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Identification of the *dadX* gene coding for the predominant isozyme of alanine racemase in *Escherichia coli K12*

J. Wild, J. Hennig, M. Łobocka, W. Walczak, and T. Kłopotowski
Instytut Biochemii i Biofizyki PAN, UL. Rakowiecka 36, PL-02-532 Warszawa, Poland

Summary. Evidence is presented that alanine racemase activity in *E. coli* K12 is due to two distinct gene products. The predominant isozyme is inducible by either alanine stereoisomer and repressible by glucose. The gene *dadX* coding for its structure is located by the *dadA* gene determining the structure of D-amino acid dehydrogenase. The regulatory site for the expression of both genes, *dadR*, is located on the other side of *dadA*. The orientation of the *dad* operon established by multiple-point crosses and deletion mapping is as follows: *fadR...dadRAX...hemA*. The *dadX* alanine racemase activity is unusually refractory to changes of incubation temperature. It differs strikingly from that of the other isozyme, probably the product of the *alr* gene. The latter isozyme shows a typical dependence upon incubation temperature. The synthesis of *alr* alanine racemase is constitutive in respect of both alanine and glucose. In *dadX* mutants, in which alanine racemase activity equals only 15% of that in wild-type cells grown in the absence of an inducer or catabolite repressor, the *dad* operon cannot be induced by D-alanine. We presume, therefore, that L-alanine is involved more directly than D-alanine in *dad* operon regulation.

Introduction

D-Alanine is a component of the peptidoglycan layer of the cells of many bacterial species. It appears to be formed directly from L-alanine by the action of alanine racemase (EC 5.1.1.1). The enzyme has been found in different bacterial genera. Homogenous alanine racemase preparations were obtained from *Bacillus subtilis* (Diven et al. 1964), *Pseudomonas putida* (Rosso et al. 1969) and two subspecies of *Escherichia coli* – W (Lambert and Neuhaus 1972b) and B (Wang and Walsh 1978). The most detailed studies were made with purified alanine racemase from *E. coli* B. This enzyme is a dimer of 100,000 daltons molecular weight composed of identical subunits each carrying one active site and one pyridoxal phosphate molecule. The enzyme is highly specific for either alanine stereoisomer (Wang and Walsh 1978).

L-Alanine can be utilized by coliform bacteria as the sole source of carbon and nitrogen. As proposed by Wijsman (1972) for *E. coli* K12, L-alanine is metabolized by the sequential action of alanine racemase and D-amino acid dehydrogenase. Wijsman showed that two mutations make *E. coli* incapable of growing without exogenous D-alanine. He claimed that the *alr* gene which he located at unit 94 of the standard *E. coli* chromosome map codes for the structure of alanine racemase. The other gene that might be involved in D-alanine production, *msuA*, was mapped by him close to the *trp* operon.

D-Amino acid dehydrogenase is coded for by the *dadA* gene which maps near the *trp* operon both in *Salmonella typhimurium* (Wild et al. 1974; Wasserman et al. 1983) and *E. coli* K12 (Wild and Kłopotowski 1981). Franklin et al. (1981) have claimed that the *dadB* gene located between *ara* and *leu* codes for another D-amino acid dehydrogenase subunit.

Very little is known about the regulation of alanine racemase synthesis. Lambert and Neuhaus (1972a) observed that growth of *E. coli* W on L-alanine increased alanine racemase activity 25-fold as compared with cells grown on glucose. Wijsman (1972) showed that either alanine isomer was able to induce alanine racemase activity in *E. coli* K12 cells. Wasserman et al. (1983) found the same with an *S. typhimurium* alanine racemase. They also observed repression of the racemase activity by glucose. The same regulatory pattern was previously observed with D-amino acid dehydrogenase in *S. typhimurium* (Wild and Kłopotowski 1975) and in *E. coli* K12 (Wild and Kłopotowski 1981; Franklin and Venables 1976).

When the experiments to be presented in this paper were close to completion Wasserman et al. (1983) reported that in *S. typhimurium* there are two distinct alanine racemase isozymes. The one that provides most of the activity is coded by the *dadB* gene located very close to *dadA*. The results of our experiments with *E. coli* K12 mutants are in agreement with these findings. We conclude that the *dadX* gene belonging to the *dad* operon codes for the predominant alanine racemase isozyme. We infer from the properties of *dadX* and polar *dadA* mutants that L-alanine is the actual inducer of the *dad* operon.

Materials and methods

Organisms. The bacterial strains employed in this study were all derivatives of *Escherichia coli* K12. We mainly used isogenic strains derived from MC4100 (Casadaban and Cohen 1979; Komeda and Iino 1979). Several authors intro-

Offprint requests to: T. Kłopotowski

Table 1. Isogenic strains of *E. coli* K12 used during this study

Strain	Change in genotype	Latest marker introduction
EC972	Standard parental strain	See below and text
EC980	*dadA278*::Mucts	L of EC972
EC982	*dadA283*::Mucts	L of EC972
EC983	*dadR1*	T of EC989 with CB29
EC989	*ΔdadA237*	CDA^r in EC972
EC990	*hemA8*	T of EC989 with CB104
EC991	*dadA262 hemA8*	CDA^r in EC990
EC1012	*ΔdadA247*	CDA^r in EC972
EC1024	*ΔdadA232*	CDA^r in EC972
EC1025	*ΔdadA238*	CDA^r in EC972
EC1026	*dadA241*	CDA^r in EC972
EC1028	*dadA261*	CDA^r in EC972
EC1034	*dadA270 dadR1*	CDA^r in EC983
EC1051	*dadA279*::Mud (Ap^r *lac*)	L of EC972
EC1082	*ΔdadA237* λd*dadA-lac*1 λM	L of EC989
EC1083	λd*dadA-lac*1 λM	L of EC972
EC1086	*dadA283*::Mucts *trpB*$^+$ *trpE6185* λ$^+$	T of EC982 with CU525 and unsollicited L
EC1091	*dadR1 fadR14*::Tn*10*	T of EC1026 with RS3044
EC1094	*Δ(fadR-dadRA)286 trpB*$^+$ *trpE6185*	Mucts eduction from EC1086
EC1608	*dadA278*::Mucts λd*dadA-lac*1 λM	L of EC980
EC1609	*dadA283*::Mucts λd*dadA-lac*1 λM	L of EC982
EC1610	λd*dadA-lac*1 λM	Mucts eduction from EC1609
EC1611	*ΔdadA247* λd*dadA-lac*1 λM	L of EC1012
EC1613	*Δ(fadR-dadRA)286 trpB*$^+$ *trpE6185* λd*dadA-lac*1 λ$^+$	L of EC1094
EC1614	*ΔdadX293 fadR14*::Tn*10*	T of EC972 with EC1705
EC1618	*ΔdadX293*	T of EC989 with EC1614

The genotype of the parental strain EC972 is as follows: *araD139 Δ(argF-lac)205 deoC1 flb-3501 metF185 ptsF25 relA1 rpsL150 trpB202*. The complete genotypes of the abbreviated phage designations used in this Table are given under Organisms in Materials and methods. CDA^r stands for isolation of a spontaneous mutation conferring resistance to β-chloro-D-alanine. L and T indicate, respectively, that lysogenization and P1*vir*-mediated transduction served for introducing the latest marker

duced corrections to the published genotype of MC4100, kindly communicated to us by Barbara Bachmann. She reminded us that its *thr-leu* region originated from *E. coli* B. We obtained MC4100 from H.E. Umbarger as CU683. By two successive diethylsulfate treatments we introduced into it *metF* and *trpB* mutations. The strain thus obtained, EC972, and its derivatives are presented in Table 1. We also used a few strains from other *E. coli* K12 pedigree branches. They are presented in Table 2.

Table 2. Strains of *E. coli* K12 from other predigree branches used during this study

Strain	Genotype	Source or derivation
CB29	*dadR1 purB52 tna trpA2 trpE3*	J. Kuhn via F. Casse
CB104	*hemA8 metB1 trpA43 lacY1 malA1 str-134*	F. Casse
CU517	*tonA mal* suIII$^+$ Mucts62*K*1010	H.E. Umbarger
CU525	*arg ilvDAC115 metE201 thi lac trpE6195*	H.E. Umbarger
EC802	*alr-1 dadX293 msuA*$^+$ *leuB6 thi his-108 ilvA634 argG66 pyrF101 trp-64 thyA6 lacY1 tonA21 tsx-95 str supE44 codA1 dra-1*	H.J.W. Wijsman as ATK111, a *thr*$^+$ transductant of TKL10
EC1089	*fadR14*::Tn*10* in CB104	Transduction of CB104 with P1 lysate from RS3044
EC1705	*fadR14*::Tn*10* in EC802	Transduction of EC802 with P1 lysate from EC1089
EC1735	*dadX*$^+$ *fadR14*::Tn*10* in EC802	Transduction of EC802 with P1 lysate from EC1089
RS3044	*fadR14*::Tn*10 dadR1 trpA62 trpE61 tna-5 pms-56*	W.D. Nunn

We used P1*vir* for generalized transduction. The defective phage λd*dadA-lac1* was constructed by Wild and Obrępalska (1982). Restriction analysis showed that lambda material from *Nu1* to *exo* was substituted by DNA from EC1051, i.e. *dadA'–trp'CBA'-lacZYA* gene fusion, its promoter-operator site *dadR* and other genes including *fadR* (J. Hennig, unpublished results). The λ*c*I857 *S7* mutant ("λM") was obtained from H.E. Umbarger. "λ$^+$" stands for wild-type phage. Mucts62K1010 ("Mucts") and Mu*d* (Ap^r*lac*) stocks were kindly provided by M. Howe and M. Casadaban, respectively. The brackets show abbreviated phage designations used in Table 1.

Media and growth conditions. Growth media and culture procedures were those described previously (Wild and Obrępalska 1982). For *dad*-controlled enzyme induction 10 mM L-alanine or 20 mM D-alanine was added to minimal media. Minimal medium supplemented with 5 mM decanoate was employed for isolation of *fadR-dadA* deletion strains. The decanoate was prepared according to Simons et al. (1980). TTC medium was prepared according to Bochner and Savageau (1977). TTC medium supplemented with 5 mM decanoate was used to differentiate between *fadR*$^+$ and *fadR* colonies. Colonies able to catabolize decanoate (*fadR*) turned red on this medium, while those unable to do so (*fadR*$^+$) remained white. TTC media supplemented with 25 mM L-alanine, D-alanine or sodium succinate were employed for identification of *dad* mutants. The *dadA* mutants unable to utilize L- or D-alanine as carbon source formed white colonies on either alanine medium and red ones on succinate medium (Wild and Kłopotowski

1981). During this study it was found that *dadX dadA*$^+$ strains formed white colonies on L-alanine and red colonies on both D-alanine and succinate TTC media.

Genetic techniques. P1*vir* phage grown by infection was used for generalized transduction as described by Miller (1972). Mu and λd*dad-lac*1 lysates were prepared by heat induction according to Howe (1973) and Miller (1972), respectively. The lysogenization and the homology-dependent transductions using λd*dadA-lac*1 phage were done as described by Wild and Obrępalska (1982). The transductants were purified by restreaking on nutrient broth plates.

To isolate Mucts phage insertions within the *dadA* locus the positive selection method of *dadA* mutants by means of β-chloro-D-alanine resistance was employed (Wild and Kłopotowski 1981). Fourteen clones from among 1500 colonies examined were simultaneously temperature-sensitive, indicating Mucts lysogeny and Dad$^-$ character. To detect the monolysogenes with a *dadA* mutation as a consequence of Mucts insertion P1-mediated transductions were performed. The strains were transduced to *dadA*$^+$ on minimal agar plates containing L-alanine as sole carbon source using P1 phage grown on strain EC972. *DadA*$^+$ transductants of two strains, EC980 and EC982, became temperature-resistant, which indicated that Mucts phage had been crossed out from its insertion site in *dadA* gene and lost. The selection of *fadR* clones capable of utilizing decanoic acid as sole carbon source and being simultaneously temperature-resistant due to Mucts phage excision served as the method for the isolation of *fadR-dadA* deletions. The strain EC1086 carrying Mucts phage inserted into the *dadA* gene was used. The selection was done at 42° C on minimal medium supplemented with 5 mM decanoic acid. DadA and FadR phenotypes were checked on TTC media as described under Media and growth conditions.

Enzyme assays. β-Galactosidase was assayed in toluenized cells with o-nitrophenol-β-D-galactoside as a substrate by the procedure of Miller (1972). The bacteria were grown from an initial OD_{650} of 0.1 for 4.5 h at 30° C or 37° C in appropriately supplemented minimal media. The activity was expressed in units as defined by Miller (1972).

Alanine racemase activity was assayed using the slightly modified method of Julius et al. (1970). Cells for the alanine racemase assays were grown overnight in appropriately supplemented minimal medium. The cells were collected and washed with 50 mM phosphate buffer, pH 7.4, by centrifugation. From the optical density of the suspension the dry weight cell content was determined using a standard curve. The cells were sonicated and cleared supernatants were used for the assay. An alternative procedure for preparing bacteria for the assay was used when less precise estimates were sufficient to distinguish between recombinant genotypes. The cells were taken directly from agar plates to the same phosphate buffer. After measuring the optical density at 650 nm the cells were toluenized as described by Wild et al. (1974) and used for the assay. One milliliter of the assay mixture contained 60 μmol Tris-HCl buffer, pH 8.9, 100 μmol L-alanine, 5 nmol pyridoxal phosphate and 0.01–0.20 ml cell-free extract or toluenized cell suspension. The reaction continued for 30 min at 37° C. It was terminated by heating the tubes for 5 min in a water bath kept at 85° C. To the control tube L-alanine was added after the heating step. For deamination of the D-alanine formed, D-amino acid oxidase from bovine kidney (Sigma, St. Louis, USA) was used. To each tube, upon cooling, 0.1 ml of the solution containing ca. 0.1 unit D-amino acid oxidase and 10 nmol FAD in 0.1 M Tris-HCl buffer, pH 8.9, was added. After 20 min at 37° C the amount of pyruvate produced was determined using the 2,4-dinitrophenylhydrazine method (Wild et al. 1974). The specific activity of alanine racemase was expressed in nmol D-alanine formed/min per mg dry weight of cells.

Results

Activity of alanine racemase in dadA mutants

We have determined the alanine racemase activity in various *dadA* mutants in order to examine whether the lesions in the gene coding for D-amino acid dehydrogenase have any effect on the synthesis of alanine racemase reported to be coded by the *alr* gene located some distance away on the chromosome (Wijsman 1972). As shown in Table 3 the basal, noninduced activity of alanine racemase was about the same in *dadA*$^+$ and in three out of four *dadA* deletion strains. In the deletion strain, EC1025, and in both *dadA*::Mucts insertion mutants (EC980 and EC982) the enzyme activity was more than 80% lower. The presence of L- or D-alanine in the glycerol-minimal growth medium increased the alanine racemase activity three to four-fold only in the strains that had normal noninduced activity. The inducing effects of alanine stereoisomers were practically the same in most of *dadA* mutants. In the *dadA*$^+$ strain D-alanine was less effective, probably due to the action of D-amino acid dehydrogenase in reducing the intracellular pool of D-alanine. In the other strains, i.e., those which had the low basal alanine racemase activity, neither alanine stereoisomer had any effect on the enzyme activity. The strain cured of Mucts phage (EC1610) had the normal activity and inducibility of alanine racemase restored. The results presented in Table 3 indicate that insertion of Mu phage or certain deletions in the *dadA* gene severely reduce alanine racemase activity and prevent its induction by alanine.

Expression of β-galactosidase in λddadA-lac1 lysogens carrying various dadA mutations in the dad operon

The fusions of the *lacZ* gene to the *dad* regulatory region were isolated previously and synthesis of β-galactosidase

Table 3. Inducibility of alanine racemase activity in *dadA* strains

Strain	Relevant genotype	Alanine racemase nmol/min/per dry wt		
		–	L-Alanine	D-Alanine
EC972	*dadA*$^+$	41	146	61
EC989	*ΔdadA237*	45	147	131
EC1012	*ΔdadA247*	41	173	205
EC1024	*ΔdadA232*	61	193	195
EC1025	*ΔdadA238*	7.1	7.9	8.9
EC980	*dadA278*::Mucts	4.9	5.8	7.6
EC982	*dadA283*::Mucts	4.0	6.2	8.2
EC1610	Mucts cured *dadA*$^+$	36	121	71

The bacteria were grown in minimal medium with glycerol as carbon source

Table 4. Expression of β-galactosidase in λd*dadA-lac*1 lysogens carrying different *dadA* mutations on the chromosome

Strain	Relevant genotype	β-Galactosidase (Units)		
		–	L-Alanine	D-Alanine
EC1083	*dadA*$^+$	245	840	617
EC1082	*ΔdadA237*	247	793	834
EC1611	*ΔdadA247*	207	824	885
EC1608	*dadA278*::Mucts	243	769	312
EC1609	*dadA283*::Mucts	240	810	267
EC1610	Mucts cured *dadA*$^+$	266	674	616
EC1613	*Δ(fadR-dadRA)286*	336	686	350

The bacteria were grown in minimal medium with glycerol as carbon source

shown to be affected by growth conditions in the same way as the synthesis of D-amino acid dehydrogenase (Wild and Obrępalska 1982). One of the *dadA-lac* fusions was transferred to λ phage. The strains carrying various mutations in the *dadA* gene were lysogenized with λ d*dadA-lac*1 phage. Measurements of β-galactosidase activity in the lysogens provided a way of monitoring *trans* effects of chromosomal lesions on expression of the *lacZ* gene fused to the *dad* regulatory region.

The results presented in Table 4 show that in all strains examined the basal activity of β-galactosidase was practically the same. In all strains the enzyme activity was induced two to four-fold by L-alanine. D-Alanine induced synthesis of β-galactosidase with the significant exception of the Mu phage insertion strains and that carrying the deletion extending through *dadR* and reaching the *fadR* gene. The lack of β-galactosidase induction by D-alanine was a *trans* effect of the mutations. Neither could the enzyme be induced by D-alanine in strain EC1051 carrying the *dadA-lac* fusion on the bacterial chromosome. Since all strains in which the fusion cannot be induced by D-alanine are lysogens of the strains with low constitutive alanine racemase activity, this low activity could be responsible for the lack of D-alanine effect. It seems likely that higher and inducible alanine racemase activity is required to provide enough L-alanine from D-alanine for the induction process. It follows, therefore, that L-alanine is the only alanine stereoisomer participating directly in *dad* operon regulation.

Catabolite repression of alanine racemase synthesis

It has been shown that the D-amino acid dehydrogenase of *S. typhimurium* as well as that of *E. coli* is subject to catabolite repression by glucose (Wild and Kłopotowski 1975, 1981). Because of the physiological relationship between D-amino acid dehydrogenase and alanine racemase, the effect of glucose on the synthesis of alanine racemase was examined.

The cells were grown in glycerol or glucose minimal media with or without L- or D-alanine. EC972 and EC989 represented the strains with normal acitivity and inducibility of alanine racemase. It can be seen from Table 5 that the presence of glucose in the growth medium reduced the enzyme activity by about 60%. Addition of L-alanine to the glucose medium resulted in induction, but its level was lower at least by a half than that in the cells grown in medium containing glycerol plus L-alanine. The observed repression of alanine racemase synthesis by glucose was about the same as glucose repressibility of D-amino acid dehydrogenase (Wild and Kłopotowski 1981). Strains EC1051, EC1613 and EC1614 were chosen to represent the mutants with low constitutive activity of alanine racemase.

As shown in Table 5 the carbon source had no effect on the low alanine racemase activity, although the cells of both strains carrying the *dadA-lac* fusion were under catabolite repression as demonstrated by β-galactosidase assays (not shown). Neither the presence of L- or D-alanine had any effect upon alanine racemase activity in these strains. The derivation and character of the *dadX* mutation will be presented in the next sections.

Location of the new gene in the dad operon

The finding that certain mutations in the *dadA* gene lower alanine racemase activity indicated that a gene distinct from *dadA* and located down-stream in the same operon might be concerned with genetic control of alanine racemase. The gene, called *dadX*, could be a positive factor in expression of the *alr* gene proposed by Wijsman (1972) to code in *E. coli* for alanine racemase. Alternatively, *dadX* could code for the major isozyme of alanine racemase and be thereby homologous with the *dadB* gene identified in *S. typhimurium* by Wasserman et al. (1983).

The notion that *dadX* is located downstream from *dadA* was first based on the assumption that the low alanine racemase activity in phage insertion mutants was due to their polar effects. More direct support for this notion would be provided by isolation and mapping of a point *dadX* mutation, as well as by delineation of a *dadRA* deletion mutation.

The *dadX293* mutation was found in strain EC802. It carries the *alr* mutation and an *msuA* allele, which together make the strain incapable of growing at 42° C unless D-alanine is provided (Wijsman 1972; personal communication). Apparently, such an allele combination makes the cell unable to produce D-alanine from L-alanine, the only source of it known so far. We examined the likely possibility that it also could carry a *dadX* mutation. For this purpose, cells of strain EC802 were used as recipients in the cross with P1 phage grown on strain EC1089. The donor carries the Tn*10* transposon in gene fadR which is 85% linked to *dadA*. The transductants capable of growing on nutrient broth plates supplemented with tetracycline were selected and examined for temperature-dependent alanine auxotrophy. It turned out that about 80% of them had lost this character. It confirmed our supposition that EC802 carries a *dadX* mutation.

To separate this mutation from the complex genetic background of EC802, P1 phage was grown on EC1705, one of the *fadR14*::Tn*10* transductants which retained the conditional D-alanine auxotrophy. Cells of strain EC972 were used as recipients. Transductants resistant to tetracycline were selected. None of them had the donor temperature-dependent D-alanine requirements. About 80% of the transductants were incapable of using L-alanine but grew well on D-alanine. Correspondingly, they formed red colonies on TTC medium with D-alanine, and white ones on the same medium with L-alanine. One such transductant was retained as strain EC1614.

Our first attempts to map *dadX* gave results that con-

Table 5. Catabolite repression of alanine racemase activity

Strain	Relevant genotype	Alanine racemase (nmol/min per mg dry weight)					
		Glycerol			Glucose		
		–	LA	DA	–	LA	DA
EC972	*dadA*$^+$	44	146	62	17	60	59
EC989	*ΔdadA237*	45	147	131	17	53	65
EC1051	*dadA279*::Mud (Ap^r*lac*)	5.8	5.8	6.5	4.9	5.9	5.4
EC1613	*Δ(fadR-dadRA)286*	5.9	6.3	6.3	5.7	6.9	6.7
EC1614	*ΔdadX293*	7.1	7.6	5.3	4.7	5.6	5.3

Cells were grown with either glycerol or glucose as carbon source LA and DA stand for L-alanine and D-alanine present in the growth media, respectively

Table 6. Assessment of the *fadR-dadR-dadA-hemA* gene order by four-point crosses

Donor: EC1091	Tn*10*	1	+	+
	fadR	*dadR*	*dadA*	*hemA*
Recipient: EC991	+	+	*262*	*8*

Recombinant class	Nonselective markers			Number of recombinants
	dadR	*dadA*	*hemA*	
1	+	+	+	0
2	+	+	*8*	0
3	+	*262*	+	3
4	+	*262*	*8*	19
5	*1*	+	+	142
6	*1*	+	*8*	309
7	*1*	*262*	+	0
8	*1*	*262*	*8*	2
			Total	475

Cells of EC991 were transduced with a P1*vir* lysate of strain EC1091. The recombinants were selected on nutrient broth plates supplemented with tetracycline and δ-aminolaevulinic acid. The *dadR1* mutants carrying *dadA*$^+$ were identified by their ability to grow on D-tryptophan as the only L-tryptophan source. In *dadA* mutants the *dadR1* character was detected by high alanine racemase activity measured in cells taken directly from colonies. The *hemA* mutants were identified on nutrient broth plates as δ-aminolaevulinic acid auxotrophs. The *dadA* mutants cannot grow on either alanine stereoisomer as carbon source

Table 7. Assessment of the *fadR-dadA-dadX* gene order by three-point crosses

Donor: EC1614	Tn*10*	+	*293*
	fadR	*dadA*	*dadX*
Recipient: EC1028	+	*261*	+

Recombinant class	Nonselective markers		Number of recombinants
	dadA	*dadX*	
1	+	+	4
2	+	*293*	123
3	*261*	+	28
4	*261*	*293*	0
		Total	155

Cells of EC1028 were transduced with a P1*vir* lysate from EC1614. The recombinants were selected on nutrient broth plates supplemented with tetracycline. The recombinants *dadA*$^+X^-$ grew on D-alanine, but not L-alanine as carbon source and those carrying the *dadA* mutation were incapable of growing on either alanine stereoisomer as carbon source. The presence of the *dadX* mutation in *dadA*$^-$ recombinants was indicated as low alanine racemase assayed in cells taken directly from purified colonies

flicted with the previously established gene order (Wild and Kłopotowski 1981). This prompted us to make several four- or three-point crosses to check the order. Cells of EC991 (*hemA8 dadA262*) and a P1 lysate of strain EC1091 (*dadR1 fadR14*::Tn*10*) were used. Tetracycline-resistant recombinants were selected and analyzed for the three nonselected characters. Table 6 presents the results. When the *dadR* and *dadA* characters were considered the rarest class indicated the order *fadR dadR dadA*. They were 95.4% and 94.9% linked to *fadR*, respectively. In the case of the *dadR* and *hemA* markers the rarest class pointed to the order *fadR dadR hemA*. The linkage value of *hemA* with *fadR* was 30.5%. With *dadA* and *hemA* nonselective markers the class with the lowest number of recombinants was in agreement with the order *fadR dadA hemA*. On the basis of this result we correct the previously reported order (Wild and Kłopotowski 1981; Wild and Obrępalska 1982) and conclude that the order is as follows: *fadR dadR dadA hemA*.

The location of *dadX* in relation to *fadR* and *dadA* was established in a cross with EC1028 as the recipient and a *Plvir* lysate of EC1614 as the donor. Tetracycline-resistant recombinants were selected. The results of the three-point cross are shown in Table 7. The rarest recombinant class points to the order *fadR dadA dadX*. The linkage of the two latter markers with *fadR* was 81.9% and 79.4%, respectively.

Taking together the results of the four-point and the three-point crosses the gene order is as follows: *fadR dadR dadA dadX hemA*.

We are working continuously on the fine genetic map of the *dad* operon. Its actual shape is presented in Fig. 1. The strain carrying the *dadA286* deletion is capable of growing on decanoate as sole carbon source, which means that the *fadR* gene product is missing. On the basis of this and other observations we conclude that a single Mucts phage

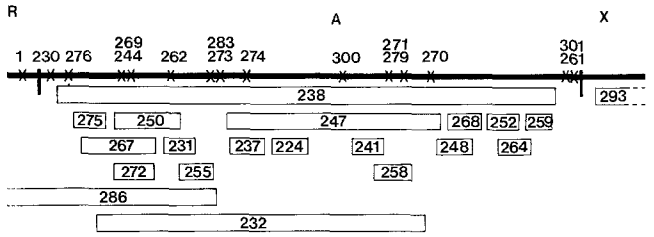

Fig. 1. Genetic map of the *dad* operon. The map was constructed on the basis of P1*vir*-mediated transductions. The homology-dependent transduction using λd*dadA-lac*1 phage helped to distinguish between *dadA279*-proximal and -distal mutations. The *dadA279* site is the junction of the *dadA-lac* fusion (Wild and Obrępalska 1982). The capital letters represent *dad* genes. The numbers stand for *dad* allele designations. The thick solid line represents the chromosome. Point mutations defined as capable of reverting to Dad$^+$ phenotype are shown with sign x. The constitutive *dadR1* mutation isolated by Kuhn and Sommerville (1971) was arbitrarily qualified as a point mutation. The open bars represent nonreverting, deletion mutations

eduction from EC1086 deleted the proximal part of *dadA*, the promoter-operator site of the *dadR* and *fadR* genes, coding for the repressor of fatty acid degradation (Simons et al. 1980). Because strains carrying *(fadR−dadRA)/286* or the *dadX293* mutation recombine yielding *dad*$^+$ offspring the *dadX* gene must be located downstream from *dadA*, which is in agreement with the conclusion of the rarest recombinant tests. Because we could not find any revertant of *dadX293*, the mutation is shown on the map as a deletion.

Properties of alanine racemase in dadX and alr mutants

Alanine racemase was assayed in the four strains carrying all combinations of the available *dadX* and *alr* alleles. Glucose was the carbon source for growth of these strains since EC802 and EC1735 cannot grow on glycerol and some other nonfermentable carbon sources. The results are shown in Table 8. There was no significant difference between the two strains carrying the wild-type *dadX*$^+$ allele. This agrees with the fact that *dadX*$^+$ completely suppresses the temperature-dependent D-alanine auxotrophy. Apparently, the D-alanine requirement of EC802 results from the combined effects of the *alr-1* and *dadX293* mutations.

We examined the supposition that temperature-sensitivity of the *dadX alr* strain reflects temperature-sensitivity of the *alr-1* mutant gene product. For this purpose alanine racemase activity in the same set of four strains was assayed at various temperatures. Figure 2 shows relative values of alanine racemase activity. The activity at 30° C was taken as 1. In the extracts of the two strains carrying the wild-type *dadX* allele the activity in the range from 30° C to 55° C changed less than 20%. The maximal activity was observed between 35° C and 50° C. The enzyme activity from *dadX alr*$^+$ cells was maximum at 45° C. At this temperature it was about 2.5-fold higher than at 30° C. The alanine racemase activity from *dadX alr* cells was highest at 35° C. At 45° C it was equal to about 20% of that in *dadX alr*$^+$ cells.

These results are in agreement with the notion that *dadX* and *alr* code for two distinct alanine racemase isozymes. The *dadX* gene product activity shows a rather unusual indifference to varying temperatures. Such behavior could

Table 8. Alanine racemase activity in *dadX* and *alr* mutants

Strain	Relevant genotype	Alanine racemase (nmol/min per mg dry wt)	
		Glucose	Glucose +L-alanine
EC972	*dadX*$^+$ *alr*$^+$	17.1	53.9
EC1735	*dadX*$^+$ *alr-1*	25.2	46.7
EC1614	*dadX293 alr*$^+$	4.6	5.6
EC802	*dadX293 alr-1*	2.0	2.0

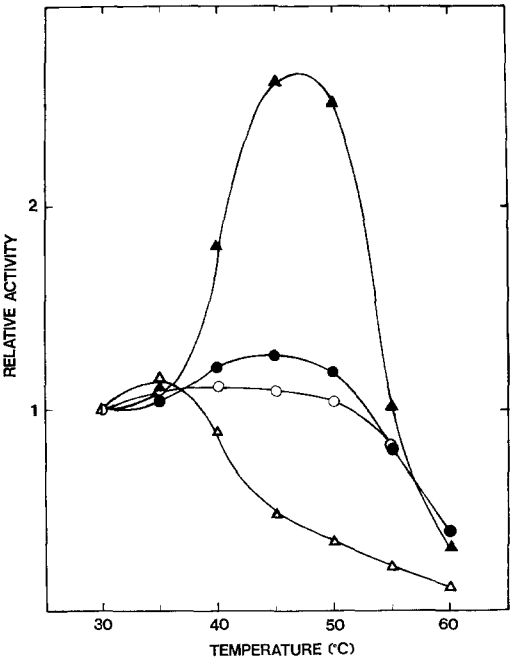

Fig. 2. Different temperature patterns of alanine racemase activity in mutants with various combinations of *alr* and *dadX* alleles. The enzyme activity was assayed in cell-free extracts of the bacteria grown in minimal medium with glucose as carbon source, L-alanine and required supplements. The relative activities are shown. The activity at 30° C was assumed to be 1 for each strain. o – EC1735 (*dadX alr-1*), ● – EC972 (*dadX*$^+$ *alr*$^+$), △ –EC802 (*dadX293 alr-1*), ▲ – EC1618 (*dadX293 alr*$^+$)

result from a colinearity of activity and denaturation temperature patterns. The behavior of the *alr* product at the varying temperatures is typical. In an *alr* mutant alanine racemase shows a significantly lower temperature optimum and this can account for the temperature-sensitivity of *dadX alr* mutants.

Thus we have found that the *dadX* and *alr* gene products differ physically, which strongly suggests that their regulatory patterns could be different also. Therefore, we interprete the lack of inducibility and repressibility of the alanine racemase isozyme present in *dadX* mutants (Table 5) as being due to the fact that *alr* gene expression is constitutive with respect to alanine induction and catabolite repression.

Alanine racemase activity is required for both L-alanine catabolism and D-alanine synthesis. The properties of *dadX alr-1* mutants indicate that growth on L-alanine as carbon source requires the *dadX* gene product. The low alanine racemase activity which is due to the *alr-1* gene product

apparently provides enough D-alanine for peptidoglycan synthesis, since the *dadX alr-1* strains grow normally unless shifted to a nonpermissive temperature.

Discussion

In this paper we present evidence that in *E. coli* K12 two gene products, those of *dadX* and *alr*, contribute to overall alanine racemase activity. We infer from the properties of the mutants lacking the *dadX* gene product that L-alanine is the only alanine stereoisomer involved directly in regulation of the *dad* operon.

We observed that in *E. coli* K12 the regulation of alanine racemase synthesis was similar to that of D-amino acid dehydrogenase. Both enzymes were inducible by L- or D-alanine and repressible by glucose present in growth media. In strains carrying the *dadX* mutation, insertions into the *dadA* gene coding for D-amino acid dehydrogenase (Wild and Kłopotowski 1981) or a deletion of the *dadR* regulatory site, alanine racemase activity was very low. It equalled about one-tenth of the noninduced activity in dad^+ strains. Moreover, it was not regulated by glucose or either alanine isomer. The ensuing supposition that the *dadX* gene could be located down-stream from *dadA* was verified in P1-mediated multiple-point crosses. We presume that it codes for the predominant alanine racemase species.

The minor alanine racemase activity remaining in strains lacking *dadX* alanine racemase is probably due to the product of the *alr* gene, identified by Wijsman (1972). Apparently, activity of this gene is not regulated by glucose or alanine.

We considered also the alternative idea of the *dadX* product being a positive factor for *alr* expression. We were able to discredit it by taking advantage of Wijsman's strain carrying the *alr-1* and *dadX* mutations. Three other strains carrying the other possible combinations of wild-type and mutant *dadX* and *alr* alleles were also used. Fortuitously, alanine racemase coded by the *alr-1* allele appeared to be more thermosensitive than the *dadX* product. Moreover, the products of wild-type *alr* and *dadX* genes, behaved distinctively, at varying incubation temperatures. If *dadX* were the presumed positive factor there would not be such a difference and the thermosensitivity of *alr-1* alanine racemase observed in the *dadX* mutant would not be suppressed by the wild-type *dadX* allele. The fact that the *dadX* temperature pattern suppressed both alr^+ and *alr-1* temperature-sensitive behavior allows rejection of the concept of the *dadX* product acting on expression of the *alr* gene.

The *dadX* mutation that we used was recovered from Wijsman's strain ATK111. This strain carries the *alr-1* mutation and *msuA* allele. Both are required for the temperature-dependent D-alanine auxotrophy of ATK111 (Wijsman 1972 and personal communication). We observed (not shown in detail) that substitution of *dadX* in ATK111 with $dadX^+$ by P1 transduction (which occurred with the 80% frequency characteristic of *fadR-dadX* linkage) resulted in suppression of the conditional D-alanine auxotrophy. It follows, therefore, that the *msuA* gene could be identical with *dadX*.

Our conclusion that *E. coli* K12 has two genes for distinct alanine racemase isozymes is in agreement with that of Wasserman et al. (1983) regarding *S. typhimurium* LT2. Their *dadB* gene would be homologous with *dadX*. We could not follow the practice of assigning identical gene names to homologous genes in the two bacterial species because in *E. coli* K12 the *dadB* designation has been given to the gene claimed to be indispensable for D-amino acid dehydrogenase activity (Franklin et al. 1981).

There is one apparent discrepancy between our results and those of Wasserman et al. They constructed a plasmid carrying the *dadB(X)*, but not *dadA* gene. In strains carrying this plasmid alanine racemase was inducible by L-alanine and repressible by glucose. Because we established the gen order *dadR-dadA-dadX* and found that the strain lacking *dadR* is completely polar, it follows that there is no promoter or regulatory site between *dadA* and *dadX*. Assuming the homology between *E. coli* and *S. typhimurium* it is hard to understand how the *S. typhimurium dadB(X)* mutant carrying a plasmid with *dadB(X)* but not *dadA* can have alanine racemase regulated by alanine. Wasserman et al. did not localize their *dadB(X)* mutations in relation to *dadR*. It may be supposed that their plasmid carries the *dad* operon with a nonpolar deletion within the *dadA* gene.

Another discrepancy is between our results on *dad* operon orientation in *S. typhimurium* (Wild and Kłopotowski 1975) and those of Wasserman et al. (1983). We paid special attention to reexamination of our previous data on *dad* orientation in *E. coli* (Wild and Kłopotowski 1981). We conclude that the gene order is *fadR-dadR-dadA-dadX-hemA* which is in agreement with the data of Wasserman et al. (1983) on *S. typhimurium dad* genes.

The important finding of our study is the lack of inducing effect of D-alanine in strains devoid of *dadX*-coded alanine racemase. It could be due to a requirement of the *dadX* product for the inducing effect of D-alanine. We favor a simpler explanation. It assumes that L-alanine is the actual inducer of the *dad* operon. The activity of alanine racemase in strains lacking the *dadX* gene product is too low and an effective intracellular concentration of L-alanine cannot be achieved in such strains.

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