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Physical Mapping of the *Escherichia coli* D-Serine Deaminase Region: Contiguity of the *dsd* Structural and Regulatory Genes

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The genes *dsdA*, *dsdO*, and *dsdC* have been located on a 3.0-kilobase pair (kb) fragment of the *Escherichia coli* chromosome by a combination of techniques. The loci were first cloned onto λ and various plasmid vectors. *dsd* hybrid plasmids were then digested with restriction enzymes, and the fragments were recloned to test for the presence of *dsdC* or *dsdA*. In one case, a 4.2-kb restriction fragment containing the *dsdA* operon was used to form a heteroduplex with a well-defined λ*dsd* deoxyribonucleic acid. The results show that *dsdA*, *dsdO*, and at least 0.6 kb of *dsdC* are present on this piece of deoxyribonucleic acid. On the basis of the mapping analysis and the molecular weight of D-serine deaminase, 1.9 kb of the 4.2-kb fragment is accounted for by the three *dsd* loci. We conclude that *dsdO* and *dsdC* are contiguous. A detailed *dsd* restriction map is presented.

Genetic evidence indicates that the structural (*dsdA*) and regulatory (*dsdC*) genes governing D-serine deaminase synthesis are very closely linked, at least 95%, by P1 transduction (24). The *dsdA* promoter-initiator region, *dsdO*, is located between them (3). Blundell (personal communication) has shown by sucrose gradient and hybridization analyses that *dsd*-specific mRNA is about 14.5S in size, as expected for a transcript of *dsdA* alone. These results suggest that the two *dsd* genes might be adjacent with no extraneous DNA between them, as is the case with *lac*, *ara*, and several other systems (12, 18). It seems reasonable that such a gene arrangement might have regulatory significance. We have therefore investigated the physical structure of the *dsd* regions carried on a pair of λ*dsd* transducing phages by heteroduplex analysis, restriction mapping, and gene cloning. A variety of hybrid plasmids were constructed containing overlapping *dsd* restriction fragments. The hybrid plasmids were used for structural comparisons, in lieu of deletion mutants. We found no more than 0.5 kilobase pair (kb) of DNA separates *dsdO* and *dsdC*.

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MATERIALS AND METHODS

Chemicals. Reagents were obtained from the following sources: CsCl, Harshaw Chemical Co.; Sarkosyl, Chemical Additive Co.; lysozyme, Calbiochem; nalidixic acid, Sigma; tetracycline hydrochloride, Pfizer; ampicillin, mitomycin, and kanamycin, Bristol Laboratories; Ficoll 400, Pharmacia Fine Chemicals; Seakem agarose, Marine Colloid, Inc.; acrylamide, *N,N'*-methylene-bisacrylamide, *N,N,N',N'*-tetramethylethylenediamine, and ammonium persulfate, Ames Co.; hydroxyapatite (Bio-Gel HT), Bio-Rad Laboratories; DNase (RNase-free) and RNase, Worthington; D-serine, Vega-Fox Biochemicals. All restriction endonucleases except *Bst*EII, as well as T4 DNA ligase, were purchased from New England Biolabs, Inc. *Bst*EII was obtained from Bethesda Research Laboratories, Inc. All other chemicals were reagent grade.

Bacterial and phage strains. The bacterial and phage strains utilized are described in Table 1.

Media. Preparation of LB plates (minimal medium plates with appropriate supplements for selection of transductants) and LB broth has been described previously (24). Preparation of Gal-TTC agar has been described (14). Drug-resistant transformants were selected on tryptone-yeast extract (TYE) agar (26) plates to which 20 μg of tetracycline or ampicillin per ml or 25 μg of kanamycin per ml had been added. Transformants of pMB9 or hybrid plasmids derived from pMB9 were tested for colicin immunity as described by Spudich et al. (34). Liquid medium for cultivation of lysogens was tryptone broth, and for transformants the medium was TYE broth supplemented with the appropriate antibiotic. Liquid medium for large-scale growth of lysogens has been described (38). Liquid medium in which cultures were grown for production of plasmid DNA was composed of 1XA minimal medium (26) supplemented with 1% glucose, 0.5% Casamino Acids, and 100 μg each of adenosine, uracil, and thiamine per ml.

Phage and plasmid DNA preparation. The procedure followed for the preparation of phage DNA was that of Zubay (39). The procedure used for the isolation of plasmid DNA has been described by Cle-

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TABLE 1. *Bacterial and phage strains*

Strain	Relevant genotype or phenotype[a]	Reference
C600	*thr leu thi rpsL*$^+$	W. K. Maas
AC6082[b]	*thr leu thi rpsL*$^+$ *dsdA*	C600
AC5020-7[c]	*dsdC*^{c}*20 dsdC201 purF*$^+$ *his supF recA nalB* Ap^s	FB5020-7 (3)
KL163	HfrH *thi thy rpsL*$^+$ *recA nalB*	W. K. Maas
EM6116	HfrH Δ(*dsdC-dsdA*) Δ(*gal-uvrB*) *relA*	FB6001 (29)
RW842	*galT*::λ *int*am29 c1857	L. W. Enquist (14)
EM6177	HfrH Δ(*dsdC-dsdA*) Δ(*gal-uvrB*) *relA* (λ/*dsdC*$^+$ *dsdO*$^+$ *dsdA*$^+$)	EM6116
λ	c1857*S*am7	(29)
λp-*dsdO*$^+$*A*$^+$	*dsdO*$^+$ *dsdA*$^+$ c1857*S*am7	y199
λy199	c1857*S*am7 *xis*6 *b*515 *b*519	(29)
λ*nin*5	c1857*S*am7 *nin*5	(29)
RR1	*leu pro thi lacY rpsL*$^+$ *hsdR hsdM* (pBR322)	H. W. Boyer (4)
TE1000	*thr leu thi rpsL*$^+$ *hsdR*$^+$ *hsdM*$^+$ (pBR322)	C600
SK1163	*thr leu thi rpsL*$^+$ *hsdR*$^+$ *hsdM*$^+$ (pMB9)	S. R. Kushner
JC1569	*leu his arg met lac recA* (pACYC177)	A. C. Y. Chang
AC9582	*thr leu thi rpsL*$^+$ *dsdA* (pAC95 *dsdO*$^+$ *dsdA*$^+$ Tc^s Ap^r)	AC6082
AC2582	*thr leu thi rpsL*$^+$ *dsdA* (pAC259 *dsdO*$^+$ *dsdA*$^+$ Tc^s Ap^r)	AC6082
AC1382	*thr leu thi rpsL*$^+$ *dsdA* (pAC13 *dsdC*$^+$ *dsdO*$^+$ *dsdA*$^+$ Tc^r colicin immune)	AC6082
AC1316	HfrH Δ(*dsdC-dsdA*) Δ(*gal-uvrB*) *rel*A (pAC13 *dsdC*$^+$ *dsdO*$^+$ *dsdA*$^+$ Tc^r colicin immune)	EM6116
AC2282	*thr leu thi rpsL*$^+$ *dsdA* (pAC221 *dsdC*$^+$ *dsdO*$^+$ *dsdA*$^+$ Tc^r colicin immune)	AC6082
AC2216	HfrH Δ(*dsdC-dsdA*) Δ(*gal-uvrB*) *relA* (pAC221 *dsdC*$^+$ *dsdO*$^+$ *dsdA*$^+$ Tc^r colicin immune)	EM6116
AC3582	*thr leu thi rpsL*$^+$ *dsdA* (pAC353 *dsdO*$^+$ *dsdA*$^+$ Km^s Ap^r)	AC6082
AC7182	*thr leu thi rpsL*$^+$ *dsdA* (pAC711 *dsdO*$^+$ *dsdA*$^+$ Km^s Ap^r)	AC6082
AC5157	*dsdC*^{c}*20 dsdC201 purF*$^+$ *his supF recA nalB* Ap^s (pAC51 *dsdO*$^+$ *dsdC*$^+$ Km^s Ap^r)	AC5020-7

[a] Bacterial nomenclature conforms to the suggestions of Demerec et al. (11) and Bachmann et al. (2); with respect to plasmid-bearing strains, nomenclature conforms to that proposed by Novick et al. (28) and Smith and Nathan (33).

[b] Strain AC6082 is a *dsdA* mutant derived from strain C600 by penicillin selection (16) after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine mutagenesis (1).

[c] Strain AC5020-7 was constructed by conjugation between FB5020-7 and KL163 with selection for resistance to nalidixic acid (5 μg/ml final concentration) (19). *recA* recombinants were identified by UV sensitivity (8).

well (9). Plasmid DNA was dialyzed against 50 mM Tris-hydrochloride (pH 8.0)–1 mM EDTA disodium salt (pH 8.0).

Heteroduplex analysis. Heteroduplex formation and visualization of DNA under the electron microscope was performed according to Davis et al. (10). Specific modifications pertaining to the preparation of phage DNA have been described (29).

Analytical slab gel electrophoresis. Digestion of 1 to 2 μg of DNA by restriction enzymes was performed as described by Meagher et al. (25). Restricted λ transducing phage DNAs were analyzed by electrophoresis through 0.8 to 1.0% agarose gels (31). Restricted plasmid DNAs were analyzed by electrophoresis through both 1.0 to 1.4% agarose gels and composite 0.5% agarose–3.5% acrylamide–0.1% bisacrylamide gels (22). A standard apparatus for vertical gels described by Studier was used (35). Electrophoresis was performed in the Tris-borate buffer of Greene et al. (17). Digest samples were mixed with a loading solution composed of 30% Ficoll 400 and 0.25% bromophenol blue in electrophoresis buffer. The size of λc1857*S*am7 (henceforth λ) DNA fragments generated by restriction endonuclease *Hin*dIII have been characterized (27), and they served as molecular weight standards on all gels of low agarose concentration. Smaller fragments of restricted pBR322 DNA were used as standards on 1.0 to 1.4% agarose gels and on composite gels (36). Visualization of bands stained with ethidium bromide (0.4 μg/ml) was performed in the manner of Helling et al. (21).

Preparative slab gel electrophoresis. Preparative electrophoresis of restriction fragments was performed by a method similar to the one described by Brown et al. (5). A 300-μg sample of digested DNA was electrophoresed on vertical 0.8% agarose gels (90 by 140 by 10 mm with a 130-mm slot). Electrophoresis was at 20 mA for 1 h, then at 50 mA until the desired band was well separated. The gel strip containing the DNA band was excised, minced, and dissolved in 5 to 10 ml of 8 N sodium perchlorate by shaking at 50°C for 15 min. The solution was cooled to room temperature, and 0.5 g (1.5-ml bed volume) of hydroxyapatite, previously equilibrated with buffer A (100 mM Tris-hydrochloride, pH 7.0–50 mM NaCl–0.1 mM disodium EDTA, pH 8.0), was added, followed by gentle mixing at room temperature for 10 min. After centrifugation at 4,500 × *g* for 10 min at room temperature, the

hydroxyapatite with the bound DNA was washed twice with 10-ml portions of 10 mM sodium phosphate (pH 7.4) by careful stirring and centrifugation as before. The DNA fragment was then eluted with 4 ml of 1 N sodium phosphate (pH 7.4) at room temperature, as described above. The supernatant was dialyzed against buffer B (50 mM Tris-hydrochloride, pH 8.0–50 mM NaCl–5 mM disodium EDTA, pH 8.0). The DNA was precipitated with ethanol, suspended in buffer B, and stored at 4°C.

Construction of hybrid plasmids. Digestion of approximately 2 μg each of plasmid and phage DNAs or a mixture of plasmid DNAs was followed by inactivation of the endonuclease at 65°C for 10 min, or phenol and ether extractions, and finally ethanol precipitation. Ligation of DNA fragments was performed according to the method of Sgaramella et al. (30); incubation was at 16°C for 20 h. Uptake of the DNA by $CaCl_2$-treated bacteria was done as described by Mandel and Higa (23). After transformation, cells were allowed to grow overnight in LB broth before plating on selective media. The rapid plasmid-screening procedure of Eckhardt (13) was used to demonstrate the presence and size of hybrid plasmids in selected clones before purification by CsCl density gradient centrifugation.

RESULTS

Isolation of a λp-*dsdO*$^+$*A*$^+$ transducing phage lacking part of *dsdC*. To facilitate fine-structure restriction mapping of the *dsd* region, we needed at least one physical marker in a *dsd* gene. Such a marker would allow recognition of *dsd* fragments in endonuclease digests. We had previously isolated a λd-*dsdC*$^+$*O*$^+$*A* phage (henceforth referred to as λd-*dsd*) which carries the entire *dsd* region (29). Since we had also obtained single lysogens with λy199 inserted into a chromosomal *dsdC* (29), we proposed to isolate a λ*dsd* phage from one of them which carried only *dsdO*$^+$ *dsdA*$^+$, the adjacent portion of *dsdC*, and approximately the same amount of chromosomal DNA distal to *dsdA* as on the λd-*dsd* phage. Digests of DNAs from the two phages would thus give similar patterns, except for material at the *dsdC* locus and to its right.

Single lysogens with λ integrated into secondary attachment sites often excise at very low frequency so that a significant portion of the progeny arise from aberrant excisions involving chromosomal DNA. Since our λy199 phages are *xis*, we expected virtually all single excisions to be aberrant. Accordingly, we plated lysates derived from several single lysogens on lawns of strain RW842 on Gal-TTC agar. Plaque-forming *int*$^+$ phages form pink haloed plaques on such plates, as the *int* product excises the λ*int* phage and reconstitutes the *gal* operon; *int* phages form clear plaques (14). We obtained phage particles with an efficiency of $1:10^6$ bacteria, and only one-third of the particles were *int*$^+$, indicating that essentially all excisions were aberrant. We purified phage from plaques and tested them for ability to transduce *dsdO*$^+$ *dsdA*$^+$, obtaining several plaque-forming *dsdO*$^+$*A*$^+$ phages, all *int*$^+$. Heteroduplex analysis of the DNA from one such phage (denoted λp-*dsd*) with λ199, λ*nin*5, and λd-*dsd* DNAs (Fig. 1) revealed it to lack part of the λ*b*2 region and to carry only about 1 kb less chromosomal DNA distal to *dsdA* than λd-*dsd* DNA. The *dsd* genes are situated on λp-*dsd* immediately adjacent to λ*att*; the *dsd* genes on λd-*dsd* are in the same orientation but 9 kb to the left. The relative position of *dsdC* is apparent from the heteroduplex of the two *dsd* transducing phage DNAs.

We previously described a dimeric phage derived from an independent λ insertion into *dsdC* which carries only 4.5 kb of bacterial DNA (29). Within the limits of heteroduplex and subsequent restriction fragment measurements (data not shown), both phages appear to have evolved from λ insertion at the same site in the activator gene. It may thus be a preferred site for λ integration.

Restriction mapping of λd-*dsdC*$^+$*O*$^+$*A*$^+$ and λp-*dsdO*$^+$*A*$^+$ phage DNAs. DNAs of λ, λd-*dsd*, and λp-*dsd* were digested with several restriction enzymes known to be useful for gene cloning. The digestion patterns of the three phages were compared. Cleavage of λd-*dsd* and λp-*dsd* DNAs by the endonuclease *Hin*dIII (Fig. 2, lanes 1, 2, and 3) showed that: (i) all four fragments arising from the region to the right of λ*att* on λd-*dsd* were the same as on λ, but only three of them were the same on λp-*dsd*; (ii) both fragments from the phage *b*2 region present in digests of λ were missing on the transducing phages; (iii) from sites located on the chromosomal insertions, five unique fragments were produced from λd-*dsd* DNA and three from λp-*dsd*; and (iv) a common 5.8-kb piece found in digests of both transducing phage DNAs did not correspond with any fragment of λ DNA. Reference to the heteroduplex measurements indicated the order of the two largest chromosomal pieces from λd-*dsd* DNA. Hybridization of λp-*dsd* DNA to that of λd-*dsd* had shown a 10.2-kb region of homology comprising most of the segment on λd-*dsd* beyond the *dsdA* operon. In addition, this analysis showed that the chromosomal substitution on λd-*dsd* originates 5.5 kb away from the left cohesive end (λ*cos*). Therefore, the fragment carrying the extreme left end of λd-*dsd* had to be longer than 5.5 kb, and the next fragment beyond this end one had to be the common piece present also on λp-*dsd*. The end fragment on *Hin*dIII-restricted λd-*dsd* DNA was estimated to be 7.0 kb.

The migration of the remaining chromosomal

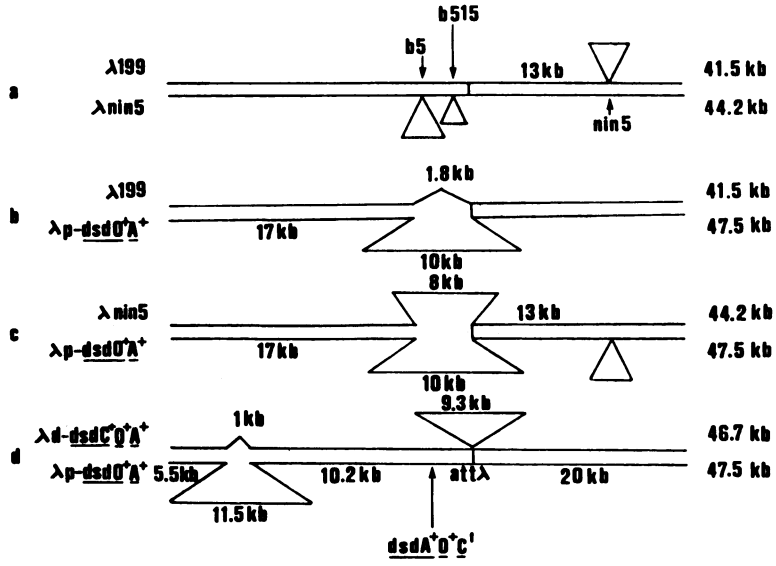

FIG. 1. *Schematic diagram of heteroduplexes between reference λ phages, λp-dsdO⁺A⁺, and λd-dsdC⁺O⁺A⁺ DNAs. Characterization of the nin5, b515, and b519 deletions illustrated in (a) have been reported (15). The measurements for (b) to (d) are presented; heteroduplexes are not shown. Coordinates are in kilobases. A vertical line through the heteroduplexes represents the relative location of the phage attachment site (λatt). An arrow indicates the relative location of the dsd region on (d).*

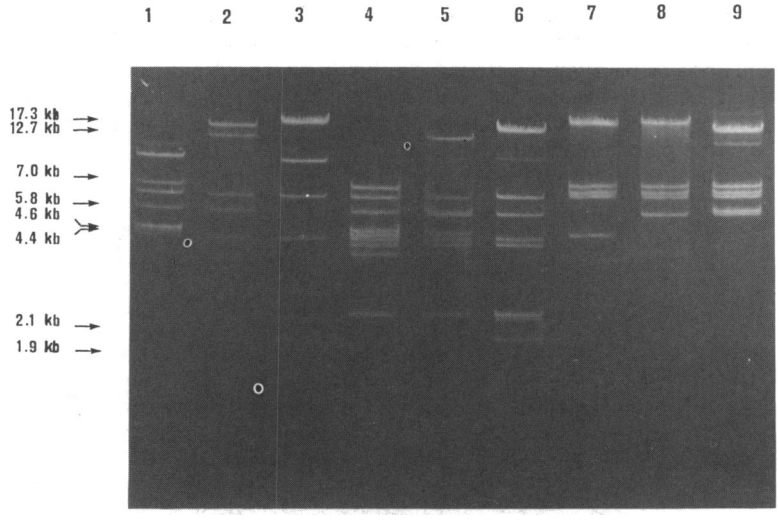

FIG. 2. *Analytical 0.8% agarose slab gel electrophoresis of restriction fragments from λdsd transducing and λ phage DNAs. The samples were prepared and electrophoresed as described in the text. Portions of digested λd-dsdC⁺O⁺A⁺ (λd-dsd) DNA were electrophoresed in slots 1, 4, and 7; similarly prepared portions of λp-dsdO⁺A⁺ (λp-dsd) and λ DNAs were electrophoresed in slots 2, 5, and 8 and 3, 6, and 9, respectively. The three were digested by HindIII (slots 1 to 3), by HindIII and BamHI (slots 4 to 6), and by BamHI (slots 7 to 9). Fragments less than 1.5 kb which are present in the digestion mixtures 1 to 7 are not apparent on this photograph. Identical 5.8-kb fragments appear after HindIII restriction of λd-dsd and λp-dsd DNAs (slots 1 and 2) due to cleavage sites located on a chromosomal region common to both.*

bands on the *Hin*dIII electrophoretic pattern of λd-*dsd* DNA indicated that each was roughly 4.5 kb in size. Two distinct bands were evident, but the slower-moving one appeared more intense than the other. Since 13.7 kb remained on the chromosomal insertion to be accounted for, three similarly sized fragments were assumed to fill this region. None of these fragments was

present in *Hin*dIII digests of λp-*dsd* DNA. Thus, one of the three comigrating pieces carried the *dsd* genes and arose from a site beyond an end of *dsdA*, present on λp-*dsd*, and from another site in close proximity to *dsdC*, present on λd-*dsd* but absent on λp-*dsd*.

*Bam*HI digestion of λd-*dsd* DNA produced two unique chromosomal pieces (Fig. 2, lanes 7, 8, and 9). They were found to be 1.2 and 4.42 kb in size and were derived from two chromosomal sites situated near λ*att* and the phage *Bam*HI site to the right of λ*att*. Neither of these fragments appeared in a *Bam*HI digest of λp-*dsd* DNA. Thus, chromosomal *Bam*HI cuts are asymmetrically positioned in the region beyond the end of *dsdC* because this portion on λd-*dsd* is not shared by λp-*dsd*. A double *Bam*HI-*Hin*dIII digestion (Fig. 2, lanes 4, 5, and 6) distinguished one of the three comigrating *Hin*dIII fragments of λd-*dsd* DNA. This double digestion showed that: (i) one of the *Hin*dIII 4.65-kb chromosomal pieces was eliminated while one remained, proving the existence of two 4.65-kb fragments; (ii) a new band corresponding to a fragment of 3.3 kb appeared; and (iii) the 1.2-kb *Bam*HI piece was conserved, indicating that *Hin*dIII cuts close to one of the *Bam*HI sites on λd-*dsd* DNA but not between them. Thus, two *Hin*dIII fragments (4.42 and 4.65 kb) from λd-*dsd* remained to be differentiated.

To learn which *Hin*dIII piece from λd-*dsd* DNA carried the *dsd* genes and the extent of *dsdC* present on this piece but not present on λp-*dsd*, a heteroduplex experiment was performed. *Hin*dIII comigrating chromosomal fragments from λd-*dsd* DNA were not resolvable by preparative slab gel electrophoresis. *Bam*HI digestion of λd-*dsd* DNA resulted in a 22.2-kb fragment comprising the left end of the phage. The remaining fragments in the *Bam*HI digest were considerably smaller; the 22.2-kb piece separated well. It was purified as described in Materials and Methods and digested by *Hin*dIII to produce four fragments. The *Hin*dIII-restricted pieces were then mixed with whole λp-*dsd* DNA to form heteroduplex molecules. Only two *Hin*dIII pieces would be capable of forming heteroduplexes with a single-stranded segment on one of the fragment's ends: the 7.0-kb end piece and the *dsd* piece. Only the *dsd* fragment would be able to hybridize to the region adjacent to λ*att* on λp-*dsd* DNA. Figure 3 shows the heteroduplex between the *dsd Hin*dIII fragment and λp-*dsd* DNA. The long single-stranded portion extending from the right half of the molecule measured 21 kb. This length indicates the relative position of λ*att*. The segment of double-stranded DNA proximal to the left side of λ*att* measured roughly 4.4 kb. Arising from the phage attachment site, a single-stranded end of the *Hin*dIII fragment was evident. Its length does not exceed 0.5 kb. This segment corresponds with *dsdC* DNA present on the fragment but missing on λp-*dsd*. Thus, these results permitted the final alignment of *Hin*dIII fragments from λd-*dsd* DNA.

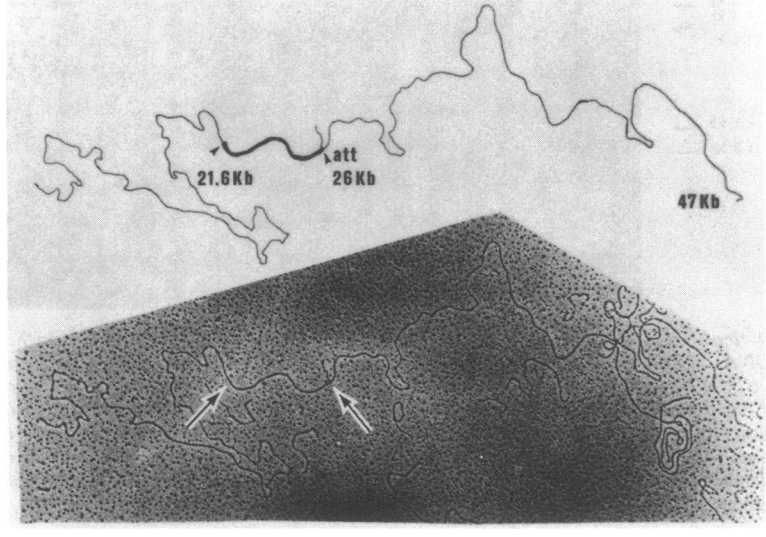

FIG. 3. *Electron micrograph of a heteroduplex between λp-dsdO⁺A⁺ DNA and a 4.4-kb HindIII fragment from λd-dsdC⁺O⁺A⁺ DNA. A tracing of the molecule is included; double-stranded DNA is represented as a heavy line; single-stranded DNA is represented as a fine line. The 0.5-kb, single-stranded, nonhomologous segment located at λatt is derived from part of dsdC present on the fragment but missing on λp-dsdO⁺A⁺.*

Cloning of a *Hin*dIII fragment of λd-*dsd* DNA onto pBR322. The cloning technique is a useful means of gene isolation. Two aspects of the technique were expected to help define the *dsd* system. First, multiple fragments resulting from digestion by different endonucleases could be cloned. The subsequently formed hybrids could be compared to determine the position of more cutting sites. Second, cloning of smaller and smaller *dsd* fragments could reveal the extent of the *dsdA* and *dsdC* genes and assist in locating *dsdO*.

The comparison of *Hin*dIII-restricted λp-*dsd* and λd-*dsd* DNAs indicated that this enzyme produced a fragment carrying the complete *dsdA* operon and some portion of *dsdC*. We cloned this piece from λd-*dsd* DNA into the *Hin*dIII site on the Ap^r Tc^r vehicle, pBR322, to obtain a source of *dsd* DNA for further restriction mapping and to ascertain whether *Hin*dIII cut *dsdC*. Ap^r/Tc^s hybrid plasmids were isolated from strain AC6082, which contained the 4.42-kb *dsd* piece in both orientations. Table 2 summarizes the physical properties of these plasmids and others described below. Each of the hybrid plasmids was 8.8 kb, but they differed phenotypically. One grew slowly on DS minimal medium and yielded a very low uninduced level of D-serine deaminase. It was designated pAC259. The pAC259 transformant of strain AC6082 was denoted AC2582. The other grew rapidly on DS medium, yielded an uninduced level of enzyme activity roughly sixfold greater than pAC259, and was designated pAC95. A transformant of strain AC6082 by the latter plasmid was denoted AC9582. Neither strain was inducible.

By performing *Bam*HI-*Sal*I double digestion of the two plasmid DNAs (data not shown), we found that the *Hin*dIII fragment was oriented such that transcription of the D-serine deaminase structural gene occurs in a clockwise direction in the case of pAC259, and counterclockwise in the case of pAC95. Both plasmid DNAs were next used as templates to program D-serine deaminase synthesis in a coupled transcription-translation system. Heincz and McFall (20) have shown that activator protein synthesis occurs in vitro during a 70-min incubation at 30°C, and that synthesis of the enzyme occurs during a subsequent 40-min incubation at 36°C. No enzyme is formed from $dsdC^+O^+A^+$ template in vitro when incubations are performed at either temperature, separately. pAC95 DNA-directed in vitro D-serine deaminase synthesis was obtained at 30 and 36°C. Preincubation at 30°C had no effect on the subsequent rate of synthesis at 36°C. In an analogous experiment, pAC259 DNA gave no enzyme at either temperature alone, or after preincubation at 30°C followed by shift to 36°C (data not shown).

TABLE 2. *Physical properties of dsd hybrid plasmids*

Plasmid	Parent vector	Cloned *dsd* fragment	Fragment size (kb)
pAC259	pBR322	*Hin*dIII, $dsdO^+$ $dsdA^+$	4.42
pAC95	pBR322	*Hin*dIII, $dsdO^+$ $dsdA^+$	4.42
pAC13	pMB9	*Bst*EII, $dsdC^+$ $dsdO^+$ $dsdA^+$	7.42
pAC221	pMB9	*Bst*EII-*Pvu*II, $dsdC^+$ $dsdO^+$ $dsdA^+$	4.3
pAC353	pACYC177	*Xho*I-*Hin*dIII, $dsdO^+$ $dsdA^+$	2.4
pAC711	pACYC177	*Xho*I-*Bgl*II, $dsdO^+$ $dsdA^+$	2.17
pAC51	pACYC177	*Sma*I-*Pvu*II, $dsdC^+$ $dsdO^+$	2.13

To evaluate the functional state of the *dsdO* locus, both plasmids were used to program D-serine deaminase in vitro after addition of increasing amounts of $dsdC^+$ extract. When the incubation was maintained at 36°C, an additional increase in enzymatic activity was obtained. These results indicate that fusion of the *dsdC* end of the *Hin*dIII fragment in one orientation onto pBR322 created a new promoter, causing anomalous *dsdA* expression in the absence of $dsdC^+$ product. Fusion of the *dsd* piece in the opposite orientation did not permit expression. Thus, in vitro enzyme synthesis programmed by the hybrid plasmids suggested that *Hin*dIII cuts *dsdC*. In addition, *dsdO* on the *Hin*dIII fragment was shown to be capable of normal function.

Formation of a $dsdA^+O^+A^+$ hybrid of pMB9. Since *Hin*dIII was shown to cut *dsdC*, we sought another restriction enzyme to clone this gene so that the location and extent of the *dsd* region could be further refined. The *dsdC* *Hin*dIII cutting site was used as a physical marker. The positions of other sites were evaluated relative to this site.

Digestions by the endonuclease *Bst*EII were performed to compare λ, λd-*dsd*, λp-*dsd*, and pAC259 DNA restriction patterns (data not shown). *Bst*EII produced five unique chromosomal bands in digests of λd-*dsd* DNA. A 2.13-kb chromosomal fragment was common to both λd-*dsd* and λp-*dsd* DNAs. There is a single *Bst*EII site beyond the 3′ end of *dsdA* on pAC259 DNA. This information indicated the presence of two *Bst*EII sites on either side of the *Hin*dIII site located to the left of the D-serine deaminase structural gene. *Bst*EII-*Hin*dIII double digestions of λd-*dsd* DNA showed that *Bst*EII cut all three comigrating chromosomal *Hin*dIII pieces.

We next used *Bst*EII to clone the entire *dsd*

region onto pMB9. *Bst*EII cleaves the vector, pMB9, at a site 3.1 kb away from the *Eco*RI cut; neither tetracycline resistance nor colicin immunity markers are disrupted. A *dsd* hybrid plasmid was isolated by selecting for resistance to tetracycline and ability to grow on DS minimal medium. It was designated pAC13 and was found to carry a 7.4-kb fragment which corresponded with a chromosomal piece present in samples of *Bst*EII-restricted λd-*dsd* DNA (data not shown). When this plasmid was used to transform a *dsd* deletion strain, EM6116, to Dsd$^+$, transformants were highly inducible. Thus, the *Bst*EII fragment contains *dsdC*$^+$ *dsdO*$^+$ *dsdA*$^+$. In vitro D-serine deaminase synthesis programmed by pAC13 DNA confirmed these physical and genetic findings (6). Figure 4 shows a restriction cleavage map of λd-*dsd* DNA; the 7.4-kb *Bst*EII piece has been offset and shows the relative location of the *dsd* region.

Cloned fragments that define the extent of the *dsd* region. The hybrid plasmids pAC259 and pAC13 were derived from overlapping fragments on λd-*dsdC*$^+$*O*$^+$*A*$^+$ DNA. They were used for further restriction mapping to determine the location of cutting sites either common to both plasmids or unique to only one. This analysis led to the formation of four smaller hybrid plasmids, which define the extent of the *dsd* region.

The restriction enzyme, *Pvu*II, was found to cut pAC13 DNA at three sites (data not shown). However, this enzyme did not cut the *Hin*dIII fragment containing the *dsdA* operon which is present on pAC259. *Pvu*II cleaves pMB9 DNA at two closely linked sites, approximately 1.91 kb from the *Eco*RI restriction site. Thus, the third *Pvu*II cut on pAC13 is situated in the vicinity of *dsdC*, between the *Hin*dIII and *Bst*EII sites. By performing a *Pvu*II-*Hin*dIII double digestion of pAC13 DNA (data not shown), we found that *Pvu*II cut 0.78 kb to the right of the *Hin*dIII site on the cloned *dsd* fragment. Therefore *Pvu*II was chosen to form a derivative plasmid of pAC13 and to determine whether the remaining *Bst*EII-*Pvu*II piece carried *dsdC* intact. A ligation mixture of pAC13 DNA, previously restricted by *Pvu*II, was used to transform the *dsdA* strain, AC6082, to Tc^r. Transformants were evaluated by the plasmid screening technique for size. An 8.4-kb hybrid plasmid designated pAC221 was isolated which contained only a single *Pvu*II cut. A pAC221 transformant of strain EM6116 (Δ*dsdCOA*/pAC221) was denoted AC2216. It was highly inducible and phenotypically identical with the

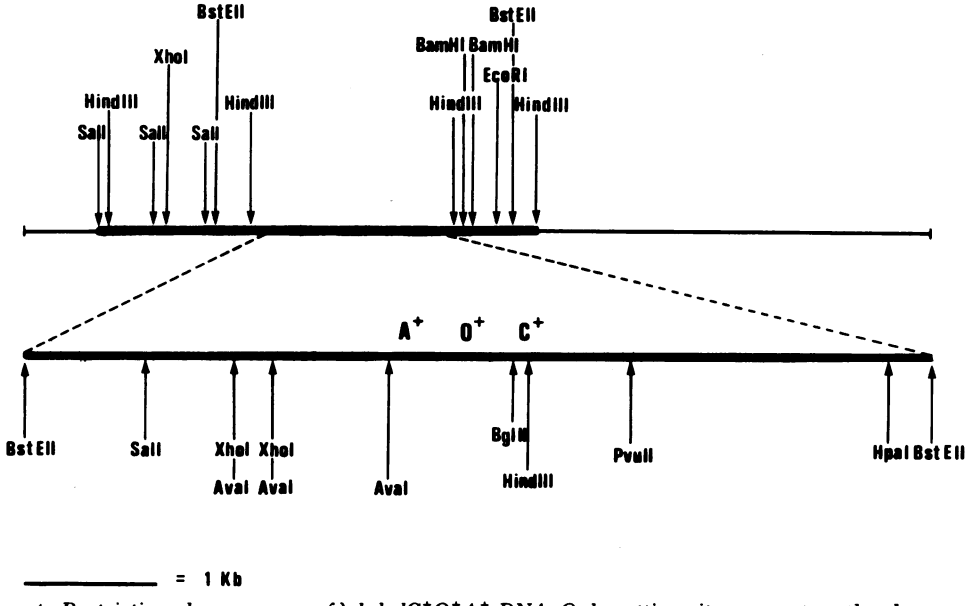

FIG. 4. *Restriction cleavage map of λd-dsdC*$^+$*O*$^+$*A*$^+$ *DNA. Only cutting sites present on the chromosomal insertion beginning at λatt are shown. The relative locations of sites on the phage are drawn to scale. PstI, EcoRI, XbaI, and KpnI do not cleave the chromosomal portion of this phage. Further restriction analysis of sites in the dsd region was performed on a cloned 7.4-kb BstEII fragment. This piece has been enlarged. The scale below shows a segment length equivalent to 1 kb on the enlarged fragment. The extent of the dsd region is defined by the second XhoI site on the left and the PvuII site on the right. The single HindIII cut identifies the location of dsdC. The genes have been drawn on the fragment to indicate their relative positions.*

pAC13 transformant of this same host, AC1316 (6). Thus, *Pvu*II does not cut *dsdC*. On the basis of the distance between this site and the *Hin*dIII site, it seems likely that *Pvu*II cuts very close to an end of *dsdC*.

Further restriction analysis which compared pAC221 DNA with that of pAC259 led to the formation of three other *dsd* hybrid plasmids by using the cloning vehicle, pACYC177. The restriction enzyme *Xho*I cuts both pAC221 and pAC259 DNAs 2.4 kb to the left of the *dsdC* *Hin*dIII site (data not shown). Plasmid pACYC177 carries *Hin*dIII and *Xho*I sites in the Km^r locus. To determine whether *Xho*I cut *dsdA*, a simultaneous *Xho*I-*Hin*dIII double digestion of pAC221 and pACYC177 DNAs was performed. The *dsd* fragment was cloned onto pACYC177, resulting in an Ap^r/Km^s hybrid plasmid called pAC353. It was identified in the recipient strain, AC6082, on the basis of its size (5.57 kb) by the plasmid screening technique. A pAC353 transformant was designated AC3582. A low level of D-serine deaminase activity was detected in uninduced concentrated log-phase cultures of strain AC3582 (6); however, in vivo it was noninducible. In vitro D-serine deaminase synthesis programmed by pAC353 DNA was obtained in the presence of $dsdC^+$ extract (6). Therefore, *Xho*I does not cut *dsdA*.

Double *Hin*dIII-*Bgl*II digestion of pAC221 and pAC353 DNAs produced a 0.225-kb common fragment (data not shown). To determine whether *dsdO* was located in this region, we cloned a *Bgl*II-*Xho*I fragment from pAC221 DNA onto *Bam*HI-*Xho*I-restricted pACYC177 DNA. A hybrid plasmid carrying the *Bgl*II-*Xho*I piece was designated pAC711 and measured 4.87 kb. If *Bgl*II cut either *dsdO* or *dsdA*, the hybrid plasmid in a *dsdA* background was expected to be Dsd^-. We found that a transformant of strain AC6082, designated AC7182, grew on DS minimal medium and was phenotypically identical to the pAC353 transformant, AC3582. Therefore, *dsdO* is situated to the left of the *Bgl*II site.

To facilitate further fine mapping of cleavage sites located between *Xho*I and *Pvu*II, a final derivative of pAC221 was created. *Sma*I was found to cut in the region of *dsdA*, 1.34 kb to the left of the *dsdC* *Hin*dIII site. There is also a *Sma*I site on pACYC177 DNA in the Km^r marker. A *Pvu*II-*Sma*I double digest of pAC221 DNA was ligated to *Sma*I-restricted pACYC177. After the standard transformation and selection process, a hybrid plasmid of 5.79 kb was obtained and designated pAC51. DNA of this plasmid was used to transform the *dsdC* $dsdA^+$ strain, AC5020-7, to Ap^r. A transformant, denoted AC5157, was phenotypically Dsd^-. In vitro synthesis at 30°C programmed by pAC51 DNA produced $dsdC^+$ activator which subsequently stimulated D-serine deaminase synthesis at 36°C after addition of a second $dsdO^+$ $dsdA^+$ template (6). Thus, pAC51 carries *dsdC* intact but not *dsdA*. However, we prepared an extract from a pAC51 transformant of the *dsd* deletion strain, EM6116, called AC5116, and found a low level of D-serine deaminase activity. S-100 extracts (20) from 100 g of cells of strain AC5116 gave 4.2 μmol of pyruvate per min of enzyme activity. Comparable crude fractions of strain EM6116 give no activity whatsoever (M. Heincz, personal communication). S-100 extracts from the same amount of cells of the pAC13 transformant strain, AC1316, gave 145.0 μmol of pyruvate per min of D-serine deaminase activity. The level of activity obtained from pAC51 is approximately 3% of that obtained from pAC13. This finding suggests that *Sma*I cuts the *dsdA* gene relatively

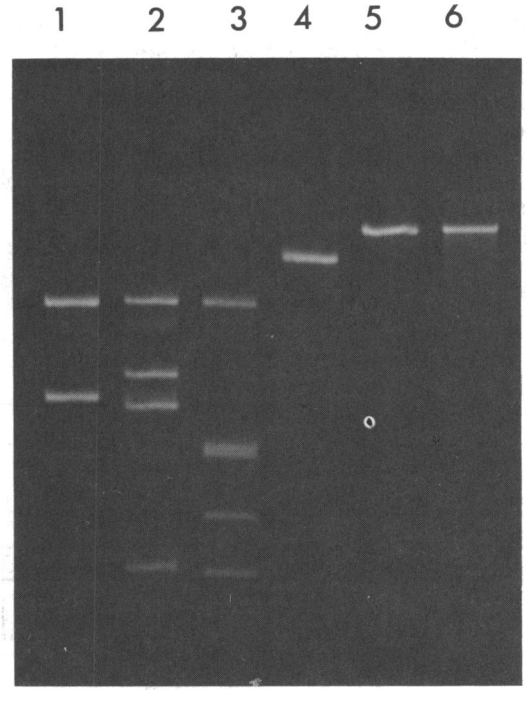

FIG. 5. *Analytical 1.0% agarose slab gel electrophoresis of endonuclease-digested dsd hybrid plasmids and pACYC177 DNAs. Conditions for electrophoresis are described in the text. Lanes 1 to 3 show HaeII restriction patterns of pACYC177, pAC353, and pAC51 DNAs, respectively. The dsdA gene is identified by the 0.538-kb HaeII fragment present on both hybrids (bottom bands in lanes 2 and 3). Slots 4 to 6 show SmaI digests of pACYC177, pAC353, and pAC51 DNAs, respectively. The two dsd plasmids carry the dsdA SmaI cutting site and are roughly the same size.*

close to the 3′ end, and that the peptide encoded by the fragment retains low catalytic activity.

The two *dsd* pieces carried on pAC353 (*Xho*I-*Hin*dIII) and pAC51 (*Sma*I-*Pvu*II) overlap. These plasmids contain very little DNA not specific for the *dsd* region. We digested them with a variety of endonucleases and compared their gel patterns with those of pACYC177 DNA. Figure 5 shows a *Hae*II digest of the two Ap^r hybrids and pACYC177 DNAs, revealing a 0.538-kb fragment from the portion of *dsdA* between the *Sma*I and *Hin*dIII sites common to both the hybrids. Analogous experiments led to the cleavage map shown in Fig. 6.

DISCUSSION

The results presented above indicate that the operons comprising the D-serine deaminase regulatory and structural genes are contiguous. *dsdA* and *dsdO* are intact on the 2.4-kb *Xho*I-*Hin*dIII fragment carried on pAC353 and pAC259, since both plasmids program D-serine deaminase synthesis in vitro and in vivo. Moreover, the fragments on these plasmids carry approximately 0.5 kb more of *dsdC* DNA than λp-*dsd* phage, as shown by the heteroduplex analysis. The *dsdA* gene codes for a protein of 45,000 daltons which requires at least 1.1 kb of DNA. Allowing an additional 0.2 kb for *dsdO* and a termination region for *dsdA*, and the 0.5 kb of *dsdC* known to be present, plus 0.1 kb for its initiation or termination region, we can account for 1.9 kb of the 2.4-kb fragment. Since a peptide with very weak enzymatic activity is specified by plasmid pAC51, which was generated from a *Sma*I cleavage site roughly 1 kb to the right of the *Xho*I site, it is likely that the DNA unaccounted for on the 2.4-kb fragment is nearly all distal to *dsdA*. With regard to *dsdC*, the size of the *Bst*EII-*Pvu*II fragment carried on pAC221 limits its extent. This fragment comprises 4.3 kb and includes an intact $dsdC^+O^+A^+$ as evidenced by its ability to program $dsdC^+$ and $dsdA^+$ expression in vivo. Since the *Pvu*II site is situated only 0.8 kb to the right of the *dsdC Hin*dIII site, the activator gene with its promoter and terminator regions should be on the order of 1.3 kb or less.

It is interesting that so many procaryotic regulatory genes coding for specific proteins are immediately adjacent to operons concerned. This is true for the *lac*, λ, and *hut* repressor genes and the *ara*, *dsd*, and *mal* activator genes (2, 32). This physical arrangement is not universal. *argR* and *trpR* are not linked to the loci they affect, *araC* is not linked to the *ara* permease gene, and *malT* is not closely linked to a second group of *mal* genes (2). However, the phenomenon of close association is obviously not random. There may be a selective advantage in terms of ability of regulatory proteins to find their sites of action if they are synthesized close by.

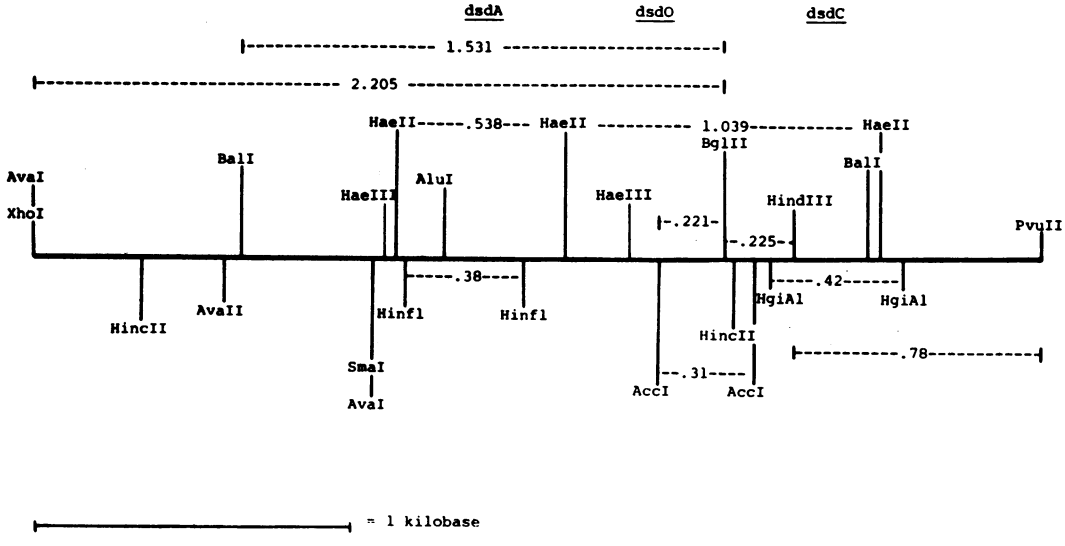

FIG. 6. *Locations of restriction cleavage sites in the dsd region. The relative position of cutting sites is drawn to scale. All digests were examined by electrophoresis on both composite agarose-acrylamide slab gels and 1.4% agarose slab gels (see the text). Molecular weight standards for each experiment consisted of restricted pBR322 DNA (36).*

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