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Two Alanine Racemase Genes in *Salmonella typhimurium* That Differ in Structure and Function

STEVEN A. WASSERMAN,* CHRISTOPHER T. WALSH, AND DAVID BOTSTEIN

Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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Mutations were isolated in a previously undescribed *Salmonella typhimurium* gene encoding an alanine racemase essential for utilization of L-alanine as a source of carbon, energy, and nitrogen. This new locus, designated *dadB*, lies within one kilobase of the D-alanine dehydrogenase locus (*dadA*), which is also required for alanine catabolism. The *dadA* and *dadB* genes are coregulated. Mutants (including insertions) lacking the *dadB* alanine racemase do not require D-alanine for growth unless a mutation is introduced at a second locus, designated *dal*. Two genes specifying alanine racemase activity were cloned from *S. typhimurium*. The two cloned DNA sequences do not cross-hybridize with each other; one was shown to contain the *dadB* gene.

Alanine racemases (EC 5.1.1.1) catalyze the interconversion of D-alanine and L-alanine. In gram-negative bacteria the biosynthesis of D-alanine and the catabolism of L-alanine both require alanine racemase activity. D-Cycloserine and other compounds that inactivate alanine racemases prevent cell wall assembly by blocking the conversion of L-alanine to D-alanine (6, 15, 30). The fact that racemase inhibitors also prevent the degradation of L-alanine (but not D-alanine) to pyruvate reveals that racemization of L-alanine to D-alanine is also obligatory for L-alanine degradation (26).

We report here the existence in *Salmonella typhimurium* of two nonhomologous alanine racemase genes, one of which clearly is associated with the catabolic function and the other of which presumably represents the biosynthetic function.

MATERIALS AND METHODS

Bacterial strains. All bacterial strains used are listed in Table 1, and all except *Escherichia coli* DB6231 are F^-. Strain constructions were done by P22 transduction, except where noted.

Bacteriophage strains. λ265 (λ *imm*434 *shn*6° p*lac*5) was constructed by crossing λ590 (λ *imm*434 *shn*6° *b*538) (22) with λGS1316 (λ *cI*857 *S*am7 p*lac*5) and screening for blue turbid plaques with *E. coli* strain DB4562 as host on a 5-bromo-4-chloro-3-indolyl-β,D-galactoside indicator plate. All lysates were prepared by the liquid NZC method of Blattner et al. (3).

P22 transductions. P22 transductions were carried out with P22 *int*3 HT 12/4 as previously described (41).

Materials. Uniformly labeled L-[^{14}C]alanine was purchased from Amersham Corp. or New England Nuclear Corp. and was purified by chromatography on Bio-Rad AG-50W-XA4 resin followed by recrystallization with unlabeled carrier L-alanine. The sources of other reagents were as follows: tetracycline and D-amino acid oxidase (EC 1.4.3.3) (15 U/mg), Sigma Chemical Co.; ethyl methane sulfonate (EMS), Eastman Organic Chemicals; restriction enzymes, the Klenow fragment of *E coli* DNA polymerase I, and T4 DNA ligase, New England Biolabs; ampicillin, Bristol Laboratories; 5-bromo-4-chloro-3-indolyl-β,D-galactoside, Bachem.

Media. Liquid media used were LB broth, M9 minimal medium supplemented with amino acids (M9-CAA), and SM buffer (35). M9 minimal plates (7) were used with carbon sources added to 0.2% (glucose) or 0.4% (pyruvate or alanine). Low-osmolarity M9 plates were made up by using only one-fourth of the standard amount of M9 buffer. Other solid media used included LB plates and λ plates (7), as well as 5-bromo-4-chloro-3-indolyl-β,D-galactoside indicator plates (19). Indicator plates for L-alanine catabolism were prepared according to the general method of Bochner and Savageau (4), using 0.2% L-alanine and 0.2% neopeptone as carbon sources. When necessary, minimal media were supplemented with L-amino acids at a concentration of 20 μg/ml. Tetracycline was used at a concentration of 25 μg/ml; ampicillin was used at 100 μg/ml.

Measurement of enzymatic activity. Alanine racemase was assayed in permeabilized cells or crude extract from sonicated cells by the radiometric method of Lambert and Neuhaus (15): this is a discontinuous, coupled assay in which radioactive D-alanine formed (step I, L-alanine to D-alanine) is converted to pyruvate by D-amino acid oxidase (step II). The specific activity of the L-[^{14}C]alanine was 5×10^5 cpm/μmol. One unit of enzyme is defined as that amount which will catalyze the formation of one micromole of product in one minute at 37°C. In crude extract we could reliably detect specific activities of greater than 0.5 mU/mg, which is 3% of the uninduced wild-type level of alanine racemase (see below). Protein was determined by the method of Lowry et al. (16).

Catabolic mutant enrichment and isolation. For cata-

TABLE 1. *S. typhimurium* LT2 and *E. coli* K-12 strains

Strain	Synonym	Genotype	Source (reference)
S. typhimurium			
DB53	TR248	*cysA348*(Am) *hisC527*(Am)	J. Roth (11)
DB1093		*cysA348*(Am) *hisC527*(Am) *dadA64*	This paper
DB4566		*hspL hspS proC9 his-2253 dhuA1 purF145 galE503*	D. Shortle (28)
DB4673	TS736	*met trp val galE hcm* Δ*malB*/F'112 *malB*$^+$ (*E. coli*)	E. T. Palva (24) via S. Kustu
DB4936	TK942	*dhuA13 hisDC129 trp-1061 dadR18*	T. Klopotowski (37) via S. Kustu
DB4939	TK649	*dadA2 dhuA1 hisCBHAFIE3501 metE368*	T. Klopotowski (40) via S. Kustu
DB4940		*cysA348*(Am) *hisC527*(Am) *zdf-1201*::Tn*10*	This paper
DB4945	TT464	*pyrF*::Tn*10*	J. Roth
DB6581	SA1889	*hemA pro purE*	K. Sanderson
DB7136		*hisC527*(Am) *leuA414*(Am)	F. Winston (41)
DB7809		*hisC527*(Am) *leuA414*(Am) *pyrF*::Tn*10*	This paper
DB7810		*hisC527*(Am) *leuA414*(Am) Δ*trp-130*	This paper
DB7811		*hisC527*(Am) *leuA414*(Am) Δ*trp-130 zdf-1201*::Tn*10*	This paper
DB7812		*hisC527*(Am) *leuA414*(Am) Δ*trp-130 zdf-1201*:Tn*10 dadA2*	This paper
DB7813		*hisC527*(Am) *leuA414*(Am) Δ*trp-130 zdf-1201*::Tn*10 dadB1*	This paper
DB7814		*hisC527*(Am) *leuA414*(Am) Δ*trp-130 dadR18*	This paper
DB7818		*hisC527*(Am) *leuA414*(Am) Δ*trp-130 dadB5*::Tn*10*Δ*16*Δ*17*	This paper
DB7819		*hisC527*(Am) *leuA414*(Am) Δ*trp-130 dadB6*::Tn*10*Δ*16*Δ*17*	This paper
DB7820		*hisC527*(Am) *leuA414*(Am) Δ*trp-130 dadB7*::Tn*10*Δ*16*Δ*17*	This paper
DB7822		*hisC527*(Am) *leuA414*(Am) Δ*trp-130 zdf-1201*::Tn*10 hemA*	This paper
DB7823		*hisC527*(Am) *leuA414*(Am) Δ*trp-130 zdf-1201*::Tn*10 dadB2*(Ts)	This paper
DB7824		*hisC527*(Am) *leuA414*(Am) Δ*trp-130 zdf-1201*::Tn*10 dadB3*	This paper
DB7830		*hisC527*(Am) *leuA414*(Am) Δ*trp-130 dadB5*::Tn*10*Δ*16*Δ*17 dal-1*	This paper
DB7838		*hisC527*(Am) *leuA414*(Am) Δ*trp-130 zdf-1201*::Tn*10 dad-52*	This paper
DB7839		*hisC527*(Am) *leuA414*(Am) Δ*trp-130 zdf-1201*::Tn*10 dadA54*	This paper
DB7840		*hisC527*(Am) *leuA414*(Am) Δ*trp-130 zdf-1201*::Tn*10 dadA62*	This paper
DB7841		*hisC527*(Am) *leuA414*(Am) Δ*trp-130 zdf-1201*::Tn*10 dadA64*	This paper
DB7842		*hisC527*(Am) *leuA414*(Am) Δ*trp-130 zdf-1201*::Tn*10 dadB4*	This paper
DB7843		*hisC527*(Am) *leuA414*(Am) Δ*trp-130 dal-1*	This paper
DB7845		*hisC527*(Am) *leuA414*(Am) Δ*trp-130 dadA2 dal-1*	This paper
DB9006		*thyA deo* Δ*trp-130*	R. Maurer (unpublished data)
E. coli			
DB4544	TKL10	*alr-1 lacY1 codA1 dra-1 tonA21 leuB6 his-108 trp-64 argG66 pyrF101 ilvA634 tsx-95 supE44 thr-1 thyA6*	H. Wijsman (36) via B. Bachmann
DB4548	BNN45	*hsdR supE44 supF met thi*	R. Davis (7)
DB4562	M182	Δ(*lacIPOZY*)X74 *galU galK rpsL*	M. Casadaban
DB4949		*alr-1 lacY1 codA1 dra-1 tonA21 leuB6 ilvA634 tsx-95 thr-1 supE44*	This paper
DB6231	PK191	Hfr Δ(*proB-lac*) X 111 *sup-56*	J. Miller

bolic mutant enrichments, exponentially growing cells were first treated with EMS for 2 h, as described by Miller (19), and grown out overnight in pyruvate minimal medium containing 0.5 mg of DL-alanine per ml. Cells were washed by centrifugation and diluted into warm, ammonia-free L-alanine minimal medium. After several generations, ampicillin was added to 225 μg/ml, and the cells were aerated for 12 h at 37°C. Surviving cells were collected by centrifugation, washed, and grown overnight in glucose-ammonia minimal medium containing 100 μg of D-alanine per ml. Diluted cells were plated on LB plates supplemented with D-alanine and replica plated. Mutants able to use pyruvate, but not L-alanine, as a carbon source were isolated and purified for further study.

Isolation of Tn*10* insertions near mutations. Tn*10* insertions near mutations interfering with L-alanine catabolism were isolated by using a transducing lysate grown on a collection of random chromosomal Tn*10* insertions (7, 12). The lysate was used in a low-multiplicity infection to cotransduce tetracycline resistance and ability to grow on L-alanine. Lysates grown on recombinants answering this double selection were tested for linkage of the Tn*10* insertion to the catabolic locus.

Strain constructions. *S. typhimurium* DB7810 was constructed from strain DB7136 by sequential transductions with lysates grown on strains DB4945 (selecting tetracycline resistance) and DB9006 (selecting *pyrF*$^+$). A cotransductant bearing the *trp* operon deletion was then purified and called DB7810. Isogenic strains containing alleles of *dadA*, *dadB*, or *hemA*

were constructed in two steps. First, the original isolates (e.g., strain DB1093) were transduced to tetracycline resistance with a lysate grown on strain DB4940. Recombinants retaining the mutant phenotype were then used as donors for cotransduction of the mutation and the selected marker, tetracycline resistance, into strain DB7810. Strain DB7814 was constructed by transducing strain DB7812 to *dadA*$^+$ (selecting ability to grow on D-alanine) and then screening recombinants for elevated levels of D-alanine dehydrogenase activity in vitro.

E. coli DB4949 was constructed from strain DB4544 by a series of steps, using P1 transduction and Hfr matings according to the methods of Miller (19). A spontaneous streptomycin-resistant strain was isolated and then transduced to *pyrF*$^+$ by P1 transduction. A *pyr*F$^+$ D-alanine requiring recombinant was then mated with Hfr strain PK191, and a *his*$^+$ *arg*$^+$ *thy*$^+$ exconjugate was isolated that retained the temperature-sensitive D-alanine requirement of strain DB4544. This isolate was called DB4949.

Manipulations of DNA. Restriction enzyme cleavages, ligations, extractions of DNA from phage, isolation of plasmid DNA, gel transfer hybridizations by the method of Southern (29), agarose gel electrophoresis, and transformations were performed as described by Davis et al. (7). Plasmid pBR322 was isolated from a transformant of *E. coli* DB4548. Fragments of DNA were purified from agarose gels by using glass beads according to the method of Vogelstein and Gillespie (33). The λ library of *S. typhimurium* LT2 DNA was a gift from R. Davis.

Isolation of clones. Exponentially growing cells of *E. coli* DB4949 were washed and suspended in 1/10 volume of SM buffer. The cells were then infected with λ *imm*434 *plac*5 (multiplicity of infection = 1) as helper for lysogenization as well as with the λ590 library (multiplicity of infection = 0.3). After 20 min at 37°C, the cells were plated at 41°C on supplemented, low-osmolar glucose minimal plates containing no D-alanine. After 24 h, 3×10^8 cells gave rise to approximately 300 colonies. These cells were then purified once selectively at 41°C on LB plates, which lack sufficient D-alanine for growth of strain DB4949. After lysogeny was tested by cross-streaking, the presumptive double lysogens were grown up in LB broth, and free phage were isolated by streaking a lawn of DB4548 cells on a 5-bromo-4-chloro-3-indolyl-β,D-galactoside plate. Clear white (i.e., helper-free) plaques were picked, and lysates were grown on strain DB4548 and tested for complementing activity by repeating the selective coinfection described above.

Isolation of Tn*10* insertions in cloned DNA. A method for isolating insertions of a modified Tn*10* element (Tn*10*Δ*16*Δ*17* [8]) in λ derivatives has been developed (R. Maurer, B. Osmond, and D. Botstein, manuscript in preparation). Insertions of this 2.9-kilobase (kb) element act as stable tetracycline resistance markers. When such insertions occur within cloned *S. typhimurium* DNA, phage can be used to transduce the insertions into the chromosome of a λ-sensitive strain of *S. typhimurium*. These insertions can then be moved by P22 general transduction into other strains of *S. typhimurium*.

We were able to identify and isolate phage with insertions in a cloned alanine racemase gene by a lysogenic selection for tetracycline resistance. Phage bearing such insertions could form tetracycline-resistant lysogens of *E. coli* DB4949, but no longer complemented its D-alanine requirement. In screening for insertions in the λ590 clone, we used λ265 as the helper phage. Upon induction of tetracycline resistant, D-alanine-requiring double lysogens of strain DB4949, derivatives of the clone could be recognized as Lac$^-$ clear plaque formers.

Subcloning of alanine racemase genes. *Hin*dIII fragments of appropriate λ clones were subcloned into the *Hin*dIII site of pBR322 by selecting ampicillin-resistant, D-alanine-independent transformants of *E. coli* DB4949. DNA coding for an alanine racemase gene was further subcloned by digestion of plasmid pSW1 with *Sal*I and *Xho*I, ligation, and then transformation as described above. Plasmids represented by pSW11 and pSW12 were found at about equal frequency.

Isolated plasmids were sequentially retransformed by ampicillin selection into *E. coli* DB4548 and then into *S. typhimurium* DB4566. Plasmids were then moved into other *S. typhimurium* strains by P22 generalized transduction with selection for ampicillin resistance. Plasmid pSW1 had a toxic effect on strains of *S. typhimurium*, but not on strains of *E. coli*. However, the smaller plasmids pSW11 and pSW12, which carry the same alanine racemase gene as pSW1, did not inhibit growth of *S. typhimurium*.

Isolation of D-alanine-requiring mutants. D-alanine-requiring mutants (Dal$^-$) were isolated from *dadB* strains after EMS treatment. A mutagenized culture of the *S. typhimurium dadB* mutant DB7818 was outgrown in glucose medium supplemented with 400 μg of D-alanine per ml. The cells were plated at 30°C on LB plates with 400 μg of D-alanine per ml and then replica plated at 41°C on LB plates with or without D-alanine. Dal$^-$ colonies were purified and investigated.

RESULTS

Catabolic mutant isolation and characterization. We began our search for alanine racemase mutants of *S. typhimurium* DB53 by EMS mutagenesis and subsequent ampicillin enrichment. Among surviving bacteria enriched for defects in the utilization of L-alanine as a carbon, nitrogen, or energy source, about 10% were unable to catabolize L-alanine. However, none of the 2,400 ampicillin survivors required D-alanine for growth, as one might have expected if they bore alanine racemase mutations.

The mutants fell into two classes. One class was able to grow on D-alanine, but not L-alanine, as a carbon or nitrogen source. The other could not use either alanine isomer as a source of carbon or nitrogen. Neither class had a defect in the assimilation of carbon from pyruvate or of nitrogen from aspartate. Four nonleaky mutants of each class were selected for further investigation. Since the second class, those unable to grow on D- or L-alanine, closely resembled the *dadA* (D-alanine dehydrogenase) mutants isolated by Klopotowski, we included one of his *dadA* mutants, TK649 (40), in our studies.

The mutants of each class were examined for

a range of possible secondary phenotypes. One of the strains was found to be temperature sensitive for L-alanine utilization, growing on L-alanine at 30°C, but not at 37°C. Growth on L-alanine at 37°C could be restored by supplementation with 25 μg of pyridoxine-hydrochloride per ml. Growth on D-alanine was unimpaired at either 30°C or 37°C. None of the other strains exhibited a temperature-sensitive phenotype or rescue by vitamin B6.

We selected for Tn*10* insertions linked to the mutations as described above. One such insertion, which was linked to all eight mutations, was used to cotransduce the mutations into an unmutagenized background. The Tn*10* insertion turned out also to be linked to Klopotowski's *dadA2* allele and was therefore further used to construct an isogenic strain, DB7812, containing the *dadA2* marker. Since the *dadA* locus has been mapped to minute 35 on the *S. typhimurium* linkage map (40), the Tn*10* insertion was given the designation *zdf-1201*::Tn*10* (see reference 7 for rules of nomenclature).

Once the isogenic strains were constructed, we used enzyme assays to probe for any correlations between growth characteristics and enzyme deficiency in the two classes of mutations. All four of the mutants showing a specific inability to grow on L-alanine exhibited abnormal alanine racemase activity (Table 2). The level in two of the four strains, DB7813 and DB7824, was so low as to be beyond the limits of detection. When the phenotypically temperature-sensitive strain DB7823 was grown at 30°C. and assayed at 25°C, the alanine racemase activity was itself temperature sensitive, falling from 50 to 9% of the wild-type (strain DB7810) level upon five min of preincubation at 37°C. In contrast, the D-alanine dehydrogenase activity in these four strains was comparable to that of the wild type.

TABLE 2. Enzymatic activity of *dad* mutants[a]

S. typhimurium strain (genotype)	Growth on:		Alanine racemase (U/mg of protein [10^3])	D-Alanine dehydrogenase (U/mg of protein)
	L-Alanine	D-Alanine		
DB7811 (*dad*$^+$)	+	+	33	33
DB7813 (*dadB1*)	−	+	<0.5	25
DB7823 (*dadB2*)	−	+	4	31
DB7824 (*dadB3*)	−	+	<0.5	24
DB7842 (*dadB4*)	−	+	3	24
DB7812 (*dadA2*)	−	−	32	<0.5
DB7838 (*dad-52*)	−	−	2	<0.5
DB7839 (*dadA54*)	−	−	31	5
DB7840 (*dadA62*)	−	−	30	2
DB7841 (*dadA64*)	−	−	25	<0.5

[a] Cells were grown to log phase at 37°C in 1.5% Casamino Acids with M9 buffer. Assays of permeabilized cells were conducted at 37°C.

A correlation between growth characteristics and enzymatic defect was also found for the four members of the other class, i.e., mutants unable to grow on either D- or L-alanine. These strains had low or undetectable amounts of D-alanine dehydrogenase activity (Table 2). They thus resembled the *dadA2* mutant DB7812 not only in growth characteristics and linkage to *zdf-1201*::Tn*10*, but also in their altered dehydrogenase levels. On the basis of these results, the three strains having wild-type racemase levels (strains DB7839, DB7840, and DB7841) were classified as having mutations only in the *dadA* gene. The fourth mutant, DB7838, had low levels of both dehydrogenase and alanine racemase. Since the mutation in this strain could not be classified phenotypically as *dadA*, its genotype was designated simply as *dad-52*. The nature of this possibly polar lesion is discussed below.

dadR mutants, isolated by Wild and Klopotowski, have levels of D-alanine dehydrogenase 4- to 32-fold higher than that in the wild type under conditions of catabolite repression (37). To study the effects of a *dadR* mutation on alanine racemase and D-alanine dehydrogenase activity, the *dadR18* allele from the Klopotowski strain TK942 was transduced into the DB7810 background (see above). When the *dadR18*-containing strain DB7814 was grown on 0.2% glucose M9 medium, the mutation was observed to elevate levels of both enzymes four- to eight-fold above levels in the *dadR*$^+$ strain DB7811.

Mapping of new loci. Since all four alanine racemase mutants were linked to a single Tn*10* insertion near *dadA* and since the wildtype activity was elevated by a *dadR* mutation, we decided to call the affected racemase locus *dadB*. The *dadB* locus could be mapped by a series of transductional crosses, because Wild et al. had already located the *dadA* locus on the *S. typhimurium* linkage map (40).

All of the *dadA* and *dadB* alleles, as well as the *dad-52* mutation, were 35 to 55% linked to the insertion *zdf-1201*::Tn*10* (Table 3). The implied proximity of *dadB* and *dadA* was confirmed by transducing strain DB7812 (*dadA2 dadB*$^+$) with phage grown on strain DB7823 [*dadA*$^+$ *dadB2*(Ts)] and selecting recombinants able to grow on L-alanine at 30°C (a permissive temperature for *dadB2*). Among 249 such *dadA*$^+$ transductants, 238 (96%) exhibited the temperature-sensitive growth response of the *dadB2* allele.

The insertion *zdf-1201*::Tn*10* could also be mapped with respect to the chromosomal map by using published data and an additional transductional cross. The linkage between *dadA* and

hemA is known from the work of Klopotowski and co-workers to be 3% (40). However, *zdf-1201*::Tn*10* is at least 40% linked to *dadA* and more than 1% linked to *hemA* (Table 3). The order clearly consistent with these data is one in which *zdf-1201*::Tn*10* lies between *dadA* and *hemA*.

Cloning of alanine racemase genes. The existence of DB4544, a D-alanine-requiring strain of *E. coli* isolated by Wijsman (36), facilitated the cloning of alanine racemase genes. Although this strain contains at least three mutations affecting alanine metabolism (36), it was nonetheless our expectation that a cloned alanine racemase gene might allow it to grow without supplemental D-alanine.

Before the cloning experiments we used P1 transduction and an Hfr cross to construct *E. coli* DB4949, which lacked some of the auxotrophic markers in strain DB4544, but retained its temperature-sensitive D-alanine requirement. The modified strain DB4949 was used as the host in a selection for lysogenic clones able to grow without D-alanine at the nonpermissive temperature of 41°C. We isolated a number of clones and, after retesting, purified five complementing phages.

A 7.4-kb *Hin*dIII fragment of *S. typhimurium* DNA was identified in three of the phages, and a nonhomologous (see below) 8.4-kb *Hin*dIII piece was found in the other two phages. Each of the two fragments was independently subcloned into the *Hin*dIII site of pBR322; plasmid pSW1 contained the 7.4-kb fragment, and pSW22 contained the 8.4-kb fragment (Fig. 1). DNA from pSW1 that retained the ability to complement the DB4949 D-alanine auxotrophy was further subcloned, producing plasmids pSW11 and pSW12 (Fig. 1).

Identification of cloned *dadB* gene. Assays revealed that the presence of plasmid pSW11 or pSW12 correlated with the appearance of an amount of alanine racemase activity about 15 times that seen without the plasmid. That the cloned gene product might be the *dadB* enzyme was suggested by the fact that the plasmid-associated racemase was subject to the same sort of induction by growth with alanine as a carbon source as was the chromosomally encoded enzyme (Table 4). Moreover, both pSW11 and pSW12, as well as pSW22 (carrying the 8.4-kb *Hin*dIII fragment), complemented (or suppressed) the inability of the *dadB* mutants to grow on L-alanine, but did not phenotypically suppress *dadA* strains.

To test whether the 7.4-kb *Hin*dIII fragment encoded *dadB*, we set out to isolate mutations in the phage carrying this insert, using a new method of local mutagenesis. As described above, we isolated stable insertions of a modified Tn*10* element in the bacterial portion of the phage DNA and transduced them into *S. typhimurium*. We characterized three such insertions (called *dadB*::Tn*10*Δ*16*Δ*17*) both in the phage and in the *S. typhimurium* chromosome.

TABLE 3. Cotransduction of *dad* alleles and *hemA* with *zdf-1201*::Tn*10*[a]

Unselected marker	No. of scored recombinants	No. of cotransductants	% Cotransduction
dadB1	1,811	838	46.3
dadB2(Ts)	99	44	44.4
dadB3	178	82	54.0
dadB4	168	85	50.6
Total			46.5
dadA2	247	89	34.6
dadA54	375	203	54.1
dadA62	386	178	46.1
dadA64	368	196	53.3
Total			48.4
dad-52	267	111	41.6
hemA	398	7	1.8

[a] In all transductions *zdf-1201*::Tn*10* was the selected marker and *S. typhimurium* DB7810 was the recipient strain.

Since the Tn*10*Δ insertions in the 7.4-kb fragment introduced new sites for cleavage by *Eco*RI and *Hin*dIII, the points of insertion could be mapped by restriction enzyme analysis of the phage DNA. The three Tn*10*Δ insertions were clustered in a 0.3-kb section of the *S. typhimurium*-derived DNA (Fig. 2). Furthermore, the insertions were localized within the same region that had been subcloned in pSW11 and pSW12 by selecting the complementing activity that the Tn*10*Δ insertions eliminated.

To confirm that the disrupted locus was in fact *dadB*, we transferred the insertions onto the *S. typhimurium* chromosome. By a series of transductions, beginning with a λ-sensitive strain of *S. typhimurium*, strain DB4673, the insertions were moved into the DB7810 background by means of the selectable tetracycline resistance gene. All three strains bearing insertions had the phenotype of tight *dadB* mutants, i.e., an ability to grow on D-alanine, but not L-alanine, as a carbon source.

There was no observable alanine racemase activity in any of the three strains when grown in Casamino Acids medium. Tetracycline resistance was tightly linked in a transductional cross

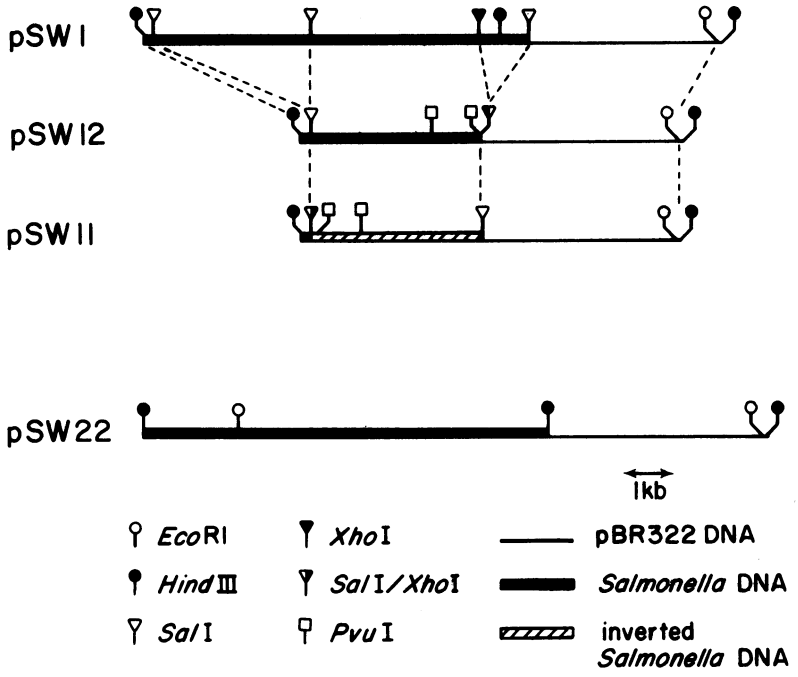

FIG. 1. Restriction maps of plasmid clones. *Hin*dIII fragments (7.4 and 8.4 kb) were ligated from phage DNA into the unique *Hin*dIII site of pBR322 to form pSW1 and pSW22, respectively. Plasmids pSW11 and pSW12 are subclones of pSW1 (see text). Sites are drawn to scale, and distances are accurate to ±0.2 kb.

to *dadA*$^+$. Strains DB7818, 7819, and 7820 were used as donors of *dadB*::Tn*10*Δ to the *dadA* strain DB1093. Among 100 tetracycline-resistant recombinants picked for each insertion, 97, 99, and 98, respectively, were *dadA*$^+$. The most important fact about the *dadB*::Tn*10*Δ mutants, however, is that they have no requirement for D-alanine, despite their lack of detectable (<3% of wild-type) alanine racemase activity.

We used gel transfer hybridization experiments (29) to demonstrate that the physical arrangement of the *dadB* insertions and the flanking cloned *S. typhimurium* DNA was not altered in the process of transduction from phage clone to chromosome. The chromosomal copy of the 7.4-kb genomic fragment was intact in strain DB7811, but had been disrupted in the *dadB*::Tn*10*Δ-bearing strain DB7818 (Fig. 3). The two *Hin*dIII fragments generated by the Tn*10*Δ insertion are the same size as those seen in the phage (Fig. 2), indicating an identical organization of the DNA.

TABLE 4. Alanine racemase activity of *dadB* clone[a]

S. typhimurium strain(plasmid)	Alanine racemase (mU/mg) in cells grown in:	
	LB broth	L-Alanine (0.4%) minimal medium
DB4566(pBR322)	15	120
DB4566(pSW11)	130	2,300
DB4566(pSW12)	150	2,000

[a] Cells were grown to exponential phase at 37°C in the presence of 100 μg of ampicillin per ml. Assays of crude extracts from sonicated cells were conducted at 37°C.

Identification of a second alanine racemase gene. Gel transfer hybridization also revealed that the 7.4- and 8.4-kb *Hin*dIII fragments were nonhomologous on the DNA level (Fig. 3). To test whether the 8.4-kb *Hin*dIII fragment included a second racemase gene, we transduced plasmid pSW22 into a *dadB* insertion background (strain DB7818) and were then able to detect alanine racemase activity (Table 5). Since the level of enzyme in our assay was low, we confirmed that the strain in fact exhibited a detectable level of alanine racemase by showing that activity in step I (racemase proper) was eliminated by 3-fluoro-L-alanine, a specific inactivator of alanine racemases (34), whereas the step II activity (D-amino acid oxidase) was not. We further showed that pyruvate formation was absolutely dependent on step II (conversion to pyruvate by D-amino acid oxidase), indicating that the extract provided racemase activity proper and not dehydrogenase or transaminase activity.

Isolation of mutants in D-alanine metabolism. We found (see above) that the *dadB* gene is not essential and therefore is not required for D-

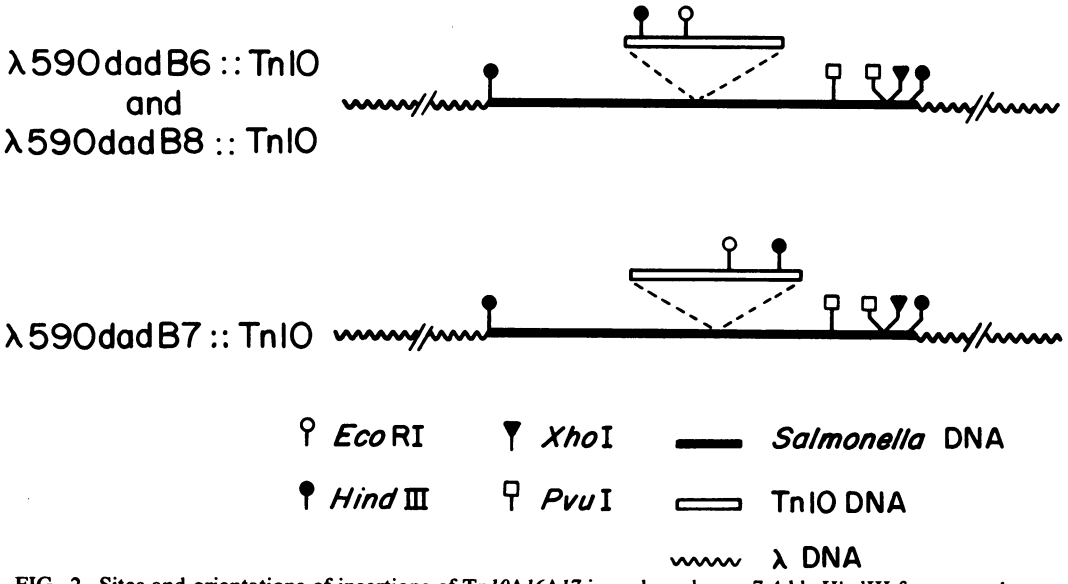

FIG. 2. Sites and orientations of insertions of Tn*10Δ16Δ17* in a phage-borne 7.4-kb *Hin*dIII fragment clone.

alanine biosynthesis. Also, we could identify two different alanine racemase-specifying clones. These two observations led us to expect that construction of a D-alanine-requiring (Dal$^-$) mutant might require the loss of both chromosomal alanine racemase genes. We therefore sought D-alanine auxotrophs in an EMS-mutagenized culture of the *dadB* strain DB7818.

Among 4,200 mutagenized colonies examined by replica plating, 7 required D-alanine at 41°C. Two of the mutants were temperature sensitive; one of these two, but none of the others, could be rescued by 2% NaCl or 20% sucrose in place of D-alanine. For all seven mutants, 20 μg of D-alanine per ml was sufficient to permit growth under nonpermissive conditions. We chose the non-temperature-sensitive strain DB7830 for further study and gave it the genotypic designation *dal-1*, since it shows a requirement for D-alanine (Dal$^-$ phenotype) in a *dadB* background. Plasmids carrying either pSW11 or pSW22 suppressed the D-alanine requirement in this strain.

Transductional crosses involving the *dal-1* mutation indicated that the new locus is unlinked to *dadB*. When the *dadA dadB*$^+$ strain DB4939 was used as a donor, 82 of 93 Dal$^+$ transductants of strain DB7830 were tetracycline sensitive, having lost the *dadB* insertion. These transductants were apparently not *dal*$^+$, since they regained their Dal$^-$ phenotype upon reintroduction of a *dadB* mutation. The majority of these Dal$^+$ *dadB*$^+$ transductants were *dadA*, showing that *dadA dal-1* double mutants do not display the Dal$^-$ phenotype. In further experiments with one such *dadA dal-1* transductant, DB7845, we found no linkage between the *dal-1* mutation and the insertion *zdf-1201*::Tn*10*.

Three-factor mapping of the *dad* region. A three-factor cross was used to orient the tightly linked *dadA* and *dadB* loci with respect to the chromosomal map. We expected that one of the

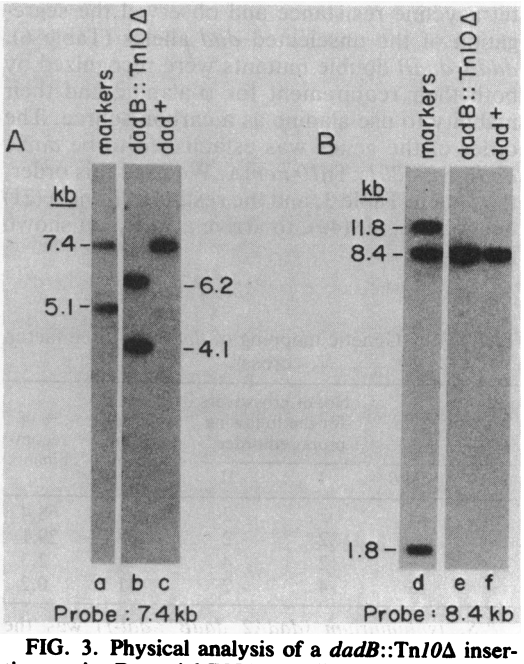

FIG. 3. Physical analysis of a *dadB*::Tn*10Δ* insertion strain. Bacterial DNA was digested with *Hin*dIII and fractionated according to size by electrophoresis on a 0.5% agarose gel. The DNA from duplicate samples was transferred to nitrocellulose and hybridized at normal stringency (7) to the indicated nick-translated probes: a 7.4-kb *Hin*dIII fragment *S. typhimurium* clone (A) or an 8.4-kb *Hin*dIII fragment *S. typhimurium* clone (B).

TABLE 5. Alanine racemase activity encoded by the 8.4-kb *Hind*III fragment[a]

S. typhimurium strain(plasmid)	*Salmonella* DNA present on plasmid	Alanine racemase (mU/mg)	Changes in step I	Changes in step II
DB7818(pBR322)	None	<0.5	None	None
DB7818(pSW12)	*dadB* subclone	1,100	None	None
DB7818(pSW22)	8.4-kb fragment	5.0	None	None
		<0.5	None	−DAAO
		<0.5	+L-Fl-ala	None
		5.0	None	+L-Fl-ala

[a] Assays were conducted at 37°C on permeabilized cells grown at 37°C on 1.5% Casamino Acids. Fluoro-L-alanine (L-Fl-ala) (5 mM) was added to the extract before incubation with substrate or just before addition of the D-amino acid oxidase (DAAO) couple to the boiled incubation mixture.

products of such a cross, a *dadA dadB* double mutant, would show the same pattern of alanine utilization as a *dadA* single mutant. Therefore, to distinguish the single and double mutants we used as the recipient in the cross a *dadA* strain carrying a mutation at the unlinked *dal-1* locus. We could detect a *dadB* recombinant in the DB7845 background (*dadA2 dadB*$^+$ *dal-1*) because the presence of mutations at both the *dadB* and *dal-1* loci results in a D-alanine auxotrophy.

With strain DB7813 (*dadA*$^+$ *dadB1 zdf-1201*::Tn*10 dal*$^+$) as the donor, we selected tetracycline resistance and observed the segregation of the unselected *dad* alleles (Table 6). *dadA dadB* double mutants were recognized by both their requirement for D-alanine and their inability to use alanine as a carbon source. The order of the genes was established to be *dadA dadB zdf-1201*::Tn*10 hemA*. We used this order, the data in Table 3, and the results of Mojica (21) and Wild et al. (40), to arrive at the map shown in Fig. 4.

TABLE 6. Genetic mapping of *dadB* by three-factor cross[a]

Unselected markers		No. of crossovers for the following proposed order:[b]		No. of recombinants	% of recombinants
dadA	*dadB*	I	II		
−	+	2	2	324	68.4
+	−	2	2	138	29.1
−	−	2	4	11	2.3
+	+	4	2	1	0.2

[a] *S. typhimurium* (*dadA2 dadB*$^+$ *dal-1*) was the recipient strain; *S. typhimurium* DB7813 (*dadA*$^+$ *dadB1 dal*$^+$) was the donor. The selected marker was tetracycline resistance (*zdf-1201*::Tn*10*). Transductants unable to grow on D-alanine as a carbon source were scored as *dadA* mutants (−); those requiring D-alanine for growth were scored as *dadB* mutants (−) (see text for explanation).

[b] Order I, *dadA dadB zdf-1201*::Tn*10 hemA*; order II, *dadB dadA zdf-1201*::Tn*10 hemA*.

DISCUSSION

We report above the isolation of mutations in a new chromosomal locus, *dadB*, which codes for a catabolic alanine racemase. These mutations arose from an enrichment in which L-alanine was the sole source of carbon, nitrogen, and energy. In our initial screen of the enriched population, we looked for strains able to use pyruvate, but not L-alanine, as a carbon and energy source. Since all of the mutants we isolated were also deficient in nitrogen assimilation from alanine, we cannot say which aspect of alanine metabolism was the effective target of the enrichment.

One of the *dadB* strains from the enrichment was a temperature-sensitive mutant with temperature-sensitive alanine racemase activity. The fact that this mutant can be phenotypically rescued by pyridoxine is consistent with it being a structural gene lesion. The *E. coli* catabolic racemase is known to have a pyridoxal phosphate cofactor (15, 34). A mutation weakening the association of pyridoxal phosphate and holoenzyme at the nonpermissive temperature might be suppressed by exogenous pyridoxine, as is known to be the case for a temperature-sensitive mutation of the *E. coli* diaminopimelate decarboxylase (5).

Since the temperature-sensitive *dadB* mutation mapped close by the other three *dadB* mutations, it is likely that all four lie in or near the structural gene. Similarly, the four *dadA* mutations are most probably structural gene lesions, since Wild and co-workers have used a temperature-sensitive mutant and deletions to localize the *dadA* gene (39, 40). We have used a three-factor cross to orient the *dadA* and *dadB* genes with respect to the known linkage map, but have not yet established an operon organization.

Although regulatory mutations in *dadR*, isolated by Klopotowski, are tightly linked to *dadA* (and therefore to *dadB*) (37), their map position and exact nature are not yet clear. Wild and

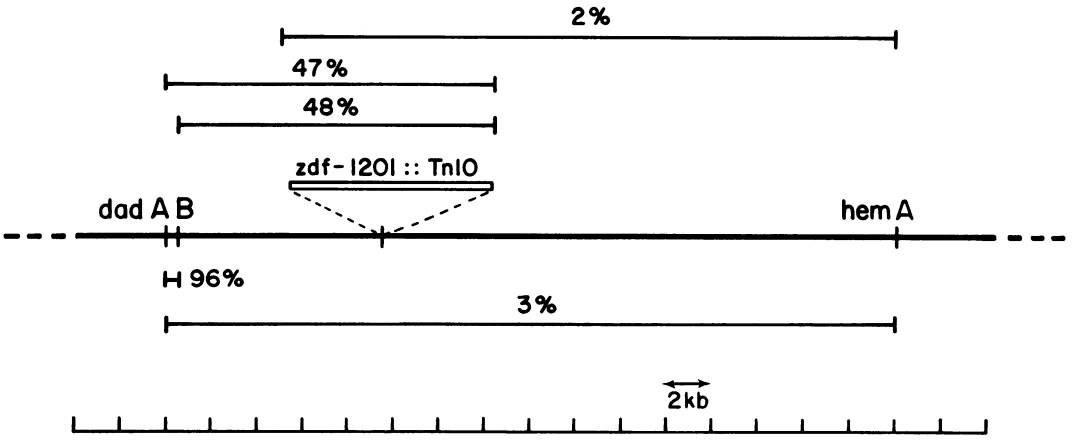

FIG. 4. Transductional map of the *dad* region of the *S. typhimurium* chromosome. For those crosses with frequencies indicated above the heavy line, Tn*10* was the selected marker. The physical distance between markers in such crosses is increased by 9.3 kb. Map distances in kilobases were calculated from cotransduction frequencies by the formula of Wu (42).

Klopotowski used the *dadA* and *dadR* loci in a three-factor cross (37). However, since the outside marker *hemA* was not selected, the cross did not distinguish on which side of *dadA* the *dadR* locus lies. The work of Wild and Obrepalska with *dadA-lac* fusion strains (39) cannot, therefore, define the absolute direction of *dadA* transcription without further mapping of *dadR*.

Strain DB7838 (*dad-52*) that we describe could have a lesion in either *dadA* or *dadB* that eliminates function in the other *dad* structural gene by a polar effect. However, the strain might also have a lesion in each gene, a deletion extending into both genes, or a mutation in a promotor or regulatory site linked to *dadA* and *dadB*.

Although supplemental D-alanine was present throughout the enrichment procedure, neither the *dadB* mutants nor other members of the enriched population required D-alanine for growth. This suggested that the alanine racemase encoded by *dadB* was not essential for D-alanine biosynthesis in *S. typhimurium*. We attempted to test this idea by isolating insertions in the *dadB* gene. Insertions which destroyed the *dadB*-complementing activity of the cloned 7.4-kb *Hin*dIII fragment were transduced into the *S. typhimurium* chromosome. As with the enrichment-generated mutants, these insertions eliminated the ability of the cells to grow on L-alanine without conferring a D-alanine requirement.

Several pieces of evidence indicate that the insertions actually physically disrupt *dadB* and, hence, that the *dadB* alanine racemase is nonessential for growth on most media. First, the three insertions are clustered, suggesting a single target. Second, the location of the insertions coincides with the same half of the insert that showed complementing activity in subcloning experiments. Lastly, DNA sequencing of the insertion-bearing region has revealed an encoded polypeptide with the same 20 amino-terminal residues as were obtained from the purified alanine racemase by protein sequencing (unpublished data).

Since D-alanine is produced even by *dadB* mutants, alternative pathways for D-alanine biosynthesis must be considered. First, D-alanine might be made from D-glutamate by a D-alanine-D-glutamate transaminase. However, evidence suggests that the function of this enzyme is to convert D-alanine to D-glutamate, for which there is no other known biosynthetic source (18, 20). D-Alanine might also arise from transamination of pyruvate by an L-amino acid, but there exists no known example of a transaminase acting on both L- and D-amino acids. Lastly, D-alanine might be synthesized by a second alanine racemase.

We now have evidence for two alanine racemase genes in *S. typhimurium*. The 7.4- and 8.4-kb *Hin*dIII fragments, which do not cross-hybridize with each other, both appear by assay to encode alanine racemases. Moreover, each complements the inability both of *E. coli* DB4949 to grow without D-alanine and of *S. typhimurium* DB7818 to grow on L-alanine. The possibility that the 8.4-kb clone encodes only a regulatory locus appears to be remote. The metabolic functions proposed for each *S. typhimurium* alanine racemase are shown in Fig. 5.

The failure of the chromosomal copy of the second alanine racemase gene to suffice for L-alanine catabolism is most likely due to the low levels of the gene product in vivo. However, when carried within the 8.4-kb *Hin*dIII fragment on a multicopy plasmid (pSW22), the second

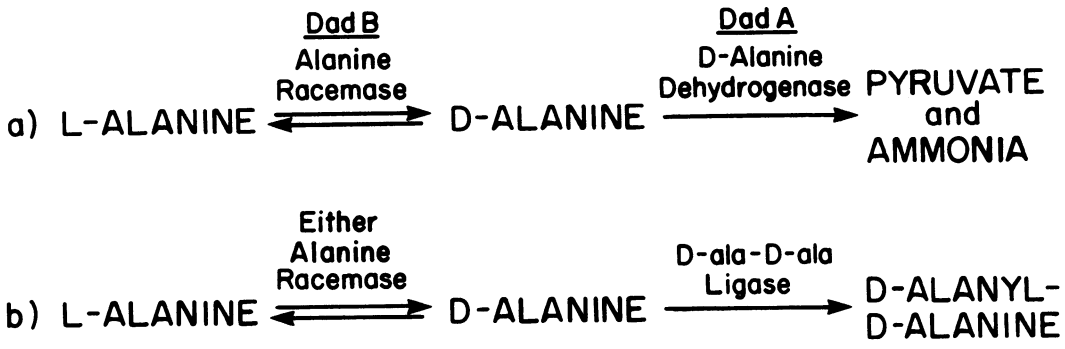

FIG. 5. Proposed alanine racemase-dependent pathways in *S. typhimurium*. (A) Utilization of L-alanine as a source of carbon, energy, and nitrogen. (B) Conversion of L-alanine into D-alanine in peptidoglycan assembly.

racemase gene does in fact complement *dadB* mutations. It should be emphasized that we have evidence for a second gene (probably, but not necessarily, the structural gene) which can provide alanine racemase activity to the cell. We do not yet know whether this activity is the primary function of this gene or whether this gene is indeed the source of biosynthetic D-alanine.

Assuming the *dadB* gene product to be only one of two alanine racemases potentially involved in D-alanine biosynthesis, we set out to isolate Dal$^-$ mutants in a *dadB* background. The *dal-1* locus did in fact act synergistically with *dadB*; loss of both loci was required for appearance of the Dal$^-$ phenotype. Starting with the *dadB* single mutant, Dal$^-$ double mutants were relatively easy to find. Previous such screens of mutagenized wild-type cultures had not been successful (unpublished data).

Work is in progress to determine whether some or all of the *dal* mutations affect the second alanine racemase gene. In the case of *dal-1*, the Dal$^-$ phenotype it confers on a *dadB* strain is rescued by either *Hin*dIII clone, as would be expected if *dal-1* affected a second racemase. However, a D-alanine transport protein (23), the D-alanine-D-alanine synthetase (17), or another cell wall locus (20) could also be the gene product affected by *dal-1*.

The discovery of two nonhomologous alanine racemase genes in *S. typhimurium* is of particular importance to the development of new antibiotics. Alanine racemases are the target of two different classes of recently developed bacteriocidal agents, the halogenated alanines and the phosphonoalanine-containing peptides (1, 13, 18, 23). If the existence of isozymes of alanine racemase with different properties proves to be widespread, it could substantially affect the design of such compounds.

There are precedents for the existence of pairs of anabolic and catabolic isozymes of bacterial enzymes, the first being the two threonine deaminases in *E. coli* (32). It is in fact likely that there are also two alanine racemase genes in *E. coli*. In the D-alanine-requiring strain of *E. coli* K-12 isolated by Wijsman (36), the apparent synergism of the mutations, their map positions, and their phenotypes indicate to us that they affect both a catabolic alanine racemase locus (temperature-sensitive mutation) and a second cell wall locus.

With *E. coli* B, Lambert and Neuhaus found only a single major alanine racemase activity in cell-free crude extract (14). However, their experiments, which included sucrose density sedimentations, heat inactivations, and polyacrylamide gel electrophoresis, relied on the same assay used here, an assay that is not accurate enough to reveal a second racemase representing less than 4% of the total activity. Not until the gene copy number was raised and the predominant catabolic activity was eliminated could we detect the minor alanine racemase in *S. typhimurium*.

Franklin and co-workers have described mutants in *Pseudomonas aeruginosa* PAO1 that resemble our *dadB* strains (25). However, unlike our mutants, these strains were isolated in the absence of any exogenous D-alanine. One cannot say, therefore, whether the residual alanine racemase activity they observed was more likely due to a leaky mutation or to a second gene. Recent work suggests that there are also two amino acid racemases in the D-cycloserine producer *Streptomyces garyphalus* (31). We believe that there may be two alanine racemase genes in all gram-negative bacteria.

In gram-positive bacteria, mutants lacking detectable alanine racemase activity have been isolated only in *Bacillus subtilis*. These strains do not require D-alanine on minimal medium unless certain L-amino acids are present (2). One relevant difference between *B. subtilis* and both *E. coli* and *S. typhimurium* is that in *B. subtilis* the alanine dehydrogenase is L-specific, rather than D-specific as in gram-negative bacteria, so that D-alanine, rather than L-alanine, must be

racemized before deamination can take place (10, 18).

For those organisms with two alanine racemases, the catabolic enzyme has probably been the sole object of study to date, since the only activity purified has been that detectable in crude lysates of wild-type cells (34), generally grown on DL-alanine. Taking advantage of the overproduction possible with cloned genes, we have undertaken the purification of both *S. typhimurium* alanine racemases with the intent of comparing their structural and catalytic differences. This should allow us to assess more completely their physiological roles in alanine metabolism and to analyze their individual susceptibilities to cell wall biosynthesis inhibitors.

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