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Identification and Control of Synthesis of the *dsdC* Activator Protein

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Operon fusions between the D-serine deaminase regulatory and structural genes and *lacZ* were constructed and used to examine the control of expression of the positive regulatory gene, *dsdC*. Merodiploid strains containing both *dsdCp*::Mu d (*lac* Ap^r) and *dsdC*$^+$*A*$^+$ produced only one-fourth as much β-galactosidase as did the haploid *dsdCp*::Mu d (*lac* Ap^r) strains, indicating that the *dsdC*$^+$ product repressed its own synthesis. The repression was reversed by D-serine. *dsdC* expression was not depressed in a *cya* background. The basal level of D-serine deaminase was the same in wild-type and *dsdCp* fusion strains. The *dsdC* gene product was identified in maxicell strains harboring *dsd* plasmids as a 34,000-dalton protein. *dsdC* gene transcription proceeded clockwise; thus, its promoter is adjacent to that of *dsdA*.

The regulatory gene *dsdC* specifies a protein activator that, together with its ligand, D-serine, is necessary for significant transcription of the adjacent D-serine deaminase operon of wild-type *Escherichia coli* K-12 to proceed (11). The cyclic AMP (cAMP)–cAMP-binding protein complex is also necessary for optimal expression of *dsdA*, although the synthesis of D-serine deaminase proceeds, at about 20% efficiency, in its absence. Presumably the catabolite control on the system is not stringent, because D-serine deaminase is an essential enzyme in minimal media when D-serine is present, regardless of carbon source. D-Serine is toxic and must be removed if the cells are to grow (16).

Most of the regulatory genes studied have been shown to be weakly expressed, and their expression is usually subject to autogenous control (4, 13). Previous experiments suggested that the *dsdC* gene behaves similarly: when the gene was cloned onto multicopy plasmids, the very low cellular level of activator was not enhanced (unpublished data). We have now used the Mu d (*lac* Ap^r) phage, kindly supplied by M. Casadaban, University of Chicago, to form *dsdC* and *dsdA* operon fusions to *lacZ*. Analysis of the fusions showed that the activator repressed its own synthesis in the absence of D-serine. In the presence of D-serine it ceased to repress synthesis, assuming its activator form. Moreover, using the maxicell system (22) with a variety of *dsd* plasmids, we were able to identify the activator and observe its induction.

MATERIALS AND METHODS

Bacterial and phage strains. Bacterial and phage strains are described in Table 1. *E. coli* CSR603 was transformed by plasmid DNAs (3) as described by Mandel and Higa (15), with selection for appropriate antibiotic resistance. The presence and size of plasmids in transformants were verified by the method of Eckhardt (9).

Chemicals. Reagents were obtained from the following sources: ampicillin, Wyeth Laboratories; 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside (X-gal), Sigma Chemical Co.; *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, K & K Laboratories, Inc.; cAMP, Calbiochem; D-serine, Vega-Fox Laboratories; L-[^{35}S]methionine, uniformly labeled, Amersham Corp. Molecular weight standards and chemicals for sodium dodecyl sulfate-polyacrylamide gel electrophoresis were from Bio-Rad Laboratories. All other reagents were of appropriate reagent grade.

Media and cell growth. The preparation of tryptone broth and plates and minimal medium and plates was described previously (20). Minimal medium was supplemented with growth requirements as needed. Glucose (0.5%) was added to tryptone broth for growth of *cya* strains. D-Serine as inducer was used at a 500-μg/ml final concentration. Antibiotics were added to final concentrations of 25 μg/ml for cultivation of cells harboring resistance markers. X-gal was added to minimal plates as described by Miller (17). To avoid the problem of D-serine toxicity, haploid *dsdC* or *dsdA* strains were grown in tryptone broth in tests for D-serine induction of β-galactosidase. *cya* strains were always grown in tryptone broth-glucose. Otherwise, all measurements were made with cells grown in minimal medium. These necessary medium variations did not introduce experimental anomalies, as the basal levels of β-galactosidase in *dsdCp*::Mu d (*lac* Ap^r) and *dsdCp*::Mu d (*lac* Ap^r)/*dsdC*$^+$*A*$^+$ fusion strains were the same in minimal medium and tryptone broth.

Genetic techniques. Dsd$^-$ strains were transduced to Dsd$^+$ with P1 and with *dsdC*$^+$*A*$^+$ *dsdA*$^+$ phage as described previously (19). Mu d (*lac* Ap^r) phage were prepared from strain MAL103, and transductions to obtain *dsd*::Mu d1 (*lac* Ap^r) fusion in strain MC4100

TABLE 1. Bacterial and phage strains

Strain	Genotype or phenotype	Source or reference
E. coli K-12		
MAL103	[Mu cts d1 (Ap^r *lac*)] [Mu cts] Δ(*proAB-lacIPOZYA*)-XIII *rpsL*	M. Casadaban (4)
MC4100	*araD139* Δ*lacU169 rpsL*	M. Casadaban (4)
EM108-1	As MC4100, but *dsdA*::Mu d1 (Ap^r *lac*)	
EM111-1	As MC4100, but *dsdA*::Mu d1 (Ap^r *lac*)	
EM145-1	As MC4100, but *dsdC*::Mu d1 (Ap^r *lac*)	
EM148-1	As MC4100, but *dsdC*::Mu d1 (Ap^r *lac*)	
EM135-1	As MC4100, but *dsdC*::Mu d1 (Ap^r *lac*)	
EM145-2	As EM145-1, plus $dsdC^+$ A^+	
EM148-2	As EM148-1, plus $dsdC^+$ A^+	
EM135-2	As EM135-1, plus $dsdC^+$ A^+	
EM145-3	As EM145-1, but *cya*	
EM148-3	As EM148-1, but *cya*	
EM135-3	As EM135-1, but *cya*	
CSR603	*recA uvrA6 phr-1*	21
AC2000	CSR603 plus pACYC177 (Ap^r)	2
AC2051	CSR603 plus pAC51 ($dsdC^+$ Ap^r)	2
AC2053	CSR603 plus pAC353 ($dsdA^+$ Ap^r)	2
AC2071	CSR603 plus pAC711 ($dsdA^+$ Ap^r)	2
AC2052	CSR603 plus pAC221 ($dsdC^+$ A^+ Tet^r)	2
AC2054	CSR603 plus pMB9 (Tet^r)	2
Phage		
λ *c*I857 *S*am7		
λ $dsdC^+$ $dsdA^+$	*c* I857 *S*am $dsdC^+$ A^+	19
λ $dsdA^+$	*c* I857 *S*am $dsdA^+$	19

were carried out by the methods of Casadaban and Cohen (5). Fusions were initially recognized on X-gal minimal plates. Mutants unable to form cAMP were obtained by first treating appropriate strains with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (1). The survivors were screened as described previously (15) for a pleiotropic (mannitol, mannose, xylose) carbohydrate utilization deficiency reversible by cAMP.

Enzyme induction and assay. Cells were cultivated for at least six generations in medium containing 500 μg of D-serine per ml to induce expression of the *dsdC* and *dsdA* genes and fusions. D-Serine deaminase and β-galactosidase production were assayed as described previously (16, 17).

Labeling of plasmid proteins in maxicells. Strain CSR603 and its plasmid-bearing derivatives were cultivated, irradiated, labeled with [^{35}S]methionine, harvested, and lysed exactly as described by Gray et al. (10) in a modification of the procedure of Sancar et al. (22). [^{35}S]methionine-labeled proteins were analyzed on 10 to 15% linear gradient sodium dodecyl sulfate-polyacrylamide gels (18). We did not measure plasmid copy numbers in the various plasmid-bearing strains. However, pAC353, pAC51, and pAC711 are all derivatives of pACYC177 with small *dsd* inserts (3) and consistently gave yields of plasmid DNA similar to that of pACYC177. The yields of pMB9 and its $dsdA^+C^+$ derivative pAC221 were consistently two- to threefold higher.

RESULTS

Isolation of strains harboring *dsdCp* and *dsdAp*::Mu d (*lac* Ap^r operon fusions. After mutagenesis of strain MC4100 with phage Mu d (*lac* Ap^r) (4) followed by a cycloserine screen (8) of Ap^r survivors for types incapable of utilizing D-serine as sole nitrogen source, we obtained 61 mutants that failed to form D-serine deaminase in tryptone broth in response to the addition of D-serine. Surprisingly, all failed to grow on minimal medium containing D-serine, indicating that they were sensitive to D-serine and therefore did not contain insertions in the *dag* locus (transport of D-serine and some other D-amino

TABLE 2. Specific activities of enzymes in fusion strains

Strain	Fusion	− D-Serine		+ D-Serine	
		D-Serine deaminase	β-Galacto-sidase	D-Serine deaminase	β-Galacto-sidase
MC4100		0.5	1.0	25	1.0
EM108-1	*dsdA*	0.5	1.2	0.5	260
EM111-1	*dsdA*	0.5	1.6	0.5	1,960
EM145-1	*dsdC*	0.5	5.0	0.5	6.2
EM148-1	*dsdC*	0.5	6.0	0.5	6.4
EM135-1	*dsdC*	0.5	7.4	0.5	7.2
EM145-2	*dsdC*	0.5	1.2	25	7.0
EM148-2	*dsdC*	0.5	1.5	25	5.0
EM135-2	*dsdC*	0.5	1.4	25	7.2
EM145-3	*dsdC*	ND[a]	4.6	ND	ND
EM148-3	*dsdC*	ND	4.0	ND	ND
EM135-3	*dsdC*	ND	5.2	ND	ND

[a] ND, Not determined.

acids [6, 7]). Six mutants showed no consistent β-galactosidase phenotype; they either grew poorly or formed large amounts of enzyme. They were suspected of being double mutants and were not studied further. Twenty-eight mutants showed no increase in β-galactosidase activity over background activity; they were assumed to have *lacZ* aligned in the wrong orientation to the *dsd* promoters and were discarded. Nineteen mutants were *dsdA* fusions, showing a very low level of enzyme in the absence of D-serine and a very high level in its presence. Of these, 8 were induced about 1,500-fold and 11 about 200-fold. We suspect that the difference between the two groups resulted from "polarity" effects inherent in phage Mu d (*lac* Ap^r) (21). The remaining six mutants showed similar low constitutive rates of β-galactosidase synthesis unaffected by D-serine. We considered that these were probably *dsdC* fusions (see below). Three *dsdCp*::Mu d (*lac* Ap^r) and two Mu *dsdAp*::Mu d (*lac* Ap^r) fusions were chosen for further study (Table 1).

It can be noted that for the *dsdA* fusions, D-serine was a gratuitous inducer of β-galactosidase. The induction ratio for the more highly inducible fusions was similar to that for β-galactosidase in wild-type strains (17); thus, *dsdAp* is a strong promoter.

Transduction analysis of Mu d (*lac* Ap^r) strains. To verify that our fusion strains contained single insertions in *dsd* genes, we first transduced the strains to Dsd^+ with P1 phage that had been grown on a Dsd^+ strain, K37. All Dsd^+ transductants were Ap^s *lac* Mu^s. The fusions were therefore *dsd* specific. They were then exposed to λ $dsdC^+A^+$ and λ $dsdA^+$ phages with and without helper phage. The *dsdA* fusions yielded Dsd^+ transductants with both phages, whereas the presumed *dsdC* fusions yielded them only with the $dsdC^+A^+$ phage. When helper phage were present in the transduction mix, no Dsd^+ types that were Ap^s *lac* were found; all transductants were apparently additions. When helper phage were omitted, about 25% of the Dsd^+ transductants were Ap^s *lac* and viable at 42°C and were apparently haploid recombinants. Thus, the fusions indeed involved the *dsdC* and *dsdA* genes, linking *lacZ* to the respective promoters.

Autogenous control of *dsdC* expression. The expression of many regulatory genes has been shown to be repressed by the gene product. To examine the control of *dsdC* expression, we compared β-galactosidase levels in haploid *dsdCp*::Mu d (*lac* Ap^r) fusion strains and in $dsdC^+A^+$ merodiploids (Table 2). The presence of an intact *dsdC* gene in the *dsdC* fusions resulted in about a fourfold decrease in the level of β-galactosidase for all three fusion strains. The repression was reversed when D-serine was present in the growth medium. Thus, in the absence of D-serine the *dsdC* gene product repressed its own synthesis.

Role of cAMP in regulation of *dsdC* gene expression. D-Serine deaminase has a catabolic function and is subject to control by the cAMP–cAMP-binding protein system (16). The results of earlier in vitro studies suggested that *dsdC* expression is not subject to catabolite control (12). To verify this point, we isolated *cya* mutants from the three *dsdC* fusion strains. The mutations had no significant effect on β-galactosidase synthesis (Table 2), indicating that the *dsdC* promoter was not subject to catabolite control.

Comparison of D-serine deaminase levels in MC4100 and the fusion strains. We were curious as to whether removal of the activator gene would affect the basal expression of *dsdA*. The uninduced level of enzyme was too low for accurate measurement with the simple toluenization assay normally used for induced and constitutive cells. Therefore, 4-liter quantities of

TABLE 3. Basal D-serine deaminase specific activity in wild-type and fusion strains

Strain	D-Serine deaminase basal sp act (U)
MC4100	1.4×10^{-2}
EM108-1	$<10^{-3}$
EM145-1	1.4×10^{-2}
EM135-1	2.6×10^{-2}

each of the strains of interest were cultivated to late log-phase growth in minimal medium, concentrated 100 times, and broken in a French pressure cell at 4,000 lb/in^2 in preparation for assay (Table 3). A *dsdA*::Mu d (*lac* Ap^r) fusion, included as a control, showed no activity. The specific activities of the wild-type and *dsdCp*::Mu d (*lac* Ap^r) fusion strains were similar, indicating that in the absence of D-serine the *dsdC* gene product did not significantly affect *dsdA* gene expression.

Identification of the *dsdC* gene product. We previously reported the construction of a number of chimeric plasmids that contain *dsd* genes (3). The *dsdC* activator protein has been partially purified (11), but the amount per cell is small, and we were not able to identify it by classical methods. We therefore transformed the maxicell strain CSR603 with several *dsd* plasmids and the parental cloning vectors. We then carried out standard maxicell experiments to determine whether we could detect production of the *dsdC* gene product in cells containing, for practical purposes, only plasmid DNA (Fig. 1). Note that the *dsdC*$^+$ *dsdA* plasmid pAC51 and the two *dsdA*$^+$ *dsdC* plasmids pAC353 and pAC711 carried very little chromosomal material not specific to the *dsd* locus. pAC221 (AC221 [*dsdC*$^+$*A*$^+$]) carried 1.5 kilobases (kb) of nonspecific material adjacent to *dsdA* that was not present on pAC353 or pAC711; the *dsdC* end was identical to that of pAC51 (3).

For the pMB9 chimera pAC221, carrying both *dsdC*$^+$ and *dsdA*$^+$ (lanes 9 and 10), there were two bands of interest. One was 47,000 daltons (47K), which corresponded to D-serine deaminase, and the other was 34K. The intensity of each band was greater relative to background activity in cells that were exposed to D-serine during growth and labeling (lane 10). pMB9 alone showed no highly labeled maxicell bands (data not shown). Lane 2 shows bands from a strain harboring the parental vector for the remaining chimeras, pACYC177. The 30K constitutive Ap^r band of these plasmids was quite strong. In the pAC51 strain labeled in the absence of D-serine (lanes 3 and 6), there was also a strong band that corresponded exactly to the size of the *Sma*I *dsdA* gene product fragment, 25.5K (3), and a weak band at 34K. For the same chimera labeled in the presence of D-serine (lane 4), there was a much stronger band at 34K. This inducible band was not present with either pAC353 or pAC711 (lanes 5, 7, 8, 11, and 12) whether or not they were labeled in the presence of D-serine. Both, however, showed the D-serine deaminase band at 47K. The presence of the unique 34K band with pAC51 and the fact that its synthesis was induced by D-serine indicate that it is the *dsdC* product.

Direction of transcription of *dsdC*. The direction of transcription of *dsdC* has not been determined. If it is opposite to that of *dsdA*, we might see *dsdC* product fragments in the pAC353 and pAC711 maxicell gels. Our most recent restriction mapping of the *dsd* region (2; A. M. Carothers and E. McFall, unpublished data) indicated that the inserts in these two plasmids are nearly the same size, 2.3 kb. There is a maximum of 0.2 kb of non-*dsd* material adjacent to *dsdA*, and the *dsdA* gene and its promoter account for 1.4 to 1.6 kb. We would therefore be

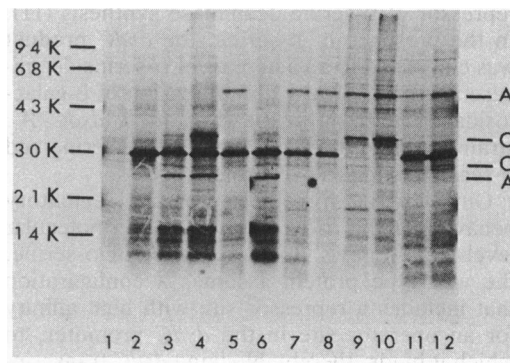

FIG. 1. Proteins programmed in maxicells by plasmids containing cloned *dsd* fragments, analyzed on 10 to 15% linear sodium dodecyl sulfate-acrylamide gradient gels. The heavy 30K band is specified by the pACYC177 Ap^r determinant and is not present in the pMB9 chimera lanes. The amount of extract layered on the gels was similar in terms of cell mass at the time of labeling, except for lanes 2 and 12, for which twice as much was used. Lanes: 1, strain CSR603; 2, CSR603(pACYC177); 3 and 6, CSR603(pAC51) labeled without D-serine; 4, CSR603(pAC51) labeled with D-serine; 5 and 8, CSR603(pAC353) labeled without D-serine; 7, CSR603(pAC353) labeled with D-serine; 9 and 10, CSR603(pAC221) labeled without and with D-serine, respectively; 11 and 12, CSR603 (pAC711) labeled without and with D-serine, respectively. This autoradiogram was developed after 7 days of exposure to the gel. A, D-Serine deaminase; A', D-serine deaminase fragment; C, activator subunit; C', activator subunit fragment. Molecular weight standards are indicated at the left.

looking for polypeptides that could be encoded by 0.5 to 0.9 kb of DNA and could appear among the proteins programmed by pAC353 and pAC711, but not by pAC51 or pACYC177. Such polypeptides can be seen in Fig. 1, lanes 5, 7, 8, 11, and 12 (pAC353 and pAC711), but not in lane 2, 3, 4, or 6 (pAC51 and pACYC177). The size of these polypeptides was 28K, which corresponds to 0.8 kb, well within the range expected.

DISCUSSION

On the basis of our present knowledge, the D-serine deaminase system appears to be controlled in a simple and efficient manner. In the absence of D-serine the *dsdC* product represses its own synthesis; in the presence of D-serine it activates D-serine deaminase synthesis. Thus, as shown above, the level of β-galactosidase in *dsdC*::Mu d1 (Ap^r *lac*) haploid strains is about fivefold higher than it is in *dsdC*::Mu d1 (Ap^r *lac*)/λ $dsdC^+A^+$ merodiploid strains. A qualitatively similar differential in the level of the *dsdC* product formed by $dsdC^+$ maxicells was observed in the presence and absence of D-serine (Fig. 1). In vitro evidence described previously indicated that in the absence of D-serine the *dsdC* product does not concomitantly act as repressor of D-serine deaminase synthesis (11). In the presence of D-serine, the *dsdC* product was converted to an activator of D-serine deaminase synthesis. Thus, the repression of β-galactosidase synthesis in the *dsdC-lac*/λ $dsdC^+A^+$ strains is lifted during growth with D-serine and D-serine deaminase is formed.

Our working hypothesis for the regulatory behavior of the *dsdC* product at the molecular level is as follows. In the absence of D-serine, the wild-type protein assumes a configuration that includes a repressor site with high affinity for an operator site in the *dsdC* promoter, to which it binds, thereby blocking *dsdC* transcription. On exposure to D-serine the protein undergoes a conformational change, which masks the repressor site and uncovers a previously masked activator site with high affinity for an initiation region in the *dsdA* promoter. The protein detaches from the *dsdC* operator and moves to the *dsdA* promoter to activate *dsdA* transcription. It is likely that our previously described *dsdC* constitutive mutants (16) specify proteins that are stabilized in the activator configuration even in the absence of D-serine. Unlike the activator constitutive mutants, *dsdA* promoter constitutive mutants are independent of the cAMP–cAMP-binding protein system as well as of D-serine production (16). These mutants probably result from changes at an RNA polymerase recognition site that bypass both activators.

The specific activity of β-galactosidase in the fusion strains is given in standard units (Table 2) (17). The phenotype of the class of *dsdA-lac* fusions represented by strain EM111-1 indicated that the *dsdA* promoter is inherently at least as strong as the *lac* promoter. Thus, the numerical values and induction ratio for β-galactosidase activity with D-serine as inducer (Table 2) are the same as those for strains with the wild-type *lac* operon (17). The expression of the *dsdC-lac* fusions, however, was very low (Table 2), with a specific activity of only 1.0 to 1.5 U in the repressed state. As we observed with the larger class of *dsdA-lac* fusion strains, such as EM108-1, polarity effects commonly resulted in the expression of genes distal to the fusions that was severalfold lower than ideal. Even assuming such a polarity factor, it was apparent that the *dsdC* promoter was very weak and that there could be only a few molecules of *dsdC* product in the repressed cell. Even so, the reason for derepression of its expression in response to D-serine was not immediately obvious. After D-serine is added to a growing culture of wild-type cells, D-serine deaminase synthesis attains its maximal rate within a few minutes (2). The level of *dsdC* product in the repressed state is therefore not limiting for the induction process, and the protein is clearly very effective both as a repressor and as an activator.

The *dsd* system shares some attributes with the well-studied *ara* system: positive control of structural gene expression, autogenous negative control of regulatory gene expression, and apparently opposite polarity of expression of structural and regulatory genes (14, 19). *dsd* differs significantly, however, in that it has no negative control on enzyme synthesis and no catabolite control on *dsdC* gene expression.

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