Escherichia coli K-12 Mutant Forming a Temperature-Sensitive D-Serine Deaminase

ELIZABETH McFALL

Department of Microbiology, New York University School of Medicine, New York, New York 10016

A single-site mutant of *Escherichia coli* K-12 able to grow in minimal medium in the presence of D-serine at 30 C but not at 42 C was isolated. The mutant forms a D-serine deaminase that is much more sensitive to thermal denaturation in vitro at temperatures above but not below 47 C than that of the wild type. No detectable enzyme is formed by the mutant at 42 C, however, and very little is formed at 37 C. The mutant enzyme is probably more sensitive to intracellular inactivation at high temperatures than the wild-type enzyme. The mutation lies in the *dsdA* region. The mutant also contains a *dsdO* mutation, which does not permit hyperinduction of D-serine deaminase synthesis.

D-Serine (DS) is bacteriostatic to *Escherichia coli* K-12: mutants that are unable to form a D-serine deaminase (Dsdase) cannot grow in minimal media containing more than 25 μg/ml (3, 4). It is therefore quite simple, by the penicillin method, to select mutants unable to form the enzyme. When the Dsdase-negative character of such mutants was mapped it was found to be located in a discrete genetic region near *aroC* and the Dsdase regulatory gene (*dsdC*) (5). I tentatively designated the mutations *dsdA*, structural gene negative, assuming that at least some of them had occurred in the Dsdase structural gene. Since negative phenotypes can result from other than structural mutations, however, it was necessary to obtain mutants that produced an altered Dsdase to define the structural gene with certainty. This paper describes the isolation, properties, and chromosomal location of a mutant strain specifying a temperature-sensitive Dsdase.

MATERIALS AND METHODS

Strains. Strain C 600, kindly supplied by J. Scott, is a threonine-, leucine-, and thiamine-requiring K-12 strain. Strain EM1400, described previously (5), is a *dsdC4* mutant of strain W3828. Strain EM1609 is a thymine, adenine, phenylalanine, and tyrosine (*thy aroC purF*) derivative of strain EM1600, itself a *dsdO6* mutant of strain W3828. Strain EM1100 is a *dsdO1* mutant of strain W3828. The *dsdO6* and *dsdO1* mutations were previously termed *dsdC6* and *dsdC1* (5). Strain EM1406 is an *aroC purF* derivative of strain EM1400, itself a *dsdC4* mutant of strain W3828 (5).

Medium. Minimal medium contained (per liter of water): 13.6 g of KH_2PO_4, 0.5 g of $(NH_4)_2SO_4$, 0.5 g of NH_4Cl, 10 g of glycerol, 40 mg of $MgSO_4 \cdot 7H_2O$

$(1.67 \times 10^{-4}$ M), and 0.5 mg of $FeCl_3$, adjusted to pH 7.0 with NaOH. Phenylalanine, tyrosine, thymine, and adenine were added to a final concentration of 50 μg/ml for growth of strains EM1609 and EM1610. DS, where indicated, was added to a final concentration of 500 μg/ml. For solid media, agar (Difco) was added to a final concentration of 15 g/liter. LB broth has been described (5).

Cell cultivation. Cells were grown at temperatures indicated in a gyratory shaking water bath (New Brunswick).

Mutagenesis and penicillin selection. Mutagenesis by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was carried out as described by Adelberg et al. (1). Penicillin selection of Dsdase-negative mutants has been described (4).

Transductions. Preparation of P1 transducing phage (P1kc) and transductions were carried out as described previously (4).

D-Serine deaminase assay. The enzyme assay (4) depends on the formation of a colored dinitrophenylhydrazone of the pyruvic acid liberated from DS by the action of the enzyme. Before assay, the bacteria are removed from the growth medium by centrifugation and suspended to an appropriate cell density in 0.07 M KPO_4 buffer (pH 7.4). The basic assay is as follows. Toluene (0.01 ml) is added to 0.3 ml of cell suspension, and the mixture is incubated at 37 C for 15 min, at which time 0.1 ml of a DS solution (10 mg/ml) is added, and incubation is continued at 37 C for 20 min. 2,4-Dinitrophenylhydrazine (0.9 ml; 0.17 mg/ml in 1.2 N HCl) is then added, and the incubation is continued at room temperature for 20 min further, at which time the reaction is stopped by the addition of 1.7 ml of 2.5 N NaOH. The optical density is read at 520 nm in a spectrophotometer (Zeiss) against an assay blank to which all components except DS have been added. The wavelength that gives the maximal reading for the reaction is actually 450, not 520 nm, but 520 nm is used because the blank

readings are rather high at 450 nm. They are quite acceptable at 520 nm. The unit of enzymic activity is 1 micromole of pyruvate liberated from DS per hour; the specific activity is the number of micromoles of pyruvate liberated per hour per milligram (dry weight) of cells. The final optical density of the assay mixture is a linear function of pyruvate concentration over the optical density range of 0 to 1.5. One milligram (dry weight) of cells corresponds to a Zeiss optical density of 1.5. The toluene step is omitted when cell extracts rather than whole cells are used. An incubation temperature of 30 rather than 37 C was used for assays involving the temperature-sensitive Dsdase.

RESULTS AND DISCUSSION

Isolation of a mutant forming a temperature-sensitive Dsdase. To obtain a mutant forming an altered but recognizable Dsdase, a culture of strain EM1609 (dsdO6 operator constitutive), which forms Dsdase at a high constitutive rate (specific activity of about 20 during logarithmic growth in minimal medium), was utilized.

It was first treated with N-methyl-N'-nitro-N-nitrosoguanidine and then put through one cycle of penicillin treatment at 42 C to screen for mutants unable to form the enzyme at high temperature only. Survivors were plated on minimal medium and incubated for growth at 30 C, and each plate was then replicated to two DS plates. One set of replica plates was incubated for growth at 42 C, and one was incubated at 30 C. Several hundred mutants unable to grow on DS plates at 42 C were obtained, and of them two did grow at 30 C. These two were purified by restreaking. They appeared to be sibs on preliminary examination. Neither formed detectable enzyme at 39 C or above, and both formed trace amounts at 37 C and about one-fifth as much as the parent at 30 C and lower temperatures. Thus only one, denoted strain EM1610, was used in further studies. The Dsdase mutation was denoted dsdA10(ts).

The Dsdase of strain EM1610. To determine whether the temperature-sensitive response of strain EM1610 to DS was due to an altered Dsdase, the rate of inactivation of its Dsdase was measured at various temperatures in cell extracts, with Dsdase from cell extracts of strain EM1609 as control. The temperature sensitivities of the Dsdases of strain EM1609 and its dsdO+ wild-type parent are identical (data not shown). A mixture of strains EM1609 and 1610 extracts at equal specific activities was included as a second control to rule out the possibility that strain EM1610 produces an inhibitor of Dsdase. The results are presented in Fig. 1. It
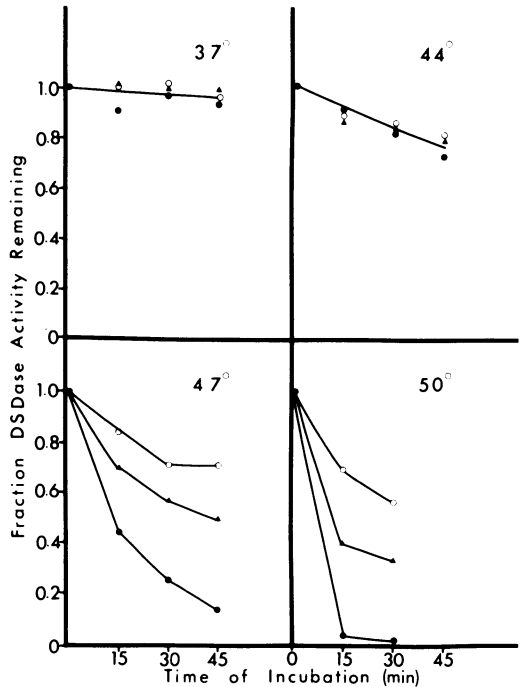

FIG. 1. Cells were grown to a density of 5 × 10⁸/ml at 30 C, concentrated 10-fold in minimal medium lacking glycerol and growth requirements, and treated for 1 min in a Branson Sonifier model no. S125 to produce cell extracts. Debris was removed by centrifugation. For heat treatment, all extracts were adjusted to a constant volume of 2.5 ml and an optical density of 9.0 at 280 nm in a Zeiss spectrophotometer to avoid nonspecific temperature effects. Samples were taken at times indicated and diluted appropriately for assay. Results are presented as fraction of untreated activity remaining at 15, 30 and 45 minutes. Symbols: ●, strain EM1610 extract; ○, strain EM1609 extract; ▲, mixed extracts of strains EM1610 and EM1609. Specific activities of Dsdase before heat treatment were about 20 for strain EM1609 extracts and 5 for strain EM1610 extracts.

may be seen that the Dsdase of strain EM1609 is fairly stable at 50 C, whereas the Dsdase of strain EM1610 is rapidly inactivated at that temperature and less rapidly inactivated at 47 C. The Dsdase of strain EM1609 was rapidly inactivated at temperatures above 55 C. Surprisingly, the Dsdase of strain EM1610 was found to be stable in cell extracts at 37 C and below and no less stable than that of strain EM1609 at 44 C. Inherent temperature sensitivity of the enzyme is thus not the reason for failure to detect it in cells growing at 42 C. Moreover, since only about 50% of the activity of the mixture was rapidly inactivated at 50 C,

it is clear that strain EM1610 does not produce a Dsdase inhibitor.

Because of the relative insensitivity of the mutant enzyme to elevated temperature in cell extracts, its sensitivity was measured in whole cells. Mutant and parent were cultivated to a cell density of 2×10^8/ml at 30 C in minimal medium, and samples were removed for assay as described above. Both were then shifted to growth at 42 C for 1 h. Samples were taken again for assay. The results are presented in Table 1. It may be seen that while the specific activity of Dsdase in the parent remained constant during growth at 42 C, the activity in the mutant was completely lost.

To determine whether the temperature sensitivity of Dsdase in growing cultures of strain EM1610 was due to a single mutation, spontaneous revertants able to grow on DS minimal medium at 42 C were selected and characterized. The results for a typical revertant are presented in Table 2. The revertant formed the enzyme at the same high constitutive rate as strain EM1609 and grew well in the presence of DS at 42 C, and enzyme from cell extracts of it showed similar kinetics of inactivation at 50 C as strain EM1609. This and other revertants did show a slightly greater Dsdase temperature sensitivity at 50 C than strain EM1609, so the reversions apparently occurred at a secondary site. They were not characterized further. The altered Dsdase phenotype in strain EM1610 is thus due to a single mutation. Very likely the structural alteration in the mutant enzyme makes it abnormally vulnerable to protease attack at temperatures above 30 C, although its inherent temperature sensitivity is not much greater than that of the parent.

Mapping of dsdA10(ts). *dsdC* specifies a regulatory substance governing the expression

TABLE 2. *Temperature sensitivity of Dsdase in EM1610 revertant*[a]

Incubation at 50 C (min)	Fraction original Dsdase activity remaining		
	EM1609	EM1610	EM1610 revertant
0	1.0	1.0	1.0
15	0.7	0.012	0.6
30	0.6	0	0.44

[a] Cell extracts of strains EM1609, EM1610, and a revertant of EM1610 were prepared and treated as described in the legend to Fig. 1. Specific activities of Dsdase before heat treatment were about 20 for strain EM1609 and the revertant of strain EM1610 and 5 for strain EM1610.

of *dsdA*, the Dsdase structural gene. It has been previously shown to be very closely linked, about 94% by P1 transduction to *dsdO* and presumed *dsdA* mutations (Dsdase negative), and about 40% linked to the left end of *aroC*. *dsdO*, which specifies the Dsdase operator, was found to be about 98% linked to presumed structural gene mutations (4). To determine the location of *dsdA10*(ts), which defines the Dsdase structural gene, strain EM1610 was transduced to *aroC*+ with P1 phage grown on strain C600 (*dsdA*+ *dsdO*+ *aroC*+). *dsdA*+ and *dsdO*+ were scored as unselected markers among the *aroC*+ recombinants. Of 604 *aroC*+ recombinants, 240 (40%) were *dsdA*+, and of these *dsdA*+ recombinants 237 (99%) were *dsdO*+. This result shows that *dsdA10*(ts) is located at about the same site as previously described Dsdase-negative mutations (4). In an attempt to order *dsdA10*(ts) vis-a-vis *dsdC* and *aroC*, strain EM1610 was transduced to *aroC*+ with P1 phage grown on strain EM1400 (*dsdA*+ *dsdC4 aroC*+), and *dsdA*+, *dsdO*+, and *dsdC4* were scored as unselected markers. Of 189 *aroC*+ transductants, 81 (43%) were *dsdA*+, and of these all were *dsdO*+ and 11 (14%) were *dsdC*+. In a second transduction, phage P1 grown on the *dsdO1dsdA*+ *aroC*+ strain EM1100 were used to transduce strain EM1406 (*dsdC4 dsdA*+ *aroC*) to *aro*+. Of 297 *aro*+ transductants, 122 (42%) were *dsdO1*, 175 (58%) were *dsdC4*, and none were *dsdC*+. Since *dsdC* and *dsdO* have previously been shown to be 95% linked by P1 transduction (4), this result indicates a gene order of *dsdC-dsdA-aroC*. Attempts to order *dsdO* relative to *dsdA* and *dsdC* by P1 transduction were unsuccessful, but methods utilizing λ phage have demonstrated the order to be *dsdC-dsdO-dsdA* (2).

Effect of the dsdO6 mutation on induction of Dsdase synthesis. Since DS is a metaboliza-

TABLE 1. *Temperature sensitivity of Dsdase in growing cells*[a]

Incubation at 42 C (min)	Fraction of Dsdase activity remaining	
	EM1609	EM1610
0	1.0	1.0
15		0.7
30		0.3
60	1.0	0

[a] The cells were cultivated in minimal medium to a density of 2×10^8/ml at 30 C and then shifted to 42 C at zero time. Specific activities of Dsdase before heat treatment were about 20 for strain EM1609 extracts and 5 for strain EM1610 extracts.
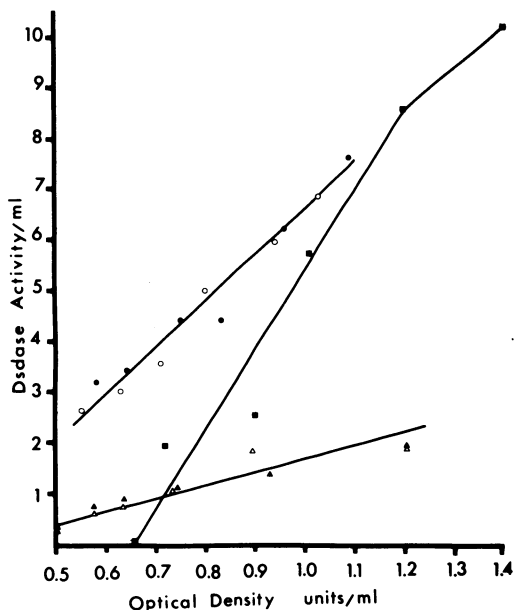
FIG. 2. *Cultures of strain EM1610 were grown to about 5 × 10⁸/ml in LB broth at 30 or 37 C. They were then divided into two portions, and DS was added to one. Growth was continued, and samples were taken from time to time for measurement of Dsdase activity after treatment with toluene. Strain W3828, the original wild-type parental strain, was included as an induction control at 30 C. Closed symbols, DS added, open symbols, no DS added. Circles, growth of strain EM1610 at 30 C; triangles, growth of strain EM1610 at 37 C; squares, growth of strain W3828 at 30 C.*

ble inducer, it has not been possible previously to determine whether *dsdO* mutations such as *dsdO6*, which result in constitutive synthesis of Dsdase at a high differential rate, permit hyperinduction. Destruction of inducer would occur too rapidly for induction to be observed. Because of the presence of the *dsdA10*(ts) mutation in strain EM1610, the differential rate of active Dsdase synthesis in this *dsdO6* strain was considerably lowered, and it was possible to test for hyperinducibility. The results are presented in Fig. 2. It may be seen that the uninduced differential rate of Dsdase synthesis was quite low at both 30 and 37 C, respectively. There would be no difficulty in detecting hyperinducibility with DS at these differential rates of synthesis. DS, however, had no effect.

There are two obvious explanations for the noninducibility of a *dsdO6* mutant strain: the *dsdO6* mutation has abolished the site of action for induction, or the mutation has caused constitutive synthesis to proceed at the maximal rate permitted by the promoter. It is not possible to decide between these alternatives at present. A third, trivial, explanation, that the mutant may have as much in vivo activity as the parent and fail to be induced further because of rapid inducer degradation, seems unlikely. The enzyme is actually much less stable in whole cells than in extracts, at least at 42 C, as shown in Table 1.

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