding L-amino acid, perhaps by transaminase A or B (19). The normal activity for D-amino-acid deamination is assumed to be "low", since *dad R* mutations lead to about a 25-fold increase in enzyme activity.

D-Cysteine and D-methionine apparently are readily transported to the inside of the cell, because *cys* and *met* mutants can grow readily on D-cysteine and D-methionine, respectively. D-tryptophan, D-histidine, D-leucine, and D-phenylalanine are assumed to be transported weakly, since mutation to *dad R* results in use of these amino acids for growth; a "high" activity of D-amino-acid deaminase would be insufficient for growth without some transport as well.

The inability of *dad R* strains to use D-tyrosine, D-valine, or D-isoleucine may mean that these amino acids are normally not transported by *E. coli*. In the middle of Fig. 2 we show the expected effect of mutation from *dadR*$^+$ to *dadR* upon D-amino-acid utilization. Mutations that lead to the ability to transport a particular D-amino acid or to an increase in transport of such an amino acid (lower part of Fig. 2) could result in the utilization of a D-amino acid by the relevant auxotroph, because the greatly increased internal concentrations of the D-amino acids would saturate the low activities of D-amino-acid oxidase characteristic of *dad R*$^+$ strains.

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