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D-Amino Acid Dehydrogenase of *Escherichia coli* K12: Positive Selection of Mutants Defective in Enzyme Activity and Localization of the Structural Gene

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Summary. A method for the positive selection of *dadA* mutants defective in D-amino acid dehydrogenase has been devised. It consists in isolating mutants resistant to β-chloro-D-alanine and screening for mutant colony color on a special agar medium. All 70 *Escherichia coli* K12 *dadA* mutants isolated either by this method or by other selection procedures map at a locus which is near to *hemA* and closely linked with *dadR*. Since some of the *dadA* mutants are thermosensitive in D-methionine utilization in vivo and have thermolabile D-amino acid dehydrogenase in vitro, it is proposed that the *dadA* gene codes for the enzyme structure. The broad substrate specificity, apparent membrane localization, inducibility by alanine, and repressibility by glucose strongly suggest that the D-amino acid dehydrogenase coded by the *dadA* gene is a species variant of the enzyme described under the same name in *Salmonella typhimurium*. It may be identical or homologous with the enzymes described under the names alaninase, D-alanine oxidase or D-alanine dehydrogenase in *E. coli* K12 or B.

Introduction

In our study on the D-amino acid dehydrogenase of *Salmonella typhimurium* (Wild et al. 1974; Wild and Kłopotowski 1975; Wild et al. 1978) we found that this enzyme is essential for biological racemization of several D-amino acids and for the metabolism of L- and D-alanine. Synthesis of the enzyme is induced by both alanine stereoisomers and repressed by glucose. The enzyme structure is coded for by gene *dadA*, which is co-transducible with *hemA*. Mutations in the *dadR* locus, which may be adjacent to *dadA*, make synthesis of the enzyme resistant to catabolite repression by glucose.

In *Escherichia coli* an activity ascribed to differently named enzymes could be analogous to that of D-amino acid dehydrogenase. In *E. coli* K12, alaninase (Beelen et al. 1973) and D-alanine dehydrogenase (Franklin and Venables 1976), and in *E. coli* B, D-alanine oxidase (Raunio and Jenkins 1973) and D-alanine dehydrogenase (Kaczorowski et al. 1975) seem to be very similar to the D-amino acid dehydrogenase of *S. typhimurium* LT2 with respect to substrate specificity, association with cell membrane and regulation of synthesis.

The only discrepancy between our results obtained with the *S. typhimurium* enzyme and those of other authors obtained with enzymes from *E. coli* K12 is the location of the gene that codes for the enzyme. In *E. coli* the gene named *alnA* (Beelen et al. 1973) or *dad* (Franklin and Venables 1976) is located between *ara* and *leu*. We mapped the *dadA* structural gene near *hemA* (Wild et al. 1974).

In this report we describe a method for positive selection of mutants lacking D-amino acid dehydrogenase, some properties of the enzyme, and show that its structural gene, *dadA*, maps on the *E. coli* K12 chromosome near the *hemA* locus.

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Materials and Methods

Strains and Genetic Techniques. Table 1 lists the strains used. EC is the stock designation of *E. coli* K12 from the Laboratory of Microbial Biochemistry, Institute of Biochemistry and Biophysics, Warsaw, Poland. Symbols for genetic markers are those used by Bachmann et al. (1976). P1*vir*-mediated transductions were performed as described by Miller (1972). Auxotrophic mutants were selected after treatment with diethylsulfate and penicillin enrichment (Roth 1970). Hemin-deficient mutants, *hemA*, were isolated according to Sasarman et al. (1970) on rich medium supplemented with 25 μg neomycin sulfate/ml. *HemA* mutants were identified on nutrient broth plates among neomycin-resistant clones as δ-aminolevulinic acid auxotrophs.

Media and Growth Conditions. The minimal citrate medium used was that described by Vogel and Bonner (1956). The minimal NC medium of Berkowitz et al. (1968) was devoid of citric acid. For growing auxotrophs, the minimal media were supplemented with 0.4 mM L-amino acids with the exception of L-valine which was added to 1 mM. Methionine auxotrophs were grown in the presence of 0.4 mM L- or D-methionine. δ-Aminolevulinic acid, 0.03 mM, was added to all media for growing *hemA* mutants. Minimal media were supplemented with 0.5% glucose, 0.5% glycerol or 20 mM L- or D-alanine as the carbon source. TTC medium was prepared according to Bochner and Savageau (1977) and was supplemented with 20 mM L-alanine, D-alanine or sodium succinate. Rich medium was made from dry broth, from Biomed (Warszawa, Poland) or Difco (Detroit, USA). Bacterial cultures were incubated with aeration at 30° C or 37° C as previously described (Wild et al. 1974).

Assay for D-Amino Acid Dehydrogenase. D-Amino acid dehydrogenase was assayed in toluenized cells using pyrophosphate buffer with 20 mM D-alanine as substrate. Formation of pyruvate was determined using 2,4-dinitrophenylhydrazine (Wild et al. 1974). When 15 mM D-histidine was used as substrate, absorption of imidazolepyruvate in alkali was measured (Wild and Kłopotowski 1975).

Chemicals. Bacto-TTC was purchased from Difco, Detroit, USA and β-chloro-D-alanine from Vega Biochemicals, Tucson, USA. Other chemicals used were common commercial products of the reagent grade purchased from Sigma Chemical Company, St. Louis, USA or Calbiochem, Los Angeles USA.

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Table 1. Strains of *Escherichia coli* K12 used or constructed during this study

Strain	Genotype	Origin
CB29	*dadR1 purB52 tna trpA2 trpE3*	J. Kuhn via F. Casse
CB104	*hemA8 lacY1 malA1 metB1 str134 trpA43*	F. Casse
CSH73	HfrH *lac Δara-leu thi*	F.G. Berger
CU4	*galT12*	H.E. Umbarger
CU683	*araD139 Δ lacU169 thi strA*	M.J. Casadaban via H.E. Umbarger
EC918	*arg ilvDAC115 metE201 thi trp*	From stock collection
EC923	*tna trpA2 trpE3*	By transduction of CB29 with CU4
EC924	*dadR1 tna trpA2 trpE3*	By transduction of CB29 with CU4
EC925	*arg hemA ilvDAC115 metE201 thi trp*	By neomycin-resistance in EC918
EC929	*arg his ilvDAC115 metE201 thi trp*	*his* in EC918
EC931	*arg dadA205 ilvDAC115 metE201 thi trp*	This paper, by DES mutagenesis of EC918
EC933	*arg dadA208 ilvDAC115 metE201 thi trp*	This paper, by DES mutagenesis of EC918
EC970	*araD139 ΔlacU169 met str thi*	By DES mutagenesis of CU683
EC972	*araD139 ΔlacU169 met str thi trp*	By DES mutagenesis of EC970
EC987	*araD139 dadA224 ΔlacU169 met str thi*	By β-chloro-D-alanine-resistance in EC972
EC988	*araD139 dadA258 ΔlacU169 met str thi*	By β-chloro-D-alanine-resistance in EC972
EC989	*araD139 dadA237 ΔlacU169 met str thi trp*	By β-chloro-D-alanine-resistance in EC972
EC990	*araD139 hemA8 ΔlacU169 met str thi trp*	By transduction of EC989 with CU104
EC991	*araD139 dadA262 hemA8 ΔlacU169 met str thi trp*	By β-chloro-D-alanine-resistance in FC990
EC1000	*araD139 dadA256 ΔlacU169 met str thi trp*	By β-chloro-D-alanine-resistance in EC972
PC0254	*gal17 lacY10 lam mal66 mtl71 phx purB23 strA64 thi78 tonA2 trp25 tsx11 tyrA50*	From Phabagen collection Utrecht, The Netherlands
RFS889	*Δ(ara-leu) Δ(gal-att – bio-uvrB) lac lam strA*	F.G. Berger

Results

Isolation and Properties of dadA Mutants

Wild-type cells of *E. coli* are capable of utilizing both L- and D-alanine as sole carbon and nitrogen sources. Our study with *S. typhimurium* showed that this capability depends upon the activity of D-amino acid dehydrogenase. Mutants lacking the enzyme, coded for by *dadA*, were unable to grow on either alanine streoisomer (Wild et al. 1978).

Table 2. Substrate specificity of D-amino acid dehydrogenase from *E. coli*[a]

Substrate	Reaction rate[b]	
	Strain EC972 (*dad*$^+$)	Strain EC989 (*dadA237*)
D-Alanine	34.1	0
D-Phenylalanine	24.7	0
D.Ethionine	14.0	0
D-Methionine	13.4	0
D-Asparagine	7.1	0
β-Chloro-D-alanine	7.0	1.1
D-Leucine	6.9	0
D-Serine	4.5	1.3
D-Histidine	4.2	0
D-Threonine	2.0	0
D-Cysteine	1.4	0
D-Arginine	1.0	0
D-Valine	0.9	0
D-Proline	0.9	0
D-Isoleucine	≦0.5	0
D-Tryptophan	≦0.5	0
D-Glutamate	≦0.5	0
D-Lysine	≦0.5	0

[a] Cells were grown overnight in NC medium supplemented with 0.5% glycerol, 20 mM L-alanine and other L-amino acids required by the mutants

[b] Activity of D-amino acid dehydrogenase was assayed at 37° C with 20 mM of indicated substrates. Keto acids formed in enzymatic reaction were determined with 2,4-dinitrophenylhydrazine as described in Materials and Methods. The reaction rates are determined as ΔOD_{520}/h/mg dry weight of cells

A positive selection method was worked out for isolating a large number of *E. coli* mutants lacking D-amino acid dehydrogenase. Advantage was taken of the inhibitory action of β-chloro-D-alanine on bacterial growth (Manning, et al. 1974). The deamination product of β-chloro-D-alanine, β-chloropyruvate, is an inhibitor of alanine racemase (Kaczorowski et al. 1974; Kaczorowski and Walsh 1975). Thus, the analog inhibits D-alanine production indirectly, which eventually prevents cell wall peptidoglycan synthesis and causes cell lysis.

β-Chloro-D-alanine is deaminated by toluenized *E. coli* cells (Table 2). Thus, mutants incapable of deaminating of the analog should be resistant to its growth inhibitory effect. The inhibitory effect was examined by applying 0.1 μmole aliquots of β-chloro-D-alanine to filter paper discs on minimal agar plates overlayed with soft agar containing wild-type or mutant cells. The analog produced inhibition zones, with a diameter of about 3.5 cm, on wild-type strains only. As expected, growth of *dadA* mutants of *S. typhimurium lacking* D-amino acid dehydrogenase activity was not inhibited by the analog. Resistant mutants were isolated by spreading about 2×10^8 cells of strain EC972 over appropriately supplemented minimal plates to which filter discs impregnated with 0.1 μmole aliquots of β-chloro-D-alanine were added. After incubation for two days at 37° C, colonies growing within the inhibition zones could be found. These colonies were picked and purified by restreaking on nutrient agar plates.

Screening of *dadA* mutants among the resistant colonies was facilitated by the use of tetrazolium agar plates (Bochner and Savageau 1977). Colonies capable of metabolizing the test substrate reduce a tetrazolium derivative and produce a deep red

farmazan, whereas colonies failing to catabolize the substrate remain white. On such plates, supplemented with either L- or D-alanine, the *dadA* mutants form white colonies and *dad*$^+$ strains red ones. The β-chloro-D-alanine-resistant mutants that formed white colonies on tetrazolium plates supplemented with L-alanine, were subsequently examined on plates identical except for substitution of succinate for L-alanine. A large fraction of the tested colonies were white on the succinate-supplemented tetrazolium plates. They were classified as mutants with defects in the bacterial respiratory enzymes. A small fraction of the resistant mutants formed red colonies on this medium. Assays of D-amino acid dehydrogenase showed that the mutants of the latter fraction were deficient in D-amino acid dehydrogenase activity.

Using this procedure 50 mutants were isolated that were unable to utilize L- and D-alanine as a carbon source and that had reduced or no D-amino acid dehydrogenase activity. Because each plate was inoculated with cells grown from a separate colony and only one Dad$^-$ clone was saved from each plate, each of the 50 mutants is different. In 46 of the mutants the enzyme activity was undetectable. They were unable to utilize D-methionine as a source of L-methionine. In four other mutants, the activity of D-amino acid dehydrogenase is significantly reduced. They could not grow on alanine isomers as a carbon source, but grew on plates containing D-methionine instead of L-methionine. As part of this selection, mutants were sought whose ability to grow on D-methionine was temperature-sensitive. Three such mutants were isolated. They could utilize D-methionine as a source of the L-amino acid at 30° C, but not at 42° C. They could not grow on plates supplemented with L- or D-alanine as a carbon source at either temperature.

In addition to the spontaneous mutants isolated by the β-chloro-D-alanine selection procedure, we obtained diethylsulfate induced mutants of EC918. After mutagenesis, the cells were subjected to penicillin enrichment and screened on alanine-tetrazolium plates. The mutants were tested for their ability to grow on minimal plates with pyruvate as a carbon source. Those which did not grow were discarded. Of the mutants unable to utilize alanine isomers but capable of utilizing pyruvate, 17 were retained.

The mutants isolated during this study have reduced or no D-amino acid dehydrogenase activity and map between the *hemA* and *purB* markers. The locus defined by these mutations was named *dadA*.

Activity and Properties of D-*Amino Acid Dehydrogenase*

To examine the substrate specificity of the *E. coli* enzyme, D-amino acid deamination rates were measured. The data presented in Table 2 show that D-alanine, D-phenylalanine, D-ethionine, D-methionine, and D-asparagine were deaminated at the highest rates. The deamination of D-leucine, D-serine, D-histidine, and D-threonine was 5–15 times slower. Keto acid formation from D-isoleucine, D-tryptophan, D-glutamate, and D-lysine was barely detectable, indicating that these D-amino acids are poor substrates for the enzyme. However, it should be noted that detection of keto acids with 2,4-dinitrophenylhydrazine is not very sensitive. To show that a particular D-amino acid has no affinity for the enzyme, more sensitive assays should be employed. Using such assays, D-tryptophan was found to be a substrate for D-amino acid dehydrogenase in *S. typhimurium* (Wild et al. 1978) and to be deaminated by *E. coli* K12 (unpublished results). In toluenized cells of mutant *dadA237* (EC989) deamination could not be detected with most of D-amino acids tested. Some keto product formation from D-serine could be due to the action of D-serine deaminase (Bloom and McFall 1975).

Table 3. D-Amino acid dehydrogenase activity in toluenized and sonicated cells[a]

Cell treatment	Activity[b]
Toluenized cells	298.0
Sonicated cells	22.0 (100)
Cell debris fraction	1.7 (8)
Membrane fraction	7.7 (35)
Supernatant after 60 min at 105,000 *g*	8.1 (37)

[a] The strain EC972 was grown for 16 h in NC medium supplemented with 0.5% glycerol, 20 mM L-alanine and the amino acids required. Cells were collected by centrifugation, washed three times with isotonic NaCl solution and resuspended in 50 mM potassium buffer, pH 7.4 to the density of 2.7 mg dry weight/ml. Toluenization and enzyme assays were performed as indicated in Material and Methods. Sonification was done in an ice bath three times for 20 s. The cell debris fraction was obtained by centrifugation at 10,000 *g* for 10 min. The cell-free extract was centrifuged at 105,000 *g* for 60 min. The sediment consisted of the membrane fraction and the supernatant contained cytoplasmic proteins and low density membrane fragments

[b] Activity was expressed in nmol of pyruvate formed/min/2.7 mg dry weight of cells or the equivalent amount of fraction material. In parentheses is given the percentage activity recovered in the sonicated fractions

The D-amino acid dehydrogenase of *S. typhimurium* was found to be an enzyme with structure-dependent activity as it was extremely sensitive to sonication of the bacteria (Wild et al. 1974). Therefore, the enzyme activity in cells permeabilized by toluene-treatment and in cell-free extracts of *E. coli* strain EC972 was compared. The results presented in Table 3 show that the rate of deamination of D-alanine by the sonicated cells was only 7% of that manifested by the toluenized cells. Toluene treatment of the sonicated cells did not increase the enzyme activity nor did the sonicate inhibit the activity in toluenized cells (data not shown). These facts support the notion that sonication disrupts a structural element required for full activity of D-amino acid dehydrogenase. In the same experiment the sedimenting fractions from the sonicate were also assayed for D-amino acid dehydrogenase activity. Only a small percentage of the total sonicate activity remained in the fraction containing cell debris. The extract devoid of the debris was centrifuged at 105,000 *g* for 60 min. The sediment, containing fragmented membranes, had about half the activity of the extract or about one-third of the total activity of the crude sonicate. We used a *lac*$^+$ *E. coli* strain to determine the effect of sonication on the activity of β-galactosidase, which is a typical soluble cytoplasmic enzyme. Idéntical treatments did not reveal any significant difference between the β-galactosidase activities in the toluenized and sonicated cells (data not shown). The observed loss of D-amino acid dehydrogenase activity upon sonication and the sedimentation of a significant fraction of it with membrane fragments indicate that in *E. coli* as well as *S. typhimurium* the activity of this enzyme is structure-dependent. Very likely, the enzyme is bound to a membrane element and its activity depends on membrane integrity.

The enzyme activity was measured in the three temperature-sensitive mutants. For that purpose they were grown at 30° C and 42° C in the inducing medium containing pyruvate and L-alanine as carbon sources. Toluenized cells were used for the

Table 4. Thermolability of D-amino acid dehydrogenase from EC1000[a]

Incubation time (min)	Specific activity[b]	
	EC972 (*dad*$^+$)	EC1000 (*dadA256*)
0	230 (100)	12.8 (5.6)
60	191 (82)	6.9 (3.0)
90	154 (67)	5.1 (2.1)
120	117 (50)	2.4 (0.9)
150	112 (48)	1.1 (0.4)

[a] The mutants were grown overnight at 30° C in rich medium supplemented with 20 mM L-alanine. The cells were collected and washed with isotonic NaCl solution on membrane filters. They were resuspended in 50 mM phosphate buffer, pH 7.4. Aliquots of the cell suspensions were taken and incubated at 41.5° C for the periods indicated. Then the cells were chilled and treated with toluene

[b] The enzyme assay was carried out at 30° C with 20 mM D-alanine as substrate. The specific activity is expressed as nmol of pyruvate formed/min/mg dry weight of cells. The relative specific activites are given in parentheses

assays, which were run at 30° C. It appeared that the mutant cells grown at 42° C did not have any measurable D-amino acid dehydrogenase activity. The enzyme level in the mutants grown at 30° C was reduced to 10% or even 1% of that of *dad*$^+$ strains. One of the mutant strains, EC1000, was used for examining the thermolability of D-amino acid dehydrogenase.

Cells of strains EC972 and EC1000 were grown in the inducing medium at 30° C and collected on membrane filters. Cells were washed with isotonic saline solution and incubated for various periods at 41.5° C. After toluene treatment the enzyme activity was assayed at 30° C using D-alanine as a substrate (Table 4). In unincubated *dadA256* cells, the dehydrogenase activity was only 6% of that found in the *dadA*$^+$ cells. Incubation of the mutant cells at 41.5° C for 150 min lowered the dehydrogenase activity by more than 90%, whereas the wild-type cells lost only about 50% of their enzyme activity. The activity remaining after treatment of the mutant cells was only 1% of that of *dadA*$^+$ cells identically treated. These results confirmed the expectation that the D-amino acid dehydrogenase of strain EC1000 is thermolabile.

Genetic Mapping of dadA Mutations

It was presumed that the D-amino acid dehydrogenases of *E. coli* and *S. typhimurium* were homologous enzymes and therefore the genes coding for their structure should be located in identical regions of the chromosome. Linkage of the mutations which cause inability to utilize alanine with the *dadR1* mutation of Kuhn and Sommerville (1971) was checked. Phage P1 was grown on strain CB29 carrying the *dadR1* mutation and used to transduce all mutants isolated during this work which are incapable of utilizing alanine as a carbon source. The crosses were made on minimal agar plates containing L-alanine as the sole carbon source. The transductants were tested for ability to utilize D-tryptophan as an L-tryptophan source a phenotype conferred by the *dadR1* mutation. All crosses gave similar results and a few typical results are shown in Table 5. The cotransduction frequencies were in the range 92%–97%. The linkage of some of the mutations with *hemA* was measured. The crosses were performed on nutrient agar plates on which *hemA* mutants do not grow unless provided with δ-aminolevulinic acid. Strain EC925 was used as recipient. The transductants were examined for their ability to utilize L-alanine. The data presented in Table 5

Table 5. Cotransducibility of *dadA*, *dadR*, and *hemA*

Strain	*dad* mutation	Cotransducibility %		
		hemA – *dadR*[a]	*hemA* – *dadA*[b]	*dadA* – *dadR*[c]
CB29	*dadR1*	42.8	–	–
EC931	*dadA205*	–	40.5	95.1
EC933	*dadA208*	–	42.6	96.6
EC987	*dadA224*	–	N.D.	93.2
EC988	*dadA258*	–	N.D.	97.0
EC989	*dadA237*	–	N.D.	96.6
EC1000	*dadA256*	–	N.D.	92.3

[a] The cross was performed on nutrient agar using EC925 (*hemA*) recipient cells and selection was made for the ability to grow in the absence of δ-aminolevulinic acid. The phage P1*vir* was grown on CB29 (*dadR1*). The ability of 100 *hemA*$^+$ transductants to grow on D-tryptophan was examined

[b] In the two crosses made on nutrient agar plates the recipient was EC925 (*hemA*) and phage P1vir lysates of EC931 and EC933 were used as donors. In both crosses 100–200 transductants were examined for the *hemA*$^+$ character on NC agar plates supplemented with 20 mM L-alanine as carbon source as well as other compounds required to satisfy the growth requirements

[c] In these transductional crosses *dadA*$^+$ was the selective marker. The crosses were performed on minimal NC agar plates supplemented with 20-mM L-alanine as a carbon source and other compounds required to satisfy the growth requirements. The P1 donor was CB29 (*dadR1*). The *dadA* mutants indicated were used as recipients. On minimal agar plates containing D-tryptophan and other supplements required for growth 50–100 transductants were examined for *dadR* mutations. N.D. not determined

indicate that the mutant loci are about 40% cotransducible. In another cross of the same recipient and on the same medium, the *dadR1* mutant was used as donor. As shown in Table 5 the linkage between *dadR1* and *hemA* was practically the same as that of the two *dadA* mutations.

The gene order on the *E. coli* chromosome map at 26 min was established as *hemA tre dadR purB* (Becerra de Lares et al. 1977). To determine the position of mutations conferring the inability to utilize alanine, two three-point crosses were done. Table 6 shows the results of a cross between strain PCO254 (*pur*B) as recipient and a phage lysate from EC991 (*dadA262 hemA*) as the donor. Among 244 *purB*$^+$ recombinants selected, 80 had acquired the *dadA* mutant character and ten of them became also *hemA*. The cotransducibility of *purB* calculated from these data was 32.6% for *dadA262* and 4.8% for the *hemA* mutation. If the gene order were *hemA dadA purB*, *dadA*$^+$ *hemA* recombinants would be rare, resulting from a quadruple crossover. Since these were the rarest class, it is concluded that this is the correct order. It is in agreement with the order established by Becerra de Lares et al. (1977) and our finding of a close linkage between *dadA* and *dadR*.

Another three-point cross was done with EC991 (*dadA262 hemA*) as recipient and CB29 (*dadR1*) as donor (Table 7). Among 301 *dadA*$^+$ recombinants, 294 received the *dadR1* mutation and 108 received the *hemA*$^+$ allele. The linkage of *dadA262* with *dadR1* and *hemA* mutations was therefore 94.2% and 32.5%, respectively. Analysis of nonselective marker distribution reveals that there was no *hemA*$^+$ *dadR*$^+$ recombinant class. The gene order *hemA dadR dadA* is indicated sinse this class would be rare, requiring a quadruple crossover.

The combined results of the two three-point crosses were consistent with the gene order *hemA dadR dadA purB* shown in Fig. 1.

Table 6. Three-point cross analysis of the *purB-dadA-hemA* region[a]

Selected marker	Unselected markers		Recombinants No.	%
	dadA	*hemA*		
purB$^+$	–	–	10	4.1
	–	+	70	28.7
	+	–	2	0.8
	+	+	162	66.3

[a] In the transduction cross the P1 donor was EC991 (*dadA262*) and the recipient PC0254. The cross was performed on minimal plates supplemented with 0.03-mM δ-amino-levulinic acid. *DadA* mutants were distinguished as being unable to grow on NC plates supplemented with L-alanine. *HemA* recombinants were detected as being unable to grow on unsupplemented nutrient broth agar

Table 7. Three-point cross analysis of the *hemA-dadR-dadA* region[a]

Selected marker	Unselected markers		Recombinants No.	%
	hemA	*dadR*		
dadA$^+$	–	–	186	61.8
	–	+	7	2.3
	+	–	108	35.9
	+	+	0	0

[a] In the transduction cross a P1 phage lysate of CB29 was used as the donor and EC931 as the recipient. The cross was made on NC plates supplemented with L-alanine. *HemA* mutants were detected as being unable to grow on nutrient broth agar. *DadR* mutants were distinguished by their ability to utilize D-tryptophan

Regulation of D-*Amino Acid Dehydrogenase Synthesis in E. coli*

In *S. typhimurium*, D-amino acid dehydrogenase is inducible by both alanine stereoisomers and repressible by glucose (Wild and Kłopotowski 1975). To examine whether in *E. coli* synthesis of the enzyme is controlled in the same way, two strains with wild-type *dad* phenotype (CU4 and EC929) and two *ara–leu* deletion mutants (CSH73 and RFS889) were chosen. The latter two strains were used because of the claims that a gene involved in D-alanine metabolism and named *alnA* (Beelen et al. 1973) or *dad* (Franklin and Venables 1976) maps between *ara* and *leu*.

The cells were grown in media with pyruvate or glucose as the main carbon source with or without L-alanine. D-Amino acid dehydrogenase was assayed in toluenized cells. No significant differences could be seen between the first two strains and the *ara–leu* deletion mutants (see Table 8). The lowest activity, in glucose-containing medium was on the average about three times lower than that in cells grown in pyruvate medium. The presence of L-alanine increased the enzyme activity about sixfold when glucose was the main carbon source or eight-fold in the pyruvate-containing medium.

This experiment shows that in *E. coli* K12 D-amino acid dehydrogenase is also inducible by L-alanine and repressible by glucose.

It was reported that in *E. coli* K12 an enzyme apparently identical with that studied in this work was inducible not only by both alanine isomers, but also by other amino acids, the most active being D-valine (Franklin and Venables 1976). We found that L-alanine was slightly more efficient for induction than D-alanine. Supplementing a pyruvate-containing medium with D-valine, D-methionine, D-asparagine or D-aspartic acid did not induce significantly the enzyme activity in strain EC918 (data not shown).

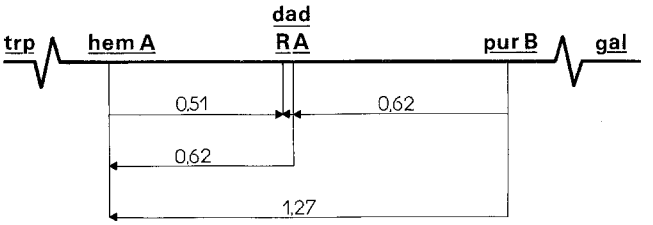

Fig. 1. Genetic map of the *hemA-purB* region of the *E. coli* K12 chromosome. The map was drawn approximately to scale. The arrows point to the unselected marker. The numbers represent intergenic distances in map min. Their values were calculated using the equation of Wu (Bachmann et al. 1976) from the cotransduction frequency data presented in Tables 5–7. The distance *dadR–dadA* is 0.03 min

Table 8. Specific activity of D-amino acid dehydrogenase in wild-type strains and *leu–ara* deletions[a]

Strain	Relevant genotype	Specific activity[b]			
		Glucose	Glucose +L-alanine	Pyruvate	Pyruvate +L-alanine
CU4	*ara*$^+$*leu*$^+$	16	49	27	392
EC929	*ara*$^+$*leu*$^+$	11	91	33	339
CSH73	*Δara–leu*	10	67	40	169
RFS889	*Δara–leu*	13	71	45	248

[a] Cells were grown overnight in NC medium supplemented with the required L-amino acids and the additions indicated: sodium pyruvate 20 mM, L-alanine 20 mM or glucose 0.5%
The activity of D-amino acid dehydrogenase was assayed at 37° C with D-histidine as a substrate

[b] Specific activity is expressed as nmol of imidazolepyruvate formed /min/mg dry weight of cells

Discussion

Activity of an enzyme that deaminates D-alanine has been described under different names in three species of enteric bacteria. Because the activity in the cells of *E. coli* K12 (Raunio and Jenkins 1973, Franklin and Venables 1976), *E. coli* B (Kaczorowski et al. 1975) and *S. typhimurium* (Wild et al. 1974) is inducible by alanine and resides in a cell membrane, it seems reasonable to presume that it is due to a species variant of the same enzyme. Identical localization of the genes coding for enzyme structure would support such a presumption. However, in *E. coli* K12, mutations mapping between *ara* and *leu* were reported to affect the structural gene of the enzyme (Beelen et al. 1973; Franklin and Venables 1976), whereas in *S. typhimurium* the gene was close to *hemA* (Wild et al. 1974).

To investigate this further, 70 mutants in two *E. coli* K12 strains were isolated with defects in the enzyme which we call D-amino acid dehydrogenase. Most of them were isolated by positive selection. We took advantage of the fact that β-chloro-D-alanine does not inhibit growth of *E. coli* unless deaminated to β-chloropyruvate, an inhibitor of alanine racemase (Kaczorowski et al. 1975). Predictably, the mutants lacking D-amino acid dehydroge-

nase were resistant to the inhibitory effect of β-chloro-D-alanine. Because only a small fraction of the resistant mutants was deficient in D-amino acid dehydrogenase, the use of a tetrazolium-containing medium (Bochner and Savageau 1977) was very helpful in the mutant selection. The procedure devised can be used for selecting *dadA* and respiratory chain mutants in any *E. coli* strain that is sensitive to β-chloro-D-alanine. It is also useful with some modification for such a selection in *S. typhimurium* strains. With these bacteria the selection for β-chloro-D-alanine mutants must be made on media containing a carbon source other than glucose (W. Walczak, personal communication).

Genetic analysis of the *E. coli* mutants deficient in D-amino acid dehydrogenase which we had obtained, revealed that all of them carried mutations in a locus homologous with *dadA* in *S. typhimurium* (Wild et al. 1974). Because some of them have a thermolabile D-amino acid dehydrogenase, we conclude that the *dadA* gene coding for the enzyme structure maps on the *E. coli* K12 chromosome in the *hemA–purB* region. The three-point cross analysis of this region indicated that the gene order is (*gal*) – *purB* – *dadA* – *dadR* – *hemA* – (*trp*).

We could not confirm the report of Franklin and Venables (1976) that deletion of a region between *ara* and *leu* in *E. coli* causes inability to utilize alanine isomers as a carbon source and loss of D-alanine deaminating activity. In our hands, two different *ara–leu* deletion strains could utilize alanine isomers, had normal D-amino acid dehydrogenase activity, and did not differ from other *E. coli* strains in inducibility of enzyme by alanine and repressibility by glucose. We tested several *dad* mutants kindly provided by Dr. W.A. Venables and found that they could not grow on either alanine or pyruvate as a carbon source, remained sensitive to β-chloro-D-alanine, had normal activity of D-amino acid dehydrogenase, as assayed by our procedure, and that the mutations responsible for this phenotype are linked with *leu* (data not shown). Thus, they differ both phenotypically and genetically from our *dadA* mutants, which grow as well as isogenic *dadA*$^+$ strains on pyruvate as a carbon source.

In other experiments reported in this paper the D-amino acid dehydrogenase activity in *E. coli* cells was shown to be very sensitive to sonication. Most likely, this is due to dependence for its activity on the structural integrity of a cell membrane to which the enzyme is bound.

The *E. coli* D-amino acid dehydrogenase has been found to have a broad substrate specificity similar to those of enzymes assayed by Franklin and Venables (1976) or *S. typhimurium* D-amino acid dehydrogenase (Wild et al. 1974). However, the controversy about D-amino acid deamination in enteric bacteria has not been investigated here. Our results show that D-amino acid dehydrogenase in *S. typhimurium* deaminates D-tryptophan (Wild et al. 1978) whereas Hadar et al. (1976) have claimed that two enzymes, one specific for D-tryptophan and another for many other D-amino acids are under the control of the *dadR* regulatory gene in *E. coli* K12. The relevant data will be presented in a future publication.

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